Gut microbiota is critical for the induction of chemotherapy-induced pain

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Chemotherapy-induced pain is a dose-limiting condition that affects 30% of patients undergoing chemotherapy. We found that gut microbiota promotes the development of chemotherapy-induced mechanical hyperalgesia. Oxaliplatin-induced mechanical hyperalgesia was reduced in germ-free mice and in mice pretreated with antibiotics. Restoring the microbiota of germ-free mice abrogated this protection. These effects appear to be mediated, in part, by TLR4 expressed on hematopoietic cells, including macrophages.

Chemotherapeutic drugs such as oxaliplatin induce a peripheral neuropathy that affects more than 30% of patients under treatment. Chemotherapy-induced peripheral neuropathy (CIPN) often presents with devastating neuropathic pain lasting from months to years, which prevents patients from receiving adequate chemotherapy dosages. Recently, gut microbiota has been shown to play a critical role in the tumor-killing effect of many chemotherapeutics drugs, including oxaliplatin. However, it is unknown whether neuropathic pain, a major side effect of chemotherapy, would be influenced by gut microbiota.

We exposed mice to a cocktail of antibiotics in drinking water (abx mice) starting 3 weeks before oxaliplatin administration and continued throughout the experiment. This treatment regimen reduced the fecal bacterial load by > 2 log-folds, decreased the diversity as measured by the Shannon index and altered the community structure of gut microbiota (Fig. 1a–c and Supplementary Fig. 1a–c), all of which are consistent with previous reports. We then exposed abx mice (fed water containing antibiotics) and H2O mice (fed regular water) to either saline or oxaliplatin treatment. While abx mice did not show changes in baseline nociceptive threshold, mechanical hyperalgesia was not detectable after the oxaliplatin therapy (Fig. 1d; ** P < 0.05, abx + oxaliplatin versus abx + saline or H2O + saline) except on day 7 (Fig. 1d). In contrast, mechanical hyperalgesia was clearly demonstrated in H2O mice (Fig. 1d; * P < 0.05, H2O + oxaliplatin versus H2O + saline), as shown in previous studies. Thus, temporary gut microbiota eradication protected those mice from developing mechanical hyperalgesia. The same phenomenon was observed in male and female mice, in rats and in a spontaneous pain-behavior test (Supplementary Fig. 2a–c). Moreover, we ruled out the possibility that mere exposure to antibiotics directly influences oxaliplatin-induced mechanical hyperalgesia, as intrathecal injection of these antibiotics did not change oxaliplatin-induced mechanical hyperalgesia (Supplementary Fig. 2d).

Next, we compared mechanical hyperalgesia in oxaliplatin-treated germ-free (GF) mice and specific pathogen-free (SPF) mice. Mechanical hyperalgesia only developed in SPF mice, but not in GF mice (Fig. 1e), indicating that, similarly to the effect of gut microbiota eradication in abx mice, the GF status also prevented oxaliplatin-induced mechanical hyperalgesia. Note that one limitation of the GF mice study was that the behavioral testing period lasted for 3 weeks, and we cannot completely rule out potential contamination of GF mice during the testing period. To confirm that gut microbiota in SPF mice could mediate oxaliplatin-induced mechanical hyperalgesia, we conventionalized GF mice to SPF status by gastric feeding of feces from SPF mice donors. Conventionalization of GF mice to SPF status abrogated the protection mediated by GF status (Fig. 1f), supporting the hypothesis that the gut microbiota in SPF mice likely mediated oxaliplatin-induced mechanical hyperalgesia.

In DRG, levels of IL-6 and TNF-α and their gene transcripts were lower in abx mice than in H2O mice after oxaliplatin therapy (Fig. 1h and Supplementary Fig. 4). These differences were only observed in DRG and not in the spinal cord. However, major immune cell proportions including T cells, B cells, monocytes, dendritic cells, NK cells and neutrophils in peripheral blood did not differ between abx mice and H2O mice (Supplementary Fig. 5). These results support the notion that DRG is a key anatomical site for the pathogenesis of CIPN. Two additional sets of data suggest that gut microbiota eradication led to dampened inflammatory responses in the DRG following exposure to oxaliplatin. First, consistent with the involvement of reactive oxygen species (ROS) in CIPN, we observed that ROS levels in DRG were lower in abx mice than in H2O mice, using L-012

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chemiluminescence as an indicator for ROS (Fig. 1i). Second, macrophage infiltration in DRG has recently been identified as a critical event in CIPN. In our experiment, DRG CD11b+ and CD45hi cells, which are presumed macrophages, were less abundant in abx mice than in H2O mice (Fig. 2a,b) after oxaliplatin therapy.

We stimulated mouse primary macrophages with oxaliplatin in the presence or absence of lipopolysaccharides (LPS), a gram negative bacterial wall component. Without LPS, oxaliplatin at 1 µM failed to stimulate macrophages to secrete IL-6 and TNF-α, and even at a higher oxaliplatin concentration (10 µM), we detected only low concentrations of IL-6 and TNF-α (Fig. 2c). In the presence of LPS, however, oxaliplatin at 1 or 10 µM robustly and dose-dependently stimulated the production of IL-6 and TNF-α (Fig. 2c). Moreover, we confirmed that the LPS and oxaliplatin concentrations we used in cell culture did not increase cell death (Supplementary Fig. 6). Therefore, oxaliplatin was ineffective in stimulating inflammatory cytokine production in vitro without the presence of LPS, indicating that a permissive signal would be required for macrophages to mount an inflammatory response against oxaliplatin.

