

GNS-infiltrating CD4⁺ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain

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We previously reported leukocytic infiltration into the lumbar spinal cord in a rodent spinal nerve L5 transection (L5Tx) neuropathic pain model. Here, we further investigated the role of infiltrating T lymphocytes in the etiology of persistent pain following L5Tx. T lymphocyte-deficient nude mice showed no evident mechanical hypersensitivity after day 3 of L5Tx compared to wild-type BALB/c mice. Through FACS analysis, we determined that significant leukocytic infiltration (CD45^{hi}) into the lumbar spinal cord peaked at day 7 post L5Tx. These infiltrating leukocytes contained predominantly CD4⁺ but not CD8⁺ T lymphocytes. B lymphocytes, natural killer cells and macrophages were not detected at day 7 post L5Tx. No differences in the activation of peripheral CD4⁺ T lymphocytes were detected in either the spleen or lumbar lymph nodes between L5Tx and sham surgery groups. Further, CD4 KO mice displayed significantly decreased mechanical hypersensitivity after day 7 of L5Tx, and adoptive transfer of CD4⁺ leukocytes reversed this effect. Decreased immunoreactivity of glial fibrillary acidic protein observed in CD4 KO mice post L5Tx indicated possible T lymphocyte-glial interactions. These results strongly support a contributing role of spinal cord-infiltrating CD4⁺ T lymphocytes *versus* peripheral CD4⁺ T lymphocytes in the maintenance of nerve injury-induced neuropathic pain.

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Introduction

Neuropathic pain, defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system [1], is one of the most devastating forms of chronic pain. The lack of a complete understanding of its etiology thwarts the treatment and prevention. Further delineat-

ing the mechanisms leading to neuropathic pain is crucial and may accelerate the design of novel, non-addictive agents. Towards this end, many rodent models of neuropathy have been developed and utilized to decipher central and peripheral nervous system mechanisms that give rise to long-lasting behavioral hypersensitivity, the animal correlate of painful neuropathy in humans [2]. A novel mechanistic theme has emerged in the last decade implicating a role of central nervous system (CNS) innate and adaptive immunity in both the generation and maintenance of chronic pain syndromes [3].

Using radiation bone marrow-chimeric rats, we have previously shown that spinal nerve L5 transection

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Abbreviations: ANOVA: analysis of variance · **CatS:** cathepsin S · **CCI:** chronic constriction injury · **GFAP:** glial fibrillary acidic protein · **IP-10:** interferon-gamma-inducible protein-10 · **L5Tx:** spinal nerve L5 transection

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(L5Tx) induced significant CNS lumbar spinal cord infiltration of peripheral immune cells compared to sham surgery. These infiltrating cells were found to co-localize with ED2⁺ macrophages and contained CD3⁺ T lymphocytes [4]. Consistent with this infiltration, we have also shown increased lumbar spinal cord expression of monocyte and T lymphocyte chemoattractants, including interferon-gamma-inducible protein-10 (IP-10), regulated upon activation normal T cell expressed and secreted (RANTES), macrophage-inflammatory protein (MIP)-1 α and MIP-1 β , and monocyte chemoattractant protein-1 (MCP-1), at 3, 7 and 14 days following L5Tx (unpublished data). The localized breakdown of the blood-spinal cord barrier following nerve transection [5] is thought to also facilitate leukocyte infiltration. In addition, we have demonstrated the presence of activated microglia expressing major histocompatibility (MHC) class II and the costimulatory molecule B7.2 (CD86) in the spinal dorsal horn following peripheral nerve injury, suggesting a role of CNS adaptive immunity [6, 7]. However, the specific role of these immune responses and CNS-infiltrating T lymphocytes in nerve injury-induced neuropathic pain development and maintenance has not been extensively investigated.

Leukocyte infiltration into the affected peripheral nerve structures during neuropathic pain has been reported in patients suffering neuropathic pain and in the sciatic nerve chronic constriction injury (CCI)-induced animal model of neuropathic pain [8, 9]. The contributing role of various types of leukocytes in pain genesis/maintenance has been demonstrated in the CCI model. Rodents with neutrophil depletion or functional T lymphocyte deficiency developed significantly reduced CCI-induced mechanical allodynia and/or thermal hyperalgesia [8, 10]. The role of infiltrating leukocytes in the CCI model of neuropathic pain was further suggested by chemotactic cytokine receptor 2 (CCR2) KO mice, which exhibit a decreased recruitment of monocytes/macrophages into the dorsal root ganglia and abrogated mechanical allodynia post CCI [11].

In the current study, we extended our initial CNS adaptive immunity observations by using quantitative FACS analyses over a time course in the mouse neuropathy model L5Tx, probed the differences in peripheral vs. infiltrating leukocytes in the same study, and addressed diverging mechanisms of initiating and maintaining nerve injury-induced behavioral hypersensitivity. Our results clearly demonstrate a role of CNS-trafficking CD4⁺ T lymphocytes in the maintenance of nerve injury-induced tactile hypersensitivity.

Results

Behavioral mechanical sensitivity of nude BALB/c mice post L5Tx

To determine the role of T lymphocytes in L5Tx-induced neuropathic pain, adult T lymphocyte-deficient nude mice on BALB/c background were subjected to L5Tx or sham surgery, and then mechanical sensitivity (indicated by the 50% threshold force for paw withdrawals) was determined by the Up-Down method using von Frey filaments as described in *Materials and methods*. All mice were baseline tested before surgery and tested again at days 1, 3, 5, 7, 11, and 14 post surgery. No significant baseline behavioral difference between wild-type (WT) and nude mice was detected. As expected, WT mice showed significant mechanical hypersensitivity throughout the observation period (up to day 14 post L5Tx). Nude mice developed mechanical hypersensitivity on day 1 post L5Tx. However, they displayed a reduced mechanical sensitivity similar to that of sham-operated animals after day 3 post surgery (Fig. 1). Two-way analysis of variance (ANOVA) revealed a statistically significant interaction between time and surgery ($p = 0.0444$), as well as significant effects of time and surgery ($p < 0.0001$ for both).

