Bacteria activate sensory neurons that modulate pain and inflammation

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ARTICLE

Host defences not necessary for S. aureus-induced pain

We determined whether key immune response pathways were necessary for S. aureus-induced pain. Innate immune cells recognize S. aureus-derived cell-wall components through TLR2 (ref. 12), leading to nuclear factor-κB (NF-κB) activation through adaptor myeloid differentiation factor 88 (MyD88). TLR2 and MyD88 protect mice against S. aureus skin infection

Pain correlates with bacterial load

To study the nature of pain caused by bacterial pathogens, we established a S. aureus infection model of the mouse hindpaw. Subcutaneous injection of LAC/USA300 (5 × 10⁶ colony-forming units (c.f.u.)), a community-associated methicillin-resistant S. aureus strain (CA-MRSA), resulted in mechanical, heat and cold hypersensitivity within 1 h that lasted for 48–72 h (Fig. 1a). This hyperalgesia peaked at 6 h after infection, and began to decrease at 24 h.

We quantified the kinetics of tissue swelling, immune activation and bacterial clearance. Tissue swelling did not correlate with pain, but showed an immediate peak after bacterial injection, and a second peak at 48 h after infection. Using flow cytometry, we found increases in myeloid immune cells in infected tissues (CD11b⁺/CD45⁺; Fig. 1b), constituted primarily of Ly6G⁺ neutrophils and Ly6C⁺ monocytes, with fewer Ly6C⁻ monocytes (Supplementary Fig. 1 and Fig. 1b). This influx began at 6 h, with a peak at 48 h after infection and a reduction at 72 h (Fig. 1b). Levels of CXCL1 (also called KC) and MCP-1, chemokines that mediate neutrophil/monocyte recruitment, peaked early (Supplementary Fig. 2), whereas levels of TNF-α and IL-1β, pro-inflammatory cytokines which directly sensitize nociceptors, also increased in infected tissue but did not correlate with hyperalgesia (Fig. 1c).

We next analysed live bacterial load in infected tissues. S. aureus recovery peaked at 6 h, and then decreased over time, similar to the time course of pain hypersensitivity (Fig. 1d). This decrease was accompanied by myeloid cell ingestion of bacteria, as detected by flow cytometry (Supplementary Fig. 1). In infected Nav1.8-Cre/TdTomato reporter mice, S. aureus (GFP–USA300) were often found in close proximity to dermal but not epidermal nociceptor fibres (Fig. 1e and Supplementary Fig. 3), indicating potential direct nerve–bacteria interactions. Taken together, hyperalgesia mirrors the time course of bacterial expansion and not tissue swelling or immune activation (see diagram in Supplementary Fig. 4).

A dense network of low- and high-threshold sensory nerves innervate peripheral tissues including the skin, respiratory tract and gastrointestinal tract, which are often exposed to bacterial pathogens. Bacterial infection induces inflammation through innate immune cell recruitment. Inflammatory pain during infection has been thought to be triggered by the action of immune-derived proteins (for example, cytokines and growth factors), lipids (for example, prostaglandins) and other mediators such as amines, potassium and protons on receptors expressed by nociceptors.

Staphylococcus aureus is a major cause of wound and surgical infections, leading to painful abscesses, cellulitis and necrotizing fasciitis. S. aureus releases toxins including haemolysins, Panton–Valentine leukocidin and phenol soluble modulins, which have roles in bacterial dissemination and tissue damage. We have now investigated the molecular mechanisms of pain generation during S. aureus infection. Unexpectedly, key immune activation pathways were not necessary for hyperalgesia during acute infection. Rather, bacteria directly activated nociceptors through N-formyl peptides and the pore-forming toxin α-haemolysin (aHL). Moreover, we find that nociceptors release neuro-peptides that modulate innate immune activation during infection.

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mice showed elevated pain-like hypersensitivity, which may reflect greater bacterial load due to reduced immune activation and bacterial clearance (Fig. 2a, b).

Neutrophils and monocytes are circulating leukocytes critical for innate immunity against S. aureus, rapidly infiltrating sites of infection to limit pathogen survival and spread4,16. We treated mice with GR1 antibody before infection, which eliminated blood-borne and splenic neutrophils and monocytes (Supplementary Fig. 6). Plantar tissue-infiltrating neutrophils and monocytes during S. aureus infection were completely depleted by GR1 (Supplementary Fig. 7; reduction of 97% of all CD45+ immune cells in infected tissues). However, instead of decreasing hyperalgesia, GR1 depletion significantly increased mechanical and heat hypersensitivity (Fig. 2c). This was accompanied by higher bacterial load, reflecting the key role of these myelomonocytic cells in combating S. aureus (Fig. 2d). We repeated the experiment using injection of heat-killed S. aureus (10⁶ c.f.u.), and found that GR1 treatment decreased tissue swelling, but did not affect pain-like hypersensitivity (Supplementary Fig. 8). The increased pain during S. aureus infection after GR1 depletion is probably linked to uncontrolled bacterial expansion (Fig. 2d), but may also reflect neutrophil analytic factors17.

