Differential effects of lipopolysaccharide on mouse sensory TRP channels

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ABSTRACT

Acute neurogenic inflammation and pain associated to bacterial infection have been traditionally ascribed to sensitization and activation of sensory nerve afferents secondary to immune cell stimulation. However, we recently showed that lipopolysaccharides (LPS) directly activate the Transient Receptor Potential channels TRPA1 in sensory neurons and TRPV4 in airway epithelial cells. Here we investigated whether LPS activates other sensory TRP channels expressed in sensory neurons. Using intracellular Ca^{2+} imaging and patch-clamp we determined the effects of LPS on recombinant TRPV1, TRPV2, TRPM3 and TRPM8, heterologously expressed in HEK293T cells. We found that LPS activates TRPV1, although with lower potency than for TRPA1. Activation of TRPV1 by LPS was not affected by mutations of residues required for activation by electrophilic agents or by diacylglycerol and capsaicin. On the other hand, LPS weakly activated TRPM3, activated TRPM8 at 25 °C, but not at 35 °C, and was ineffective on TRPV2. Experiments performed in mouse dorsal root ganglion (DRG) neurons revealed that genetic ablation of *Trpa1* did not abolish the responses to LPS, but remain detected in 30% of capsaicin-sensitive cells. The population of neurons responding to LPS was dramatically lower in double *Trpa1*/*Trpv1* KO neurons. Our results show that, in addition to TRPA1, other TRP channels in sensory neurons can be targets of LPS, suggesting that they may contribute to trigger and regulate innate defenses against gram-negative bacterial infections.

1. INTRODUCTION

The detection of pathogen-associated molecules relies on pattern recognition receptors (PRR) that identify specific molecular motifs, highly conserved across species [1]. Among these PRRs, Toll-like receptors (TLR) have been described as sentinels for distinct viral and bacterial components [2]. For instance, lipopolysaccharides (LPS), a major component of the wall of gram-negative bacteria, are recognized by TLR4 in antigen presenting cells and innate immune cells [3]. Upon LPS ligation, TLR4 activation triggers the production of pro-inflammatory cytokines and chemokines (TNFα, interleukin (IL)-6, IL-1) that induce immune-mediated inflammation through the recruitment of leukocytes [3, 4].

Pain associated to infections was first described as sensitization and activation of nociceptors by inflammatory mediators found in the cytokine cocktail secreted by immune cells [5, 6]. However, recent evidence indicates that bacterial components can directly sensitize and activate sensory afferent neurons. For instance, it has been proposed that bacterial-derived *N*-formyl peptides, pore-forming toxins and LPS produce pain by inducing depolarization and firing in nociceptive neurons [7-9]. Furthermore, we recently reported that the cation channel TRPA1 can be activated by LPS, leading to pain and neurogenic inflammation in mice [10] and to aversive responses in *Drosophila melanogaster* [11]. In the former study, we found that *E. coli* LPS induces a concentration dependent activation of mouse sensory neurons isolated from nodose and trigeminal ganglia. Up to the concentrations of 3 and 10 µg/ml the responses to LPS occurred exclusively in neurons responsive to the TRPA1 agonist cinnamaldehyde. However, at higher concentrations (> 20 µg/ml), LPS induced responses in neurons that did not express TRPA1 (cinnamaldehyde-insensitive). Furthermore, the genetic ablation of *Trpa1* significantly reduced, but did not completely abolish, the responses to LPS. These data demonstrate that TRPA1 is not the only excitatory Ca^{2+} permeable channel mediating LPS effects.

Ligand promiscuity has risen as one of the key features of the TRP channel superfamily [12-16]. For instance, allyl isothiocyanate (AITC), initially described as a TRPA1 specific agonist, can also activate TRPV1 at higher concentrations, leading to aversive and pain responses and visceral irritation [17]. Furthermore, we have recently shown that epithelial TRPV4 is activated by LPS, inducing nitric oxide production and increased mucociliary beat frequency [18].

In this study, we tested the hypothesis that other sensory TRP channels may be involved in the TRPA1-independent effects of LPS. For this, we first characterized the effects of LPS on recombinant TRPV1, TRPV2, TRPM3 and TRPM8 channels heterologously expressed in HEK293T cells. We found that TRPV1 and TRPM3 are also sensitive to LPS, although at higher concentrations than for TRPA1. On the other hand, TRPM8 responded to LPS only at temperatures lower than physiological ones, and TRPV2 appeared to be LPS-insensitive. Using freshly isolated dorsal root ganglion neurons, we determined the relative roles of these channels *ex vivo*, by comparing the LPS-induced responses in sensory neurons isolated from wild type, *Trpa1* KO, *Trpv1* KO and double *Trpa1/Trpv1* KO mice. Together with our previous results [10], we conclude that TRPA1 and TRPV1 are the main contributors to the acute responses to LPS in sensory neurons at physiological temperatures.

