Microglia and monocytes synergistically promote the transition from acute to chronic pain after nerve injury

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Microglia and peripheral monocytes contribute to hypersensitivity in rodent models of neuropathic pain. However, the precise respective function of microglia and peripheral monocytes has not been investigated in these models. To address this question, here we combined transgenic mice and pharmacological tools to specifically and temporally control the depletion of microglia and monocytes in a mouse model of spinal nerve transection (SNT). We found that although microglia and monocytes are required during the initiation of mechanical allodynia or thermal hyperalgesia, these cells may not be as important for the maintenance of hypersensitivity. Moreover, we demonstrated that either resident microglia or peripheral monocytes are sufficient in gating neuropathic pain after SNT. We propose that resident microglia and peripheral monocytes act synergistically to initiate hypersensitivity and promote the transition from acute to chronic pain after peripheral nerve injury.
Neuropathic pain is a chronic pain state resulting from peripheral or central nerve injury due to trauma (for example, amputation and nerve injury) or systemic disease (for example, diabetes, viral infection and cancer)\(^1,2\). A key event in neuropathic pain is the transition from acute to chronic pain; however, the underlying mechanisms of this transition remain largely unknown. Our understanding of neuropathic pain mechanisms has expanded from being focused largely on neurocentric mechanisms to include neuro-glial interactions\(^3,4\). Spinal glia including astrocytes and microglia become activated after peripheral nerve injury, subsequently contributing to chronic pain by releasing a number of glial mediators that sensitize spinal neurons\(^6,7,8\). In addition, peripheral nerve injury recruits circulating monocytes to the injury sites, which can also release proinflammatory mediators that cause neuronal hyperactivity in the periphery\(^9\). These studies raise the intriguing possibility that central resident microglia and peripheral monocytes may control the transition from the acute to chronic pain.

Microglia comprise a unique subset of glial cells (5–10%) as the principal immune cells in the central nervous system (CNS). Resting microglia have highly dynamic processes by which they survey the microenvironment in the brain and spinal cord\(^11,12\). After peripheral nerve injury, spinal microglia transform from resting to reactive states, exhibiting marked changes in cell surface protein expression, and releasing a variety of proinflammatory mediators\(^16,17,18\). Inhibiting these microglia-derived molecules strongly suppresses pain-like hypersensitivity, suggesting that microglial response is a critical component of neuropathic pain. However, no evidence directly addresses the exact role of microglia in the initiation or maintenance of neuropathic pain.

Peripheral nerve injury is also associated with the recruitment of peripheral monocytes and their infiltration into the CNS parenchyma. Indeed, it was demonstrated that blood-borne circulating monocytes infiltrate into the spinal cord and play an essential role in neuropathic pain development\(^20\). However, the role of peripheral monocytes in neuropathic pain is still debated\(^21,22\). Nevertheless, the importance of peripheral monocytes in pain-like hypersensitivity is evident from the studies showing dissociation between activation of spinal microglia and chronic pain in certain animal models of peripheral nerve injury\(^23,24\) and in chemotherapy-induced pain\(^25\). So far, no report has pinpointed the specific role of resident microglia and peripheral monocytes in neuropathic hypersensitivity.

Using transgenic mice that enabled us to ablate resident microglia and peripheral monocytes in a temporally controlled fashion, we delineated the time window during which microglia and monocytes are required for the development of neuropathic pain in a mouse model of spinal nerve transection (SNT). In addition, we combined pharmacological and genetic tools to ablate resident microglia and peripheral monocytes, and determined their respective functions in neuropathic hypersensitivity. Our results indicate that depletion of both resident microglia and peripheral monocytes completely prevented the development of neuropathic pain. However, either resident microglia or peripheral macrophages are critical for the initiation of neuropathic pain, suggesting that they act synergistically to promote the transition from acute to chronic pain after peripheral nerve injury.