To analyse the contribution of the immune system to pain further, we examined S. aureus infection in NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (also called NOD scid gamma) mice, which are deficient in natural killer, T and B cells19. Infection-induced mechanical and heat hyperalgesia did not differ between NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ and wild-type NOD mice (Supplementary Fig. 9). To control for strain-dependent differences, we also analysed C57BL/6 congenic Rag1-/- mice, which are deficient in mature T and B cells19. Pain-like hypersensitivity did not differ after S. aureus infection in C57BL/6 Rag1-/- mice compared to C57BL/6 wild-type mice (Supplementary Fig. 9). On the basis of these results, we conclude that adaptive immunity through T and B cells is not required for acute bacterial pain.

**Formyl peptides and α-haemolysin activate nociceptors**

The strong correlation between pain and bacterial load indicated that nociceptors directly interact with bacteria during pathogen invasion.

To test this, we applied heat-killed bacteria on dorsal root ganglia (DRG) sensory neurons. Heat-killed S. aureus induced a robust calcium flux response in a subset of neurons that also responded to capsaicin, which activates transient receptor potential V1 (TRPV1) (Fig. 3a). Heat-killed S. aureus application also induced action-potential firing in capsaicin-responsive DRG neurons (Fig. 3b). Extending these results, we found that several other strains of heat-killed bacteria caused calcium flux in DRG neurons (Fig. 3c, responsive cells: heat-killed S. aureus = 152 of 1,046; heat-killed Streptococcus pneumoniae = 82 of 968; heat-killed Listeria monocytogenes = 67 of 852; heat-killed Mycoplasma fermentans = 9 of 339; heat-killed Helicobacter pylori = 85 of 1,365; heat-killed Pseudomonas aeruginosa = 14 of 269; heat-killed Escherichia coli = 3 of 233). Nav1.8-Cre/TdTomato reporter mice were used to genetically mark nociceptors20 and all bacteria-evoked neuronal responses were within the Nav1.8-Cre/TdTomato population (Supplementary Fig. 10). Patterns of nociceptor responsiveness to particular bacteria differed, indicating strain-specific ligands acting through disparate mechanisms (Supplementary Fig. 11). Intraplantar injection of different heat-killed bacterial strains (10⁸ c.f.u.) induced acute pain responses similar to the relative efficacies of noci-peptide-induced calcium flux in a subset of DRG neurons that also responded to capsaicin, which activates transient receptor potential V1 (TRPV1) (Fig. 3a). Heat-killed S. aureus application also induced action-potential firing in capsaicin-responsive DRG neurons (Fig. 3b). Extending these results, we found that several other strains of heat-killed bacteria caused calcium flux in DRG neurons (Fig. 3c, responsive cells: heat-killed S. aureus = 152 of 1,046; heat-killed Streptococcus pneumoniae = 82 of 968; heat-killed Listeria monocytogenes = 67 of 852; heat-killed Mycoplasma fermentans = 9 of 339; heat-killed Helicobacter pylori = 85 of 1,365; heat-killed Pseudomonas aeruginosa = 14 of 269; heat-killed Escherichia coli = 3 of 233). Nav1.8-Cre/TdTomato reporter mice were used to genetically mark nociceptors20 and all bacteria-evoked neuronal responses were within the Nav1.8-Cre/TdTomato population (Supplementary Fig. 10). Patterns of nociceptor responsiveness to particular bacteria differed, indicating strain-specific ligands acting through disparate mechanisms (Supplementary Fig. 11). Intraplantar injection of different heat-killed bacterial strains (10⁸ c.f.u.) induced acute pain responses similar to the relative efficacies of noci-peptide-induced calcium flux in a subset of DRG neurons that also responded to capsaicin, which activates transient receptor potential V1 (TRPV1) (Fig. 3a). Heat-killed S. aureus application also induced action-potential firing in capsaicin-responsive DRG neurons (Fig. 3b). Extending these results, we found that several other strains of heat-killed bacteria caused calcium flux in DRG neurons (Fig. 3c, responsive cells: heat-killed S. aureus = 152 of 1,046; heat-killed Streptococcus pneumoniae = 82 of 968; heat-killed Listeria monocytogenes = 67 of 852; heat-killed Mycoplasma fermentans = 9 of 339; heat-killed Helicobacter pylori = 85 of 1,365; heat-killed Pseudomonas aeruginosa = 14 of 269; heat-killed Escherichia coli = 3 of 233). Nav1.8-Cre/TdTomato reporter mice were used to genetically mark nociceptors20 and all bacteria-evoked neuronal responses were within the Nav1.8-Cre/TdTomato population (Supplementary Fig. 10). Patterns of nociceptor responsiveness to particular bacteria differed, indicating strain-specific ligands acting through disparate mechanisms (Supplementary Fig. 11). Intraplantar injection of different heat-killed bacterial strains (10⁸ c.f.u.) induced acute pain responses similar to the relative efficacies of noci-peptide-induced calcium flux in a subset of DRG neurons that also responded to capsaicin, which activates transient receptor potential V1 (TRPV1) (Fig. 3a). Heat-killed S. aureus application also induced action-potential firing in capsaicin-responsive DRG neurons (Fig. 3b). Extending these results, we found that several other strains of heat-killed bacteria caused calcium flux in DRG neurons (Fig. 3c, responsive cells: heat-killed S. aureus = 152 of 1,046; heat-killed Streptococcus pneumoniae = 82 of 968; heat-killed Listeria monocytogenes = 67 of 852; heat-killed Mycoplasma fermentans = 9 of 339; heat-killed Helicobacter pylori = 85 of 1,365; heat-killed Pseudomonas aeruginosa = 14 of 269; heat-killed Escherichia coli = 3 of 233).
to capsaicin and to allyl isothiocyanate (AITC), a TRPA1 ligand (Fig. 3e and Supplementary Fig. 14). Moreover, unformylated MIFL activated fewer DRG neurons than fMLF (Fig. 3e), similar to formyl H. pylori (Fig. 4d), indicating that this mechanism of nociceptor activation is separate from heat-stable elements (Fig. 3). HL also evoked action potentials in DRG-responsive neurons (Supplementary Fig. 21). HL induced a dose-dependent calcium flux in DRG neurons (half-maximum effective concentration (EC50) 356 nM, Fig. 4b). Live bacteria actively release formyl peptides22 and secrete a host of virulence factors, including pore-forming toxins (PFTs), to facilitate tissue dissemination23-25. We found that S. aureus culture supernatant induced calcium flux in DRG neurons (Supplementary Fig. 20).
Finally, we found that an isogenic *S. aureus* mutant devoid of zHL expression caused significantly less hyperalgesia than wild-type bacteria (Fig. 4h). Therefore, we conclude that zHL contributes to pain during *S. aureus* infection in a manner dependent on pore formation.

