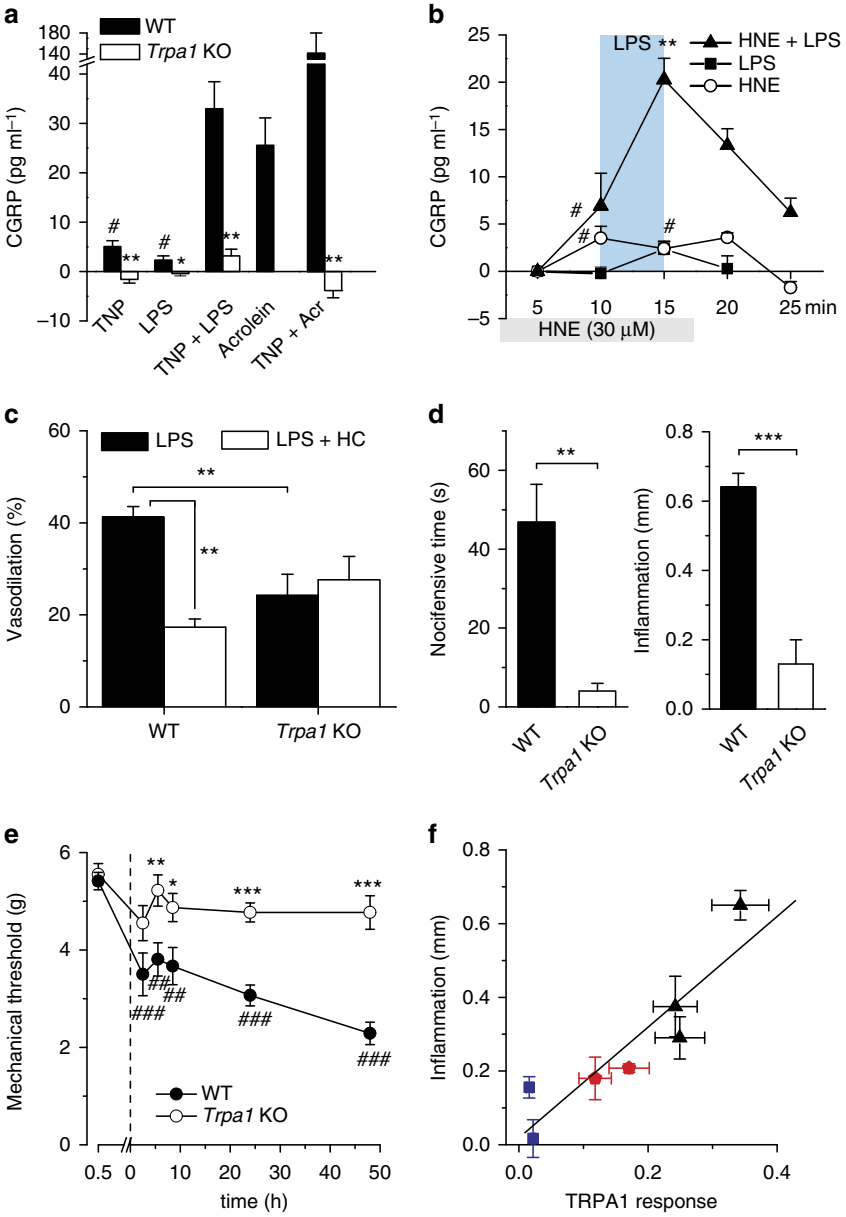

Figure 7 | Neurogenic inflammation and pain by LPS depend on TRPA1 activity. (**a**) Trinitrophenol (TNP, 50 μM) and LPS (100 μg ml^{-1}) evoke (mean ± s.e.m.) modest but significant ($^{\#}P<0.05$, Wilcoxon test, $n=12/8$) tracheal CGRP release; preincubation with TNP potentiates response to LPS and acrolein (30 μM). *Trpa1* KO mice do not respond to LPS ($^{*}P<0.05$, analysis of variance (ANOVA) Fisher's least significant difference (LSD) test, $n=4$), TNP or its co-application with LPS or acrolein ($^{**}P<0.01$). (ANOVA Fisher's LSD test, $n=8, 4, 4$). (**b**) LPS and 4-hydroxy-2-nonenal (HNE) evoke small but significant ($^{\#}P<0.05$) CGRP responses. Preincubation with low concentration of HNE potentiates tracheal CGRP release evoked by LPS ($^{**}P<0.01$, ANOVA Fisher's LSD test, $n=4$, mean ± s.e.m.). (**c**) Summary of effect of LPS (100 μg ml^{-1}) perfusion on mesenteric artery diameter (mean ± s.e.m.) from WT ($n=7$) and *Trpa1* KO ($n=8$) mice in control condition and in the presence of HC-030031 (10 μM). Arteries precontracted with the alpha1 adrenergic agonist phenylephrine (2 μM). Statistical differences were evaluated with an unpaired *t*-test. (**d**) The nocifensive response and the acute local inflammation produced by intraplantar LPS injection (5 μg μl^{-1} in 10 μl) were nearly abolished in *Trpa1* KO animals ($n=6$) compared with WT littermates ($n=7$) (unpaired *t*-test, values are mean ± s.e.m.). (**e**) Mechanical paw withdrawal threshold (mean ± s.e.m.) to mechanical stimulation before and after intraplantar injection of LPS (5 μg μl^{-1}) in WT ($n=14$) and *Trpa1* KO ($n=14$) animals. The asterisks reflect the differences in threshold between WT and KO (unpaired *t*-test). Comparison between baseline and the different time points gave significant differences ($^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$) in WT animals only (one-way ANOVA with Bonferroni's correction). (**f**) Correlation between the hind paw inflammation (mean ± s.e.m.) induced by LPSs of different shapes (blue for cylindrical, red for semiconical and black for conical; $n=5$–9) with the potency for TRPA1 activation (mean ± s.e.m.) estimated from the amplitude of Ca^{2+} response evoked in mTRPA1 cells; Fig. 5d).

developed normally in *Tlr4* KO mice following LPS injection (Supplementary Fig. 14). Notably, mechanical hyperalgesia resolved more rapidly in *Tlr4* KO mice compared with wild type, suggesting that immune response pathways are engaged by LPS in a slower timescale compared with TRPA1 activation.

