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Peripheral Nerve Resident Macrophages and Schwann Cells Mediate Cancer-induced Pain

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Abstract

Although macrophages (MΦ) are known to play a central role in neuropathic pain, their contribution to cancer pain has not been established. Here we report that depletion of sciatic nerve resident MΦs (rMΦ) in mice attenuates mechanical/cold hypersensitivity and spontaneous pain evoked by intraplantar injection of melanoma or lung carcinoma cells. MΦ-colony stimulating factor (M-CSF) was upregulated in the sciatic nerve trunk and mediated cancer-evoked pain *via* rMΦ expansion, transient receptor potential ankyrin 1 (TRPA1) activation, and oxidative stress. Targeted deletion of *Trpa1* revealed a key role for Schwann cell TRPA1 in sciatic nerve rMΦ expansion and pain-like behaviors. Depletion of rMΦs in a medial portion of the sciatic nerve prevented pain-like behaviors. Collectively, we identified a feed-forward pathway involving M-CSF, rMΦ, oxidative stress and Schwann cell TRPA1 that operates throughout the nerve trunk to signal cancer-evoked pain.

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Declaration of Interests

F.D.L., P.G., R.N. are founding scientists of FloNext Srl. N.W.B. is a founding scientist of Endosome Therapeutics Inc. The other authors declare that they have no competing interests.

Keywords

Cancer pain; Resident macrophages; Macrophage-colony stimulating factor; Transient Receptor Potential Ankyrin 1 (TRPA1); Schwann cells; Oxidative stress

Introduction

Pain is a common and devastating symptom of cancer, which afflicts 70–90% of cancer patients and can diminish the quality of life more than the cancer itself (1). However, cancer pain remains incompletely understood and poorly managed, thus representing a major unmet medical need (2). A series of cytokines, chemokines, and their receptors have been proposed to contribute to signal cancer pain (3). These include interleukin 1 β (IL-1 β), IL-6, tumor necrosis factor α (TNF- α), the C-X-C motif chemokine ligand (CXCL) 8 (CXCL8), CXCL13, CXC receptor (CXCR) 4 (CXCR4) and CXCL12/CXCR4. Although some of these cytokines may directly or indirectly attract inflammatory and immune cells, the implication of macrophages (M Φ s) in cancer pain remains unknown.

In contrast, the role of M Φ s in neuropathic pain associated with nerve injury has been extensively investigated, and distinct M Φ -dependent proalgesic pathways have been identified in the central and peripheral nervous systems (4–11). Recruitment and expansion of proalgesic M Φ s can be due to the predominant contribution of the upregulation of chemokine (C-C motif) ligand 2 (CCL2) and its receptor (CCR2) (4–6), or to the release of M Φ -colony stimulating factor (M-CSF) (7–9). Furthermore, peripheral neuropathy can be associated with different M Φ subpopulations (12). Thus, neuropathic pain may be promoted by hematogenous monocytes, which, recruited on demand after neural injury, rapidly invade the damaged peripheral nerves as mature and activated M Φ s (5,6), and by resident M Φ s (rM Φ s), which are constitutively present inside the *epineurium*, and may undergo a time-dependent expansion when activated by pathological insults (8,11).

Transient receptor potential ankyrin 1 (TRPA1), a proalgesic ion channel expressed in a subset of primary sensory neurons (13), is uniquely sensitive to oxidative stress byproducts (14). In a mouse model of trigeminal neuralgia, TRPA1 was proposed to signal mechanical allodynia elicited by oxidative stress generated by M Φ s recruited by CCL2 at the site of nerve injury (5). However, a later study, which used a murine model of partial nerve ligation, clarified that TRPA1 expressed in Schwann cells is interposed between M Φ -evoked oxidative stress and the pain generating neuronal channel (6). In fact, Schwann cell TRPA1, by eliciting oxidative stress *via* a Ca²⁺- and NADPH oxidase 1 (NOX1)-dependent mechanism, initiates an amplification loop which sustains neuroinflammation and targets neuronal TRPA1 to signal pain (6). We recently reported that genetic deletion or pharmacological inhibition of TRPA1 attenuated mechanical/cold hypersensitivity and spontaneous nociception in a syngeneic orthotopic model of solid cancer pain induced by inoculation of B16-F10 melanoma cells in the mouse hindpaw (15). However, the mechanism by which TRPA1 mediates mechanical/cold hypersensitivity and spontaneous nociception associated with tumor growth is unknown.

The aim of the present study was two-fold. In mouse models of cancer pain, we first explored the implication of MΦs in mechanical/cold hypersensitivity and spontaneous nociception. Then, we investigated the role of Schwann cell TRPA1 in the MΦ-dependent pain-like behaviors. In addition to revealing an essential function of sciatic nerve rMΦs, results obtained after B16-F10 melanoma cell inoculation revealed the role of Schwann cell TRPA1 to release M-CSF, which sustains rMΦ expansion, and to generate the oxidative stress that targets the neuronal TRPA1 to signal pain. Neuroinflammation and mechanical/cold hypersensitivity and spontaneous nociception are maintained by a feed-forward mechanism which requires the continuous interaction between Schwann cell TRPA1 and expanded rMΦs throughout the entire sciatic nerve trunk. Furthermore, the inoculation of Lewis lung carcinoma (LLC1) cells in the mouse hindpaw evoked mechanical allodynia that was attenuated by elimination of rMΦ or Schwann cell TRPA1. Thus, M-CSF, rMΦs, oxidative stress, and Schwann cell TRPA1 signal mechanical/cold hypersensitivity and spontaneous nociception in different mouse tumors and may represent potential targets for the treatment of cancer pain.

Materials and methods

Study design

The group size of $n = 6$ animals for behavioral experiments was determined by sample size estimation using G*Power (v3.1) (16) to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Allocation concealment of mice to vehicle(s) or treatment(s) group was performed using a randomization procedure (<http://www.randomizer.org/>). The assessors were blinded to the identity (genetic background or allocation to treatment group) of the animals. No animals were excluded from experiments.

All experiments and sample collections were carried out according to the European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. All animal studies were approved by Animal Ethics Committee of the University of Florence and the Italian Ministry of Health (permit #579/2017-PR). The behavioral studies followed the animal research reporting *in vivo* experiment (ARRIVE) guidelines.

Animals

Adult C57BL/6J (male and female 20–25 g, 5–6 weeks), littermate wild type (*Trpa1*^{+/+}) and TRPA1-deficient (*Trpa1*^{-/-}) mice (male, 25–30 g, 6–8 weeks), generated by heterozygotes on a C57BL/6J background (B6.129P-Trpa1^{tm1Kyk}/J; RRID:IMSR_JAX:006401 Jackson Laboratories) (6), were used. To generate mice in which the *Trpa1* gene was conditionally silenced in Schwann cells/oligodendrocytes, homozygous 129S-Trpa1^{tm2Kyk}/J (floxed TRPA1, *Trpa1*^{fl/fl}, Stock No: 008649, RRID:IMSR_JAX:008649 Jackson Laboratories), were crossed with hemizygous B6.Cg-Tg(Plp1-CreERT)3Pop/J mice (*Plp1-Cre*^{ERT}, Stock No: 005975, RRID:IMSR_JAX:005975 Jackson Laboratories), expressing a tamoxifen-inducible Cre in myelinating cells (proteolipid protein myelin 1, Plp1) (6). The progeny (*Plp1-Cre*; *Trpa1*^{fl/fl}) was genotyped by standard PCR for *Trpa1* and *Plp1-Cre*^{ERT} (6). Mice negative for *Plp1-Cre*^{ERT} (*Plp1-Cre*^{ERT-/-}; *Trpa1*^{fl/fl}) were used as control. Both positive and

negative mice to *Cre^{ERT}* and homozygous for floxed *Trpa1* (*Plp1-Cre^{ERT+};Trpa1^{fl/fl}* and *Plp1-Cre^{ERT-};Trpa1^{fl/fl}*, respectively) were treated with tamoxifen (i.p., 1 mg/100 μ l in corn oil, once a day, for 5 consecutive days) (6), resulting in Cre-mediated ablation of *Trpa1* in PLP-expressing Schwann cells/oligodendrocytes. Successful Cre-driven deletion of TRPA1 mRNA was confirmed by RT-qPCR (6). To selectively delete the *Trpa1* gene in primary sensory neurons, 129S-*Trpa1^{tm2Kykw/J}* mice (floxed *Trpa1*, *Trpa1^{fl/fl}*, Stock No: 008649; Jackson Laboratories), which possess loxP sites on either side of the S5/S6 transmembrane domains of the *Trpa1* gene, were crossed with hemizygous Advillin-Cre male mice (8). The progeny (*Adv-Cre;Trpa1^{fl/fl}*) were genotyped by standard PCR for *Trpa1* and Advillin-Cre. Mice negative for Advillin-Cre (*Adv-Cre⁻;Trpa1^{fl/fl}*) were used as control. Successful Advillin-Cre driven deletion of TRPA1 mRNA was confirmed by RT-qPCR. To evaluate the involvement of MΦs, transgenic Macrophage Fas-Induced Apoptosis (MaFIA) mice (C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J, stock No: 005070, RRID:IMSR_JAX:005070, Jackson Laboratories) were used. These transgenic mice express a mutant human FK506 binding protein 1A, 12kDa (FKBP12)-Fas inducible suicide/apoptotic system, driven by the mouse *Csf1r* promoter conjugated with a green fluorescent protein (GFP), which preferentially binds the B/B dimerizing agent (B/B-HmD, AP20187) (Diatech Labline s.r.l.). Treatment of mice with AP20187 induces the dimerization of the suicide protein to activate the cytoplasmic FKBP12-Fas fragments, leading to the apoptosis of transgene-expressing cells and consequent macrophage depletion (17).

At least 1 hour before behavioral experiments, mice were acclimatized to the experimental room and the evaluations were performed between 9:00 AM and 5:00 PM. Animals were anesthetized with a mixture of ketamine and xylazine (90 mg/kg and 3 mg/kg, respectively, i.p.) and euthanized with inhaled CO₂ plus 10–50% O₂.

