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The Contributing Role of CD14 in Toll-Like Receptor 4 Dependent Neuropathic Pain

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Abstract

We have previously demonstrated that central nervous system (CNS) toll-like receptor 4 (TLR4) plays a key role in the development of behavioral hypersensitivity in a rodent model of neuropathic pain, spinal nerve L5 transection (L5Tx). TLR4 is a well-known receptor for lipopolysaccharide (LPS) in innate immune responses. In the current study, we further investigated the role of CD14, an accessory molecule in the LPS-TLR4 signaling pathway, in the development of L5Tx-induced neuropathic pain. CD14 knockout (KO) mice displayed significantly decreased behavioral sensitivity (mechanical allodynia and thermal hyperalgesia) as early as day 1 post-L5Tx, indicating a nociceptive role of CD14. By flow cytometric analyses, we observed significantly elevated microglial surface CD14 expression in the ipsilateral lumbar spinal cord 3 days post-L5Tx, as well as remarkable increases in microglial size (via forward scatter (FSC)) and granularity (via side scatter (SSC)). Further, intrathecal injection of soluble CD14 induced significantly greater mechanical hypersensitivity in wild type (C3H/HeN) mice compared to TLR4-deficient (C3H/HeJ) mice. Together, these data demonstrate that CD14 plays a contributing role in TLR4-dependent nerve injury-induced neuropathic pain.

Keywords

Spinal nerve transection; TLR4; Mechanical allodynia and Thermal hyperalgesia

Introduction

The innate immune response is the first line of defense against infectious disease. Although previously thought to be a nonspecific process engaged only by peripheral organs to maintain homeostasis after stress and injury, there is now increasing evidence suggesting that the central nervous system (CNS) mounts an organized innate immune response during systemic infection and neuronal injury (Aravalli et al., 2007, Muzio et al., 2007). Evidence indicates that the

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injured CNS (e.g., in stroke, Alzheimer's disease, and other neurodegenerative diseases) requires an innate immune intervention in order to limit damage and initiate tissue repair for the clearance of apoptotic cells and toxic debris such as myelin, amyloid fibrils, and damaged neurons (Wyss-Coray and Mucke, 2002). Investigations have also focused on the role of CNS immune responses after nerve injury that leads to behavioral hypersensitivity, a hallmark of neuropathic pain. A current theory for the etiology of neuropathic pain involves CNS immune activation with cytokine/chemokine production inducing the expression of final common pain mediators such as glutamate and nitric oxide (DeLeo and Yezierski, 2001, Watkins et al., 2003, Wieseler-Frank et al., 2005).

Toll-like receptors (TLRs), a family of pathogen recognition receptors of the innate immune system, have been found to be expressed by multiple types of immune cells, including CNS microglia (Aravalli et al., 2007, Krishnan et al., 2007, Miyake, 2007). In particular, TLR4 is a transmembrane protein specialized in the recognition of the bacterial endotoxin, lipopolysaccharide (LPS) (Miyake, 2004). Our previous study evaluated the role of CNS TLR4 in the development of behavioral hypersensitivity in a rodent model of neuropathic pain, spinal nerve L5 transection (L5Tx) (Tanga et al., 2005). We demonstrated that mice that lack functional TLR4 and rats intrathecally administered with antisense oligodeoxynucleotide (ODN) to TLR4 exhibited attenuated nerve injury-induced behavioral hypersensitivity in both the initiation and maintenance phases post-surgery (Tanga et al., 2005). Recently, Bettoni et al. have reported that repeated administration of a potent TLR4 antagonist (FP-1) resulted in relief of both thermal hyperalgesia and mechanical allodynia in mice with painful neuropathy (Isabella Bettoni, 2008). TLR4-mediated attenuation of neuropathic pain behaviors was further attributed to decreased CNS proinflammatory cytokine expression post-L5Tx (Tanga et al., 2005). However, the initial signal(s) triggering TLR4 activation and signaling/accessory molecules involved in TLR4 activation were not elucidated in the previous study.

It has been well accepted that recognition of bacterial LPS by TLR4 is greatly amplified by an accessory protein, CD14, which is also a pathogen recognition molecule. CD14, serving as a carrier linking LPS to cell surface TLR4 (Miyake, 2004), is expressed by mature myeloid cells (including CNS microglia) and also exists in a secreted form in the circulation (Landmann et al., 2000). Mice with a targeted mutation of CD14 were found to be highly resistant to LPS-induced endotoxic shock (Haziot et al., 1996). We have previously observed an increase of CD14 mRNA levels post-L5Tx in the lumbar spinal cord of rats (Tanga et al., 2004). However, it was not clear what the role of CD14 is in L5Tx-induced neuropathic pain and the relationship between CD14 and TLR4 in the development of neuropathic pain following nerve injury.

