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ORIGINAL ARTICLE

Cellular Inflammatory Response of the Spleen After Acute Spinal Cord Injury in Rat

Feng Wu,[1] Xiao-Yan Ding,[2] Xiao-Hui Li,[3] Min-Jie Gong,[4] Jia-Qi An,[4] Jiang-Hua Lai,[5,7] and Sheng-Li Huang[6,7]

Abstract— Spinal cord injury (SCI) involves both primary and secondary damages. After the phase of primary injury, a series of inflammatory responses initiate, which belong to the secondary injury. There has been little investigation into the cellular inflammatory response of the spleen to SCI. To disclose the impact of SCI on the spleen, we examined the inflammatory reactions of the spleen during the acute phase of SCI in rat. Adult rats were used as experimental animals and divided into un-injured, sham, and SCI groups ($n = 36$). Contusion injuries were produced at the T3 vertebral level. Spinal cords were harvested 6 h, 24 h, 48 h, 72 h, 120 h, and 168 h after surgery and were prepared for immunohistochemistry. Spleen wet weight was measured. Blood and spleens were prepared for quantitative analyses. The spleen index was significantly decreased in the SCI groups. Immunohistochemical results showed an increase of the infiltrating cells in the spinal cord tissues from SCI rats at all time points, peaking in 72 h post injury. In the blood, T and B lymphocytes significantly decreased in the SCI group as compared with the sham group, while monocyte increased. Surprisingly, in the SCI group, neutrophil initially decreased and subsequently tended to return toward baseline levels, then remained elevated until the end of the study. Spleen analyses revealed a significant increase in monocyte and neutrophil but a minor (not statistically significant) reduction in T and B lymphocytes. Our

Feng Wu and Xiao-Yan Ding contributed equally to this work.

[1] Center of Teaching and Experiment for Medical Post Graduates, School of Medicine, Xi'an Jiaotong University, Xi'an, 710061, China
[2] Department of Ophthalmology, Xi'an No. 3 Hospital, Xi'an, 710021, China
[3] Department of Radiology, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, 710004, China
[4] Department of Neurology, The First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, 7100061, China
[5] Key Laboratory of Ministry of Health for Forensic Sciences, Xi'an Jiaotong University, Xi'an, 710061, China
[6] Department of Orthopaedics, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, 710004, China
[7] To whom correspondence should be addressed to Jiang-Hua Lai at Key Laboratory of Ministry of Health for Forensic Sciences, Xi'an Jiaotong University, Xi'an, 710061, China. E-mail: Laijh1011@mail.xjtu.edu.cn; and Sheng-Li Huang at Department of Orthopaedics, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, 710004, China. E-mail: huangshengli@xjtu.edu.cn

data show that the four most prevalent inflammatory cells infiltrate the spinal cord after injury. Increased levels of inflammatory cells (monocyte and neutrophil) in the blood and spleen appear to be very sensitive to SCI. The spleen plays a critical role in the acute phase of SCI.

KEY WORDS: spinal cord injury; spleen; immune; inflammatory; acute phase.

INTRODUCTION

Spinal cord injury (SCI) is a devastating condition that affects sensation and locomotor function, for which there is still no effective therapy and its prognosis is often poor [1, 2]. Therefore, it is still major challenges today [3–22]. Pathophysiologically, SCI involves primary and secondary damages. It can generate profound changes of inflammatory cells in the primary injury site, consequently giving rise to secondary damage in the spinal cord [23].

The secondary injury may be related to inflammatory response and the immune system. Recent studies have revealed a close relationship between the immune system and the nervous system [24, 25]. Interestingly, the spinal cord has a more pronounced inflammatory response to injury than the brain, which special property may make the spinal cord particularly vulnerable to secondary lesion processes [26]. Therefore, when and where the inflammatory cells change is an important point to consider. SCI activates resident inflammatory cells including neutrophils, monocytes, and T and B lymphocytes. The first wave of infiltrating immune cells is neutrophils, the peak within the spinal cord around 1 day post injury. Neutrophils decrease within a week of injury coincident with increased monocyte infiltration into the spinal cord [27]. T lymphocytes progressively increase after injury within the first week and predominantly within the epicenter [28]. And, activation of B cells is parallel to T cell [29].