Cancer cell inoculation

Naïve (CRL-6475; RRID:CVCL_0159 American Type Culture Collection, ATCC) and green fluorescent protein (GFP) expressing B16-F10 murine melanoma and Lewis lung carcinoma (LLC1, CRL-1642, RRID:CVCL_4358 ATCC) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (10000 U/100 μ g/mL) at 37 °C with 5% CO₂ in a humidified atmosphere and were used without further authentication. For inoculation, 20 μ l of B16-F10 melanoma (2×10^5 cells) or LLC1 (5×10^5 cells) cells were suspended in phosphate buffer saline (PBS) and injected into the plantar region of the mouse right hindpaw (15,18). Control groups (sham) were injected with 20 μ l of PBS containing B16-F10 melanoma (2×10^5 cells) or LLC1 (5×10^5 cells) cells killed by quickly freezing and thawing them twice without cryoprotection. B16-F10 and LLC1 cell lines are isogenic with the C57BL/6 mouse strain.

Treatment protocols and behavioral assays (mechanical allodynia, cold response and spontaneous nociception)

See Supplemental Materials and Methods for experimental details

Cell cultures

Human Schwann cells (HSC, #P10351, Innoprot) were cultured in Schwann cell medium (#P60123, Innoprot) according to the manufacturer's protocol. Human Schwann cells used in the study are primary cells. Cells were passaged at 90% confluency and discarded after 20 passages. Approval by the local Ethic committee and written informed consent were obtained by the vendor. All cells were used when received without further authentication. Mouse Schwann cells were isolated from sciatic nerves of C57BL/6J mice. Briefly, the *epineurium* was removed, and nerve explants were divided into 1 mm segments and dissociated enzymatically using collagenase (0.05%) and hyaluronidase (0.1%) in Hank's Balanced Salt Solution (HBSS, 2 hours, 37 °C). Cells were collected by centrifugation (800 rpm, 10 minutes, room temperature) and the pellet was resuspended and cultured in DMEM containing: 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin. Three days later, cytosine arabinoside (10 mM) was added to remove fibroblasts. All cells were cultured in an atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were used after 15 days of culture.

Sciatic nerve explant culture

See Supplemental Materials and Methods for experimental details

H₂O₂ assay

See Supplemental Materials and Methods for experimental details

ELISA assays (Multi-analyte, M-CSF, PDL-1)

See Supplemental Materials and Methods for experimental details

Immunofluorescence

See Supplemental Materials and Methods for experimental details

Real-Time PCR

See Supplemental Materials and Methods for experimental details

Statistical analysis

Results are shown as the mean and standard error of the mean (SEM). The statistical significance of differences between groups was assessed using Student's t-test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc where appropriate. Statistical analyses were performed on raw data using Prism 8 (GraphPad Software Inc.). P values less than 0.05 (P<0.05) were considered significant.

Results

Neural resident macrophages mediate cancer pain

Intraplantar (i.pl.) inoculation of B16-F10 melanoma cells into the hindpaw of C57BL/6J mice induced a time-dependent increase in paw thickness, mainly due to tumor growth (Figures 1A and 1B). Tumor progression was associated with a parallel increase in

mechanical/cold hypersensitivity as well as spontaneous nociception in the ipsilateral paw (Figures 1C–1E) and in the number of F4/80⁺ monocytes and MΦs within the tumor and the adjacent tissue (tMΦs) (Figure 1F) and inside the ipsilateral sciatic nerve trunk (rMΦs) (Figure 1G). Since no gender differences were observed in cancer growth, mechanical/cold hypersensitivity or spontaneous nociception, male mice were used in subsequent experiments (Figures 1B–1E). Control (sham) mice inoculated with dead cells did not develop an increase in paw volume, mechanical/cold hypersensitivity or spontaneous nociception (Figures 1B–1E).

To explore the role of MΦs in cancer-evoked mechanical/cold hypersensitivity and spontaneous nociception, cancer cells were inoculated in MΦ Fas-Induced Apoptosis (MaFIA) transgenic mice. The inoculation of cancer cells in MaFIA mouse hindpaw induced a time-dependent increase in paw thickness, mechanical/cold hypersensitivity, and spontaneous nociception, like those observed in C57BL/6J mice (Figures 1H–1K). After inoculation, MaFIA mice received daily injections (day 10–14) of AP20187 (2 mg/kg, intraperitoneal, i.p.) to induce apoptosis in the entire MΦ population. After AP20187-treatment, MaFIA mice exhibited a marked reduction in the number of GFP⁺/F4/80⁺ cells in both the tumor and sciatic nerve (Figure 1L) in mechanical/cold hypersensitivity and spontaneous nociception (Figures 1H–1K).

To determine whether tMΦs or rMΦs contribute to cancer-evoked mechanical/cold hypersensitivity and spontaneous nociception, MΦ-depleted tumor-bearing MaFIA mice were inoculated (day 14) in the hindpaw with F4/80⁺ cells harvested from the peritoneum of naïve C57BL/6J mice to reconstitute the tMΦ population. This intervention, while repopulating F4/80⁺ cells within the tumor microenvironment, failed to repopulate the number of F4/80⁺ cells within the sciatic nerve trunk (Figure 2A) and to restore mechanical/cold hypersensitivity and spontaneous nociception (Figures 2B–2D). In other experiments, tumor-bearing MaFIA mice received a local injection (60 µg, 6 µl, i.pl.) of AP20187 for 5 consecutive days (day 10–14). This local treatment depleted the tumor GFP⁺/F4/80⁺ (tMΦ) cells, but did not diminish the increased sciatic nerve GFP⁺/F4/80⁺ (rMΦ) cells (Figure 2E) mechanical/cold hypersensitivity and spontaneous nociception (Figures 2F–2H). Although several cellular and humoral factors of the nerve and tumor microenvironment may increase pain signals, our data implicate a major role for rMΦs in mechanical/cold hypersensitivity and spontaneous nociception evoked by cancer in mice.

In a model of neuropathic pain evoked by partial sciatic nerve ligation, we and others have shown that the MΦs responsible for the allodynia quickly accumulate inside the injured sciatic nerve trunk (4,6,19,20). This fast recruitment was eliminated by treatment with liposome-encapsulated clodronate (LCL), which rapidly depletes circulating monocytes (21) and attenuates the number of MΦs recruited at the site of the injury (6). However, in the present model of cancer pain, daily LCL (i.p., one day before and day 1–14 after cancer cell inoculation), while markedly reducing tumor F4/80⁺ cells, did not affect sciatic nerve F4/80⁺ cells, paw thickness, or mechanical/cold hypersensitivity (Figures S1A–S1C). These findings further support the role of the expanded sciatic nerve rMΦs, but not hematogenous MΦs which accumulate from the blood stream into the tumor microenvironment, to sustain cancer-evoked allodynia.

M-CSF promotes macrophage expansion and cancer-evoked allodynia

Next, we investigated the mechanisms underlying the rM Φ expansion responsible for cancer-evoked mechanical allodynia. We used a multiplex array to profile chemokines and cytokines in the tumor and sciatic nerve at day 14 after cancer cell inoculation in C57BL/6J mice. Except for IL-1 α , IL-6, IL-10, IL-12, transforming growth factor-1 β (TGF-1 β , and granulocyte-CSF (G-CSF), which were unchanged in tumor homogenates, the other mediators, including IL-1 β , IL-2, IL-4, IL-17A, interferon- γ (IFN- γ), TNF- α , CCL2, CCL3, CCL4, M-CSF, and GM-CSF, were increased both in tumor and sciatic nerve homogenates (Figure 3A). Mounting evidence suggests that melanoma cells express the checkpoint inhibitory protein PD-L1 (CD274), which suppresses T cell function and induces immune tolerance *via* its cognate receptor, PD-1 (22,23). We found that PD-L1 levels were increased in the tumor tissue but not in sciatic nerve at day 14 after melanoma cell inoculation in C57BL/6J mice (Figure 3B). Due to the negative result in the sciatic nerve and finding that PD-L1 has been shown to inhibit spontaneous pain and allodynia in melanoma-bearing mice (24), this was not further explored.

Among the number of cytokines/chemokines increased in the neuronal and tumor tissue, we first focused on CCL2 because of its prominent role in augmenting M Φ numbers at sites of nerve injury, thereby contributing to neuropathic pain (4,8). CCL2 blockade favors tumor escape from the primary sites in a mouse model of melanoma (25). However, the contribution of CCL2 in pain evoked by cancer has not been explored. In contrast to the prominent role in M Φ recruitment and allodynia after nerve injury (4–6), CCL2 does not seem to contribute to cancer pain, given that treatment with a neutralizing anti-CCL2 monoclonal antibody (mAb) did not affect tumor growth, mechanical/cold hypersensitivity (Figure S2A), or the number of either tM Φ s or rM Φ s (Figures S2B and S2C).

In addition to CCL2, M-CSF has been shown to mediate both M Φ increase and allodynia in some neuropathic pain models (7–9). In the present model of cancer-evoked pain, we found that M-CSF levels were markedly increased in both tumor and sciatic nerve homogenate at day 14 after B16-F10 melanoma cell inoculation (Figure 3C). Targeting the M-CSF signal with the M-CSF receptor (M-CSFR) antagonist, PLX3397, or a neutralizing anti-M-CSF mAb, which did not affect tumor growth (Figures 3D and 3J), markedly attenuated the number of both tM Φ s and rM Φ s (Figures 3H, 3I, 3N and 3O), and mechanical/cold hypersensitivity (Figures 3E, 3F, 3K and 3L). Accumulation of F4/80⁺ cells in the sciatic nerve was associated with a robust increase in oxidative stress (H₂O₂), which was reduced by PLX3397 (Figure 3G) or an anti-M-CSF mAb (Figure 3M). Thus, M-CSF mediates cancer-evoked rM Φ expansion and the ensuing allodynia.

M-CSF induces allodynia and neuroinflammation *via* TRPA1

In addition to M-CSF, the CSF family includes G-CSF and GM-CSF, which were found to regulate tumor-nerve interactions, remodeling of peripheral nerves, and sensitization of damage-sensory neurons in a mouse sarcoma model of bone tumor (26). G-CSF and GM-CSF levels were increased in sciatic nerve trunk homogenates at day 14 after cancer cell inoculation (Figure 3A). However, in contrast to M-CSF, neutralizing anti-G-CSF or anti-

GM-CSF mAbs did not affect cancer-evoked mechanical/cold hypersensitivity (Figures S2D and S2E), reinforcing the pivotal role of M-CSF in cancer-induced pain.