2. MATERIALS AND METHODS

2.1. Reagents

Reagents were purchased from Sigma-Aldrich (Bornem, Belgium), unless stated otherwise.

2.2. Animals

Trpv1 knock-out (KO) mice were obtained from The Jackson Laboratory (http://jaxmice.jax.org/strain/003770.html) and *Trpa1* KO and double *Trpv1*/*Trpa1* KO mice have been described earlier [17, 19]. All knockout strains were backcrossed at least nine times

into the C57BL/6J background, and C57BL/6J mice were used as wild type (WT) 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 male 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 (P021/2012).

2.3. Cell culture and transfection

Human embryonic kidney cells, HEK293T, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator with 10% CO_2. For intracellular Ca^{2+} imaging and whole-cell patch-clamp experiments, HEK293T cells were transiently transfected with mouse TRPM3α2, human TRPV2, TRPM8 or TRPV1 in the bicistronic pCAGGS/IRES-GFP vector, or with mouse TRPV1-S513Y or TRPV1-C157A in the bicistronic pIres2GFP vector, using Mirus TransIT-293 (Mirus Corporation; Madison, WI, USA). For whole-cell patch-clamp experiments with mouse TRPM3α2, a stably expressing HEK293T cell line was used.

2.4. Isolation and primary culture of dorsal root ganglion neurons

DRG neurons from adult (postnatal weeks 8–12) mice were cultured using a variant of a method previously described [20]. Briefly, DRGs were bilaterally excised under a dissection microscope, washed in 10% fetal calf serum Neurobasal A Medium (basal medium) and then incubated at 37 °C in a mix of collagenase of 1 mg/ml (Gibco, Gent, Belgium) and dispase of 2.5 mg/ml (Gibco) for 45 min. Digested ganglia were gently washed twice with basal medium and mechanically dissociated by mixing with syringes fitted with increasing needle gauges. Neurons were seeded on poly-l-ornithine/laminin-coated glass bottom chambers (Fluorodish

WPI, Hertfordshire, UK) and cultured for 12–18 h at 37 °C in B27-supplemented Neurobasal A medium (Invitrogen, Gent, Belgium) containing GDNF of 2 ng/ml (Invitrogen) and NT4 of 10 ng/ml (Peprotech, London, UK).

2.5. Intracellular Ca^{2+} imaging experiments

Cells were incubated at 37 °C with 2 μM of Fura-2AM ester for 30 min before the recordings. Intracellular Ca^{2+} concentration ($[Ca^{2+}]$) was measured on an Olympus Cell-M system. Fluorescence was measured during excitation at 340 nm and 380 nm, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (F_{340}/F_{380}) was monitored. Intracellular Ca^{2+} concentrations were calculated using the formula described below, as described by Grynkiewicz and colleagues [21]:

$$[Ca^{2+}] = K_{eff}\frac{R - R_{min}}{R_{max} - R}$$

where R denotes the ratio between the fluorescence intensities measured at 340 nm (F_{340}) and 380 nm (F_{380}); R_{min} is the value of this ratio measured in Ca^{2+}-free medium containing 10 mM EGTA; R_{max} corresponds to the ratio measured using high Ca^{2+} concentration (10 mM)-containing medium; and K_{eff} is the effective dissociation constant.

K_{eff} is determined by:

$$K_{eff} = K_D\frac{R_{max} + \alpha}{R_{min} + \alpha}$$

where K_D is the dissociation constant of Fura-2AM for Ca^{2+} and α is the 'isocoefficient'. The value of α is calculated from: $F_{isosb} = F_{340} + \alpha F_{380}$, and correspond to a value for which, the isosbestic point (F_{isosb}) becomes independent of the values of F_{340} and F_{380} [22].

Experiments were performed using the standard Krebs solution containing (in mM): 150 NaCl; 6 KCl; 10 HEPES; 1.5 $CaCl_2$; 1 $MgCl_2$; 10 glucose monohydrate; pH adjusted to 7.4 with NaOH. The data were classified semi-automatically using a function programmed in MATLAB

(MathWorks, MA) and analyzed with Origin 7.0 (OriginLab Corporation, Northamptom, MA, USA).