In the present study, we used *in vivo* murine models to investigate the specific role of CD14 and its interaction with TLR4 in the development of behavioral hypersensitivity induced by peripheral nerve injury. Our results demonstrate a contributing role of CNS CD14 in the development of behavioral hypersensitivity post-nerve injury. Data further suggest that the nociceptive role of CD14 is mediated primarily through TLR4-dependent pathways.

Experimental procedures

Animals

Male C57BL/6 mice were purchased from Charles River (Wilmington, MA) and the National Cancer Institute (NCI, Frederick, MD). Male CD14 Knockout (KO) mice on the C57BL/6 background were obtained from the Jackson laboratory (Bar Harbor, Maine). Male C3H/HeJ and C3H/HeN (mammary tumor virus (MTV) negative) mice (7-8 week old) were purchased from NCI (Frederick, MD). C3H/HeJ mice are TLR4 deficient due to a spontaneous missense point mutation Pro → His at position 712 in the third exon of the TLR4 gene (Poltorak et al., 1998). C3H/HeN mice are the respective wild type controls. Mice were allowed to habituate

to the Dartmouth-Hitchcock Medical Center animal facility for at least 1 week before being used in experiments. They were group-housed (3-4 per cage) with food and water *ad libitum* and maintained on a 12-h light/dark cycle. Mice were 8-9 weeks old when used. The Institutional Animal Care and Use Committee (IACUC) at Dartmouth College approved all experimental procedures.

Spinal nerve L5 transection (L5Tx)

Surgery was conducted following previously published procedures (Tanga et al., 2005). Briefly, mice were anesthetized by inhalation of halothane or isoflurane (4% for induction and 2% for maintenance) in 100% O₂. To expose the L5 spinal nerve, a 1-2 cm skin incision overlying the L4-S1 section was made, the muscle tissue was separated and retracted from the left superior articular processes and the transverse process. The L6 transverse process was removed 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.

Behavioral tests

For all the behavioral tests, animals were previously baseline tested before L5Tx or CD14 injection, and tested at selected times post-surgery or post-CD14 injection. The person performing the behavioral tests was blinded to the experimental groups.

Mechanical sensitivity—The tactile sensitivity response was used and measured as the direct pressure stimulus required to elicit foot-withdrawal in non-restrained conditions. Tests for animals subjected to surgery were conducted in the morning between 8:30–11:30 AM. Tests for animals subjected to intrathecal injection were conducted during the daytime between 8:30 AM–5:00 PM. Animals were habituated to the testing apparatus for at least 40 min before testing. For mechanical allodynia (allodynia – increased response to a non-noxious stimulus, used in Figure 1A), each animal was subjected to the stimulation of 2 von Frey filaments (0.008 g and 0.02 g, Stoelting, Wood Dale, IL); each filament was pressed to the point of 30° bending against the plantar surface of the ipsilateral hind paw. The testing paradigm consists of 3 sets of 10 stimulations with each filament (total 30 stimulations for each). Each set of 10 stimulations was separated by approximately 10 minutes to decrease possible sensitization. Allodynia was recorded as the numbers of paw withdrawals out of 30 stimuli. For testing animals without nerve injury (in Figure 5) in which small gauge filaments would not elicit sufficient responses for quantifying, each animal was subjected to the stimulation of a series of von Frey filaments ranging from 0.008 g 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 (Chaplan et al., 1994). The fifty percent threshold force needed for paw withdrawal was calculated for each mouse and used to represent the mechanical sensitivity of this animal.

Thermal sensitivity—Two independent tests were used to determine thermal hyperalgesia (increased response to a noxious stimulus, Figure 1B and C) as described previously (Tanga et al., 2005). *Hargreaves' thermal hyperalgesia test*: the thermal nociceptive latency was measured as the paw-withdrawal to radiant heat (Hargreaves et al., 1988) using the PAW Thermal Stimulator System 106 (UCSD, San Diego, CA, USA) described in detail previously (Lacroix-Fralish et al., 2006a). *Tail-flick test*: Thermal nociception was measured as the latency of tail withdrawal from 49°C hot water (with two third of the tail was immersed into the hot water). Three paw-withdrawal and tail-flick latencies were obtained per animal for each testing

session. The average latencies were used for final data analysis. For the results presented in Figure 1, each animal was tested in the following sequence: mechanical allodynia, Hargreaves' thermal hyperalgesia test and tail-flick test. Mice were acclimated to the testing chambers of the PAW Thermal Stimulator System for 40-60 min before being subjected to the Hargreaves' thermal hyperalgesia test. Following the Hargreaves' thermal hyperalgesia test, mice were given about 30 min to rest before subjected to the tail-flick test. It is commonly accepted that the Hargreaves test measures thermal sensitivity of the hind paw area affected by the injured spinal nerve in an unrestrained animal; while the tail flick test determines the general thermal sensitivity of the animal.

