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Master's Thesis in Medicine

Spinal Cord T-Cell Infiltration in the Spared Nerve Injury Model of Neuropathic Pain: a Time Course Study

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ABSTRACT

Background

Neuropathic pain, resulting from a lesion to the somatosensory nervous system, causes considerable disability and affects up to 8% of the European population. Nevertheless, the current treatments are often unsatisfactory and new therapeutic targets are actively sought. In this regard, several seminal studies have claimed that T-lymphocytes infiltrate the spinal cord after peripheral nerve injury and that they may contribute to sensory hypersensitivity in rodents. However, no systematic description of the time course or cell types that characterize this infiltration has yet been published, although this constitutes a rational first step toward an overall understanding of the impact of spinal T-cells in neuropathic pain. Thus, in the present study we explored spinal T-cell infiltration using the spared nerve injury (SNI) model of neuropathic pain in rodents.

Materials and Methods

Lumbar spinal cords of adult Sprague Dawley rats were collected at days 2, 7, 21 and 42 after SNI or sham operation (4 animals in each group) and were analysed using immunofluorescence detecting T-cell proteins CD2 and CD8 as well as glial markers Iba1 and GFAP. Western blot was employed to compare the expression of CD2 in the ipsilateral versus contralateral dorsal horn of SNI rats. Fluorescent cells were counted by a blinded investigator and the intensity of bands was quantified using ImageJ sofware. Statistical analysis was performed on GraphPadPrism software with statistical significance set at $p<0.05$.

Results

Iba1 signal was increased in the dorsal horn ipsilateral to injury confirming that SNI surgery produced neuroinflammation. Only a small number of CD2- and CD8-positive cells were found in the spinal cord at the various time points, and there was no significant difference between SNI and sham animals. On the other hand, CD2 protein was significantly higher ipsilaterally than contralaterally to injury in SNI rats.

Conclusion

Neuroinflammation occured without an increase of T-cells in the spinal cord of neuropathic animals, suggesting that if central lymphocytic traffic has a role in promoting neuropathic pain, it is likely not a major contributor in the SNI model.

Keywords : Neuropathic Pain, T-Cell, Spinal Cord, Rat, Microglia.

INTRODUCTION

Chronic Pain and Central Sensitization

Chronic pain is a major health issue that impacts all aspects of the patients' lives. The definition of pain as specified by the International Association for the study of Pain (IASP) is « An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage » (Merskey and Bogduk, 1994). In Europe, 19% of adults suffer for more than 6 months from moderate to severe pain, which affects the quality of their social and working lives (Breivik et al, 2006). Thus, the economic burden of chronic pain is colossal: in the United States its direct costs exceed $100 billion, more than those of cancer, coronary heart disease, and acquired immunodeficiency syndrome (AIDS) combined (McCarberg et al, 2011).

Chronic pain differs from acute pain in many ways. Nociception, which is the neural process of encoding noxious stimuli (Merskey and Bogduk, 1994), triggers protective autonomic and behavioural reflex responses, that optimize the avoidance of injury. The evolutionary usefulness of pain is limited in time and disappears when the insult to the tissues has resolved. The persistence of pain after healing of the original lesion is a key feature of many chronic pain syndromes, where pain extends from being solely a symptom toward a pathology *per se*. The complex process leading to chronicization involves changes within the brain, spinal cord and peripheral primary sensory neurons in the dorsal root ganglia (DRG) (Julius and Basmaum, 2001). These changes can be roughly divided into two different entities. First, peripheral sensitization represents a lowering in threshold of activation and an increase in responsiveness of the nociceptors, the peripheral sensory receptors encoding noxious stimuli. In contrast, central sensitization is an amplified excitability of neurons within the central nervous system (CNS) which may become independent from the persistence of the injury that originally caused it (Latremolière and Woolf, 2009). Changes in central sensory neurons include the development of spontaneous activity, a reduction in their activation threshold and an enlargement of their receptive field, the area in which stimulation leads to their activation. There are several clinical forms of chronic pain in which central sensitization plays a role, including inflammatory pain, migraine, irritable bowel syndrome and neuropathic pain (Latremolière and Woolf, 2009).

Neuropathic Pain and Glial Theory of Central Sensitization

Neuropathic pain is a form of chronic pain that results from a lesion or disease of the somatosensory nervous system, either peripheral or central (Merskey and Bogduk, 1994). In Europe, two studies have shown a prevalence of neuropathic pain among the general population of 7% and 8% respectively (Bouhassira et al, 2008 ; Torrance et al, 2006).

A wealth of data has been released about the role of neuronal components in neuropathic pain, such as sodium channels, neurotransmitters or dendritic connectivity. Interestingly, increased attention has recently been given to the role of glial and inflammatory cells in central sensitization underlying neuropathic pain. In 1991, Garrison et al were the first to describe an increase in astrocytic activation in the ipsilateral grey matter of the spinal cord following chronic constriction injury (CCI). These glial changes were correlated to the degree of behavioural hyperalgesia, suggesting a possible role for non-neuronal CNS-cells in the physiopathology of neuropathic pain (Garrison et al, 1991). Since then, many studies have provided convergent data indicating that a variety of peripheral noxious stimuli trigger astrocyte activation in the spinal cord. Similarly, microglia, the resident macrophage-like cells of the CNS, have been shown to be activated in the ipsilateral grey matter of the spinal cord following peripheral nerve injury. The current view is that microglial activation occurs at an early phase post-injury and is transient, whereas astrocytic activation starts later and lasts longer (Gosselin et al, 2010). It was shown that, in addition to qualitative changes, there was also a marked increase in the total number of microglia in the dorsal horn in the spared nerve injury (SNI) model of neuropathic pain (Beggs and Salter, 2007). This raised the question about the origins of the additional microglia. While microglial proliferation has been reported in the dorsal horn of SNI animals (Suter et al, 2009), another possibility is the occuring of an infiltration and differentiation of bone marrow-derived circulating monocytes into the CNS. Zhang et al used rat chimeras exposed to peripheral nerve injury to show that, in addition to resident microglial reaction, hematogenous monocytes infiltrate the spinal cord, proliferate and differentiate into microglia (Zhang et al, 2007).

Beyond monocytic invasion, DeLeo's group found that some of the leukocytes that trafficked to the ipsilateral grey matter of the spinal cord in response to L5 spinal nerve

transection expressed the T-lymphocyte marker CD3 and exhibited a lymphocyte-like morphology (Schweitzer et al, 2002). This study opened the doors for a new range of work aiming to characterize the transit of lymphocytes into the uninjured CNS triggered by peripheral nerve injury and understand its effect on the development of pain.

Spinal Lymphocytes and Neuropathic Pain

Scarce but convergent series of data indicate that lymphocytes infiltrate the spinal cord in neuropathic pain. Hu et al (2007) compared the leukocyte traffic to the spinal cord in the CCI and nerve transection models of neuropathic pain, both of them applied to the sciatic nerve, just above its trifurcation. Their study showed that one week after nerve injury, the number of α/β TCR positive T-cells in the lumbar spinal cord was increased after CCI, but not after nerve transection. Most of these T-cells were CD8+, with only a minority of CD4+ cells. Moreover, Cao and DeLeo used fluorescence activated cell sorting (FACS) to establish a time course of leukocyte infiltration into the lumbar spinal cord after spinal nerve L5 transection (L5Tx). They found that L5Tx surgery induced an increase in infiltrating peripheral leukocytes peaking at day 7 and decreasing by day 14 post-injury. CD4+ T-cells were the most prevalent cell type within the infiltrating population. In contrast to other studies, no marcrophages or CD8+ lymphocytes were detected in the population of infiltrating leukocytes (Cao and DeLeo, 2008). Finally, Costigan et al (2009), using CD2 immunohistochemistry, found that the number of T-lymphocytes increased 10-fold in the ipsilateral dorsal horn of adult SNI rats compared to naive animals, at 7 days post-injury.

