Flow cytometry analysis of inflammatory cells isolated from the sciatic nerve and
DRG after chronic constriction injury in mice

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Running head: Assessment of cellular responses to nerve injury in the sciatic nerve and DRG by
flow cytometry

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Abbreviations: DRG (dorsal root ganglion); PWT: paw withdrawal thresholds; CCI: Chronic
constriction injury; FACS: flow cytometry cell sorting; PFA: paraformaldehyde; DAPI: 4', 6-
Diamidino-2-Phenylindole; BSA: bovine serum albumin; HEPES: 4-(2-hydroxyethyl)-1-
piperazineethanesulfonic acid; EDTA: ethylenediaminetetraacetic acid

Abstract:

Background: Cellular responses to nerve injury play a central role in the pathogenesis of neuropathic pain. However, the analysis of site specific cellular responses to nerve injury and neuropathic pain is limited to immunohistochemistry staining with numerous limitations. **New**

Methods: We proposed to apply flow cytometry to overcome some of the limitations and developed two protocols for isolation of cells from small specimens of the sciatic nerve and dorsal root ganglion (DRG) in mice.

Results and comparasion with existing methods: We found that both the non-enzymatic and enzymatic approaches were highly effective in harvesting a sufficient number of cells for flow cytometry analysis in normal and pathological conditions.

The total number of cells in the injury site of the sciatic and its DRGs increased significantly 14 days after chronic constriction injury (CCI) of the sciatic nerve, compared to sham surgery control or the contralateral control. The enzymatic approach yielded a significantly higher total number of cells and CD45 negative cells, suggesting that this approach allows for harvest of more resident cells, compared to the non-enzymatic method. The percentage of CD45⁺/CD11b⁺ cells was significantly increased in the sciatic nerve but not in the DRG. These results were consistent with both protocols. **Conclusions:** We thus offer two simple and effective protocols that allow for application of flow cytometry to the investigation of cellular and molecular mechanisms of neuropathic pain.

Key words: Nerve injury; Chronic Pain; Neuropathic pain; Flow cytometry; Sciatic nerve; Dorsal root ganglion (DRG); Enzymatic dissociation; Non-enzymatic dissociation; Mice.

Introduction

Neuropathic pain is defined as pain due to a lesion or disease of the somatosensory nervous system. It is one of the most common, debilitating, and costly pain conditions (O'Connor, 2009; Toth et al., 2009). Clinically, it is commonly seen after peripheral nerve injuries (Baron, 2006). A key process that leads to neuropathic pain is the inflammatory changes surrounding the injured or disordered nerves (Basbaum et al., 2009). These changes are featured by cellular infiltration from the blood circulation and activation of the infiltrating and resident inflammatory cells, such as neutrophils, macrophages, and CD4⁺ T-cells, and their release of inflammatory mediators, such as IL-1, IL-6, and TNF- α , that act on their molecular targets locally to cause peripheral sensitization with increased sensitivity to noxious and non-noxious stimulation. Hyperactivity in nociceptors may further induce secondary changes in neurons, immune cells (microglia), and glial cells (astrocytes) in the spinal cord and brain, and lead to central sensitization (Baron, 2006; Lees et al., 2015; Ren and Dubner, 2010). Clearly, cellular responses to nerve injury play a central role in the pathogenesis of neuropathic pain.

It remains to be determined as to how the cellular responses lead to the development of neuropathic pain (Lees et al., 2015; Ren and Dubner, 2010). A better understanding of these cellular response processes may help uncover new and more effective therapeutic strategies for neuropathic pain. Unfortunately, our understanding of the cellular processes is limited by the lack of sensitive and quantitative methodology. Immunohistochemistry staining is currently used to evaluate the cellular responses in and around a peripheral nerve after nerve injury (Kim and Moalem-Taylor, 2011). While recognizing the advantages of this technique such as the maintenance of the integrity and spatial orientation of the cells, it also has several significant