**Results**

**CX3CR1\(^+\) cell depletion prevents hypersensitivity after SNT.** The chemokine receptor CX3CR1 is predominantly expressed by microglia in the CNS, but is also found in a subset of monocytes, macrophages, natural killer cells and dendritic cells in the periphery\(^26\). To study the role of CX3CR1\(^+\) cells in neuropathic pain, we used a strategy to temporally and specifically express the diphtheria toxin receptor (DTR) in CX3CR1\(^+\) cells by crossing CX3CR1\(^{CreER}\) mice with Rosa26-stop-DTR mice (CX3CR1\(^{CreER}\) \(\times\) R26\(^{+}\)/DTR\(^+\)) and subsequent induction of cre recombinase by tamoxifen (TM) injection\(^27\). Therefore, we were able to control the ablation of CX3CR1\(^+\) cells by diphtheria toxin (DT) application and directly investigated the temporal role of CX3CR1\(^+\) cells in chronic pain behaviours after L4 SNT, a well-established mouse model of neuropathic pain. TM (150 mg kg\(^{-1}\) in corn oil, 4 doses with 2-day intervals) or corn oil control was intraperitonially (i.p.) injected to CX3CR1\(^{CreER}\) \(\times\) R26\(^{DTR}\)/+ mice before SNT and DT (50 µg kg\(^{-1}\) i.p., 3 days after last TM dose) was administered 1 day before and 1 day after SNT to deplete CX3CR1\(^+\) cells (Fig. 1a). At postoperative day 3 (POD3), we examined the CNS microglia and dorsal root ganglia (DRG) macrophages by immunostaining for Iba1, as well as blood monocytes that express CX3CR1 by flow cytometry (Fig. 1b–d). CD11b, another marker for microglia and macrophages, was also used to confirm the ablation efficiency in spinal dorsal horn (Supplementary Fig. 1a,b). In CX3CR1\(^{CreER}\) \(\times\) R26\(^{DTR}\)/+ mice without TM-induced DT expression (control), SNT markedly increased the number of microglia in the ipsilateral dorsal horn and resident macrophages in DRGs compared with contralateral sides at POD3. However, in CX3CR1\(^{CreER}\) \(\times\) R26\(^{DTR}\)/+ mice with both TM and DT injection (ablation), spinal microglia and DRG macrophages in both contralateral and ipsilateral sides were largely depleted (Fig. 1b,c and Supplementary Fig. 1a,c). In these CX3CR1\(^{CreER}\) mice in which the CreER-encoding gene was followed by an IRES-EYFP element\(^27\), a subset of CD11b\(^+\)/EYFP\(^+\) positive blood cells that are monocytes with high CX3CR1 expression was also depleted (Fig. 1d and Supplementary Fig. 1d). In addition, microglia were depleted in most supraspinal brain regions, such as the rostral ventromedial medulla, anterior cingulate cortex and hippocampus (Supplementary Fig. 1b,c). Together, these results indicate that our ablation strategy was able to successfully deplete CX3CR1\(^+\) cells, including microglia in the brain and spinal cord, DRG macrophages and CX3CR1\(^+\) monocytes.

Next, we wanted to know whether depletion of CX3CR1\(^+\) cells affected mouse pain behaviours. Firstly, we measured acute pain behaviours in mice with CX3CR1\(^+\) cell depletion. We found that CX3CR1\(^+\) cell depletion did not alter acute pain responses to either mechanical or thermal stimulation (POD0, Fig. 1e,f). Also, there was no difference in tail flick tests between control and ablation groups (Supplementary Fig. 2a,b). Motor coordination in the rotarod test was similar between the two groups, although inter-session motor learning was impaired in mice with CX3CR1\(^+\) cell depletion compared with control mice. Both mechanical allodynia and thermal hyperalgesia were prevented after SNT by such ablation and lasted at least 2 weeks (Fig. 1e,f). A recent report showed a sex difference in the role of microglia in mechanical allodynia in male and female mice 7 days after nerve injury\(^28\). Interestingly, we found that mechanical and thermal hypersensitivity following SNT were completely abolished in mice with CX3CR1\(^+\) cell depletion compared with control mice. Both mechanical allodynia and thermal hyperalgesia were prevented after SNT by such ablation and lasted at least 2 weeks (Fig. 1e,f). A recent report showed a sex difference in the role of microglia in mechanical allodynia in male and female mice 7 days after nerve injury\(^28\). Interestingly, we found that mechanical and thermal hypersensitivity following SNT were completely abolished in both male and female mice with CX3CR1\(^+\) cell-depleted at POD3 compared with control mice (Supplementary Fig. 3). These results suggest that CX3CR1\(^+\) cells equally participated in the neuropathic pain development in both male and female mice.

The CX3CR1\(^+\) cell depletion strategy using DT in CX3CR1\(^{CreER}\) \(\times\) R26\(^{DTR}\)/+ mice induces microglial cell death.
and may cause local inflammatory environment change. Thus, we introduced a second microglia- and macrophage-manipulating method to confirm the role of CX3CR1$^+$ cell population in neuropathic pain (Supplementary Fig. 4). Colony-stimulating factor 1 receptor (CSF1R), a key regulator of myeloid lineage cells, is required for microglia survival in the adult brain, and CSF1R inhibitor PLX3397 is able to deplete microglia in the brain$^{29}$. Since PLX3397 is not commercially available, here we blocked the

**Figure 1 | Ablation of CX3CR1$^+$ cell population prevents neuropathic pain development.** (a) An experimental diagram showing the timeline of drug treatments, SNT surgery, immunostaining and behavioural tests. (b) Confocal images showing Iba1-positive microglia in the dorsal horn of CX3CR1CreER$^+$:R26DTR$^+$ mice on the ipsilateral and contralateral side at POD3 after SNT in control and ablation mice. Scale bar, 100 μm. Left and right: representative higher-magnification images from boxed regions showing microglial morphology. Scale bar, 20 μm. (c) Representative images of Iba-1-stained macrophages from both contralateral and ipsilateral L4 DRG in ablation and control group. Scale bar, 50 μm. (d) Flow cytometry signatures of blood monocytes depicting CD11b$^+$ CX3CR1$^+$ population in ablation and control groups. The CX3CR1 expression level was indicated by intensity of EYFP fluorescence ($n = 3$ for each group). (e,f) Behavioural assays showing mechanical allodynia (e) and thermal hyperalgesia (f) in ablation and control groups. (Data represent mean ± s.e.m., $n = 9$ for ablation group and 7 for control group. ***$P < 0.001$, **$P < 0.01$, ablation ipsi versus control ipsi for both mechanical, U-test, and thermal response, t-test.) CX3CR1CreER$^+$:R26DTR$^+$ mice with DT only were considered to be controls and mice with both TM + DT treatment was ablation group.
CSF-1 pathway by neutralizing CSF-1 with antibody (200 ng in 5 μl ACSF) through daily intrathecal injections from POD0 to POD5 after SNT surgery (Supplementary Fig. 4a). We found that neutralizing CSF-1 antibody was not able to completely ablate microglia and macrophages, but it suppressed microglia and macrophage numbers by reducing their proliferation, which is consistent with a recent study showing that CSF-1 signalling is critical for microglial activation after peripheral nerve injury30. The proliferation of spinal microglia and DRG macrophages was examined by a proliferating marker Ki-67 staining at POD3 after SNT. Compared with vehicle-treated mice (CX3CR1CreER+/+ mice), the number of Ki-67+ proliferating dorsal horn microglia or DRG macrophages were markedly reduced in CSF-1 antibody-treated mice (Supplementary Fig. 4b,c). Consistently, we found that although the vehicle-treated mice developed chronic neuropathic pain normally, both mechanical allodynia and thermal hyperalgesia were significantly reversed in the CSF-1-neutralizing antibody-treated mice (Supplementary Fig. 4d,e). These results confirmed that manipulation of the number of spinal microglia and DRG macrophages is able to affect neuropathic pain development and replicate some aspects of CX3CR1+ cell depletion strategy using CX3CR1CreER+/−;R26iDTR+/+ mice.

