TRP CHANNELS IN INFLAMMATION AND IMMUNITY

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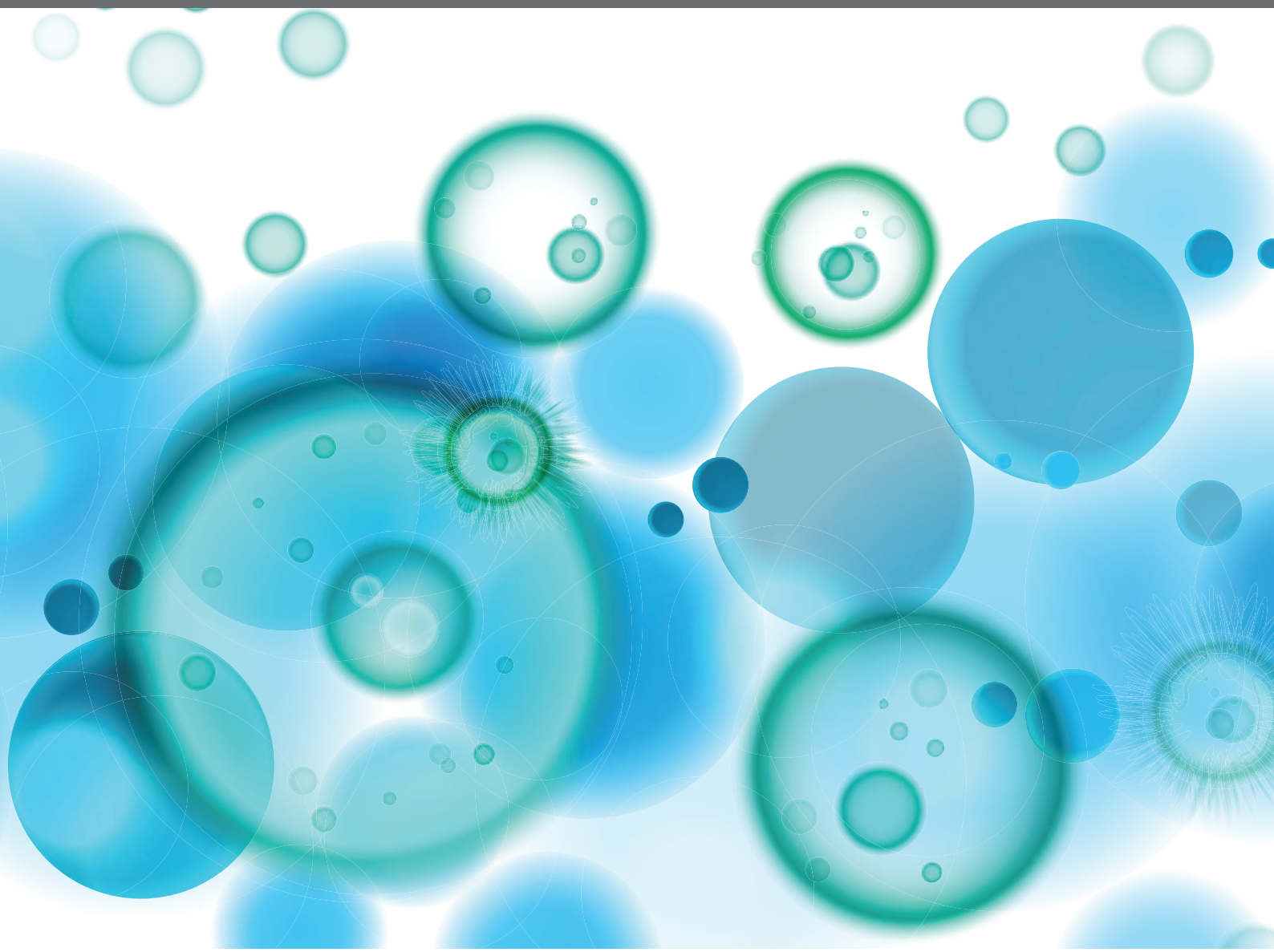

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TRP CHANNELS IN INFLAMMATION AND IMMUNITY

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EDITORIAL
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Editorial: TRP Channels in Inflammation and Immunity

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Editorial on the Research Topic

TRP Channels in Inflammation and Immunity

TRP channels respond to a variety of chemical and physical stimuli derived from harmful agents and contribute to increased intracellular cation concentrations. TRP channel-mediated effects in immune cells are diverse and range from regulation of cell migration and phagocytosis, to the production and release of inflammatory mediators. Furthermore, these channels mediate an active crosstalk between epithelial cells, neuronal tissue, and the immune cells that orchestrate immune responses to tissue damage or infection. The articles published in this special issue review current thinking with regards to the role of TRP channels in innate immune cells, covering from the prototypical phagocytic cells, neutrophils, and macrophages, to other innate immune cells like mast cells and natural killer cells. Original articles and reviews highlight the involvement of TRP channels in regulating the inflammatory properties of innate immune cells, and how these processes shape the outcome of diseases including inflammatory bowel disease, lung disease, allergic disorders, infectious and autoimmune diseases. Because of the wide functional role of TRP channels in inflammation and immunity, authors discuss the use of specific agonists or antagonists and the potential for development of novel treatments for infectious and/or inflammatory diseases.

In the special issue, several original papers focus on TRP channels in neutrophils. The work by Robledo-Avila et al. adds new knowledge to the role of TRPM2 channels in neutrophils during a L. monocytogenes infection. They show that a role of TRPM2 channels is to contain the inflammatory response to a L. monocytogenes infection. Accordingly, TRPM2$^{-/-}$ mice develop septic shock and TRPM2$^{-/-}$ neutrophils acquire a hyperinflammatory profile. The increased inflammatory properties of TRPM2$^{-/-}$ neutrophils correlate with dysregulated cytoplasmic concentration of Ca^{2+}, which potentiate membrane depolarization and accounts for the higher susceptibility of TRPM2$^{-/-}$ mice to infection. Whereas the importance of TRPM2 in regulating the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in neutrophil function has been studied, TRPM2 as non-selective cation channel, is also permeable to Na^+. However, the impact of this channel in modulating the intracellular Na^+ concentration ($[Na^+]_i$) is unknown. The original research article by Najder et al. exams for the first time how knockdown of TRPM2 may affect the intracellular Na^+ homeostasis and chemotaxis of neutrophils. The authors found that knockout of TRPM2 channel results in altered neutrophil $[Na^+]_i$, likely by indirectly modulating the Na^+ transport protein NCX1. Therefore, they postulated that TRPM2 channel regulation of cation balances may be essential under inflammatory environment.

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TRPM2 channel is widely expressed in immune cells and it is firmly established that the channel can be activated by intracellular adenosine 5′-diphosphoribose (ADPR). However, additional NAD derivatives including NAADP and cADPR have been considered TRPM2 agonists, but the activating mechanism and activation site of the channel for these nucleotides remain controversial. In a review article Fliegert et al. critically summarize the literature regarding the role for cADPR as an agonist of TRPM2 channel, with an emphasis on recent structural data. Authors conclude from the literature that removal of contaminating ADPR prevents activation of TRPM2 by commercial cADPR, nonetheless, the idea that cADPR could affect TRPM2 channel remains unsettled.

Another original research article by Nadolni et al. investigates the role of TRPM7 channels in neutrophil inflammatory recruitment with a particular focus on the kinase domain of this channel. They found that mice whose TRPM7 channels bear an inactive kinase domain are recruited less efficiently in a peritonitis model. This is underpinned by *in vitro* studies that reveal reduced chemotaxis and reactive oxygen species production in the presence of TRPM7 channel or kinase blockers.

TRPV4, members of the vanilloid (TRPV) subfamily of TRP channels, are widely expressed mechanosensitive channels also found in many (innate) immune cells, where they contribute to their activation and differentiation. Immune cells are challenged by many mechanical cues that may originate from the blood stream during the recruitment process, from the surrounding extracellular matrix or be a consequence of the respective organ function such as ventilation or heartbeat. The new concept of mechanoimmunology attempts to bring the function of immune cells and their response to mechanical cues in a causal relationship. Two reviews are evaluating this idea with TRPV4 channels being the common denominator. Michalick and Kuebler provide an overview on how the mechano-sensitivity of TRPV4 channels ("mechanoTRPV4") impacts on their role in immune cells ("immunoTRPV4"). Scheraga et al. discus the link between mechanics and signaling in a narrower context for macrophages and lung injury. Furthermore, Alpizar et al. report in an original research that TRPV4 mediates LPS-induction of inflammatory innate immune responses in urothelial cells. TRPV4 is highly expressed in urothelial cells and is known to play a role in sensing the normal filling state of the bladder and the mechanisms of bladder voiding. The regulatory role of TRPV4 on the increase in proinflammatory cytokine expression induced by bacterial LPS is a novel finding.

Mast cells and basophils are essential drivers of allergic and anaphylactic reactions. Two original papers focus on the role of TRPC channels in these innate immune cell types. Tsvilovskyy et al. nicely demonstrate that TRPC channels contribute at least partially to mast cell activation *via* the Mas-related G-protein coupled receptor member B2 (MrgprB2), a mast cell-specific receptor for basic secretagogues, such as the widely used compound 48/80 or substance P. On the other hand, mast cell activation following FcεRI stimulation is not affected by TRPC channels. Authors support their claim that TPRC channels might be associated with systemic pseudo-allergic reactions by using double ($Trpc1/4^{-/-}$) and triple ($Trpc1/4/6^{-/-}$) knockout mouse models and a thorough comparison of bone marrow derived mast cells versus peritoneal mast cells. In the other study, Bacsa et al. elegantly test the principal possibility of pharmaco-optogenetic modulation of the function of immune cells using a recently established TRPC3/6/7 selective photochromic benzimidazole agonist OptoBI-1. The researchers demonstrated that the rat basophilic cell line, RBL-2H3, lacks noticeable Ca^{2+}/NFAT signaling in response to OptoBI-1 photocycling. Different genetic modifications of these cells, by introduction of recombinant benzimidazole-sensitive TRPC isoforms (TRPC3/6/7), revealed that exclusively the single expression of TRPC6 generates OptoBI sensitivity suitable for opto-chemical control of NFAT1 activity. These results provide the first proof-of-concept for efficient chemo-genetic targeting of Ca^{2+} signaling in basophils and transcriptional regulation based on TRPC photo-pharmacology.

Unlike most TRP channels, which are expressed at the plasma membrane, endolysosomal TRP mucolipin channel family (TRPML), are found in the endolysosomal system. Endosomes and lysosomes are involved in many aspects of immune cell function, such as phagocytosis, antigen presentation and processing by antigen-presenting cells, release of proinflammatory mediators. Consequently, TRPML channels must play critical roles in inflammation and immunity. In this special issue, Spix et al. critically review the multiple functions of TRPML channels in regulating immune responses. This includes the TRPML1-mediated modulation of secretory lysosomes, granzyme B content, tuning of effector function in NK cells, TRPML1-dependent directional dendritic cell (DC) migration and DC chemotaxis, and the role of TRPML2 in chemokine release from LPS-stimulated macrophages. However, they noted that expression and the functional roles for TRPML channels on other very diverse immune cell types (e.g. basophils, eosinophils, monocytes and T cells) remain to be elucidated. With a more specific focus on TRPML channels on NK cell functionality and cancer immunotherapy, Clement et al. provide a concise review of lysosome biogenesis in NK cells. They further highlight methodological advances and new specific pharmacological modulators generated in the last years and their potential for future studies.

In addition, Santoni et al. review the impact of TRPML channels on mammalian anti-viral responses as well as on NK cell function. They highlight, on the one hand, an essential role of TRPML1 and 2 channels in the promotion of virus entry and infectivity and, on the other hand, the contribution of TRPML channels enhancing antiviral innate and possibly adaptive immune responses by regulating TLR signaling in different innate immune cells. Thus, TRPML channels might form a double-edged sword in the innate immune response to viral infections.

There is an emerging theme that TRP channels play a diverse yet poorly defined role in the immune– nonimmune cell crosstalk during inflammatory processes. Chen et al. review and summarize the plethora of findings linking the functions of TRP channels in immune cells as well as sensory nerves in the pathogenesis of inflammatory bowel disease. Similarly, the review by Silverman et al. elaborates further on how neuronal TRP channels drive the inflammatory processes in general. These reviews clearly highlight the significance of TRP channels in inflammatory processes, but they also reveal that there are glaring mechanistic gaps in our

understanding of how TRP channels and their downstream electrical signals regulate these inflammatory processes – much work is needed to understand how TRP channels regulate intracellular signaling in immune cells and how their activities help coordinate the complex cellular networks that underly tissue inflammation.

In summary, this special issue of Frontiers in Immunology presents a comprehensive overview of the most recent data on the role of several TRP channel families in innate immunity. It highlights the importance of these proteins as potential drug targets for future therapeutics against allergic reactions, inflammatory or infectious diseases.

AUTHOR CONTRIBUTIONS

All authors listed made direct intellectual contribution to the work, wrote, edited, and approved the final manuscript for publication.

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ORIGINAL RESEARCH
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The TRPM2 Ion Channel Regulates Inflammatory Functions of Neutrophils During *Listeria monocytogenes* Infection

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During infection, phagocytic cells pursue homeostasis in the host via multiple mechanisms that control microbial invasion. Neutrophils respond to infection by exerting a variety of cellular processes, including chemotaxis, activation, phagocytosis, degranulation and the generation of reactive oxygen species (ROS). Calcium (Ca^{2+}) signaling and the activation of specific Ca^{2+} channels are required for most antimicrobial effector functions of neutrophils. The transient receptor potential melastatin-2 (TRPM2) cation channel has been proposed to play important roles in modulating Ca^{2+} mobilization and oxidative stress in neutrophils. In the present study, we use a mouse model of *Listeria monocytogenes* infection to define the role of TRPM2 in the regulation of neutrophils' functions during infection. We show that the susceptibility of $Trpm2^{-/-}$ mice to *L. monocytogenes* infection is characterized by increased migration rates of neutrophils and monocytes to the liver and spleen in the first 24 h. During the acute phase of *L. monocytogenes* infection, $Trpm2^{-/-}$ mice developed septic shock, characterized by increased serum levels of TNF-α, IL-6, and IL-10. Furthermore, *in vivo* depletion of neutrophils demonstrated a critical role of these immune cells in regulating acute inflammation in $Trpm2^{-/-}$ infected mice. Gene expression and inflammatory cytokine analyses of infected tissues further confirmed the hyperinflammatory profile of $Trpm2^{-/-}$ neutrophils. Finally, the increased inflammatory properties of $Trpm2^{-/-}$ neutrophils correlated with the dysregulated cytoplasmic concentration of Ca^{2+} and potentiated membrane depolarization, in response to *L. monocytogenes*. In conclusion, our findings suggest that the TRPM2 channel plays critical functional roles in regulating the inflammatory properties of neutrophils and preventing tissue damage during *Listeria* infection.

Keywords: TRPM2, *Listeria monocytogenes*, neutrophils, inflammation, systemic inflammation

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INTRODUCTION

Polymorphonuclear cells (PMNs), commonly called neutrophils, are the first line of defense of the host against microbial infections (1–3). During an active infection, neutrophils migrate to the site of inflammation following chemotactic gradients of chemokines and pathogen-associated molecular patterns (PAMPs) (4, 5). Neutrophils eliminate pathogens through phagocytosis, granule release

or the production of neutrophil extracellular traps (NETs) (1, 6), these mechanisms are in part regulated by mobilization of calcium (Ca^{2+}) and the subsequent Ca^{2+} signaling events (7, 8).

Although Ca^{2+} release-activated channels (CRAC) are considered the main ion channels responsible for the regulation of Ca^{2+} entry in immune cells (7, 9), the transient receptor potential (TRP) superfamily have emerged as crucial ion channels that regulate specific cell processes in myeloid cells (10–12). Particularly, the transient receptor potential melastatin 2 (TRPM2), has been proposed to regulate inflammatory responses in myeloid cells (13–16). TRPM2 is a Ca^{2+} permeable, non-selective cation channel, which is activated by ADP-ribose (ADPR), temperature, oxidative stress and Ca^{2+} (17). TRPM2 is highly expressed in dendritic cells, macrophages, and neutrophils (10). Activation of TRPM2 results in transport of Ca^{2+} across the plasma membrane and cytosolic Ca^{2+} release in lysosomes (18). Studies using inflammatory models of TRPM2-genetically deficient mice ($Trpm2^{-/-}$) have revealed the importance of this channel in the immunity of the host (13). A previous study showed that $Trpm2^{-/-}$ mice are susceptible to *L. monocytogenes* infection, presumably through reduced production of IL-12 and IFN-γ, suggesting a defective interplay between innate and adaptive immune responses in the $Trpm2^{-/-}$ mice (19). However, recent work supports the hypothesis that TRPM2 functions as a negative regulator of inflammation. Favoring this premise, $Trpm2^{-/-}$ mice challenged with lipopolysaccharide (LPS) developed greater inflammatory responses, which correlated with a reduced survival rate as compared to WT mice (20). Moreover, $Trpm2^{-/-}$ mice displayed increased vascular damage due to exacerbated migration of neutrophils into the tissue in a model of neutrophil-mediated vascular injury (16). Results from our group also showed that loss of TRPM2 yielded increased production of inflammatory mediators and M1 polarization of macrophages during *H. pylori* infection, which correlated with greater gastric inflammation in chronically *H. pylori*-infected $Trpm2^{-/-}$ mice (14). Yet, the mechanisms by which TRPM2 regulates the development of the inflammatory response during bacterial infections are not fully understood.

Here, we aimed to elucidate the cellular innate inflammatory mechanisms responsible for the increased susceptibility of $Trpm2^{-/-}$ mice to infection with *L. monocytogenes*. We found that lethality in $Trpm2^{-/-}$ mice infected with *L. monocytogenes* is caused by the overwhelming bacterial burden in the liver and spleen, which follows increased neutrophil and monocyte recruitment to the liver. The systemic inflammatory response elicited in the absence of TRPM2 in infected mice culminated in septic shock. Unexpectedly, depletion of neutrophils in $Trpm2^{-/-}$ mice rendered resistance to *L. monocytogenes* infection and a reduced inflammatory cytokine storm in these mice. The highly inflammatory profile of $Trpm2^{-/-}$ neutrophils was further confirmed *in vitro*, linked to augmented Ca^{2+} entry and the enhanced membrane depolarization triggered by bacteria in these cells. Together, our results suggest an essential functional role for TRPM2 channel in the regulation of neutrophils' inflammatory responses that follow bacterial infection.

MATERIALS AND METHODS

Mice Strains

Wild-type (WT) C57BL/6, B6N.129S2-*Ncf1*^{tm1Shl}/J ($Nox2^{-/-}$), and B6.129S4-*Ccr2*^{tm1Ifc}/J ($Ccr2^{-/-}$) were originally purchased from Jackson's Laboratory. $Trpm2^{-/-}$ mice were backcrossed for over 10 generations into the C57BL/6J genetic background (21) and were originally donated by Dr. Y. Mori, University of Kyoto, Japan. All animals were bred and maintained in Nationwide Children's Hospital vivarium.

Bacterial Culture

Listeria monocytogenes 10403S and *Listeria monocytogenes* Xen-32 (constitutively bioluminescent) were cultured in Brain-Hearth infusion broth (Difco) at 37°C for 4–6 h, bacterial concentration was adjusted before each experiment based on absorbance at 600 nm.

L. monocytogenes Infection Model

C57BL/6 (WT) and $Trpm2^{-/-}$ mice were intravenously (*i.v.*) infected with 10^4 colony-forming units (CFU) of *L. monocytogenes*, and the animals were monitored for up to 8 days post-infection (dpi). Some WT or $Trpm2^{-/-}$ mice were *i.v.* injected with 250 μg of anti-Ly6G (1A8) or anti-Gr1 (RB6-8C5) 24 h before infection and 48 h post-infection (hpi). Results were graphed using Kaplan-Meier curves.

Tracking of *L. monocytogenes* Spreading *in vivo*

WT and $Trpm2^{-/-}$ mice were *i.v.* infected with 10^8 CFU of *L. monocytogenes* Xen-32 as previously described (22). Mice were anesthetized with isoflurane 4 h post-infection and bacterial dissemination was tracked using Xenogen IVIS Imaging System (Perkin Elmer Inc.). Photons were measured during 1 min exposure by keeping the animals in the ventral position. Following the procedure, mice were euthanized and livers and spleens were collected. Bacterial burden within the infected organs was also measured by a 30 s exposure on the Xenogen IVIS system. Photon emissions were quantified with Living Image software (Caliper Life Science).

Isolation of Mouse Neutrophils

Mouse bone marrow cells were isolated from femurs and tibiae. Polymorphonuclear cells (PMN) were purified by negative selection (Stemcell Technologies) according to the manufacturer's directions. For inflammatory neutrophils, mice were injected intraperitoneally (*i.p.*) with 1 ml of 4% of thioglycolate, 24 h later peritoneal contents were collected and neutrophils were purified by positive selection using biotinylated anti-Ly-6G (Biolegend) and MACS streptavidin-microbeads (Miltenyi Biotec), following the manufacturer's instructions.

Quantitation of Total Superoxide Species

A total of 10^5 neutrophils were seeded into the wells of 96 well black plates and incubated for 10 min at 37°C. Some cells were pretreated with 10^{-5} M Diphenyleneiodonium chloride (DPI) (Tocris Bioscience), for 10 min at 37°C, followed by the addition of 10^{-4} M of luminol (Sigma Aldrich). Cells were

incubated for 5 min at 37°C, and then, stimulated with 10^{-7} M of phorbol 12-myristate 13-acetate (PMA) (Acros organics) or *L. monocytogenes* with a multiplicity of infection (MOI) of 10. The kinetics of luminescence were measured using the Synergy H1 multi-mode plate reader (Biotek). The area under the curve (AUC) was calculated with GraphPad Prism software V8.0.1 (San Diego, CA).

Bacterial Burden Quantitation

WT and Trpm2$^{-/-}$ mice were *i.v.* infected with 10^4 CFU, animals were euthanized at 18 and 72 h post-infection (hpi), followed by the dissection of liver and spleen, organs were mashed and CFU quantified by serial dilution and plating on LB agar. The CFU were calculated and normalized to the weight of the organs. In some experiments, WT or Trpm2$^{-/-}$ mice were injected *i.v.* with anti-Ly6G antibodies 1 day before bacterial infection and 2 days after bacterial infection for depletion of neutrophils.

Cytokine Quantitation

For *in vivo* quantitation of inflammatory cytokines response, WT and Trpm2$^{-/-}$ mice were *i.v.* infected with 10^4 CFU of *L. monocytogenes*, blood samples were collected 18 and 72 hpi, Cytometric Bead Array (CBA) assay was performed to quantitate TNF-α, IL-6, IL-10, CCL2, IL-12, and IFN-γ according to the manufacturer's directions (BD), the samples were acquired with an LSR II flow cytometer (BD) and analyzed using the FCAP Array software V3.0 (BD).

For the *ex vivo* experiments, bone marrow from WT or Trpm2$^{-/-}$ mice was collected and cultured for 5 days in presence of 10 nM of M-CSF (Biolegend) to induce macrophage (M*Φ*) differentiation. 10^6 M*Φ* were then seeded and stimulated with 100 ng/ml of LPS or with *L. monocytogenes* (MOI of 5) overnight, the supernatants were collected and stored at −80°C. Bone marrow neutrophils were purified by negative selection, as described above, and 10^6 neutrophils were seeded and stimulated with *L. monocytogenes* (MOI of 10) for 4 h, the supernatants were collected and stored at −80°C. Inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6, IL-10) were quantitated in supernatants from cell cultures of M*Φ* and neutrophils by using the LEGENDplex mouse inflammation panel (Biolegend), the assay was performed according to the manufacturer's directions. The samples were acquired with an LSR II flow cytometer and analyzed using the LEGENDplex data analysis software (Biolegend).

Immunofluorescence and Histopathology

WT, Trpm2$^{-/-}$ or mice subjected to neutrophil depletion (anti-Ly6G 1 day before infection and 2 days post-infection) were infected with *L. monocytogenes*, 72 hpi livers and spleens were dissected, fixed, embedded in paraffin or optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Sections were cut (4 μm) and stained with rabbit anti-Listeria (Abcam) 1:100, rat anti-Ly6G (Biolegend) 1:100 or rat anti-Ly6C (Santa Cruz Biotechnology) 1:100 plus chicken anti-rabbit IgG Alexa Fluor 488 (Invitrogen) 1:500 and goat anti-rat IgG Alexa Fluor 594 (Invitrogen) 1:500. The slides were prepared with fluoroshield mounting medium with DAPI (Abcam). For the histopathological analysis, paraffin sections of spleens and livers were stained with Hematoxylin and Eosin (H&E) and analyzed by microscopy. The bacterial abscesses were counted in the median lobe of the livers at 72 hpi, whereas in the spleens the severity of the infection was evaluated by comparing the percentage of necrotic follicles.

Flow Cytometry

WT or Trpm2$^{-/-}$ mice were infected with 10^4 CFU of *L. monocytogenes i.v.*, liver or spleen were collected 18 and 72 hpi, the organs were disrupted and leukocytes were purified using Percoll 33% as previously described (23). Cells were stained with antibodies (dilution 1:100) (Biolegend) against CD45 Brilliant Violet 510, CD11b Brilliant Violet 785, Ly6G Brilliant Violet 605, Ly6C Brilliant Violet 421, F4/80 Brilliant Violet 711 and Live/Dead Blue (Invitrogen). Stained cells were acquired in an LSR II flow cytometer (BD) and analyzed with FlowJo V10.1 (Tree Star). The gating strategy for neutrophils was as follows: singlets>live cells>CD45+ >CD11+, Ly6G^{high} Ly6C^{int}. The gating strategy for inflammatory macrophages was: singlets>live cells>CD45+ >CD11+, Ly6G- Ly6C^{high}, F4/80-. Relative cell counts were assessed by using countBright absolute counting beads (Thermofisher).

Degranulation Assay

WT and Trpm2$^{-/-}$ bone marrow neutrophils were stimulated with 100 nM of PMA for 30 min, then fixed and stained with anti-mouse CD63 APC/Cy7 (Biolegend) 1:50. The cells were acquired by flow cytometry and analyzed with FlowJo software.

Cell Death and NETosis Assay

WT or Trpm2$^{-/-}$ bone marrow neutrophils were stimulated with 1 mM of H_2O_2 or *L. monocytogenes* (MOI of 10), dead cells were identified by staining with 1 μM of SyTOX green (impermeant to live cells). Neutrophils were acquired by flow cytometry and analyzed with FlowJo software. For the analysis of extracellular DNA in a plate, WT, Trpm2$^{-/-}$ or Nox2$^{-/-}$ bone marrow neutrophils were stimulated with 100 nM of PMA. Next, 1 μM of SyTOX green was added and the kinetic of extracellular DNA release was measured by a fluorescence plate reader (488/525 nm). The formation of Neutrophil Extracellular Traps (NETs) was evaluated by immunofluorescence. WT or Trpm2$^{-/-}$ bone marrow neutrophils were seeded in coverslips and stimulated with 100 nM of PMA, 1 mM of H_2O_2 or *L. monocytogenes* (MOI of 10) for 3 h, cells were fixed and stained with rabbit anti-mouse Neutrophil Elastase (Abcam) 1:100, wheat germ agglutinin (WGA) Oregon 488 (Thermofisher) 1:1,000, Hoechst 33342 (Thermofisher) and goat anti-rabbit Alexa Fluor 594 (Abcam) 1:500. The slides were mounted with fluoroshield mounting medium (Abcam) and visualized by confocal microscopy.

Intracellular Antimicrobial Killing Assay

10^6 WT or Trpm2$^{-/-}$ bone marrow neutrophils were infected with *L. monocytogenes* with a multiplicity of infection (MOI) of 10 for 45 min, cells were centrifuged at 335 × g for 5 min and the supernatant was removed. Infected neutrophils were seeded in 24 well plates and incubated for 3 h at 37°C. To quantify

intracellular bacteria, neutrophils were lysed with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO), and free bacteria were quantified by serial dilution on LB agar plates. Relative percent of antimicrobial killing was calculated dividing the inverse CFU obtained from WT neutrophils by CFU from Trpm2$^{-/-}$ neutrophils and multiplied by 100.

Measurement of Cytosolic Ca^{2+} in Neutrophils

For the analysis of intracellular Ca^{2+} mobilization by flow cytometry, bone marrow neutrophils were freshly isolated and stained with 10^{-6} M Fluo-4 AM (Invitrogen, Eugene, OR) for 45 min in the dark at RT, then cells were resuspended in Hank's Balanced Salt Solution (HBSS) buffer and aliquots were prepared with 5×10^5 cells/tube, for some experiments, neutrophils were preincubated with 3×10^{-3} M EGTA. The kinetics of Ca^{2+} were recorded by collecting baseline levels, followed by the addition of 10^{-7} M N-formyl-methionyl-leucyl-phenylalanine (fMLP), (10^{-3}, 5×10^{-3} or 10^{-2} M) H_2O_2, or *L. monocytogenes* (MOI of 10), the accumulation of intracellular free Ca^{2+} was assessed by FACS with an LSR II cytometer (BD, Franklin Lakes, NJ) for up to 300 s. The results were analyzed using FlowJo (Ashland, OR) and GraphPad Prism (San Diego, CA). For quantitative evaluation of Ca^{2+} responses, the areas under the curve (AUC) were calculated for each trace by using GraphPad Prism. For microscopic visualization of cytosolic Ca^{2+}, bone marrow neutrophils were stained with 10^{-6} M Rhod-2 AM (Invitrogen, Eugene OR) for 45 min in the dark at RT, then cells were resuspended in HBSS buffer. Neutrophils were seeded in microscopy slides for 10 min at 37°C, the basal fluorescence was adjusted prior the stimulation with *L. monocytogenes* (MOI of 10), and the kinetics of intracellular Ca^{2+} were recorded up to 4 min by using a Zeiss LSM800 confocal microscope. Images and videos were analyzed using ImageJ (NIH, USA).

Measurement of Membrane Potential

Bone marrow neutrophils were loaded with 10^{-6} M $DiBAC_4(3)$ (Thermofisher), for 30 min at 37°C, then cells were transferred to flow cytometer tubes in aliquots of 10^6 neutrophils. The dye enters to depolarized cells, exhibiting an enhance in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence.

The kinetics of membrane potential were started by the acquisition of basal levels (up to 60 s), followed by the addition of 10^{-7} M PMA or *L. monocytogenes* (MOI of 10), the curves were recorded up to 600 or 300 s, respectively, by using an LSR II cytometer (488, 505 LP 530/30 BP). The analysis was performed using FlowJo and GraphPad software. A quantitative analysis of membrane depolarization was obtained calculating the AUC by using GraphPad Prism software.

qPCR

WT, Trpm2$^{-/-}$ or mice treated with anti-Ly6G, were infected with *L. monocytogenes*, 72 h later, livers were collected, and a small portion was lysed with QIAzol (Qiagen). RNA was purified with columns (Qiagen) and cDNA was prepared with Super Script II reverse transcriptase (Thermofisher). For qPCR, 96 well plates were used with a customized Taqman design (Thermofisher, 4391528). Data were normalized using 18s RNA as a housekeeping gene and the fold changes were made by comparing non-infected with infected samples.

Statistical Analysis

Data analysis were performed by using GraphPad Prism 8 (San Diego CA). Statistical evaluation was performed with ANOVA one way with Dunnett's, Tukey's or Sidak tests for comparison of multiple groups and Welch's *t*-test or multiple *t*-tests for comparing two data sets. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Trpm2$^{-/-}$ Mice Are Susceptible to *L. monocytogenes* Infection

The mouse model of systemic listeriosis has been used as a powerful tool to study innate and adaptive immune responses for decades (24). In the acute inflammatory phase, myeloid cells have a critical role in controlling the bacterial burden of *L. monocytogenes* infection (25, 26). To determine how TRPM2 channel modulates the innate inflammatory response induced against *L. monocytogenes* infection, we infected C57BL/6 (WT), Trpm2$^{-/-}$ or Ccr2$^{-/-}$ mice with a sublethal dose of *L. monocytogenes* (10^4 CFU) and evaluated the susceptibility of Trpm2$^{-/-}$ mice to infection. The Ccr2$^{-/-}$ mice were used as a control, because the strain reported susceptibility to *L. monocytogenes* infection (27). As expected, WT mice were resistant to *L. monocytogenes* infection and 100% of the animals survived for the length of the experiment (8 dpi). In contrast, Ccr2$^{-/-}$ mice were highly susceptible to *L. monocytogenes* infection and did not survive beyond 5 dpi, as reported previously (27). Similar to the Ccr2$^{-/-}$ group, only 30% of the Trpm2$^{-/-}$ mice survived 5 dpi and by 6 dpi 100% of this group had succumbed to the acute infection (**Figure 1A**). The Ccr2$^{-/-}$ and Trpm2$^{-/-}$ mice showed increased bacterial burden at 18 and 72 hpi in liver (**Figure 1B**) and spleen (**Figure 1C**) compared to WT mice. To analyze the time course of *L. monocytogenes* colonization and bacterial spreading in mice, we infected the animals using luminescently labeled *L. monocytogenes* Xen-32 and followed luminescence emission *in vivo* at 6 hpi. Trpm2$^{-/-}$ mice showed greater levels of luminescence than WT mice when visualized in the ventral position (**Supplementary Figure 1A**), the relative luminescence was quantitated as shown (**Supplementary Figure 1B**). In addition, liver and spleen were dissected, and bacterial burden, was visualized in the organs *ex vivo* (**Supplementary Figures 1C–E**). Livers from Trpm2$^{-/-}$ showed significantly higher bacterial burden, as compared to WT mice. Interestingly, spleens from WT mice showed greater concentration of *L. monocytogenes* spleens from Trpm2$^{-/-}$ mice, suggesting bacterial containment in the spleen of the WT mice (**Supplementary Figures 1C,E**), as opposed to extensive bacterial spreading from the spleen to the liver in the Trpm2$^{-/-}$ mice (**Supplementary Figures 1C,E**) at 18 and 72 hpi.

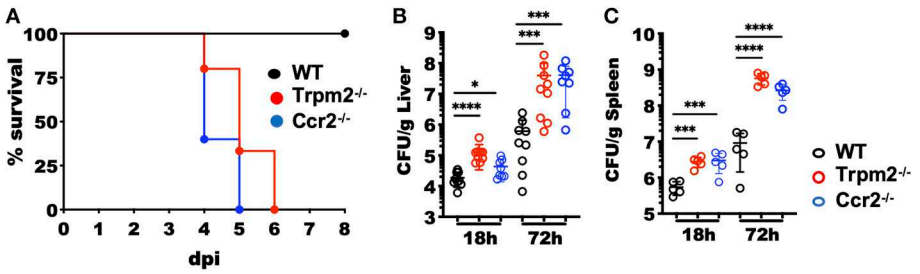

FIGURE 1 | Increased susceptibility of Trpm2$^{-/-}$ mice to *L. monocytogenes* infection. **(A)** Mice were *i.v.* infected with 10^4 CFU of *L. monocytogenes* and survival monitored up to 8 days post-infection (dpi). Survival is represented by Kaplan–Meier survival curves for WT ($n = 11$), Ccr2$^{-/-}$ ($n = 5$) and Trpm2$^{-/-}$ ($n = 15$). Bacterial burden of mice infected with *L. monocytogenes* is shown at 18 and 72 hpi in **(B)** liver (WT and Trpm2$^{-/-}$ $n = 9$, Ccr2$^{-/-}$ $n = 8$) and **(C)** spleen ($n = 5$). Graphs show mean ± SD, statistical analysis was performed using ANOVA one way and Dunnett's multiple comparison test (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

TRPM2$^{-/-}$ Phagocytes Differentially Migrate to the Site of Inflammation

To determine the contribution of phagocytic cells to the susceptibility of Trpm2$^{-/-}$ mice during *L. monocytogenes* infection, we analyzed the migration kinetics of myeloid cells to the site of infection. To this end, we collected the liver of WT and Trpm2$^{-/-}$ infected mice at 18 and 72 hpi. Trpm2$^{-/-}$ mice showed a greater number of neutrophils than WT at 18 hpi; however, the total number of neutrophils in the WT was only slightly larger at 72 hpi, but not significantly different than in Trpm2$^{-/-}$ mice (**Figures 2A,B**). Trpm2$^{-/-}$ mice also showed increased recruitment of inflammatory monocytes at 18 hpi as compared to WT, but WT mice reached a significantly larger number of inflammatory monocytes at 72 hpi (**Figures 2C,D**). Immunostainings of the liver from infected mice at 72 hpi showed an increased number of bacteria in the Trpm2$^{-/-}$ organs, correlating with the larger amounts of recruited neutrophils (**Supplementary Figure 1F**). Similarly, increased recruitment of monocytes in the liver of Trpm2$^{-/-}$ mice was observed (**Supplementary Figure 1G**), suggesting that myeloid cells may be participating in the exacerbated inflammatory responses of TRPM2 deficient mice upon *L. monocytogenes infection.*

Trpm2$^{-/-}$ Mice Infected With *L. monocytogenes* Develop Systemic Inflammation

Because Trpm2$^{-/-}$ mice were highly susceptible to *L. monocytogenes* infection and succumbed as early as 4 dpi, we investigated whether these mice were undergoing septic shock. To achieve this goal, we analyzed the inflammatory cytokine profile of WT and Trpm2$^{-/-}$ mice infected with *L. monocytogenes* at 18 and 72 hpi. Both, WT and Trpm2$^{-/-}$ mice had no significant differences in blood levels of TNF-α at 18 hpi. However, blood levels of TNF-α were 5-fold increased in Trpm2$^{-/-}$ mice, as compared to WT mice, at 72 hpi (**Figure 2E**). Trpm2$^{-/-}$ mice also showed increased levels of IL-6 as early as 18 hpi (**Figure 2F**) and IL-6, IL-10 and CCL2 at 72hpi (**Figures 2G,H**), compared to WT mice. In contrast to a previous report (19), we did not find differences between WT and Trpm2$^{-/-}$ mice in the blood levels of IFN-γ or IL-12 (**Figures 2I,J**). Augmented levels of IL-6 and TNF-α in Trpm2$^{-/-}$ mice suggest a systemic inflammation in these animals, which likely results in a lethal septic shock during the course of *L. monocytogenes* infection.

TRPM2 Ion Channel Regulates the Inflammatory Response in Neutrophils

To best define the specific contribution of phagocytes to the inflammatory response induced by *L. monocytogenes* in the Trpm2$^{-/-}$ mice, we either depleted both neutrophils and monocytes or neutrophils only by treating the mice with antibodies anti-Gr-1 (RB6-8C5) or anti-Ly6G (1A8), respectively. Next, we infected the mice with *L. monocytogenes* and evaluated the susceptibility of depleted mice to the infection. To confirm the efficacy of cell depletion in the mice, we performed flow cytometry evaluation of cells from the liver of infected mice after cell depletion (**Figures 3A,B**). The treatment with anti-Ly6G effectively depleted the population of neutrophils in WT and Trpm2$^{-/-}$ mice.

Depletion of neutrophils (anti-Ly6G), slightly increased susceptibility of WT mice to the bacterial infection (**Figure 3C**), a similar response to *L. monocytogenes* infection was previously described in neutrophil depleted mice (27). However, depletion of monocytes/neutrophils (anti-Gr1) rendered WT mice considerably more susceptible to *L. monocytogenes* infection (**Figure 3C**). Interestingly, neutrophils depletion in the Trpm2$^{-/-}$ mice, increased their resistance to *L. monocytogenes* infection (**Figure 3D**), suggesting a predominant role for TRPM2$^{-/-}$ neutrophils in orchestrating the inflammatory response elicited upon *L. monocytogenes* infection. The depletion of neutrophils/monocytes in Trpm2$^{-/-}$ did not significantly change the susceptibility of the mice compared to non-depleted Trpm2$^{-/-}$ mice, and those animals did not survive beyond day 5 after infection.

Examination of bacterial burden in livers from neutrophil depleted WT mice showed an increase in bacterial CFU at 18 hpi which reached the bacterial burden observed in Trpm2$^{-/-}$ mice without any depletion. Neutrophils depletion in the Trpm2$^{-/-}$ mice resulted in further increased bacterial burden in the liver

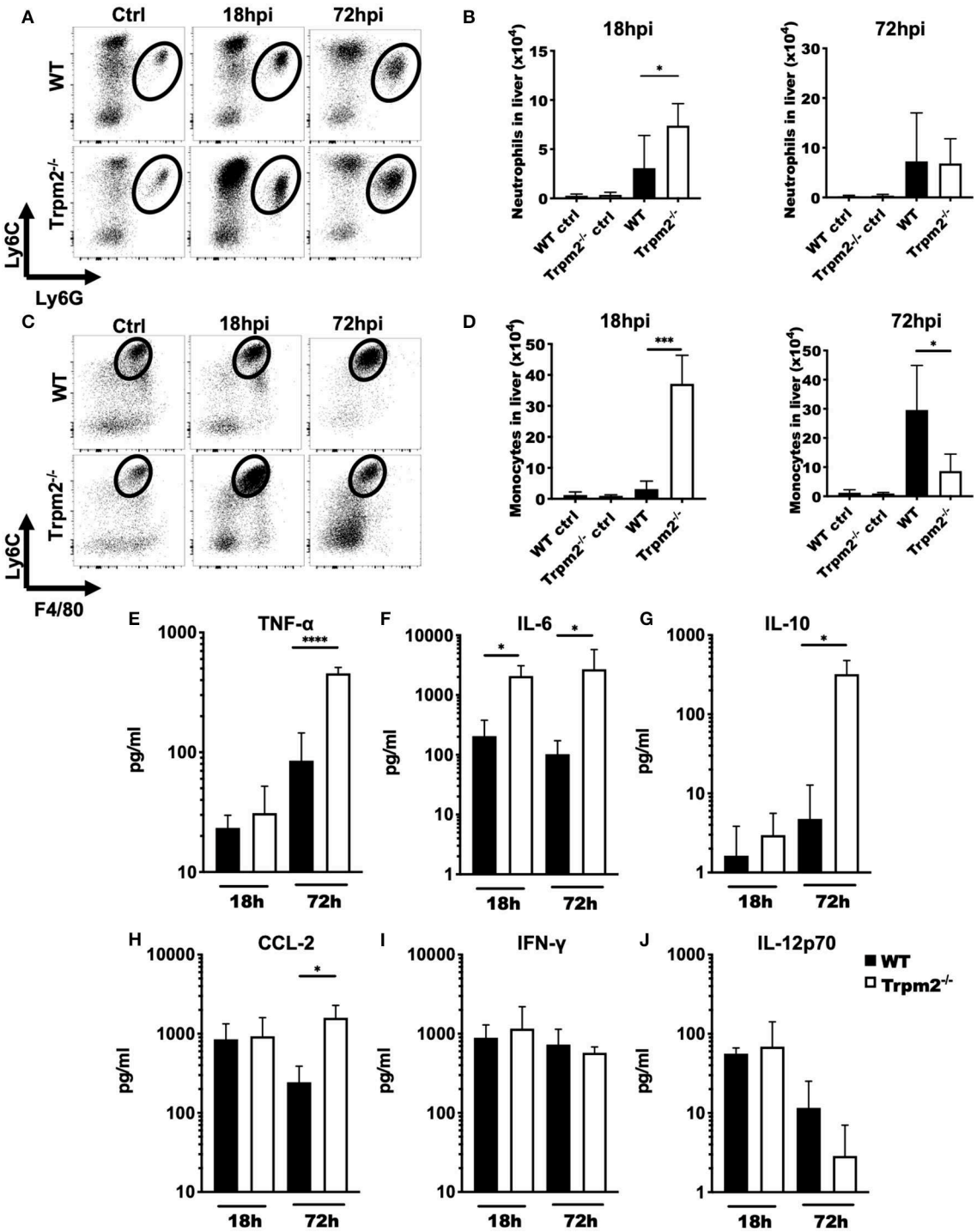

FIGURE 2 | Trpm2$^{-/-}$ inflammatory phagocytes migrate to infected organs and contribute to the development of systemic inflammation. WT and Trpm2$^{-/-}$ mice were infected with *L. monocytogenes*. Livers were collected and disrupted, hepatocytes and erythrocytes were removed, and the remaining cells were immunostained. **(A)** Dot plots show the distribution of neutrophils (CD45^{+}/CD11b^{high}/Ly6G^{high}/Ly6C^{int}) in liver at 18 and 72 hpi in WT and Trpm2$^{-/-}$ mice, **(B)** graphs show the total number of neutrophils in liver at 18 or 72 hpi ($n = 5$). **(C)** Distribution of monocytes in the liver at 18 and 72 hpi, **(D)** and the total number of monocytes in the liver ($n = 5$). Graphs show mean ± SD, the statistical analysis was performed using Welch's *t*-test (*$p < 0.05$, ***$p < 0.001$). **(E–J)** WT and Trpm2$^{-/-}$ mice were infected with *L. monocytogenes* and blood was collected at 18 or 72 hpi, then serum was separated and TNF-α, IL-6, IL-10, CCL-2, IFN-γ, or IL-12p70 were quantified by flow cytometry ($n = 5$). Graphs show mean ± SD, the statistical analysis was performed using Welch's *t*-test (*$p < 0.05$, ****$p < 0.001$).

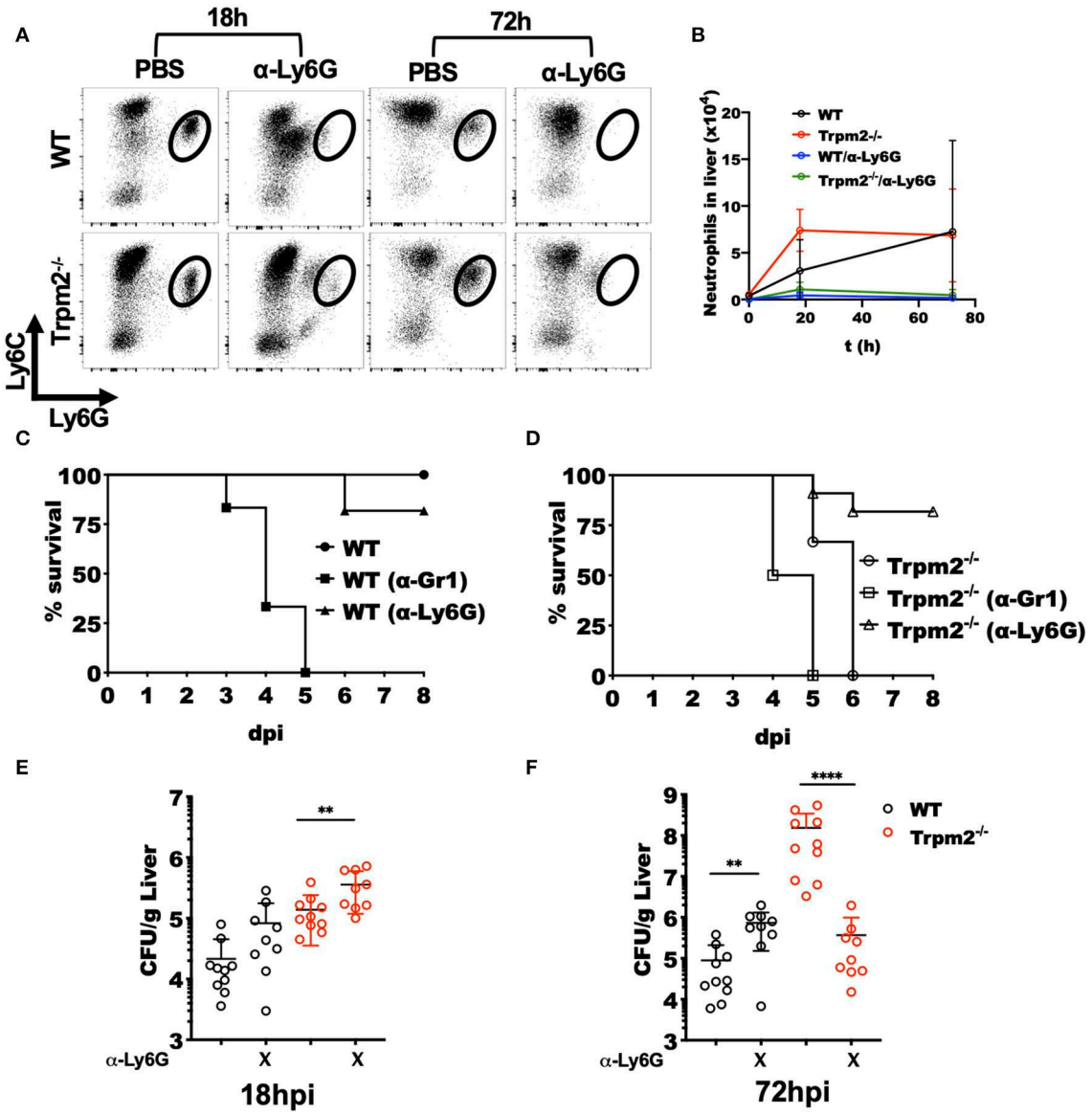

FIGURE 3 | Depletion of Trpm2$^{-/-}$ neutrophils promotes resistance to *L. monocytogenes* infection. Neutrophils (anti-Ly6G) were depleted in WT or Trpm2$^{-/-}$ mice and infected with *L. monocytogenes*, the liver was collected at 18 or 72 hpi, and flow cytometry was performed to analyze inflammatory neutrophils. **(A)** The dot plots show the distribution of neutrophils in infected mice. **(B)** The graph shows kinetics of neutrophils total counts in liver for the groups of mice described in **(A)** ($n = 5$), mean ± SD. Survival analysis was performed on **(C)** WT or **(D)** Trpm2$^{-/-}$ mice with neutrophils depletion (anti-Ly6G) or with neutrophils/monocytes depletion (anti-Gr1) up to day 8. WT ($n = 6$), Trpm2$^{-/-}$ ($n = 6$), anti-Gr1 ($n = 6$ for WT and Trpm2$^{-/-}$) and anti-Ly6G ($n = 11$ for WT and Trpm2$^{-/-}$). The bacterial burden was quantified in liver at **(E)** 18 hpi or **(F)** 72 hpi ($n = 9$). Bar graphs show mean ± SD. The statistical analysis was performed using Welch's *t*-test (**$p < 0.01$, ****$p < 0.0001$).

compared to neutrophil depleted WT (**Figure 3E**). At 72 hpi, WT mice depleted for neutrophils had a slightly greater bacterial burden than the non-depleted WT mice. Interestingly, infected Trpm2$^{-/-}$ mice treated with anti-Ly6G had a reduced bacterial burden at 72 hpi (**Figure 3F**), suggesting that the expression of TRPM2 ion channel in neutrophils impedes bacterial dissemination during the infection with *L. monocytogenes*.

We next analyzed the immunopathology of spleens and livers from infected mice at 24 or 72 hpi by H&E staining. Low magnification images of spleens from infected Trpm2$^{-/-}$ mice at 72 hpi, exhibited large areas of necrosis compared to WT mice (**Figure 4A**). Depletion of neutrophils in WT mice increased the areas of necrosis in the spleen. Interestingly, spleens from neutrophil-depleted Trpm2$^{-/-}$ mice showed less necrotic areas in the spleen. Similar results were observed in the liver, where, Trpm2$^{-/-}$ mice showed numerous abscesses (**Figure 4B**), however, the absence of neutrophils in Trpm2$^{-/-}$ mice reduced the number of abscesses in the liver.

The microscopic images of infected spleens had minimal pathological changes at 18 hpi in both, WT and Trpm2$^{-/-}$ mice (data not shown), however, Trpm2$^{-/-}$ mice had large areas of caseous necrosis in the germinal center zones at 72

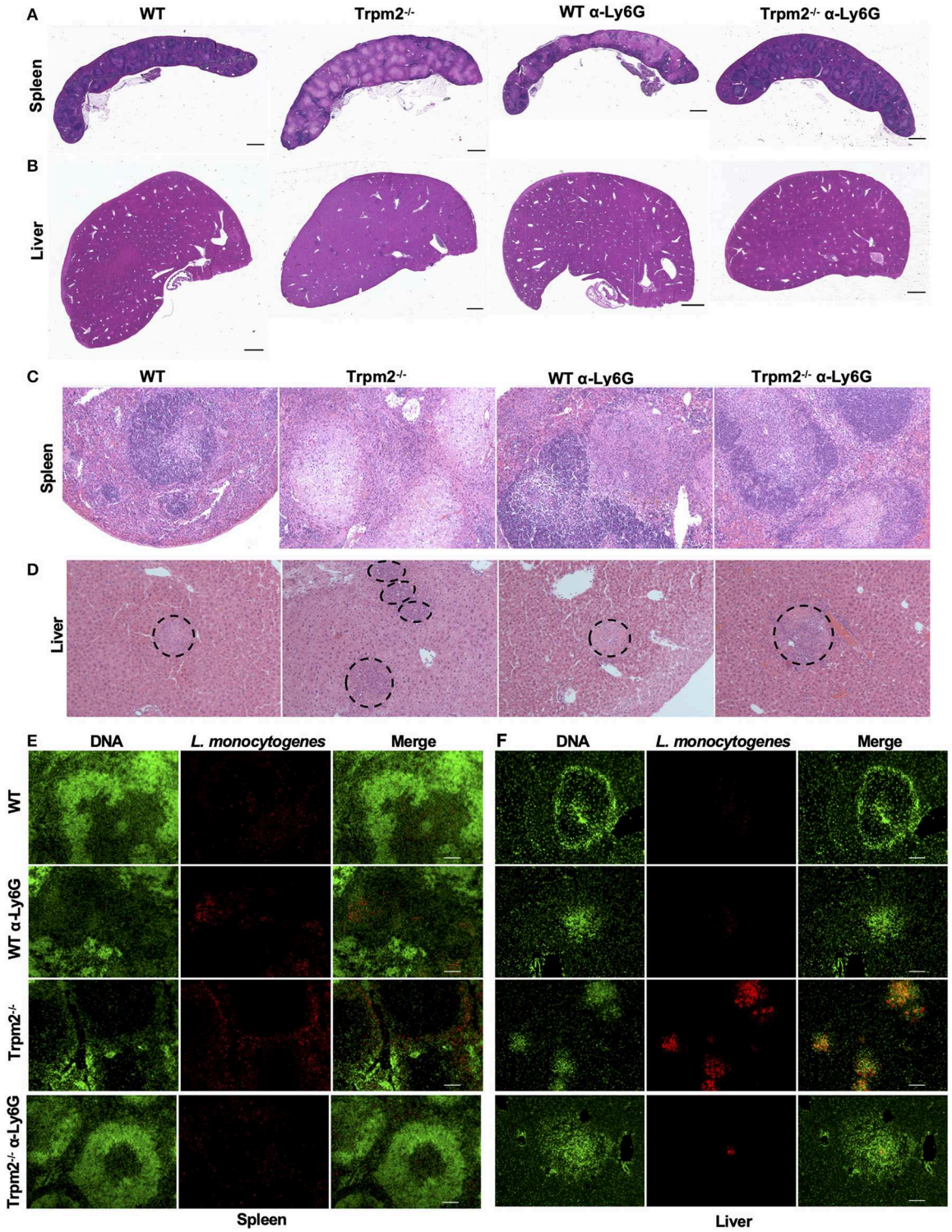

FIGURE 4 | Depletion of neutrophils in Trpm2$^{-/-}$ mice infected with *L. monocytogenes* reduces the bacterial burden and tissue damage. Neutrophils (anti-Ly6G) were depleted in WT or Trpm2$^{-/-}$ mice, spleens and livers were dissected at 72 hpi, embedded in paraffin and stained with H&E. **(A)** Spleen and **(B)** liver of infected mice. Trpm2$^{-/-}$ mice exhibited large areas of necrosis in the spleen and numerous abscesses in the liver compared to WT, depletion of neutrophils increased the areas of necrosis in spleen of WT mice while neutrophil depletion reduced the damage in Trpm2$^{-/-}$ mice (scale bar indicates 1 mm). **(C)** The spleen of infected mice at 10X magnification, Trpm2$^{-/-}$ mice showed a large area of necrosis in the B cell zone than other groups. **(D)** The livers of infected mice, dotted circles show the area of abscesses, depletion of neutrophils in Trpm2$^{-/-}$ mice reduced the number of the abscesses. **(E)** Spleens or **(F)** livers were stained with DAPI (green) and anti-*L. monocytogenes* antibody (red) and visualized at 10X of magnification, Trpm2$^{-/-}$ showed more positive staining areas for *L. monocytogenes* bacteria.

hpi (**Figure 4C**), such tissue damage was significantly smaller in infected WT mice and neutrophil-depleted Trpm2$^{-/-}$ mice. Consistently, infected Trpm2$^{-/-}$ mice displayed a greater percentage of spleen follicles with necrosis than WT mice. Interestingly, the depletion of neutrophils reduced the number of necrotic areas in the Trpm2$^{-/-}$ mice, reaching similar levels as WT mice (**Supplementary Figure 2A**). Bacterial accumulation in the liver was observed in the abscess-like structures in the Trpm2$^{-/-}$ mice (**Figure 4D** dotted circles). At 72 hpi Trpm2$^{-/-}$ mice showed a large number of abscesses in the liver. In contrast, the livers from Trpm2$^{-/-}$ mice that were depleted of neutrophils showed a significantly reduced number of abscesses compared to the Trpm2$^{-/-}$ mice counterpart without neutrophils depletion (**Figure 4D** and **Supplementary Figure 2B**), suggesting that Trpm2$^{-/-}$ neutrophils may facilitate bacterial dissemination and subsequent tissue damage in these animals.

To correlate bacterial tissue invasion with the observed tissue pathology, we used immunofluorescence to localize *L. monocytogenes* in the infected organs. The immunostaining revealed large spots of bacterial accumulation (red) in the spleens of Trpm2$^{-/-}$ mice at 72 hpi, and the bacterial distribution was mainly localized in the white pulp (**Figure 4E**). Whereas, WT or neutrophil depleted WT mice showed reduced areas of bacterial accumulation, as compared to Trpm2$^{-/-}$ mice, depletion of neutrophils in Trpm2$^{-/-}$ mice resulted in even further reduction in bacterial dissemination in the spleen during the acute infection. Similar results were observed in livers of mice infected with *L. monocytogenes*, where Trpm2$^{-/-}$ mice had a larger number of abscesses compared to WT mice (**Figure 4F**). Depletion of neutrophils in WT mice did not increase the dissemination of *L. monocytogenes* in the liver. However, depletion of neutrophils in Trpm2$^{-/-}$ mice, drastically reduced the areas of infection in the liver, suggesting that deficiency of TRPM2 ion channel in neutrophils promotes bacterial dissemination in spleen and liver of mice infected with *L monocytogenes*.

Depletion of Neutrophils Prevents Systemic Inflammation in Infected Trpm2$^{-/-}$ Mice

Because depletion of neutrophils in the Trpm2$^{-/-}$ mice resulted in increased survival to *L. monocytogenes* infection, we sought to analyze the inflammatory microenvironment in the liver of these mice. We collected liver tissue from each group at 72 hpi and performed gene expression array analysis of selected inflammatory mediators. We found that Trpm2$^{-/-}$ mice expressed increased levels of IL-23-α, Camp (Cathelicidin), IL-10, Csf3, and Ccl3, as compared to the other groups. Moreover, WT mice showed greater levels of Cxcl9, Elane (NE), IFN-γ, and Cxcl10, as compared to Trpm2$^{-/-}$ mice (**Figure 5A**). Depletion of neutrophils in WT or Trpm2$^{-/-}$ mice also modified the inflammatory liver microenvironment following infection. WT mice treated with anti-Ly6G showed increased NE, Cathelicidin, and MPO, but reduced Cxcl9 and IFN-γ. In addition, depletion of neutrophils in Trpm2$^{-/-}$ mice increased the expression levels of Cxcl9, IFN-γ, and Cxcl10 but reduced the expression of IL-10 and IL-23-α compared to non-neutrophil depleted Trpm2$^{-/-}$ mice (**Figure 5A**).

Due to infection lethality in some of our experimental groups, we hypothesized that those mice were undergoing septic shock. To test this, we analyzed the levels of various cytokines known to be involved in septic shock syndrome, including: TNF-α, IL-6, IL-10, CCL-2, IFN-γ, and IL-12 in the blood of mice infected with *L. monocytogenes*. Blood collected at 72 hpi from neutrophil depleted Trpm2$^{-/-}$ mice contained reduced levels of TNF-α, as compared with non-neutrophil-depleted Trpm2$^{-/-}$ mice (**Figure 5B**). The blood levels of TNF-α did not change significantly in neutrophil depleted WT mice, when compared to non-depleted WT group. Depletion of neutrophils in the WT mice initially yielded decreased levels in IL-6 at 18 hpi, but the levels of IL-6 were slightly higher in this group at 72 h (**Figure 5C**). Contrasting the results in the WT group, neutrophil depletion in Trpm2$^{-/-}$ mice resulted in lower levels of IL-6 at 18 and 72 hpi (**Figure 5C**). Neutrophils depletion prevented the significant increase in IL-10 (**Figure 5D**) and CCL-2 (**Figure 5E**) blood levels seen with the Trpm2$^{-/-}$ mice at 72 hpi. Interestingly, depletion of neutrophils in WT or Trpm2$^{-/-}$ mice reduced slightly, but not significantly, the levels of IFN-γ at 18 and 72 hpi (**Figure 5F**). Similarly, the blood levels of IL-12 were only marginally reduced in both groups, neutrophil-depleted WT and neutrophil-depleted Trpm2$^{-/-}$ mice at 18 hpi (**Figure 5G**), but no difference was observed at 72 hpi, as compared to non-depleted groups. These data suggest that the presence of neutrophils may be less impactful on the production of regulatory cytokines IFN-γ and IL-12, but TRPM2 function in neutrophils is critical to determine the course of inflammation during *L. monocytogenes* infection.

TRPM2 Function Regulates Antimicrobial Responses in Neutrophils

Our *in vivo* studies strongly suggest that neutrophils regulate the systemic inflammatory response, and therefore, determine the fate of the animals upon *L. monocytogenes* infection. We then questioned how the TRPM2 ion channel could be involved in the regulation of inflammation and whether such influence was due to alterations in the antimicrobial mechanisms of neutrophils. To address these questions, we purified peritoneal neutrophils and measured the neutrophils' oxidative response. We found that Trpm2$^{-/-}$ neutrophils produced more oxidative products when cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (**Figure 6A**) or *L. monocytogenes* compared to WT neutrophils (**Figure 6B**). The analysis of the area under the curve (AUC) of the ROS kinetics showed statistical significance between WT and Trpm2$^{-/-}$ neutrophils (**Figure 6C**).

Next, we analyzed the mobilization of primary granules by measuring the expression of the primary granules marker CD63 (LAMP-3) in the cell membrane, and found that Trpm2$^{-/-}$ neutrophils released more primary granules than WT after the cells were stimulated with PMA (**Figures 6D,E**), suggesting that the lack of TRPM2 induces changes in cytoskeleton and

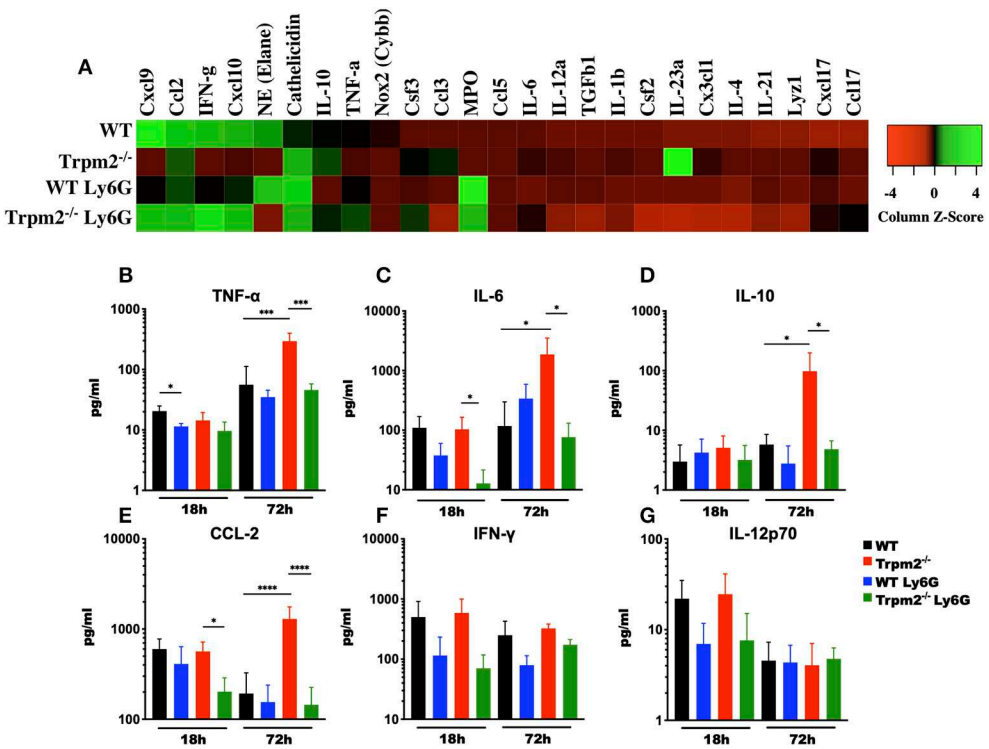

FIGURE 5 | Depletion of neutrophils prevents the systemic inflammation in Trpm2$^{-/-}$ mice infected with *L. monocytogenes*. **(A)** Gene expression analysis was performed on RNA collected from livers of WT ($n = 3$) and Trpm2$^{-/-}$ ($n = 3$) mice, with or without depletion of neutrophils (WT $n = 3$, Trpm2$^{-/-}$ $n = 2$), at 72 hpi. **(B–G)** Serum from infected mice was collected at 18 and 72 hpi, TNF-α, IL-6, IL-10, CCL-2, IFN-γ, or IL-12p70 were quantified by flow cytometry ($n = 5$). Bar graphs show mean ± SD. The statistical analysis was performed using ANOVA one way and Tukey's multiple comparison test (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$).

increased mobilization of intracellular vesicles in neutrophils. Due to the extensive tissue damage observed in Trpm2$^{-/-}$ mice infected with *L. monocytogenes*, we sought to analyze the capabilities of Trpm2$^{-/-}$ neutrophils to kill the bacteria. Surprisingly, we found that Trpm2$^{-/-}$ neutrophils were more efficient at killing *L. monocytogenes* compared to WT neutrophils (**Figure 6F**).

TRPM2 Channel Deficiency Results in Hyper Inflammatory Cytokine Response of Phagocytes

To analyze whether the phagocytes were involved in the production of the inflammatory cytokines detected in mice infected with *L. monocytogenes*, bone marrow derived macrophages (MΦ) and neutrophils isolated from the bone marrow, were stimulated *in vitro* with LPS or with *L. monocytogenes,* and then the levels of TNF-α, IL-6, IL-1β, IL-1α, and IL-10 cytokines determined. Trpm2$^{-/-}$ and WT MΦ showed similar levels of TNF-α under *L. monocytogenes* infection or LPS stimulation (**Figure 7A**). WT and Trpm2$^{-/-}$ MΦ showed almost undetectable levels of IL-6 when cells were infected with *L. monocytogenes*, but Trpm2$^{-/-}$ MΦ showed statistically significant greater levels of IL-6 under LPS stimulation, used as control (**Figure 7B**). In addition, *L. monocytogenes* induced significantly higher levels of IL-1β (**Figure 7C**) and IL-1α (**Figure 7D**) in Trpm2$^{-/-}$ MΦ. Interestingly, the regulatory cytokine IL-10 was reduced in Trpm2$^{-/-}$ MΦ infected with *L. monocytogenes* (**Figure 7E**), suggesting that Trpm2$^{-/-}$ MΦ are more pro-inflammatory than WT MΦ. Furthermore, WT and Trpm2$^{-/-}$ neutrophils showed similar levels of TNF-α (**Figure 7F**) and IL-6 (**Figure 7G**) under infection with *L. monocytogenes*. However, the cytokines derived from the inflammasome, IL-1β (**Figure 7H**) and IL-1α (**Figure 7I**) were elevated in Trpm2$^{-/-}$ neutrophils. No differences were observed in the production of IL-10 (**Figure 7J**). Our results suggest a functional role for the TRPM2 cation channel in regulating the production of inflammatory cytokines in phagocytes. Thus, the expression of TRPM2 channel may prevent excessive local and systemic inflammatory responses mediated by immune phagocytes.

TRPM2 Regulates Cell Death Pathways in Neutrophils

The excesses of ROS products in Trpm2$^{-/-}$ neutrophils suggested a potential reduction in the survival rate, and consequently, accelerated cell death of these cells. To evaluate the effect of increased ROS production in cell death activation pathways of Trpm2$^{-/-}$ neutrophils, we determined the production of Neutrophils Extracellular Traps (NETs), which is linked to NETosis, a unique cell death pathway in neutrophils (28). NETosis in turn is associated to increased

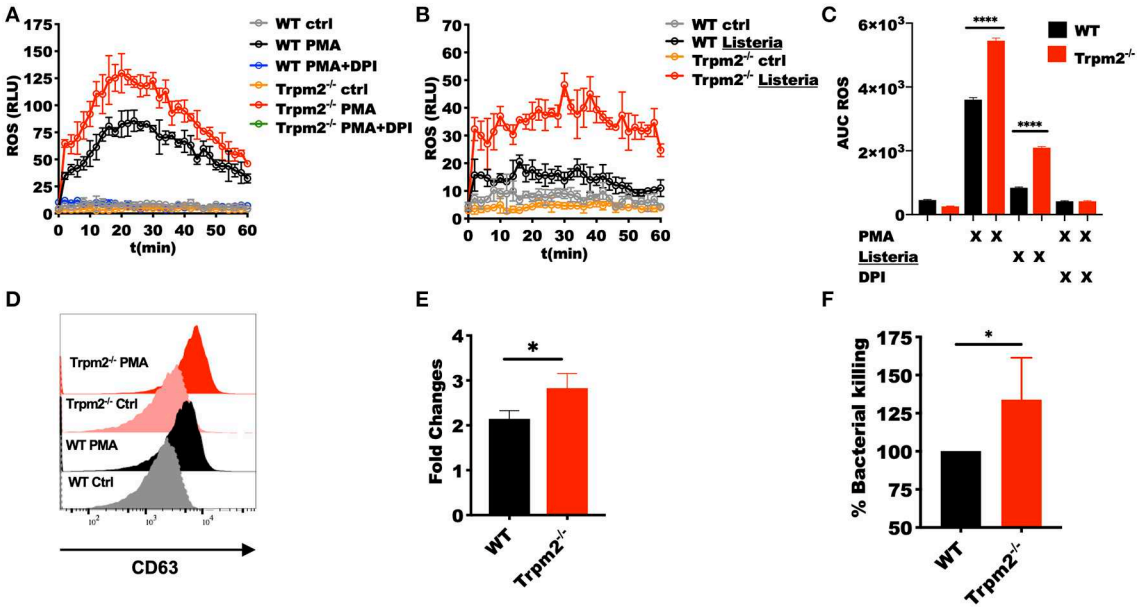

FIGURE 6 | Trpm2$^{-/-}$ phagocytes are hyperresponsive to *L. monocytogenes* infection. WT and Trpm2$^{-/-}$ peritoneal neutrophils were stimulated with **(A)** 100 nM PMA or **(B)** *L. monocytogenes* at MOI of 10, some cells were pretreated with 10 μM of DPI. Oxidative burst was measured by adding 100 μM of luminol, chemiluminescence was measured on a plate reader ($n = 3$, mean ± SD). **(C)** The bar graphs show the area under the curve (AUC) of the ROS curves ($n = 3$, mean ± SD, ****$p < 0.0001$). **(D)** Bone marrow neutrophils were stimulated with 100 nM of PMA and primary granules release was analyzed by measuring the expression of CD63 on the cell surface 30 min after stimulation. **(E)** Shows the fold changes of WT and Trpm2$^{-/-}$ granules release ($n = 3$, mean ± SD, *$p < 0.05$). **(F)** The intracellular antimicrobial killing was measured in WT and Trpm2$^{-/-}$ neutrophils infected with *L. monocytogenes* (3 h), the graph shows the relative % killing of Trpm2$^{-/-}$ neutrophils compared to WT ($n = 8$, mean ± SD, *$p < 0.05$). The statistical analysis was performed using Welch's *t*-test (*$p < 0.05$).

chronic inflammation (29, 30). To evaluate NET formation, we stimulated WT and Trpm2$^{-/-}$ neutrophils with H_2O_2, a recognized activator of TRPM2, then detected extracellular DNA and neutrophil elastase by immunofluorescence. We observed fewer Trpm2$^{-/-}$ neutrophils than WT producing NET fibers. Next, we stimulated the cells with PMA, or *L. monocytogenes* and found that Trpm2$^{-/-}$ neutrophils were similarly capable of producing NET structures as compared to WT neutrophils (**Figure 8A**). In order to have a quantitative measurement of NETs, we analyzed the production of extracellular DNA by stimulating WT, Trpm2$^{-/-}$ or Nox2$^{-/-}$ neutrophils with PMA, dead cells and extracellular DNA were stained with SyTOX green (non-cell-permeable dye) and read by a fluorescence plate reader. We found that Trpm2$^{-/-}$ neutrophils released more extracellular DNA than WT (**Figure 8B**). Because neutrophil NADPH oxidase can stimulate NETosis (31), neutrophils from Nox2$^{-/-}$ mice were used as a negative control (32). As expected, NADPH deficient neutrophils did not produce NETs when stimulated with PMA (**Figure 8B**). Next, we evaluated the kinetics of cell death in cellular suspension by flow cytometry and confirmed that Trpm2$^{-/-}$ neutrophils had increased cell death rate compared to WT, which peaked as early as 2 or 3 h when the cells were stimulated with either H_2O_2 (**Figure 8C**) or with *L. monocytogenes* (**Figure 8D**), respectively. Altogether, these findings suggest that TRPM2 channel functions as a negative regulator of antimicrobial inflammatory responses and NADPH-dependent cell death pathways in neutrophils.

TRPM2 Regulates Ca^{2+} Signaling and Membrane Potential in Neutrophils

The main known function of the TRPM2 ion channel is the modulation of Ca^{2+} entry, and consequently, it is expected that TRPM2 deficiency will impact major Ca^{2+} dependent cellular functions, including cell migration (33), oxidative stress (34, 35) and NADPH oxidase mediated cell death pathways (34). Our results indicate that TRPM2-deficient neutrophils exhibited increased inflammatory responses, which are mostly dependent on Ca^{2+} signaling (8). Therefore, we sought to determine the contribution of TRPM2 to Ca^{2+} entry triggered by PAMPs recognition in neutrophils. First, we stained neutrophils with Rhod-2 AM and analyzed the kinetic of intracellular Ca^{2+} mobilization by confocal microscopy. We observed that both, WT and Trpm2$^{-/-}$ neutrophils were able to rapidly increase the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]i$) when the cells were stimulated with *L. monocytogenes* (**Figure 9A**). To best resolve the kinetics of Ca^{2+} mobilization during the time course of infection, we next stained the neutrophils with Fluo-4 AM and continuously recorded the events for 5 min by flow cytometry. Unexpectedly, Trpm2$^{-/-}$ neutrophils stimulated with fMLP, responded with greater levels of $[Ca^{2+}]i$ than WT neutrophils (**Figure 9B**). Similar results were obtained when neutrophils were stimulated with *L. monocytogenes* (**Figure 9C**). When Ca^{2+} was depleted from the media by the addition of EGTA, Trpm2$^{-/-}$ neutrophils showed slightly higher intracellular Ca^{2+} release than WT neutrophils under stimulation with

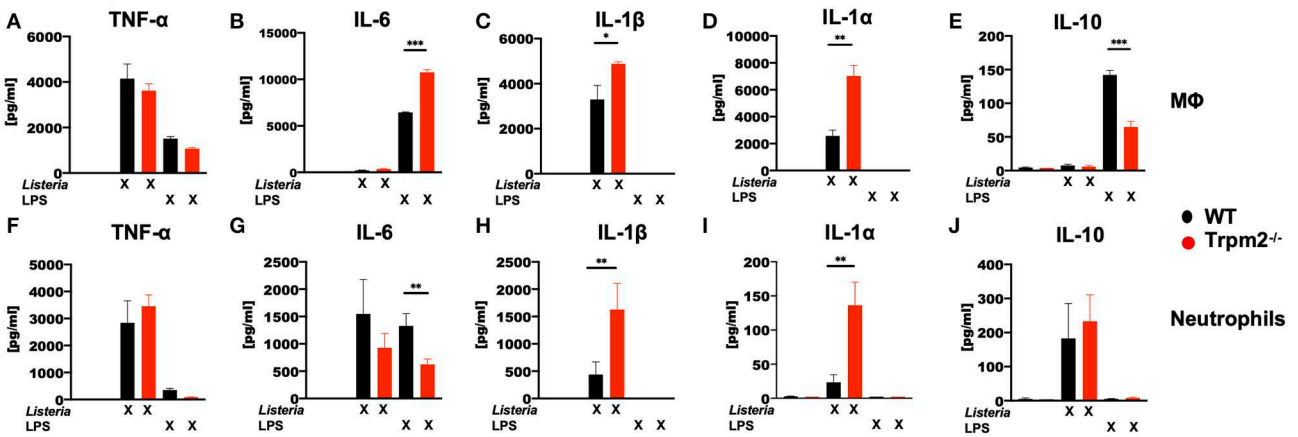

FIGURE 7 | *L. monocytogenes* induces a hyperinflammatory cytokine profile in Trpm2$^{-/-}$ phagocytes. WT or Trpm2$^{-/-}$ bone marrow-derived macrophages (MΦ) were stimulated with 100 ng/ml of LPS or infected with *L. monocytogenes* (MOI of 5) overnight, the supernatants were collected and **(A)** TNF-α, **(B)** IL-6, **(C)** IL-1β, **(D)** IL-1α, and **(E)** IL-10 were quantified by flow cytometry ($n = 3$). WT or Trpm2$^{-/-}$ bone marrow neutrophils were stimulated with 100 ng/ml of LPS or infected with *L. monocytogenes* (MOI of 10) for 4 h, the supernatants were collected and **(F)** TNF-α, **(G)** IL-6, **(H)** IL-1β, **(I)** IL-1α, and **(J)** IL-10 were quantified by flow cytometry ($n = 4$). Bar graphs represent the mean ± SD, the statistical analysis was performed by using Welch's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

fMLP (**Figure 9D**) or *L. monocytogenes* (**Figure 9E**). In neutrophils stimulated with H_2O_2 the concentration of 1mM, was insufficient to induce Ca^{2+} entry in neutrophils (**Supplementary Figure 3A**). However, Trpm2$^{-/-}$ neutrophils showed a reduction of [Ca^{2+}]i when the cells were stimulated with 5 mM of H_2O_2 (**Supplementary Figure 3B**) or with 10 mM of H_2O_2 (**Supplementary Figure 3C**), as compared to WT. The calculation of the area under the curve (AUC) showed statistical significance between WT and Trpm2$^{-/-}$ neutrophils stimulated with fMLP (**Supplementary Figure 3D**), *L. monocytogenes* (**Supplementary Figure 3E**) or H_2O_2 (**Supplementary Figure 3F**). These findings suggest that the absence of TRPM2 ion channel in neutrophils modifies the regulation of PAMPs-dependent mobilization of Ca^{2+}, possibly due to the engagement of alternative Ca^{2+} entry channels as a compensatory mechanism to maintain the Ca^{2+} homeostasis in neutrophils.

Because changes in the cellular membrane potential is a key physiological response in immune cells, which is tightly linked to in and out ion mobilization, and during the induction of oxidative stress (36). We evaluated the impact of TRPM2 function on the relative membrane potential of neutrophils directly in response to *L. monocytogenes* infection *in vitro*. To achieve this goal, we loaded the cells with the slow-response potential-sensitive probe $DiBAC_4(3)$, which responds to increases in cell depolarization by an increment in fluorescence (36). We recorded the fluorescence kinetics by flow cytometry and found that when stimulated with PMA, WT neutrophils had increased membrane depolarization, as compared to Trpm2$^{-/-}$ neutrophils (**Figure 9F**). The analysis of the AUC indicated statistical significant difference between WT and Trpm2$^{-/-}$ neutrophils stimulated with PMA (**Supplementary Figure 3G**). Trpm2$^{-/-}$ neutrophils however, showed significant increases in membrane depolarization relative to WT cells, when stimulated with *L. monocytogenes* (**Figure 9G** and **Supplementary Figure 3H**). In contrast, Nox2$^{-/-}$ neutrophils, which lack NADPH oxidase function, did not considerably increase membrane depolarization (**Figure 9G**). These results suggest that *L. monocytogenes* induces extensive membrane depolarization of neutrophils, and that TRPM2 regulates membrane depolarization in neutrophils, partially dependent on the NADPH oxidase complex induced by *L. monocytogenes*. To further analyze the impact of Ca^{2+} mobilization on membrane potential, we pretreated WT or Trpm2$^{-/-}$ neutrophils with 2-APB, a wide spectrum inhibitor of TRP channels, and then stimulated the cells with *L. monocytogenes* (**Figure 9H**). Inhibition of generic TRP channels prevented membrane depolarization of both, WT and Trpm2$^{-/-}$ neutrophils (**Supplementary Figure 3I**).

Moreover, pretreatment with Xestospongin C, an inhibitor of the IP_3-dependent Ca^{2+} release pathway, slightly reduced membrane depolarization in WT neutrophils activated with *L. monocytogenes* (**Figure 8I**). Interestingly, Trpm2$^{-/-}$ neutrophils treated with Xestospongin C reduced membrane depolarization levels similar to that of WT neutrophils, suggesting that in the absence of TRPM2 channel activity over-activation of the IP_3-dependent Ca^{2+} release pathway, and subsequent store-operated Ca^{2+} entry may occur. Overall, our data suggest that the absence of TRPM2 channels in neutrophils causes an increase in membrane depolarization and Ca^{2+} overload, which favors the cascade of hyperinflammatory signals observed in the Trpm2$^{-/-}$ neutrophils.

