Characterization of lumbar lymph node, a CNS-specific draining lymph node, after spinal cord injury in rats

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Short report

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Abstract

Regional tissue-draining lymph nodes are the major active site after inflammation that generate primary immune responses according to lymphatic system pathways. However, CNS specific draining lymph nodes have not characterized individually after spinal cord injury (SCI). Therefore, we examined the morphofunctional state of the Lumbar lymph node, the closest CNS-draining lymph node adjacent to the lesion site in thoracic spinal cord injury. Findings: Lumbar lymph nodes isolated 7 days after low thoracic spinal cord injury were compared with sham control and intact control rats. The significant enlargement was observed in lumbar lymph nodes in SCI group accompanied by increased total cell number, while there was a significantly higher cell death rate. Besides, the proliferation test performed on lymphocytes lumbar lymph nodes one week after SCI revealed accelerated proliferation rate compared to sham and intact control groups, which was associated with significant elevation of IFN-γ/IL4 ratio, which confirms adaptive immunity is biased towards the Th1 pro-inflammatory responses. Conclusions: Accordingly, we conclude that the lumbar lymph node is an important CNS-draining lymph node for understanding the immunopathology of SCI, which needs to be considered as a major active lymphoid organ adjacent to the lesion site.

Introduction

Tissue-draining lymph nodes, which could serve as sinks for cellular and solute transportation, are essential active-site for generating immune responses during inflammations, as well as in maintaining tolerance to minimize tissue damage [1-4]. The central nervous system-draining lymph nodes are thus likely vital for the balancing of protective versus harmful immune responses in experimental models of neuroinflammation [5-8]; however, surprisingly *identification and functional characterization of* CNS-draining lymph nodes during spinal cord injury have remained unclear. Although previous studies have addressed the different response of lymph nodes according to anatomical sites and lymphatic drainage pathways after spinal cord injury [9], attention specifically to spinal cord-draining lymph nodes in searching for immune-cell gateways into and out of injury site during immune response after spinal cord injury has not been considered precisely. Accordingly, the determination of spinal cord-draining lymph nodes, as well as assessment of cellular immune responses in these specific lymph nodes, will shed new light on the etiology of neuroinflammatory processes after spinal cord injury.

Lumbar lymph nodes (also called para-aortic lymph nodes), which are located para-aortically where the abdominal aorta bifurcates into the iliac arteries, capture spinal cord-derived antigens. Carbon particles accumulated in the lumbar lymph nodes in rats following tracer infusion into the subarachnoid space at cisterna magna [10]. Although recent articles focused on the role of cervical lymph nodes as a CNS-draining lymph node, lumbar lymph nodes demonstrated higher proliferative responses with broad-range reactivity against CNS antigens compared with the cervical lymph nodes [5]. Surgical excision of lumbar lymph nodes and cervical lymph nodes prior to experimental allergic encephalomyelitis (EAE) induction, reduces EAE severity by interfering with immunological priming [5], supports the concept that the CNS-draining lymph nodes are essential in the induction and severity of neuroinflammation versus non-

draining lymph nodes [11]. Moreover, interestingly, lumbar lymph node-originated Lymphocytes at dorsal root leptomeninges has an important role in the development of chronic mechanical allodynia after tibial nerve injuries in rat, whereas lymphadenectomy of cervical and other CNS non-draining lymph node did not show any significant effect [6]. This implies that the nearest CNS-draining lymph node has the most potent immune-boosting capabilities against injury. However, most of the immunological studies after spinal cord injury performed on cervical and other CNS non-draining lymph node [12] or lumbar lymph node cells were pooled with other non-draining lymph node cells [13]; however, there are just a few studies examining individually lumbar lymph nodes response after spinal cord injury in acute-phase to show functional relevance of the spinal cord-draining lumbar lymph nodes [9].