limitations. First, it does not allow simultaneous staining of cells with more than 3 antibodies. This limits its ability to reveal the relationships between various cell types at any given point in time. Second, it does not allow accurate quantification of the percentage and total number of different types of cells in the tissue specimens simultaneously. Third, some of the cells may not be recognized by immunohistochemistry due to the lack of working antibodies as a result of specific treatment, such as fixation, for immunohistochemical staining. Fourth, immunohistochemistry is labor-intensive and time-consuming. In order to overcome some of these limitations, we proposed to test the feasibility of applying multi-parameter flow cytometry (MP-FCM) for cellular analysis. Flow cytometry is a powerful tool for rapid analysis of cellular populations on a cell-by-cell basis. A flow cytometer can be described as an “automated microscope” with the advantages of high objectivity, efficiency, accuracy, and sensitivity (Leonard et al., 2016). Each individual cell can be characterized based on its size, shape, granularity of the intracellular particles, fluorescence color, and intensity of the fluorescence signal (Leonard et al., 2016). Although protocols for isolation of cells from spinal cord or brain with different digestion methods have been utilized to analyze infiltrating cells in the central nerve system for several decades (Liu et al., 2010; Liu et al., 2015b; Ponomarev et al., 2005; Sedgwick et al., 1998), there are no published protocols for the isolation of murine cells around an small peripheral nerve and its DRG for flow cytometry. It is not known if it is technically feasible and reliable to isolate sufficient numbers of cells from small specimens of the sciatic nerve and/or DRG in mouse for this purpose. In order to increase the number of cells, previous reports of flow cytometry analysis of the sciatic nerve and DRG had to pool tissue specimens (even from rats) or use enzymatic digestion (Eckert et al., 1999; Labuz et al., 2009; Labuz et al., 2010; Liou et al., 2011; Morin et al., 2007; Peluffo et al., 2015), which may compromise cell

surface marker staining and affect result interpretation (Garcia et al., 2013; Liou et al., 2011). In order to develop methodologies that allow for accurate and reliable analysis of the cellular responses to peripheral nerve injury, we examined two protocols based on our previous experiences and published reports for the isolation of oligodendrocytes, microglia and leukocytes from the central nerve system (Butovsky et al., 2014; Garcia et al., 2013, 2014; Liu et al., 2010; Liu et al., 2015b). One protocol employed mechanical homogenization of specimens from the injured nerve or its DRG while the other involved mild enzymatic dissociation followed by homogenization. We used the mouse model of neuropathic pain consequent to chronic constriction injury of the sciatic nerve. Since the specimens from the injured sciatic nerve and DRG are small, we tested if it is possible to isolate enough cells for flow cytometry analysis and examined the differences between the two protocols.

Material and methods

Animals:

Wild-type C57BL/6 mice were obtained from our breeding colony originally purchased from Jackson lab at the Biological Resources Unit, Cleveland Clinic, Lerner Research Institute. The mice were group housed (4–5/cage) in standard cages in a colony room maintained on a 12-hour reversed light/dark cycle. All mice had continuous access to food and water throughout the study. Similarly, mice were handled and adapted to the testing environment for 1 week prior to initiation of the experiments. All behavioral testing procedures were conducted between 08:00 and 13:00h. Mice of both sexes were used in this study. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics

of the Institutional Animal Care and Use Committee at Cleveland Clinic (IACUC, Permit Number: 2014-1360). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Chronic constriction injury (CCI) of the sciatic nerve

CCI was performed in mice following the established surgical procedures (Liu et al., 2015a). Briefly, under pentobarbital anesthesia (50 mg/kg, i.p.), the right sciatic nerve was exposed at the mid-thigh level by freeing the adhering fascia between the gluteus and biceps femoris. Three ligatures (4/0 chromic gut) were tied loosely around the sciatic nerve at 1-mm intervals above the nerve's trifurcation. The ligatures constricted only about 1/3–1/4 of diameter of nerve and produced a brief twitch in the muscle around the exposure. The surgical side was defined as the ipsilateral side and the contralateral side was used as control in the following analyses. Muscle and skin layers were finally closed with surgical staples. Sham surgeries involved identical procedures, but without ligation of the sciatic nerve.

Behavioral Tests

The sensitivity to noxious stimulation was determined by measuring the paw withdrawal thresholds (PWT) in response to mechanical and thermal stimulation to the right and left hind paws.

Thermal Hyperalgesia (Hargreaves test) (Hargreaves et al., 1988)

Animals were allowed to habituate in the environment for at least 60 min prior to the behavioral test. Each mouse was placed in a box (22 × 12 × 12 cm) containing a smooth glass floor (Stoelting, Wood Dale, IL, USA) (Shen et al., 2012, 2013). The temperature of the glass

was measured and maintained at $27\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. A heat source (Stoelting, Wood Dale, IL, USA) was focused on a portion of the hindpaw and a radiant thermal stimulus was delivered. The stimulus shut off automatically when the hindpaw moved or 20 seconds had passed to prevent tissue injury. The procedure was repeated three times at 5 min intervals to avoid sensitization and withdrawal latencies were averaged and recorded.

