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Analyses Of Spinal Cord Mononuclear Cells Following Spinal Nerve L5 Transection-Induced Neuropathic Pain In Wild Type, CD4 Knockout, And CD40 Knockout Mice

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Analyses of Spinal Cord Mononuclear Cells Following Spinal Nerve L5 Transection-Induced Neuropathic Pain in Wild Type, CD4 Knockout, and CD40 Knockout Mice

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by

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I. ABSTRACT

$CD4^+$ T cells and CD40, highly expressed in activated microglia, along with microglia themselves have been demonstrated to contribute to mechanical hypersensitivity in a murine model of neuropathic pain, spinal nerve L5 transection (L5Tx). This study investigated whether CD4 and CD40 mediate their effects by affecting spinal cord microglial responses and/or leukocyte infiltration into the spinal cord. L5Tx was performed on wild type (WT), CD4 knockout (KO), and CD40 KO mice. Mononuclear cells from the lumbar spinal cord were collected and the total number of microglia ($CD45^{lo}CD11b^+$) and infiltrating leukocytes ($CD45^{hi}$) were analyzed in a time course study via flow cytometry. In WT mice, L5Tx significantly increased the total number of microglial cells in the ipsilateral side of the lumbar spinal cord at day 3 and day 7 post-surgery. Similar changes in microglial numbers were observed in CD4 KO mice at day 7 post-L5Tx but not in CD40 KO mice. Post-L5Tx, WT mice displayed elevated numbers of infiltrating leukocytes in the ipsilateral side of the lumbar spinal cord. Only minimal increases in infiltrating leukocytes were found in CD4 KO and CD40 KO mice. The current data suggest CD40 may have greater involvement than CD4 in peripheral nerve injury-induced neuropathic pain.

II. INTRODUCTION

The quality of life for many across the globe has been severely impacted by neuropathic pain, one of the most debilitating manifestations of chronic pain. The prevalence rates of neuropathic pain from general population studies have ranged from estimates of 1-2% up to 8% worldwide (Smith and Torrance 2010). However, such pain is often left untreated or mistreated. As a result, long-term disability and depression are common as is the overuse of diagnostic services and procedures, hospitalizations, surgery, and inappropriate medication (DeLeo and Winkelstein 2002). Opioids such as methadone or morphine are often prescribed to temporarily alleviate pain sensations by binding to opioid-specific receptors in the central and peripheral nervous systems to inhibit nociceptive activity (DeLeo and Winkelstein 2002). While drugs like opioids are effective at reducing the perception of pain, they also are highly addictive and can cause severe withdrawal symptoms such as tremors, nausea, and anxiety. Tricylclic antidepressants (TCAs) such as amitriptyline, imipramine, and nortriptyline have also proved effective at reducing neuropathic pain symptoms (Gelder et al. 2005). However, in overdose, TCAs are cardiotoxic, prolonging heart rhythms and increasing myocardial irritability usually leading to death (Gelder et al. 2005). By using animal models, the biological mechanisms underlying neuropathic pain may be better examined to help develop efficient medicinal treatment options accompanied by less detrimental consequences.

II.1 Neuropathic Pain

To comprehend the features of neuropathic pain, it is first necessary to explain how pain is generated and identify its two main types (acute and chronic). The induction of nociception occurs when specific stimuli evoke a response activating specialized areas on nociceptive nerve terminals. The terminals encourage electrical impulse (or action potential) conduction, along the nociceptive fiber to the spinal cord dorsal horn (Milligan and Watkins 2009). The electrical signals then enter the brain from the spinal cord where the sensation may be perceived as a state of pain and expressed as either acute or chronic depending on the signals' duration. Acute pain is an essential defense mechanism warning against existing or imminent damage to the physiological functioning of the normal body system (Tsuda et al. 2005). In contrast, chronic pain is considered a reflection of atypical functioning of a pathologically altered nervous system, serving no established defensive or otherwise helpful function (Tsuda et al. 2005). The duration of chronic pain lasts longer than any identifiable continuous injury or inflammation (Wang and Wang 2003). One kind of chronic pain, identified as "neuropathic," is initiated by a primary lesion or dysfunction expressed in the nervous system (Backonja 2003). Neuropathic pain is often manifested when peripheral nerves are damaged through surgery, bone compression in cancer, diabetes, "channelopathies," and autoimmune diseases (Tsuda et al. 2005, Campbell and Meyer 2006). Common sensations reported by patients include those of burning, tingling, electric shock-like, or "pins and needles" (Galluzzi 2005). These feelings may develop due to an altered or inhibited pain perception pathway when a damaged nerve cannot properly send electrical

signals from the peripheral nervous system (PNS) to the central nervous system (CNS) and vice-versa. While nerve regeneration is often attempted, there is great risk the affected neurons will become sensitized and display low thresholds of excitability, thereby firing impulses more often. Several symptoms of this agonizing state include spontaneous pain, or pain independent of a stimulus; hyperalgesia, an increased response to noxious stimuli; and allodynia, a pain response to normally innocuous stimuli (Moalem et al. 2004). In several studies using the spinal nerve L5 transection (L5Tx) murine model of neuropathic pain, subjects displayed significant mechanical allodynia along with thermal and mechanical hyperalgesia for a duration of at least four weeks (Tanga et al. 2005, Cao et al. 2009a). By using this model to mimic neuropathic pain, it was found that inflammation in the CNS and resulting neuroimmune activation, proposed to be by way of glia and infiltrating leukocytes, could influence behavioral hypersensitivity at least in animals (DeLeo et al. 2004). Due to neuropathic pain's association with such a wide array of diseases and disorders, the study of the CNS's immunological responses to lesions/disruptions associated with neuropathic pain may provide the information required to develop improved avenues of treatment.

II.2 Microglia

When a nerve injury occurs, the resulting molecular and cellular changes may influence neuronal plasticity and anatomical reorganization throughout the peripheral and central nervous systems (Woolf and Salter 2000). An injured CNS may use inflammation, an inherent immune response, to detect and rapidly react to microbial

invasion or chemical/physical lesions (Muzio et al. 2007). Microglia, one type of glial cell derived from bone marrow, produce some of the first notable active immune responses (DeLeo and Yezierski 2001). These cells limit nerve damage in a manner similar to that of tissue macrophages as both cell types are derived from monocytes. Microglia initiate CNS tissue repair for the clearance of apoptotic cells and toxic debris such as free-floating myelin, amyloid fibrils, and fragmented neurons (Muzio et al. 2007).

Microglia exist in two functional states: surveillance and activation. Microglial cells in healthy tissue allow their cell bodies to remain at rest while their fine branching processes undergo continuous rebuilding (Hanisch and Kettenmann 2007). This limited motion is used to constantly scan their territory and prepare their receptors for adjacent neuronal/glial signaling (Hanisch and Kettenmann 2007). An influx of infectious microbes (possibly projecting a specific ligand that can bind to receptors such as P2X4 and toll-like receptor 4 (TLR4)), ATP released from dead cells, and serum factors leaking out into the extracellular environment resulting from a breakdown of the blood spinal cord/brain barrier or severe tissue damage (a disruption in signaling) may activate the microglia (Tsuda et al. 2003, Hanisch and Kettenmann 2007, Eroglu and Barres 2010). While surveillance microglia appear somewhat stationary, activated microglia are able to identify an injured site and accumulate there. Here, the microglia proliferate, phagocytose dead/dying cells and debris, express activation surface molecules (such as major histocompatibility complex (MHC) class II, CD11b, B7.2, and CD40), and secrete soluble proinflammatory cytokines and chemokines upon the activation of TLRs (Sweitzer et al. 2002, Cao and DeLeo 2008a, Zhu and Paul 2008, Cao et al. 2009a).