Lumbar spinal cord-infiltrating T lymphocytes post L5Tx

To further analyze the spinal cord-infiltrating T lymphocytes, we first determined the time course of leukocyte infiltration into the lumbar spinal cord *via*

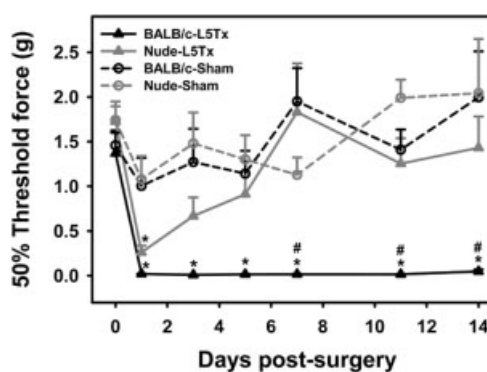


Figure 1. Mechanical sensitivity of nude/BALB/c mice post L5Tx or sham surgery. Adult male nude/BALB/c mice and BALB/c WT controls were subjected to L5Tx or sham surgery. Mechanical sensitivity was tested with von Frey filaments using the Up-Down method before and after surgery. Mechanical sensitivity was presented as the 50% threshold force for each animal ($n = 8$, mean \pm SEM). * indicates significant differences between L5Tx- and sham-operated mice within nude or WT groups ($p < 0.05$). # indicates significant differences between nude and WT mice subjected to the same surgery ($p < 0.05$).

FACS analysis. We isolated total lumbar spinal cord cells 1, 3, 7 and 14 days post L5Tx and labeled them with anti-mouse CD45-PE antibody for flow cytometry. CD45^{hi} populations were identified as the infiltrating leukocytes (Fig. 2A, B). L5Tx induced a transient increase in the infiltration of peripheral leukocytes that peaked at day 7 and diminished by day 14 within both sham and L5Tx surgery groups (Fig. 2C; two-way ANOVA, $p_{\text{time}} < 0.0010$). At day 7, the L5Tx group had significantly higher infiltration in both the ipsilateral (day 7 vs. days 1, 3 and 14) and contralateral (day 7 vs. day 14) sides of lumbar spinal cord, while no statistically significant increases at day 7 were seen within the sham groups. Further, no differences between ipsilateral vs. contralateral lumbar spinal cord within either group were observed. The infiltration appeared to be localized to the lumbar region, since no evident infiltration was observed in the remote cervical region of spinal cord in either sham- or L5Tx-operated mice compared to naive spinal cord (Fig. 2B). Because of these findings, the following studies were focused on the lumbar spinal cord at day 7 post injury.

To further assess the phenotype of L5Tx-induced infiltrating leukocytes, particularly the subtypes of T lymphocytes, mononuclear cells of lumbar spinal cord isolated 7 days post sham or L5Tx surgery were

subjected to multiple fluorescence labeling. Due to limited cell yield from each animal and lack of significant side differences in the CD45^{hi} population (Fig. 2C), spinal cords from three or four animals were pooled together and not separated into ipsilateral and contralateral sides for analysis during these experiments. CD45^{hi} cells were first identified (Fig. 3A) and then, within this population, CD4⁺ T lymphocytes (CD3⁺CD4⁺), CD8⁺ T lymphocytes (CD3⁺CD8⁺), B lymphocytes (CD3⁻CD19⁺), natural killer (NK) cells (CD3⁻panNK⁺), and macrophages (CD3⁻CD11b⁺I-A^{d+}) were further delineated. CD4⁺ T lymphocytes (CD3⁺CD4⁺) were identified as the most consistent and dominant cell type within the infiltrating populations in both L5Tx and sham groups of samples from three independent experiments (Fig. 3B and data not shown). CD8⁺ T lymphocytes and other types of leukocytes were not detectable at this time point. To confirm that the identification of CD3⁺CD4⁺ T lymphocyte was specific, CD4 KO mice were subjected to sham and L5Tx surgeries and the CD45^{hi} population from lumbar spinal cords 7 days post surgery was analyzed similarly. No CD3⁺CD4⁺ population was detected in CD4 KO mice (Fig. 3B, right panel).

To visualize the infiltrating CD4⁺ T lymphocytes, L5 spinal cord sections from three BALB/c mice at day 7