TRPA1 is not critical for LPS systemic inflammatory response. As deletion of TRPA1 produces such prominent effects on nociceptive and neurovascular response to LPS, we wondered whether other, well-established, systemic responses to LPS were affected as well. In mice, i.p. application of high doses of LPS

produces a profound and long-lasting hypothermia[44]. As previously described, the injection of 3 mg kg^{-1} *E. coli* LPS produced a marked hypothermia in WT mice (Supplementary Fig. 15). The severity and time course of this hypothermic response was nearly identical in *Trpa1* KO littermates.

Activation of TLR4 by LPS is known to potently activate immune cells (for example, hepatic macrophages) to produce and release proinflammatory cytokines such as TNF-α and IL-6 (refs 4,45). Indeed, i.p. LPS treatment (0.75 and 1 mg kg^{-1} for 1 h) increased dose dependently circulating serum IL-6 levels from an undetectable baseline after saline injection (Supplementary Fig. 16a). As was the case for thermoregulatory responses, we did not find differences in the levels of IL-6 between both genotypes of mice. Moreover, LPS treatment (1 mg kg^{-1} for 1 h) also increased IL-6 messenger RNA levels in the liver in both types of mice similarly (Supplementary Fig. 16b).

Discussion

Here we demonstrate that TRPA1 channels are key participants in the biological response to LPS, a microbial signature molecule, translating the presence of elevated LPS into a rapid nociceptor response and neurogenic inflammation. Therefore, LPS appears as a totally novel class of biological toxin among the large number of natural compounds that are detected by TRPA1 channels (reviewed by Nilius *et al.*[16]). The ancient nature of LPS and the fact that the role of TRPA1 in chemical avoidance is conserved from flies to mammals underscores the evolutionary significance of this alert mechanism in animals[14,46–48]. It is noteworthy that bacterial sepsis is accompanied by severe redox imbalance, favouring the oxidant state[49], a condition that also leads to robust TRPA1 channel activation[24,50].

Our findings also raise the question of how this channel can function as such an extremely broadly tuned chemo-nocisensor. In the light of previous studies and of the present results, it seems clear that rather than using specific molecular determinants (that is, the key and lock concept), TRPA1 activation relies on the general physico-chemical properties of the agonist. Whereas highly reactive electrophilic irritants are detected by their capacity to modify covalently key amino-acid residues in the amino terminus of the TRPA1 channel[47,51], our results suggest that LPS modulates TRPA1 activity by inducing mechanical perturbations in the plasma membrane. This result is in line with the known mechanosensing function of TRPA1 in visceral[52] and somatic[53,54] neurons. The activation of TRPA1 by LPS in cell-free membrane patches indicates that the mechanism underlying this effect does not require other intermediate molecules, but additional experiments, including the characterization of LPS action after incorporation of TRPA1 into artificial lipid bilayers of defined composition, are needed to substantiate this point. Nevertheless, it is noteworthy that different forms of LPS activated TRPA1 in proportion to their ability to produce inflammation *in vivo*, which suggests a close relation between both processes. Recently, Hardie and Franze[55] showed, in a very elegant way, that activation of light-sensitive TRP and TRPL channels in *Drosophila* photoreceptors is mediated by rapid mechanical perturbation of the plasma membrane caused by phosphatidylinositol 4,5-bisphosphate (PIP_2) depletion downstream of PLC activation by light. This study is very relevant to the hypothetical activation of TRPA1 under pathophysiological conditions because TRPA1 is also activated by G-protein-coupled receptors that activate PLC and deplete PIP_2 (refs 15,23). In the context of infection, the production of some inflammatory mediators (for example, bradykinin and trypsin) that couple to Gq/11 will also lead to PIP_2 depletion. Thus, the mechanical perturbations produced by LPS hypothesized in our study could act synergistically with those produced by changes in PIP_2 levels, leading to TRPA1 gating.

Sensory neurons express a number of TLR4 signalling elements, including TLR4, making it plausible that TLR4 signalling participates in sensory responses to LPS[11]. Indeed, several recent studies have implicated TLR4 in rapid responses to LPS in sensory neurons, including the sensitization of TRPV1 channels[10]. However, a number of results clearly indicate that TLR4 is dispensable for the excitatory actions of LPS on TRPA1. First and foremost, responses in sensory neurons from *Tlr4* KO mice were indistinguishable from those observed in WT animals. Second, incubation with a specific inhibitor of TLR4 signalling did not affect responses to LPS in TRPA1-expressing neurons or recombinant TRPA1 channels. Moreover, acute pain and tissue swelling peaked normally in *Tlr4* KO mice *in vivo*. Obviously, under *in vivo* conditions, circulating LPS will engage the available TLR4 machinery, including the production of proinflammatory cytokines, which could participate in the slow sensitization of nociceptors. Consistent with this view, we observed a faster resolution of the mechanical hypersensitivity, following local LPS injection, in *Tlr4* KO mice. *In vivo*, we envision both effector pathways of LPS acting synergistically on somatic and visceral neurons.

