Depletion of Foxp3+ regulatory T cells increases severity of mechanical allodynia and significantly alters systemic cytokine levels following peripheral nerve injury

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Abstract:

Neuropathic pain is a debilitating condition caused by damage to the somatosensory nervous system, such as peripheral nerve injury. The immune system, and in particular the adaptive T cell response, plays a key role in mediating such pain. Regulatory T (Treg) cells are a small subpopulation of inhibitory T cells that prevent autoimmunity, limit immunopathology and maintain immune homeostasis. Here, we investigated the effects of conditional depletion of Treg cells on mechanical allodynia and serum cytokines in mice with chronic constriction injury (CCI) of the sciatic nerve, an animal model of neuropathic pain. We demonstrate that CCI induced the infiltration of small numbers of Treg cells within effected neuronal tissue. Utilising the transgenic DEREG (DEpletion of REGulatory T cells) mice, we confirmed effective depletion of Foxp3+ Treg cells by diphtheria toxin injections. Following CCI we observed a transient, though significant, increase in pain hypersensitivity for Treg-depleted DEREG mice compared to non-Treg-depleted mice. Analysis of systemic cytokine levels demonstrated significant changes in serum cytokine expression profiles. In particular, we observed significant increases in systemic concentration of RANTES, IL-2 and IL-5, and significant decreases in IL-12 and IFN-γ in nerve-injured Treg-depleted DEREG mice. Further analysis indicated a substantial increase in the serum concentration of IL-12p40 as a direct result of Treg cell depletion. These results suggest that depletion of Foxp3+ Treg cells promote nerve injury-induced pain hypersensitivity, partially by inducing altered systemic concentrations of cytokines, which may act to regulate neuropathic pain.

Keywords: Treg; DEREG; chronic constriction injury; neuropathic pain; systemic cytokines.

Abbreviations: Chronic constriction injury (CCI), partial sciatic nerve ligation (PSNL), diphtheria toxin (DT), green fluorescent protein (GFP), sciatic nerve (SN), dorsal root ganglion (DRG), spinal cord (SC), wild type (WT), phosphate buffered saline (PBS), room temperature (RT), Roswell Park Memorial Institute medium (RPMI), enzyme-linked immunosorbent assay (ELISA), analysis of variance (ANOVA), standard error of the mean (SEM).
**Introduction**

Development of neuropathic pain following nerve injury is now understood to be a neuroimmune disorder involving inappropriate activation of the immune system [1]. Immune cells such as neutrophils, macrophages, CD4+ T-cells and immune-like glial cells (microglia and astrocytes) are known to respond to nerve injury by altering intercellular signalling and by migrating to areas of inflammation within the peripheral and central nervous system [2]. The precise nature of immune cell-based intercellular signalling relevant to the development of neuropathic pain is yet to be fully defined, but experimental evidence indicates that cytokine-mediated signalling is critical [3].

We have previously shown that CD4+ T-cells contribute to the development of neuropathic pain [4]. Signalling between CD4+ T-cells and other immune cells within the nervous system via the release of cytokines is an active area of research and a target for the development of clinical therapies [5]. During the adaptive T-cell response, conventional CD4+ T-cells are transformed into a variety of effector CD4+ T-cell subsets (e.g. Th (T-helper) 1, Th2, Th9 and Th17), and each of these secrete different cytokines. Regulatory Foxp3+ T (Treg) cells play a key role in determining the activity of CD4+ T-cells by regulating the development and proliferation of cells within each Th subset, and therefore have a substantial influence on CD4+ T-cell cytokine secretion. Treg cells also substantially influence cytokine secretion from other relevant immune populations such as dendritic cells and macrophages. Therefore, we hypothesise that systemic depletion of Treg cells would have a significant impact on systemic cytokine profile, which may influence neuropathic pain due to nerve injury.