Beyond the sole description of lymphocytic infiltration, work has been conducted to understand the function of central T-lymphocytes in neuropathic pain. Genetically-modified mice with T-lymphocyte deficiency were used to explore the contribution of T-cells in the onset of neuropathic pain. Both the *Rag1* null (Costigan et al, 2009) and CD4 knock-out (Cao and DeLeo, 2008) strains showed a significantly decreased mechanical hypersensitivity after SNI or L5Tx, respectively, compared to wild type animals, and this effect was reversed by an adoptive transfer of CD4-positive leucocytes (Cao and DeLeo, 2008). The next step was to differentiate between the involvement of peripheral versus central lymphocytes in the development of neuropathic pain. No difference was found in the activation of peripheral CD4-positive T-lymphocytes in the spleen or lymph nodes between L5Tx and sham surgery groups. Furthermore, it was shown that, while

microglial signal increased after injury as predicted, GFAP signal in the ipsilateral dorsal horn was decreased in CD4 KO mice compared to wild type mice after L5Tx surgery, indicating possible specific T-lymphocyte-astrocyte interactions (Cao and DeLeo, 2008).

In the context of a study on the effects of peripheral nerve injury on the blood-spinal cord barrier (BSCB), CD2- and CD3-positive T-cells infiltrated the ipsilateral side of the spinal cord 7 days after partial sciatic nerve ligation and an intrathecal injection of TGF-β prevented the entrance of peripheral lymphocytes, as well as preventing other BSCB dysfunctions (Echeverry et al, 2011).

However, some studies contradicted these results. Kim and Moalem-Taylor (2011) found a significant increase in the number of infiltrating leukocytes in the ipsilateral sciatic nerve and DRGs in mice that underwent partial ligation of the sciatic nerve, including CD3+ T-cells, but no lymphocytes were found in the spinal cord at any time points post-injury.

Thus, only a few studies have shown a lymphocytic infiltration in the spinal cord following peripheral nerve injury, and there is only limited agreement on the subsets of T-cells involved. Furthermore, no systematic description of the time course with a detailed characterization of infiltrating cells and their protein expression-profile has been published yet, although this seems to be the rational first step to an overall understanding of the phenomenon.

The « T-cell Family »

T-cells exist in a variety of subtypes, with very different properties and functions. Briefly, stem cells originating from the bone marrow migrate to the thymus, where they differentiate into T-lymphocytes. The majority of immature thymocytes express CD4 and CD8 markers as well as the T-cell receptor (TCR). In the process of differentiation, T-cells loose either the CD4 or CD8 expression to become mature lymphocytes (Male, 1999).

Cytotoxic T-cells can recognize and destroy virally infected cells and allogeneic cells, and are characterized by their expression of CD8 glycoprotein, a co-receptor of the TCR. They recognize antigens associated with major histocompatibility complex (MHC) class 1 on the surface of target cells (Male, 1999 ; Bierer et al, 1989). Conversely, T helper (T_H)-cells assist B-cells in their activation processes, and help them produce antibodies.

T_H-cells express CD4 and recognize antigens associated with MHC class 2 on the surface of antigen presenting cells (Male, 1999 ; Bierer et al, 1989). Additionally, T_H-cells divide into many subtypes, including T_H1, T_H2 and T_H17, which can be differentiated according to their cytokine profile. The T_H1 subpopulation promotes the cellular immune system whereas T_H2-cells induce the production of antibodies (Male, 1999).

Working Hypothesis and Objectives

The hypothesis behind this project is that different T-cell populations might infiltrate the spinal cord at different time points post-injury, and that finally, there may be a resolution of the process, explicited by a decreasing number of lymphocytes in the spinal cord at later time points. In order to explore this hypothesis, we used immunofluorescence stainings and western blotting to compare the number of T-cells (CD2- and CD8-positive) present in various lumbar spinal cord regions of rats at days 2, 7, 21 and 42 after SNI or sham surgery.

MATERIALS AND METHODS

Animal Surgery

We used the spared nerve injury (SNI) model of neuropathic pain as previously described (Decosterd and Woolf, 2000). Briefly, adult male Sprague Dawley rats were anesthetized using isoflurane. An incision was made through the skin and biceps femoris muscle to expose the sciatic nerve at the level of its trifurcation into 3 branches : the sural, common peroneal and tibial nerves. The sural nerve was left intact, whereas the two others were ligated and cut distally to the ligation and a few millimeters of each distal nerve was removed. The muscle and skin were then closed separately. The same procedure was followed for the sham surgery, but the nerves were not cut. A silk was laid next to the trifurcation to reduce the difference between sham and SNI.

Tissue Preparation

Twenty-five mg of Bromodeoxyuridine (BrdU) in Phosphate buffered saline (PBS) + 0.007 N NaOH were injected intraperitoneally (i.p.) 2 hours before sacrifice. The rats were terminally anesthetized by an i.p. injection of 50 mg of pentobarbital. The right atrium was slit open in order to let the blood out, and an other incision was made in the left ventricle, where a blunt needle was inserted for perfusion. First, ice cold PBS was infused for 1 minute to rinse out the blood, and then ice cold 4% paraformaldehyde (PFA)/PBS for 15 minutes. The ipsilateral sciatic nerve was dissected and exposed up to the L4-L5-L6 spinal nerves where it originates. Laminectomy was performed to expose the spinal cord, and the L4-L5 lumbar section was collected and post-fixed in 4% PFA/PBS at 4°C overnight. The lumbar samples were then transferred to 20% sucrose in PBS at 4°C overnight for cryoprotection and rapidly frozen.

Thirty µm slices were cut using a cryostat and stored in freezing solution (30% ethylene glycol and 30% glycerol in PBS) in wells respecting the craniocaudal distribution of the slices.

Brains, thymuses and neuromas at the site of the SNI were also collected in some animals to be used as positive controls for the antibodies, and were subjected to the same conditioning as the spinal cords, but were directly mounted on slides after being cut.

Immunofluorescence

Slices were incubated in blocking solution (10% normal goat serum (NGS), 0.05% triton and 0.05% azide in PBS) for 30 minutes and incubated overnight at 4°C in blocking solution with primary antibody (table 1.1). They were washed in PBS and incubated in blocking solution with secondary antibody (table 1.2) for 1 hour at room temperature (RT). Diamidinophenylindole (DAPI) was applied on the slices for nuclear labelling before a final wash in PBS. The lumbar spinal cord sections were then mounted on slides with Mowiol mounting medium and stored at 4°C.

Antibody	**Target**	**Concentration**	**Source**
Mouse anti CD2	T-cells	1 : 250	Serotec, UK
Rabbit anti-CD2	T-cells	1 : 100	Santa Cruz, USA
Mouse anti-CD8	CD8+ T-cells	1 : 250	Abcam, USA
Mouse anti-CD4	CD4+ T-cells	1 :100	Abcam, USA
Rabbit anti-Iba1	Activated microglia	1 : 2000	Wako, USA
Mouse anti-GFAP	Activated astrocytes	1 : 1500	Millipore, USA
Rat anti-BrdU	Proliferating cells	1 : 500	Abcam, USA

Table 1.1 : Primary antibodies used for immunofluorescence.

Antibody	**Concentration**	**Source**
Alexa 488-labelled goat anti-mouse	1 : 500	Molecular Probes, UK
Alexa 488-labelled donkey anti-rabbit	1 : 500	Molecular Probes, UK
Cy3-labelled donkey anti-mouse	1 : 500	Jackson, USA
Cy3-labelled donkey anti-rat	1 : 500	Jackson, USA

Table 1.2 : Secondary antibodies used for immunofluorescence.

Tyramide Signal Amplification Immunofluorescence

We used lumbar spinal cord sections prepared as described above. Endogenous peroxidase activity was blocked using 3% H_2O_2 or phenylhydrazine. Blocking and primary antibody incubation were carried out as described above. The slices were incubated in 1 : 200 Goat anti-rabbit/biotin (Vector, USA) or 1 : 200 Goat anti-mouse/biotine (Vector, USA) for 1 hour at RT and 1 : 100 HRP-conjugated streptavidin for 30 minutes at RT. Each incubation was followed by washing in PBS. TSA™ Plus

Cyanine 3 System (PerkinElmer, USA) was used. 1 : 100 tyramide-Cy3 was applied on the sections for 3 minutes, before they were washed. Slides were dried and protected from quenching of fluorescence with Mowiol, before being cover-slipped.