We next studied the mechanism by which M-CSF causes mechanical allodynia and neuroinflammation. Local injection of M-CSF (1–100 ng, 20 μ l, i.pl.) induced a dose-dependent mechanical allodynia lasting for 6 hours (Figure 4A). Pretreatment with an anti-M-CSF mAb prevented the development of mechanical allodynia and the increase in F4/80⁺ cells in the ipsilateral sciatic nerve trunk (Figures 4B and 4C). The involvement of M Φ s in the M-CSF-induced nociception was shown by using M Φ -depleted MaFIA mice, in which the M-CSF injection failed to induce mechanical allodynia (Figure 4D), whereas G-CSF and GM-CSF still elicited allodynia (Figures 4E and 4F). We previously reported that cancer-evoked allodynia induced by B16-F10 melanoma cell inoculation was absent in *Trpa1*^{-/-} mice (15). To investigate the role of TRPA1 in M-CSF-induced mechanical allodynia and neuroinflammation, M-CSF was injected in *Trpa1*^{+/+} and *Trpa1*^{-/-} mice. In *Trpa1*^{-/-} mice, M-CSF failed to induce mechanical allodynia and F4/80⁺ cells in the sciatic nerve (Figures 4G and 4H). In contrast, allodynia evoked by G-CSF and GM-CSF was unaffected by TRPA1 deletion (Figure 4I).

M-CSF from Schwann cells sustains allodynia

As monocytes are known to release M-CSF (27), we tested whether the M-CSF in the paw and sciatic nerve originated from M Φ s. M-CSF was measured in the tumor and sciatic nerve from tumor-bearing and M Φ -depleted MaFIA mice. AP12087 treatment, while reducing both tM Φ s and rM Φ s, left unchanged the M-CSF content in the ipsilateral sciatic nerve and tumor homogenates (Figure 5A). Schwann cells, which ensheath nerve fibers and represent 90% of the nucleated cells of nerve fibers are known to release M-CSF (28,29). We confirmed that both human and mouse cultured Schwann cells express M-CSF mRNA (Figure 5B). We then explored the mechanism of M-CSF release from Schwann cells. TRPA1 stimulation has been reported to promote oxidative stress (6,30) and oxidants may release members of the CSF family (31). Exposure of cultured human Schwann cells to the non-reactive (PF-4840154) or reactive (H₂O₂) agonists increased M-CSF levels in the cell supernatant, responses that were attenuated by the TRPA1 antagonist, A967079, and PF-4840154-evoked release of M-CSF was reduced by the antioxidant, phenyl-N-tert-butyl-nitron (PBN) (Figure 5C). This finding implicates an autocrine pathway that upon Schwann cell TRPA1 activation elicits M-CSF release mediated by ROS generation.

To further explore the contribution of Schwann cell and neuronal TRPA1 in M-CSF release in sciatic nerve, we used *Plp1-Cre*^{ERT+};*Trpa1*^{fl/fl} and *Adv-Cre*⁺;*Trpa1*^{fl/fl} mice, which harbor a selective deletion of TRPA1 in the Schwann cell/oligodendrocyte lineage and in primary sensory neurons, respectively. At day 14 after cancer cell inoculation, M-CSF levels in the ipsilateral sciatic nerve, but not in the tumor, of *Plp1-Cre*^{ERT+};*Trpa1*^{fl/fl} mice, were reduced, while in *Adv-Cre*⁺;*Trpa1*^{fl/fl} they were preserved in both the sciatic nerve and tumor, compared to control mice (Figures 5D and 5E). This finding suggests that Schwann cell TRPA1 is a major driver of neuronal M-CSF released in tumor-bearing mice.

To further support the role of Schwann cell TRPA1 in M-CSF release, the cytokine content was measured in the sciatic nerve explant derived from *Plp1-Cre*^{ERT+};*Trpa1*^{fl/fl} and *Adv-Cre*

⁺; *Trpa1^{fl/fl}* after exposure to H₂O₂. Upon stimulation, the increase in M-CSF content in nerve explant from control mice was markedly reduced in *Plp1-Cre^{ERT+};Trpa1^{fl/fl}*, but unaffected in *Adv-Cre⁺;Trpa1^{fl/fl}*, thus supporting the role of TRPA1 Schwann cell in M-CSF release (Figure 5F). In addition, in the sciatic nerve explant culture from naïve MaFIA mice, the exposure to H₂O₂ increased the number of GFP⁺ cells (Figure 5G), an effect that was attenuated by A967079, PBN and PLX3397 (Figure 5G).

Schwann cell TRPA1 mediates neuroinflammation, which sustains cancer allodynia

TRPA1 is implicated in cancer pain (15). However, the underlying mechanism by which TRPA1 sustains chronic cancer allodynia is unknown. Our results show that cancer pain entails rMΦ expansion in the sciatic nerve and the release of M-CSF, which elicits TRPA1-dependent mechanical allodynia and neuroinflammation. We investigated whether TRPA1 promotes the cancer-evoked neuroinflammation that causes chronic allodynia. B16-F10 melanoma cell inoculation in *Trpa1^{-/-}* mice did not induce cancer-evoked mechanical allodynia (Figure 6A). The increased number of rMΦs in *Trpa1^{+/+}* mice was markedly reduced in *Trpa1^{-/-}* mice (Figure 6B), while the number of tMΦs remained equally elevated in both mouse strains (Figure S3A). In addition, we found that the high levels of the oxidative stress marker, H₂O₂, detected in ipsilateral sciatic nerve homogenates of *Trpa1^{+/+}* mice, were absent in *Trpa1^{-/-}* mice (Figure 6C). In partial contrast with this observation, a single injection (i.p. day 14 after inoculation) of the TRPA1 antagonist A967079, or the antioxidant PBN, transiently and completely reversed mechanical allodynia and the increase in H₂O₂ levels (Figures 6D, 6G, 6F and 6I), but failed to affect the number of rMΦs (Figures 6E and 6H) and tMΦs (Figures S3B and S3C). Accordingly, a single injection (i.p. 1 h post M-CSF) of A967079 and PBN transiently reversed mechanical allodynia (Figure 6J) without decreasing the number of rMΦs (Figure 6K). This observation indicates that a short-term inhibition of the TRPA1 channel or oxidative stress, while efficiently abating allodynia, is not sufficient to attenuate the cellular neuroinflammatory response.

We then investigated the role of Schwann cell TRPA1 in orchestrating mechanical/cold hypersensitivity, spontaneous nociception, and neuroinflammation. After B16-F10 melanoma cell inoculation, *Plp1-Cre^{ERT+};Trpa1^{fl/fl}* mice showed a reduction in mechanical/cold hypersensitivity and spontaneous nociception, as well as in the number of rMΦs (Figures 6L–6N) and H₂O₂ levels in the ipsilateral sciatic nerve compared to control mice (Figure 6O). However, the number of tMΦs was unchanged (Figure S3D). In addition, intraplantar injection of B16-F10 melanoma cells significantly increased the expression of ATF3 mRNA (Figure S3E), a marker of nerve injury (32) in DRG neurons ipsilateral to the injected paw, thus suggesting that melanoma can activate an injury response similar to that evoked by axotomy (33). ATF3 mRNA expression in DRGs from *Plp1-Cre^{ERT+};Trpa1^{fl/fl}* was markedly reduced compared to control mice (Figure S3E), further strengthening the contribution of Schwann cell TRPA1 in cancer-evoked neuroinflammation and hypersensitivity.

To investigate the contribution of neuronal TRPA1, we studied *Adv-Cre⁺;Trpa1^{fl/fl}*. In these mice, mechanical/cold hypersensitivity and spontaneous nociception were reduced (Figures S3F and S3G), the number of rMΦs (Figure S3H), tMΦs (Figure S3I) and the H₂O₂ levels in

the sciatic nerve (Figure S3J) were unaffected. Thus, in contrast to the Schwann cell TRPA1, the channel expressed by nociceptors signals pain, but does not contribute to neuroinflammation.

rMΦs and Schwann cell TRPA1 mediates allodynia in a second cancer pain model

To obtain further evidence on the role of rMΦs and the Schwann cell TRPA1 channel in cancer-related mechanical allodynia, we used a soft tissue tumor/metastasis model induced by intraplantar inoculation of LLC1 cells, which mimics behavioral and functional changes similar to a metastatic bone cancer pain model (34). Inoculation of these cells, but not killed cells, elicited a progressive and rapid cancer growth that was associated to temporarily similar increases in mechanical allodynia (Figures S4A and S4B) and in the number of tMΦs of rMΦs (Figures S4C and S4D) in C57BL/6J mice. No sex differences in cancer growth and mechanical hypersensitivity were observed (Figures S4A and S4B). Thus, also in this case, male mice were used in subsequent experiments. The MΦ role was confirmed by using MaFIA mice, which, after intraplantar inoculation of LCC1 cells, developed a time-dependent increase in paw thickness and mechanical allodynia similar to that observed in C57BL/6J mice (Figures S4E and S4F). However, MaFIA mice receiving daily injections (day 4–8) of AP20187 exhibited a normal cancer growth (Figure S4E) but a markedly reduced mechanical allodynia (Figure S4F) and GFP⁺/F4/80⁺ cells in both the sciatic nerve and tumor (Figures S4G and S4H). Robust evidence of the key role of TRPA1 derived from the observation that *Trpa1*^{-/-} mice inoculated with LLC1 cells showed normal tumor growth (Figure S4I) and increased number of tMΦs (Figure S4L) comparable to *Trpa1*^{+/+} mice. However, in *Trpa1*^{-/-} mice, mechanical allodynia (Figure S4J) and rMΦ expansion (Figure S4K) were remarkably reduced. Similar results were obtained with *Plp1-Cre*^{ERT+}/*Trpa1*^{fl/fl} mice, in which mechanical allodynia (Figure S4N) and increase in rMΦ number (Figure S4O), but not increase in paw thickness (Figure S4M) and tMΦ number (Figure S4P), were attenuated as compared to control. Reported data strengthen the hypothesis that rMΦs and Schwann cell TRPA1 are a common mechanism to sustain neuroinflammation and pain in different types of mouse cancer.

The site where resident macrophages mediate allodynia

The importance of dorsal root ganglia (DRG) MΦs in neuropathic pain has been reported previously (11). Thus, the presence of MΦs in DRGs was explored in our model. By quantifying F4/80⁺ cells, we observed an increase in MΦ number in ipsilateral lumbar (L4–L6) DRGs from MaFIA mice 14 days after melanoma cell inoculation, which, after treatment with systemic AP20187, was slightly, although significantly, decreased by about 30% (Figures S5).