We have previously demonstrated that enrichment of Treg cells with CD28 superagonist attenuated mechanical allodynia in a chronic constriction injury (CCI) model in rats, whilst suppression of Treg cells using anti-CD25 antibody increased the severity of mechanical allodynia in a partial sciatic nerve ligation (PSNL) model in mice [6]. These results suggest that Treg cells influence the development of neuropathic pain. Targeting the CD25 receptor is not specific for Treg
cells and therefore, we sought out a more effective depletion method. The DEREG (DEpletion of REGulatory T cells) mouse model, in which cells express a transgene containing the human diphtheria toxin (DT) receptor tagged with green fluorescence protein (GFP) under the control of the Foxp3 promoter, is a useful option for studying the systemic effect of Treg cell depletion in injury and disease [7,8]. Treatment of these mice with diphtheria toxin leads to a transient but almost complete depletion of Treg cells [9]. Here, we demonstrate that specific depletion of Treg cells in DEREG mice increases the severity of mechanical allodynia associated with sciatic nerve (SN) injury and also results in significant changes in serum cytokine profile, which may play a role in the development of neuropathic pain.
1. **Materials and Methods**

2.1 *Animals*

Experiments were carried out on male C57BL/6 wild-type (WT) or DEREG mice [9] (Animal Resource Centre, Moss Vale, NSW, Australia). Animals were housed with free access to food and water and maintained on a 12:12-hour light/dark cycle. All animal experiments were approved by the Animal Care and Ethics Committee of UNSW, Australia, and followed guidelines issued by the International Association for the Study of Pain.

2.2 *Chronic constriction injury and Treg cell depletion*

Mice in the Treg cell depletion treatment group were given intraperitoneal (i.p.) injections of 500ng/ml of diphtheria toxin (Merck Millipore, Kilsyth, VIC, Australia) in 100µl of phosphate buffered saline (PBS) once a day on the two days prior to CCI, whilst control mice received vehicle injections. On the day of surgery mice were anaesthetized with isoflurane (Delvet, Seven Hills, NSW, Australia), and the left SN exposed. Two chromic gut ligatures (7-0, Ethicon, Somerville, NJ, USA) were tied loosely around the SN at 1-mm intervals, proximal to the trifurcation, to just occlude but not arrest epineural blood flow [10]. In sham operated mice, the left SN was exposed, but not ligated. The muscle layers were closed with sutures (Mersilk, 5-0, Ethicon, Somerville, NJ, USA), and the skin fastened with staples (Autoclip, 9 mm; BD Diagnostics, North Ryde, NSW, Australia). Mice in the Treg cell depletion group were given two additional injections of 500ng/ml of DT on days 5 and 9, whilst control mice received vehicle injections. All mice were culled for analysis of Treg cell depletion and serum cytokine concentration on day 10. For Foxp3 immunohistochemistry and Western blot analysis, C57BL/6 mice underwent CCI or sham operation, as described above, and were sacrificed on day 10 post-surgery.
2.3 **Mechanical allodynia**

Mechanical allodynia was assessed on days 2, 4, 6 and 8 of the 10 day experimental period by placing animals on an elevated wire mesh and stimulating the plantar surface of both the left and right hind paws, using manual von Frey hairs. The up-down method [11] was used with the following filament scale (0.07g, 0.16g, 0.4g, 0.6g, 1g, 1.4g, 2g, 4g, 6g) and testing initiated using the 1g filament. Each paw was tested by holding filament for 3-4 seconds. A positive response was recorded if mouse licked paw, flinched or lifted its leg. Ambulation or unrelated movement was not recorded as a positive response. At least 5 minutes was elapsed between subsequent paw tests. We then calculated the 50% paw withdrawal threshold using the formula and table previously published [11]. The assessor of mechanical allodynia was blinded to the identity of the animals during the testing process.