DISCUSSION

The activation of TRPM2 cation channel has emerged as an important cell-mechanism that regulates inflammation in phagocytes (13, 14, 16, 21, 37, 38). Because TRPM2 has

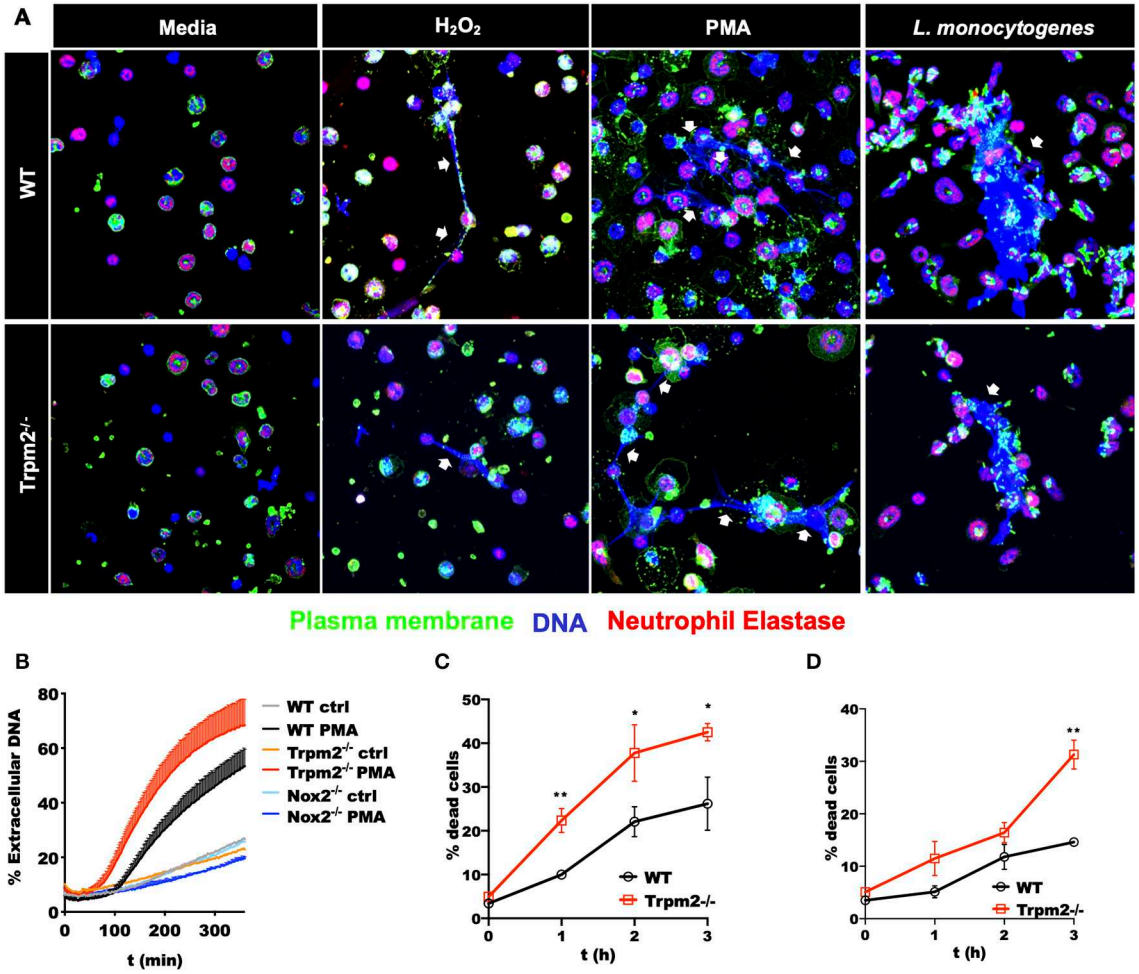

FIGURE 8 | Stimulated Trpm2$^{-/-}$ neutrophils undergo accelerated cell death. **(A)** The formation of NETs was induced by stimulating WT or Trpm2$^{-/-}$ neutrophils with H_2O_2, PMA or *L. monocytogenes* for 3 h. The NETs structures (white arrows) were visualized by co-localized staining of Neutrophil Elastase and DNA (Hoechst 33342). The plasma membrane was stained using the lectin wheat germ agglutinin (WGA). Both WT and Trpm2$^{-/-}$ neutrophils produced NETs under the stimulation with H_2O_2, PMA or *L. monocytogenes*. **(B)** WT, Trpm2$^{-/-}$ or Nox2$^{-/-}$ neutrophils were stimulated with PMA, SyTOX green (cell-impermeant dye) was added and kinetic of extracellular DNA (NETosis) was measured by fluorescence plate reader, the graph represents the mean ± SD. WT or Trpm2$^{-/-}$ neutrophils were stimulated with **(C)** 1 mM of H_2O_2 or **(D)** *L. monocytogenes*, dead cells were identified by staining the cells in suspension with SyTOX green and analyzed by flow cytometry. The graphs show the % of dead cells in WT and Trpm2$^{-/-}$ neutrophils after normalization with their respective unstimulated controls. The graphs show the mean ± SD ($n = 3$), the statistical analysis was performed using multiple *t*-tests (*$p < 0.05$, **$p < 0.01$).

been associated with oxidative responses, it becomes critical to understand the functional role of this channel in modulating antimicrobial effector mechanisms of phagocytic cells. Here, we focused on defining the specific contribution of the TRPM2 channel to the antimicrobial and inflammatory function of neutrophils in response to *L. monocytogenes* infection.

Previously, Knowles et al. reported that Trpm2$^{-/-}$ mice were more susceptible than WT mice to *L. monocytogenes* infection (19). This report suggested that increased susceptibility was due to reduced production of IFN-γ, but when the mice received recombinant IFN-γ prior to infection, the Trpm2$^{-/-}$ mice recovered resistance to *L. monocytogenes* infection (19). However, the study did not address how phagocytes participate in the pathobiology of the infection or the induction of systemic inflammation by *L. monocytogenes* infection in Trpm2$^{-/-}$ mice. Unlike the Knowles et al. work, we focused our studies on the acute phase of the infection in an effort to understand the cellular events leading the mice to develop a systemic failure. We confirmed that indeed Trpm2$^{-/-}$ mice were more susceptible than WT mice to *L. monocytogenes* infection. Since Trpm2$^{-/-}$ mice developed systemic inflammation and succumbed at the acute phase of the infection, we first investigated how phagocytic cells contribute to the pathobiology leading to lethality in these mice. Furthermore, investigating the migration dynamics of phagocytic cells upon *L. monocytogenes* infection, we observed increased neutrophil and monocyte migration during the first 24 hpi in the liver of Trpm2$^{-/-}$ mice, as compared to WT mice. Those inflammatory cells persisted in the liver of Trpm2$^{-/-}$ mice

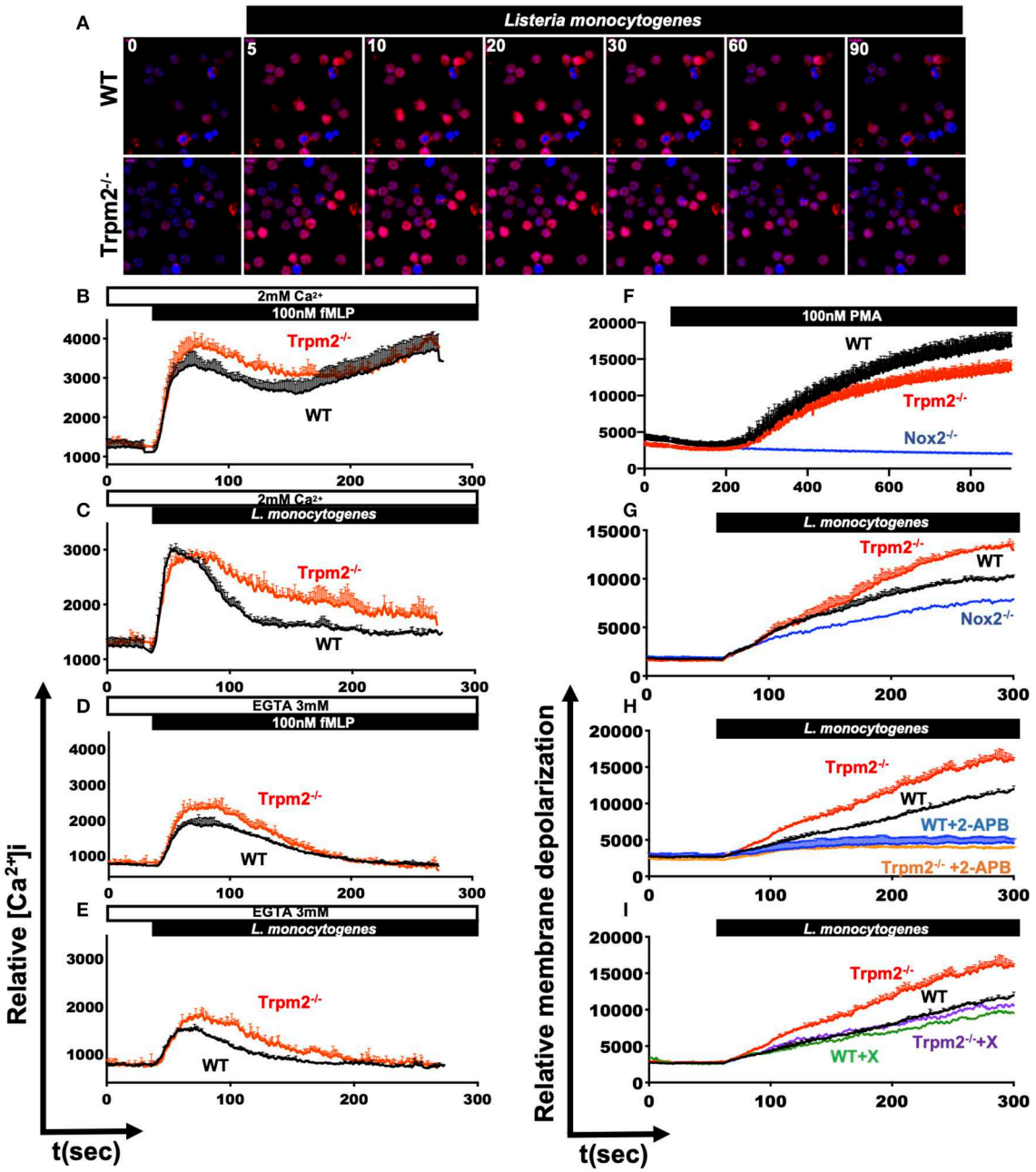

FIGURE 9 | Dysregulated mobilization of Ca^{2+} in Trpm2$^{-/-}$ neutrophils promotes increased membrane depolarization. WT or Trpm2$^{-/-}$ neutrophils were stained with Rhod-2 AM and stimulated with *L. monocytogenes*, the kinetic was recorded by confocal microscopy, **(A)** shows the cytoplasmic levels of Ca^{2+} at 0, 5, 10, 20, 30, 60, and 90 s after stimulation. Neutrophils were stained with Fluo-4 AM and the experiments were recorded by flow cytometry, WT (black lines) or Trpm2$^{-/-}$ (red lines) neutrophils were stimulated with **(B)** fMLP or **(C)** *L. monocytogenes* in media containing 2 mM of Ca^{2+}. In some experiments, EGTA was added to the media to deplete free Ca^{2+}, and the neutrophils were stimulated with **(D)** fMLP or **(E)** *L. monocytogenes,* Trpm2$^{-/-}$ showed high levels of intracellular Ca^{2+} release. Graphs show mean ± SD (above), $n = 3$. For evaluation of membrane potential, WT and Trpm2$^{-/-}$ neutrophils were stained with $DiBAC_4(3)$, the experiments were recorded by flow cytometry. WT and Trpm2$^{-/-}$ neutrophils were stimulated with **(F)** PMA or **(G)** *L. monocytogenes,* Trpm2$^{-/-}$ neutrophils showed higher levels of membrane depolarization than WT under *L. monocytogenes* stimulation. For some experiments, neutrophils were treated with **(H)** 2-APB or **(I)** Xestospongin C and stimulated with *L. monocytogenes*. Graphs show mean ± SD (above), $n = 3$.

longer than in WT mice, along with the deteriorating symptoms in those animals.

In addition to the cellular response, it is known that some inflammatory cytokines are critical for controlling the infection with *L. monocytogenes*, including IFN-γ (39), IL-12 (40), TNF-α (41) IL-6 (42), however, high levels of those inflammatory mediators can result in a lethal cytokine storm (43). In this study, the systemic inflammatory response developed by Trpm2$^{-/-}$

mice was characterized by elevated levels of TNF-α, IL-6, IL-10, CCL-2 in blood, but surprisingly, no differences were observed in IFN-γ or IL-12 within the first 72 h post-infection. Nonetheless, better prognosis markers during bacterial infections may be IL-10 and IL-6 (44). Increased blood levels of IL-6 and IL-10 have been clinically related to the high rate of mortality in patients with severe sepsis (44–46). Also, systemic levels of IL-10 appear to facilitate bacterial persistence and dissemination within the host during infections caused by intracellular bacteria or by pathogens that modulate the inflammatory responses (47, 48). Indeed, increased levels of IL-10 have been linked to the progression of *L. monocytogenes* infections (48). It is, therefore, possible that increased production of IL-10 in the Trpm2$^{-/-}$ mice, rather than a deficiency in IFN-γ or IL-12, may be associated to the susceptibility of the Trpm2$^{-/-}$ mice upon *L. monocytogenes* infection.

Previously, neutrophils had been considered not to be essential in the natural resistance against *L. monocytogenes* infection (27), however, another group demonstrated the importance of neutrophils during the primary and secondary responses against *L. monocytogenes* (25). Our results are in good agreement with those later findings. Initially, we did not see increased susceptibility in WT mice after neutrophil depletion, however, neutrophil-depleted Trpm2$^{-/-}$ mice developed resistance to *L. monocytogenes* infection. The extended survival of neutrophil-depleted Trpm2$^{-/-}$ mice was also accompanied by reduced levels of bacterial burden in the liver and the spleen. Similarly, the levels of systemic inflammatory cytokines were reduced in Trpm2$^{-/-}$ mice without neutrophils, suggesting that TRPM2$^{-/-}$ neutrophils promote a state of hyper-inflammation, possibly related directly to ion homeostasis imbalance in these cells, which might contribute to the dissemination of *L. monocytogenes*.

Similar exacerbated inflammatory response was already observed in the Trpm2$^{-/-}$ mice by our group, in a model of gastric infection by *H. pylori* infection (14), and by other investigators using distinct models of infection including, lung infection induced by *P. aeruginosa* (38), sepsis-induced by *E. coli* (49) or polymicrobial sepsis (50). Moreover, non-infectious models of inflammation have also added experimental evidence demonstrating that TRPM2 deficient animals cannot efficiently control inflammatory responses. For example, Trpm2$^{-/-}$ mice succumbed to LPS challenge in a lung inflammatory model (20), Also, skin inflammation is aggravated in Trpm2$^{-/-}$ mice in a model induced by LPS and TNF-α (16). In all these referenced studies, the increased inflammation was a consistent feature observed in the absence of TRPM2 ion channel, further supporting the paradigm of an anti-inflammatory functional role of TRPM2 (20, 51), as opposed to the paradigm favoring a pro-inflammatory function for TRPM2 in phagocytes (21, 51).

The infection with *L. monocytogenes* in Trpm2$^{-/-}$ mice was characterized by neutrophilia, bacterial dissemination and acute tissue pathology in the liver and spleen of these mice, therefore, we sought to determine how the TRPM2 cation channel might regulate the antimicrobial response and inflammation in neutrophils. Our initial findings revealed that Trpm2$^{-/-}$ neutrophils had increased effector functions, which included augmented production of ROS, enhanced released of primary granules, and likely, increased production of NETs than WT neutrophils, in response to *L. monocytogenes* infection. Altogether, these data suggested a direct functional role for the TRPM2 ion channel in the regulation of neutrophil's antimicrobial and inflammatory pathways. Despite the increased susceptibility of Trpm2$^{-/-}$ mice to *L. monocytogenes* infection, TRPM2$^{-/-}$ neutrophils exhibited increased capacity to kill these bacteria *in vitro*, which suggested that uncontrolled inflammation, rather than deficient bacterial killing, is responsible for exacerbated pathology resulting in the death of the Trpm2$^{-/-}$ mice. Our previous findings showed that Trpm2$^{-/-}$ macrophages had a similar marked inflammatory profile, which was associated with increased chronic gastric inflammation induced by *H. pylori* infection in Trpm2$^{-/-}$ mice (14). It is, therefore, possible that in addition to neutrophils, macrophages may also contribute to the hyper inflammation observed in the tissue microenvironment upon bacterial infection in Trpm2$^{-/-}$ mice. Notably, neutrophils also showed increased levels of cytosolic Ca^{2+} upon bacterial stimulation, in agreement with our published findings in TRPM2 deficient macrophages (14).

Stimulated neutrophils activate the membrane-associated NADPH oxidase (NOX2) resulting in a powerful oxidative burst, which constitute the central host defense mechanism in neutrophils (36, 52). We and others have proposed that activation of TRPM2 cation channel function downregulates NADPH oxidase activity, via a mechanism linked to membrane depolarization in phagocytes. Thus, activation of TRPM2 results in dampening of the NADPH oxidase-mediated ROS production through depolarization of the plasma membrane in WT phagocytes (20), whereas PMA or bacterial stimulation of Trpm2$^{-/-}$ macrophages yielded increased levels of ROS (14). Therefore, as expected, the absence of TRPM2 channel in the Trpm2$^{-/-}$ neutrophils also resulted in elevated NADPH oxidase activity and abundant ROS production, which correlated with the increase in membrane depolarization upon *L. monocytogenes* stimulation, as we show in **Figures 9F–I**, and likely the augmented NETosis observed in **Figure 8**, in response to PMA stimulation. Furthermore, the excessive membrane depolarization was prevented when 2-APB was added to Trpm2$^{-/-}$ neutrophils, suggesting that in the absence of TRPM2 ion channels, additional plasma membrane channels, including members of the TRP family (10–12) or store-operated Ca^{2+} channels (7, 9), can be activated, and likely compensate for the entry of Ca^{2+} in these cells. The activation of plasma membrane Ca^{2+} channels will contribute to Ca^{2+} overloading and the overall hyperresponsiveness of Trpm2$^{-/-}$ neutrophils. Consequently, the lack of TRPM2 channel-mediated function will result in the increased inflammation observed in Trpm2$^{-/-}$ mice under *L. monocytogenes* infection.

Hence, TRPM2-mediated calcium influx in neutrophils is an essential mechanism for the regulation of the antimicrobial and inflammatory effector functions of these cells, including oxidative stress responses. The role of TRPM2 as an oxidant sensor has been extensively demonstrated in multiple cell types, including cancer cells [reviewed in (51)], where inhibition of the channel

causes dysfunctional cellular bioenergetics, increased production of ROS, and impaired DNA repair leading to increased cell death (53, 54). It is therefore possible that, in addition to modulating Ca^{2+} mobilization, membrane depolarization, and NADPH activity, TRPM2 also controls neutrophils' oxidative burst, and oxidant dependent cell death, by scavenging the excess of harmful oxidants, such as H_2O_2 produced in response to bacterial stimulation. Altogether, the multiple key functions of TRPM2 appear to define the dynamic effector response of neutrophils during the onset of infection and/or inflammatory processes.

In summary, we propose that the TRPM2 ion channel functions as a global modulator of inflammation in neutrophils, by reducing the oxidative response and regulating Ca^{2+} influx and membrane depolarization in these cells. Thereby, TRPM2 could be a target aiming to modulate the pathology of inflammatory diseases were neutrophils are critical mediators of inflammation and aggravated tissue damage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The Institutional Animal Care and Use Committee (IACUC) at the Abigail Wexner Research Institute of Nationwide Children's Hospital approved all animal experiments to ensure the humane care and ethical use of animals (IACUC protocol # 00505AR). All mice studies were performed in strict accordance with the National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals [DHSS Publication No. (NIH) 85–23].

AUTHOR CONTRIBUTIONS

FR-A and SP-S conceived the study, designed the experiments, and wrote the manuscript. FR-A, JR-R, and KB performed the experiments. FR-A analyzed the data and prepared the figures. FR-A, JR-R, KB, and SP-S edited the manuscript. SP-S provided financial resources and supervised the investigation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00097/full#supplementary-material

Supplementary Figure 1 | Faster dissemination of *L. monocytogenes* in $Trpm2^{-/-}$ mice induces an acute myeloid inflammatory response in the liver. **(A)** WT and $Trpm2^{-/-}$ mice were infected with *L. monocytogenes* Xen-32 and luminescence was visualized in ventral position at 6 hpi, **(B)** luminescence was quantified as photons/s ($n = 9$). **(C)** Liver ($n = 10$) and spleen ($n = 10$) were dissected and visualized, graphs show the photons/s of **(D)** liver and **(E)** spleen. Frozen sections of livers from mice infected with *L. monocytogenes* at 72 hpi were stained with anti-*L. monocytogenes*, anti-Ly6G or anti-Ly6C, **(F)** shows the interaction of neutrophils with *L. monocytogenes* in the liver and **(G)** the interaction between monocytes and *L. monocytogenes*. Images were acquired at 10X ($*p < 0.05$, $**p < 0.01$).

Supplementary Figure 2 | Depletion of neutrophils in $Trpm2^{-/-}$ mice results in reduced tissue pathology. Neutrophils (anti-Ly6G) were depleted in WT or $Trpm2^{-/-}$ mice 1 day prior to infection and 2 days after infection, spleens and livers were dissected at 72 hpi, embedded in paraffin and stained with H&E. **(A)** The graph shows the percentage of spleen follicles with necrosis at 72 hpi ($n = 3$). The percentage of follicles with necrosis in the spleen was significantly larger in $Trpm2^{-/-}$ mice compared to WT, the depletion of neutrophils however, reduced the percentage of follicles with necrosis in the spleen of $Trpm2^{-/-}$ mice. **(B)** The quantitation of abscesses/median lobe in livers of mice 72 hpi ($n = 3$) showed a significantly increased number of abscesses in the liver of $Trpm2^{-/-}$ mice compared to WT, but the depletion of neutrophils drastically reduced the number of abscesses in livers from $Trpm2^{-/-}$ mice **(B)**. The bar graphs show mean ± SD, the statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison tests ($*p < 0.05$).

Supplementary Figure 3 | *L. monocytogenes* induces increased cytosolic levels of Ca^{2+} and membrane depolarization in $Trpm2^{-/-}$ neutrophils. Bone marrow neutrophils were stained with Fluo-4 AM, aliquoted in HBSS containing Ca^{2+} and Mg^{2+} and stimulated with **(A)** 10 mM H_2O_2, **(B)** 5 mM H_2O_2 or **(C)** 1 mM H_2O_2. The kinetics of intracellular Ca^{2+} levels were recorded by flow cytometry up to 300 s ($n = 3$), the kinetics are shown as mean ± SD (upper). The areas under the curve (AUC) of kinetics of intracellular Ca^{2+} were quantified (the kinetics are shown in the **Figure 9**), The bar graphs show the AUC of neutrophils stimulated with **(D)** fMLP or **(E)** *L. monocytogenes*, in media containing Ca^{2+} or when Ca^{2+} was depleted by the addition of EGTA ($n = 3$). The **(F)** shows the AUC of the kinetics of intracellular Ca^{2+} when neutrophils were stimulated with 10, 5, or 1 mM of H_2O_2 ($n = 3$), the graphs show the mean ± SD, the statistical analysis was performed with Welch's *t*-test ($**p < 0.01$, $***p < 0.001$). Bone marrow neutrophils were stained with $Dibac_4(3)$ in order to analyze membrane depolarization by flow cytometry. The kinetics of membrane depolarization are shown in **Figures 9F–I**. The bar graphs show the AUC of the kinetics of membrane depolarization when neutrophils were stimulated with **(G)** PMA, **(H)** *L. monocytogenes*, or **(I)** *L. monocytogenes* plus 2-APB or Xestospongin C. The graphs show the mean ± SD ($n = 3$). The statistical analysis was performed by Welch's *t*-test **(G,H)** or with ANOVA one-way and Sidak multiple comparisons ($****p < 0.0001$).

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REVIEW
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Transient Receptor Potential Channels and Inflammatory Bowel Disease

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The transient receptor potential (TRP) cation channels are present in abundance across the gastrointestinal (GI) tract, serving as detectors for a variety of stimuli and secondary transducers for G-protein coupled receptors. The activation of TRP channels triggers neurogenic inflammation with related neuropeptides and initiates immune reactions by extra-neuronally regulating immune cells, contributing to the GI homeostasis. However, under pathological conditions, such as inflammatory bowel disease (IBD), TRP channels are involved in intestinal inflammation. An increasing number of human and animal studies have indicated that TRP channels are correlated to the visceral hypersensitivity (VHS) and immune pathogenesis in IBD, leading to an exacerbation or amelioration of the VHS or intestinal inflammation. Thus, TRP channels are a promising target for novel therapeutic methods for IBD. In this review, we comprehensively summarize the functions of TRP channels, especially their potential roles in immunity and IBD. Additionally, we discuss the contradictory findings of prior studies and offer new insights with regard to future research.

Keywords: transient receptor potential channels, gastrointestinal tract, neurogenic inflammation, immune cells, inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing GI inflammatory disorder, comprising of Crohn's disease (CD) and ulcerative colitis (UC). It is acknowledged that IBD is related to inappropriate immunity, commensal bacteria, genetics, and environmental factors. The exact pathogenesis of IBD, however, remains unknown (1). Nowadays, various receptors in the gastrointestinal (GI) tract are proposed to play a role in the pathophysiology of IBD, amongst which transient receptor potential (TRP) ion channels have been identified and are considered to be potentially effective. TRP channels are polymodal ion channels that serve as sensors for chemical noxious and physical stimuli. These channels are widely distributed in the GI tract and exert various effects, contributing to the somatic and visceral nociception and the maintenance of physiological function of the GI tact (2). The activation of TRP channels can evoke neurogenic inflammation, namely the inflammation initiated by the local release of immunomodulatory neuropeptides, including calcitonin-gene-related peptide (CGRP) and substance P (SP) released by unmyelinated afferent neurons (3, 4). Some TRP channels are also expressed in multiple immune cells, and are primarily responsible for modulating actions, such as cytokine release, cell migration, and phagocytic activity (2). Therefore, numerous studies have indicated that TRP channels are mainly

involved in the visceral hypersensitivity (VHS) and immune pathogenesis of IBD due to their comprehensive functions of sensors and immunomodulators. Different subtypes of TRP channels seem to have distinct effects. Here, we briefly review the correlation between TRP channels and IBD with a focus on TRPV1, TRPA1, TRPV4, TRPM2, and TRPM8, which have been documented to be the most relevant TRP channels in IBD.

TRP CHANNELS IN THE GI TRACT AND RELATED NEUROPEPTIDES

In the GI tract, TRP channels are mainly expressed on the extrinsic primary afferent nerves with some on epithelial, endocrine cells, and intrinsic enteric neurons (5, 6). Intriguingly, 97% of TRPA1-positive ($TRPA1^+$) afferents co-express TRPV1, and 30% of the TRPV1-positive ($TRPV1^+$) neurons co-express TRPA1, hinting at the potential interaction between the two channels (5, 7). Capsaicin is a significant agonist for TRPV1 with an exquisite selectivity and allyl isothiocyanate (AITC), the pungent ingredient in garlic, is the prototypical agonist for TRPA1 (8, 9). TRPV4 colocalizes with TRPV1, TRPA1, and protease-activated receptors 2 (PAR-2) in the GI tract, in response to strong acidosis, hypotonicity, warmth, and mechanical stimuli (10, 11). TRPM2 is sensitive to heat stimulus while TRPM8 is essential to cold-induced pain (12). Most of TRP channels are non-selective cation channels and show the permeability to calcium ion (Ca^{2+}). Upon stimulation, TRP channels in afferents can lead to autonomic reflex responses by transmitting signals to the central nervous system. Meanwhile, TRP channels can transduce sensory signal of G-protein coupled receptors (GPCRs) based on the phosphorylation sites in N-terminus for serine and threonine protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC) (11, 13).

Additionally, TRP channels in the GI tract can mediate the crosstalk between the nervous and immune systems by modulating the release of neuropeptides. TRPV1, TRPA1, and TRPV4 are especially often found to colocalize with CGRP and SP (6, 14).

CGRP, which is generated from the alternative RNA processing of the gene for calcitonin, serves as a potent peptide vasodilator and is involved in the transmission of nociception (15). CGRP plays a protective role in the inflammation and inhibits the capacity of immune cells. For dendritic cells (DCs) and macrophages, CGRP could restrain their ability in the presentation of antigens and the secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (16, 17). CGRP also downregulated DCs' responses to Toll-like receptor 4 (TLR4), a receptor for lipopolysaccharides (LPS) which is an abundant outer wall glycolipid of Gram-negative bacteria (18). CGRP can exert an inhibitory effect on the activation and chemotaxis of neutrophils (19), and inhibit neutrophil-mediated killing of bacteria mainly through suppressing the activity of the bactericidal enzyme myeloperoxidase (MPO) in a dose-dependent manner (20). CGRP was found to restrain group 2 innate lymphoid cells proliferation and type 2 innate immune responses (21), and be required for the induction of protective innate type 17 immunity after the activation of cutaneous $TRPV1^+$ neurons (22). Furthermore, CGRP could induce the upregulation of interleukin (IL)- 10 and was beneficial in preserving mucosal integrity and limiting tissue damage (16). These observations demonstrate the negative regulatory role of CGRP in innate immunity and the benefits of CGRP in the GI tract. Conversely, GRCP was reported to be capable of stimulating T-cell migration and promoting the release of interferon gamma (IFN-γ) and IL-2 from T-helper cells (23, 24). In IBD patients, the expression of CGRP in the colonic mucosa was significantly increased and was closely associated with the severity of disease (25, 26). Therefore, CGRP might also play a part in the pro-inflammatory process.

SP belongs to tachykinin family. The receptor for SP is the neurokinin 1 receptor (NK-1R) (15). Similar to GCRP, SP serves as a potent vasodilator and the SP-induced vasodilatation is based on nitric oxide (NO) release (27). Of interest, high-dose CGRP was reported to restrain the SP-evoked vasodilation but facilitate SP-evoked plasma protein extravasation (28), suggesting a crosstalk between CGRP and SP. The expression of SP and NK-1R has been well-documented in DCs, monocytes, eosinophils, neutrophils, mast cells, natural killer cells, and T cells, enabling SP to regulate functions of different types of immune cell (29). SP also modulated the immune response to microbial infection (29). It was recently demonstrated that SP could promote the migration and activation of mast cells, inducing the release of multiple pro-inflammatory cytokines and chemokines (30). Noteworthy, SP directly caused the secretion of IL-8 in human colonic epithelial cell lines (31), hinting at the potential pro-inflammatory role of SP in intestine. SP was detected to be elevated in tissue extracts from the colon and rectum of IBD patients, and the level of SP was correlated with disease activity (32). However, in animal studies, SP ameliorated dextran sulfate sodium (DSS)-induced colitis by promoting the enrichment of M2 macrophages and regulatory T cells, or maintaining barrier structure and regulating immune response (33, 34). Such results remind us of the possible protective effect of SP in colitis. Taking these contradictory observations with regard to the properties of CGRP and SP into consideration, we can conclude that the neurogenic inflammation triggered by TRP channels has bidirectional functions on immunity and colitis. However, the exact function of neuropeptides on a certain physical or pathological condition has not yet been discovered and further studies are required.

TRP CHANNELS IN IMMUNE CELLS

Besides the roles in nervous system of the GI tract, TRP channels are also expressed in immune cells and directly contribute to immune responses. In bone marrow-derived macrophages, the TRPV1 expression was increased and intracellular Ca^{2+}-transients were triggered after oxidized low-density lipoprotein (ox-LDL)-stimulation (35). TRPV1 could dose-dependently modulate the level of inducible NO synthase in stimulated peritoneal macrophages through the inhibition of nuclear factor kappa B (NF-κB), thus influencing the secretion of

pro-inflammatory cytokines involved in this pathway (2). In a sepsis model, the LPS-stimulated peritoneal macrophages showed an impaired phagocytosis when TRPV1 was knock-out (36), suggesting the putative role of TRPV1 to potentiate macrophages. In $CD4^+$ T cells, TRPV1 was associated with T cell receptor (TCR) and facilitated TCR-induced Ca^{2+} inflow (37), and the activity of $CD4^+$ T cells was impaired via the inhibition of TRPV1 (38). The activation of TRPV1 was also reported to enhance leukocyte rolling and adhesion (39). These data indicated the possible pro-inflammatory properties of TRPV1 in immune cells. Intriguingly, TRPV1 activation can trigger the production of the endocannabinoid anandamide, which increases the level of regulatory CX3CR1 (hi) macrophages in the gut and enhances their immunosuppressive activity (40). In DCs, TRPV1 mediates the downregulation of TLR4/NF-κB signaling pathway that leads to the maturation of DCs (41). A recent research concerning lethal Staphylococcus aureus pneumonia stated that the activation of $TRPV1^+$ nociceptors by capsaicin could suppress cytokine release, inhibit the recruitment and surveillance of neutrophils, and alter lung γδ T cell numbers; thus impairing lung bacterial clearance (42). As discussed above, TRPV1 is an important immunomodulator that regulates the activation and function of immune cells.

As for TRPA1, its expression was increased in stimulated T cells, and TRPA1 was vital for the T cell activation and release of cytokines like TNF-α, IFN-γ and IL-2 (43). TRPA1 also expresses in mast cells and DCs (44). In TRPA1-knockout ($Trpa1^{-/-}$) mice, mast cells, leukocytes, and T cells, together with the expression of IL-1β, IL-6, IL-17, IL-22, and IL-23 were decreased in the lesions of skin (45), indicating the ability of TRPA1 to induce inflammation through these immune cells. In addition to its pro-inflammatory function, the activation of TRPA1 could suppress the pro-inflammatory effect of LPS-stimulated peritoneal macrophages by decreasing the level of NO, which is an abundant pro-inflammatory mediator (46). Taken together, TRPA1 has the ability to regulate immune cells in diverse manner. The crosstalk between TRP channels and bacteria is noteworthy. It was discovered that LPS interacted with TLR4 on the $TRPV1^+$ afferent neurons. This then activated or sensitized TRPV1 via its phosphorylation binding sites through PKC, thus resulting in an increased release of CGRP (17, 47). Antagonists for TRPV1 and CGRP could reverse LPS-induced motility disturbance of the intestine (48). Another study showed that a probiotic bacterium named Lactobacillus reuteri and its condition medium dose-dependently reduced the capsaicin- and distension- evoked firing of jejunal spinal afferents in mice (49), revealing the engagement of afferents in bacteria-induced GI sensory-motor dysfunction. TRPA1 in nociceptive neurons could be sensitized by LPS in a TLR4-independent manner during inflammation, causing pain, CGRP release, and vasodilation (50). Therefore, it was hypothesized that TRP channels may be able to directly or indirectly interact with microbiota or their products in the gut, thus influencing the release of neuropeptides and contributing to the maintenance of gut homeostasis.

The activation of TRPV4 increased intracellular Ca^{2+} concentration in LPS-treated macrophages and potentiated macrophage (51), while downregulation of TRPV4 subsequently impaired the phagocytosis of macrophages (52). In neutrophils, TRPV4 was essential for inflammatory responses, such as the neutrophil adhesion, chemotaxis, and formation of reactive oxygen species (53). TRPV4-mediated Ca^{2+} influx in T cells was also capable of inducing the proliferation of T cells and the secretion of TNF-α, IFN-γ, and IL-2 *in vitro* (54). Recent data revealed that TRPV4 could promote the phagocytosis of mouse $CD11c^+$ bone marrow-derived cells (55). These findings clearly highlight the critical pro-inflammatory role of TRPV4 in immune cells.

Regarding TRPM2, it was demonstrated that the lack of this channel in LPS-stimulated monocytes cell line reduced the release of TNF-α, IL-6, IL-8, and IL-10 (56). TRPM2-associated Ca^{2+} signaling was essential in the transmigration and cytotoxicity of neutrophils (57, 58), the proliferation of T cells, and the release of pro-inflammatory cytokines (59). For TRPM8, the activation by menthol in murine peritoneal macrophages increased IL-10 expression and decreased TNF-α release, thus exerting an anti-inflammatory effect (60). TRPM8-knockout ($Trpm8^{-/-}$) peritoneal macrophages exhibited an impaired phagocytic activity while the phagocytosis was enhanced in WT peritoneal macrophages after the activation of TRPM8 (60). Consistently, the activation of TRPM8 was reported to restrain the release of pro-inflammatory mediators in monocytes and lymphocytes, and $Trpm8^{-/-}$ $CD11c^+$ DCs showed hyperinflammatory responses to TLR-stimulation (61, 62). In T cells, the inhibition of TRPM8 suppressed murine T-cell activation and the release of IL-2 and IL-6 (63). Overall, TRPM2 appears to potentiate inflammatory effects of immune cells while TRPM8 often performs anti-inflammatory roles.

TRP CHANNELS IN INFLAMMATORY VISCERAL HYPERSENSITIVITY OF IBD

Due to their immunomodulatory function via neuropeptides and immune cells, TRP channels are associated with GI immunity and inflammation. Notably, it is found that their expression has been altered in IBD patients and colitis models (**Table 1**), suggesting an involvement of TRP channels in IBD. In particular, IBD patients are associated with a visceral hypersensitivity (VHS), which is featured of an aberrant and chronic visceral pain (5, 12). As visceral nociceptors, TRP channels are proposed to be responsible for VHS in IBD. Since TRP channels serve as secondary transducers for GPCRs, some mediators that act on GPCRs subsequently activate or sensitize TRP channels, resulting in aberrant sensation. Through this mechanism, pro-inflammatory mediators secreted during colitis, such as bradykinin, serotonin (5-hydroxytryptamine, 5-HT), cytokines, adenosine triphosphate, prostaglandins, and epinephrine can lead to the inflammatory VHS (5). A number of researches have been conducted to explore the definite role of TRP channels in colitis and VHS (**Table 2**).

TRPV1

TRPV1 channel is closely linked to VHS. It was found that some patients with quiescent IBD still complained about

TABLE 1 | The expression of TRP channels in colonic tissue of IBD patients and colitis models.

	TRPV1	TRPA1	TRPV4	TRPM2	TRPM8
IBD patients					
UC	UP (64–68) NS (69) DOWN (70, 71)	UP (68)	UP (70, 72) NS (73)	NA	NA
CD	UP (64–66) DOWN (68, 71)	UP (68, 71, 74, 75)	UP (72, 76) NS (73)	NA	UP (77)
Animal models					
DSS-treated mice	UP (68, 78–80) NS (71)	UP (71)	UP (73, 81)	NA	UP (77, 82)
DSS-treated rats	UP (83)	NA	NA	NA	NA
TNBS-treated mice	UP (84)	NA	UP (72)	NA	UP (77, 82)
DNBS-treated mice	NA	UP (85)	NA	NA	NA
TNBS-treated rats	UP (86) NS (87)	UP (88, 89)	NA	UP (90)	NA
Mustard oil-treated mice	NS (91)	UP (91)	NA	NA	NA

UC, ulcerative colitis; CD, Crohn's disease; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DNBS, dinitrobenzene sulfonic acid; UP, upregulated; DOWN, downregulated; NS, no significant difference; NA, not available.

TABLE 2 | The function of TRP channels in VHS of colitis models.

Pro-VHS function			
TRP channel	**Colitis model**	**Result**	**References**
TRPV1	DSS-treated mice	Increased VHS which could be enhanced by the agonist for TRPV1	(92)
	DSS- and TNBS- treated rat	Increased VHS which could be relieved by the antagonist for TRPV1	(83, 86, 87, 93)
	$Trpv1^{-/-}$ mice with DSS-induced colitis	Decreased VHS compared to WT mice	(78)
TRPA1	DSS-treated mice	Increased VHS which could be enhanced by the agonist or be relieved by the antagonist for TRPA1	(94)
	TNBS-treated rat	Increased VHS which could be enhanced by the agonist or be relieved by the antagonist for TRPA1	(88, 89, 95, 96)
	$Trpa1^{-/-}$ mice with TNBS-induced colitis	Decreased VHS compared to WT mice	(97, 98)
TRPV4	TNBS-treated mice	Increased VHS which could be relieved by the antagonist for TRPV4	(72)
	$Trpv4^{-/-}$ mice	Decreased VHS compared to WT mice	(99)
TRPM2	TNBS-treated rat	Increased VHS which could be relieved by the antagonist for TRPM2	(90)
	$Trpm2^{-/-}$ mice with TNBS-induced colitis	Decreased VHS compared to WT mice	(90)
TRPM8	DSS- and TNBS- treated mice	Increased VHS which could be enhanced by the agonist or be relieved by the antagonist for TRPM8	(82)
	$Trpm8^{-/-}$ mice	VHS was only decreased under higher level of stimuli compared to $Trpv1^{-/-}$ and $Trpv4^{-/-}$ mice	(100)
Anti-VHS function			
TRPM8	WT mice	The function of TRPV1 and TRPA1 was inhibited by TRPM8 activation	(101)
	TNBS-treated rat	Increased VHS which could be relieved by the agonist for TRPM8	(102)

VHS, visceral hypersensitivity; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzenesulfonic acid; WT, wild-type.

abdominal pain, and the severity of their symptoms was correlated to the increased $TRPV1^{+}$ fibers in colonic mucosa (64). In animal studies, the behavioral responses to intracolonic capsaicin administration and the expression of spinal cord neuronal c-Fos, which is a marker of neuronal excitation, were increased in DSS-treated mice (92). Yang et al. (83) reported that an oral administration of curcumin, which is clinical valuable for the treatment of IBD (103), in DSS-treated rats could significantly ameliorate visceral hyperalgesia through inhibiting phosphorylation of TRPV1, indicating a nociceptive effect of TRPV1. Likewise, Phillis et al. (93) revealed that TRPV1 antagonist remarkably reduced the mechanosensory response to the stimulus in a dose-dependent manner in rats with DSS-induced colitis. Visceral hyperalgesia and increased

visceromotor response (VMR) were also confirmed in rats with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (86, 87), while TRPV1 antagonist (JYL1421) could prevent and relieve the VHS (86). Additional studies exhibited that TRPV1-knockout (*Trpv1*$^{-/-}$) mice conferred a resistance to colorectal distension (78, 104), and VHS was enhanced by inflammatory mediators, such as bradykinin, 5-HT, histamine, and prostaglandin E2 (PGE2) in WT mice but not in *Trpv1*$^{-/-}$ mice (12). SP was demonstrated to enhance the sensitivity and function of TRPV1 in DSS-induced colitis and *in vitro* (78), suggesting the engagement of neuropeptides in VHS. Noteworthy, the augmented activity of pelvic nerve afferents after TRPV1 activation in DSS-treated rats was more prominent on the first day post DSS-treatment, in comparison to the eighth day (92). Similarly, the levels of TRPV1 and TRPA1 messenger RNA (mRNA) in mice were upregulated in mustard oil (MO)-induced colitis within 6 h but decreased 24- and 72-h after MO-injection (91). Therefore, it can be hypothesized that the excitatory mechanism modulated by TRPV1 mainly particulate during early stage of experimental colitis.

TRPA1

TRPA1 could contribute to colorectal contraction and enhanced VMR to intracolonic AITC, which were detectable in TNBS-induced colitis. These actions could be suppressed by intrathecal pretreatment with a TRPA1 antisense oligodeoxynucleotide, and were absent in *Trpa1*$^{-/-}$ mice (88, 89, 97, 98). Likewise, AITC enhanced the sensitivity of colon and the expression of c-Fos in spinal cord of DSS-treated mice (94). During TNBS-induced colitis, the production of hydrogen peroxide (H_2O_2) was enhanced due to the infiltration of white blood cells and the presence of oxidative stress. The increased H_2O_2 then activated TRPA1 and led to the hypersensitivity of VMR (95). The aberrant GI motility might result from the effects of PGE2 induced by TRPA1 activation (105). Similarly, inflammatory mediators, such as bradykinin and 5-HT could lead to an increased visceral mechano-sensitivity in a TRPA1-associated manner (97, 106). These data suggest a close link between TRPA1 and pro-inflammatory cytokines, both of which contribute to visceral hyperalgesia. Of interest, Vermeulen et al. (96) reported that a combined application of antagonists for TRPV1 and TRPA1 could reduce the VMR more effectively in TNBS-induced colitis, in comparison to targeting either TRPV1 or TRPA1 alone. Such evidence appears to provide an inspiring therapeutic method for inflammatory VHS.

TRPV4

TRPV4 is vital for a mechanically-evoked visceral pain in the GI tract (76). It was found that TRPV4 co-expressed with PAR-2 and pretreatment of PAR-2 agonist enhanced TRPV4 activity and hypersensitivity, while the inhibition of PKA and PKC restrained this effect (99, 107). Also, 5-HT and histamine improved TRPV4-induced hypersensitivity for colorectal distention in mice (108). These results indicate the responses of TRPV4 to inflammatory mediators through GPCR signaling pathway. A selective blockade of TRPV4 was subsequently evident to alleviate intestinal inflammation and pain in TNBS-treated mice (72). Therefore, the pro-hypersensitivity function of TRPV4 during colitis is relatively clear.

TRPM2

In TNBS-induced rat colitis, the VMR was enhanced and could be ameliorated by an oral administration of TRPM2 antagonist (econazole) (90). However, in TRPM2-knockout (*Trpm2*$^{-/-}$) mice, the TNBS-induced VMR was less severe compared to WT mice (90), showing a potential facilitating role of TRPM2 in VHS.

TRPM8

Menthol, serving as the agonist for TRPM8, has been applied to relieve abdominal discomfort and pain in traditional Chinese medicine, suggesting that TRPM8 activation can diminish visceral pain perception (12). In TNBS-induced colitis, the colonic mechano-hypersensitivity was remarkably suppressed by a combined adoption of peppermint and caraway oil, which are agonists for TRPM8 (102). Harrington et al. (101) demonstrated that TRPM8 activation restrained the downstream chemosensory and mechanosensory actions of TRPA1 and TRPV1 to agonists in colonic afferents, stating the potential function of TRPM8 for inhibiting TRPV1 and TRPA1. In contrast, it was showed that the TRPM8 agonist (WS-12) enhanced visceral pain response while a pretreatment of TRPM8 antagonist inhibited the hypersensitivity (82). Another study reported that in *Trpm8*$^{-/-}$ mice, VMR only decreased when the pressure level of colorectal distension was quite high; but in *Trpv1*$^{-/-}$ and *Trpv4*$^{-/-}$ mice, VMR was remarkably decreased in all pressure ranges (100). Of note, both bradykinin and histamine were found to suppress TRPM8 mainly via the G-protein subunit Gα which inhibited ion channel activity of TRPM8 (109), indicating the ability of inflammatory mediators to desensitize TRPM8 and inhibit its function. Such a mechanism may account for the TRPM8-associated enhanced-VHS during inflammation.

TRP CHANNELS IN IMMUNE PATHOGENESIS OF IBD

In addition to the roles in inflammatory VHS, the potential engagement of TRP channels in the immune pathogenesis of IBD has been highlighted in human and animal studies (**Table 3**).

TRPV1

The TRPV1^{+} fibers were increased in the colonic mucosa of IBD patients, along with non-neuronal TRPV1 immunoreactivity (65, 66). Further study confirmed an increased expression of TRPV1 in inflamed tissue of active UC patients compared with non-inflamed tissue, being associated with a relapse and continuous activity of disease (64, 67). However, a downregulated expression of TRPV1 was also revealed in colonic biopsies from UC and CD patients (68, 70, 71), and Rizopoulos et al. (70) found no significant correlation between TRPV1 expression and clinical features in UC patients. In experimental colitis models, TRPV1 expression was also found to be altered (68, 78–80, 83, 84, 86) (**Table 1**). Kihara et al. (110) subcutaneously injected noxious-dose capsaicin into neonatal rats to chemically denervate the TRPV1 channel, revealing that the denervated rats exhibited a

TABLE 3 | The function of TRP channels in pathophysiology of colitis models.

Pro-inflammatory function			
TRP channel	**Colitis model**	**Result**	**References**
TRPV1	DSS-treated mice	Chemically denervation of TRPV1, the antagonist for TRPV1, and the Trpa1-knockout alleviated colitis	(110–114)
	TNBS-treated rat	The antagonist for TRPV1 alleviated colitis	(115)
	TLR-4$^{-/-}$ mice with TNBS-induced colitis	Downregulated TRPV1 expression and alleviated colitis compared to WT mice	(84)
	Toxin-A treated isolated rat ileal segment	Aggravated inflammation which could be enhanced by the agonist or be alleviated by the antagonist for TRPV1	(116)
	T cell-transfer mice colitis	Genetic or pharmacological inhibition of TRPV1 in T cell or colonic tissue resulted in less severe colitis	(117, 118)
TRPA1	DSS-treated mice	The Trpa1-knockout and the antagonist for TRPA1 alleviated colitis	(119)
	TNBS-treated mice	The Trpa1-knockout and the antagonist for TRPA1 alleviated colitis	(119)
TRPV4	DSS-treated mice	The agonist for TRPV4 aggravated colitis and the Trpv4-knockout alleviated colitis	(73, 81)
	TNBS-treated mice	The antagonist for TRPV4 alleviated colitis	(72)
TRPM2	DSS-treated mice	The Trpm2-knockout alleviated colitis	(120)
Anti-inflammatory function			
TRPV1	DSS-treated rat	The agonist for TRPV1 alleviated colitis and chemically denervation of TRPV1 aggravated colitis	(121)
	TNBS-treated rat	The agonist for TRPV1 alleviated colitis	(122, 123)
	DNBS-treated mice	The Trpv1-knockout aggravated colitis	(124)
	Oxazolone-treated mice	Chemically denervation of TRPV1 aggravated colitis	(125)
	Iodoacetamide-treated rat	Chemically denervation of TRPV1 aggravated colitis	(126)
	Formalin-treated rabbit	Chemically denervation of TRPV1 aggravated colitis	(127)
TRPA1	DSS-treated mice	The Trpa1-knockout and the antagonist for TRPA1 aggravated colitis; the agonist for TRPA1 alleviated colitis	(71, 85)
	T cell-transfer mice colitis	TRPV1^{+}TRPA1^{-} T cells induced more severe colitis compared to TRPV1^{+}TRPA1^{+} T cells	(68)
	TNBS-treated mice	The Trpa1-knockout aggravated the fibrosis in colitis	(57, 67)
	DNBS-treated mice	The agonist for TRPA1 alleviated colitis	(85)
TRPM8	TNBS-treated mice	The agonist for TRPM8 alleviated colitis	(77)
	DSS-treated mice	The Trpm8-knockout aggravated colitis and the agonist for TRPM8 alleviated colitis; adoptive transfer of TRPM8$^{-/-}$ macrophages in mice induced more severe colitis compared to WT macrophages	(60, 77)

DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DNBS, dinitrobenzene sulfonic acid; WT, wild-type.

lower severity of DSS-induced colitis compared with the control group. Similarly, it was showed that an oral administration of capsazepine (CPZ), which is a specific antagonist for TRPV1, significantly reduced the overall macroscopic epithelial damage in mice colonic tissue after intraperitoneal DSS-administration (111). In *Trpv1*$^{-/-}$ mice, the DSS-induced colitis was less severe (112, 113), and a DSS-associated upregulation of SP-positive fibers was reduced (114), demonstrating a crosstalk between TRPV1 and neurogenic inflammation in colitis. In addition, it was reported that rats with TNBS-induced colitis exhibited a reduction of macroscopic damage score and MPO activity after CPZ enema (115). Recent data pointed that TLR4-knockout mice showed a less inflammatory infiltration and a decreased expression of TRPV1 in TNBS-induced colitis, indicating one possible function of TLR4 for mediating TRPV1 signaling under inflammatory conditions (84). As for other animal models, McVey et al. (116) suggested that an intraluminal administration of capsaicin in isolated ileal segments of rats led to an intestinal inflammation which could be reduced by CPZ. In T-cell-transfer colitis model, the activation of TRPV1 tended to exacerbate the intestinal inflammation, while the colitis was less severe when the TRPV1 in T cell was genetically or pharmacologically inhibited. The pro-inflammatory property of TRPV1 in T cells may be associated with the release of TNF-α, IFN-γ, IL-2, IL-4, IL-10, and IL-17 (117). In another study, capsaicin-induced TRPV1^{+} fibers-denervation ameliorated the intestinal inflammation in the T-cell-transfer colitis model (118), but the suppressive effect of noxious-dose capsaicin pretreatment only existed in 7–8 weeks old mice for several weeks after T-cell transfer, and these mice eventually developed colitis (128). It was further revealed that the severity of TNBS-induced colitis in the TRPV1^{+} fibers-denervated rats was drastically increased within 3–7 days after TNBS administration. Nevertheless, no significant difference of the colitis was found between denervated rats and normal rats in 14–21 days (129), reinforcing the concept that TRPV1^{+} fibers are involved in the early steps of colitis. Taken

together, these reports indicate that the activation of TRPV1 in colon is essential for the propagation of intestinal inflammation, and it might be a proximal event in the inflammatory process.

Noteworthy, several publications have reported the protective effects of TRPV1 in experimental colitis. It was exhibited that a local application of capsaicin and exogenous administration of CGRP ameliorated the colonic lesions in TNBS-induced rat colitis (122, 123). Massa et al. (124) stated that *Trpv1*$^{-/-}$ mice exhibited a worse outcome of colitis and lower expression of anti-inflammatory neuropeptides, such as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP), while the NF-κB and STAT3 signaling pathways were demonstrated to be enhanced (130). Moreover, TRPV1 was reported to restrain the initiation and progression of colon cancer (130). In DSS-treated rats, a daily administration of capsaicin was able to reduce the severity of colitis, while a desensitization of TRPV1^{+}-fibers dramatically worsened the inflammation (121). A protective role of TRPV1 was also identified in oxazolone-induced mice colitis, iodoacetamide-induced rat acute colitis, and formalin-induced rabbit acute colitis (125–127). The rather ambiguous findings concerning the roles of TRPV1 in colitis required further scrutiny.

Intriguingly, the expression of the TRPV1 was increased in the distal colon and rectum compared to the proximal colon in mice, and similar proximodistal gradient of CGRP/SP was detected (79, 131). Considering the anatomical distribution pattern of UC that often exhibits an ascending inflammation from rectal to proximal colon (4), the increased activity of TRPV1 and neuropeptides in distal colon might give rise to the increased susceptibility of distal colon to colitis and promote the spread of ascending inflammation. Such observations hint at the correlation between the diverse expression of TRP channels in the GI tract and the anatomical distribution pattern of IBD. Differences of microbial composition in certain gut regions and the crosstalk between microbiota and TRP channels are also likely to underlie the IBD anatomical distribution (6, 132). Likewise, it is reasonable to hypothesize that the diverse function of TRP channels in immune cells may be responsible for the distinct pathological pattern of UC and CD. A clear elucidation of this issue can facilitate a better understanding of the TRP channels and pathogenesis involved in IBD.

TRPA1

The studies regarding the role of TRPA1 in IBD all showed a upregulated TRPA1 expression in the colonic tissue of IBD patients (**Table 1**). In animal studies, mice with experimental colitis exhibited an increased TRPA1-mediated colonic neuropeptide release, while the experimental colitis appeared to be less severe after the inhibition of TRPA1 by the antagonist or genetic depletion (119). Additional studies suggested a protective role of TRPA1 in the GI tract. Pagano et al. (85) demonstrated that Cannabidivarin, a potent agonist of TRPA1, was able to attenuate the intestinal inflammation in biopsies from pediatric patients with active UC. In dinitrobenzene sulfonic acid (DNBS)- and DSS-treated mice, Cannabidivarin could also ameliorate neutrophil infiltration, intestinal permeability, cytokine production, and alter the dysregulation of gut microbiota (85). Kun et al. (71) reported that the ablation of TRPA1 aggravated DSS-induced colitis and the activation of TRPA1 reduced the release of neuropeptides, cytokines, and chemokines, such as IL-1β and macrophage chemoattractant protein-1 (MCP-1). Further support showed that TRPA1 activation reduced the level of TNF-α in colitis (2). Given that macrophage is the major producer for TNF-α, it may be that the TRPA1 in macrophages can suppress the release of TNF-α and modulate the anti-colitogenic effect, albeit the definite mechanism remains unclear. In addition, some evidence indicated that the expression of TRPA1 was increased in colonic stenotic regions of CD patients. The extent of intestinal inflammation and fibrotic changes in TNBS-treated TRPA1$^{-/-}$ mice were more prominent compare to WT mice and the fibrosis could not be suppressed by inhibitors. The underlying mechanism was considered to be based on the anti-fibrotic role of TRPA1 in intestinal myofibroblasts (74, 75). These observations hint a novel therapeutic target to relieve the fibrosis in IBD.

Whilst considering the highly co-expressive nature of TRPV1 and TRPA1 in colonic afferents, it is interesting to shed light on the interaction between TRPV1 and TRPA1 in colitis. A stimulation of TRPA1 in dorsal root ganglia could result in the activation of PKA and subsequent phosphorylation of TRPV1 (133), while the activation of TRPV1 in afferents could desensitize TRPA1 through phosphatidylinositol biphosphate (PIP_2) depletion (134). In IBD patients, a vast infiltration of TRPV1^{+}TRPA1^{+} T cells had been identified in inflamed colonic tissue (68). Bertin et al. (68) found that TRPV1^{+}TRPA1^{-} T cells were able to enhance T-cell receptor-induced Ca^{2+} influx and aggravated intestinal inflammation in IL-10 knockout mice and T-cell-transfer colitis models compared to TRPV1^{+}TRPA1^{+} T cells. However, the colitogenic properties of TRPV1^{+}TRPA1^{-} T cells were abrogated with pharmacological inhibition or genetic deletion of TRPV1 (68, 117), suggesting that TRPA1 inhibited TRPV1 activity in CD4^{+} T cells and consequently restrained the activity of CD4^{+} T cells. Thus, the role of TRPA1 in colitis could be either protective or damaging.

TRPV4

The TRPV4 mRNA expression and TRPV4 immunoreactivity in colon were remarkably upregulated in IBD patients (70, 72, 76), in particular, serosal blood vessels with active inflammation were more densely innervated by TRPV4-positive fibers, which often co-localized with the infiltrating CD45^{+} cells (73, 76). Meanwhile, TRPV4 activation could recruit macrophages and other immune cells through the induction of chemokines, such as IL-8 and MCP-1 (73). D'Aldebert et al. (73) indicated the upregulated colonic TRPV4 expression in DSS-treated mice. Intracolonic administration of the TRPV4 agonists (4alpha-phorbol-12,13-didecanoate or GSK1016790A) in mice activated NF-κB and activator protein 1 (AP-1) signaling pathway, resulting in exacerbated DSS-induced colitis and even transiently increased the paracellular permeability of epithelium and blood vessel, while TRPV4-knockout mice conferred a strong resistance to the colitis (73, 81). These results prove the deleterious effects of TRPV4 on mucosal inflammation. Conversely, a systemic or local administration of RN1734, a selective TRPV4

antagonist, remarkably relieved the TNBS-induced colitis (72), suggesting the benefit of attenuating inflammation through blocking TRPV4. The medications aiming at TRPV4 might be capable of alleviating intestinal inflammation in IBD.

TRPM2

The expression of TRPM2 in distal colon was increased in TNBS-treated rats (90). In *Trpm2*$^{-/-}$ mice, Yamamoto et al. (120) exhibited that the infiltration of immune cells and the severity of intestinal inflammation were ameliorated in DSS-induced colitis. The underlying mechanism might be that the Ca^{2+} influx was impaired in *Trpm2*$^{-/-}$ macrophages, thus affecting the activation of NF-κB pathway (120). This evidence reminds us that TRPM2 can exert pro-inflammatory effects in the colitis via its essential role in macrophages and NF-κB signaling pathway.

TRPM8

TRPM8 expression was demonstrated to be upregulated in IBD patients and in DSS- or TNBS-treated mice (77). The activation of TRPM8 with icilin significantly attenuated the experimental colitis, but *Trpm8*$^{-/-}$ mice were quite susceptible to colitis (62, 77). It was considered that TRPM8 performed its protective role in the intestine via restraining the release of TNF-α, IL-1, IL-6, and MCP-1, and inducing the release of CGRP (62, 77). TRPM8 activation could also reduce the TRPV1-dependent CGRP release in the gut (77), showing the ability of TRPM8 to suppress the TRPV1-associated inflammatory cascade. The reconstitution of *Trpm8*$^{-/-}$ macrophages in mice exerted a deleterious effect on DSS-induced colitis (60), exhibiting a protective property of TRPM8 in macrophages. These findings reinforce the anti-colitogenic function of TRPM8. Agonists for TRPM8 possibly serve as therapeutic strategies for alleviating intestinal inflammation.

POSSIBLE FACTORS BEHIND PRIOR CONTRADICTORY RESULTS

According to aforementioned researches, the roles of a certain type of TRP channels in IBD and experimental colitis tended to be bidirectional or even conflicting.

The human studies mainly concentrated on the expression of TRP channels in the colonic tissue of IBD patients, however, the results of these studies appeared to be contradictory, especially concerning the expression of TRPV1. Actually, TRP channels are widely but anatomically distinctly distributed in various tissues and cell types in the GI tract (6). The expression and function of TRP channels may also be diverse in different subtypes and phases of IBD, and vary among individuals (79). However, the tissue samples of previous studies were acquired at multiple sites of the GI tract and the sample sizes were relatively small. Therefore, further researches that collect sufficient samples from a certain GI region and separately analyze the expression of TRP channels in UC and CD are warranted. Noteworthy, in IBD genome-wide association studies (GWAS), no single nucleotide polymorphism of TRP channel- related genes has been identified in correlation with IBD (135). However, the functions of TRP channels in the GI tract are tightly associated with the content in GI lumen and molecules that possess significant polymorphisms in IBD GWAS, such as TLR4 (136). Additional IBD GWAS studies are needed to uncover specific factors including dietary intake or microbiota in IBD patients in order to explore the definite role of TRP channels polymorphisms in IBD.

Amongst the animal studies regarding TRP channels, researches on TRPV1 and TRPA1 were dominated, thus the majority of conflicting data was related to the functions of these two channels in experimental colitis. Many elements were probably responsible for the paradox.

First, the limitations of the animal models and experimental methods applied in the studies should be considered. Unfortunately, the ideal IBD models that completely mimic the multifactorial chronic disease do not exist and the pathophysiological mechanisms underlying different models are diverse. Also, animals of different strains, species, or ages have their distinct susceptibility to the stimulus, resulting in various demonstrations in experimental colitis (137). Due to the variety of animals and colitis models being used in prior studies, the animal models with distinct characteristics *per se* might accidentally account for the discrepant actions of TRP channels. Meanwhile, the different experimental methodology and drug administration could lead to opposing results. For example, capsaicin, the agonist for TRPV1, has dual effects that the low-dose capsaicin only affects a variable number of TRPV1-expressing nerves, while the high-dose capsaicin results in nerve desensitization (6), indicating the influence of the dose of stimuli on TRP channels. Moreover, the function of TRP channels might be affected by the changes in the microenvironment of the gut induced by agonists or antagonists (138), thus masking the true effects of TRP channels. It was revealed that TRP channels activation could be achieved via overexpression, phosphorylation, or recruitment to the plasma membrane (68). Additional experiments regarding the mode of TRP channels activation induced by specific stimulus may provide a rational view on the interaction between the stimulus and TRP channels.

Second, in addition to the exogenous stimuli applied in studies, there appears to be various endogenous ligands acting on TRP channels, thus influencing the results of experiments. Compounds, such as prostaglandin metabolites, nerve growth factor, and products of oxidative stress can mediate TRPV1 and TRPA1 (5, 95, 139), making it difficult to attribute the results observed in studies to the stimulation of exogenous chemicals or to the stimulation of endogenous mediators. Actually, besides the administration of exogenous stimuli for TRP channels, the activation of TRP channels in IBD is also based on the stimulating effects of multiple endogenous mediators which are synthesized and released within the progress of colitis. Some of these compounds may potentialize TRP channels via the GPCR pathway (5). Hence, it is likely that TRP channels play a role not only in the initiation but also in the regulation of the intestinal inflammation, while the exact mechanism is unclear and needs further explorations.

Third, the functions of neurogenic inflammation and immune responses triggered by TRP channels activation are complicated. The neurogenic inflammation is featured of the release of

CGRP and SP, but the effects of these two neuropeptides on intestinal inflammation were not clearly elucidated and tended to be contradictory. The differences in the concentration of neuropeptides and the expression of receptors might contribute to the discrepancy (4). SP was reported to sensitize TRPV1 during colitis and affect the functions of TRPV1 (78), suggesting a possible feedback sensitization loop between neuropeptides and TRP channels. In addition, a range of evidence showed that neuropeptides, such as somatostatin, galanin, opioid peptides, VIP, and PACAP could participate in the inflammation and regulate the inflammatory responses (130, 140). It is warranted to explore whether there is an association between these neuropeptides and TRP channels in colitis. As for immunity, besides the TRP channels-expressing immune cells, some non-immune cells may have TRP channels in the colitis. For example, the expression and function of TRPA1 were identified in fibroblasts which could transform into myofibroblasts and contribute to the regulation of intestinal inflammation (74, 75, 105). However, the definite involvement of myofibroblasts in colitis was poorly understood. Additional explorations are necessary to reveal other TRP channels-expressing cells that play a role in colitis.

Fourth, the TRP channels may interact with various cellular pathways. For instance, the inhibition of TRPV1 could lead to an increased availability of anandamide, and then induced downstream effects on NF-κB and TNF-α and affected bowel motility via the receptor for anandamide (78, 124). Meanwhile, anandamide could also act on TRPV1 and regulate a protection against intestinal inflammation (78, 124), suggesting a potential synergy between TRP channels and other cellular pathways in some settings. Similar to the interaction between TRPV1 and TRPA1, the possible crosstalk between other subtypes of TRP channels is also worth noting. Further studies are warranted to elucidate the comprehensive regulatory network induced by the stimulation of TRP channels in colitis.

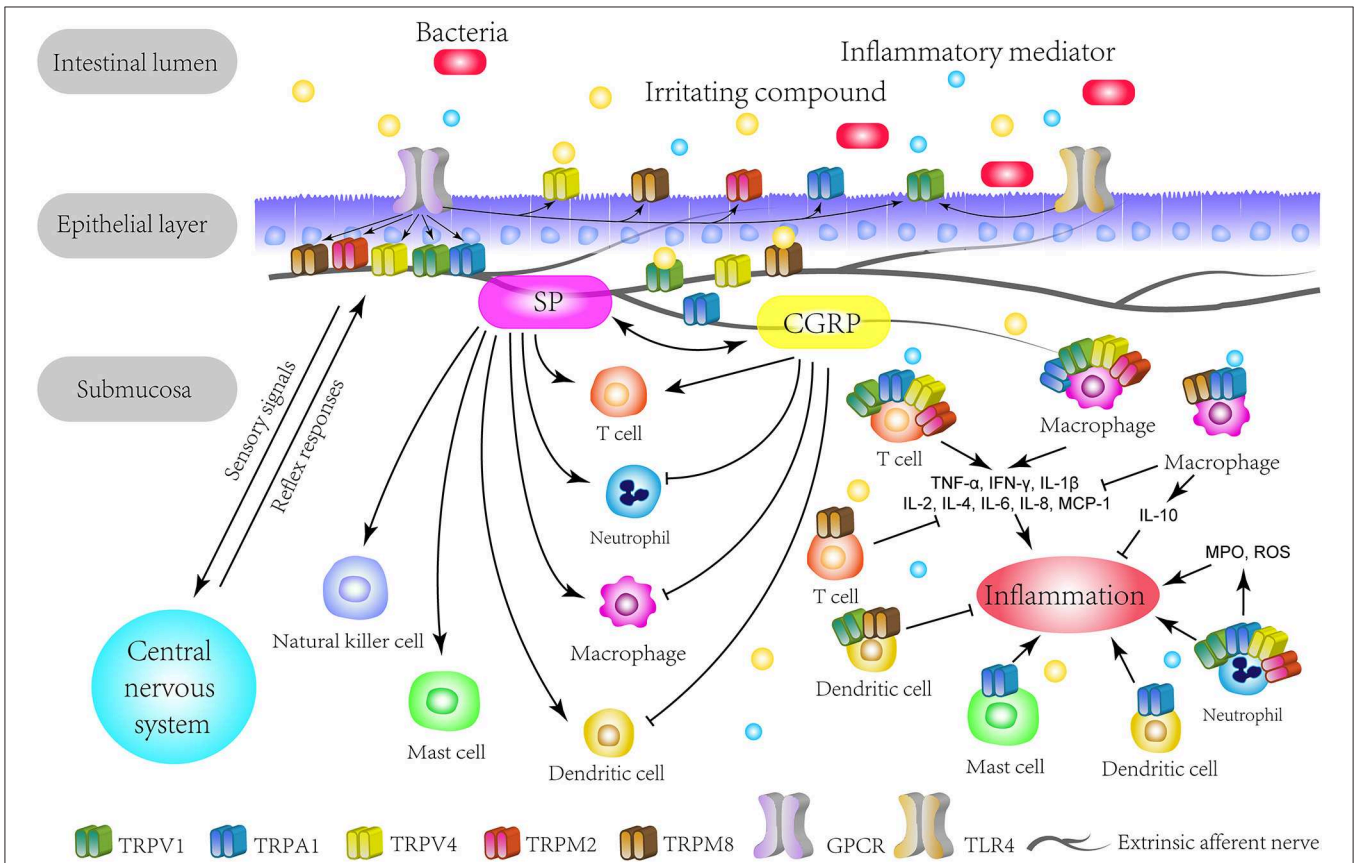

FIGURE 1 | The overview of TRP channels involved in IBD. The TRP channels mainly express themselves on extrinsic primary afferents in the intestine. TRP channels directly detect various stimuli in the intestinal lumen and act as secondary transducers for GPCR. Specially, TRPV1 and TRPA1 can crosstalk with microbiota through TLR4 or in a TLR4-independent manner. Upon activation, TRP channels transduce the sensory signal to the central nervous system and lead to autonomic reflex responses. This mechanism could be enhanced by inflammatory mediators and be responsible for the visceral hypersensitivity on pathological conditions. Meanwhile, the activation of TRP channels triggers neurogenic inflammation with neuropeptides, such as CGRP and SP, which can interact with immune cells. On the other hand, TRP channels express on multiple immune cells and regulate their functions, thus promoting or restraining the initiation or process of inflammation. Therefore, based on the immunomodulatory effects, TRP channels play a role in the immune pathogenesis of IBD. GPCR, G-protein coupled receptors; TLR4, Toll-like receptor 4; SP, substance P; CGRP, calcitonin-gene-related peptide; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; MCP-1, macrophage chemoattractant protein-1; MPO, myeloperoxidase; ROS, reactive oxygen species.

In general, the stimulation of TRP channels and a serial cascade of events are like double-edged swords in the intestinal inflammation that exert colitogenic or anti-colitogenic effects in different situations, influenced by a myriad of interactions amongst stimuli, neuropeptides, and the immunity. It is a challenge to figure out the accurate action of a certain type of TRP channels in a specific immune or cellular pathway. Intriguingly, Cohen et al. (22) applied *in vivo* optogenetic strategy to selectively stimulate cutaneous $TRPV1^+$-neuron and showed that the afferent activation was based on a nerve reflex. In this study, the activation of TRPV1 was in the absence of other inflammatory stimuli, thus specifically demonstrating the precise role of TRPV1 in the afferents and its immunity-triggering effects. Considering the intense interactions amongst various factors in previous researches, utilizing novel technologies that can efficiently eliminate interferences is a promising strategy for further studies on TRP channels.

THERAPEUTIC VALUES OF TRP CHANNELS FOR IBD

As discussed, the effects of TRP channels in IBD have been increasingly appreciated, it is intriguing for researchers to explore their therapeutic values for relieving inflammatory VHS and intestinal inflammation. Pharmacologically, the modulating agents for TRP channels include antagonists and stimulant agonists (141). In particular, antagonists for TRP channels exert a specific effect on modification of ion channel, and stimulant agonists facilitate the desensitization of sensitive afferents (142). However, owing to the wide distribution and various physiological roles of TRP channels within and outside the GI tract (143), the modulation of TRP channels may result in pronounced side effects, such as hyper-thermic effect and impaired injurious-heat perception generated by TRPV1 antagonists (144, 145). Therefore, it is vital to develop the stimulus-specific blockers for TRP channels that specifically act on the aberrant function while sparing the physiological function.

Besides targeting TRP channels directly, it is worth noting that aiming at the stimulus and downstream pathways for GPCRs tends to be another valuable method of restraining the action of TRP channels, especially in the inflammatory process. A novel class of endogenous lipid mediators named resolvin, which are generated from immune cells, such as eosinophils and neutrophils, are of particular interest and have the ability to suppress the function of TRP channels including TRPV1, TRPA1, and TRPV4 (146, 147). The anti-inflammatory effects of resolvin are likely based on the activation of inhibitory GPCR that subsequently suppresses the GPCR-associated sensitization or activation of TRP channels (148), showing the feasibility for

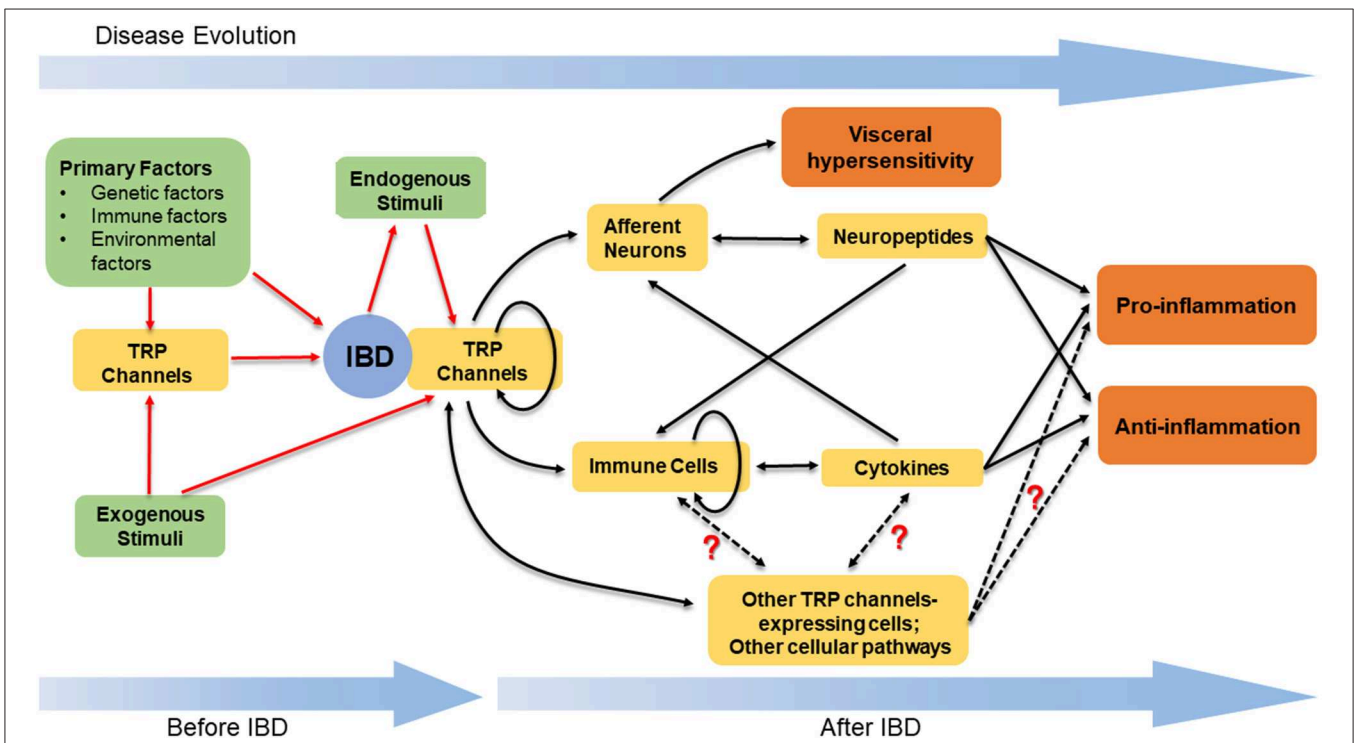

FIGURE 2 | The stimulation of TRP channels and the downstream regulatory network in IBD. TRP channels, together with other primary factors, play a role in the pathogenesis of IBD. The stimulation of TRP channels is based on the exogenous stimuli and the endogenous stimuli. The latter mainly refer to the mediators synthesized and released within the progress of colitis. The activated TRP channels can induce the release of neuropeptides and cytokines, thus leading to the pro- or anti-inflammatory effects. In addition to neurons and immune cells, there are other TRP channels-expressing cells and cellular pathways contributing to regulate the intestinal inflammation, while the definite functions of these cells and pathways are unclear. The complicated crosstalk amongst the neuropeptides, cytokines, TRP channels-expressing cells, and diverse cellular pathways results in the various but elusive effects induced by the stimulation of TRP channels in IBD.

inhibiting TRP channels through regulating GPCRs. Overall, treatment strategies targeting TRP channels and their signaling pathways predict a promising future for alleviating the symptoms and improving the prognosis of IBD. More studies are warranted to identify the efficacy and safety of these therapeutic approaches.

To conclude, TRP channels are not only widely distributed on neurons in the GI tract, functioning as detectors for stimuli and triggers for neurogenic inflammation, but also expressed in multiple immune cells and modulate immune responses (**Figure 1**). Accumulated evidence has supported an important association between TRP channels and IBD. Although different types of TRP channels exert distinct effects, it is evident that TRP channels are involved in the VHS and the pathogenesis of IBD through a complicated and elusive regulatory network (**Figure 2**). The inhibition or activation of selected TRP channels can restrain the development of VHS and inflammation in the context of colitis. Therefore, TRP antagonists and agonists tend to constitute an attractive target in IBD treatment and need further attention.

AUTHOR CONTRIBUTIONS

YC and HZ reviewed the literature and outlined the overall manuscript. YC wrote the manuscript. JM and MZ helped to write the manuscript. AM and HZ revised the manuscript. HZ supervised the preparation of the draft and edited it and worked as a corresponding author. All authors approved the final version.

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REVIEW
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TRPML Cation Channels in Inflammation and Immunity

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Background: In 1883, Ilya Mechnikov discovered phagocytes and established the concept of phagocytosis by macrophages. In 1908, he was awarded the Nobel Prize in Physiology/Medicine for his findings, which laid the foundations for today's understanding of the innate immune response. Only in the 1960s, Max Cooper and Robert Good significantly advanced our understanding of the immune system by demonstrating that B- and T-cells cooperate to regulate the adaptive immune response. Both, innate and adaptive immune response are essential to effectively protect the individual against infectious agents, such as viruses, bacterial or insect toxins, or allergens. Innate immune responses occur rapidly upon exposure to noxious or infectious agents or organisms, in contrast to the adaptive immune system that needs days rather than hours to develop and acts primarily on the basis of antigen-specific receptors expressed on the surface of B- and T-lymphocytes. In recent years, it has become evident that endosomes and lysosomes are involved in many aspects of immune cell function, such as phagocytosis, antigen presentation and processing by antigen-presenting cells, release of proinflammatory mediators, e.g., by mast cells, or secretion of the pore-forming protein perforin by cytotoxic T lymphocytes. Several lysosomal storage disorders (LSDs) have been associated with defects in immune system function or immune system hyperactivity, such as Gaucher, Fabry, or Niemann-Pick type C1 disease, mucopolysaccharidoses (MPS), gangliosidosis, or juvenile neuronal ceroid lipofuscinosis (JNCL). Beside accumulating evidence on the importance of endolysosomes in immune cell function, recent results suggest direct roles of endolysosomal ion channels, such as the TRPML channels (mucolipins), which are members of the transient receptor potential (TRP) superfamily of non-selective cation channels, for different aspects of immune cell function. The aim of this review is to discuss the current knowledge about the roles of TRPML channels in inflammation and immunity, and to assess their potential as drug targets to influence immune cell functions.

Advances: Examples of recently established roles of TRPML channels in immune system function and immune response include the TRPML1-mediated modulation of secretory lysosomes, granzyme B content, and tuning of effector function in NK cells, TRPML1-dependent directional dendritic cell (DC) migration and DC chemotaxis, and the role of TRPML2 in chemokine release from LPS-stimulated macrophages.

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Outlook: Although our understanding of the functional roles of TRPML channels in inflammation and immunity is still in its infancy, a few interesting findings have been made in the past years, encouraging further and more detailed work on the role of TRPMLs, e.g., in intracellular trafficking and release of chemokines, cytokines, or granzyme B, or in phagocytosis and bacterial toxin and virus trafficking through the endolysosomal machinery.

Keywords: immune system, immune cells, TRPML cation channels, mucolipin, lysosome

INTRODUCTION

TRP channels are a very diverse and heterogenous group of cation channels. With few exceptions, the majority of them are expressed at the plasma membrane. One subfamily of the TRP channels, the mucolipin or TRPML/MCOLN subfamily comprises three members in mammalian genomes, TRPML1, 2, and 3 which are all found to be expressed in the endolysosomal system, i.e., in early and late endosomes, in recycling endosomes and in lysosomes to various degrees. All three channels are regulated by the phosphoinositide $PI(3,5)P_2$, a major component of endolysosomal membranes and by luminal pH (1, 3–5). Thus, TRPML1 is most active at highly acidic pH as found in lysosomes while TRPML2 and TRPML3 are more active at higher pH as it occurs in early and recycling endosomes (1, 3–5). TRPML1 activity already decreases under mild acidic conditions (pH 5.5; **Figure 1**) and is lowest at neutral conditions (4). A recent hypothesis suggested that elevated lysosomal pH may hyperactivate the TRPML1 calcium channel (2), is not supported by endolysosomal patch-clamp evidence (**Figure 1**).

Several functional roles have been proposed for TRPML1, e.g., in gastric acid secretion by parietal cells (6, 7), as a ROS sensor in lysosomes to regulate autophagy (8, 9), as an autophagy regulator through calcineurin and TFEB (10), or in membrane repair, e.g., repair of the sarcolemma to prevent muscular dystrophy (11). Loss or dysfunction of TRPML1 causes the rare lysosomal storage disorder mucolipidosis type IV, major hallmarks of which are severe neuro- and retinal degeneration, mental and psychomotor retardation, hypotonia, achlorhydria, and premature death. The observation that macromolecules, e.g., certain lipids (sphingolipids, phospholipids) and mucopolysaccharides as well as metals like iron or zinc accumulate in patient cells suggested roles for TRPML1 in metal cation release, in addition to the release of calcium and other cations from lysosomes as well as a critical function for the overall integrity of lysosomes including their roles in intracellular trafficking, fission/fusion and autophagy. While most research has focused on TRPML1, due to its clear association with human disease, function and pathophysiological relevance of the related channels TRPML2 and TRPML3 are less well-understood, with no links to human (genetic) disease so far. In mice, gain-of-function variants of TRPML3 have been shown to cause deafness, circling behavior, and coat color dilution due to the loss of inner ear hair cells and melanocytes following intracellular calcium overload. (12–16). $TRPML2^{-/-}$ and $TRPML3^{-/-}$ mice are viable and according to genomic databases, human TRPML2 and TRPML3 knockouts do also exist. Nothing however is known about health issues they may have. In contrast to the ubiquitously expressed TRPML1, the expression of TRPML2 and TRPML3 is restricted to certain cell types including a number of immune cells. Recently, several roles of immune cell function could be linked to TRPML channels, e.g., a role of TRPML1 for the phagocytosis of large particles (17), the migration of dendritic cells (18), or for the tuning of the functional potential in self-KIR+ natural killer (NK) cells (19) while TRPML2 was found to play a role in chemokine and cytokine secretion by macrophages (5, 20). In the following, the current state of knowledge on TRPML channel expression and function in different immune cells shall be discussed (**Figure 2**).

CHAPTER I: INNATE IMMUNE SYSTEM

Macrophages

Macrophages are phagocytic cells. They express receptors on their surface called pattern recognition receptors (PRRs), which are able to detect varying molecular structures of microbes, referred to as pathogen-associated molecular pattern (PAMP). One example for a PRR are the toll-like receptors (TLRs). Sun et al. (20) have recently discovered that activation of these receptors leads to a strong increase of both mRNA and protein levels of the TRPML2 channel. They tested different TLR activators, including LPS (a component of bacteria that activates TLR4), zymosan A (a component of fungi that activates TLR2), loxoribine which activates TLR7, and resiquimod (R848) which activates TLR7/8. The latter two are involved in recognizing viruses. In all cases and both in primary (murine microglia) and cultured cells (RAW 264.7) an upregulation of TRPML2 was found, suggesting that the TRPML2 channel is involved in the host defense against different pathogens like bacteria, viruses and fungi.