While each of these actions appears beneficial for the healing and protection of damaged tissue, there is growing evidence to suggest that continued microglial activation may be detrimental to the elimination of neuronal pain signaling.

II.3 CD40 and Neuropathic Pain

As microglia are activated in the CNS, they become antigen-presenting cells (APCs) and display elevated CD40 expression (Kornbluth 2000). CD40 is classified as a 48kD cell surface receptor in the tumor necrosis factor (TNF) receptor superfamily that is activated by CD40 ligand (CD154) (Grewal and Flavell 1998). Besides being present in microglial cell systems, CD40 is also expressed by B cells, macrophages, Langerhans cells, endothelial cells, and thymic epithelial cells (Togo et al. 2000). It is known that CD40-CD154 ligation activates a series of microglial signaling pathways that include the release of nitric oxide (NO), the expression of cyclooxygenase-2, the secretion of chemokines/cytokines (IL-10, IL-12, TNFα, IFNγ, MCP-1), and increased antigen presentation (Matyszak et al. 1999, Tan et al. 1999, Jana et al. 2001, D'Aversa et al. 2002, Okuno et al. 2004, Townsend et al. 2005). Increased CD40 expression is often observed in several CNS diseases including multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, and HIV-1 encephalitis (Togo et al. 2000, Cao et al. 2009a). Microglia expressing CD40 at sites of nerve damage are linked to the development of mechanical hypersensitivity in a neuropathic pain model (Cao et al. 2009a). However, the exact mechanism underlying such CD40 association is not well understood.

II.4 $CD4^+$ T Cells and Neuropathic Pain

In general, there are two major types of T cells, $CD4^+$ and $CD8^+$ T cells, with a ratio between these two groups at approximately 2:1. While T cells, like microglia, originate in bone marrow, they become mature in the thymus before leaving to assist in adaptive immunity (Schwarz and Bhandoola 2006). $CD4^+$ T cells are the center of the adaptive immune response. During an immune response, $CD4^+$ T cells differentiate into at least two subsets (type 1 and type 2 helper T cells) with different functional capabilities and cytokine profiles (Mosmann et al. 1986). $CD4^+$ T cells are able to help B cells make antibodies, influence the development of enhanced microbicidal activity by macrophages, recruit other white blood cells to sites of infection and inflammation, and, by producing proinflammatory cytokines and chemokines, coordinate extensive immune protection (Zhu and Paul 2008). In several studies it has been shown that while T cells, particularly $CD4^+$ T cells, attempt to gather more immunological support to respond to nerve injury, they actually contribute to the nociceptive hypersensitivity associated with neuropathic pain (Moalem et al. 2004, Cao and DeLeo 2008b, Costigan et al. 2009). In Moalem's study (2004), the systematic adoptive transfer of type 1 helper $CD4^+$ T cells (one sub-group of $CD4^+$ T cells) to nerve-injured nude rats reinstated allodynia. These results were further supported by Cao and DeLeo's study (2008b) showing that attenuated allodynia in CD4 KO mice could be restored through the systematic adoptive transfer of $CD4^+$ T cells.

In addition, following peripheral nerve injury, $CD4^+$ T cells tend to migrate toward areas of microglial activation suggesting a link between the infiltration of $CD4^+$ T

cells and microglia (Cao and DeLeo 2008b). It has been suggested that $CD4^+$ T cells are able to enter the CNS through the blood-spinal cord barrier using leukocyte extravasation, a process involving rolling and later tight adhesion by immune cells (Gordh et al. 2006, Costigan et al. 2009). These actions involve various proinflammatory cytokines and chemokines, eventually leading to endothelial transmigration (Costigan et al. 2009). Due to the presence of ligand CD154 on the activated $CD4^+$ T cells and their rapid trafficking into sites of degradation alongside activated microglia, it has been theorized that the CD40-CD154 interaction contributes to $CD4^+$ T cell and microglia immune signaling.

II.5 CD40-CD154 Interaction

The interaction of CD40 with its ligand CD154, a 34-39 kD surface protein primarily present on activated $CD4^+$ T cells, allows for the development of the acquired immune response, including both humoral and cell-mediated immune responses (Quezada et al. 2004). The CD40-CD154 interaction is associated with the release of several chemokines and cytokines which could potentially signal infiltrating leukocytes including T cells. The pathway's influence on neuropathic pain manifestation has not yet been thoroughly investigated. However, by determining the temporal and spatial relationship between $CD40^+$ microglia and infiltrating $CD4^+$ T cells in a nerve-damaged section of the CNS, it may help to identify key signaling components of the CD40-CD154 interaction that influence the progress of the chronic pain state.

II.6 Murine Models of Neuropathic Pain

Over the years, several models of neuropathic pain have been developed in rodents to mimic chronic pain that later provokes extensive behavioral hypersensitivity, as experienced by humans (Wang and Wang 2003). Both central and peripheral pain models have been shown to be effective in producing behaviors reflective of the neuropathic pain state. Peripheral nerve injury-induced neuropathic pain usually involves manipulation (nerve ligation (clamping), cryoneurolysis (freezing), and transection (severance)) of selected peripheral nerves such as sciatic or spinal nerves (Wang and Wang 2003).

The model of neuropathic pain utilized in the current study is the spinal nerve L5 transection (L5Tx). The L5Tx protocol is widely used based upon its inter-experimenter reliability through the surgical procedure. This model has been shown to produce robust mechanical and thermal hypersensitivity (including both allodynia and hyperalgesia) as well as activation of both astrocytes and microglia in the lumbar spinal cord (DeLeo and Winkelstein 2002, Sweitzer et al. 2002). Recently, it was demonstrated that there are significant increases of $CD40^+$ microglia and infiltrating $CD4^+$ T cells in the lumbar spinal cord post-L5Tx and that both microglial CD40 and $CD4^+$ T cells contribute to the maintenance of L5Tx-induced mechanical hypersensitivity, with an earlier involvement of microglial CD40 than $CD4^+$ T cells (Cao and DeLeo 2008b, Cao et al. 2009a).

III. HYPOTHESIS AND SPECIFIC AIMS

This research was designed to investigate the mediating effects of CD4 and CD40 on L5Tx-induced microglial responses and leukocyte infiltration in the lumbar spinal cord. It was hypothesized that L5Tx significantly increases the numbers of microglia and infiltrating T cells in the ipsilateral side of the lumbar spinal cord post-L5Tx in wild type (WT) mice, while reduced numbers of microglia and/or infiltrating leukocytes would be observed in CD4 KO and CD40 KO mice post-L5Tx. The hypothesis was tested through two specific aims: 1) To determine total numbers of microglia present in the lumbar spinal cord over a specific time period post-L5Tx in WT, CD40 KO, and CD4 KO mice; and 2) To determine total numbers of infiltrating leukocytes present in the lumbar spinal cord of these genotypes over a specific time interval post-L5Tx. In addition, both CD40 and CD4 play their roles primarily in the maintenance phase of neuropathic pain in the L5Tx model of neuropathic pain (Cao and DeLeo 2008b, Cao et al. 2009a). The involvement of microglia interacting with infiltrating leukocytes in the development of neuropathic pain can be further elucidated from this study.