2.4 **Immunofluorescent staining**

Inguinal lymph nodes were removed under isoflurane anaesthesia and collected in 4% paraformaldehyde. Tissue was fixed overnight in 4°C, before being transferred to 30% sucrose/PBS for cryo-protection. Tissue was placed in Tissue Tek® O.C.T. compound (Sakura, AJ Alphen aan den Rijn, The Netherlands), and frozen at -20°C prior to being sectioned using a cryostat in 10µm sections, and subsequently transferred to coated glass slides. Slides were stored at -20°C until staining. For removal of nervous tissues, mice were killed by lethabarb injection and transcardially perfused with saline containing heparin followed by 4% paraformaldehyde. Following perfusion, left and right SNs, left and right L3-L5 dorsal root ganglion (DRG) and lumbar spinal cords (SC) of each mouse were harvested and post-fixed overnight in 4% paraformaldehyde in PBS at 4°C and later stored in 30% sucrose solution. Tissues were embedded in paraffin and cut into 10-15µm sections before being mounted on gelatine-coated slides. Formalin-fixed, paraffin embedded tissues
were subjected to antigen retrieval in citrate buffer (pH 6.0) at 95°C for 15 minutes. Cryosections or paraffin-embedded sections were permeabilised in 0.1% Triton-X-100/PBS for 30 minutes and blocked for 30 minutes in 0.1%-Tween-20/5%-serum/PBS. Then rat anti-Foxp3 (FJK-16s; 1:25; eBioscience, San Diego, CA, USA) primary antibody was applied overnight at 4°C, and rabbit anti-GFP (A11122; 1:500; Life Technologies, Mulgrave, VIC, Australia) primary antibody was applied for 1 hour at room temperature (RT). This was followed by washings with PBS + 0.05% Tween-20 and incubation with secondary antibody for 30 minutes – 1 hour at RT, protected from light. The secondary antibodies used were Cy2-conjugated donkey anti-rat secondary antibody (1:100; Jackson Laboratories, Bar Harbor, ME, USA) and anti-rabbit Alexa Fluor 488 (1:1000; Life Technologies, Mulgrave, VIC, Australia). Subsequent to incubation with secondary antibody, sections were washed with PBS + 0.05% Tween-20. Prolong gold anti-fade with DAPI (Life Technologies, Mulgrave, VIC, Australia) was then applied, and sections were sealed with a coverslip for microscopic analysis. Sections were viewed and images were captured using an Olympus fluorescent microscope, and cells were counted using NIH ImageJ software.

2.5 Flow-cytometry

Inguinal lymph nodes were removed under isoflurane anaesthesia and placed in PBS. A single-cell suspension was created by grinding lymph nodes with a plastic pestle within a 1ml centrifuge tube, then passing cells through a 40µm cell strainer (BD Bioscience, Franklins Lakes, NJ, USA). For results relating to Figure 2b and c, cells were incubated at 37°C in Roswell Park Memorial Institute medium (RPMI) (Life Technologies, Mulgrave, VIC, Australia) for 1 hour, to allow adherence and therefore removal of monocytes. After incubation, cells were counted and re-suspended in flow cytometer staining buffer. Cell surface markers were stained for 30 minutes whilst protected from light with rat anti-mouse CD4-PE-Cy7 (RM4-5; 1:1000; eBioscience, San Diego, CA, USA) and rat anti-mouse CD25-APC (PC61.5; 1:1000; eBioscience, San Diego, CA, USA), or suitable isotope controls. Cells were then washed and analysed using a BD FACS Canto II.
and DIVA software (BD Bioscience, Franklin Lakes, NJ, USA). Final analysis and plot formatting was performed using FlowJo software (Tree Star, Ashland, OR, USA).

### 2.6 Cytokine assays

Under isoflurane anesthesia, mice were killed by lethabarb injection. The heart was exposed and blood extracted using a syringe and needle, and then transferred to an eppendorf tube. The volume of collected blood was >200µl per mice. Whole blood was allowed to clot for at least 1 hour at RT before centrifugation. The serum supernatant was removed and transferred to a fresh eppendorf tube, then stored at -80°C. For analysis using the Bio-plex 23 cytokine mouse array (Bio-Rad, Hercules, CA, USA), serum was diluted 1:4 and analysed following the manufacturer’s instructions. The plates were read using the Bioplex reader (Bio-Rad, Hercules, CA, USA), and results were converted to a pg/ml concentration for all of the cytokines tested. The concentration detected within the mouse serum for several of the 23 cytokines included in the assay was below the limit of detection and no meaningful data were attained for these cytokines (IL-3, GM-CSF and Eotaxin). Further analysis of cytokines using enzyme-linked immunosorbent assay (ELISA) was performed with the IL-12p40 and IL-23 Quantikine kits following the manufacturer’s instructions (R&D systems, Minneapolis, MN, USA). Serum was diluted 1:2 for ELISA assays. Data was converted to a pg/ml concentration using a SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