The binding of such particles to the PRRs or the binding of IgG-opsonized particles to the FCγ-receptor trigger an important process in the innate immune response: phagocytosis. During this process the particle is surrounded by the macrophage with the help of extensions of the cytoplasm (i.e., pseudopods) and is completely enclosed with membrane in structures called phagosomes. These phagosomes mature by fusing with lysosomes into phagolysosomes, which have an acidic luminal pH and contain hydrolytic enzymes to break down the engulfed particles. Samie et al. (17) have first shown that TRPML1 regulates the ingestion of large particles by providing the membrane for the cell surface, necessary for phagosome formation. They suggested the following cascade of events and signaling pathways: after

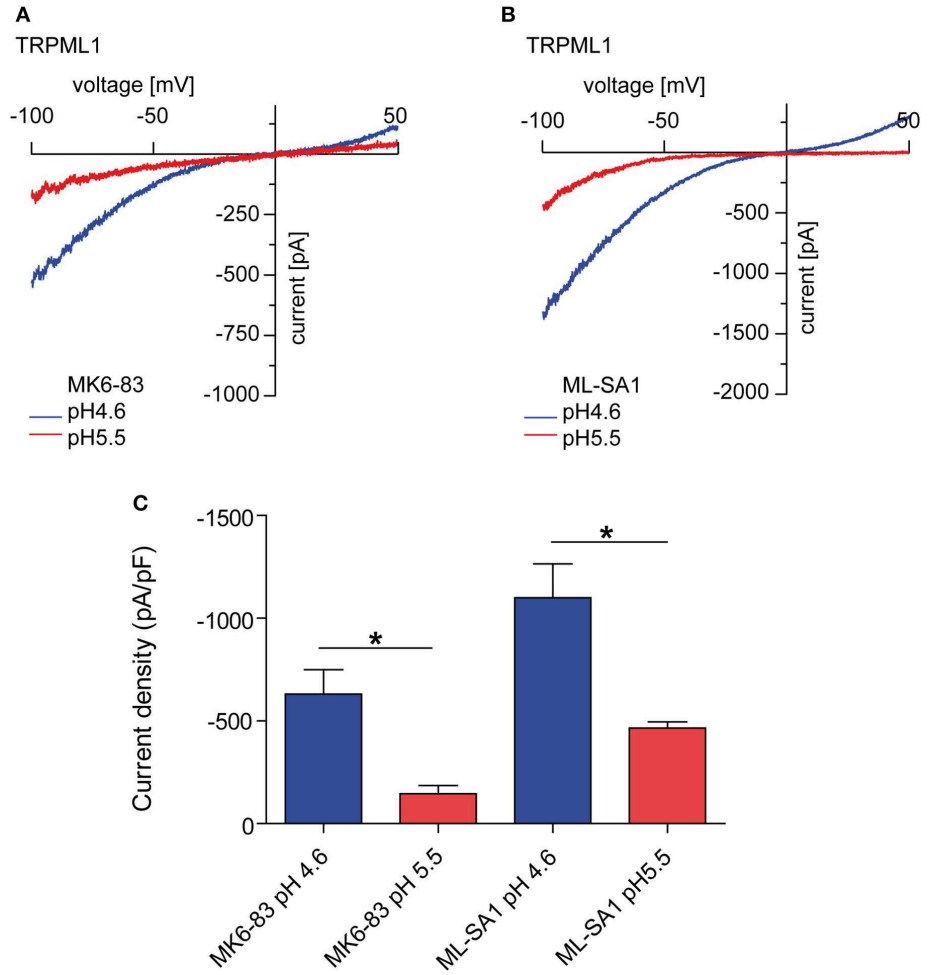

FIGURE 1 | Endolysosomal patch-clamp experiments showing TRPML1 currents induced by MK6-83 and ML-SA1 (10 μM, each) at different luminal pH. **(A)** Representative MK6-83 elicited currents from TRPML1 expressing HEK293 cells at pH 4.6 or pH 5.5. **(B)** Representative ML-SA1 elicited currents from TRPML1 expressing HEK293 cells at pH 4.6 or 5.5. **(C)** Statistical summary of data as shown in **(A,B)**. Shown are average current densities at −100 mV (mean ± SEM). In all statistical analyses, the mean values of independent experiments ($n > 4$, each) are shown as indicated. *$p < 0.05$, Student's *t*-test, unpaired.

particle binding of the phagocytic cell the endolysosomal PI-5 kinase, PIKfyve is stimulated and phosphorylates PI(3)P to form $PI(3,5)P_2$, which is then present in sufficient amounts to activate the TRPML1 channel in lysosomes. The resulting Ca^{2+} release induces lysosomal exocytosis at the site of the formation of the phagocytic cup. Such fusion events with the plasma membrane increase the surface area of the phagocytosing macrophage which is essential for the engulfment of large particles. Thus, TRPML1 plays a crucial role in the initial steps of phagocytosis and enables the innate immune system to eliminate large particles quickly (17). However, the TRPML1 channel does not only seem to be involved in phagosome formation but also in phagosome maturation, i.e., the fusion process of phagosomes with lysosomes. This was proposed by Dayam et al. (21) who analyzed phagocytosis in TRPML1-silenced or PIKfyve-inhibited cells in which the phagolysosomal biogenesis was impaired, because the phagosomes and lysosomes were not able to fuse after docking. The phagolysosome maturation could be rescued by Ca^{2+} ionophores like ionomycin. This suggested that both PIKfyve and TRPML1, more specifically the TRPML1-mediated Ca^{2+} release from lysosomes, were key mediators in phagosome maturation. PIKfyve would presumably act upstream of TRPML1, since it produces the endogenous TRPML1 activator $PI(3,5)P_2$ (21). Impaired ability to eliminate the ingested bacteria was also observed in PIKfyve and TRPML1 deficient macrophages. This is in line with previous findings showing reduced levels of cathepsin D, a common lysosomal enzyme, in phagosomes of PIKfyve inhibited cells (22). Mycobacteria for example, interfere with the PI(3)P metabolism and thereby induce an impaired phagosome maturation, enabling them to prevent degradation through fusion with lysosomes (23).

Furthermore, TRPML1 has been found to enhance the degradative function of lysosomes during phagocytosis through TFEB (24). Gray et al. speculated that the protein phosphatase calcineurin, activated by the Ca^{2+} release through TRPML1, dephosphorylates TFEB to induce its translocation to the nucleus.

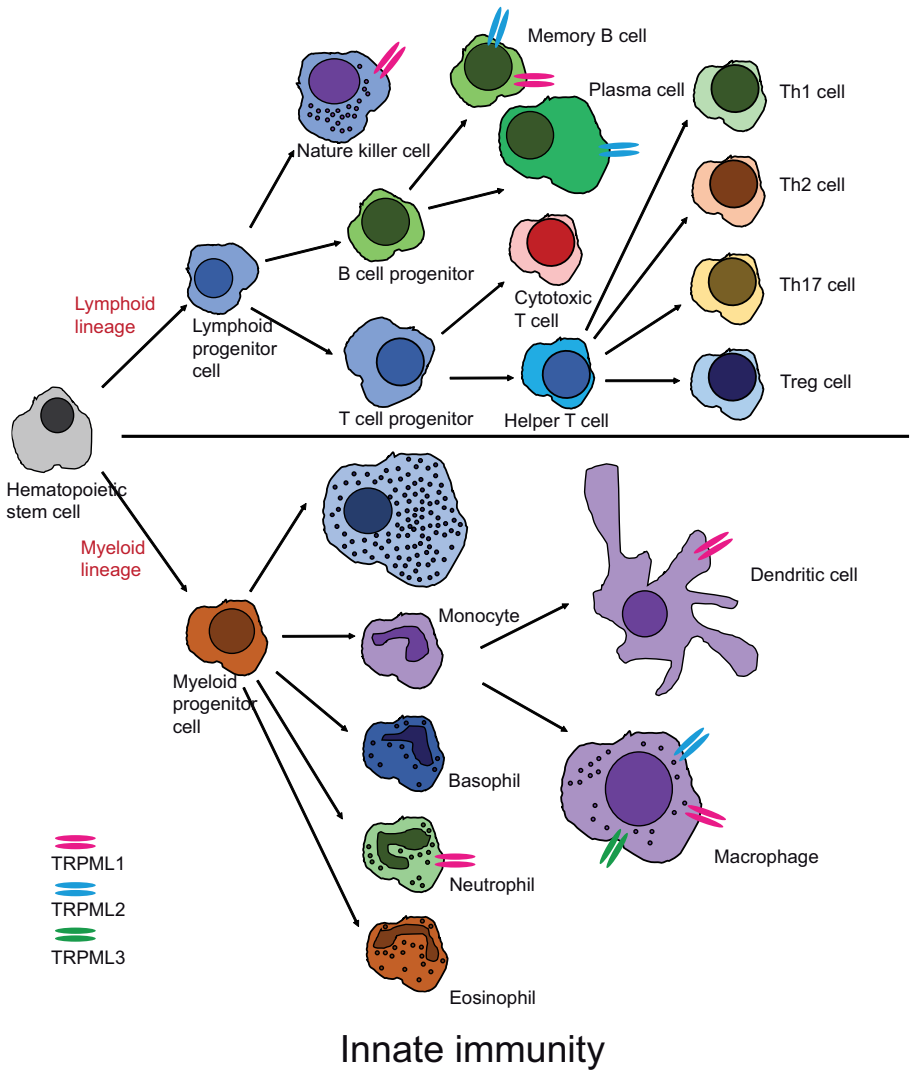

FIGURE 2 | Cartoon showing expression of TRPML channels in different cells of the innate and adaptive immune system. Expression has either been confirmed by direct patch-clamp experimentation of endo-lysosomes isolated from these cells or by other published experimental evidence as outlined in chapter I and II.

There, it acts as a transcription factor for the lysosomal enzyme cathepsin D and the H subunit of the V-ATPase, which regulates the acidic and hydrolytic environment in the phagolysosomes. This enhances the degradation ability of the existing lysosomes, so that bacteria are eliminated more efficiently in the phagolysosomes. They showed this for opsonic phagocytosis mediated by the FCγ-receptor and for non-opsonic phagocytosis as well. Hence, the TRPML1 channel may not only be necessary for the initial steps of phagocytosis but also for later steps in the phagocytosis process like phagosome maturation and efficient degradation of engulfed particles. This is important, in particular when large numbers of bacteria are present.

Cathepsins and their proteolytic functions are essential for the degradation of bacteria captured in lysosomes during phagocytosis. The role of different cathepsins has been studied in great detail. Cathepsin D, besides its degradative capacity, may also induce bacterial killing by activating apoptosis in alveolar macrophages after take-up of pneumococci by phagosomes (25). Mice lacking cathepsin E are more sensitive to infections by Staphylococcus aureus and Porphyromonas gingivalis (26). Qi et al. (27) detected a role of cathepsin B in the defense of macrophages against Francisella novicida. Thus, bone marrow-derived macrophages (BMDM) from cathepsin B deficient mice were able to clear bacteria more efficiently and these mice were protected from lethality (27) which may be due to the down-regulation of mTOR activity and prevention of TRPML1 degradation (28). Decreased mTOR activity means that TFEB is no longer kept in the cytosol by phosphorylation but tanslocates

into the nucleus (29), where it promotes the transcription of genes encoding lysosomal proteins and the kinase ULK1 as inducer of autophagy. In addition, TRPML1 contributes to the regulation of TFEB through the protein phosphatase calcineurin. Both, enhanced lysosomal biogenesis and induction of autophagy promote the ability of cathepsin B deficient macrophages to get rid of ingested bacteria. TRPML3 has also been shown by several groups to be involved in autophagy (30–32), suggesting that as described above for TRPML1 it may play similar roles in certain cell types. Evidence for an evolutionary conserved role of TRPMLs in bacterial clearance by macrophages comes from drosophila work. Thus, Wong et al. (33) found that flies lacking trpml in macrophages exhibited compromised clearance of *E. coli*, a phenotype similar to the one observed in macrophages deficient of ClC-b, the drosophila homolog of the mammalian late-endosomal/lysosomal Cl^-/H^+ transporter CLCN7. Wong et al. further showed that ClC-b-mediated Cl^- transport into endolysosomes was necessary for the accumulation of luminal Ca^{2+}, which, when released through TRPML, drives the delivery of phagocytic cargo to lysosomes for degradation. Beside phagocytosis, macrophages play another important role within the innate immune system as they produce and secrete a variety of cytokines and chemokines after stimulation. Inflammatory cytokines and chemokines are signaling molecules that attract other immune cells to the inflammation herd. Apart from this, cytokines may also determine the polarization state of macrophages, where classically activated macrophages (M1) are stimulated by interferon-γ, TLR ligands, or microbial substrates like LPS. This is connected with an increased production of proinflammatory cytokines, such as TNF-α, IL-6, IL-1, IL-23 and reactive oxygen/nitrogen species. These macrophages have a high pro-inflammatory and microbicidal activity. Alternatively, activated macrophages (M2) are stimulated by IL-4 and IL-13 and are involved in tissue repair, suppression of inflammation and tumor progression by secreting the anti-inflammatory cytokines IL-10 and TGF-β. A third class of macrophages are regarded as tumor-associated macrophages. They differentiate from circulating monocytes after migrating into tumor tissue upon stimulation with IL-4, IL-10, or IL-13 and exhibit pro-tumorigenic functions (34, 35).

The release of cytokines by macrophages is a highly regulated process. Time, volume and site of release must be controlled, but first cytokines need to be transported to the plasma membrane. To this end, they make use of the cell's trafficking machinery including the trans-Golgi network (TGN) and the endolysosomal system, especially recycling endosomes (36). In the latter ones high levels of TRPML2 are found (37, 38). Sun et al. demonstrated that lack of TRPML2 in BMDM leads to an intracellular accumulation and decreased secretion of CCL2 [chemokine (C-C motif) ligand 2, also called monocyte chemoattractant protein 1 (MCP1)] (20). They also found reduced macrophage recruitment in $TRPML2^{-/-}$ mice after LPS stimulation which is in accordance with the role of CCL2 as chemoattractant recruiting additional immune cells to the site of inflammation (39). The link between TRPML2 and CCL2 was further investigated by Plesch et al. who developed ML2-SA1, a selective agonist for TRPML2, and found that activation of the channel directly stimulates the secretion of CCL2 from BMDM. The release is most likely mediated via the early/recycling endosomal pathway, since activation of TRPML2 by ML2-SA1 promotes trafficking through this pathway. In addition, Plesch et al. found that direct activation of TRPML2 leads to enhanced recruitment of macrophages (5). These findings by Sun et al. and Plesch et al. strongly suggest that TRPML2 plays a crucial role in the release of CCL2 and likely other chemokines as well as in the stimulation of macrophage migration.

Neutrophils

Neutrophils are another essential cell type in the innate immune system. They are the first cells to arrive at the site of inflammation or infection as they have a high chemotactic ability (40). They carry out numerous functions: First, they express and secrete cytokines that recruit more immune cells like macrophages to amplify the inflammatory response (41). Second, they perform phagocytosis of pathogens resulting in phagosome formation and fusion with lysosomes to kill engulfed bacteria (41, 42). Third, they release a variety of antimicrobial proteins (cathepsins, neutrophil elastase, lysozyme, NADPH oxidase) that help eliminate the pathogens (41).

In 2017, Dayam et al. showed the lipid kinase PIKfyve to play an essential role in coordinating various neutrophil functions. As mentioned above, PIKfyve is responsible for the synthesis of $PI(3,5)P_2$, an endogenous activator of TRPML channels that results in lysosomal Ca^{2+} release also in neutrophils. The authors further found that the PIKfyve-TRPML1-Ca^{2+} axis regulates phagosome maturation, i.e., the fusion of phagosomes and lysosomes. They also found that inhibition of this axis blocks phagosome maturation (43). These findings are in line with their previous works and other works by Kim et al. (22), reporting on the same PIKfyve-TRPML1-Ca^{2+} pathway to trigger phagosome-lysosome-fusion in macrophages (21, 22). Furthermore, Dayam et al. found that PIKfyve activity is essential for ROS generation and chemotaxis mediated through the stimulation of Rac GTPases. Taken together these data suggest that PIKfyve and TRPML1 are important regulators of several neutrophil functions that are critical for the rapid response of the innate immune system (43).

Dendritic Cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) which play key roles in the adaptive immune response (44, 45). After capturing pathogenic antigens via macropinocytosis, immature DCs turn into mature DCs. They start to process and express high level of antigens, stimulatory molecules, and cytokines that are able to induce the T-cell response after migrating to lymph nodes, where they present the antigens to activate T-cells (46–48). Intracellular Ca^{2+} signaling from endolysosomal TRPML1 channel is involved in DC functions, such as regulating TLRs for nucleic acid sensing and DC migration (18). TLRs can recognize structurally conserved molecules from pathogens. Membrane lipids from pathogens can be recognized by cell surface located TLR1, TLR2, TLR4, and TLR6 while nucleic acids from pathogens can be recognized by intracellular located TLR3, TLR7, TLR8, and TLR9 (49–51). TRPML1 has been

reported to be involved in the TLR7 response to single strand RNA (ssRNA). Li et al. demonstrated that loss of TRPML1 function or inhibition of $PI(3,5)P_2$ generating PIKfyve blocks the transportation of ssRNA into lysosomes while activation of TRPML1 by the TRPML channel agonist ML-SA1 enhances this process. Impaired transportation of ssRNA leads to an impaired TLR7 response, demonstrating that the $P(3,5)P_2$-TRPML1 axis plays an important role in this process (52).

Regarding the role of TRPML1 in DC migration, Bretou et al. (18) found that after the down regulation of macropinocytosis in DCs upon sensing the pathogens, lysosomal calcium signaling through TRPML1 regulates DC chemotaxis and migration to lymph nodes by controlling the motor protein myosin II retrograde flow at the cell rear to induce fast and directional migration. TRPML1 mediated calcium signaling further initiates TFEB translocation from cytoplasm to nucleus which will further maintain TRPML1 expression forming a positive feedback loop mediated by the TFEB-TRPML1 axis. Therefore, activation of the TFEB-TRPML1 axis via the inhibition of macropinocytosis is a critical step to switch DCs from patrolling mode to fast migration mode and homing into lymph nodes (18).

Natural Killer Cells

Natural killer (NK) cells differentiate from the same lymphoid progenitor as T- and B-cells but are classified as innate immunity lymphocytes because of their rapid response to pathogens, especially viruses and fungi. NK cells are also functionally active against tumor cells (53, 54). Killing of target cells is mediated by cytotoxic factors, such as perforin and granzymes which are secreted from lysosome related organelles called lytic granules in NK cells (55, 56). Besides killing target cells directly through cytotoxicity, NK cells can indirectly contribute to immune defense via secretion of cytokines, such as interferon-γ (IFN-γ) or tumor necrosis factor–α (TNF-α) to regulate antigen-presenting cell function and T cell responses (57). NK cell activity is regulated by the dynamic balance between activating and inhibitory signals generated from a combination of germ-line encoded receptors which recognize the ligands expressed on the target cell surface. These determine whether or not the NK cell will kill the target cell (58, 59). Major histocompatibility complex (MHC) class I molecules are antigen-presenting molecules and critical for adaptive immune responses, which can be recognized by inhibitory receptors, such as killer cell Ig-like receptors (KIR), Ly49, and CD94/NKG2A on NK cells (60, 61). Decrease of MHC class I expression occurs when cells are infected by virus or under cellular transformation. This situation is called "missing-self" and can be detected by NK cells and promote NK cell cytotoxicity and cytokine production to selectively kill the target cell (57, 62). A process called NK cell education is the interaction between self-MHC and inhibitory receptors on NK cells to calibrate NK cell effector capacities (63). Goodridge et al. have recently reported that TRPML1 is involved in this process by modulating secretory lysosomes, granzyme B content, and by regulating effector function in NK cells (19). Goodridge et al. found that silencing of TRPML1 or pharmacological interference with PIKfyve resulted in enlarged lysosomes with increased granzyme B content and higher effector function, representing the educated state of NK cells. Therefore, these findings establish a link between NK cell education and remodeling of the lysosomal compartment. They also suggest a potential way to increase NK cell function via manipulating calcium homeostasis within lysosome related organelles (19).

CHAPTER II: ADAPTIVE IMMUNE SYSTEM

B Cells

B cells form a vital part of the adaptive immune system. They cooperate with other immune cells, such as T-cells, macrophages, and dendritic cells to eliminate foreign antigens. B-cells operate by producing and secreting millions of different antibody

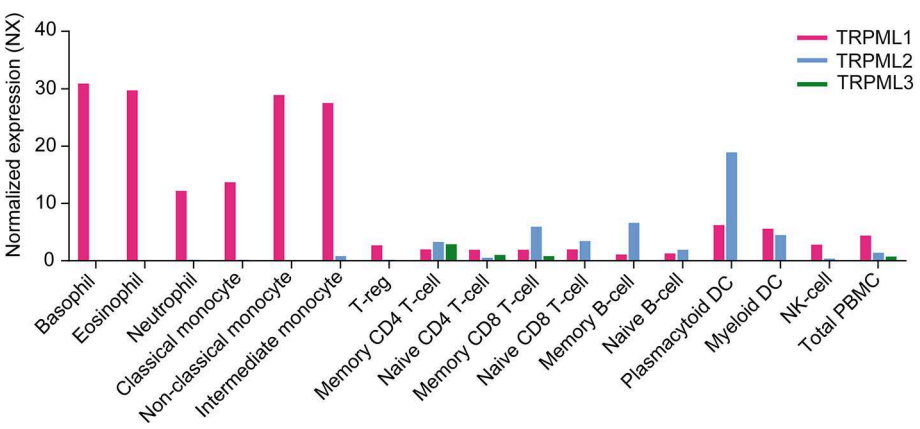

FIGURE 3 | Gene expression level in various human immune cells showing the RNA sequencing data averaged from three different sources (Internally generated Human Protein Atlas (HPA) as well as data generated by Monaco et al. and Schmiedel et al.) Data are shown as normalized expression (NX) (71, 72), resulting from an internal normalization pipeline for the different cell types and total peripheral blood mononuclear cells (PBMC). The NX value for each gene represents the maximum NX value in the three data sources (Based on data from Human Protein Atlas: Blood Cell Type Expression (RNA) of MCOLN1/MCOLN2/MCOLN3 available from: https://www.proteinatlas.org).

molecules, which in turn recognize and respond to a pathogen or foreign antigen. This response is partially mediated by an integral membrane protein called the B-cell receptor (BCR) (64, 65). BCRs are specialized receptors, structurally composed of two Ig light chains, two Ig heavy chains, and two heterodimers Igα and Igβ (65). B-cells have a specialized lysosomal compartment in which antigens deriving from endocytosed BCRs are loaded onto MHC class II. Upon BCR engagement, this compartment undergoes a regulated transformation linked to the *de-novo* formation of multi-vesicular bodies (MVBs) which mature from tubulo-vacuolar early endosomes by a process of remodeling (66, 67). Although these processes are thought to be crucial for the role of B-lymphocytes in immune-modulatory and antigen processing, the molecular pathways underlying the regulation and formation of the specialized lysosomal compartment within B-cells are still poorly understood (66). Specifically, the distribution of MHC class II products over endolysosomal compartments including MVBs in response to BCR engagement remains a matter of debate (68).

Song et al. (66) have verified the expression of TRPML1 and TRPML2 in B-lymphocytes. Their results however indicate that TPRML1 deficient B-lymphocytes are not linked to gross changes in the lysosomal compartment. This is in accordance with the finding that MLIV patients do not exhibit any obvious abnormalities in lymphocyte function, nor do they have obvious immune function defects, arguing for compensatory mechanisms. The authors suggested that normal lysosomal compartments seen in lymphocytes with TRPML1 deficiency may be due to a role of TRPML2 in compensating for the loss of TRPML1 function, and therefore postulated overlapping functions of TRPML1 and TRPML2 in B-lymphocytes. In contrast, expression of TRPML3 had not been demonstrated for B cells (66).

Normal immune response is dependent on an intact development of B-lymphocytes and mutations of genes involved in B-cell differentiation distort this process. Bruton's tyrosine kinase (Btk) gene, that is part of the Tec family of cytoplasmic non-receptor protein-tyrosine kinases, encodes for one of these crucial molecules. Mutations in this gene lead to a partial blockage between the pre- and pro-B-lymphocyte stage, and a complete blockage between the pre- and mature B-lymphocyte stage, leading to X-linked immunodeficiency pathologies in mice and to X-linked agammaglobulinemia in humans. A more severe phenotype is observed in humans as compared to mice because only a partial blockage is established between the pre- and mature B-cell stage in the latter (69).

The phosphorylation and activation of Btk is dependent on plasma membrane (PM) localization. BCR engagement by an antigen results in the activation of PI3K, generating the phosphoinositide phosphatidylinositol-3,4,5-trisphosphate (PIP3). Btk, along with other signaling proteins, are then recruited, as a result of PIP3 accumulation to the PM. Subsequent binding of the pleckstrin homology (PH) domain to PIP3 is a pre-requisite for Btk activation. Even though the molecular mechanisms which target Btk to the cell surface remain largely unclear, the PH domain seems to play an important role. As Btk is a cytoplasmic tyrosinase kinase, loss of Btk leads to dysregulation in downstream signaling pathways, impacting several effector molecules (70).

Lindvall et al. have shown that TRPML2 is expressed in T-, B-, myeloma, and mastocytoma cell lines, in addition to whole primary splenocytes. TRPML2 was also shown to be expressed at pre-B cell, mature B-cell, and plasma cell stages, and in splenic T1 B-lymphocyte cell populations. TRPML2 was up-regulated in both Btk-defective and wild-type splenic primary mouse B-cells post-stimulation with either phorbol-12-myristate-13-acetate (PMA) plus ionomycin or anti-IgM while it was downregulated by a factor of four in unstimulated Btk-defective splenic primary mouse B-cells. The authors further proposed a role of Btk in B-cells in suppressing the activation of TRPML2. However, these results await further confirmation.

CONCLUSIONS AND OUTLOOK

Functional expression of TRPML channels has been demonstrated for a number of cells belonging to both the innate and the adaptive immune system, in particular macrophages, dendritic cells, neutrophils, NK cells, and B lymphocytes as outlined above. Based on gene expression data from different sources (**Figure 3**), TRPML channels may however be functionally active in many more cells of the immune system, such as basophils, eosinophils, monocytes as well as CD4+ and CD8+ T cells. Which functional roles they have in these very diverse immune cell types remains to be elucidated as well as potential functional differences between the different TRPML channels which may act as homomers or heteromers in some of these cells.

AUTHOR CONTRIBUTIONS

All authors wrote and discussed the manuscript. Y-KC and C-CC provided the patch-clamp data in **Figure 1**.

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MINI REVIEW
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TRPV4—A Missing Link Between Mechanosensation and Immunity

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Transient receptor potential vanilloid-type 4 (TRPV4) cation channel is widely expressed in all tissues as well as in immune cells and its function as mechanosensitive Ca^{2+} channel seems to be conserved throughout all mammalian species. Of late, emerging evidence has implicated TRPV4 in the activation and differentiation of innate immune cells, especially in neutrophils, monocytes, and macrophages. As such, TRPV4 has been shown to mediate neutrophil adhesion and chemotaxis, as well as production of reactive oxygen species in response to pro-inflammatory stimuli. In macrophages, TRPV4 mediates formation of both reactive oxygen and nitrogen species, and regulates phagocytosis, thus facilitating bacterial clearance and resolution of infection. Importantly, TRPV4 may present a missing link between mechanical forces and immune responses. This connection has been exemplary highlighted by the demonstrated role of TRPV4 in macrophage activation and subsequent induction of lung injury following mechanical overventilation. Mechanosensation via TRPV4 is also expected to activate innate immune cells and establish a pro-inflammatory loop in fibrotic diseases with increased deposition of extracellular matrix (ECM) and substrate stiffness. Likewise, TRPV4 may be activated by cell migration through the endothelium or the extracellular matrix, or even by circulating immune cells squeezing through the narrow passages of the pulmonary or systemic capillary bed, a process that has recently been linked to neutrophil priming and depriming. Here, we provide an overview over the emerging role of TRPV4 in innate immune responses and highlight two distinct modes for the activation of TRPV4 by either mechanical forces ("mechanoTRPV4") or by pathogens ("immunoTRPV4").

Keywords: TRPV4, mechanosensation, innate immunity, infection, host defense, inflammation

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INTRODUCTION

Mechanotransduction is a multistep process to convert mechanical stimuli into biochemical signals that elicit specific cellular effector functions. Over the past decade many key players in this mechanosensitive machinery have been identified, e.g., ion channels like transient receptor potential vanilloid-type 4 cation channel (TRPV4) (1, 2), PIEZO (3–5) and epithelial Na^+ channel (ENaC) (6), cellular microcompartments such as primary cilia (7) or caveolae (8), or integrins which can sense the stiffness of the extracellular matrix (ECM) (9).

In injury, infection, or cancer immune cells are attracted by biochemical cues, yet during invasion of the affected tissue they encounter changes in the biophysical properties of the microenvironment, which in turn affect their functions. This emerging role of mechanical forces in immune responses has been termed mechanoimmunology (10). Moreover, in organs with a

high dynamic mechanical load such as the lung or the heart immune cells face rapid changes in mechanical forces during the respiratory or cardiac cycle; yet relatively little is known about the effects of such mechanical cues on the innate immune system. During cell adhesion and migration immune cells are exposed to various biophysical stimuli including shear (in the circulation as well as during cell adhesion), deformation (when squeezing though narrow capillary passages or in transmigration), or cyclic mechanical stretch (in lung and heart as a result of ventilation or cardiac function). Although mechanical forces have been shown to impact signaling during adaptive immune processes (11–13), their effects on innate immunity are rarely considered and remain poorly understood. Recent studies, however, have implicated TRPV4 function in the regulation of innate immune responses (14–16). In the present review, we connect this emerging evidence with the established role of TRPV4 in mechanosensation to propose a novel concept of mechanoimmunology via TRPV4.

MechanoTRPV4

The polymodal and non-selective TRPV4 cation channel, originally described by Strotmann et al. (17) and Liedtke et al. (18) in 2000, has been implicated over the past decades to act as a cellular mechanosensor in response to mechanical forces such as shear, stretch, osmotic swelling and shrinking, stiffening, and surface expansion (19–27) and is ubiquitously expressed in a wide range of cell types, including parenchymal cells such as smooth muscle cells, fibroblasts, epithelial cells, and endothelial cells as well as in immune cells, including macrophages, neutrophils (14, 16, 27–33).

TRPV4 activation mediates the influx of extracellular Ca^{2+}, which can in turn activate Ca^{2+}-triggered signaling cascades resulting in changes in transcription, vesicular transport, or cytoskeletal remodeling. The molecular mechanism how TRPV4 is activated by mechanical forces is currently not completely understood. At present, models for either direct or indirect mechanical activation of TRPV4 have been proposed (34, 35). The concept of direct activation is based on the assumption that an initial deformation of the plasma membrane's lipid bilayer will cause an expansion in cross sectional area, which creates a membrane tension-dependent energy difference followed by conformational change of the ion channel and thereby promotes force activation, as previously described for KCNK4 potassium channels by Brohawn et al. (36). This concept is supported by studies of Loukin et al. (37) who showed that TRPV4 can be activated by pipette suction in the presence of enzyme inhibitors in Xenopus oocytes, thus excluding mechanisms of indirect activation (37).

The concept of indirect TRPV4 activation follows the notion that TRPV4 is rather mechanically gated via intracellular signaling pathways such as integrin signaling, intracellular messengers and kinases, or simply by changes in surface expression (38, 39). Therefore, it has been demonstrated that forces applied to β_1-integrins result in ultra-rapid activation of Ca^{2+} influx through TRPV4 channels and that the TRPV4 channels are rather activated by mechanical strain in the cytoskeletal backbone of the focal adhesion than by deformation of the lipid bilayer or peripheral cortical cytoskeleton (40). Such localized indirect activation is proposed to cause highly compartmentalized TRPV4-mediated Ca^{2+} signaling at focal adhesions and facilitates downstream activation of additional β_1-integrins (integrin-to-integrin signaling) and leads to cell reorientation (40, 41). Moreover, several studies have shown that TRPV4 activation in response to osmotic or mechanical stress depends on formation of intracellular mechanomessengers, like lipid metabolites as arachidonic acid and its derivative 5′,6′-epoxyeicosatrienoic acid, and PIP_2 (21, 42–45). Additionally, calmodulin as a classical second messenger binds to TRPV4 and mediates Ca^{2+} influx by conformational change and dissociation of its N- and C-terminus (20). It also has been observed that several protein kinases affect the activity of TRPV4 and/or facilitates binding to anchoring proteins (AKAPs) and F-actin and stabilize the channel in the plasma membrane (24, 27, 46–48). As such, a series of intracellular signaling cascades have been identified that modulate TRPV4 activity and may serve as pathways for indirect TRPV4 activation by mechanical forces.

In addition to the intracellular signaling pathways activating TRPV4, mechanical forces can affect TRPV4 trafficking and upregulate surface expression of the channel by recruitment from intracellular pools of TRPV4 to the plasma membrane via mechanoreceptive structures like calveolae, integrins, or adherens junctions (8, 26, 32, 41). Subcellular localization and trafficking of TRPV4 have been proposed to depend on pre- and post-translational modifications, like alternative splicing (49), nitrosylation, glycosylation and phosphorylation (27, 47, 50, 51). As these mechanisms were, however, largely identified using fluorescent-tagged overexpression systems their exact role in the trafficking of endogenous TRPV4 still remains incompletely understood.

In summary, TRPV4 may not only respond to diverse triggers via various modes (direct and indirect) of activation, diverse signaling pathways and protein modifications, but also different mechanisms to increase the abundance of open Ca^{2+} channels at the plasma membrane, respectively (**Figure 1**).

ImmunoTRPV4

The role of TRPV4 in the innate immune system was first recognized more than a decade ago due to its thermosensitivity. Increments in body temperature in response to infection (i.e., fever) are important activators of the immune system with an evolutionary conserved role in host defense (52, 53). TRPV4 became first implicated in thermo-dependent immune modulation based on its role in thermal hyperalgesia (54–56). At the same time, similar effects were reported for other Ca^{2+} channels, such as the temperature-dependent activation of stromal interaction molecule (STIM) 1, which induces Ca^{2+} influx and in turn modulates gene expression and immune functions (57). These studies unveiled for the first time a direct link between Ca^{2+} channels, temperature, and immune function.

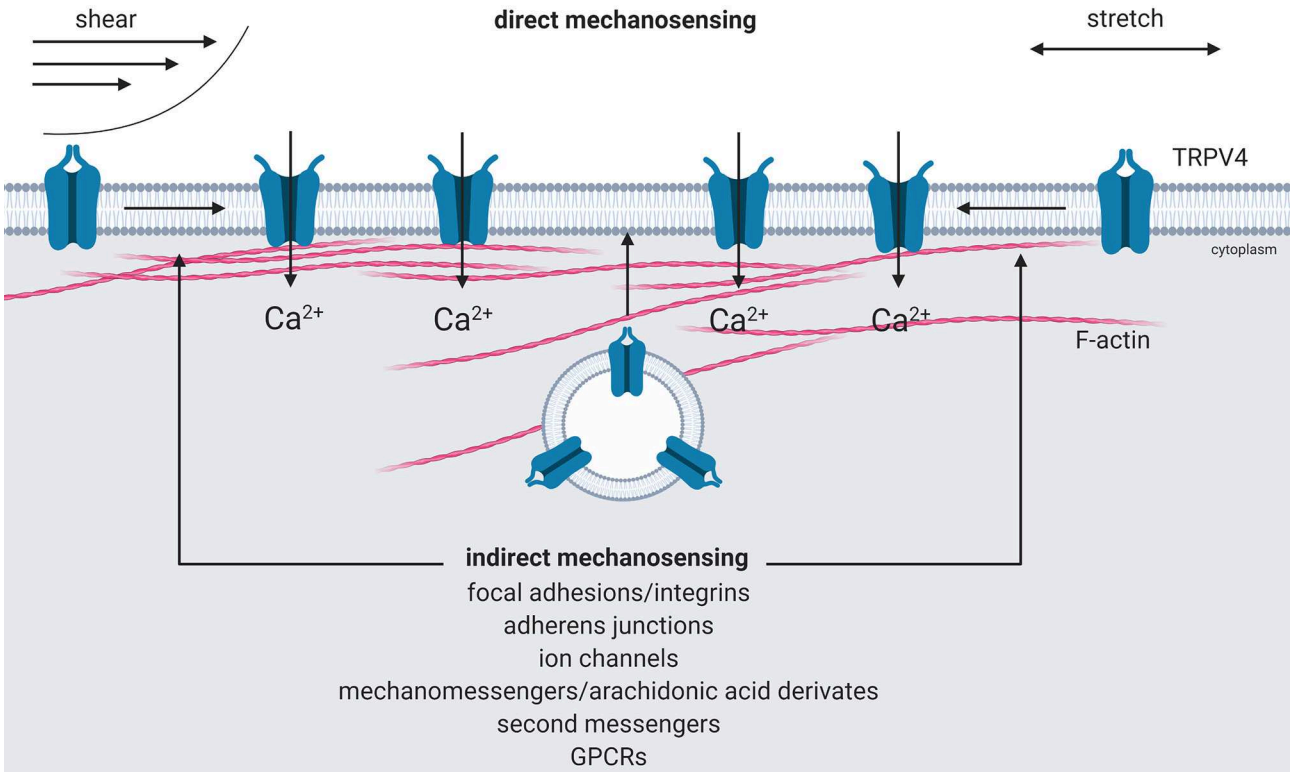

FIGURE 1 | Activation of mechanoTRPV4. Direct activation of TRPV4 by shear or stretch forces results in an expansion in cross sectional area that creates a tension-dependent energy difference and leads to conformational changes of the channel by force activation (direct mechanosensing). Indirect activation is mediated by intracellular signaling cascades triggered via mechanosensitive focal adhesions or adherens junctions, ion channels, by intracellular mechano- or second messengers, G-protein-coupled receptors, e.g., protease-activated receptors that either activate TRPV4 or recruit it from intracellular pools to the plasma membrane (indirect mechanosensing). Created with BioRender.com.

In 2008, Spinsanti and colleagues for the first time detected high expression levels of TRPV4 in human leukocytes (58). Subsequent functional studies from our group identified an important role for TRPV4 in regulating key neutrophil functions in response to pro-inflammatory stimuli like production of reactive oxygen species, cell adhesion, or migration (16). *In vivo*, *Trpv4*-deficient mice showed a marked protection from acute lung injury in two independent studies following either acid-induced or chlorine-induced lung injury (16, 59, 60). These effects were replicated by pharmacological inhibition of TRPV4, which similar to *Trpv4* deficiency attenuated characteristic signs of lung injury including hypoxemia, reduced compliance, edema formation, histological evidence of lung injury, and last not least, neutrophil infiltration and the release of pro-inflammatory cytokines (27, 59). Bone marrow chimeras from *Trpv4*-deficient and corresponding wild type mice revealed that the barrier protective effects in *Trpv4*-deficient mice was mostly attributable to a lack of TRPV4 in parenchymal tissue (presumably most relevant in endothelial cells), whereas TRPV4 deficiency in hematopoietic blood cells primarily reduced neutrophil infiltration into the injured lung (16). As such, it remains to be shown to which extent TRPV4-mediated activation of neutrophils affects organ function *in vivo*. In principle, these findings demonstrate that TRPV4 regulates neutrophil adhesion and migration, whereas in barrier forming cells like endothelial and epithelial cells, TRPV4 acts as a door opener for protein and fluid extravasation.

In macrophages, TRPV4 mediates both pro-inflammatory functions including phagocytosis, adhesion, and reactive oxygen species production, as well as anti-inflammatory effects and secretion of pro-resolution cytokines and bacterial clearance (14, 15). As such, macrophage TRPV4 may exert both protective and detrimental effects to the host tissue, by facilitating bacterial clearance in infection while promoting parenchymal injury in sterile inflammation (16, 27, 59). In a recent study, a similar double-edged role of mechanosennsation in the modulation of the innate immune response to sterile inflammation vs. bacterial infection was reported for another emerging mechanoimmunological cation channel, PIEZO1 (5).

An important additional role of TRPV4 in innate immunity relates to expression and function in the vascular endothelium, which by way of lining the inner surface of blood vessels and regulating cell adhesion and migration via expression of adhesion molecules acts as a gate keeper and controls the access for cells of the innate (and adaptive) immune system to sites of inflammation (61). TRPV4 activation in lung endothelial cells has

been shown to increase vascular permeability (16, 27, 29), in part via disintegration of cell junctions (62) and degradation of ECM components and non-matrix components like integrins and VE-cadherins by matrix metalloproteinases (MMPs) MMP2 and MMP9 (63), and in part by calmodulin-dependent activation of the endothelial contractile machinery (64). As a consequence of these effects, TRPV4 activation results in endothelial detachment from the basal lamina, consecutive disruption of the endothelial barrier, and ultimately edema formation (65, 66). These effects seem to be most prominent in the pulmonary microvasculature (67), which may be related to TRPV4's roles in immunity and defense on the one, and the fact that the alveolo-capillary barrier presents a large surface for pathogen invasion on the other hand.

In lung alveolar epithelium, TRPV4 has been shown to act as a critical regular of epithelial barrier function, but at the same time revealed protective effects by increasing in bacterial clearance in large airways (31, 68, 69). All these findings identify TRPV4 as an important regulator of innate host defense responses including the regulation of phagocytes such as neutrophils and macrophages, but also of barrier forming epithelial and endothelial cells.

TRPV4 IN HOST DEFENSE

In line with its role in innate immune cells, TRPV4 has been implicated in different scenarios of host defense. In a murine model of *Streptococcus pneumoniae* infection TRPV4-deficiency prevented leukocyte infiltration, reduced bacterial load in the alveolar space, and attenuated characteristic features of lung injury (70). While these data unequivocally highlight the role of TRPV4 in host defense against gram-positive bacteria, the mechanism of TRPV4 activation by such bacteria or their pore-forming toxins such as pneumolysin (*S. pneumoniae*), α-hemolysin (*S. aureus*), or listeriolysin O (*L. monocytogenes*) is still unclear (70–72).

In gram-negative infections with bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, TRPV4 activation by ECM stiffening during infection synergizes with LPS-stimulated TLR4 activation of p38 and thereby promotes host defense and resolution from lung injury (15, 73). Conversely, activation of protease-activated receptor (PAR)-2 by thrombin suppresses TRPV4 activity in macrophages and resolves lung injury (74). Similarly, PARs are also activated by neutrophil elastase (NE), matrix metalloproteases (MMPs) or other microbial proteases (33, 75–79) which has been implicated to degrade ECM and thereby causing remodeling and matrix stiffening during infection (33, 63, 73, 80).

Albeit the number of studies on TRPV4 in immune cells is still limited, TRPV4 emerges as a regulator of innate immunity and host defense and may sense mechanical changes of the extracellular environment during inflammation. Therefore, occurring mechanical forces are crucial for TRPV4-mediated immune response and regulate both pro- and anti-inflammatory effects, which may have both beneficial effects in terms of bacterial clearance and resolution from injury.

TRPV4 IN MECHANOSENSATION OF IMMUNE CELLS

Mechanical forces generated by hemodynamic forces or a factor of ECM composition under physiological and pathophysiological conditions are acting on immune cells and can be subclassified in (i) mechanical stretch by shape changes during cell passage through narrow capillary segments, (ii) shear stress acting on circulating or adherent immune cells as a function of blood flow or viscosity, and (iii) changes in substrate stiffness of the ECM induced by inflammation (81, 82).

In particular, mechanical stretch has been implicated as a central component in pathological processes at the alveolo-capillary barrier of the lung where it has been extensively studied in the context of ventilator-induced lung injury (VILI). In endothelial and epithelial cells, TRPV4 has been shown to become activated during mechanical stretch as exerted by mechanical (over-) ventilation leading to Ca^{2+} influx and subsequent loss of lung barrier integrity and the release of cytokines (27, 29, 68). For macrophages, TRPV4 function has been shown to be critical in the pro-inflammatory response to mechanical ventilation (14). As shown by Hamanaka and colleagues in studies on isolated-perfused mouse lungs, replacement of wild type with *Trpv4*-deficient macrophages in wild type lungs was sufficient to attenuate classical features of VILI, a finding that was linked to stretch-induced and TRPV4-dependent intracellular Ca^{2+} signaling, and the subsequent formation of reactive oxygen and nitrogen species *in vitro*.

In neutrophils, transmigration during VILI has so far largely been considered as a response secondary to the mechanical stretch on parenchymal cells (16, 83). In contrast to the systemic circulation where neutrophil adhesion and migration are primarily localized to postcapillary venules and mediated by adhesion molecules (61), the initial mechanism of neutrophil sequestration in the lung is largely based on cytoskeletal rearrangement and formation of F-actin rims which increase cellular stiffness and as a result, decrease their ability to change their shape from spherical to elliptical. These changes in deformability prevents activated neutrophils to pass through the narrow capillary segments of the alveolo-capillary network where they get trapped at sites of inflammation (84). While neutrophil stiffening was originally considered an irreversible feature, recent studies suggest that alternating neutrophil stiffening and softening can drive the dynamic oscillation of neutrophils between the activated/primed and deactivated/deprimed state (85). Since F-actin has been shown to bind to activated TRPV4, it can be speculated that this priming/depriming occurs as a function of TRPV4 activation secondary to the formation of F-actin rims (24). Given the implications of such mechanical effects due to e.g., changes in neutrophil shape and stiffness not only on neutrophil kinetics through the vascular system but also on their biological responsiveness in health and disease (86), the molecular dissection of the underlying signaling pathways and the potential link to TRPV4 mechanosensation may be of considerable scientific interest and relevance.

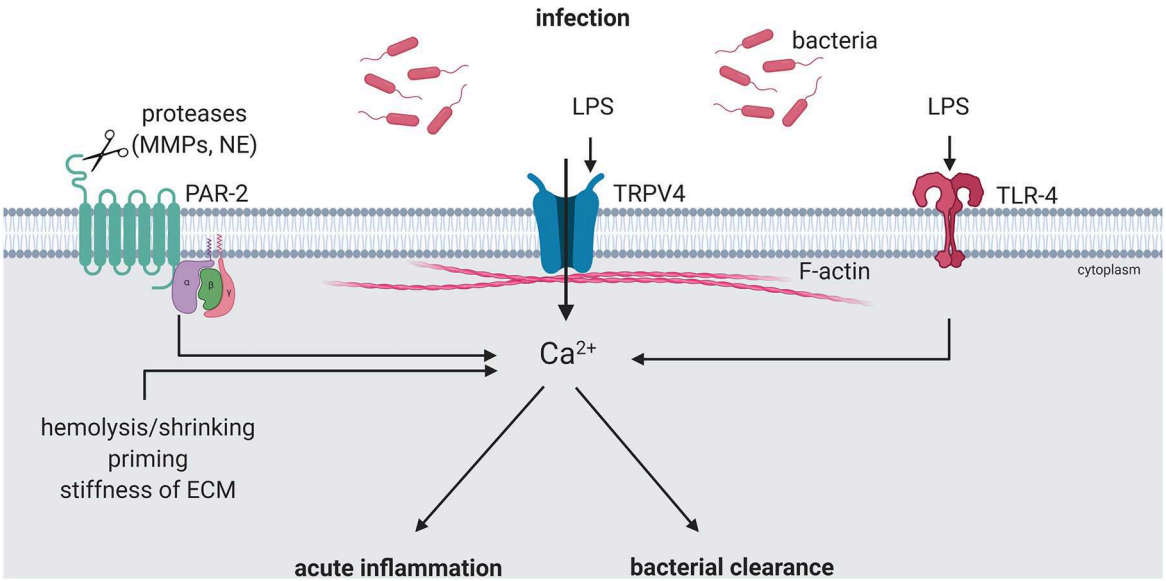

FIGURE 2 | ImmunoTRPV4 activation during infection. Pathogen-associated signals, e.g., lipopolysaccharide (LPS) can modulate TRPV4 function directly or indirectly. Direct activation can lead to pro-inflammatory phenotypes and boost the response in acute inflammation. Activation of TLR-4 by LPS and cleavage of PAR-2 by release matrix metalloproteases (MMPs) and/or neutrophil elastase (NE) during inflammation can modulate the TRPV4-mediated immune response toward an anti-inflammatory phenotype and induce bacterial clearance. Created with BioRender.com.

Shear stress in the vasculature is a result of blood flow velocity, vessel diameter, and blood viscosity and primarily acts on endothelial cells outlining the vessel lumen. Endothelial cells respond to shear stress by segregation of TRPV4 channels from β-catenin following relocating TRPV4 from adherens junctions to focal adhesions of the basal membrane which in turn increases endothelial permeability by destabilization of junctional complexes and Ca^{2+}-mediated cytoskeletal remodeling (87). While it is reasonable to expect that circulating immune cells tethering or adhering to the vascular wall experience likewise considerable degrees of vascular shear stress, the effects of fluid shear stress on innate immune cells have so far not been extensively addressed. In the alveolar compartment, alveolar macrophages have been shown to contribute to VILI by secretion of pro-inflammatory mediators in a TRPV4-dependent manner (14). This effect is presumably predominantly caused by stretch rather than shear effects. Nevertheless, it is conceivable that shear-dependent activation of macrophages may become relevant in conditions of alveolar fluid accumulation when fluid will cyclically shift in and out of the alveolus resulting in considerable shear forces exerted not only on alveolar epithelial cells but also on alveolar macrophages (88, 89).

Finally, substrate stiffness is regulated by the composition of the ECM which changes as a function of physiological (development, aging) and pathophysiological (atherosclerosis, hypertension, fibrosis) processes (90–93). TRPV4 has been identified as a major mechanosensor for substrate stiffness, but so far this function has been exclusively attributed to parenchymal cells (30, 31, 94–96). Yet, it is fair to speculate that changes in substrate stiffness will similarly affect the mechanical forces that act upon immune cells during the processes of adhesion and transmigration, which accordingly may affect TRPV4-dependent cellular responses. Conversely, TRPV4-mediated activation of immune cells may in turn affect local ECM structure and composition by secretion of MMPs. As such, TRPV4 may play an important role in proteolytic disruption of ECM, cell-cell, and cell-matrix interaction by MMPs that is required for effective immune cell extravasation to sites of injury and inflammation (63). By similar mechanisms, TRPV4 may also contribute to chronic parenchymal remodeling, explaining its prominent role in tissue fibrosis in a positive feedback of substrate stiffening and TRPV4-mediated pro-fibrotic effects (30, 97).

In line with a critical role of substrate stiffness for immune cell function, the Ca^{2+} response to LPS in of macrophages were shown to correlate with substrate stiffness. Notably, such substrate stiffness-dependent modulation of macrophage signaling can alter macrophage phenotype toward an anti-inflammatory phenotype (M2) initiating bacteria clearance and resolution of lung injury (15, 73).

SUMMARY AND CONCLUSION

This review provides an update on the role of TRPV4 in mechanosensation ("mechanoTRPV4") on the one and inflammation and host defense ("immunoTRPV4") on the other hand with the aim to point toward a possible, albeit still speculative, role of TRPV4 in mechanoimmunology.

Activation of mechanoTRPV4 directly impacts host defense in that it reduces endothelial and epithelial barrier function, but

at the same time promotes the infiltration of innate immune cells and the release of pro- and anti-inflammatory cytokines (15, 16, 27). In addition, TRPV4 activation can regulate the delivery of circulating immune cells to local sites of inflammation and infection by mediating vasodilation (98, 99). Activation of immunoTRPV4 triggers and promotes inflammation and has emerged as a key regulator of bacterial clearance. Importantly, mechanosensitive and immunoregulatory functions of TRPV4 may not be distinct, but intrinsically linked, thus opening a new view on mechanoregulation of immune responses. This concept has already been well-established for epithelial and endothelial cells, where mechanical activation of TRPV4 has emerged a major regulator of barrier function and inflammatory responses. Yet, this notion has also been demonstrated for innate immune cells such as macrophages where Ca^{2+} signaling as a function of TRPV4-mediated sensing of substrate stiffness has been shown to shift the immune response from pro-inflammatory to an anti-inflammatory and resolving phenotype (15, 73) (**Figure 2**). Similar scenarios of mechanoregulation of immune cell function via TRPV4 may relate to a variety of scenarios where immune cells undergo changes in mechanical stress, such as during cell adhesion (shear stress) and transmigration (substrate stiffness, shape change), capillary transit (shape change) as well as tissue strain e.g., in mechanically ventilated lungs (stretch). So far, the link between mechanoTRPV4 and immunoTRPV4 has not been characterized in detail, but may provide for important insights into the regulation of innate immunity and host defense and as such, for the development of novel preventive, therapeutic, or adjuvant strategies in inflammatory and infectious diseases such as sepsis, pneumonia, or sterile inflammation as in VILI.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Analysis of Mrgprb2 Receptor-Evoked Ca^{2+} Signaling in Bone Marrow Derived (BMMC) and Peritoneal (PMC) Mast Cells of TRPC-Deficient Mice

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Mast cells are a heterogeneous group of immune cells. The simplest and commonly accepted classification divides them in two groups according to their protease content. We have compared the action of diverse secretagogues on bone marrow derived (BMMC) and peritoneal (PMC) mast cells which represent classical models of mucosal and connective tissue type mast cells in mice. Whereas, antigen stimulation of the FcεRI receptors was similarly effective in triggering elevations of free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in both BMMC and PMC, robust $[Ca^{2+}]_i$ rise following Endothelin-1 stimulation was observed only in a fraction of BMMC. Leukotriene C4 activating cysteinyl leukotriene type I receptors failed to evoke $[Ca^{2+}]_i$ rise in either mast cell model. Stimulation of the recently identified target of many small-molecule drugs associated with systemic pseudo-allergic reactions, Mrgprb2, with compound 48/80, a mast cell activator with unknown receptor studied for many years, triggered Ca^{2+} oscillations in BMMC and robust $[Ca^{2+}]_i$ rise in PMCs similarly to that evoked by FcεRI stimulation. $[Ca^{2+}]_i$ rise in PMC could also be evoked by other Mrgprb2 agonists such as Tubocurarine, LL-37, and Substance P. The extent of $[Ca^{2+}]_i$ rise correlated with mast cell degranulation. Expression analysis of TRPC channels as potential candidates mediating agonist evoked Ca^{2+} entry revealed the presence of transcripts of all members of the TRPC subfamily of TRP channels in PMCs. The amplitude and AUC of compound 48/80-evoked $[Ca^{2+}]_i$ rise was reduced by ~20% in PMC from *Trpc1/4/6*$^{-/-}$ mice compared to *Trpc1/4*$^{-/-}$ littermatched control mice, whereas FcεRI-evoked $[Ca^{2+}]_i$ rise was unaltered. Whole-cell patch clamp recordings showed that the reduction in compound 48/80-evoked $[Ca^{2+}]_i$ rise in *Trpc1/4/6*$^{-/-}$ PMC was accompanied by a reduced amplitude of Compound 48/80-induced cation currents which exhibited typical features of TRPC currents. Together, this study demonstrates that PMC are an appropriate mast cell model to study mechanisms of Mrgprb2 receptor-mediated mast cell activation, and

it reveals that TRPC channels contribute at least partially to Mrgprb2-mediated mast cellactivation but not following FcεRI stimulation. However, the channels conducting most of the Ca^{2+} entry in mast cells triggered by Mrgprb2 receptor stimulation remains to be identified.

Keywords: mast cells degranulation, secretagogues, connective tissue type mast cells, mucosal tissue type mast cells, TRPC channels, Mrgprb2 receptor, intracellular calcium

INTRODUCTION

Mast cells play a very important role in innate and adaptive immunity by their capability of quick and massive release of granules containing preformed inflammatory mediators and proteases (1) as well as by the capability to secrete a broad spectrum of cytokines and growth factors (2). The canonical way of mast cell activation is their participation in an immediate allergic reaction of anaphylactic type involving a specific antigen-induced crosslinking of surface IgE molecules bound to high-affinity receptors for IgE (FcεRI). However, in clinical practice there are a lot of examples of mast cell participation in less specific pseudoallergic reactions. Recently, a pivotal role of a Mrgpr in initiation of such reactions was shown (3, 4).

In addition to these pathophysiological processes, mast cells represent a first line of immune defense against pathogens (5), particularly parasites (6), determine immune tolerance (7) and play an important role in wound healing (8) or angiogenesis (9). Proteases released from mast cells are responsible for exogenous toxin inactivation (10).

From direct measurements of mast cell degranulation it is well-known that this process strongly depends on elevation in the concentration of free intracellular calcium ions ($[Ca^{2+}]_i$) (11). Numerous experiments demonstrate that particularly Ca^{2+} influx from extracellular space is indispensable for mast cell activation [for review see (12)]. Activation of Fcε receptor for IgE (FcεRI) stimulation by antigens is well-described as a trigger to evoke elevation of $[Ca^{2+}]_i$ in mast cells, but numerous other agonists including adenosine, endothelin 1 (ET-1), Leukotriene C4 (LTC_4), lysophosphatidylcholine (LysoPC), sphingosine-1-phosphate (S1P) or Substance P have been reported to lead to mast cell activation in a Ca^{2+} dependent manner (12–14). The receptors triggered by these agonists can either potentiate FcεRI-mediated mast cell activation or act by themselves, and stimulate the release of mast cell mediators, using different signaling cascades.

Recently, Mrgprb2 was identified as the target of many small-molecule drugs associated with systemic pseudo-allergic reactions and as the receptor for Compound 48/80, a Ca^{2+} mobilizing mast cell agonist known for years (4). Already in 1974, it was reported that the intravenous administration of Compound 48/80 in dogs triggers an increase of histamine levels in the plasma (15), and some years later, it was shown that Compound 48/80 induces histamine release from rat mast cells (16). Mast cell responses elicited by either FcεRI or Mrgprb2 stimulation differ significantly from each other in many aspects (17, 18), and the molecular constituents of Ca^{2+} entry channels leading to elevation of $[Ca^{2+}]_i$ following stimulation of Mrgprb2 receptors are unknown. Nevertheless, receptor stimulation leads to a Ca^{2+} influx in rat peritoneal mast cells (19). One type of channel activated by Compound 48/80 is voltage and IP_3-independent with a 50 pS conductance (20, 21). A more detailed characterization of the inward current through these channels revealed that it has a ratio of Ca^{2+} to Na^+ permeability (P_{Ca}/P_{Na}) of 0.55. All seven members of the TRPC subgroup of Transient Receptor Potential (TRP) channel protein family were reported to be expressed in several types of mast cells [for review see (14, 22)]. As TRPC channels represent receptor-operated non-selective cation channels that can be activated by stimulation of various $G_{q/11}$-coupled receptors, it can be speculated whether TRPCs might be involved downstream of Mrgprb2 activation by agonists such as compound 48/80.

Mast cells are classified according to their neutral proteases content: TC mast cells (expressing tryptase and chymotryptic proteinase) and T mast cells (expressing only tryptase) (23) which are also referred as connective tissue-type and mucosal-type mouse mast cells (24). These two distinct mast cells types have different responsivity to different stimuli including Compound 48/80 (25). In the presented work we investigated mast cell activation and Ca^{2+} homeostasis in two classical, well-established models of murine mast cell models: Bone marrow derived mast cells (BMMC) and Peritoneal mast cells (PMC)—representing primary cell models of mucosal and connective tissue type mast cells, respectively. We performed a systematical checkup of different mast cell secretagogues to test their ability to evoke $[Ca^{2+}]_i$ rise and degranulation in BMMC and PMC. Whereas, antigen stimulation resulted in robust $[Ca^{2+}]_i$ rise in both BMMC and PMC, we observed much weaker responses with all other agonists in BMMC compared to PMC. PMC showed prominent reactions to Compound 48/80 as well as other Mrgprb2 agonists including Tubocurarine, LL-37 and Substance P. Thus, PMC represent an ideal cell model for comparison of Ca^{2+}-dependent mast cell activation elicited by stimulation of FcεRI and by Mrgprb2 receptors. We found an expression of all members of the TRPC protein family in PMC. Experiments using PMC isolated from mice lacking TRPC1, TRPC4, and TRPC6 proteins (*Trpc1/4/6*$^{-/-}$ mice) revealed a functional role of these proteins as constituents of non-selective cation channels activated by Mrgprb2 receptor stimulation, that contribute at least partially to Mrgprb2 receptor-mediated $[Ca^{2+}]_i$ rise and degranulation in PMC mast cells.

METHODS

Peritoneal Mast Cell (PMC) Isolation and Culture

Male mice with C57Bl6/N genetic background at the age of 8–14 weeks were used for our experiments. A double knockout mouse line *Trpc1/4*$^{-/-}$ (DKO) and a triple knockout mouse line *Trpc1/4/6*$^{-/-}$ (TKO) was generated by intercrossing mice of the three mouse lines lacking expression of TRPC1 (26), TRPC4 (27), and TRPC6 (28), respectively. Each had been backcrossed to the C57Bl6/N strain (Charles River) for at least 7 generations before they were used to generate the *Trpc1/4*$^{-/-}$ (DKO) and *Trpc1/4/6*$^{-/-}$ (TKO) knockout lines. For comparative Ca^{2+} imaging experiments we used littermatched *Trpc1/4/6*$^{-/-}$ (TKO) and *Trpc1/4*$^{-/-}$; *Trpc6*$^{+/+}$ (DKO) offspring derived from intercrosses of *Trpc1/4*$^{-/-}$; *Trpc6*$^{+/-}$ mice. Animal husbandry and experimental procedures were performed in accordance with local and European Union animal welfare standards.

For the isolation of PMC, peritoneal cells were washed from the peritoneal cavity using peritoneal lavage technique (29). Suspension of peritoneal cells isolated from 2 to 3 mice was pooled together. Cells were centrifuged at ∼300 × g and resuspended in RPMI Medium supplemented with 20% Fetal Calf Serum (FCS), 1% PenStrep, 10 ng/ml IL-3, and 30 ng/ml SCF. The cells were further cultured in 5% CO_2 at 37°C. On the 2nd day of cultivation all non-adherent cells were discarded. The cells were split and transferred into a new flask on day 10. PMC were used for experiments between 14 and 16 days of culturing. Flow cytometry analysis identified 98.5–99.5% cells to be double-positive for FcεRI and c-Kit and could be ranked as mast cells.

Bone Marrow Derived Mast Cells (BMMC) Culture

Both hind paws were used to dissect femur bones. After cleaning of the bones from connective and muscle tissues, two incisions at distal and proximal sides of each bone were made. Bone marrow was released by two short centrifugation steps (30 s at 5,000 rpm and 120 s at 2,000 rpm). Bone marrow cells were cultured at 1 × 10^6 cells/ml in IMDM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin at 37°C, 5% CO_2. The medium was additionally supplemented with 2 ng/ml IL-3 and 5 ng/ml SCF. The cells were split twice a week and cultured for 6–12 weeks before the experiments (30).

Expression Analysis

RNA isolation was performed using the RNeasy Mini kit (Qiagen) according to manufacturer's protocol. To avoid false signals originating from genomic DNA, on-column DNA-se digestion was performed and intron spanning RT-PCR primers were used. cDNA synthesis was performed with the SensiFAST cDNA synthesis kit (Bioline) according to manufacturer's instructions. Primers were designed with the Roche® online tool and primer pairs with efficiency between 90 and 110% were used. Quantitative expression analysis was performed using the Universal Probe system (Roche) with the corresponding FastStart Essential DNA Probes Master (Roche) on a LightCycler 96 Instrument (Roche). Expression levels of housekeeping genes (Cxxc1, Aip, H3f3a) were also measured. Primer sequences and probe number for TRPC1 were 5′-ctgaaggatgtgcgagaggt-3′ (fw) and 5′-cacgccagcaagaaaagc-3′ (rev), for TRPC2 were 5′-tccttg tcttcctcggagtc-3′ (fw) and 5′-ttcacagatagggcactggac-3′ (rev), for TRPC3 were 5′-ggtgaactgaaagaaatcaagca-3′ (fw) and 5′-cgtcg cttggctcttatctt-3′ (rev), for TRPC4 were 5′-aaacttttggttcagaaagg tgtc-3′ (fw) and 5′-acagttacagcggacctcgt-3′ (rev), for TRPC5 were 5′-ggcgatgcattactctacgc-3′ (fw) and 5′-gctaagcagaagttccacagc-3′ (rev), for TRPC6 were 5′-aggcaaaaggttagcgacaa-3′ (fw) and 5′-ggcataaaagtcatcttgctgaa-3′ (rev), for TRPC7 were 5′-aatgg cgatgtgaacttgc-3′ (fw) and 5′-gtttgattcggctcagacttg-3′ (rev), for Cxcc1 5′-TAGTGCCGACCGCTGACT-3′ (fw) and 5′-GGCCT CTCCCCTAACTGAAT-3′ (rev), for Aip 5′-ACCAGTCATC CACCAAGAGG-3′ (fw) and 5′-AGGCGATGGCGTCATAGTA-3′, for H3f3a 5′-GCCATCTTTCAATTGTGTTCG-3′ (fw) and 5′-AGCCATGGTAAGGACACCTC-3′ (rev).

Calcium Imaging

For the measurements of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) cells were pre-loaded with a ratiometric calcium sensitive dye, Fura-2. For this purpose the cells were incubated 30 min at room temperature in Physiological Salt Solution (PSS) supplemented with 2.5 μM Fura-2 acetoxymethyl ester. PSS composition was (in mM): NaCl 135; KCl 6; $MgCl_2$ 1.2; $CaCl_2$ 2; HEPES 10; glucose 12. The cells were mounted on the stage of an inverted Fluorescence microscope Axio Observer-A1 (Zeiss Jena, Germany) equipped with 40x Fluar oil Objective (Zeiss, Germany) and imaged using a CCD camera Axiocam MRm5 (Carl Zeiss GmbH, Germany). Cytoplasmic Fura-2 was excited using a light source, Lambda DG-4 Plus (Sutter Instrument, USA). Fluorescence signal was measured at 510 nm during alternate excitation at 340 and 380 nm. The Light Source and the camera were controlled by the Axiovision 4.8.2 software (Zeiss, Germany) through synchronization interface SVB-1 (Zeiss, Germany). After correction for the background fluorescence signals, the fluorescence ratio (F340/F380) was analyzed using Origin (8.5) software (Northampton, USA). For antigen stimulation experiments the PMC were pretreated over night with 300 ng/ml anti-DNP IgE. Experiments were performed at 22–25°C. Ca^{2+} imaging experiments in **Figures 4C–H** and **Figures 4I–N** were performed at different Ca^{2+} imaging setups with different light sources and optical properties of the setups. Therefore, the amplitudes of F340/F380 ratios obtained in *Trpc1/4*$^{-/-}$ DKO PMC vary accordingly and cannot be directly compared between the two sets of experiments.

Electrophysiological Experiments

Transmembrane currents were measured using EPC- 10 (HEKA Elektronik, Lambrecht, Germany) "patch-clamp" amplifier in the whole-cell configuration. The ramp protocol consisted of a 400-ms ramp from −100 to +100 mV (holding potential of 0 mV) applied at 0.5 Hz. Recordings were started immediately after achievement of whole-cell configuration. The standard extracellular solution for patch-clamp contained in mM: NaCl 135; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 12; HEPES 10; pH

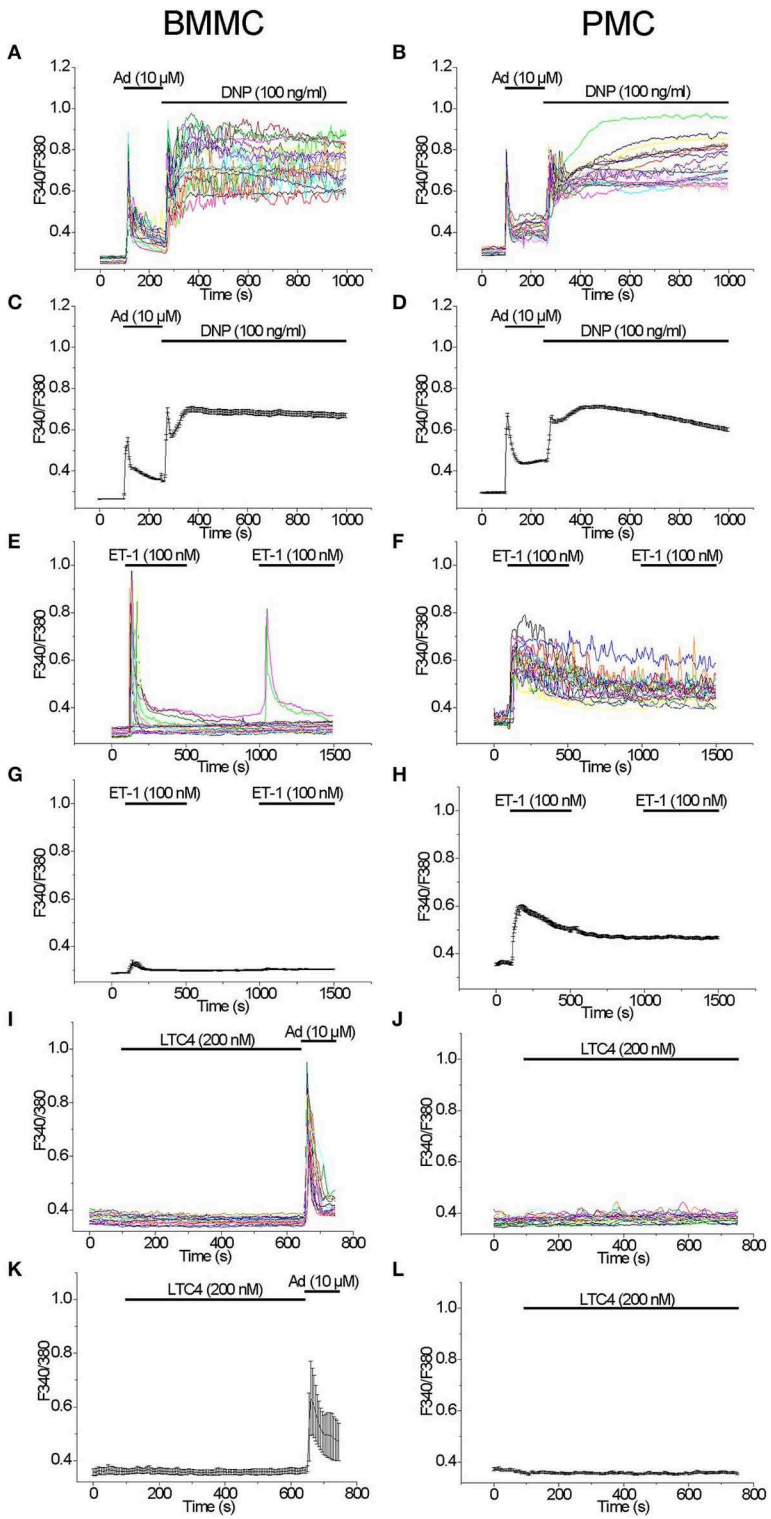

FIGURE 1 | Comparison of $[Ca^{2+}]_i$ rise induced by different agonists in BMMC and PMC. Measurements of $[Ca^{2+}]_i$ changes performed with Fura-2 and presented as F340/F380 fluorescence ratio in BMMC **(A,C,E,G,I,K)** and PMC **(B,D,F,H,J,L)** isolated from WT mice. Representative traces ($n = 20$ each panel) of $[Ca^{2+}]_i$ changes (error bars indicate S.E.M.) induced by application of: 10 µM Adenosine (Ad) and subsequently DNP (100 ng/ml) **(A–D)**, 100 nM Endothelin-1 (ET-1) **(E–H)**, 200 nM LTC4 **(I–L)**. The measurements were performed in 3–5 independent cell preparations. At the end of recordings, control reactions were elicited by application of 10 µM adenosine (Ad) **(I,K)**.

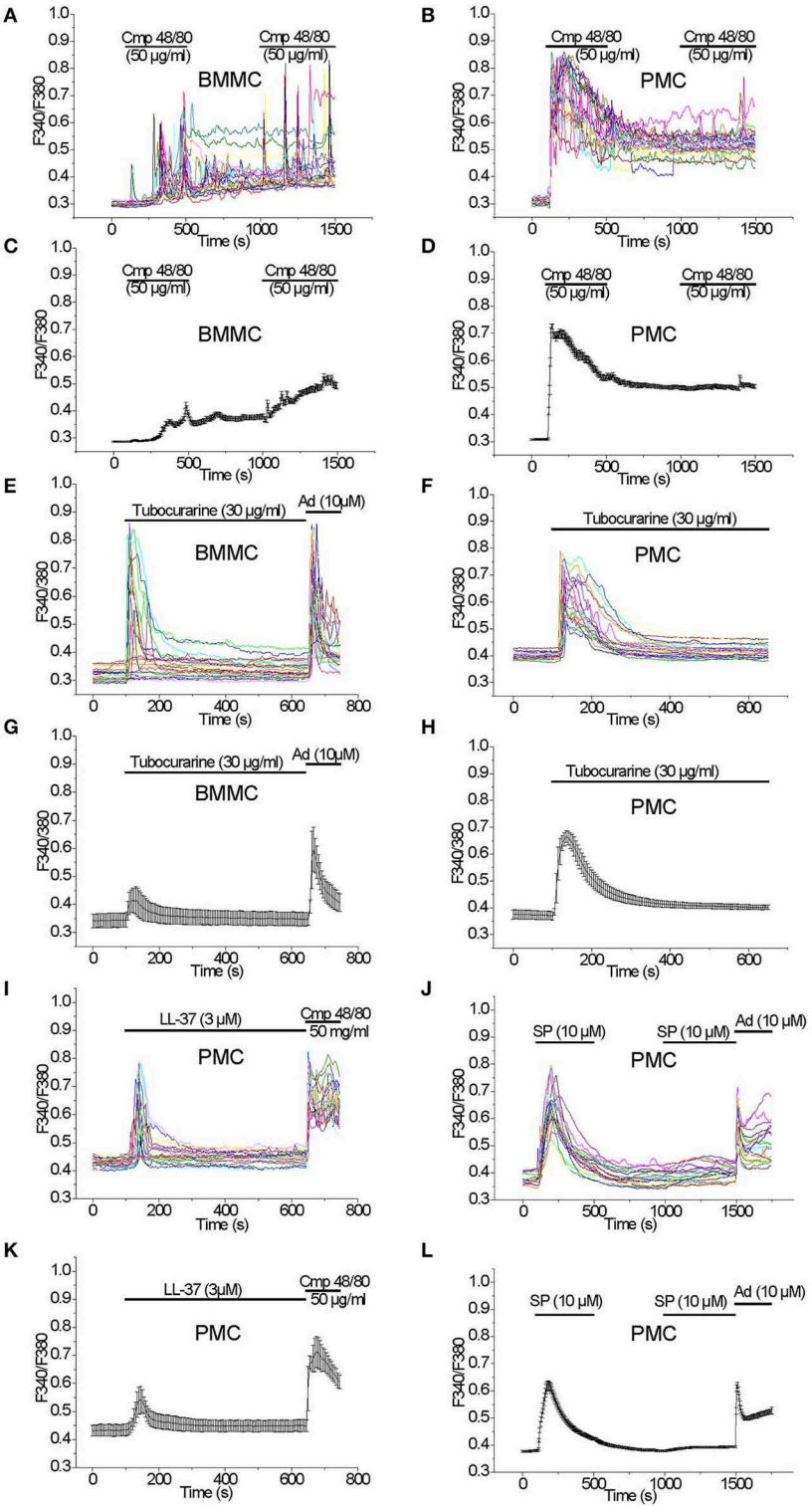

FIGURE 2 | $[Ca^{2+}]_i$ rise induced by Mrgprb2 agonists in BMMC and PMC. Measurements of $[Ca^{2+}]_i$ changes performed with Fura-2 and presented as F340/F380 fluorescence ratio in BMMC **(A,C,E,G)** and PMC **(B,D,F,H–L)** isolated from WT mice. Representative traces ($n = 20$ each panel) of $[Ca^{2+}]_i$ changes (error bars indicate S.E.M.) induced by application of: 50 µg/ml Compound 48/80 **(A–D)**, 30 µg/ml Tubocurarine **(E–H)**, 3 µM LL-37**(I,K)**, 10 µM Substance P **(J,L)**. The measurements were performed in 3–5 independent cell preparations. At the end of recordings, control reactions were elicited by application of 10 µM adenosine "Ad" **(E,G,J,L)** or by application of 50 µM Compound 48/80 "Cmp" **(I,K)**.

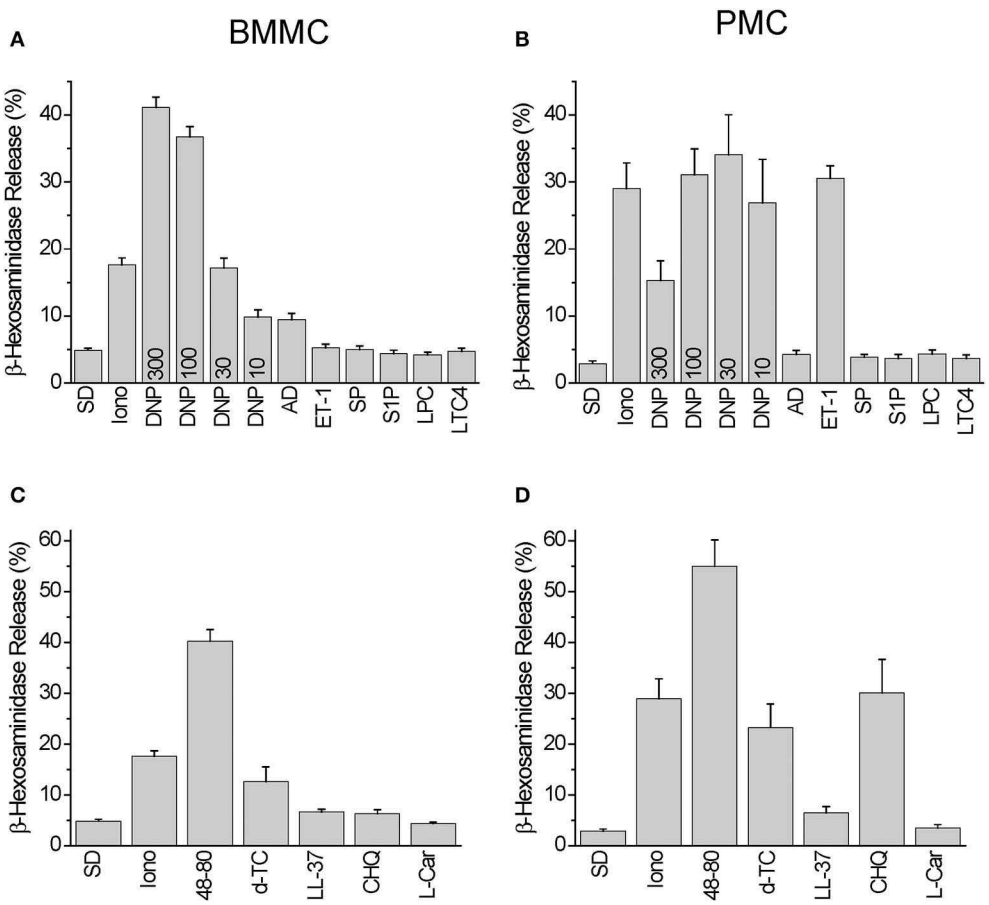

FIGURE 3 | Degranulation induced by Mrgprb2 agonists in BMMC and PMC in comparison with other established mast cell activators. Degranulation of BMMC **(A,C)** or PMC **(B,D)** isolated from wild type C57Bl6/N mice was measured using β-hexosaminidase release assay and presented as % of degranulation. Five **(A,C)** and six **(B,D)** independent preparations were measured. The PMC were stimulated with: vehicle solution (SD- spontaneous degranulation), 10 μM of ionomycin (Iono), 10 ng/ml DNP (DNP10), 30 ng/ml DNP (DNP30), 100 ng/ml DNP (DNP100), 300 ng/ml DNP (DNP300), 10 μM Adenosin (AD), 100 nM Endothelin−1 (ET-1), 10 μM Substance P (SP), 10 μM Sphingosine-1-phosphate (S1P), 10 μM Lysophosphatidylcholine (LPC), 200 nM Leukotriene C4 (LTC4), 50 μg/ml Compound 48/80 (48-80), 30 μg/ml Tubocurarine (d-TC), 3 μM LL-37 (LL-37), 500 μM Chloroquine (CHQ), 300 μM L-Carnosine (L-Car).

7.4, with NaOH. Na^+ and Ca^{2+}-zero extracellular solution contained in mM: NMDG 135; KCl 6; $MgCl_2$ 1.2; glucose 10; HEPES 10; pH 7.4 (with HCl). The pipette solution for whole-cell measurements contained in mM: CsCl 80; MgATP 1; creatine 5; glucose 5; HEPES 10; BAPTA [1,2-bis(2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid] 10; $CaCl_2$ 4.6; pH 7.4 (with CsOH).

Beta-Hexosaminidase Release PMC Degranulation Assay

PMC were centrifuged at 300 × g for 5 min and re-suspended in Tyrode solution containing in mM: NaCl 130; KCl 5; $CaCl_2$ 1.4; $MgCl_2$ 1; glucose 5,6; HEPES 10, 0,1% Bovine Serum Albumine (Fraction V); pH 7.4 (with NaOH). The cells were seeded in a V-bottom 96-well plate (2×10^5 cells/well). All experimental conditions were performed in duplicates. Degranulation was induced by incubation of PMC in the presence of the agonists during 45 min at 37°C and 5% CO_2. Cells were centrifuged at 300 g for 5 min at 4°C. The supernatants were separated and the cell pellets were lysed in Tyrode solution supplemented with 1% Triton-X 100 during 5 min at room temperature. The amount of released β-hexosaminidase enzyme was quantified by spectrophotometric analysis of 4-Nitrophenyl N-acetyl-β-D-glucosaminide (pNAG) hydrolysis, as previously described (29). In short, the cell lysates and supernatants were incubated separately with 2 mM pNAG for 1 h at 37°C and the reaction was stopped by adding 200 mM glycine (pH 10.7 with NaOH). Hydrolysis rate of pNAG was quantified by colorimetric measurements at 405 nm using the NanoQuant Infinite M200pro (Tecan, Switzerland) spectrophotometer. For the background correction the 630 nm absorbance was subtracted from the 405 nm absorbance values. The percentage β- hexosaminidase release was calculated as the absorbance ratio of the supernatant to the sum of supernatant and lysate. If not stated otherwise, all chemicals used in this work were purchased from Sigma.

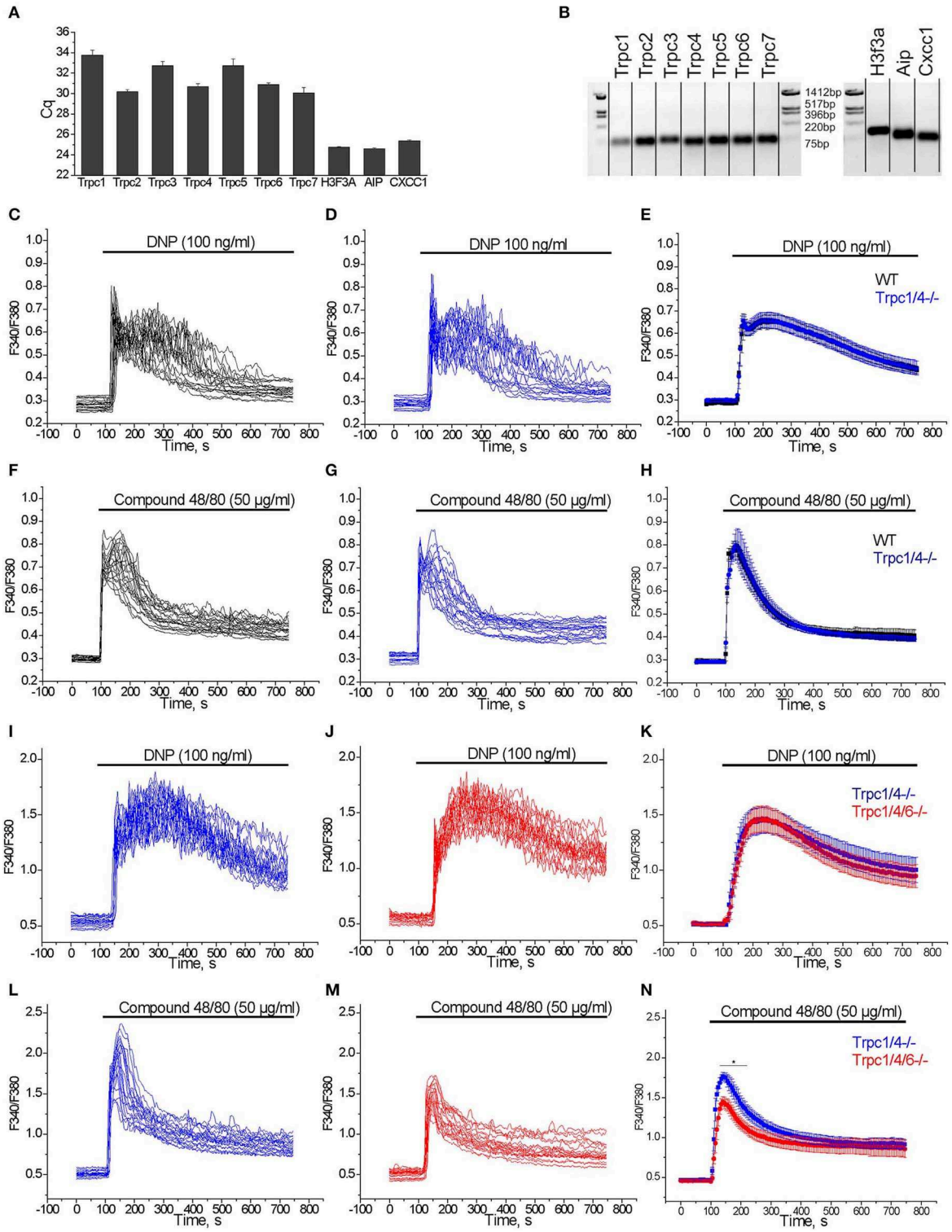

FIGURE 4 | (A) RT-qPCR expression analysis of Trpc transcripts in PMC. Cq values (mean ± S.E.M.) were obtained from duplicates of 3 independent wild type PMC preparations. **(B)** RT-qPCR end products are visualized by agarose gel (2%) electrophoresis. The amplicons correspond to the calculated sizes: Trpc1- (65 bp), Trpc2- (87 bp), Trpc3- (75 bp), Trpc4- (60 bp), Trpc5- (63 bp), Trpc6- (61 bp), Trpc7- (64 bp), H3f3a - (90 bp), Aip - (86 bp), Cxcc1 - (66 bp). **(C–H)** Measurements of $[Ca^{2+}]_i$ changes performed with Fura-2 presented as F340/F380 fluorescence ratio in PMC isolated from WT (C57Bl6/N, black) and *Trpc1/4*$^{-/-}$ (DKO) mice (backcrossed

(Continued)

FIGURE 4 | for seven generations on a C57Bl6/N background, blue). Representative traces ($n = 20$) of $[Ca^{2+}]_i$ changes induced by application of 100 ng/ml DNP **(C,D)** or 50 μg/ml Compound 48/80 **(F,G)** as indicated by horizontal bars. Mean values of $[Ca^{2+}]_i$ changes (error bars indicate S.E.M.) induced by application of 100 ng/ml DNP **(E)** or 50 μg/ml Compound 48/80 **(H)**. For the statistical analysis an average of mean traces obtained from 4 independent preparations was calculated. In each preparation at least 200 cells were imaged and analyzed. **(I–N)** Measurements of $[Ca^{2+}]_i$ changes performed with Fura-2 and presented as F340/F380 fluorescence ratio in PMC isolated from *Trpc1/4/6*$^{-/-}$ (TKO) mice (red) and *Trpc1/4*$^{-/-}$ (DKO) littermates (genotype *Trpc1/4*$^{-/-}$; *Trpc6*$^{+/+}$, blue) and. Representative traces ($n = 20$) of $[Ca^{2+}]_i$ changes induced by application of 100 ng/ml DNP **(I,J)** or 50 μg/ml Compound 48/80 **(L,M)** as indicated by horizontal bars. Mean values of $[Ca^{2+}]_i$ changes (error bars indicate S.E.M.) induced by application of 100 ng/ml DNP **(K)** or by application of 50 μg/ml Compound 48/80 **(N)**. For the statistical analysis an average of mean traces obtained from 5 **(K)** to 6 **(N)** independent preparations was calculated. In each preparation at least 200 cells were imaged and analyzed.