Repopulated CX3CR1+ cells cannot recover hypersensitivity. A unique property of microglia and macrophages is their capacity to quickly repopulate the CNS after ablation29,31. Here we allowed the repopulation of spinal microglia and DRG macrophages and examined their function in neuropathic pain using CX3CR1CreER+/−;R26iDTR+/+ mice after SNT. We found that microglia in the spinal dorsal horn repopulated rapidly after their complete depletion at POD3. In both contralateral and ipsilateral dorsal horn, spinal microglia repopulated quickly at POD7 and POD14 in CX3CR1 cell-ablated mice (Fig. 2a,b). The morphology of the newly repopulated spinal microglia differed from that in age-matched control mice, exhibiting shorter processes and fewer branches at POD7 (Supplementary Fig. 5). In addition, macrophages in the injured L4 DRG also showed rapid repopulation at POD7 and POD14 (Fig. 2c,d). These results indicate that both spinal microglia and DRG macrophages undergo rapid repopulation after their depletion by DT in CX3CR1CreER+/−;R26iDTR+/+ mice. Although microglia and macrophages repopulated in the spinal cord and DRG within a week, the reduction in pain-like behaviours in CX3CR1 cell ablation mice was long-lasting (Fig. 1e,f). Therefore, these results suggest that repopulated microglia and macrophages after SNT are unable to re-establish neuropathic hypersensitivity.

To test whether repopulated microglia and macrophages are functional in the development of neuropathic pain, we depleted CX3CR1+ cells and allowed a week for their repopulation. Then SNT was performed in these mice (Fig. 2e). We confirmed that the number of repopulated spinal microglia and DRG macrophages in CX3CR1 cell-ablated mice was comparable to that in control mice (Fig. 2e). In these mice with repopulated microglia and macrophages (post ablation), we performed SNT and then neuropathic pain behaviours were tested. We found that mechanical allodynia developed normally in these mice and there was no difference in hypersensitivity between control and post-ablation groups (Fig. 2f). These results suggest that repopulated microglia and macrophages are functional being able to initiate neuropathic pain after peripheral nerve injury. Thus, we demonstrate that repopulated microglia and macrophages are able to engage in the development of neuropathic pain after de novo but not pre-existent nerve injury. These results suggest a critical period when signals derived from peripheral nerve injury, such as CSF-1 or ATP30,32, are able to recruit microglia to develop neuropathic pain-like hypersensitivity.

Early ablation of CX3CR1+ cells reverses hypersensitivity. Depletion of CX3CR1+ cells abolished neuropathic pain behaviours after peripheral nerve injury (Fig. 1). However, the precise role of these cells at different stages of neuropathic pain development is still unknown. Taking advantage of CX3CR1CreER+/−;R26iDTR+/+ mice, we were able to temporally ablate CX3CR1+ cells by DT injection in the early (POD3−5) and late (POD7−9) stages after SNT. Neuropathic hypersensitivity after SNT gradually developed from POD1−3 and was fully established after POD7. Consistently, the number of microglia in the ipsilateral spinal cord and L4 DRG increased during neuropathic pain development and peaked at POD7 (Fig. 2a−d).

To directly assess the role of CX3CR1+ cells in the development and initiation of neuropathic pain, we administered DT (i.p., 50 μg kg−1) at POD3 and POD5 (DT3&5) after SNT and then examined the depletion of microglia and macrophages, as well as chronic pain behaviours (Fig. 3a). As expected, DT at POD3 and 5 completely ablated spinal microglia (Fig. 3b,c) and most DRG macrophages at POD7 (Supplementary Fig. 6). Interestingly, behavioural experiments revealed that mechanical allodynia was significantly reversed at POD7 (Fig. 3d). In addition, the reduced mechanical allodynia was sustained, lasting up to POD14 (Fig. 3d), although microglia had largely repopulated the spinal cord by this stage (data not shown). Consistently, thermal hyperalgesia was also significantly reversed when CX3CR1 cells were ablated at POD3 and POD5 after SNT (Supplementary Fig. 7a,b). These results indicate that CX3CR1+ cells are critical during the initiation of neuropathic pain.