Mechanical Allodynia

Animals were placed in individual $10 \times 10 \times 15$ cm plastic boxes on an elevated metal mesh floor and allowed to acclimate for at least 30 min before test. Mechanical sensitivity was tested by using von Frey sensory evaluator filaments (Stoelting, Wood Dale, IL, USA) (Shen et al., 2012, 2013). Filaments were applied to the plantar surface of the right hind paw in ascending order of force until the filament bent and was held there for ~3 seconds or until a paw withdrawal response took place. Upon a paw withdrawal response, the filaments were applied in descending order, beginning with the next thinner filament until there was no withdrawal response. The threshold was the thinnest filament to evoke a paw withdrawal response. The procedure was repeated three times at 5 min intervals to avoid sensitization and the withdrawal thresholds were averaged and recorded (mean $\pm$ SEM).

Immunohistochemistry and quantitation of immunoreactivity staining

Immunohistochemistry of CD68 staining in the sciatic nerve and DRG were performed as we have described previously (Liu et al., 2010; Liu et al., 2006). Briefly, animals were sacrificed by intracardiac perfusion with ice-cold PBS, followed by 4% paraformaldehyde (PFA) solution under deep anesthesia (Phenobarbital Sodium). The sciatic nerve and DRG were rapidly dissected and post-fixed in 4% PFA. The tissues were washed with PBS and placed in 30% of

sucrose buffer at 4°C until it sank. Afterwards, 10-um-thick sections were cut on a cyrostat machine (Leica Microsystems) and stored at -20°C.

For immunohistochemistry staining, the tissue slides were rinsed in PBST (1xPBS+0.05% TritonX-100), blocked by incubation with 10% goat serum and 1% bovine serum album (BSA) at room temperature for 1 h, and then incubated overnight at 4°C with primary Abs for CD68 (AbD, MCA-1957, 1:1000). The next day, tissues were incubated with Alexa 488-anti-rat antibody (Invitrogen) for 1 hour. The immunofluorescence stained tissues were mounted with VECTASHIELD HardSet Anti-fade mounting medium with 4', 6-Diamidino-2-Phenylindole (DAPI) (H-1500, Vector lab). Omission of the primary antibody served as negative control. The stained sections were examined with a fluorescence microscope (Leica, Wetzlar, Germany), and images were captured with confocal microscopy (Leica, Germany).

Quantitation of immunoreactivity: The quantification of percentage area occupied by immunoreactivity was performed as previously described (Liu et al., 2010; Liu et al., 2006). Briefly, 40x pictures of the sciatic nerve (near the constriction area for ipsilateral side) and DRG were captured by confocal microscopy and used for quantification. Digitized images were analyzed with National Institutes of Health ImageJ1.34s software. A thresholding procedure was established to determine the proportion of immunoreactive area within each fixed field of view. These parameters were then held constant for each set of images obtained at equal objectives and light intensities. The data represent the mean immunoreactivity area in the sciatic nerve and DRG.

Dissociation of cells from the injured sciatic nerve and the DRG

Non-enzymatic dissociation protocol

Materials

1xHBSS free of calcium, magnesium chloride and magnesium sulfate (Gibco)

FBS (Invitrogen)

DNase (Sigma)

70% ethanol

1M HEPES (Invitrogen)

0.5M EDTA (Invitrogen)

Syringe 1ml (BD)

Needle 21G and 23G (BD)

Cell strainer cap (Falcon)

Desktop centrifuge (Eppendorf) or similar

Additional reagents and equipment for anesthesia (Isoflurane induction chamber)

Solution A: 1x HBSS/ 25mM HEPES/10% FBS/10ug/ml DNase

Procedures:

1. Anesthetize the mouse with isoflurane in the presence of oxygen in an induction chamber. Perform cardiac perfusion with 1x HBSS by cutting the thoracic cavity open and making a small incision in the right atrium of the heart. Inject 15–20mL of ice cold 1x HBSS into the left ventricle to completely remove blood from tissues.
2. Carefully dissect part of the sciatic nerve (approximately 1 cm long surrounding the ligation site) and its DRGs (L3-5) . Take controls from the contralateral side, as well as from both sides of the sham control mice.
3. Transfer the single sciatic nerve and 3 DRGs separately to 1.5ml Eppendoff tube containing solution A and keep on ice until all tissues are collected.