### 2.7 Western blot

Mice were killed by lethabarb injection and transcardially perfused with saline containing heparin. Following saline perfusion, the lumbar SC, left SN and L3-L5 DRGs of nerve-injured and sham-operated mice were taken and immediately snap frozen in liquid nitrogen. Mice tissues were then weighed and resuspended in TM buffer (50 mM Tris Cl, pH 7.5, 10 mM Magnesium Sulfate)
containing protease inhibitors before being homogenised for 2 minutes in a Precellys homogeniser
(Bertin Corp, Washington DC, USA). Tissues were then incubated for 20 minutes at 4°C before
centrifugation at 4°C for 20 minutes at 11,000 rpm. The supernatants were collected and protein
concentration was quantified using an EZQ protein quantification assay (Life Technologies,
Mulgrave, VIC, Australia). Protein extracts were then loaded (10µg of protein per lane) into 4-12%
Bis-Tris gels and separated using SDS-polyacrylamide gel electrophoresis at 200V for 45 minutes.
The gel was then blotted onto polyvinylidene difluoride membranes using the iBlot system (Life
Technologies) according to manufacturer’s instructions. Chemiluminescent detection of protein
expression was performed using an anti-rabbit Western Breeze kit (Invitrogen). Membranes were
blocked in the provided blocking solution for 30 minutes before incubated with the primary
antibodies overnight at 4°C. The primary antibodies used were rabbit anti-mouse GAPDH (ab9485;
Abcam, Cambridge, UK) for housekeeping gene diluted at 1:4000 and a rabbit anti-mouse Foxp3
antibody (ab54501; Abcam, Cambridge, UK) diluted 1:1000. The membranes were then washed
before incubation with the provided secondary antibody for 1 hour at room temperature. This was
followed by 5-minute incubation with the chemiluminescent substrate provided in the kit. Protein
expression was then visualised and quantified using a ChemiDoc Imaging System (Bio-Rad).

2.8 Statistical analysis

For analysis of mechanical allodynia data, non-parametric Kruskal-Wallis test followed by
Dunn’s multiple comparison was performed to determine differences in paw withdrawal threshold
between more than 2 groups. Mann-Whitney analysis was performed to determine specific
differences between 2 groups. For analysis of parametric data, two-way analysis of variance
(ANOVA) followed by Bonferroni’s multiple comparisons test was performed for flow cytometric
data, and one-way ANOVA followed by Tukey’s multiple comparisons test was performed for
cytokine assay data. For analysis of western blot and immunohistochemical data, comparison
between the ipsilateral side and contralateral side or sham-operated of each tissue was performed
using unpaired Students t-test. In all cases, a probability of 0.05 or less was considered statistically significant.
3. Results

3.1 Foxp3+ Treg cells infiltrate the nervous system following peripheral nerve injury

In order to assess the involvement of Tregs in the CCI model of neuropathic pain, we first examined SN, DRG and SC for Foxp3-expressing cells following injury. Tissue was collected 10 days after injury and analysed using immunofluorescent staining. We found a small, though significant, infiltration of Foxp3-expressing cells to all these tissues in the ipsilateral injured side when compared to the non-injured contralateral side (Figure 1a-b). In the SN, infiltration was most prominent at the site of injury but also elevated proximal and distal to the injury (Figure 1b). A significant increase in the presence of Foxp3-expressing cells was also demonstrated in the DRG and SC of the ipsilateral side compared to the contralateral side (Figure 1b).

To confirm Foxp3 expression, protein from the SN, DRG and SC of injured and sham-operated mice was analysed by western blot (Figure 1c). In both the SN and SC there was a significant increase in the expression of Foxp3 in nerve-injured mice compared to the sham-operated group, suggesting that Foxp3 positive cells infiltrated the SN and SC following peripheral nerve injury. Although there was a higher expression of Foxp3 protein in the DRG of injured animals, the difference between the injured and sham groups was not found to be significant (Figure 1c).

We then examined the inguinal lymph nodes of CCI-injured DEREG mice. The ipsilateral inguinal lymph node is located adjacent to the injury site and lymph nodes from the ipsilateral side were found to contain approximately fourfold greater cells than lymph nodes taken from the contralateral side (Figure 1d), indicating a significant immune response. This agrees with our previously published results showing substantial immune cell infiltration following CCI [12]. In the DEREG mouse model, Foxp3+ Treg cells can be tracked due to co-expression of GFP [9]. We looked at the percentage of cells from the total cell population of the lymph nodes, which were GFP+/Foxp3+ Treg cells, and found approximately 3% in both the ipsilateral and contralateral populations (Figure 1d). Together, these results suggest that the absolute number of Tregs in the
lymph node adjacent to the injury was approximately fourfold that of the lymph node from the contralateral side.