IV. METHODS

IV.1 Experimental Design

During previous studies, increased microglial CD40 surface molecule expression was observed at day 3 and day 7 post-L5Tx and the most significant leukocyte infiltration was seen to occur on day 7 post-L5Tx with $CD4^+$ T lymphocytes as the main infiltrating type of cell (Cao and DeLeo 2008b, Cao et al. 2009a). In the current study, mice of three genotypes (WT, CD40 KO, and CD4 KO) were used. Three to five animals were randomly divided into either the L5Tx group or the sham surgery group. At selected times (days 0, 1, 3, 7, and 14 post-surgery), lumbar spinal cord mononuclear cells were harvested, categorically pooled, and analyzed via flow cytometry using monoclonal antibodies (mAbs) against CD45 and CD11b. CD45, a protein tyrosine phosphotase, regulates sarcoma kinases required for T and B cell receptor signal transduction. It is expressed by all types of leukocytes, including microglia (Kung et al. 2000). It has been established that CD45 can be used as a marker to distinguish infiltrating leukocytes from the CNS resident, monocyte-derived microglia by flow cytometry, with $CD45^{hi}$ (high level of CD45 expression) representing infiltrating leukocytes and $CD45^{lo}$ (low level of CD45 expression) indicating microglia (Badie and Schartner 2000, Ford et al. 1995, Sedgwick et al. 1991). CD11b, a component of complement receptor 3 (CR3), is an antigen characteristic of mature bone marrow-derived myeloid cells including microglia (Prakash et al. 1998). Thus, in this study, a low level of CD45 expression and positive expression of CD11b ($CD45^{lo}CD11b^+$) together are used to identify microglia and a high level of CD45 expression ($CD45^{hi}$) is used to identify infiltrating leukocytes (Figure 1).

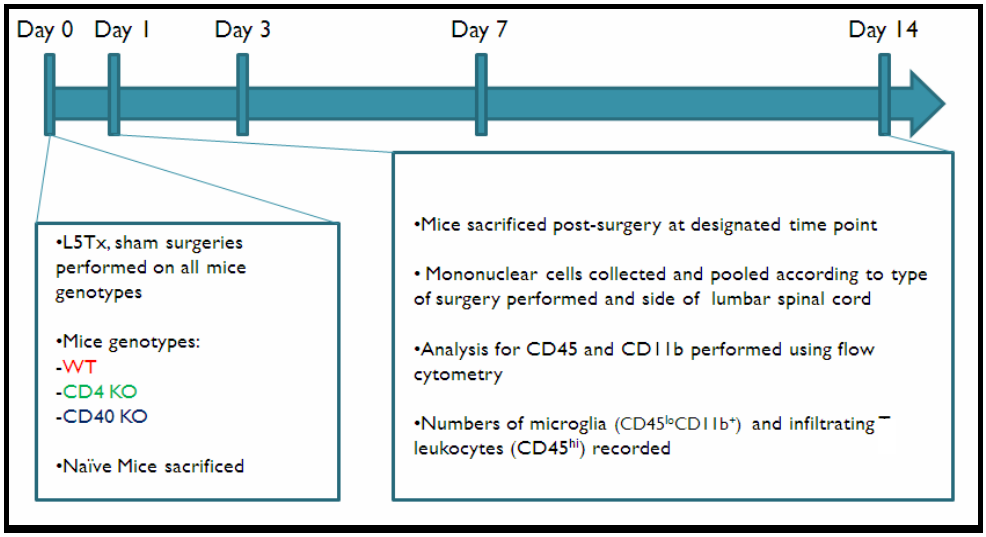

Figure 1. Experimental Design (Please see text for detail).

IV.2 Animals

Mice were housed in groups of three, four, or five according to sex in standard isolation cages with food and water available *ad libitum*. Cages were changed once a week and after each surgery to avoid contamination and prevent infection of surgical wounds. The Institutional Animal Care and Use Committee (IACUC) at UNE approved all experimental procedures used in this research. In this study, three types of mice were used. Adult WT BALB/c mice were purchased from the National Cancer Institute (NCI, Frederick, MD, USA). The animals were allowed to habituate for at least one week in the animal facility before use in experimentation. Breeding pairs for BALB/c CD4 KO mice

were originally obtained from Dr. William Lee in the Wadsworth Center of the New York State Department of Health, bred in the Dartmouth-Hitchcock Medical Center, and are currently maintained in the UNE animal facility. Breeding pairs for BALB/c CD40 KO mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in the animal facility at UNE. Mice were 8-10 weeks old upon the initiation of each experimental procedure. Both CD4 KO and CD40 KO mice are immunocompromised due to the loss of molecules critical in eliciting effective adaptive immune responses. In particular, CD4 KO mice lack mature $CD4^+$ T cells (Ganta et al. 2004). However, possible changes in the numbers of CNS glial cells (including both microglia and astrocytes) have not been investigated previously. Both male and female mice were used throughout the current study. In previous studies, no differences in L5Tx-induced mechanical hypersensitivity were observed between the sexes in WT, CD4 KO, or CD40 KO mice (Cao and DeLeo 2008b, Cao et al. 2009a). Also, at the basal level, there were no differences in mechanical sensitivity among these mice.

IV.3 Spinal Nerve L5 Transection (L5Tx)

Three to five WT, CD40 KO, or CD4 KO mice were randomly selected into L5Tx surgery, sham surgery, and naïve (no surgery) groups. L5Tx and sham surgeries were performed, as previously described by Cao et al. (2008b), using aseptic techniques. The animals were anesthetized with isoflurene (4% for induction and 2.5% for maintenance) delivered in oxygen at a flow rate of 70 vol/min. Prior to the surgery for each mouse, the hair of the lower back was removed and the area was cleaned with betadine. A 1-2 cm

long incision was made along the mid-line of the back over the L5-L6 vertebra area. Muscle fibers were pulled away from the L6 transverse process and both spinal nerves L4 and L5 were exposed. L5 was transected and a 0.5-1 mm piece of the severed L5 was removed to prevent the nerve from reconnecting. For mice undergoing sham surgery, the L5 was only exposed and not transected. The incision was flushed with sterile saline before closure. The fascia and muscle layers were sutured using the soft silk 6-0 suture. The skin was closed with 3-0 sutures.

IV.4 Lumbar Spinal Cord Mononuclear Cell Preparation

Prior to euthanization, the animals were massed and inspected for any unexpected abnormalities that may have developed following surgery or while held in the animal facility. Mice were euthanized by CO_2 asphyxiation and transcardially perfused with a 0.1 M phosphate-buffered saline (PBS, pH 7.4; between 50 and 150 ml per mouse). After decapitation, the spinal cord was harvested from each individual mouse. The lumbar enlargement portion of the spinal cord (L4-L6) was isolated and separated into ipsilateral and contralateral (relative to injury) segments. Lumbar spinal cord mononuclear cells were further prepared according to a previously published method (Cao and DeLeo 2008b). Lumbar spinal cord pieces from 3-5 animals (same treatment and same side of the lumbar spinal cord) were pooled together, homogenized in PBS, filtered through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA) and pelleted through centrifugation. In order to obtain mononuclear cells from the spinal cord tissue, Percoll gradients of 40% and 70% were used (800 g at 24°C for 40 minutes, without braking).