Our observation that LPS excites somatic and visceral nociceptor neurons via a mechanism dependent on TRPA1 activation and independent of TLR4 can readily explain acute pain and neurogenic inflammation induced by LPS. Similar concentrations of LPS have been used to evoke pain behaviour in experimental animals[42]. The activation of TLR4 occurs at lower LPS levels than those required to activate TRPA1 channels. However, severe endotoxemia can bring plasma LPS levels into the micromolar range[56], levels also encountered in urine and locally inflamed tissues[57]. Moreover, we found strong (that is, 10-fold) potentiation of LPS-induced activation of TRPA1 by other TRPA1 agonists, including endogenous modulators (for example, HNE), suggesting that in the context of a bacterial infection *in vivo*, the levels of LPS required to activate TRPA1 may be lower than what we found experimentally. It should be noted that the *ex vivo* preparations we used (that is, the superfused trachea and the isolated tongue), in contrast to the skin *in vivo*, cannot reflect secondary inflammatory reactions, like plasma extravasation, accumulation of inflammatory mediators (for example, bradykinin, thrombocytes and immune cells), which, together, may complement or amplify, like we found with HNE or acrolein, the excitatory effects of LPS on TRPA1 to activate nociceptors[58]. The modest but significant effect of LPS alone on tracheal CGRP release is not surprising, because even prostaglandin E2, the most widely acknowledged nociceptor sensitizer, shows no effect of its own on nociceptor discharge and CGRP release but 'only' facilitates heat and chemical responses[59]. Finally, these avascular *ex vivo* preparations cannot be compared directly with dissociated sensory neurons in culture with respect to chemosensitivity, because the nerve endings do not reach the very surface of the mucosa, formed by epithelial cells and their tight junctions.

TRPA1-mediated calcium influx in sensory terminals leads to release of neuropeptides[13,24], thereby triggering rapid TRPA1-dependent vasodilation, local inflammation and sustained mechanical hyperalgesia. Recently, it was established that TRPA1-mediated substance P release in the colon plays a necessary role in experimental colitis[60], providing further support for a causal link between TRPA1 channels and neuroimmune processes. Other studies have indicated the essential role of TRPA1 in the pathophysiology of asthma[37], a disease with a significant immunological component.

We found that in the absence of TRPA1 the proinflammatory and pro-algesic actions of LPS are severely curtailed. Interaction

of LPS with the MD-2–TLR4 complex leading to activation of the transcription factor nuclear factor-kappaB (NF-κB) and cytokine production is the canonical pathway of LPS signalling[2]. The identification of TRPA1 as a functional target for LPS may represent an important missing link between the stimulation of afferent sensory fibres and the immune responses during a bacterial challenge. We tentatively conclude that TRPA1 channels and pattern receptors like TLR4 act as synergic sensory mechanisms, alerting the host about the presence of pathogens, allergens and environmental irritants[3]. TRPA1 would be involved in the early steps of the response cascade, sensing potentially damaging compounds, in parallel with the well-established production of proinflammatory cytokines. The recent discovery of activation of the NF-κB signalling pathway following TRPA1 activation provides independent support to our view[61]. Thus, identification of TRPA1 as a new target for LPS opens novel avenues for the treatment of adverse biological responses of Gram-negative bacterial infections. Finally, the recent report describing the activation of nociceptors by *N*-formylated peptides and the pore-forming toxin α − haemolysin isolated from bacteria, by mechanisms independent of immune pathways[62], suggests that direct activation of nociceptors by bacterial products may be a more general signalling mechanism than previously recognized.

Methods

Animals. Experiments were performed in accordance with the guidelines established by the European Communities Council Directive (86/609/ECC), by Spanish Royal Decree 1201/2005 and the Ethics Committee of the Catholic University, Leuven.: *Tlr4* KO mice on a C57BL/6 background, originally generated by Hoshino *et al.*[63] were kindly provided by C Guerri Centro de Investigación Príncipe Felipe (CIPF) and by H Hammad and B Lambrecht (VIB, Ghent, Belgium). *Trpa1* KO mice were provided by D Corey[64]. *Trpv1* KO mice, originally generated in D Julius laboratory[18], were obtained from Jackson.

Neuronal culture. Neuronal culturing methods were similar to those used previously[65,66]. TG and nodose ganglia were isolated from young adult male mice (postnatal months 1–3), WT and *Trpa1* KO siblings[64], *Tlr4* KO (ref. 63) and *Trpv1* KO (ref. 18), and incubated for 1 h with 0.07% collagenase XI + 0.3% Dispase at 37 °C. Thereafter, ganglia were mechanically dissociated and cultured in minimal essential medium with Earle's balanced salt solution and L-Glutamine (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% minimal essential medium vitamin solution (Invitrogen), 100 μg ml^{-1} penicillin/streptomycin and 100 ng ml^{-1} nerve growth factor (NGF). Cells were plated on poly-L-lysine-coated glass coverslips and used after 1 day in culture.