2.6. Whole-cell patch-clamp experiments

Whole-cell membrane currents were measured with an EPC-10 patch-clamp amplifier and the Pulse (HEKA, Lambrecht/Pfalz, Germany) and Clampex (axon instruments, Sunnyvale CA, US) softwares. Currents were digitally filtered at 3 kHz, acquired 20 kHz and stored for off-line analysis on a personal computer. Cells were recorded in an extracellular solution containing (in mM): 156 NaCl; 10 HEPES; 1.5 $CaCl_2$; 1 $MgCl_2$; 10 glucose monohydrate; pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM): 145 Cs-Aspartate; 10 EGTA; 10 HEPES; 1 $MgCl_2$; pH adjusted to 7.2 with CsOH. Non-transfected HEK293T cells were used as control. Whole-cell currents were elicited using 200 ms-long voltage ramps applied every 2 s from a holding potential of 0 mV from -110 mV to +110 mV. Time courses of current amplitudes were plotted as the current measured at -75 mV and +75 mV. Unless otherwise mentioned, all measurements were performed at 35 °C.

2.7. Data analysis and statistics

Electrophysiological data were analyzed using the WinASCD software (Guy Droogmans, KU Leuven) and Origin 9.0 (OriginLab). Origin 9.0 was also used for statistical analysis and data display. Pooled data are expressed as mean ± s.e.m.

3. RESULTS

3.1. TRPV1 is activated by LPS

First, we sought to determine whether LPS activates TRPV1 using intracellular Ca^{2+} imaging and whole-cell patch-clamp recordings in HEK293T cells transfected with human TRPV1. In accordance with our previous report [10], LPS was ineffective at the low concentration of

6 µg/ml. However, at higher concentrations it induced intracellular Ca^{2+} increase in TRPV1-expressing cells (capsaicin-sensitive; Fig. 1A and B). Notably, the amplitude of the responses did not follow a simple dependence with the LPS concentration. Indeed, the magnitude reached a plateau between 20 and 100 µg/ml but displayed a dramatic increase beyond 100 µg/ml (Fig. 1B). We found that non-transfected HEK293T cells (capsaicin-insensitive) responded to LPS at concentrations of 60 µg/ml or higher, in a concentration-dependent manner. This indicates that for those concentrations the responses of TRPV1-transfected cells were partly mediated by a mechanism independent of TRPV1 (Fig. 1B). At high concentrations (>100 µg/ml), LPS has been previously shown to insert spontaneously in lipid membranes [23], inducing membrane disorder [24] and ultimately compromising membrane integrity [25]. Thus, LPS-induced disruption of the plasma membrane could facilitate the passive non-selective permeation of Ca^{2+} from the extracellular solution, resulting in non-specific Ca^{2+} increase in non-transfected cells. Activation of TRPV1 currents was demonstrated in whole-cell patch-clamp recordings using 20 µg/ml LPS, a concentration at which we observed Ca^{2+} increase specifically in TRPV1-expressing cells. LPS activated TRPV1 in a fast and reversible manner (Fig. 1C), significantly increasing both inward and outward currents (P = 0.0187 and 0.0010, respectively; n = 13; Fig. 1D).

To gain insight in the mechanism of TRPV1 activation by LPS we investigated whether amino acid residues required for activation by electrophilic compounds or vanilloids are required for this effect. Replacing Cys 157 in the N-terminus tail to alanine is known to abrogate the response to allicin and 2-aminoethyl methanethiosulfonate (MTSEA) [26]. Cells transfected with the C157A mutant displayed robust responses to LPS (Fig. 2A). The TRPV1 antagonist SB366791 (1 µM) [27] completely abrogated LPS-induced responses of this mutant (Fig. 2A). Likewise, mutation of Ser 513, which is known to abrogate the responses to capsaicin but not to heat [28], did not affect the responses to LPS (Fig. 2C). Interestingly, the antagonist

SB366791 did not prevent the activation of the S513Y mutant by LPS (Fig. 2D), which suggests that this compound acts on the vanilloid binding site.