Statistics

For statistical analysis, Origin 8.5 (Northampton, USA) and Microsoft Excel 2010 software were used. For the determination of significant differences of mean values obtained from two groups, a two-sample Student's *t*-test was used ($p < 0.05$ for significance). *n* indicates the number of individual experiments unless otherwise stated.

RESULTS

Comparison of Ca^{2+}-Dependent MC Activators

We have compared the ability of well-known secretagogues to elevate $[Ca^{2+}]_i$ in BMMC and PMC. Application of Adenosine (10 μM) with the subsequent application of the antigen DNP (100 ng/ml) as previously described (30) led to a typical biphasic reaction with comparable amplitudes evoked by either agonist in both BMMC and PMC (**Figures 1A–D**). Acute application of Endothelin 1 (100 nM) evoked a transient response of high amplitude only in some BMMC (**Figure 1E**). The probability of response to the second application of Endothelin-1 (100 nM) in BMMC was much lower in comparison to the first one (**Figure 1E**). In contrast to BMMC, acute application of Endothelin-1 evoked a massive synchronized response of high amplitude in all tested PMC (**Figure 1F**). The removal of the agonist as well as the recurrent application of the same agonist concentration showed no visible effect (**Figure 1F**). In average, Endothelin-1 evoked a much more pronounced rise in $[Ca^{2+}]_i$ in PMC in comparison to BMMC (**Figures 1G,H**). It is published, that activation of cysteinyl leukotriene type I (cysLT1) receptors with Leukotriene C4 (LTC_4, 160 nM) in RBL2H3 cells evokes a series of oscillations in $[Ca^{2+}]_i$ involving calcium release activated Ca^{2+} influx (31). We tested LTC_4 (200 nM) in both BMMC and PMC but did not observe any rise in $[Ca^{2+}]_i$ (**Figures 1I–L**).

Testing of the Ca^{2+} Mobilizing Ability of Mrgprb2 Receptor Agonists in BMMC and PMC

Acute application of the Mrgprb2 receptor agonist Compound 48/80 (50 μg/ml) evoked a delayed oscillatory non-synchronous $[Ca^{2+}]_i$ elevation in BMMC which did not return to the baseline after the agonist removal. A second application of the agonist elicited an additional $[Ca^{2+}]_i$ elevation (**Figures 2A,C**). In PMC, acute application of Compound 48/80 (50 μg/ml) evoked an immediate, synchronous and prominent $[Ca^{2+}]_i$ elevation which also did not get back to baseline levels after the agonist removal. In contrast to BMMC, a second application of the agonist did not elicit an additional $[Ca^{2+}]_i$ elevation (**Figures 2B,D**). Based on this robust $[Ca^{2+}]_i$ rise evoked by Compound 48/80, we tested other Mrgprb2 receptor agonists. Acute application of Tubocurarine (30 μg/ml) evoked an immediate and prominent $[Ca^{2+}]_i$ elevation in BMMC which was not observed in all tested cells (**Figures 2E,G**). In PMC, acute application of Tubocurarine (30 μg/ml) evoked an immediate synchronous prominent $[Ca^{2+}]_i$ elevation which was observed in all tested cells (**Figures 2F,H**).

Acute application of LL-37 (3 μM), which is an antimicrobial peptide known to activate Mrgprb2 receptors (32), evoked an immediate synchronous $[Ca^{2+}]_i$ elevation in PMC which was observed in all tested cells (**Figures 2I,K**). In contrast, application of LL-37 (3 μM) evoked no significant $[Ca^{2+}]_i$ elevation in BMMC (**Figures S1A,B**).

Acute application of Substance P (10 μM), which can activate MRGPRX2 receptors (33) in addition to NK receptors (34), evoked an immediate synchronous prominent $[Ca^{2+}]_i$ elevation in PMC, which was observed in all tested cells. A second application of the agonist did not elicit a significant $[Ca^{2+}]_i$ elevation (**Figures 2J,L**). In BMMC, an acute application of Substance P (10 μM) did not evoke a significant elevation of the $[Ca^{2+}]_i$ (data not shown).

Acute application of the dipeptide L-Carnosine (β-Alanyl-l-histidine) can activate Mrgprd receptors (35) and β-Alanin was reported to activate primary sensory neurons (36). We tested the effect of L-Carnosine in both mast cell types, and found that application of L-Carnosine (1 μM followed by 10 μM) evoked no significant $[Ca^{2+}]_i$ elevation in BMMC (**Figures S1C,E**). Also in PMC, no significant $[Ca^{2+}]_i$ elevation was observed following application of either 1 or 10 μM of L-Carnosine (**Figures S1D,F**).

Analysis of Degranulation Evoked by Mrgprb2 Receptor Agonists in Comparison With Other Mast Cells Secretagogues in BMMC and PMC

Among the Mrgprb2 receptor agonists used in the Ca^{2+} imaging experiments we have observed degranulation reactions in BMMC, which were significantly higher compared to spontaneous degranulation after stimulation with 10–300 ng/ml DNP, 10 μM Adenosine, 50 μg/ml Compound 48/80, and 30 μg/ml Tubocurarine. Degranulation reaction was negligible in response to 100 nM Endothelin-1, 10 μM Substance P, 10 μM Sphingosine-1-phosphate, 10 μM Lysophosphatidylcholine,

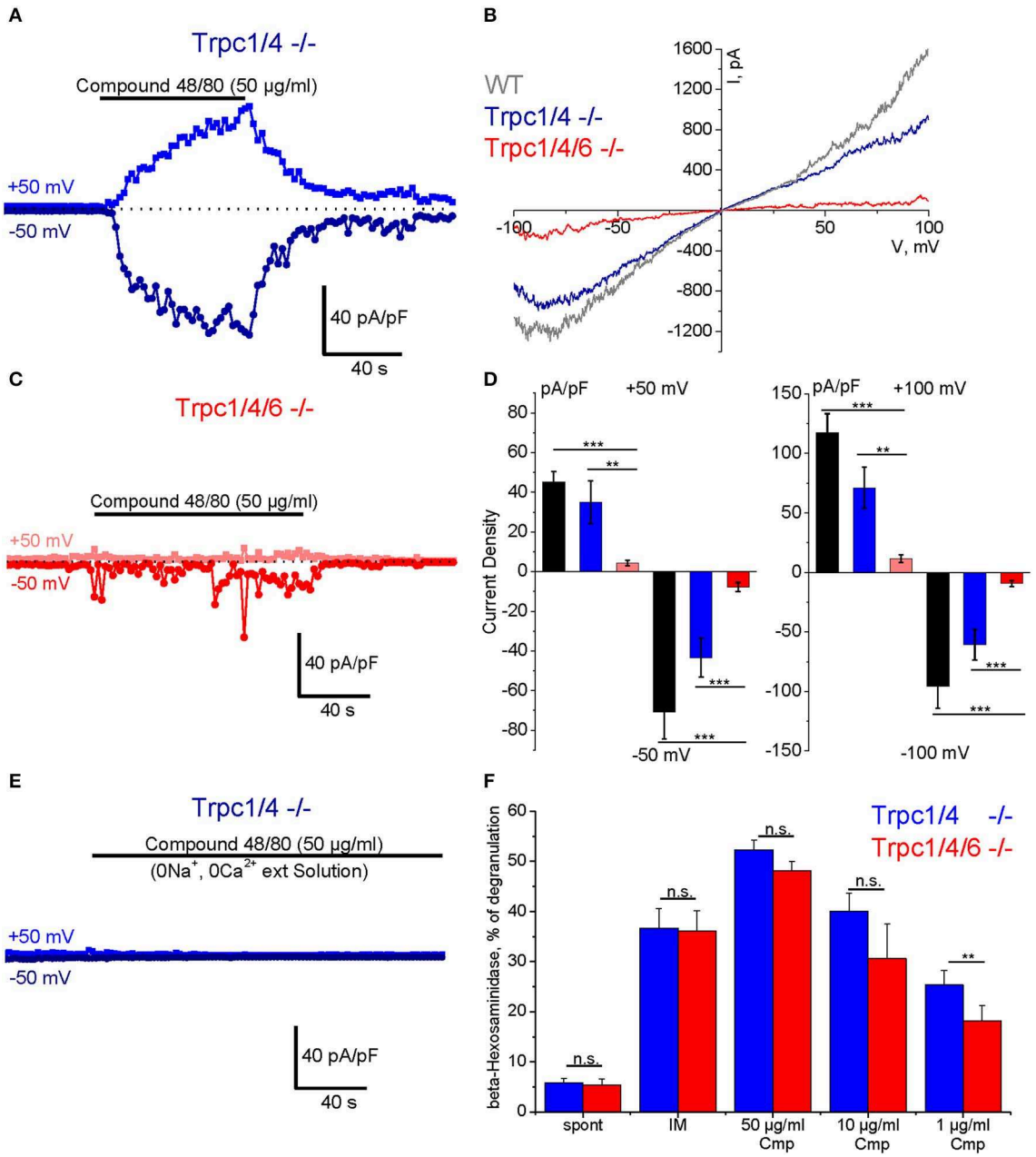

FIGURE 5 | Compound 48/80-induced whole-cell transmembrane currents **(A–E)** and degranulation **(F)** in PMC isolated from TRPC-deficient mice. **(A)** Representative time course of the amplitude of inward (dark blue) and outward (blue) currents measured at −50 and +50 mV, respectively in PMC isolated from *Trpc1/4*$^{-/-}$ (DKO) mice (DKO, genotype *Trpc1/4*$^{-/-}$; *Trpc6*$^{+/+}$). The currents were elicited by acute application of Compound 48/80 (50 µg/ml) as indicated by horizontal bar. **(C)** Representative time course of the amplitude of inward (red) and outward (pink) currents measured at −50 and +50 mV, respectively in PMC isolated from *Trpc1/4/6*$^{-/-}$ (TKO) mice. The currents were elicited by acute application of Compound 48/80 (50 µg/ml) as indicated by horizontal bar. Current amplitude is normalized to the cell capacitance and presented as pA/pF. **(B)** Representative current-voltage relationship curves measured in PMC isolated from wild type (WT, gray), *Trpc1/4*$^{-/-}$ (DKO, genotype *Trpc1/4*$^{-/-}$; *Trpc6*$^{+/+}$, blue) and from *Trpc1/4/6*$^{-/-}$ (TKO) mice (red). The curves correspond to the measurements presented in **(A,C)** at the current's maximal values. **(D)** Statistical bar diagrams of the maximal current density measured at +50/−50 mV (left panel) and at +100/−100 mV (right panel) shows the mean (vertical bars) and S.E.M. (error bars) values calculated for PMC isolated from WT (gray and black bars) Trpc1/4 double deficient (blue and dark blue) and Trpc1/4/6 deficient (pink and red bars) mice. The measurements were performed with PMC originating from three independent preparation and 15–20 individual cells were analyzed. Horizontal bars indicate significance (**$p < 0.01$ and ***$p < 0.001$). **(E)** Representative time course of the amplitude of inward (dark blue) and outward (blue) currents measured at −50 and +50 mV, respectively in PMC isolated from *Trpc1/4*$^{-/-}$ (DKO) mice. Compound 48/80 was applied in Ca^{2+}- and Na^{+}-free external solution in concentration 50 µg/ml as indicated by horizontal bar. **(F)** Degranulation of PMC measured using ß-hexosaminidase release assay and presented as % of degranulation. PMC were isolated from *Trpc1/4*$^{-/-}$ (DKO) mice (blue bars) and *Trpc1/4/6*$^{-/-}$ (TKO) (red bars) mice and stimulated with different concentrations of Compound 48/80 (Cmp). Positive (IM: −10 µM of ionomycine) and negative controls (spont- spontaneous release) were also measured.

200 nM Leukotriene C4, 3 μM LL-37, 500 μM Chloroquine, and 300 μM L-Carnosine, respectively (**Figures 3A,C**). Stimulation with Ionomycin (10 μM) served as a control.

In PMC, we found degranulation reactions significantly higher than those occurring spontaneously after stimulation with 10–300 ng/ml DNP, 100 nM Endothelin-1, 50 μg/ml Compound 48/80, 30 μg/ml Tubocurarine, 3 μM LL-37, and 500 μM Chloroquine. Degranulation reactions were negligible after application of 10 μM Adenosine, 10 μM Substance P, 10 μM Sphingosine-1-phosphate, 10 μM Lysophosphatidylcholine, 200 nM Leukotriene C4, and 300 μM L-Carnosine, respectively (**Figures 3B,D**).

Based on these results we can conclude that BMMC exhibit an increased degranulation response compared to PMC following stimulation with 10 μM Adenosine ($p = 0.007$), and PMC exhibit a larger response to 100 nM Endothelin-1 (p = 8e-07) and 500 μM Chloroquine ($p = 0.001$). The Mrgprb2 receptor agonist Compound 48/80 evoked similar extents of degranulation in both PMC and BMMC.

Analysis of TRPC Expression in PMC

We studied the expression of transcripts encoding TRPC channel proteins by RT-qPCR in PMC. As shown in **Figure 4A**, all TRPCs transcripts are detected in PMC, with Trpc2, Trpc4, Trpc6, Trpc7 transcripts being more abundant compared to Trpc1, Trpc3, and Trpc5. The relative expression is presented as Cq values which are reverse proportional to the level of expression. Endpoint PCR products were visualized by gel electrophoresis and amplicons of the expected size were obtained (**Figure 4B**).

Partial Contribution of TRPC Channels to $[Ca^{2+}]_i$ Elevation Elicited by Mrgprb2 Receptor Stimulation in PMC

From former experiments using PMC from various Trpc knockout mouse lines we had evidence that Ca^{2+} elevation evoked by several agonists were reduced in mast cells of *Trpc1/4/5/6*$^{-/-}$ quadruple knockout mice (37), as well as in*Trpc1/4/6*$^{-/-}$ triple and *Trpc1/4*$^{-/-}$ double knockout mice. The mast cells for these experiments had been isolated from mouse lines on a mixed C57Bl6/N × 129SvJ genetic background and compared to PMC of mice from the F1 generation of C57Bl6/N × 129SvJ matings as controls (data not shown). However, when we analyzed calcium transients measured in PMC from *Trpc1/4*$^{-/-}$ double knockout (DKO) mice on C57Bl6/N background (mated from *Trpc1*$^{-/-}$ and *Trpc4*$^{-/-}$ single knockout mice after 7 generation backcrossing) we found that $[Ca^{2+}]_i$ elevation evoked by FcεRI stimulation by acute application of DNP (100 ng/ml) was statistically not significantly different compared to those in PMC isolated from C57Bl6/N wild type controls (**Figures 4C–E**). Similarly, Ca^{2+} transients observed in PMC in response to acute application of 50 μg/ml of Compound 48/80 were also statistically not significantly different in PMC isolated from *Trpc1/4*$^{-/-}$ DKO mice in comparison to C57Bl6/N wild type PMC (**Figures 4F–H**).

Since the responses to FcεRI and Mrgprb2 receptor stimulation were identical in PMC from *Trpc1/4*$^{-/-}$ DKO and WT mice, we then compared PMC from *Trpc1/4/6*$^{-/-}$ (TKO) knockout mice and used littermatched *Trpc1/4*$^{-/-}$ DKO mice as controls (see methods for breeding scheme). Calcium transients evoked in PMC in response to acute application of DNP (100 ng/ml) were statistically not significantly different in PMC isolated from *Trpc1/4/6*$^{-/-}$ (TKO) mice or *Trpc1/4*$^{-/-}$ DKO mice (**Figures 4I–K**). In contrast, peak calcium transients evoked in response to acute application of Compound 48/80 (50 μg/ml) were significantly lower (by 24%) in PMC from *Trpc1/4/6*$^{-/-}$ (TKO) mice in comparison to those in PMC isolated from *Trpc1/4*$^{-/-}$ (DKO) mice (**Figures 4L–N**). The AUC of the Ca^{2+} transients was reduced by 20 % in average.

Characterization of Compound 48/80-Induced Currents in PMC

To study Compound 48/80-induced transmembrane ionic currents in PMC we applied the standard patch clamp voltage clamp technique in the whole-cell configuration. The cells were perfused with Cs^+-based pipette solution to block K^+ channels, and $[Ca^{2+}]_i$ was strongly buffered at a concentration close to 100 nM. A standard external physiological salt solution was used for these experiments. The cells were kept at a holding potential of 0 mV and periodical 400 ms depolarizing ramp pulses (−100 to +100 mV) were applied at a frequency of 0.5 Hz. In response to external application of Compound 48/80 (50 μg/ml) we observed a slowly developing current, which reached a plateau phase after 1–2 min and showed an almost linear current-voltage relationship and a reversal potential near 0 mV (**Figures 5A,B**). Agonist removal led to gradual reduction of the current which returned almost to the basal level during 1–3 min (**Figure 5A**). These currents were observed in PMC isolated from WT (C57Bl6/N, black bars in **Figure 5D**) as well as from *Trpc1/4*$^{-/-}$ (DKO) mice, and their amplitude was not different in PMC of these two genotypes. However, the additional deletion of TRPC6 proteins in PMC of *Trpc1/4/6*$^{-/-}$ (TKO) mice significantly decreased the amplitude of the inward and outward currents compared to PMC from *Trpc1/4*$^{-/-}$ (DKO) or C57Bl6/N mice as indicated in representative traces (**Figure 5C**) and in the statistical analysis performed at +50, −50, +100, −100 mV (**Figure 5D**). The current in PMC from *Trpc1/4*$^{-/-}$DKO mice was also almost entirely abolished if an extracellular solution lacking Na^+ and Ca^{2+} ions was used (**Figure 5E**).

Participation of TRPC6 in Compound 48/80 Induced Degranulation in PMC

A β-hexosaminidase assay was performed in PMC of *Trpc1/4/6*$^{-/-}$ (TKO) knockout mice and litter-matched *Trpc1/4*$^{-/-}$ DKO mice. Whereas, no difference in degranulation was observed when the cells were stimulated with 50 μg/ml and 10 μg/ml Compound 48/80, the measurements revealed a significant reduction (28% in average) of Compound 48/80-induced degranulation in PMC lacking TRPC1/TRPC4/TRPC6 proteins when a concentration of 1 μg/ml of Compound 48/80 was used (**Figure 5F**).

DISCUSSION

In this study we comparatively analyzed numerous secretagogues in two primary murine mast cell (MC) models: peritoneal mast cells (PMC), as an example of connective tissue type MC (CTTMCs) (38), and bone marrow-derived and *in vitro* matured mast cells (BMMC) (39) which belong to the mucosal type MCs (MMCs). We provide experimental evidence for at least a partial contribution of TRPC channel proteins to Ca^{2+}-dependent mast cell activation in PMC evoked by the Mrgprb2 agonist Compound 48/80.

Characteristics of CTTMC and MMC Mast Cell Models

With a comparative analysis of two culture-matured and tissue-derived mast cell models we aimed to functionally evaluate the suitability of these primary mast cells for the analysis of Ca^{2+}-dependent mast cell activation *in vitro*. The main difference between the connective tissue and mucosal type mast cells is the variety of proteases they express. Thus, mature connective-tissue mast cells express chymases known as mouse mast-cell protease-4 (MMCP-4), -5, and -2, as well as tryptases MMCP-6 and -7, and carboxypeptidase A. Mast cells of the mucosal type preferentially express proteases such as MMCP-1 and -2 as seen in rat mast cells (40, 41). In addition, connective-tissue-type mast cells express little or no NDST-1(*N* -deacetylase/*N* -sulphotransferase-2), but contain large amounts of the transcript encoding NDST-2 (42). It is also known that PMC express a wider range of Toll-like receptors (TLRs), and secrete significantly more cytokines in response to TLR ligand stimulation compared to BMMC or to immortalized mast cell lines (43). The expression of distinct proteases can be well illustrated by the fact that *Mcpt5-Cre* (mast-cell protease-5) transgenic mice enable gene inactivation only in connective tissue mast cells but not in mucosal mast cells (44). In contrast to extensive characterization of connective tissue and mucosal type mast cells with respect to their enzymatic repertoire, there is very little information regarding their responses to the numerous published Ca^{2+} mobilizing mast cell activators. In particular, the activity of these Ca^{2+} mobilizing mast cell activators has not been comparatively characterized in BMMC and PMC as the two primary murine mast cell models (12, 45), which were studied very frequently after gene-deficient mouse lines became increasingly available.

Features of the Agonist-Evoked Ca^{2+} Rise in BMMC and PMC

An elevation of the $[Ca^{2+}]_i$ in mast cells, which is a key signal for mast cell activation, can be triggered not only by activation of Fcε receptor for IgEs (FcεRI) with antigens but has also been reported after exposure to numerous agonists such as adenosine, Endothelin-1 (ET-1), Leukotriene C4 (LTC_4), lysophosphatidylcholine (LysoPC), sphingosine-1-phosphate (S1P) or Substance P and even others (14). In our study we found that, whereas antigen stimulation of FcεRI was similarly effective in triggering $[Ca^{2+}]_i$ elevations in PMC and BMMC, other agonists including Endothelin-1 (ET-1, 100 nM) evoked much more prominent responses in PMC in comparison to BMMC or did not evoke any $[Ca^{2+}]_i$ rise in either mast cell model. As expected, the magnitude of $[Ca^{2+}]_i$ rise correlated with extent of mast cell degranulation. Similar results were obtained in a comparison of Endothelin-1 (ET-1)-induced degranulation in fetal skin-derived cultured mast cells (FSMCs) and BMMC, as ET-1 induced degranulation in FSMC but not in BMMC (46). The cysteinyl leukotriene receptor I (cysLT1) agonist, leukotriene C4, did not produce any $[Ca^{2+}]_i$ rise or degranulation response neither in BMMC nor in PMC at least in the concentration of 200 nM. This is in contrast to published data that show that LTC_4 (160 nM) is quite effective in producing $[Ca^{2+}]_i$ rise in other mast cell models such as RBL2H3 cells (31). Notably, LTC_4 plays a central role in activation of human nasal polyp–derived mast cells in a paracrine way (47). Obviously, murine BMMC and PMC are not suitable models to study Ca^{2+} dependent mast cell function that are evoked by Cysteinyl leukotriene receptors and triggered by LTC_4.

Mrgprb2-Mediated Ca^{2+} Signaling in BMMC and PMC

Compound 48/80 is well-known as a Ca^{2+}-dependent mast cell activator for years, but the receptor mediating Ca^{2+} entry and mast cell activation by this agonist and a range of other cationic substances termed basic secretagogues was identified in 2015 (4). We found that the Mrgprb2 agonist Compound 48/80 evoked a significant $[Ca^{2+}]_i$ rise and degranulation response in PMC and BMMC, but these responses were much weaker in BMMC. Other agonists of Mrgprb2 receptors such as Tubocurarine or LL-37 and Chloroquine, which are reported to activate other Mrgpr receptors (48), demonstrated a similar relation regarding their action in BMMC vs. PMC suggesting that the expression level or coupling of Mrgprb2 receptors or of the signaling molecules downstream differs between these two types of mast cells. Substance P evoked a $[Ca^{2+}]_i$ rise only in connective tissue type PMC and did not produce any significant degranulation in these cells. The functional target of Substance P in PMC and mast cells in general is not clearly defined. Substance P is able to activate the human ortholog of Mrgprb2, MRGPRX2 (33), but not the mouse Mrgprb2 receptor (49). In addition, Substance P is also capable of stimulating NK receptors (34) which are also expressed in TC mast cells (50).

In general, our data demonstrate a much higher efficiency of Mrgpr agonists in PMC to induce $[Ca^{2+}]_i$ elevation and degranulation in comparison to BMMC. These results are in agreement with those of a previous report, in which it has been shown that Mrgprb2 is specifically expressed on connective tissue mast cells but not on mucosal mast cells in mice (4).

Contribution of TRPC Channels to Mrgprb2-Mediated Ca^{2+} Rise in PMC

Our expression analysis revealed that transcripts of all members of the TRPC family of transient receptor potential (TRP)

channels could be detected in PMC. Since we had evidence that Ca^{2+} elevation evoked by various agonists leading to Phospholipase C activation were reduced in PMC mast cells of *Trpc1/4/5/6*$^{-/-}$ quadruple knockout mice (37) and also *Trpc1/4/6*$^{-/-}$ triple knockout mice both on a mixed C57Bl6/N × 129SvJ genetic background compared to PMC of corresponding wild type control mice, we subsequently studied *Trpc1/4/6*$^{-/-}$ mice that were obtained after seven generation of backcrosses into the C57Bl6/N background, which were then compared to littermatched *Trpc1/4*$^{-/-}$ controls (see Methods for details of breeding). We found that FcεRI-mediated Ca^{2+} rise was not significantly different in PMC from *Trpc1/4/6*$^{-/-}$ mice compared to PMC from either C57Bl6/N or *Trpc1/4*$^{-/-}$ littermatched controls indicating the importance of the genetic background for studies evaluating Ca^{2+} signaling in murine PMC mast cells. Nevertheless, Compound 48/80-evoked $[Ca^{2+}]_i$ transients were reduced by ∼20% in PMC from *Trpc1/4/6*$^{-/-}$ mice which goes along with the abrogation of Compound 48/80-evoked cation currents and an according reduction of mast cell degranulation. In the past, many studies about the role of TRPC channels in mast cells have been performed using immortalized mast cell lines (14). There are only a few reports about the role of TRPC channels and particularly TRPC1, TRPC4, and TRPC6 in mast cell activation. Altered calcium responses in Lyn-deficient mast cells are at least partially attributed to a Lyn-dependent role in maintaining basal expression of Trpc4 (51). In BMMC from TRPC1 knockout mice, an unexpected increase in antigen-evoked interleukin and TNFα secretion was reported (52). TRPC1 and TRPC6 were found to be expressed in human mast cell lines, but a contribution of Orai and not TRPC channels to FcεRI-mediated calcium signaling was demonstrated recently (53). These data are similar to our findings showing that TRPC6 plays a significant role specifically in Mrgprb2- but not FcεRI-mediated $[Ca^{2+}]_i$ elevation in murine PMC. In murine mast cells the Orai1 knock out (54, 55) and knock down of Orai1 in human lung mast cells (56) reduced FcεRI-mediated calcium rise and FcεRI-dependent mediator release, respectively, whereas these responses was increased in PMC of Orai2 knock out mice (57).

Our study shows that in PMC from *Trpc1/4/6*$^{-/-}$ mice the FcεRI-mediated Ca^{2+} rise is unchanged and Mrgprb2-mediated Ca^{2+} rise is partially reduced. The signaling events downstream of Mrgprb2 receptor activation that specifically engage these TRPC channels remain unclear, but it needs to be mentioned that there is a growing experimental evidence of important differences between MRGPRX2- and FcεRI-mediated mast cell activation and degranulation (17). Notably, FcεRI-mediated mast cell activation involves a robust inflammatory reaction by synthesis of cytokines and chemokines, and this reaction has been shown to be limited in MRGPRX2-mediated activation (17). Classically, IgE-dependent mast cell activation occurs via FcεRI with the subsequent tyrosine phosphorylation of multiple proteins including phospholipase Cγ1 the activation of which leads to multiple downstream events including an increase in cytosolic calcium levels. There is evidence that Mrgpr activation involves activation of phospholipase C which leads to DAG formation. For example, beta-defensins are proinflammatory pruritogens that activate Mrgprs (58) and act chemotactic for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway (59). Diacylglycerol (DAG) formed by phospholipase C could be a signal activating TRPC3/6 channels (60) also in the plasma membrane of mast cells.

The molecular constituents of the channels engaged following Mrgprb2 receptor activation have not been identified. However, it was reported already long time ago that Compound 48/80 activates a Ca^{2+} influx in rat peritoneal mast cells (17). One type of channel responsible for the Ca^{2+} influx followed Compound 48/80 stimulation is voltage–independent and has a relatively low selectivity for Ca^{2+} over Na^{+} (19–21), which closely resembles the properties of agonist-operated TRPC cation channels. In our study in PMC we observed Compound 48/80–induced currents using patch-clamp recordings. These currents exhibited a nearly linear current-voltage relationship with a reversal potential close to 0 mV resembling typical TRPC-mediated currents described in numerous cell models (61). This current was almost absent when an external solution lacking both Ca^{2+} and Na^{+} ions was used demonstrating the cationic nature of Compound 48/80–induced currents. This current was strongly reduced in PMC from *Trpc1/4/6*$^{-/-}$ mice implying that the Compound 48/80-induced Ca^{2+} elevation might be attributed to the Ca^{2+} influx through TRPC6-containing channels in murine PMC. The contribution of TRPC6 to the compound 48/80-evoked current was elaborated by genetic deletion of TRPC6 expression. The deduced concept of TRPC6 as an indispensable constituent of cation channels activated by compound 48/80 in PMCs could be corroborated in future studies by experimental evidence showing that acute blockage of these currents using TRPC6-specific antagonists such as Pyrazolo [1,5-a] pyrimidines antagonist (62) or BI 749327 (63).

Taken together, our data revealed that PMC are an appropriate mast cell model to study mechanisms of Mrgprb2 receptor-mediated mast cell activation and that TRPC proteins contribute at least partially to the activation of PMC evoked by stimulation of Mrgprb2 receptors. However, the channels conducting most of the Ca^{2+} entry triggered by Mrgprb2 receptor stimulation still remain unknown. PMC express plenty of Ca^{2+}-permeable cation channels. We have recently excluded a significant participation of TRPV1, TRPV2, and TRPV4 channels in both FcεRI- and Mrgprb2- mediated activation of PMC (64). At the same time Orai1, Orai2, and Orai3 proteins are abundantly expressed in PMC, and at least Orai2 has a critical role in receptor- and store-operated Ca^{2+}entry in murine PMC (57), whereas the contribution of Orai1 and Orai3 proteins in Mrgprb2-evoked mast cell activation in general remains to be identified.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regierungspräsidium Karlsruhe, Abteilung 3.

AUTHOR CONTRIBUTIONS

VT: experimental design, Ca^{2+} imaging, degranulation assay, patch-clamp, and manuscript writing. AS-L: Ca^{2+} imaging and degranulation assay. JA: Ca^{2+} imaging. CR: qPCR. LB and AD: Trpc1 and Trpc6 mouse models. MF: concept and experimental design, data analysis and interpretation, funding, and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00564/full#supplementary-material

Figure S1 | Mrgprb2 agonists without Ca^{2+} mobilizing effects in BMMC or PMC murine mast cells. Measurements of $[Ca^{2+}]_i$ changes performed with Fura-2 and presented as F340/F380 fluorescence ratio in BMMC **(A–C,E)** and PMC **(D,F)** isolated from WT mice. Representative traces ($n = 20$ each panel) of $[Ca^{2+}]_i$ changes (error bars indicate S.E.M.) induced by application of 3 μM of LL-37 **(A,B)**, 1 μM L-Carnosine **(C,E)**, and 10 μM of L-Carnosine **(D,F)**. The measurements were performed in 3–5 independent cell preparations. At the end of recordings, control reactions were elicited by application of 10 μM adenosine "Ad" **(A–C,E)** or by application of 50 μM Compound 48/80 "Cmp" **(D,F)**.

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REVIEW
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TRP Channels as Interior Designers: Remodeling the Endolysosomal Compartment in Natural Killer Cells

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Cytotoxic lymphocytes, including natural killer (NK) cells and T cells are distinguished by their ability to eliminate target cells through release of secretory lysosomes. Conventional lysosomes and secretory lysosomes are part of the pleomorphic endolysosomal system and characterized by its highly dynamic nature. Several calcium-permeable TRP calcium channels play an essential role in endolysosomal calcium signaling to ensure proper function of these organelles. In NK cells, the expression of self MHC-specific inhibitory receptors dynamically tunes their secretory potential in a non-transcriptional, calcium-dependent manner. New insights suggest that TRPML1-mediated lysosomal calcium fluxes are tightly interconnected to NK cell functionality through modulation of granzyme B and perforin content of the secretory lysosome. Lysosomal TRP channels show a subset-specific expression pattern during NK differentiation, which is paralleled with gradually increased loading of effector molecules in secretory lysosomes. Methodological advances, including organellar patch-clamping, specific pharmacological modulators, and genetically-encoded calcium indicators open up new possibilities to investigate how TRP channels influence communication between intracellular organelles in immune cells. This review discusses our current understanding of lysosome biogenesis in NK cells with an emphasis on the TRP mucolipin family and the implications for NK cell functionality and cancer immunotherapy.

Keywords: NK cell, TRP cation channel, secretory lysosomes, cytotoxic lymphocytes, mucolipin, calcium signaling

THE SECRETORY LYSOSOME AT THE HEART OF INTRACELLULAR CALCIUM SIGNALING IN NK CELLS

Malignant or virally-infected cells can be eradicated by T cells, due to surface presentation of peptide-loaded major histocompatibility complex (MHC) class I molecules (1, 2). However, some cells escape T cell recognition through down-regulated expression of MHC class I molecules, due to

specific genetic alterations, cellular stress, or intracellular retention by viral proteins. This potential loophole of the body's defense is guarded by natural killer (NK) cells that express MHC binding inhibitory receptors. Therefore, in contrast to T cells, it is the loss of MHC expression that can elicit a cytotoxic response by NK cells (3). NK cells also recognize discontinuity such as expression of stress ligands commonly displayed on virally infected cells through a broad array of activating receptors (4). Hence, NK cell responses are counterbalanced by combinations of various inhibitory and activating signals (5, 6). The net outcome of these signaling cascades that drive these responses is reflected in a shift of the cytoplasmic calcium homeostasis. Unopposed triggering of NK cells by ligands for activating receptors evokes strong calcium signals in the cytoplasm of NK cells (7–9).

At the interface between the effector and target cell lysosome-related organelles polarize and fuse with the plasma membrane in a highly-directed, and calcium-dependent manner (2, 10, 11). The release of lysosome-related organelles (or lytic granules), hereafter referred to as secretory lysosomes, represents one of the classic killing mechanisms of NK and T cells and is mostly driven by ER-derived calcium stores (11, 12). Successful release of cytotoxic molecules marks a crucial step in the destruction of the target cell (13). Additional evidence suggests that a single NK cell degranulation event is sufficient to trigger target cell apoptosis, as elucidated by elegant live cell imaging (14).

However, the underlying global calcium signals are far more complex and require not just the ER-derived calcium, but also involve calcium signals from the acidic calcium stores (15). The importance of calcium signals derived from the acidic, endolysosomal calcium stores, as a basis for communication between organelles and coordination of metabolism and transport processes has become increasingly appreciated in the context of disease and immune cell function (16–19). A multitude of metabolites can be exchanged via physical inter-organelle contact sites, which, in turn, influences calcium channel activity, and can help to coordinate calcium homeostasis, in order to establish proper immune cell responses. In cytotoxic lymphocytes, changes in the lysosomal signaling capacity directly correlates with immune cell effector responses (20–25). Therefore, lysosomal calcium channels emerge as novel targets for genetic and pharmacological interventions aiming to boost immune effector cell function in cancer immunotherapy.

This review focus on the role of transient receptor potential (TRP) calcium channels in the remodeling of the endolysosomal compartment in NK cells and in orchestrating organelle communication in cytotoxic lymphocytes. It is important to point out that the lysosomal calcium channel repertoire is highly diverse. In addition to TRPs, which are the focus of the current review, they also comprise two pore channels (TPCs) and the ATP-gated P2X channel family (16). In T cells the TPC calcium channel, TPC2, has been detected on secretory lysosomes and TPC2-activation stimulates the calcium-dependent exocytosis of the these organelles (26). TPC biology and their role in the endolysosomal system are reviewed elsewhere (27, 28).

THE LYSOSOMAL COMPARTMENT IN CYTOTOXIC LYMPHOCYTES

Early studies have revealed important morphological features and the functional principle of secretory lysosomes in cytotoxic immune cells. Electron microscopy enabled examination of these organelles in great detail, and as the name implies, morphologically they resemble conventional lysosomes in many aspects. Yet, there is a large degree of heterogeneity within the pool of lysosomes, as discussed later in this section (29, 30). Moreover, both conventional lysosomes and secretory lysosomes share vesicular delivery pathways from the Golgi and represent the terminal part of the endosomal pathway (30–32). This model is based on the immuno-EM detection of lytic effector molecules, an electron-dense core and acid hydrolases in the same compartment, which supports a common identity. Hence, secretory lysosomes in T and NK cells are lysosomes with a luminal secretory unit, the dense core, which contains the effector molecules and is releasable during degranulation (33–35). The electron dense core of secretory lysosomes is mainly composed of serglycin, an anionic chondroitin-sulfate coated proteoglycan, regarded to function as safe storage site for cationic granzymes and perforin (36, 37). Lysosomes are a heterogenous group of organelles distinguished on the basis of their luminal pH, morphology, degradative capacity, and subcellular positioning (38). Furthermore, the secretory lysosomes of NK cells have been described as a multi-facetted pool of organelles and are commonly divided into three classes (33). Type I granules appear to have an electron-dense core with relatively little cortex. In comparison, type II granules are distinguished with varying morphology and inclusion of vesicles and membrane whorls. First, the intermediate type of granules combines both phenotypes and can appear with multiple dense-core structures and vesicular, multilamellar cortex. Second, it has been speculated that type I granules might represent a more mature stage, whereas, intermediate and type II might resemble immature stages in the cycle of granule biogenesis (39). The interpretation of this model must be treated with some caution, since NK cell specific data has mostly been obtained from investigations on two-dimensional images of rat NK cell lines or on PBMC fractions. In future studies the implementation of correlative-light electron microscopy and three-dimensional EM-tomography of primary well-defined NK cell subsets might provide additional insights on the heterogeneity of dense core granules.

REMODELING OF SECRETORY LYSOSOMES IN NK CELLS

Lysosomes in NK and T cells have dual functionality. These acidic organelles harbor both the enzymatic activity of a conventional lysosome and cytotoxic molecules, most importantly granzyme B and perforin, used for killing target cells by apoptosis. Target cell recognition unleashes a complex signaling cascade in cytotoxic lymphocytes, which culminates in the formation of an immunological synapse (IS). Most importantly, PLCγ-generated

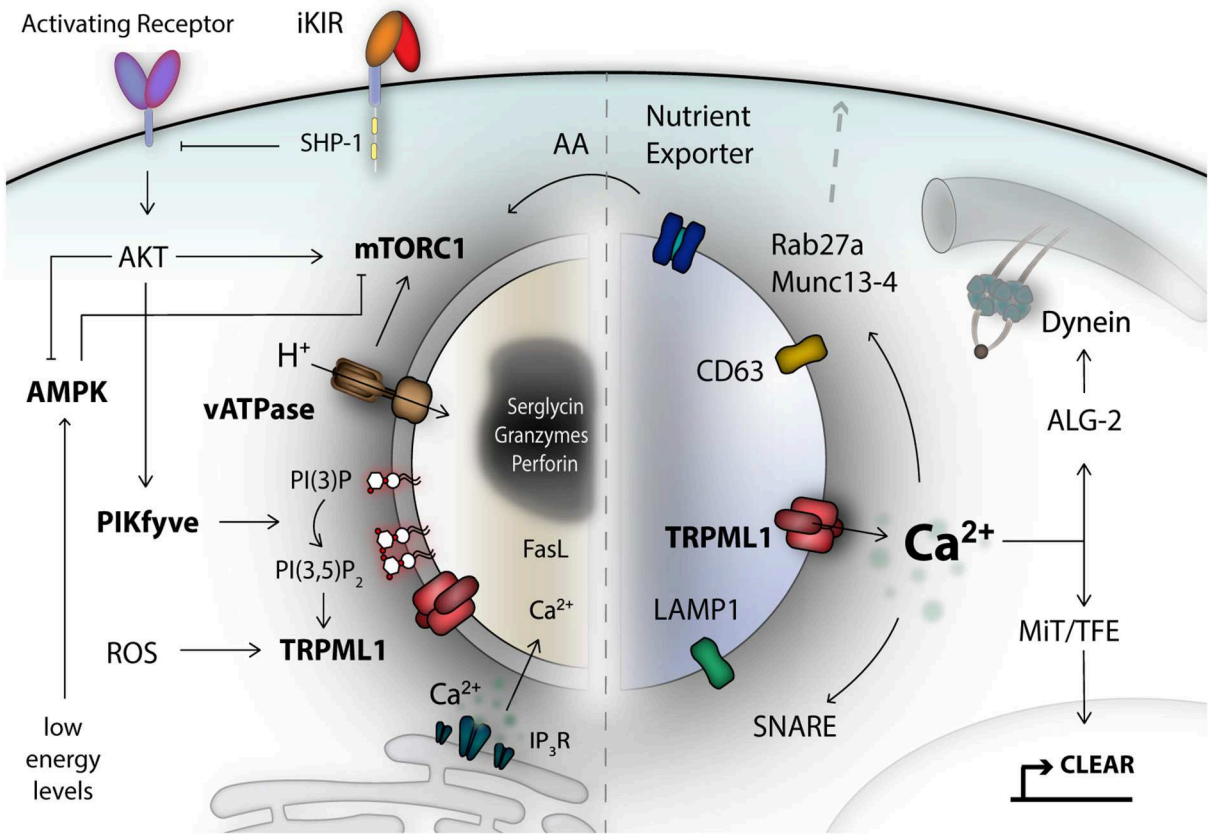

FIGURE 1 | The lysosomal compartment is an important signaling hub and integrates a diverse range of signals. Secretory lysosomes are dual-functional organelles consisting of a lysosomal limiting membrane and a proteoglycan electron-dense core as safe storage unit for effector molecules like granzymes and perforin. Many different signals form the cell surface, or from the inside, converge at the limiting lysosomal membrane and can be detected by specialized metabolic-, energetic-, stress-, pH-, and lipid-moiety-sensor proteins. For NK cells, one of the central metabolic sensors is called mTOR complex 1, which can detect amino acids (AA) and growth factor signals. AMP-activated kinase (AMPK) reacts to stress signals, such as reactive-oxygen species (ROS) and can trigger autophagy induction to recover nutrients. A remarkable class of signal integrators, is the transient receptor potential (TRP) channel family, most importantly TRPML1, localized on the lysosomal membrane. TRP channels can integrate signals of diverse nature, translated into calcium signals. TRPML1 calcium signals control lysosomal trafficking membrane dynamics and TFEB-dependent activation of the CLEAR gene network. A network of genes associated with lysosomal biogenesis and autophagy, and commonly regulated by transcription factors of the MiT/TFE family. Lysosomal calcium signals and lipid membrane composition, as well as integral lysosomal surface proteins are essential for the recruitment of e.g., motor proteins, the small Rab27a GTPase, Munc 13-4, and SNARE proteins as mediators of plasma membrane fusion. Altogether, these are critical components for orchestrating exocytosis of secretory lysosomes in NK cells. iKIR, inhibitory killer immunoglobulin-like receptors.

calcium signals derived from IP_3-receptors (Inositol triphosphate receptor) at the Endoplasmic reticulum (ER) are one of the key downstream signals that are paramount in orchestrating the directed NK cell degranulation at the IS (40–42). Cytotoxic lymphocytes recruit further proteins to the lysosomal surface, which primes these organelles for exocytosis. In NK cells, among these accessory proteins are the small GTPase Rab27a and its effector Munc13-4, which interact with the SNARE proteins syntaxin11 and VAMP7 on the plasma membrane (43–46). The Rab27a-dependent mode of secretion discriminates secretory lysosomes from conventional lysosomes (45). These peripheral proteins on the cytoplasmic side of the secretory lysosome are needed to initiate transportation toward the IS, followed by a docking step and fusion with the plasma membrane (10, 11, 47, 48). By contrast, specialized secretory cells, such as insulin secreting β cells of the pancreas, contain conventional lysosomes for digestion of endocytosed cargo, alongside a designated pool of vesicles for regulated release of their secretory products.

Naïve T cells undergo an initial priming phase that requires stimulation of the TCR in order to initiate secretory lysosome formation along with accumulation of effector molecules (49). In contrast to naïve $CD8^+$ T cells, NK cells generate granzyme B and perforin containing secretory lysosomes in response to cytokines alone, without the need for specific ligation of an activating receptor (30, 33, 50). The cytotoxic potential of NK cells develops gradually during differentiation and is reflected in the content of effector molecules and the accumulation of large secretory lysosomes (37, 51, 52). In addition to this gradual acquisition of effector function, NK cells tune their functional potential against self MHC in a non-transcriptional process termed education (53–57). Inhibitory interactions with self-MHC translate into a predictable, quantitative relationship

between self-recognition and effector potential. Paradoxically, a lack of constitutive inhibitory self-interaction is associated with hyporesponsiveness of NK cell subsets to various stimuli, while the presence of self-interactions is associated with gain in functionality. Although there are several models that encompass cellular events that are critical for NK cell education, the mechanism behind the functional calibration against MHC has remained unclear. Recent work from our laboratory established a direct link between inhibitory signaling and the size of the secretory lysosomes in NK cells (25). We found that educated NK cells contain more granzyme B stored in a unique pool of dense core secretory lysosomes. Furthermore, as we shall discuss in more detail below, pharmacological and genetic interference pointed to a previously unknown upstream role for Transient Receptor Potential Mucolipin-1 (TRPML1) activity in the remodeling of secretory lysosomes in NK cells. Altogether, new mechanistic insights were gained from studying NK cell education, which demonstrated that tonic inhibitory receptor input from the plasma membrane can affect critical processes in the endolysosomal system, with sustained impact on the calcium signaling capacity from the acidic compartment.

LYSOSOMES AT THE CENTER STAGE OF METABOLIC REGULATION

The highly complex nature of the lysosomal compartment and how it shapes cellular calcium signaling was initially largely overlooked and lysosomes were simply regarded as terminal unit for degradation of endocytosed material. The original concept is reflected in its name "*lysis*- and *-some*" derived from the Greek for "digestive body" (58). Since then, our view on lysosomes has changed dramatically, from a waste disposal site to a multifunctional signaling hub, indispensable for cellular calcium signaling and killing ability of cytotoxic lymphocytes, and at the center stage of metabolic control (**Figure 1**). Secretory lysosomes can be seen as a two-component organelle combining the luminal constituents and outer limiting membrane of a conventional lysosome with an electron dense-core, harboring toxic effector molecules (33–35). Intriguingly, there are mechanisms in place to maintain lysosomal integrity, due to their cytotoxic load (59). Irrevocably damaged lysosomes will be subject to lysophagy (60, 61).

Secretory lysosomes are organelles with dual functionality and they have a similar biogenesis as conventional lysosomes. Lysosomal biogenesis is a highly dynamic process, which incorporates a myriad of different cellular signaling pathways and metabolic conditions, which are surveyed by intracellular metabolic sensor proteins. One of the key metabolic sensors is called mechanistic target of Rapamycin (mTOR) (62, 63). NK cell maturation and responsiveness to cytokine-mediated activation and proliferation is critically dependent on mTOR (64, 65). The active mTOR kinase complex is recruited to the lysosomal surface in order to sense nutrient and growth factor input (63, 66). During starvation, a lack of nutrients and low energy levels are detected by AMPK. A complex signaling cascade, encompassing AMPK and mTORC1 and lysosomal pH changes, promote lysosomal biogenesis and autophagy in a coordinated fashion, allowing recovery of nutrients (67). The reformation of lysosomes after termination of autophagy has been linked to reactivation of mTORC1 (68). Moreover, this process also integrates transcription factor cues, such as TFEB, which regulates expression of a network of genes for lysosomal biogenesis and autophagy, termed the CLEAR network (69). Nutrient levels like cholesterol can also be sensed and regulate lysosome motility via TRPML1-derived calcium signals and subsequent ALG-2-dependent dynein engagement (70). Damaged mitochondria can prompt TRPML1 activation by reactive-oxygen species (ROS) and orchestrate lysosomal adaptation to clear damaged mitochondria via autophagy, known as mitophagy (71). Altogether, this illustrates a cross-talk of fundamental metabolic-, pathogenic- and stress-signals at the lysosome, which are jointly integrated and aim to establish a stable lysosomal number matching the cellular needs (72, 73).

THE LYSOSOME AS A DYNAMIC FUNCTIONAL UNIT IN THE ENDOLYSOSOMAL SYSTEM

The classical pathway of endocytic cargo trafficking from the early endosome to the lysosome is based on a gradual maturation model. First, endocytosed material is collected in the early endosome, which then is subject of recycling back to the plasma membrane or undergoes ESCRT-complex-mediated sorting of ubiquitinylated proteins into intralumenal vesicles (ILV) (74, 75). During the transition from early to late endosome, endosomal proteins are retracted and newly synthesized lysosomal hydrolases, and in the case of secretory lysosomes, also effector molecules, are received from the Golgi complex. Due to homotypic fusions between LEs, an increasing size and a higher density of ILVs dominate the appearance of late endosomes or so-called multi-vesicular bodies (MVB). Late endosomal acidification and increase of the luminal calcium concentration through intensified ER-contact sites, is another prerequisite prior to fusion with a lysosome (30, 76–78). In a final step a temporary hybrid organelle called endolysosome is generated via transient kiss-and-run or full-fusion events between late endosomes and lysosomes. Concurrently, this marks the final step in the degradative pathway and commences the lysosomal reformation from the endolysosome (67, 79–81). Moreover, the lysosomal number and size, as well as positioning within the cells are critical parameters (70, 73, 82). The constant adaptation of the lysosomal compartment, regulated by the cellular demands, is imperative for proper cellular function. Since only membranes in close proximity can undergo fusion, lysosomal motility is required for degradation of damaged organelles and endocytosed cargo. Lysosomal trafficking is coordinated by kinesin and dynein proteins, which are recruited to the peripheral lysosomal membrane in a calcium- or nutrient-dependent manner (70, 83, 84). The very same proteins involved in tethering motor proteins to lysosomes are indispensable for granule convergence to the MTOC (Microtubule-organizing center) in NK cells (85). From this perspective, lysosomes can be

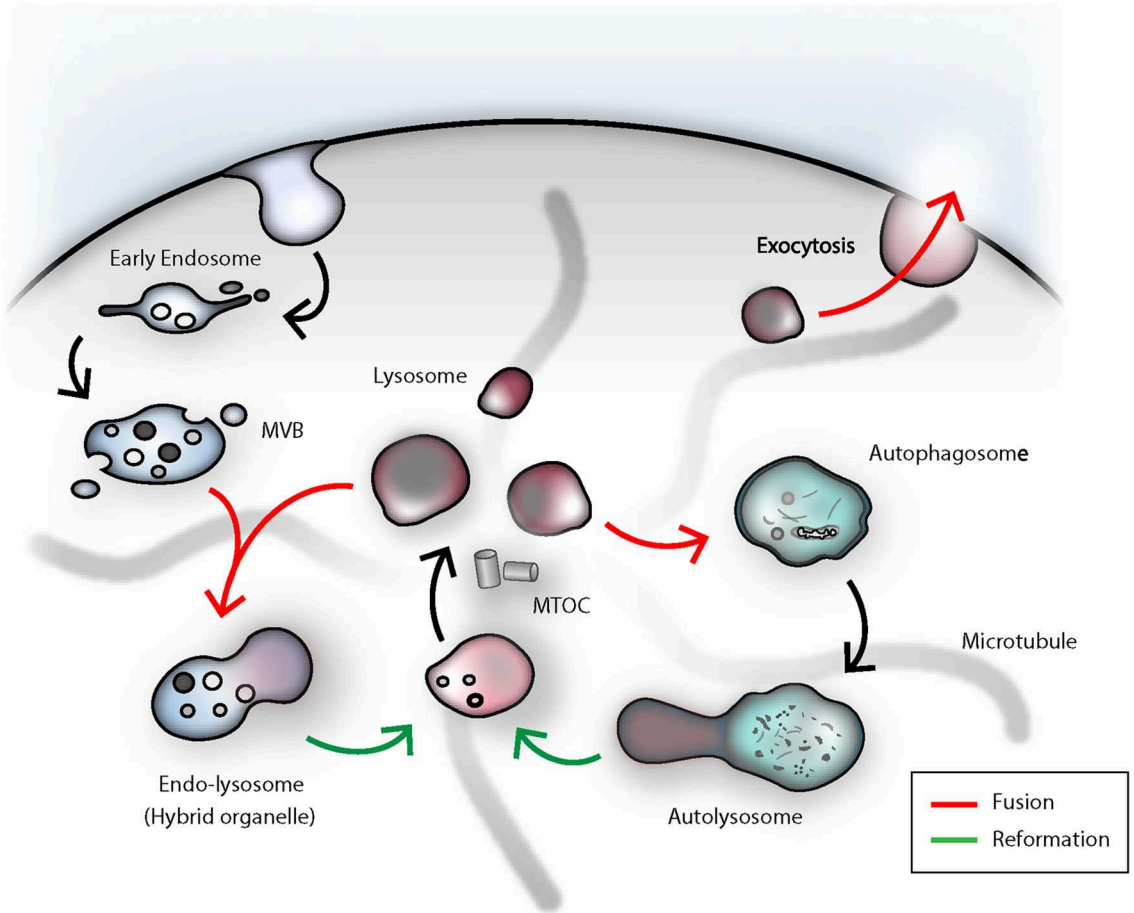

FIGURE 2 | The dense-core secretory lysosomes are the terminal storage unit for acidic hydrolases and effector molecules in NK cells. The lysosome as such is a highly dynamic compartment and can engage in several fusion events in order to deliver its degradative capacity for endocytosis, autophagy or killing of target cells by NK cells. Termination of signal transduction is an important task for the orchestration of NK cell effector functions. Early endosomal cargo can be propagated by gradual-maturation of an early endosome to a late endosome, which finally can fuse with a lysosome for degradation by acidic hydrolases. As a result, an endolysosomal hybrid organelle is formed. Lysosomes can be reformed through tubulation from the latter compartment and leads to formation of a proto-lysosome, which undergoes further maturation and content condensation. In analogy to that, lysosomes can also engage in autophagy. In immune cells, autophagy fulfills the important task of eradicating worn-out mitochondria. Damaged organelles or proteins can be entrapped in autophagosomes, ultimately fusing with lysosomes. Upon termination of autophagy, again, through tubulation and membrane extrusions, consumed proto-lysosomes are regenerated and mature to lysosomes. During target cell killing, NK cells go through step-wise receptor cross-linking, granule polarization and eventually lysosome-related organelle fusion with the plasma membrane at the immunological synapse. During this process, the effector molecules and the electron-dense core can be ejected and the lysosomal membrane will be recovered through endocytosis. MVB, multi-vesicular body; MTOC, microtubule-organizing center.

regarded as dynamic functional unit, which can be transported on microtubule tracks to the place of action (84). Mechanistically, however, the process of lysosomal fusion and fission is not well-defined. Hitherto, several mechanisms have been postulated and will most likely be context-dependent, or apply only to a fraction of lysosomes within a given cell (86). In fact, the number of freely available lysosomes in a cell is depleted, each time, a lysosome undergoes homotypic or heterotypic fusion with late endosomes or autophagosomes, or else is secreted (**Figure 2**). The actual biogenesis and size modulation of new lysosomes from existing lysosomes or transient hybrid organelles has been understood as a reformation process. Lysosomal reformation, by fission, from an existing late endosomal- or autophagosomal-pool can take place through tubulation and subsequent calcium-dependent scission of these tubular membrane extrusions, which bridge the donor endolysosome and the newly formed protolysosomes (80, 87, 88).

Above, we have discussed the regulation and function of conventional lysosomes as well as some specific features of secretory lysosomes found predominantly in immune effector cells such as T and NK cells. The lysosomal compartment in cytotoxic lymphocytes is dominated by secretory lysosomes (35). It is therefore reasonable to assume that secretory lysosomes carry out all the classic lysosomal functions, such as metabolic control, degradation of endocytosed cargo and autophagy, on

top of their unique role in the killing of target cells. Whereas, T cells have to undergo antigen-driven activation in order to form dense-core secretory lysosomes (49), all mature NK cells contain preformed dense-core secretory lysosomes with high levels of perforin and granzyme B (50, 51). Notably, $CD56^{bright}$ NK cells, which are less differentiated NK cells, drastically upregulate the granular content upon short term exposure to IL-15 (89). It is difficult to discriminate conventional lysosomes from secretory lysosomes solely based on morphological features, since the electron-dense core of secretory lysosomes can have a wide variety of shapes (33, 35). Instead, secretory lysosomes can be discriminated from conventional lysosomes based on the unique protein machinery involved in the tightly regulated mode of secretion (35). The main evidence for this notion can be deduced from genetic diseases that only impair the secretion of secretory lysosomes, but not the secretion of conventional lysosomes. Mutation of the RAB27A gene, leads to immunodeficiencies and hypopigmentation. The manifested disease phenotype is known as Griscellis syndrome, which only strikes and compromises secretion of secretory lysosomes in immune cells and melanocytes (90). Similarly, much could be learned about the specific function of secretory lysosomes from studies in patients with Chediak-Higashi Syndrome (CHS). The phenotype of CHS includes defects in skin pigmentation and immunodeficiencies due to malfunction of the release of secretory lysosomes with no impact on conventional lysosome function (35, 91, 92). Altogether, both disease phenotypes indicate a common theme of secretion that involves an exclusive secretory apparatus, which is used by secretory lysosomes, as found in cytotoxic lymphocytes and melanocytes (10). From an evolutionary viewpoint, the development of the secretory lysosomes, probably originated from conventional lysosomes, and give cytotoxic lymphocytes and other cell types the ability to control the surface mobilization of lysosomal membrane proteins and soluble, luminal effector molecules in a spatiotemporal fashion (35, 93).

CALCIUM SIGNALS FINE TUNE FUNCTIONAL RESPONSES IN IMMUNE EFFECTOR CELLS

The ER is the most-studied and well-established principal calcium source in NK cells. Stimulation of NK cells triggers ER-derived calcium efflux (40–42) Ultimately, this results in depletion of ER calcium stores, which subsequently triggers a well characterized process, called store-operated calcium entry (SOCE), and replenishes ER calcium levels by tapping into extracellular sources of calcium via ER-plasma membrane contact sites (94, 95). Defects in this process, strongly affect NK cell degranulation and cytokine production (96). The contribution of individual calcium sources and the interplay between the ER calcium stores and the acidic, lysosomal compartment, as well as various other intracellular calcium sources is covered in several excellent reviews (97–99). Increasing evidence suggests that the acidic compartment of the endolysosomal system plays an important role in intracellular calcium signaling. The latter compartment contains high amounts of luminal calcium, quantified at around 0.5 mM, which corresponds to calcium concentrations similar to the ER at steady-state conditions (100–102). However, when compared with the low cytosolic calcium concentration of approximately 100 nM, it becomes apparent that signals derived from endolysosomal calcium flux can influence cytosolic events. It is not surprising that permeabilization of lysosomes strongly raises cytosolic calcium levels and can provoke secondary ER-derived calcium flux likely at membrane contact sites between the two organelles (15, 97). Conversely, the refilling of lysosomal calcium stores may also have important implications for signaling. ER-derived calcium signals can be sequestered by lysosomes and, consequently, alter cytosolic calcium signals (103, 104). Indeed, there is evidence that the ER is the principal calcium source for filling lysosomes (105). However, the molecular mechanisms underlying lysosome calcium uptake are unclear. Most evidence suggests that lysosomal calcium uptake occurs through Ca^{2+}-H^{+} exchangers but these genes have yet to be identified in humans (106). Alternative routes involve the Parkinson's related protein, ATP13A2 (107). Many endolysosomal processes are coordinated by calcium fluxes (108, 109). These encompass lysosomal positioning, reformation and membrane fission or fusion events (67, 69, 70, 88). Calcium channels of the TRP family are one of the most versatile group of ion channels in terms of integration of disparate signals, including phosphatidylinositol phosphate levels and pH-changes. The lysosome fulfills fundamental functions for the immune system, including antigen processing for MHC class II presentation, the release of cytotoxic granules and influences migration of immune cells (110). Many of these cytosolic events are centered around the calcium-permeable TRP channel family (111).

Lysosomal calcium signals are essential for proper target cell killing by NK cells (20, 25). Alterations of lysosomal calcium signaling, as seen during lysosomal storage disorders, mitigate proper execution of NK cell effector functions, due to lysosomal impairment (20, 101). In addition, eradication of ROS by mitophagy, a process dependent on lysosomes, contributes to survival of virus-specific NK cells during NK cell memory formation (112). The number and size of lysosomes may vary drastically between the resting state of immune cells in the blood stream and during tumor-challenge within the debilitating, nutrient-depleted tumor microenvironment (113). In the next section, we will elaborate on the molecular pathways by which the lysosome-derived calcium signals affect the outcome of immune effector cell responses.

FUNCTION OF DISTINCT TRP CHANNELS IN CYTOTOXIC LYMPHOCYTES

The transient receptor potential (TRP) channels comprises a functionally versatile superfamily of 28 disparate members and are classified in six subfamilies. All TRP channels possess six putative transmembrane domains and assemble as tetrameric complexes with cation-permeable pores (114). The channel activity underlies a wide variety of stimuli and can integrate

several signals at once. The TRP superfamily shows diverse ion selectivity, and hence, are not just exclusive calcium channels (115, 116). TRP cation-channels are known to be broadly expressed among immune cells (111). Yet, the repertoire of TRP channels in immune cells, especially NK cells, remains poorly characterized. TRPML1, TRPML2, and TRPM2 in particular have been implicated in the regulation of innate immunity, and NK cell effector functions.

Cation-channels are the door opener of immune reactions, since calcium-mobilization is crucial for proper lymphocyte activation and function, such as degranulation, cytokine-production, and proliferation (117). TRP channels have the capability to directly alter the intracellular calcium levels and consequently modulate fundamental signaling processes that orchestrate, among other things, differentiation, migration, and cytotoxic effector responses of dendritic cells, neutrophils, macrophages, NK cells, $CD8^+$ T cells and mast cells (21, 118–120). There are several excellent and comprehensive reviews describing the clinical picture of TRP channelopathies and the impact on the immune system (111, 116, 117, 121–124). TRP channels moved into the spotlight of pharmaceutical research due to their pathophysiological role in the sensory nervous system with implications on chronic pain disorders (121). Congenital mutations in TRP channels can lead to severe disease phenotypes and can affect disparate tissues (116). This facet is also reflected in the name of two TRP subfamilies, the -mucolipin (TRPML) and -polycystic subfamily (TRPP), which were both named according to the related human diseases: mucolipidosis and polycystic kidney disease (121). Derailed TRP channel sensitization can contribute to the secretion of neuropeptides, such as CGRP and substance P, which can trigger neurogenic inflammatory responses. As a result, these neuropeptides can locally modulate vascular permeability, leukocyte migration and immune cell activation (125). The underlying inflammatory processes are complex and have been associated with miscellaneous diseases, in particular, inflammatory bowel disease, asthma, arthritis, experimental autoimmune encephalomyelitis and anaphylaxis [reviewed in (111)].

The detailed mapping and functional characterization of TRP channels in primary human immune cells is still incomplete. New evidence based on gene-deletion studies helped to uncover the role of TRP channels in the immune system and in inflammatory conditions. For example, the targeted-deletion of TRPM7 in T cells in a mouse model, leads to reduced peripheral T cell numbers due to impaired T cell selection and development in the thymus (126). TRPM7-mutant T cells showed altered migratory behavior and affected mice suffered from pulmonary inflammation, most likely based on defects in Fas-dependent T cell apoptosis (123). These findings underline the importance of TRPM7 in lymphocytes and were extended in DT40 chicken B cells, where genetic deletion of TRPM7 leads to increased cell death and compromised B cell proliferation (127). Further mouse studies have shown alterations in innate immune cell function, such as mast cell responses toward IgE and monocyte-derived chemokine release, by genetic manipulation of TRPM channels (117). Taken together, TRP channels can influence the response of individual immune cells and contribute to the homeostasis of inflammation in health and disease. Mutations or impairment of single TRP channels can lead to serious congenital diseases and have also been associated with the pathophysiologic course of various inflammatory diseases (111, 121). All these properties make TRP proteins attractive targets for immunomodulatory drugs and genetic manipulations.

Exposure of NK cells to activating stimuli triggers complex signal transduction and provokes effector responses (6). A diverse range of input signals converge at the level of the lysosome, which subsequently orchestrates appropriate cellular responses (72, 73, 128). We propose that the nature of these upstream signals, in concert with the highly dynamic nature of the lysosome, are imprinted through remodeling of the lysosomal membrane and the components making up the dense-core. Therefore, the composition of the lysosomal compartment has profound impact on immune cell responses that extends beyond the release of the effector molecules stored in the granules (25). This notion is supported by the fact that lysosomal constituents provide a critical molecular wiring, translating upstream signaling into appropriate downstream functionality, with TRP channels acting as key signal integrators (116). In essence, the lysosomal compartment is a tunable signaling hub and can be remodeled by external stimuli or rational manipulation.

TRPML1

TRPML1 is a non-selective calcium-permeable TRP cation channel resident in the lysosomal compartment. Mutations in the MCOLN1 gene, which encodes TRPML1, lead to Mucolipidosis type IV which is a lysosomal storage disorder. Affected patients present with severe neurological and ophthalmological symptoms (129). On a molecular level, loss of mucolipin-1 activity impairs lipid efflux from the lysosomal compartment and results in abnormal endolysosomal transport (130). Phosphatidylinositol-(3,5)-bisphosphate ($PI(3,5)P_2$) has been identified as an endogenous agonist of the mucolipin TRP channel subfamily and can trigger lysosomal calcium efflux (131). $PI(3,5)P_2$ is produced by a phosphatidylinositol-(3)P 5-kinase termed PIKfyve (132). The role of PIKfyve, upstream of TRPML1, as a regulator of lysosomal positioning and fusion has been well-established (70, 133, 134). On the other hand, $PI(4,5)P_2$, which can be found as transient pools on lysosomes and autophagosomes (87, 135), and sphingomyelin have antagonistic properties on TRPML1 activity (136, 137) (**Figure 3**).

As demonstrated in cell-free assays, luminal calcium is a prerequisite for proper formation of dense-core lysosomes from the endolysosomal compartment (80). Potentially, activation of TRPML1 by $PI(3,5)P_2$, together with low lysosomal pH, may trigger luminal calcium efflux and recruitment of calmodulin and other hitherto unidentified protein machineries to the surface of the endolysosome, which then may facilitate membrane tubulation or scission between the parental organelle and newly formed protolysosome (67, 138, 139). Thus, TRPML1 may directly alter the luminal calcium concentration and influence lysosomal size and re-formation (88, 134). Activation of TRPML1 requires a close spatiotemporal adjustment of agonistic $PI(3,5)P_2$,

antagonistic PI(4,5)P_2 and sphingomyelin concentrations in the lysosomal membrane. Therefore, lysosomal fission and fusion events are precisely coordinated by lysosomal membrane lipid concentrations.

TRPML1-dependent lysosomal calcium efflux is also involved in the activation of the transcription factor TFEB. Originally, TFEB was known to be a mTOR-dependent master regulator of a gene network (CLEAR network), associated with lysosomal biogenesis and autophagy (69, 140). The classical role of the microphthalmia/transcription factor E MiT/TFE- family of transcription factors has been extended, since the two members, TFEB and TFE3, have been linked as transcriptional regulators of innate and adaptive immune responses (73, 141). In macrophages, pathogen challenge and engagement of Fcγ- or toll like receptors initiates TFEB and TFE3-dependent expression of lysosomal genes, autophagy-related genes and pro-inflammatory effector molecules, such as CCL5, TNFα and IL-1β or IL-6 (142–145). Interestingly, MCOLN1, the gene coding for TRPML1 is under the transcriptional control of TFEB itself (140). Thus, in regard to immune cell activation, the TRPML1-TFEB axis might also have important implication for the transcriptional regulation of NK cell effector functions. Acute activation of NK cells could trigger TRPML1-mediated lysosomal calcium flux. Subsequently, these calcium signals can lead to nuclear translocation of TFEB/TFE3, which initiates TRPML1 upregulation and secretory lysosomal biogenesis and reformation, as well as transcription of additional pro-inflammatory molecules, similar to what has been demonstrated in macrophages. In NK cells, the exact pathways and regulatory networks of the MiT/TFE transcription factors have yet to be dissected.

Since TRPML1 is such a central player in the regulation of lysosomal biogenesis and secretory lysosomal function, it is not surprising that this channel protein might have important implications for the immune system, as already demonstrated in NK cells and murine macrophages (25, 88). Based on RNA sequencing data, TRPML1 is expressed at relatively constant levels across mature and immature NK cell subsets (25). Our laboratory recently demonstrated that the highly functional educated NK cells (express inhibitory KIR for self HLA class I) have larger dense-core lysosomes, which converge closer to the MTOC and accumulate more lysosomal calcium and granzyme B, as compared to NK cells that lack inhibitory receptors to self HLA and therefore receive tonic activation input. The phenotype of educated NK cells could be mimicked by pharmacological as well as genetic interference of the PIKfyve-TRPML1 axis and the lysosomal calcium content. These results point to a role for lysosomal calcium and the TRPML1 calcium channel in the remodeling of dense-core lysosomes.

TRPML2

TRPML2 is the second member of the Mucolipin subfamily of TRP channels. As all members of the mucolipin subfamily TRPML2 also exhibits a pore domain and is permeable to sodium and calcium. TRPML2 is encoded by the MCOLN2 gene, the expression pattern of which seems to be more restricted to the lymphoid and myeloid tissue. More specifically this selected set of tissues includes thymus, spleen and immune cells. From all three members of the mucolipin subfamily, TRPML2 shows the most limited tissue distribution, compared to the other members of the subfamily (146). Based on electrophysiological data, TRPML2 activity is reduced at low luminal pH and reaches its maximum at neutral luminal pH (24). TRPML2 can be activated as previously introduced for TRPML1, by the phosphatidylinositol-(3,5)-phosphate PI(3,5)P2 (131, 147). On the other hand in structural studies on TRPML2 it was claimed that the pre-pore regions of TRPML1 and TRPML2 are structurally more similar to each other than to TRPML3 under acidic conditions (148). While TRPML1 activity reaches is maximum at highly acidic luminal pH, TRPML3 maximal activity is found at neutral pH. Both TRPML2 and 3, in contrast to TRPML1 reside in early and recycling endosomal compartments hence would be exposed to less acidic conditions. In conclusion, the early endosomal and recycling endosomes provide a neutral pH, which promotes TRPML2 activity (24).

The selective expression of MCOLN2 in lymphatic and myeloid cells suggests that this calcium channel is involved in regulating immune cell function (149). TRPML2 resides in Arf6$^+$ recycling endosomes and is associated with recycling of plasma membrane proteins (150). In murine macrophages TRPML2 expression is connected to activation status and modulates the recruitment and chemokine secretion in response to pro-inflammatory stimuli. The latter effect has been shown by knock-out studies and upon specific pharmacological intervention (24, 151). There are many outstanding questions regarding the role of the intracellular TRPML2 localization and its impact on TRPML1 activity and effector functions in general. TRPML2 is linked to the recycling endosomes and observations support a role in the regulation of innate immune cell function (149). In NK cells, MCOLN2 expression reaches its peak in the terminally differentiated adaptive CD57$^+$ NKG2C$^+$ NK cell subset but is also detected in various NK cell lines (152, 153). It is tempting to speculate that TRPML2 modulates chemokine secretion in activated NK cells and perhaps to a greater extent in adaptive NK cells.

TRPM2

In addition to the mucolipins, the transient receptor potential melastatin 2 (TRPM2) channel can be detected in a wide range of immune cells and has implications for granzyme B secretion and antitumor activity of NK cells among others (21, 111, 118). TRPM2 is a calcium-permeable cation channel and responsive to reactive oxygen species (ROS) and the second messenger ADP-ribose (ADPR) (154, 155). Furthermore, the channel can be synergistically gated by a family of second messengers, derived from the ectoenzyme CD38, which comprises: cyclic ADP-ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and ADPR (154–157). TRPM2 is resident in the plasma membrane and can also be found on the lysosomal membrane (157, 158). Several studies outlined the proinflammatory character of TRPM2 and its importance for innate and adaptive immune cells (118, 120, 156). A study examined the effects of ADPR on TRPM2 modulation in murine NK cells and found that TRPM2 knock-out mice exhibit a defect in their antitumor responses (21). The cytotoxic activity

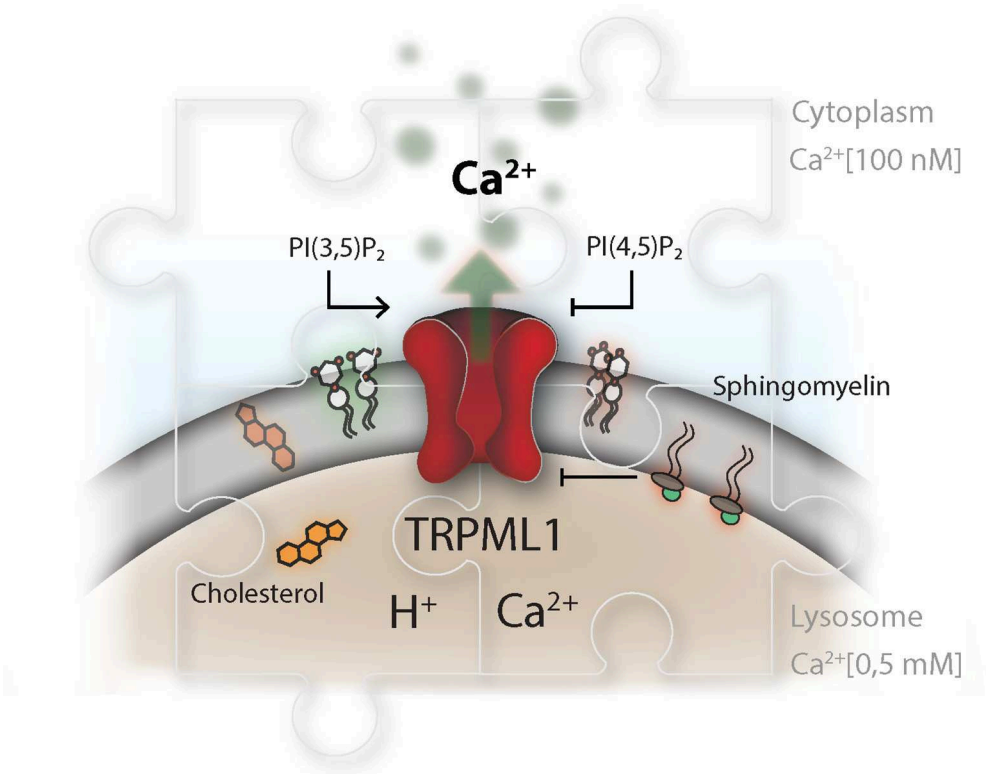

FIGURE 3 | TRP channels as master regulators in the endolysosomal compartment. The TRP channel conductance is affected by a broad range of stimuli and integrates information about pH and signals from within the lipid bilayer, as well as the surrounding cytosol and vesicular lumen. This is illustrated for TRPML1, which is described to be activated by phosphatidylinositol-(3,5)-bisphosphate, $PI(3,5)P_2$, and inhibited by phosphatidylinositol-(4,5)-bisphosphate. $PI(3,5)P_2$ and $PI(4,5)P_2$ play an delicate role in controlling lysosomal membrane dynamics, hence their synthesis needs to be highly coordinated in time and space. Disparity in the concentration of TRP ligands, in the context of lysosomal storage disorders, impairs proper lysosomal lipid transfer, trafficking and calcium signaling capacity. Niemann-Pieck disease type A/B is characterized by lysosomal accumulation of sphingomyelins (SM). In this disease, SM has been suggested to be the causing agent for the grave pathogenic conditions due to its presumable antagonistic function on TRPML1. On the other site, Niemann Pieck type C is denoted by a defect in cholesterol transporters and might also affect SM levels in the lysosome.

of NK cells strongly correlates with their ability to secrete granzyme B and perforin-containing granules. Upon tumor encounter, CD38 and TRPM2 localizes to secretory lysosomes in mouse NK cells (21, 159). Mechanistically, CD38-derived ADPR has been associated with sustained TRPM2-derived calcium mobilization, which is a prerequisite for granule polarization and degranulation (21). Intervention of ADPR production by ADPR antagonists or genetic manipulation have been associated with lower intracellular calcium mobilization and reduced effector responses in mouse NK and T cells (21, 119). Similar data was obtained in T cells, where NAADP was associated with granule polarization and directed degranulation (26). The studies on TRPM2 showed a direct link of how a cation channel can modulate crucial processes like antitumor responses (21, 119).

Collectively, the studies discussed above highlight a potentially important role for lysosomal calcium channels, particularly TRP channels, in the formation and remodeling of secretory lysosomes and in tuning various effector functions in immune cells. However, more data from cells of human origin are needed to draw conclusions about the pharmacological benefit and potential of TRP agonists and antagonists in humans. The lack of specific antibodies and low expression levels of most TRP channels make the work with TRP channels challenging. Therefore, as we shall discuss in the next section, new tools are needed to accelerate the accumulation of knowledge in the field (160).

METHODOLOGICAL ADVANCES FOR ASSESSING THE FUNCTION OF TRP CHANNELS IN IMMUNE CELLS

The tight connection between the endolysosomal system and the pathogenesis of metabolic, neurological and infectious human diseases has motivated increasing efforts to advance the tool-box for studies of lysosomal biology. Over 70 lysosomal storage diseases (LSD) have been described and linked to mutations in lysosomal proteins, highlighting the urgency to decipher the lysosomal function more in depth (161–163). Overall, the lack of knowledge of the precise function of malfunctional lysosomal gene products results in poorly available treatment options (163). Early experiments mapping the proteome of

isolated secretory lysosomes from NK cells generated valuable information about granule specific proteins (164). Similar but more targeted approaches were applied to make the endolysosomal membrane composition more accessible and revealed the identity of more than 70 lysosomal ion channels and transporter proteins (165, 166).

Typically, electrophysiological properties, such as the ion selectivity of channel proteins are studied by patch-clamping, but this has remained challenging for intracellular, endolysosomal proteins. The combination of genetic manipulation, or advancement thereof with small molecules, which selectively enlarge endolysosomal organelles, together with patch-clamp technology enabled interrogation of the properties of such intracellular ion channels in great detail also in immune cells (167–169). In particular, the research on the mucolipin family of TRP cation channels gained momentum by the development of selective pharmacological agonists. These small molecules can also assist in validating established patch-clamp approaches or function as lead structures for potential drug candidates, respectively (22, 170, 171). The development of a series of genetically-encoded calcium indicators (GECI), such as the FRET-based Cameleon-type (172) or the single GFP-based GCaMP-family (173, 174), fused to specific TRP channels, hold promise to delineate calcium signals in immune cells with subcellular resolution in time. The functional principle of the GCaMP reporters is based on circulary permutated EFGP (cpEGFP), N-terminally fused to the M13 peptide obtained from myosin light chain kinase and C-terminally connected to calmodulin (CaM) (175). Initially, the reporter exhibits weak fluorescent activity, but upon calcium binding to the CaM domain, CaM binds to the M13-peptide and the cpEGFP undergoes a conformational change, which induces a drastic increase in fluorescent activity (173, 175, 176). The advantage of these sensors lies in their stable expression levels and the possibility to target them to subcellular organelles like lysosomes (69, 136). Modifications of the GFP-based GECIs, can also be used as organellar-entrapped sensors, to survey luminal calcium concentrations (177). Many of these calcium reporters have a single wavelength and can be combined with further reporters like genetically-encoded pH sensors (178). Further novel technologies, like the usage of induced pluripotent stem cell (iPSC)-derived NK cells can help to circumvent the challenging gene-editing of primary NK cells. iPSCs can more easily be genetically manipulated and function as a human model system for studying TRP channels in NK cells (179, 180). Altogether, these advances hold promise to generate ground-breaking discoveries concerning the spatiotemporal contribution of TRP channels to the dynamic modulation of immune cell function.

CONCLUDING REMARKS

Recent experimental data has challenged our current understanding of lysosomes. Until recently, these organelles have been seen as a static terminal storage unit of the endosomal pathway, providing degradative capacity to the cell. In cytotoxic lymphocytes, lysosomes act also as secretory lysosomes, which can be released upon target cell encounter. The lysosomal homeostasis and biogenesis are complex and calcium signaling plays a critical role. In NK cells the lysosomal compartment undergoes constant remodeling that is under influence by surface receptors binding to self MHC molecules. Specifically, inhibitory signaling through KIR and CD94/NKG2A during NK cell education is tightly associated with retention of lysosomal matrix proteins and accumulation of perforin and granzyme B in a unique pool of dense-core secretory lysosomes. The consequence is an enhanced lysosomal signaling capacity and increased NK cell effector responses. The role of TRP channel members as regulators of lysosomal calcium fluxes and the subsequent effects on lysosomal fission and fusion events are gaining increasing attention. New tools, including highly specific small molecule TRP agonists and antagonists, as well as organelle-specific, genetically-encoded calcium sensors hold promise to advance the field.

Biological insights into the dynamic regulation of lysosomal signaling may pave the way for new means to boost function of cytotoxic lymphocytes in cancer immunotherapy. In this context, TRP channels represent promising targets for manipulation by means of pharmacological or genetic perturbation (25). The mucolipin subfamily of TRP cation channels could potentially be targeted to modify the rate of lysosomal biogenesis or, as described for TRPML2 in macrophages, to modulate the secretion of chemokines in immune effector cells (24, 151). It may also be possible to build functional potential in lymphocytes through manipulation of the lysosomal matrix, which influence loading of positively charged effector molecules such as granzyme B as well as calcium (181, 182). Another attractive possibility is to indirectly affect lysosomal signaling capacity through tuning of NK cell education by agonistic stimulation of inhibitory or activating receptors on NK cells. A key challenge for all these efforts is to determine the right kinetics since long term silencing/inhibition of calcium channels may be detrimental to the cell. Intermittent, pulsatile interference may be superior to chronic engagement given the rapid calibration of immune cell reactivity to external input (183). Despite these challenges, the insight that TRP channels contribute to remodeling of the interior of immune effector cells hold promise for the discovery of new therapeutic targets in cancer immunotherapy.