Cells below the 40% Percoll layer and above the 70% Percoll layer were collected. The total cell number of each sample was determined by using a hemocytometer with Trypan Blue (Sigma, St. Louis, MO, USA) before staining for flow cytometric analysis.

IV.5 Flow Cytometry

The collected mononuclear cells were labeled with mAbs for flow cytometric analysis following a published procedure (Cao and DeLeo 2008b). To prevent non-specific binding, cell surface Fc receptors were blocked with staining buffer (2% fetal bovine serum and 0.09% NaN_3 in PBS) containing anti-mouse-CD16/CD32 (2.4G2, BD Biosciences). After 30 minutes of incubation, combinations of fluorescence-labeled mAbs: APC-anti mouse CD45 (clone 30-F11 eBiosciences) and PE-anti mouse CD11b (clone M1/70 eBiosciences) were added to each tube. The cells were then incubated on ice for another 30 minutes. The cells were washed twice with PBS and centrifuged at 2000 g at 4°C for 5 minutes each. All labeled cells were re-suspended in 1% formaldehyde/PBS and kept on ice until analysis. All samples were analyzed using an Accuri C6 flow cytometer with CFlow software (Accuri Cytometers Inc., Ann Arbor, MI, USA). Non-stained cells were included in each run as controls. All files collected with the flow cytometer were further analyzed using the FlowJo 7.6 (tree Star, Sanford, NJ, USA). During data analysis, total cell population was identified based on the FSC (forward scatter) vs. SSC (side scatter) plot of each sample (Figure 2 top panel). FSC is mostly diffracted light from the cell and is proportional to cell-surface area or size; SSC is mostly refracted and reflected light and is proportional to cell granularity or internal

complexity. As described in section IV.1 and in previous studies (Cao and DeLeo 2008b, Cao et al. 2009a), cells with low levels of CD45 expression and positive for CD11b ($CD45^{lo}CD11b^{+}$) were identified as microglial cells (Figure 2 lower left), and cells with high levels of CD45 expression ($CD45^{hi}$) were identified as infiltrating leukocytes (Figure 2 lower right). For each sample, the total numbers of microglial cells and infiltrating leukocytes were further calculated based on the respective percentages of these populations within the total cells of each sample (obtained via flow cytometric analysis) and the total number of cells of each sample (obtained via counting using a hemocytometer – See Section IV.4).

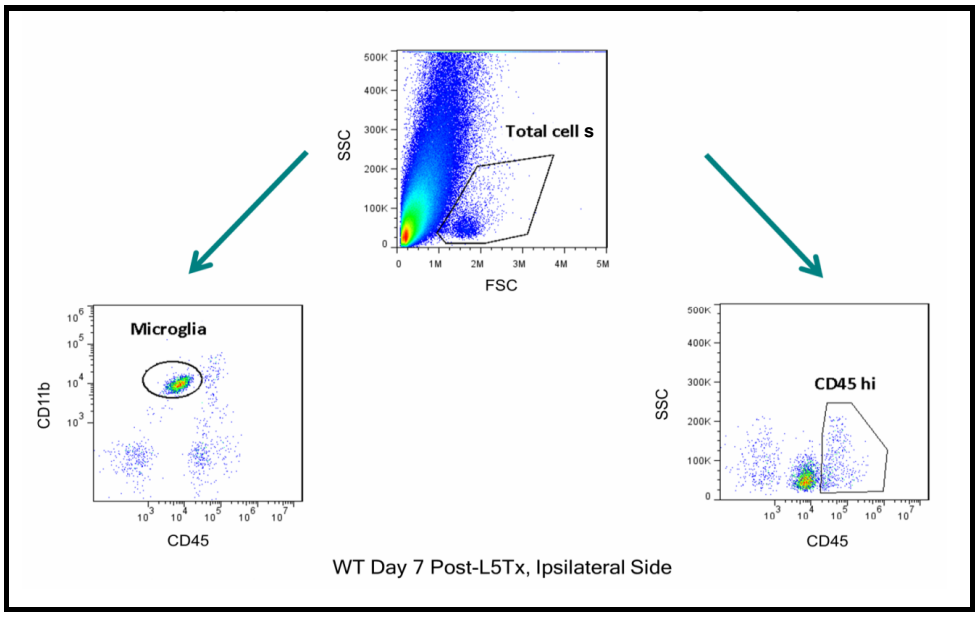

Figure 2. Phenotypes of Lumbar Spinal Cord Microglia and Infiltrating Leukocytes.

Mononuclear cells were isolated from pooled lumbar spinal cord tissue (3-5 mice per group) at days 0, 1, 3, 7, and 14 post-sham or L5Tx surgery. Mononuclear cells were stained with mAbs, APC-anti mouse CD45 and PE-anti mouse CD11b to identify cell populations of interest via flow cytometric analysis. An example of how each sample was analyzed is shown in Figure 2 using a sample collected from the ipsilateral side of the lumbar spinal cord of WT mice at day 7 post-L5Tx. On the top, a typical FSC vs. SSC plot of each sample is shown and the total mononuclear cell population is identified as "Total cells". On the lower left side, "Total cells" are shown in the CD11b vs. CD45 plot and microglia ($CD45^{lo}CD11b^{+}$) are identified. On the lower right side, "Total cells" are shown in the SSC vs. CD45 plot and infiltrating leukocytes ($CD45^{hi}$) are identified.

IV.6 Statistical Analysis

The SigmaPlot 10.0 (Systat Software, San Jose, CA, USA) was used to graph the data and statistical analyses were performed with the SigmaStat 3.5 (Systat Software). One-way analysis of variance (ANOVA) was performed and followed by the Student-Newman-Keuls (SNK) post hoc test. All data are presented as mean ± SEM when applicable. Statistical significance was defined at $p < 0.05$.

V. RESULTS

V.1 Total Numbers of Microglia in the Lumbar Spinal Cord Post-L5Tx

Activated microglia have been shown to proliferate and migrate to areas of injury to help phagocytose debris and produce several bioactive molecules (Langmann, 2007). To begin investigating the role of CD4 (particularly, $CD4^+$ T cells) and CD40 in lumbar spinal cord microglial responses following L5Tx-induced neuropathic pain, an examination of total numbers of microglia in the lumbar spinal cord subsequent to both L5Tx and sham surgery in WT, CD4 KO, and CD40 KO mice was performed. Adult male and female BALB/c mice with different genotypes (WT, CD4 KO, or CD40 KO) were subjected to either L5Tx or sham surgery. Lumbar spinal cord mononuclear cells were harvested (pooled from 3-5 mice for each treatment) at specific time points post-surgery and analyzed via flow cytometry. Changes of the number of total mononuclear cells are shown in Figure 3. L5Tx induced a general trend of increase in the total mononuclear cell number at days 3 and 7 in WT mice, particularly in the ipsilateral side of the lumbar spinal cord. The data for the WT mice represent the average of at least three sets of experiments and no statistical significance was detected at this time. $CD45^{lo}CD11b^+$ populations were identified as microglial cells and the total number of microglia from each sample was calculated based on the total number of mononuclear cells collected from each sample (as illustrated in Figure 2 and detailed in Materials and Methods, and graphed in Figure 3). As expected, a distinct, brief increase in the total number of microglia in the ipsilateral side of the lumbar spinal cord was observed at day 3 (2079.78 ± 1383.30 cells) and day 7 (1707.71 ± 731.12 cells) post-L5Tx compared to

the mice subjected to the sham operation. Throughout the study, the sham surgery did not induce a detectable increase of microglial numbers (Figure 4). Preliminary statistical analyses with the data thus far from the WT mice using a two-way ANOVA and SNK post hoc test indicated a significant time effect ($p_{time} = 0.013$) and potential group difference ($p_{group} = 0.135$).