Cell culture. CHO-K1 cells, from the American Type Culture Collection, were cultured in DMEM containing 10% of fetal bovine serum, 2% glutamax (Invitrogen), 1% non-essential amino acids (Invitrogen) and 200 μg ml^{-1} penicillin/streptomycin. An inducible CHO-K1 cell line stably expressing mouse TRPA1 channels was kindly provided by Ardem Patapoutian (The Scripps Research Institute, USA)[13] and cultured as detailed for the parental cell line. Expression of TRPA1 was induced by application of 0.25–0.5 μg ml^{-1} tetracycline 4–18 h before recording. HEK-293 cells, from the European Collection of Cell Cultures (Salisbury, UK), were seeded in 18 mm glass coverslips coated with poly-L-lysine (0.1 mg ml^{-1}) and grown in DMEM containing 10% (v/v) human serum, 2 mM L-glutamine, 2 U ml^{-1} penicillin and 2 mg ml^{-1} streptomycin at 37 °C in a humidity-controlled incubator with 10% CO_2. Human TRPA1, TRPV1, TRPM8 and TRPV2 were cloned in the pAGGS-IRES-GFP vector and transiently transfected in HEK-293 cells using Trans-IT-293 reagents (Mirus, Madison, MI, USA). Transfected cells were identified by the expression of GFP (green fluorescent protein).

Calcium imaging. Calcium-imaging experiments were conducted with the fluorescent indicator Fura-2. Cells were incubated with 5 μM Fura-2-acetoxymethylester (Invitrogen) for 60 min at 37 °C. Fluorescence measurements were made with a Zeiss Axioskop FS (Germany) upright microscope fitted with an ORCA ER charge-coupled device camera (Hamamatsu, Japan). Fura-2 was excited at 340 and 380 nm with a rapid switching monochromator (TILL Photonics, Germany). Mean fluorescence intensity ratios (F340/F380) were displayed online with Metafluor software (Molecular Devices, Sunnyvale, USA). The control bath solution contained (in mM) 140 NaCl, 3 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 10 HEPES and 10 glucose, and was adjusted to a pH of 7.4 with NaOH. Bath temperature was maintained at 34–36 °C by a custom-built Peltier device.

Patch-clamp electrophysiology. Whole-cell voltage-clamp recordings were performed with standard patch pipettes (3–6 MΩ resistance) made of borosilicate glass capillaries (Harvard Apparatus Ltd, UK) and contained (in mM) 140 NaCl, 5 CsCl, 10 EGTA, 10 HEPES, (305 mOsm kg^{-1}; pH 7.4, adjusted with NaOH). The control bath solution was kept at room temperature except for HC-030031 experiments (32 ± 1 °C). During calcium-free experiments, the bath solution was as follows (in mM): 140 NaCl, 3 KCl, 1.3 $MgCl_2$, 10 HEPES, 1 EGTA, 10 glucose. Current signals were recorded with an Axopatch 200B patch-clamp amplifier (Molecular Devices). Stimulus delivery and data acquisition were performed using pClamp 9 software (Molecular Devices). Current development was monitored with repetitive (0.5 Hz) injections of 500 ms duration voltage ramps from − 100 to + 100 mV, and the voltage steps consisted in a series of 400 ms duration steps ranging from − 150 to + 175 mV followed by an invariant pulse to − 75 mV (400 ms). The holding potential was set to 0 mV.

Outside-out patch recordings on mTRPA1 cells were performed in the absence of intracellular and extracellular Ca^{2+}. Patch pipettes (3–6 MΩ resistance) contained the standard internal solution (see above) and the calcium-free bath solution (see above). Currents were sampled at 20 kHz and low-pass filtered at 2 kHz. Current development was continuously monitored with repetitive (0.5 Hz) injections of 500 ms duration voltage ramps from − 100 to + 100 mV from a holding potential of 0 mV.

Patch-clamp data were analysed with WinASCD software written by Dr Guy Droogmans (ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip) and Origin 7.0 (OriginLab Corporation).

Current-clamp recordings of cultured nodose neurons. Perforated-patch current-clamp recordings were performed on cultured nodose neurons from adult mice. Standard patch pipettes (2–5 MΩ) were made of borosilicate glass capillaries (Harvard Apparatus Ltd) and contained (in mM) 105 K gluconate, 35 KCl, 10 NaCl, 10 HEPES, 0.1 EGTA and 1 mg ml^{-1} amphotericin B, pH 7.4. The external solution was identical to the one used in voltage-clamp recordings. Before electrical recordings, neurons expressing TRPA1 were identified with Ca^{2+} microfluorimetry (see above) by their response to 10 μM nifedipine, a potent, reversible and non-electrophilic activator of TRPA1 channels[22].

Recordings of lingual nerve afferents. The animals were killed by exposure to CO_2 and quickly decapitated. The tongue was carefully dissected, split at the midline and placed in a recording chamber and continuously perfused (3 ml min^{-1}) with physiological saline (in mM): NaCl 128, KCl 5, NaH_2PO_4 1, $NaHCO_3$ 26, $CaCl_2$ 2.4, $MgCl_2$ 1.3 and glucose 10. This solution was gassed with carbogen to pH 7.4. Temperature of the solution was kept around 34 °C and could be modified with a water-cooled, home-designed, Peltier device. Fine filaments of the lingual nerve were split with fine forceps and placed on a platinum electrode for extracellular recording. Electrical signals were alternating current-amplified and high pass filtered. Data were captured and analysed using a CED 1401 interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Single units were detected online using a threshold-based criterion and sorted manually afterwards to remove artefacts.

Arterial pressure myography. Mice were killed by CO_2 inhalation and decapitation. The mesenteric bed was removed and transferred to ice-cold solution (in mM): NaCl 124, $NaHCO_3$ 25, KCl 5.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 (pH 7.4 when aerated with 95% O_2–5% CO_2). Second- and third-branch arteries were removed, dissected free of the adherent fat, cannulated and mounted in a pressure myograph (Danish Myotechnology, Aarhus, Denmark) for measurement of diameter. Before testing the effect of LPS or HC-030031 on arterial tone, arteries were allowed to equilibrate at 70 mm Hg and 37 °C, and after 60 min were precontracted with phenylephrine 2 μM.