The spleen is a vital immune organ, which is a reservoir for immune cells, including T and B lymphocytes and macrophages. Unfortunately, little is known about how the spleen is affected in the injury process after SCI. It would be of interest to examine cellular inflammation and immune response of the spleen after acute spinal cord injury. The aim of this study is to characterize cell inflammatory responses in the spleen during the acute phase of SCI.

MATERIALS AND METHODS

Adult male Sprague Dawley rats weighing 220–260 g were used. The study was approved by the Animal Experiment Committee of Xi'an Jiaotong University (No. 2013117). All experimental procedures were carried out in compliance with the guidelines established by the National Institutes of Health (NIH).

Materials and Reagents

A FACSCalibur flow cytometer (BD Biosciences, New Jersey, USA) was used in the experiment. SCI impactor NYU was obtained from a weight-drop device, New York University (New York, USA). Mouse anti-rat CD3-FITC, mouse anti-rat CD45RA-PE, mouse anti-rat CD11b-PE, mouse anti-rat Ly6G-FITC, and FACS Lysing Solution were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Anti-CD3 and anti-CD11b were obtained from Biorbyt Ltd. (San Francisco, California, USA). Anti-Ly6G and anti-CD45RA were purchased from GeneTex Inc. (Alton Pkwy, Los Angeles, California, USA).

Animal Grouping

A total number of 108 rats were randomly divided into three groups: un-injured group, sham group, and SCI group, with 36 in each group. Rats in the un-injured group were anesthetized but did not undergo surgery. The sham group received laminectomy with the spinal cord un-injured. In the SCI group, a severe high thoracic (T3) injury was induced. Six animals in each group were randomly selected for tissue collection at 6 h, 24 h, 48 h, 72 h, 120 h, and 168 h after surgery.

Spinal Cord Contusion

The animal models of SCI were made following the procedures described earlier [7, 11]. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital (15 g/l) at a dose of 30 mg/kg body weight. Using routine aseptic technique, a laminectomy was performed at the vertebral level T3 to expose the dorsal spinal cord. After a laminectomy of the T3 lamina, a spinal contusion was made by the SCI impactor, with a 10-g rod dropping from a height of 50 mm. All animals that received impact

exhibited complete hindlimb paralysis. After surgery, rats were housed in individual cages and had free access to food and water. After SCI, bladders were manually expressed twice daily for the duration of the study. The sham group underwent T3 laminectomy, with the exposed spinal cord left intact. The sham-operated rats exhibited essentially normal over-ground locomotion. All animals were given a subcutaneous injection of buprenorphine (0.05 mg/kg) twice a day for 2 days after surgery.

Tissue Collection

Tissues were obtained at hours 6, 24, 48, 72, 120, and 168 after surgery, which the time points were established according to our experience and previously described [7, 30]. Six animals in each group were randomly selected at the abovementioned time points. The animals were deeply anesthetized using pentobarbital, the body weight of each animal was measured, and approximately 3 ml of whole blood was collected by cardiac puncture under aseptic conditions. The blood was diluted into a tube containing heparin to prevent clotting for flow cytometry. After blood collection, a laparotomy was performed to expose the spleen, and the spleen was excised and its weight was measured. The collected spleens were prepared for subsequent flow cytometry analysis.

Following blood and spleen sample collection, rats were perfused intraventricularly with ice-cold phosphate-buffered saline (PBS) to remove blood till the lungs turned white, and then perfused with freshly 4% paraformaldehyde in PBS. The T2–T4 segment of the spinal cord was then harvested and postfixed in the same fixative for 24 h. A 2-mm segment centered at the contusion epicenter was embedded in paraffin and was then sectioned using a sliding microtome at a thickness of 5 μm.

Determination of Spleen Index

The spleen index was calculated according to the following formula: spleen index (mg/g) = spleen weight (mg) / animal body weight (g).