Due to the limited available numbers of CD4 KO and CD40 KO mice, only one complete experimental set for each of these KO mice was performed. The results suggest both CD4 and CD40 are involved in microglial responses following L5Tx. In CD4 KO mice, increased numbers of lumbar spinal cord microglia in the ipsilateral side were observed only at day 7, not day 3, post-L5Tx compared to the sham surgery (Figure 5). This elevation appeared to be comparable to that of WT mice at the same time (WT = 1707.71 ± 731.12 cells, CD4 KO = 3380.00 cells). While in CD40 KO mice, the total number of microglial cells appeared to be less in naïve mice (left side = 189.07 cells, and right side = 95.22 cells) compared to that of WT (left side = 481.50 ± 243.50 cells, and right side = 669.34 ± 302.86 cells) and CD4 KO (left side = 469.96 ± 444.48 cells, and right side = 255.78 ± 200.22 cells) mice. There was also no L5Tx-induced ipsilateral side-specific increase of total numbers of microglia (Figure 5). Further, unlike WT and CD4 KO mice, in CD40 KO mice, a trend of increase in the total number of lumbar spinal cord microglia in both ipsilateral and contralateral sides was noticed in animals subjected to L5Tx at day 7 and day 14 post-surgery.

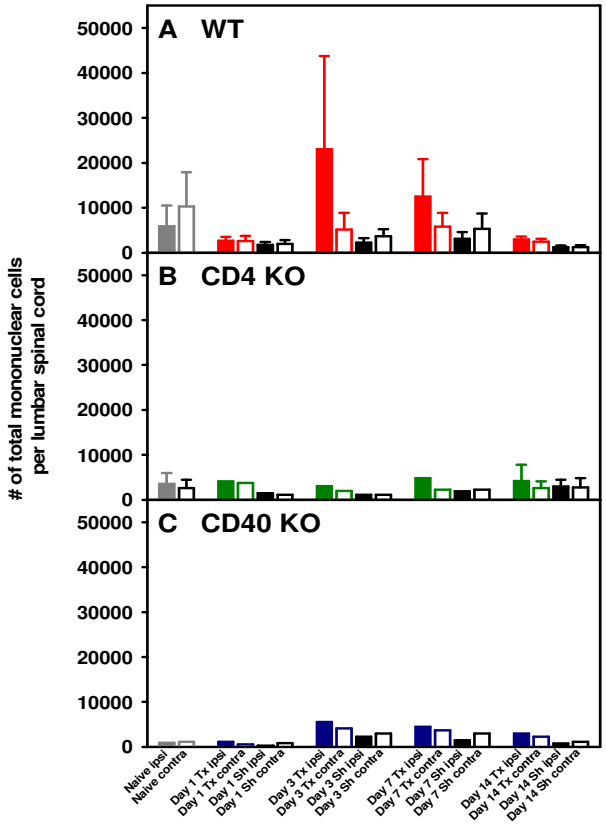

Figure 3: Total numbers of mononuclear cells in the lumbar spinal cord post-L5Tx in WT, CD4 KO, and CD40 KO mice. Mononuclear cells from pooled lumbar spinal cord samples (4 per treatment, per side) of each type of mice were collected and counted using a hemocytometer with trypan blue. Total numbers of mononuclear cells per lumbar spinal cord (mean ± SEM) are presented. In A, n = 3-4 per group; in B, for "Naïve" and "Day 14" groups, n = 2, and the rest, n = 1; and in C, n = 1 for all groups. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

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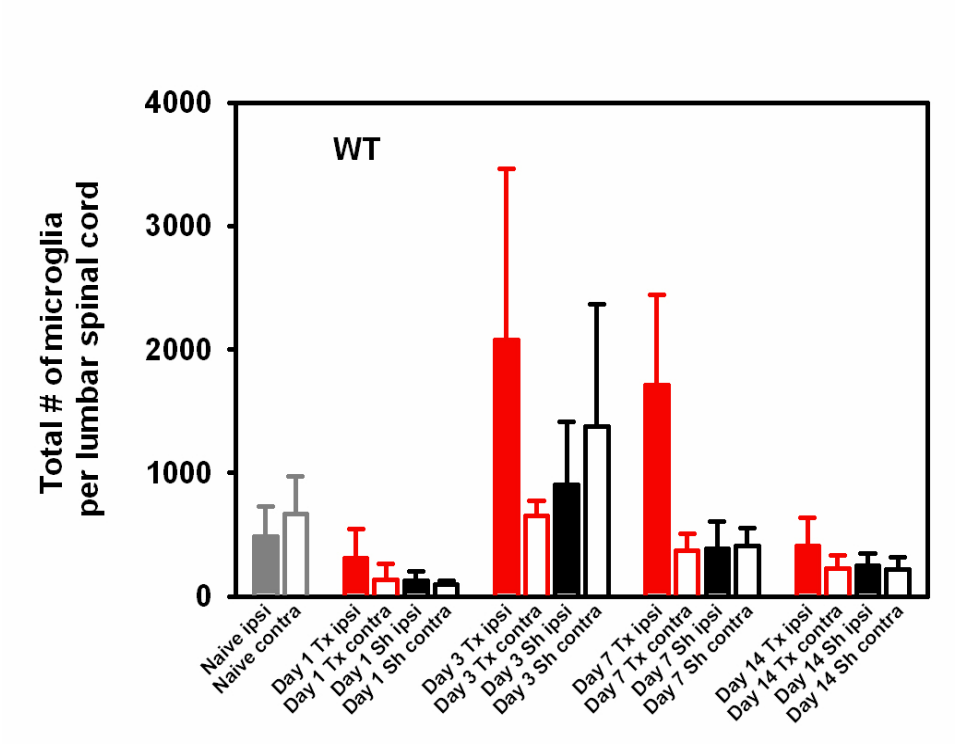

Figure 4: Total numbers of microglia in the lumbar spinal cord post-L5Tx in WT mice. Mononuclear cells from pooled lumbar spinal cord samples (4 per treatment, per side) of WT mice were collected and analyzed via flow cytometry using mAbs against CD45 and CD11b. A total of 4 independent experiments were performed and at least 3 complete sets of data were collected. Total numbers of microglia ($CD45^{lo}CD11b^{+}$) per lumbar spinal cord (mean ± SEM) are presented. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