AUTHOR CONTRIBUTIONS

DC wrote the manuscript and made the illustrations. All authors contributed to the conceptual framework of the review and to the writing of the manuscript.

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Involvement of the TRPML Mucolipin Channels in Viral Infections and Anti-viral Innate Immune Responses

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The TRPML channels (TRPML1, TRPML2, and TRPML3), belonging to the mucolipin TRP subfamily, primary localize to a population of membrane-bonded vesicles along the endocytosis, and exocytosis pathways. Human viruses enter host cells by plasma membrane penetration or by receptor-mediated endocytosis. TRPML2 enhances the infectivity of a number of enveloped viruses by promoting virus vesicular trafficking and escape from endosomal compartment. TRPML2 expression is stimulated by interferon and by several toll like receptor (TLR) activators, suggesting a possible role in the activation of the innate immune response. Noteworthy, TRPML1 plays a major role in single strand RNA/DNA trafficking into lysosomes and the lack of TRPML1 impairs the TLR-7 and TLR-9 ligand transportation to lysosomes resulting in decreased dendritic cell maturation/activation and migration to the lymph nodes. TRPML channels are also expressed by natural killer (NK) cells, a subset of innate lymphocytes with an essential role during viral infections; recent findings have indicated a role of TRPML1-mediated modulation of secretory lysosomes in NK cells education. Moreover, as also NK cells express TLR recognizing viral pattern, an increased TLR-mediated activation of cytokine production can be envisaged, suggesting a dual role in the NK cell-mediated antiviral responses. Overall, TRPML channels might play a double-edged sword in resistance to viral infections: on one side they can promote virus cellular entry and infectivity; on the other side, by regulating TLR responses in the various immune cells, they contribute to enhance antiviral innate and possibly adaptive immune responses.

Keywords: TRP channel, mucolipin, innate immunity, viral infection, endolysosome

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DISCOVERY AND CHARACTERIZATION OF MUCOLIPIN CHANNELS

The transient receptor potential mucolipin channels (TRPML) are non-selective cation channels that conduct Ca^{2+} and monovalent cation currents from the lumen to the cytoplasm (1, 2). These channels are tetramers, consisting of proteins with six transmembrane-spanning domains and amino- and carboxy-terminal tails oriented toward the cytosol (3). Each subunit has six

transmembrane segments (S1–S6) and a pore-loop between S5 and S6, which forms a voltage-sensor-like domain and a pore domain. A long extracellular linker between S1 and S2, "polycystin-mucolipin domain" is identified (4). There are three TRPML subtypes sharing ∼40% amino acid sequence identity (2). TRPMLs play a role in membrane trafficking (1, 2, 5), autophagy (6, 7), exocytosis (8), and ion homeostasis (9).

The *MCOLN1* gene encoding TRPML1 is located on human chromosome 19. No splicing variants have been found in humans, whereas splice variants were described in mice (10). The *MCOLN2* gene encoding TRPML2 is located on human chromosome 1 and only one TRPML2 isoform showing 60% amino acid homology with TRPML1, has been detected in humans. The human *MCOLN3* gene maps on the short arm of chromosome 1.

TRPML1 is expressed in a number of tissues including adrenal gland, lung, bladder and placenta as well as in thymus, spleen and immune cells (11–13). Mutations in *MCOLN1* gene cause a lysosomal storage disorder called mucolipidosis type IV (MLIV). Over 95% of patients with MLIV have loss of functional mutations in MCOLN1 (11–13). Many patients carry mutations that introduce premature stop signals in *MCOLN1*, thus the TRPML1 protein is completely absent, or abnormally short and it lacks the ion conducting pore (13–15). Some patients show single point mutations in *MCONL1* that maintain the open reading frame but lead to a incorrect location or to the production of a TRPML1 inactive form (11–14, 16–18). TRPML2 mRNA is mainly detected in lymphocytes and other cells of the immune system (19). In addition, TRPML2 was found to be overexpressed in aggressive human glioblastoma (20). TRPML3 is mainly expressed in cochlear and vestibular sensory hair cells and melanocytes (21). Two TRPML3 spontaneous gain-of-function mutations (A419P and I362T) called varitint-waddler mutations cause deafness and coat color dilution in mice (22–26).

TRPML1 is activated by phosphatidylinositol-3,5-biphosphate (PtdIns(3, 5)P2) (15, 21, 27–29). Moreover, TRPML1 has an intraluminal loop whose protonation stimulates channel activation (24, 30, 31). It is inhibited by phosphatidylinositol-4,5-biphosphate (PtdIns(4, 5)P2), sphingomyelins, and lysosomal adenosine (28, 29). PtdIns(3, 5)P2 is able to activate also TRPML2 and TRPML3. Na^+ removal or less acidic/neutral pH activate TRPML3 and TRPML2, respectively (32, 33). Among synthetic activators currently available ML-SA1 activates TRPML1, TRPML2, and TRPML3 in human; ML2-SA1 is TRPML2 specific; MK6-83 activates TRPML1 and TRPML3 (15, 21, 28, 32). There are several synthetic inhibitors (ML-SIs); however, they are unable to discriminate the TRPML isoforms from each other (7, 8). Therefore, PtdIns(3, 5)P2 seems to have a central role in activating the TRPML family. This is a low-abundance endolysosome-specific phosphoinositide, produced by PtdIns (3) P5-kinase (PIKfyve). In the immune response, PtdIns(3, 5)P2 is responsible for the fusion of phagosomes with lysosomes to form phagolysosomes, which are essential for the digestion of engulfed pathogens (11, 12, 21). It should be noted that the phagosome acidification is allowed because PtdIns(3, 5)P2 activates TRPML1 channel by directly binding to its N-terminus (34).

SUBCELLULAR LOCALIZATION OF TRPML CHANNELS

TRPMLs primarily localize to vesicles along the endocytosis and exocytosis pathways. TRPML1 is localized in the lysosome-associated membrane protein $(Lamp\text{-}1)^+$ or $Rab7^+$ late endosomal and lysosomal (LEL) compartment (2, 26, 35, 36). Late endosomes have an acidic pH of 5.5–6.0, and lysosomes have a more acidic pH of 4.5–5.0 (37–39), a necessary condition to maintain the activity of lysosomal hydrolases (40). The lysosomal localization of TRPML1 protein is likely mediated by clathrin adaptor AP2-dependent internalization from the plasma membrane and/or AP1/AP3-dependent trafficking from the trans-Golgi network (41). Moreover, TRPML1 functions as a key lysosomal Ca^{2+} channel controlling both lysosome biogenesis and reformation, crucial events for cellular homeostasis (42). TRPML1 also regulates focal exocytosis and phagosome biogenesis. Phagocytic ingestion of large particles activates a PtdIns(3, 5)P2- and Ca^{2+}-dependent exocytosis pathway necessary for pseudopod extension and for leading to clearance of senescent and apoptotic cells *in vivo* (8).

Similar to TRPML1, TRPML2, and TRPML3 co-localize with Lamp-1 and Rab7 in the LEL compartment (41).

Antigen presentation is central in activating adaptive immunity and is mainly mediated by professional antigen-presenting cells including dendritic cells (DCs) and macrophages. In mouse macrophages TRPML1 co-localizes with the MHC-II molecules (43), and by heteromeric interactions with TRPML2 (44) that also contributes to MHC-II/antigen complex formation. The $TRPML2^+$ vesicles colocalize with CD63, Lamp-1 and Lamp-3, and Rab11; they induce accumulation of LysoTracker (3, 45), indicating that a fraction of TRPML2 is present in LEL. Numerous proteins, including MHC-I, CD59, interleukin-2 receptor, β_1-integrins, and many glycosylphosphatidylinositol-anchored proteins (GPI-APs), travel along the Arf6-regulated pathway (46–48), and co-localize with TRPML2. In addition, Arf6 mutations induce sequestration of TRPML2, MHC-I, and GPI-APs into the same enlarged vacuolar organelles (49), suggesting that TRPML2 uses the Arf6 pathway to cycle between the plasma membrane and recycling endosomes. TRPML2 overexpression induces a strong activation of Arf6, while the inactive form of TRPML2 ($D^{463}D$/KK) delays the recycling of internalized GPI-APs back to the plasma membrane (49). TRPML2 has been also suggested to participate in the regulation of the lysosomal compartment of B-lymphocytes (45).

Much less is known about the localization and function of TRPML3. TRPML3 is localized in the ELs, early endosomal (EEs), and plasma membrane compartments. Moreover, TRPML3 regulates endocytosis, membrane trafficking and autophagy (50).

GENERAL MECHANISMS FOR VIRUS ENTRY INTO HOST CELLS

Viruses have developed different mechanisms and molecules to interact with proteins, lipids and sugar moieties expressed on the surface of host cells, which generally trigger virion uptake

through the endosomal system (51, 52). Different endocytic cell routes for virus entry have been reported (53–58). Numerous host factors are involved in the viral uptake, including coat proteins (clathrin and caveolin), scission factors (dynamin 2), and regulatory and trafficking factors (Ras, Ras-related C3 botulinum toxin substrate 1, cell division control protein 42 homolog, and phosphatidylinositol 3-kinase,RabGTPases, etc.) (59). Endocytosis is a dynamic process and involves recycling, trafficking, maturation and fusion of endocytic vesicles (60). Viruses that are running the endocytic gauntlet, need to escape the endosome before being recycled back into the extracellular space (61–63), or degradation in the lysosome. Thus, enveloped viruses (e.g., *Filoviridae*, *Arenaviridae*, and *Orthomyxoviridae*) fuse the viral envelope with an endosome membrane, releasing their genomic content into the cytoplasm. Non-enveloped viruses (e.g., *Adenoviridae*, *Parvoviridae*, and *Picornaviridae*) use membrane-modifying proteins which can physically pierce the endosomal membrane to allow release of the genomic content into the cytoplasm and receptor switching to facilitate the viral endosomal escape (64). Another feature of viral endosomal penetration is the ability to co-opt membrane damage responses of the target cell, by recruiting host phospholipases (*Picornavirus*) or inducing lysosomal/autophagosomal exocytosis (*Adenovirus*) (65).

Among the receptors involved in virus uptake and anti-viral immune responses, a role for TRPMLs in virus infection as well as in the activation of innate immune responses has recently been suggested.

TRPMLs ENHANCE VIRUS INFECTIVITY BY INCREASING THE TRAFFICKING EFFICIENCY OF ENDOCYTOSED VIRUSES

Recently, it has been found that TRPML2 channel is one of the interferon (IFN)-stimulating genes (ISGs). However, as several ISGs, TRPML2 enhances the infectivity of the yellow fever virus, the *Zika virus*, *the influenza A virus* (IAV) and the equine arteritis virus, while no effect on the Venezuelan equine encephalitis virus, respiratory syncytial virus, or vesicular stomatitis virus has been reported (65–67). Human A549 lung adenocarcinoma cells, stably transfected with TRPML2, result in enhanced IAV infectivity and infectious virus production. Moreover, knockout of TRPML2 in A549 and U-2 OS osteosarcoma cells caused a reduction of viral infection. In addition, treatment of THP-1 monocytes with IFN-γ, poly (I:C) or LPS (68, 69) enhanced TRPML2 protein expression.

Specifically, TRPML2 promotes virus trafficking from early to late endosomes and causes an enhanced viral release into the cytosol and a consequent escape from endosomal compartments; thus, it promotes a productive infection. This process requires TRPML2 channel activity, but doesn't involve the antiviral IFN signaling pathways, and broadly is applied to enveloped RNA viruses that are transported to late endosomes by infection. TRPML2 doesn't modulate antiviral signaling in IFN-responsive A549 cells: indeed, no differences in MX1, interferon induced transmembrane protein 3, or Interferon alpha inducible protein 27as well as IFNB1 and ISG induction were found in TRPML2-expressing cells infected with either IAV or *Sindbis virus*. Both IAV and *Sindbis virus* infections were enhanced by TRPML2. Accordingly, no IAV infection has been evidenced in TRPML2-DD/KK dominant negative mutant stably-transfected A549 cells. Similarly, TRPML3 increased IAV infection in ectopically expressing cells (65).

Overall, these data suggest that TRPML-mediated increase of viral infection is not linked to impairment of IFN or ISG induction.

During virus life cycle, TRPML2 expression increases at the early, but not at late post-entry stages. No major effects on adhesion of IAV on A549 cell surface or virus particle endocytosed cells, have been observed, whereas TRPML2 affected virus vesicular trafficking by promoting the efficiency of IAV trafficking to late endosomes or by preventing virion degradation (65), with more IAV fused within the endosomes in ectopic TRPML2-expressing cells. This effect has been evidenced only with viruses requiring TRPML2-dependent transport to endocytic carrier vesicles/late endosomes.

In this regard, a rare genetic variant of human TRPML2, which induces a lysine/glutamine or arginine change at 370 aa in TM3 and TM4 domains of TRPML2 protein (MCOLN2-K370Q), failed to enhance TRPML2-mediated viral infection when ectopically expressed. Intriguingly, the frequency of this variant is rather low (about 3%), but increases (11%) in some African populations (65).

TRPML2 CHANNEL TRIGGERS ANTI-VIRAL INNATE IMMUNE RESPONSES

Innate immune activation is based on the ability of the host to recognize pathogens through specific pathogen recognition receptors such as TLR, NOD-like receptors, lectin-like receptors and RIG-1 receptors. Engagement of these receptors activates the production of cytokines, chemokines, and interferons that by binding to their cognate receptors, signal through the JAK-STAT pathways and transcriptionally induce hundreds of ISGs (66, 67). Recent evidence indicates that TRPML2 is expressed at low levels in resting RAW 264.7 macrophages, but its expression is strongly induced upon TLR activation, with no effect on TRPML1 or TRPML3 (68). These data have been also confirmed in bone marrow and alveolar macrophages as well as in microglia from mice treated with a panel of TLR activators, including zymosan (TLR2 ligand), PolyI:C (TLR3 ligand), LPS (TLR4 ligand), R-848 (TLR7/8 ligand), and Imiquimod (TLR7 ligand). Endogenous TRPML2 co-localizes with perinuclear vesicles that also contain the transferrin receptor and likely correspond to recycling endosomes.

It is therefore interesting to consider the implications of TRPML2 up-regulation during *in vivo* viral infections, when IFN

is produced and triggers a number of responses. In non-immune cells, basal or IFN-induced TRPML2 expression may lead to enhanced viral uptake thus promoting virus infection. However, in immune cells expressing higher levels of basal TRPML2 (68, 70, 71), TRPML2-mediated increased viral uptake also results in increased PAMP receptor engagement and activation, stronger immune response, and subsequently improved viral clearance. In this regard, apilimod, an inhibitor of PIKfyve by functioning as activator of the TRPML2 channels, blocks the entry and the infection of the Ebola virus and the Marburg virus in Huh 7 liver, in Vero E6 kidney cells and in human primary macrophages. Apilimod also blocked Ebola-glycoprotein-virus like particle (VLP) entry and VLP infection (72).

Infection of mouse bone marrow derived macrophages (BMDM) with the intracellular *Mycobacterium smegmatis* induces TRPML2 expression, suggesting that TRPML2 up-regulation, occurs not only in response to purified TLR ligands but also to live pathogens. In addition, TRPML2 knocked-out mice treated for 24 h with LPS showed reduced expression of chemokine (C-C motif) ligand (CCL) 3, CCL5, chemokine (C-X-C motif) ligand 2 and intercellular adhesion molecule 1. Moreover, the amount of secreted CCL2 a chemokine released via the early/recycling endosomalwas significantly reduced in the supernatants from LPS-treated TRPML2$^{-/-}$ BMDM. Similarly, ML2-SA1 a new TRPML2 agonist, stimulated CCL2 release by LPS-activated WT but not TRPML2$^{-/-}$macrophages (73). ML2-SA1 treatment also promoted macrophage migration (32), and macrophage and neutrophil migration, in response to LPS, was reduced in TRPML2 knocked-out mice (68).

INVOLVEMENT OF TRPML1 IN THE REGULATION OF TLR RESPONSES IN DC: POTENTIAL ROLE IN THE ANTIVIRAL ADAPTIVE IMMUNITY?

Dendritic cells play an important role in the beginning of specific immune responses. Immature DCs patrol the tissues and check for the antigen presence by continuously internalizing extracellular material mainly via micropinocytosis (32, 74). Sensing of pathogen/danger signals triggers the DC maturation, reduces the antigen uptake, and up-regulates the membrane expression of CC-chemokine receptor 7 that binds to CCL21 and CCL19 chemokines, driving DCs to lymph nodes, where they present the antigen to T cells (75, 76). Recent evidence indicates that activating the TRPML1-transcription factor EB (TFEB), by regulating TRPML1 gene expression, allows DCs to switch from a tissue-patrolling mode to a fast migratory mode in order to reach the lymph nodes (77). Upon microbial sensing, lysosomal calcium is released by TRPML1, which in turn activates myosin II at the cell rear, promoting fast and directional migration. Lysosomal calcium also induces the activation of TFEB, that at steady state is phosphorylated by the mammalian target of rapamycin complex 1 (mTORC1) and remains in the cytosol (78), but due to the dephoshorylation induced by the TRPML1-mediated calcium efflux-activated calcineurin (79), TFEB translocates into the nucleus where it regulates the TRPML1 expression. Of interest, sensing of bacterial or viral products also induced the TFEB translocation from the cytosol to the nucleus with consequent expression of a network of genes involved in lysosome activity, biogenesis, and secretion (80–83).

Toll like receptors play a crucial role in the early host detection of invading viruses (84–87). In particular, TLR-7 and TLR-9 recognize single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), respectively, in the endolysosomes (84–86). A recent report demonstrates the involvement of TRPML1 in TLR7-mediated DC responses by faciliting ssRNA trafficking into lysosomes. TRPML1$^{-/-}$ DCs showed impaired TLR7 responses to ssRNA, while a mucolipin agonist specifically enhanced TLR7 responses to ssRNAs. In addition, the inhibition of PtdIns(3, 5)P2 generation, that binds directly to TRPML1 and induces the Ca^{2+} release, completely inhibited TLR7 responses to ssRNA in DCs (88). Confocal analyses showed that ssRNA transportation to lysosomes in DCs was impaired by a PIKfyve inhibitor as well as by the lack of TRPML1. Moreover, in TRPML1$^{-/-}$bone marrow derived-DCs (BM-DCs) RNA transportation to lysosomes was more severely impaired than DNA transportation. TLR9 responses to CpG-A were also significantly impaired in TRPML1$^{-/-}$ Bone Marrow-conventional DCs (BM-cDCs) and plasmocytoid-DC (pDCs) by the PIKfyve inhibitor, suggesting that TRPML1 has a role in CpG-A transportation to lysosomes. However, CpG-A transportation to lysosomes was only transiently halted in TRPML1$^{-/-}$ BM-cDCs, suggesting a redundant role of TRPML1 in this pathway. Conversely, TLR9 responses to CpG-B were not altered in TRPML1$^{-/-}$ BM-cDCs and pDCs (77, 88). Impaired TLR7 and TLR9 responses in TRPML1$^{-/-}$ BM-cDCs stimulated with ssRNAor CpG-Awas associated with reduced IL-6, TNF-α and IFN-α production. In addition, the PIKfyve inhibitor induced an impairment of TLR7 responses to ssRNA in BM-pDCs, while only reduced production of IFN-α in response to TLR9 stimulation by CpG-A was observed.

A role for TRPML channels in CpG-A transportation is not limited to TRPML1, but it has been described also for TRPML2 and TRPML3 (88).

Collectively, these findings suggest that TRPML channels, by enhancing TLR responses and promoting DC maturation/activation, play a critical role in stimulating anti-viral adaptive immune responses. In this regard, it has recently been discovered that TRPML2 increases the expression of B7 costimulatory molecules on DC via TFEB activation and simultaneously induces CD8 T cell proliferation and cytolytic activity in an antigen-specific manner (89).

TRPML1 AND TRPML2 REGULATE OF NK CELL FUNCTIONS: A DUAL ROLE IN ANTIVIRAL NK CELL RESPONSES?

Natural killers are a subset of innate lymphoid cells that have the ability to recognize and eliminate infected cells. Moreover, they can secrete anti-viral cytokines such as IFN-γ and TNF-α and chemokines to recruit and instruct other immune cell types

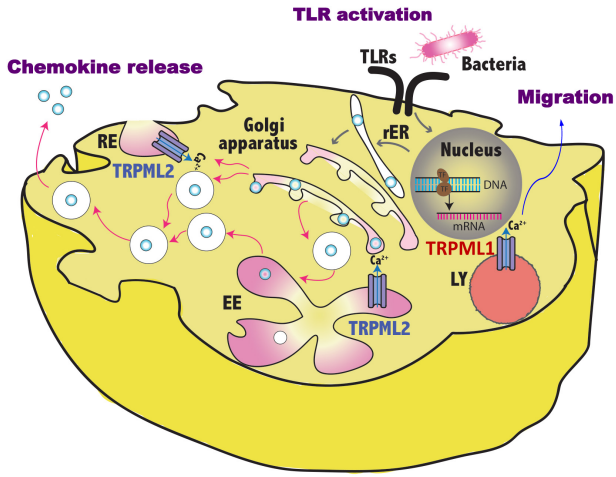

FIGURE 1 | Schematic representation of TRPML1 and TRPML2 involvement in the regulation of vesicles trafficking induced by pathogen sensing. By controlling the fluxes of vesicular Ca^{2+}, TRPML1 promotes in dendritic cells the activation of myosin II that leads to fast and directional migration whereas TRPML2 regulates in macrophages the fission/fusion processes of transport vesicles and so the release of chemokines in the environment. TLR, toll like receptor; RE, recycling endosome, LY, lysosome; EE, early endosome; rER, rough endoplasmic reticulum.

(87). The activation of NK cell depends on a delicate balance between activating and inhibitory signals, the latter mainly being transduced by (KIRs, CD94/NKG2A) receptors for class I MHC. The interaction of MHC I inhibitory receptors with their self-ligands also results in the acquisition of the effector potential, a process called NK cell education (90, 91). The recognition of abnormal self on virus-infected cells triggers a number of non-MHC I-restricted activating receptors such as NKG2D, MHC I-related molecules MHC class I chain-related protein A and B (MICA and MICB) and UL16 binding proteins (ULBPs), DNAX Accessory Molecule-1and the NCR. These activating receptors function mainly in a cooperative manner to overcome the inhibitory signals of KIR and CD94-NKG2A receptors (92).

As other innate immune effector cells, NK cells can also sense viral patterns by means of TLRs. Indeed TLR3, TLR 7, TLR 8, and TLR 9 have been detected in human NK cells and the engagement by their respective ligands leads to production IFN-γ and CSFs and chemokines. Moreover, antiviral NK cell-mediated reactivity strongly relies on the cross-talk with other innate immune cells, including DCs and macrophages which can promote NK cell effector functions and proliferation by secreting cytokines such as IFN-α/β, IL-12, and IL-15, respectively (93–95).

Recent findings demonstrated that TRPML1 is expressed at mRNA level in both educated (KIR^+) and not educated (KIR^-) NK cell subsets. Interestingly, pharmacological inhibition of PtdIns(3, 5)P2 synthesis, or genetic silencing of TRPML1, resulted in enlargement of lysosomal compartment, increased granzyme B (GZB), and enhanced specific degranulation and IFN-γ production. On the contrary, stimulation of NK cells with the TRPML agonist, MK6-83, induced the loss of GZB and decreased degranulation and IFN-γ production in response to K562 cells. Overall, these findings suggest an important role of TRPML1-mediated modulation of secretory lysosomes in NK cell education (96). Recent evidence reported that NK cells also express high levels of TRPML2, which further increase during NK cell differentiation. Silencing of TRPML2 leads to slight enhanced NK cell degranulation and to the production of IFN-γ (96).

Finally, although no evidence is available in the literature so far, a role for TRPML in promoting NK cell-mediated TLR responses to viral patterns can be envisaged (97).

Collectively, these findings suggest that TRPML channels negatively affecting NK cell education and promoting TLR activation, play a dual role in NK cell-mediated antiviral responses.

CONCLUSION

Several evidences indicate that TRPMLs play a crucial role in membrane trafficking, autophagy, exocytosis and ion homeostasis. Thanks to these functions, TRPML1 and TRPML2 have been found to be involved in the entry and trafficking of virus by promoting virus infectivity and productive infection. Conversely, these receptors are also expressed on innate immune cells where they stimulate the transport of viral patterns and therefore the cognition by their respective receptors present in the endosomal compartment. In DCs, this results in enhanced TLR responses that lead to increased DC maturation, production of IFNs, inflammatory cytokines/chemokines and migration with consequent activation of the anti-viral adaptive immune responses (**Figure 1**). Recent findings indicate that on NK cells, which also express TRPML1 and TRPLM2 as well as the TLRs recognizing the viral nucleic acids, TRPML1 impairs NK cell education and functional activity by modulating the secretory lysosomes, thus suggesting a dual role in the NK cell-mediated antiviral responses.

For what concern TRPML3 role in infections, it has been demonstrated in bladder epithelial cells that TRPML3, by mediating efflux of Ca^{2+} ions from lysosomes, promotes the expulsion of exosome-encased bacteria (98). However, little is known about its functions in viral infections. At this regard, findings showed that TRPML3 increased IAV infections in ectopically expressing cells (65). Moreover, it has been recruited in the autophagosome upon induction of autophagy (50) and this suggests that it could participate to the xenophagy. Regarding the viral infections, autophagy can be either pro-viral or anti-viral. Some virus exploit the autophagy machinery for their intracellular survival, while other express specific protein to evade autophagy and propagate in host cells (99). Thus, an important role of TRPML3 in viral infections cannot be excluded; however, additional findings are required to further clarify this issue.

AUTHOR CONTRIBUTIONS

GS and AS drafted the manuscript. GS conceived and designed the study. MM, CA, MN, and MS critically revised the manuscript.

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TRPV4 Mediates Acute Bladder Responses to Bacterial Lipopolysaccharides

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Urinary tract infections (UTI) affect a large proportion of the population, causing among other symptoms, more frequent and urgent micturition. Previous studies reported that the gram-negative bacterial wall component lipopolysaccharides (LPS) trigger acute epithelial and bladder voiding responses, but the underlying mechanisms remain unknown. The cation channel TRPV4 is implicated in the regulation of the bladder voiding. Since TRPV4 is activated by LPS in airway epithelial cells, we sought to determine whether this channel plays a role in LPS-induced responses in urothelial cells (UCs). We found that human-derived UCs display a fast increase in intracellular Ca^{2+} concentration upon acute application of *Escherichia coli* LPS. Such responses were detected also in freshly isolated mouse UCs, and found to be dependent on TRPV4, but not to require the canonical TLR4 signaling pathway of LPS detection. Confocal microscopy experiments revealed that TRPV4 is dispensable for LPS-induced nuclear translocation of NF-κB in mouse UCs. On the other hand, quantitative RT PCR determinations showed an enhanced LPS-induced production of proinflammatory cytokines in TRPV4-deficient UCs. Cystometry experiments in anesthetized wild type mice revealed that acute intravesical instillation of LPS rapidly increases voiding frequency. This effect was not observed in TRPV4-deficient animals, but was largely preserved in *Tlr4* KO and *Trpa1* KO mice. Our results suggest that activation of TRPV4 by LPS in UCs regulates the proinflammatory response and contributes to LPS-induced increase in voiding frequency. These findings further support the concept that TRP channels are sensors of LPS, mediating fast innate immunity mechanisms against gram-negative bacteria.

Keywords: LPS, TRPV4, bladder, urothelial cells, cystitis

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INTRODUCTION

Epithelial cells lining the bladder and upper urinary tracts play key roles in defensive mechanisms against urinary tract infections (UTI). In addition to serving as physical barrier, urothelial cells (UCs) act as first responders upon recognition of several virulence factors, such as hemolysin and cytotoxic necrotizing factor, the siderophore aerobactin, bacterial capsules and lipopolysaccharide (LPS) (1). UCs host several defensive responses against invading bacteria, of which the most common is uropathogenic *Escherichia coli*

(2, 3). These include the production of specific soluble epithelial cell-derived mediators (i.e.: lactoferrin, soluble IgA, Tamm-Horsfall protein, lipocalin), bactericidal antimocrobial peptides (defensins, cathelicidin), and bacterial clearance by urothelium exfoliation mechanisms (3). In addition, LPS recognition by Toll-like receptor 4 (TLR4) activates the transcriptional factor NF-κB, which regulates the expression of several immunomodulatory cytokines (4), causing the infiltration of inflammatory cells, and eventually edema and hemorrhage (5–8). These histological changes in the bladder wall are accompanied by burning sensation during urination, low-abdominal pain and frequent urge to urinate, typical symptoms of UTI (9).

Interestingly, there is evidence for rather fast bladder responses to LPS. For instance, acute application of LPS induces an increase of intracellular Ca^{2+} concentration within minutes and secretion of the proinflammatory cytokine IL-6 in a grade II human carcinoma bladder epithelium cell line (10). Furthermore, an increased mouse bladder voiding frequency was observed 1 h after direct intravesical instillation of LPS, that is, sooner than any histological inflammatory changes have been detected (11). Although these effects may be regarded as early bladder defensive responses against bacterial infection based LPS detection, the underlying mechanisms remain unknown.

We recently showed that airway epithelial cells respond to LPS with an elevation of cytosolic Ca^{2+} via the activation of the Transient Receptor Potential Vanilloid 4 (TRPV4) cation channel. This triggers protective responses such as production of bactericidal nitric oxide and increased ciliary beat frequency within a few minutes (12). Similar to airway epithelial cells, UCs have a prominent expression of TRPV4, and activation of this channel has been implicated in the mechanisms of bladder voiding (13, 14).

Thus, in this study we tested the hypothesis that LPS activates TRPV4 in UCs, which might lead to immediate increase in intracellular Ca^{2+} concentration, regulation of cytokine production and to changes in the bladder voiding pattern. To do this, we performed intracellular Ca^{2+} imaging experiments and NF-κB nuclear translocation and cytokine expression determinations in freshly isolated UCs. We found that *E. coli* LPS activates TRPV4 in UCs independent of the TLR4 signaling pathway. Activation of TRPV4 did not have an impact in NF-κB nuclear translocation, but we obtained evidence for a regulatory role of TRPV4 on the increase in proinflammatory cytokine expression induced by LPS. Finally, using cystometry in anesthetized mice, we found that TRPV4, but not TLR4, is required for a fast increase in voiding frequency triggered by intravesical instillation of LPS.

MATERIALS AND METHODS

Animals

Trpv4 KO mice were kindly provided by Prof W. Liedtke (Duke University, Durham, NC). *Tlr4* KO (B6.B10ScN-Tlr4lps-del/JthJ) mice were purchased at Charles River Laboratories (Chatillon-sur-Chalaronne, France). These knockout strains were backcrossed at least ten times into the C57BL/6J background, and C57BL/6J mice were used as wild type controls. Mice of all genotypes were housed under identical conditions, with a maximum of four animals per cage on a 12-h light-dark cycle and with food and water *ad libitum*. Ten- to twelve-week-old female mice were used in all experiments. All animal experiments were carried out in accordance with the European Union Community Council guidelines and were approved by the local ethics committee.

Primary Urothelial Cells Culture

Isolation and culture of mouse urothelial cells was performed as previously described by others (15). After euthanasia, bladders were quickly removed, cut open, and stretched out on a Sylgard-coated dish containing MEM (Invitrogen, Merelbeke, Belgium) with 2.5 mg/ml dispase (Invitrogen) for 2 h at room temperature. After incubation, the urothelium was gently scraped from the underlying tissue, treated with trypsin-EDTA (Invitrogen) for 15 min, and resuspended in defined keratinocyte serum-free medium (Invitrogen). The cell suspension was plated on collagen (type IV; Sigma, Overijse, Belgium)-coated coverslips. Cells were used for experiments 16 h after isolation.

Human urothelial cells were collected from bladder cancer patients undergoing cystectomy, as previously described (16). Briefly, healthy bladder strips were incubated overnight at 4°C in sterile HBSS-based stripping solution containing 14 mM HEPES, 20 KIU aprotinin, 0.1% EDTA, pH 7.6. After incubation, the urothelium was gently scraped, treated with collagenase for 15 min, and resuspended in keratinocyte medium (Invitrogen). Cells were seeded in gelatin-coated glass coverslip and used for experiments 16 h after isolation.

Intracellular Ca^{2+} Imaging

Urothelial cells were incubated with Fura-2 acetoxymethyl (2 μM) ester for 30 min at 37°C. During recordings cells were perfused by gravity via a multi-barreled pipette tip with bath solutions prepared in Krebs, containing (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, 10 glucose and titrated to 7.4 with NaOH. Intracellular Ca^{2+} concentration was monitored through the ratio of fluorescence measured upon alternating illumination at 340 and 380 nm using an MT-10 illumination system and the Xcellence pro software (Olympus Belgium N.V., Berchem, Belgium).

Quantitative Real-Time PCR

Total RNA from cultured urothelial cells was extracted using the RNeasy Mini Kit (Qiagen, Antwerp, Belgium), following manufacturer's protocol. RNA concentration was determined in a micro-volume spectrophotometer DropSense16 (Trinean NV, Gent, Belgium). cDNA synthesis was performed with 1μg of total RNA using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Diegem, Belgium). Quantitative PCR reactions (20 μl), containing 3 μl cDNA template (diluted 1:5), Universal TaqMan MasterMix (2x concentrated, Life Technologies), specific TaqMan probes (**Table 1**, 20× concentrated, Life Technologies) and H_2O, were performed with the 7500 Fast Real-Time PCR System (Life Technologies). Reactions were made using the following program: 50°C for 2 min and 95°C for 10 min,

TABLE 1 | List of TaqMan probes.

Gene name	Probe ID (in Applied Biosystems)
Trpv2	Mm00449223_m1
Trpv4	Mm00499025_m1
Trpm7	Mm00457998_m1
Tlr4	Mm00445273_m1
Gapdh	Mm99999915_g1
Il-1b	Mm00434228_m1
Cxcl-1	Mm04207460_m1
Cxcl-2	Mm00436450_m1
Tnf	Mm00443258_m1

followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Non-template controls (NTCs) were used as negative controls in every experiment.

Confocal Microscopy

Freshly isolated urothelial cells from wild type, *Trpv4* KO and *Tlr4* KO mice were seeded in glass coverslips and exposed to LPS (20 μg/ml) for 30 min. After treatment, cells were fixed with cold paraformaldehyde and permeabilized with 0.2% Triton X-100. Primary antibody against p65 NF-κB (1:250; Cell Signaling #4764) or S534-phosphorylated p65 NF-κB (1:800; Cell Signaling #3033) was incubated overnight at 4°C, followed by anti-rabbit Alexa Fluor 633 (1:600; A21070, Invitrogen) for 1 h at room temperature. Coverslips were mounted in glass slides using DAPI-containing mounting solution (VectaShield, Vector Laboratories, Burlingame, CA, United States). Confocal images were obtained from ten randomly selected fields from three independent experiments using the optimal pinhole size for the 63X oil objective on a Zeiss LSM 880-Airyscan (Carl Zeiss AG, Oberkochen, Germany). Images were analyzed using Fiji software (17) as described before (18).

Cystometry

Catheter implantation and intravesical pressure recordings were performed as previously described (19). All recordings were performed under urethane anesthesia, and body temperature was maintained at 37°C using a heating lamp and an animal temperature controller (World Precision Instruments, Hertfordshire, United Kingdom). Bladders were infused with saline at a constant rate (20 μl/min), inducing repetitive cycles of bladder filling and voiding. After an equilibration period of 60 min, baseline intravesical pressures were recorded for 30 min. The infusion fluid was then switched to a solution containing LPS in saline, and intravesical pressures were measured again for 30 min. In a group of animals HC-067047 (1 ml at 100 μM = 2.35 mg/kg) was administered intraperitoneally 30 min prior recordings.

Statistics

Magnitudes were statistically compared using GraphPad Prism version 7.0d for MacOS, GraphPad Software, La Jolla, CA, United States[1] ([1]www.graphpad.com). The non-parametric Wilcoxon and Mann-Whitney *U* tests were used to determine statistically significant changes and differences in medians, respectively. Differences were considered to be statistically significant when $P < 0.05$ (labeled as * in the Figures), but $P < 0.01$ (**) was also indicated when applicable.

RESULTS

TRPV4 Mediates LPS-Induced Ca^{2+} Entry in Freshly Isolated Urothelial Cells

To determine whether LPS induces acute responses in freshly isolated human urothelial cells (hUCs) we isolated hUCs from bladder strips obtained after cystectomy and followed the dynamics of intracellular Ca^{2+} concentration using Fura-2. We explored the effects of LPS in the micromolar range of concentrations to be able to compare our results to those previously reported (10, 20). We found that acute application of *E. coli* LPS (20 μg/ml) induced a significant increase in the intracellular Ca^{2+} concentration in 41% (72 out of 173) of cells responding to the TRPV4-specific agonist GSK1016790A. The rise in intracellular Ca^{2+} concentration appears rapidly after LPS application and was quickly reversed upon LPS washout (**Figure 1A**). Pharmacological inhibition of TRPV4 with the specific antagonist HC-067047 (21) strongly suppressed LPS- and GSK1016790A-induced responses (**Figures 1B,C**), indicating that TRPV4 activation is necessary for the immediate response of hUCs to LPS.

We next tested the effects of LPS on freshly isolated mouse UC (mUCs), because this preparation was more readily available than human-derived cells, and the possibility to study cells from genetically modified animals. LPS triggered intracellular Ca^{2+} responses in a subpopulation of mUCs derived from wild type (WT) animals. LPS (20 μg/ml) induced a fast and reversible increase in the intracellular Ca^{2+} concentration in 78% (110 out of 141, average amplitude 0.37 ± 0.03 μM) of mUCs responsive to the TRPV4 agonist GSK1016790A (10 nM; **Figure 2A**). The amplitude of these responses and the proportion of LPS-sensitive cells were concentration-dependent, with effective concentrations (EC_{50}) of 7.1 ± 1.1 and 7.7 ± 0.3 μg/ml, respectively and Hill coefficients of 1.6 ± 0.4 and 1.9 ± 0.2, respectively (**Figures 2B,C**). LPS largely failed to increase the intracellular Ca^{2+} concentration in mUCs in the presence of the TRPV4 inhibitor HC-067047 (10 μM) (**Figures 2D,F**). mUCs harvested from *Trpv4*-deficient animals were also largely insensitive to LPS (**Figures 2E,F**). As expected, *Trpv4*-deficient mUCs were also unresponsive to the TRPV4 agonists GSK1016790A and 4αPDD (**Figures 2E,G**). To assess the possibility that the lack of TRPV4 results in a generalized loss of cellular responsiveness, we evaluated the activation of other Ca^{2+}-permeable channels expressed in mUCs cells, namely TRPV2 (22) and purinergic receptors (23). Application of the TRPV2 activator cannabis oil or ATP induced acute and reversible increases in cytoplasmic Ca^{2+} concentration (**Figure 2E**). Altogether, these results demonstrate that, as shown above in hUCs, TRPV4 mediates the LPS-induced acute increase of intracellular Ca^{2+} concentration in mUCs.

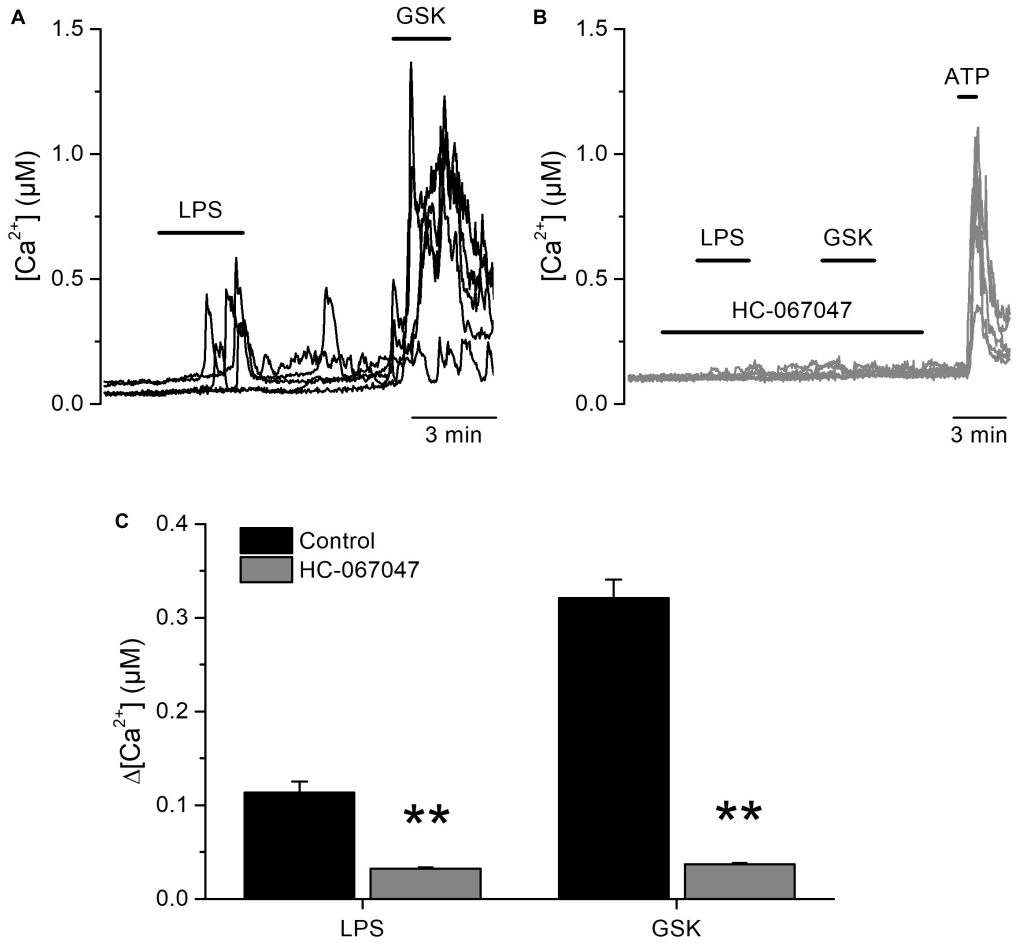

FIGURE 1 | Acute TRPV4-dependent stimulation of human urothelial cells by *E. coli* LPS. **(A,B)** Representative traces of intracellular Ca^{2+} signals recorded in human UCs. LPS (20 μg/ml) and GSK1016790A (GSK, 10 nM) were perfused in control **(A)**, or in the presence of the TRPV4 inhibitor HC-067047 (10 μM) **(B)**. ATP (100 μM) was applied at the end of the latter experiments to assess cellular responsiveness. **(C)** Average amplitudes of intracellular Ca^{2+} responses evoked by LPS (20 μg/ml) or GSK1016790A (GSK, 10 nM) in the absence (n = 173) of presence (n = 280) of HC-067047 (10 μM). $^{**}P < 0.01$, Mann–Whitney U test.

Although *E. coli* accounts for 75–90% of UTI, the bacterial spectrum differs in immunocompromised hosts (3). For instance, the opportunistic gram-negative bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are common causative agents during nosocomial UTI (2, 24). Thus, we tested whether also LPS from these bacterial strains are capable of activating TRPV4 in mUCs. We found that LPS (20 μg/ml) extracted from *K. pneumoniae* or *P. aeruginosa* induced robust increases in intracellular Ca^{2+} concentration in 80% (72 out of 90 mUCs) and 47% (42 out of 88 mUCs), respectively (**Figures 3A,B**). Although the amplitudes of the responses elicited by these two LPS were not significantly different from each other (*K. pneumoniae* LPS: 0.14 ± 0.01 μM (n = 72), vs. *P. aeruginosa* LPS: 0.12 ± 0.02 μM (n = 42); P = 0.24, two-tailed unpaired t test), they were significantly smaller than the amplitude of the response evoked by *E. coli* LPS (*E. coli*: 0.38 ± 0.03 μM (n = 68); $P < 0.0001$, Tukey's multiple comparison test). LPS from *K. pneumoniae* and *P. aeruginosa* largely failed to increase the intracellular Ca^{2+} concentration in mUCs isolated from *Trpv4* KO mice (**Figures 3C,D**), confirming that TRPV4 is necessary for acute responses to LPS.

Next, we tested whether the canonical immune receptor TLR4 contributes to the acute responses of mUCs to LPS. We found that 65% (99 out of 151) of the mUCs isolated from *Tlr4* KO mice exhibited an increase in intracellular Ca^{2+} concentration upon LPS application (**Figures 4A,C**). As found in WT cells, both the amplitude of the LPS-induced intracellular Ca^{2+} responses (**Figures 4B,C**) and the percentage of LPS-responding cells (1.3%; 2 out of 148) were significantly smaller in the presence of the TRPV4 inhibitor HC-067047, compared to untreated *Tlr4* KO mUCs ($P < 0.001$, Dunn's multiple comparison test and $P < 0.0001$, Fisher's exact test, respectively). Interestingly, *Tlr4*-deficient mUCs exhibited a lower fraction of responding cells (65%) and smaller amplitude of responses to LPS (0.27 ± 0.01 μM) in comparison to WT mUCs (P = 0.019, Fisher's exact test and P = 0.0008, two-tailed unpaired t test, respectively). Furthermore, we found that also the responses to GSK1016790A were significantly smaller in *Tlr4*-deficient cells (WT: 0.79 ± 0.05

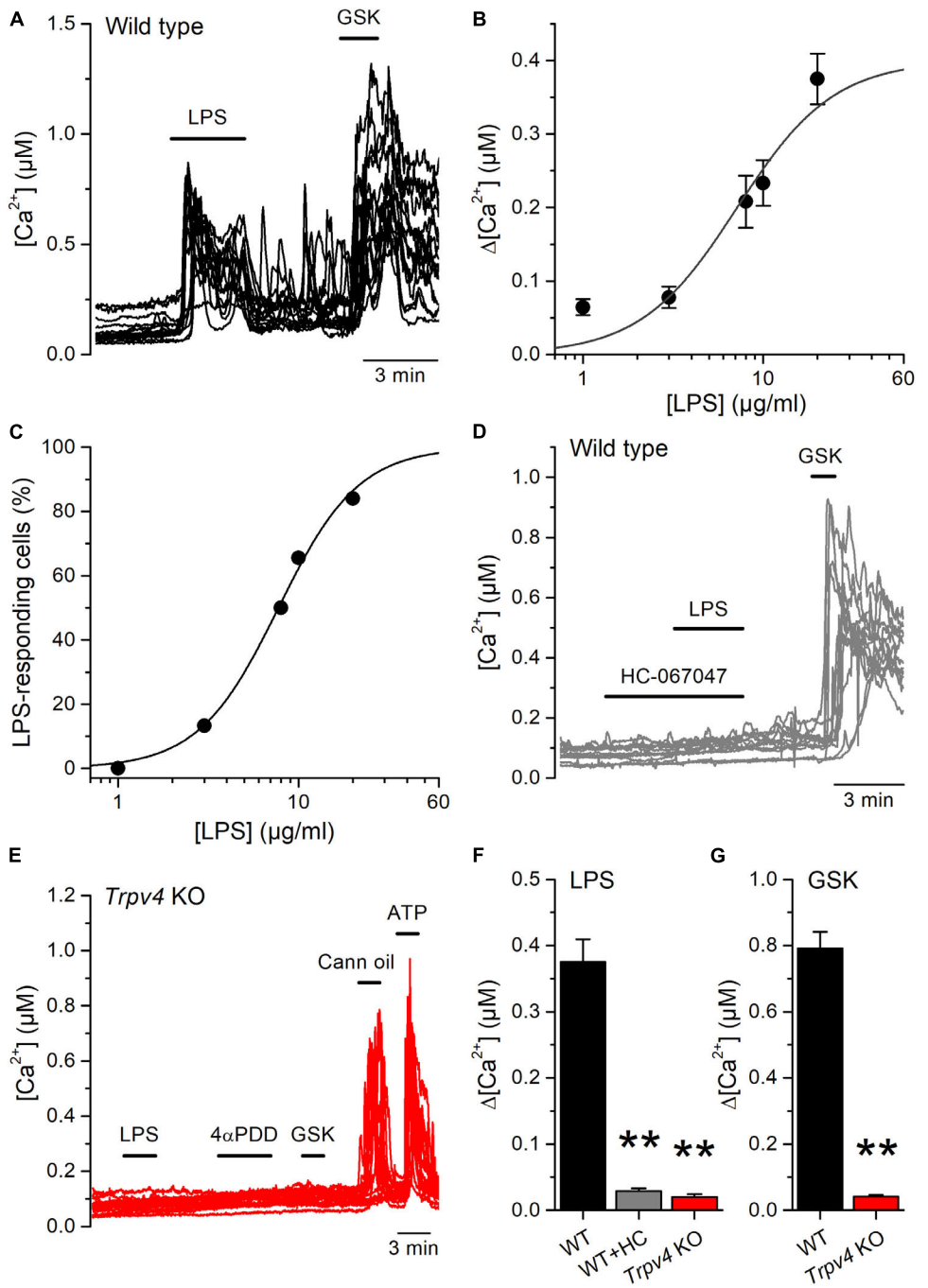

FIGURE 2 | Acute TRPV4-dependent stimulation of mouse urothelial cells by *E. coli* LPS. **(A)** Representative intracellular Ca^{2+} signals recorded in urothelial cells isolated from wild type mice, showing the responses to acute application of LPS (20 μg/ml) or GSK1016790A (GSK, 10 nM). **(B,C)** Concentration dependences of the amplitude (B) and percentage of occurrence **(C)** of LPS-evoked intracellular Ca^{2+} responses in TRPV4-expressing cells (responsive to GSK1016790A 10 nM). The solid lines represent the fits with Hill equations. $n > 80$ cells per data point. **(D)** Representative intracellular Ca^{2+} signals recorded in WT mUCs to which LPS (20 μg/ml) was applied in the presence of the TRPV4 inhibitor HC-067047 (10 μM). GSK1016790A (GSK, 10 nM) was later perfused to assess functional expression of TRPV4. **(E)** Representative intracellular Ca^{2+} signals recorded in urothelial cells isolated from *Trpv4* KO mice. LPS was applied at a concentration of 20 μg/ml. The TRPV4 agonists 4αPDD (2 μM) and GSK1016790A (GSK, 10 nM) were used to evaluate the functional expression of TRPV4. A TRPV2 agonist (cannabis oil, 100 μM) and ATP (10 μM) were used to assess cell responsiveness. **(F,G)** Average amplitude of responses to LPS (20 μg/ml) **(F)** and GSK1016790A (10 nM) **(G)** in urothelial cells isolated from wild type (WT, black bars) and *Trpv4* KO (red bars) mice. HC, HC-067047 (10 μM). The ** symbols indicate $P < 0.01$ with a Dunn's multiple comparison test.

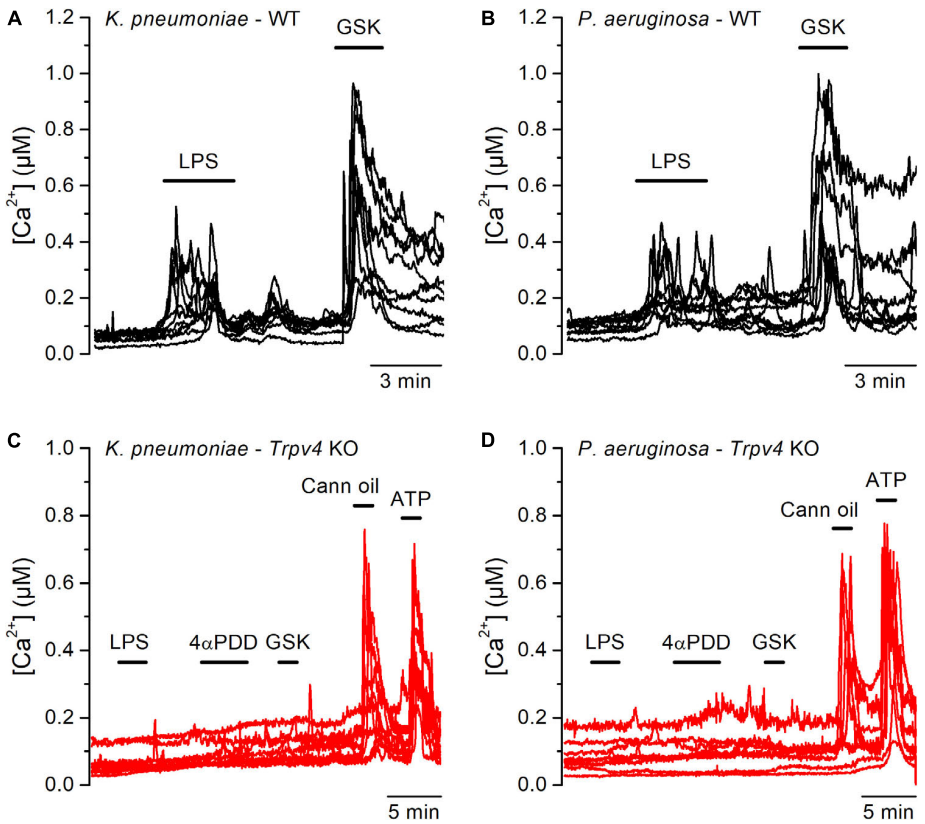

FIGURE 3 | Acute TRPV4-dependent stimulation of mouse urothelial cells by *K. pneumoniae* and *P. aeruginosa* LPS. **(A–D)** Representative intracellular Ca^{2+} signals recorded in urothelial cells isolated from WT (panels **A** and **B**) or *Trpv4* KO (panels **C** and **D**) mice. LPS extracted from *K. pneumoniae* **(A,C)** or *P. aeruginosa* **(B,D)** were applied at a concentration of 20 μg/ml. The TRPV4 agonists 4αPDD (2 μM) and GSK1016790A (GSK, 10 nM) were used to evaluate the functional expression of TRPV4. A TRPV2 agonist (cannabis oil, 100 μM) and ATP (10 μM) were used to assess responsiveness of *Trpv4* KO-derived cells.

μM vs. *Tlr4* KO: 0.49 ± 0.02 μM; $P < 0.0001$, two-tailed unpaired *t* test). These weaker TRPV4-mediated responses in *Tlr4* KO mUCs may be related to the lower expression levels of TRPV4, since comparative transcripts analysis showed significant lower expression of *Trpv4* (and *Trpv2*) in *Tlr4* KO than in WT mUCs (**Figure 4D**). Altogether, these findings confirm that TRPV4 is the main contributor to the fast increase in intracellular Ca^{2+} concentration induced by LPS in mUCs and demonstrate that the TLR4 signaling pathway is not critically required for LPS-induced activation of TRPV4.

LPS-Induced Inflammatory Gene Expression Is Enhanced in Trpv4-Deficient Urothelial Cells

Lipopolysaccharides triggers an increase in intracellular Ca^{2+} concentration that leads to Ca^{2+}-dependent phosphorylation of NF-κB, inducing its nuclear translocation and a consequent increased production of proinflammatory cytokines in a line of human carcinoma bladder epithelial cells and in macrophages (10, 25). Thus, we sought to determine whether the TRPV4-mediated increase in intracellular Ca^{2+} induced by LPS triggers NF-κB translocation in mUCs. For this, we incubated these cells with LPS during 30 min and quantified the presence of NF-κB in the nucleus. Untreated WT mUCs exhibited a scattered distribution of NF-κB, with similar expression in the nucleus and the cytoplasm, whereas LPS-treated cells displayed a significant increase in nuclear NF-κB and S534-phosphorylated p65 subunit (**Figures 5A–C** and **Supplementary Figures 1**, **2**). *Trpv4*-deficient mUCs showed results similar to WT cells, but *Tlr4* KO mUCs appeared unresponsive to LPS (**Figures 5A–C** and **Supplementary Figures 1**, **2**).

We also tested for the implication of TRPV4 in the regulation of the expression of pro-inflammatory cytokines. We found that the LPS challenge induced a significantly larger increase in *Il-6*, *Cxcl-1*, *Cxcl-2,* and *Tnf* transcript levels in TRPV4-deficient mUC than in WT mUC (**Figure 6**). Interestingly, LPS-treated *Tlr4* KO mUC did not display *Il-6* upregulation, but did show about 10-fold increased expression of *Cxcl-1*, *Cxcl-2,* and *Tnf* (**Figure 6**).

TRPV4 Is Required for LPS-Induced Increase in Bladder Voiding Frequency

Finally, we determined whether intravesical infusion of LPS produces acute changes in the mouse bladder voiding reflex using cystometry *in vivo* (19). Given that ablation of TRPV4 induces a reduction of the voiding frequency in mice (21), we hypothesized

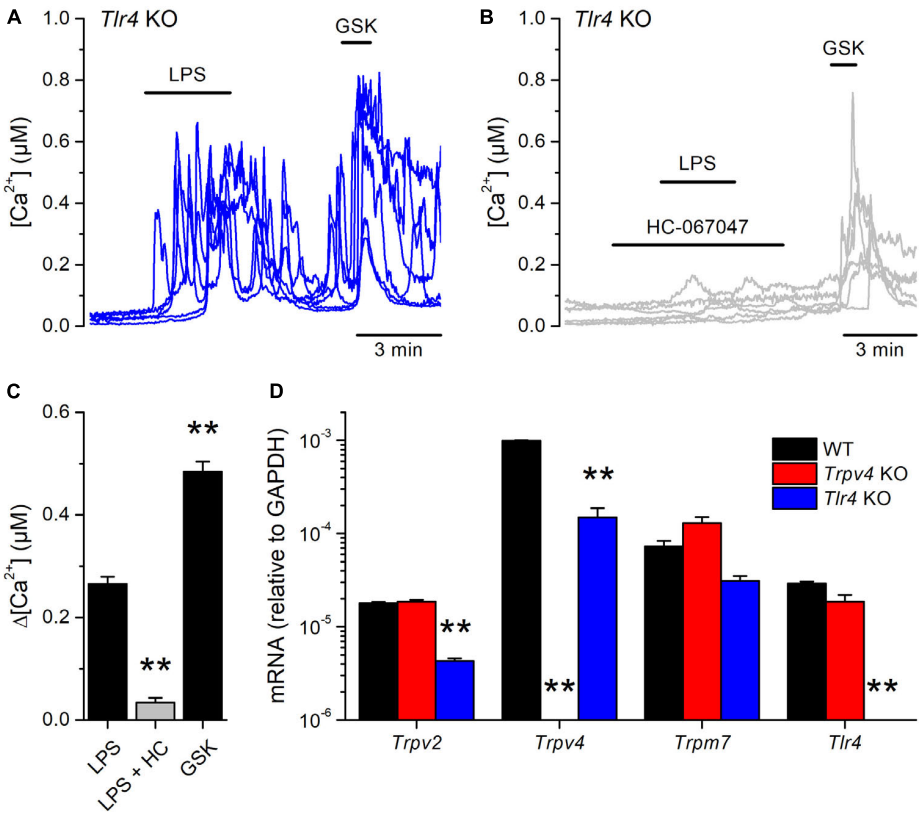

FIGURE 4 | TLR4 is dispensable for the acute response to *E. coli* LPS in mouse urothelial cells. **(A,B)** Representative intracellular Ca^{2+} signals recorded in urothelial cells isolated from *Tlr4* KO mice. LPS (20 μg/ml) was perfused in control **(A)** or in the presence of the TRPV4 inhibitor HC-067047 (10 μM) **(B)**. GSK1016790A (GSK, 10 nM) was applied to assess the functional expression of TRPV4. **(C)** Average amplitude of responses to LPS (20 μg/ml) in control (n = 151) or in the presence of HC-067047 (HC, 10 μM) (n = 148) in urothelial cells isolated from *Tlr4* KO mice. The average amplitude of the responses to GSK1016790A (GSK, 10 nM) in control (n = 151) is also shown for comparison. The ** symbols indicate $P < 0.01$ with a Dunn's multiple comparison test. **(D)** Expression levels of *Trpv2*, *Trpv4*, *Trpm7* and *Tlr4* mRNA transcripts in urothelial cells derived from WT, *Trpv4* KO and *Tlr4* KO mice. The bars represent mean ± SEM, (n = 3). The ** symbols indicate $P < 0.01$ with a Tukey's multiple comparison test.

that LPS-induced activation of the channel may produce the opposite effect.

During infusion of saline solution at a rate of 20 μl/min *Trpv4* KO mice displayed a basal voiding frequency that was significantly lower than that of WT animals (**Figures 7A,B**; P = 0.0011), as previously reported (13, 26). Acute intravesical administration of LPS (200 μg/ml in saline) via the infusion catheter induced an increase in the voiding frequency in WT ($P = 8 \times 10^{-4}$), but not in *Trpv4*-deficient mice (P = 0.74) (**Figures 7A,C**).

The lack of effect of LPS in the latter could be related to the lower rate of administration of this compound to the bladder, given the lower basal voiding frequency in these animals. To assess this possibility, we performed another series of experiments in WT mice using an intravesical infusion rate 8 μl/min to adjust the basal voiding frequency in these animals to values closer to that of *Trpv4* KO mice (**Figure 7B**). In this condition, LPS increased the voiding frequency to levels similar to those found in WT animals at a perfusion rate of 20 μl/min (P = 0.48; **Figure 7C**). WT mice under pharmacological inhibition of TRPV4 by intraperitoneal administration of HC-067047 (2.35 mg/kg) displayed a basal voiding frequency that was not different from that of control animals (**Figure 7B**; P = 0.18). However, instillation of LPS did not result in changes in voiding frequency in HC-067047-treated mice (P = 0.58; **Figure 7C**), which is consistent with the result obtained in *Trpv4* KO animals.

We also tested the implication of TLR4 in the voiding response by performing experiments in *Tlr4* KO mice. These animals displayed a basal voiding frequency and response to LPS that were not different from those of WT mice (**Figures 7B,C**). Finally, given its reported role in acute inflammation and pain responses to LPS (27), we assessed the implication of TRPA1. Mice deficient of this channel showed no difference in basal voiding frequency or response to LPS with respect to WT animals (**Figures 7B,C**).

DISCUSSION

The quick defensive responses of cells lining the urinary tract and bladder wall are determinant in limiting further bacterial colonization and tissue damage. Of particular interest in this

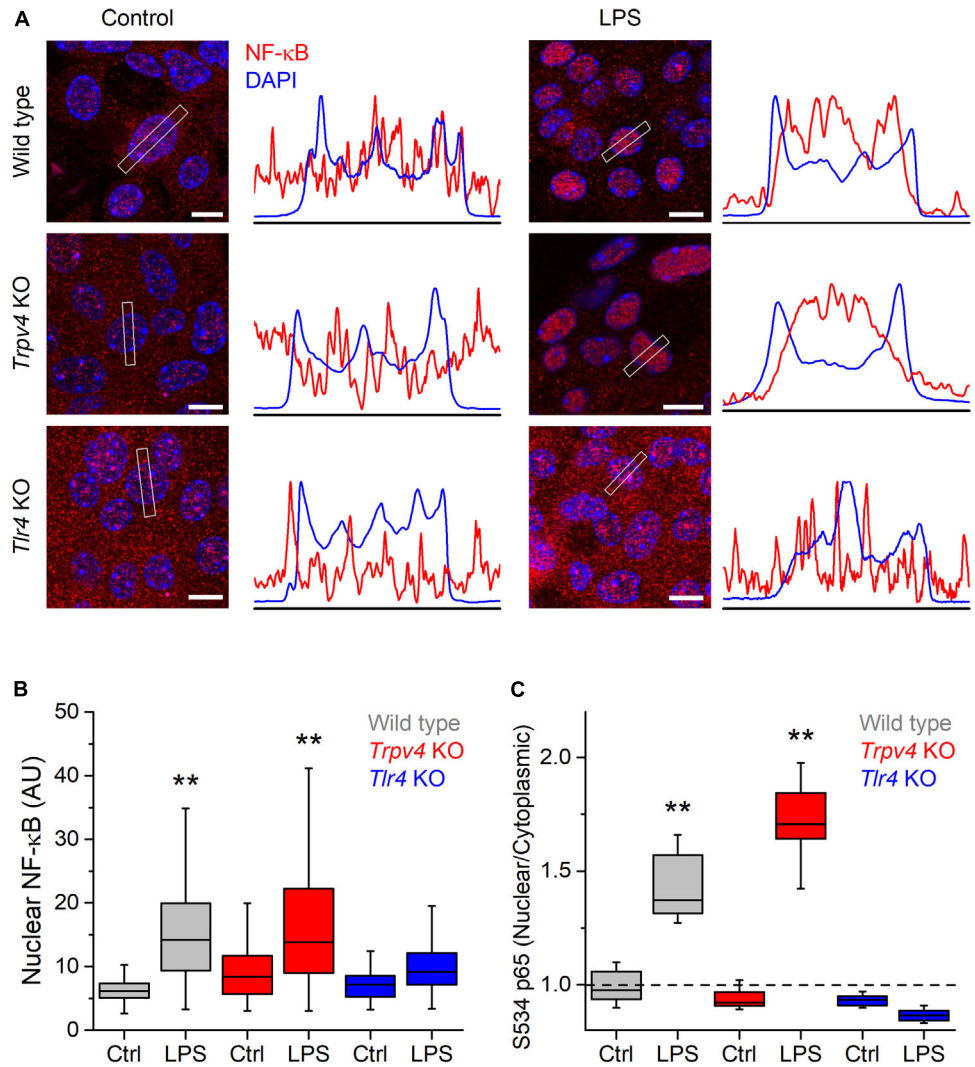

FIGURE 5 | TRPV4 is not required for the phosphorylation and nuclear translocation of NF-κB induced by *E. coli* LPS in mouse urothelial cells. **(A)** Representative confocal immunofluorescence microscopy images of fixed mUCs in control or 30 min after treatment with LPS (20 μg/ml). Cells were stained with NF-κB p65 (red) and DAPI nuclear staining (blue). Scale bar, 10 μm. The average linear intensity along the gray rectangle is represented next to each image. **(B)** Mean intensity of nuclear NF-κB p65 staining in mUCs in control or 30 min after treatment with LPS (20 μg/ml, 30 min). The data is represented in a box plot indicating the median (black line), the 25–75 percentiles (box) and the 10–90 percentile range (whiskers). The ** symbols indicate $P < 0.01$ and with a Tukey's multiple comparison test (the *n* values (control/LPS) are 53/100 for WT, 96/92 for *Trpv4* KO and 88/103 for *Tlr4* KO). **(C)** Average nuclear/cytoplasmic ratio of S534 p65 subunit in mUCs in control or 30 min after treatment with LPS (20 μg/ml, 30 min). The data is represented in a box plot indicating the median (black line), the 25–75 percentiles (box) and the 10–90 percentile range (whiskers). The ** symbols indicate $P < 0.01$, with a Tukey's multiple comparison test. At least eight randomly selected images were analyzed per conditions from three independent experiments.

regard are the fast increases in intracellular Ca^{2+} concentration and in proinflammatory gene expression induced by acute LPS application that were reported in a carcinoma human bladder epithelium cell line (10). In this study we found that fast LPS-induced Ca^{2+} responses are present also in primary cultured UCs isolated from human and mouse bladder samples, and obtained pharmacologic and genetic evidence indicating that TRPV4 activation is a necessary factor for this effect in both cell types.

Of note, we found that mUCs express mRNA of TRPV2 and TRPM7, two channels that were reported to mediate Ca^{2+} influx required for TLR4-dependent cytokine production in macrophages (25, 28). However, these channels do not play a critical role in the generation of the fast Ca^{2+} responses to LPS in mUCs, because these cells fail to respond when TRPV4 function is ablated. The activation of TRPM7 by LPS in macrophages occurs through interaction with the adaptor protein CD14, which facilitates the inclusion of this channel in the TLR4-MD2-CD14 complex (25). This mechanism is unlikely to operate in UCs since they lack expression of CD14 (29). Regarding TRPV2, we have previously reported that this channel is larger insensitive to LPS (30), and therefore is not expected to contribute to LPS-induced Ca^{2+} responses. Importantly, our data in *Tlr4* KO mUCs

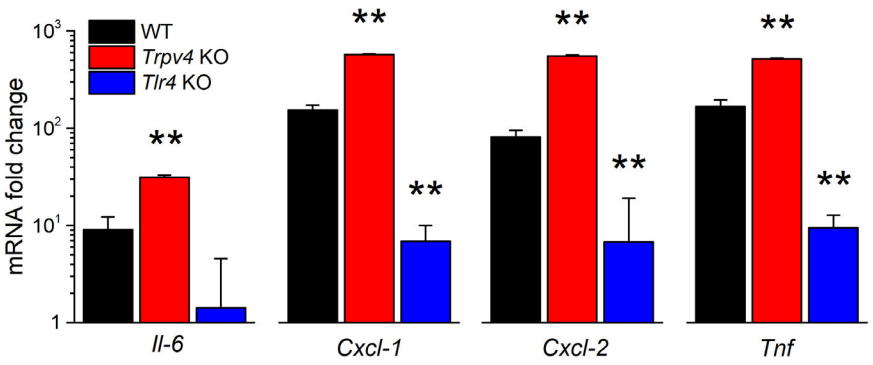

FIGURE 6 | Differential regulation of LPS-induced cytokine gene expression by TRPV4 and TLR4 in mUCs. Fold change of the expression levels of pro-inflammatory genes in urothelial cells derived from WT, *Trpv4* KO and *Tlr4* KO mice and treated with *E. coli* LPS (20 μg/ml, 30 min). The data is presented as mean ± SEM. of the expression levels relative to the respective values obtained in the untreated condition ($n = 3$). The ** symbols indicate $P < 0.01$, with a Tukey's multiple comparison test.

demonstrate that this receptor is not critical for LPS-induced activation of TRPV4. These conclusions fall in line with our previous observations in airway epithelial cells (12), suggesting for a general role of TRPV4 as LPS effector in epithelial cells.

Another feature that recapitulates what was found in airway epithelial cells (12) is that LPS elicits intracellular Ca^{2+} responses only in a fraction of UCs responding to the potent TRPV4 agonist GSK1016790A. This is probably due to the fact that LPS is a weaker channel agonist (31). The apparent effective stimulatory concentration of LPS on TRPV4 in mUCs we report here (∼7 μg/ml) is very similar to that previously found in airway epithelial cells (11 μg/ml) (12). These values are in the same range as that reported for the broadly tuned chemosensory channel TRPA1 (32) (∼3 μg/ml) (27), and lower than for the other sensory TRP channels, TRPV1, TRPV2, TRPM3, and TRPM8 (30). This confirms TRPA1 and TRPV4 as the most sensitive to LPS of the sensory TRP channels studied so far in this respect.

The effect of endotoxins on intracellular Ca^{2+} levels in mUCs and the implication of TRPV4 were confirmed for LPS extracted from *K. pneumoniae* and *P. aeruginosa*, which are two of the most frequent bacterial strains associated with the presence of indwelling urinary catheters (33). The weaker action of LPS from these strains compared to that of *E. coli*-derived LPS in activating TRPV4 is reminiscent of the stronger agonist effect of *E. coli* in TRPA1 (27). The molecular mechanism underlying TRPV4 activation by LPS remains to be fully clarified. However, because of the widely reported mechanosensitivity of this channel (34–36), we hypothesize that it could be similar as that operating in TRPA1, i.e., via the detection of LPS-induced mechanical perturbations in the plasma membrane (27, 37–39).

Our results show that TRPV4 activation does not influence the level of TLR4-dependent phosphorylation of NF-κB nor its translocation to the nucleus observed after 30 min stimulation with LPS. On the other hand, TRPV4-deficient cells displayed enhanced LPS-induced gene expression of the proinflammatory cytokines *Il-6*, *Cxcl-1*, *Cxcl-2*, *Tnf*, irrespective of whether this depended fully or not on TLR4 activation. The fact that this was also observed for *Il-6* and *Cxcl-1*, although not for *Cxcl-2*, in mouse tracheobronchial cells, suggests that the increase in Ca^{2+}/Na^{+} and/or the depolarization resulting from TRPV4 activation can have a negative regulatory action on LPS-induced gene expression of some proinflammatory cytokines in epithelial cells. The elucidation of the mechanism underlying this regulation requires further investigation.

The effects of luminal LPS in the urinary bladder are not restricted to long-term histological inflammatory changes in the bladder wall, but also to an immediate increase in voiding frequency (11). Our cystometry experiments demonstrate that TRPV4 is a key mediator of the LPS-induced acute increase in voiding frequency. Notably, these effects are already established within minutes of LPS installation, a time-frame in which histological inflammatory changes are not seen yet (6). The rapidity of these events is likely linked to the activation of sensory fibers densely innervating the bladder wall. However, the full abrogation of changes in voiding frequency in *Trpv4* KO mice excludes the direct interaction of LPS with TRPA1 (27) in sensory afferents as the trigger for changes in voiding pattern. Instead, TRPV4 activation may lead to the release of H_2O_2 (40), a known activator of TRPA1 (41, 42). However, the lack of difference in the voiding response to intravesical LPS administration between *Trpa1* KO and WT animals indicates that TRPA1 channels are not implicated. Alternatively, the activation of TRPV4 in UCs by LPS may induce the release of ATP (13, 36, 43), triggering afferent pathways through activation of purinergic receptors (13).

Altogether, our results suggest that UCs can recognize bacterial colonization through TRPV4 activation by LPS. The increase in voiding frequency induced by LPS may explain the strong urge to void frequently during cystitis, even immediately after the bladder is emptied. Together with UCs exfoliation during cystitis (44, 45), the increased flushing activity mediated by TRPV4 may contribute to the elimination of the pathogen from the bladder lumen.

In more general terms, our present findings further support the notion that TRP channels may function as sensors of bacterial endotoxins, playing crucial roles in the timely detection of

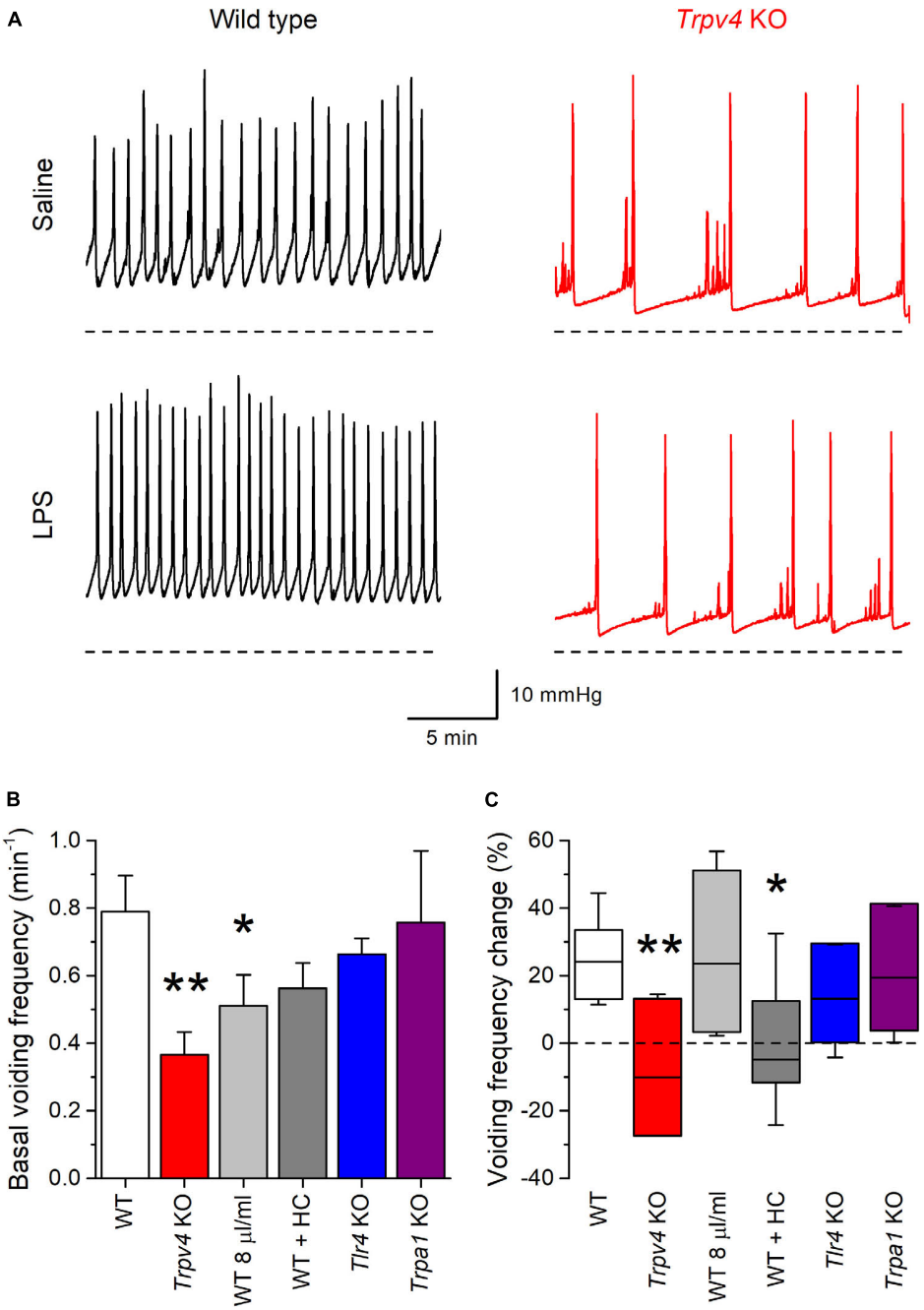

FIGURE 7 | TRPV4 mediates the increase of mouse bladder voiding frequency induced by intravesical instillation of *E. coli* LPS. **(A)** Traces of intravesical pressure recordings performed in wild type (black) and *Trpv4* KO (red) mice, during infusion of control saline (NaCl 0.9%) or *E. coli* LPS (200 μg/ml). **(B)** Basal voiding frequency of WT (n = 14), *Trpv4* KO (n = 9), WT perfused at a slow rate of 8 μl/min (n = 8), WT administered the TRPV4 inhibitor HC-067047 (HC, 2.35 mg/kg, n = 6), *Tlr4* KO (n = 9) and *Trpa1* KO (n = 7) mice. The data is presented as mean ± SEM. The * and ** symbols indicate $P < 0.05$ and $P < 0.01$, respectively with a Mann–Whitney U test vs. WT. **(C)** Change in the voiding frequency determined over 20 min after the first 10 min of intravesical instillation of *E. coli* LPS (200 μg/ml). The data is represented as the median (black line), the 25–75 percentiles (box) and the 95% confidence interval (whiskers). The * and ** symbols indicate $P < 0.05$ and $P < 0.01$, respectively with a Mann–Whitney U test vs. WT. WT (n = 13), *Trpv4* KO (n = 9), WT perfused at a slow rate of 8 μl/min (n = 7), WT administered the TRPV4 inhibitor HC-067047 (HC, 2.35 mg/kg, n = 6), *Tlr4* KO (n = 9), and *Trpa1* KO (n = 7).

invading gram-negative bacteria (38). Importantly, they indicate that the pharmacological inhibition of TRPV4, which has been suggested for the treatment of several airway diseases (46–48), as well as of cyclophosphamide-induced cystitis (21), may lead to undesirably decreased defensive responses of the bladder against rather common bacterial infections.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the first and corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the KU Leuven Ethics Committee.

AUTHOR CONTRIBUTIONS

YA and KT conceived and designed the project, and wrote the manuscript. JF and YA isolated and cultured the human urothelial cells. YA conducted and analyzed the Ca^{2+} imaging experiments. RN and YA performed the confocal imaging and quantitative RT-PCR. PU, SP, and YA designed and conducted the cystometry recordings. AS contributed to the data analyses. DD, TG, WE, and TV contributed to the interpretation of data. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00799/full#supplementary-material

FIGURE S1 | TRPV4 is not required for the LPS-induced nuclear translocation of the p65 subunit of NF-κB in mouse urothelial cells. Single-color confocal images of NF-κB p65 (red) and DAPI nuclear staining (blue). The corresponding merged images are shown in **Figure 5A**. Scale bar, 10 μm.

FIGURE S2 | TRPV4 is not required for the LPS-induced phosphorylation of S534 p65 subunit of NF-κB in mouse urothelial cells. Representative confocal immunofluorescence microscopy images of fixed mUCs in control or 30 min after treatment with LPS (20 μg/ml). Cells were stained with phospho p65 NF-κB (red) and DAPI nuclear staining (blue). Scale bar, 10 μm. The histograms show the corresponding pixel intensity distribution (8-bit scale) of phospho p65 NF-κB staining in the cytoplasmic area (gray-shaded bars) or within the nuclear area (blue bars). Ratios represented in **Figure 5C** were calculated using the mean intensity values from the pixel intensity distribution in these two areas.

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MINI REVIEW
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The Role of TRPV4 in Regulating Innate Immune Cell Function in Lung Inflammation

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Ion channels/pumps are essential regulators of innate immune cell function. Macrophages have been increasingly recognized to have phenotypic plasticity and location-specific functions in the lung. Transient receptor potential vanilloid 4 (TRPV4) function in lung injury has been shown to be stimulus- and cell-type specific. In the current review, we discuss the importance of TRPV4 in macrophages and its role in phagocytosis and cytokine secretion in acute lung injury/acute respiratory distress syndrome (ARDS). Furthermore, TRPV4 controls a MAPK molecular switch from predominately c-Jun N-terminal kinase, JNK activation, to that of p38 activation, that mediates phagocytosis and cytokine secretion in a matrix stiffness-dependent manner. Expanding knowledge regarding the downstream mechanisms by which TRPV4 acts to tailor macrophage function in pulmonary inflammatory diseases will allow for formulation of novel therapeutics.

Keywords: TRPV4 (transient receptor potential vanilloid-4), macrophage, innate immunity, lung inflammation and injury, MAPK

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INTRODUCTION

Ion channels and transporters are rapidly being recognized as essential for basic physiological functions of immune cells (1, 2). However, gaps in knowledge remain on the intracellular molecular mechanisms by which ion channels contribute to immune cell function. Calcium and other cations (such as sodium and potassium) have been shown to act as second messengers to regulate innate immune cell function and activation (3). For example, macrophage migration, polarization, phagocytosis, and cytokine secretion have been shown to be regulated through calcium (4–6). One such mechanism of calcium regulation in the cell is through the calcium permeable cation channel transient receptor potential vanilloid 4 (TRPV4). TRPV4 is a mechanosensitive cation channel that is essential for macrophage activation functions such as macrophage phagocytosis and cytokine secretion in a matrix stiffness-dependent manner (7, 8). The current review focuses on experimental data illustrating the importance of TRPV4 on immune cell function. We appreciate all the important contributions to the literature in this field, however given space considerations we have focused on what is perceived to be directly relevant to this review.

MACROPHAGE HETEROGENEITY IN THE LUNG

The lung is constantly exposed to inhaled particles and pathogens from the environment (9). Hence, lung innate immunity needs to be tightly regulated and phenotypically plastic in order to simultaneously maintain homeostasis and clear foreign invaders (10). Recently published data

characterize macrophage phenotypic subsets based on their location in the lung [alveolar (AM) vs. interstitial (IM)] in naïve and injured (LPS/bleomycin treatment) conditions (11–13). The alveolar and interstitial subsets have been further divided based on site of origin into "resident" and "recruited" macrophage populations after injury or inflammation, by fate mapping, and lineage tracing models (11, 14–16). "Resident" macrophages populate the lung a few days after birth from fetal monocytes, and self-renew after injury (14, 15, 17). In contrast, recruited lung macrophages populate the lung only after injury and are derived from circulating monocytes that originated from the bone marrow (14, 15, 18). Despite this anatomic lineage and genomic classification, the *in vivo* biologic functions of both resident/recruited alveolar and interstitial macrophage populations are not fully understood. The microenvironment plays a key role in reprograming the monocyte/macrophage phenotypic response to lung injury (19, 20). Reprograming of the macrophage phenotype is not as simple as the classically-defined *in vitro* M1/M2 paradigm characterized by surface marker labeling (21–24). The molecular pathways *in vivo* by which the macrophage phenotype and function change in response to the microenvironment have yet to be fully described.