Immunohistochemistry

For immunohistochemistry, the sagittal tissue sections were deparaffinized in xylene and rehydrated. The sections were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity and were then placed in 0.1 mol/l citric acid buffer solution (pH 7.42) and kept in microwave at 92–98 °C for 13 min for antigen retrieval. After cooling at room temperature, the sections were washed with PBS three times for 5 min each time, incubated with 10% goat serum for 15 min, and were then incubated with one of the following primary antibodies: CD3 (1:50), CD45RA (1:100), CD11b (1:50), and LY6G (1:100), followed by an overnight incubation at 4 °C with primary antibodies. After that, the sections were washed well with PBS three times, incubated with goat horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse for 30 min at 37 °C, and washed again with PBS three times. HRP was detected with freshly prepared 3,3′-diaminobenzidine solution. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were processed according to the same protocol, but the primary antibodies were omitted.

Flow Cytometry from Blood Cells

Anticoagulated blood (400 μl) was extracted and diluted 1:10 in FACS lysing solution for 10 min at room temperature, and samples were centrifuged at 1500×*g* for 5 min at 4 °C. The pelleted cells were washed with PBS three times. Cell viability was assessed by 0.04% Trypan blue dye exclusion. The number of viable cells was quantified at 1×10^6 ml^{-1}. Next, the samples were stained with CD3-FITC, CD45RA-PE, CD11b-PE, and Ly6G-FITC (mouse anti-rat CD3-FITC for T cells, mouse anti-rat CD45RA-PE for B cells, mouse anti-rat CD11b-PE for macrophages, and mouse anti-rat Ly6G-FITC for neutrophils) for 30 min at 4 °C. An LSR II flow cytometer was used for data acquisition, where at least 10,000 events of viable cells were collected. CellQuest 3.1 was used for data

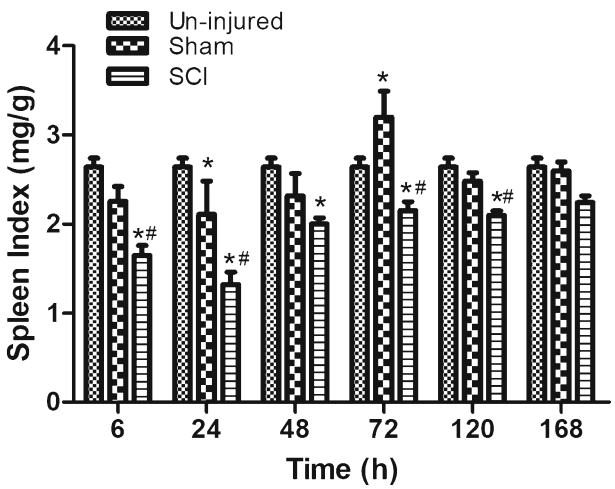

Fig. 1. Spleen index after spinal cord injury at T3 levels. *$p < 0.05$ *versus* un-injured, #$p < 0.05$ *versus* sham.

analysis, and gating of positive populations was performed based on appropriate isotype-matched antibody controls. For each sample, a negative control was prepared where no antigen was added to the culture.

Flow Cytometry from Spleen Cells

The spleen was transferred in PBS and mechanically dissociated through a 200-mesh stainless steel sieve. An amount of 600-μl homogenate was extracted, and red blood cells were lysed as described above. Antibodies were added and allowed to incubate for 30 min at room temperature. A FACSCalibur flow cytometer was used to analyze samples.

Statistical Analysis

Statistical analyses were performed using the SPSS 21.0 software package. Data normality was assessed using the Shapiro-Wilk test. All results were expressed as mean ± SEM. Student's *t* test was performed when comparing two independent samples. One-way analysis of variance was used to compare means of three samples. Significance was set at $p < 0.05$ for all analyses.