In this study, we have applied a clinically relevant spinal cord injury contusion model [14] to detail lumbar lymph nodes characterization in acute phase after spinal cord injury. We presented the enlargement of lumbar lymph node, which was accompanied by increased total cell numbers with higher apoptotic cells after spinal cord injury. Using Carboxyfluorescein succinimidyl ester (CFSE) flow cytometry analysis revealed neuroinflammation in acute-phase after SCI with enhanced lumbar lymph node T lymphocyte reactivity resulted in increased proliferative responses against selective T-cell mitogen. We confirmed spinal cord injury in acute phase can influence cytokine secretion patterns of T cells in lumbar lymph nodes. Our data recommended that the specification of lumbar lymph node, as the nearest CNS-draining lymph node to spinal injury site, need to be considered for evaluating immune response following spinal cord injury.

Materials And Methods

Experimental animals

Female Sprague-Dawley rats 11 weeks were purchased from Razi Institute (Tehran, Iran). After arrival, all rats were housed in standard control caging at the standard condition of temperature (21±1°C) and controlled humidity. All rats were given ad libitum access to food and water over the study period. The rats were housed one per wire cage. Animals were randomly divided into three groups: Spinal cord injured animals (SCI) (n=6), Sham control (laminectomy alone without spinal cord injury, n=6); and intact control group (n=6). All surgical and postoperative care procedures were performed in accordance with the Animal Ethics Committee of Tarbiat Modares University, Tehran, Iran.

Animal surgical procedures

Rats were anesthetized with a mixture of xylazine (100-150 mg/kg) and ketamine (60-90 mg/kg) by intraperitoneal (IP) injection, and laminectomy performed at the T9-T10 thoracic level to expose the spinal cord. The vertebral column was stabilized using clamps on T8 and T11 vertebrae, and rats received low-level thoracic injury by dropping a 10-g weight (a 2-mm metal rod) from 12.5 mm height centered above the spinal cord. Afterward, the impact rod was immediately detached following the injury,

and overlying muscle layers and skin were sutured. During recovery, animals were placed on heating pads and monitored. The animals were housed individually in cages and bladders of rats were expressed manually daily until they recovered their function. Antibiotic (Cefazolin, Jaber Ibn Hayan Co., Tehran) was given with a dose of 50 mg/kg of body weight once a day for the first 7 days post-surgery to prevent infection. Moreover, 6 ml of sterile saline was injected subcutaneously for three days. Rats always had ad libitum access to food and water, with some food pellets placed at the bottom of each cage to have easy access to the pellets. The sham groups underwent laminectomy similar to that performed in the SCI group, but without impact injury.

Lumbar lymph node isolation

Rats were deeply anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (10 mg/kg) one week after thoracic spinal cord injury. For lumbar lymph nodes isolation, each rat was put in its prone position, a median incision was made into the skin and peritoneum of the lower abdomen to open the abdominal cavity, and the intestines were then retracted to have access to the rat lumbar lymph nodes, which could be observed singly or in pairs located along each side of the distal abdominal aorta at the *bifurcation* into the iliac arteries [10]. All the Lumbar lymph nodes were isolated.

Lumbar lymph node size and weight estimation

Lumbar lymph nodes were harvested, any additional connective tissue and fat covering the lymph node were removed, and all lumbar lymph nodes weighed. Lymph node size was calculated using a two-dimensional area of lymph nodes.

Total cell number, cell viability, and cell death rate measurement

Single-cell suspensions were isolated from excised lumbar lymph nodes by forcing the tissues through a fine wire mesh. Cells were washed and resuspended in PBS containing 10% FCS. Total cell number was enumerated with Cellometer (Nexcelom), an automated cell counter. The cell viability and cell death rate were also determined using acridine orange/propidium iodide (AO/PI) assay. Cell counts were performed in duplicates.