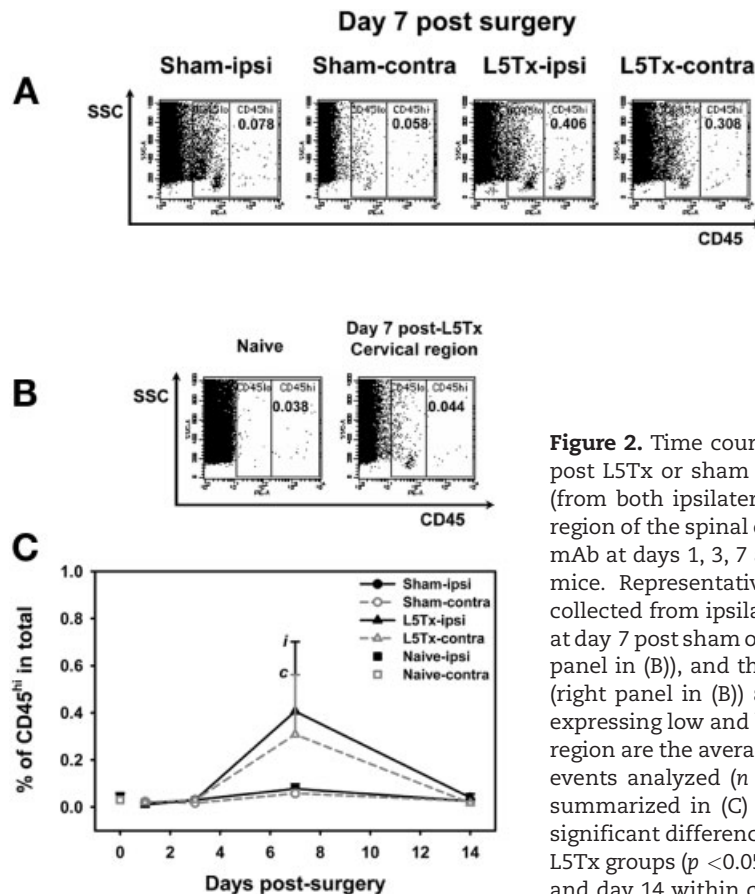


Figure 2. Time course of leukocyte infiltration into the lumbar spinal cord post L5Tx or sham surgery in BALB/c mice. Total lumbar spinal cord cells (from both ipsilateral and contralateral sides) and cells from the cervical region of the spinal cord were isolated and labeled with anti-mouse CD45-PE mAb at days 1, 3, 7 and 14 post sham or L5Tx surgery in adult male BALB/c mice. Representative side scatter (SSC) vs. CD45 plots for total events collected from ipsilateral (ipsi) or contralateral (contra) lumbar spinal cords at day 7 post sham or L5Tx operation (A), naive lumbar spinal cord tissue (left panel in (B)), and the cervical region of the spinal cord on day 7 post L5Tx (right panel in (B)) are presented. CD45^{lo} and CD45^{hi} indicate populations expressing low and high levels of CD45, respectively. Numbers in the CD45^{hi} region are the average percentages of the CD45^{hi} population within the total events analyzed ($n = 2-5$ per group). Time course of CD45^{hi} expression is summarized in (C) and data are presented as mean \pm SEM. "i" indicates significant differences between day 7 and days 1, 3 and 14 within ipsilateral L5Tx groups ($p < 0.05$); and "c" indicates significant difference between day 7 and day 14 within contralateral L5Tx groups ($p < 0.05$).

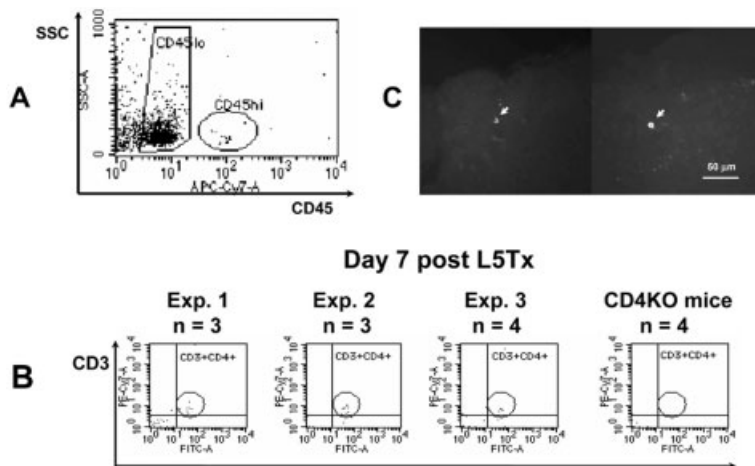


Figure 3. Phenotype of lumbar spinal cord-infiltrating T lymphocytes 7 days post L5Tx in BALB/c mice. Mononuclear cells were isolated from pooled lumbar spinal cord tissue (three or four mice per group) at day 7 post sham or L5Tx surgery in adult male BALB/c mice. The CD45^{hi} population was first identified as shown in (A), and then was analyzed in CD3 vs. CD4 dot plots. Results from three independent experiments are presented in (B) ("n" indicates numbers of animal used for each group; circle indicates CD3⁺CD4⁺ population). Similar samples were also obtained from CD4 KO mice that underwent L5Tx to ensure the specific identification of CD3⁺CD4⁺ T lymphocytes (far right panel in (B)). Fluorescent immunohistochemical staining for CD4 was performed in the L5 lumbar spinal cord of mice 7 days post L5Tx. CD4⁺ cells with typical T lymphocyte morphology were identified mostly in the ipsilateral dorsal horn region. Representative photomicrographs of CD4⁺ T lymphocytes in the ipsilateral dorsal horn region (arrows) are shown in (C) (40×, scale bar = 50 μm).

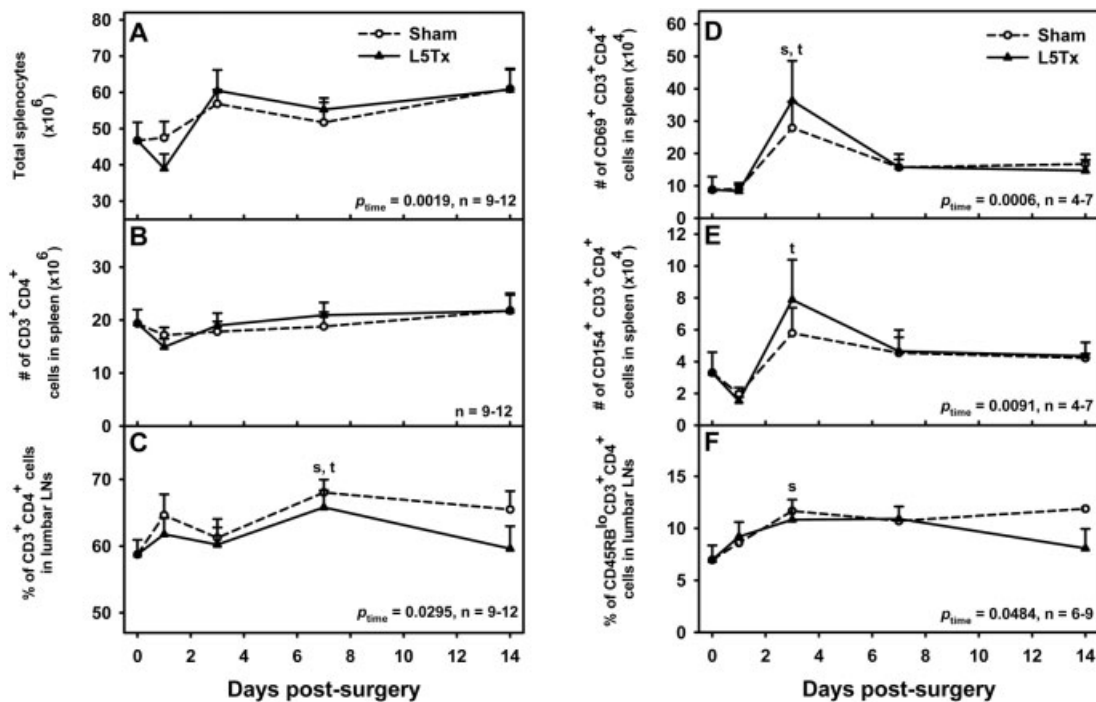


Figure 4. Changes in CD4⁺ T lymphocytes in the peripheral lymphoid organs post L5Tx or sham surgery in BALB/c mice. Total cells isolated from spleen and lumbar LN of sham- and L5Tx-operated mice were labeled with mAb and analyzed via FACS. Changes in the numbers of total splenocytes (A), numbers of splenic CD4⁺ T lymphocytes (CD3⁺CD4⁺) (B), and percentages of CD4⁺ T lymphocytes in the lumbar LN (C) after surgeries are presented in the left column. Activation of splenic and LN CD3⁺CD4⁺ cells were further determined through their surface expression of CD69, CD154 and CD45RB. Representative data are shown in (D–F) in the right column. Numbers of animals per group are indicated in each graph. Data are presented as mean ± SEM and "s" or "t" denote significant differences between the selected time and day 0 within sham and L5Tx groups, respectively (*p* < 0.05).