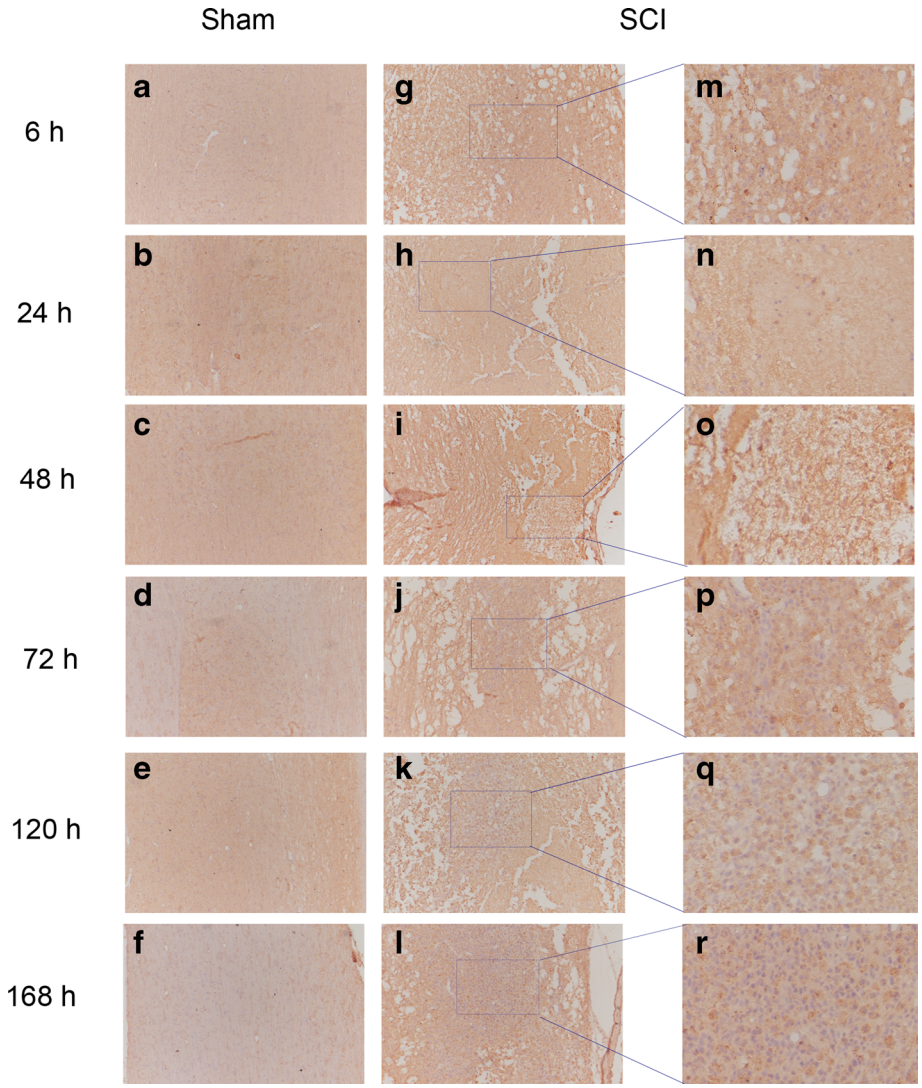

Fig. 2. Representative T lymphocytes in the spinal cord tissue. **a**–**f** Sham, × 100. **g**–**l** Injured cord, × 100. **m**–**r** Injured cord, × 200.

RESULT

Spleen Index

Observation of the spleen surface revealed that the spleen of post-SCI rat was smaller than that of the control. The decrease of the spleen index in SCI rats, in comparison to un-injured and sham-operated rats, remained significant ($p < 0.05$) in 120 h post injury; then, the index returned toward the pre-injury level by 168 h post injury (Fig. 1). Importantly, decreased spleen index was not seen following the surgical laminectomy, indicating that the decrease of the spleen index was induced by SCI but not by the general surgical trauma. Interestingly, no difference in spleen index was found between the SCI group and the sham group at 168 h post SCI ($p > 0.05$), indicating that the spleen was mostly affected at the early phase of SCI rather than but not the late phase.

Immunohistochemistry

In the spinal cord, most of the infiltrating cells concentrated around the lesion epicenter, the majority of which appeared located in the spinal white matter. The infiltrating cells increased significantly in the SCI rats than in the sham-operated or un-injured rats at all time points, peaking in 72 h post injury (Figs. 2, 3, 4, and 5).