Immunohistochemistry

Tissue preparation, blocking and primary antibody incubation was achieved as described for immunofluorescence protocol. The slices were incubated at RT in 1 : 200 Goat anti-rabbit/biotin (Vector, USA) or 1 : 200 Goat anti-mouse/biotine (Vector, USA) for 1 hour, in the A+B mix from the Vectastain ABC kit (Vector, USA) for 30 minutes and in a DAB-Nickel solution until color appears. Each incubation was followed by washing in PBS. The sections were dehydrated in baths with increasing concentrations of ethanol, with two final baths of toluene. The mounting was carried out using Permount™ Mounting Medium.

Citrate Buffer Antigen Revealing

Slides were plunged into boiling 0.01 M citrate buffer for 30 seconds and then directly into PBS. Immunofluorescence was then carried out as described above.

BrdU Immunostaining

In order to denature the DNA, the slices were incubated as followed : PBS at RT for 5 minutes, HCl 1N on ice for 10 minutes, HCl 2N on ice for 10 minutes, HCl 2N 20 minutes at 37°C, twice in borate buffer 0.1M at pH 8.5 and RT for 5 minutes. Then, immunofluorescence was carried out as described above.

Cell Counting

Six 30µm-thick L4-L5 spinal cord transverse sections from each animal were stained by immunofluorescence for CD2 or CD8 as described above. The investigator was blinded to the sample identity and counted fluorescent cells in each region as illustrated in Figure 1, using an Axio Imager.Z1 fluorescence microsocope (Zeiss, Germany). Spinal cord sections were viewed with the x20 objective and the counting was done « on view » directly at the microscope.

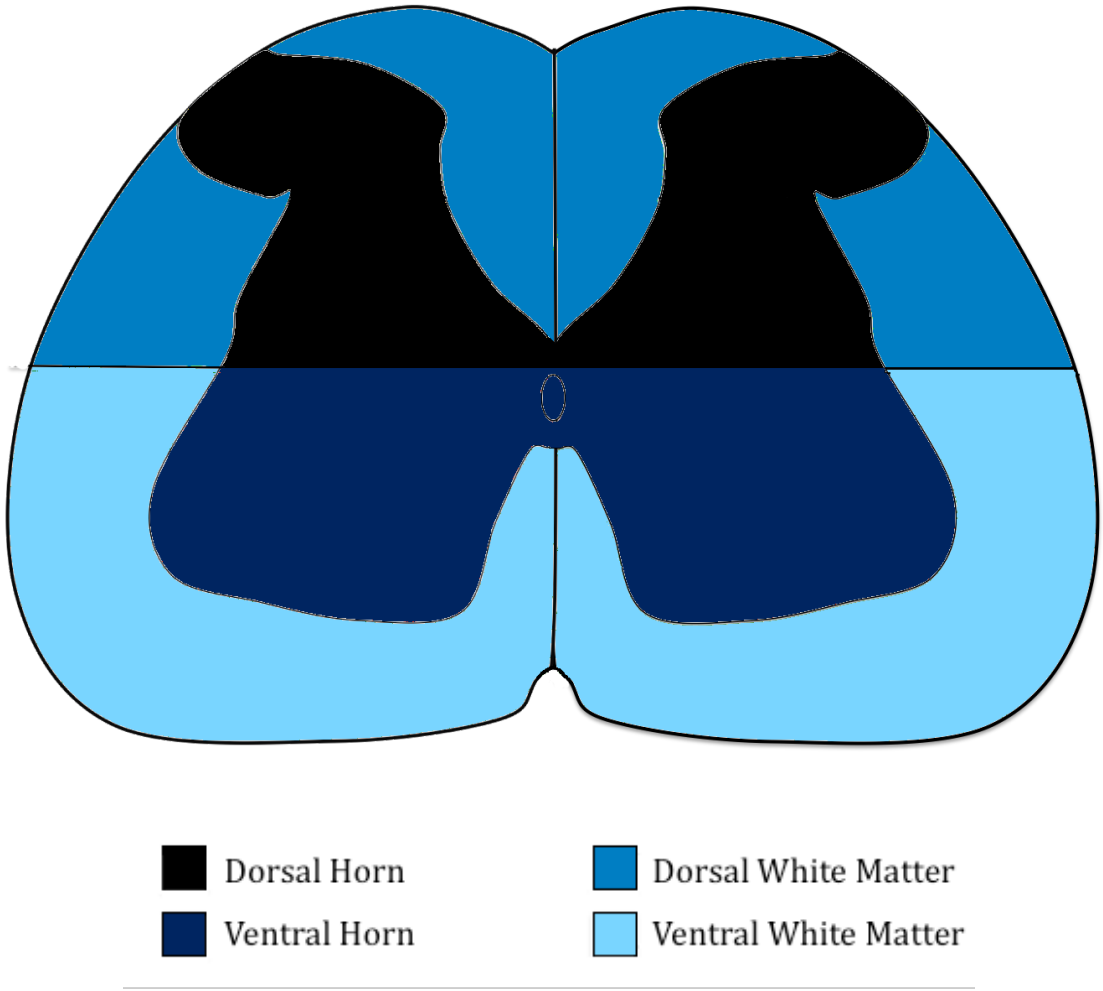


Fig. 1: Definition of regions for cell counting.

Iba1 and GFAP Signal Quantification

Three Iba1- and GFAP-labelled lumbar sections from each SNI or sham-operated animals were viewed under an Axio Imager.Z1 fluorescence microsocope (Zeiss, Germany). Pictures of the ipsilateral dorsal horn were taken with the x20 objective, keeping the same fluorescence intensity and exposure time for every picture. For Iba1 signal quantification, the contrast of all the images was enhanced using GNU Image Manipulation Program software (www.gimp.org), the labelled area was quantified and expressed as a percentage of total area using ImageJ software (National Institute of Health. http://rsbweb.nih.gov/ij). GFAP signal quantification was achieved with ImageJ by calculating three parameters : the mean intensity (mean grey value) and relative area of the GFAP signal as well as the number of GFAP-labelled particles. For the two latter parameters, a binary image was previously created.

Western Blot

One week after SNI, the rats were terminally anesthetized by an i.p. injection of 50 mg of pentobarbital and then sacrificed by decapitation. Spinal cords were dissected to collect the L4-L5 lumbar enlargement. Samples were subjected to protein extraction and protein concentration for each sample was measured by the Bradford protein assay. Nine µg of protein from each sample with loading buffer were loaded in the wells of a 10% acrylamide gel. To prevent multimerization of our target protein, we added extra SDS into the loading buffer, boiled the samples for 10 minutes at 100°C and used PBS twice more concentrated for washing and in incubations. After migration and transfer on Immun-Blot® PVDF membrane (Bio-Rad, Germany), blocking was performed in 5% non-fat milk for 5 minutes at RT followed by incubation in 1 :200 Rabbit anti-CD2 (Santa Cruz, USA) in milk overnight at 4°C. For detection, membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1 :10000 goat anti-rabbit/HRP, Dako, Denmark) for 30 minutes at RT. Washings were made 5 times between each incubation in PBS 2x with tween0.1%. Signals were revealed using enhanced chemiluminescence (ECL) reagent (West Dura, Pierce, USA). A luminescent image analyser (LAS-4000, Fujifilm, Japan) was used for imaging. The membrane was then stained with Coomassie Blue for total protein quantification and normalisation.

Statistical Analysis

All data are expressed as means ±SEM. For immunofluorescence, sections from the same level of the L4-L5 spinal cord were pooled for each animal and data from the ipsilateral side were compared to the contralateral side and to sham-operated rats. Four animals were used in each group. For Western blot analysis, four animals were used in each group and data were analysed by comparing the ipsilateral side to the contralateral side. Data were analysed using unpaired two-tailed Student's t-test when comparing sham versus SNI, and paired two-tailed Student's t-test when comparing ipsilateral versus contralateral, with GraphPad Prism software (GraphPad Prism, San Diego, USA). A difference was considered significant for $P<0.05$. Western blot bands were quantified using ImageJ software.