Flow cytometry

Mononuclear cell preparation from mouse lumbar spinal cord tissue for flow cytometry was described in detail previously (Cao and DeLeo, 2008). Briefly, mice were euthanized by CO₂ and transcardially perfused with 0.1M Phosphate-buffered saline (PBS). The ipsilateral and contralateral (relative to the injury) lumbar spinal cords were harvested. Tissue from pooled mice (4 per group) were homogenized and filtered through a 70-micron cell strainer (BD Biosciences, San Diego, CA). Following PBS wash, cells were subjected to discontinuous Percoll (Amersham, Piscataway, NJ) gradients, and cells of the mononuclear cell layer (between 40% and 70% Percoll) were collected. Total mononuclear cell number was determined using a hemocytometer (Fisher Scientific, Hampton, NH) with trypan blue (Sigma, St Louis, MO) before staining for flow cytometric analyses.

Following the final wash with PBS, cells were resuspended in staining buffer (2% fetal bovine serum (HyClone, Logan, UT) and 0.09% NaN₃ in PBS) containing anti-mouse-CD16/CD32 (1 µg/50 µl/tube) to prevent non-specific binding. After 30 min incubation, combinations of fluorescence-labeled monoclonal antibodies (mAbs): APC-Cy7-anti mouse CD45, PE-Cy7-anti mouse CD11b (BD Biosciences), and FITC-anti mouse CD14 (eBioscience, San Diego, CA) were added into each tube and cells were incubated on ice for another 30 min. After washing twice with PBS, all stained cells were finally resuspended in 1% formaldehyde / PBS and stored at 4 °C until analysis. Up to 10,000 events from each sample were analyzed using a FACSCanto flow cytometer with Cell Quest software (BD Biosciences). Non-stained cells and proper isotype controls were included in each run as the negative control and reference for data analysis. In general, forward scatter (FSC) is proportional to cell size; the larger the cell, the higher the detected signal. Side scatter (SSC) is proportional to cell complexity; the greater granularity of the cell, the higher the detected signal. Thus, parameters measured in FSC and SSC are often used to indicate the "morphological" features of the cell analyzed. All presented illustrations for flow cytometry analysis were generated using WinList software (Verity Software House, Topsham, ME)

Intrathecal administration of CD14

Intrathecal injection was performed with a 10-µl Hamilton syringe attached to a 30G needle inserted into the subarachnoid space through the space between the L5 and L6 vertebrae as described previously (Hylden and Wilcox, 1980). C3H/HeJ and C3H/HeN mice were briefly anesthetized by inhalation of isoflurane (4% for induction and 2% for maintenance) in 100% O₂, and then intrathecally injected with 5 µl of one of the following solutions: 1) 500 ng of soluble CD14 in the form of mouse CD14-human IgG Fc fusion protein (the only commercially available soluble mouse CD14; R&D Systems, Minneapolis, MN), 2) 500 ng of human IgG Fc control (Calbiocam-EMD, San Diego, CA). All drugs were prepared in sterile 0.1% bovine serum albumin (BSA)/PBS. CD14 doses were chosen based on our previous experience using intrathecal drug administration (Cauwels et al., 1999a, Lacroix-Fralish et al., 2006b) and pilot dose response experiments. The experimenter injecting the mice was blinded to the type of solution being used. Mice were allowed to recover from anesthesia (mice were awake usually

within 5 min post-injection) and then subjected to von Frey filament test for mechanical sensitivity post-injection. LPS - injected animals (i.t. 10 µg per mouse, *Salmonella enterica* serotype typhimurium, Sigma) were used as positive controls.

Statistical Analysis

All data were graphed with SigmaPlot 9.0 (Systat Software, Inc. San Jose, CA) and analyzed with GraphPad Prism 4 (GraphPad Software Inc. San Diego, CA) or SigmaStat 3.5 (Systat Software, Inc. San Jose, CA). Appropriate one-way analysis of variance or two-way analysis of variance (ANOVA) was performed followed by Bonferroni (GraphPad Prism 4) or Student-Newman-Keuls (SigmaStat 3.5) *Post hoc* analysis. All data are presented as mean ± SEM when applicable. $p < 0.05$ was considered as statistically significant.