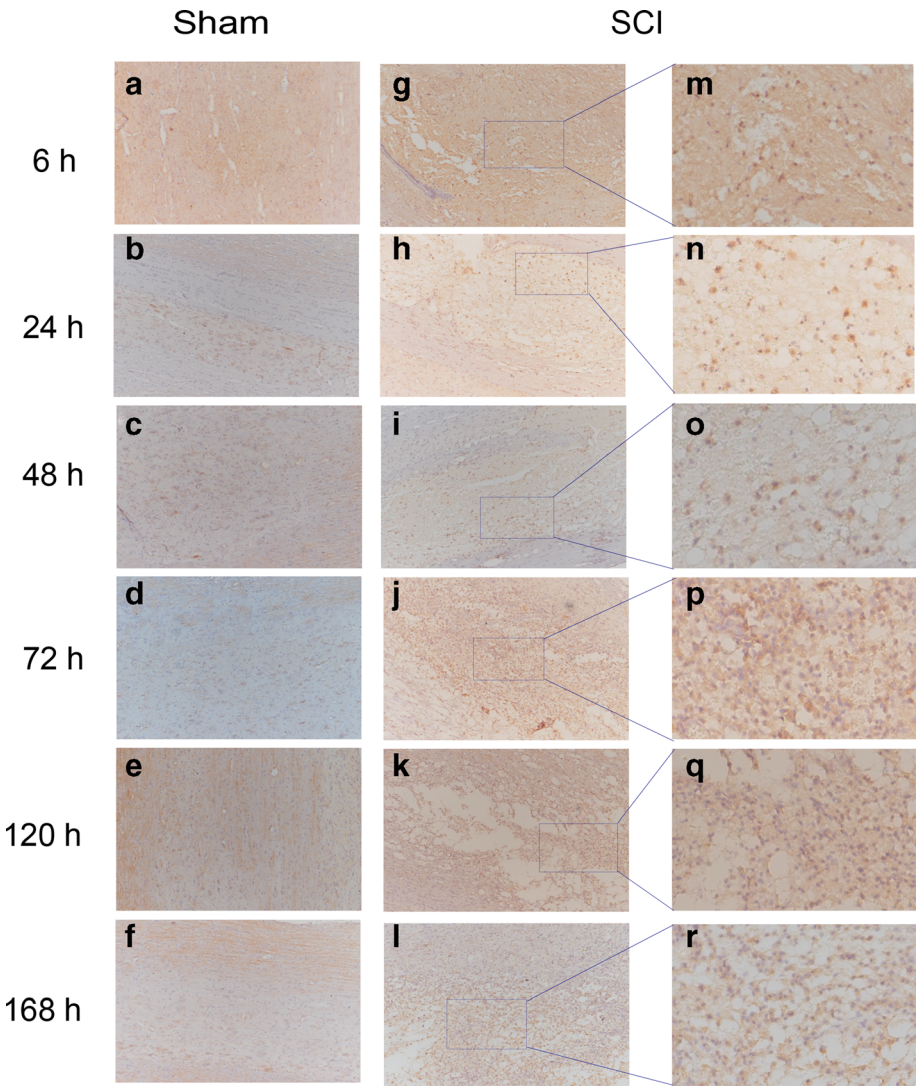

Fig. 3. Representative B lymphocytes in the spinal cord tissue. **a**–**f** Sham, × 100. **g**–**l** Injured cord, × 100. **m**–**r** Injured cord, × 200.