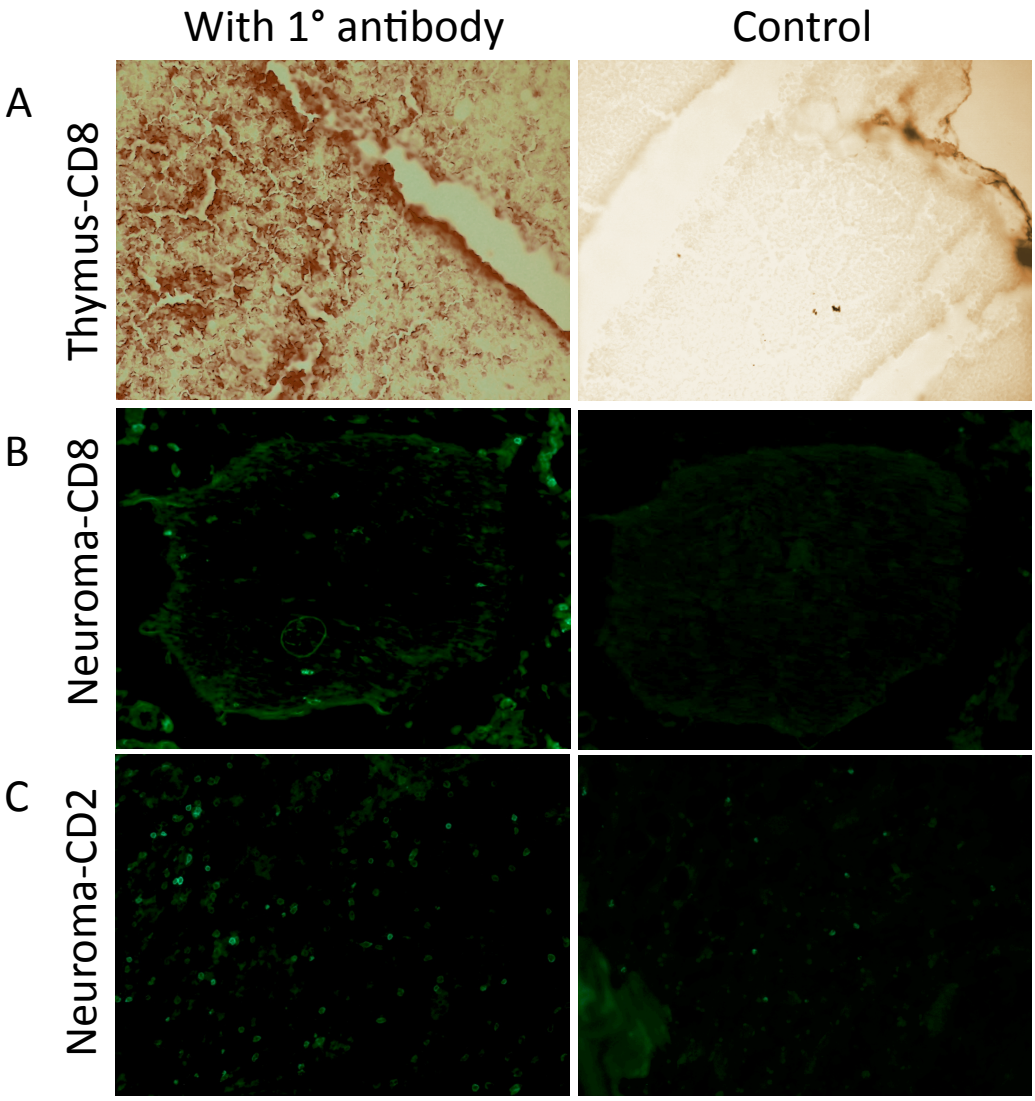
RESULTS

Antibody Sensitivity Testing

To test the sensitivity of our antibodies we needed positive controls, i.e. tissues containing T-cell clusters. The most obvious choice was the thymus, where T-lymphocytes mature. We also collected neuromas, the area of local inflammation of the sciatic nerve at the site of the surgery. As the thymus presents a high degree of autofluorescence, we used DAB-based immunohistochemistry for CD2 (Santa Cruz), CD4 and CD8 at different concentrations. In addition, we stained neuroma sections using immunofluorescence for the same antibodies. This first trial showed that our CD8 antibody gave a specific signal, targeting the periphery of small cells consistent with a lymphocytic morphology (Fig. 2*A, B*). On the other hand, CD2 (Santa Cruz) and CD4 antibodies gave no specific signals.

We hypothetized that this lack of signal could possibly be due to a formaldehyde-induced cross-linking of proteins, thus masking our target epitopes. We therefore proceeded with the citrate buffer antigen retrieval protocole, in the hope of overcoming this problem. Another possibility was that the target protein amount was too low in comparison to the antibody's sensitivity, so we tried using the TSA™ technology, which is known to have a much higher sensitivity than standard immunofluorescence. However, neither method was found to produce a specific signal with the CD2 (Santa Cruz) and CD4 antibodies. We therefore used a CD2 antibody from another source (Serotec), which gave a specific signal at the periphery of small cells on neuroma sections (Fig. 2*C*).

For Western Blotting, we tested the Santa Cruz CD2 antibody on thymus protein extract. This produced a single band around 45 kDa (molecular weight of non-glycosylated CD2 is around 38 kDa) (Fig. 2*D*).



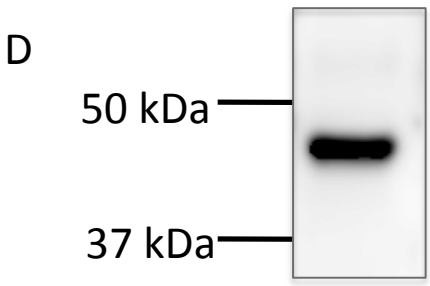


Fig.2: Antibody sensitivity tests. A: Immunohistochemistry on rat thymus with (left), or without (right), primary anti-CD8 antibody. **B, C**: Immunofluorescence on neuroma 2 days after SNI with green-labeled CD2 or CD8 T-cell marker (left), and control without primary antibody (right). Small cells consistent with a lymphocytic morphology are targeted**. D**: Western Blot analysis of thymus protein extract with anti-CD2 antibody, showing a single band around 45 kDa.

SNI Induced Neuroinflammation in the Spinal Cord

The SNI model of neuropathic pain has been used in our group and in other laboratories for many years and has shown to be very robust, the animals invariably developing hyperalgesia and allodynia. Thus, we did not perform behavioural tests on the rats. To show that there was indeed a neuroinflammation in the spinal cord, we targeted reactive microglia and astrocytes with anti-Iba1 and -GFAP antibodies respectively, using immunofluorescence (Fig.3). The area labelled with Iba1 was significantly increased in the ipsilateral dorsal horn of SNI compared to sham-operated rats 7 and 42 days after injury ($p<0.0001$ and $p=0.007$, respectively). There was no difference at day 2, and it was impossible to analyse the data of day 21 because of technical problems. The intensity of the GFAP signal was only higher in the dorsal horn of SNI than sham animals 2 days after injury ($p=0.03$). Neither the labelled area nor the number of GFAP-positive particles was different between the two groups at any time point. Two days after injury, there were numerous nuclei with a BrdU signal in the dorsal horn, ipsilaterally to injury only. All of these cells expressed the microglial marker Iba1 (Fig.4).

The Number of T-cells was not Increased in the Spinal Cord Following SNI

To determine whether there was a T-cell infiltration in the spinal cord following SNI, we used CD2 immunofluorescence (Fig. 5*A*). We found no significant difference in the number of CD2-positive T-cells in most regions of the lumbar spinal cord and at most time points, neither when comparing sham and SNI animals, nor the ipsilateral and contralateral sides of the spinal cord to the lesion (Fig. 6*A-D*). However, there were significantly more T-cells in the ipsilateral than the contralateral dorsal horn of sham-operated animals 2 days after injury ($p=0.03$; Fig. 6A), in the ipsilateral than the contralateral dorsal horn of SNI-operated animals 21 days after injury ($p=0.04$; Fig. 6A), and in the ipsilateral ventral white matter of SNI-operated animals compared to the contralateral side and to sham ($p=0.02$ and $p=0.005$; respectively) 7 days after surgery (Fig. 6D). Only a small number of fluorescent cells were detected in each region, with a general trend for T-cells to be in the grey rather than in the white matter, and in the dorsal rather than in the ventral horn.