post L5Tx were labeled with anti-CD4 mAb via fluorescence immunohistochemistry. CD4⁺ cells with typical T lymphocyte morphology were observed mostly in the ipsilateral dorsal horn region (representative photomicrographs are shown in Fig. 3C). Although some CD4⁺ cells were also seen in the ventral horns of both the ipsilateral and contralateral side of L5 spinal cord (no evident difference in numbers), no CD4⁺ cells were detected in the contralateral side of the dorsal horn following peripheral nerve injury.

Peripheral CD4⁺ T lymphocyte responses

CD4⁺ T lymphocytes in the spleen and local draining lumbar lymph nodes (LN) in sham and L5Tx mice were analyzed to determine whether there were any associated changes in the periphery. Total numbers of splenocytes, but not splenic CD4⁺ T lymphocytes, were increased with time post L5Tx or sham surgery, with the peak level at day 3 [Fig. 4A, B; in (A) two-way ANOVA $p_{\text{time}} = 0.0019$]. The percentage of CD4⁺ T lymphocytes in lumbar LN significantly increased and peaked at day 7 post both surgeries (Fig. 4C; two-way ANOVA $p_{\text{time}} = 0.0295$). Further, in both spleen and lumbar LN, a slight but significant activation of CD4⁺ T lymphocytes was detected after both surgeries as indicated by increased surface expression of CD69 and CD154 (Fig. 4D, E; two-way ANOVA, in (D) $p_{\text{time}} = 0.0006$, and in (E) $p_{\text{time}} = 0.0091$) for splenic cells, and of CD45RB^{lo} for both splenic (data not shown, two-way ANOVA, $p_{\text{time}} = 0.0003$) and LN cells (Fig. 4F; two-way ANOVA, $p_{\text{time}} = 0.0484$). However, no differences between L5Tx and sham surgery were identified in all cases. Additionally, no differences between L5Tx and sham surgeries in the percentages of other subtypes of leukocytes (CD8⁺ T lymphocytes (CD3⁺CD8⁺), B lymphocytes (CD3⁻CD19⁺), NK cells (CD3⁻panNK⁺), and macrophages (CD3⁻CD11b⁺I-A^{d+})) were detected in the spleens at day 7 post surgery (data not shown).

Behavioral mechanical sensitivity of CD4 KO BALB/c mice post L5Tx

To further determine the role of CD4⁺ T lymphocytes in L5Tx-induced neuropathic pain, adult CD4 KO BALB/c mice, which lack functional CD4⁺ T lymphocytes, were subjected to L5Tx or sham surgery. All mice were baseline tested before surgery and repeatedly tested at days 1, 3, 5, 7, 11, 14, 18 and 21 post surgery for their mechanical sensitivity using the Up-Down method as described in *Materials and methods*. No significant baseline behavioral difference between WT and CD4 KO mice was detected. Although L5Tx induced similar mechanical hypersensitivity in both CD4 KO mice and WT mice initially (days 0–5), CD4 KO mice displayed

significantly reduced mechanical hypersensitivity at a later phase (after day 7) post nerve injury (Fig. 5A; two-way ANOVA analysis: $p_{\text{time}} \times \text{surgery} < 0.0001$; $p_{\text{time}} < 0.0001$, and $p_{\text{surgery}} < 0.0001$). CD4 KO mice adoptively transferred with 3×10^6 negatively selected CD4⁺ leukocytes showed heightened mechanical sensitivity compared to CD4 KO mice without transfer (Fig. 5B; two-way ANOVA analysis: $p_{\text{time}} \times \text{treatment} < 0.0206$; $p_{\text{time}} < 0.0271$, and $p_{\text{treatment}} < 0.0001$). Similarly, CD4 KO mice receiving 20×10^6 mixed splenic and LN leukocytes also showed increased behavioral sensitivity compared to CD4 KO mice without transfer (data not shown).

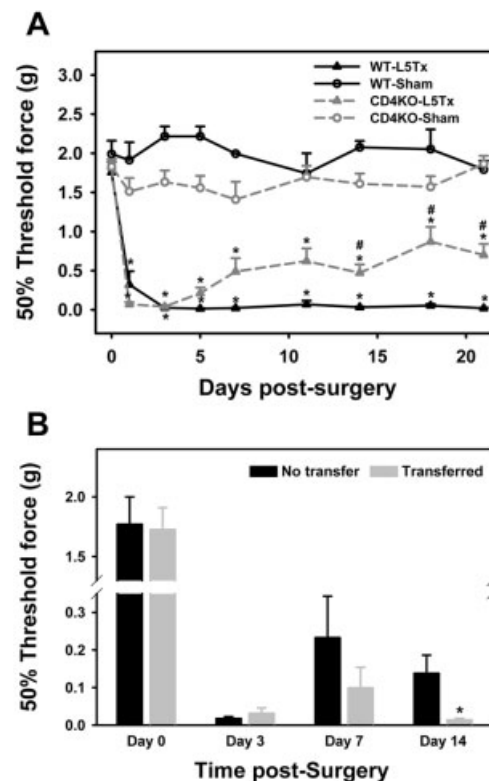


Figure 5. Mechanical sensitivity of CD4 KO BALB/c mice post L5Tx or sham surgery. (A) Adult male and female CD4 KO BALB/c mice ($n = 18$ per group) and male BALB/c WT controls ($n = 7$ per group) were subjected to L5Tx or sham surgery. Mechanical sensitivity was tested with von Frey filaments using the Up-Down method before and after surgery. Mechanical sensitivity was presented as the 50% threshold force for each animal (mean \pm SEM). * indicates significant differences between L5Tx- and sham-operated mice within CD4 KO or WT groups ($p < 0.05$). # indicates significant differences between CD4 KO and WT mice subjected to the same surgery ($p < 0.05$). (B) Mechanical sensitivity is shown in CD4 KO mice (black bar, $n = 4$) and CD4 KO mice adoptively transferred with CD4⁺ leukocytes (gray bar, $n = 6$) subjected to L5Tx at different times after surgery. Results of behavioral tests are presented as described in (A). * indicates significant differences between non-transferred group and CD4⁺ leukocyte-transferred group ($p < 0.05$).