3.2 Depletion of Foxp3+ Treg cells in nerve-injured DEREGR mice

Our previous study demonstrated that suppression of CD25+ cells (including Treg cells) following intraperitoneal injection of anti-CD25 antibody increased the severity of mechanical allodynia due to PSNL [6]. A more targeted approach was adopted to deplete Treg cells (DEREG mice). Treg cells were depleted prior to CCI and further depleted over 10 days following CCI. Immuno-fluorescent staining with an anti-GFP antibody demonstrated that within the inguinal lymph nodes of control non-depleted DEREG mice, there was a significant population of GFP+(Foxp3+) cells. However, repeated treatment of DEREG mice with DT led to the disappearance of the GFP+(Foxp3+) cell population analysed at day 10 (Figure 2a). A further indication that Treg cells were successfully depleted was demonstrated by flow cytometric analysis of CD25+GFP+(Foxp3+) compartment of CD4+ positive cells in inguinal lymph nodes. Within the control group approximately 6.5% of CD4+ cells were found to be CD25+GFP+(Foxp3+), conversely DEREG mice which received DT were found to have approximately 0.2% CD25+GFP+(Foxp3+) on day 10 (Figure 2b and 2c).

We also observed that CD25-GFP+(Foxp3+) cells, sometimes referred to as central or resting Treg cells [13], were similarly depleted. Conversely, the proportion of CD25+GFP-(Foxp3-) cells significantly increased (Figure 2b and 2c). The CD25+GFP-(Foxp3-) cells most likely represent an increase in effector T-cells which has been demonstrated previously following suppression of Treg cells [14]. Taken together, these results clearly demonstrated a highly significant depletion of Foxp3+ Treg cells within the DT-treated DEREG mice during the 10 day treatment period.

3.2 Conditional ablation of Treg cells enhances mechanical pain hypersensitivity
To test whether specific depletion of Treg cells would increase the severity of mechanical allodynia following peripheral nerve injury, we used the CCI model in DEREG mice. We compared paw withdrawal thresholds in response to mechanical stimuli (von Frey filaments) in wild type mice given i.p. injections of PBS + sham surgery (WT+PBS+SHAM), DEREG mice given i.p. injections of PBS + CCI (DEREG+PBS+CCI) and DEREG mice given i.p. injections of DT + CCI (DEREG+DT+CCI). Kruskal-Wallis non-parametric analysis with Dunn’s multiple comparison gave a significant difference in ipsilateral hind paw mechanical allodynia between sham and both treatment groups at day 2 and between sham and DT treated animals at day 6. Further analysis using a Mann-Whitney test showed that both groups of nerve-injured DEREG mice had significantly reduced paw withdrawal thresholds on the ipsilateral side after CCI on days 2, 6 and 8 (Figure 3a). Mann-Whitney analysis specifically comparing (DEREG+PBS+CCI) vs (DEREG+DT+CCI) gave a significant difference between these specific treatment groups at day 2 (Figure 3a). These results demonstrated that the Treg-depleted group experienced significantly greater severity of mechanical allodynia at this time point compared to the non-depleted group. There was also an observable, but not significant, increase in the severity of mechanical allodynia in Treg-depleted mice compared to non-depleted mice at day 6 (Figure 3a). There was no significant difference in mechanical allodynia in the uninjured contralateral paw at any time point between any groups (Figure 3b). The lack of mechanical allodynia in the contralateral paw of DEREG mice treated with DT also demonstrated that Treg depletion on its own doesn’t alter paw withdrawal thresholds. To further confirm that DT injection by itself, in the absence of Treg depletion, doesn’t induce changes in pain sensitivity, we compared nerve-injured WT mice given i.p. injections of DT (WT+CCI+DT) and DEREG mice given i.p. injections of PBS (DEREG+CCI+PBS). There was no significant difference in mechanical allodynia in mice due to administration of DT alone (Figure 3c). Taken together, these results suggest that Treg cell depletion increases the severity of mechanical allodynia experienced by mice subjected to CCI.
3.3 Altered cytokine profile in nerve-injured Treg-depleted mice

Pain hypersensitivity results from mechanism dictated, at least in part, by cytokine signalling. Treg cells regulate the cytokine secretion of a range of immune cells. We hypothesised that systemic depletion of Treg cells would affect systemic cytokine concentrations, which may be associated with the development of pain hypersensitivity. Analysis of 23 cytokines within the serum of mice from each treatment group was assessed using the Bio-Plex Pro™ mouse cytokine 23-plex assay (Bio-rad). Interestingly, we found a significant change in the concentration of 6 of the cytokines which were tested (Figure 4).