We posit that this permissive signal is likely to be LPS derived from gut microbiota. To examine this hypothesis, we first measured serum and DRG LPS levels in abx mice and H2O mice with oxaliplatin or saline treatment. Oxaliplatin treatment increased the serum and DRG LPS levels in both groups of mice compared to saline treatment. However, the serum and DRG LPS levels were significantly higher in H2O mice than in abx mice following oxaliplatin treatment (Fig. 2d). Next, we examined whether exogenous LPS administration could abrogate the protection of oxaliplatin-induced mechanical hyperalgesia by gut microbiota eradication. Exogenous LPS administration by gastric gavage to abx mice indeed abrogated the protection offered by gut microbiota eradication (Fig. 3a). Since LPS is a ligand of Toll-like receptor 4 (Tlr4), we compared the development of mechanical hyperalgesia following oxaliplatin treatment in Tlr4 knockout (Tlr4−/−) mice and littermate heterozygous (Tlr4+/−) mice. Oxaliplatin-induced mechanical hyperalgesia was substantially less severe in Tlr4−/− mice than in their littermate Tlr4+/− counterparts during the entire observation period (Fig. 3b), which is in agreement with previous findings on other models of CIPN.

**Figure 1** Temporary eradication of gut microbiota prevents oxaliplatin-induced mechanical hyperalgesia (a–c). Impacts of antibiotic water feeding on mice gut microbiota. Fecal samples were obtained after 3 weeks of antibiotic water (abx mice, n = 10) or regular water (H2O mice, n = 10) followed by DNA isolation from these samples. (a) Feeding mice antibiotics reduced bacterial load as determined by semiquantitative real-time PCR. (b) Feeding mice antibiotics reduced the α diversity of microbiota. (c) Feeding mice antibiotics altered bacterial community structure as shown in the phylogenetic analysis. (d) Gut microbiota eradication prevented the development of oxaliplatin-induced mechanical hyperalgesia. Mice were fed on antibiotics water (abx) or regular water (H2O) before oxaliplatin or saline treatment (as control). Hindpaw mechanical withdrawal threshold (HWT) was examined at indicated timepoints after oxaliplatin therapy (n = 6 mice in each group; *P < 0.05, H2O + oxaliplatin vs. abx + oxaliplatin; **P > 0.05, abx + oxaliplatin vs. abx + saline or H2O + saline). (e) GF status protected mice from oxaliplatin-induced mechanical hyperalgesia. GF or SPF mice were given oxaliplatin or saline. HWT was examined at indicated timepoints after oxaliplatin therapy (n = 7 mice per group). (f) Conventional GF mice, feces from SPF mice were diluted with PBS and administered daily via gastric gavage for 3 weeks. GF and conventional GF (GF conv) mice were treated with oxaliplatin or saline. Conventionalization of GF mice abrogated the protection offered by GF status (*P < 0.05, GF conv + oxaliplatin vs. GF + oxaliplatin; n = 8 GF + oxaliplatin, 7 GF + saline, 7 GF conv + oxaliplatin, 6 GF conv + saline). (g) Gut microbiota eradication did not change tissue oxaliplatin distribution (n = 6 mice per group). Spinal cord (SC), serum and DRG concentrations of platinum were determined by inductively coupled plasma mass spectrometry (ICP-MS). Two-way ANOVA suggested abx treatment did not change tissue platinum distribution (P = 0.42) while tissue type had significant influence on platinum levels (P = 0.001). Post hoc t tests (P values in the figure) were performed to identify the impact of tissue type on platinum levels. (h) DRG cytokine levels for IL-6 and TNF-α were lower in mice with eradicated gut microbiota than in mice with normal gut microbiota (n = 6 mice per group); one-way ANOVA indicated a significant difference in DRG samples and subsequent post hoc t tests determined the difference between the abx and H2O groups. Sera and spinal cord levels for IL-6 and TNF-α were not significantly different among groups (n = 6 mice per group; one-way ANOVA for sera IL-6 samples, P = 0.58; spinal cord IL-6 samples, P = 0.06; sera TNF-α samples, P = 0.88; spinal cord TNF-α samples, P = 0.75). (i) Reduced ROS in DRG from mice with eradicated gut microbiota, ROS levels were determined with an IVIS Spectrum in vivo imaging system using L-012 as a chemiluminescent probe (n = 6 mice per group; one-way ANOVA followed by post hoc tests). Error bars in a and d–i indicate mean ± s.e.m.; in b, box plot bottoms and tops indicate 25th and 75th percentiles, respectively, whiskers indicate minima and maxima, and center lines indicate medians.
levels in serum and DRG. Mice were fed antibiotics water (abx) or regular water (H2O) before oxaliplatin treatment. (a) Dot plots show representative stainings for macrophages (CD11b+ and CD45hi cells) of each group. Insets show CD11b+CD45hi cells. Percentages represent CD11b+CD45hi cells among all DRG cells. (b) Percentages of macrophages in DRG cells were significantly higher in the H2O + oxaliplatin group than in the abx + oxaliplatin group (n = 6 each group; P = 0.0001 by one-way ANOVA; post hoc t-test, P = 0.009). (c) A permissive effect of LPS on production of IL-6 and TNF-α in macrophages. Macrophages were collected from peritoneal cavity for flow cytometry staining 10 d after the initiation of oxaliplatin or saline treatment. LPS levels in culture supernatant were determined at 24 h and LPS stimulation with ELISA (enzyme-linked immunosorbent assay). One-way ANOVA followed by post hoc t test suggested that significant differences exist among three groups at given LPS concentration (* P < 0.05 vs. oxaliplatin 0 µM, ** P < 0.05 vs. oxaliplatin 1 µM). Data represent quadruplicate wells. (d) LPS (endotoxin) levels in serum and DRG. Mice were fed antibiotics water (abx) or regular water (H2O) before oxaliplatin treatment or saline treatment as control. Serum and DRG samples were collected and processed for endotoxin assay 10 d after the initiation of oxaliplatin or saline treatment. LPS levels in sera and DRG were higher in the H2O + oxaliplatin group than in the abx + oxaliplatin group (one-way ANOVA followed by post hoc t test found significant differences present among all groups; n = 6 mice per group). Error bars in b and d indicate mean ± s.e.m.; in c, box plot bottoms and tops indicate 25th and 75th percentiles, respectively, whiskers indicate minima and maxima, and center lines indicate medians.