**Nociceptor neuropeptides regulate inflammation**

Nociceptor activation results in release of neuropeptides from peripheral terminals which can induce vasodilation and neurogenic inflammation. To understand the role of nociceptors in modulating the immune response, we generated Nav1.8-Cre/diphtheria toxin A (DTA) mice to specifically ablate these cells. Nav1.8-Cre/DTA DRG neurons did not show calcium flux upon stimulation with heat-killed bacteria (Fig. 5a). Mechanical and thermal hypersensitivity after *S. aureus* infection was abolished in Nav1.8-Cre/DTA mice, indicating that Nav1.8-lineage neurons are the major cell type mediating bacterial pain (Fig. 5b). Granulocytes were found 24 h after infection near Nav1.8-Cre/TdTomato nerve fibres (Supplementary Fig. 24), and when co-cultured, DRG neurons also formed close contacts with neutrophils and macrophages (Supplementary Fig. 25), raising the question of whether nociceptors may act on immune cells. After infection, Nav1.8-Cre/DTA mice displayed significantly increased tissue swelling relative to control littermates (Fig. 5c). Bacterial load did not differ significantly between the mice (Supplementary Fig. 26). Nav1.8-Cre/DTA mice showed increased infiltration of neutrophils/monocytes at infection sites (Fig. 5d). The poikilothermal lymph node which drains the footpad was significantly larger, by weight and cellularity, in Nav1.8-Cre/DTA mice relative to control littermates following *S. aureus* infection (Fig. 5e and Supplementary Fig. 27). There was a difference in baseline lymph node size, but the increase was substantially greater in Nav1.8-Cre/DTA than control mice after infection. Nav1.8-Cre/DTA tissues also showed increased levels of...
TNF-α (Supplementary Fig. 28), a cytokine that drives lymphadenopathy (hypertrophy) of the draining lymph node during bacterial infection34. This lymphadenopathy was localized, as downstream inguinal lymph nodes and spleens were not enlarged in infected Nav1.8-Cre/DTomato+ nociceptors from dorsal root, trigeminal and nodose ganglia (Fig. 6a). Neuropeptide expression levels were ranked based on a neuropeptide database (http://www.neuropeptides.nl; Fig. 6b, full data set in Supplementary Fig. 29). Microarray data from innate immune cell subsets35 were analysed for neuropeptide receptor levels. CGRP, galanin and somatostatin receptors showed the highest expression in neutrophils, monocytes and macrophages; these neuropeptides were also highly expressed in purified nociceptors (Fig. 6b, full data set in Supplementary Fig. 30). In vitro, we found that CGRP, galanin and somatostatin all suppressed TNF-α release from macrophages stimulated with heat-killed S. aureus or lipoteichoic acid (Fig. 6c, complete analysis in Supplementary Fig. 31). Furthermore, S. aureus supernatant and zHL induced CGRP release from DRG neurons in a dose-dependent manner (Fig. 6d). CGRP injection during S. aureus infection did not alter inflammation at the infection site, but significantly suppressed lymphadenopathy of the draining lymph nodes (Fig. 6e and Supplementary Fig. 32). Therefore, upon infection, nociceptors may release neuropeptides that directly modulate innate immune activation.