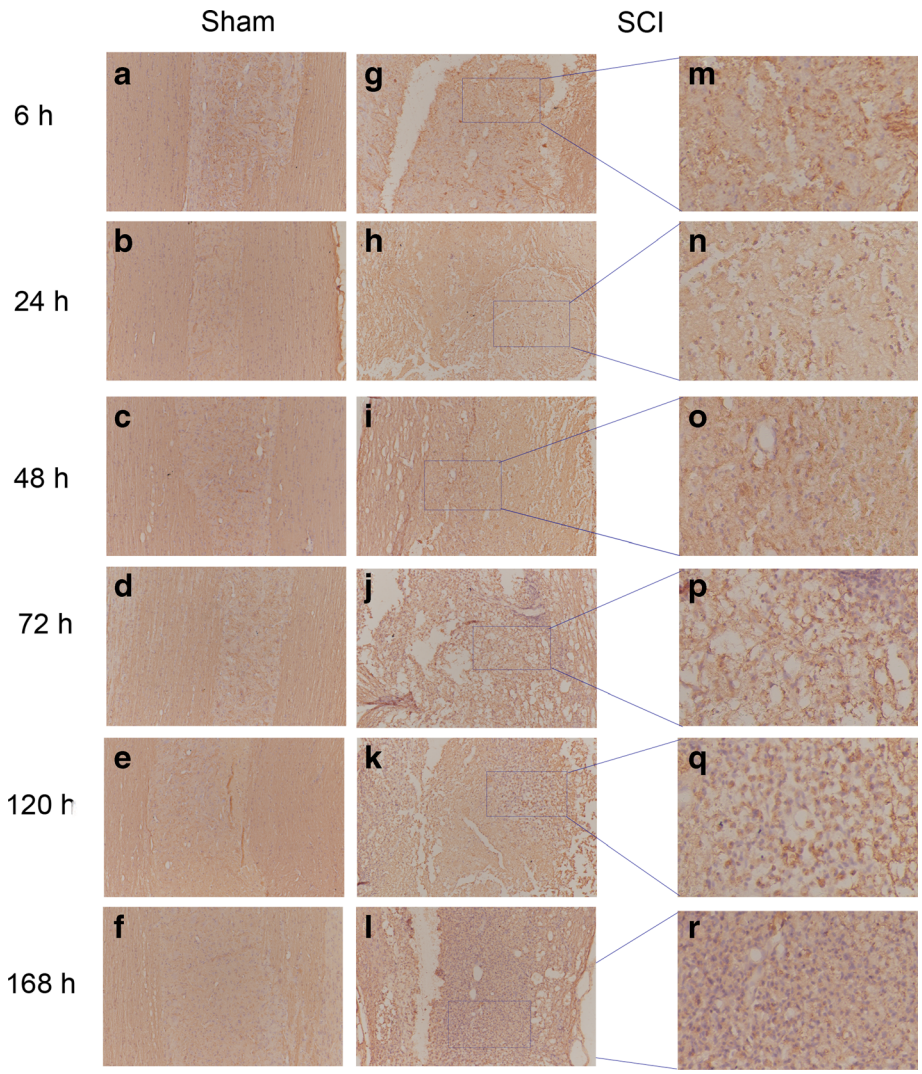

Fig. 4. Representative monocytes in the spinal cord tissue. **a**–**f** Sham, × 100. **g**–**l** Injured cord, × 100. **m**–**r** Injured cord, × 200.

Flow cytometry

T Cell

The T cell level in the blood from the SCI rats decreased at 6 h post injury, returning to baseline by 168 h. The cell counts dropped significantly at 24 h, compared with sham-operated rats (Fig. 6). Despite T cells were decreased in the spleen at 24 h and 48 h, the T cell counts at all time points post SCI were not significantly different among the groups ($p > 0.05$) (Fig. 7).

B Cell

In the blood from the SCI rats, the B cell level decreased at 6 h post injury, reaching peak at 48 h and maintaining for an additional 48 h, after which it disappeared (Fig. 6). In the SCI rat spleen, the B cell level initially decreased at 24 h post SCI but increased significantly at 168 h, as compared with sham-operated rats ($p < 0.05$) (Fig. 7).

Monocyte

Compared with that in the sham and un-injured groups, the monocyte level in the blood from the SCI group maintained to be increased at all time points, with only the increase at 6 h not statistically significant ($p > 0.05$) (Fig. 6). In the spleen, the monocyte level was elevated at 48 h post injury, with levels rising through 168 h and peaking in 72 h post injury (Fig. 7).