Results

Behavioral sensitivity of CD14 KO mice

To investigate the involvement of CD14 in nerve injury - induced behavioral sensitivity, both CD14 KO mice on C57BL/6 background and C57BL/6 WT mice that underwent L5Tx or sham surgery were tested for their mechanical and thermal sensitivity. CD14 KO mice displayed significantly attenuated mechanical allodynia compared to the WT controls from day 1 to day 14 post-L5Tx (tested with von Frey filaments 0.02 g and 0.008 g). Similar results were obtained from the two filaments used and only results with 0.02 g are shown in Figure 1A (Two-way ANOVA, $p_{\text{time}} < 0.0001$, $p_{\text{surgery}} < 0.0001$, and $p_{\text{time} \times \text{surgery}} < 0.0001$). CD14 KO mice also showed decreased thermal hypersensitivity compared to the wild type control group post-L5Tx in both the Hargreaves' thermal hyperalgesia and the tail-flick tests (Figure 1B and C, Two-way ANOVA, in B: $p_{\text{time}} < 0.0001$, $p_{\text{surgery}} < 0.0001$, and $p_{\text{time} \times \text{surgery}} < 0.0001$, and in C: $p_{\text{time}} < 0.0001$, $p_{\text{surgery}} < 0.0001$, and $p_{\text{time} \times \text{surgery}} = 0.0001$). Interestingly, slightly but significantly increased behavioral sensitivities was observed in the “CD14 KO L5Tx” mice compared to the “CD14 KO sham” mice in all three behavioral tests. CD14 KO mice that underwent sham surgery exhibited similar mechanical and thermal sensitivities compared to the wild type sham controls (Figure 1).

Lumbar spinal cord microglial CD14 expression post-L5Tx

It is known that CD14 is expressed primarily by cells from monocytic origin, mainly microglia in the spinal cord. To establish a mechanistic association between behavioral sensitivity and CD14 expression, we determined microglial CD14 expression post-L5Tx by flow cytometry. A previous time course study (from day 0 to day 14 post-surgery) showed an increase of lumbar spinal cord CD14 mRNA expression by day 3 post-L5Tx (Tanga et al., 2004). A preliminary time course study (from day 0 to day 14 post-surgery) indicated that measurable changes of microglial CD14 was observed at day 3 post-L5Tx. Mononuclear cells were collected from both ipsilateral- and contralateral- lumbar spinal cord regions from pooled (4 per treatment in each experiment and 3 independent experiments were performed) mice. For naïve mice, left and right sides of lumbar spinal cord tissue were collected separately. CD45^{lo}CD11b⁺ cells were identified as microglia (Figure 2A). There was a significant increase in the total number of microglia in the ipsilateral lumbar spinal cord post-L5Tx compared to the contralateral side of transected mice and either groups of naïve samples (Figure 2B, One way ANOVA, $p = 0.057$, *t*-tests were performed between indicated groups). The sham-operated ipsilateral and contralateral spinal cords were not significantly different from each other and the naïve samples. Interestingly, we also observed “morphological” changes of microglia 3 days post-L5Tx based on the profiles in SSC vs. FSC dot plot by flow cytometric analysis. Microglia from the ipsilateral lumbar spinal cord displayed significant increases in both their size (FSC) and granularity (SSC) at day 3 post-surgery compared to microglia from the contralateral side of transected mice, sham and naïve mice (Figure 3A and data not shown. The numbers of

microglia with this phenotype, termed “enlarged microglia”, were determined and shown in Figure 3B (One-way ANOVA, $p = 0.022$).

Further analysis of lumbar spinal cord microglial CD14 expression via flow cytometry demonstrated that microglial surface CD14 expression significantly increased in the ipsilateral lumbar spinal cord post-L5Tx compared to the contralateral lumbar spinal cord (Figure 4, One-way ANOVA, $p = 0.005$). Expression of CD14 by splenocytes was also determined by flow cytometry and no significant increase of CD14 expression was observed post-L5Tx (data not shown).

CD14-induced mechanical hypersensitivity in TLR4 deficient mice

To further determine whether CNS CD14-dependent increased behavioral sensitivity is mediated through TLR4, we tested tactile mechanical sensitivity of C3H/HeJ mice (that have a spontaneously developed TLR4 point mutation) following intrathecal (i.t.) delivery of soluble CD14 (a mouse CD14-human IgG Fc fusion protein). Optimal CD14 dosage (500 ng per mouse) and time point (1 hr post-injection) were determined in a series of pilot experiments with various doses of CD14 and time points following i.t. injection. Vehicle alone-injected animals were also included in the pilot experiment and no significant behavioral differences between vehicle and human IgG Fc treatments were observed. In C3H/HeN (WT control) mice, CD14 induced significantly increased mechanical sensitivity compared to either non-treated mice or mice injected with human IgG Fc control, as well as compared to C3H/HeJ (TLR4 deficient) mice i.t. administered with the same dose of soluble CD14 (Figure 5, one-way ANOVA: $p_{groups} < 0.001$). Interestingly, human IgG Fc alone also induced significantly increased mechanical sensitivity in C3H/HeN but not C3H/HeJ mice, but with less magnitude than soluble CD14 treatment in C3H/HeN mice. Further, compared to naïve C3H/HeJ mice, soluble CD14 induced slightly but significantly increased mechanical sensitivity in the TLR4-deficient C3H/HeJ mice, which was not significantly different from human IgG Fc treatment in these mice. In addition, mice from each genotype were intrathecally injected with LPS to verify their differential responses to LPS as a result of their individual TLR4 phenotypes (Figure 5).