Assay of TLR4 activity. Activation of TLR4 by different forms of ultrapure LPS was tested in HEK-Blue-hTLR4 cells (InvivoGen, San Diego, USA). These HEK-293 cells stably express human TLR4, MD-2, CD14 and an NF-κB-inducible alkaline phosphatase reporter gene system. Expression of secreted alkaline phosphatase was detected with Quanti-Blue (InvivoGen) and quantified by reading the absorbance with a spectrophotometer at 620–655 nm. Cells were maintained in complete DMEM with selective antibiotics, as described by the manufacturer's instructions (InvivoGen). For testing, cells were plated on 96-well plates at 25,000 cells per well, and were incubated with different concentrations of LPS for 16 h.

Analysis of IL-6 levels. WT and TRPA1 KO mice were injected i.p. with 0.75 or 1 mg kg^{-1} *E. coli* LPS 055:B5 (Sigma-Aldrich) or saline and killed by decapitation 1 h later. Serum and liver were quickly collected and immediately frozen and stored at − 80 °C. IL-6 serum levels were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, USA).

Liver analysis of IL-6 messenger RNA levels was performed by reverse transcriptase PCR. Total RNA was extracted using Trizol Reagent (Invitrogen). Reverse transcriptase PCR was performed with a specific set of primers: forward: 5′-GCCACTCCTTCTGTGACTCCAGCT-3′.

Table 1 | List of LPS reagents used in the study, purity and supplier.

Source organism	Serotype	Extraction method	Impurities	Company	Catalogue #
LPS					
Escherichia coli	0127:B8	Phenol extraction	<3% protein	Sigma-Aldrich	L3129
E. coli	0127:B8	Gel-filtration chromatography	<1% protein	Sigma-Aldrich	L3137
E. coli	055:B5	Phenol extraction	<3% protein	Sigma-Aldrich	L2880
E. coli	0111:B4	Phenol-TEA-DOC extraction	Ultrapure	InvivoGen	tlrl-pelps
Salmonella enterica	Minnesota	Phenol-TEA-DOC extraction	Ultrapure	InvivoGen	tlrl-smlps
S. enterica	Minnesota	Gel-filtration chromatography	<1% protein	Sigma-Aldrich	L2137
S. enterica	Typhimurium	Gel-filtration chromatography	<1% protein	Sigma-Aldrich	L2262
Rhodobacter sphaeroides		Phenol-TEA-DOC extraction	Ultrapure	InvivoGen	tlrl-rslps
Klebsiella pneumoniae	—	Phenol extraction	<3% protein	Sigma-Aldrich	L4268
Serratia marcescens	—	Phenol extraction	<3% protein	Sigma-Aldrich	L6136
Pseudomonas aeruginosa	10	Gel-filtration chromatography	<3% protein	Sigma-Aldrich	L8643
Neisseria meningitidis	—	Phenol extraction	Ultrapure	Finlay Institute, Cuba	—
Lipid A					
Diphosphoryl lipid A	*E. coli* F583 Rd mutant	Mild acid, heat chromatography		Sigma-Aldrich	L5399
Monophosphoryl lipid A	Synthetic			InvivoGen	Tlrl-mpls

The LPS forms purchased from InvivoGen do not specify the degree of proteinaceous impurities. The label 'Ultrapure' indicates that they are specific for TRLR4 and do not activate TLR2 signalling.

Reverse: 5′-ACCACTTCACAAGTCGGAGGCTT-3′. Hypoxanthine guanine phosphoribosyl-transferase was used as a housekeeping gene. The amplified products were resolved in a 2% agarose gel and quantified by densitometry using a digital imaging system (Molecular Analyst, Bio-Rad). The PCR products were digested with specific restriction enzymes to determine their specificity.

CGRP enzyme immunoassay and analysis. The CGRP content of the incubation fluid was measured using a commercial enzyme immunoassay kits with a detection threshold of 2 pg ml^{-1} (Bertin Pharma, Montigny-le-Bretonneux, France). For this purpose, 100 µl fluid samples were stored on ice and mixed, immediately following the trachea incubation period, with 25 µl of fivefold concentrated commercial CGRP enzyme immunoassay buffer that contained a cocktail of peptidase inhibitors. The CGRP contents were determined after the end of the experiment; the antibody reactions took place overnight. Enzyme immunoassay plates were scored photometrically using a microplate reader (Dynatech, UK). All results are presented as measured by the enzyme immunoassay in pg CGRP per ml incubation fluid. To reduce interindividual and day-to-day baseline variability, the data were normalized to the second baseline value (before any stimulation). This value was subtracted from all data points so that only the absolute change in CGRP release (Δ pg ml^{-1}) is displayed in the figures.

Statistical comparisons were performed using the Statistica 7 software (Statsoft, Tulsa, USA). The baseline-normalized ΔCGRP values were entered into a one-way analysis of variance followed by Fisher's least significant difference test, focusing on the peak values of stimulated CGRP release; $^{*}P<0.05$ was considered significant. Data points represent means ± s.e.m. of the given number (n) of experiments.

Behavioural testing. Behavioural studies were performed on littermates that were wild type or homozygous null at the *Trpa1* or *Tlr4* allele. All experiments were scored by a tester blind to genotype and to drugs injected. A total of 51 wild type, 25 *Trpa1* KO and 6 *Tlr4* KO male mice (2–3 months old) were used.