In relation to the chemokine response, there was no significant change in the level of MIP-1α, MCP-1 and neutrophil attracting KC between the treatment groups (data not shown). However, the chemokine RANTES showed a significantly increased systemic concentration in Treg-depleted nerve-injured mice when compared to both sham control and non-Treg-depleted nerve-injured mice (Figure 4a).

There was no significant change in serum concentrations of most cytokines associated with the Th2 immune response (IL-4, IL-6, IL-10 or IL-13), but we found a very significant increase in IL-5 in Treg-depleted nerve-injured mice when compared to the other groups (Figure 4b). There was no change in Th9 subset-associated IL-9 concentration and no change in the Th17 T-cell subset-associated IL-17 concentration (data not shown). There was no significant changes in the Th1-associated cytokines IL-1α, IL-1β or TNF-α (data not shown), but we did observe a significant increase in the systemic concentration of IL-2 in Treg-depleted nerve-injured mice when compared to the non-Treg-depleted nerve-injured mice (Figure 4c). We also found the concentration of IL-12 was significantly reduced, and this decrease was mirrored by a similarly significant decrease in the concentration of IFN-γ in Treg-depleted nerve-injured mice when compared to the sham control group (Figure 4d and 4e). These results may be complementary as downstream signalling of IL-12 is a major activator of IFN-γ secretion [15]. The Bio-plex assay also included analysis of IL-12p40,
which is the β-chain of IL-12. Intriguingly, we detected that the concentration of this protein more than doubled following CCI in Treg-depleted mice (Figure 4f).

Considering we had detected a reduced concentration of IL-12 under these conditions, an increase in IL-12p40 was somewhat surprising. However, IL-12p40 is also the β-chain of the cytokine IL-23. Since IL-23 was not included in the Bioplex assay, we sought to detect the systemic concentration of IL-23 using a specific ELISA. Firstly, we confirmed that the concentration of IL-12p40 was in fact elevated in Treg-depleted nerve-injured mice by testing serum from new groups of mice and using a specific ELISA kit for IL-12p40 (Quantikine) (Figure 5a). We then performed an ELISA on the same serum testing for IL-23. Contrary to expectations, we found that IL-23 was significantly elevated in non-depleted nerve-injured mice compared to Treg-depleted nerve-injured mice (Figure 5b). Since our Bio-plex results indicated that IL-12 was reduced following Treg cell depletion and CCI and our ELISA results indicated that IL-23 was also reduced in these conditions, the doubling in IL-12p40 concentration might be in the form of a monomer, the poorly described IL-12p40 homodimer, or a component of an undescribed cytokine. To determine the specific role of the CCI, we tested serum from mice with only Treg cell depletion and no CCI and compared to control non-depleted mice. We found that the IL-12p40 concentration doubled as a direct result of Treg cell depletion (Figure 5c).
4. Discussion

In this study, we demonstrate infiltration of the SN, DRG and SC by a small number of Treg cells following CCI of the sciatic nerve, suggesting that these cells may play a role in regulating the inflammatory response within these tissues [12]. Neuropathic pain following peripheral nerve injury can be modulated by T cells. Indeed, the introduction of pro-inflammatory T-cells into athymic nude rats increased mechanical allodynia and thermal hyperalgesia, whilst the introduction of anti-inflammatory T-cells decreased pain hypersensitivity [4]. Furthermore, depletion of CD25+ cells, including Treg cells, resulted in increased pain hypersensitivity associated with peripheral nerve injury [6]. Here, we show that a more targeted approach to Treg cell depletion results in short term enhanced mechanical allodynia. In addition, we describe for the first time significant changes in systemic cytokine profile following Treg cell depletion and CCI. We found that serum concentrations of RANTES, IL-5 and IL-2 were all significantly elevated, whereas IL-12 and IFN-γ were, somewhat unexpectedly, significantly reduced in Treg-depleted nerve-injured mice. Strikingly, we also observed a highly significant increase in the concentration of IL-12p40, which was found to be directly related to Treg cell depletion.