4. Chop the tissues with small scissors into small pieces.
5. Homogenize the tissue with 1ml syringe attached with 21G needle, then switch to 23G needle to continue homogenization as previously described (Butovsky et al., 2014).
Make sure to use new and sterile syringe and needles for each sample. Take out the white fiber-like debris before moving to the next step.
6. Filter the homogenized solution through a cell strainer cap to a new 1.5ml Eppendoff tube .
7. Quickly centrifuge down the cells (room temperature at high speed (12000rpm) desktop centrifuge for 10 seconds).
8. Aspirate supernatant and suspend cell pellet in 1ml of FACS buffer and centrifuge down again (repeat step 5). These cells are ready for staining.
9. Check for sufficient cell yield and viability by counting cells on a hemocytometer in trypan blue.

During cell isolation, cell suspensions obtained by syringe homogenization should appear as clear solutions without visible pieces of tissues. After centrifugation, a white cell pellet at the bottom of the Eppendoff tube may contain a small amount of myelin, which will not affect the staining of cell surface markers or clog the flow cytometry equipment. It is important to use trypan blue staining to differentiate viable cells from myelin debris and dead cells during cell counting.

Enzymatic dissociation protocol

Materials

We chose papain as it is a relative mild digestive enzyme that may dissociate cells from the CNS tissue without significantly compromising cell surface markers (Liu et al., 2015b). In addition to the materials used for the homogenization protocols, we used the following materials.

1. Papain Tissue Dissociation Solution (Papain (15U/ml) /DNAase (10ug/ml), /) (Papain, Roche; DNase, Sigma)
2. Oven with rotating tube holders

Procedures

1. Prepare Papain solution as described above and previously published (Butovsky et al., 2014; Liu et al., 2015b); divide into aliquots in 5 ml tubes, and stored at -20C for later use.
2. Perform the tissue collecting procedures described above with one exception in Step 2: replacing **Solution A** to **Papain Solution** before collecting the tissues (200ul of papain solution for each sciatic nerve or 3 DRG samples (L3-5) combined).
3. Chop the tissues with small scissors into small pieces.
4. Close eppendoff tube and put in oven rotating at 37°C for 30 minutes (digestion)
5. Stop the digestion by adding the equal amount of solution A
6. Follow Steps 4-8 described for the non-enzymatic dissociation protocol.

Cell surface antigens staining and flow cytometry analysis

Staining of cells with fluorescent antibodies for flow cytometric analysis allows determination of various cell populations within a given sample using different fluorochromes. It is possible to determine the absolute number of cells of each population present in any given samples by cell counting prior to staining.

Materials

FACS buffer (1xPBS/1%BSA/0.05%NaN₃/0.05mM EDTA)

1.5mL micro centrifuge tubes

FC block: rat anti-mouse CD16/CD32 clone 2.4G2 in FACS buffer (BioLegend)

Fluorescent antibodies (All from BioLegend: CD45APC, clone: 30-F11; CD11bBV650, clone:

M1/70; Ly6GAPCCY7, clone 1A8; F4/80PECy7, clone: BM8) (Supplementary data 1)

2% PFA (Sigma)

Fortessa flow cytometer (BD Biosciences) or similar

FlowJo analysis software

Blocking and staining

1. Quickly centrifuge down the cells, and aspirate supernatant and suspend cells in FC block solution. Incubate for 5–10 minutes at room temperature or 30 minutes on ice.
2. Use blood cells lysed from peripheral blood for single staining control (Liu et al., 2010).
3. Prepare dilutions of antibodies in cell staining buffer in a final volume accommodating for 50 μ L per sample. Add respective antibodies to each staining control sample with a final volume of 50 μ L. All antibodies should be titrated prior to conducting the experiment to ensure optimal results.
4. Incubate cells in antibody mix for 10 minutes at room temperature or 30 minutes on ice in the dark.
5. Wash cells with 1mL FACS buffer.
6. Quickly centrifuge down. Aspirate supernatant and suspend cells in 100 μ L FACS buffer and add 100 μ L 2% PFA for a final concentration of 1% PFA.
7. Store fluorescently labeled cells at 4°C, which could be read using a flow cytometer within one week.

Analysis

1. Compensate all fluorochromes by plotting SSC-A by FSC-A and gating on leukocyte population as shown at Figure 2B, using parameters for peripheral blood cell gating; proceed to gate the total leukocyte population (Fig. 2) and label it “Lym”.
2. From “Lym” gating, plot FSC-W by FSC-H to gate on the density population as “Singlets” (Supplement 2). From “Singlets” gating, make a plot showing CD45 by CD11b to determine the percent of infiltrating CD45⁺/CD11b⁺ cells and Ly6G population in CD11b positive cells (Supplement 2).