CFSE assay

Single-cell suspensions were generated from excised lumbar lymph nodes within seven days after SCI from all groups. The cells in RPMI-1640 medium (GIBCO, New York) supplemented with 10% fetal bovine

serum (Gibco, New York) were cultured in flat-bottomed wells on a 96-well microtiter plate. Cells (2×10^5 cells per well) were cultured for 72 hours in an antigen-free medium (unstimulated control) or together with PHA (10 µg/ml) at 37°C in 5% CO_2. After rinsing with RPMI-1640, the cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE) (CellTrace TM CFSE Cell Proliferation Kit, Invitrogen, Molecular Probes, USA). The cells were stained at a final concentration of 1 µM, then incubated for 72 h at 37°C and unstained cells were used to distinguish the background autofluorescence. The unstimulated cells from lumbar lymph nodes were used as a reference to fix the zero point of the peak for the undivided population in FlowJo software (FlowJo, Ashland, OR, USA). The area of lymphocytes was gated according to light scattering characteristics (size/granularity). Ten thousand events were acquired for each sample on FACS CanII instrument (BD Company, USA) and data were analyzed by FlowJo software.

Cytokine analysis

ELISA kits (DuoSet; R&D System, Oxon, UK) were used to evaluate the concentrations of either IL-4 (lower and upper quantitation limits were 15.625 and 1000 pg/ml, respectively), and IFN-γ (lower and upper quantitation limits were 39.1 and 2500 pg/ml, respectively; samples were diluted 1:30). Mononuclear cells collected from lumbar lymph nodes of animal groups 7 days following thoracic spinal cord injury were stimulated with PHA (10µg/ml) in 96-well plates for 72h and then levels in the culture supernatants were determined using ELISA kits as described by the manufacturer.

Statistical analysis

All Statistical analysis was performed with GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA). Data distribution was assessed by the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for the analysis of data. Data were presented as mean ± SD. P values less than 0.05 were considered statistically significant.

Results

Lumbar lymph node size, weight, and cell numbers changes

To evaluate lumbar lymph nodes morphological changes after spinal cord injury, lymph nodes harvested one week following spinal cord injury and lymph nodes size and weight compared with sham and intact control groups. Our results revealed significant enlargement ($p<0.01$) in lumbar lymph nodes in SCI group accompanied by increased weight ($p<0.01$). On the contrary, there was no significant difference between sham and intact control groups (Fig. 1a-c).

Determination of total cell numbers along with cell viability and cell death rate in enlarged lumbar lymph nodes was carried out by acridine orange/propidium iodide assay (Fig. 1d). We found that thoracic spinal

cord injury results in a significant increase in total cell numbers compared to sham and intact control groups (p<0.01) (Fig. 1e); however, cell death percentage was also enhanced in lumbar lymph node cells after SCI (p<0.05) (Fig. 1f).

Proliferative response to Phytohemagglutinin (PHA)

To identify T lymphocyte function, important part of adaptive immune system, in enlarged lumbar lymph node following spinal cord injury, the proliferative efficacy of lumbar lymph node lymphocytes in response to Phytohemagglutinin (PHA), a T-lymphocyte mitogen, was assessed one week after spinal cord injury by CFSE assay in experimental groups (Fig. 2a).

Quantitative analysis of division index, the average number of cell divisions that a cell in the original population has undergone, showed a significant increase in response to PHA stimulation in SCI group compared to sham and intact control groups (p<0.01) (Fig. 2b). Moreover, a similar pattern of response was demonstrated related to the proliferation index, the total number of divisions divided by the number of cells that went into division, which increased against PHA in SCI rats in comparison with sham and intact controls (p<0.05 and p<0.01, respectively) (Fig. 2c). The obtained data showed markedly high lymphocyte reactivity in the lumbar lymph nodes in acute phase after spinal cord injury. Moreover, there was no significant difference between sham and intact control groups in response to PHA (Fig. 2a, b).