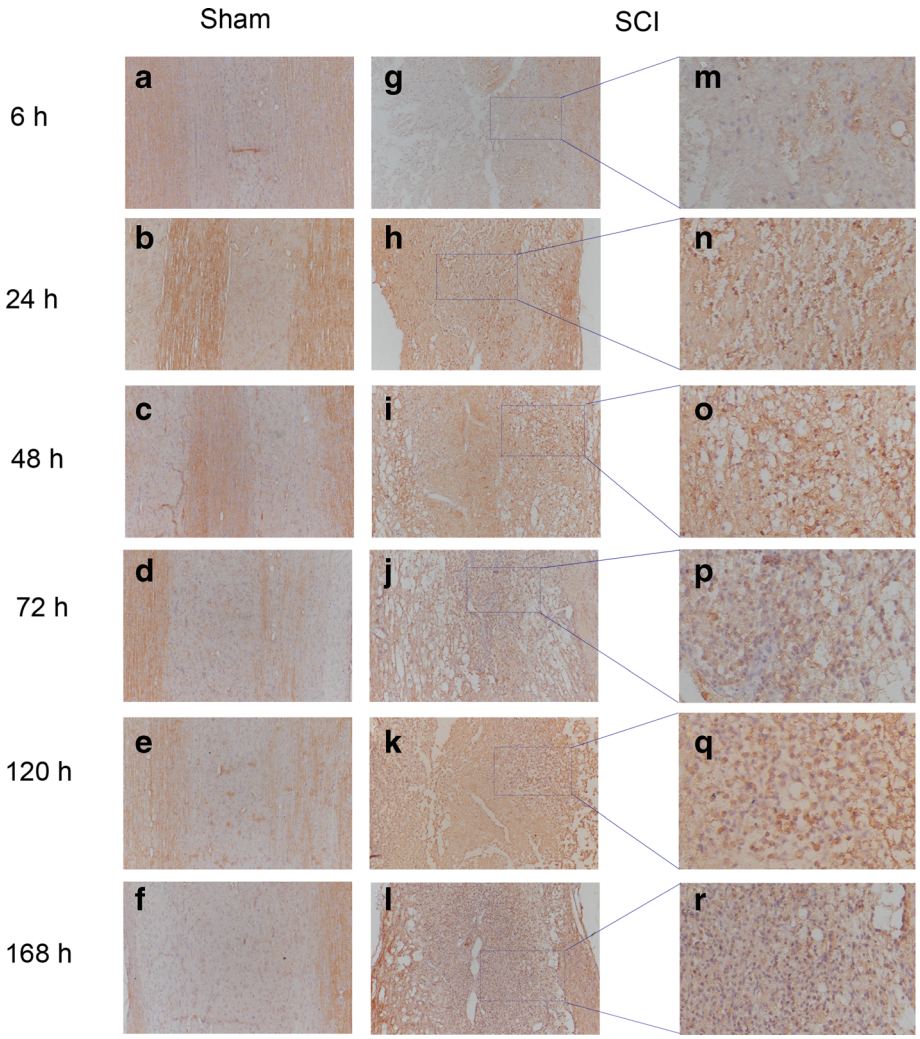

Fig. 5. Representative neutrophils in the spinal cord tissue. **a**–**f** Sham, × 100. **g**–**l** Injured cord, × 100. **m**–**r** Injured cord, × 200.

Neutrophil

In the blood from the sham group and SCI group, as indicated by the consistently lower neutrophil levels in 6 h post injury, neutrophil returns toward normal by 24 h post injury, with levels rising through 168 h (Fig. 6). In the spleen, neutrophil was elevated at 6 h post injury, with levels rising through 168 h and peaking in 72 h post injury ($p < 0.05$) (Fig. 7).

DISCUSSION

The present study assessed the impact of acute SCI on the cellular inflammatory response of the spleen. Using a spinal cord contusion model with severe injury at the T3 level, we demonstrated rapid splenic atrophy in the acute phase after SCI. Increased cellular levels, relative to those in the sham animals, were detected in the injured spinal cords during the acute phase of SCI. Particularly evident was an increase in inflammatory cells from 6 h after injury in the acute period of inflammation. In addition, in parallel with a decrease in spleen size after SCI, a marked decrease in the number of blood lymphocytes was detected.

The inflammatory cells were assessed by histology and flow cytometry. The acute splenic atrophy and changes have been reported in models of acute brain ischemia [31], but they are rarely documented in early SCI. Our experimental results show that SCI can cause a significant decrease in rat spleen index in its acute phase. The reduced