Experiments with MΦ-depleted MaFIA mice highlighted the key role of rMΦs to sustain mechanical/cold hypersensitivity and spontaneous nociception (Figure 2). However, the precise anatomical site where rMΦs mediate allodynia remains unknown. To address this issue, we used MΦ-depleted tumor-bearing MaFIA mice. Mice received AP20187 for 5 days (daily, day 10–14) by perineural injection at three adjacent sites (each at a ~2 mm distance) of the ipsilateral sciatic nerve trunk (from ~10 to ~14 mm from the paw surface) (Figure 7A). Perineural treatment elicited a substantial reduction in mechanical/cold hypersensitivity

and spontaneous nociception (Figure 7B, 7D and 7E) and a marked decrease in the number of GFP⁺ cells in the portion corresponding to the sciatic nerve segment that had been treated with AP20187 (Figure 7C). The number of GFP⁺ cells in both the distal and proximal portion to that treated with AP20187 was similar in MaFIA mice that received either the dimerizing agent or its vehicle (Figure 7C). The number of GFP⁺ cells in the paw (tMΦs) was similar in mice treated with AP20187 or vehicle (Figure 7F). These data indicate that depletion of rMΦs in a limited portion of the ipsilateral sciatic nerve is necessary and sufficient to interrupt the signaling pathway that entails MΦs and Schwann cell interaction to mediate mechanical/cold hypersensitivity and spontaneous nociception.

Discussion

Although pain is a debilitating symptom of cancer and a major medical condition that warrants a better understanding of its underlying mechanism (2), little is known about the role of MΦs in cancer pain. Herein, we reveal the key role of MΦs and, in particular, of rMΦs, in sustaining mechanical/cold hypersensitivity and spontaneous nociception in mouse models of cancer pain. A series of findings support this conclusion. Melanoma B16-F10 cell growth in the mouse hindpaw was associated with a time-dependent and parallel increase in mechanical/cold hypersensitivity and spontaneous nociception and in the number of tMΦs and rMΦs. Depletion of the two MΦ populations by the homodimerizer agent, AP20187, in MaFIA mice abolished the mechanical/cold hypersensitivity and spontaneous nociception. In neuropathic pain models, including partial nerve injury (6,35), constriction of the infraorbital nerve (5), and diabetic neuropathy (36), clodronate, which depletes circulating monocytes, simultaneously removed MΦs and abated mechanical allodynia. In contrast, in the present cancer pain model, clodronate markedly reduced tMΦs, but affected neither rMΦ expansion nor mechanical/cold hypersensitivity. Additional evidence that cancer-evoked mechanical/cold hypersensitivity and spontaneous nociception is dependent on rMΦs was obtained in MΦ-depleted MaFIA mice, whose tumor microenvironment, but not the nerve bundle, was replenished with peritoneal MΦs from donor mice. In these mice mechanical/cold hypersensitivity and spontaneous nociception were not restored. Conversely, mechanical/cold hypersensitivity and spontaneous nociception were unaffected by local AP20187 treatment in the hindpaw, which depleted tMΦs, but not rMΦs. Thus, mechanical/cold hypersensitivity and spontaneous nociception are elicited by the expansion of rMΦs, whereas tMΦs, which are recruited from the blood circulation, are not relevant. In this respect, the present cancer pain model shows some similarity with certain models of neuropathic pain, such as those produced by the spared nerve injury and the spinal nerve transection, where rMΦs have been identified as the proinflammatory cellular component responsible for allodynia (11,37). The observation that melanoma cell inoculation increased the staining for the nerve injury biomarker, ATF3 (38), in DRG neurons ipsilateral to the tumor supports the presence of a neuropathic component orchestrated by Schwann cell TRPA1 in the present cancer pain model. Further support is given by the observation of increased MΦs in DRGs, a finding also reported in neuropathic pain models (11). However, as treatment with AP20187 abated mechanical/cold hypersensitivity and spontaneous nociception and only partially reduced DRG MΦs, the role of such a subpopulation in promoting mechanical/cold hypersensitivity and spontaneous nociception appears limited in

cancer. Elimination of mechanical allodynia in MΦ-depleted MaFIA mice with LLC1 supports the hypothesis that the role of MΦs in cancer pain is not unique to the B16-F10 melanoma model, but is essential in various types of murine tumors.

The analysis of a proinflammatory cytokine panel in melanoma homogenates showed a pronounced increase in CCL2 and M-CSF levels, whereas a more diffuse augmentation of most cytokines, including CCL2 and M-CSF, was observed in sciatic nerve homogenates. While an anti-CCL2 mAb did not affect mechanical/cold hypersensitivity, both a M-CSFR antagonist and an anti-M-CSF mAb robustly attenuated mechanical/cold hypersensitivity. Targeting the M-CSF signaling not only reduced mechanical/cold hypersensitivity, but also inhibited neuroinflammation, as after immunological or pharmacological blockade of the M-CSF signal both MΦ expansion and increased oxidative stress (H₂O₂ levels) in the sciatic nerve homogenates were attenuated. M-CSF has been shown to induce mechanical allodynia after intrathecal administration (7). We showed that intraplantar M-CSF elicits mechanical allodynia in mice. Although the entire family of CSF chemokines exhibits a similar proalgesic activity (39–41), only M-CSF, and not G-CSF or MG-CSF, produced MΦ-dependent allodynia. Together, these findings support the role of M-CSF in cancer pain and indicate another common feature between the present cancer pain model and certain (8,9,11), but not other (4–6), neuropathic pain models where MΦ chemoattraction was driven by CCL2.

We recently reported a major role for TRPA1 in mechanical/cold hypersensitivity and spontaneous nociception induced by melanoma cell inoculation in mice (15). Thus, we hypothesized that TRPA1 is implicated in the rMΦs/M-CSF-mediated pro-allodynic pathway. After confirming that *Trpa1*^{-/-} mice did not develop mechanical hypersensitivity after B16-F10 inoculation, we found that TRPA1 deletion abolished allodynia evoked by (intraplantar) M-CSF, but not by G-CSF or MG-CSF. These results reveal a cause-effect relationship between M-CSF and TRPA1 in pain. Therefore, we investigated how M-CSF elicits TRPA1-dependent allodynia. The inability of M-CSF to evoke allodynia in *Trpa1*^{-/-} mice was associated with failure to expand rMΦs and to increase H₂O₂ levels in the sciatic nerve. Thus, elimination of TRPA1 blunted the neuroinflammatory response evoked by M-CSF. The observation that rMΦ expansion was attenuated by TRPA1 deletion implies that channel activation is required to increase rMΦ number. This finding was unexpected, as MΦs express M-CSFR (42), and therefore, in principle, M-CSF might increase their number by direct activation of its cognate receptor on rMΦs, without the involvement of TRPA1. This apparently contradictory finding was addressed by using multiple tools, including MaFIA mice, cultured human and mouse Schwann cells, and an *ex vivo* sciatic nerve mouse explant culture.

Cancer cells (43), monocytes (27), DRG neurons (11), and Schwann cells (44) can all express and release M-CSF. The increased M-CSF levels evoked by cancer growth were not reduced in either the mouse hindpaw or sciatic nerve in MΦ-depleted MaFIA mice, indicating that MΦs are not involved in cancer-associated secretion of the chemokine. We corroborated the previous findings (44), showing that M-CSF is expressed by either mouse or human Schwann cells in culture. We also showed that the TRPA1 agonist H₂O₂, which is increased in the sciatic nerve of mice with cancer, elicited a TRPA1-dependent release of M-

CSF from cultured Schwann cells. Furthermore, in an *ex vivo* sciatic nerve explant culture, rMΦ expansion elicited by H₂O₂ was attenuated by a M-CSFR antagonist, a TRPA1 antagonist and an antioxidant. As H₂O₂ increases MΦs at sites of injury (6,45), the inhibitory action of the antioxidant on rMΦ expansion was expected. However, the ability of M-CSFR and TRPA1 antagonists to similarly attenuate rMΦ expansion and M-CSF release from Schwann cells suggests a feed-forward mechanism, which entails the following steps: oxidative stress from cancer tissue targets Schwann cell TRPA1 to release M-CSF which elicits rMΦ expansion within the sciatic nerve trunk. Expanded rMΦs, *via* their own oxidative stress, perpetuate this pathway, which sustains allodynia and spontaneous pain (Figure 7G).

Direct assay of M-CSF in the hindpaw and sciatic nerve provides support to this hypothesis. In mice with a Cre-mediated deletion of *Trpa1* in the Schwann cell/oligodendrocyte lineage (*Plp1-Cre^{ERT+}/Trpa1^{fl/fl}*), cancer-evoked increase in tumor M-CSF was unchanged, whereas a marked reduction in the M-CSF levels was observed in the ipsilateral sciatic nerve. Importantly, M-CSF release elicited by H₂O₂ stimulation was markedly attenuated in the sciatic nerve explant from *Plp1-Cre^{ERT+}/Trpa1^{fl/fl}* mice. Thus, while M-CSF of the tumor microenvironment does not contribute to the proalgesic rMΦ expansion, the Schwann cell-mediated M-CSF is essential for the cancer-evoked allodynia.

The proalgesic pathways that include glial cell activation, inflammatory cell expansion, and increased proinflammatory mediators in the central and peripheral nervous systems, collectively referred to as neuroinflammation, have been extensively characterized as a major underlying mechanism of neuropathic pain associated with a variety of neuropathological conditions (4–6,8,11,20). Although several cell types and proalgesic mediators have been proposed, the neuroinflammatory pathways that drive cancer pain remain uncertain (46,47). Here, we identified the key role of M-CSF, released following Schwann cell TRPA1 activation, in sustaining cancer-evoked rMΦ expansion and the ensuing mechanical/cold hypersensitivity and spontaneous nociception. Supporting evidence derives from the observation that deletion of TRPA1 in Schwann cell/oligodendrocyte lineage attenuates neuroinflammation and mechanical/cold hypersensitivity and spontaneous nociception in the melanoma mouse model of cancer pain. In contrast, deletion of TRPA1 in primary sensory neurons attenuates mechanical allodynia but not neuroinflammation. Thus, we hypothesize that, although neuronal TRPA1 is the final target of the proalgesic signaling pathway, the feed-forward mechanism that encompasses M-CSF, rMΦs oxidative stress, and Schwann cell/TRPA1 is needed to chronically sustain mechanical/cold hypersensitivity and spontaneous nociception (Figure 7G).