IFN-γ and IL-4 secretion

Considering the importance of IFN- γ and IL-4 effects in multiple immune processes and pathologies after spinal cord injury [11, 34], produced by CD4 *Th1 and Th2 correspondingly,* we measured IFN- γ and IL-4 concentration secreted by mononuclear cells of lumbar lymph nodes in experimental rats. Mononuclear cells were collected following 7 days after thoracic spinal cord injury, cocultured with PHA for 72 hours, and then the supernatants were tested by Sandwich ELISA to examine alternation in cytokines production.

Our results showed that IFN-γ secretion was significantly increased in response to PHA in lumbar lymph node cells after spinal cord injury in the SCI group in comparison with sham and intact control groups (p<0.01) (Fig. 2d). Furthermore, ELISA results revealed no significant difference in IL-4 secretion in response to PHA in the SCI group compared to non-injured sham and intact groups (Fig. 2e). However, IFN-γ/IL-4 ratio against PHA was enhanced substantially after spinal cord injury in lumbar lymph node cells (p<0.05) (Fig. 2f).

Discussion

Despite the key role of tissue-draining lymph nodes in the pathogenesis and orchestrate of inflammation cascade by eliciting anti-CNS immune responses [6-8], CNS specific draining lymph nodes have been not considered after spinal cord injury according to anatomical pathways to the injury site. As a result, to elucidate the mechanisms may adaptive immune system operate in the pathogenesis of neuroinflammation in spinal cord injury, looking for active CNS draining lymph nodes, particularly adjacent to injury site, will open a new gateway to understand the patrolling role of immune cells after SCI and may provide a valuable approach to treat SCI-associated neuroinflammatory conditions. Lumbar lymph node, the closest secondary lymphoid organ into the injury site after thoracic spinal cord injury, is a reservoir for CSF-drained solutes and cells [10, 15, 16]. Moreover, the results of the current literature highlighted the role of the lumbar lymph node in severity and induction of injury in the EAE model and T lymphocyte-*neuroimmune* actions underlying neuropathic *pain* in rodent models, which surgical excision of lumbar lymph node attenuated these pathological features [6]; however, there are just few studies concerning the anatomical importance of spinal cord- draining lymph nodes after spinal cord injury with no attention to lumbar lymph node cellular change and function [9]. Therefore, characterization of lumbar lymph node changes in acute phase after spinal cord injury as well as evaluation of its function will facilitate to understand better the immunopathology of SCI.

Here, we showed that lumbar lymph nodes enlarged distinctively in acute phase after thoracic contusion spinal cord injury in rats compared to sham control and non-injured rat. We found that this enlargement was associated with increasing total cell number in lumbar lymph node following SCI confirming inflammatory patterns of morphological change. Our findings are in line with previous studies showing enlargement of the regional lymph nodes in response to animal models of inflammatory diseases such as in EAE, Autoimmune Retinal Inflammation, airway inflammation and skin dermatitis [17-22]. Furthermore, in agreement with our data, which indicate a considerable surge in a number of cells in the enlarged lumbar lymph node, it has been demonstrated an increased number of immune cells in lumbar lymph node with significant weight gain 7 days after tibial nerve injury [6]. In contrast, it has been shown cervical Lymph node shrinkage in high-level thoracic SCI (TH1) versus low-level thoracic SCI (TH9) depends on the level of SCI in mice by defining level-dependence of spinal cord injury-induced immune deficiency syndrome (SCI-IDS) [12]; whereas they described cell loss in lymph nodes on 3 days after SCI. Furthermore, the patterns of shrinkage were not reported in the lumbar lymph node, as an adjacent major CNS-draining lymph node in thoracic spinal cord injury. Additionally, we found that in parallel to increased cell number in lumbar lymph node, the number of apoptotic cells also increased in lumbar lymph node on 7 days after injury. In agreement with our data, it has been recently demonstrated that thoracic spinal cord injury could enhance significantly apoptosis in spleen 3 and 7 days after thoracic spinal cord injury in rats [23, 24]. Therefore, results obtained *from the present study* revealed a possible mechanism by which anatomical position and lymphatic pathways of CNS regional draining lymph nodes to the spinal cord could originate the differential immune response to spinal cord injury. Furthermore, our results are in parallel to previous reports that showed progressively increase of infiltrated lymphocytes into spinal cord injury site within the first week-post injury [25]; which could be correlated with lumbar lymph node enlargement as the closest CNS darning lymph node to the injury site.