RANTES is a ligand for the CCR5 receptor, which is known to be expressed by glial cells within the central nervous system. Recent research indicates that RANTES is associated with increased pain hypersensitivity and mechanical allodynia [16]. The RANTES antagonist met-RANTES was also demonstrated to alleviate mechanical allodynia [17]. Furthermore, elevated RANTES has been detected in painful intervertebral disc in humans [18]. Therefore, increased circulating levels of RANTES could exacerbate or contribute to the development of neuropathic pain.

Treg cells are known to express the RANTES receptor CCR5, which is thought to be critical for their homing ability [19]. Similarly, the IL-5 receptor IL-5rα is also strongly expressed in effector Treg cells activated by IL-4 and antigen [20]. IL-5, a key cytokine secreted by the Th2 subset of effector T-cells, was also detected at significantly elevated concentrations in Treg-depleted
nerve-injured mice. As far as we know, increased IL-5 has not been implicated in neuropathic pain. We speculate that depletion of Treg cells may contribute to the increased level of circulating RANTES and IL-5 due to the reduced availability of the CCR5 and IL5rα receptors. This assumption is based on the precedent that systemic concentrations of IL-2 are known to be elevated in circumstances of immune activation and Treg cell depletion due to a reduction in the availability of the IL-2 receptor [21]. Agreeing with these findings, our results also demonstrate an increase in circulating IL-2 concentration following Treg cell depletion in nerve-injured mice. While some studies have reported an anti-nociceptive effect of IL-2 involving binding to opioid receptors [22], others have shown significantly increased levels of IL-2 mRNA and protein in patients with painful peripheral neuropathy as compared to patients with painless neuropathy and healthy control subjects [23]. Thus, the role of IL-2 in neuropathic pain remains unclear. Considering both IL-5 and IL-2 have significant roles in adaptive immunity, further investigation is warranted.

The complementary reduction in IL-12 and IFN-γ concentration in the serum suggests that these results are connected. IL-12 is the key activator of the Th1 cell subset, and IFN-γ the key cytokine expressed by this subset. It has previously been demonstrated that IFN-γ promotes neuropathic pain through the activation of spinal microglia [24]. Therefore, reduced IFN-γ would potentially have the effect of alleviating mechanical allodynia in nerve-injured mice, which is contrary to our behavioural findings. However, the role of IFN-γ signalling in neuropathic pain has been demonstrated in the spinal cord, and whether similar changes in the levels of serum cytokines occur in the spinal cord remains to be elucidated.

Depletion of Treg cells specifically results in a more than doubling in the concentration of IL-12p40. We assume this is in the form of IL-12p40 monomer, homodimer, or undescribed cytokine because we observed a reduction in IL-12 and IL-23 concentrations and these are the only other cytokines known to contain IL-12p40. To our knowledge, this is a novel finding that may have significant implications. The IL-12 family of cytokines is unique in the sharing of different chains between heterodimer members (IL-12, IL-23, IL-27 and IL-35). All members of the IL-12 family
have independent and critical roles in the regulation of the immune system [25]. The IL-12p40 homodimer, though not generally recognized as a cytokine in its own right, is present in serum at substantial concentrations [26] and is known to antagonize the IL-12 and IL-23 receptors due to the presence of its IL-12Rβ1 receptor chain [27]. Interestingly, treatment of mice with recombinant IL-12p40 homodimer substantially reduced IFN-γ production during acute endotoxemia [26] due to antagonism of the IL-12 receptor. Antagonism of the IL-12 receptor by elevated concentration of IL-12p40 homodimer following Treg depletion may also be the reason we observed a reduction in circulating IFN-γ.

In a study of cerebrospinal fluid from patients with suspected multiple sclerosis, IL-12p40 was found to be an excellent biomarker for distinguishing between clinically isolated syndrome and other neurological disorders [28]. IL-12p40 may also have a specific role to play in the development of neuropathic pain as it had been shown to induce nitric oxide synthesis and NF-κB activation in microglia [29]. IL-12p40 was also reported to suppress the expression of the Treg cell transcription factor Foxp3 in naïve mouse splenocytes due to the induction of nitric oxide [30]. Therefore, we speculate that substantially increased IL-12p40 concentration in serum may contribute to the increased mechanical allodynia we observed, however, there is an alternative opinion that IL-12p40 may actually have anti-nociceptive properties [31]. Nevertheless, our finding of up-regulation of IL-12p40 as a result of Treg cell depletion, in association with reduced IL-12 and IL-23, further informs the intriguing interplay between members of the IL-12 family.