A limitation to the translational value of the cancer model used in the present study is the relatively low incidence of pain reported in patients with early stage melanoma (48). However, the rapid cancer growth and prominent inflammatory response of our model may recapitulate the pain reported in metastatic melanoma, where >50% of patients require palliative care and morphine treatment (49–52). The most relevant findings were replicated in another model of cancer pain by using LLC1 cells inoculated in the mouse paw. However, the fact that lung carcinomas usually metastasize to bones (53) and not to skin should be considered an additional limitation of the present study. The essential role of rMΦs and

Schwann cell TRPA1 in the murine LLC1 model is indicated by the observation that mechanical allodynia and expansion in rMΦs were attenuated in both *Trpa1*^{-/-} and *Plp1-Cre^{ERT+}/Trpa1^{fl/fl}* mice. The selective elimination of rMΦs in mice with a Cre-mediated Schwann cell deletion of *Trpa1*, associated with eradication of allodynia, supports the crucial role of the Schwann cell channel in orchestrating neuroinflammation and pain not only in melanoma, but also in LLC1 models. Although we identified the macrophage subpopulation implicated in mechanical/cold hypersensitivity and spontaneous nociception, we cannot exclude the contribution of other local autocrine and paracrine factors, which may act upstream or downstream of macrophage expansion to sustain cancer pain.

Two additional findings of our study are difficult to interpret. The first relates to the different effects produced by TRPA1 pharmacological antagonism *vs.* those elicited by TRPA1 genetic deletion. While *Trpa1*^{-/-} mice showed a marked attenuation of both mechanical allodynia and neuroinflammation, systemic exposure to a TRPA1 antagonist efficiently abrogated allodynia, but did not affect rMΦ expansion. To explain this apparent contradiction, the different time courses of these interventions should be considered. In fact, *Trpa1*^{-/-} mice harbor a permanent channel deletion, whereas the short half-life of the TRPA1 antagonist, A967079, provides brief channel inhibition. Thus, the transient (a few hours) blockade of the neuronal channel is sufficient to briefly reverse allodynia, but cannot provide the prolonged inhibition (some days) of the Schwann cell TRPA1, which is necessary to attenuate the rMΦ expansion and the ensuing allodynia. This interpretation is further supported by the observation that treatment of MaFIA mice with AP20187, which depletes MΦs for several days, elicits a prolonged attenuation of mechanical/cold hypersensitivity and spontaneous nociception, probably because the rMΦ/Schwann cell TRPA1 feed-forward mechanism is switched off for a prolonged period of time.

The second unsettled finding relates to the anatomical site of action where expanded rMΦs sustain allodynia. In models of MΦ-dependent neuropathic pain, MΦ depletion at the site of nerve injury efficiently attenuated allodynia (5,6,37). However, in other models, a series of direct and indirect evidence showed that selective elimination of rMΦs at the site of the damaged nerve did not affect allodynia, which suggested that rMΦ expansion in the DRG was required (11,35). In the present model of cancer pain, we asked which was the anatomical site where the M-CSF/rMΦ/Schwann cell TRPA1 pathway must operate to sustain allodynia. Because of the technical complications inherent to the elimination of DRG rMΦs, we chose to examine this issue by depleting rMΦs in an anatomically well-identified segment of the sciatic nerve. The observation that a selective rMΦ depletion in a ~4 mm portion of the sciatic nerve trunk abrogated mechanical/cold hypersensitivity and spontaneous nociception proposes a spatial constraint in the neuroinflammatory mechanism that sustains mechanical/cold hypersensitivity and spontaneous nociception. As rMΦ expansion was unaltered in the proximal and distal untreated segments, the amplification loop consisting of rMΦs and Schwann cell TRPA1 that communicates *via* M-CSF and oxidative stress must function by contiguity throughout the entire sciatic nerve to warrant that the mechanical/cold stimulus applied to the mouse paw is conveyed centrally as an allodynic signal.

The soma of DRG neurons does not participate in the central conduction of action potentials (54). Instead, sensory impulses from peripheral terminals continue directly into the spinal cord and do not depolarize the soma (55). However, the present findings do not exclude the possibility that DRG cells, with the cooperation of surrounding satellite cells and local expanded rMΦs, are implicated in magnifying the sensory impulse that conveys mechanical/cold hypersensitivity and spontaneous nociception. Nevertheless, the present discovery of the role of the neuroinflammatory and proalgesic pathway that entails M-CSF, rMΦs, oxidative stress and Schwann cell TRPA1 in two different types of murine cancer offers novel targets for the identification of better and safer treatments for cancer pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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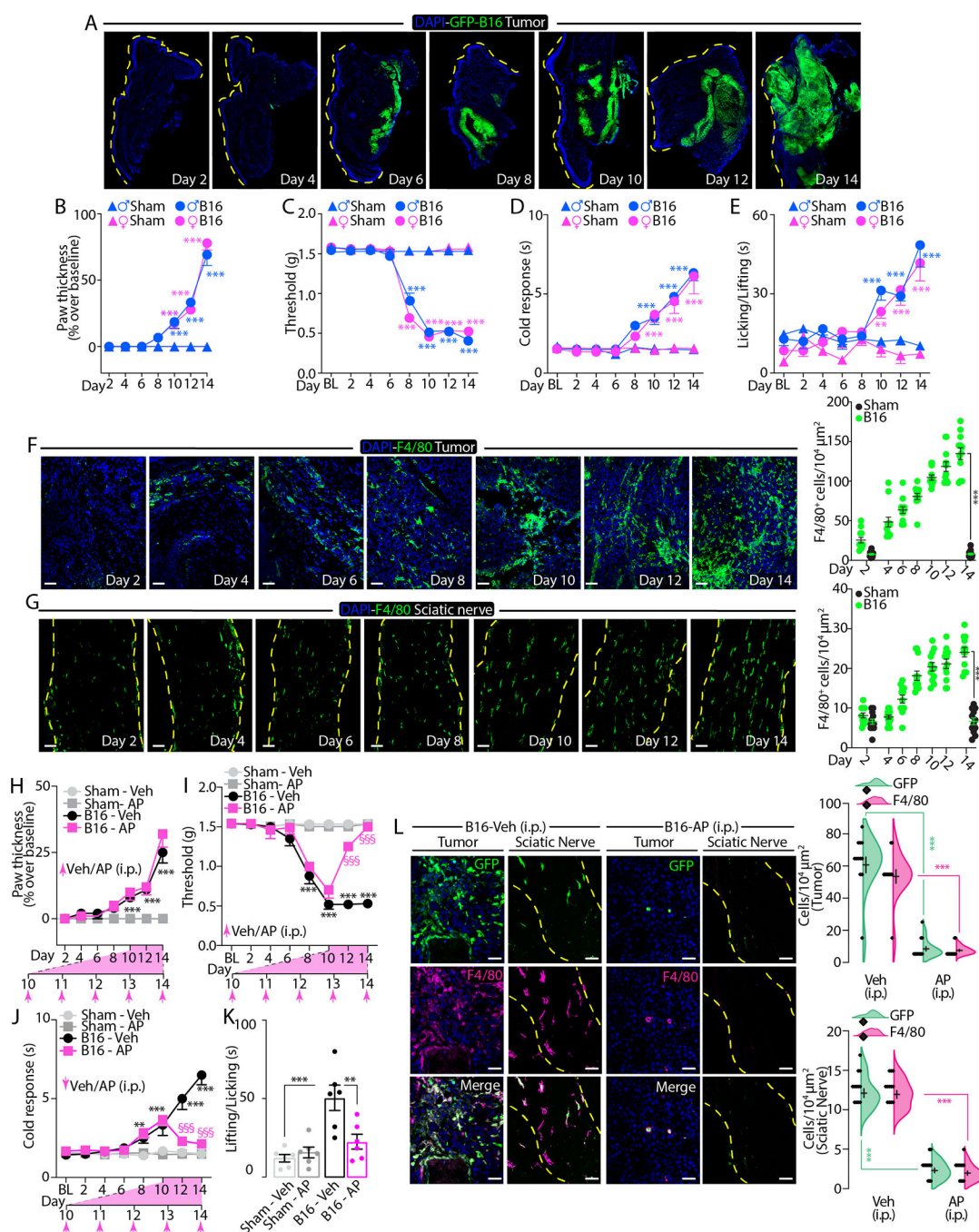
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Significance

Schwann cell TRPA1 sustains cancer pain through release of M-CSF and oxidative stress, which promote the expansion and the proalgesic actions of intraneural macrophages.



nerve (L) after B16 cell inoculation or sham in MaFIA mice treated with AP12087 (AP) or Veh (i.p.). BL, baseline. Pink arrows: time of treatment with Veh/AP. Yellow dashed line delimits hindpaw epidermis (A) and *epineurium* (G, L). Scale bar 50 μm . N=6 mice. *** $P < 0.001$ to Sham, Sham-Veh, GFP Veh, F4/80 Veh; §§§ $P < 0.001$ to B16-Veh. Data are presented as mean $\pm$ SEM, data points overlaid (F, G, K, L). Two-way (B-J) and one-way (K, L) ANOVA and Bonferroni post hoc test.