3.2. TRPM3 is activated by LPS

To determine the effect of LPS on TRPM3 channels we used HEK293T cells transfected with the mouse TRPM3α2 isoform, and the functional expression was assessed from the response to the TRPM3 agonist pregnenolone sulfate [29]. TRPM3-expressing cells were sensitive to 20 µg/ml LPS in a reversible manner (Fig. 3A). LPS effects in transfected cells reached a plateau between 60 and 100 µg/ml, and drastically increased at 200 µg/ml (Fig. 3B). Notably, at doses of 100 µg/ml of LPS or higher, the amplitudes of these responses were indistinguishable from those of the 'non-specific' effects observed in non-transfected cells (Fig. 3B). We sought to confirm the activation of TRPM3 by LPS by measuring whole-cell currents in a stably transfected cell line expressing the mouse TRPM3α2 isoform. Application of 20 µg/ml LPS induced a clear and reversible activation of TRPM3 (Fig. 3C), significantly increasing both inward and outward currents (P = 0.00037 and 0.0028, respectively; n = 6; Fig. 3D).

3.3. LPS activates TRPM8 at cold temperatures

Next, we investigated whether TRPM8 can be activated by LPS. HEK293T cells transfected with human TRPM8 were largely insensitive to LPS in experiments performed at 35 °C (Fig. 4A). Only at a concentration of 100 µg/ml the responses were significantly larger than those evoked in non-transfected cells (Fig. 4B). Accordingly, 20 µg/ml LPS induced no significant increase of inward whole-cell currents in TRPM8-transfected cells (P = 0.078; n = 6) and only a minor increase was observed at positive potentials (P = 0.030; n = 6; Fig. 4C, D). However, TRPM8 channel gating is the result of an intricate interaction between chemical stimuli and physical stimuli [30]. For instance, the agonistic effect of menthol was more evident when

examined at lower temperatures or higher voltages [31]. High temperatures shift the open probability of the TRPM8 channel to positive voltages, which makes it hard to identify agonistic effects of chemicals in such conditions. Indeed, decreasing the bath solution to 25 °C induced responses in TRPM8-transfected cells, but also endowed these cells with the ability to respond to 20 µg/ml LPS (Fig. 4A). In series of experiments that were fully conducted at 25 °C, we found that a large proportion (50 to 78%) of TRPM8-expressing cells responded to LPS in a dose dependent manner (Fig. 5A, B). At this temperature, non-transfected cells only responded at the highest concentration tested (200 µg/ml). We confirmed this result with whole-cell current measurements in TRPM8 transfected cells (Fig. 5C, D). At 25 °C, we detected significant increases in inward and outward currents upon application of 20 µg/ml LPS (P = 0.0248 and 0.00093, respectively; n = 9; Fig. 5D). Thus, compared to TRPA1, TRPV1 and TRPM3, TRPM8 seems to be largely insensitive to LPS at physiological temperatures, but is sensitized to LPS by cold.

3.4. TRPV2 is largely insensitive to LPS

Intracellular Ca^{2+} imaging experiments revealed that cells transfected with TRPV2 did not respond to 20 µg/ml LPS, but to the known activator cannabis oil (Fig. 6A). A screening of the effects of LPS at higher concentrations revealed that only at 200 µg/ml LPS-induced responses of TRPV2-transfected cells were stronger than in non-transfected cells (Fig. 6B). These results demonstrate that TRPV2 is largely insensitive to LPS.

3.5. Roles of the main nociceptive TRP channels in LPS effects on mouse DRG neurons

Finally, we determined the effects of LPS on mouse DRG neurons, and guided by the above results we focused on the relative contribution of TRPA1, TRPV1 and TRPM3. We found that *E. coli* LPS induces a fast and reversible stimulation of DRG neurons isolated from wild type mice (Fig. 7A), similar to our previous results in nodose and trigeminal ganglion neurons [10].

This effect was concentration-dependent and occurred at concentrations equal or higher than 6 µg/ml (Fig. 7B). Almost 50% of the cells responding at the highest concentration tested (60 µg/ml). Notably, we found that in the subpopulation of neurons insensitive to CA but sensitive to capsaicin, 20% (11 out of 52) of the cells reacted to the application of 60 µg/ml LPS (Fig. 7A, red trace). This suggested that TRPV1 may partly mediate the responses to LPS in DRG neurons. Alternatively, desensitization of TRPA1 after LPS-induced activation may have reduced the responses to CA, resulting in an underestimation of the TRPA1-expressing neuron population. Thus, to directly test the contribution of TRPA1, we carried out similar experiments in neurons isolated from *Trpa1* KO mice. LPS activated a significantly lower proportion of neurons from *Trpa1* KO mice, but this reduction was less prominent with increasing concentrations (Fig. 7B, C). This indicates that TRPA1 is the major mediator of the responses to low concentrations of LPS and that other TRP channels may be implicated in the responses to LPS at high concentrations. The fact that 16% (52 out of 329) of the capsaicin-sensitive *Trpa1* KO neurons were stimulated by LPS suggests for a role of TRPV1 in these responses (Fig. 7C and D). When inquiring about a possible role of TRPM3 in capsaicin-insensitive neurons we found that only 1 out of 129 cells reacted to both LPS and PS (Fig. 7C, D). Responses to 20 µg/ml LPS were drastically abrogated in DRG neurons from *Trpa1/Trpv1* KO mice, and higher concentration of LPS (60 µg/ml) activated only 4.3% (50 out of 1154) of the total population (Fig. 7D). Taken together, these results show that TRPA1 and TRPV1 are the major contributors to LPS-induced responses in DRG neurons.