We also used CD8 immunofluorescence to assess if the cytotoxic subtype of T-cells penetrated preferentially into the spinal cord after SNI (Fig. 5*B*). There was no significant infiltration, neither when sham animals nor the contralateral side were used

as control. The number of CD8-positive T-cells was very small, with an average of less than four cells counted per spinal cord transverse section (Fig. 7*A*-*D*).

CD2 Protein was Significantly Higher Ipsilaterally to Injury than Contralaterally

To confirm these results with another technique, we compared the amount of CD2 protein in the dorsal quadrants of the spinal cord ipsilaterally and contralaterally to injury. The normalized amount of CD2 protein was higher ipsilaterally to injury, and the difference between sides was statistically significant ($p=0.03$; Fig. 8). We did not include sham-operated rats in this experiment in order to spare animals.

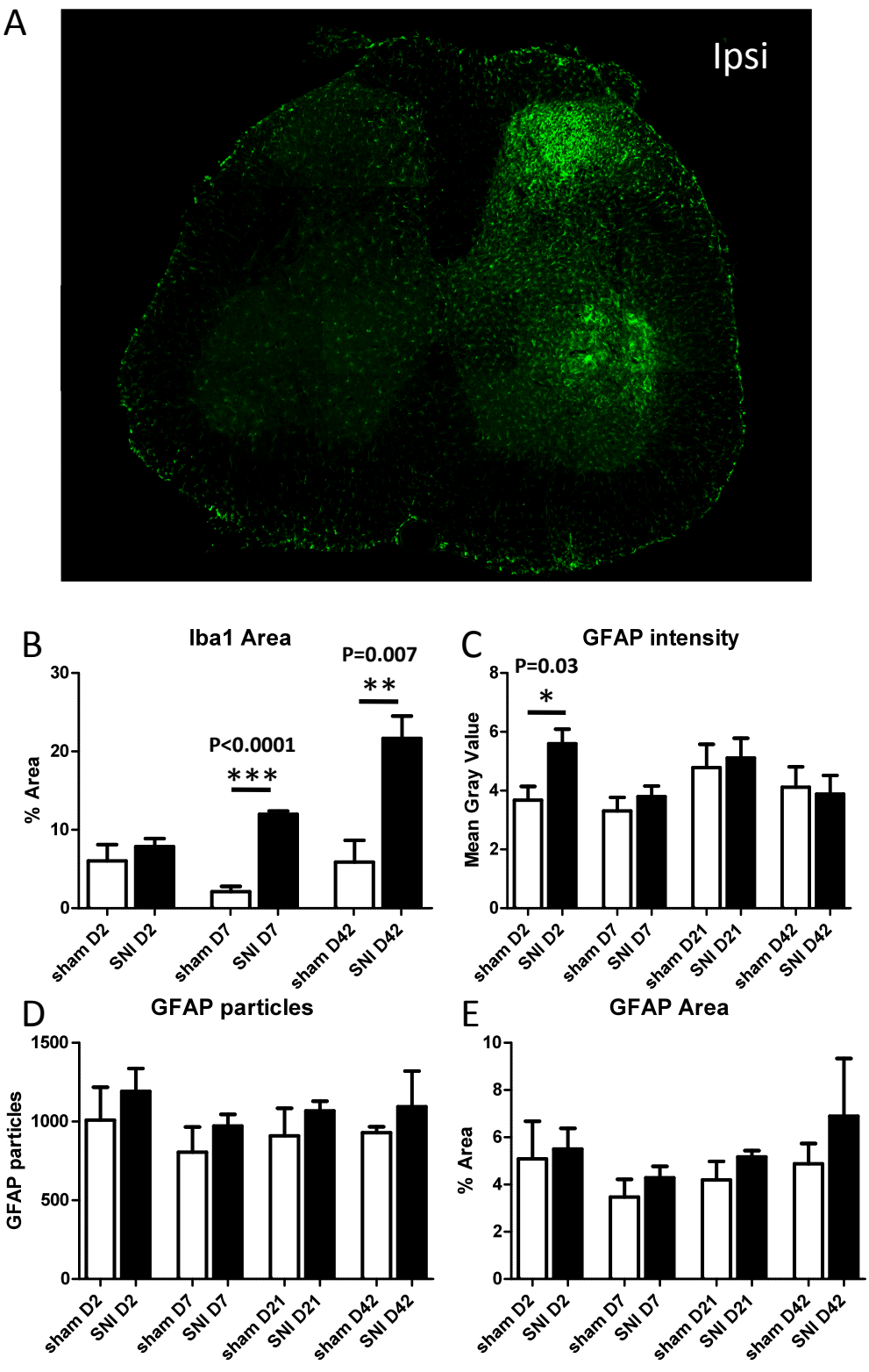


Fig. 3: Neuroinflammation in the spinal cord following SNI. A: Representative immunofluorescence on a lumbar section 7 days after injury showing the microglial activation marker Iba1 in green. There is a prononced reaction in the dorsal and ventral horns ipsilateral to SNI. **B-E**: Time course of microglial (Iba1) and astrocytic (GFAP) activation in the ipsilateral dorsal horn. SNI: black, sham: white. N = 4/group. Values are expressed as means ± SEM. *, p<0.05 ; **, p<0.01 ; ***, p<0.001. Student's t-test, sham vs SNI.