**Discussion**

We analysed mechanisms responsible for nociceptor activation during S. aureus infection, which commonly causes pain. We found that bacterial-derived factors directly activate nociceptors and contribute to hyperalgesia in vivo. N-formylated peptides and the PFT zHL induced direct neuronal responses through distinct mechanisms: for myel peptides through FPR1 and zHL through pore assembly leading to ionic influx (diagram, Supplementary Fig. 33).

Direct activation of nociceptors by bacteria is probably a major mechanism leading to pain, especially early in S. aureus infection during active pathogen expansion (Figs 1, 3 and 4). After immune cell infiltration, bacteria are largely eliminated, concurrent with TNF-α production, and although pain is reduced it remains (Fig. 1). Immune-mediated mechanisms may have a role during these later time points. Live infection is complex and our blockade of host defences may also enhance direct pathogen-mediated mechanisms.

Pathogen virulence and immunogenicity probably contribute to the degree of direct nociceptor activation. USA300/LAC is a highly virulent strain, expressing several PFTs in addition to zHL including β-haemolysin, γ-haemolysin, Panton–Valentine leukocidin and phenol soluble modulins7–9. S. aureus also possesses effective immune evasion mechanisms, including toxins that lyse immune cells, staphylococcal protein A which impairs antibody function, and complement evasion strategies3,36. Thus, additional elements from the bacterial proteome may exist that activate nociceptors. E. coli and other Gram-negative bacteria also induce painful infections, and lipopolysaccharides have been found to sensitize TRPV1 (ref. 15). The balance of nociceptor activation by pathogenic and immune mechanisms may differ between dissimilar pathogenic bacteria species.

Although peripheral nociceptor activation contributes to neurogenic inflammation, inducing vasodilatation and capillary permeability35, we found that ablation led to increased immune influx and lymphadenopathy, implying pain-mediated immune suppression. Our data support a role for neuropeptides in regulating innate immune activation that occurs later than the acute vascular phase of inflammation. Receptors for CGRP, galanin and somatostatin are expressed by myeloid immune subsets, and these neuropeptides have been shown to have inhibitory functions on immunity37–39. In particular, CGRP dampens TNF-α transcription in dendritic cells through a cAMP-dependent repressor mechanism37,40. CGRP downregulates cytokine levels in endotoxic shock41, and conversely, Trpv1−/− mice display increased inflammation during sepsis42.

Potent immunomodulatory neural reflex circuits also exist that maintain immune homeostasis41. In Caenorhabditis elegans, a sensory neural circuit suppresses innate immunity and modulates survival during bacterial infection41. In mammals, vagal efferents suppress splenic macrophage activity, protecting against bacterial endotoxic shock43,45, and activation of liver autonomic fibres modulates NK T-cell activity, leading to increased bacterial infection44. It has been proposed that sensory neurons initiate mammalian neural circuits43, but a direct immunosuppressive role of nociceptive fibres, as revealed here, was not suspected. Lymph-node swelling during infection is often accompanied by pain, and nociceptor activation may limit immune influx into lymph nodes. Both nociceptive and autonomic fibres innervate lymph nodes46; therefore, lymph-node suppression may act through local neuropeptide release or initiation of autonomic reflex circuits. Highly pathogenic bacterial strains may have evolved the ability to exploit these neural-mediated immune regulation pathways for virulence and spread within infected tissues, by producing more nociceptor activation and greater immunosuppression.