4. DISCUSSION

The initial hypothesis for pain associated to bacterial infection featured the sensitization and activation processes in nociceptors by pro-inflammatory mediators secreted by leukocytes in response to bacterial endotoxins [5, 6]. However, the identification of TLR4 and the LPS

binding protein CD14 in TRPV1-expressing trigeminal nociceptor [32] raised the possibility of a direct recognition of LPS by sensory afferents. Indeed, subsequent studies demonstrated that LPS can directly stimulate sensory neurons and sensitize TRPV1 to capsaicin-evoked intracellular Ca^{2+} increase and CGRP release [8, 9]. Although interfering with TLR4 signaling prevented LPS-induced sensitization, no specific molecular mechanism has been described to link TLR4 stimulation and the rather fast activation of sensory neurons.

We have recently shown that the broadly tuned chemonociceptor TRPA1 is a key player in some biological responses to bacterial endotoxins [10]. Specifically, LPS induced a robust and sharp Ca^{2+} increase in mouse trigeminal and nodose ganglion sensory neurons, leading to acute pain and neurogenic inflammation. Surprisingly, genetic ablation of *Trpa1*, but not of *Tlr4*, strongly reduced LPS-evoked neuronal responses at doses of LPS close to the EC_{50} for TRPA1 activation (~3 µg/ml). However, responses to higher doses of LPS were not limited to TRPA1-expressing neurons, suggesting that other TRP channels were involved in LPS-induced Ca^{2+} increase.

DRG neurons receive sensory input from a large fraction of our body, as they innervate abdominal viscera and a major proportion of the skin. Some of these organs, i.e. the bladder, the gut and the skin, are constantly exposed to the risk of bacterial infection and are some of the first fronts of action of the innate immunity mechanisms. However, neither the responses of DRG neurons to LPS, nor the possible underlying contributions of sensory TRP channels in these cells have been reported. For this reason, and for the advantage of the higher yield and purity of the DRG neuron cultures we used this preparation to determine the relative role of sensory TRP channels in the responses to LPS over a broad range of concentrations.

We found that DRG neurons isolated from WT mice responded to LPS in a reversible and concentration-dependent manner. As previously reported for trigeminal and nodose neurons [10], the proportion of DRG neurons responding to LPS is dramatically lower in *Trpa1* KO

than in WT mice. Yet, a significant fraction of *Trpa1* KO neurons responded to 20 and 60 µg/ml LPS.

Robust intracellular Ca^{2+} increase in response to LPS has been reported to occur in endothelial cells (ECs) through TRPC6 [33], a non-selective Ca^{2+}-permeable channel from the TRP superfamily. It has been proposed that ligation of LPS to the component of the TLR4 complex CD14 in ECs, induces the hydrolysis of phosphatidyl inositol-4,5-biphosphate ($PI(4,5)P_2$) by phospholipase C leading to formation of diacylglycerol (DAG), which further activates TRPC6 [34]. This TRPC6-dependent mechanism is unlikely to operate in sensory neurons (DRG and trigeminal) due to the lack of expression of the channel [35]. Strikingly, LPS was virtually ineffective in neurons isolated from double *Trpa1*/*Trpv1* KO mice, strongly indicating for a role of TRPV1. Accordingly, we found that LPS can trigger significantly stronger responses in HEK293T cells transfected with human TRPV1 than in non-transfected cells. However, TRPV1 seems to require higher concentrations of LPS than TRPA1 to be activated.