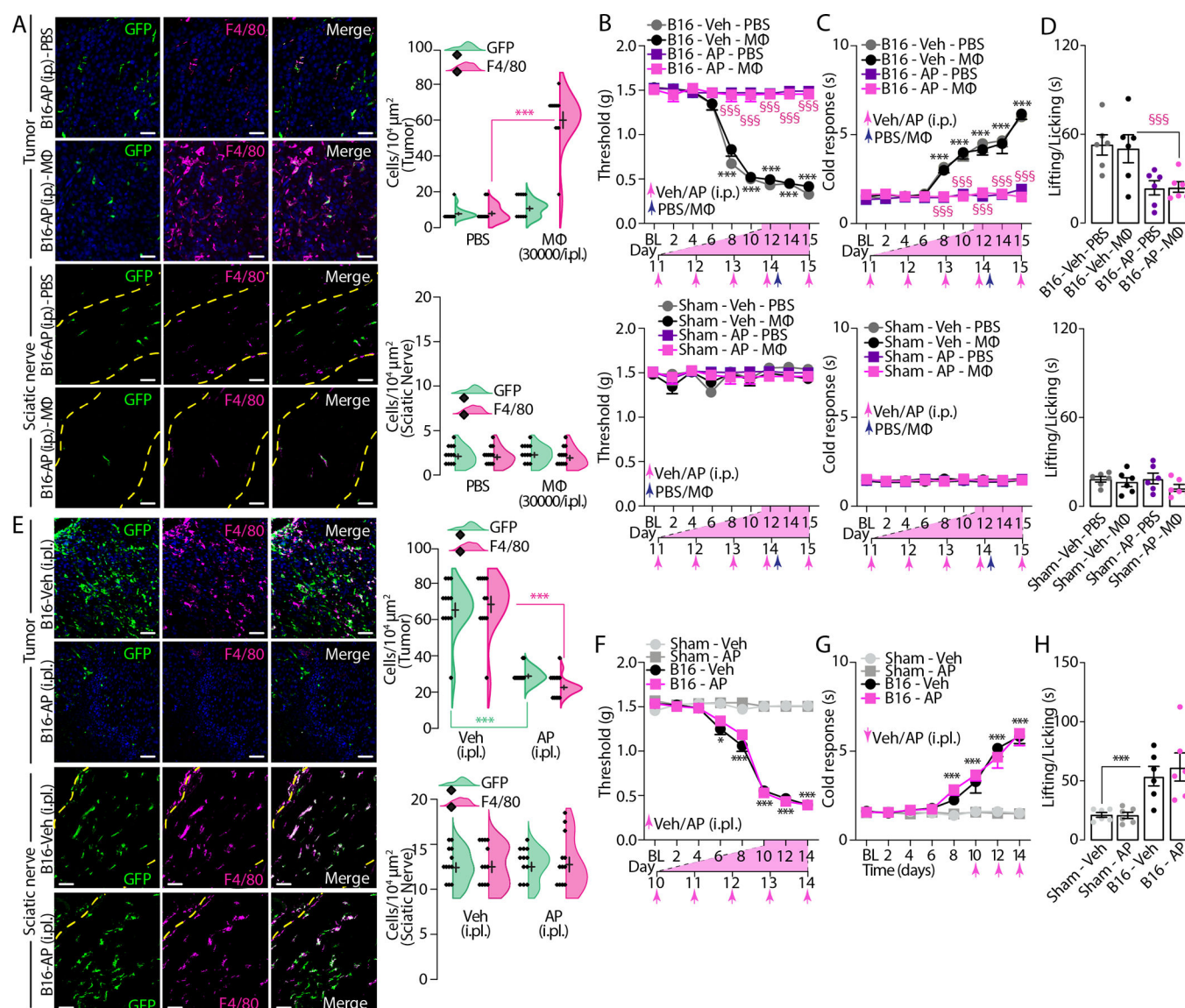


Figure 2. Resident macrophages are responsible for cancer-evoked mechanical allodynia, cold response and spontaneous nociception.

(A) Typical images and data of GFP⁺ and F4/80⁺ cells in the tumor and ipsilateral sciatic nerve and (B) mechanical allodynia, (C) cold response and (D) spontaneous nociception after B16-F10 melanoma (B16) cell inoculation or sham in MaFIA mice, treated with AP12087 (AP) or Veh (i.p.) and inoculated (i.p., at day 14) with MΦs or PBS. (E) Typical images and data of GFP⁺ and F4/80⁺ cells in the tumor and sciatic nerve and (F) mechanical allodynia, (G) cold response and (H) spontaneous nociception after B16 cell inoculation or sham in MaFIA mice treated with AP or Veh (i.p.). BL, baseline. Pink arrows: days of treatment; blue arrow: day of MΦ/PBS inoculation. Yellow dashed line delimits the epineurium. Scale bar 50 μm. N=6 mice. *P < 0.05, ***P < 0.001 to F4/80 PBS, F4/80 Veh, GFP Veh, Sham-Veh; §§§P < 0.001 to B16-Veh-MΦ. Data are presented as mean ± SEM, data points overlaid (A, E, D, H). Two-way (B, C, F, G) and one-way (A, D, E, H) ANOVA and Bonferroni post hoc test.

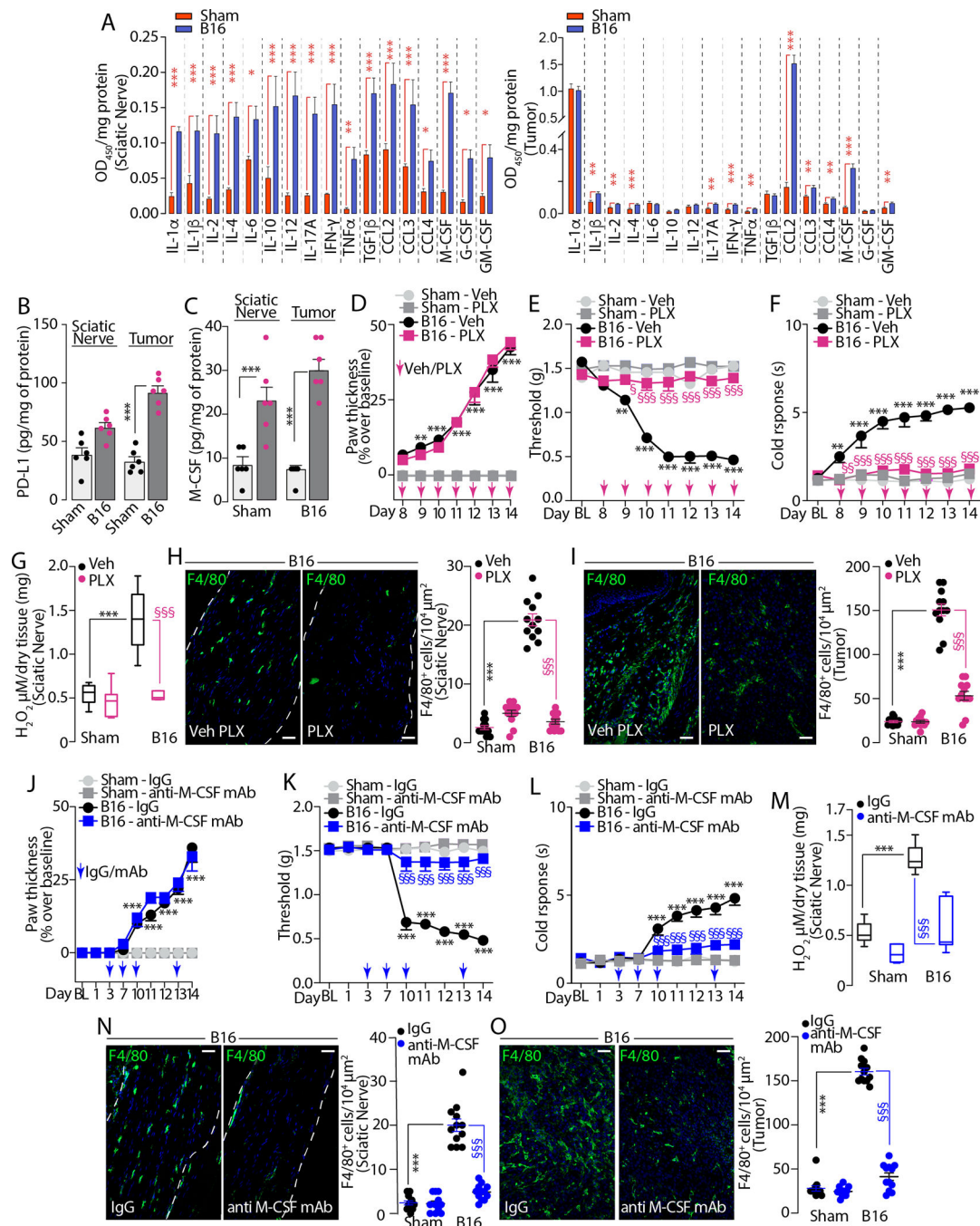


Figure 3. M-CSF promotes macrophage expansion and cancer-evoked mechanical allodynia and cold response.

Cytokines/chemokines array profile (A), PD-L1 (B) and M-CSF (C) content in sciatic nerve and tumor after B16-F10 melanoma (B16) cell inoculation or sham in C57BL/6J mice. Paw thickness (D), mechanical allodynia (E), cold response (F), H₂O₂ content (G), and typical images and data of F4/80⁺ cells in sciatic nerve (H) and tumor (I) after B16 cell inoculation or sham in C57BL/6J mice after PLX3397 (PLX) or Veh (i.p.). Paw thickness (J), mechanical allodynia (K), cold response (L), H₂O₂ content (M) and typical images and data of F4/80⁺ cell in sciatic nerve (N) and tumor (O) after B16 cell inoculation or sham in

C57BL/6J mice treated with an anti-M-CSF mAb or IgG2B (IgG) (i.p.). Assays were performed at day 14 from B16 cell inoculation or sham. BL, baseline. Pink arrows: treatment with PLX/Veh; blue arrow: treatment with anti-M-CSF mAb/IgG. Dashed line delimits the *epineurium*. Scale bar 50 μ m. N=6 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ to Sham, Sham-Veh, Sham-IgG; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ to B16-Veh and B16-IgG. Data are presented as mean $\pm$ SEM, data points overlaid (B, C, H, I, N, O). (G, M) Box plots 25th and 75th percentile, min and max values and data points overlaid. Student's t-test (A, B, C); Two-way (D, E, F, J, K, L) and one-way (G, H, I, M, N, O) ANOVA and Bonferroni post hoc test.