In conclusion, our data demonstrate an important interaction between the nervous system and the immune system in neuropathic pain involving systemic cytokine-mediated signalling. Future studies will determine whether changes in circulating cytokines are reflective of local concentration along the pain pathway; at the site of nerve injury, the dorsal root ganglia, or the dorsal horn of the spinal cord. However, it is clear that dysregulation of the immune system alters the systemic cytokine profile, which may subsequently affect local neuroinflammatory responses and neuropathic pain due to nerve injury.
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5. **Figure Legends**

**Fig. 1**

Foxp3 expression following peripheral nerve injury. (a) Images of nervous tissues taken 10 days after CCI. Foxp3-expressing cells (indicated by white arrows) are present in the injured sciatic nerve (SN), dorsal root ganglia (DRG) and dorsal horn of the spinal cord (SC). All scale bars represent 100μm. (b) A histogram showing mean and SEM of Foxp3-positive cell counts at different tissue sites. (n=4; **** P < 0.0001). (c) Western Blot analysis of Foxp3 (47kDa) and GAPDH (37kDa), which is used as a loading control. Density of each Foxp3 band was calculated relative to GAPDH and the histogram shows means and SEM. (n=5; *P<0.05 and *** P< 0.001). (d) Histograms showing means and SEM of total number of live cells isolated from ipsilateral and contralateral inguinal lymph nodes and percentage of GFP+ cells sorted from total single cell population of inguinal lymph nodes (n=3; ** P< 0.01).

**Fig. 2**

Treg depletion following treatment with DT. (a) Immunofluorescent staining of inguinal lymph node from DEREG mice. Positive cells stained with a halo like ring around the outside of cells (arrows in left image) in PBS treated mice were absent from DEREG mice treated with DT (right image). (b) Representative dot plot graphs of flow cytometry data of CD4+ cells sorted for GFP (x-axis) and CD25 (y-axis) from inguinal lymph nodes of DEREG mice treated with PBS (Left graph) or DT (Right graph). (c) Histogram showing mean and SEM comparing different compartments of dot plot graphs in (b) (n≥3; * p<0.05; ** p<0.01, **** p<0.0001).

**Fig. 3**

Analysis of mechanical allodynia. (a) Graph showing mean and SEM of progressive 50% paw withdrawal threshold in the ipsilateral paw following CCI or sham operation in WT or DEREG mice treated with either PBS or DT (n=10; # = sig. diff. between WT+SHAM+PBS vs DEREG+CCI+PBS, * = sig. diff. between WT+SHAM+PBS vs DEREG+CCI+DT, + = sig. diff.
between DEREG+CCI+PBS vs DEREG+CCI+DT; * or # p<0.05; ++ p<0.01, *** or ### p<0.001).

(b) Graph showing mean and SEM of 50% paw withdrawal threshold in the contralateral paw following CCI or sham operation in WT or DEREG mice treated with either PBS or DT (n=10). (c) Graph showing mean and SEM of progressive 50% paw withdrawal threshold in the ipsilateral paw following CCI in WT mice treated with DT and DEREG mice treated with PBS (n=6).

Fig. 4

Bio-plex analysis of the serum concentration (pg/ml) of cytokines. (a-f) Histograms showing mean and SEM of serum concentrations for indicated cytokines (n≥5; * = sig. diff. between WT+SHAM+PBS vs DEREG+CCI+DT, # = sig. diff. between DEREG+CCI+PBS vs DEREG+CCI+DT; * or # p<0.05; ** or ## p<0.01, *** or ### p<0.001).

Fig. 5

ELISA of serum concentration (pg/ml) of IL-12p40 and IL-23. (a) Histograms showing mean and SEM of serum concentrations for IL-12p40 (n≥5; * = sig. diff. between WT+DT+SHAM vs DEREG+CCI+DT, # = sig. diff. between WT+PBS +CCI vs DEREG+DT+CCI; **** or #### p<0.0001). (b) Histograms showing mean and SEM of serum concentrations for IL-23 (n≥4; σ = sig. diff. between WT+DT+SHAM vs WT+PBS+CCI, + = sig. diff. between WT+PBS +CCI vs DEREG+DT+CCI; σ p<0.05; ++ p<0.01). (c) Histograms showing mean and SEM of serum concentrations for IL-12p40 (n≥5; ~ = sig. diff. between DEREG+ PBS vs DEREG+DT; ~~~~ p<0.0001).
6. References