To identify the probable function corresponding to enlargement, lumbar lymph nodes were isolated from animals one week after spinal cord injury and proliferative responses, as well as cytokine production, were measured in response to Phytohemagglutinin (PHA), a selective T-lymphocyte mitogen. In the present study, we showed that thoracic spinal cord injury augmented proliferative response in lumbar lymph nodes cell accompanied by a change in cytokine production. In parallel to our findings, it has been demonstrated increased lymph node cell proliferation one week after spinal cord injury in rats in response to T-lymphocyte mitogen accompanied by significant up-regulation of IL2 receptors, a critical receptor for lymphocyte proliferation [13]. Interestingly, in this study lymph node cells were obtained by pooling lumbar lymph node (also known as periaortic lymph node) with cervical, peritracheal, axillary, brachial, popliteal, and inguinal lymph nodes. Moreover, previous studies also showed that enhanced proliferative responses in lumbar lymph node cells in neuroinflammatory conditions 7 days after nerve injury [6]; however, in literature reported level-dependence of spinal cord Injury immune deficiency syndrome, the proliferative response of lymph node cells has not been evaluated [12].Additionally, it has been shown that lumbar lymph node has higher proliferative activity than other lymph nodes in response to CNS antigens after EAE induction [5], which confirm difference in response to CNS derived antigens between the lymph nodes from different anatomical sites; while the importance of anatomical location of lumbar lymph node has not been considered for immune response analysis in spinal cord injury studies. Our results revealed lumbar lymph node as an active site adjacent to thoracic spinal cord injury, which could respond to CSF drainage from inflamed CNS.

We next investigated the pattern of cytokine production in lumbar lymph node after spinal cord injury to understand the interaction between injury site and the nearest lymphoid organ which could characterize better primary immune responses which principally occur in a local draining lymph node. Our findings demonstated a significant elevation of the IFN-γ/IL4 ratio in lumbar lymph node cells stimulated with PHA, confirmed *cytokine production* toward a Th1 *pattern. It has been shown* after SCI, adaptive immunity is biased towards the Th1 pro-inflammatory responses [26], which could be the source of pathological overwhelming increase in the Th1:Th2 cytokine ratios after spinal cord injury. Besides, our previous studies have shown that lymph node cells stimulated with MBP produce more IFN-γ after thoracic spinal cord injury [27, 28]. Interestingly, it has been demonstrated adoptive cell transfer of immune cells obtained from pooling lumbar lymph node with other lymph nodes at 7 days post-injury, but not at later post-injury intervals, from spinal cord injury donors to naive recipient rats resulted in transient hind limb paraparesis and ataxic gait [29]; may imply the importance of 7-days post injury time point with a shift in the predominant T-cell subtype form Th1 to Th2. In another interesting study performed at 7 days post injury time point, lumbar lymph node responded significantly less than cervical lymph node against the neoantigen ovalbumin (OVA), exhibited acute down-regulation of antibody production related to catecholamines immunosuppression effects after spinal cord injury [9]. It could be concluded that the major site of that antibody reduction might be originated from lumbar lymph node function, as surgical removal of sympathetic inputs to the cervical lymph nodes failed to reverse decreased antibody level after spinal cord injury. However further studies need to be carried out in this regard to understand the exact mechanisms of spinal cord injury-induced immune deficiency syndrome.