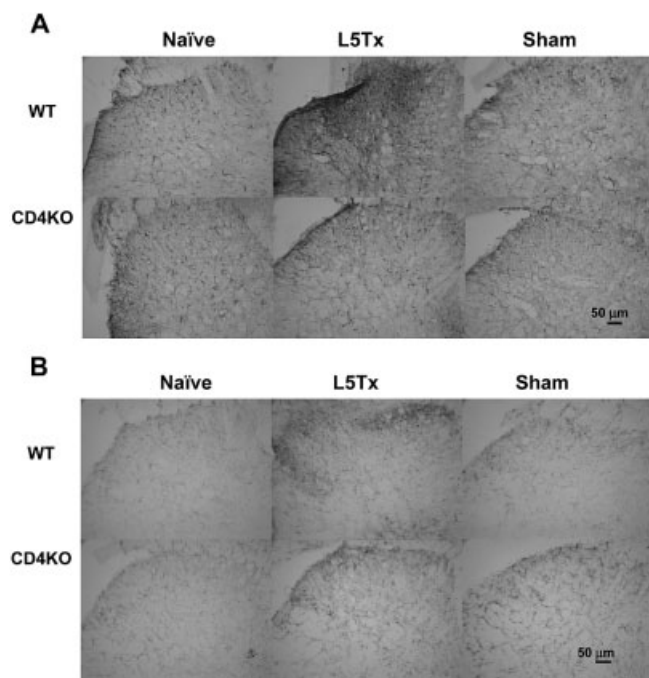


Figure 6. Glial responses in the L5 lumbar spinal cord dorsal horn post L5Tx or sham surgery in WT and CD4 KO BALB/c mice. L5 lumbar spinal cord sections were prepared from both WT and CD4 KO mice 7 days post L5Tx or sham surgery and from naive mice (two to three mice per group). Immunohistochemical staining for (A) GFAP (astrocyte marker) and (B) CD11b (microglial marker) were performed separately. Representative photomicrographs of the ipsilateral dorsal horn region of L5 lumbar spinal cord are shown here (20 \times , scale bar = 50 μ m).

Lumbar spinal cord glial responses post L5Tx

It has been shown that glial activation post L5Tx plays a key role in L5Tx-induced behavioral hypersensitivity [3]. Therefore, we examined astrocytic and microglial responses at day 7 post L5Tx in both WT and CD4 KO mice *via* immunohistochemistry. Naive, L5Tx- or sham-operated WT and CD4 KO mice were used ($n = 2$ for both WT and CD4 KO naive groups, and sham-operated CD4 KO group; $n = 3$ for both WT and CD4 KO L5Tx groups, and sham-operated WT group). Anti-glial fibrillary acidic protein (GFAP) and anti-CD11b antibodies were used for labeling astrocytes and microglia, respectively. We focused on the ipsilateral dorsal horn region, which previously showed consistent and significant increases in the immunoreactivity of both GFAP and CD11b post L5Tx [12]. As predicted, L5Tx induced significantly increased immunoreactivity of both GFAP and CD11b in the ipsilateral L5 dorsal horn region in the WT BALB/c mice (Fig. 6A and B top panel). Similarly, significantly increased expression of CD11b in CD4 KO mice was also observed, while no significant increase in GFAP expression was detected in CD4 KO mice at the same time (Fig. 6A and B lower panel).

Discussion

It has been clearly demonstrated that nerve injury-induced behavioral hypersensitivity can be divided into at least two phases: an initiation phase (from day 0 to \sim day 5) and a maintenance phase (after \sim day 5) based on the pattern of nociceptive behaviors and the animals' responses to selective drug treatments [13–15]. The initiation phase may be more reflective of an acute pain mechanism that transfers to a chronic phase if unabated. Our results demonstrate a contributing role of CNS-infiltrating CD4⁺ T lymphocytes in maintaining tactile hypersensitivity following peripheral nerve injury. No differences in peripheral CD4⁺ T lymphocyte responses between L5Tx and sham surgery groups were observed. Our previous studies of MHC class II KO mice, which have decreased mature CD4⁺ T lymphocytes and showed reduced L5Tx-induced hypersensitivity [7], support the current findings.

CD4⁺ T lymphocytes continuously circulate through the CNS to provide immunological surveillance [16]. However, CD4⁺ T lymphocytes are also linked to various CNS pathological conditions. CD4⁺ T lymphocyte-mediated autoimmune responses are vital in the pathogenesis of multiple sclerosis and, to some extent, Alzheimer's disease [17, 18]. Alternatively, CNS-infiltrating activated CD4⁺ T lymphocytes promote facial motoneuron survival following axotomy injury [19], possibly *via* their brain-derived neurotrophic factor (BDNF) production [20]. The report that intraperitoneal injection of *in vitro* driven type 1 helper T (Th1) lymphocytes induced nociceptive hypersensitivity post CCI in athymic nude rats ($rnu^{-/-}$), while type 2 helper T lymphocytes prevented the behavioral hypersensitivity in the heterozygous $rnu^{+/-}$ rats [8], provided initial evidence of the involvement of CD4⁺ T lymphocytes in neuropathic pain. We and others have also previously detected CD4⁺ cells in the lumbar spinal cord or dorsal root ganglia in animal models of neuropathic pain, which were thought to be mostly CD4-expressing macrophages/microglia [7, 21]. In the current study, we clearly detected CD3⁺CD4⁺ T lymphocytes in the lumbar spinal cord post L5Tx at day 7 post L5Tx *via* both FACS and immunohistochemistry. Our results from CD4 KO mice further confirm the contributing role of CD4⁺ T lymphocytes in the later phase of neuropathic pain progression. Interestingly, a recent report suggests that the presence of CD4⁺ T lymphocytes in the gut exhibits an antinociceptive effect in a mouse model of visceral pain, possibly through their release of endorphins [22]. Together with our current findings, these results highlight a complex role of the immune system, particularly CD4⁺ T lymphocytes, in pain modulation. Additionally, we had previously demonstrated macrophage-like cell infiltration at early time points after

peripheral nerve injury using radiation bone marrow-chimeric rats [4]. The disparity between that and our current study may relate to the differences in species (rats vs. mice) and method of detection (immunohistochemistry vs. FACS).