Regarding the mechanisms underlying activation of TRPV1 by LPS one could hypothesize that, as shown in ECs [34], activation of TLR4 in sensory neurons leads to the production of DAG, which is known to be an endogenous agonist of TRPV1. However, DAG activation of TRPV1 is absent in the S513 mutant [36], but this mutation did not abrogate LPS-induced TRPV1 activation. We also found that LPS effects are not mediated by residues required for activation by allicin and MTSEA, thus excluding the production of electrophilic compounds as intermediaries triggering TRPV1 activation upon stimulation with LPS.

Interestingly, lysophosphatidic acid (LPA), an endogenous lipid associated with neuropathic pain, activates TRPV1 by electrostatic interaction with positive charges within the proximal C-terminus of the channel [37, 38]. Although, the lipidic nature of LPA might suggest that these residues are crucial for the LPS-induced activation, there are several important limitations that hamper a direct interaction of these region in TRPV1 with the LPS molecule, and thus a role in

the activation of TRPV1. First, the interaction is described to occur between the LPA present in the inner leaflet of the plasma membrane and the proximal C-terminus of TRPV1 (mainly Lys-710 for rat TRPV1, Lys-711 for mouse/human TRPV1) [37]. The spatial dimensions of LPS, however, constrains its interaction with the outer leaflet of the plasma membrane, and flipping mechanisms accommodating the large O-polysaccharide are unlikely to operate in mammalian cells. Secondly, TRPV1 activation by LPA (and LPA analogs) was clearly demonstrated to be restricted to fatty acids containing one monounsaturated C18 (Δ9, 10 or 11) hydrocarbon chain. *E. coli* LPS, however, typically contains six saturated C14 or C12 hydroxy acyl chains, further ruling out a similar interaction as described for LPA and other lipids alike.

Of note, we obtained results that shed additional light on the mechanism of action of the TRPV1 inhibitor SB366791. The loss of inhibitory effect in the capsaicin-insensitive S513Y mutant strongly indicates that SB366791 is a true competitive antagonist, with partial or total occupancy of the vanilloid binding site. Thus, the capsaicin binding pocket acts as the spatial entity for the allosteric inhibition of acid, heat [27] and LPS.

The differences in sensitivity to LPS observed between TRPA1 and TRPV1 may explain why the nocifensive responses to LPS are considerably reduced in *Trpa1* KO mice [10]. Nonetheless, these nocifensive responses were reported at submicromolar concentrations of LPS. Although actual contents of LPS during local infections have not been reported in literature, severe endotoxemia rises LPS plasma levels up to micromolar ranges (equivalent to 100 µg/ml), which can be higher within the spatially-constrained inflamed tissue (e.g.: in micrometer-thin mucosal layers at epithelial barriers or acute dental abscesses were LPS accumulates in very small volumes) [39, 40]. Thus, in the context of bacterial infection, TRPV1 may directly contribute to LPS-induced pain and inflammation. In addition, hydrolysis of $PI(4,5)P_2$ upon TRPV1 activation, with subsequent production of DAG and inositol 1,4,5-triphosphate (IP_3), may also explain the sensitization reported by others [8, 9]. LPS may also

decrease the threshold for TRPV1 activation by temperature, acidification and inflammatory mediators.

Our data from double *Trpa1*/*Trpv1* KO predicted a minor contribution of other mechanisms. Overall, the results of our experiments in HEK293T cells heterologously expressing other sensory TRPs were in agreement with this contention. For instance, we found that TRPV2 seems to be largely insensitive to LPS. On the other hand, despite of detecting clear stimulation of TRPM3-expressing cells, these responses were indistinguishable from non-transfected cells beyond a concentration of 60 µg/ml. This may indicate that TRPM3 is less sensitive to LPS than TRPA1 and TRPV1. Another reason for the lack of relevance of TRPM3 to the responses of DRG neurons to LPS could be that the expression of TRPM3 was restricted to only 26% of the total population of neurons isolated from double *Trpa1*/*Trpv1* KO mice. We have to note, however, that this figure is smaller than the 70% value previously reported for WT neurons [41]. Whether this discrepancy is due to different culture conditions or relates to a double *Trpa1*/*Trpv1* KO phenotype remains to be investigated.

Finally, we found that at 35 °C the responses of HEK293T cells transfected with TRPM8 to LPS were virtually indistinguishable from those of non-transfected cells. This indicates that TRPM8 does not contribute to neuronal responses to LPS at normal physiological temperatures. Interestingly, at 25 °C a large proportion of TRPM8-expressing cells responded to LPS, in a dose dependent manner. Similar temperature dependent effects have been described for other chemical agonists such as menthol and icilin [31, 42, 43]. This effect arises from the intricate interaction between chemical and physical stimuli contributing to TRPM8 channel gating [30]. At 35 °C the agonistic effect exerted by LPS was likely obscured due to an insufficient shift of the voltage dependent activation curve. Decreasing the temperature to 25 °C, shifts the open probability to more negative voltages, thus revealing the agonistic effect of LPS on TRPM8.