Our data reveal an unsuspected mechanism for pain induction during bacterial infection: a direct pathogen-mediated activation of nociceptors. This neuron–pathogen interaction leads to a downregulation of the local inflammatory response. The nervous system therefore has direct sensory and modulatory roles in host–pathogen interactions during acute staphylococcal infection.
METHODS SUMMARY

All experiments were conducted according to institutional animal care guidelines. The following mouse strains were used: C57BL/6, C57BL/6 Tlr2−/−, C57BL/6 Myd88−/−, C57BL/6 Rag1−/−, NOD wild type, NOD.Cg-Pkdcltm1Cm/Mmu.J spl1 (Jackson Laboratories); C57BL/6 wild type, C57BL/6 Fpr1−/− (Taconic); NavI.8-Cre/TdTomato, NavI.8-Cre/DTA mice (NavI.8-Cre (ref. 20) from R. Kuner, Heidelberg University). Bacterial strains: S. aureus LAC and isogenic LAC deficient in σHl, described previously. Recombinant σHl and σHl(H35L) are described previously. For infection, 5 × 10^6 c.f.u. of S. aureus were injected into the plantar surface. For GFP–LAC/USA300 generation, statistical analysis, bacterial infection, formylpeptide synthesis, behaviour, microscopy, microarrays, neuronal cultures, electrophysiology, calcium imaging, flow cytometry, see Methods.

Full Methods and any associated references are available in the online version of the paper.

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METHODS

Mice. C57BL/6, C57BL/6 Tsl2−/−, C57BL/6 Myd88−/−, C57BL/6 Rag1−/−, C57BL/6 TdTomato reporter mice (alb1 line), C57BL/6 Zsgreen mice, C57BL/6 DTA reporter mice, C57BL/6 TdTomato reporter mice, NOD.Cg-Pkdcrd−/−Ibp−/−l2Zig1−/−/Saj and NOD wild-type mice were purchased from Jackson Laboratories; C57BL/6 Fpr1−/−, C57BL/6 wild-type controls were purchased from Taconic Farms. Nav1.8-Cre (Sns-Cre) mice were a gift from R. Kuner (University of Heidelberg). Nav1.8-Cre mice were bred with C57BL/6 TdTomato, C57BL/6 Zsgreen mice to generate Nav1.8-Cre/TdTomato and Nav1.8-Cre/Zsgreen mice. Nav1.8-Cre mice were bred with C57BL/6 DTA−/− mice to generate nociceptor-deficient Nav1.8-Cre−/−DTA−/− and control littermates (Nav1.8-Cre−/−DTA−/−). For infection and behavioural experiments, adult, 7–14-week-old male mice were used. For acute pain assessment, 9–11-week-old male mice were used. For when age-matched, male and female Nav1.8-Cre/DTA with control littermates were used. All bacterial and animal experiments were conducted according to institutional animal care and safety guidelines and with IACUC approval at Boston Children’s Hospital and Harvard Medical School.

Statistical analysis. Sample sizes for all experiments were chosen according to standard practice in the field. Bar and line graphs are plotted as mean ± standard error (s.e.m.), and in some cases, individual mice are plotted as dots. ‘n’ represents the number of mice used in each group. Statistical analysis of behavioural data of mechanical, heat, and cold, as well as tissue swelling time courses was conducted by two-way repeated measures ANOVA, with Bonferroni post-tests conducted for each time point tested. In these experimental analyses, saline-injected mice or wild-type mice served as control groups for each statistical comparison.

For electrophysiology, significance was calculated using the Mann–Whitney U-test. Statistical comparisons of acute nociceptive behaviour (total time licking/biting in 20 min), tissue bacterial load, tissue swelling measurements, immune cell influx, neuronal responses by calcium imaging, CGRP and TNF-α levels were by unpaired, student’s t-test. In acute pain analysis, saline-injected mice were compared to treatment groups. In CGRP and TNF analysis, buffer-treated controls were used for comparisons. In calcium imaging, the proportion of neuronal responses from at least three fields was quantified. Data were plotted using Prism (Graphpad).

Behavioural analysis. All animals were acclimatized to the behavioural testing apparatus used on at least three habituation sessions. At least two baseline measures were obtained for each behavioural test before testing. To measure mechanical sensitivity, animals were placed on an elevated wire grid and the lateral plantar surface of the hindpaw stimulated with von Frey monofilaments (0.007–1.3 mN). Spikes were identified using Mobius software (Alpha Med Scientific), and histological reconstruction was conducted in an open-field arena. Activity measures obtained for when age-matched, male and female Nav1.8-Cre/DTA with control littermates were used. All bacterial and animal experiments were conducted according to institutional animal care and safety guidelines and with IACUC approval at Boston Children’s Hospital and Harvard Medical School.