Multiple factors may be involved in regulating migration of CD4⁺ T lymphocytes into the CNS and their localization within the CNS. Differential expression in cytokine/chemokine receptor and/or adhesion molecules on infiltrating CD4⁺ T lymphocytes determines their ability to migrate into the CNS and their specific interactions with CNS-resident cells [23, 24]. Increased lumbar spinal cord IP-10 expression post L5Tx (unpublished data) would suggest a Th1 phenotype of the observed CNS-infiltrating CD4⁺ T lymphocytes, which is supported by the fact that adoptive transfer of Th1 cells produced hypersensitivity in nude rats following CCI [8]. High concentrations of extracellular glutamate have been known to induce neuronal damage and central nociceptive sensitization. Expression of both constitutive and inducible metabotropic glutamate receptors by T lymphocytes may promote neuron-T lymphocyte interactions in the CNS [25]. Antigen-specific interactions between CD4⁺ T lymphocytes and activated resident microglia may induce the formation of CNS immunosynapses [26] and further modulate T lymphocyte-mediated CNS adaptive immunity.

The specific mechanisms of how infiltrating leukocytes mediate neuropathic pain are unknown. We suspect that the CNS local environment (cellular and soluble factors, such as CNS glia and their secreted products) is actively involved in the actions of infiltrating CD4⁺ T lymphocytes and the resulting sequelae. The involvement of local factors also explains the long-lasting pro-nociceptive effects resulting from the relatively temporary infiltration of CD4⁺ T lymphocytes into the spinal cord. Our immunohistochemical study illustrated that the majority of infiltrating CD4⁺ T lymphocytes were observed in the dorsal horn region of the ipsilateral spinal cord, which is also the area showing the most significant activation of astrocytes and microglia post L5Tx [12]. It is reasonable to hypothesize that infiltrating CD4⁺ T lymphocytes interact with CNS local glia and induce further glial responses, such as production of pro-inflammatory mediators by glial cells. It has been well established that nitric oxide (NO), prostaglandin E₂ (PGE₂), the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , and chemokines can be linked to spinal sensitization and the pathogenesis of persistent pain state [27].

A recent publication by Clark *et al.* [28] reported that peripheral nerve injury led to the expression of the lysosomal cysteine protease cathepsin S (CatS) in activated microglia in the ipsilateral dorsal horn, with expression peaking at day 7 and remaining high on

day 14. CatS was shown to further release the soluble chemokine fractalkine, which in turn, initiated downstream pro-inflammatory responses. Since the maximal CD4⁺ T lymphocyte infiltration was observed at day 7, it is likely that CD4⁺ T lymphocyte-microglia interaction results in the microglial secretion of CatS and further pro-inflammatory responses in maintaining neuropathic behaviors. Future studies will be conducted to test this pathway.

The lack of a detectable significant difference in microglial CD11b expression between WT and CD4 KO mice only indicates that changes in multiple microglial factors (other than CD11b) may exist from CD4⁺ T lymphocytes-microglia interaction, *e.g.* Iba-1 or MHC class II expression. The observed differential immunoreactivity in GFAP between WT and CD4 KO mice is consistent with the concept that astrocytes have been associated with the maintenance of L5Tx-induced neuropathic pain [3]. Our previous observations of increases in the costimulatory molecule B7.2 and MHC class II in the lumbar spinal cord post L5Tx and significantly decreased pain behaviors post L5Tx in MHC class II KO mice [4, 6, 7] support the involvement of T lymphocyte-mediated adaptive immunity in pain.

Interestingly, Schwartz *et al.* have reported that passive administration of T lymphocytes specific to self antigens, at selected times, could promote the recovery from both post partial optic nerve crush injury [29] and post incomplete spinal cord injury [30] in rodents, supporting the concept of protective autoimmunity. Whether the observed CD4⁺ T lymphocytes in the spinal cord post L5Tx respond to specific CNS antigen(s) and whether this possible T cell specificity plays a role in maintaining behavioral hypersensitivity requires further investigation. Alternatively, we hypothesize that the T lymphocyte infiltration following nerve injury is a protective mechanism to promote neuronal survival but may also concomitantly induce neuronal sensitization.

The finding that CD4 KO mice did not display complete reversal of L5Tx-induced behavioral hypersensitivity indicates that L5Tx-induced neuropathic pain is partially modulated by CD4⁺ T lymphocytes. It has been established that activation of both astrocytes and microglia plays crucial roles in the etiology of neuropathic pain following nerve injury [3]. The finding that the numbers of both astrocytes and microglia in adult nude mice (13-wk) were significantly reduced (31 and 33%, respectively) compared to normal BALB/c mice in the CNS [31] may explain the further reduction of mechanical hypersensitivity in nude mice compared to CD4 KO mice post L5Tx and warrants further investigation. It is also possible that other non-immunological mutations in the nude mouse could be responsible for these differential changes in mechanical hypersensitivity. However, no other pain sensation/transmission-

related mutations (other than the difference in glial numbers mentioned above) in nude mice have been detected so far.

Finally, current treatments for neuropathic pain are not universally effective and are often associated with a myriad of side effects such as physical dependence, hyperalgesia, tolerance, and sedation. Innovative approaches to reduce CD4⁺ T lymphocyte infiltration by chemokine/cytokine inhibition or regulating interactions between infiltrating CD4⁺ T lymphocytes and their CNS targets may reduce CD4⁺ T lymphocyte-mediated CNS responses after nerve injury, and thus can be used in chronic neuropathic pain management.