3. From “Singlets”, make a plot showing counts by CD45, CD11b, Ly6G and F4/80 to determine the intensity and percent of cells.

Statistical analysis

The paw withdrawal thresholds to mechanical and thermal stimulation were expressed as mean $\pm$ SEM. Statistical analyses were made using two-way analysis of variance (ANOVA) followed by paired comparisons with Bonferroni corrections when comparisons were made between more than 3 groups. The total numbers of cells, CD68+ cells and the percentage of cells were expressed as mean $\pm$ SEM. Statistical analyses were made using un-paired T test when comparisons were made between two different groups. Graphpad Prism 3 was used for the analysis. $P < 0.05$ was considered statistically significant (* $P < 0.05$).

Results:

Neuropathic pain behaviors induced by CCI of the sciatic nerve

CCI of the right sciatic nerve was performed in C57BL/6J mice. All mice exhibited neuropathic pain symptoms defined by the development of mechanical and thermal pain hypersensitivity of the hind-paw in the injured (ipsilateral) side but not in the contralateral side or in sham-operated mice (Fig. 1) as indicated by a profound decrease of paw withdrawal latency to the mechanical (Fig. 1A) or thermal (Fig. 1B) stimulation in the ipsilateral side. The significant reduction of the mechanical threshold or thermal threshold started 5 days after CCI, peaked at 14 days and persisted to the end of the experiments (28 days).

Figure 1 Pain behaviors after CCI of the sciatic nerve in C57BL/6J mice. Paw withdrawal thresholds ($\text{mean} \pm \text{s.e.m}$) to mechanical (A) and thermal (B) stimulation were significantly lower in the ipsilateral hind-paw, compared to the contralateral side. The decrease started from day 5,

peaked at day 14 and persisted through the end of the experiment (day 28). (<P0.05, two-way ANOVA, followed by Bonferroni's post-test, n=10).*

Immunohistology analysis of neuroinflammation in the injured sciatic nerve and its DRGs

CD68 antibody was used to identify activated macrophages in and around the sciatic nerve and DRG. The percent of CD68 immunoreactive area was comparable between the sham control and the contralateral side (Supplement 3 and data not shown). In contrast, it was dramatically increased in the injured sciatic nerve but not in the DRGs, compared to the sham control. The increase started at post-injury day 3 (data not shown) and peaked at day 14 (Supplement 3, $P<0.001$, $n=4$). These results indicate that neuroinflammation occurs specifically in injured sciatic nerve and is correlated to the development of neuropathic pain which is consistent to previous report (Basbaum et al., 2009).

Successful isolation of infiltrated cells from the sciatic nerve and DRGs

To accurately account the total number and percentage of each cell population in the sciatic nerve and DRG, flow cytometry analysis is expected to be a better choice than immunohistochemical staining. The specimens were collected from a single sciatic nerve (SN) or a combination of 3 DRGs (L3-5) from both sides of the mouse 14 days after CCI or sham surgery. Both enzymatic and non-enzymatic isolation protocols were used to isolate the cells for flow cytometric characterization. The flow cytometry data demonstrated the feasibility of successful staining of surface cell markers that allowed for accurate quantification and comparison of inflammatory cells from different specimens (Figs. 2-5). Cells in the sciatic nerve and DRG were properly gated based on peripheral blood cell gating albeit their morphology were different from peripheral blood cells (from side scatter and forward scatter (Fig. 2B and C)). Morphological differences were also noted between cell populations in the sciatic nerve and those in the DRG. Consistent results were produced by both methods (Fig. 2C). The enzymatic method produced a greater total numbers of cells from the sciatic nerve (2.79 ± 0.72 million vs 1.17 ± 0.03 million) and DRGs (1.08 ± 0.38 million vs 0.39 ± 0.03 million), compared to the non-enzymatic method (Fig. 2D).

Figure 2. Isolation and characterization of cells from the sciatic nerve and DRG.

Cells from a single ipsilateral sciatic nerve or dorsal root ganglion (DRGs, L3-5) 14 days after CCI were isolated by either the non-enzymatic or enzymatic method. A. Workflow for cell isolation through non-enzymatic dissociation (green and black arrows) and enzymatic dissociation (green and red arrows). B. Flow cytometry characterization of cells from the peripheral blood samples. C. Flow cytometry characterization of cells from the sciatic nerve and DRGs. The cells were gated based on peripheral blood cell gating. Side scatter and forward scatter revealed morphological characters of the infiltrated cells in the sciatic nerve and DRG.