Finally, our findings confirm and extend the importance of characterizing the lumbar lymph node as one of the important regional secondary lymphoid organs in acute phase of spinal cord injury. While cervical lymph node was known to receive and process antigens draining from the brain and spinal cord [30], our data directly showed remarkable changes in the lumbar lymph node morphofunctional state, which indicate a major role of lumbar lymph node as a spinal cord-draining lymph node, that could receive and process antigens from lesion site in thoracic spinal cord injury. Our data is in line with this concept that primary immune responses are generated followed by lymphocytes activation and differentiation by antigen-presenting cells in adjacent regional lymph nodes [3, 4, 11]. According to these results, we need to consider the lumbar lymph node as a gateway for understanding how immune response shapes following thoracic spinal cord injury.

Abbreviations

CFSE: carboxyfluorescein succinimidyl ester; IL: interleukin; INF: interferon; PHA: Phytohemagglutinin; SCI: spinal cord injury.

Declarations

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Authors' contributions

HA carried out the study design, the experimental animal studies, statistical analyses, and drafted the manuscript. LK carried out the experimental animal studies, statistical analyses, and drafted the manuscript. AP, MF, and TT contributed to the conceptualization of the studies, coordination of the study, and reviewed and edited the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animal experiments were performed in accordance with approved animal protocols and guidelines established by Animal Ethics Committee of Tarbiat Modares University, Tehran, Iran. Consent to participate for human subjects is not applicable in this study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