MACROPHAGE FUNCTION AND SIGNALING IN ACUTE LUNG INJURY

Other literature has extensively characterized the important macrophage mediated mechanisms of chronic lung injury. Herein, this review will focus on the role of macrophages in acute lung injury. Macrophages are the most abundant, and critical cells, that maintain homeostasis in the lung (9). Macrophages have also been shown to play an important role in orchestrating the acute lung injury and repair process (25). Acute lung injury, both from non-infectious and infectious inflammation is a complex process. Acute lung injury is a consequence of endothelial and/or alveolar epithelial injury, followed by recruitment, and accumulation of inflammatory cells in the injured/stiffened alveolus (26–28). Macrophages have surface receptors that recognize pathogen (PAMPs) and/or damage-associated molecular patterns (DAMPs) to recruit inflammatory cells (e.g., neutrophils, recruited alveolar/interstitial macrophages) and coordinate both activation and cessation of inflammation (29). Macrophages function to phagocytize invading organisms, apoptotic cells/neutrophils, or particles. In addition, macrophages secrete, and respond to pro- and anti-inflammatory cytokines and chemokines (e.g., IL-1β, TNF-α, IL-8, IL-6, IL-10) (30–34). Activation of macrophages in response to infection occurs in part through coordination of activation of key stress activated pathways including Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Interferon regulatory factor 3 (IRF3), Stimulator of interferon genes (STING), and Mitogen activated protein kinases (MAPKs) (35). The MAPK family (i.e., p38, ERK, and JNK) exhibits functional cross-talk and redundant functions in inflammation (35). p38 and JNK have been shown to be activated in lung injury in response to infection and to be important in macrophage activation functions such as phagocytosis (36). Control of persistent activation of MAPKs is mainly regulated by the phosphatases, dual-specificity serine threonine phosphatases/MAPK phosphatases (DUSPs/MKPs) (37, 38). It has been described that increased lung stiffness regulates MAPK/phosphatase cross-talk, while others have shown that alveolar vessel wall stiffness increases >10-fold (from 3 to 45 kPa) after intratracheal LPS-induced lung injury in mice (39), thus providing mechanistic insight into how macrophages can respond to cues from the injured lung. However, distinct mechanisms whereby increased lung tissue stiffness/injury controls the MAPK and phosphatase cross-talk is poorly understood.

THE TRPV4 CHANNEL AND MECHANOBIOLOGY OF THE LUNG

Transient receptor potential (TRP) channels are a family of 6 transmembrane domain proteins that are permeable to multiple cations including calcium (40). TRP channels are widely expressed in multiple tissues and cell-types with varied physiologic functions (40). Specifically, the TRP family member, TRPV4, is a ubiquitously-expressed, plasma membrane-based, calcium-permeable channel that is sensitized and activated by both chemical [5,6-Epoxyeicosatrienoic acid (EET), 4 alpha-phorbol 12,13-didecanoate (4-αPDD)] and physical stimuli (temperature, stretch, and hypotonicity) (40–43). TRPV4 can initiate intracellular, celltype and context-specific signals that depend on local increases in intracellular calcium which could act as a second messenger, and/or induce heterodimerization with other channels, activate kinases, and/or directly interact with cytoskeletal proteins via intracellular amino-(NH_2) and carboxy-(COOH) terminal tails (44–47).

It has been increasingly recognized that cellular responses depend on the biophysical properties of the surrounding lung tissue environment (48, 49). Thus, mechanical cues from lung tissue stretch/stiffness can alter cellular responses to soluble mediators (e.g., growth factors, cytokines, chemokines) resulting in cellular dysfunction and disease. The mechanosensitive channel, TRPV4 has been implicated in mouse models of lung injury/fibrosis, which include hydrochloric acid, pulmonary edema, ventilator-associated lung parenchymal overdistension, and from our group, pulmonary fibrosis (50–53). The recent mini-review by Michalick and Kuebler in Frontiers Immunology further supports the concept that TRPV4 may connect mechanosensation to immunity in the lung (54). Given TRPV4's published role on regulating activity and infectivity of RNA viruses such as Zika, it remains possible that TRPV4 plays a role in the profound lung injury observed in the current SARS-CoV-2 pandemic (55). Conflicting data exist on the role of TRPV4 in mouse models of lung inflammation/injury, which seem to depend on the inciting agent, mechanism of injury, and the effector cell type (50–53). In ventilator-induced lung injury, macrophage TRPV4 has shown to exacerbate the lung injury (51, 52). Similarly in acid-induced lung injury, TRPV4 also exacerbates the lung injury (53). Furthermore, a recent

study using a single pharmacologic inhibitor of TRPV4 revealed decreased lung injury after intratracheal instillation of LPS for 24 h (56). Our data, in a clinically relevant infectious model of lung injury, support the hypothesis that TRPV4 is protective from injury (7). In support of our findings, epithelial cell TRPV4 similarly protects the lung, but in a somewhat distinct, rapid direct LPS-induced lung injury model (3 h) (57). Despite the conflicting data on the role of TRPV4 in mouse models of lung injury, some consensus exists on the importance of TRPV4 in macrophage signaling (7, 8, 52). Further understanding of the molecular mechanisms by which macrophage TRPV4 is involved in the pathogenesis of lung injury will allow for a therapeutic target to ameliorate lung injury.

TRPV4 IN MACROPHAGES AND LUNG INJURY

The calcium ion channel, TRPV4 is an essential mechanosensor that is required for effective phagocytosis *in vitro* and protects against infection-associated lung injury *in vivo* (7, 8). TRPV4 in macrophages have been shown to exacerbate ventilator-associated lung injury, and macrophages are key effector cells in the lung injury process (51, 52). Calcium has long been described as a mediator of many discrete steps in the phagocytic process (58). In addition, effective phagocytosis requires cytoskeletal rearrangements and direct interaction with the biophysical properties of the matrix (19, 20, 59). However, the key regulatory ion channels/pumps by which calcium influx into the cell is controlled during phagocytosis is not fully elucidated.

Published work first revealed that differentiated murine bone marrow-derived macrophages (BMDMs) express equal amounts of TRPV4 that was functionally active with or without LPS. TRPV4 in macrophages functions to effectively phagocytize both non-opsonized, *E. coli* bacteria, and opsonized (FcR-dependent), IgG-coated latex beads, *in vitro* in response to LPS (8). The LPS-stimulated phagocytic response was induced, in our hands, under conditions of pathophysiologic-range extracellular matrix stiffnesses, in the range noted in inflamed or injured lung ($\geq$8–25 kPa) (16). TRPV4 had no effect on basal phagocytosis. In addition, TRPV4 downregulated LPS-induced IL-1β secretion and upregulated IL-10 secretion. Further, this TRPV4 mediated anti-inflammatory cytokine profile ($\downarrow$ IL-1β, $\uparrow$ IL-10) was dependent on pathophysiologic-range matrix stiffness. To apply *in vivo* relevance, TRPV4 was found to be required for effective alveolar macrophage phagocytosis of IgG-coated latex beads in live mice *in vivo* (8). Taken together, TRPV4 is necessary for effective opsonized and non-opsonized macrophage phagocytosis and an anti-inflammatory cytokine profile, in a stiffness-dependent manner *in vitro* and *in vivo* (8).

To expand on this work, TRPV4's *in vivo* relevance to human disease, and molecular mechanism by which TRPV4 mediates its phagocytic and cytokine secretory effects was determined. TRPV4 was found to function to protect the lung from injury in an experimental model of *Pseudomonas aeruginosa* pneumonia in intact mice (7). Lung injury was measured by (i) inflammatory cell infiltration, (ii) total protein in whole lung lavage, (iii) cytokine secretion, and (iv) lung parenchymal consolidation. In addition, TRPV4 was required for effective clearance of the *P. aeruginosa* bacteria as measured by colony forming units retained in the lung in WT, as compared to global TRPV4 KO mice. Next, macrophages were identified as the critical cell type required to clear the *P. aeruginosa* infection by flow cytometric techniques (7).

To determine the molecular mechanism by which TRPV4 protects the lung from injury and clears bacteria, putative intracellular signaling pathways were investigated that are known to regulate LPS signals in macrophages (60). TRPV4 controlled molecular switching from predominate JNK activation to that of p38, in a stiffness-dependent manner. Since MAPK phosphorylation occurs commonly through the phosphatase family DUSPs/MKPs, it was postulated that DUSPs controlled the TRPV4-mediated MAPK molecular switch. TRPV4 acted to increase DUSP1 and then functioned to selectively dephosphorylate/deactivate JNK. Hence, the TRPV4-mediated MAPK molecular switch was found to be controlled through DUSP1 in a stiffness-dependent manner. TRPV4 additionally enhanced p38 activation thereby driving effective phagocytosis, while inhibiting JNK thereby decreasing pro-inflammatory cytokine secretion (IL-6, CCL2, and CXCL1). Finally, TRPV4 is also required for macrophage phagocytosis and p38 activation in healthy human monocyte-derived macrophages. Taken together, published work shows that TRPV4 in macrophages protected the lung from infection-associated lung injury through regulation of MAPK activation switching via DUSP1 (7). TRPV4 provides a novel mechanistic link between the mechanoenvironmental properties of the lung and innate immune cell function (**Figure 1**).

IMPORTANT FUTURE DIRECTIONS

Ongoing questions remain regarding the molecular mechanism by which TRPV4 activity is regulated or how TRPV4 is directly activated. In addition, the molecular signals by which TRPV4 regulates the MAPK molecular switch remain unknown. Since TRPV4 can directly interact with signaling molecules via its amino (NH_2) and carboxy (COOH) terminal intracellular tails, the key signaling molecules that interact with TRPV4 to enhance macrophage phagocytosis and limit cytokine secretion is an active area of investigation. It remains possible that the TRPV4 interacting partners are not TLR4-dependent, as other data suggests that TRPV4 interacts with PI3K to mediate pulmonary fibrosis (61). The role of TRPV4 in different macrophage populations after infection remains an important question. Other cation/calcium channels (e.g., Piezo) have been shown to have an effect on immune cells in a mechanosensitive manner and interestingly recent work demonstrates TRPV4 is required for Piezo1-induced pancreatitis (62, 63). The molecular pathways *in vivo* by which the macrophage phenotype and function change in response to the microenvironment have yet to be fully described. For example, it will also be interesting in the future to explore

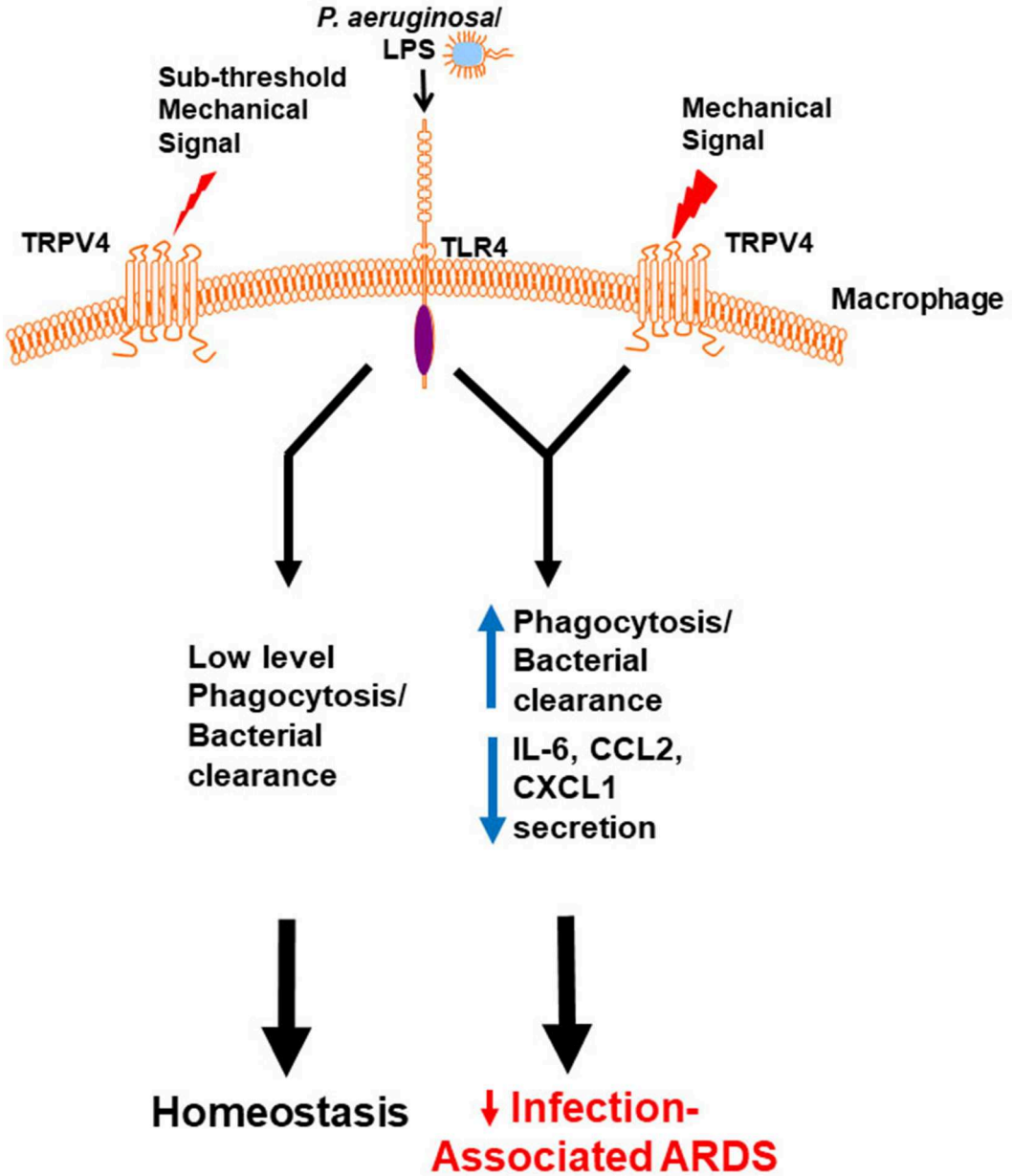

FIGURE 1 | Working model demonstrates that a mechanical signal through TRPV4 regulates the LPS response. Our data shows in the **absence** of an above threshold mechanical signal, TRPV4 does not influence the minimal LPS/TLR4 response, leading to low level phagocytosis/bacterial clearance, and resultant lung homeostasis. In the **presence** of an above threshold mechanical signal, TRPV4 modulates with the LPS/TLR4 response to increase phagocytosis and decrease pro-inflammatory cytokine secretion, thereby protecting the lung from infection-associated injury/ARDS (7). Adapted from original publication in *The Journal of Immunology*. Copyright© 2020 The American Association of Immunologists, Inc.

TRPV4's action in response to (a) other types of infectious stimuli (e.g., Gram positive organisms, viral infections such as SARS-CoV-2), (b) sex differences, and (c) transcriptional/epigenetic mechanisms. An important goal of future work is to integrate mouse and human macrophage experiments, however there is limitation to this approach. It is well-known that mouse models do not fully recapitulate human disease, and the mouse immune system is programmed differently than that of humans (64). In order to circumvent this limitation, investigators utilize human diseased tissue which may provide insight into disease mechanisms, however it is usually in an *in vitro* setting. Hence, data obtained from mouse models and human disease tissue have their own independent strengths. Therefore, it is important to interpret the findings in a contextual nature to determine the relevance to mechanisms of human disease and design targeted pharmacologic therapies.

CONCLUSION

In summary, macrophages function in the lung to maintain homeostasis and clear environmental particles and pathogens. Extensive macrophage heterogeneity and plasticity allows for fine-tuning of the inflammatory response upon inflammation or infection. Ion channels have been shown to play a key role in regulating innate immune function and contribute to the pathogenesis of inflammatory/infectious lung diseases. The cation channel TRPV4 has been implicated in lung diseases associated with parenchymal stretch and inflammation or infection. The data outlined in this review show the importance of macrophage TRPV4 in response to infection and lung injury. The data shows that TRPV4 is **(a)** functionally active in macrophages, **(b)** required for effective non-opsonized and opsonized phagocytosis *in vitro* and *in vivo*, and **(c)** required for secretion of an anti-inflammatory cytokine profile by macrophages. These phagocytic and cytokine effects in macrophages were both dependent on matrix stiffness in the range of injured or fibrotic lung (8). In addition, TRPV4 **(a)** protects the lung from injury after *P. aeruginosa* pneumonia, **(b)** mediates the lung injury effects through MAPK molecular switching, and **(c)** is required for effective macrophage phagocytosis in human macrophages. This MAPK switching effect in macrophages was also dependent on matrix stiffness in the range of injured or fibrotic lung (7). Collectively, TRPV4 is shown to play a novel role in protecting the lung from infection-associated lung injury by regulating the phagocytic and cytokine secretory response to infection, and therefore may be a potential therapeutic target in the pathogenesis of acute lung injury.

AUTHOR CONTRIBUTIONS

RS, BS, LG, and MO reviewed the literature and wrote the paper. All authors contributed to the article and approved the submitted version.

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Does Cyclic ADP-Ribose (cADPR) Activate the Non-selective Cation Channel TRPM2?

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TRPM2 is a non-selective, Ca^{2+}-permeable cation channel widely expressed in immune cells. It is firmly established that the channel can be activated by intracellular adenosine 5′-diphosphoribose (ADPR). Until recent cryo-EM structures have exhibited an additional nucleotide binding site in the N-terminus of the channel, this activation was thought to occur via binding to a C-terminal domain of the channel that is highly homologous to the ADPR pyrophosphatase NudT9. Over the years it has been controversially discussed whether the Ca^{2+} mobilizing second messenger cyclic ADP ribose (cADPR) might also directly activate Ca^{2+} entry via TRPM2. Here we will review the status of this discussion.

Keywords: TRPM2, ion channel, calcium, signal transduction, cADPR

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INTRODUCTION

TRPM2 is a non-selective, Ca^{2+}-permeable cation channel expressed in immune cells like monocytes (1, 2), macrophages (3–5), neutrophils (6–9), dendritic cells (10) and effector T cells (11). The channel plays a role in the inflammatory response by modulating differentiation (10), cell migration and chemotaxis (7, 10, 12), cytokine (11) and chemokine secretion (1) and is regulated in a complex manner integrating inputs from the physical environment of the cell like temperature (13) and pH (14) with intracellular second messengers like Ca^{2+} (15, 16) and adenine nucleotides. Since cloning of TRPM2 over 20 years ago (17), a number of adenine nucleotides have been proposed to affect TRPM2. While ADPR and 2′-deoxy-ADPR (18) are firmly established as TRPM2 agonists, the roles of NAADP and cADPR in activation of the channel remain controversial. In this review we want to summarize the literature regarding the role cADPR with an emphasis on recent (structural) data.

In 2001 Perraud et al. found that the cytosolic C-terminus of TRPM2 (at that time known as LTRPC2) contains a Nudix box motif (19). This sequence motif is known from a huge superfamily of proteins, many of them pyrophosphorylases that hydrolyse "nucleoside diphosphates linked to a residue X" (hence the name NudiX) [reviewed in Srouji et al. (20)]. Nudix pyrophosphorylases differ largely with regard to substrate specificity with some of them having only a single substrate while others hydrolyse a broad range of dinucleotides. By sequence analysis Perraud et al. discovered the gene for an enzyme, now known as NudT9, that exhibits 50% sequence homology to TRPM2. By testing a number of potential substrates, they identified adenosine 5′-diphosphoribose (ADPR) as its substrate (19). ADPR is a cellular nucleotide that can arise from hydrolysis of NAD by the NAD glycohydrolase CD38 (21, 22) or may be cleaved from poly-ADP-ribosylated or mono-ADP-ribosylated proteins (23). Perraud et al. also demonstrated that ADPR can activate TRPM2 in a Ca^{2+}-dependent manner supposedly by binding to its C-terminal NudT9 homology domain (19).

Kolisek et al. later found that cyclic ADP ribose (cADPR), another metabolite of NAD, is also able to activate TRPM2 (24). cADPR is a second messenger in a number of different cell types [reviewed in Lee (25)] including cells of the immune system like T cells (26) and neutrophils (27) that mobilizes Ca^{2+} from intracellular stores (26). The cellular target for cADPR remains still elusive. While photoaffinity labeling with a cADPR analog in sea urchins pointed to a receptor with a molecular weight of 100–140 kDa which has so far escaped identification (28), most data for higher animals indicate that Ca^{2+} release by cADPR involves ryanodine receptors type 2 (29) or type 3 (30). This might be by displacement of FKBP12.6 from the ryanodine receptors resulting in an increased open probability (31, 32) or by indirect mechanisms like increasing store loading by stimulating activity of the ER Ca^{2+} pump SERCA (33–35).

In addition to releasing Ca^{2+} from intracellular stores, it has also been shown that cADPR can trigger Ca^{2+} entry via the plasma membrane. In Jurkat T cells microinjection of cADPR activates Ca^{2+} entry over the plasma membrane (36) and in neutrophiles the cADPR antagonist 8-Br-cADPR inhibits Ca^{2+}-influx in response to the chemotactic peptide fMLP (27). So far it is unclear whether this Ca^{2+} influx works via activation of capacitative Ca^{2+} entry via the STIM/Orai system (37) or involves additional Ca^{2+} channels directly activated by cADPR. These findings made the observation that cADPR might activate TRPM2 especially interesting.

Activation of TRPM2 by cADPR requires exceedingly high concentrations (EC_{50} 700 μM) of cADPR and even at 3 mM cADPR in the patch pipette the current was only about 5% of the current evoked by ADPR in low micromolar concentrations (24). Cellular concentrations of cADPR determined in the past by us and others using either HPLC (26, 38) or an enzymatic cycling assay (39–41) are significantly lower. This makes it highly unlikely that cADPR alone can contribute to Ca^{2+} entry by activation of TRPM2. On the other hand did cADPR shift the concentration-response for ADPR by two orders of magnitude from an EC_{50} of 12 μM in the absence of cADPR to 90 nM in the presence of 10 μM cADPR, resulting in the hypothesis that cADPR and ADPR may activate TRPM2 synergistically (24).

POTENTIAL BINDING SITE OF cADPR AT TRPM2

A synergism between cADPR and ADPR raises the question of the binding site. 8-Br-cADPR, an antagonist of cADPR (42) inhibited activation of TRPM2 by cADPR but not by ADPR, whereas AMP, one of the products of the enzyme NudT9, inhibited activation by ADPR but not by cADPR, indicating that ADPR and cADPR do not act via the same binding site. Since AMP affects activation by ADPR it seems that ADPR binds to the NudT9H domain, whereas cADPR would bind to a distinct site for which it competes with 8-Br-cADPR. First indications of a secondary nucleotide binding site came from work on TRPM2 from the sea anemone *Nematostella vectensis* (nvTRPM2). nvTRPM2 also features a Nudix domain, but Kühn et al. showed that removal of this NudT9H domain does not interfere with gating of the channel by ADPR, but that the domain is catalytically active and breaks down ADPR (43). This led them to propose that the ADPR binding site for nvTRPM2 is separate from the NudT9H domain. Later they showed that the NudT9H domain of nvTRPM2 while not required for activation by ADPR can contribute to gating. While in nvTRPM2, activation by ADPR is not affected by removal of the NudT9H domain, activation by inosine 5′-diphosphoribose (IDPR) is abrogated. This shows that the second binding sites can modulate channel activity and exhibits a different agonist selectivity (44). During the last year a number of cryo-EM structures of TRPM2 from different species became available (45–48). One especially interesting finding from the studies by Huang et al. was the identification of additional nucleotide binding sites in TRPM2 from zebra fish (drTRPM2) (47) and humans (48). Located between the first two melastatin homology regions (MHR1/MHR2) in the cytosolic N-terminus of the channel they observed an ADPR molecule in a horseshoe-like conformation that resembles the conformation of cADPR in both zebra fish and human TRPM2 (**Figure 1**). In human TRPM2 they were able to resolve an additional ADPR molecule in the NudT9H domain which exhibited in contrast to the horse-shoe-like ADPR in the MHR1/MHR2 binding site an elongated confirmation (**Figure 1**). They also solved a structure of TRPM2 in an inhibited state with the cADPR antagonist 8-Br-cADPR in the presence of Ca^{2+}. The functional role of these distinct nucleotide binding sites remains controversial. While Huang et al. observed a loss of activity when mutating the MHR1/MHR2 binding site as well as when removing the NudT9H domain in both zebra fish (47) as well as human TRPM2 (48), Wang et al. did neither observe any ADPR related electron density in the MHR1/MHR2 domain nor did they see an effect of the mutation of this site (46).

TEMPERATURE DEPENDENCY OF cADPR MEDIATED TRPM2 ACTIVATION

Togashi et al. first noticed that TRPM2 can be activated by heat above a threshold temperature of 35°C with currents increasing up to 42°C (13). While ADPR activated TRPM2 already at 25°C, the currents were largely enhanced when the temperature was increased to 35°C and above. In contrast to Kolisek et al. Togashi et al. did not observe any cADPR evoked currents at 25°C but found that 100 μM cADPR in the pipette largely enhanced TRPM2 currents evoked by heat. This effect was absent in cells expressing a TRPM2 variant lacking the NudT9H domain (13).

Due to its labile N1-glycosidic bond cADPR is prone to hydrolysis to ADPR. At room temperature and under slightly acidic conditions its half-life is 10 days which decreases to 24 h at 37°C (49). Even frozen solutions of cADPR have been shown to slowly degrade to ADPR at -20°C (50). cADPR is also subject to enzymatic hydrolysis to ADPR by CD38 and CD157/Bst-1 which besides NAD glycohydrolase and ADP-ribosyl cyclase activity also exhibit cADPR hydrolase activity (51–53). Hydrolysis of the pyrophosphate in cADPR by an Mn^{2+}-dependent ADP-ribose/CDP-alcohol pyrophosphatase yields N^1-(5-phosphoribosyl)-AMP resulting in breakdown of cADPR

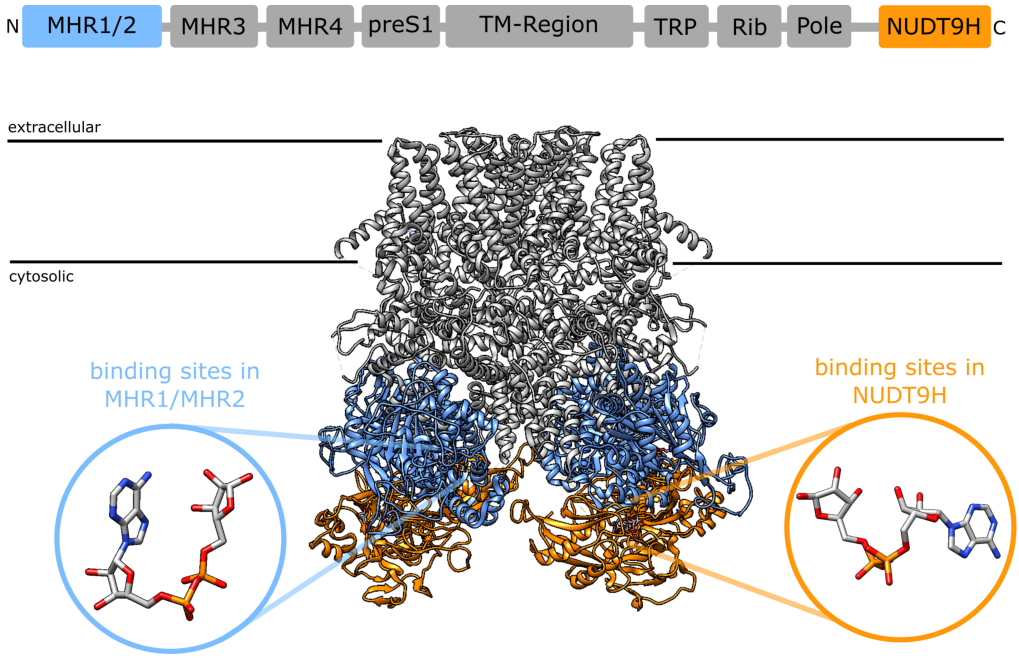

FIGURE 1 | Domain structure of TRPM2 [after (46)] and location of the two nucleotide binding sites in the cryoEM structure of human TRPM2 [pdb: 6PUS, (48)]: the recently identified N-terminal binding site between the MHR1 and MHR2 domains (blue) and the established C-terminal binding site in the NudT9H domain. The insets show the different conformation of ADPR in the binding sites as determined from the cryoEM structure, the ADPR in the N-terminal binding site assumes a horseshoe like conformation whereas the ADPR in in the NudT9H domain has a more elongated conformation. The depicted ADPR molecules have been reoriented relative to the TRPM2 structure to better illustrate the difference in conformation between the two binding sites. In a structure that has been solved in the presence of 8-Br-cADPR, 8-Br-cADPR occupies the N-terminal binding site between MHR1/MHR2 (48).

without production of ADPR (54). Increasing temperature accelerates chemical and enzymatic turnover but due to the rapid kinetics it appears unlikely that increased hydrolysis of cADPR to ADPR is responsible for the results observed by Togashi et al. In addition Yu et al. demonstrated that wildtype HEK293 cells do not express CD38 or CD157/Bst-1 nor do they show turnover of cADPR to ADPR over the time course of a typical patch-clamp experiment (55).

CONTAMINATION OF COMMERCIAL cADPR PREPARATIONS

A complicating factor in interpreting the results from Kolisek et al. and Togashi et al. is, that commercial preparations of cADPR from one of the major suppliers are often partially degraded and contain significant amounts of ADPR. Heiner et al. noticed high currents when infusing cADPR into human neutrophils which prompted them to check their solutions for ADPR contaminations by HPLC (56). They found that even freshly prepared solutions from several batches of commercial cADPR, contained roughly 25% ADPR. When they incubated the contaminated cADPR with nucleotide pyrophosphatase thereby fully converting ADPR to AMP and ribose 5-phosphate, the ADPR-free cADPR did no longer evoke TRPM2 currents in the granulocytes (56). When using a commercial preparation of cADPR Tóth et al. also observed activation of TRPM2 by "cADPR" in inside-out patches from *Xenopus oocytes*, but analysis of the composition of the "cADPR" preparation by thin layer chromatography showed that in addition to cADPR it contained roughly 20% ADPR (57). Selective hydrolysis of ADPR by nucleotide pyrophosphatase, without degradation of cADPR (58), resulted in a complete loss of TRPM2 activation. Both groups noticed that the loss in channel activation was not due to inhibition by AMP as addition of the same amount of AMP to ADPR did not affect activation of the channel by ADPR (56, 57). Interestingly, in contrast to previous reports that showed inhibition by AMP with an IC_{50} of 70 μM (24) and later of 10 μM (8) Tóth et al. didn't observe any inhibition of human TRPM2 expressed in Xenopus oocytes by AMP up to 200 μM (57).

Like for cADPR commercial preparations also 8-Br-cADPR often contains significant amounts of 8-Br-ADPR (>20%). The observation that 8-Br-ADPR is a low affinity competitive antagonist for ADPR on TRPM2 (7) [IC_{50} ~300 μM (18)] further complicates interpretation of reports of selective inhibition of cADPR-mediated activation of TRPM2 (24). One conceivable explanation for these results might be that the administration of an excess of 8-Br-ADPR (from the 8-Br-cADPR) to a small amount of ADPR (as a contaminant in cADPR) is effectively preventing channel activation, while it has no effect on activation of TRPM2 by 100 μM ADPR. This could also explain how 8-Br-cADPR exerts its effects on H_2O_2-mediated activation of TRPM2 (24). Interestingly it has been

shown recently that a variant of nvTRPM2 lacking the NudT9H domain can be activated by 8-Br-ADPR acting as a low affinity partial agonist (59), indicating that 8-Br-ADPR may bind to the N-terminal nucleotide binding domain of TRPM2. This and the amount of 8-Br-ADPR in commercial preparations of 8-Br-cADPR raises the question whether the resolution of the current cryo-EM structures is sufficient to exclude the possibility that the nucleotide observed in the N-terminal binding site in the pdb structure 6PUU (48) is not 8-Br-cADPR but 8-Br-ADPR in a horseshoe-like conformation.

Interestingly a relatively recent paper by Yu et al. again seems to demonstrate activation of human TRPM2 overexpressed in HEK cells by cADPR (55). The concentration-response curve for cADPR was shifted to the right with an EC_{50} of 250 μM compared to 40 μM for ADPR. In stark contrast to what has been observed by Kolisek et al. (24) the maximal currents for ADPR and cADPR were similar (55). They tried to account for the problems with ADPR contaminations described above by using cADPR they either synthesized themselves or purified from commercial cADPR and demonstrated purity by mass spectrometry. Although the previous data by Kolisek et al. indicated that cADPR binds to a different site than ADPR they assumed binding to the NUDT9H domain which they confirmed by showing the binding to the isolated NUDT9H domain using surface plasmon resonance. Using molecular dynamics simulation they identified a number of residues involved in binding to cADPR and ADPR. Mutations of some of these residues exhibited differential effects on channel activation by either ligand (55). It is really interesting to see, that even more than 10 years after Heiner et al. (56) and Tóth et al. (57) convincingly demonstrated that removal of contaminating ADPR prevents activation of TRPM2 by commercial cADPR, the idea that cADPR could affect TRPM2 still lingers on.

To avoid misleading results in the future, we consider it of utmost importance to always keep in mind both, the possibility that commercial preparations of cADPR can contain significant amounts of ADPR (even despite the advertised purity) and the limited stability of cADPR in solution, even when frozen. When working with cADPR we would therefore highly recommend to (i) purify commercial preparations before use, and (ii) to test for degradation of cADPR in solution routinely by using a suitable HPLC system.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Role of the Intracellular Sodium Homeostasis in Chemotaxis of Activated Murine Neutrophils

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The importance of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophil function has been intensely studied. However, the role of the intracellular Na^+ concentration ($[Na^+]_i$) which is closely linked to the intracellular Ca^{2+} regulation has been largely overlooked. The $[Na^+]_i$ is regulated by Na^+ transport proteins such as the Na^+/Ca^{2+}-exchanger (NCX1), Na^+/K^+-ATPase, and Na^+-permeable, transient receptor potential melastatin 2 (TRPM2) channel. Stimulating with either N-formylmethionine-leucyl-phenylalanine (fMLF) or complement protein C5a causes distinct changes of the $[Na^+]_i$. fMLF induces a sustained increase of $[Na^+]_i$, surprisingly, reaching higher values in $TRPM2^{-/-}$ neutrophils. This outcome is unexpected and remains unexplained. In both genotypes, C5a elicits only a transient rise of the $[Na^+]_i$. The difference in $[Na^+]_i$ measured at $t = 10$ min after stimulation is inversely related to neutrophil chemotaxis. Neutrophil chemotaxis is more efficient in C5a than in an fMLF gradient. Moreover, lowering the extracellular Na^+ concentration from 140 to 72 mM improves chemotaxis of WT but not of $TRPM2^{-/-}$ neutrophils. Increasing the $[Na^+]_i$ by inhibiting the Na^+/K^+-ATPase results in disrupted chemotaxis. This is most likely due to the impact of the altered Na^+ homeostasis and presumably NCX1 function whose expression was shown by means of qPCR and which critically relies on proper extra- to intracellular Na^+ concentration gradients. Increasing the $[Na^+]_i$ by a few mmol/l may suffice to switch its transport mode from forward (Ca^{2+}-efflux) to reverse (Ca^{2+}-influx) mode. The role of NCX1 in neutrophil chemotaxis is corroborated by its blocker, which also causes a complete inhibition of chemotaxis.

Keywords: neutrophil, chemotaxis, intracellular sodium, NCX1, TRP channels

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INTRODUCTION

Neutrophil migration, adhesion, neutrophil extracellular trap formation, bacterial killing, and production of reactive oxygen species (ROS) require coordinated ion fluxes (1–6). Ca^{2+} is among the best studied ions in this context (7, 8). It is a potent, multifunctional second messenger and its increase in the cytoplasm is a hallmark of neutrophil activation. An increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is caused by release of Ca^{2+} from intracellular stores and by receptor- or store-operated Ca^{2+} entry (ROCE or SOCE, respectively). This relies to

a large extent on transient receptor potential (TRP) and Orai channels present in the plasma membrane of neutrophils (9–13). Ca^{2+} influx was shown to be a necessary factor in neutrophil chemotaxis almost half century ago (14), but only recently the changes in $[Ca^{2+}]_i$ and its intracellular gradient was visualized and confirmed as a decisive factor in neutrophil chemotaxis (15). A gradient of the $[Ca^{2+}]_i$ was also shown to be modulated by TRPC1 channel in murine neutrophils (4). TRPC1 knock-out and its effect on Ca^{2+} homeostasis hinders neutrophil response to N-formylmethionine-leucyl-phenylalanine (fMLF).

However, an increase of the $[Ca^{2+}]_i$ is not the only ionic event upon cell stimulation. Neutrophil activation with fMLF leads also to an increase of the intracellular Na^+ concentration ($[Na^+]_i$) (16). Importantly, the extracellular Na^+ concentration ($[Na^+]_o$) also influences the $[Na^+]_i$. In normal tissue $[Na^+]_o$ equals its concentration in blood (∼145 mM), but it can be lower in conditions like hypoxia (17, 18). In inflammation and tumor the $[Na^+]_o$ is usually increased (19, 20).

There are several Na^+ transport proteins, which can account for the cell responses to changes of the extracellular ion composition or stimulation with chemoattractant. One of them is the non-selective transient receptor potential melastatin 2 (TRPM2) cation channel, which is highly expressed in neutrophils (21). Due to its presumed role as a ROS sensor, TRPM2 emerged as a putative mediator of inflammatory responses in monocytes and neutrophils (22). However, the role of TRPM2 in immune cells is rather complex (23, 24). Whether the channel has a pro- or anti-inflammatory function is still disputed, especially regarding neutrophil migratory abilities (8, 22, 23, 25). Moreover, the pH sensitivity of TRPM2 also raises the question whether the channel is still active in the acidic microenvironment of an inflammation (26–28).

Another Na^+ transport protein, Na^+/Ca^{2+}-exchanger 1 (NCX1) stands for one of the major Ca^{2+} extrusion mechanisms in many cell types (29). It removes one Ca^{2+} in exchange for 3 Na^+ ions transported into the cell (*forward* mode). Depending on the ion concentrations and membrane potential, it can also operate in *reverse* (Ca^{2+} influx) mode. Subtle changes of the ion composition and/or cell membrane potential are sufficient to alternate the NCX1 modes. In neutrophils, NCX1 was shown to contribute to Ca^{2+} influx (30) and membrane repolarization (31).

Finally, the activity of the NADPH-oxidase 2 (NOX2) leads to release of protons into the cytoplasm. In activated neutrophils, phosphorylation of NOX2 subunits leads to enzyme activation and assembly in the plasma membrane or in membranes of secondary granules (32, 33). Active NOX2 depolarizes the membrane through electron extrusion (34–36). The subsequent H^+ removal is mediated not only by H^+ channels and pumps but also by using the driving force of the Na^+ gradient which fuels the Na^+/H^+- exchanger (NHE1) (37). Depolarization of the membrane also affects the electrogenic activity of the NCX1, which in effect moves one positive charge in or out of the cell affecting also Ca^{2+} flux (29). This further supports the importance of Na^+ homeostasis in neutrophil function.

Using two *end-target* chemoattractants, fMLF (formylated peptide) and C5a (complement molecule) we analyzed neutrophil chemotaxis and changes of the $[Na^+]_i$ in neutrophils. For the first time, we show that neutrophil chemotaxis is modulated by the extra- and intracellular Na^+ concentration. We suggest that proteins expressed in neutrophils and involved in Na^+ homeostasis, especially NCX1, may contribute to neutrophil response upon chemoattractant stimulation. Also, knock-out of the Na^+-permeable TRPM2 channel results in altered neutrophil $[Na^+]_i$, likely by indirectly modulating NCX1. The involvement of TRPM2 channel in neutrophil function in physiological conditions is rather minor, but may be decisive in an inflammatory environment.

MATERIALS AND METHODS

Reagents

Reagents were purchased from SigmaAldrich®, Steinheim, Germany if not indicated otherwise.

Animals

$TRPM2^{-/-}$ mice were generated in a C57BL/6J background as described previously (22). $TRPM2^{+/+}$ and $TRPM2^{-/-}$ mice were derived from heterozygous mating. PCR using genomic DNA as template was performed to confirm the genotypes. C57BL/6J, $TRPM2^{+/+}$ and littermate $TRPM2^{-/-}$ mice (8–12 weeks of age) were used in the study. Mice were euthanized by cervical dislocation. Experimental protocols were approved by the local committee for animal care with permit number: 84-02.05.50.15.010.

Cell Culture

Myelomonocytic leukemia cells (WEHI-3B) were cultured in bicarbonate-buffered Dulbecco's Modified Eagle Medium (DMEM, Merck Chemicals GmbH, Darmstadt, Germany) medium supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS) (Gibco/ThermoFisher Scientific, Darmstadt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. Four days after cells reached confluence, the supernatant was collected, filtered and stored at −20°C. Since bone-marrow derived cells comprise also undifferentiated progenitors, medium from cultivated WEHI-3B cells served as a source rich in G-CSF and Il-3 and was used for neutrophil differentiation (38).

Isolation of Murine Neutrophils

Mouse bone marrow was prepared from femurs and tibiae. Hind limbs were sterilized with 70% ethanol and dissected bones were flushed via a 0.4 mm needle with Ca^{2+}/Mg^{2+}-free HBSS ($HBSS^{-/-}$, Merck Chemicals GmbH, Darmstadt, Germany) containing 25 mM HEPES and 10% FBS. Bone marrow cell suspension was dispersed and filtered with a 70 µm cell strainer and centrifuged at 4°C, 200 g for 10 min. Pelleted cells were resuspended in 1 ml $HBSS^{-/-}$ with 25 mM HEPES and put on Histopaque®1077/1119 layers. Cells were then centrifuged at 400 *g*, for 30 min. The layer of granulocytes was taken and washed twice in $HBSS^{-/-}$ with 25 mM HEPES and 10% FBS (centrifuged at 4°C, 200 g, 10 min). We use $HBSS^{-/-}$ for isolating and processing the neutrophils to avoid neutrophil attaching

to the labware. Cells were resuspended in bicarbonate-buffered RPMI-1640 medium with L-glutamine, 10% FBS, 10% WEHI-3B-conditioned medium, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell suspension was put in 10 cm Petri dishes and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2.

Measurements of the $[Na^+]_i$

Ibidi µ-Slides I (ibidi GmbH, Gräfelfing, Germany) were used for sodium measurements. Slides were coated with 1 µg/cm^2 fibronectin for 1 h at RT and washed twice with HEPES-buffered Ringer's solution (140 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, 5.5 mM glucose, pH 7.4) before the experiments. When experiments were done with a Ringer's solution with 72 or 5 mM Na^+, we isosmotically replaced NaCl by 68 and 135 mM NMDG-Cl, respectively. Neutrophils were resuspended in 2 ml Ringer's solution and about 1 × 10^6 cells were used for a single experiment. Cells were incubated with 5 µM Asante NaTRIUM Green-2 AM dye (ANG-2 AM) (TEFLabs Inc., Austin, USA) in Ringer's solution for 1 h at RT, put onto µ-Slides and allowed to adhere at 37°C for 10 min. The slide was washed twice with 1 ml pre-warmed Ringer's solution and mounted on the Axiovert 200 microscope (Carl Zeiss AG, Oberkochen, Germany) connected to the perfusion system, monochromator (Visitron System, Puchheim, Germany) and CCD camera (pco.edge 5.5) (PCO AG, Kelheim, Germany). A 100×oil objective (Plan Apo) was used for the observation. The filter set consisted of a T525lpxr beam splitter and an ET560/50m emission filter (Chroma Technology GmbH, Olching, Germany). Image acquisition was controlled by VisiView software (Visitron Systems GmbH, Puchheim, Germany). Excitation wavelength was set to 520 nm with 200 ms exposure time, binning 2. Images were acquired in 20 s intervals.

Experiments started after a short superfusion period with Ringer's solution until cells showed a stable fluorescent signal before the experimental solutions were applied. Where indicated, neutrophils were stimulated with 1 µM fMLF or 60 nM C5a in the superfusion solution or treated with 100 µM ouabain to inhibit the Na^+/K^+-ATPase and/or 10 µM KB-R7943 to inhibit the Na^+/Ca^{2+} exchanger. Each assay lasted 10 min and was followed by a calibration. To this end, cells were sequentially superfused with solutions of different Na^+ concentrations. Standard solutions were prepared by mixing equimolar NMDG-Cl (Na^+-free) and Na^+-containing solutions. 0, 10, and 20 mM Na^+ solutions were used and ionic concentrations were verified with ABL800 blood analyzer (Radiometer Medical ApS, Brønshøj, Denmark). All calibration solutions contained 50 µM amphotericin B to permeabilize the membranes and 100 µM ouabain to inhibit the Na^+/K^+-ATPase. Experiments were conducted at 37°C. Acquired time-lapse stacks were analyzed using ImageJ software. After background subtraction, the total projected area and fluorescence of the cells in the field of view were analyzed (≥5 cells for each N; $N = 3$). The product of area and intensity was then compared with linear standard curves derived from the calibration values for each cell.

Measurements of the $[Ca^{2+}]_i$

Ibidi µ-Slides I (ibidi GmbH, Gräfelfing, Germany) were prepared as described above [see section Measurements of the $[Na^+]_i$]. About 1× 10^6 neutrophils were used for each experiment. Cells were incubated with 3 µM Fura-2 AM (Biomol GmbH, Hamburg, Germany) for 25 min at 4°C in the dark. After incubation, neutrophils were seeded onto Ibidi µ-Slides I and incubated for 10 min in a warming cabinet and then washed with pre-warmed Ringer's solution. Measurements were performed with the same superfusion system as described above [see section Measurements of the $[Na^+]_i$]. 40× oil objective, beam splitter 400dclp and D510/40m emission filter (Chroma Technology GmbH, Olching, Germany) were used. Experiments were conducted at 37°C. Two excitation wavelengths were applied: 340 and 380 nm with 100 ms exposure time each, binning 2. Emission was measured at 510 nm. Acquisition was set for every other 20 s. Neutrophils were firstly superfused with Ringer's solution followed by Na^+-free solution (140 mM NMDG-Cl). In next experimental phase, cells were either superfused with 1 µM fMLF only, or 1 µM fMLF with 10 µM KB-R7943. Each experiment was calibrated by applying Ringer's solution containing 1 µM ionomycin with 5 mM Ca^{2+} or Ca^{2+}-free solution with 5 mM EGTA. Fluorescence intensity for each excitation wavelength for cells in the field of view was analyzed using VisiView software (Visitron Systems GmbH, Puchheim, Germany). Intensity values were background corrected and absolute values were calculated as described before (39).

Chemotaxis Assays in a 3D Collagen Matrix

For chemotaxis assays, ibidi µ-Slides Chemotaxis 2D/3D (ibidi GmbH, Gräfelfing, Germany) were used as described previously (40). Slides were coated with 1 µg/cm^2 fibronectin (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h. Following overnight incubation cells were resuspended in HEPES-buffered Ringer's solution (140 mM NaCl or 72 mM Na^+ and 68 mM NMDG-Cl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, 5.5 mM glucose; pH 7.4) and seeded in a three-dimensional collagen I (2.1 mg/ml) (Corning®, Kaiserslautern, Germany) matrix in $HBSS^{+/+}$. The chemotactic gradients were built by adding the attractants to one of the chambers. Two *end-target* chemoattractants were used in our chemotaxis assays: fMLF (1 µM) and (recombinant murine) C5a (60 nM) (Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany). Optimal chemoattractant concentrations were chosen based on neutrophil chemotaxis indices (CI) obtained in experiments with increasing concentrations of fMLF (10, 100 nM, 1, 33 µM) and C5a (6, 60, 370 nM). Live-cell imaging was performed using phase-contrast microscopy and video cameras (models XC-ST70CE and XC-77CE) at 37°C. Using HiPic Image acquisition software (Hamamatsu Photonics, Herrsching am Ammersee, Germany) time-lapse settings were set for every 5 s for 30 min. Acquired stacks were analyzed with Amira software (Thermo Fisher Scientific, Darmstadt, Germany). Amira files were evaluated using ImageJ (National Institutes of Health, Maryland, USA) and a self-made plugin (courtesy of Peter Dieterich, Dresden). Cell velocity was calculated by applying a three-point difference quotient and CI was calculated as the migration in the

direction of the chemical gradient divided by the total path length (4, 8, 40, 41).

Reverse Transcription and Quantitative PCR (RT-qPCR)

Total RNA was isolated using TRIzol reagent and chloroform phase separation (ThermoFisher Scientific, Darmstadt, Germany). The RNA concentration was measured with a spectrophotometer and 2 µg of total RNA were reverse transcribed. SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific, Darmstadt, Germany) was used for the reaction. cDNA samples were diluted in water to the final volume of 50 µl. For qPCR analysis, 2 µl of cDNA template was used in 10 µl of final reaction solution. PowerUp™ SYBR™ Green Master Mix (Applied Biosystems®/ThermoFisher Scientific, Darmstadt, Germany) was used for qPCR. We applied the following reaction protocol: an initial hold step (95°C, 10 min) and 40 cycles of a three-step amplification (95°C, 30 s; 55°C, 25 s; 72°C, 60 s). An additional melting curve stage was implemented for product control. Murine 18S rRNA transcript (m18S rRNA) served as endogenous reference. We used the following forward and reverse primers: 5′-GTA ACC CGT TGA ACC CCA TT-3′ and 5′- CCA TCC AAT CGG TAG TAG CG-3′ (42). NCX1 primers were designed to span the region of alternative splicing specific for BD exons specific for NCX1.3 and NCX1.7 (43) (also: GenBank: AF108396.1). Forward and reverse primer sequences were: 5′- TCT CCC TTG TGC TTG AGG AAC-3′ and 5′- AGC CAC CTT TCA ATC CTC TTC T-3′. NCX1 mRNA expression was calculated from mean Ct values of technical duplicates and subtraction of mean Ct for 18S rRNA transcript (ΔCt). Values transformed to $2^{-\Delta Ct}$ were used for statistical analysis. Relative normalized transcript expression in TRPM2$^{-/-}$ neutrophils was calculated in relation to TRPM2$^{+/+}$ ($2^{-\Delta\Delta Ct}$) (44).

Statistical Analysis

All data is shown as mean ± SEM. For comparison between two groups, statistical analysis was performed using a two-tailed Student's *t*-test or Mann-Whitney *U*-test when data was not normally distributed. In experiments comparing more than 2 groups 1-way ANOVA or repeated measures ANOVA was used, with Tukey or Dunn *post-hoc* tests. For all tests, $p < 0.05$ were considered statistically significant. "*N*" stands for number of animals and "*n*" designates number of single cells analyzed.

RESULTS

fMLF Stimulation Causes a Sustained Increase of the $[Na^+]_i$ in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ Neutrophils

Like most chemoattractant receptors, fMLF receptor (FPR1) activation leads to Ca^{2+} store release and an increase of the $[Ca^{2+}]_i$ (45). Further Ca^{2+} influx is mediated by opening of plasma membrane channels, among others non-selective TRP channels, allowing for concurrent Na^+ influx. TRPM2 not only allows Ca^{2+} and Na^+ influx, but its gating mechanism

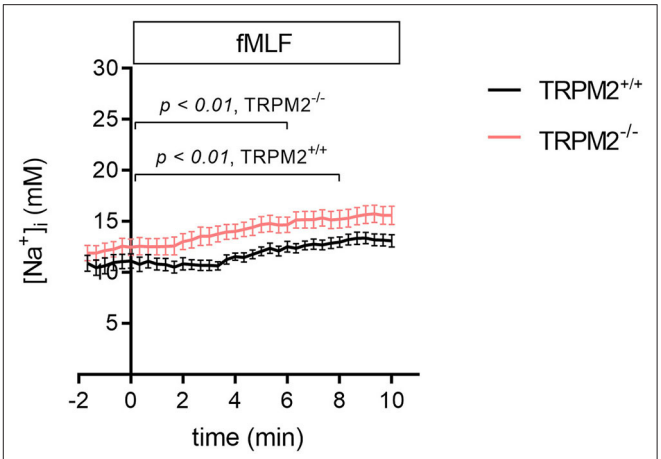

FIGURE 1 | $[Na^+]_i$ upon fMLF stimulation in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils. fMLF stimulation leads to an increase of the $[Na^+]_i$ in neutrophils. The increase of the $[Na^+]_i$ is attenuated in the presence of TRPM2 channels. Repeated measures ANOVA was used to analyze whether time points of significant increase of the $[Na^+]_i$ differ between genotypes. It is evident that the earlier concentration rise leads to higher $[Na^+]_i$ after 10 min in TRPM2$^{-/-}$ neutrophils. One micromolar fMLF was added at $t = 0$. For TRPM2$^{+/+}$: $N = 3$, $n = 23$; for TRPM2$^{-/-}$: $N = 3$, $n = 24$.

requires Ca^{2+} ions and an increase of cytoplasmic ADP-ribose (46, 47). It seems that an increased $[Ca^{2+}]_i$ would serve as a constant positive feedback. However, at the same time, the resulting membrane depolarization dampens cation influx. The high $[Ca^{2+}]_i$ may also promote NCX1 forward mode in order to resolve the neutrophil activation. The activity and role of NCX1 in neutrophils, however, has not been determined unequivocally (30, 31). TRPM2 and NCX1 are highly dependent on the intra- and extracellular ionic composition. It is known that the rise of the $[Ca^{2+}]_i$ upon fMLF stimulation is somewhat diminished in TRPM2$^{-/-}$ neutrophils (22, 48) which in turn may influence the NCX1 activity.

Previous experiments studying the $[Na^+]_i$ upon fMLF stimulation in human neutrophils showed an increase by about 8.5 ± 3.2 mM Na^+ within 5 min (16). Using the novel Na^+ indicator, ANG-2 AM, we compared the time course of changes of the $[Na^+]_i$ in murine TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils over a 10 min time span. Knowing that TRPM2 is permeable for both Ca^{2+} and Na^+ ions, we assumed that in the absence of the channel the increase of $[Na^+]_i$ will be hampered. However, this is not the case. A steady increase of the $[Na^+]_i$ is observed in both genotypes with the $[Na^+]_i$ reaching higher values in TRPM2$^{-/-}$ neutrophils within 10 min (15.6 ± 0.9 vs. 13.1 ± 0.6) (**Figure 1**). This difference is mainly due to the earlier rise of the $[Na^+]_i$ as determined by repeated measures ANOVA. There is a significant increase of the $[Na^+]_i$ in TRPM2$^{-/-}$ neutrophils after 6 min and only after 8 min in the presence of channel. This suggests that the channel prevents the rapid increase of the $[Na^+]_i$ during the first minute of fMLF stimulation. In the absence of the TRPM2 due to possible diminished membrane depolarization, Na^+ influx can be facilitated and mediated through other transport proteins.

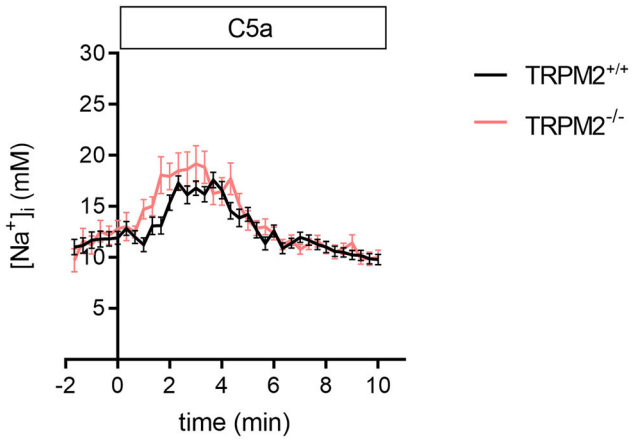

FIGURE 2 | $[Na^+]_i$ upon C5a stimulation in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils. C5a stimulation causes a rapid and pronounced increase of the $[Na^+]_i$, but in contrast to fMLF, the $[Na^+]_i$ returns to a basal levels within 5 min. The time course of the $[Na^+]_i$ changes is similar in neutrophils of both genotypes. Sixty nanomolar C5a was added at $t = 0$. For TRPM2$^{+/+}$ $N = 3$, $n = 33$; for TRPM2$^{-/-}$ $N = 3$, $n = 30$.

C5a Stimulation Causes a Rapid, but Transient Increase of the $[Na^+]_i$ in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ Neutrophils

C5a is an end-target chemoattractant and its mechanism of action after binding to C5aR resembles to a large extent the molecular pathway of FPR1 activation (45). C5aR1 also activates a phospholipase C β signaling pathway causing an increase of the $[Ca^{2+}]_i$ (45, 49). To our knowledge, there is no data showing the $[Na^+]_i$ in neutrophils upon C5a stimulation.

Although C5a and fMLF employ similar signaling pathways, the temporal changes of the $[Na^+]_i$ upon C5a stimulation differ from those induced by fMLF (**Figure 2**). The most striking difference is that the $[Na^+]_i$ increases only transiently upon C5a stimulation. C5a causes a rapid increase of the $[Na^+]_i$ (+5.6 mM Na^+ after 3 min) but in contrast to fMLF, also a fast recovery to the basal level (~11 mM Na^+). The curves are similar for TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils. There is no significant difference between the genotypes in curve slopes (only slightly faster increase in TRPM2$^{-/-}$ neutrophils) and $[Na^+]_i$ at the end of the observation. The $[Na^+]_i$ measurements for fMLF and C5a indicate the different Na^+ regulation upon stimulation with these chemoattractants. We therefore tested whether the differences of the $[Na^+]_i$ may directly or indirectly affect neutrophil function or reflect the fact that FPR1 and C5aR1 cause distinct ionic responses.

Impact of Na^+ Transport Proteins on the $[Na^+]_i$ of Neutrophils

The Na^+/K^+-ATPase and NCX1 are two neutrophil membrane proteins involved in Na^+ homeostasis. The Na^+/K^+-ATPase sustains the Na^+ gradient by extruding intracellular Na^+ in exchange for extracellular K^+ with a stoichiometry of 3:2. NCX1 is also a ubiquitous protein and has varied functions depending on cell type. Its role in activated neutrophils is not yet well-established.

Both transport proteins differ in their affinity and capacity. Constitutively active, high-affinity and low capacity Na^+/K^+-ATPase is less susceptible to environmental disturbances, but low-affinity high-capacity NCX1 may be critical for the cell under stress conditions. Ouabain is a widely used inhibitor of the Na^+/K^+-ATPase with anti-inflammatory properties. In neutrophils, ouabain diminishes CD18 expression, but the exact mechanism is not yet described (50).

KB-R7943 is a pharmacological inhibitor of the cardiac NCX1.1 splice variant in *forward* mode. However, it inhibits both *forward* and *reverse* modes of NCX1.3 and NCX1.7 splice variants, which are expressed in many non-excitable cells (IC_{50} = 2.9 and 2.4 μM, respectively) (51).

To further elucidate mechanisms underlying the $[Na^+]_i$ homeostasis in TRPM2$^{-/-}$ neutrophils, we inhibited Na^+/K^+-ATPase with 100 μM ouabain and measured the $[Na^+]_i$. As expected, the $[Na^+]_i$ of fMLF-stimulated neutrophils increases to much higher values in the presence of ouabain than in the presence of fMLF alone (**Figure 3A** vs. **Figure 1**). However, there is almost no difference between TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils (20.9 ± 1 mM vs. 21.7 ± 1.5 mM). This increase confirms the activity of the Na^+/K^+-ATPase pump, which is responsible for extruding Na^+ from the cytosol of neutrophils.

Using KB-R7943 alone abolishes the fMLF-induced rise of the $[Na^+]_i$. In TRPM2$^{+/+}$ neutrophils, the NCX1 inhibitor lowers the final sodium concentration (**Figure 3B**). In the absence of TRPM2 channels, fMLF stimulation and NCX1 inhibition lead to a higher final $[Na^+]_i$. The time course of the $[Na^+]_i$ changes is consistent with NCX1 operating in the forward mode in TRPM2$^{+/+}$ neutrophils during the entire observation period. Initially, TRPM2$^{-/-}$ neutrophils behave identically. However, there is no further decrease of the $[Na^+]_i$ in TRPM2$^{+/+}$ neutrophils. This temporal distinction in neutrophil $[Na^+]_i$ may suggest altered NCX1 function in neutrophils lacking TRPM2 channel.

When neutrophils are superfused with a combination of 1 μM fMLF, 100 μM ouabain and the NCX1 inhibitor KB-R7943 (10 μM), the $[Na^+]_i$ in TRPM2$^{+/+}$ neutrophils is lower than with fMLF and ouabain only (from 20.9 ± 1 mM down to 16.5 ± 1.2 mM). In contrast, in TRPM2$^{-/-}$ neutrophils the $[Na^+]_i$ rises even higher (from 21.7 ± 1.5 mM up to 25.4 ± 1.7 mM) (**Figure 3C**). This further supports different activity of NCX1 in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils, however, may also indicate the compensatory action of other Na^+ transport protein.

Decrease of Extracellular $[Na^+]$ Causes a Decrease of the Neutrophil Intracellular $[Na^+]$

To analyze the impact of decreasing the $[Na^+]_o$ we measured the $[Na^+]_i$ of neutrophils that were stimulated with fMLF in Ringer's solution with reduced Na^+ concentration. Lowering the $[Na^+]_o$ to 72 or 5 mM causes a fall of the $[Na^+]_i$ compared to the control situation (**Figure 4**). One of the possible explanations of these

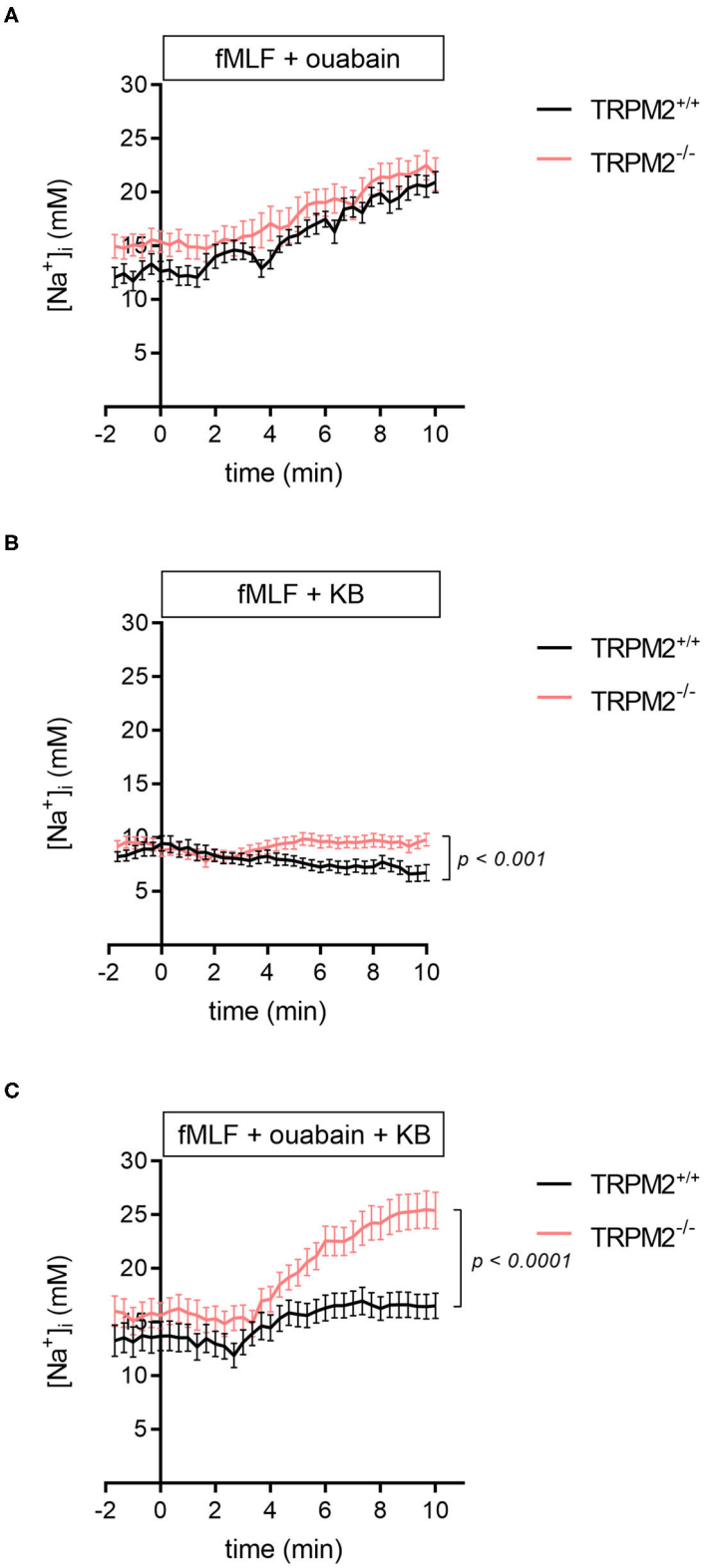

FIGURE 3 | $[Na^+]_i$ upon fMLF stimulation and addition of Na^+/K^+-ATPase and/or NCX1 inhibitor. **(A)** In $TRPM2^{+/+}$ and $TRPM2^{-/-}$ neutrophils, inhibition of the Na^+/K^+-ATPase leads to an increase of the $[Na^+]_i$ to similar end values. **(B)** Upon the stimulation with fMLF and inhibition of NCX1, the $[Na^+]_i$ decreases in $TRPM2^{+/+}$

(Continued)

FIGURE 3 | but not in TRPM2$^{-/-}$ neutrophils, reaching significantly lower values after 10 min (6.7 ± 0.8 mM vs. 9.8 ± 0.6 mM; $p < 0.001$). **(C)** Comparison of the $[Na^+]_i$ upon fMLF stimulation with Na^+/K^+-ATPase and NCX1 inhibitors in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils. Following 10 min of stimulation with fMLF, the $[Na^+]_i$ in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils reaches 16.5 ± 1.2 mM and 25.4 ± 1.7 mM, respectively. fMLF and inhibitors were added at $t = 0$. $N = 3$, $n \geq 22$.

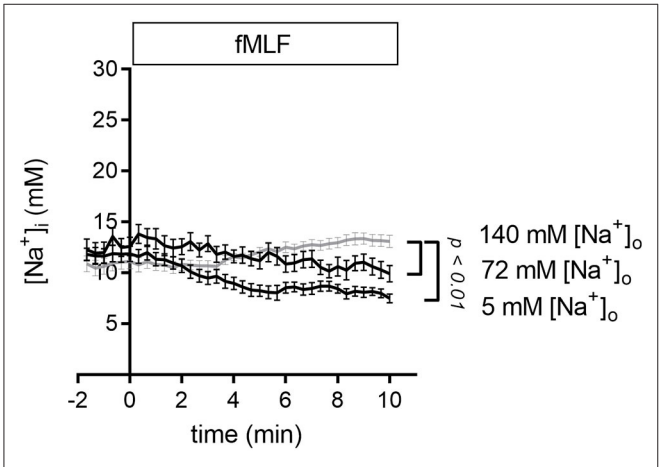

FIGURE 4 | $[Na^+]_i$ upon fMLF stimulation in Ringer's solutions with reduced Na^+ concentrations. Stimulation of neutrophils with fMLF in Ringer's solution with reduced extracellular $[Na^+]$ leads to a decrease of the $[Na^+]_i$. For comparison, we replotted the results of TRPM2$^{+/+}$ neutrophils stimulated in the presence of 140 mM Na^+ (gray curve) already depicted in **Figure 1**. fMLF and solutions of low Na^+ were added at $t = 0$. $N = 3$, $n \geq 23$.

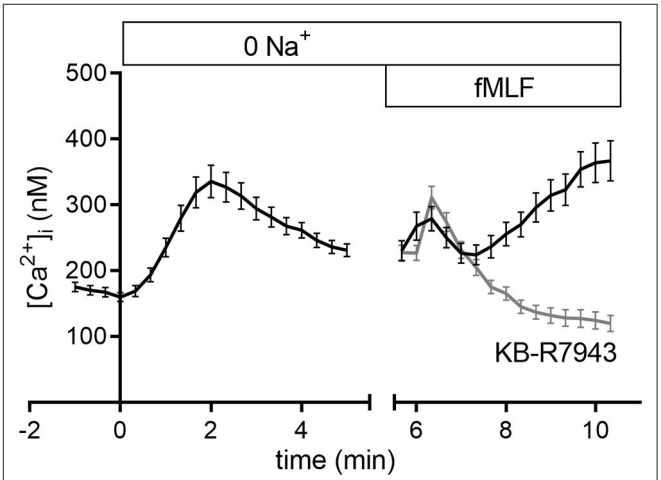

FIGURE 5 | Intracellular Ca^{2+} concentration in nominally Na^+-free Ringer's solution upon fMLF stimulation with or without NCX1 inhibition. The increase of the $[Ca^{2+}]_i$ upon removal of extracellular Na^+ indicates the expression and activity of the NCX1. Subsequent stimulation in Na^+-free solution leads to a transient increase of the $[Ca^{2+}]_i$ followed by sustained rise. Concurrent inhibition of the NCX1 prevents this increase of the $[Ca^{2+}]_i$. $N = 3$; $n \geq 100$.

results is that less Na^+ is entering the cell via NCX1 activity so that the extrusion of the Na^+ ions dominates.

Removal of Extracellular Na^+ Causes an Increase of the Neutrophil $[Ca^{2+}]_i$

To further analyze NCX function and elucidate the impact of extracellular Na^+ on the intracellular Ca^{2+} concentration, we superfused neutrophils with a nominally Na^+-free Ringer's solution and measured the $[Ca^{2+}]_i$ (**Figure 5**). The nominal removal of extracellular Na^+ leads to an increase of the $[Ca^{2+}]_i$ which is indicative of NCX1 activity in neutrophils. Subsequent stimulation with fMLF induces a biphasic increase of the $[Ca^{2+}]_i$. A short rapid peak is followed by a subsequent slow increase of the $[Ca^{2+}]_i$. However, inhibition of the NCX1 prevents the rise of the $[Ca^{2+}]_i$ and rather leads to a decrease of the $[Ca^{2+}]_i$. This suggests that in a nominally Na^+-free solution NCX1 is active in the *reverse* mode.

C5a Causes More Robust Chemotaxis of Murine neutrophils Than fMLF

One of the pivotal neutrophil functions is to rapidly reach the site of injury or inflammation. The ability to follow chemoattractant gradients can be measured *in vitro* using various migration assays. In our experimental setting, neutrophils were exposed to chemoattractant (1 μM fMLF or 60 nM C5a) gradients after they had been embedded within a 3D collagen I matrix, to mimic their physiological environment in a tissue.

fMLF induces directional migration of neutrophils to a lesser extent than C5a (**Figure 6**). Both chemoattractants are used in previously established optimal concentrations (data not shown). Cell trajectories and the distance covered toward fMLF over time differ from those in a C5a gradient. Chemotaxis toward C5a is not only more prominent, but also different between the genotypes. TRPM2$^{-/-}$ neutrophils follow the C5a gradient slightly better than TRPM2$^{+/+}$. This difference, not revealed in our previous studies using three mice of each genotype (8), became apparent after more than doubling the number of mice analyzed. It supports the view that TRPM2 mitigates neutrophil migration (25).

Lowering the $[Na^+]_o$ Augments Neutrophil Chemotaxis in fMLF Gradient

Assuming that the differences in neutrophil response may depend on the Na^+ homeostasis, we analyzed neutrophil chemotaxis in an fMLF gradient in an isosmotic, low sodium (72 mM Na^+) solution. Under these conditions, the neutrophils adapt by lowering their $[Na^+]_i$; the $[Na^+]_i$ reaches only ∼10 mM as compared to 13.1 ± 0.6 mM when using the regular Ringer's solution (see **Figure 4**). It is known that lowering of the $[Na^+]_o$ does not affect the number of FPR1 receptors in the membrane, but may increase receptor affinity (52). To elucidate whether lower extracellular Na^+ is beneficial for neutrophil chemotaxis we compared the chemotaxis index (CI) of fMLF-stimulated neutrophils in 140 and 72 mM $[Na^+]_o$.

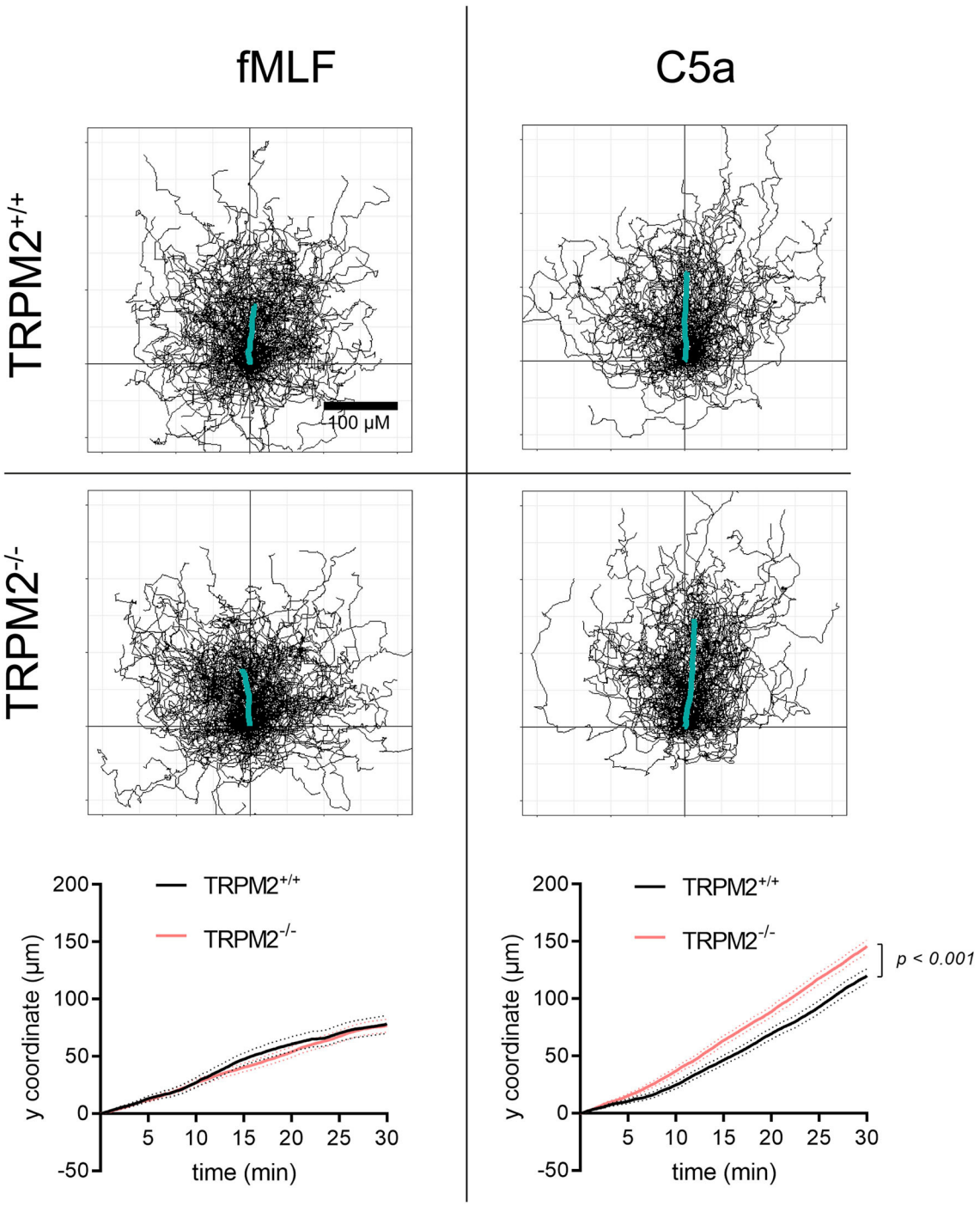

FIGURE 6 | Trajectories of TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils in fMLF and C5a gradients. fMLF induces less robust chemotaxis than C5a as shown by trajectories and mean distances covered toward the chemoattractants. Trajectories are normalized to common starting points, and the thick lines represent the averaged cell path of the entire cell populations. There are no differences between genotypes in the fMLF gradient. In a C5a gradient, TRPM2$^{-/-}$ neutrophils have a higher chemotaxis index (not shown) and cover longer distances toward the chemoattractant (fMLF: TRPM2$^{+/+}$, $N = 7$, $n = 140$, TRPM2$^{-/-}$, $N = 8$, $n = 160$; C5a: TRPM2$^{+/+}$, TRPM2$^{-/-}$, $N = 6$, $n = 120$).

TRPM2$^{+/+}$ neutrophils follow the fMLF gradient better when the Na^+ concentration is reduced to 72 mM (**Figures 7**, **8**). The CI of TRPM2$^{+/+}$ is increased in solution of reduced Na^+, TRPM2$^{-/-}$ neutrophils, however, cover longer distances, which is evident by more "spread" trajectories (**Figure 7**) and higher velocity (**Figure 8**). These results indicate that in the presence

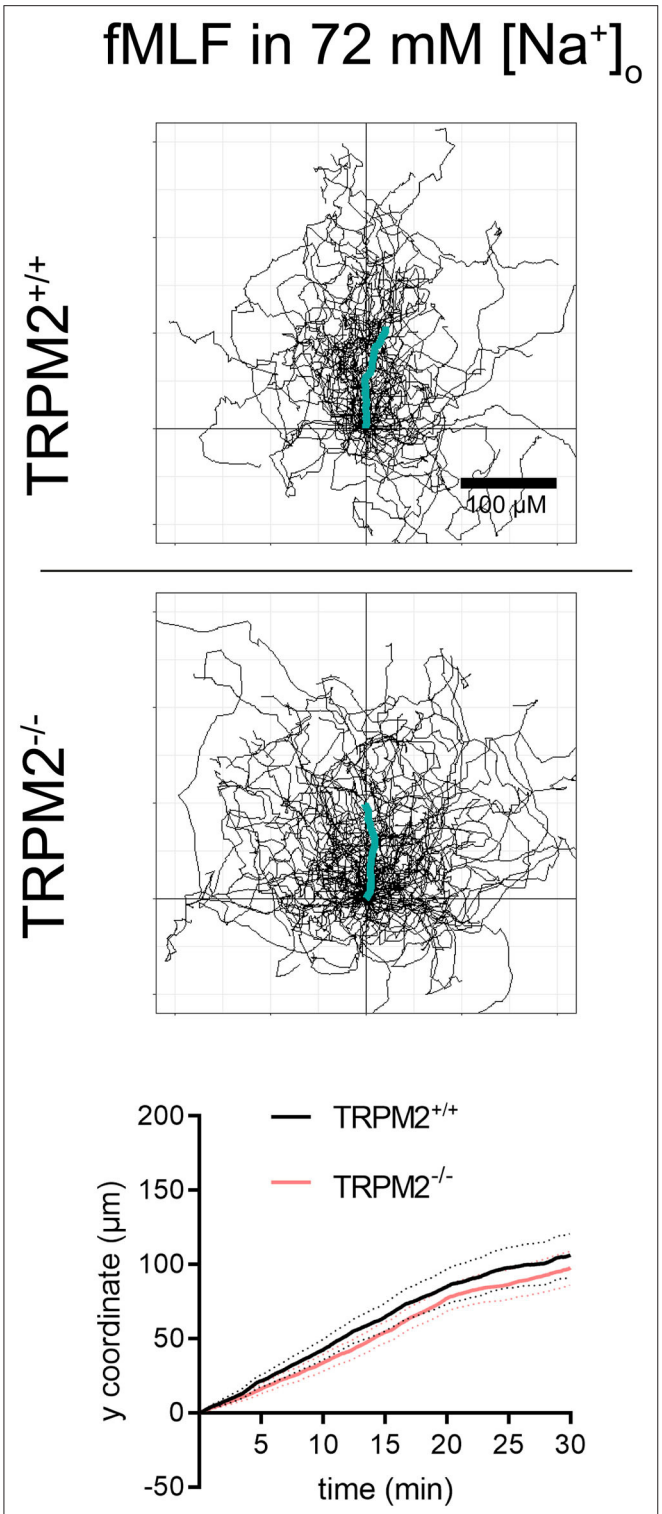

FIGURE 7 | Neutrophil migratory parameters in an fMLF gradient and 72 mM extracellular sodium. Cell trajectories and mean distances covered toward fMLF ($TRPM2^{+/+}$, $N = 3$, $n = 60$, $TRPM2^{-/-}$, $N = 4$, $n = 80$).

of the TRPM2 channel, lower $[Na^+]_i$ improves neutrophil directionality. However, in the absence of the channel, only random cell migration is increased.

Blocking the Na^+/K^+-ATPase and NCX1 Restrains Neutrophil Chemotaxis

To analyze the consequences of the increased $[Na^+]_i$ in C5a-stimulated neutrophils, we inhibited the Na^+/K^+-ATPase of neutrophils with 100 µM ouabain (**Figure 9**). Inhibition of the Na^+/K^+-ATPase diminishes neutrophil chemotaxis. Surprisingly, mean velocity values are very similar to those upon C5a stimulation only (ctrl). Addition of 10 µM KB-R7943 strongly impedes neutrophil chemotaxis and velocity.

NCX1 Splice Variants Containing BD Exons Are Expressed in Neutrophils

KB-R7943 was developed as a reverse mode NCX1 inhibitor, although it can also inhibit the forward mode. The IC_{50} of forward mode inhibition depends on the NCX1 splice variants (51, 53). Splice variants containing BD exons [predominantly NCX1.3, NCX1.7 (51)] are almost completely inhibited in the forward mode by the concentration used in our $[Na^+]_i$ measurements (10 µM KB-R7943). To confirm the presence of NCX1 splice variants sensitive to inhibition of both modes, we analyzed the expression of BD exons by means of qPCR. As shown in **Figure 10**, the BD transcript is expressed in both neutrophil genotypes. Expression values calculated by housekeeping gene Ct value subtraction and conversion to $2^{-\Delta Ct}$ expression level do not differ ($p = 0.19$, data not shown) and normalized relative expression ($2^{-\Delta\Delta Ct}$) is not different in neutrophils from both genotypes.

$[Na^+]_i$ Is a Factor Involved in Neutrophil Chemotaxis

Finally, taking under consideration all chemotaxis assays and $[Na^+]_i$ measurements performed under similar conditions, we observed a correlation between the $[Na^+]_i$ and neutrophil chemotaxis index (**Figure 11**). The higher $[Na^+]_i$ is associated with lower CI. In contrast, there is only a minor impact of the $[Na^+]_i$ on cell velocity. Thus, chemotaxis appears to be exquisitely sensitive to changes of the $[Na^+]_i$ which in neutrophils is controlled by Na^+-transport proteins presumably, to the large extent by NCX1.

DISCUSSION

The intracellular Na^+ homeostasis in neutrophils is tightly coupled with Ca^{2+} and H^+ fluxes. Since the concentrations of these ions change drastically when neutrophils are activated, it is not a surprise that Na^+-transport proteins also play a role in neutrophil function.