These data represent at least three individual experiments. D. The total numbers of cells (mean $\pm$ s.e.m) were quantified by a hemocytometer (N=4 for each group). $P < 0.05$ or $P < 0.01$, comparisons between the two methods.

Inflammatory CD45⁺ and CD11b⁺ cells in the sciatic nerve and DRGs

We stained the isolated cells with different cell surface specific markers such as CD45, CD11b, Ly6G and others antibodies to identify different cell populations. Both the non-enzymatic and enzymatic methods allowed for successful staining with CD45 and CD11b antibodies (Fig. 3 and 4). Compared to the non-enzymatic approach, the enzymatic approach yielded a lower percentage of CD45 positive cells and a higher percentage of CD45 negative cells from both the sciatic nerve and DRGs (Fig. 3A and B). Quantitative data from four experiments revealed significant differences in the mean percentage of CD45 positive cells in the sciatic nerve and DRG between the two different methods (Fig 3B) ($P < 0.001$). Compared to the non-enzymatic approach, the enzymatic method yielded a significantly higher total numbers of CD45 positive cells from the sciatic nerve ($n=4$, $P < 0.01$), but not from the DRGs ($n=4$, $P > 0.05$) (Fig 3C), suggesting that inflammatory CD45⁺ cells was injury site-specific.

Figure 3. CD45⁺ and CD45 negative cells from the sciatic nerve and DRG. A. The non-enzymatic dissociation yielded a higher percentage of CD45⁺ cells in both the sciatic nerve (SN, upper) and dorsal root ganglion (DRG, lower), compared to the enzymatic approach. These data represent three individual experiments. B. Summary data showing that the non-enzymatic approach yielded a significantly higher percentage of CD45⁺ cells (mean $\pm$ s.e.m) from the sciatic nerve and DRG, compared to the enzymatic approach ($P<0.001$, $N=4$ for each group). C. The total numbers of CD45⁺ cells in the sciatic nerve and DRG. The enzymatic approach yielded a significantly higher total number of CD45⁺ cells (mean $\pm$ s.e.m.) in the sciatic nerve ($P<0.01$), but not in the DRG ($P>0.05$, $N=4$ for each group).

Further analysis of the CD45⁺ cell population with myeloid marker CD11b indicated that the majority of CD45⁺ cells were also CD11b⁺ (Fig. 4). Such cells were observed in specimens

from the injured sciatic nerve, but not from the sciatic nerve of the contralateral side (Fig. 4) nor from those of the sham control (data not shown). There was no difference in the percentage of CD11b⁺ cells in the DRG between ipsilateral and contralateral sides. Thus, the inflammatory CD11b⁺ cells was injury site-specific. This is consistent with results of the immunohistochemical staining of activated macrophage marker CD68 (Supplement 1). Compared to the enzymatic method, the non-enzymatic method yielded a much higher percentage of CD45⁺/ CD11b⁺ cells in the SN (92% vs 56%) and DRGs (60% vs 25%) ipsilateral to the CCI injury (Fig. 4).

B

Figure 4. *CD45⁺/CD11b⁺ cells from the sciatic nerve and DRG. The non-enzymatic approach yielded a significantly higher percentage of CD45⁺/CD11b⁺ cells in the ipsilateral SN and DRG*

($P < 0.001$), compared to the enzymatic approach. A significantly higher percentage of $CD45^+ / CD11b^+$ cells was observed in the ipsilateral sciatic nerve ($P < 0.01$), but not in the DRG ($P > 0.05$), compared to the contralateral side. These experiments were repeated at least three times.

Effects of enzymatic isolation on cell surface markers

Cells dissociated from the ipsilateral sciatic nerve and DRG were also stained with CD11b, Ly6G for neutrophil and F4/80 antibodies for macrophage (Fig 5). The two dissociation methods yielded different percentage of CD11b, Ly6G, and F4/80 positive cells from the sciatic nerve and DRG 14 day after CCI. Papain digestion compromised the staining of Ly6G and F4/80 cell surface markers, as evidenced by the absence of the second peak on the right in blue. Papain digestion did not affect CD11b staining.

Figure 5. Effects of papain digestion on the staining of CD11b, Ly6G, and F480 cell surface markers. Papain digestion (blue line) did not affect the staining of CD11b⁺ cells, but compromised the staining of Ly6G and F4/80 positive cells from the sciatic nerve (SN) and dorsal root ganglion (DRG), compared to the non-enzymatic approach (red line). These data represent at least three individual experiments.