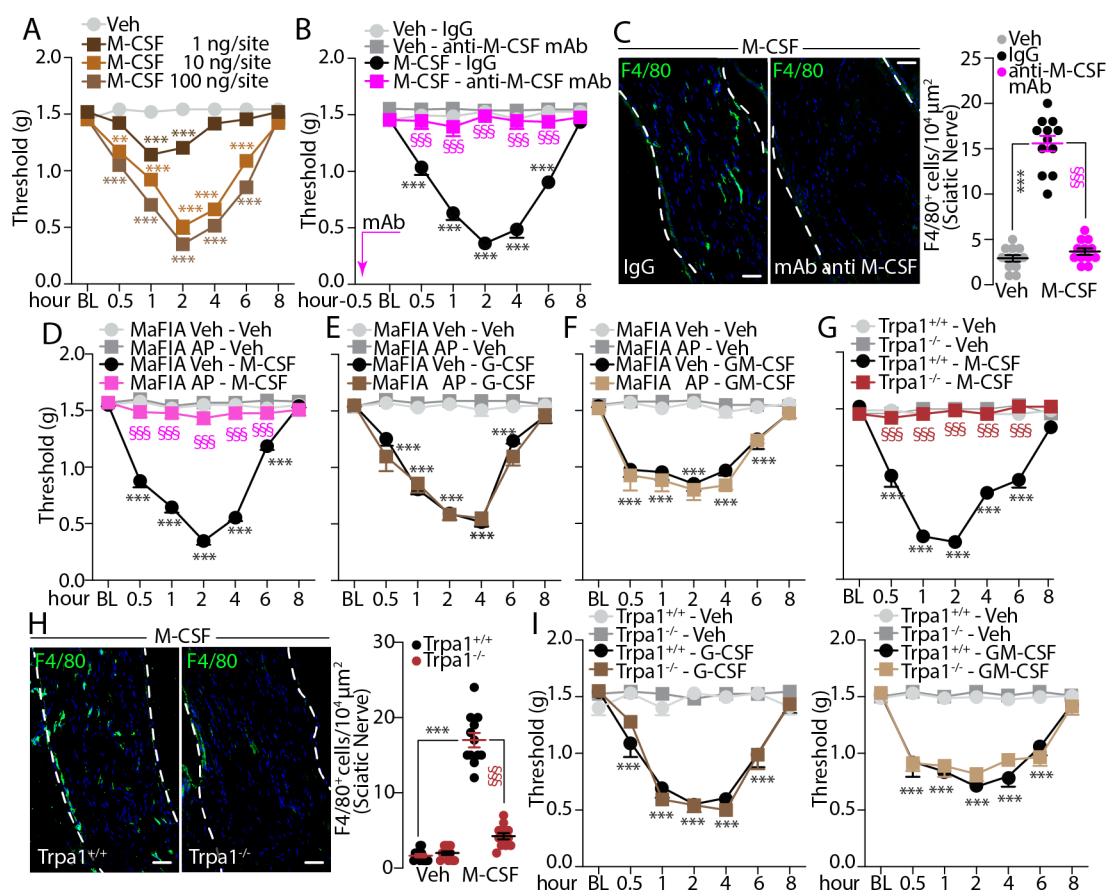


Figure 4. M-CSF induces allodynia and neuroinflammation via TRPA1.

(A) Mechanical allodynia in C57BL/6J mice after M-CSF or Veh (i.p.) and (B) pretreated with an anti-M-CSF mAb or IgG2B (IgG) (i.p.). (C) Typical images and data of F4/80⁺ cell in sciatic nerve of C57BL/6J mice after M-CSF (i.p.) and pretreated with an anti-M-CSF mAb or IgG (i.p.). Mechanical allodynia induced by M-CSF (D), G-CSF (E) and GM-CSF (F) or Veh (i.p.) in MaFIA mice treated with AP12087 (AP) or Veh (i.p.). (G) Mechanical allodynia, (H) typical images and data of F4/80⁺ cell in sciatic nerve after M-CSF or Veh (i.p.) in *Trpa1*^{+/+} and *Trpa1*^{-/-} mice. (I) Mechanical allodynia after G-CSF, GM-CSF or Veh (i.p.) in *Trpa1*^{+/+} and *Trpa1*^{-/-} mice. BL, baseline. Dashed line delimits the *epineurium*. Scale bar 50 μ m. N=6 mice. ** P < 0.01, *** P < 0.001 to Veh, Veh-IgG, MaFIA Veh-Veh, *Trpa1*^{+/+}-Veh; §§§ P < 0.001 to M-CSF-IgG, MaFIA Veh-M-CSF, *Trpa1*^{+/+}-M-CSF. Data are presented as mean $\pm$ SEM, data points overlaid (C, H). Two-way (A, B, D, E, F, G, I) and one-way (C, H) ANOVA and Bonferroni post hoc test.

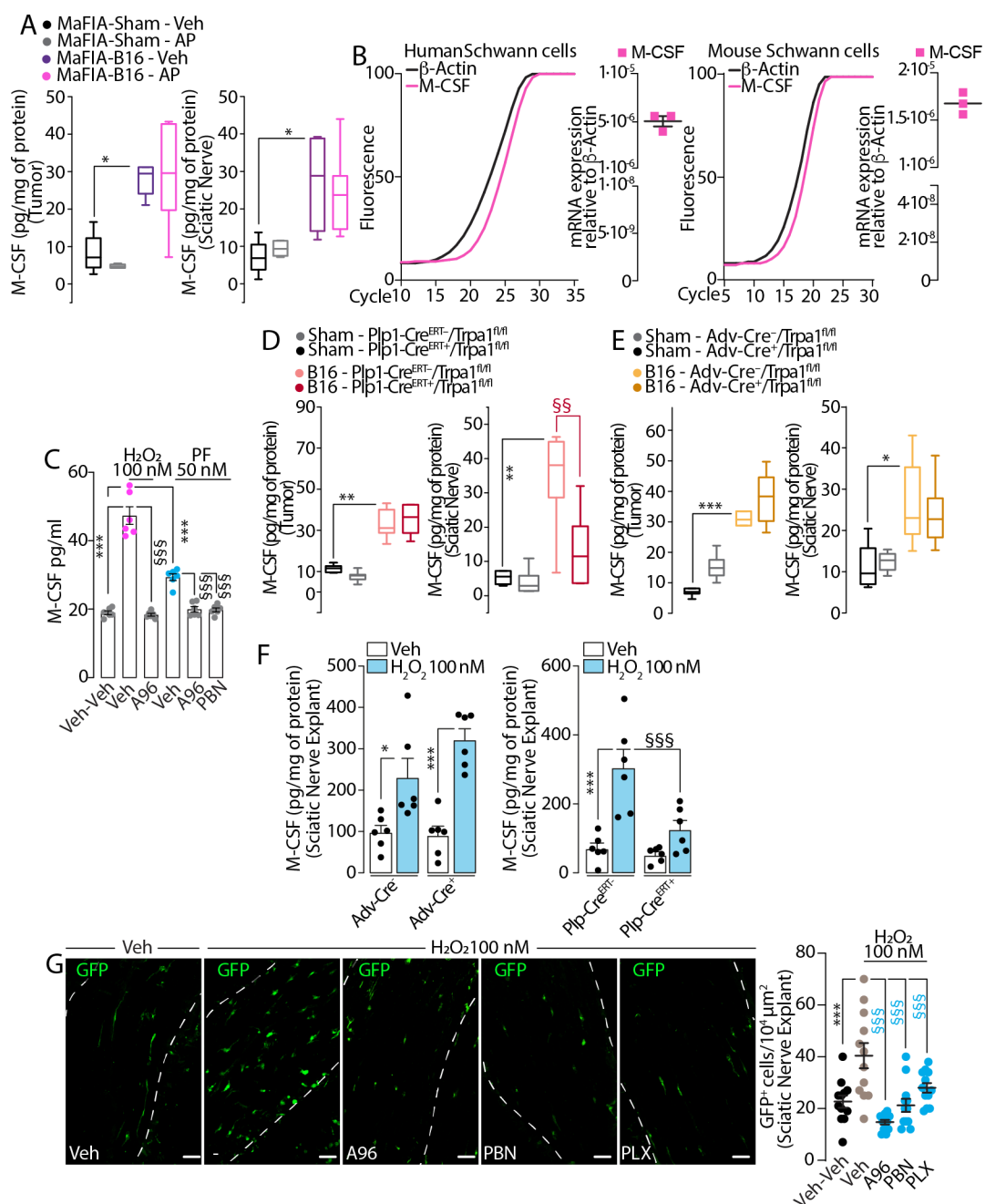


Figure 5. Schwann cell TRPA1 stimulation releases M-CSF.

(A) M-CSF content in tumor and sciatic nerve after B16-F10 melanoma (B16) cell inoculation or sham in MaFIA mice after AP12087 (AP) or Veh (i.p.). (B) M-CSF mRNA relative expression in human and mouse Schwann cells. (C) M-CSF content in human Schwann cells stimulated with H₂O₂, PF-4840154 (PF) or Veh in presence of A967079 (A96), PBN or Veh. M-CSF content in tumor and sciatic nerve at day 14 after B16 cell inoculation or sham in *Plp1-Cre^{ERT}/ERT⁺/Trpa1^{fl/fl}* (D) and *Adv-Cre⁺/Cre⁺/Trpa1^{fl/fl}* (E). (F) M-CSF content in sciatic nerve explant from *Plp1-Cre^{ERT}/ERT⁺/Trpa1^{fl/fl}* and *Adv-Cre⁺/Cre⁺/Trpa1^{fl/fl}* mice after H₂O₂ or Veh exposure. (G) Typical images and data of GFP⁺ cells in

sciatic nerve explant from MaFIA mice after H₂O₂ or Veh exposure, in the presence of A96, PBN, PLX or Veh. Dashed line delimits the *epineurium*. Scale bar 50 μ m. N=6 mice (A, D, E), N=3 experiments (B, C, F, G). * P < 0.05, ** P < 0.01, *** P < 0.001 to MaFIA-Sham-Veh, Veh-Veh, Sham-*Plp1-Cre^{ERT}/Trpa1^{fl/fl}*, Sham-*Adv-Cre⁻/Trpa1^{fl/fl}*; §§ P < 0.01, §§§ P < 0.001 to Veh H₂O₂ and B16-*Plp1-Cre^{ERT}/Trpa1^{fl/fl}*. (A, D, E) Box plots 25th and 75th percentile, min and max values and data points overlaid. Data are presented as mean $\pm$ SEM, data points overlaid (B, C, F, G). (A, C-G) One-way ANOVA and Bonferroni post hoc test.