Discussion

The current study investigated the role of CD14 in nerve injury-induced behavioral hypersensitivity using a murine model of neuropathic pain. We had previously shown the direct involvement of TLR4 in the development of neuropathic pain (Tanga et al., 2005). The function of CD14 and CD14/TLR4 pathways had not been previously investigated in the etiology of neuropathic pain. The current results indicate a contributing role of CD14 in the development of nerve injury-induced persistent pain but not in the nociception of physiological pain. The observation that soluble CD14 induced significantly greater increase in the mechanical sensitivity in WT C3H/HeN mice compared to the TLR4 deficient C3H/HeJ mice suggests that the nociceptive role of CD14 is mediated primarily through TLR4-dependent pathways. Whether the involvement of CD14 is in both initiation and maintenance of neuropathic pain or is restricted to the initiation of neuropathic pain requires further investigation.

It has been well characterized that CD14 plays a crucial role in peripheral innate immunity through recognizing bacterial and yeast cell components (Landmann et al., 2000). Emerging evidence also indicates the role of innate immune responses and CD14 in the CNS. The presence of CD14 has been detected in both circumventricular organs in the brain and microglial cells in the CNS, and systemic challenge with LPS can significantly increase CNS CD14 expression in rodents (Rivest, 2003). Elevated CD14 expression is also observed during non-infectious situations (Bas et al., 2004), including inflammatory responses induced by nerve injury and neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis

(Fassbender et al., 2004, Tanga et al., 2004, Lutterotti et al., 2006). In addition, CD14 exists in two forms: membrane-bound and soluble CD14. Both membrane-bound and soluble CD14 are capable of delivering LPS to TLR4 and initiate the activation of TLR4 signaling pathway (Miyake, 2004). The observed significant increase of lumbar spinal cord microglial surface CD14 expression post-L5Tx suggests that microglial membrane-bound CD14 is most likely to be involved in the nociceptive role of CD14 after nerve injury. By using FACs analyses, this study focused only on the anchored form on the surface of microglia. However, CD14 can be released from the surface of microglia, (as well as from the surface of infiltrating and peripheral leukocytes) (Cauwels et al., 1999b). Thus, soluble CD14 may also contribute to enhanced nociceptive processing.

It should be noted that our behavioral data showed that CD14 deficiency did not completely reverse nerve injury-induced behavioral hypersensitivity (including both mechanical and thermal sensitivity, Figure 1). This observation reminds us of the complexity of the underlying mechanisms of neuropathic pain. Growing evidence indicates that multiple mediators together with CD14 contribute to nerve injury-induced behavioral hypersensitivity. It has been documented that microglial purinergic receptor-mediated pathways, chemokines involving neuronal-glial interaction (such as fractalkine), factors regulating glial recruitment (such as monocyte chemoattractant protein-1 (MCP-1), and spinal cord infiltrating leukocytes are involved in the development of neuropathic pain to various extents (Abbadie et al., 2003, Tsuda et al., 2003, Chessell et al., 2005, Milligan et al., 2005, Cao and DeLeo, 2008).

It is not surprising that CD14-mediated hypersensitivity can be TLR4 independent, which is suggested by our observation of a slight but significant increase in mechanical sensitivity in TLR4-deficient C3H/HeJ mice following CD14 injection (Figure 5). TLRs including both TLR4 and TLR2 are known co-receptors linking pathogen-stimulated CD14-NF- κ B activation (Landmann et al., 2000). TLR2 has been reported to be critical in the development of spinal nerve transection-induced mechanical and thermal hypersensitivity (Kim et al., 2007). The involvement of other TLRs, such as TLR2, in CD14-mediated nociception warrants further investigation. It is likely that similar signaling pathways as those in pathogen-stimulated CD14-TLR-NF- κ B activation are involved following peripheral nerve injury. We hypothesize that activation of CD14-TLR pathway may then lead to astrocytic activation, cytokine/chemokine expression and neuroinflammation (trafficking of leukocytes into the CNS). This process would lead to a subsequent release of pain mediators, such as glutamate and nitric oxide followed by neuronal sensitization, the electrophysiological read-out leading to behavioral hypersensitivity.