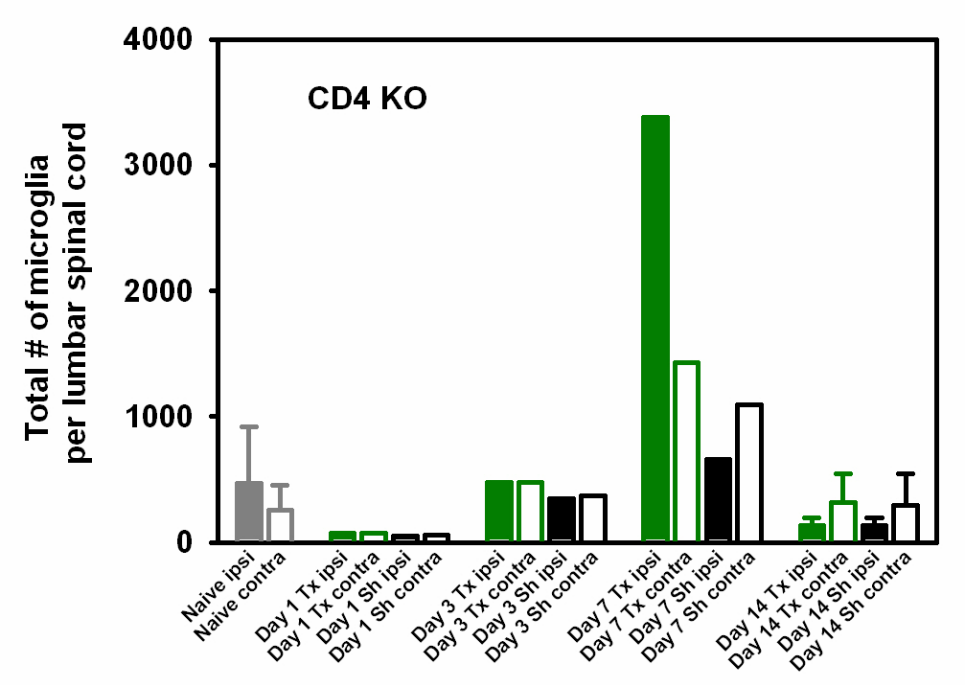

Figure 5: Total numbers of microglia in the lumbar spinal cord post-L5Tx in CD4 KO mice. Mononuclear cells from pooled lumbar spinal cord samples (3-5 per treatment, per side) of CD4 KO mice were collected and analyzed via flow cytometry using mAbs against CD45 and CD11b (for "Naïve" and "Day 14" groups, n = 2, and the rest, n = 1). Total numbers of microglia ($CD45^{lo}CD11b^{+}$) per lumbar spinal cord (mean ± SEM) are presented. Note that the same scale for the y-axis as that in Figure 4 is used in order to compare the data collected from mice with different genotypes. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

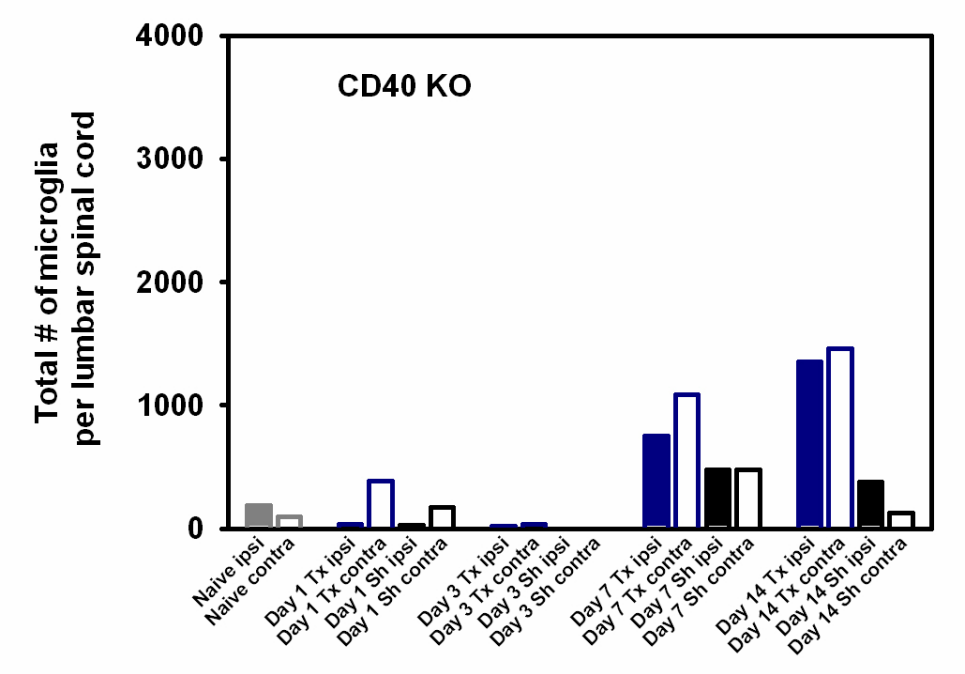

Figure 6: Total numbers of microglia in the lumbar spinal cord post-L5Tx in CD40 KO mice. Mononuclear cells from pooled lumbar spinal cord samples (3-5 per treatment, per side) of CD4 KO mice were collected and analyzed via flow cytometry using monoclonal antibodies against CD45 and CD11b (n = 1). Total numbers of microglia ($CD45^{lo}CD11b^{+}$) per lumbar spinal cord are presented. Note that the same scale for the y-axis as that in Figure 4 is used in order to compare the data collected from mice with different genotypes. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

V.2 Total Numbers of Infiltrating Leukocytes in the Lumbar Spinal Cord Post-L5Tx

Infiltrating leukocytes (primarily $CD4^+$ T lymphocytes and macrophages) have been detected in the lumbar spinal cord following peripheral nerve injury and these infiltrating leukocytes have been associated with the development of neuropathic pain (Cao and DeLeo 2008b, Costigan et al. 2009). To examine whether CD4 and CD40 are involved in the leukocyte infiltration of the lumbar spinal cord following L5Tx, an analysis of total numbers of lumbar spinal cord infiltrating leukocytes in WT, CD4 KO, and CD40 KO mice at selected times following either L5Tx or sham surgery was performed. Flow cytometric data collected from the same samples in the experiment, as described in V.1, were used in this investigation. $CD45^{hi}$ populations were identified as infiltrating leukocytes and the total number of infiltrating leukocytes from each sample was calculated based on the total number of mononuclear cells collected from each sample (as illustrated in Figure 2 and detailed in Materials and Methods, and graphed in Figure 3). In WT mice, a noticeable increase of infiltrating leukocytes was detected in the ipsilateral side of the lumbar spinal cord at days 3 and 7 post-L5Tx but not post-sham surgery (Figure 7). Preliminary statistical analyses with the data from WT mice using a two-way ANOVA and SNK post hoc test indicated potential time effect ($p_{time} = 0.115$) and group differences ($p_{group} = 0.084$). In contrast, no significant increase of the numbers of infiltrating leukocytes was observed at either day 3 or day 7 post-L5Tx in CD4 KO mice (Figure 8). Similarly, there is no detectable increase of leukocyte infiltration in CD40 KO mice post-L5Tx (Figure 9).