These results suggest that gut microbiota influenced the development of mechanical hyperalgesia following oxaliplatin therapy through an LPS–TLR4 pathway. To determine the contribution of TLR4 expressed on hematopoietic versus nonhematopoietic cells to oxaliplatin-induced mechanical hyperalgesia, we carried out a set of experiments using bone marrow chimeric mice. By using reciprocal bone marrow transplantation between wild-type (WT) and Tlr4−/− mice, we generated bone marrow chimeric mice that only expressed TLR4 on hematopoietic cells and not on nonhematopoietic cells (Fig. 3c,d). Successful generation of bone marrow chimera was confirmed by finding that more than 80% and 95% of CD11b+ cells in DRG and peripheral blood, respectively, were from the donor origin (Fig. 3d and Supplementary Fig. 7). We also found that, when hematopoietic cells were from Tlr4−/− donors, host mice were protected from oxaliplatin-induced mechanical hyperalgesia regardless of whether their nonhematopoietic cells expressed TLR4 (Fig. 3e). These results indicate that the TLR4 expressed on hematopoietic cells was responsible for oxaliplatin-induced mechanical hyperalgesia.

We found that gut microbiota eradication or GF status prevents the development of mechanical hyperalgesia through its impact on DRG inflammatory responses to oxaliplatin. The exact mechanism underlying this role of gut microbiota remains to be elucidated. Recent studies show that gut microbiota determines the functional maturation of microglia in the central nervous system15, promotes neutrophils migration in inflammatory responses16 and, notably, facilitates the migration of monocytes to kidneys following ischemic-reperfusion injury17. The present results demonstrate that gut microbiota is indispensable in oxaliplatin-induced mechanical hyperalgesia. LPS has been shown to be one of the key factors derived from gut microbiota that determine autoimmunity and inflammation18. Our data indicate that LPS enabled and augmented macrophages to secret inflammatory cytokines in response to oxaliplatin exposure, showing a synergy between signals derived from gut microbiota and the impact of oxaliplatin itself on immune cells. This synergy was further supported by the findings that LPS administration abrogated the protective effect offered by gut microbiota eradication (Fig. 3a).

Our findings further demonstrate that oxaliplatin is dependent on gut microbiota to exert not only its tumor-killing effect19 but also its crucial impact on the development of mechanical hyperalgesia, a major side effect associated with chemotherapy. These findings are not mutually exclusive to previous findings that oxaliplatin has neurotoxicity7. In addition, oxaliplatin may directly alter the gut microbiota that contributes to the development of chemotherapy-induced mechanical hyperalgesia. Previously, Amaral et al. found that carrageenan-induced inflammatory pain was attenuated in GF mice, suggesting a key role of gut microbiota in mediating inflammatory pain20. Our findings indicate that gut microbiota also plays a key role in mechanical hyperalgesia induced by oxaliplatin. Future studies are needed to examine whether gut microbiota is implicated in mechanical hyperalgesia induced by other chemotherapy agents.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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Figure 3 TLR4 on hematopoietic cells is critical for oxaliplatin-induced mechanical hyperalgesia. (a) Exogenous administration of LPS through gastric gavage reversed the effect of gut microbiota eradication. Mice were fed antibiotic water (abx) or regular water (H₂O), followed by oxaliplatin injection. LPS (3 mg/kg) or normal saline was administered on days of oxaliplatin injection and twice weekly afterwards (*P < 0.05, abx + oxaliplatin with LPS gavage vs. abx + oxaliplatin; saline gavage, n = 7 mice per group. (b) Tlr4−/− knockout mice did not develop oxaliplatin-induced mechanical hyperalgesia. Tlr4−/− littermate heterozygous (Tlr4+/−) mice were treated with oxaliplatin or saline and were examined at indicated timepoints for HWT (n = 6 mice per group; *P < 0.05, WT + oxaliplatin vs. all other groups). (c,d) Generation of bone marrow (BM) chimeras. (c) Flowchart for BM chimeric mice generation. Recipients were irradiated with 500 rad × 2, followed by BM cell injection. Donor BM cells were derived from Tlr4−/− or WT mice and were injected to WT or Tlr4−/− recipients using a crossover study design. CD 45.1 and CD45.2 congenic markers were used to distinguish between donor-derived and recipient-derived hematopoietic cells. (d) Confirmation of successful generation of BM chimeras. DRG samples were collected and stained for CD45.1 and CD45.2 congenic markers 14 weeks after BM transplantation. Contour plots were gated on CD11b+ and CD3− cells. Each panel represents 6 independent stainings. (e) TLR4 on hematopoietic cells is critical for oxaliplatin-induced mechanical hyperalgesia. BM chimeric mice generated as shown in c and d were treated with oxaliplatin (n = 6 mice per group). In Tlr4−/− to WT mice, hematopoietic cells were from Tlr4−/− donors. These mice were protected from oxaliplatin-induced mechanical hyperalgesia despite the absence of TLR4 on host-derived radio-resistant cells (*P < 0.05, Tlr4−/− to WT vs. WT to WT) In contrast, in WT to Tlr4−/− mice, hematopoietic cells were from WT donors; these mice developed oxaliplatin-induced mechanical hyperalgesia despite the absence of Tlr4 on host-derived radio-resistant cells (**P > 0.05, WT to Tlr4−/− vs. WT to WT). Data are shown as mean ± s.e.m.