Materials and methods

Animals

Male BALB/c mice (7–8 wk old) were purchased from Charles River (Wilmington, MA) or the National Cancer Institute (NCI, Frederick, MD). Male nude BALB/c mice (7–8 wk old) were purchased from NCI. These animals were allowed to habituate to the animal facility for at least 1 wk before being used in experiments. Breeding pairs for CD4 KO mice were obtained from Dr. William T. Lee of Wadsworth Center, New York State Department of Health, and maintained in the Dartmouth-Hitchcock Medical Center animal facility. Dr. Lee originally obtained homozygous BALB/c CD4 KO mice from Dr. Steve Reiner at the University of Pennsylvania who generated these mice from CD4 KO mice with C57BL/6/Sv129 background [32] (mice from the fifth backcross were used in this original publication). In Dr. Lee's laboratory, only occasionally (less than three times) BALB/c CD4 KO mice were backcrossed to BALB/c mice when the mouse line was getting low, and then they were inter-crossed regularly. The mouse line is randomly screened by PCR and FACS of peripheral blood. All mice used in the current experiments are from the first and second generations of breeding since the breeding pairs arrived at Dartmouth. Both male and female CD4 KO mice were used due to limited production of male mice. No significant behavioral differences in mechanical sensitivity were observed between sexes (data not shown). T lymphocyte deficiency in nude mice and CD4 deficiency in CD4 KO mice were further confirmed at the end of experiments through FACS analysis of blood leukocyte CD3 and CD4 expression, respectively. All mice were group-housed (3–4 per cage) with food and water *ad libitum*. All animals were maintained on a 12-h light/dark cycle. Mice were 8–9 wk old when used and about 21–25 g per mouse. The Institutional Animal Care and Use Committee (IACUC) at Dartmouth College approved all experimental procedures.

Spinal nerve L5 transection

Surgery was conducted following a previously published procedure [33]. Briefly, mice were anesthetized by inhalation of halothane (4% for induction and 2% for maintenance) in 100% O₂. To expose the spinal nerve L5, a 1–2-cm skin incision overlying the L4-S1 section was made and the muscle tissue

was separated and retracted from the left superior articular processes and the transverse process. The L6 transverse process was cleaned and the spinal nerves L4 and L5 were exposed. The L5 spinal nerve was gently separated from the L4 spinal nerve, lifted slightly, and transected with removal of a 1–2-mm segment of nerve (to prevent reconnection). The wound was irrigated with sterile saline, and closed using a 6-0 silk suture for the fascia and a 3-0 polyester suture for skin. Sham surgery was performed identically except that the L5 spinal nerve was not manipulated in any way following the partial laminectomy. Mice were randomly selected into either sham or L5Tx groups.

Assessment of mechanical sensitivity

The tactile sensitivity response was measured as the direct pressure stimulus required to elicit foot withdrawal in non-restrained conditions. All tests were conducted in the morning between 8:30 and 11:30 am. Animals were habituated to the testing apparatus for at least 40 min before testing. Each animal was subjected to the stimulation of a series of von Frey filaments (Stoelting, Wood Dale, IL) ranging from 0.008 to 2 g (log force 1.65, 2.36, 2.44, 2.83, 3.22, 3.84, 4.08, 4.17 and 4.31) using the Up-Down paradigm (detailed in [34]). The selected von Frey filament was pressed against the plantar surface of the ipsilateral (side of surgery) hind paw to the point of 30° bending for 3 s. Paw withdrawal response was considered as the positive response. The 50% threshold force needed for paw withdrawal was calculated for each mouse and used to represent the mechanical sensitivity of this animal. For each experiment, animals were previously baseline tested before L5Tx and tested at selected times post surgery. The person performing the behavioral tests was blinded to the experimental groups.

Sample preparation for flow cytometry

Mice were euthanized by CO₂ and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4; between 50 and 100 mL per mouse), and then the spinal cords, spleens and lumbar local LN were harvested. The ipsilateral and contralateral (relative to the injury) portions of the lumbar region of each spinal cord were further dissected in selected experiments. In some experiments (as described in the Results), a remote region of spinal cord (cervical section of spinal cord) was also collected.

To obtain total cells from spinal cord sections, spleen and LN, each tissue was homogenized and filtered through a 70-μm cell strainer (BD Biosciences, San Diego, CA). After cells were washed with PBS, they were ready for staining. Red blood cells in splenocytes were lysed with a lysing buffer containing ammonium chloride. Total splenocyte numbers were then determined using a hemacytometer (Fisher Scientific, Hampton, NH) with Trypan blue (Sigma, St. Louis, MO) before staining for FACS. All spinal cord and LN cells were collected and used for staining.

To obtain mononuclear cells from spinal cord tissue, discontinuous Percoll gradients were used following a published method [35]. Due to the limited numbers of mononuclear cells recovered from each tissue piece from a single animal, total cell suspensions prepared (as described

Table 1. Combinations of primary antibodies used for FACS

Experiment	section in text	Primary antibody combination
(i) Initial determination of lumbar spinal cord leukocyte infiltration	Section 2 of Results	anti-mouse CD45-PE
(ii) Phenotypic analysis of lumbar spinal cord-infiltrating and splenic leukocytes	Section 2 of Results and Section 3 of Results data not shown	Tube 1: anti-mouse CD45-APC-Cy7, anti-mouse CD3-perCP (or -PE-Cy7), anti-mouse CD4-FITC, anti-mouse CD8-PE Tube 2: anti-mouse CD45-APC-Cy7, anti-mouse CD3-perCP, anti-mouse panNK-FITC, anti-mouse CD19-PE Tube 3: anti-mouse CD45-APC-Cy7, anti-mouse CD3-perCP, anti-mouse CD11b-FITC, anti-mouse I-A ^d -PE
(iii) Phenotypic analysis of CD4 ⁺ T lymphocytes in spleen and lumbar LN	Section 3 of Results	Tube 1: anti-mouse CD4-APC-Cy7-5, anti-mouse CD3-PE-Cy7, anti-mouse CD4-FITC, anti-mouse CD45RB-biotin Tube 2 ^{a)} : anti-mouse CD45-APC-Cy7, anti-mouse CD3-PE-Cy7, anti-mouse CD4-FITC, anti-mouse CD154-PE, anti-mouse CD69-biotin

a) For LN, only tube 1 was used due to the limited numbers of cells available. All mAb were from BD Biosciences (San Diego, CA) except for anti-mouse CD69-biotin mAb which was from eBioscience (San Diego, CA).