Studies on NCX1 in neutrophils show that the exchanger partially contributes to Ca^{2+} influx in activated neutrophils (30, 31). Membrane potential as well as the extra- and intracellular concentrations of Na^+ and Ca^{2+} ions determine the NCX1 transport mode. Neutrophil stimulation or the harsh inflammatory extracellular environment cause alterations of the above-mentioned variables. Importantly, only slight changes of the $[Na^+]_i$ in neutrophils or of their membrane potential, determine NCX1 mode and thus, its consequence

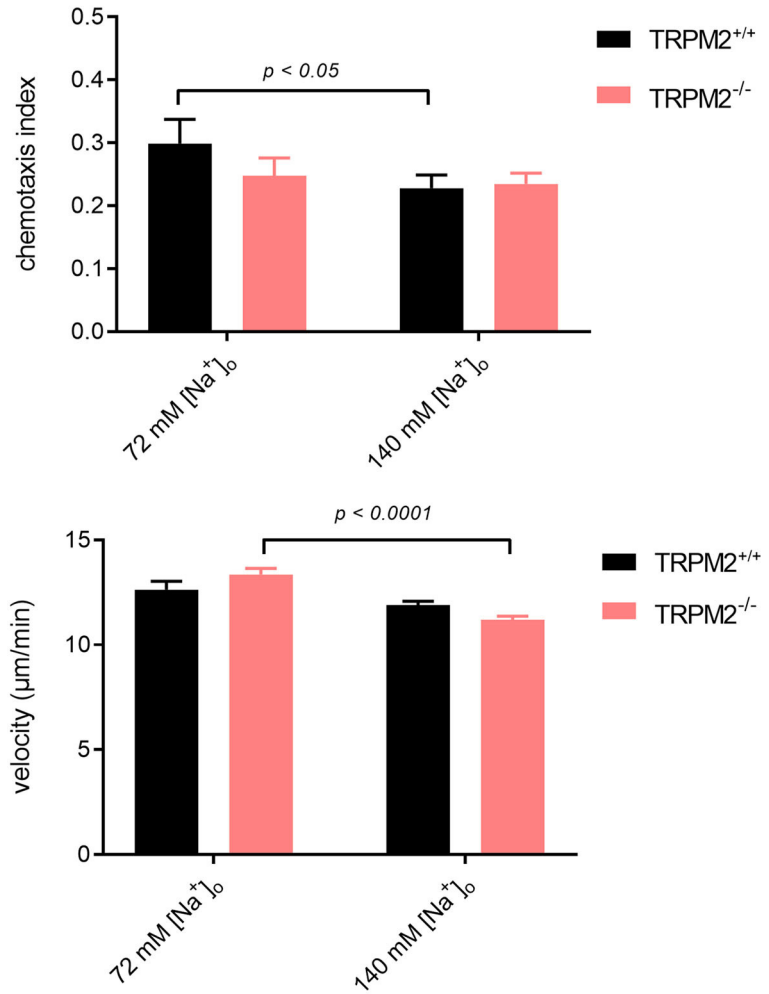

FIGURE 8 | Neutrophil chemotaxis indices and velocity upon stimulation with fMLF in different $[Na^+]_o$. Comparison of chemotaxis indices and mean velocities in low and physiological $[Na^+]_o$. In the lower sodium concentration, $TRPM2^{+/+}$ neutrophils follow the fMLF gradient better, but $TRPM2^{-/-}$ neutrophils, despite increased velocity, show no improvement in chemotaxis ($TRPM2^{+/+}$, $N = 3$, $n = 60$, $TRPM2^{-/-}$, $N = 4$, $n = 80$).

for Na^+/Ca^{2+} homeostasis. Assuming the neutrophil resting membrane potential to be at −60 mV (54), a $[Na^+]_o$ of 140 mM and a free intra-/extracellular $[Ca^{2+}]$ of 100 nM/1.2 mM, respectively, an increase of the $[Na^+]_i$ from 12 to 13 mM results in a driving force for NCX1 *reverse* mode as calculated from ΔG_{NCX} (55). At $[Na^+]_i = 11$ mM, a depolarization of the membrane potential to−47 mV also leads to mode switch (29). In our studies, we show that neutrophil $[Na^+]_i$ is remarkably close to the values which determine NCX1 modes. Therefore, we presume that already slight differences of the $[Na^+]_i$ in $TRPM2^{+/+}$ and $TRPM2^{-/-}$ neutrophils may in some circumstances affect neutrophil behavior, likely due to altered NCX-mediated Ca^{2+} influx. The contribution of NCX to Ca^{2+} influx may become even more important during the respiratory burst of primed, maximally stimulated neutrophils when their membrane potential may be as high as +60 mV (56).

Despite the fact that Na^+ is the electrolyte with the highest concentration in the plasma and the interstitial fluid, there is only scarce data on the Na^+ homeostasis in neutrophils. Previous studies using neutrophils and differentiated HL-60 cells have shown a rise of the $[Na^+]_i$ upon fMLF stimulation. Researchers concluded that influx of both Ca^{2+} and Na^+ is pivotal for neutrophil exocytosis and production of O_2^- (16, 57). In many cell types, an increased $[Na^+]_i$ is associated with pathological conditions like ischemia and excessive ROS production (58), but

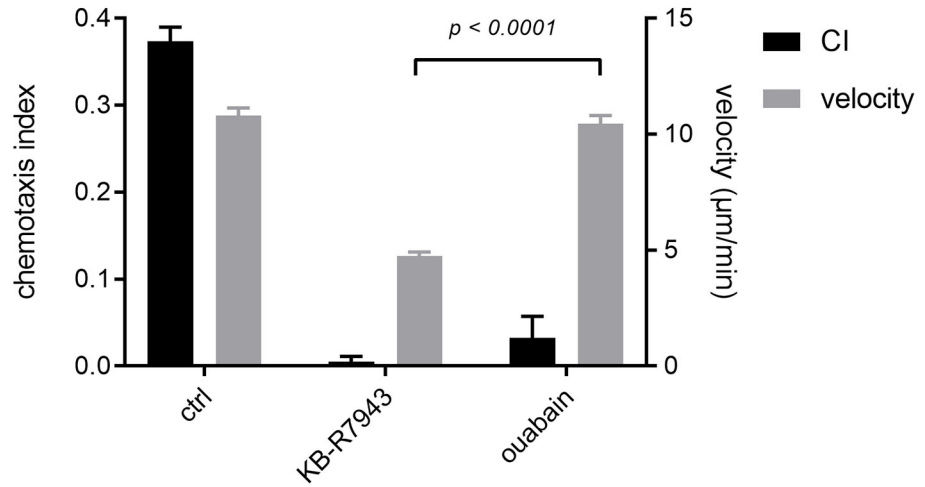

FIGURE 9 | Neutrophil chemotaxis indices and velocity upon stimulation with C5a and inhibition of Na^+/K^+-ATPase or NCX1. Na^+/K^+-ATPase and NCX1 inhibitors almost completely abrogate neutrophil chemotaxis. Inhibiting NCX1 with KB-R7943 affects neutrophil velocity much stronger than ouabain. For clarity, only the significant difference between velocity values using inhibitors is shown ($N = 3$, $n = 60$).

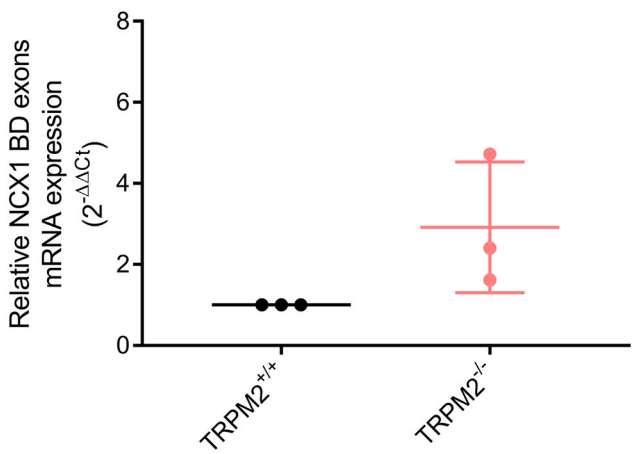

FIGURE 10 | mRNA expression of NCX1 region of alternative splicing containing BD exons. NCX1 splice variants sensitive to inhibition with 10 µM KB-R7943 in reverse and forward mode are expressed in neutrophils ($N = 3$).

the consequences of the $[Na^+]_i$ rise for neutrophil chemotaxis were left unexplored.

By elucidating the changes of the $[Na^+]_i$ in neutrophils, we may now suggest that Na^+ transport proteins contribute to alternations in $[Ca^{2+}]_i$ and neutrophil chemotaxis. In this context it was intriguing to observe that the $[Na^+]_i$ rises more strongly in $TRPM2^{-/-}$ than in WT neutrophils following fMLF stimulation.

Putative explanation could be based on the TRPM2 involvement in the regulation of neutrophil membrane potential. We can hypothesize that upon fMLF stimulation, the lack of the channel renders neutrophil membrane permissive for Na^+ influx, but it is not the case upon C5a stimulation. Chemoattractant's different impact on the neutrophil membrane potential may result in somewhat surprising outcomes.

Unfortunately, the unexpected $[Na^+]_i$ rise upon fMLF stimulation in $TRPM2^{-/-}$ neutrophils remains unexplained. Also, there is no impact of the higher $[Na^+]_i$ on $TRPM2^{-/-}$ chemotaxis in fMLF gradient. The prominent increase of $[Na^+]_i$ upon KB-R7943 and ouabain treatment in $TRPM2^{-/-}$, but not in WT neutrophils suggests altered NCX1 activity, however this assumption needs to be corroborated. Upon C5a stimulation, despite similar $[Na^+]_i$ courses, $TRPM2^{-/-}$ neutrophils migrate further in chemoattractant direction. The complexity of mechanisms allow us only to speculate and the detailed explanation requires additional studies. Our ongoing and future analysis of absolute neutrophil membrane potential values may shed some light on this matter. We presume that channel knock out results in altered membrane potential and/or compensatory activities of other ion transport proteins (presumably NCX1). Worth noting, $TRPM2^{-/-}$ neutrophils often display slightly higher basal $[Na^+]_i$. Whether this is caused by permissive resting membrane potential or for e.g., different basal NCX1 activity is yet to be explored.

Despite the outlying results of the neutrophils lacking TRPM2 channel, in our view, Na^+ indirectly affects neutrophil migration (*via* Ca^{2+} modulation) and may directly and specifically affect neutrophil chemotaxis (directed migration).

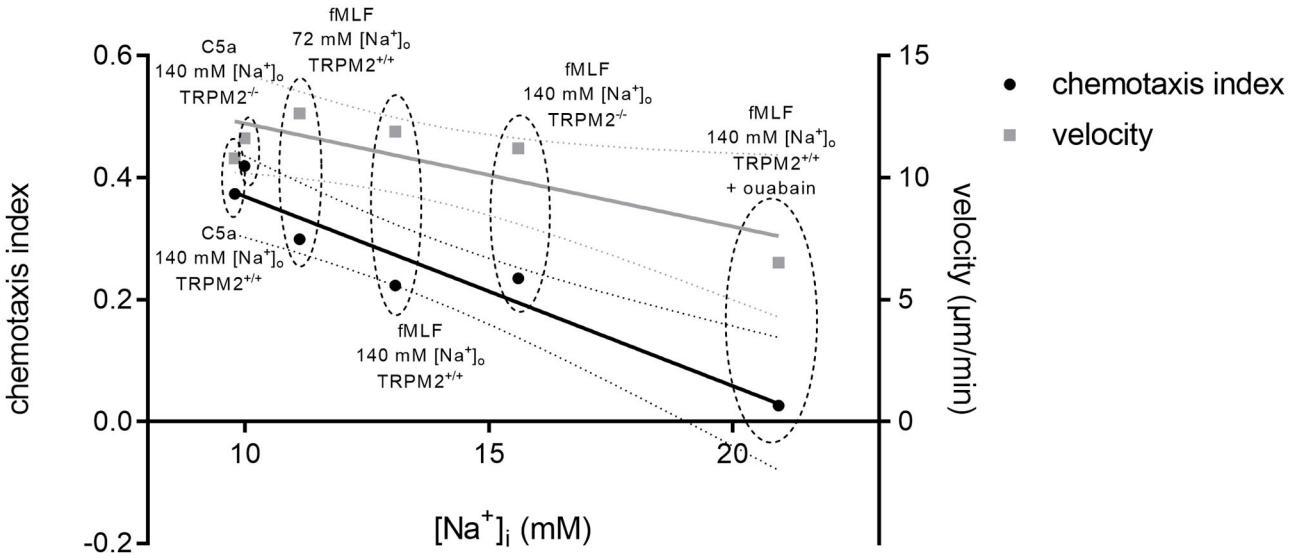

FIGURE 11 | Chemotaxis and mean velocity values plotted against the respective $[Na^+]_i$ after 10 min of chemoattractant stimulation. Summary of $[Na^+]_i$ and chemotaxis index in TRPM2$^{+/+}$ and TRPM2$^{-/-}$ neutrophils following stimulation with fMLF or C5a. The curves reveal an inverse correlation between the $[Na^+]_i$ and neutrophil chemotaxis (Pearson's correlation coefficient ($r = -0.9584$; $p = 0.0026$)). Neutrophil velocity seems to be affected to a lesser extent by $[Na^+]_i$ (with Pearson's correlation coefficient ($r = -0.8307$; $p = 0.0405$)). Dotted lines represent confidence intervals (95%). CI and velocity values from respective assays are encircled and described.

Two chemoattractants, fMLF and C5a, differently influence the $[Na^+]_i$ and neutrophil chemotaxis, supporting our assumptions about a role of Na^+ ions in directed migration of neutrophils. A sustained increase of the $[Na^+]_i$ upon fMLF stimulation correlates with worse chemotaxis. On the contrary, C5a induces only a transient increase of the $[Na^+]_i$ with rapid recovery to the basal $[Na^+]_i$, which coincides with efficient chemotaxis. The notion that the $[Na^+]_i$ is more critical for chemotaxis rather than for migration itself is supported by the fact that inhibiting the Na^+/K^+-ATPase abolishes neutrophil chemotaxis almost completely, but has a much lesser impact on neutrophil velocity. Thus, despite obvious Na^+ influence on Ca^{2+} homeostasis, we suggest the distinct roles of Na^+ and Ca^{2+} ions in neutrophil function. We propose that Na^+ gradients may strongly influence neutrophil directionality, while Ca^{2+} is pivotal for neutrophil migration.

This leads to another, yet still quite speculative explanation for the importance of the Na^+ homeostasis for neutrophil chemotaxis. It relates to the fact that type A GPCRs including chemoattractant receptors are allosterically inhibited by Na^+ bound to the receptor protein. GPCRs are constitutively active, but Na^+ ions keep the receptors in an inactive state. This was also shown for FPR and C5R1 where Na^+ ions act as an allosteric inverse agonist (59). Upon binding of the ligand, e.g., fMLF, Na^+ has to leave its binding site in the GPCR in order to allow its full activation. One could therefore speculate that local changes of the Na^+ concentration and thereby changes of the transmembranous Na^+ gradient can modulate the activity of chemoattractant receptors (60). However, so far it is unknown whether the transmembranous Na^+ gradient and/or the cell membrane potential contribute to this unbinding step. If this were the case, our results could be interpreted such that the lower $[Na^+]_i$ following the stimulation with C5a activation would help to remove allosteric inhibition and allow a stronger receptor activation than after stimulation with fMLF. Consequently, chemotaxis toward C5a is more efficient.

In our view the $[Na^+]_i$ impacts indirectly on neutrophil migration and chemotaxis by regulating the NCX1. When NCX1 is inhibited, neutrophil migration is severely impaired. A likely explanation for the importance of the NCX1 function is its role in maintaining the proper gradients of the intracellular Ca^{2+} concentration that are found in many migrating cells including neutrophils (4, 5). However, the use of inhibitors has several caveats. KB-R7943, a presumed inhibitor of NCX1, was shown to have off-targets. At concentrations used in our studies, KB-R7943 also may inhibit TRPC channels expressed in neutrophil such as TRPC3 and TRPC6. This leads to diminished Ca^{2+} influx, what can be partially the cause of the $[Ca^{2+}]_i$ drop upon KB-R7943 addition to fMLF-stimulated neutrophils as shown in **Figure 5**. In astrocytes, KB-R7943 was also shown to inhibit SOCE (61). With these assumptions, we cannot dismiss the possibility that the used inhibitor affects neutrophil migration through several targets. On the other hand, we showed previously that Ca^{2+} signaling of murine neutrophils is independent of TRPC6 channels (40) and that TRPC3 channel mRNA is expressed only at a very low level (4). Moreover, ion substitution assays as well as the measurements of the $[Na^+]_i$ and the $[Ca^{2+}]_i$ provide strong support for NCX1 expression and activity in neutrophils and

show that Na^+ influences $[Ca^{2+}]_i$ and neutrophil response. The novel assumption derived from our studies is that although Na^+ fluxes (or the NCX1 activity) affect important Ca^{2+} homeostasis, the former seems to be pivotal for the *directed* neutrophil migration. Moreover, according to our findings, altered $[Na^+]_o$ in inflammation and tumor environment may be considered as a factor, which (even if indirectly) modulates neutrophil migration and chemotaxis.

Besides Na^+ and Ca^{2+} transport, neutrophil activation induces H^+ flux. Transient neutrophil intracellular acidification and depolarization are due to NOX2 activity and resolved predominantly by H_v1 channel (62, 63). However, another Na^+-dependent exchanger, NHE1, contributes to the electroneutral removal of H^+. NHE1 was shown to be involved in production of arachidonic acid derivatives in neutrophils (64) and polarization of migrating cells (65, 66). Our own studies show that NHE1 contributes to neutrophil chemotaxis and regulation of intracellular pH (pH_i) (unpublished data). This Na^+-dependent pH_i regulation has to be considered when interpreting the $[Ca^{2+}]_i$ changes upon Na^+ removal. The anticipated decrease pH_i may have caused an inhibition of NCX1 and thereby caused an only transient increase of the $[Ca^{2+}]_i$ shown in **Figure 5**.

Taken together, our study shows that TRPM2 channels have a subtle impact on neutrophil migration and chemotaxis by paradoxically regulating the $[Na^+]_i$ of neutrophils. However, the exact mechanism of $[Na^+]_i$ regulation by TRPM2, requires further studies. On the other hand, NCX1 activity and its driving force, the transmembranous Na^+ gradient, appears to be an important factor for neutrophil function. The complexity of the mechanisms in which Na^+ is involved, does not allow for simple assumptions. However, the present and future studies will help to understand the interwoven aspects of ionic homeostasis in activated neutrophils.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Natur, Umwelt und Verbraucherschutz NRW permit number: 84-02.05.50.15.010.

AUTHOR CONTRIBUTIONS

KN, MR, ML, JS, LO, SSc, SSa, ZP, and EB performed the experiments. KN made statistical analyses. KN and AS designed the study and wrote the manuscript. All authors edited the manuscript and approved the submitted version.

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REVIEW
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Involvement of Neural Transient Receptor Potential Channels in Peripheral Inflammation

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Transient receptor potential (TRP) channels are a superfamily of non-selective cation channels that act as polymodal sensors in many tissues throughout mammalian organisms. In the context of ion channels, they are unique for their broad diversity of activation mechanisms and their cation selectivity. TRP channels are involved in a diverse range of physiological processes including chemical sensing, nociception, and mediating cytokine release. They also play an important role in the regulation of inflammation through sensory function and the release of neuropeptides. In this review, we discuss the functional contribution of a subset of TRP channels (TRPV1, TRPV4, TRPM3, TRPM8, and TRPA1) that are involved in the body's immune responses, particularly in relation to inflammation. We focus on these five TRP channels because, in addition to being expressed in many somatic cell types, these channels are also expressed on peripheral ganglia and nerves that innervate visceral organs and tissues throughout the body. Activation of these neural TRP channels enables crosstalk between neurons, immune cells, and epithelial cells to regulate a wide range of inflammatory actions. TRP channels act either through direct effects on cation levels or through indirect modulation of intracellular pathways to trigger pro- or anti-inflammatory mechanisms, depending on the inflammatory disease context. The expression of TRP channels on both neural and immune cells has made them an attractive drug target in diseases involving inflammation. Future work in this domain will likely yield important new pathways and therapies for the treatment of a broad range of disorders including colitis, dermatitis, sepsis, asthma, and pain.

Keywords: pain, itch, thermal sensing, nervous system, vagus nerve, cytokine

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INTRODUCTION

Transient receptor potential (TRP) channels are polymodal calcium-permeable cation channels that broadly act as cellular sensors. Mammalian TRP channels consist of 28 members and can be grouped into six main families: TRP ankrin (TRPA), TRP canonical (TRPC), TRP melastatin (TRPM), TRP mucolipins (TRPML), TRP polycystin (TRPP), and TRP vanilloid (TRPV). For the

purposes of this review, we will focus on specific channels within the TRPA, TRPM, and TRPV families that have documented roles and mechanisms relevant to inflammation. There is already an extensive body of literature covering the many different TRP families, their protein structures, and their specific functions, therefore, the goal of this review is to highlight a specific set of TRP channels that are expressed in the peripheral nervous system and have been linked to immune system responses. These TRP channels, specifically TRPA1, TRPM3, TRPM8, TRPV1, and TRPV4 are expressed on peripheral nerves and neurons that communicate with the immune system and major peripheral organs to regulate inflammatory responses (1–10) (**Figure 1**).

We will focus on the broad role of these neural TRP channels as well as their role on neurons and peripheral nerves in mediating the crosstalk between the nervous system and immune system, particularly in the context of inflammation. The activation of TRP channels has an increasingly recognized role in a wide range of inflammatory disorders and therefore may be suitable as potential targets for therapeutic intervention.

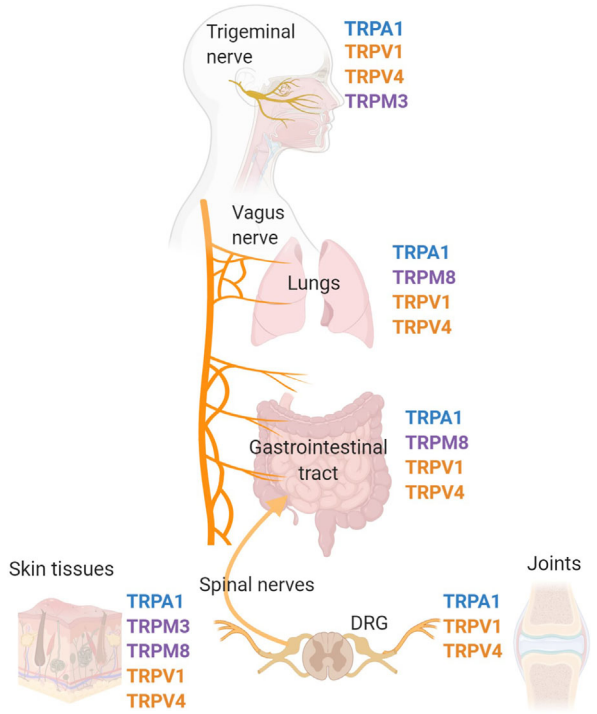

FIGURE 1 | TRP channel-expressing peripheral nerves innervate major organs of the body and effect inflammation. Pre-clinical and clinical studies have identified that the TRP channels, TRPV1, TRPV4, TRPM3, TRPM8, and TRPA1 are expressed on a variety of cell types, specifically sensory nerves that innervate peripheral tissues throughout the body. Major sites of TRP channel expression include the trigeminal nerve, vagus nerve, dorsal root ganglia and associated spinal nerves. The trigeminal nerve is the fifth cranial nerve innervating the face and sinus, with the cell bodies located in the trigeminal ganglia. The vagus nerve is the tenth cranial nerve which innervates many peripheral organs including the lungs and gastrointestinal tract. Spinal nerves, with cell bodies located in the dorsal root ganglia (DRG), innervate many tissues in the periphery including the skin, joints, and colon.

TRP CHANNEL FUNCTION AND EXPRESSION

TRP "Vanilloid" Channels

The Transient Receptor Potential Vanilloid (TRPV) channel subfamily consists of six members: TRPV1-V6, with TRPV1-4 classified as the thermo-TRPs that are activated by heat in heterologous expression systems (11). TRPV proteins contain seven hydrophobic domains with six spanning the cellular membrane (S1–S6) and the seventh hydrophobic domain, as well as the C- and N- termini, located within the cell (12).

TRPV1

TRPV1 is a nonselective, calcium permeable, cation channel, and the first member for the TRPV family of ion channels discovered. Activated by a multitude of endogenous and exogenous compounds, TRPV1 is the most extensively studied of the TRPV channels (**Figure 2**) (1). Endogenous endocannabinoids, anandamide (13), N-arachidonoyl-dopamine (14), as well as endogenous lipoxygenase products, 12-(S)-hydroperoxyeicosatetraenoic acid, and leukotriene B4 (15), serve as ligands to TRPV1. Exogenous chemicals such as resiniferatoxin, olvanil, and most prominently, capsaicin, the main irritant found in hot chili peppers (16), also effectively activate TRPV1. In addition to these substances, temperatures greater than 43°C and acidic conditions with a pH lower than 6.0, can induce TRPV1 activation leading to a burning sensation and pain (17–19).

The initial discovery of TRPV1 was through the sequencing of genes expressed in dorsal root ganglion neurons (DRG) (16). Subsequently, it has been reported that the majority of DRG neurons express TRPV1 (2). In addition to DRGs, TRPV1 is highly expressed in nodose ganglia (NG) and trigeminal ganglia (TG), specifically on unmyelinated C- and thinly myelinated Aδ-type sensory nerve fibers (20). In the central nervous system (CNS), TRPV1 is expressed on dopaminergic neurons of the substantia nigra, hippocampal pyramidal neurons, hypothalamic neurons, locus coeruleus neurons, and the cerebral cortex (21). TRPV1 is also found to be expressed on a variety of non-neuronal cell types, including immune cells such as T lymphocytes (22), macrophages (23), and dendritic cells (24). Non-neural and non-immune cell expression of TRPV1 can be found on keratinocytes (25), bladder urothelium (26), smooth

Abbreviations: AITC, allyl isothiocyanate; CFA, complete Freund's adjuvant; CGRP, calcitonin gene-related peptide; IBD, inflammatory bowel disease; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; NKA, neurokinin A; ROS, reactive oxygen species; SP, substance P; TNF, tumor necrosis factor.

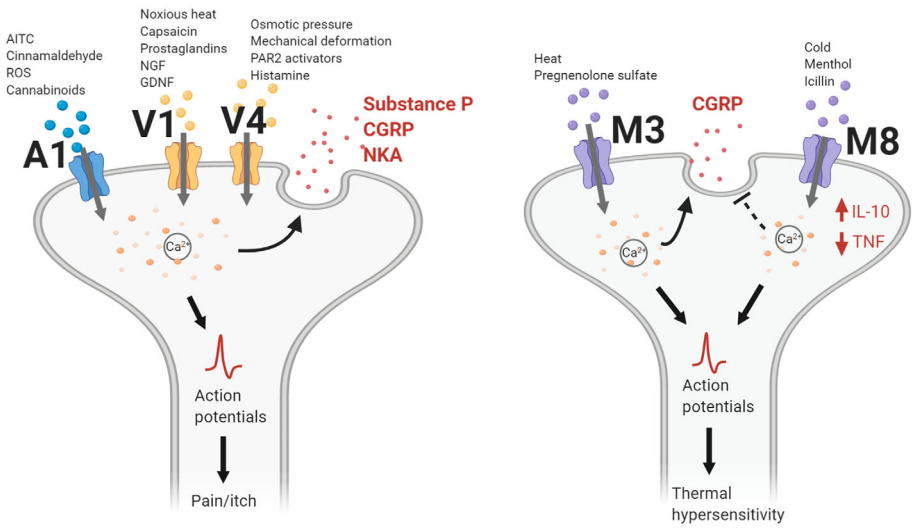

FIGURE 2 | TRP channel actions contributing to neurogenic inflammation. TRPA1, TRPV1, and TRPV4 channels gate cations following activation by their respective chemical agonists, temperature changes, or mechanical stimulation. Intracellular calcium levels increase and lead to the release of neuropeptides such as calcitonin gene-related peptide (CGRP), substance P (SP), or neurokinin A (NKA). Nerve action potentials trigger the sensation of pain or itch. For TRPM3, heat changes and pregnenolone sulfate activate the channel to gate cations, leading to CGRP release. Conversely, TRPM8 activation inhibits CGRP release while increasing levels of interleukin-10 (IL-10) and decreasing levels of tumor necrosis factor (TNF). Action potentials generated following TRPM3/8 channel activation lead to changes in thermal sensitivity. AITC, allyl isothiocyanate; GDNF, glial cell line-derived neurotrophic factor; NGF, nerve growth factor; PAR2, protease-activated receptor 2; ROS, reactive oxidative species.

muscle (27), hepatocytes (28), pancreatic β-cells (29), and endothelial cells (30).

TRPV4

TRPV4 is a nonselective, moderately calcium permeable, cation channel. It has a homo-dimeric tetramer structure with the TRPV family standard of six transmembrane segments. The pore loop is between segments 5 and 6 and both the C- and N-termini are located within the cytoplasm (31). The channel contains six ankyrin repeats and the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), can bind to this site inhibiting the channel (32). Similar to TRPV1, TRPV4 is expressed on a variety of cells such as neurons, leukocytes (33), T cells (34), and macrophages (35). In the brain, neurons and glial cells in the hippocampus, cortex, thalamus, cerebellum (36), and hypothalamus (37) all express TRPV4. In the peripheral nervous system, TRPV4 is extensively expressed on DRG, NG, and TG neurons (38, 39) (**Figure 1**). DRG neurons containing this channel are found innervating the spinal dorsal horn (40), gastrointestinal tract (38), skin (41), and liver (42). TRPV4-positive nerve fibers have been also found to innervate arrector pili smooth muscle of the skin, sweat glands, intestines, and blood vessels, and dura mater (39, 43).

TRP "Melastatin" Channels

The transient receptor potential melastatin (TRPM) subfamily has been regarded as the most diverse group of TRP channels, comprised of eight nonselective cation channels: TRPM1, TRPM2, TRPM3, TRMP4, TRPM5, TRPM6/7, and TRPM8 (3). These channels were first identified as the protein that decreases in expression in highly metastatic melanoma cell lines (44). Structurally akin to those of voltage-gated channels, all TRPM channels possess six transmembrane domains, a TRP helix, and a cytoplasmic N- and C-terminal (45, 46). The widely expressed family of TRPV channels has been discovered to contribute toward a variety of physiological functions from sensing oxidative stress, temperature changes, and cell swelling. While several of these channels play a crucial role in the nervous system (e.g., neuroinflammation), TRPM3 and TRPM8 are the most prominent channels among sensory nerves, particularly in the skin, sinuses, lungs, and the gastrointestinal tract (7, 47–52) (**Figure 1**).

TRPM3

First discovered through residual heat sensitivity testing in TRPV1 KO mice, the transient receptor potential melastatin-3 (TRPM3) is a non-selective calcium cation channel that has been recently observed to play a crucial role in noxious heat detection (45, 47, 48). TRPM3 is widely expressed in both neuronal and non-neuronal tissue, such as in brain and spinal tissues, retinal (53), pituitary, kidney, and testes (49). Through *in situ* hybridization and RT-qPCR of TRPM3 mRNA, studies have shown abundant expression in both TG and DRG sensory neurons (**Figure 1**) (54). A large majority of TRPM3-expressing neurons are also responsive to capsaicin, demonstrating that it is often co-expressed with TRPV1. The functional detection of

TRPM3 has been identified using calcium imaging through chemical activation on DRG cells (45).

TRPM8

TRPM8 is a nonselective, calcium-permeable cation channel. Among the thermal-sensing TRP channels, the TRPM8 channel is notable for detecting cold temperatures (8–26°C) and contributing to the cooling sensation by chemicals such as menthol and icilin (**Figure 2**). TRPM8 has been found to be expressed on C- and Aδ- sensory nerve fibers, as well as DRG and TG neurons (48). In the CNS, expression of TRPM8 has been found on hypothalamic and hindbrain nuclei responsible for autonomic thermoregulation (55). In addition to neuronal cell types, TRPM8 is also expressed on macrophages and pulmonary epithelial cells. Activation of TRPM8 on macrophages has been shown to induce an anti-inflammatory response with the increased release of interleukin 10 (IL-10) and decreased release of tumor necrosis factor (TNF). In contrast, activation of TRPM8 on pulmonary epithelial cells increases the expression of pro-inflammatory cytokines such as TNF and interleukin 1 (IL-1) (50).

TRP "Ankyrin" Channels

Transient receptor potential ankyrin 1 (TRPA1) is the only member of the TRPA family. TRPA1 is a polymodal cation channel that is made up of approximately 1,100 amino acids, with roughly 80% of its molecular mass located in the large intracellular domain (56) and a 14 ankyrin repeat in its structure (50, 57). When first described, TRPA1 was reported to sense cold temperatures (<17°); however, it has since been found to additionally sense heat, a common function of TRPV1 and TRPM3 as mentioned above (58–61). Some evidence indicates that the role of TRPA1 as a bidirectional thermo-sensor is due to different channel conformations, and that its heat sensing properties are dependent on its redox state and ligands (60). Along with its role as a thermo-sensor, TRPA1 also responds to mechanical stimuli *via* membrane stress, in a redox state dependent manner (62, 63).TRPA1 is predominantly expressed on myelinated Aδ- and unmyelinated C-fibers of peripheral nerves. Protein expression is found on both cell bodies of DRG, NG, and TG neurons, as well as on the axons of spinal nerves, the vagus nerve, and trigeminal nerve (**Figure 1**) (4, 64). Although not extensively studied, some TRPA1 expression has also been found in regions of the brain such as the somatosensory cortex, and cerebellum (65, 66). Additionally, TRPA1 channels can be found on non-neuronal cell types. TRPA1 was first cloned in fibroblasts (67) and has since been found to be expressed on T-cells, macrophages, endothelial cells, epithelial cells, and smooth muscle cells (50, 68–71). Along with its role as a thermo- and mechanosensory, TRPA1 is activated by a wide variety of chemical stimuli such as cinnamaldehyde, allyl isothiocyanate (AITC), allicin, hydrogen peroxide, oxygen (O_2), nitroxyl (HNO), methylglyoxal, and endotoxin (lipopolysaccharide; LPS) (**Figure 2**) (4, 72–75). Many of these activators have been found to also play a role in modulating inflammatory responses.

NEURAL TRP CHANNELS IN THE CONTEXT OF INFLAMMATION

TRPV1

TRPV1 has been found to be have a key role in inflammation, being linked to both pro- and anti- inflammatory mechanisms. Noxious heat, which can cause cell damage and even death, is a mediator of TRPV1 activation inducing hyperalgesia or pain (**Figure 2**). Increased thermal sensitivity of the TRPV1 channel is mediated by bradykinin and nerve growth factor (NGF) *via* the hydrolysis of intracellular phosphatidylinositol 4,5-bisphosphate (PIP2) (76). After retrograde transport of NGF in peripheral nerves thermal hypersensitivity is maintained *via* changes in TRPV1 expression through the activation of p38, a regulator of pro-inflammatory cytokines TNF, IL-1β, and cyclooxygenase-2 (77). Glial cell line-derived neurotrophic factor (GDNF) family members have also been shown to activate TRPV1 on DRGs, leading to increased thermal hyperalgesia (78). Additionally, protein kinase A (PKA) increases TRPV1's sensitivity to heat and capsaicin through phosphorylation of TRPV1 on Ser-502 (79, 80). PKA can reduce a desensitized state of TRPV1 through the phosphorylation of Thr-370 and Ser-116, (81). In rats in order for TRPV1 to respond to capsaicin Calmodulin-kinase II must be phosphorylated on Ser-502 and Thr-704 (80). Similarly, protein kinase C (PKC), sensitizes TRPV1 when phosphorylated. This occurs when inflammatory mediators prostaglandin E2 and prostaglandin I2 signal through the prostaglandin EP1 and prostacyclin receptors in a PKC dependent manner (82). Adenosine triphosphate (ATP), released from damaged cells after trauma, also activates and sensitizes these channels through P2Y receptors. In such instances, the threshold temperature for TRPV1 was decreased enough to activate the channel under normal physiological conditions (82, 83). It has been demonstrated that some inflammatory mediators, including TNF, NGF and ATP, promote the recruitment of TRPV1 to the surface of the cell, while other mediators, such as bradykinin and GDNFs, act by decreasing the activation threshold of the channel without affecting expression density (84, 85). Furthermore, additional inflammatory mediators released by macrophages and neutrophils such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) can directly activate TRPV1 on afferent vagus neurons (86). During inflammation, acidic conditions can also be produced within the affected tissue activating TRPV1, leading to TRPV1 sensitization and pain (87, 88).

TRPV1 expression and functionality on neurons is significantly altered depending on the inflammatory condition (**Table 1**). For example, mice injected with cerulean to induce acute pancreatic inflammation, display an increase in TRPV1 excitability and mRNA expression on NG and DRG neurons (126). Cerulean also leads to leukotriene B4 production by acinar pancreas cells, activating TRPV1 on sensory afferents (89). Treatment with capsazepine, a TRPV1 antagonist, was capable of reducing myeloperoxidase activity and histological severity of acute pancreatitis (127). These findings were further corroborated utilizing intraperitoneal injection of AMG 9810, another TRPV1

TABLE 1 | Pro- and anti-inflammatory TRP channel functions in inflammatory conditions.

TRP Channel	Conditions	Pro-/Anti-Inflammatory	Functionality	References
TRPV1	Pancreatitis	Pro-	Increases histological damage and release of SP, triggering nociception	(89–90)
	Pulmonary inflammation	Pro-/Anti-	CGRP and SP release induces bronchial constriction and elicits coughing reflex/Activation decreases allergic airway inflammation	(24, 91–92)
	Lung Injury	Anti-	Somatostatin is released to diminish neurogenic inflammation and appears to reduce bronchial hypersensitivity	(93, 94)
	Atopic Dermatitis	Pro-	Contributes to itching sensation and dermatitis clinical severity	(95–96)
	Arthritis	Pro-	Upregulation of pro-inflammatory cytokine release, knee joint swelling, and thermal hyperalgesia	(97)
	Sepsis	Anti-	Upregulates anti-inflammatory IL-10 and attenuation of pro-inflammatory CGRP, TNF, and IL-6	(98–100)
	Carditis/Ischemic Injury	Anti-	SP release which increases IL-10 and reduces TNF levels, ROS, and neutrophil infiltration	(101)
TRPV4	Colitis/IBD	Pro-	Release of CGRP and SP in hypotonic and irritant conditions and contributes to mechanical hyperalgesia	(102–103)
	Itch	Pro-	Mediates pruritus through cutaneous application of agonists and serotonin and histamine-dependent itch in sunburn and chronic itch	(104–105)
	Sepsis	Pro-	Inhibition of channel significantly decreased systemic cytokines and maintained endothelial cell function	(9, 106)
TRPM3	Thermal Hyperalgesia	Pro-	Produces and augments TRPV1/TRPA1 heat-induced nociception in inflamed tissues and mediates CGRP release	(8, 47–49, 54)
TRPM8	Colitis	Anti-	Suppression of pro-inflammatory cytokine release in colitis model and diminishes TRPV1-mediated CGRP release	(7, 52, 107)
	Chronic Neuropathic Pain	Pro-/Anti-	Reduction of thermal and mechanical hyperalgesia, enhances cold hypersensitivity	(5, 108, 109)
	Asthma	Pro-	Increased pro-inflammatory IL-6 and IL-8 release in bronchial tissue	(110–111)
TRPA1	Headache/migraine	Pro-	Increased vasodilation and release of CGRP and SP producing migraine like behaviors	(112–113)
	Allergic Contact Dermatitis	Pro-	CGRP and SP release produces thermal or mechanical hypersensitivity and activation *via* co-localized G-protein coupled receptors	(4, 114–115)
	Acute Lung Injury	Pro-	Releases of pro-inflammatory neuropeptides (CGRP, SP, NKA), ROS, and triggers a cough reflex	(4, 64, 72, 116)
	Asthma	Pro-	Agonist stimulation can induce asthma, increasing bradykinin and ROS	(86, 117–119)
	Colitis/IBD	Pro-/Anti-	AITC administration induced pro-inflammatory IBD conditions/Diminishes histological damage through CGRP release, decreases pro-inflammatory cytokines and oxidative stress	(3, 4, 120–121)
	Arthritis	Pro-	Increases cold and mechanical hypersensitivity in CFA-induced arthritic models, antagonists reduces cartilage, edema, and SP release in paw	(122–123)
	Sepsis/Endotoxemia	Anti-	Attenuates disease severity through modulating release of cytokines IL-1β and IL-6 in mice and decreases serum TNF	(124–125)

antagonist, resulting in diminished pain behaviors in mice (126). Similarly, in a the trinitrobenzene sulfonic acid pre-clinical model of chronic pancreatitis TRPV1 mRNA and protein expression is increased, as is capsaicin-induced activation. This model also induces an increase in the proportion of capsaicin sensitive pancreas-specific DRG neurons, as well as the over expression of NGF, artemin, and GDNF (90, 128). The increased expression of NGF and the GDNF artemin may thus activate TRPV1 on intrapancreatic nerves resulting in the release of substance P (SP) and the maintenance of the disease state (129).

In the airways, enhancement of the coughing reflex in humans is associated with an augmented expression of TRPV1 channels on sensory nerves (91, 130). Inhalation of capsaicin has been linked to an increased cough sensitivity in patients with both asthma and chronic obstructive pulmonary disease (COPD; **Table 1**) (24, 131, 132). Similarly, TRPV1 expression and capsaicin sensitivity was increased in myelinated pulmonary afferents in the rat model of ovalbumin-induced airway inflammation (92). Due to the implication of TRPV1 in the initiation of cough, TRPV1 antagonists have been utilized as a treatment to effectively block this reflex (133–135). It has been proposed that TRPV1 activation on C-type fibers releases SP and calcitonin gene-related peptide (CGRP) neuropeptides to induce neurogenic inflammation and airway smooth muscle contraction, thus activating retinoic acid receptors (RARs) to elicit a cough response (136). In models of bacterial lung infections and pneumonia ablation, of TRPV1-positive nerves increase survival, cytokine induction, and bacterial clearance of Staphylococcus aureus pneumonia from the lungs. TRPV1-positive fibers of the vagus nerve in this model release CGRP inducing immunosuppression (137). In contrast, TRPV1 is protective in the LPS-induced model of lung injury. Depletion of TRPV1 causes increased disease severity, with elevated inflammation and bronchial hypersensitivity. When activated during a LPS-induced model of lung injury TRPV1-positive neurons release somatostatin (SST), which acts to diminish neurogenic inflammation (93). Treatment with TRPV1 agonists have additionally been beneficial in treating the ovalbumin-induced allergic airway inflammation. It is believed that SST and CGRP release decrease neutrophil influx and cytokine release (94).

In patients with inflammatory bowel disease (IBD), TRPV1 immunoreactivity is greatly increased in the colonic nerve fibers

(138). In a mouse model of IBD, dextran sulfate sodium (DSS)-consuming mice displayed an increase in pelvic afferent activity in response to capsaicin compared to normal mice (139). In gastro-esophageal reflux disease, patients have displayed increased TRPV1 fiber expression in their inflamed esophagus (140).

Similarly, in the skin, an increase in TRPV1 sensitivity and expression of TRPV1 is found in atopic dermatitis (**Table 1;** AD) (95). Phospholipase A2 and 12-Lipoxygenase activation of TRPV1 on histamine-sensitive C nerve fibers have been shown to lead to itching sensation (141). Interleukin 31 (IL-31), an inflammatory cytokine, also induces TRPV1-dependent itch, as cutaneous neurons and DRG neurons co-express the IL-31 receptor and TRPV1. TRPV1 deficient mice also display significantly reduced itching in the presence of IL-31 (96). Furthermore, PAC-14028, a potent TRPV1 antagonist, accelerates skin barrier recovery from tape-stripping-induced damage on hairless mice, as well as in both the *Dermatophagoides farinae* and oxazolone-induced dermatitis models. In addition to the accelerated function, PAC-14028 alleviates IgE increase, mast cell degranulation, scratching behavior, and dermatitis clinical severity (95).

In arthritis, inhibition of TRPV1 has identified it as a potential target for therapeutic interventions. In the complete Freund's adjuvant (CFA) pre-clinical model of arthritis, capsaicin depletion of TRPV1-positive cells, reduces arthritis severity and depletes neuropeptide levels. The depletion of these cells occurs due to a significant increase in intracellular calcium. TRPV1 responds to this influx by desensitizing itself to capsaicin activation, preventing cytotoxic amounts of calcium ions from entering the cell (142). In TRPV1 knockout (KO) mice, swelling of the knee joint and hyperpermeability were reduced. When TNF is directly injected into the knee joint, TRPV1 KO mice have decreased thermal hyperalgesia and joint swelling (97). Additionally, CFA induced arthritis causes a significant increase in TRPV1 expression on the overall proportion of unmyelinated nerves innervating the paw and on DRG neurons (143, 144).

In systemic inflammatory diseases such as sepsis, TRPV1 has an inconsistent role (**Table 1**) (20, 145). In a rat model of endotoxin induced sepsis, pretreatment with capsaicin increases anti-inflammatory cytokine IL-10 levels, and attenuation of CGRP, TNF, and interleukin 6 (IL-6) cytokines (98). In agreement with these findings blocking TRPV1 with capsazepine increases LPS induced hypotension, and mortality rates (146). TRPV1 KO mice further corroborate this, exhibiting elevated hypotension, hypothermia, cytokine levels, organ dysfunction, and mortality in mice with endotoxemia and polymicrobial sepsis *via* cecal ligation puncture (CLP) (99, 147). These studies suggest that TRPV1's anti-inflammatory role in sepsis, is in modulating nitric oxide (NO), ROS, and TNF (99, 145). However, a contradictory study has also shown that in the same CLP model of sepsis blocking TRPV1 activity with capsazepine attenuates systemic inflammation, multiple organ damage, and mortality (100). The inconsistency in these studies may be a result of capsazepine's dual ability to antagonize TRPV1 and agonize TRPA1 at similar concentrations, leading to a profound desensitization of not only TRPA1 but the nociceptive neuron (148).

TRPV1-positive sensory nerves innervating the heart have a beneficial role in cardiac inflammation. Genetic depletion of TRPV1 results in excessive inflammation, left ventricular remodeling, and deteriorated cardiac function after myocardial infarction in mice (149). Administration of a TRPV1 antagonist elevates myocardial damage in isolated wild-type hearts, suggesting that TRPV1 may have a protective effect in ischemia-reperfusion injury, with links to the release of SP (129). In ischemia-reperfusion injury CGRP and SP increase the release of anti-inflammatory IL-10, and reduce TNF level, lowering ROS and neutrophil infiltration (150).

The role of TRPV1 in inflammation is complex, with a dependence on disease and tissue-specific actions. Many preclinical studies utilize total body TRPV1 KO mice to elucidate its role in inflammation. However, given the sizable contrast in these findings, further study into the role of TRPV1 in mediating inflammation would benefit from selective knockdown or optogenetic manipulations in different cell types. It is posited that TRPV1 on sensory neurons plays a pro-nociceptive role in acute tissue injury, but an antinociceptive role in chronic conditions (20). Cell type-specific, particularly neuronal, TRPV1 modulation may prove useful in combating the extensive list of inflammatory diseases.

Despite the need for more comprehensive studies on understanding TRPV1 as a potential therapy, TRPA1 been regarded as a therapeutic target for pain and inflammation since the mid-20th century (101). Clinically, desensitization of TRPV1-expressing sensory nerves using high doses of capsaicin has been utilized as a treatment for patients with disorders such as psoriasis, osteoarthritis, cutaneous allergic reactions, pruritus, and peripheral neuropathy (151–153). Though capsaicin has demonstrated clinical benefits, in the 1990s there was a shift away from desensitization due to a common side effect, an intense burning sensation (154) with a prolonged effective duration (155). TRPV1 competitive antagonists, due to their reversible nature and lack of burning, then began to receive attention as potential anti-inflammatory and analgesic therapies.

Clinical trials of such agents have demonstrated varied results based on the disorder in question. TRPV1 antagonists have failed to show benefits in chronic cough and related disorders, such as COPD (156–158). Furthermore, these antagonists demonstrated no significant benefits for patients with seasonal allergic rhinitis (159). On the other hand, TRPV1 antagonists have recently demonstrated efficacy in molar extraction pain (160), mild-to-moderate AD (161), pain/stiffness in knee osteoarthritis (162, 163), and gastroesophageal reflux disease pain (164). Recently completed trials of these compounds examine their usage in patients with rosacea (NCT02583009), seborrheic dermatitis (NCT02749383), and skin pruritus (NCT02565134). Interestingly, many clinical trials utilizing TRPV1 antagonists reported that enrolled patients became hyperthermic after administration (19, 164–166), even to the point of trial termination (167). This significant side effect, related to

thermal sensitivity, has stalled clinical studies of TRPV1 antagonists (168, 169).

TRPV4

Similar to other TRP channels, different inflammatory molecules can affect the expression and signaling of TRPV4. Inflammatory cytokines such as IL-1β and interleukin 17 (IL-17) increase TRPV4 mRNA levels in DRG neurons (170) and NGF increases TRPV4 expression in the urothelium (171). Ischemia increases expression in astrocytes (172), while TNF, high-fat and high-alcohol diet (HFA) induce chronic pancreatitis leading to TRPV4 expression in pancreatic stellate cells (173). In patients with active colitis (**Table 1**), tissue samples indicate significant TRPV4 expression on nerve fibers innervating the outer layers of the colon (38). In addition, the inflammatory skin conditions papulopustular rosacea and phymatous rosacea facial, as well as COPD exhibit increased TRPV4 expression in the skin and lungs, respectively (174, 175).

The role of TRPV4 in inflammation has been extensively linked to the Protease-activated Receptor 2 (**Figure 2**; PAR2). PAR2 agonists activate and sensitize TRPV4 in DRG neurons. During intraplanar injection of PAR2 agonist mechanical hyperalgesia and increased pain sensitivity to TRPV4 agonists 4αPDD and hypotonic solutions is induced. 4αPDD and hypotonic solutions additionally stimulate the release of CGRP and SP, with increased sensitivity by the application of PAR2 agonists. Depletion of TRPV4 ablates this PAR2 agonist-induced mechanical hyperalgesia and sensitization (102). Such TRPV4-dependent sensitization is apparent in DRG neurons innervating the mouse colon (176). In addition, PAR2 activation can induce sustained activation of TRPV4 through the production of endogenous agonists, which are believed to increase the duration of PAR2's proinflammatory effects. This was confirmed using a PAR2-induced paw edema model. When TRPV4 is genetically deleted, paw edema is significantly reduced (103).

The role of TRPV4 in inflammation has not only been linked to PAR2, but to histamine and serotonin as well. Colonic DRG neurons pretreated with histamine and serotonin increased TRPV4 agonist 4α-Phorbol 12,13-didecanoate (4αPDD) induced neural firing *via* PKC, phospholipase C-beta, mitogen-activated protein kinase kinase, and PLA2-dependent pathways. By blocking TRPV4 using siRNA, visceral hypersensitivity induced by histamine or serotonin is significantly reduced, indicating a histamine- or serotonin-mediated response dependent upon TRPV4 in sensory neurons (177). Both serotonin and histamine have also been associated with the induction and exacerbation of pruritus' or itch responses. When administering serotonin intradermally, inhibition of TRPV4 through genetic deletion or pharmacologic block, reduced itch behavior significantly in comparison to control mice (104). This work has led to the idea that serotonin-evoked pruritus could be mediated by TRPV4 expressed on DRG neurons. In response to acute administration of histamine, no significant differences were found in itch behavior when TRPV4 is inhibited. However, in a chronic setting, histaminergic pruritogens induced itching behaviors were decreased when TRPV4 is blocked, specifically on keratinocytes. This may indicate TRPV4 has a different role in modulating an itch reflex based on the cell type it is expressed on (178). Additionally, TRPV4 has a direct link to itch *via* agonist application. Subcutaneous injection of GSK1016790A, a TRPV4 agonist for example, induces itching behavior in mice (179). Interestingly, in sunburn, in which itch is a common symptom, TRPV4 expression and proalgesic mediator endothelin-1 are enhanced in both humans and mice. Following sunburn, keratinocyte-specific TRPV4 KOs have decreased IL-6 release, with a decrease in the number of recruited neutrophils and macrophages (105). The mechanism for this release has been shown to involve TRPV4-mediated ATP production, stimulation of the P2Y11 receptor, and results in the release of IL-6 and interleukin 8 (IL-8) through the p38 mitogen-activated protein kinases-nuclear factor-κB signaling pathway (180).

In models of sepsis, inhibition of TRPV4 through genetic deletion or pharmacological block has been shown to be protective. In both LPS and CLP models of sepsis inhibition of TRPV4 significantly decreased systemic cytokines, maintained endothelial cell function, and reduced mortality in mice (9). However, there has been contrasting reports on the role of TRPV4 in sepsis, other studies have shown that inhibition has no significant effect on sepsis pathology (106). This inconsistency was found to be due to the antagonist dosage used. That is, excessively low or excessively high doses of TRPV4 agonists cannot effectively treat sepsis. It is hypothesized that a balance in TRPV4 activation is necessary for optimal improvement in sepsis severity (181).

TRPM3

TRPM3 is primarily linked to the induction of thermal hyperalgesia, a common tissue-level responses to inflammation. Endogenous TRPM3 agonists, such as the neurosteroid pregnenolone sulfate (PS) lowers the thermal response threshold of TRPM3, allowing activation at temperatures as low as 37°C (**Figure 2**) (47). In inflamed regions, this heat sensitization induces a thermal hyperalgesia commonly seen in inflammation. Injection of CFA is commonly used in models of peripheral inflammation and arthritis, in which thermal hyperalgesia often occurs. The role of TRPM3 role in thermal hyperalgesia has been demonstrated through behavioral studies utilizing TRPM3 KO mice. TRPM3 KO mice exhibit increased latency of withdrawal response to a heat test (47). Pretreatment with primidone, an established TRPM3 inhibitor, produces similar responses to the TRPM3 KO studies by preventing CFA-induced heat sensitization (116). Furthermore, TRPM3-deleted mice do not develop thermal hyperalgesia during inflammation, strengthening the channel's link to inflammatory pain signaling in response to heat (47).

Interestingly, a recent study investigating the CFA-induced model of peripheral inflammation through hind paw injection, revealed that inflammation significantly upregulated TRPM3 mRNA independent of temperature sensing TRP channels, TRPA1 and TRPV1, in DRG neurons innervating the inflamed tissue (8). Additionally, in this model, it has been demonstrated that TRPM3 can augment the responses of both TRPV1 and

TRPA1 (8). When isosakuranetin, a TRPM3 specific agonist, is applied to inflamed cutaneous tissue there is a simultaneous reduction in TRPM3 agonist responsiveness as well as a diminished responsiveness to capsaicin and mustard oil, both well-defined TRPV1- and TRPA1-agonists (8). This identifies a potential new role for TRPM3 in inflammation, with the upregulation of TRPM3 playing a key role in thermal hypersensitization (**Table 1**) of DRG neurons co-expressing TRPV1 and TRPA1. Together this is strong evidence that TRPM3 functions primarily as a heat-induced pain sensor in inflammatory conditions.

Viewing TRPM3 as a potential target for inflammation-associated thermal hyperalgesia, certain plant metabolite flavonoids, such as naringenin, ononetin, isosakuranetin, and FDA approved drugs, such as anticonvulsant primidone, may be viable therapeutic options. Thus far, these molecules have shown promise in blocking heat-dependent and PS-induced outward calcium currents within *in vitro* DRG neurons (116, 182). In behavioral experiments, flavanones, and primidone attenuated TRPM3 activation through PS-intraplantar injection by inducing increasingly latent responses to noxious heat (116, 182).

Endogenous regulation of TRPM3 is largely influenced by phosphoinositides (PIPs), such as phosphatidylinositol 4,5-bisphosphate (PIP2) and less abundant phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Whole-cell, patch-clamp recordings demonstrated that decreasing PIP2 concentrations inhibited TRPM3 activity and can be restored through ATP-dependent re-synthesis of phosphoinositides. The restoration process can be attributed to the synthesis of PIPs, in which the increased surge of cytosolic ATP stimulates kinase activity to synthesize PIPs which stimulate TRPM3 (183). Points of negative TRPM3 regulation through depleting PIP concentrations have been observed to occur through phosphatases, notably PIP 5-phosphatases. Antagonists of TRPM3 downregulate the inflammatory channel through the three major Gs, Gq, and Gi/o-coupled G protein-coupled receptors (GPCR), such as Gq-coupled M1 muscarinic, Gi/o-coupled opioid (μ) gamma-aminobutyric acid B, and Gs-coupled adenosine A2B receptors, have shown direct interaction of the Gβγ subunits with TRPM3 (184). Activation of these receptors with their agonists oxoremorine-M, DAMGO ([D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin), baclofen, and adenosine, respectively, strongly inhibited TRPM3 PS-evoked calcium response on DRG neurons (183, 185). However, it is important to note that the current studies observing the molecular regulation of TRPM3 measure channel activity primarily through PS stimulation, and the implications of these mechanisms modulating TRPM3 thermosensitivity requires additional investigation.

TRPM8

The key functionalities of TRPM8 in sensing both innocuous and noxious cold (8–26°C) have been shown to play a major physiological role in inflammation, thermoregulation, itch, and migraines (**Table 1**). Numerous studies examining the role of TRPM8 in inflammatory conditions, such as chronic neuropathic pain, noxious cold, and colitis, have demonstrated an upregulation of TRPM8 expression (7, 50–52). One mechanism by which the upregulation of channel expression affects inflammatory conditions is through mediating the release of inflammatory cytokines. Prolonged cold stress (4°C) triggers an increase in rodent hypothalamic TRPM8 expression and corresponds to the decrease of pro-inflammatory cytokine TNF (52). Evidence suggests cytokine regulation through TRPM8 occurs through its interactions with nuclear factor kappa-light chain-enhancer of activated B cells (NFκB), the nuclear import receptor controlling TNF levels. This mechanism could mediate how TNF levels are decreased in response to cold-stress and menthol (52). TRPM8 can also mediate inflammation through crosstalk with other TRP channels. Numerous studies (6, 7, 186) have exhibited how TRPM8 activation suppresses TRPV1-mediated inflammatory neuropeptide, CGRP, release. TRPA1, which also plays a role in the release of inflammatory neuropeptides and pain hypersensitivity during inflammation, has also been observed to become desensitized to exogenous irritant and agonist AITC on sensory neurons that have been pre-treated with icilin. This was confirmed in TRPA1 KO mice, in which icilin induced neuronal activation of the splanchnic nerve is unchanged when compared to wild type mice (107, 186). Thus, TRPM8 may serve an anti-inflammatory function to balance the pro-inflammatory responses of TRPV1 and TRPA1, mediating chemosensory deactivation and inflammatory neuropeptide release.

In preclinical murine models of colitis, TRPM8 appears to regulate inflammation through direct mediation of inflammatory cytokines. Significant upregulation of TRPM8 expression was observed in both chemically induced colitis within mice (trinitrobenzene sulfonic acid; TNBS– and DSS-treatment) and non-inflamed colonic tissue from Crohn's disease patients (7). Macroscopically, the simultaneous treatment of TRPM8 agonist icilin with TNBS and DSS appeared to substantially diminish colitis-associated histological damage in comparison to TNBS and DSS treatment alone (7). Profiling the effects of icilin on the cytokine distribution revealed a significant reduction in pro-inflammatory cytokines and chemokines, chemokine (C-X_motif) ligand 1, IL-6, monocyte chemoattracted protein-1, IL-1α, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and interleukin 12 p40, which likely contributed to attenuated histological damage (7, 187). In mediating CGRP release of other TRP channels, TRPM8 modulation appears to also play a pro-nociceptive role in colonic mechanosensitivity alongside TRPA1 and TRPV4. Pharmacological blockage using AMTB and genetic KOs of the channel produced significant inhibition of distension-induced CGRP release at high pressure (150 mmHg), whereas TRPA1 and TRPV4 inhibition produced a significant attenuation at a lower pressure (90 mmHg) (63). This suggests that TRPM8 works in concert alongside TRPA1 and TRPV4 in controlling CGRP-mediated, colonic mechanosensitivity, however, signals pain at extreme noxious distension levels. The aforementioned crosstalk with TRPV1 appears to be potentially relevant in TRPM8's regulation of colitis. Capsaicin activation of TRPV1 is known to significantly elevate CGRP levels within the colon, however, prior activation of TRPM8 has been shown to attenuate this response (7). Additionally, icilin treatment and menthol

enemas both attenuate TRPV1-mediated release of CGRP in both healthy colonic tissue stimulated with capsaicin and inflammation-induced release (7, 187).

In neuropathic injury models, there has been evidence that TRPM8 plays a role in decreasing mechanical allodynia and thermal hypersensitivity while simultaneously enhancing cold sensitivity. Behavioral experiments generally demonstrate how neuropathic injury models, such as chronic constriction injury (CCI) of the sciatic nerve or spinal nerve ligation (SNL), contributes to cold hypersensitivity post-treatment (5, 108, 109). An original study posited that cold allodynia after nerve injury occurred independently of TRPM8 activity, as the decrease in TRPM8 mRNA levels post-CCI surgery did not correlate with sustained levels of cold hypersensitivity (108); however, more recent experiments have all noted a positive correlation in elevated TRPM8 expression and cold hypersensitivity. Elevated TRPM8 expression in these studies were detected through western blot and mRNA analysis (5, 108, 109). These discrepancies could be attributed to a lack of functional TRPM8 measurements, mRNA levels do not necessarily represent TRPM8 protein or functional expression. TRPM8 has also been suggested to modulate the reflex sensitization to thermal and mechanical stimuli in nerve injury. Injection or topical application of menthol can trigger nociceptive pain in neuropathically injured rodents but is also known to attenuate thermal hyperalgesia and mechanical allodynia (5). Intrathecal injection of TRPM8 antagonist AMTB produces the opposite effect in CCI rodents, causing an increase in thermal hypersensitivity and a decrease in cold sensitivity (109). As a therapeutic measure, TRPM8 activation or inhibition to produce a satisfactory analgesic response is case-dependent on the type of temperature-induced nociception. It has been proposed that low concentrations of menthol may be appropriate to produce sufficient analgesic responses without evoking pain (108).

As with many TRP channels, TRPM8 does not have only anti-inflammatory properties but pro-inflammatory properties as well. For example, asthmatic patients have an upregulation of TRPM8 expression in bronchial epithelial cells and sputum (110, 188). Unlike the analgesic effects produced in colitis or nerve injury, TRPM8 function in the lungs triggers bronchial inflammation through prolonged cold air inhalation. These responses can be replicated in models of pulmonary cold exposure and menthol treatment, causing a significant increase in IL-6, IL-8, and interleukin 25/thymic stromal lymphopoietin receptor (TSLP) mRNA expression (110, 111).

Notable advances in the inclusion of TRPM8 as a potential therapeutic target have been made recently through both chemical antagonists and molecular inhibition through GPCRs. GPCRs known to mediate TRPM8 functionality (e.g., bradykinin and histamine receptors) have been noted to do so through two mechanisms. Continuous activation of phospholipase C by G-α-q depletes PIP2 concentrations in the membrane, which leads to the inhibition of TRPM8-mediated currents (189, 190). The agonistic effect of PIP2 on TRPM8 has been seen through the restoration of TRPM8 in PIP2-depleted membranes through the addition of an aqueous PIP2 analog and activation through high concentrations of PIP2 at warm temperatures 37°C (190). Conclusions are drawn from the inhibition of TRPM8 through PIP2 depletion may likely serve as a method of desensitization/adaptation to continuous cold stimuli in the environment (190). Direct interactions with the G-α-q subunit are an additional mechanism responsible for TRPM8 thermosensitivity in peripheral sensory neurons (191). G-α-q KOs, lowered the TRPM8 potentiation threshold to higher temperatures and increased TRPM8-dependent firing rates in cold conditions, without interfering with PIP2 hydrolysis, indicating the direct inhibitory activity of the G-α-subunit (191, 192). Additional chimeric experiments of G-α-q revealed effector binding sites directly on TRPM8 affirming the direct inhibitory activity for TRPM8 activation (191, 192).

Certain inflammatory mediators can serve as inhibitors of TRPM8 due to their G-α-q-linked GPCRs. Inflammatory mediators possessing Gαq-linked receptors, such as bradykinin and histamine, have shown to inhibit TRPM8-mediated responses to cold temperatures and enhance heat responses on cold-sensitive peripheral fibers. Notably, this response occurred alongside the inhibition of numerous downstream effector proteins, including PKC and phospholipase C, suggesting the independent inhibition of TRPM8 by activated G-α-q (192). This response was not seen in G-α-q KOs, demonstrating G-α-q as a crucial step in gating TRPM8. Additionally, the presence of activated G-α-q desensitizes TRPM8 to positive regulator PIP2, creating a synergistic mechanism to enhance TRPM8 inhibition *via* PIP2 depletion (193). Depending on the inflammatory context, certain conditions that mediate the release of G-α-q-linked inflammatory chemicals possess the ability to counter regulate the anti-inflammatory functions of TRPM8. Thus, we note that multiple mechanisms exist to regulate TRPM8 activity, however, conditions that trigger G-α-q activity may result in reduced TRPM8-dependent anti-inflammatory responses.

Multiple efforts toward the identification of selective TRPM8 antagonists for treating inflammation, chronic pain, and cold-hypersensitivity have led to potential candidates but none have reached clinical settings. Various molecular candidates (arylglycine derivative, benzothiophene-derived phosphonate esters, benzimidazole variants) have been identified to strongly inhibit TRPM8-mediated currents in *in vitro* calcium flux assays, as well as suppress *in vivo* icilin-induced "wet-dog shaking" behavior in a dose-dependent manner (194–196). Among these small molecule inhibitors, studies relating to the benzimidazole derivatives generated greater than 80% inhibition in neuropathic CCI models for cold allodynia in a dose-dependent manner (195, 196). Another noted side effect related to the application of these antagonists is the lowering of core body temperature. Menthol and icilin treatment naturally raise the core body temperature, and certain antagonists have been shown to cause hypothermia by counteracting TRPM8 activation (197, 198). Overall, current drug candidates have been demonstrated to be potent inhibitors of TRPM8 activity through basic *in vitro* and *in vivo* assays, with potential therapeutic implications in inflammation induced cold-induced nociception and allodynia.

TRPA1

TRPA1 has been shown to have a prominent role in inflammation, both through its expression which can be modulated by inflammatory mediators, and as a regulator of inflammatory signaling. TNF, a well-defined pro-inflammatory cytokine, upregulates the trafficking of TRPA1 to the cell membrane, increasing its membrane expression in peripheral nerves (85). Additionally, when exposing neuronal and epithelial cell lines to viruses such as rhinovirus, respiratory syncytial virus, and measles, both TRPA1 protein and mRNA expression are upregulated (199, 200). Interestingly, neutralizing IL-6 and IL-8 in viral cocultures with neurons blocks the upregulation of TRPA1, indicating that both IL-6 and IL-8 may also play a direct role in increasing TRPA1 expression. In the cerulean-induced pancreatic inflammation model, TRPA1 expression is upregulated on pancreatic sensory nerves (201). Similarly, TRPA1 expression is increased in CFA and nerve injury models of inflammation (202). In many of these models, the upregulation and activation of TRPA1 is accompanied by the release of the generally pro-inflammatory neuropeptides, CGRP, SP, and neurokinin A (NKA) (57, 203). Activation of TRPA1 on TG nerves *via* environmental irritants contributes to this release of SP and CGRP along with increased meningeal vasodilation, which are implicated in migraine pathophysiology (112, 204). Many TRPA1 agonists that are linked to the release of SP and CGRP have also been shown to induce migraine or headache behaviors (113, 205). Interestingly, TRPA1 agonists have not only demonstrated pro–inflammatory properties, but anti-inflammatory as well depending on location and disease context.

Many TRPA1 agonists have been shown to induce a local inflammatory response when topically applied to skin. AITC, the molecule responsible for the pungent taste of mustard, horseradish, and wasabi, for example induces the release of CGRP and SP, causing thermal and mechanical hypersensitivity, also known as an increased pain sensitivity, associated with inflammation (4). Cinnamaldehyde, the molecule that gives cinnamon its flavor and odor, also induces acute skin inflammation when applied topically. Mice receiving topical cinnamaldehyde exhibit edema formation and dermal leukocyte infiltration (206). Additionally, many contact dermatitis reactions are TRPA1 mediated. Xylene and toluene are common solvents that can induces a significant inflammatory response of edema and pain when exposed to skin. However, when the skin of TRPA1 deficient mice were exposed to xylene or toluene, the inflammatory response was ablated, and when wild-type mice were treated with a TRPA1 antagonist orally (HC-030031) the inflammation of the skin was significant reduced (207).

As previously mentioned, itch is a common symptom of skin irritation and dermal inflammation associated with different conditions, such as allergic contact dermatitis (**Table 1**; ACD), AD, and psoriasis. Itch warns against harmful environmental irritant, and the urge to scratch is an evolutionary mechanism to remove irritants from the affected area, characterized by swelling and infiltration of immune cells such as lymphocytes (114). In histamine-independent itch, it has been found that itch related GPCRs activate TRPA1 to initiate a local inflammatory response. These itch GPCRs (G protein-coupled bile acid receptor 1, TSLP, MAS-related G protein coupled member A3, and MAS-related G protein coupled member C11) co-localize with TRPA1 on cutaneous sensory nerves (115, 208). In ACD, a major obstacle is histamine-independent inflammation. ACD is a common inflammatory skin condition caused by hypersensitivity to allergens (114, 209). In both acute and chronic models of murine contact dermatitis, symptoms of ACD were significantly decreased with both pharmacological inhibition as well as genetic ablation of TRPA1. When inhibiting TRPA1, it was found that local levels of proinflammatory cytokines interleukin 4, IL-6, and chemokine (c-x-c motif) ligand 2 were decreased along with dermatitis scores, edema, swelling, and T cell infiltration (209). In psoriasis ROS and RNS play a critical role in its pathology inducing oxidative and nitrosative stress activating TRPA1 channels on sensory nerves innervating the skin, causing the release of SP and CGRP. In a preclinical model of psoriasis, TRPA1 antagonists significantly inhibited itching, however it Is important to note that long term treatment with a TRPA1 antagonist or TRPA1 deletion is actually associated in increased psoriasis skin phenotype (210).The differences in long term vs. short term treatment may be linked to TRPA1 on other cell types, as additional studies have shown a role for TRPA1 on immune cells (71, 210–212).

Similar to its role in dermal inflammation, TRPA1 plays a key role in pulmonary inflammation. TRPA1 is expressed on afferent vagus neurons, specifically located on Aδ-, and C-fibers, which densely innervate the lungs. Noxious irritants in the air such as heavy metals, general anesthetics, cigarette smoke, and tear gas have been shown to activate TRPA1. Once activated, these TRPA1-positive neurons locally release of CGRP, SP, and NKA to induce inflammation, bronchoconstriction, vasodilation, and infiltration of immune cells (4, 64, 72). In addition, pulmonary TRPA1 stimulation can induce airway reflex responses such as coughing (117). Experimentally, multiple exogenous TRPA1 agonists have been used in animal and human models of cough such as, citric acid, cinnamaldehyde, and AITC. These responses are dose dependent and significantly reduced with the inhalation of TRPA1 antagonist HC-030031 (117, 213, 214). Endogenous TRPA1 agonists are also known induce cough, which occurs in response to tissue inflammation in conjunction with diseases such as asthma.

In asthmatic patients, the airway is hyperreactive and can cause bronchoconstriction. Many TRPA1 agonists listed above can induce asthma, such as cigarette smoke and the leading cause of occupational asthma, toluene diisocyanate is a strong TRPA1 stimulant. Endogenous activators of TRPA1 have also been shown to induce asthma. Asthmatic lungs exhibit increases in bradykinin, 4-hydroxynonenal and ROS, which are all TRPA1 agonists. This increase in ROS in turn leads to elevated oxidative stress (86, 117, 215–217). In murine and rat, ovalbumin-induced models of asthma, a decrease in late asthma response symptoms is seen post TRPA1 antagonist treatment (118). In TRPA1-deleted mice, airway infiltration of leukocytes is significantly reduced, as are the levels proinflammatory cytokines interleukin 5, interleukin 13, and TNF (4, 118, 119, 217).

Unlike its role in dermal and pulmonary inflammation, TRPA1 has been found to have contradictory pro- and anti-inflammatory roles in gastrointestinal inflammation. TRPA1 is widely expressed in the gut, with functional expression found on neurons innervating the intestine and mucosal endocrine cells. In patients with Crohn's disease (CD) and ulcerative colitis (UC), as well as preclinical models of IBD TRPA1 expression is upregulated in colonic tissue (3). In animal models of colitis, TRPA1 has an inconsistent role inflammation. For example, genetic deletion of TRPA1 has been found to both decrease and aggravate the disease (120, 212, 218), while blocking TRPA1 with an antagonist and activating with an agonist can be protective (120, 218). These contradictory occurrences may be due the opposing inflammatory effects of GCRP and SP in the gut. Activation of TRPA1 on afferent nerve fibers innervating the colon as well as DRG and NG neurons induce the release both CGRP and SP. However, in the gut the role of CGRP in inflammation is reversed compared to other locations of inflammation. In experimental models of colitis, it is widely accepted that CGRP is protective and a lack of CGRP increases the susceptibility to spontaneous colitis, as well as experimentally induced colin damage. A local block of the receptor for CGRP, calcitonin receptor-like receptor, also increases colitis severity (4, 10, 121, 219). Different IBD models as well as the stage of the disease could also be the source of these contradictions, AITC induced IBD, indicates TRPA1 has having pro-inflammatory properties, as does the TNBS induced model of colitis (3, 10, 220, 221). The DSS-induced model of colitis has also shown contrasting results, with TRPA1 genetic deletion or treatment with antagonists increasing disease severity in some cases, but alleviating disease severity in others (212, 221). In addition, treatment with cannabiderol and cannabidivarin, both non-psychotropic cannabinoids, and TRPA1 agonists have shown anti-inflammatory effects, decreasing disease severity in the dinitrobenzene sulfonic acid model of IBD (3, 222, 223). Other TRPA1 agonists have also found to be anti-inflammatory when used to treat intestinal inflammation. Carvacrol and carvacryl acetate were used as effective treatments of intestinal inflammation, decreasing levels of pro-inflammatory cytokines, and oxidative stress. Blocking carvacrol and carvacryl acetate treatment with a TRPA1 antagonist, reversed these beneficial effects (224, 225). Overall, TRPA1 is an interesting target for treating gastrointestinal inflammation, however, more studies are needed to fully understand its role in this context.

In models of arthritis, TRPA1 has been linked both to joint inflammation and hyperalgesia (**Table 1**). In many studies using monoiodoacetate and CFA models of arthritis, genetic depletion of TRPA1 or antagonist inhibition, have resulted in significant reduction in cold and mechanical hypersensitivity (122, 123, 226–228). In arthritis-induced joint edema, erosion, and inflammation, TRPA1 does not have a well-defined role, with conflicting results reported. In some studies, TRPA1 was not found to have a prominent role in knee joint swelling or paw edema as genetically deleting TRPA1 and blocking with an antagonist had no effect on TNF and CFA induced joint swelling (122). In contrast, other studies have also shown that either genetic deletion or blocking of TRPA1 reduces changes in cartilage, edema, and SP release in the paw (123, 227–229). Although the extent of the TRPA1 channel contribution to arthritis is not conclusive, the current evidence shows the potential for further study to understand and potentially target TRPA1 as an arthritis therapy.

Unlike the previous inflammatory disease models, with localized regions of inflammation, models of systemic inflammation have shown TRPA1 to be generally protective. Treatment with TRPA1 agonists, cinnamaldehyde, AITC, fentamate non-steroidal anti-inflammatory drugs, and carnosol have all been shown to be protective in inflammation. In the CLP model of sepsis, blocking TRPA1 increases disease severity as well as the levels cytokines IL-1β and IL-6 in mice (**Table 1**) (124). In an LPS induced model of inflammation, oral administration of cinnamaldehyde deceases NO, TNF, high mobility group box protein 1, interleukin 18 and other inflammatory mediators in serum and plasma (230, 231). Cinnamaldehyde can also attenuate apoptosis and promote neuronal survival in DRGs due to oxidative stress, by inhibiting NFκB and decreasing ROS (232). Furthermore, direct activation of TRPA1 on the cervical vagus nerve by the optopharmacological molecule optovin reduces systemic inflammation induced by LPS by significantly decreasing serum TNF (125). Interestingly, LPS itself has recently been found to directly activate TRPA1 directly on sensory neurons, inducing a calcium influx, along with vasodilation, pain, and the release of CGRP in a TRPA1 dependent manner (73).

Based on many of the preclinical discoveries previously mentioned, TRPA1 has been increasingly identified as a potential therapeutic target for pain and inflammation. Between 2015 and 2019, twenty-eight patent applications were filed for TRPA1 antagonists, many of which were aimed at treating pain, airway respiratory diseases, and dermatological disorders. Currently, to our knowledge, only five of the twenty-eight patented molecules have gone on to clinical trials, with only a few making it to phase II and none reaching phase III. Translation of TRPA1 antagonists from pre-clinical to clinical results remains challenging, some have shown strong activity in humans with reduced or no antagonist activity in mice or rats. Additionally, as TRPA1 is expressed in a wide variety of tissues with varying roles in inflammation, this makes it difficult to determine safety and efficacy without very targeted approaches (233, 234). Current preclinical models lack tissue specificity for *in vivo* modeling. Similar to TRPV1 preclinical studies, many preclinical models utilize whole-body genetic deletion when evaluating the role of TRPA1 in inflammation. Additional studies looking into tissue-specific genetic modification and activation would greatly benefit in identifying the potential role of TRPA1 as a therapeutic target in inflammation. Despite these challenges, TRPA1 remains a key target for treating inflammation with the potential for more disease and tissue-specific targeted therapies.

CONCLUSIONS

The broad selectivity and polymodal nature of TRP channels make them critically important in sensory transduction and integration. Their presence on various immune cells (e.g., macrophages, T-cells) and wide distribution across sensory nerves (**Figure 1**) suggests that they may be important conduits for neural-immune crosstalk. Moreover, because their activation often leads to the release of neuropeptides linked to neurogenic inflammation (**Figure 2**), their expression on peripheral nerve and on epithelial cells may be key factor in a wide range of conditions and inflammatory disorders that we have discussed in this review including sepsis, arthritis, asthma, colitis, pain, and dermatitis (**Table 1**).

Interestingly, there is some discrepancy with the understanding of TRP channels role in many of these inflammatory disorders. Both role and specificity have been disputed which could be influenced by multiple variables. First, in preclinical disease models, the availability of multiple different models for an individual disease adds to the variability of TRP channel contributions in different disease contexts. Second, there is a lack of appropriate genetic models for many of these diseases. Given the broad range of tissue expression and polymodal activation of TRP channels, utilizing whole-body genetic deletion models often results in differing outcomes, when compared to cell-type or tissue-specific genetic manipulations. As observed with TRPV1 and TRPA1, the site of the inflammation in addition to how widespread it is (local vs. systemic) influences its pro- or anti-inflammatory function (**Table 1**). Cell and region-specific KO models would greatly enhance our understanding of how TRP channels modulate inflammation, which would additionally allow for better targeted therapeutics. In addition, as TRP channels are activated by a wide range of agonists, both exogenous and endogenous, it is important to understand how inflammatory responses differ depending on disease states and depending on the location within the body. An emerging area of interest within TRP channel research is focused on how the nervous system deciphers immune-related signals to initiate, trigger, and modulate inflammation. TRP channels, including those discussed in this review, play an important role in this communication between the nervous and immune systems. As the field advances to discover more about the mechanisms and roles of TRPV, TRPM, and TRPA channels, it will be exciting to see if targeted drug development based on a better mechanistic understanding can provide therapies to treat the assortment of conditions involving TRP channels.

AUTHOR CONTRIBUTIONS

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Pharmaco-Optogenetic Targeting of TRPC Activity Allows for Precise Control Over Mast Cell NFAT Signaling

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Canonical transient receptor potential (TRPC) channels are considered as elements of the immune cell Ca^{2+} handling machinery. We therefore hypothesized that TRPC photopharmacology may enable uniquely specific modulation of immune responses. Utilizing a recently established TRPC3/6/7 selective, photochromic benzimidazole agonist OptoBI-1, we set out to test this concept for mast cell NFAT signaling. RBL-2H3 mast cells were found to express TRPC3 and TRPC7 mRNA but lacked appreciable Ca^{2+} /NFAT signaling in response to OptoBI-1 photocycling. Genetic modification of the cells by introduction of single recombinant TRPC isoforms revealed that exclusively TRPC6 expression generated OptoBI-1 sensitivity suitable for opto-chemical control of NFAT1 activity. Expression of any of three benzimidazole-sensitive TRPC isoforms (TRPC3/6/7) reconstituted plasma membrane TRPC conductances in RBL cells, and expression of TRPC6 or TRPC7 enabled light-mediated generation of temporally defined Ca^{2+} signaling patterns. Nonetheless, only cells overexpressing TRPC6 retained essentially low basal levels of NFAT activity and displayed rapid and efficient NFAT nuclear translocation upon OptoBI-1 photocycling. Hence, genetic modification of the mast cells' TRPC expression pattern by the introduction of TRPC6 enables highly specific opto-chemical control over Ca^{2+} transcription coupling in these immune cells.

Keywords: canonical transient receptor potential channels, mast cells, opto-chemical immunomodulation, NFAT nuclear translocation, photopharmacology, OptoBI-1

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INTRODUCTION

Photopharmacology and optogenetics have emerged as experimental strategies that allow for exceptionally precise interference with tissue functions (1, 2). These technologies have proven particularly successful in elucidating basic principles of communication within complex signaling networks and are suggested as a prospective basis for light-mediated computer-cell/tissue interfaces in the context of synthetic biology (3). So far optogenetic and chemo-optogenetic have unequivocally made significant contributions to our understanding of neuronal circuits and provided important insights into the complex orchestration of immune reactions. Moreover, the

feasibility of optogenetic manipulation of immune responses has repeatedly been demonstrated in a therapeutic context (4–7).

We have recently developed a new photopharmacological tool that allows for specific, light-assisted control over TRPC3/6/7 conductances in native tissues (8). Here we set out to test the suitability of this new approach for modulation of immune responses, using the well-characterized RBL-2H3 mast cell model. Mast cells are tissue-resident and confer innate and adaptive immune reactions, thereby playing a pivotal role in allergic disorders, cancer, and autoimmune diseases (9). Mast cells mediate IgE-dependent allergic reactions by release of inflammatory mediators *via* degranulation, production of inflammatory lipids, and production of cytokines (10) and have long been recognized as a critical component of the tumor microenvironment in a number of cancer types (11–13). Based on the complex and essentially two-faced function of these tumor resident immune cells (12), therapeutic targeting of these cells requires uniquely specific approaches. High precision, local modulation of tumor-resident immune cells might represent a novel strategy for adjuvant immunotherapy in cancers (13). One approach to achieve sufficient specificity of immunomodulation is based on the finding that Ca^{2+} downstream signaling is strictly dependent on temporal signaling features (14, 15). Hence, the controlled sculpturing of immune cell Ca^{2+} signals is expected to enable control over immune responses in a uniquely specific manner. So far, the therapeutic modulation of immune cell functions by light has focused mainly on the major player in immune cell Ca^{2+} handling, the STIM/Orai Ca^{2+} entry complex (7). Nonetheless, other Ca^{2+} signaling elements may similarly serve as suitable targets for optical approaches. Although many other plasma membrane transporters and channels, including K^+ channels and TRP channels are reportedly critical for immune cell activation (16–18), these molecular players have so far not been considered and tested as targets.

For canonical transient receptor potential (TRPC) channels a contribution to Ca^{2+} signaling in immune cells has repeatedly been suggested (19). While several studies provide evidence for a contribution of TRPC1 and TRPC5 proteins to store-operated calcium signaling in rat (RBL-2H3) and mouse bone marrow-derived mast cells (BMMC), the exact function of TRPC isoforms in mast cells remains largely elusive (20–22). Interestingly, TRPC3/6/7 protein complexes were found to interact with fyn kinase during FcεRI-mediated mast cell activation (23). However, a more recent study argues against the contribution of TRPC3/6 channels to FcεRI-mediated Ca^{2+} signaling in primary human lung mast cells as well as in LAD2 human mast cells, suggesting exclusively Orai but not TRPC may be considered as a target for the control of mast cells in allergic disease (24). Nonetheless, in clear contrast to a lack of linkage to FcεRI stimulation, TRPC1, TRPC4, and TRPC6 proteins have been shown to confer downstream signaling in Mrgprb2-mediated mast cell activation of murine peritoneal mast cells (25). Thus, the efficacy and specificity of TRPC generated Ca^{2+} signals in terms of their coupling to downstream effectors might be strictly dependent on the mechanism and mode of activation.

In the present study, we explored the functional consequences of direct, lipid-independent control of TRPC3/6/7 conductances in RBL-2H3 mast cells by a new photochromic benzimidazole agonist (OptoBI-1). We report that overexpression of the TRPC6 isoform, and thus specific modification of the TRPC expression pattern of RBL-2H3 cells, allows for efficient and temporally precise control over NFAT1 signaling by light. Our results provide the first proof of concept for efficient chemo-genetic targeting of mast cell Ca^{2+} signaling and transcriptional regulation based on TRPC photopharmacology.