Further research will be required to determine whether this sharp temperature dependence is relevant for LPS signaling at cold temperatures.

The spectrum of mechanisms of activation of nociceptors has been recently expanded to several bacterial-derived compounds. Chiu *et al.* have recently reported that *N*-formylated peptides (*N*-FP) and the pore-forming toxin (PFT) αHL from gram-positive bacteria contribute to hyperalgesia *in vivo*, although through distinct mechanisms [7]. *N*-FP, are recognized by G-protein-coupled formyl peptide receptor 1 [44] found in DRG neurons and promotes mechanical hypersensitivity [7]. On the other hand, PFT αHL, a toxin actively secreted by live bacteria [45], assembles into heptameric pores that allows non-selective Ca^{2+} entry and consequent DRG depolarization [46]. These may constitute mechanisms parallel to TRP channel activation, leading to efficient acute responses to bacterial infection.

In conclusion, we have demonstrated that TLR4 is far from being the sole effector of LPS. Although our data confirms that TRPA1 activation remains the most sensitive sensory TRP channel in sensory neurons, TRPV1 may also contribute to pain sensation, in particular at higher doses. At present, the biological implications of the differential activation of TRPA1 and TRPV1 by LPS remain unknown. However, we can speculate that the recruitment of the large TRPV1-expressing subpopulation at higher LPS concentrations may serve as additional information about the magnitude of the infection to the host defense mechanisms.

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AUTHOR CONTRIBUTION

B.B. conducted the electrophysiological recordings. Y.A.A. and A.S. conducted Ca^{2+} imaging experiments. B.B., Y.A.A. and K.T. wrote the manuscript. A.L.-R. and T.V. contributed to the interpretation of data. K.T. conceived, designed and supervised the project. All authors edited and accepted the final version of the manuscript.

CONFLICT OF INTEREST

No conflict of interest is declared by the authors.

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FIGURE LEGENDS

Fig. 1. LPS activates TRPV1 in HEK-293T cells. (A) Average time course of intracellular Ca^{2+} concentration in HEK-293T cells exposed to LPS (20 µg/ml) and capsaicin (Caps, 1 µM). The thick lines represent the means (black, TRPV1-transfected cells; blue, non-transfected cells) and the grey and cyan bands represent the corresponding means ± s.e.m (n = 100 and 62 for transfected and non-transfected cells, respectively). (B) Average amplitude of intracellular Ca^{2+} increase during application of LPS in TRPV1-expressing cells (capsaicin-responsive, n > 42) and non-transfected HEK293T cells (capsaicin-unresponsive, n > 61). (C) Time course of TRPV1 current responses to 20 µg/ml LPS and 1 µM capsaicin (Caps) monitored at -75 and +75 mV. The colored data points correspond to the current traces shown in the inset. (D) Average amplitude of the currents in baseline condition (black bar) and during extracellular application of 20 µg/ml LPS (red bar) in TRPV1-expressing cells. All experiments were performed at 35 °C. *, $P < 0.05$; one-tailed paired *t* test.

Fig. 2. TRPV1 activation by LPS does not require electrophilic residues. (A, B) Representative intracellular Ca^{2+} concentration responses to 20 µg/ml LPS and 1 µM capsaicin (Caps) in cells expressing the TRPV1-C157A mutant (black traces). In A, the gray traces correspond to cells unresponsive to capsaicin. In B, cells were perfused with the TRPV1 antagonist SB366791 (1 µM) prior to LPS application. (C, D) Representative intracellular Ca^{2+} concentration responses to 20 µg/ml LPS, 1 µM capsaicin (Caps) and heat (45 °C) in cells expressing the capsaicin-insensitive TRPV1-S513Y mutant (black traces). In C, the gray lines represent cells unresponsive to heat. In D, cells were perfused with 1 µM SB366791 prior to LPS application.