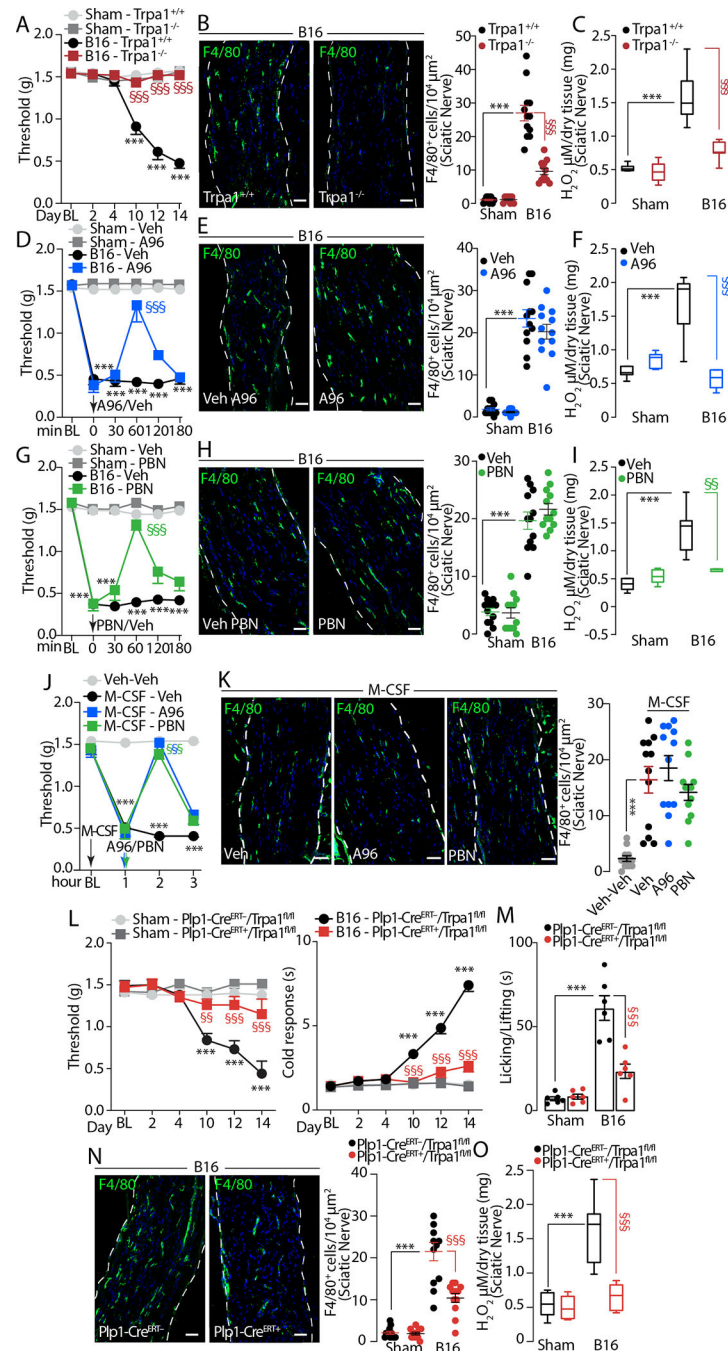


Figure 6. Schwann cell TRPA1 mediates neuroinflammation and cancer-evoked allodynia.

(A) Time-dependent mechanical allodynia, (B) typical images and data of F4/80⁺ cells and, (C) H₂O₂ content in sciatic nerve after B16-F10 melanoma (B16) cell inoculation or sham in *Trpa1*^{+/+} and *Trpa1*^{-/-} mice. (D) Mechanical allodynia, (E) typical images and data of F4/80⁺ cells and (F) H₂O₂ content in sciatic nerve in C57BL/6J mice at day 14 after B16 cells inoculation or sham and after A967079 (A96) or Veh (i.p.). (G) Mechanical allodynia, (H) typical images and data of F4/80⁺ cell and (I) H₂O₂ content in sciatic nerve in C57BL/6J mice at day 14 after B16 cell inoculation or sham and after PBN or Veh (i.p.). (J)

Mechanical allodynia, (K) typical images and data of F4/80⁺ cell in sciatic nerve after M-CSF or Veh (i.p.) in C57BL/6J mice treated with A96, PBN or Veh (i.p.). (L) Mechanical allodynia, cold response and (M) spontaneous nociception after B16 cell inoculation or sham in *Plp1-Cre^{ERT}/ERT⁺/Trpa1^{fl/fl}*. (N) Typical images and data of F4/80⁺ cells and (O) H₂O₂ content in sciatic nerve after B16 cell inoculation or sham in *Plp1-Cre^{ERT}/ERT⁺/Trpa1^{fl/fl}* mice. BL, baseline. Dashed line delimits the *epineurium*. Scale bar 50 μ m. N=6 mice. *** $P < 0.001$ to Sham-*Trpa1^{+/+}*, Sham-Veh, Veh-Veh, Sham-*Plp1-Cre^{ERT}/Trpa1^{fl/fl}*; §§ $P < 0.01$, §§§ $P < 0.001$ to B16-*Trpa1^{+/+}*, B16-Veh, M-CSF-Veh, B16-*Plp1-Cre^{ERT}/Trpa1^{fl/fl}*. Data are presented as mean $\pm$ SEM, data points overlaid (B, E, H, K, M, N). (C, F, I, O) Box plots 25th and 75th percentile, min and max values and data points overlaid. Two-way (A, D, G, J, L) and one-way (B, C, E, F, H, I, K, M, N, O) ANOVA and Bonferroni post hoc test.

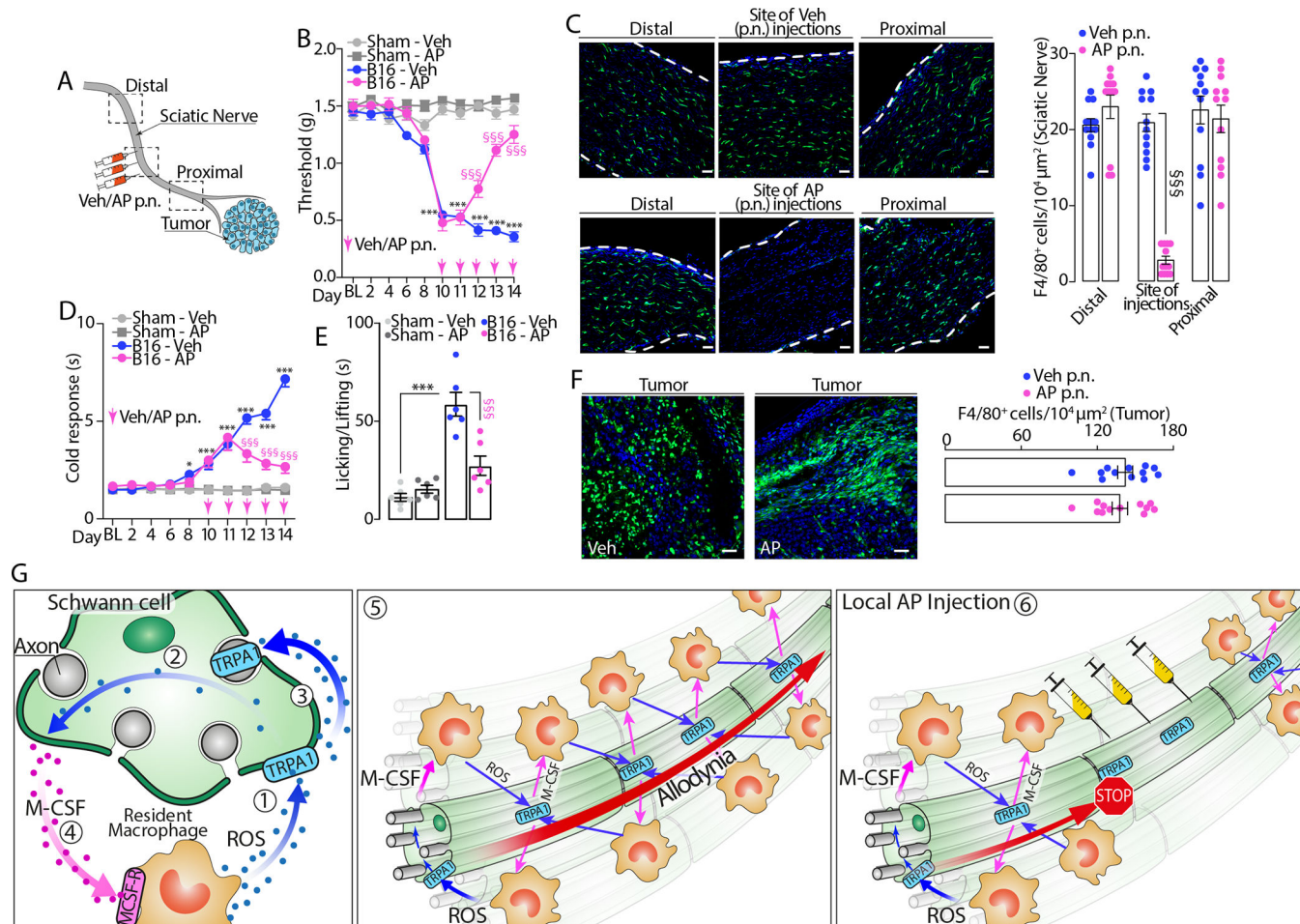


Figure 7. Segmental resident macrophages depletion switches off cancer-evoked mechanical allodynia, cold response and spontaneous nociception.

(A) Illustration of three local perineural (p.n.) injections of AP12087 (AP) or Veh in sciatic nerve after B16-F10 melanoma (B16) cell inoculation in MaFIA mice. Mechanical allodynia (B), cold response (D) and spontaneous nociception (E), typical images and data of F4/80⁺ cell in sciatic nerve (C) and tumor (F) after B16 cell inoculation or sham in MaFIA mice treated with AP or Veh (p.n.). BL. Baseline. Pink arrows: treatment with Veh/AP. Dashed line delimits the epineurium. Scale bar 50 μ m. N=6 mice. * $P < 0.05$, *** $P < 0.001$ to Sham-Veh; §§§ $P < 0.001$ to B16-Veh. Data are presented as mean $\pm$ SEM, data points overlaid (C, E, F). Two-way (B, D) and one-way (C, E, F) ANOVA and Bonferroni post hoc test. (G) Illustration of the feed-forward mechanism that sustains cancer pain: (1) expanded sciatic nerve resident macrophages (rMΦs) by their own oxidative burst target Schwann cell TRPA1; (2) Schwann cell TRPA1 amplifies the oxidative burst; (3) to release M-CSF which sustains further rMΦ expansion, and (4) to target neuronal TRPA1 which signals allodynia. The paracrine pathway, which entails Schwann cell TRPA1 activation, M-CSF release and signaling and rMΦ expansion, and Schwann cell- and rMΦs-dependent oxidative burst (5) must be present along the entire sciatic nerve to sustain mechanical allodynia, as (6) the

disruption of the feed-forward mechanism by segmental depletion of rMΦs switches off the pain-like response.

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