Next, we asked whether CX3CR1+ cells are required for the maintenance of neuropathic hypersensitivity. To this end, we ablated microglia and macrophages when neuropathic pain was fully developed. DT was administered (i.p., 50 μg kg−1) at POD7 and POD9 (DT7&9), which completely depleted spinal microglia and most DRG macrophages at POD11 (Fig. 3b,c and Supplementary Fig. 6). Surprisingly, when we examined the pain-like behaviours in these mice, we found that mechanical allodynia was only transiently reversed but then maintained to a comparable level as those without CX3CR1+ cell ablation at POD11 (Fig. 3e). In addition, the mechanical allodynia persisted at least to POD14 in those mice with CX3CR1+ cell ablation (Fig. 3e). Consistently, thermal hyperalgesia in mice, in which CX3CR1+ cells were ablated at POD7 and 9, was also only transiently reversed compared with those in control mice without cell ablation (Supplementary Fig. 7a,c). Interestingly, in female mice, thermal hyperalgesia but not mechanical allodynia was transiently reversed after DT injection at POD7 and POD9 (Supplementary Fig. 8), suggesting that sexual dimorphism may differentiate CX3CR1+ cell’s function in different modalities of neuropathic pain. Taken together, our results suggest that CX3CR1+ cells participate in the initiation of the neuropathic pain state, but are only transiently required in the maintenance of neuropathic pain.

In particular, there during a critical time window of at POD0–POD5, when CX3CR1+ cells promote the transition from acute to chronic pain after peripheral nerve injury.

Depletion of monocytes did not alter hypersensitivity. CX3CR1 is expressed in CNS microglia, DRG macrophages and circulating monocytes26. Our above results demonstrated the pivotal function of CX3CR1+ cells in neuropathic pain. However, the respective role of peripheral monocytes and microglia in neuropathic hypersensitivity remains unknown. To address this question,
Clodronate liposomes were used to deplete blood and tissue phagocytes including monocytes and macrophages\(^{33,34}\) (Fig. 4a). In wild-type mice, Iba-1 positive cells (presumably resident macrophages) in the spleen and liver were completely depleted after clodronate treatment (Supplementary Fig. 9). However, clodronate liposomes were unable to deplete spinal microglia (Fig. 4a,b). The DRG macrophages were also preserved after the application of clodronate liposomes (Fig. 4a,b), suggesting that clodronate liposomes were not able to penetrate into the DRG, or macrophages in the DRG were unable to phagocytose clodronate liposomes. We tested the effect of clodronate liposomes (16 ml kg\(^{-1}\), i.p. twice at 3 days intervals) on blood CX3CR1\(^{+}\) cell depletion using CX3CR1\(^{CreER/+}\) mice. Indeed, CD11b\(^{+}\)CX3CR1\(^{+}\) cells were largely ablated in the blood 2 days after the last clodronate liposome treatment (Fig. 4c). We further confirmed the depletion of blood monocytes by clodronate at POD3 after SNT using F4/80 (mature macrophage marker) and Gr-1 (also known as Ly-6, a marker of monocytes, neutrophils and eosinophils) staining in flow cytometry (Supplementary Fig. 10a,b). The results show that the Gr-1\(^{low}\) F4/80\(^{high}\) cells that correspond to mature blood monocytes\(^{35}\) were reliably depleted by clodronate liposomes. However, there was increased CD11b\(^{+}\)CX3CR1\(^{-}\)F4/80\(^{low}\)Gr-1\(^{high}\) population after clodronate treatment, which might be repopulated monocytes\(^{36}\). These results show that application of clodronate liposomes depleted CD11b\(^{+}\)CX3CR1\(^{+}\) monocytes but not DRG macrophages or spinal microglia.

It has been reported that blood monocytes can infiltrate the spinal dorsal horn and contribute to pain-like hypersensitivity after peripheral nerve injury\(^{20}\). Here we wanted to directly address the role of peripheral monocytes in neuropathic pain by testing pain-like behaviours after clodronate liposome treatment. Clodronate liposomes (16 ml kg\(^{-1}\), i.p.) were applied to wild-type mice. Indeed, CD11b\(^{+}\)CD11c\(^{+}\) monocytes in the blood were depleted. However, in the spinal dorsal horn, CD11b\(^{+}\)CD11c\(^{-}\) monocytes were not depleted, indicating that monocytes from blood were not able to penetrate into the spinal cord. These results suggest that peripheral monocytes contribute to pain-like hypersensitivity after peripheral nerve injury.
Ablation of resident microglia delays hypersensitivity. Peripheral monocytes are reported to infiltrate the spinal cord to participate in hypersensitivity after peripheral nerve injury. To further dissect the respective role of peripheral monocytes and resident microglia in neuropathic pain, we again used CX3CR1CreER/+:R26iDTR/+ mice, which enabled us to exclusively deplete resident microglia in the CNS but not blood CX3CR1+ cells. This is because resident microglia show a much slower turnover, whereas blood CX3CR1+ cells have substantially rapid turnover and are replenished frequently. To this end, we applied TM (i.p., 150 mg kg−1) to CX3CR1CreER/+:R26iDTR/+ mice and allowed a 3-week interval of peripheral turnover before DT injection (Fig. 5a). We found that spinal microglia at POD3 after SNT were largely depleted (Fig. 5b), with efficiencies comparable to the total CX3CR1+ cell depletion. However, macrophages in L4 DRG in these mice were similar to non-depleted control mice at POD3 (Fig. 5c, d).