Discussion

This is the first report of methodological feasibility of isolating cells from the small sciatic nerve and DRGs in mice for flow cytometry analysis. We tested and compared the non-enzymatic isolation and enzymatic methods and demonstrated that flow cytometry is suitable for quantitative analysis of cellular responses to peripheral nerve injury and neuropathic pain. We described in detail two tissue preparation protocols that allowed for successful analysis of cellular responses around an individual nerve or its DRGs in relation to development of pain behavior after CCI injury of the sciatic nerve. The enzymatic approach yielded a much larger total number of cells than the non-enzymatic approach. However, the latter produced a significantly high percentage of CD45⁺ cells, the majority of which were CD11b⁺. Although we only provided examples of successful staining of cell surface markers, this new approach of isolating cells from the sciatic nerve and DRG can potentially allow for simultaneous and accurate analysis of more than 9 different cell surface markers. The flow cytometry machine (LSRFortessa) allows for detection of 9 to 12 different fluorochromes staining for different cell surface markers at the same time, thus allowing differentiation of at least 9 different cell surface markers simultaneously from the same sample as long as fluorochromes are chosen wisely. These findings provide a new and powerful tool to investigate site-specific and quantitative cellular responses to peripheral nerve injury and overcome a major barrier to better understanding the precise cellular and molecular mechanisms of neuropathic pain.

The key to successful flow cytometry analysis is to obtain a sufficient number of cells from specimens of a small nerve injury site and the DRG without affecting cell surface markers. Previous reports of flow cytometry for sciatic nerve had to pool specimens (even from rat tissues) (Eckert et al., 1999), and use a variety of enzymes to digest tissues (Labuz et al., 2009; Labuz et al., 2010; Liou et al., 2011; Morin et al., 2007), or chop the specimen and pass it

through a cell strainer (Peluffo et al., 2015). Consequently, it was difficult to determine the exact production of cells and efficiency of cell surface staining of each method and to reliably investigate the cellular responses to the nerve injury. The use of enzymatic digestion may compromise cell surface marker and lead to misinterpretation of results (Garcia et al., 2013; Liou et al., 2011). Here we examined both the non-enzymatic and enzymatic approaches and compared the outcomes of cell isolation from the mouse sciatic nerve injury site and the DRGs. We also, based on our previous experiences (Butovsky et al., 2014; Liu et al., 2015b), simplified the protocols for both approaches and made it easier and more reliable to perform (6 steps for the non-enzymatic method and 7 steps for the enzymatic method) (Fig. 2A). Our data clearly showed that even simple mechanical homogenization without enzymatic digestion produced a sufficient number of cells from a single sciatic nerve or its DRGs in normal healthy mice (sham and/or contralateral side controls) and mice with CCI injury (Figures. 2-4). Homogenization with a syringe after the chopping or digestion of the specimen is a key step to harvest almost all infiltrated cells from the sciatic nerve and DRG for flow cytometry analysis. This step differentiates the current protocols from those previously reported, where the specimens were chopped or digested with various enzymes (Labuz et al., 2009; Labuz et al., 2010; Liou et al., 2011; Peluffo et al., 2015). Syringe homogenization dramatically increased the number of isolated cells (Data not shown).

CD11b⁺ cells constituted the majority of cells around the injured sciatic nerve (Fig. 4). This is consistent with the data of immunohistochemical staining of activated macrophage marker CD68 (Supplement 3). These cells have been reported to play an important role in the development of neuropathic pain. There are several sub-populations of CD11b⁺ cells such as neutrophils, activated and non-activated monocyte, and dendritic cells. Flow cytometry allows

differentiation and analysis of these populations simultaneously by staining specific cell surface markers such as Ly6G, F4/80 or CD11C (Fig. 5, and data not shown). In addition, Treg cells are a small but very important population that has been indicated in the regulation of neuropathic pain. These cells can be further classified into different populations based on the expression and flow cytometric analysis of CD4, CD25 and foxp3; this is not readily achieved by immunohistochemical staining (Lees et al., 2015).

Multicolor flow cytometry has been used to analyze changes of cell populations in the blood after peripheral nerve injury and development of neuropathic pain (Luchting et al., 2015; Perera et al., 2015). However, such an approach suffers from the lack of site specificity. Immunohistochemistry is very site-specific but is limited by many factors, one of which is the lack of suitable antibodies for staining. Flow cytometry analysis of specimens from the injury site overcomes many of the limitations of immunohistochemical staining and makes it possible to accurately and quantitatively analyze the number and composition of a large variety of cells simultaneously. This method also allows for sorting of a single cell population and studying of specific signaling pathways of a particular cell type. The combination of these advantages promises a powerful strategy for the investigation of neuroinflammation and neuropathic pain.