METHODS

Reagents and Construct

All reagents used were of molecular biology grade, purchased from Sigma-Aldrich Handels GmbH unless specified otherwise (Vienna, Austria). CMV-R-GECO1.2 construct (#45494) was obtained from Addgene. The following constructs were generated: YFP-TRPC3, CFP-TRPC3, YFP-TRPC6, CFP-TRPC6-hTRPC3 (Q13507-3) and hTRPC6 (Q9Y210-1) genes were cloned into peYFP-C1 and peCFP-C1 vectors using EcoRI/XbaI cloning sites. The mCherry-NFAT1 was prepared by replacing CFP by mCherry in a CFP-NFAT (in peCFP-C1 vector) construct. The TRPC7-mseCFP construct (Q9WVC5-1 cloned into pCI-neo vector) was kindly provided by Prof. Yasuo Mori (Kyoto University, Japan). OptoBI-1 was synthesized at Bio-Techne (Bristol, GB).

Cell Culture and Genetic Manipulations

All experiments were performed in rat basophilic leukemia cells (RBL-2H3) cells, and no special ethical considerations were applied. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/ml), penicillin (100 U/ml), L-glutamine (2 mmol/L), and HEPES (10 mmol/L). Cells were maintained in an incubator at 37°C, 5% CO_2. Plasmids were delivered into RBL-2H3 cells by electroporation in a 4-mm electroporation cuvette (Bio-Rad, US), by adding plasmid (5 µg mCherry-NFAT1 and R-GECO; 20 µg TRPC constructs) to 4×10^6 cells in 400 µl Opti-MEM® and applying 200 V and 950 µF in a Gene Pulser II (Bio-Rad, UK). After electroporation cells were transferred from the cuvette onto coverslips in a 35 mm dish containing cell culture medium. Experiments were performed 16–24 h after electroporation.

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from cell lysates using EXTRACTME TOTAL RNA KIT with 1% ß-Mercaptoethanol (Blirt) and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in a thermal cycler (Bio-Rad) according to the manufacturer's protocol. qPCR was performed using GoTaq® qPCR and RT-qPCR Mix (Promega) in a LightCycler C1000 Thermal Touch, Thermal Cycler CFX96™ Real Time System (Bio Rad). Relative

expression of the target gene was normalized to rat *ß-Actin* as a reference gene. Specific Primers used for rat Trpc genes are listed below.

CDS TRPCs	Accession number	Orientation	Primer sequence (5'-3')
rTrpC1	NM_053558.1	Forward	AGGTGACTTGAACATAAATTGCGT
		Reverse	TCCATAAGTTTCTGACAACCGT
rTrpC2	NM_022638.3	Forward	ATCCCCTTTCGCCCAACTG
		Reverse	ATCGGAGCCTGATTCCAGCAG
rTrpC3	NM_021771.2	Forward	GACGCAGTACGGCAACATCC
		Reverse	ACCTCCAGATGCTCATTGCC
rTrpC4	NM_080396.1	Forward	AAACGAAATGTCAACGCCCC
		Reverse	TCTCGGCTTCCTCCAGAGAT
rTrpC5	NM_080898.2	Forward	CATCGAGATGACCACAGCGA
		Reverse	GGGAAGCCATCGTACCACAA
rTrpC6	NM_053559.1	Forward	GTGAACGAAGGGGAGCTGAA
		Reverse	GCGGCTTTCCTCTTGTTTCG
rTrpC7	NM_001191691.2	Forward	GGGGTCCTGCCTACATGTTC
		Reverse	CCCATGTAGTCCACGCAGTT
rActin		Forward	CGATATCGCTGCGCTCGT
		Reverse	ATACCCACCATCACACACCCTG
rGapdh	NM_017008.4	Forward	CCTTCTCTTGTGACAAAGTGGACAT
		Reverse	GCTTCCCATTCTCAGCCTTGA

Electrophysiology

Whole-cell electrophysiology was performed at room temperature. RBL-2H3 cells were seeded on coverslips 24 h prior to the experiments. Coverslips were mounted in a cell bath on an inverted Axiovert 200 microscope (Zeiss). YFP-TRPC3, YFP-TRPC6 and TRPC7-CFP transfected cells were identified by their green and blue fluorescence when illuminated at 500 nm or 436 nm using Oligochrome light source (FEI, Germany). Whole-cell measurements were performed using an Axopatch 200B amplifier (Molecular Devices) connected with a Digidata-1440A Digitizer (Axon Instruments). Signals were low-pass filtered at 2 kHz and digitized with 8 kHz. The application of linear voltage-ramp protocols ranging from −130 to +80 mV (holding potential 0 mV) was controlled by Clampex 11.0 (Axon Instruments) software. Currents at −90 and +70 mV were plotted against time and normalized by capacitance. Maximal current–voltage relationships from −130 to +80 mV were subtracted and normalized by capacitance. Illumination protocol for photopharmacological measurements was applied as for Ca^{2+} imaging. Patch pipettes were pulled from thin-wall filament glass capillaries (Harvard Apparatus, W3 30-0068), using a Sutter Instruments P1000 puller and BOX filaments FB245B to a resistance of 2–4 MΩ. Cells were kept in Tyrode solution during the experiments. Pipette solution contained (in mM): 120 cesium methanesulfonate, 20 CsCl, 15 HEPES, 5 MgCl2, 3 EGTA, titrated to pH 7.3 with CsOH. The osmolarity of all solutions was between 290 and 315 mOsm.

$[Ca^{2+}]_i$ Imaging

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were monitored using red-shifted genetically encoded Ca^{2+} sensor (R-GECO1.2). Briefly, RBL-2H3 cells overexpressing R-GECO with YFP-TRPC3, YFP-TRPC6 or TRPC7-CFP, were washed twice with experimental buffer (EB composition in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose and 1 $CaCl_2$ (pH 7.4, adjusted with NaOH). Coverslips were mounted in a cell bath containing EB and 10 μM OptoBI-1 on an inverted microscope (Olympus IX71, Germany) with 40 × 1.3 N.A. oil-immersion objective. Cells were excited to follow the R-GECO signal using 577/25 nm filters *via* TILL Oligochrome light source (FEI, Germany) and fluorescent images were captured every second at 632 nm (using 632/60 nm emission filter, Chroma Technology, VT, USA) with an ORCA-03G digital CCD camera (Hamamatsu, Japan) using Live Acquisition 2.6 software (FEI, Germany). The *cis* isomerization of OptoBI-1 compound was triggered by exposure to 10 s illumination period at 365 nm, and subsequent reversal of OptoBI-1 to *trans* conformation was achieved with 430 nm light exposure to 10 s period. The cycling of UV (365 nm; violet) and blue light (430 nm; blue) illuminations were repeated three times. The interval between each illumination cycle was 60 s. All experiments were performed at room temperature. Detailed photocycling protocol is described in (26).

NFAT Nuclear Translocation

Translocation of NFAT in mCherry-NFAT1 used as a control and YFP-TRPC3, YFP-TRPC6 and TRPC7-CFP overexpressed in RBL-2H3 cells was observed using an inverted microscope (Olympus IX71, Germany) equipped with a 40 × 1.3 NA oil immersion objective. During the recordings using Live Acquisition v2.6 software (TILL Photonics FEI Company, Gräfelfing Germany), the excitation of mCherry was achieved using 577/25 nm filter and fluorescent images were captured every 2 s at 632 nm (using 632/60 nm emission filter Chroma Technology, VT, USA) with an ORCA-05G digital CCD camera (Hamamatsu, Herrsching am Ammersee, Germany). ImageJ 1.51n software was used to measure the fluorescence intensity in the nucleus and cytoplasm before and after stimulation with 10 μM OptoBI-1. These values (nucleus/cytosol) were then plotted using the SigmaPlot 14.1 software (Systat Software Inc.). The translocation of mCherry-NFAT1 in RBL-2H3 cells was observed for 12 min.

Fluorescence Imaging

Images from RBL-2H3 cells overexpressing CFP-TRPC3, CFP-TRPC6 and TRPC7-CFP were taken with the Zeiss array confocal laser scanning microscope (ACLSM, Zeiss Axiovert 200 M) using a 100×/1.45 oil immersion objective (Zeiss Microsystems, Jena, Germany). Illumination at 445 nm with an argon laser system (series 543, CVI Melles Griot, CA, USA) and emissions collected with a CCD camera (CoolSnap HQ2, Photometrics, Tucson, Arizona, USA). Image analysis was performed in ImageJ open source imaging analysis software (https://fiji.sc/).

Statistical Analysis

Data analyses and graphical display were performed using Clampfit 11 (Axon Instruments) and SigmaPlot 14.1 (Systat Software Inc.). Data are presented as mean values ± S.E.M. Primarily, a Shapiro–Wilk test was conducted to test for

normality of the value distribution. Whenever a normal distribution criterion was met, we used ANOVA to analyze the statistical significance. In general, differences were considered significant at $p < 0.05$ and indicated for individual comparisons in figures (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

Benzimidazole (OptoBI-1)-Mediated Control Over RBL-2H3 Mast Cell Ca^{2+} Signaling Requires Expression of Recombinant TRPC6 and TRPC7

Mammalian mast cells have been shown to express an array of TRPC gene products (27). Our initial experiments to explore the sensitivity of mast cell Ca^{2+} signaling to a new photochromic TRPC ligand (OptoBI-1, **Figure 1**), clearly indicated that endogenous expression of benzimidazole TRPC target channels (8) is below the threshold for effective photopharmacological intervention in RBL-2H3 mast cells. Nonetheless, our analysis of the expression profile for TRPC subtypes in RBL-2H3 cells revealed that besides TRPC1 and TRPC4 also two potential benzimidazole targets, *i.e.* TRPC3 and TRPC7, were expressed at the mRNA level, while TRPC6 expression was not detectable (**Supplementary Figure 1**). Consistent with the lack of Ca^{2+} signals generated in native RBL cells by OptoBI-1 photocycling (**Figure 1**; control), we failed to detect TRPC3 by immunoblotting. Unfortunately, a suitable antibody for TRPC7 detection is currently not available. Next, we attempted to reconstitute OptoBI-1 sensitive cation conductances in RBL-2H3 cells by overexpression of a single benzimidazole responsive TRPC proteins (TRPC3/6/7). Mast cells were genetically modified to express single OptoBI-1 target channels in combination with R-GECO as a reporter for cytosolic Ca^{2+} changes (8, 26). The overexpression of single TRPC proteins allowed us to investigate the consequences of photoactivation for reconstitution of each TRPC channel subtype in our mast cell model. Genetically modified RBL-2H3 cells were exposed to photocycling of OptoBI-1 to induce defined pattern of transient rises in cytosolic Ca^{2+} as shown in **Figure 1**. These changes were modest in cells overexpressing TRPC3 but profound in cells expressing TRPC6 or TRPC7. OptoBI-1-induced peak values of normalized R-GECO fluorescence in TRPC6 (3.29 ± 0.26; n = 23) and TRPC7 (4.4 ± 0.56; n = 21) overexpressing RBL cells remain below the maximum SOCE-mediated signals achieved by thapsigargin (1 μM, 6.2 ± 0.14; n = 7), given as a reference stimulus. Of note, both "on" and "off" kinetic of light-controlled Ca^{2+} changes were essentially fast allowing for precise control over signal frequency and duration. For TRPC6 the reversal of cellular Ca^{2+} levels upon channel deactivation was incomplete and showed a tendency to remain elevated above control levels between consecutive photocycles. Importantly, TRPC6 as well as TRPC7 expression enabled the light-mediated generation of temporally defined Ca^{2+} signaling pattern, with TRPC7 producing the largest UV light-induced rise in cytoplasmic Ca^{2+} during the first illumination cycle as well as the most prominent desensitization during consecutive photocyling.

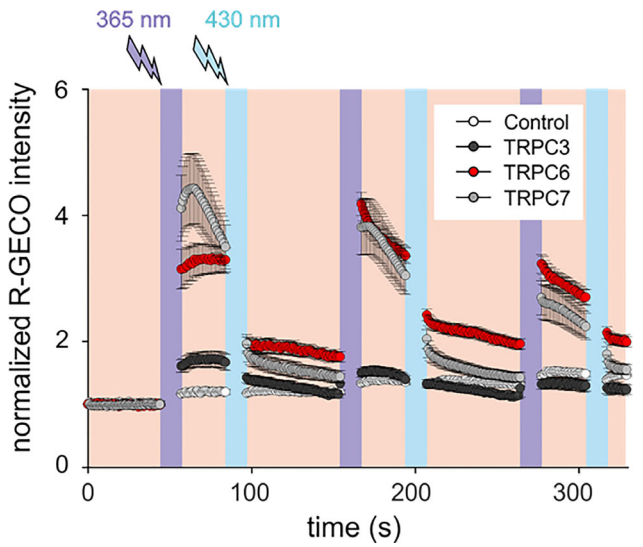

FIGURE 1 | OptoBI-1 photocycling induced Ca^{2+} signaling triggered by overexpressed TRPC3, TRPC6 or TRPC7 channels in RBL-2H3 cells. Comparison of responses in cells overexpressing YFP-TRPC3, YFP-TRPC6 or TRPC7-CFP together with R-GECO as a Ca^{2+} reporter. Cells expressing R-GECO was only used as a control. Time courses (mean ± SEM) of R-GECO fluorescence intensity (red, 577 nm) during photoactivation of TRPC3 (black, n = 8 cells), TRPC6 (red, n = 23 cells), TRPC7 (gray, n = 21 cells), and controls (white, n = 10 cells). *Cis-trans* OptoBI-1 photocycling was repeated three times, by illuminating cells with UV (365 nm, 10 s; violet) followed by blue light (430 nm, 10 s, blue).

Recombinant TRPC3, TRPC6, and TRPC7 Are Similarly Targeted to the Plasma Membrane but Produce Divergent Levels of OptoBI-1-Sensitive Cation Conductances

To better understand the mechanistic basis of the observed differences in reconstitution of OptoBI-1 sensitive Ca^{2+} signaling by the individual TRPC isoforms in RBL-2H3 cells, we continued with examination of the cellular localization and characterization of the membrane conductances generated with recombinant TRPC3/6/7. As shown in **Figure 2A** and **Supplementary Figure 2A**, recombinant TRPC channels fused to fluorescent markers (CFP or YFP) were similarly targeted to the plasma membrane. All cells expressing a TRPC fusion construct exhibited clear plasma membrane localized fluorescence. Of note, modification of RBL-2H3 cells to overexpress single TRPC-CFP fusion constructs did not affect cell morphology (**Figure 2A**) or promoted signs of degranulation. The OptoBI-1-induced TRPC conductances were quantified by electrophysiology applying the same OptoBI-1 photocycling protocol as in experiments measuring cytoplasmic Ca^{2+} rises (**Figure 2B**). All three TRPC isoforms reconstituted cation conductances with features

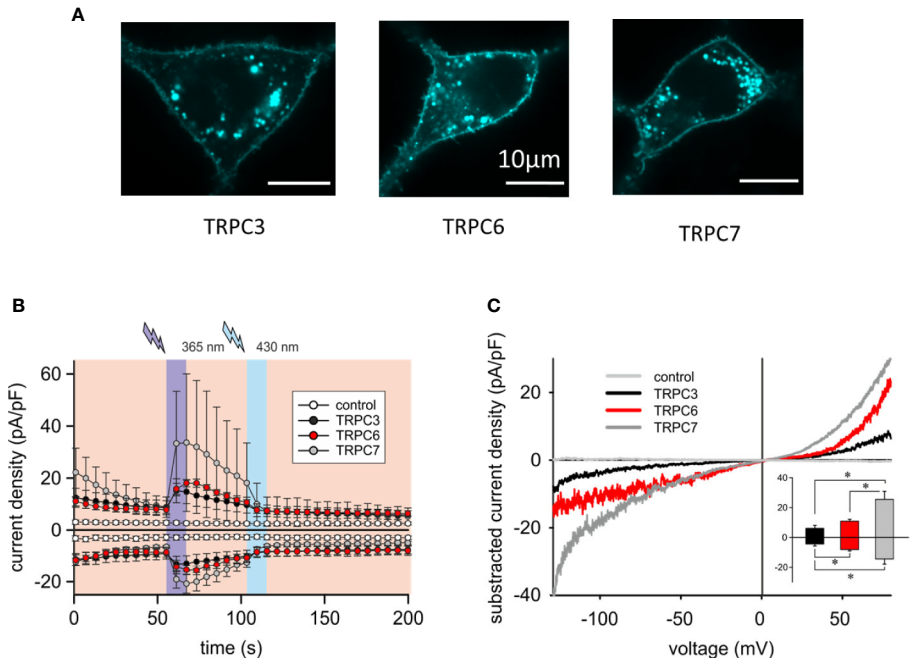

FIGURE 2 | Overexpressed TRPC3/6/7 proteins are targeted to the plasma membrane of RBL-2H3 cells and generate cation conductances with divergent efficiency. **(A)** Representative epifluorescence images of RBL-2H3 cells expressing CFP-TRPC3 (n = 57), CFP-TRPC6 (n = 26) and TRPC7-CFP (n = 45), respectively. Scale bar = 10 µm. **(B)** Time courses of control (sham transfected, white, n = 6 cells), YFP-TRPC3 (black, n = 6 cells), YFP-TRPC6 (red, n = 8 cells) and TRPC7-CFP (grey, n = 8 cells) conductances recorded at –90 to +70 mV during repetitive photoconversion of OptoBI-1. Light illumination cycling is indicated as violet (365 nm) and blue (430 nm). **(C)** Representative net I–V relations (I_{max} – I_{basal}) of OptoBI-1 induced currents in YFP-TRPC3 (black), YFP-TRPC6 (red), TRPC7-CFP (dark grey) transfected RBL-2H3 cells applying voltage-ramp protocols. Insert: Current density of net, maximum responses obtained at –90 to +70 mV (mean ± SEM). Statistical significance was tested by two tailed t-test (normally distributed values) or Mann–Whitney tests (non-normally distributed values), *p < 0.05, if not indicated differences are not significant (p > 0.05).

consistent with those recently described for OptoBI-1-activated recombinant channels in HEK293 cells (8). These features included the I–V characteristics (**Figure 2C**) and current inactivation/desensitization, consistent with previous reports on benzimidazole agonists as well as photochromic actuators (28, 29). In full agreement with the OptoBI-1-induced Ca^{2+}signaling, recombinant TRPC3 produced the smallest conductance, while TRPC7 expressing cells showed the largest current density after photoactivation (**Figure 2C**). Hence, the superior impact of TRPC6 and TRPC7 on mast cell Ca^{2+} homeostasis corresponds to larger cation conductances generated by these isoforms in RBL-2H3 cells combined with the reported higher Ca^{2+} permeability as compared to TRPC3 (30).

Genetic Modification of RBL-2H3 Cells to Overexpress TRPC6 Enables Control Over Cellular NFAT Activity by Light

In a next step we explored whether the generation of OptoBI-1 sensitivity in RBL-2H3 by overexpression of a single TRPC isoform is suitable to gain control over downstream Ca^{2+}-dependent gene transcription. We set out to test the concept of opto-chemical control over NFAT1 activity by genetic modification of the mast cell's TRPC expression pattern. NFAT1 nuclear translocation was recorded by expressing mCherry-NFAT1 fusion protein as a reporter, and applying the above-described protocol of repetitive photoactivation (three flashes of UV illumination). The extent of NFAT activation was quantified before and 12 min after initiation of the channel activation/deactivation cycles. Basal NFAT1 translocation was generally increased in all cells modified to overexpress TRPC channels. Nonetheless, this increase was modest with TRPC6 expression (**Figure 3A**) and repetitive, transient activation of the TRPC6 conductance by light resulted in a robust and highly significant NFAT1 nuclear translocation (**Figure 3** and **Supplementary Video 1**). By contrast cell expressing TRPC3 or TRPC7 displayed basal nuclear NFAT1 localization at levels comparable to that maximally achieved by TRPC6 activation. This phenomenon may be related to differences in basal channel activity, with an essentially low constitutive activity of TRPC6 (31), RBL cells expressing this channel subtype, displayed the lowest basal conductance measured immediately upon obtaining whole cell configuration in the absence of OptoBI-1 (**Supplementary Figure 3**). Importantly, only overexpression of TRPC6 channel in RBL-2H3 cells enabled precise control over mast cells Ca^{2+} transcription coupling by the photochromic benzimidazole OptoBI-1.

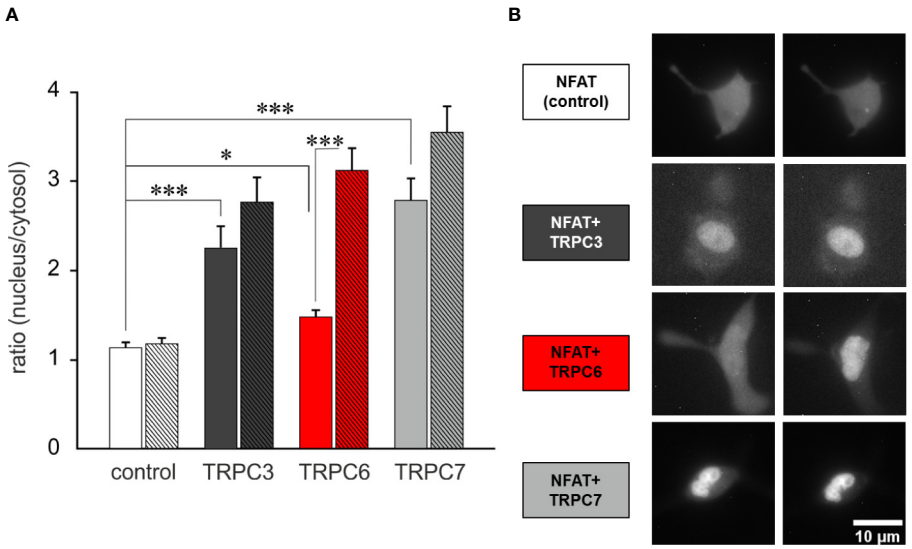

FIGURE 3 | TRPC6 expression enables OptoBI-1-mediated control of NFAT1 activity in RBL-2H3 cells by light. **(A)** NFAT1 nuclear translocation in RBL-2H3 cells overexpressing either mCherry-NFAT1 only (control; white, n = 30) or mCherry-NFAT1 along with YFP-TRPC3 (black, n=17), YFP-TRPC6 (red, n = 31) or with TRPC7-CFP (gray, n = 21). Mean ± SEM nucleus/cytosol fluorescence intensity ratio. The translocation of mCherry-NFAT1 was monitored during OptoBI-1 photoconversion, applying three photocycles of illumination (three Flashes) as indicated. Statistical significance was tested by One-Way ANOVA (Holm–Sidak test for normally distributed values) or One-Way ANOVA for Ranks (Dunn's test for non-normally distributed values), *$p < 0.05$, ***$p < 0.001$, if not indicated—not significant ($p > 0.05$). **(B)** Images of NFAT1 translocation in RBL-2H3 cells expressing mCherry-NFAT1 only (control; white) or coexpressing mCherry-NFAT1 and YFP-TRPC3 (black), YFP-TRPC6 (red) or TRPC7-CFP (grey) before (0 min) and after (12 min) three cycles of UV (365 nm, 10 s) and blue light (430 nm, 10 s) illuminations. Scale bar represents 5 µm.

DISCUSSION

With the present study, we provide evidence for practicability of chemo/pharmaco-optogenetic modulation of immune cells function. This strategy is based on targeting overexpressed TRPC6 channels by the photochromic benzimidazole OptoBI-1. We report proof of this concept by demonstrating control over NFAT1 activation in RBL-2H3 by light.

RBL-2H3 cells were found to express TRPC proteins, specifically TRPC3 and TRPC7, which reportedly confer sensitivity to benzimidazole photopharmacology (8). However, this endogenous TRPC expression pattern was insufficient to generate significant cellular Ca^{2+} signals and NFAT1 translocation in response to OptoBI-1 photocycling. Lack of benzimidazole sensitivity of native RBL-2H3 mast cells may be explained by essentially low TRPC expression at the protein level. TRPC3 protein was indeed barely detectable by immunoblotting (not shown), while a test for TRPC7 protein expression was hindered by the lack of an appropriate antibody. Nonetheless, it appears reasonable to assume that the expression of endogenous benzimidazole target channels (TRPC3/6/7) is below the threshold for coupling to the Ca^{2+}/CaN/NFAT pathway. Of note, in certain cellular settings TRPC3-generated Ca^{2+} signals failed to serve as an upstream trigger signal for NFAT activation (32). Nonetheless, the coupling of TRPC activity to downstream effectors may be strictly dependent on their mode of activation (33). In a recent study, we were able to demonstrate the linkage of recombinant TRPC channels to the calcineurin (CaN)/NFAT pathway in HEK293 cells (26). Expression of a TRPC3 gain-of-function mutant displaying enhanced benzimidazole sensitivity, was found to enable control of NFAT activity by OptoBI-1 (26). Consequently, we explored the option to achieve high precision control over mast cell NFAT signaling by combining photopharmacology and genetic modification. To do so, we altered mast cell TRPC expression by individual overexpression of benzimidazole-sensitive TRPC isoforms. Overexpression of recombinant TRPC3, TRPC6, or TRPC7 channels in RBL-2H3 cells reconstituted TRPC conductances with a clear order of efficacy with the largest OptoBI-1-induced current densities measured in TRPC7 expressing cells, whereas recombinant TRPC3 produced only a modest benzimidazole-sensitive conductance. All recombinant channel isoforms were found well targeted to the mast cell plasma membrane. The reason for the substantial difference observed upon reconstitution of TRPC3 and TRPC7 in RBL-2H3 cells remains unclear. Light-activated TRPC conductances displayed marked inactivation/desensitization as reported previously for benzimidazole agonists as well as lipid actuators (28, 29). The prominent inactivation observed for TRPC7, may in part be explained by the high current density, considering a current- and/or Ca^{2+}-dependent mechanism. Consistent with the higher Ca^{2+} selectivity of TRPC6 and TRPC7 channels, surmounting that of TRPC3 (34), OptoBI-1-photocycling exerted a profound impact on mast cell Ca^{2+} levels when cells expressed either TRPC6 or

TRPC7, but not with TRPC3 expression. Thus, genetic modification of mast cells to overexpress TRPC6 or TRPC7 channels generated OptoBI-1 sensitivity that allows temporal sculpturing of Ca^{2+} signals in these immune cells. Notably, membrane currents and cytosolic Ca^{2+} levels did not decline synchronously upon fast, light-induced deactivation as previously reported for TRPC3 mutants in the HEK293 expression system (26). Remarkably long-lasting Ca^{2+} elevations were triggered by repetitive TRPC6 activation. This phenomenon may be related to cellular localization of these channels relative to major Ca^{2+} extrusion systems. Since TRPC6 has repeatedly been found co-localized with NCX1 (35–37), it is tempting to speculate that Na^{+} loading during TRPC6 activation might counteract NCX1-mediated Ca^{2+} extrusion thereby generating tonic elevation of basal Ca^{2+} upon repetitive activation. Importantly, only the introduction of an expression pattern featuring TRPC6 as the prominent species, provided a basis for efficient optical control over NFAT activation in the mast cells. Of note, all three benzimidazole-sensitive isoforms were found to communicate with the CaN/NFAT pathway, albeit in a divergent manner. TRPC3 and TRPC7 overexpressing mast cells displayed significant constitutive levels of NFAT1 nuclear localization, indicating the generation of a tonic increase of NFAT dephosphorylation, due to Ca^{2+} signals that arise from the constitutive activity of the overexpressed TRPC channels. Constitutive inward currents generated by TRPC3 and TRPC7 expression may results in tonic NFAT1 activation and preclude further control of NFAT transcriptional activity by photocycling of OptoBI-1. In RBL cells overexpressing TRPC6, which displays essentially low constitutive activity (31), basal NFAT1 translocation remained close to controls, and short pulsatile activation-deactivation of TRPC6 channels by OptoBI-1 photocycling resulted in rapid and significant NFAT1 nuclear translocation (within 10 min—see video in **Supplementary Video 1**). It is important to note that OptoBI-1 activation of TRPC6 may generate channel features and local Ca^{2+} entry pattern different from those of TRPC channels activated in response to receptor-phospholipase C pathways. The complex cascades inevitably linked to receptor-mediated activation of TRPC channels have been reported to modify the coupling between TRPC and NFAT signaling in cardiac muscle (38). To this end, the molecular basis of the observed efficient linkage between OptoBI-1-activated TRPC6 conductances and NFAT1 nuclear translocation in RBL-2H3 remains elusive. Nonetheless, our results demonstrate the general ability of TRPC6 to serve an important function, which is executed mainly by STIM/Orai channels in immune cells. Importantly, NFAT1 nuclear translocation triggered by TRPC-mediated Ca^{2+} entry, both by constitutive channel activity as well as short, transient pulses of channel activation using OptoBI-1 photocycling is potentially devoid of eliciting excessive exocytosis/degranulation, and may thereby enable a more specific modulation of immune responses as expected from interventions targeting the STIM/Orai machinery. This concept may be of relevance for the development of highly specific interventions to modify immune reactions of mast cells but also other immune cells, which play a complex, dual role in cancer pathology. It remains to be clarified if the here described concept of immunomodulation by TRPC photopharmacology can be adopted for control of other immune cells and as a basis of therapeutic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BB: Ca^{2+} imaging, manuscript writing, data analysis. AG: Ca^{2+} imaging, NFAT translocation, data analysis. DK: NFAT translocation, data analysis. PW: qPCR, primer design. TR: fluorescence microscopy. RM: experimental design. OT: patch-clamp, data analysis. KG: concept, experimental design, funding, and manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.613194/full#supplementary-material

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TRPM7 Kinase Is Essential for Neutrophil Recruitment and Function *via* Regulation of Akt/mTOR Signaling

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During inflammation, neutrophils are one of the first responding cells of innate immunity, contributing to a fast clearance of infection and return to homeostasis. However, excessive neutrophil infiltration accelerates unsolicited disproportionate inflammation for instance in autoimmune diseases such as rheumatoid arthritis. The *transient-receptor-potential* channel-kinase TRPM7 is an essential regulator of immune system homeostasis. Naïve murine T cells with genetic inactivation of the TRPM7 enzyme, due to a point mutation at the active site, are unable to differentiate into pro-inflammatory T cells, whereas regulatory T cells develop normally. Moreover, TRPM7 is vital for lipopolysaccharides (LPS)-induced activation of murine macrophages. Within this study, we show that the channel-kinase TRPM7 is functionally expressed in neutrophils and has an important impact on neutrophil recruitment during inflammation. We find that human neutrophils cannot transmigrate along a CXCL8 chemokine gradient or produce reactive oxygen species in response to gram-negative bacterial lipopolysaccharide LPS, if TRPM7 channel or kinase activity are blocked. Using a recently identified TRPM7 kinase inhibitor, TG100-115, as well as murine neutrophils with genetic ablation of the kinase activity, we confirm the importance of both TRPM7 channel and kinase function in murine neutrophil transmigration and unravel that TRPM7 kinase affects Akt1/mTOR signaling thereby regulating neutrophil transmigration and effector function. Hence, TRPM7 represents an interesting potential target to treat unwanted excessive neutrophil invasion.

Keywords: TRPM7, ion channel, kinase, neutrophils (PMNs), innate immunity, inflammation, chemotaxis, migration

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INTRODUCTION

The melastatin-like *transient-receptor-potential* (TRP) cation channel, TRPM7, is a bifunctional protein fusing a channel pore selective for divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}) to a C-terminal alpha-type serine/threonine kinase (1–4). TRPM7 channel and kinase activities are interdependent in that Mg^{2+} enters through the channel pore and the kinase domain requires Mg^{2+} ions for its activity (5). Thus, suppression of the channel activity might also reduce kinase function (6). Known substrates of the TRPM7 kinase include annexin A1, myosin II, Rho A, and SMAD2 (5, 7, 8). TRPM7 has been proposed to be essential for lymphocyte proliferation and development (9, 10). We recently established that TRPM7 kinase activity regulates differentiation of T lymphocytes toward pro-inflammatory T_H17 cells, while anti-inflammatory regulatory T cells remain unaffected (7). Thereby the channel-kinase reins immune system homeostasis (5, 7, 11). Moreover, TRPM7 is indispensable for lipopolysaccharides (LPS)-induced activation of murine macrophages (12) and has previously been suggested to promote the differentiation of M1 over M2 macrophages (13). The role of TRPM7 in neutrophil function, though, remains unclear. Neutrophils are the most abundant leukocytes in blood and one of the first responding cells during acute inflammation. Therefore, they are essential players in innate immunity (14, 15). Following activation, neutrophils migrate toward the inflammatory site, where they eliminate pathogens *via* phagocytosis, thereby helping to restore tissue homeostasis. Another key function of neutrophils is the secretion of various cytokines and antimicrobial factors as well as the production of reactive oxygen species (ROS), killing invading pathogens (16). To prevent the spread of the infection, neutrophils are able to release so called neutrophil extracellular traps (NETs), which in turn contribute not only to bacterial clearance but also support blood vessel occlusion and immunothrombosis (17). It is well established that Ca^{2+} signaling is pivotal for the recruitment cascade and activation of neutrophils, highlighting the importance of ion channels for neutrophil function (18, 19). TRPM7 channel activity has been implicated as a regulator of cell migration by facilitating Ca^{2+} oscillations (20–22). Moreover, TRPM7 was suggested to be involved in neutrophil chemotaxis, adhesion and invasiveness with, yet, rather conflicting results (23–25). The precise mechanism how TRPM7 might be involved in neutrophil transmigration and activity remains to be explored. Patients with inherited neutrophil deficiencies suffer from severe infections, underscoring the importance of this cell type in immune defense (14). Moreover, unwarranted neutrophil infiltration accelerates tissue damage due to unsolicited inflammation in inflammatory disorders such as multiple sclerosis or rheumatoid arthritis (26, 27). Thus, it is critical to gain a better understanding of the role of TRPM7 channel and kinase activities in the signaling cascades triggering neutrophil recruitment.

Using pharmacologic blockade of TRPM7 channel or kinase as well as genetic inhibition of TRPM7 kinase activity, we here resolve the indispensable role of TRPM7 in human and murine neutrophil transmigration and production of reactive oxygen species. We further identify a potential underlying molecular mechanism, with Akt1 as novel downstream target of the TRPM7 kinase.

MATERIAL AND METHODS

Mice and *In Vivo* Experiment

Trpm7tm1.1Mkma C56BL/6 (Trpm7$^{R/R}$) mice (28) were obtained from RIKEN, Japan and housed in single ventilated cages at the animal facility of the Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Munich, Germany. All animal experiments were performed in accordance with the EU Animal Welfare Act and were approved by the District Government of Upper Bavaria, Germany, on animal care (permit no. 55.2-1-54–2532–163–2015). Six- to twelve-week-old male and female mice were used for all experiments.

For TNF-α induced peritonitis *Trpm7$^{R/R}$* and littermate control animals were injected i.p. with 0.9% NaCl (unstimulated) or TNF-α (500 ng, R&D Systems) and sacrificed 4 h later. Peritoneal lavage was performed using ice cold PBS and the number of extravasated neutrophils was evaluated by flow cytometry (CytoFlex S, Beckmann Coulter) and analyzed using FlowJo software. Flow-Count Fluorospheres (Beckman Coulter, Brea, USA) were added to each sample to accurately count the cells and to calculate the total number of transmigrated neutrophils (**Figure S1**). Neutrophils were defined as $CD11b^+$, $Ly6G^+$ population (rat anti mouse CD11b, clone M1/70, rat anti-Ly6G, clone 1A8, all 5µg/ml, BioLegend).

Neutrophil Isolation

Human neutrophils were extracted from whole blood of healthy volunteer blood donors using Polymorphprep (AXIS-SHIELD PoC AS) or EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies Inc.). Blood sampling was approved by the ethic committee from the Ludwig-Maximilians-Universität München (Az. 611-15).

Bone marrow (BM) derived murine neutrophils were isolated from the femurs and tibiae of *Trpm7$^{R/R}$* and control littermates in the C57BL/6J background. Murine neutrophils were purified from BM using EasySep™ Mouse Neutrophil Enrichment Kit (STEMCELL Technologies Inc.).

After isolation, cells were resuspended in HBSS buffer [containing 0.1% of glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25% BSA, and 10 mM HEPES (Sigma-Aldrich), pH 7.4].

White blood cell counts were analyzed from whole blood of *Trpm7$^{R/R}$* and control mice using a hematology analyzer (IDEXX ProCyte Dx).

Electrophysiology

Human and murine neutrophils were isolated as described above and verified *via* Alexa Fluor® 488 anti-human CD16 or PE anti-mouse Ly6G antibody staining (clone: 3G8, BioLegend, 5µg/ml), respectively, (clone: 1A8, BioLegend, 10µg/ml) using an inverted AxioVert Microscope and Zen software (Zeiss). Patch-clamp

experiments in whole-cell configuration were performed as follows: currents were elicited by a ramp protocol from −100 mV to + 100 mV over 50 ms acquired at 0.5 Hz and a holding potential of 0 mV. Inward current amplitudes were extracted at −80 mV, outward currents at +80 mV and plotted *versus* time in seconds (s). Initial capacitative currents were subtracted and data were normalized to cell size as pA/pF. Capacitance was measured using the automated capacitance cancellation function of the EPC-10 (HEKA, Lambrecht, Germany). Values over time were normalized to the cell size measured immediately after whole-cell break-in. Nominally Mg^{2+}-free extracellular solution contained (in mM): 140 NaCl, 3 CaCl2, 2.8 KCl, 10 HEPES-NaOH, 11 Gluc (pH 7.2, 300 mOsm). Standard intracellular solution contained (in mM): 120 Cs-glutamate, 8 NaCl, 10 HEPES, 10 Cs-EGTA, 5 EDTA (pH 7.2, 300 mOsm). Solutions were adjusted to 300 mOsm using a Vapro 5520 osmometer (Wescor Inc). NS8593 (30 μM, Alomone labs) was added to the bath solution at least 15 min prior to electrophysiological recordings. TG100-115 {[3-(2,4-diamino-6-(3-hydroxyphenyl)pteridin-7-yl)phenol]; 20 μM, Selleckchem} was added to the bath solution at least 30 min prior to electrophysiological recordings.

Transwell Assay

Human neutrophils were isolated using Polymorphprep, resuspended in HBSS and incubated for 30 min at 37°C with either DMSO (Ctrl.), NS8593 (30 μM), TG (20 μM), or a combination of IPI-549 (160 nM, Selleckchem) and Nemiralisib (100 nM, Selleckchem), respectively. $3x10^5$ cells were then added to the upper compartment of the transwell filters (5 μm pore size, Corning) placed in a 24 well plate and allowed to migrate toward CXCL8 gradient in the lower compartment (10nM in HBSS, Peprotech) for 45 min at 37°C. HBSS alone was used as a negative control (unstimulated). The transwell assay was also performed using isolated murine neutrophils. Cells were incubated with DMSO (Ctrl) or NS8593 (30 μM) for 30 min at 37°C and CXCL1 (10 nM, PeproTech) was used as chemoattractant. Migrated cells were collected, stained for CD15 (clone HI98) and CD66b (clone G10F5, human) or against Ly6G (clone 1A8, murine, all 5 μg/ml, BioLegend), respectively. Cell numbers were quantified by flow cytometry (CytoFlex S, Beckmann Coulter) and Flow-Count Fluorospheres (Beckman Coulter, Brea, USA). FlowJo software was used to analyze flow cytometry data.

Phagocytosis Assay

Heparinized human whole blood was pre-treated with DMSO, NS8593 (30 μM), TG100-115 (20 μM), or a combination of IPI-549 (160 nM) and nemiralisib (100 nM), respectively for 30 min at 37°C and then incubated with fluorescent *Escherichia coli* bio particles (pHrodo green *E. coli* BioParticle Phagocytosis Kit for flow cytometry, Thermo Fisher Scientific) for 30 min at 37°C. Phagocytosis was stopped and cells were fixed according to the manufacturer's protocol. As control, whole blood was incubated with the particles for 30 min at 4°C. Samples were analyzed by flow cytometry (CytoFlex S, Beckmann Coulter) and FlowJo software. $CD15^+/CD66b^+$ populations were defined as neutrophils.

2',7'-Dichlorofluorescin Diacetate Assay

For measurement of cellular reactive oxygen species (ROS) in human neutrophils 2',7'-dichlorofluorescin diacetate assay (DCFDA) Cellular ROS Detection Assay Kit (Abcam, catalog# ab113851) was used according to the manufacturer's instructions. Cells were stained and afterward preincubated with DMSO, NS8593 (30 μM, Alomone labs), TG100-115 (20 μM, Selleckchem) or a combination of IPI-549 (160 nM, Selleckchem) and Nemiralisib (100 nM, Selleckchem) for 30 min. Cells were then stimulated with LPS (10 ng/ml) for 15, 30, 60, and 90 min. Fluorescence was detected with a PolarStar Omega (BMG LABTECH) plate reader. After background subtraction ratio of relative fluorescence intensity of control and treated cell was calculated.

Bio-Plex Assay

For Bio-Plex Pro™ Cell Signaling Assay (Bio-Rad) human and murine neutrophils were pre-incubated with DMSO, NS8593 (30 μM, Alomone Labs), TG100-115 (20 μM, Selleckchem) or a combination of IPI-549 (160 nM, Selleckchem) and nemiralisib (100 nM, Selleckchem) for 30 min and then treated with LPS (10 ng/ml, Sigma-Aldrich) for 30 min at 37°C. Cells were then washed and lysed using Cell Signaling Reagent Kit (Bio-Rad, catalog #171-304006M). Protein amount was measured using *DC*™ protein assay kit II (Bio-Rad, catalog #500-0012). Afterward samples were stored at −80°C. Collected samples were processed for the assay according to manufacturer's instructions with following phosphoprotein targets: Akt (Ser^{473}, catalog #171-V50001M), Erk1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}, catalog #171-V50006M), NF-κB p65 (Ser^{536}, catalog #171-V50013M), mTOR (Ser^{2448}, catalog #171-V50033M).

Statistical Analysis

All experimental data are presented as mean ± standard error of the mean (s.e.m.). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, LLC). Comparisons between two groups were carried out using Student's *t*-test. Differences between more than two groups were compared either by one-way or two-way analysis of variance (ANOVA), respectively, and Sidak's multiple comparison test was used as a *post-hoc* test. Each experiment was performed independently at least in triplicate.

RESULTS

TRPM7 Is Essential for Human Neutrophil Chemotaxis and ROS Production

To validate functional expression of TRPM7 in primary human neutrophils isolated from whole blood of healthy donors (**Figure 1A**), we subjected $CD16^+$ neutrophils to whole-cell patch-clamp recordings (**Figure 1B**, left panel). We confirmed TRPM7-like current activity upon depletion of intracellular magnesium (Mg^{2+}) and Mg-ATP (**Figure 1B**, middle and right panel), using our standard internal and external solutions (see *Methods*). TRPM7 currents were inhibited by the pre-incubation of cells with the

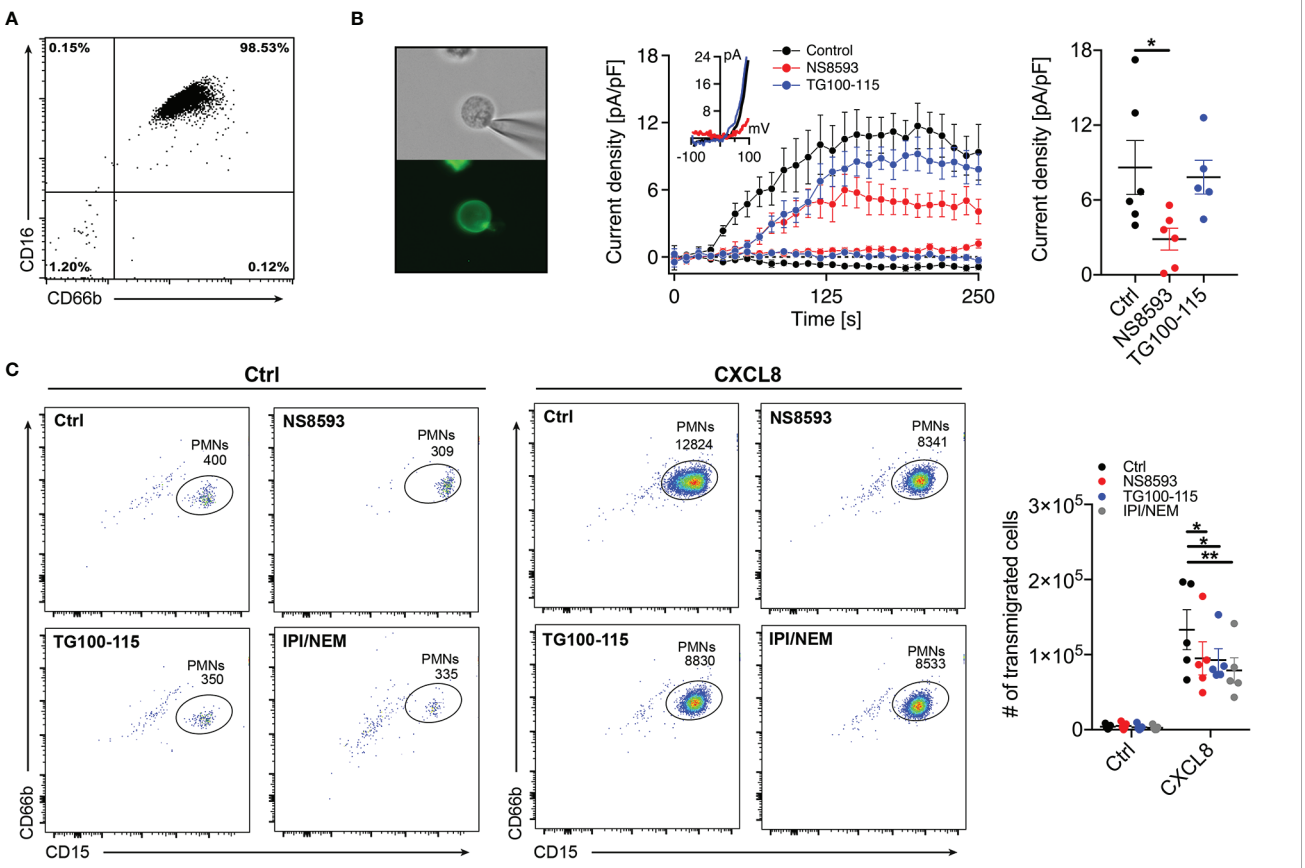

FIGURE 1 | TRPM7 is essential for human neutrophil transmigration. **(A)** Representative purity of primary human neutrophils isolated from whole blood using magnetic cell sorting. **(B)** Representative human neutrophils stained with Alexa Fluor-488 conjugated anti-CD16 antibody (left panel). Whole-cell patch clamp analysis of TRPM7 ion channel activity. TRPM7 current densities in human neutrophils treated with 30 µM NS8593 (NS8593, red circles, $n = 6$), 20 µM TG100-115 (TG, blue circles, n = 5), or without (Ctrl, black circles, $n = 6$) were plotted *versus* time of the experiment in seconds (s). Error bars indicate s.e.m. Representative current-voltage relationships extracted at 250 s of human neutrophils treated with NS8593 (red), TG100-115 (blue) or without (black) (middle panel). Quantification of the current density extracted at +80 mV and displayed as pA/pF at 250 s of human neutrophils treated with NS8593 (NS8593, red, $n = 6$), 20 µM TG100-115 (TG, blue, n = 5), or without (Ctrl, black, $n = 7$) (right panel). Data are shown as mean ± s.e.m. *p < 0.05, one-way ANOVA. **(C)** Representative dot plot analysis (left and middle panel) and quantification (right panel) of neutrophil chemotaxis toward a CXCL8 gradient (10 nM) in a transwell assay. Human neutrophils were pretreated with NS8593 (30 µM, red), TG100-115 (20 µM, blue), a combination of IPI-549 and nemiralisib (IPI/NEM, 160/100 nM, gray) or dissolvent (Ctrl, black), for 30 min prior to CXCL8 or saline (Ctrl) exposure. Two-way repeated measurements ANOVA, Sidak's multiple comparison, *p < 0.05, **p < 0.01.

known TRPM7 channel inhibitor NS8593 (30 µM) for 15 min, further confirming TRPM7 channel activity in primary human neutrophils (**Figure 1B**, red trace). In contrast, the recently described TRPM7 kinase inhibitor, TG100-115 (29) (20 µM, 30 min pre-incubation), did not affect channel activity in our patch-clamp recordings (**Figure 1B**, blue trace).

To investigate whether TRPM7 is involved in the regulation of neutrophil function, we first analyzed chemotactic properties of isolated human neutrophils in a transwell assay in response to saline (**Figure 1C**, Ctrl, left panels) or the chemokine interleukin 8 (CXCL8 (30, 31),) (**Figure 1C**, middle panel, 10 nM, 45 min) by flow cytometry. Inhibition of TRPM7 channel activity, using NS8593 (30 µM, 30 min pre-incubation), resulted in significantly reduced neutrophil numbers migrating toward a CXCL8 gradient compared to controls (**Figure 1C**, right panel). Interestingly, TRPM7 kinase blockade, using TG100-115 (20 µM, 30 min pre-incubation), ensued a significant reduction in neutrophil CXCL8-triggered chemotaxis as well. Originally, TG100-115 was identified as potent inhibitor of phospoinositol-3-kinases (PI3K), with particular affinity to PI3K-γ and -δ isoforms. Therefore, to account for its off-target effects *via* PI3K, we added respective controls IPI-549 (anti PI3K-γ) and Nemiralisib (anti PI3K-δ) (IPI/NEM, 160 nM/100 nM, 30 min pre-incubation) (**Figure 1C**, right panel). Notably, also inhibition of PI3K-γ and -δ isoforms resulted in significantly reduced chemotactic properties. These results suggest that TRPM7 and PI3 kinases contribute to chemotaxis with a yet undefined role of TRPM7 kinase activity.

Another important physiological function of neutrophils is the killing of invading pathogens *via* phagocytosis and reactive

oxygen burst. In order to elucidate whether this functionality is also affected by TRPM7 blockade, we evaluated phagocytic activity and ROS production of human neutrophils in the presence of TRPM7 inhibitors. To test for phagocytic activity, we incubated human whole blood from healthy donors with fluorescent *E. coli* particles and analyzed phagocytosis by flow cytometry. While phagocytosis was not affected by TRPM7 channel blockade (NS8593, 30 μM) or kinase inhibition using TG100-115 (20 μM, blue), or a combination of IPI-549 and nemiralisib (IPI/NEM, 160/100 nM, gray) (**Figure 2A**), we found differences in ROS production in response to the bacterial lipopolysaccharid LPS. TRPM7 channel blockade using NS8593 (30 μM) and TRPM7 kinase blockade using TG100-115 (20 μM) resulted in significantly less ROS production upon stimulation in human neutrophils compared to control cells (**Figure 2B**). Comparable to chemotaxis, ROS production was also affected in neutrophils by the inhibition of PI3K-γ and -δ isoforms demonstrating that TRPM7 and PI3K-γ and -δ isoforms mediate similar neutrophil functions suggesting that TRPM7 and PI3K-γ and -δ isoforms might follow analogous or even sequential signaling pathways.

TRPM7 Regulates Human Neutrophil Function *via* NFκB, Erk, and Akt/mTOR Signaling Pathways

To further unravel the mechanism and signaling pathways by which TRPM7 channel and/or kinase blockade alters neutrophil function, we analyzed the effect of the different TRPM7 moieties on cellular signaling. Typically, chemotaxis and migration of neutrophils is regulated by LPS-triggered pathways followed by NFκB activation (32, 33). For cell motility and chemotaxis also Erk1-dependent signaling cascades are essential (34). Aside of NFκB and Erk1, PI3K/Akt/mTor signaling pathways regulate neutrophil function and recruitment (35, 36). Therefore, we employed a bead-based Bio-Plex assay (Bio-Rad) to simultaneously measure the phosphorylation status of multiple signaling proteins in the same sample (37). We examined phosphorylation levels of NFκB (p65 Ser^{536}), ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), and mTOR (Ser^{2448}) in LPS stimulated primary human neutrophils (**Figures 2C–F**). TRPM7 channel blockade using NS8593 (30 μM) revealed a reduction in NFκB, ERK1, and Akt/mTOR-dependent signaling, whereas TRPM7 kinase blockade using TG100-115 (20 μM) only led to a reduction in Akt-dependent signaling. Interestingly, inhibition of PI3K-γ and -δ using IPI-549 and Nemiralisib (IPI/NEM, 160 nM/100 nM) did not show any impact (**Figures 2C–F**) on the phosphorylation status of the detected proteins in human neutrophils. This indicates that TRPM7 might not directly signal through activation of PI3K-γ and -δ in human neutrophils or that other isoforms can compensate, at least for the investigated downstream signaling molecules. The impact of PI3K-γ and -δ blockade on chemotaxis and ROS production might not involve the analyzed signaling targets and thus might not be compensated for.

TRPM7 Kinase Is Essential for Neutrophil Transmigration and Infiltration in an *In Vivo* Peritonitis Model

To further elucidate the role of TRPM7 kinase activity for neutrophil function, we next analyzed whether genetic inactivation of the TRPM7 kinase conveys similar effects on neutrophil chemotaxis and transmigration as pharmacologic blockade by TG100-115. We took advantage of a mouse model carrying a point mutation at the active site of the enzyme. Mutating lysine at position 1646 to arginine ($Trpm7^{R/R}$) disrupts ATP binding and thereby kinase activity (28). We have previously shown, that this mutation affectively abolishes phosphotransferase activity, while leaving the channel function of TRPM7 intact (7). Here, analogously to human neutophils, we subjected $CD16^+$ bone marrow derived murine neutrophils (**Figure 3A**) from $Trpm7^{+/+}$ and $Trpm7^{R/R}$ mice to whole-cell patch-clamp recordings (**Figure 3B**). We confirmed TRPM7-like current activity upon depletion of intracellular Mg^{2+} and Mg-ATP (**Figure 3B**, middle and right panel), using our standard internal and external solutions (see *Methods*). TRPM7-like currents were blocked by the application of the known TRPM7 channel inhibitor NS8593 (30 μM, pre-incubation for 15 min), further confirming TRPM7 channel activity in primary murine neutrophils (**Figure 3B**, red traces). Thus, our electrophysiologic analysis established the functional expression of TRPM7 on murine bone marrow derived neutrophils, with similar current development and amplitude as well as inhibition in response to NS8593 between the two genotypes (**Figure 3B**). This further indicates that in neutrophils the channel function is independent of its kinase activity.

To further delineate whether neutrophil transmigration is regulated by TRPM7 channel and/or kinase activities, we perfomed a transmigration-assay using primary bone marrow-derived neutrophils isolated from TRPM7 kinase-deficient ($Trpm7^{R/R}$) and recpective wild-type ($TRPM7^{+/+}$) mice. We analyzed chemotactic properties of murine neutrophils in a transwell assay (**Figure 3C**) in response to saline or CXCL1 (keratinocytes-derived chemokine, KC), the murine counterpart to CXCL8 (**Figure 3C**, left panel, 10 nM, 45 min) by flow cytometry. Inhibition of TRPM7 channel activity, using NS8593 (30 μM, 30 min pre-incubation), resulted in reduced $Trpm7^{+/+}$ neutrophil numbers migrating toward a CXCL1 gradient compared to controls, albeit not significantly (**Figure 3C**, right panel). Interestingly, TRPM7 kinase-deficient $Trpm7^{R/R}$ neutrophils ensued a significant reduction in neutrophil CXCL1-triggered chemotaxis. Additional inhibition of TRPM7 channel activity using NS8593 even further reduced the numbers of migrating $Trpm7^{R/R}$ neutrophils in response to CXCL1. These results suggest that both TRPM7 kinase and channel activity contribute to chemotaxis of murine neutrophils.

In order to apprehend whether circulating neutrophils or other leukocyte numbers were different in $Trpm7^{R/R}$ mice compared to $Trpm7^{+/+}$, we analyzed the distribution of white blood cells in whole blood performing a differential blood count *via* a hematology analyzer (IDEXX ProCyte Dx). Notably, white

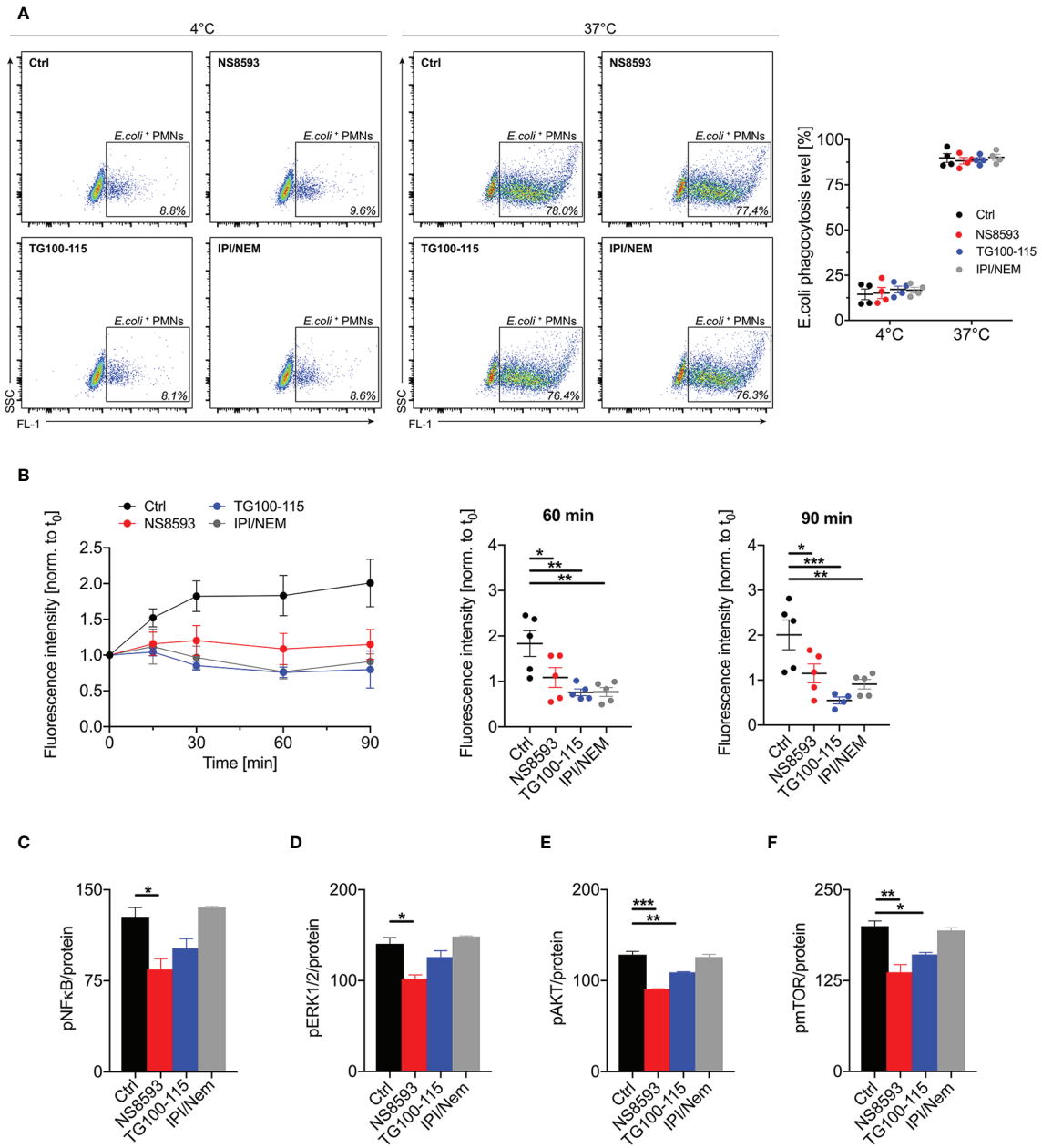

FIGURE 2 | TRPM7 activity is dispensable for phagocytosis but indispensable for reactive oxygen species (ROS) production of human neutrophils. **(A)** Phagocytic activity of neutrophils was measured using fluorescent *Escherichia coli* particles together with human whole blood pre-incubated with NS8593 (30 μM, red), TG100-115 (20 μM, blue), or a combination of IPI-549 and nemiralisib (IPI/NEM, 160/100 nM, gray) for 30 min or vehicle (Ctrl, black) and analyzed by flow cytometry (n = 5). Representative dot plot analysis (left panel) and quantification of phagocytic activity (right panel). Data are shown as mean ± s.e.m., two-way repeated measurements ANOVA, Sidak's multiple comparison. **(B)** Effects of TRPM7 channel and kinase blockade on lipopolysaccharides (LPS)-triggered ROS production. Human neutrophils were pretreated with or without (Ctrl, black), NS8593 (30 μM, red), TG100-115 (20 μM, blue), or a combination of IPI-549 and nemiralisib (IPI/NEM, 160/100 nM, gray) for 30 min and then incubated with LPS (10 ng/ml) for 0, 15, 30, 60 and 90 min. Intracellular ROS levels over time (left panel) and quantification at 60 min (middle panel) and 90 min (right panel). Data are normalized to t_0 and represented as mean ± s.e.m.; n=5. Statistics: one-way ANOVA *p < 0.05, ** p < 0.01, ***p < 0.005. **(C–F)** Assessment of the activity of the cell signaling molecules NFκB, Erk1/2, Akt1, and mTOR exploiting a Bio-Plex assay and phospho-specific antibodies. Primary human neutrophils were pre-incubated with or without (Ctrl, black) the TRPM7 inhibitor NS8593 (30 μM, red), the TRPM7 kinase blocker TG100-115 (20 μM, blue), or a combination of IPI and NEM (160 and 100 nM, gray), respectively, for 30 min prior to stimulation with 10 ng/ml LPS. Phosphorylation status of human neutrophils upon stimulation with 10 ng/ml LPS for 30 min of **(A)** NFκB p65 (Ser536), **(B)** Erk1/2 (Thr202/Tyr204, Thr185/Tyr187), **(C)** Akt (Ser473), and **(D)** mTOR (Ser2448), were analyzed. Data were normalized to protein content and presented as mean ± s.e.m.; biological n = 3 and measured in duplicates. Statistics: one-way ANOVA *p < 0.05, **p < 0.01, ***p < 0.005.

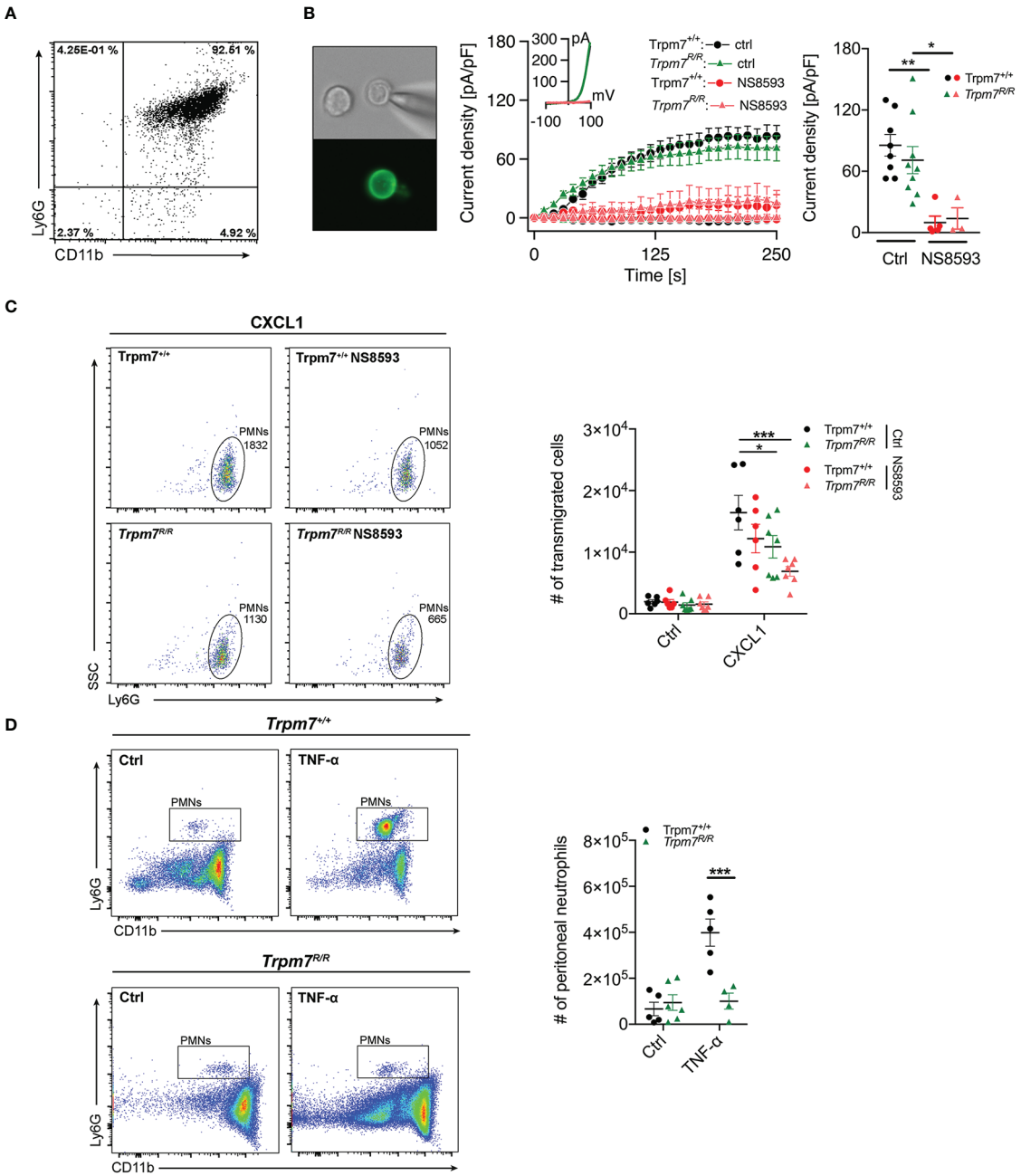

FIGURE 3 | TRPM7 kinase is essential for neutrophil chemotaxis and infiltration in an *in vivo* murine peritonitis model. **(A)** Representative purity of primary bone marrow derived murine neutrophils isolated from Trpm7$^{+/+}$ and *Trpm7*$^{R/R}$ mice using magnetic cell sorting. **(B)** Representative murine neutrophil stained with PE conjugated anti-Ly6G antibody (left panel). Whole-cell patch clamp analysis of TRPM7 ion channel activity. Primary murine neutrophils were treated with or without NS8593 (30 μM). TRPM7 current densities in neutrophils isolated from Trpm7$^{+/+}$ mice (circles) without (black, n = 8) and with NS8593 treatment (red, n = 5) as well as from *Trpm7*$^{R/R}$ mice (triangles) without (green, n = 9) or with NS8593 (red, n = 3) were averaged and plotted *versus* time of the experiment in seconds (s) (left panel). Error bars indicate s.e.m. Representative current-voltage relationships extracted at 250 s of murine neutrophils (middle panel). Quantification of the current density extracted at +80 mV and displayed as average current density (pA/pF) at 250 s (right panels). Data are shown as mean ± s.e.m. Statistics: one-way ANOVA *p < 0.05, **p < 0.01. Note: there is no difference in TRPM7 current development or amplitude between the two genotypes. **(C)** Representative dot plot analysis (left and middle panel) and quantification (right panel) of murine neutrophil chemotaxis toward a CXCL1 gradient (10 nM) in a transwell assay. Two-way repeated measurements ANOVA, Sidak's multiple comparison, *p < 0.05, ***p < 0.001. **(D)** Murine TNF-α peritonitis model. Saline (Ctrl), or TNF-α (500 ng) were injected intra-peritoneally into Trpm7$^{+/+}$ (black) and *Trpm7*$^{R/R}$ (green) mice. Numbers of recruited neutrophils in the peritoneum were assessed 4 h later (n = 4–6 mice per group) using flow cytometry. Representative dot plot analyses of recruited neutrophils (left panel) and quantification (right panel). Data are shown as mean ± s.e.m. Statistics: two-way ANOVA, Sidak's multiple comparison. ***p ≤ 0.001.

blood cell counts were similar between *Trpm7*$^{R/R}$ mice and Trpm7$^{+/+}$ controls (**Figure S1A**), indicating no major differences in the composition of circulating leukocytes. To finally understand the impact of TRPM7 kinase moiety on the function of neutrophils *in vivo*, we employed a TNF-α–induced peritonitis model using TRPM7 kinase-deficient (*Trpm7*$^{R/R}$) mice. The intraperitoneal application of TNF-α triggers the recruitment of circulating leukocytes to sites of inflammation through upregulation of endothelial-specific adhesion relevant molecules (38). We pre-treated Trpm7$^{+/+}$ or *Trpm7*$^{R/R}$ mice with saline only (Ctrl) or TNF-α (500 ng) intraperitoneally (i.p.), respectively. Four hours post injection, we analyzed neutrophil infiltration into the peritoneal cavity *via* flow cytometry (**Figure 3D**, left panels). In Trpm7$^{+/+}$ mice, i.p. injection of TNF-α dramatically increased the number of neutrophils in the peritoneal cavity compared to control mice (**Figure 3D**, right panels). In contrast, neutrophils from *Trpm7*$^{R/R}$ mice showed a dramatic reduction in neutrophil transmigration into the peritoneal cavity upon TNF-α stimulation (**Figure 3D**), suggesting a critical role of TRPM7 kinase in this process.

TRPM7 Kinase Regulates Neutrophil Function *via* Activation of Akt/mTOR Pathways

To further deliniate the signaling pathways by which TRPM7 channel or kinase alter murine neutrophil function, we analyzed the effect of pharmacologic modulation and genetic TRPM7 kinase inhibition on cellular signaling. Bone marrow derived murine neutrophils from Trpm7$^{+/+}$ and *Trpm7*$^{R/R}$ mice were used to analyze the phosphorylation status of NFκB (p65 Ser^{536}), ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), and mTOR (Ser^{2448}) upon LPS stimulation. In analogy to human neutrophils, TRPM7 channel and kinase were blocked in neutrophils derived from Trpm7$^{+/+}$ mice using either NS8593 (30 μM) or TG100-115 (20 μM). We observed reduced NFκB (p65 Ser^{536}) phosphorylation in NS8593 and in TG100-115 treated cells, whereas NFκB signaling was redusced albeit not significatly altered in *Trpm7*$^{R/R}$ neutrophils (**Figure 4A**). We found that neither neutrophils from *Trpm7*$^{R/R}$ nor NS8593 or TG100-115 treated Trpm7$^{+/+}$ neutrophils showed significant differences in ERK1/2 signaling (**Figure 4B**). Pharmacologic TRPM7 channel blockade, TRPM7 kinase blockade as well as genetic inactivation of the kinase (*Trpm7*$^{R/R}$) led to significant reduction in Akt/mTOR-dependent signaling (**Figures 4C, D**). Notably, the application of the channel blocker NS8593 resulted in a similar LPS-induced phosphorylation status compared to *Trpm7*$^{R/R}$ controls for all signaling proteins, further suggesting that TRPM7 channel blockade might also affect kinase activity. To confirm that the effects of pharmacologic inhibition of the TRPM7 channel or kinase using NS8593 or TG100-115 on the respective signaling proteins was not due to off-target effects, we incubated *Trpm7*$^{R/R}$ neutrophils for 30 min with NS8593 or TG100-115 prior to LPS stimulation. Indeed, the pharmacologic blockade of TRPM7 kinase by TG100-115 in *Trpm7*$^{R/R}$ neutrophils did not further reduce the phosphorylation status of NFκB (p65 Ser^{536}), ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), or mTOR (Ser^{2448}) (**Figures 4E–H**) compared to *Trpm7*$^{R/R}$ controls. Similar results were found for application of the channel blocker NS8593 (**Figures 4E–H**).

Together our findings indicate that the TRPM7 kinase regulates neutrophil function *via* activation of Akt/mTOR pathways, while channel activity controls neutrophil activity also through NFκB-dependent pathways, most likely due to providing essential calcium and magnesium (**Figure 4I**).

DISCUSSION

Excessive neutrophil infiltration accelerates tissue damage due to unrestrained inflammation in pro-inflammatory as well as autoinnmue diseases (26, 27). Therfore, it is crucial to understand the mechanisms that regulate neutrophil function and migration. Previously, TRPM7 was suggested to be involved in CD147-triggered Ca^{2+}-induced chemotaxis, adhesion, and invasiveness of a human neutrophil cell line as silencing TRPM7 *via* siRNA led to a reduction of neutrophil adhesion (23). However, the precise mechanism how TRPM7 might be involved in neutrophil recruitment still remains to be explored. Utilizing a heterozygous TRPM7 kinase-deficient mouse model, *Trpm7*$^{+/DK}$ (39), it was shown that channel and or kinase activity might affect neutrophil rolling and recruitment (25). Consequently, authors concluded that the channel-kinase TRPM7 could have a protective role in cardiovascular inflammation and fibrosis (25). As in the respective model also the channel activity is reduced (39, 40) it is critical to gain a better understanding of the role of TRPM7 channel and kinase activities in the signaling cascades triggering neutrophil recruitment. We here show that pharmacologic and genetic inhibition of TRPM7 kinase affects human and murine neutrophil function. We further identify a potential underlying molecular mechanism, with Akt1 as novel downstream target of the TRPM7 kinase in neutrophils.

Along with other ion channels, TRPM7 has been linked to migration and motility of various different cell types (41). It has been suggested that TRPM7 channel activity is aiding calcium (Ca^{2+}) flickers thus steering fibroblast cell migration (22). We here propose, that in addition to facilitating Ca^{2+} or Mg^{2+} influx, TRPM7 might affect migration and motility *via* its kinase domain. TRPM7 might do so *via* direct phosphorylation of myosin II (29, 42). Recently, a compound with TRPM7 kinase inhibitory activity was identified in a small molecule library screen. TG100-115 was the most potent inhibitor in the screen, significantly decreasing cell migration and invasion of breast cancer cells and inhibiting TRPM7 kinase-dependent myosin IIA phosphorylation (29). Although TG100-115 has been reported to suppress TRPM7 ion channel activity (29), in our hands the kinase inhibitor had no effect on ion channel function. Originally, TG100-115 was shown to inhibit PI3K-γ and -δ (43), to which its broad anti-inflammatory and anti-cancerous as well as cardio-protective effects were attributed (43, 44). However, at least in part, this could also be due to its TRPM7 kinase inhibition. To assess whether the observed effects of

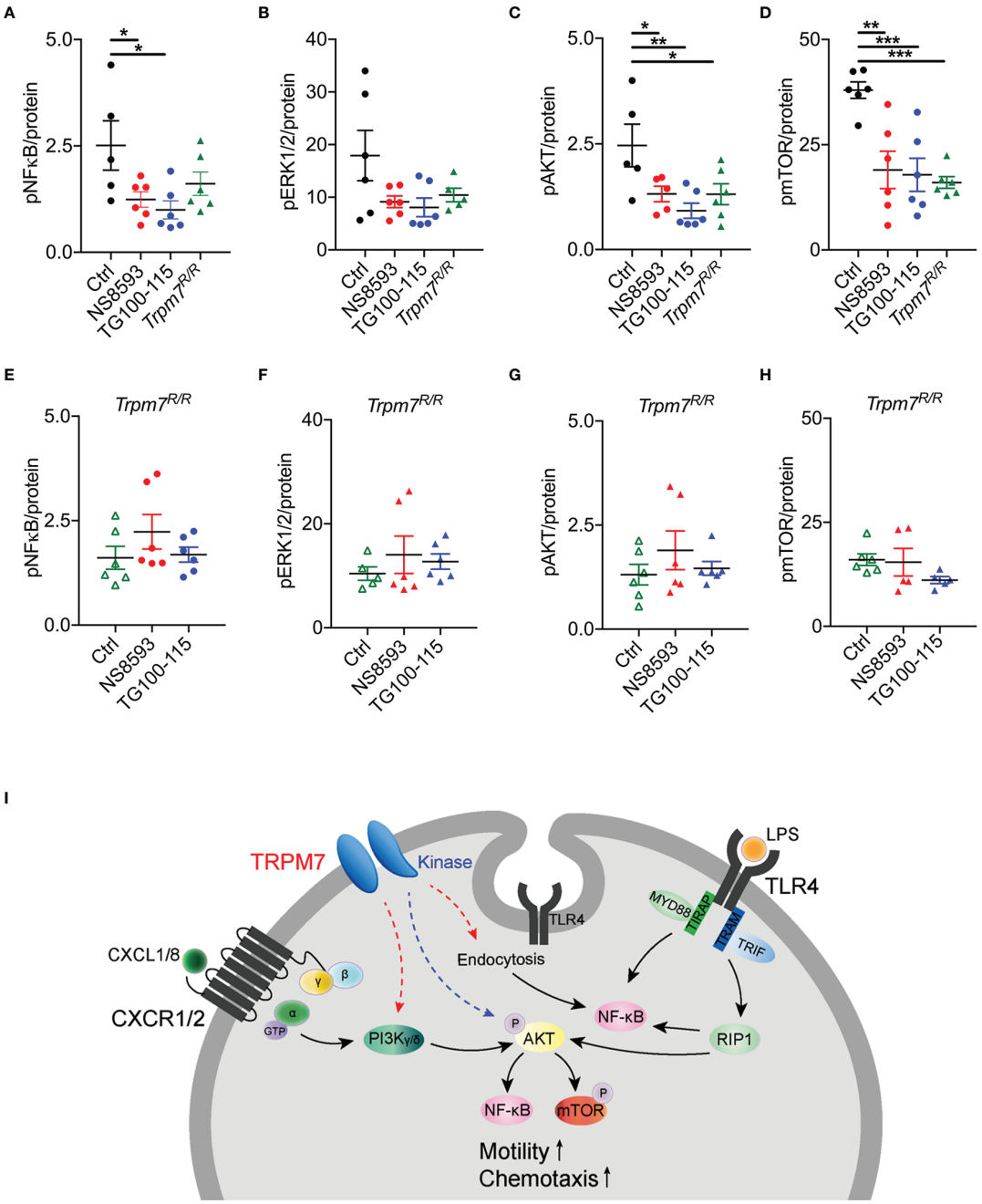

FIGURE 4 | TRPM7 regulates neutrophil function *via* NFκB and Akt/mTOR signaling pathways. Assessment of the activity of the cell signaling molecules NFκB, Erk1/2, Akt1, and mTOR utilizing a Bio-Plex assay and phospho-specific antibodies on lysates of bone marrow derived murine neutrophils of Trpm7$^{+/+}$ (black) and *Trpm7*$^{R/R}$ (green) mice. Trpm7$^{+/+}$ and *Trpm7*$^{R/R}$ neutrophils were pre-incubated with or without (control, black) the TRPM7 inhibitor NS8593 (30 μM, red), the TRPM7 kinase blocker TG100-115 (20 μM, blue), or a combination of IPI and NEM (160 and 100 nM, gray), respectively, for 30 min. Presented data depict the phosphorylation status upon stimulation with 10 ng/ml LPS for 30 min of **(A, E)** NFκB p65 (Ser536), **(B, F)** Erk1/2 (Thr202/Tyr204, Thr185/Tyr187), **(C, G)** Akt (Ser473), and **(D, H)** mTOR (Ser2448). For comparison results from control *Trpm7*$^{R/R}$ neutrophils (open green triangle) were taken from the respective panels above. Data are normalized to protein content and represented as mean ± s.e.m.; n = 3, measured in duplicates; a total number of 5–6 mice were used for each genotype. Statistics: one-way ANOVA *p < 0.05, **p < 0.01, ***p < 0.005. **(I)** Molecular model illustrating the role of TRPM7 in neutrophil chemotaxis and function: in macrophages, LPS-dependent endocytosis of TLR4 and subsequent NFκB activation was shown to depend on TRPM7 (12). We confirm a similar pathway involving TRPM7 channel activity in neutrophils (red dashed arrow). Previously, TRPM7 channel moiety was suggested to be positioned alongside the PI3K signaling axis (red dashed arrow). In neutrophils, PI3K isoforms are activated *via* CXCL8 and CXCR1/2, further triggering the activation of Akt1, NFκB, and mTOR signaling cascades. Alternatively, we propose a model in which TRPM7 kinase is directly or indirectly phosphorylating Akt1, thereby controlling NFκB and mTOR signaling pathways (blue dashed arrow).

TG100-115 are primarily due to PI3K-γ/δ blockade, we applied distinct PI3K-γ/δ inhibitors (IPI, NEM) with similar IC_{50} values (43). While affecting neutrophil chemotaxis in response to a CXCL8 gradient, as well as the production of reactive oxygen species (ROS) upon stimulation with the gram-negative bacterial lipopolysaccharide LPS, PI3K-γ/δ inhibitors did not decrease the LPS-triggered phosphorylation of NFκB, ERK1/2, Akt1, or mTOR. Blockade of TRPM7 kinase using TG100-115, however, affected neutrophil chemotaxis as well as ROS production in response to LPS most likely due to a reduction in Akt/mTOR signaling pathways. Interestingly, TRPM7 inhibition with the channel blocker NS8593 resulted in a similar reduction of Akt/mTOR signaling pathways, culminating in diminished neutrophil chemotaxis and ROS production. It is therefore tempting to speculate that TRPM7 channel blockade also reduces kinase activity and that the Mg^{2+} entering through the channel is required for optimal kinase function. TRPM7 kinase-deficient murine neutrophils also displayed reduced chemotaxis toward a CXCL1 gradient, which was further decreased upon TRPM7 channel blockade using NS8593, suggesting that TRPM7 channel function, at least in part, acts independent of TRPM7 kinase due to ion conductance. Previously, TRPM7 activity has already been implicated alongside PI3 kinase signaling pathways (10, 45, 46), however, as of now the impact of TRPM7 channel *versus* kinase moiety on different PI3K isoforms remains to be established. We here used PI3K isoform specific inhibitors as well as genetic inactivation of TRPM7 kinase to shed light on their interrelationship. In human LPS-treated neutrophils downstream analysis of signaling targets did not reveal a direct functional connection between TRPM7 and PI3K-γ/δ. However, functional analyses of neutrophil chemotaxis and ROS production indicated a potential interdependence. One possible explanation could be that, in CXCL1-triggered chemotaxis as well as LPS-induced ROS production, signaling molecules depending on PI3K-γ/δ pathways cannot be remunerated for, while the LPS-dependent phosphorylation of NFkB, Erk1/2, Akt1, and mTOR was compensated by other PI3K isoforms. Accordingly, TRPM7 kinase might directly or indirectly phosphorylate Akt1 and thereby control complex PI3 kinase signaling pathways while the channel function may indirectly act on PI3 kinase signaling *via* providing essential Ca^{2+} and/or Mg^{2+} for proper kinase activity (**Figure 4I**).

Taken together, we have shown that TRPM7 regulates several neutrophil effector functions including transmigration, extravasation, and ROS production. In addition, we were able to determine that TRPM7 kinase activity leads to Akt/mTOR activation, while the TRPM7 channel moiety also mediates activation of NFκB and ERK1/2. Finally, we demonstrate that TRPM7 and PI3K-γ/δ are not directly linked *via* those signaling pathways. Additional work is needed to further sort out how TRPM7 regulates neutrophil effector functions and how TRPM7 and PI3K-γ/δ cooperate in mediating those neutrophil functions. To finally understand whether TPRM7 kinase activity also affects neutrophil transmigration *in vivo*, we employed a TNF-dependent peritonitis model (47). While this short-term model does not exclude potential effects TRPM7 might have on other cell types, including the migration of other immune cells at later stages, it emphasizes the importance of TRPM7 kinase in regulating neutrophil effector functions and highlights the urgent need for more specific pharmacological inhibitors targeting the TRPM7 kinase moiety.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee from the Ludwig-Maximilians-Universität München (Az. 611-15). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by District Government of Upper Bavaria, Germany. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

WN conceived and performed experiments, analyzed data, and wrote the manuscript. RI conceived and performed experiments and analyzed data. KH performed experiments and analyzed data. MF, MR, and SR performed experiments. MM provided reagents. IB, TG, and MS provided their expertise and feedback and revised the manuscript. SZ conceived and supervised experiments, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.606893/full#supplementary-material

Supplementary Figure 1 | (A) Differential blood count of Trpm7$^{+/+}$ and *Trpm7*$^{R/R}$ mice. Representative dot plot of the gating schemes of the individual white blood cell types (left panel). Quantification of basal neutrophil, lymphocyte, monocyte, and eosinophil count in whole blood of Trpm7$^{+/+}$ (black, n = 4) and *Trpm7*$^{R/R}$ (green, n = 5) mice. Data are presented as 1000 (K) cells/µl and indicate mean ± s.e.m. **(B-D)** Gating schemes obtained using flow cytometry from individual figures as indicated.

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