Also, blood CD11b+ CX3CR1+ cells were largely preserved (Supplementary Fig. 11a, b). Therefore, it seemed that a 3-week interval between TM and DT application was able to selectively deplete CNS microglia but not blood CX3CR1+ monocytes and DRG macrophages in CX3CR1CreER/+:R26iDTR/+ mice. Since the depletion of monocytes by clodronate did not affect neuropathic pain development (Fig. 4), we surmised that microglial ablation might replicate the reversed pain phenotype in CX3CR1+ cell-depleted mice (Fig. 1). To test this idea, we examined the pain behaviours in these CX3CR1CreER/+:R26iDTR/+ mice with resident microglial ablation after SNT. To our surprise, we found

**Figure 3 | Early but not late CX3CR1+ cell ablation reverses neuropathic pain.** (a) An experimental design showing the timeline for drug treatments, SNT surgery, immunostaining and behavioural assays to identify the critical window for microglia in SNT-induced neuropathic pain. For early phase: DT3&5, DT was applied at POD3 and 5. For late phase: DT7&9, DT was applied at POD7 and 9. (b) Confocal images of Iba1 staining showing microglial ablation in dorsal horn at POD7 in DT3&5 group and at POD11 in DT7&9 group. Representative higher-magnification images from boxed regions in larger images showing microglial morphology. Scale bar, 100 μm. (c) Pooled results showing the effect of ablation in the spinal dorsal horn (n = 3 mice for each group, 3 images for each animal, ***P < 0.001, ablation versus control, t-test). (d) Measurement of mechanical allodynia in the two temporal models of ablation (DT3&5 group (n = 12 for DT3&5 and 7 for control) and (e) DT7&9 (n = 8 for DT7&9 and 8 for control). ***P < 0.001, **P < 0.01, ablation versus control ipsilateral side, U-test. Data represent mean ± s.e.m. CX3CR1CreER/+:R26iDTR/+ mice with DT only were considered to be controls and mice with both TM + DT treatment was ablation group.
that mice without microglia showed attenuated pain-like hypersensitivity only at POD1–3 (Fig. 5c). From POD5–14, the mechanical allodynia gradually returned to the similar level as those control mice with normal resident microglia (Fig. 5e). Consistently, thermal hyperalgesia in the microglial ablation mice after SNT were only reduced at POD1–3 but not at POD5–14 (Supplementary Fig. 11a,c). To test the possibility that this delayed hypersensitivity might be due to the potential secondary inflammation after microglia ablation, we examined the inflammatory cytokines interleukin (IL)-1β in control and microglial ablation mice using western blot. We found that IL-1β expression was not altered after microglia ablation in sham control. Also, IL-1β expression was equally upregulated at POD3 after SNT in mice with or without microglial ablation (Supplementary Figs 12a,b and 14). These results suggest that secondary inflammation after microglia ablation may explain the delayed hypersensitivity in microglia-depleted mice. Interestingly, we found astrocyte marker glial fibrillary acidic protein (GFAP) was significantly increased after microglial ablation in sham control and also at POD3 after SNT (Supplementary Figs 12a,c and 14). Nevertheless, our results unexpectedly found that the selective depletion of CNS microglia delayed but did not reverse neuropathic hypersensitivity after peripheral nerve injury.

Monocytes do not infiltrate into the dorsal horn after SNT. The recovery of pain-like hypersensitivity after SNT in CNS microglia-ablated mice could be due to the monocyte infiltration since peripheral monocytes are known to infiltrate into spinal cord after bone marrow transplantation29. However, the 3-week interval between TM and DT application in CX3CR1CreER+/tdTomato mice was able to largely deplete the spinal microglia after SNT (Fig. 5b,d), suggesting that there was no infiltration of peripheral monocytes into the spinal cord after peripheral nerve injury. To further examine the possibility of monocyte infiltration after SNT, we crossed a cre-inducible RFP (tdTomato) reporter (R26iDTR/+) mice with CX3CR1CreER+/tdTomato (R26iDTR/RFP+) mice to generate CX3CR1CreER+/tdTomato mice. We then monitored tdTomato+ cells that are CX3CR1+ either at 3 days or 3 weeks after TM treatment. As expected, we found that <2% of CD11b+ CX3CR1+ monocytes were labelled with tdTomato 3 weeks after TM treatment, while over 90% of CD11b+ CX3CR1+ positive cells were tdTomato positive at 3 days after TM treatment (Fig. 6a). Therefore, blood CX3CR1+ cells were indeed replaced within a 3-week period.

We then examined the monocyte infiltration in CX3CR1CreER+/R26iDTR/+ mice after SNT. If there was monocyte infiltration, we would have observed Iba1+ tdTomato- cells in the ipsilateral spinal cord after SNT. However, we found that at POD3 after SNT, all microglia stained with Iba1 in both ipsilateral and contralateral dorsal horn were also labelled with tdTomato (Fig. 6b). These results further confirm that there is no infiltration of blood monocytes to the spinal dorsal horn after SNT in our study.