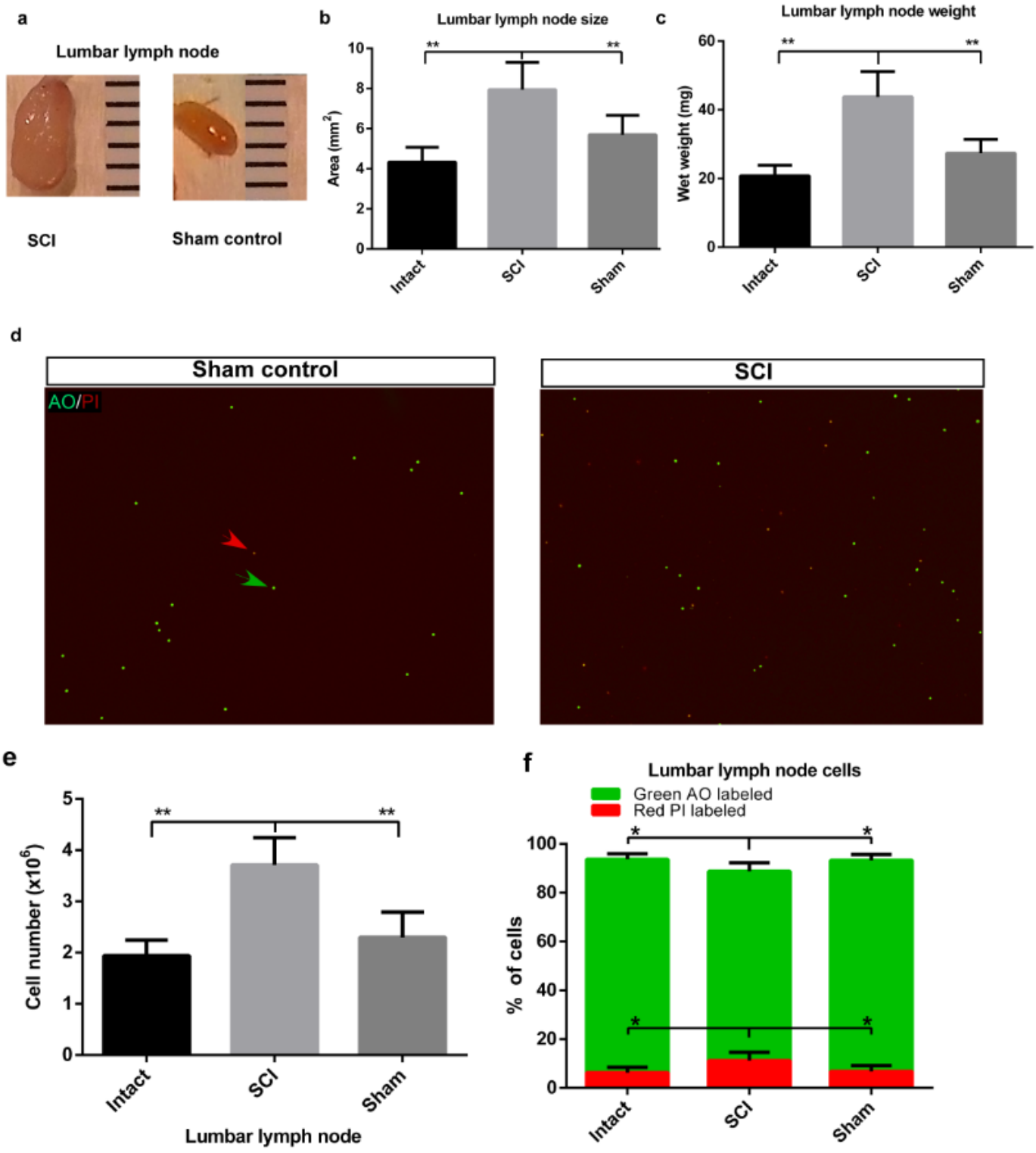

Figure 1

Spinal cord injury caused enlarged lumbar lymph node accompanied by increased absolute cell number. a Representative images showing lumbar lymph nodes. Lumbar lymph node isolated from spinal cord injury group (SCI), surgery control group (Sham) and, intact control group at one week after spinal cord injury. The scale is mm b Bar diagram represents the average (n=6 rat/group) lumbar lymph node size from spinal cord groups at one week after spinal cord injury. c Bar diagram shows the average of lumbar

lymph node weight in SCI, Sham and non-injured intact groups (n=6 rat/groups). d Representative fluorescent staining images of acridine orange (green) and propidium iodide (red) represent live and dead cells, respectively; the automatic measurement of lumbar lymph node cells after spinal cord injury on spinal cord injury (SCI) and sham control (Sham) groups 7 days after spinal cord injury. e Bar diagram shows the average of lumbar total lymph node cell count in SCI, Sham and non-injured intact groups at one week after spinal cord injury. f In the bar diagram, the percentage of living cells is represented in green bars and the percentage of dead cells in red bars on the bottom of the green bars. Data are expressed as mean ± SD. Asterix indicates significant differences (one-way ANOVA, Tukey's post hoc test. (*p<0.05 and **p<0.01). AO: acridine orange; PI: propidium iodide.