Neuronal cultures and calcium imaging. Dorsal root ganglia (DRG) from adult mice (7–12 weeks) were dissected into neurobasal-A medium (Life Technologies), dissociated in 0.25 % trypsin/0.1 % EDTA, triturated with glass Pasteur pipettes of decreasing size, DRG were cultured on glass coverslips in neurobasal-A medium with 50 ng ml−1 nerve growth factor (NGF) plus penicillin/streptomycin (Life Technologies). For bacterial co-cultures, Nav1.8-Cre/TdTomato or Nav1.8-Cre/ZsGreen DRG neurons were plated in poly-L-lysine, laminin pre-coated, 8-well chamber slides (Lab-Tek) overnight at 37°C for 16 h. GFP−/− or CgTMTM-labelled S. pneumoniae were added in neurobasal-A to DRG neurons for 2 h at 37°C, co-cultures were fixed with 4% PFA and mounted in Vectashield (Vector Labs) for microscopy. DRG neurons were used for calcium imaging and electrophysiology 16–48 h after plating.

For calcium imaging, cells were loaded with 10 μM Fura-2–AM (Life Technologies) at 37°C for 45 min in neurobasal-A medium, washed into Standard Extracellular Solution (SES, 145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH 7.5), and imaged at room temperature. Cells were illuminated by an ultraviolet light source (Xenon lamp, 75 W, Nikon), 340 nm and 380 nm excitation alternated by a LEP MAC 5000 filter wheel (Spectra services), and fluorescence emission captured by Cool SNAP ES camera (Princeton Instruments). 340/380 ratio images were processed, background corrected, and analysed with IPLab software (Scientific Analytics). Microsoft Excel was used for further analyses (Microsoft). Ligands were flowed directly onto neurons using perfusion barrels followed by buffer washout and further application, or applied to the culture bath at the beginning of imaging. In some experiments, 1 μM capsaicin (Tocris), 100 μM ATIC (Sigma) or 40 μM Ki (Sigma) was applied after bacterial ligands.

Electrophysiology. Whole-cell transmembrane voltages of DRG neurons were recorded at room temperature (21 ± 1°C) in the current-clamp mode using an Axopatch 200A amplifier (Molecular Devices). The internal pipette solution consisted of (in mM): 140 KCl, 5 NaCl, 2 MgCl2, 0.5 CaCl2, 5 EGTA, 10 HEPES, 3 Na2ATP and 0.1 MgGTP (pH 7.4 with KOH). The extracellular solution consisted of the following: 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES and 10 glucose (pH 7.4 with NaOH). Data were sampled at 5–10 kHz and analysed using pCLAMP 10.2 (Molecular Devices) software. Resting membrane potentials of DRG neurons were typically −60 to −63 mV and cell bodies were 15–25 μm.

For multi-electrode arrays, DRG neurons were isolated as described, plated on MED-P515A 64-electrode probes, and recorded using a MED64 device (Alpha Med Scientific). Recombinant hβ1H was applied at 30 μg ml−1 concentration. Spikes were identified using Mobius software (Alpha Med Scientific), and histogram analysis was performed in Matlab (Mathworks).

Bacterial ligands. For heat-killed bacteria experiments, to standardize titres used for differential comparisons we used bacteria purchased from InvivoGen (10×106 c.f.u., each heat-treated for 30 min at 120°C). Heat-killed S. aureus, American-type tissue culture (ATCC) strain 6539 Rosenbach; heat-killed S. pneumoniae, NCTC7466; L. monocytogenes, ATCC strain 19998; E. coli, ATCC strain 11637; heat-killed L. monocytogenes, strain 9686P; heat-killed M. fermentans, ATCC strain 19998; heat-killed P. aeruginosa, strain ATCC strain BAA-47. For calcium imaging, bacteria were dissolved in SES at 10−5 c.f.u. ml−1. We note that bacterial addition did not affect pH, which remained at pH 7.5. For infections, heat-killed bacteria strains were dissolved in 0.9% saline, 20 μl heat-killed bacteria (105 c.f.u. total) was injected into the hindpaw using a Hamilton syringe fitted with a 26-gauge needle.

N-formyl peptides were synthesized by Foci solid-phase peptide synthesis and the peptides were formulated by 2,2,2-trifluoroethyl formate13. HLPC resulted in the pure compounds fMLF, fMIFL and fMMIF, which were confirmed by mass spectroscopy38. Peptides were dissolved at 10 mM concentration dissolved in DMSO (Thermo Fisher) at −20°C. For calcium imaging, fMLF, fMIFL and fMMIF were dissolved in SES to 1 μM concentration. For intraperitoneal injections, fMLF and fMIFL were dissolved in 0.9% saline, and 1 μg of fMLF (2.1 nmol) or 1.3 μg of fMIFL (2.04 nmol) injected in a 20 μl volume.
For *S. aureus* supernatant collection, LAC bacteria were cultivated overnight in TSB, removed by centrifugation, and resulting supernatant diluted to 5% in SES buffer for application to DRG neurons. Recombinant *S. aureus* zHL and mutant H35L (zHL(H35L)) were generated and purified as previously described. Commercial zHL (Sigma) induced comparable results *in vitro* and *in vivo*. In some imaging experiments, zHL was bath applied or flowed directly onto neurons at 10 μM zHL (Fig. 4a and Supplementary Fig. 20). For pain behavioural studies, zHL and zHL(H35L) dissolved in 0.9% saline was injected in 20 μl volume into the hindpaw at described doses. For heat inactivation, the same batch of DMLM or recombinant zHL was split into two aliquots, one of which was treated at 100 °C for 30 min. Untreated or heat-treated substances were used for mass spectrometry and pain hypersensitivity studies.