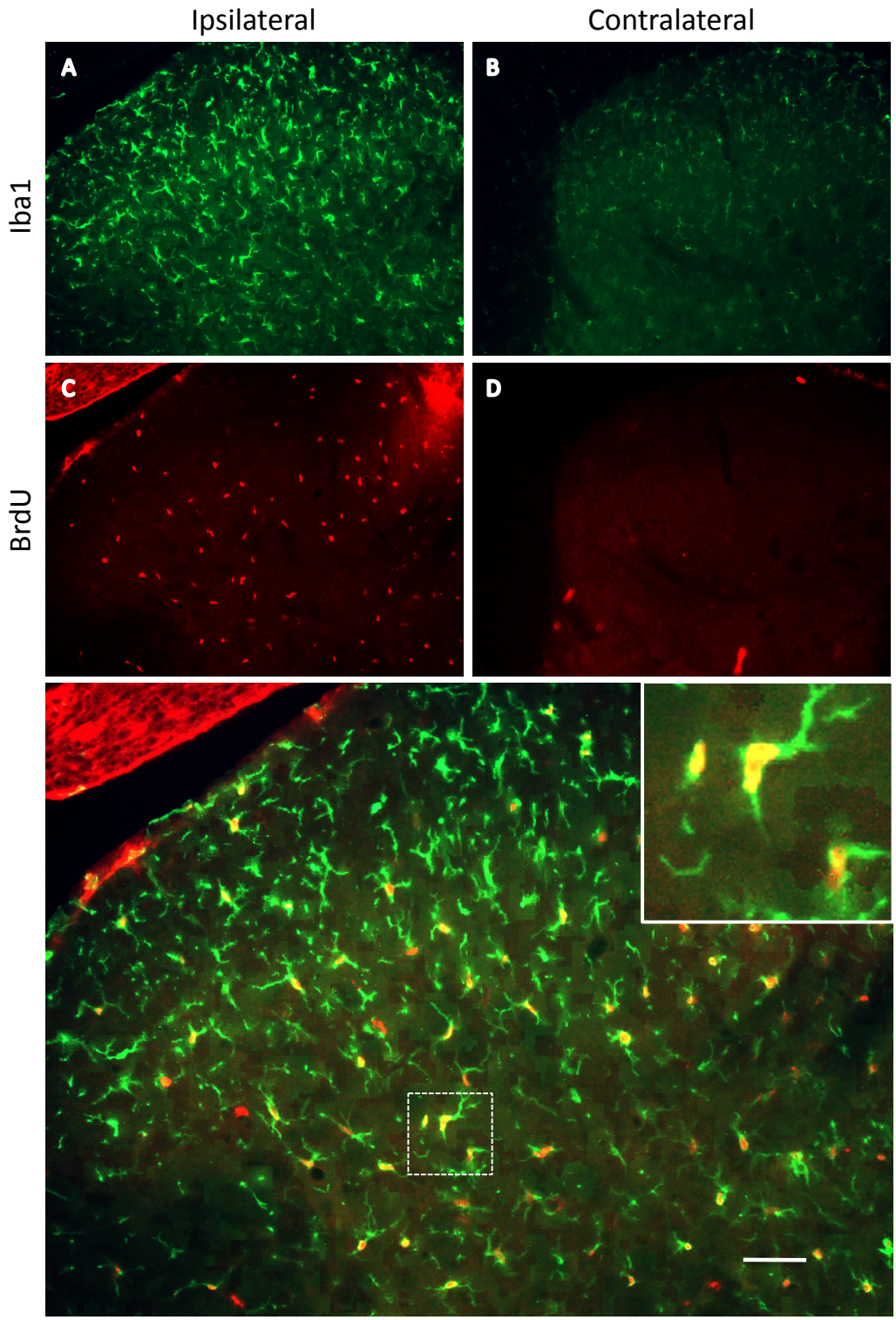
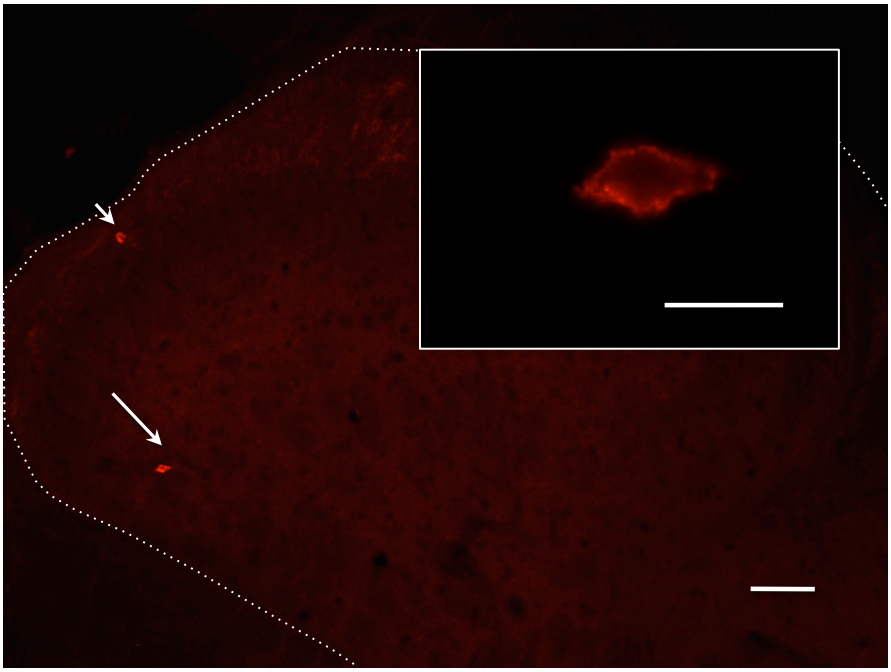


Fig 4: The proliferation marker BrdU colocalizes with Iba1 in the ipsilateral dorsal horn 2 days after injury. A-D: Iba1 (green) and BrdU (red) in the ipsilateral (A and C) and contralateral (B and D) dorsal horn 2 days after SNI surgery. There is a marked microglial reaction and cell proliferation ipsilaterally to injury. **E**: The two markers colocalize almost systematically, indicating microglial proliferation following peripheral nerve injury. Insert: magnification of the region inside the dotted square. Scale bar: 50 µm.

A



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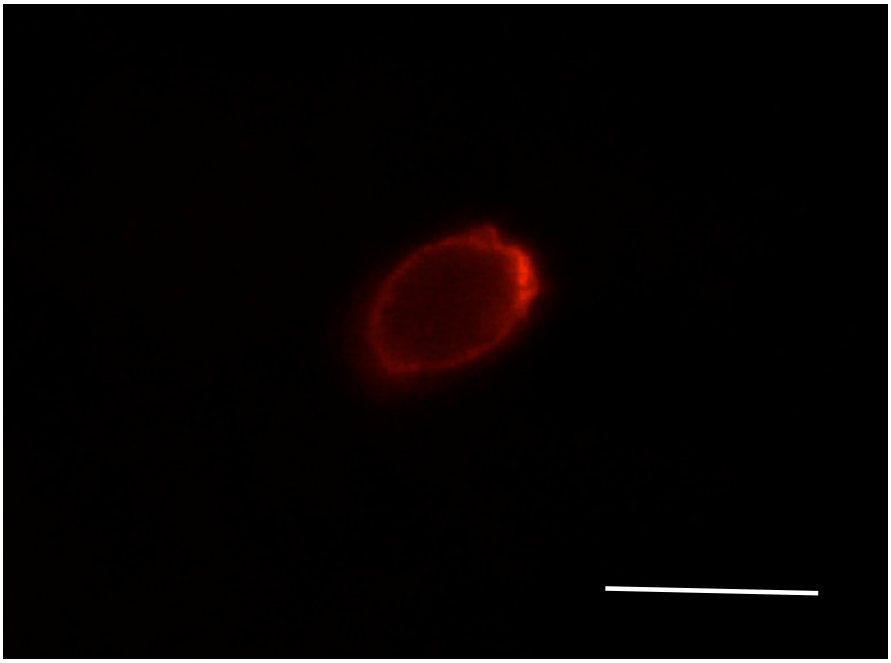

Fig 5: T-cells in the spinal cord following SNI. A: Representative immunofluorescence of the ipsilateral dorsal horn of an SNI animal at day 7 post-injury, with the CD2 marker in red. White arrows indicate T-cells. The insert is a magnification of the cell pointed by the longest arrow. Scale bar main image, 50 µm. Scale bar insert, 10 µm. **B**: High magnification of a representative CD8-positive cell. Scale bar, 10 µm.