AUTHOR CONTRIBUTIONS
S.S. and J.M. conceived the project and wrote the manuscript. S.S., G.L., Z.Y., W.D., S.T., H.K. and M.M. conducted the experiments. C.R. conducted the bioluminescence study. F.C. carried out the mass spectrometry study. P.H. performed part of the animal study. J.D. contributed to manuscript preparation. K.H. assisted the bone marrow chimera analysis. Z.X. contributed to the Tlr4-knockout mice experiment. D.K. contributed to the analysis of gene sequencing data. B.H. and L.C. contributed intellectually to the project.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All procedures and animal use were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC) and were in accordance with the guidelines established by NIH and the International Association for the Study of Pain. Conventional SPF C56BL/7 mice and Sprague Dawley rats were purchased from the Jackson Laboratory and Charles Rivers Laboratory, respectively. All initial group assignments were randomized. Except for the indicated experiment using female mice, we used male mice (6–12 weeks of age) for the study. For gut microbiota eradication, SPF mice and rats were provided, *ad libitum*, with drinking water containing 0.5 g/L ampicillin, 0.5 g/L neomycin, 0.5 g/L metronidazole and 0.25 g/L vancomycin (all antibiotics from Sigma-Aldrich) with 3 g/L artificial sweetener Splenda, for 3 weeks. Antibiotics were removed once the antibiotic levels were undetectable.

For the righting reflex and hind paw stepping tests, mice were restrained in a plastic tube, with the tube’s opening facing downward. A glass bead was placed in the opening and the mouse was released. The time for the mouse to right itself or pull the bead into the tube when released from the plastic tube was recorded. For the vocalization test, mice were restrained in a plastic tube with a fine mesh at both ends to prevent escape and autopsies. Mice were killed by cervical dislocation, dissection, and preservation for histological analysis.

DNA extraction and quantification of fecal bacteria. Fecal DNA was extracted with DNA Stool Kit (MoBio) according to the manufacturer’s instructions. The abundance of eubacteria in feces was measured by qPCR using a StepOnePlus instrument (Applied Biosystems) with the fecal DNA and 16S rRNA gene primers for eubacteria. The sequences of the primers are: ACT CCT ACG GGA GGC AGC AGT (UniF 340) and ATT ACC GCGGCT GGC (UniR 514). The real-time PCR program started with an initial step at 95 °C for 10 min, followed by 40 cycles of 95 °C 30 s and 60 °C 1 min. RT-PCR was done using SYBR Green Mastermix (Qiagen). Bacterial numbers was determined using standard curves constructed with DNA of *Escherichia coli* strain DH10B as reference bacteria. The 16S rRNA gene sequencing and data analysis were performed by BGI America (Cambridge, MA). Briefly, after ampiclon based sequencing, OTUs were clustered using USEARCH (7.0.1090) with a 97% threshold. Greengene V201305 was used as reference database for taxonomic classification. The MixOmics package for R was used for PLS-DA analysis. OTU rank curve was generated in R (V3.1.1). The tag number of each taxonomic rank (phylum, class, order, family, genus, species) or OTU in different samples were summarized in histograms generated in R (V3.1.1) 2–4. Sequencing data are available through NCBI BioProject PRJNA388399.

Behavioral tests. All behavioral experiments were carried out by investigators blind to treatment conditions. Animals were habituated to the test environment for two consecutive days (30 min per day) before baseline testing.

Mechanical withdrawal threshold. A von Frey filament was perpendicularly applied to the plantar surface of each hind paw using an up-and-down approach22.23. A threshold force of response (in g) was defined as the first filament that evoked at least two withdrawals out of five applications. Seven or fewer withdrawals were applied to animals. Facial grooming was evaluated by counting the number of forepaw rubs and hindpaw scratches in a 10-min period24. GF animal testing was performed in a facility room with HEPA air filter and a testing hood with laminar flow. Test performers wore sterile gown and gloves. Testing enclosures were autoclaved before use. Of note, the GF behavioral testing period occurred over 3 weeks. We could not completely rule out potential contamination occurred during the testing period, despite surveillance Quantitative PCR was negative for contamination.

Bioluminescence assay. Mice were injected with 100 mg/kg luminescent probe L-012 (Wako, Richmond, VA) 3 days before oxaliplatin injection. Within 3–5 min after injection, mice were killed for analysis and (lumbar) L3–L5 DRGs were obtained for an IVIS Spectrum (Xenogen, Alameda, CA) study. L-012 shows luminescence after chemical reaction with ROS. To calculate bioluminescence, radiant (photons/s/cm²/sr) was obtained within 15 min after L-012 injection.

Platinum concentration by ICP-MS analysis. Each tissue sample was transferred to a glass sample vial and the sample weight was recorded. The sample was then digested with 1.5:4% HNO₃:HCl to 20 mL volume at 37 °C overnight. Elemental analysis was completed using ICP-MS (Agilent Technologies, 8800 ICP-MS-QQQ). Calibration standards were prepared from serial dilutions of 1-µg/g and 10-µg/g Pt (NIST SRM 3140) solutions. Briefly, 10–25, 50–75, 100–150- and 200-µg/g standards were prepared by diluting 0.10, 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 mL of a 1-ng/g Pt solution. Each prepared standard was transferred to an 18-mL LDPE sample vial. The standards were diluted to a total of 10 mL using 1.5% HNO₃:HCl solution. The weights of the empty vial, the vial containing the sample and the vial containing the final dilution were recorded. The exact concentration in each standard was determined by difference. The three analyzed elements were platinum (Analyte), lutetium (internal standard) and dysprosium (internal standard). Samples were run in triplicate, with each one or the triplicate consisting of 10 measurement repetitions. A calibration curve was constructed from the external calibration samples. The limit of detection (LOD) for the sample run was 0.48 pg/g. The limit of quantitation (LOQ) was also determined using the formula above, with k = 10. The LOQ for the ICP-MS run was 1.6 pg/g.