**Whole-cell imaging of calcium flux.** DRG neurons were seeded in B27 supplemented Neurobasal-A at 2,000 neurons per well in laminin-coated 384-well microplates (Greiner) at 37 °C for 24 h. Neurons were loaded with Fura-2 AM for 30 min at 37 °C, then washed twice with HBSS (Life Technologies). A Hamamatsu FDSS 7000EX kinetic reader was used to dispense ligands onto DRG neurons and calcium flux recorded every 1.9 s at room temperature for 30 min total. To evaluate the role of extracellular cations, cells were analysed in calcium/magnesium-free HBSS (Life Technologies). For related experiments, EDTA (5 mM) or HBSS was added to wells during recording to evaluate zHL flux.

**Immune stimulation and neuroimmune co-cultures.** For peritoneal macrophages, 0.5 ml Brewer’s thiglyglycolate solution (2%) was injected i.p. into mice; 4 days later, animals were killed and peritoneal cavities flushed using 10 ml DMEM/10% fetal calf serum (FCS). Cells were plated in 96-well plates at 5,000 cells per well. For bone-marrow-derived macrophages, tibia and femurs were flushed using a 27 gauge needle, and bone marrow plated in 15-cm Petri dishes in DMEM/10%FCS/50 μM β-mercaptoethanol/20% 1929 conditioned media for 7 days. Differentiated macrophages were digested from plates using non-enzymatic dissociation media in HBSS (Sigma), and plated into 96-well plates at 5,000 cells per well in DMEM/10%FCS/50 μM β-mercaptoethanol. Anti-CD11b (rat), somatostatin and galanin (Tocris) were used at different concentrations in macrophage stimulation media in HBSS (Sigma) and plated into 96-well plates at 5,000 cells per well in well in DMEM/10%FCS/50 μM β-mercaptoethanol. Anti-CD11b (rat), somatostatin and galanin (Tocris) were used at different concentrations in macrophage stimulation media. Peritoneal macrophages were stimulated with 10 c.f.u. per ml heat-killed *S. aureus* (Invivogen) and bone marrow macrophages stimulated with 1 μg ml⁻¹ *S. aureus*-derived lipoteichoic acid (Invivogen) for 16 h in DMEM/10% FCS; TNF-α levels in culture supernatant were determined using an ELISA kit (Biolegend).

For neuron-macrophage co-cultures, DRG neurons were isolated and bone marrow macrophages derived as described above, re-suspended together in neurobasal-A/2% FCS/50 μM β-mercaptoethanol, and co-plated at a density of 2,000 neurons + 10,000 macrophages per well in polylaminin/poly-l-lysine-coated 8-well chamber slides (Lab-Tek). co-cultures were fixed with 4% PFA after 20 h. For neuron-neutrophil co-cultures, DRG neurons were cultured overnight in 8-well chamber slides at 2,000 cells per well. Neutrophils were isolated as described briefly: briefly, mouse bone marrow from tibias and femurs was depleted of red blood cells with ammonium-chloride-potassium buffer (0.15 M NaCl, 10 mM KHCO₃, 0.1 mM EDTA), run over a discontinuous 52%, 69%, 78% percoll gradient for 30 min at 1,500g. The 69%/78% interface and 78% layer containing neutrophils were collected, washed, re-suspended in neurobasal medium/2% FCS/50 μM β-mercaptoethanol and added to DRG neurons cultured for 24 h, and stained using LPS and LTA according to the digestion and the process was repeated until all lymph node fragments were completely digested. Cells were filtered through 80 μm nylon mesh and counted by haemocytometer. 5 × 10⁶ cells were incubated with 50 μl diluted antibodies (TCRγ, CD11b, Ly6C, Ly6G, CD19, antibodies from Biolegend) for 20 min at 4 °C in FACS buffer before acquisition on a FACSCalibur or FACSaria II (BD Biosciences). Neutrophils were considered CD11b-Ly6G⁺, monocytes CD11b–Ly6C⁺Ly6G⁻, B cells CD19⁻, T cells TCRβ⁻. Flow cytometry data were analysed using Flowjo software (TreeStar).