Synergistic action of microglia and monocytes. So far, we have shown that depletion of CX3CR1+ cells abolished neuropathic pain (Fig. 1), while peripheral monocytes or microglia depletion did not affect (Fig. 4) or only delayed the development of pain-like hypersensitivity after SNT (Fig. 5), respectively. These results strongly suggest that both resident microglia and monocytes are required for the full development of neuropathic pain. To test this idea, we applied clodronate liposomes to deplete...
monocytes in mice with ablated resident microglia, and tested their pain behaviours. Clodronate liposomes were applied 3 days before and immediately after SNT while DT was administrated at 1 day before and POD1 after SNT. Our data show that DRG macrophage depletion did not occur in both our peripheral (Fig. 4c) and central (Fig. 5c) ablation model. However, the number of DRG macrophages was drastically reduced in our combined ablation model (Fig. 7c). These results suggest that DRG macrophages are CX3CR1+/+ and are slowly replenished by blood monocytes (data not shown). Therefore, clodronate depleted the infiltration source (blood monocytes), resulting in an overall reduction in number of DRG macrophages. Future investigations on the specific contribution of DRG macrophages to neuropathic pain are warranted.

Next, we examined pain behaviours in CX3CR1CreER+/+;R26DTR+/+, mice with both clodronate and DT treatment. Consistent with those in the total CX3CR1+/+ cell-depleted mice (Fig. 1e), we found that both mechanical allodynia (Fig. 7e) and thermal hyperalgesia (Supplementary Fig. 13) after SNT were completely prevented after clodronate and DT treatment compared with control mice without the treatment. These results corroborate the data obtained from total CX3CR1+/+ cell-depleted mice, indicating that both peripheral monocytes and resident microglia are required for the development of neuropathic pain. Therefore, the two populations of CX3CR1+/+ cells may synergistically promote the transition from acute pain to chronic pain after peripheral nerve injury.

**Discussion**

Currently available treatments for chronic neuropathic pain typically show limited efficacy in a majority of patients. Therefore, comprehensive studies of chronic pain pathogenesis are required to identify novel therapeutic targets and develop a better treatment for neuropathic pain. It is well known that spinal microglia and peripheral monocytes contribute to neuropathic pain development after peripheral nerve injury. Indeed, systemic inhibition of microglia and macrophages by the broad inhibitor minocycline, attenuated neuropathic pain behaviours. However, the temporal and spatial function of resident microglia and peripheral monocytes in the development of neuropathic pain has not been elucidated. Using CX3CR1CreER+/+;R26DTR+/+, mice, combined with clodronate treatment enabled us to deplete microglia and monocytes in a controllable fashion. We discovered a critical time window for microglia and monocytes in promoting the transition from acute to chronic pain after peripheral nerve injury. Moreover, we demonstrated that resident microglia and peripheral monocytes synergistically initiate pain-like hypersensitivity after peripheral nerve injury. These novel findings provide a rationale for targeting both microglia and macrophages to prevent the development of neuropathic pain. Our understanding of the exact role of microglia and monocytes in...
the initiation and maintenance of neuropathic pain was limited due to our inability to manipulate these cells in a temporally distinct manner. To circumvent this limitation, we took advantage of CX3CR1CreER+/R26tdTomato+ reporter mice showing the CD11b+/CX3CR1+ population at 3 days and 3 weeks post tamoxifen administration (n = 3 for each time point). (b) Iba1 staining (green) showing all spinal dorsal horn microglia were tdTomato (red) positive at POD3 after SNT in CX3CR1CreER+/R26tdTomato+ mice with 3-week interval between TM and DT treatment. The results indicate no peripheral monocyte infiltration to spinal cord at POD3 after SNT. Higher-magnification images from boxed regions in larger images showing microglial morphology (n = 3 mice for each group, n = 3 images for each animal). Scale bar, 100 μm.

Figure 6 | There is no infiltration of blood monocytes in the dorsal horn after SNT. (a) Representative blood cytometry data from CX3CR1CreER+/R26tdTomato+ reporter mice showing the CD11b+/CX3CR1+ population at 3 days and 3 weeks post tamoxifen administration (n = 3 for each time point). (b) Iba1 staining (green) showing all spinal dorsal horn microglia were tdTomato (red) positive at POD3 after SNT in CX3CR1CreER+/R26tdTomato+ mice with 3-week interval between TM and DT treatment. The results indicate no peripheral monocyte infiltration to spinal cord at POD3 after SNT. Higher-magnification images from boxed regions in larger images showing microglial morphology (n = 3 mice for each group, n = 3 images for each animal). Scale bar, 100 μm.

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thermal hyperalgesia) during the pain maintenance phase but not during the initiation phase (Supplementary Figs 3 and 8). These results suggest that microglia equally participated in the mechanical pain development in both male and female mice. Moreover, our results suggest that sexual dimorphism may play a differential role in the different pain modalities. The significance and underlying mechanism for the role of gender differences in pain initiation versus maintenance warrants further investigation.