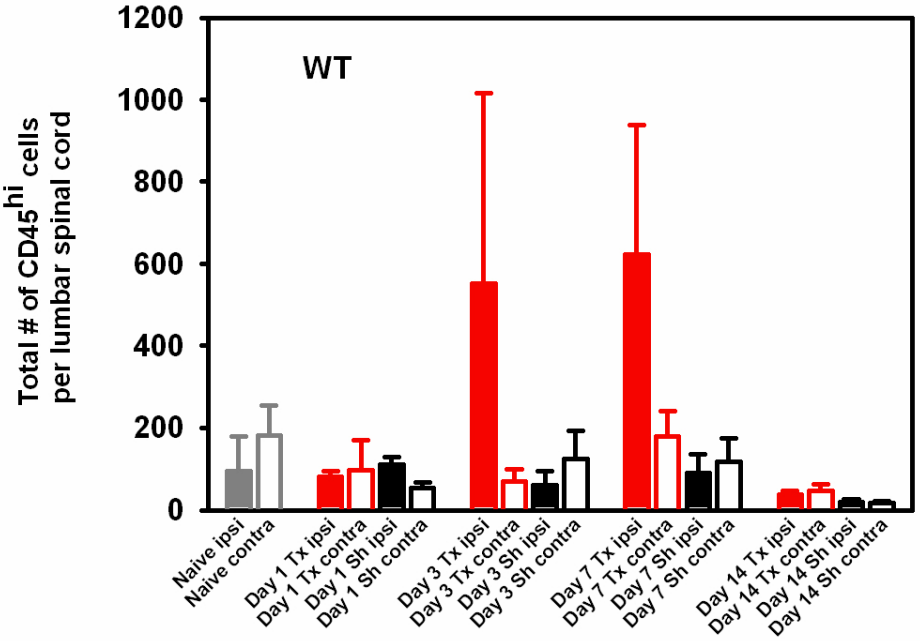

Figure 7: Total numbers of infiltrating leukocytes in the lumbar spinal cord post-L5Tx in WT mice. Mononuclear cells from pooled lumbar spinal cord samples (4 per treatment, per side) of WT mice were collected and analyzed via flow cytometry using mAbs against CD45 and CD11b. A total of four independent experiments were performed and at least 3 complete sets of data were collected. Total numbers of infiltrating leukocytes ($CD45^{hi}$) per lumbar spinal cord (mean ± SEM) are presented. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

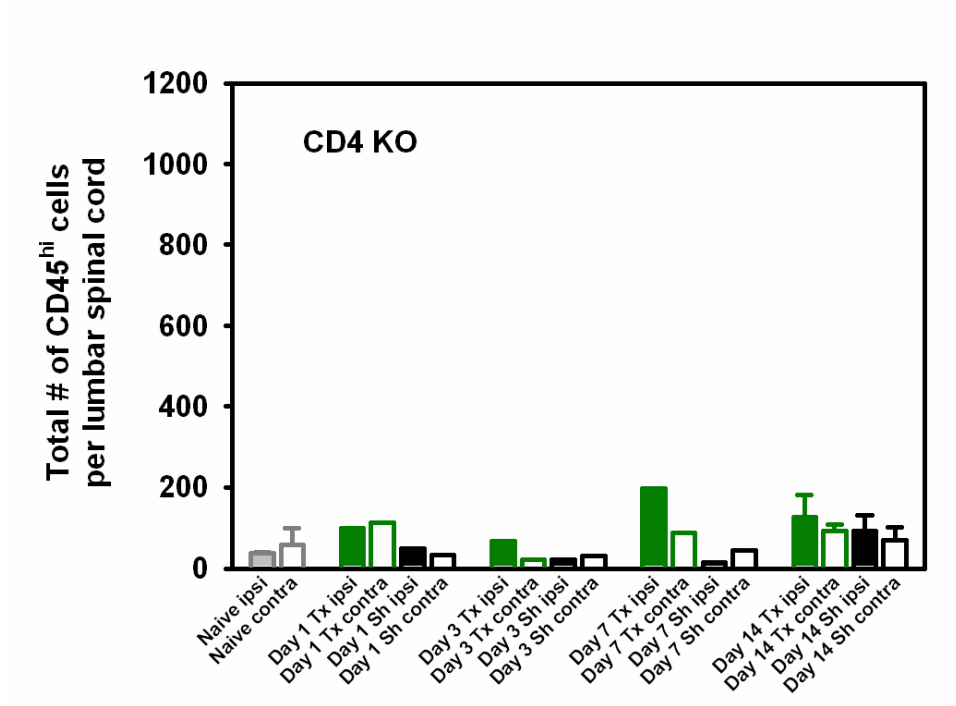

Figure 8: Total numbers of infiltrating leukocytes in the lumbar spinal cord post-L5Tx in CD4 KO mice. Mononuclear cells from pooled lumbar spinal cord samples (3-5 per treatment, per side) of CD4 KO mice were collected and analyzed via flow cytometry using mAbs against CD45 and CD11b (for "Naive" and "Day 14" groups, n = 2, and the rest, n = 1). Total numbers of infiltrating leukocytes ($CD45^{hi}$) per lumbar spinal cord (mean ± SEM) are presented. Note that the same scale for the y-axis as that in Figure 7 is used in order to compare the data collected from mice with different genotypes. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

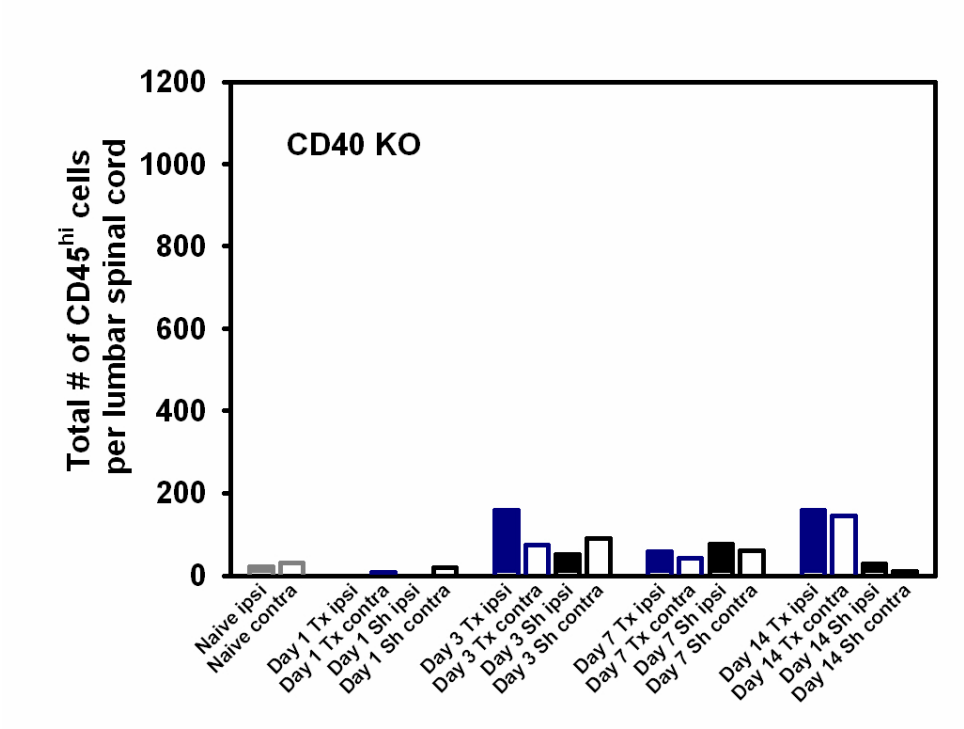

Figure 9: Total numbers of infiltrating leukocytes in the lumbar spinal cord post-L5Tx in CD40 mice. Mononuclear cells from pooled lumbar spinal cord samples (3-5 per treatment, per side) of CD40 KO mice were collected and analyzed via flow cytometry using monoclonal antibodies against CD45 and CD11b (n = 1). Total numbers of infiltrating leukocytes ($CD45^{hi}$) per lumbar spinal cord are presented. Note that the same scale for the y-axis as that in Figure 7 is used in order to compare the data collected from mice with different genotypes. Tx = L5Tx, Sh = Sham, and Naïve = no surgery was performed; ipsi = ipsilateral side and contra = contralateral side. For naïve mice, ipsi = left side and contra = right side.