It is generally accepted that the harvesting time of leukocytes should take less than 4 hours to keep the cells alive for proper analysis (Garcia et al., 2013). Our protocols are simple and quick to perform (Fig. 2A), primarily as a result of modification and simplification of both our own and other previously reported procedures (Butovsky et al., 2014; Garcia et al., 2013, 2014; Liu et al., 2010; Liu et al., 2015b). We shortened the time of leukocyte isolation from the sciatic nerve and DRG by collecting the tissues directly to the buffer used for the next step and keeping them on ice until all samples from an animal were collected. It took approximately 5

minutes for sedation, perfusion, and tissue collection and 10 minutes for homogenization, filtration, centrifugation, and washing per mouse. The staining of cells for flow cytometry took about 40 minutes for 10 samples. The entire procedure to process 5–10 samples took 1-2 hours.

The number of inflammatory cells in the injury site increased dramatically at day 14 after CCI injury, as exemplified by data of immunohistological staining and flow cytometry (Supplement 3, Fig. 4). Consistent with previous report from immunohistochemical studies (Kim and Moalem-Taylor, 2011), the majority of inflammatory cells was CD11b⁺ activated myeloid cells which was also CD68 positive (Supplement 3, Fig. 4). Without enzymatic digestion, a sufficient number of cells was harvested from a single sciatic nerve after sham surgery or from the sciatic nerve contralateral to the CCI (Fig. 2, or data not shown). In contrast, more than 10 times the number of cells (1×10^6) were found in specimens from the injured sciatic nerve 14 days after CCI (Fig. 2D). The enzymatic approach dramatically increased the total number of cells (Fig 2D) as well as total number of CD45⁺ cells (Fig. 2C). The enzymatic approach also dramatically increased the percentage of CD45 negative resident cells from both the sciatic nerve and DRG (Fig. 2C). As a result, the percentage of inflammatory CD45⁺ cells in the enzymatic group were significantly lower compared to the non-enzymatic group (Fig. 2C). The enzymatic approach yielded a significantly higher total number of CD45⁺ cells in the sciatic nerve, but not in the DRG, compared to the non-enzymatic approach (Fig. 2C). It appears that resident cells are difficult to isolate without enzymatic digestion, which facilitates dissociation of resident cells from each other. The increased percentage and total number of resident cells after papain digestion contributed to the increased total number of cells.

Thus, our protocols allow for harvesting a sufficient number of cells from specimens of a single mouse sciatic nerve and make it feasible to apply flow cytometry to the analysis of

neuroinflammation around small nerves and their DRGs. The two protocols may be selected to serve differential purposes. The non-enzymatic approach is particularly suitable for the analysis of infiltrating cells and the enzymatic method may be considered for sorting and analyzing resident cell population because it significantly increased the total number of resident cells. In addition, this approach may be beneficial for analysis at single cell level for sorting majority population CD11b⁺ cells. However, sorting other than CD11b⁺ cells can be a big challenge given the small percentage of CD45^{Hi} CD11b⁻ population in this model.

One of the concerns with enzymatic digestion is that it may compromise cell surface markers (Garcia et al., 2013). Thus we tested whether papain affects the leukocyte cell surface markers and found that it did not affect the staining of CD11b, CD45 and Gr1 (Figs 5, and data not shown). It did, however, compromised the staining of Ly6G and F4/80 (Fig. 5) and CD4, CD8, and CD3 (data not shown) demonstrated from our observation and other reports (Liou et al., 2011). Therefore, the non-enzymatic approach is the preferred method to produce accurate and reliable results for the analysis of cellular responses to nerve injury.

Although we only used the CCI model of neuropathic pain for these experiments in mice, our protocols are applicable to other animal models of pain that are associated with neuroinflammation. Our protocols may also be applicable to small biopsy specimens, in addition to blood samples, from patients for the analysis of cellular responses to internal and external environmental insults. Such clinical applications would allow investigations of the cellular and molecular mechanisms of the disease processes, such as complex regional pain syndrome and idiopathic neuropathies, for each individual patient and enable accurate diagnosis based on objective pathological findings.

Author contributions:

LL was responsible for day-to-day management of the project, designed and performed the experiment, analyzed the flow data in FlowJo software, and performed the statistical analysis. YY and LF performed the CCI surgery and assisted with cell isolation. LF and MC performed the cell counting and quantification of immunoreactivity area. JC supervised the project and directed the overall project. LL drafted and revised the manuscript and JC revised and finalized it. All authors read and approved the final manuscript.

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