Thus far, we have demonstrated the involvement of both TLR4 and CD14 in nerve injury-induced neuropathic pain. To date no endogenous ligand(s) that initiates the CD14-TLR4 signaling pathway post-nerve injury has been identified. Numerous cellular products released during inflammation and tissue damage, including heat shock proteins (HSPs), extracellular matrix degradation products, high-mobility group box 1 (HMGB1) and β -defensins, so-called danger signals, have been reported as endogenous ligands for TLR4 (Miyake, 2007). In particular, various types of HSPs can stimulate/enhance microglial production of proinflammatory cytokines and phagocytosis *in vitro* (Kakimura et al., 2002). CD14 was found to be essential in HSP60 mediated signaling in human mononuclear cells (Kol et al., 2000). HSPs would be one of several valid candidates to investigate in future studies.

We are the first to report the observation of a morphological change in lumbar spinal cord microglia post-L5Tx using flow cytometry, i.e. the CD45^{lo}CD11b⁺ population display increases in both SSC and FSC post-L5Tx compared to sham surgery in flow cytometric analyses. These microglial changes strongly indicate a transformation of microglia from a “resting” or “surveying” state into a reactive phenotype (Ponomarev et al., 2005, Hanisch and

Kettenmann, 2007) after nerve injury-induced neuropathic pain. The associated cytoarchitectural changes during the dynamics of microglial activation as described by Dailey's group (Stence et al., 2001) need to be further defined.

In summary, our data indicate a nociceptive role of CD14 in the development of neuropathic pain and provide a further link between CD14 and TLR4 in chronic pain. These data provide additional evidence that CNS innate immune responses occur and can be deleterious to the long-term behavioral sequelae after peripheral nerve injury. However, the recognition that innate immunity also has a beneficial role to an organism complicates simple extrapolation to inhibiting these pathways as a novel treatment for chronic pain syndromes (Tahara et al., 2006, Stenzel et al., 2008). Further investigation is needed to reveal the molecular basis of the CD14-TLR4 mediated nociceptive pathway in order to determine whether fine-tuned regulation of this pathway can be utilized to develop novel targets for the treatment of chronic pain syndromes.

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References

- Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci U S A* 2003;100:7947–7952. [PubMed: 12808141]
- Aravalli RN, Peterson PK, Lokensgard JR. Toll-like receptors in defense and damage of the central nervous system. *J Neuroimmune Pharmacol* 2007;2:297–312. [PubMed: 18040848]
- Bas S, Gauthier BR, Spenato U, Stingelin S, Gabay C. CD14 Is an Acute-Phase Protein. *J Immunol* 2004;172:4470–4479. [PubMed: 15034063]
- Cao L, DeLeo JA. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *Eur J Immunol* 2008;38:448–458. [PubMed: 18196515]
- Cauwels A, Frei K, Sansano S, Fearn C, Ulevitch R, Zimmerli W, Landmann R. The Origin and Function of Soluble CD14 in Experimental Bacterial Meningitis. *J Immunol* 1999a;162:4762–4772. [PubMed: 10202018]
- Cauwels A, Frei K, Sansano S, Fearn C, Ulevitch R, Zimmerli W, Landmann R. The origin and function of soluble CD14 in experimental bacterial meningitis. *J Immunol* 1999b;162:4762–4772. [PubMed: 10202018]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994;53:55–63. [PubMed: 7990513]
- Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* 2005;114:386–396. [PubMed: 15777864]
- DeLeo JA, Yezierski RP. The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* 2001;90:1–6. [PubMed: 11166964]
- Fassbender K, Walter S, Kuhl S, Landmann R, Ishii K, Bertsch T, Stalder AK, Muehlhauser F, Liu Y, Ulmer AJ, Rivest S, Lentschat A, Gulbins E, Jucker M, Staufenbiel M, Brechtel K, Walter J, Multhaup G, Penke B, Adachi Y, Hartmann T, Beyreuther K. The LPS receptor (CD14) links innate immunity with Alzheimer's disease. *FASEB J* 2004;18:203–205. [PubMed: 14597556]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007;10:1387–1394. [PubMed: 17965659]

- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88. [PubMed: 3340425]
- Haziot A, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL, Goyert SM. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 1996;4:407–414. [PubMed: 8612135]
- Hylden JL, Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 1980;67:313–316. [PubMed: 6893963]
- Bettoni, Isabella; C, F.; R, C.; G, F.; G, G.; P, F.; C, B. Glial TLR4 receptor as new target to treat neuropathic pain: Efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* 2008;56:1312–1319. [PubMed: 18615568]
- Kakimura JI, Kitamura Y, Takata K, Umeki M, Suzuki S, Shibagaki K, Taniguchi T, Nomura Y, Gebicke-Haerter PJ, Smith MA, Perry G, Shimohama S. Microglial activation and amyloid- β clearance induced by exogenous heat-shock proteins. *FASEB J* 2002;16:601–603. [PubMed: 11919167]
- Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB, Lee SJ. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem* 2007;282:14975–14983. [PubMed: 17355971]
- Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Cutting Edge: Heat Shock Protein (HSP) 60 Activates the Innate Immune Response: CD14 Is an Essential Receptor for HSP60 Activation of Mononuclear Cells. *J Immunol* 2000;164:13–17. [PubMed: 10604986]
- Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S. Toll-like receptor signal transduction. *Exp Mol Med* 2007;39:421–438. [PubMed: 17934330]
- Lacroix-Fralish ML, Tawfik VL, Nutile-McMenemy N, DeLeo JA. Progesterone mediates gonadal hormone differences in tactile and thermal hypersensitivity following L5 nerve root ligation in female rats. *Neuroscience* 2006a;138:601–608. [PubMed: 16413124]
- Lacroix-Fralish ML, Tawfik VL, Nutile-McMenemy N, Harris BT, Deleo JA. Differential regulation of neuregulin 1 expression by progesterone in astrocytes and neurons. *Neuron Glia Biol* 2006b;2:227–234. [PubMed: 18049715]
- Landmann R, Muller B, Zimmerli W. CD14, new aspects of ligand and signal diversity. *Microbes Infect* 2000;2:295–304. [PubMed: 10758406]
- Lutterotti A, Kuenz B, Gredler V, Khalil M, Ehling R, Gneiss C, Egg R, Deisenhammer F, Berger T, Reindl M. Increased serum levels of soluble CD14 indicate stable multiple sclerosis. *J Neuroimmunol* 2006;181:145–149. [PubMed: 17046070]
- Milligan E, Zapata V, Schoeniger D, Chacur M, Green P, Poole S, Martin D, Maier SF, Watkins LR. An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. *Eur J Neurosci* 2005;22:2775–2782. [PubMed: 16324111]
- Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* 2004;12:186–192. [PubMed: 15051069]
- Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 2007;19:3–10. [PubMed: 17275324]
- Muzio L, Martino G, Furlan R. Multifaceted aspects of inflammation in multiple sclerosis: The role of microglia. *J Neuroimmunol* 2007;191:39–44. [PubMed: 17936915]
- Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science* 1998;282:2085–2088. [PubMed: 9851930]
- Ponomarev ED, Shriver LP, Maresz K, Dittel BN. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J Neurosci Res* 2005;81:374–389. [PubMed: 15959904]
- Rivest S. Molecular insights on the cerebral innate immune system. *Brain Behav Immun* 2003;17:13–19. [PubMed: 12615045]
- Stence N, Waite M, Dailey ME. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia* 2001;33:256–266. [PubMed: 11241743]
- Stenzel W, Soltek S, Sanchez-Ruiz M, Akira S, Miletic H, Schluter D, Deckert M. Both TLR2 and TLR4 are required for the effective immune response in *Staphylococcus aureus*-induced experimental murine brain abscess. *Am J Pathol* 2008;172:132–145. [PubMed: 18165267]

- Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 2006;129:3006–3019. [PubMed: 16984903]
- Tanga FY, Natile-McMenemy N, DeLeo JA. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 2005;102:5856–5861. [PubMed: 15809417]
- Tanga FY, Raghavendra V, DeLeo JA. Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int* 2004;45:397–407. [PubMed: 15145554]
- Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 2003;424:778–783. [PubMed: 12917686]
- Watkins LR, Milligan ED, Maier SF. Glial proinflammatory cytokines mediate exaggerated pain states: implications for clinical pain. *Adv Exp Med Biol* 2003;521:1–21. [PubMed: 12617561]
- Wieseler-Frank J, Maier SF, Watkins LR. Central proinflammatory cytokines and pain enhancement. *Neurosignals* 2005;14:166–174. [PubMed: 16215299]
- Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease--a double-edged sword. *Neuron* 2002;35:419–432. [PubMed: 12165466]

Comprehensive list of abbreviations

CNS	Central nervous system
L5Tx	Spinal nerve L5 transection
TLR	Toll-like Receptor
LPS	Lipopolysaccharide
KO	Knockout
FSC	Forward scatter
SSC	Side scatter
ODN	Oligodeoxynucleotide
MTV	Mammary tumor virus
NCI	National Cancer Institute
PBS	Phosphate-buffered saline
mAbs	Monoclonal antibodies
BSA	

	Bovine serum albumin
MCP-1	Monocyte chemoattractant protein-1
HSP	Heat shock protein
HMGB1	High-mobility group box 1

Figure 1. Behavioral sensitivity of CD14 KO mice

CD14 KO mice on the C57BL/6 background and wild type control mice were subjected to either L5Tx or sham surgery. All mice were tested for mechanical allodynia with von Frey filaments (A), thermal hyperalgesia using both the Hargreaves test (B) and the tail flick test (C). Data are presented as mean $\pm$ SEM. Numbers of animals used in each group are indicated in the graph (n). Significant differences ($p < 0.05$ via *post-hoc* test) between the “CD14 mice-L5Tx” and “C57BL/6-L5Tx” groups at each time point are indicated with *. # indicates the significant differences ($p < 0.05$ via *post-hoc* test) between “CD14 mice-L5Tx” and “CD14 mice-Sham” groups at each time point.