VI. DISCUSSION

In this study, the mononuclear cells from pooled mouse lumbar spinal cord (L4-L6) sections were collected and stained with mAbs for the analysis of microglial and infiltrating leukocyte numbers in a mouse model of neuropathic pain, L5Tx. The experiment thus far has indicated a general trend of activated microglial presence at the site of injury over a two week period across WT and CD4 KO but not CD40 KO genotypes. A similar trend for the infiltrating leukocytes was seen in WT mice but not in the CD4 KO or CD40 KO mice.

As reported previously, an increase of total numbers of lumbar spinal cord microglia at days 3 and 7 post-L5Tx in the WT mice was observed (Cao 2009b). This is attributed to an increase in the rapid proliferation and migration towards the site of injury (specifically, the ipsilateral side of the lumbar spinal cord) of activated microglia. Earlier, it was demonstrated that both $CD4^+$ T cells and microglial CD40 contribute to the development of L5Tx-induced mechanical hypersensitivity, particularly during the maintenance phase (Cao and DeLeo 2008b, Cao et al. 2009a). Reduced total numbers of spinal cord microglial cells in both CD4 KO and CD40 KO mice following nerve injury suggest that both CD40 and CD4 can mediate L5Tx-induced mechanical hypersensitivity through activating microglia. Interaction between infiltrating $CD4^+$ T cells and microglia, which may be mediated through CD40 ligation by CD154 on $CD4^+$ T cells, will be further examined. It appears CD40 plays a greater role in the microglial activation than CD4 since an increased number of microglia was found at day 7 post-L5Tx in CD4 KO mice, while no significant increase of microglial numbers was

observed at either day 3 or day 7 post-L5Tx in CD40 KO mice. Moreover, a noticeable increase of microglial numbers in both ipsilateral and contralateral sides of the lumbar spinal cord at day 7 and day 14 post-L5Tx was observed in CD40 KO mice. Although confirmation through further experimentation is necessary, this late increase may suggest the involvement of other factors in microglial activation. Additionally, the microglial count for the CD40 KO mice maintained consistently lower values than those observed for the WT and the CD4 KO mice. This indicates a potential intrinsic defect in microglial development may exist and could further contribute to the reduced hypersensitivity post-L5Tx in CD40 KO mice (Cao 2009a).

As expected, L5Tx induced a significant increase of leukocyte infiltration in WT mice in the ipsilateral side of lumbar spinal cord, which peaks at day 3 and day 7 post-L5Tx. Both $CD4^+$ T cells and macrophages, particularly $CD4^+$ T cells, have been shown to infiltrate into the spinal cord following peripheral nerve injury and contribute to the progress of neuropathic pain (Moalem et al. 2004, Cao and DeLeo 2008b, Costigan et al. 2009). Consistent with this, the data showed that CD4 KO mice (that lack $CD4^+$ T cells) had significantly reduced numbers of infiltrating leukocytes following L5Tx. The reduction of infiltrating leukocyte numbers in CD40 KO mice post-L5Tx further suggests that microglial CD40 is critical in leukocyte infiltration into the spinal cord. Based on the previous observation by Cao et al. (2009a), 10-20% of activated microglia expressed detectable CD40. Reduced CD40-mediated microglial activation and the resultant reduction of proinflammatory cytokine/chemokine production in CD40 KO mice are the most likely causes of the decreased leukocyte infiltration. However, indirect effects from

the interaction between microglia and astrocytes or between microglia and neurons are also possible (Milligan and Watkins 2009). These relationships may be further examined in future studies.

Microglia appear to be one type of target cells for T cells upon their infiltration into the CNS (Costigan et al. 2009). The temporal relationships observed between the microglial response and the infiltration of leukocytes support an interaction between microglial CD40 and infiltrating $CD4^+$ T cells, possibly through CD40-CD154 ligation, following peripheral nerve injury. The CD40-CD154 interaction has been shown to be involved in the inflammatory and neurotoxic pathways in Alzheimer's disease, multiple sclerosis, and HIV (Calingasan et al. 2002). Whether CD40-CD154 ligation between microglia and infiltrating $CD4^+$ T cells plays a role in the pathophysiology of peripheral nerve injury-induced neuropathic pain will be investigated in the future.

Within the collected mononuclear cells, significant numbers of cells are not microglia or infiltrating leukocytes (when numbers of total mononuclear cells shown in Figure 3, numbers of microglial cells in Figures 4-6, and infiltrating leukocytes in Figures 7-9 are compared). However, microglia and infiltrating leukocytes were suggested to be the major cell types within the mononuclear cells prepared using Percoll gradient (Ponomarev et al. 2004). It is believed that astrocytes and certain groups of neurons were also harvested under this experimental condition. Detailed phenotyping of mononuclear cells will be performed in the future to explain this observation.

Based on the results thus far, besides the continuation of data collection for the current study, upcoming research may focus on the roles of CD40 and CD4 in other

aspects of microglial responses following L5Tx. This could be performed using both *in vivo* and *in vitro* approaches. For example, morphological changes, migration and proliferation of microglia, expression of selected surface molecules by microglia, and microglial cytokine/chemokine secretion can be examined. Further, the interactions between microglia and astrocytes, microglia and neurons, $CD4^+$ T cells and astrocytes, as well as $CD4^+$ T cells and neurons, are areas warranting greater investigation. It is known that there is a significant activation of astrocytes following peripheral nerve injury and it has been proposed that astrocytes are mainly involved in the maintenance of neuropathic pain (Milligan and Watkins 2009). In addition, reduced astrocytic response was observed in CD4 KO mice 7 days post-L5Tx compared to WT mice (Cao and DeLeo 2008b). Therefore, it is not surprising that multi-cellular interactions can modify neuronal activities and contribute to the development of neuropathic pain following peripheral nerve injury.

In summary, the results suggest both CD4 (particularly $CD4^+$ T cells) and CD40 are involved in the development of L5Tx-induced neuropathic pain through the inhibition of lumbar spinal cord microglial activation and leukocyte infiltration into the spinal cord. CD40 seems to play a greater role in controlling microglial responses following L5Tx. Further elucidation of the underlying mechanisms could aid in the development of new drug targets for the treatment of peripheral nerve injury-induced neuropathic pain.

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