To measure acute nocifensive responses, mice were placed within transparent plastic cylinders and allowed to accommodate for 2 h. LPS (5 µg µl^{-1}) was prepared in PBS and delivered via intraplantar injection in a volume of 10 µl using a 30 G needle coupled to a Hamilton syringe. Immediately after injection, nocifensive time was scored as the total time spent licking, lifting, guarding, shaking or biting of the injected hind paw over a period of 10 min. A separate group of animals received injection of vehicle alone.

Mechanical thresholds were determined with Von Frey hairs using a modified version of Dixon's up-down method[67]. Procedures and intraplantar dosis of LPS were identical to those used for nocifensive responses. Mice were placed in transparent plastic cylinders (13 cm diameter × 8 cm height) on a metal mesh platform and calibrated von Frey filaments were applied to the plantar surface of the hind paw for up to 2 s with sufficient force to cause the filament to bow. Brisk withdrawal of the hind paw during or immediately after application was considered a positive response. The threshold force required to elicit withdrawal of the paw (50% hind paw withdrawal) was determined from two separate trials. Data presented are from both left and right hind paws averaged together, as there were no laterality effects.

To examine the effects of LPS on body temperature (Tb), animals (adult male mice) were adapted to a Plexiglass chamber (15 × 15 × 30 cm^3) in a room at 23 ± 1 °C. Baseline rectal temperature was recorded with a thermoprobe connected to a digital thermometer (Lendhermack, model IM902C). At time zero (10:00 day time), immediately after a second measure of Tb, vehicle (PBS) or 3 mg kg^{-1} of *E. coli* LPS were injected i.p. Thereafter, Tb was monitored at 1 h interval for 7 h, and the following morning.

Chemical reagents. LPS from *E. coli*, *S. typhimurium*, *S. minnesota* and *P. aeruginosa* (purified by gel-filtration chromatography), *and K. pneumoniae*, *S. marcescens* (purified by phenol extraction) were purchased from Sigma-Aldrich. *N. meningitidis* LPS was purified by phenol extraction at the Center for Vaccines Research and Production (Finlay Institute, Havana, Cuba). The quality of this LPS (40.5×10^6 EU ml^{-1}) was determined with a Kinetic-QCL Limulus amebocyte lysate, using the programme Wiea QCL and a Lonza kit. LPSs were dissolved as a stock solution in endotoxin-free distilled water, or PBS for behavioural experiments, at a concentration of 5 mg ml^{-1}. Other ultrapure forms of LPS were purchased from InvivoGen (San Diego, USA), obtained by repeated phenol extractions and prepared as sterile stock solutions with sterile water and diluted right before use. These included LPS from *E. coli* strain 0111:B4, *S. minnesota* and *R. sphaeroides*. Table 1 summarizes the source and characteristics of the different LPS used.

Diphosphoryl lipid A (from *E. coli* F583 Rd mutant, Sigma) was dissolved in a glass tube at a concentration of 1 mg ml^{-1} in chloroform/methanol (3:1 v/v). The suspension was heated up to 40 °C and solvent was dried by rotary evaporation under a stream of N_2. The glass tubes were supplemented with extracellular solution and subjected to sonication for 40 min. Synthetic monophosphoryl lipid A (InvivoGen) was prepared as a 1-mg ml^{-1} solution in dimethyl sulphoxide (DMSO). Control experiments contained the same concentration of vehicle. CLI-095 (InvivoGen), also known as TAK-242, was prepared as a 3-mM stock solution in DMSO. Polymyxin B (InvivoGen) was prepared as a 50-mg ml^{-1} stock in distilled water. AITC, CA, TNP and HNE were stored as stock solutions and diluted immediately before the experiments. HC-030031 was purchased from Tocris and stored in a DMSO solution at 100 mM.

Data analysis. Data are reported as mean ± s.e.m. Dose–response data were normalized to the maximum response of the drug in each case and then were fitted with the Hill equation using the Levenberg–Marquardt method implemented in Origin 7.0 software. The s.e.m. were used as weights in fitting where applicable. When comparing two means, statistical significance ($P<0.05$) was assessed by Student's two-tailed t-test. When comparing proportions between two experimental groups (for example, percentage of responding neurons), we used Fisher's exact test.

References

1. Murray, P. R., Rosenthal, K. S. & Pfaller, M. A. *Medical Microbiology* (Elsevier, 2009).
2. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124,** 783–801 (2006).
3. Ronald, P. C. & Beutler, B. Plant and animal sensors of conserved microbial signatures. *Science* **330,** 1061–1064 (2010).
4. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat. Rev. Immunol.* **4,** 499–511 (2004).
5. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282,** 2085–2088 (1998).