Flow cytometry. For DRG staining, mice were anesthetized by isoflurane anesthesia followed by perfusion through left ventricle cannulation with a 20-gauge needle. For each mouse, 20 mL of ice-cold normal saline were perfused to minimize blood contamination in the sample. Six DRG samples were obtained from each mouse. DRG samples were minced and digested with Liberase TL (Roche) at 37 °C for 60 min in RPMI-1640 medium with intermitten vortex. The samples were then filtered through a nylon membrane with 60-µm pores, followed by centrifugation. A Percoll (Sigma) gradient (70–40%) was used for cell isolation. For peripheral blood staining, we used 20 µL of peripheral blood obtained from retro-orbital veins. For cultured macrophage staining, macrophages cultured in RPMI-1640 were washed in PBS three times, followed by

LPS (endotoxin) assay. All materials used for both sample preparation and testing were pyrogen-free. LPS concentrations in plasma and tissue homogenate were measured by an endotoxin assay based on a limulus amebocyte lysate (LAL) assay (Pierce LAL Chromogenic Endotoxin Quantitation Kit). Samples were diluted in pyrogen-free water and heated to 70 °C for 10 min to inactivate inhibitor agents that could interfere with the assay. All samples were tested in triplicate, and results were accepted when the intra-assay coefficient of variation was 15%. The endotoxin content was expressed as endotoxin units per milliliter (EU/mL) or endotoxin units per 100 mg of tissue.
a brief trypsin treatment to detach cells from culture plate. Flow cytometry staining was performed at room temperature (22–24 °C) for 10 min, except for annexin V and 7-AAD staining. Antibodies (anti-CD3, catalog #100235; anti-CD4, catalog #100413; anti-CD11b, catalog #101222; anti-CD35, catalog #137617; anti-CD11c, catalog #117309; anti-Ly-6G, catalog #127623; anti-annexin V, catalog #640905; and anti-7-AAD, catalog #420403) were obtained from BioLegend. For annexin V and 7-AAD staining, binding buffers containing 140 mM NaCl, 4 mM KCl, 0.75 mM MgCl₂, 2.5 mM CaCl₂ and 10 mM HEPES in DDW were used at 0.2 mL per sample. Annexin V and 7-AAD were added to the sample 10 min before acquisition by flow cytometer. All samples were acquired by LSR II (BD) flow cytometer. Data were analyzed with Flowjo (FlowJo) software.

Macrophage isolation and culture²⁶. Mice were killed for analysis and soaked in 70% ethanol. We injected 10 mL of cold PBS to the peritoneal cavity, followed by needle aspiration. Aspirated fluid was centrifuged and cells (including neutrophils and macrophages) were resuspended in DMEM medium. A total of 4 × 10⁵ cells/well was added to a 24-well plate. The cells were allowed to adhere to the culture plate for 2 h at 37 °C. Nonadherent cells were removed by gently washing three times with warm PBS. Adherent cells were macrophages and were used for subsequent culture in the presence of LPS and oxaliplatin at desired concentrations.

Statistical analysis and sample size. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications²⁷. Data distribution was assumed to be normal, but this was not formally tested. Behavioral data were analyzed using two-way ANOVA, repeated across timepoints and groups. One-way ANOVA was used to analyze the data from various assays (qPCR, ELISA). Post hoc Waller-Duncan k-ratio t-tests (two-tailed) were performed to determine the source(s) of differences. SPSS 12.0 software was used for the statistical analyses. All data were expressed as mean ± s.e.m. and the statistically significant level was set at P < 0.05. A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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- Each figure legend should ideally contain an exact sample size (n) for each experimental group/condition, where n is an exact number and not a range, a clear definition of how n is defined (for example x cells from x slices from x animals from x litters, collected over x days), a description of the statistical test used, the results of the tests, any descriptive statistics and clearly defined error bars if applicable.

- For any experiments using custom statistics, please indicate the test used and stats obtained for each experiment.

- Each figure legend should include a statement of how many times the experiment shown was replicated in the lab; the details of sample collection should be sufficiently clear so that the replicability of the experiment is obvious to the reader.

- For experiments reported in the text but not in the figures, please use the paragraph number instead of the figure number.

Note: Mean and standard deviation are not appropriate on small samples, and plotting independent data points is usually more informative. When technical replicates are reported, error and significance measures reflect the experimental variability and not the variability of the biological process; it is misleading not to state this clearly.

| TEST USED | n | DESCRIPTIVE STATS (AVERAGE, VARIANCE) | P VALUE | DEGREES OF FREEDOM & F/Z/T/R/ETC VALUE |
|-----------|---|-------------------------------------|--------|--------------------------------------|
| one-way ANOVA | 9, 9, 10, 15 mice from at least 3 litters/group | error bars are mean +/- SEM | p = 0.044 | F(3, 36) = 2.97 |
| Results para 6 | 15 slices from 10 mice | error bars are mean +/- SEM | p = 0.0006 | t(28) = 2.808 |
| unpaired t-test | 6, 6 mice from two groups | mean +/- SEM | p<0.0001 | t(10)=20.09 |

Example:

1a one-way ANOVA
Fig legend
Results para 6
para 8
Fig legend
Methods
Methods
Fig legend
Fig legend
Para #
Para #
Para #

Nature Neuroscience: doi:10.1038/nn.4606
| Figure Number | Test Used  | n  | Descriptive Stats (Average, Variance) | p Value | Degrees of Freedom & F/t/z/R/ETC Value |
|---------------|-----------|----|--------------------------------------|---------|---------------------------------------|
| 1b            | unpaired t-test | 10,10 | mice from two groups | Para # | mean +/- SEM | p < 0.0001 | t(10) = 9.523 | Fig. Legend |
| 1d            | two-way ANOVA | 8,8,8 | mice from four groups | Para # | mean +/- SEM | p < 0.001 | F(3,28) = 14.15 | Fig. Legend |
| 1d            | two-way ANOVA | 8,8 | mice from three groups | Para # | mean +/- SEM | p = 0.3954 | F(2,21) = 0.97 | Fig. Legend |
| 1e            | two-way ANOVA | 7,7,7,7 | mice from four groups | Para # | mean +/- SEM | p < 0.001 | F(3,24) = 12.68 | Fig. Legend |
| 1f            | two-way ANOVA | 8,7,6 | mice from four groups | Para # | mean +/- SEM | p < 0.0001 | F(3,24) = 64.28 | Fig. Legend |
| 1g            | two-way ANOVA, abx vs H2O | 6,6,6,6 | two groups of mice | mean +/- SEM | p = 0.42 | F(1,20) = 0.006 | Fig. Legend |
| 1g            | two-way ANOVA, different tissues | 6,6,6,6 | three different types of tissues | mean +/- SEM | p < 0.001 | F(2,20) = 65.42 | Fig. Legend |
| 1g            | unpaired t-test, | 6,6 | two types of tissues | mean +/- SEM | p = 0.004 | t(10) = 4.422 | Fig. Legend |
| 1g            | unpaired t-test | 6,6 | two types of tissues | mean +/- SEM | p = 0.002 | t(10) = 7.143 | Fig. Legend |
| 1g            | unpaired t-test | 6,6 | two types of tissues | mean +/- SEM | p = 0.004 | t(10) = 6.704 | Fig. Legend |
| 1g            | unpaired t-test | 6,6 | two types of tissues | mean +/- SEM | p = 0.005 | t(10) = 7.019 | Fig. Legend |
| 1i            | one-way ANOVA | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.0001 | F(3,20) = 14.31 | Fig. Legend |
| 1i            | unpaired t-test | 6,6 | mice from two groups | mean +/- SEM | p = 0.004 | t(10) = 3.943 | Fig. Legend |
| 1h            | one-way ANOVA, sera IL-6 | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.58 | F(3,20) = 0.67 | Fig. Legend |
| 1h            | one-way ANOVA, DRG IL-6 | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.0001 | F(3,20) = 73.01 | Fig. Legend |
| 1h            | one-way ANOVA, spinal cord IL-6 | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.06 | F(3,20) = 2.91 | Fig. Legend |
| 1h            | one-way ANOVA, sera TNF | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.88 | F(3,20) = 0.22 | Fig. Legend |
| 1h            | one-way ANOVA, DRG TNF | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.0001 | F(3,20) = 21.73 | Fig. Legend |
| 1h            | one-way ANOVA, SC TNF | 6,6,6,6 | mice from four groups | mean +/- SEM | p = 0.75 | F(3,20) = 0.41 | Fig. Legend |
| 1h            | unpaired t-test | 6,6 | mice from two groups | mean +/- SEM | p = 0.004 | t(10) = 3.664 | Fig. Legend |
| 1h            | unpaired t-test | 6,6 | mice from two groups | mean +/- SEM | p = 0.005 | t(10) = 4.211 | Fig. Legend |

Nature Neuroscience: doi:10.1038/jn.4606
| Fig. | Method          | Fig. | Legend | n | Description | Mean +/- SEM | p-value | F(3,20) | t(10) | t(6) | t(6) | t(6) |
|------|-----------------|------|--------|---|-------------|--------------|----------|---------|------|-----|-----|-----|
| 2b   | One-way anova   | Fig. | Legend | 6,6,6,6 | cells from four groups | mean +/- SEM | p=0.001 | F(3,20)=14.96 | Fig. Legend |
| 2b   | Unpaired t-test | Fig. | Legend | 6,6 | cells from two groups | mean +/- SEM | p=0.009 | Fig. Legend |
| 2c   | One-way anova, IL-6 panel, LPS 10 | Fig. | Legend | 4,4,4 | three groups of cells | mean +/- SEM | p=0.0026 | Fig. Legend |
| 2c   | One-way anova, IL-6 panel, LPS 30 | Fig. | Legend | 4,4,4 | three groups of cells | mean +/- SEM | p=0.0001 | Fig. Legend |
| 2c   | One-way anova, TNF panel, LPS 10 | Fig. | Legend | 4,4,4 | three groups of cells | mean +/- SEM | p=0.0001 | Fig. Legend |
| 2c   | One-way anova, TNF panel, LPS 30 | Fig. | Legend | 4,4,4 | three groups of cells | mean +/- SEM | p=0.0002 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells; LPS at 10; IL-6 levels oxa 0 vs oxa 1 | mean +/- SEM | p=0.04 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells LPS at 10; IL-6 levels oxa 1 vs oxa 10 | mean +/- SEM | p=0.04 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of LPS at 30; IL-6 levels oxa 0 vs oxa 1 | mean +/- SEM | p=0.024 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of LPS at 30; IL-6 levels oxa 1 vs oxa 10 | mean +/- SEM | p=0.014 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells; LPS at 10; TNF levels oxa 1 vs oxa 1 | mean +/- SEM | p=0.02 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells; LPS at 10; TNF levels oxa 1 vs oxa 10 | mean +/- SEM | p=0.0005 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells; LPS at 30; TNF levels oxa 0 vs oxa 1 | mean +/- SEM | p=0.027 | Fig. Legend |
| 2c   | Unpaired t-test | Fig. | Legend | 4,4 | two groups of cells; LPS at 30; TNF levels oxa 1 vs oxa 10 | mean +/- SEM | p=0.016 | Fig. Legend |
| 2d   | One-way anova, serum LPS panel | Fig. | Legend | 6,6,6,6 | four groups of mice | mean +/- SEM | p=0.0001 | F(3,20)=40.09 | Fig. Legend |
| 2d   | One-way anova, DRG LPS panel | Fig. | Legend | 6,6,6,6 | four groups of mice | mean +/- SEM | p=0.0001 | F(3,20)=19.81 | Fig. Legend |
| 2d   | Unpaired t-test | Fig. | Legend | 6,6 | two groups of mice | mean +/- SEM | p=0.01 | Fig. Legend |
| 2d   | Unpaired t-test | Fig. | Legend | 6,6 | two groups of mice | mean +/- SEM | p=0.02 | Fig. Legend |
| 2d   | Unpaired t-test | Fig. | Legend | 6,6 | two groups of mice | mean +/- SEM | p=0.01 | Fig. Legend |
| 2d   | Unpaired t-test | Fig. | Legend | 6,6 | two groups of mice | mean +/- SEM | p=0.07 | Fig. Legend |
**Representative figures**