**Immunostaining and microscopy.** Mice were transcardially perfused with PBS followed by 4% PFA/PBS (Sigma). Plantar tissue was dissected, post-fixed for 2 h, cryoprotected in 30% sucrose/PBS, embedded, and frozen in Optimal cutting temperature compound (OCT, Electron Microscopy Sciences). Tissues were stored at −80 °C until sectioning. Cryosections were cut at 50 μm thickness onto Superfrost plus slides (Thermo Fisher). For haematolxyn and eosin, sections were dehydrated by sequential alcohol steps, mounted in Permament medium (Thermo Fisher), and imaged by light microscopy on a BZ-II analyser at ×15 magnification (Keyence). For immunostaining, sections were stained with GR1-Alexa 647 antibody (Biolegend, 1:100) for 2 h at room temperature, mounted in Vectashield with DAPI/1% DAPI (6,7-diamino-2-phenylindole staining solution) and scanned using a confocal microscope (Carl Zeiss). ×10 Zeiss EC-plan-NEOFLAR dry and ×63 Zeiss plan-Apochromat oil objectives were used, with z-stacks of 40 μm total imaged at 1 μm steps; maximum projection images were exported for presentation. For DRG, tissues were dissected, post-fixed, and embedded in OCT as described above. 14-μm cryosections were cut and stained with rabbit anti-CD11b (Millipore, PC205L, 1:500) followed by Alexa 488 goat anti-rabbit IgG (Life Technologies, 1:1,000) or chicken anti-neurilfluorescence (Millipore, AB5539, 1:500), followed by Alexa 488 anti-print (Life Technologies, 1:1,000) or chicken anti-neurilfluorescence (Millipore, AB5539, 1:500). Sections were mounted in Vectashield with DAPI (Vector Labs), and imaged by Eclipse 50i epifluorescence microscope.

**Flow-cytometric purification of nociceptors and ADAM10 staining.** DRG, trigeminal, or nodose ganglia from Nav1.8-Cre/TdTomato mice were dissected, dissociated into single cells by enzymatic digestion (1 mg ml⁻¹ collagenase A plus 2.4 U ml⁻¹ dispase II (Roche Applied Sciences) in HEPES buffered saline (Sigma)) for 60 min at 37 °C, filtered through a 70-μm filter, and stained with DAPI (Sigma, 20 ng ml⁻¹), as a dead cell stain in HBSS/0.5% BSA. Nociceptors were sorted on a FACSaria II machine (Bectin Dickinson) using a yellow–green laser to detect TdTomato fluorescence (genes, Fig. 6, Supplementary Fig. 14). To determine purity, cells were sorted into neurobasal-A and plated on laminin-coated 384-well microarrays, neurons were sorted directly into Qiaozao reagent (Qiaozao). For ADAM10 surface staining, Nav1.8-Cre/TdTomato DRG were dissociated and stained with rat anti-ADAM10 ecdotomain antibody (clone 139712, R&D systems; reviewed, 1DegreeBio) or with rat IgG2A isotype control (clone RTK2758, leaf purified, Biolegend) for 1 h on ice.
(5 μg ml⁻¹ each). After washing with HBSS, cells were incubated with Alexa 488 goat anti-rat IgG (4 μg ml⁻¹, Life technologies) for 30 min on ice. After two washes with HBSS, cells were re-suspended in HBSS/0.5% BSA and analysed on a BD FACSaria II machine. Flow cytometry data was analysed using FlowJo (Treestar).

**Microarray analysis.** Total RNA was extracted by sequential Qiazol extraction and purification through the RNeasy micro kit with on column genomic DNA digestion (Qiagen). RNA quality was determined by an Agilent 2100 Bioanalyzer using the RNA Pico Chip (Agilent). RNA was amplified into cDNA using the Ambion wild-type expression kit for whole transcript expression arrays, with Poly-A controls from the Affymetrix Genechip Eukaryotic Poly-A RNA control kit. The Affymetrix Genechip WT terminal labelling kit was used for fragmentation, biotin labelling. Affymetrix GeneChip Hybridization control kit and the Affymetrix GeneChip Hybridization, wash, stain kit was used to hybridize samples to Affymetrix Mouse Gene ST 1.0 GeneChips, fluidics performed on the Affymetrix Genechip Fluidics Station 450, and scanned using Affymetrix Genechip Scanner 7G (Affymetrix). Microarray work was conducted at the Boston Children’s Hospital IDDRC Molecular Genetics Core, which is supported by NIH-P30-HD 18655. Affymetrix CEL files were normalized with the robust multi-array average (RMA) algorithm with quantile normalization, background correction, and median scaling. The ImmGen data set was also analysed (GEO accession number GSE15907).

Heat maps were generated using GenePattern platform (Broad institute, MIT). Nociceptor microarray data sets are deposited at the GEO database under accession number GSE46546.

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