Figure 2. Identification of lumbar spinal cord microglia post-L5Tx

C57BL/6 mice were subjected to either L5Tx or sham surgery. Mononuclear cells from lumbar spinal cord of pooled animals (4 mice per sample group) were collected 3 days post-surgery and analyzed by flow cytometry with mAbs against CD45, CD11b and CD14. Figure 2 presents the changes of total lumbar spinal cord microglia (other parameters presented in Figure 3 and 4 respectively). Representative dot plots from flow cytometric analyses are shown in A: left panel – SSC vs. FSC dot plot showing total collected lumbar spinal cord mononuclear cells from naive mice, which is indicated in the R1 region; right panel – gated on R1 region, CD11b vs. CD45 dot plot showing CD45^{lo}CD11b⁺ microglia (in the circled region) from the same mice. Numbers of total lumbar spinal cord microglia per mouse were calculated (from three independent experiments) and are shown in B. Data are presented as mean $\pm$ SEM. + denotes the significant differences between the indicated groups via *t*-test, $p < 0.05$. ipsi = ipsilateral side and contra = contralateral side.

Figure 3. “Morphological” phenotype of lumbar spinal cord microglia post-L5Tx

Mononuclear cells were collected from lumbar spinal cord of pooled animals and analyzed by flow cytometry as described in Figure 2. Representative SSC vs. FSC dot plots of identified CD45^{lo}CD11b⁺ microglia (as described in Figure 2A right panel) from selected groups (from left to right) – naïve-left, day 3 sham-ipsi, and day 3 L5Tx-ipsi are shown in A. Microglia from naïve animals are contained mostly in the circle R4. Microglia outside circle R4 express both increased granularity and size compared to the cells within R4, so called with “enlarged” phenotype. Average percentage of “enlarged” microglia in total microglia of each group is presented within each plot. Numbers of total “enlarged” microglia per mouse were calculated (from three independent experiments) and are shown in B. Data are presented as mean $\pm$ SEM. * indicates the significant differences between the “L5Tx-ipsi” group and all other groups via *post-hoc* test, $p < 0.05$. ipsi = ipsilateral side and contra = contralateral side.

Figure 4. CD14 expression of lumbar spinal cord microglia post-L5Tx

Mononuclear cells were collected from lumbar spinal cord of pooled animals and analyzed by flow cytometry as described in Figure 2. Representative CD11b vs. CD14 dot plots of identified CD45^{lo}CD11b⁺ microglia (as described in Figure 2A right panel) are shown in A: left panel – microglial cells without CD14 staining (i.e. CD45 and CD11b staining only) and right panel – microglia from ipsilateral side of L5Tx mice with CD14 staining. CD14⁺ microglia are shown in the rectangular region. Numbers of total CD14⁺ microglia per mouse were calculated (from three independent experiments) and are shown in B. Data are presented as mean $\pm$ SEM. * indicates the significant differences between “L5Tx-ipsi” group and all other groups via *post-hoc* test, $p < 0.05$. ipsi = ipsilateral side and contra = contralateral side.

Figure 5. Mechanical sensitivity of TLR4 mutant mice post-CD14 injection

Tactile mechanical sensitivity was determined in both C3H/HeJ mice (TLR4 deficient, in grey) and the wild type control mice (C3H/HeN in white) 1 hr following intrathecal (i.t.) administration of either CD14-human IgG Fc fusion protein (labeled as CD14) or human IgG Fc (labeled as IgG) (both at 500 ng / 5 μ l / mouse). Baseline mechanical sensitivities of each strain of mice were assessed and all available data are shown here (Naïve). Numbers of animals for each group are the following, in C3H/HeN mice: Naïve group - 42, IgG treated group - 13, and CD14 treated group - 9; in C3H/HeJ mice: Naïve group - 30, IgG treated group - 4, and CD14 treated group - 9. All data are presented as mean $\pm$ SEM. As positive controls, mice from each strain were i.t. injected with 10 μ g of LPS and tested 1 hr post-injection (n = 1 for C3H/HeN mice and n = 2 for C3H/HeJ mice). Statistical analysis was performed using all experimental groups (not including LPS groups). # indicates significant differences between the indicated group and all other groups via *post-hoc* test; * indicates the significant differences between indicated group and the naïve group of the same mouse strain via *post-hoc* test; $p < 0.05$.