1. Are any representative images shown (including Western blots and immunohistochemistry/staining) in the paper?
   
   If so, what figure(s)?

2. For each representative image, is there a clear statement of how many times this experiment was successfully repeated and a discussion of any limitations in repeatability?
   
   If so, where is this reported (section, paragraph #)?

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| Fig | Test | Groups | Mean +/- SEM | p-Value | t-Value or F-Value |
|-----|------|--------|--------------|---------|-------------------|
| 2d  | unpaired t-test | 6,6 | two groups of mice | p=0.003 | t(10)=4.348 |
| 2d  | unpaired t-test | 6,6 | two groups of mice | p=0.005 | t(10)=3.736 |
| 3a  | two-way ANOVA | 7,7,7,7 | four groups of mice | p=0.001 | F(3,24)=40 |
| 3b  | two-way ANOVA | 6,6,6,6 | four groups of mice | p=0.0056 | F(3,20)=5.6 |
| 3e  | two-way ANOVA | 6,6,6,6 | four groups of mice | p=0.0001 | F(3,21)=16.33 |
| 6   | two-way ANOVA | 6,6 | two groups of mice | p=0.8 | F(1,10)=0.8 |

Supplementary Figure 2d

- Fig 2d: Representative figures shown staining represent six individual animals in each group. Summary of Fig2a staining were plotted in Fig 2b.
### Statistics and general methods

1. **Is there a justification of the sample size?**
   *If so, how was it justified?*
   *Where (section, paragraph #)?*
   
   Even if no sample size calculation was performed, authors should report why the sample size is adequate to measure their effect size.
   
   We did not perform power analysis to determine the sample size. However, we referred to previously published studies on animal models of chemotherapy induced mechanical hyperalgesia to determine our sample size.

2. **Are statistical tests justified as appropriate for every figure?**
   *Where (section, paragraph #)?*
   
   a. **If there is a section summarizing the statistical methods in the methods, is the statistical test for each experiment clearly defined?**
   
   b. **Do the data meet the assumptions of the specific statistical test you chose (e.g. normality for a parametric test)?**
   *Where is this described (section, paragraph #)?*

   c. **Is there any estimate of variance within each group of data?**
   *Is the variance similar between groups that are being statistically compared?*
   *Where is this described (section, paragraph #)?*

   d. **Are tests specified as one- or two-sided?**

   e. **Are there adjustments for multiple comparisons?**

3. **To promote transparency, *Nature Neuroscience* has stopped allowing bar graphs to report statistics in the papers it publishes. If you have bar graphs in your paper, please make sure to switch them to dot-plots (with central and dispersion statistics displayed) or to box-and-whisker plots to show data distributions.**

4. **Are criteria for excluding data points reported?**
   *Was this criterion established prior to data collection?*
   *Where is this described (section, paragraph #)?*

5. **Define the method of randomization used to assign subjects (or samples) to the experimental groups and to collect and process data.**
   *If no randomization was used, state so.*
   *Where does this appear (section, paragraph #)?*
|   |   |   |
|---|---|---|
| 6. | Is a statement of the extent to which investigator knew the group allocation during the experiment and in assessing outcome included? | Yes, in Methods. |
|   | If no blinding was done, state so. |   |
|   | Where (section, paragraph #)? |   |
| 7. | For experiments in live vertebrates, is a statement of compliance with ethical guidelines/regulations included? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 8. | Is the species of the animals used reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 9. | Is the strain of the animals (including background strains of KO/transgenic animals used) reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 10. | Is the sex of the animals/subjects used reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 11. | Is the age of the animals/subjects reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 12. | For animals housed in a vivarium, is the light/dark cycle reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 13. | For animals housed in a vivarium, is the housing group (i.e. number of animals per cage) reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 14. | For behavioral experiments, is the time of day reported (e.g. light or dark cycle)? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
| 15. | Is the previous history of the animals/subjects (e.g. prior drug administration, surgery, behavioral testing) reported? | Yes, in Materials and methods, paragraph #1. |
|   | Where (section, paragraph #)? |   |
|   | a. If multiple behavioral tests were conducted in the same group of animals, is this reported? | No multiple behavioral tests in the same group of animals. |
|   | Where (section, paragraph #)? |   |
| 16. | If any animals/subjects were excluded from analysis, is this reported? | No animal excluded. |
|   | Where (section, paragraph #)? |   |
Reagents

1. Have antibodies been validated for use in the system under study (assay and species)?

   a. Is antibody catalog number given?
      Where does this appear (section, paragraph #)?

   b. Where were the validation data reported (citation, supplementary information, Antibodypedia)?
      Where does this appear (section, paragraph #)?