above) from three to four mice were pooled together for processing. Briefly, after washing, the cell pellet was resuspended in 8 mL 40% Percoll [100% Percoll was prepared by mixing one part 10 × PBS with nine parts Percoll solution (Amersham, Piscataway, NJ)] in 15-mL conical tubes, and then centrifuged at 800 × g, 22°C for 30 min without breaking. After removal of the top layer of myelin debris and supernatant, the cell pellet was resuspended in 8 mL 70% Percoll and centrifuged again as above. The resulting mononuclear cell fraction (top layer) was carefully collected and cells were further washed twice with PBS containing 3% fetal bovine serum (FBS; HyClone, Logan, UT) followed by PBS. Total mononuclear cell numbers were determined using a hemacytometer with Trypan blue before staining for FACS.

Flow cytometry

Following the final wash with PBS in the cell preparation above, cells were resuspended in staining buffer (2% FBS and 0.09% NaN₃ in PBS) containing anti-mouse-CD16/CD32 antibody (1 µg/50 µL/tube), and incubated on ice for 30 min to block Fc receptors. Then, combinations of two to five fluorescent-labeled or biotinylated mAb were added into each tube and cells were incubated on ice for another 30 min. After washing twice with PBS, if a biotinylated primary antibody was used, the secondary reagent streptavidin-perCP (BD Biosciences) was added and cells were again incubated on ice for 30 min, and then washed twice with PBS. All stained cells were finally resuspended in 1% formaldehyde/PBS and stored at 4°C until analysis. The combinations of primary mAb used are shown in detail in Table 1. After the completion of staining, a total of 100 000 events (for total cells from lumbar spinal cord), up to 10 000 events (for spinal cord mononuclear cells and cells from lumbar LN) or 30 000 events (for

splenocytes) from each sample were analyzed using a FACSCanto flow cytometer with CellQuest software (BD Biosciences). Non-stained cells from the same tissue with the same preparation were included in each run as the negative control and reference for data analysis.

Leukocyte isolation and mouse adoptive transfer

Splenocytes and LN (axillary and inguinal) cells were aseptically isolated as described above from 7-wk-old female BALB/c mice. All cells were pooled together in sterile Dulbecco's PBS (DPBS) containing 2% FBS after the final wash at 1 × 10⁸ cells/mL. One portion of cells was kept on ice for later use. The remaining cells were subjected to further CD4⁺ cell isolation by a Robosep automated cell separator using the EasySep negative selection mouse CD4⁺ T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). Collected CD4⁺ cells and non-separated total leukocytes were resuspended in sterile PBS for adoptive transfer. CD4 KO mice (male and female) at 6–8 wks of age were used as recipient mice. CD4⁺ cells were intravenously (i.v.) injected at 3 × 10⁶ cells/200 µL per mouse, and total leukocytes were i.v. injected at 20 × 10⁶ cells/200 µL per mouse. Non-injected CD4 KO mice served as controls. CD4⁺ T cell contents in both types of injected cells were determined by FACS using anti-CD3-PE-Cy7 and anti-CD4-FITC mAb (BD Biosciences). CD4⁺ cell and CD3⁺CD4⁺ cell contents were at 34.31 and 24.93% in total leukocytes, respectively. CD4⁺ cell and CD3⁺CD4⁺ cell contents were at 96.58 and 74.80% in isolated CD4⁺ cells, respectively. Mice underwent L5Tx surgery on day 8 post transfer and were monitored for their mechanical sensitivity up to day 14 post surgery as described earlier. One or two mice from each transfer group were sampled on the day of surgery and upon the completion of

behavioral testing to verify the positive existence of CD4⁺ cells in peripheral blood, LN and spleen *via* FACS.

Immunohistochemistry

WT and CD4 KO mice subjected to L5Tx or sham surgery were deeply anesthetized by inhalation of isoflurane (4%) in 100% O₂. Mice were transcardially perfused with 0.1 M PBS (pH 7.4, ~100 mL per mouse) followed by 4% formaldehyde in 0.1 M PBS (between 100 and 150 mL per mouse). The L5 segment of lumbar spinal cord was harvested and cryo-protected in 30% sucrose for at least 72 h at 4°C. Tissue was then freeze-mounted in OCT embedding medium (Sakura Finetek, Torrance, CA) on cork blocks for cryostat sectioning. Immunohistochemistry was performed on 20- μ m L5 spinal cord sections mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Fluorescent immunohistochemistry for CD4 was conducted as previously described [4]. FITC-labeled anti-mouse CD4 antibody (BD Biosciences) was used at a 1 : 50 dilution. Slices were examined using an Olympus fluorescence microscope (U-ULH) (Olympus Optical Co., Ltd., Japan) with an Olympus Q-FIRE camera. Light level immunohistochemistry for glial cells was conducted using an avidin-biotinylated enzyme complex technique (Vectox Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) as the substrate, as described in [36]. Antibodies for CD11b (for microglia; from Serotec, Raleigh, NC) and GFAP (for astrocytes; from DAKO UK Ltd., UK) were used at 1 : 100 and 1 : 5000 dilution, respectively. Images of all sections were taken *via* an Olympus fluorescence microscope (U-ULH) (Olympus Optical Co.) with an Olympus Q-FIRE camera and then analyzed with SigmaScan Pro 5 (Aspire Software International, Ashburn, VA) for the total number of pixels in the ipsilateral dorsal horn region. Average numbers of pixels from three to four slices of each animal were used to represent the immunoreactivity of each animal.

Statistical analysis

All data were graphed with SigmaPlot 9.0 (Systat Software Inc., San Jose, CA) and analyzed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). For comparison of mechanical sensitivity and cellular phenotypes, appropriate two-way ANOVA with time and surgery as factors was performed followed by Bonferroni *post hoc* analysis. All data are presented as mean \pm SEM when applicable. $p < 0.05$ was considered as statistically significant.

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