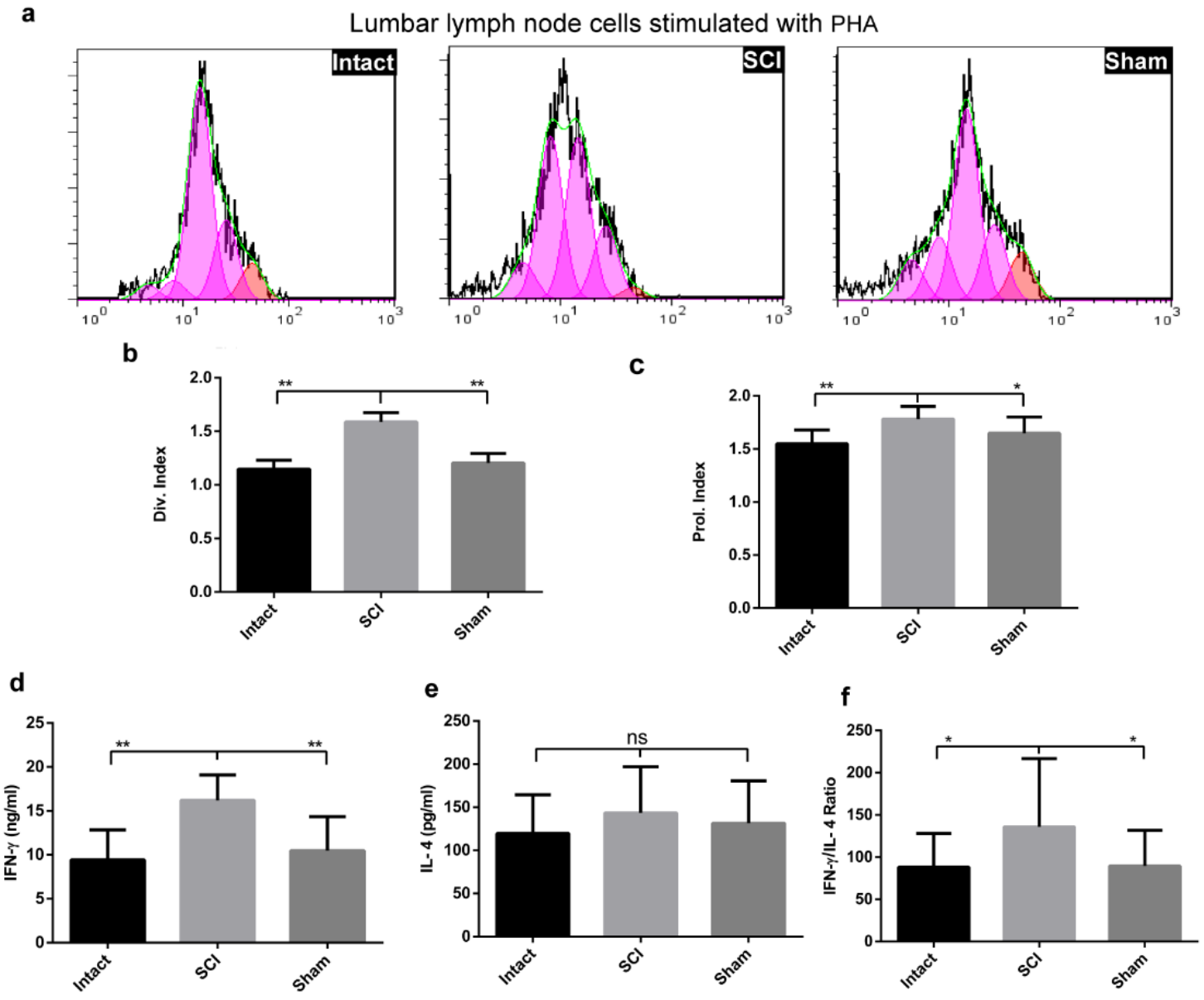

Figure 2

Increased proliferative activity of lumbar lymph node cells associated with increased IFN-γ/IL4 ratio indicateTh1/Th2 imbalance in lumbar lymph nodes one week after spinal cord injury. a: Representative flow cytometric analysis of CFSE-labeled lymphocytes of lumbar lymph node cells. Cells were isolated

from spinal cord injury group (SCI), surgery control (Sham), and intact control groups at one week after spinal cord injury, were stained with CFSE and subjected to proliferation in a 96-well plate stimulated with PHA (10μg/ml) as a polyclonal T-cell mitogen for 72h. Representative histograms indicating the multiple peaks at consecutive generations of divisions are shown. b, c: Bar diagrams show the average of division index (Div. index) and proliferation index (Prol. Index) in SCI, Sham and, intact groups (n=6 rat/groups). CFSE cell proliferation data were quantitatively analyzed by FlowJo software. d, e Bar diagrams show the average of IFN-γ and IL-4 concentration in culture supernatants secreted by mononuclear cells isolated from lumbar lymph nodes one week after spinal cord injury and stimulated with PHA (10μg/ml) in 96-well plates for 72h as determined by Sandwich ELISA. f Representative bar diagram shows the IFN-γ/IL4 ratio obtained from the IFN-γ and IL-4 concentration in culture supernatants. Values represent the mean ± SD of six rats for each group. Asterix indicates significant differences (one-way ANOVA, Tukey's post hoc test. (*p <0.05, **p<0.01). CFSE: Carboxyfluorescein succinimidyl ester; PHA: phytohemagglutinin.