2. Cell line identity

   a. Are any cell lines used in this paper listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample?
      Where (section, paragraph #)?

   b. If yes, include in the Methods section a scientific justification of their use—indicate here in which section and paragraph the justification can be found.

   c. For each cell line, include in the Methods section a statement that specifies:
      - the source of the cell lines
      - have the cell lines been authenticated? If so, by which method?
      - have the cell lines been tested for mycoplasma contamination?
      Where (section, paragraph #)?
Data availability

Provide a Data availability statement in the Methods section under “Data availability”, which should include, where applicable:

• Accession codes for deposited data
• Other unique identifiers (such as DOIs and hyperlinks for any other datasets)
• At a minimum, a statement confirming that all relevant data are available from the authors
• Formal citations of datasets that are assigned DOIs
• A statement regarding data available in the manuscript as source data
• A statement regarding data available with restrictions

See our data availability and data citations policy page for more information.

Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Microarray data

Deposition is strongly recommended for many other datasets for which structured public repositories exist; more details on our data policy are available here. We encourage the provision of other source data in supplementary information or in unstructured repositories such as Figshare and Dryad.

We encourage publication of Data Descriptors (see Scientific Data) to maximize data reuse.

Where is the Data Availability statement provided (section, paragraph #)?

Computer code/software

Any custom algorithm/software that is central to the methods must be supplied by the authors in a usable and readable form for readers at the time of publication. However, referees may ask for this information at any time during the review process.

1. Identify all custom software or scripts that were required to conduct the study and where in the procedures each was used.

2. If computer code was used to generate results that are central to the paper’s conclusions, include a statement in the Methods section under “Code availability” to indicate whether and how the code can be accessed. Include version information as necessary and any restrictions on availability.

Human subjects
1. Which IRB approved the protocol?  
Where is this stated (section, paragraph #)?

2. Is demographic information on all subjects provided?  
Where (section, paragraph #)?

3. Is the number of human subjects, their age and sex clearly defined?  
Where (section, paragraph #)?

4. Are the inclusion and exclusion criteria (if any) clearly specified?  
Where (section, paragraph #)?

5. How well were the groups matched?  
Where is this information described (section, paragraph #)?

6. Is a statement included confirming that informed consent was obtained from all subjects?  
Where (section, paragraph #)?

7. For publication of patient photos, is a statement included confirming that consent to publish was obtained?  
Where (section, paragraph #)?

**fMRI studies**

For papers reporting functional imaging (fMRI) results please ensure that these minimal reporting guidelines are met and that all this information is clearly provided in the methods:

1. Were any subjects scanned but then rejected for the analysis after the data was collected?  
   a. If yes, is the number rejected and reasons for rejection described?  
      Where (section, paragraph #)?

2. Is the number of blocks, trials or experimental units per session and/or subjects specified?  
   Where (section, paragraph #)?

3. Is the length of each trial and interval between trials specified?  

4. Is a blocked, event-related, or mixed design being used? If applicable, please specify the block length or how the event-related or mixed design was optimized.
5. Is the task design clearly described?  
   Where (section, paragraph #)?

6. How was behavioral performance measured?

7. Is an ANOVA or factorial design being used?

8. For data acquisition, is a whole brain scan used?  
   If not, state area of acquisition.
   
   a. How was this region determined?

9. Is the field strength (in Tesla) of the MRI system stated?  
   
   a. Is the pulse sequence type (gradient/spin echo, EPI/spiral) stated?
   
   b. Are the field-of-view, matrix size, slice thickness, and TE/TR/flip angle clearly stated?

10. Are the software and specific parameters (model/functions, smoothing kernel size if applicable, etc.) used for data processing and pre-processing clearly stated?

11. Is the coordinate space for the anatomical/functional imaging data clearly defined as subject/native space or standardized stereotaxic space, e.g., original Talairach, MNI305, ICBM152, etc? Where (section, paragraph #)?

12. If there was data normalization/standardization to a specific space template, are the type of transformation (linear vs. nonlinear) used and image types being transformed clearly described? Where (section, paragraph #)?

13. How were anatomical locations determined, e.g., via an automated labeling algorithm (AAL), standardized coordinate database (Talairach daemon), probabilistic atlases, etc.?

14. Were any additional regressors (behavioral covariates, motion etc) used?

15. Is the contrast construction clearly defined?

16. Is a mixed/random effects or fixed inference used?  
   
   a. If fixed effects inference used, is this justified?

17. Were repeated measures used (multiple measurements per subject)?
a. If so, are the method to account for within subject correlation and the assumptions made about variance clearly stated?

18. If the threshold used for inference and visualization in figures varies, is this clearly stated?

19. Are statistical inferences corrected for multiple comparisons?
   a. If not, is this labeled as uncorrected?

20. Are the results based on an ROI (region of interest) analysis?
   a. If so, is the rationale clearly described?
   b. How were the ROI’s defined (functional vs anatomical localization)?

21. Is there correction for multiple comparisons within each voxel?

22. For cluster-wise significance, is the cluster-defining threshold and the corrected significance level defined?

› Additional comments

Additional Comments