Fig. 3. TRPM3 is activated by LPS in HEK-293T cells. (A) Average time course of intracellular Ca^{2+} concentration in TRPM3-expressing cells exposed to LPS (20 µg/ml) and 40 µM pregnenolone sulfate (PS). The thick line corresponds to the mean and the gray band

represent the means ± s.e.m. (n = 249). (B) Average amplitude of intracellular Ca^{2+} increase during application of LPS at different concentrations in TRPM3-expressing cells (PS-responsive, n > 68) and non-transfected HEK293T cells (PS-unresponsive, n > 19). (C) Time course of TRPM3 current responses to 20 µg/ml LPS and 40 µM PS monitored at -75 and +75 mV. The colored data points correspond to the current traces shown in the inset. (D) Average amplitude of the currents in baseline condition (black bar) and during extracellular application of 20 µg/ml LPS (red bar) in TRPM3-expressing cells. All experiments were performed at 35 °C. *, $P < 0.05$; one-tailed paired *t* test.

Fig. 4. TRPM8 is not activated by LPS at physiological temperature. (A) Average time course of intracellular Ca^{2+} concentration in HEK-293T cells exposed to LPS (20 µg/ml) and menthol (100 µM). The thick traces represent the means (black, TRPM8-transfected cells; blue, non-transfected cells) and the gray and cyan bands represent the corresponding means ± s.e.m. (n = 93 and 18 for transfected and non-transfected cells, respectively). The upper panel represents the temperature of the perfused solutions. (B) Average amplitude of intracellular Ca^{2+} increase during application of increasing concentrations of LPS at 35 °C in TRPM8-expressing cells (menthol-responsive, n > 54) and in non-transfected cells (menthol-unresponsive, n > 30). (C) Time course of TRPM8 current responses to 20 µg/ml LPS and 100 µM menthol monitored at -75 and +75 mV. Currents were measured at 35 °C until indicated by the 21 °C application bar. The colored data points correspond to the current traces shown in the inset. (D) Average amplitude of the currents in baseline condition (black bar) and during extracellular application of 20 µg/ml LPS (red bar) in TRPM8-expressing cells. *, $P < 0.05$; one-tailed paired *t* test.

Fig. 5. TRPM8 activation by LPS at cold temperature. (A) Ratiometric intracellular Ca^{2+} measurements showing the effects of 20 µg/ml LPS and 100 µM menthol on HEK-293T cells. The thick traces represent the means (black, TRPM8-transfected cells; blue, non-transfected

cells) and the gray and cyan bands represent the corresponding means ± s.e.m. (n = 136 and 51 for transfected and non-transfected cells, respectively) (B) Average amplitude of intracellular Ca^{2+} increase during application of increasing concentrations of LPS at 25 °C in TRPM8-expressing cells (menthol-responsive, n > 53) and in non-transfected cells (menthol-unresponsive, n > 30). (C) Time course of TRPM8 current responses to 20 µg/ml LPS and 100 µM menthol monitored at -75 and +75 mV. Currents were measured at 25 °C. The colored data points correspond to the current traces shown in the inset. (D) Average amplitude of the currents in baseline condition (black bar) and during extracellular application of 20 µg/ml LPS (red bar) in TRPM8-expressing cells. *, $P < 0.05$; one-tailed paired *t* test.

Fig. 6. TRPV2 is largely insensitive to LPS. (A) Ratiometric intracellular Ca^{2+} measurements showing the effects of 20 µg/ml LPS and 100 µM cannabis oil on HEK-293T cells. The thick trace represents the mean and the gray band represents the corresponding mean ± s.e.m. (n = 110) (B) Average amplitude of intracellular Ca^{2+} increase during application of increasing concentrations of LPS in TRPV2-expressing cells (cannabis oil-responsive, n > 63) and in non-transfected cells (cannabis oil-unresponsive, n > 40). All experiments were performed at 35 °C.

Fig. 7. Main role of TRPA1 and TRPV1 in the responses of mouse DRG neurons to LPS. (A, C) Representative traces of intracellular Ca^{2+} levels in DRG neurons isolated from wild type mice (A) or *Trpa1* KO mice (C). The horizontal bars indicate the application of LPS (60 µg/ml), cinnamaldehyde (CA, 100 µM), capsaicin (Caps, 1 µM), pregnenolone sulfate (PS, 40 µM) and Krebs containing high K^+ concentration (45 mM, K^+). (B) Percentage of cells isolated from wild type (n > 291), *Trpa1* KO (n > 263) and double *Trpv1/Trpa1* KO (n > 734) mice responding to different concentration of LPS. (D) Percentage of cells isolated from *Trpa1* KO mice responding to different concentrations of LPS in the TRPV1-expressing (capsaicin-responsive, n > 67) and TRPM3-expressing (PS-responsive, n > 21) populations. All experiments were performed at 35 °C.