Compared with the well-documented function of microglia in neuropathic pain, the role of monocytes in neuropathic pain is still uncertain. Clodronate depletion of peripheral macrophages and monocytes reduced thermal hyperalgesia and Wallerian degeneration in one study\(^22\) although the same treatment had no effect on mechanical allodynia in another study\(^21\). In contrast, a recent study using bone marrow transplantation provided support to the idea that circulating blood monocytes are able to infiltrate into the spinal cord and thus contribute to neuropathic pain via central sensitization\(^20\). In our study, we found that sole depletion of peripheral macrophages had a limited effect on pain-like hypersensitivity. With liposomal clodronate treatment, we could specifically deplete most CX3CR1 \(^+\) monocytes from the blood but not CNS microglia. Under this condition, the mice without blood monocytes developed neuropathic pain normally after SNT. Moreover, we have strong evidence showing that there was no infiltration of peripheral microglia in the spinal cord after peripheral nerve injury. Therefore, the infiltrated monocytes observed in the study could be due to the irradiation chimerism protocol that may lead to non-physiological transmigration of cells into the spinal cord\(^37\). However, we cannot exclude the possibility that the CX3CR1\(^{CreER}/:+\).R26\(^{RFP}/+\) mice used in this study lack a copy of the endogenous CX3CR1 gene and may account for the lack of monocyte infiltration observed. Our study suggests that even without peripheral monocytes, resident microglia are sufficient to initiate neuropathic pain. However, we were surprised that depletion of resident microglia did not abolish, but only delayed the development of neuropathic pain. Hence, peripheral monocytes are capable of playing an equally important role in initiating neuropathic pain and are able to help facilitate neuropathic pain even in the absence of resident microglia. Together, our results strongly indicate that microglia and monocytes synergistically promote the transition from acute to chronic pain after peripheral nerve injury. Indeed, we found that neuropathic pain-like hypersensitivity was completely reversed in CX3CR1\(^+\) cell-ablated mice and in the microglia-ablated mice treated with clodronate liposomes. Future studies are needed to address the molecular mechanisms underlying synergistic interaction between resident microglia and peripheral monocytes in gating the neuropathic hypersensitivity.

Our results are novel in that we were able to dissect the respective roles of resident microglia and peripheral monocytes in neuropathic pain using a combination of genetic and pharmacological tools. The limited effect of microglial ablation on pain behaviours was unexpected, considering that numerous studies have proved the critical function of microglia in
neuropathic pain. However, since most studies were not able to distinguish the particular molecules in microglia versus monocytes and macrophages, the interpretation of microglial function in those studies should be interpreted with caution. Although our monocyte depletion study indeed supports the important function of microglia, we believe that both microglia and monocytes work in concert to initiate neuropathic pain after nerve injury. However, peripheral monocytes alone may be capable of initiating pain under conditions where microglial activation is minimal23,25. A caveat is that we also found astrocye activation in the spinal cord after microglial depletion. This result may complicate the explanation for the delayed pain-like hypersensitivity caused by monocytes alone, considering the critical role of astrocytes in neuropathic pain maintenance5,53. However, since there is no obvious pain-like hypersensitivity despite the astrocye activation in microglia ablation mice in sham control, we suspect that the reactive astrocytes may not directly account for delayed hypersensitivity after microglia depletion. In sum, our current study demonstrates that microglia and monocytes participate in the initiation of pain-like hypersensitivity but may not be as critical for its maintenance after peripheral nerve injury. In addition, either resident microglia or peripheral monocytes are sufficient to initiate neuropathic pain and thus they synergistically promote the transition from acute to chronic neuropathic pain. Our results provide a rationale for early intervention of pain development targeting both resident microglia and peripheral monocytes.

Methods

Animals. Mice (7-12 weeks old) were used in accordance with institutional guidelines as approved by the animal care and use committee at Rutgers University. C57BL/6J (Charles River) and CX3CR1GFPP/− mice were used as wild-type control. CX3CR1CreERT2;EYFP+/− mice were obtained from Dr Wen-Biao Gan at New York University. The mice were crossed with R26RtdTomato+/− or CX3CR1CreERT2/+ (purchased from Jax lab) to obtain CX3CR1CreERT2;R26RtdTomato+/− or CX3CR1CreERT2−/−, respectively. Male mice were used throughout the study, unless the use of female mice was specifically indicated. Mice were assigned to experimental groups randomly within a litter. Experimenter was blind to drug treatments.

Surgery. Lumbar 4 SNT was done in 7- to 9-week-old mice. SNT surgery was performed under 2% isoflurane anaesthesia. An incision was made along the mid-line of the lumbar spine. The left paraspinal muscles in front of the pelvic bone were separated to expose the L5 transverse process. The L5 transverse process was removed to expose L4 spinal nerve. The L4 spinal nerve was separated and transplanted and removed 1-1.5 mm from the end to DRG. The wound was then irrigated with sterile PBS and closed with #6 silk sutures for the muscles and #5 silk sutures for the skin. POD represents the post-operative day following SNT and all the experimental timelines are in reference to POD0, which is the day of SNT surgery.

CX3CR1 ablation. TM (Sigma) was administered as a solution in corn oil (Sigma) to mice over 6 weeks old by i.p. injection. Animals received four doses of TM (150 mg.kg−1, 20 mg.ml−1 in corn oil) at 48-h intervals. For total CX3CR1+/− cell ablation, two doses of DT (Sigma, Catalogue #D0564, 50 μg.kg−1, 2.5 μg.ml−1 in PBS) were given at 3 and 5 days after the last TM treatment. For microglia ablation, the interval between the last TM and the first DT was 3 weeks. Mice administered with DT only (without TM) were used as control for all ablation experiments.

Monocyte depletion. Liposome-encapsulated clodronate was used to deplete phagocytic macrophages. Clodronate liposomes (15 ml.kg−1, ClodronateLiposomes.com) were i.p. injected 3 days before and immediately after the SNT surgery.