6. Watkins, L. R. *et al.* Neurocircuitry of illness-induced hyperalgesia. *Brain Res.* **639,** 283–299 (1994).
7. Yilmaz, M. S. *et al.* Lipopolysaccharide-induced hypotension is mediated by a neural pathway involving the vagus nerve, the nucleus tractus solitarius and alpha-adrenergic receptors in the preoptic anterior hypothalamic area. *J. Neuroimmunol.* **203,** 39–49 (2008).
8. Romanovsky, A. A., Simons, C. T., Szekely, M. & Kulchitsky, V. A. The vagus nerve in the thermoregulatory response to systemic inflammation. *Am. J. Physiol.* **273,** R407–R413 (1997).
9. Maier, S. F., Goehler, L. E., Fleshner, M. & Watkins, L. R. The role of the vagus nerve in cytokine-to-brain communication. *Ann. N. Y. Acad. Sci.* **840,** 289–300 (1998).
10. Diogenes, A., Ferraz, C. C., Akopian, A. N., Henry, M. A. & Hargreaves, K. M. LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. *J. Dent. Res.* **90,** 759–764 (2011).
11. Ochoa-Cortes, F. *et al.* Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. *Am. J. Physiol Gastrointest. Liver Physiol.* **299,** G723–G732 (2010).
12. Hosoi, T., Okuma, Y., Matsuda, T. & Nomura, Y. Novel pathway for LPS-induced afferent vagus nerve activation: possible role of nodose ganglion. *Auton. Neurosci.* **120,** 104–107 (2005).
13. Story, G. M. *et al.* ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112,** 819–829 (2003).
14. Jordt, S. E. *et al.* Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427,** 260–265 (2004).
15. Bautista, D. M. *et al.* TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124,** 1269–1282 (2006).
16. Nilius, B., Appendino, G. & Owsianik, G. The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflugers Arch.* **464,** 425–458 (2012).
17. McNamara, C. R. *et al.* TRPA1 mediates formalin-induced pain. *Proc. Natl Acad. Sci. USA* **104,** 13525–13530 (2007).
18. Caterina, M. J. *et al.* Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288,** 306–313 (2000).
19. Doerner, J. F., Gisselmann, G., Hatt, H. & Wetzel, C. H. Transient receptor potential channel A1 is directly gated by calcium ions. *J. Biol. Chem.* **282,** 13180 9 (2007).
20. Zurborg, S., Yurgionas, B., Jira, J. A., Caspani, O. & Heppenstall, P. A. Direct activation of the ion channel TRPA1 by Ca(2+). *Nat. Neurosci.* **10,** 277–279 (2007).
21. Karashima, Y. *et al.* Bimodal action of menthol on the transient receptor potential channel TRPA1. *J. Neurosci.* **27,** 9874–9884 (2007).
22. Fajardo, O., Meseguer, V., Belmonte, C. & Viana, F. TRPA1 channels: novel targets of 1,4-dihydropyridines. *Channels (Austin)* **2,** 429–438 (2008).
23. Dai, Y. *et al.* Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J. Clin. Invest.* **117,** 1979–1987 (2007).
24. Trevisani, M. *et al.* 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proc. Natl Acad. Sci. USA* **104,** 13519–13524 (2007).
25. Macpherson, L. J. *et al.* An ion channel essential for sensing chemical damage. *J. Neurosci.* **27,** 11412–11415 (2007).
26. Raetz, C. R. & Whitfield, C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71,** 635–700 (2002).
27. Brandenburg, K., Schromm, A. B. & Gutsmann, T. Endotoxins: relationship between structure, function, and activity. *Subcell. Biochem.* **53,** 53–67 (2010).
28. Netea, M. G., van Deuren, M., Kullberg, B. J., Cavaillon, J. M. & van der Meer, J. W. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? *Trends Immunol.* **23,** 135–139 (2002).
29. Martinac, B., Adler, J. & Kung, C. Mechanosensitive ion channels of E. coli activated by amphipaths. *Nature* **348,** 261–263 (1990).
30. Hill, K. & Schaefer, M. TRPA1 is differentially modulated by the amphipathic molecules trinitrophenol and chlorpromazine. *J. Biol. Chem.* **282,** 7145–7153 (2007).
31. Matsunaga, N., Tsuchimori, N., Matsumoto, T. & Ii, M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol. Pharmacol.* **79,** 34–41 (2011).
32. Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K. & Raetz, C. R. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J. Biol Chem.* **266,** 19490–19498 (1991).
33. Morrison, D. C. & Jacobs, D. M. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* **13,** 813–818 (1976).
34. Bautista, D. M. *et al.* Pungent products from garlic activate the sensory ion channel TRPA1. *Proc. Natl Acad. Sci. USA* **102,** 12248–12252 (2005).
35. Pozsgai, G. *et al.* Evidence for the pathophysiological relevance of TRPA1 receptors in the cardiovascular system in vivo. *Cardiovasc. Res.* **87,** 760–768 (2010).
36. Earley, S. TRPA1 channels in the vasculature. *Br. J. Pharmacol.* **167,** 13–22 (2012).
37. Caceres, A. I. *et al.* A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc. Natl Acad. Sci. USA* **106,** 9099–9104 (2009).
38. Hua, X. Y., Chen, P., Fox, A. & Myers, R. R. Involvement of cytokines in lipopolysaccharide-induced facilitation of CGRP release from capsaicin-sensitive nerves in the trachea: studies with interleukin-1beta and tumor necrosis factor-alpha. *J. Neurosci.* **16,** 4742–4748 (1996).
39. Kichko, T. I. & Reeh, P. W. TRPV1 controls acid- and heat-induced calcitonin gene-related peptide release and sensitization by bradykinin in the isolated mouse trachea. *Eur. J. Neurosci.* **29,** 1896–1904 (2009).
40. Landry, D. W. & Oliver, J. A. The pathogenesis of vasodilatory shock. *N. Engl. J. Med.* **345,** 588–595 (2001).
41. Ferreira, S. H., Lorenzetti, B. B. & Poole, S. Bradykinin initiates cytokine-mediated inflammatory hyperalgesia. *Br. J. Pharmacol.* **110,** 1227–1231 (1993).
42. Kanaan, S. A. *et al.* Endotoxin-induced local inflammation and hyperalgesia in rats and mice: a new model for inflammatory pain. *Pain* **66,** 373–379 (1996).
43. Andersson, D. A., Gentry, C. & Bevan, S. TRPA1 has a key role in the somatic pro-nociceptive actions of hydrogen sulfide. *PLoS One* **7,** e46917 (2012).
44. Rudaya, A. Y., Steiner, A. A., Robbins, J. R., Dragic, A. S. & Romanovsky, A. A. Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289,** R1244–R1252 (2005).
45. Ulich, T. R., Guo, K. Z., Remick, D., Del Castillo, J. & Yin, S. M. Endotoxin-induced cytokine gene expression in vivo. III. IL-6 mRNA and serum protein expression and the in vivo hematologic effects of IL-6. *J. Immunol.* **146,** 2316–2323 (1991).
46. Kim, S. H. *et al.* Drosophila TRPA1 channel mediates chemical avoidance in gustatory receptor neurons. *Proc. Natl Acad. Sci. USA* **107,** 8440–8445 (2010).
47. Macpherson, L. J. *et al.* Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445,** 541–545 (2007).
48. Kang, K. *et al.* Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. *Nature* **464,** 597–600 (2010).
49. Bosmann, M. & Ward, P. A. The inflammatory response in sepsis. *Trends Immunol.* **34,** 129–136 (2013).
50. Takahashi, N. *et al.* TRPA1 underlies a sensing mechanism for O2. *Nat. Chem. Biol.* **7,** 701–711 (2011).
51. Hinman, A., Chuang, H. H., Bautista, D. M. & Julius, D. TRP channel activation by reversible covalent modification. *Proc. Natl Acad. Sci. USA* **103,** 19564–19568 (2006).
52. Brierley, S. M. *et al.* The ion channel TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli. *Gastroenterology* **137,** 2084–2095 (2009).
53. Vilceanu, D. & Stucky, C. L. TRPA1 mediates mechanical currents in the plasma membrane of mouse sensory neurons. *PLoS One* **5,** e12177 (2010).
54. Kwan, K. Y., Glazer, J. M., Corey, D. P., Rice, F. L. & Stucky, C. L. TRPA1 modulates mechanotransduction in cutaneous sensory neurons. *J. Neurosci.* **29,** 4808–4819 (2009).
55. Hardie, R. C. & Franze, K. Photomechanical responses in Drosophila photoreceptors. *Science* **338,** 260–263 (2012).
56. Hurley, J. C. Endotoxemia: methods of detection and clinical correlates. *Clin. Microbiol. Rev.* **8,** 268–292 (1995).
57. Jacinto, R. C. *et al.* Quantification of endotoxins in necrotic root canals from symptomatic and asymptomatic teeth. *J. Med. Microbiol.* **54,** 777–783 (2005).
58. Basbaum, A. I., Bautista, D. M., Scherrer, G. & Julius, D. Cellular and molecular mechanisms of pain. *Cell* **139,** 267–284 (2009).
59. Derow, A., Izydorczyk, I., Kuhn, A., Reeh, P. W. & Petho, G. Prostaglandin E(2) and I(2) facilitate noxious heat-induced spike discharge but not iCGRP release from rat cutaneous nociceptors. *Life Sci.* **81,** 1685–1693 (2007).
60. Engel, M. A. *et al.* TRPA1 and substance P mediate colitis in mice. *Gastroenterology* **141,** 1346–1358 (2011).
61. Hatano, N. *et al.* Hypoxia-inducible factor-1alpha (HIF1alpha) switches on transient receptor potential ankyrin repeat 1 (TRPA1) gene expression via a hypoxia response element-like motif to modulate cytokine release. *J. Biol. Chem.* **287,** 31962–31972 (2012).
62. Chiu, I. M. *et al.* Bacteria activate sensory neurons that modulate pain and inflammation. *Nature* **501,** 52–57 (2013).
63. Hoshino, K. *et al.* Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162,** 3749–3752 (1999).
64. Kwan, K. Y. *et al.* TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50,** 277–289 (2006).
65. Madrid, R. *et al.* Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. *J. Neurosci.* **26,** 12512–12525 (2006).