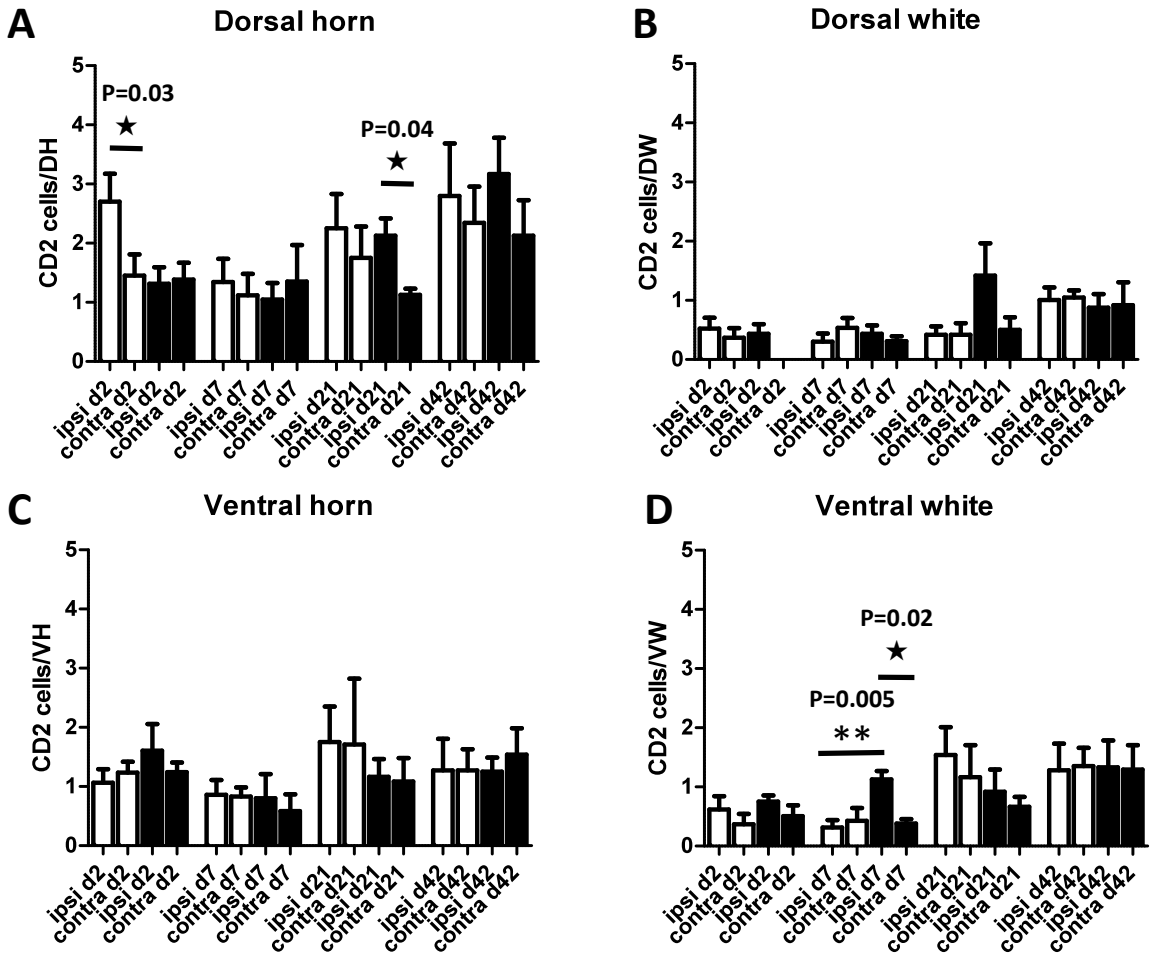

Fig. 6: Time course of CD2-positive T-cell distribution in the lumbar spinal cord. A-D: bar histograms showing the number of CD2+ cells detected in SNI (black) or sham (white) animals, at days 2, 7, 21 and 42, ipsilateral (ipsi) or contralateral (contra) to injury. Four regions of the spinal cords were individualized in each histogram: *A*, the dorsal horn (DH), *B*, the dorsal white matter (DW), *C*, the ventral horn (VH), *D*, the ventral white matter (VW). N = 4/group. Values are expressed as means ± SEM. **, $p<0.01$ Student's t-test, sham vs SNI. ★, $p<0.05$ Student's t-test, ipsilateral vs contralateral.

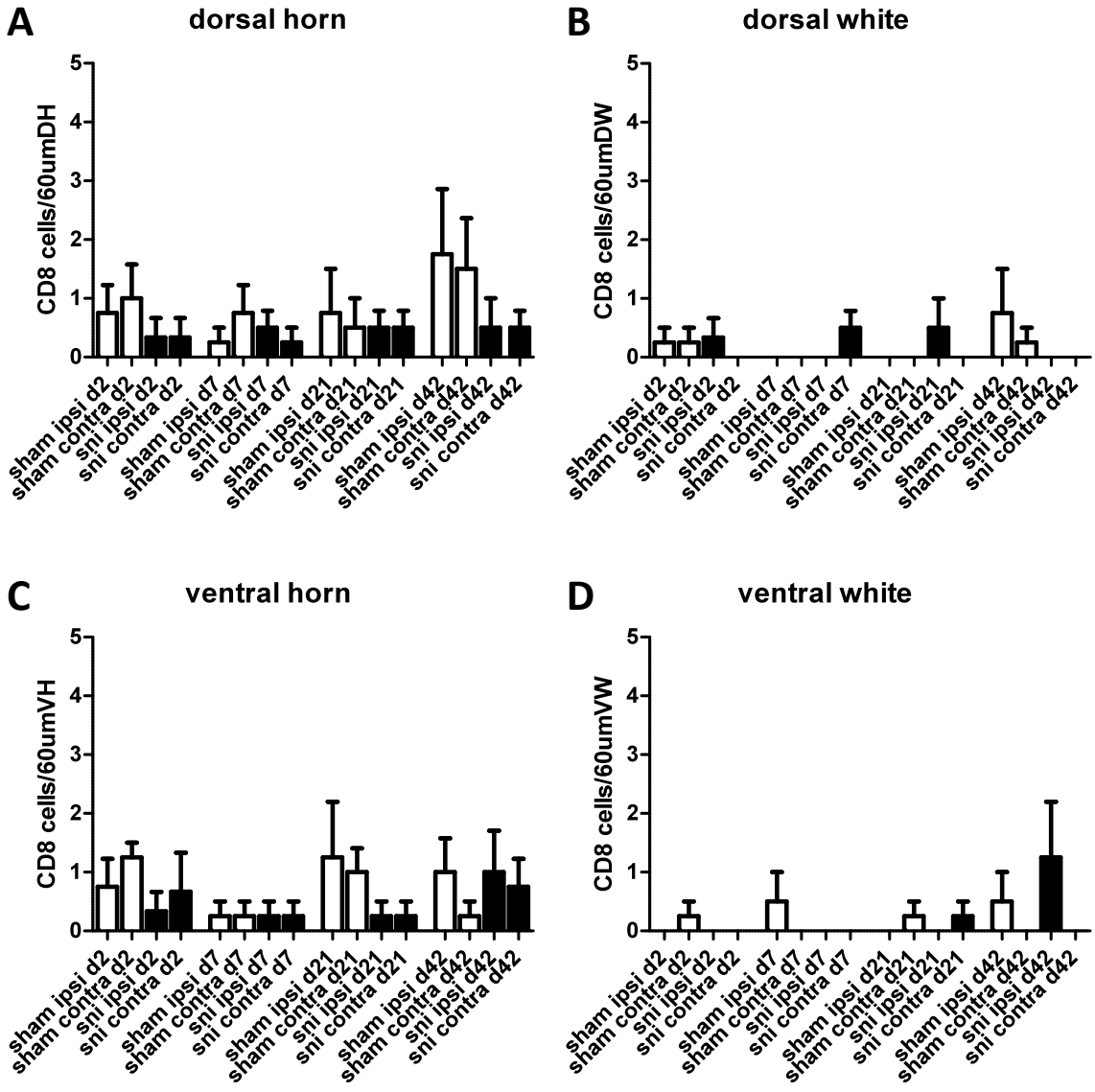

Fig. 7: Time course of CD8-positive T-cell distribution in the lumbar spinal cord. A-D: bar histograms showing the number of CD8+ cells detected in SNI (black) or sham (white) animals, at days 2, 7, 21 and 42, ipsilateral (ipsi) or contralateral (contra) to injury. Four regions of the spinal cords were individualized in each histogram: *A*, the dorsal horn (DH), *B*, the dorsal white matter (DW), *C*, the ventral horn (VH), *D*, the ventral white matter (VW). In several cases, no cells were detected. N = 4/group. Values are expressed as means ± SEM of the sum of cells counted in 2 lumbar sections of 30µm/animal.

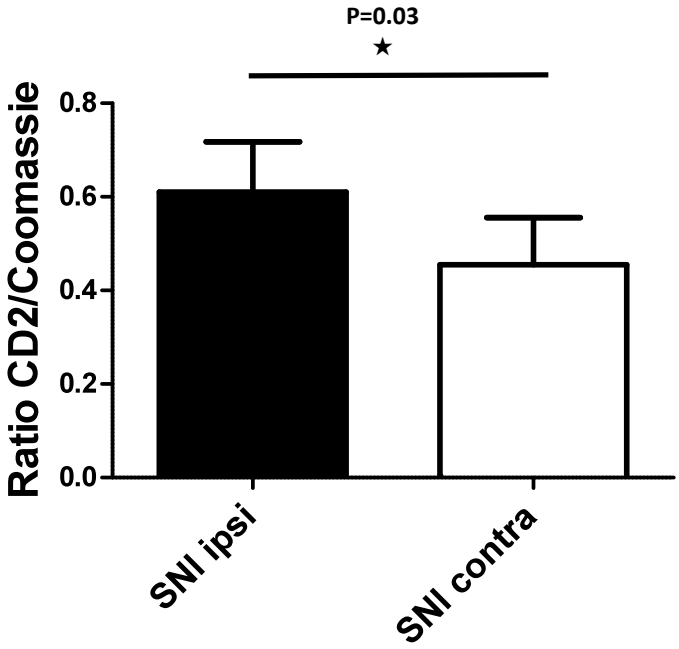

Fig. 8: CD2 protein in the dorsal horn after SNI. The means of the ratios are significantly higher for ipsilateral than for contralateral dorsal horns, when using paired, rather than unpaired, two-tailed Student's t-test. N = 4. Values are expressed as means ± SEM. ★, $p<0.05$ Student's t-test, ipsilateral vs contralateral.