CSF-1-neutralizing antibody treatment. CSF-1 antibody (200 μg in 5 μl ACSF, R&D #AF4161) or the vehicle (ACSF) were daily injected intrathecially by direct lumbar puncture between L3 and L6 vertebral of the spine, using a 10-μl Hamilton syringe (Hamilton Bonaduz AG) with a 31G needle. Successful lumbar puncture was identified by tail reflex.

Behavioural measurement. Mechanical allosthesia was measured by measuring the paw withdraw threshold, with a set of Von Frey filaments (0.04-2 g; North Coast medical). Mice were placed on an elevated metal grid. The filament was applied to the plantar surface at a vertical angle for up to 3 s from the bottom. Fifty percent withdraw threshold values were determined using the up–down method54. The average withdrawal latency of the four trials was recorded as the response latency.

Tail flick was assessed using the same radiant heat source (ITC Inc life science). Mice were restrained in a cylindrical holder with the tail hung out. Heat was applied to the tail at ~2 cm from the rear end. The machine automatically detects the tail latency as the light/heat beam travels unharmed after the tail moves away.

The rotoarod tests were performed using a four-lane Rotarod apparatus (Med Associates Inc). The rotoarod speed started from 4 rounds per minute and uniformly accelerated to 40 rounds per minute in 5 min. Each mouse was tested for 3 times with 5-min interval.

Fluorescent immunostaining. Mice were deeply anaesthetized with isoflurane (5% in O2) and perfused transcardially with 20 ml PBS followed by 20 ml of cold 4% paraformaldehyde (PFA) in PBS containing 1.5% picric acid. The spinal cord and DRG were removed and post-fixed with the same 4% PFA for 4-6 h at 4°C. The samples were then transferred to 30% sucrose in PBS overnight. Sample sections (14 μm in thickness) were prepared on gelatin-coated glass slide with a cryostat (Leica). The sections were blocked with 5% goat serum and 0.3% Triton X-100 (Sigma) in TBS buffer for 60 min, and then incubated overnight at 4°C with primary antibody for rabbit-anti-Iba1 (1:1,000, Wako Chemicals, Catalogue #019-17941), rat-anti-CF121b (1:200, Biologic, Catalogue #101202) and rabbit-anti-Ki-67 (1:500, Abcam, Catalogue #16667). The sections were then incubated for 60 min at room temperature, with secondary antibodies (1:500, Alexa Fluor 594, Life Technologies). The sections were mounted with Fluoromount-G (SouthernBiotech) and fluorescent images were obtained with a confocal microscope (LSM510, Zeiss). Cell counting and fluorescent signal intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Note that the EYPF signal in CX3CR1 cells in tissue obtained from CX3CR1CreERT2;R26RtdTomato+/− mice was too weak. Hence, Iba1 staining was performed as described above. The Iba1 staining images were represented in green channel (using Image J) for consistency.

Monocyte flow cytometry. Whole mouse blood was collected and monocytes were separated from erythrocyte and granulocyte on a Ficoll (GE Healthcare) gradient. Separated monocytes were washed with Hank’s Balanced Salt Solution and then incubated with 2% goat serum for 10 min, and single stain with allophycocyanin (APC)-conjugated CD11b antibody (50 μg.ml−1, Biolegend). The sections were incubated with fluorophore- and fluorophore-conjugated antibodies for 30 min then washed twice with PBS. The sections were mounted with Fluoromount-G (SouthernBiotech) and fluorescent images were obtained with a confocal microscope (LSM510, Zeiss). Cell counting and fluorescent signal intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Western blot. Under isoflurane anaesthesia, lumbar 4–5 spinal dorsal horn in ipsilateral were collected in different treatment groups of mice. The tissues were homogenized and sonicated on ice in SDS lysis buffer with protease inhibitor cocktail (Roche Molecular Biochemicals) and phosphatase inhibitor, followed by centrifugation at 13,000 r.p.m. for 20 min at 4°C to obtain supernatant containing protein. Equal concentration of protein from different supernatant were loaded and 5% SDS–PAG electrophoresis was performed. The proteins were transferred to a PVDF membrane (Bio-Rad), blots were blocked and incubated at 4°C overnight with primary antibodies, rabbit anti-IL-1β (Abcam, 1:2,000), mouse anti-GEAP (Cell Signalling Technology, 1:2,000) and mouse anti-β-actin (Cell Signalling Technology, 1:2,000). Following which, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (secondary antibody, 1:5,000). Jackson Immune Research Laboratory) for 1 h at 25°C and washed. The immune complex on the membrane was detected by SuperSignal West Femto Maximum Sensitivity Substrate (34,095; Thermo Scientific) and captured on ImageQuantLAS4000 (Fujifilm Life Science). Integrated optical density was determined using ImageJ 1.48 (NIH). Standard curves were constructed to establish that we operated within the linear range of the detection method.

Statistical analysis. Quantification of Iba1 cells was done with ImageJ software (NIH Image). Data were presented as mean ± s.e.m. Student’s t-test and Wilcoxon rank-sum test (U-test) were used to establish significance. No statistical methods were used to predetermine sample sizes.
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experiments; U.B.E. and M.M. assisted with the experiments; W.-B.G. kindly provided CX3CR1CreER mice, some experimental design and expert discussion of the project; L.-J.W. conceived the study, supervised the overall project, designed the experiments and wrote the manuscript.

Additional information
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