66. Fajardo, O., Meseguer, V., Belmonte, C. & Viana, F. TRPA1 channels mediate cold temperature sensing in mammalian vagal sensory neurons: pharmacological and genetic evidence. *J. Neurosci.* **28,** 7863–7875 (2008).
67. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M. & Yaksh, T. L. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53,** 55–63 (1994).

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Author contributions

V.M. initiated and coordinated the project, performed most patch-clamp experiments, calcium-imaging experiments in TG neurons and contributed to the final version of the manuscript; Y.A.A. characterized the responses to different forms of LPS, effects of LPS on human TRPs, the effect of polymixin B and performed behavioural experiments; E.L. performed current-clamp and calcium-imaging experiments in nodose neurons, S.T. performed measurements of LPS effects on mesenteric arteries, B.D. performed experiments on lingual nerve fibres; O.F. measured LPS effects on transfected cells, J.M. tested the interaction between LPS and TNP and helped in behavioural experiments; C.F. performed most behavioural experiments; A.T. provided reagents; T.K. measured GCRP release from tracheal sensory terminals; B.N. measured thermoregulatory responses and IL-6 release; A.S. characterized the responses to different forms of LPS in sensory neurons; R.S., P.R., M.T.P., J.R.L., T.V. and C.B. supervised experiments in their laboratories and contributed to the final version of the manuscript. K.T. analysed single-channel data, performed behavioural experiments, coordinated the project and drafted the article and F.V. coordinated the project, analysed data and drafted the article.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications.

Competing financial interests: V.M., C.B. and F.V. are inventors on a patent (WO/2013/038046), held by the Universidad Miguel Hernández and Consejo Superior de Investigaciones Científicas, related to the use of TRPA1 antagonists for the treatment of symptoms caused by bacterial infections or bacterial endotoxins. The remaining authors declare no competing financial interests.

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