DISCUSSION

A new perspective in research on neuropathic pain was opened by several seminal studies showing an infiltration of T-lymphocytes into the spinal cord after peripheral nerve injury in various animal models. However, no systematic description of the time course or cell types that characterize this infiltration has yet been published, although this might be the rational first step towards an overall understanding of the impact of T-cells on sensory function. Thus, the present study was undertaken with the aim of providing a comprehensive time course of T-cell infiltration in SNI.

In contrast with previous studies (Schweitzer et al, 2002 ; Hu et al, 2007 ; Cao and DeLeo, 2008 ; Costigan et al, 2009 ; Echeverry et al, 2011) but in agreement with others (Kim and Moalem Taylor, 2011 ; Austin et al, 2012), we did not find a significant increase in the number of spinal T-lymphocytes in the ipsilateral spinal cord after SNI compared to sham rats or the contralateral side of neuropathic animals. Therefore, we were unable to characterize the types of penetrating T-cells.

There are several possible reasons that could explain the discrepancy between our results and the studies mentioned above. First, in view the well-documented robustness of th SNI model, we did not confirm the development of hypersensitivity by performing behavioural tests. However, there was a marked neuroinflammation with increased Iba1 signal and microglial proliferation in the ipsilateral side of the spinal cord of SNI animals, which strongly prompt us to consider that peripheral nerve injury did produce neuropathic pain. The lack of difference in the GFAP signal only emphasizes the difficulty of showing the subtle changes occurring in astrocytes after peripheral nerve injury.

Small variances in the employed experimental protocol can lead to substantial differences in the observed outcome. For example, it was claimed that there was a differential inflammatory response in the spinal cord following two neuropathic pain models, where transection of the sciatic nerve did not result in the entrance of new T-lymphocytes but CCI did (Hu et al, 2007). The type of animal, model of pain and experimental techniques vary between the above-mentioned studies and our own, thus possibly explaining the disparity between the results. Nevertheless, we used the same

rat strain, pain paradigm and antibodies as others who reported 10 times more T-cells in the ipsilateral spinal cord of SNI rats than in our study (Costigan et al, 2009). Other factors such as the level of stress of the animals, differences in the way the SNI was performed or undisclosed details in the immunofluorescence protocol might therefore have impacted the results.

A limitation of our study is that we did not investigate the rostrocaudal distribution of T-cells, and nor did we normalize the exact segmental division within the L4-L5 region at which we counted the cells in the different animals. Most T-cells that Costigan et al found were located in the L4-L5 region, and their number was much lower above or below this part of the spinal cord (Costigan et al, 2009). Since the level of the spinal cord considered seemed to affect the number of T-cells counted, it could be interesting in future studies to sample more precisely at the same lumbar level for each animal.

Even though the number of T-cells was not greater in the ipsilateral spinal cord of SNI animals for most time points and regions considered, there were three exceptions. First, in SNI rats at day 21 after injury, the number of CD2-positive cells in the ipsilateral dorsal horn was significantly higher than on the contralateral side ($p=0.04$; Fig. 6A), but not in the sham-operated animals. Second, at day 7 after injury, there was a significant increase in CD2-positive cells in the ventral white matter compared to the contralateral side and sham animals ($p=0.02$ and $p=0.005$, respectively ; Fig.6D). Third, in the dorsal horn of sham-operated animals, the ipsilateral side contained more CD2-positive T-cells than the contralateral side, 2 days after injury ($p=0.03$; Fig. 6A). Although those results are statistically significant, their biological relevance is to be questioned for two reasons.

First, their profile in the time course does not point to a phenomenon caused by the nerve injury. Indeed, at day 21 after injury, despite the significantly higher number of T-cells in the ipsilateral dorsal horn compared to the contralateral side in SNI rats, there was no difference between sham and SNI animals. Thus, rather than neuropathic injury, it is more likely that a statistical sampling effect drives the mathematical significance (Fig. 6A). As for the statistically significant increase of T-cells in the ventral white matter one week after injury, it is unsure whether neuroinflammatory reaction located in the ventral white matter might influence pain behaviour. The biological mechanisms causing a higher number of CD2-positive cells to be present in the sham ipsilateral dorsal horn but not in the SNI are also unclear. In the three cases, statistical significance

can be further explained by the problem of multiple comparisons. Indeed, we compared many pairs of data, so there is an increased likelihood that some of them will have a p-value of less than 0.05.

Second, the number of T-cells was very limited in all cases. Indeed, in each 30 µm thick section of the spinal cord, there were less than 3 CD2-positive cells *per* side and time point in the dorsal horn, and less than 2 in the other regions considered. Thus, even if there was a statistically significant increase in the number of T-cells in ipsilateral regions of the spinal cord as mentioned above, the biological importance of these results is unclear. A better characterization of the phenotype of these cells, for example their naive/activated status, would help to understand the role they may play in neuropathic pain. However, this might be arduous to achieve considering their scarcity.

In contrast with the number of T-cells, the quantity of CD2 protein was moderately but significantly increased in the ipsilateral versus contralateral dorsal quadrants of SNI animals' spinal cords, 7 days after injury ($p=0.03$; Fig. 8). Statistical significance was achieved only when using paired, rather than unpaired, Student's t-test. The use of a paired t-test was appropriate when comparing ipsilateral versus contralateral spinal cords, because pairs of data come from the same animal. Several reasons can be evoked to understand the differential results between western blot and immunofluorescence. First, proteins are processed differently with the two techniques, so that the epitopes might be slightly changed. We also used a different anti-CD2 antibody for immunofluorescence or western-blotting, possibly recognizing somewhat different proteins. Additionally, we did not count the numerous fluorescent CD2-positive cells that were located in the meninges, whereas these membranes were not removed from the spinal cord samples used for the western-blot. Altogether, our immunofluorescence data revealed very few T-cells in the spinal cord after SNI, whereas our western blot results suggested that peripheral nerve injury induced an increase in a T-cell marker. Thus, we cannot draw a definitive conclusion on the subject. However, both techniques corroborate the fact that there was not a massive spinal infiltration of T-lymphocytes.

Despite being partially negative, our results bring to light some interesting facts. Indeed, it has been proposed that centrally infiltrating T-cells contribute to the development of mechanical hypersensitivity (Cao and DeLeo, 2008; Costigan et al, 2009), possibly by activating spinal cord glia, thus leading to the production of proinflammatory mediators

(Cao and DeLeo, 2008 ; Cao et al, 2009). However, we demonstrated that, following SNI, there could be an important glial activation and proliferation in the spinal cord with little or no central T-cell infiltration. This suggests that, if central lymphocytic traffic plays a role in promoting neuropathic pain, it is not a major contributor in the SNI model. Moreover, the main argument of the above-cited authors is that T-cell deficient animals display a reduced mechanical hypersensitivity after nerve injury. However, as this lack of functional T-lymphocytes occurs systemically and is not specific to the CNS, its effect on pain can be entirely explained by peripheral mechanisms. Indeed, our present data in neuromas as well as previous work has revealed that the inflammatory process at the site of injury included the presence of numerous T-cells in the injured nerve, and that they contributed to neuropathic pain (Moalem et al, 2004 ; Kleinschnitz et al, 2006 ; Kim and Moalem Taylor, 2011). Thus, while T-lymphocytes seem to take a part in neuropathic pain, their role appears to be greater in the peripheral nervous system, and their implication in the spinal cord mechanisms leading to central sensitization has yet to be convincingly proven.

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