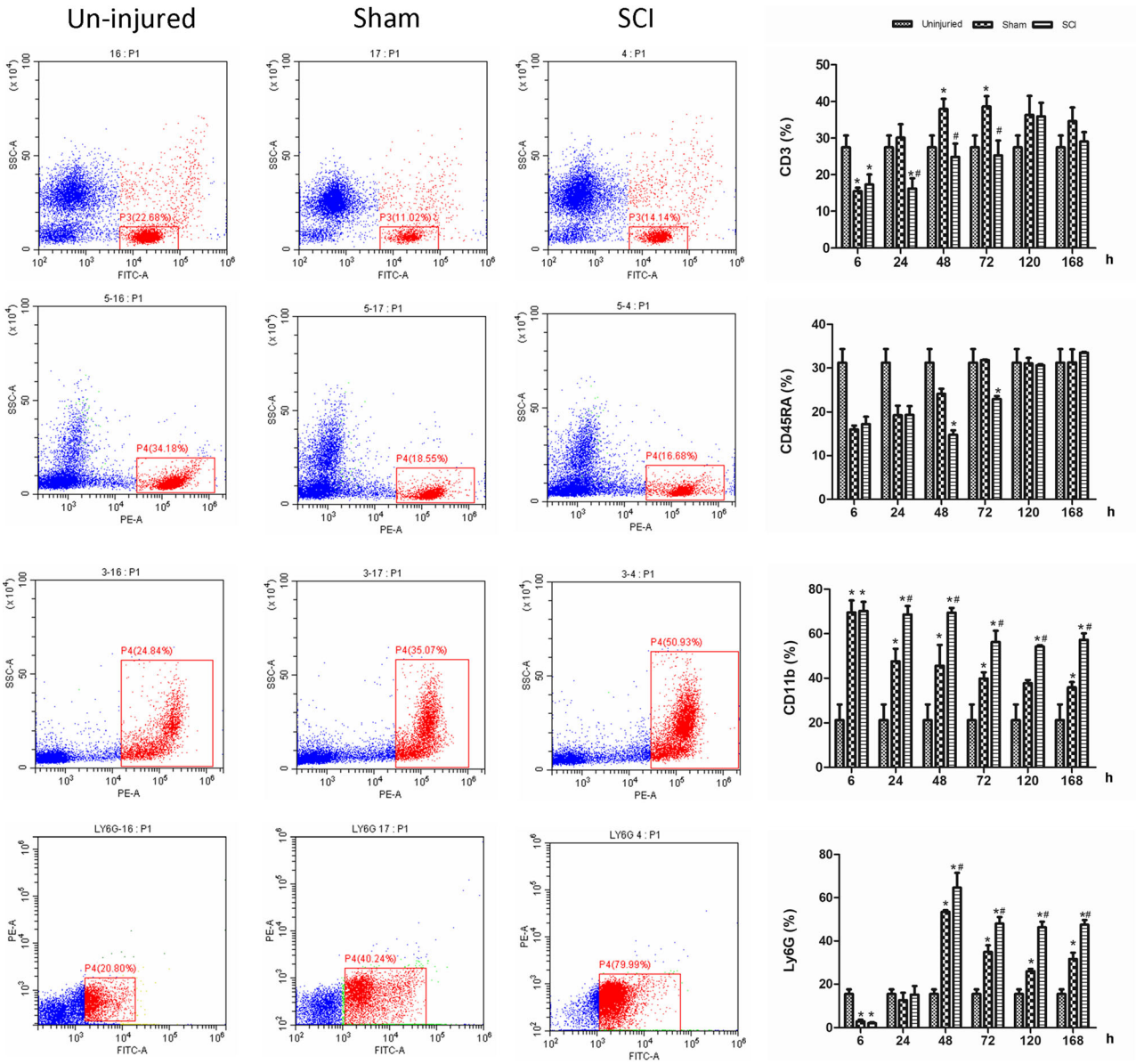

Fig. 6. Time course of changes of the four most prevalent inflammatory cells recorded in the blood. $^{*}p < 0.05$ *versus* un-injured, $^{\#}p < 0.05$ *versus* sham.

weight of the spleen of SCI rats suggests an increased mobilization of inflammatory cells after SCI. To evaluate the impact of SCI on spleen function in an acute phase, specific immune cell types including T lymphocyte, B lymphocyte, and monocyte were detected in the blood, spleen, and spinal cord, since the spleen is a main organ where these cells reside [32]. As we all know, B lymphocyte is critical to antibody-mediated immunity, and T lymphocyte is needed in cell-mediated immunity. Changes of these cells were observed in the SCI rats, including a significant elevation of T and B cells 24 h or 48 h in the spinal cord after SCI and a decrease of T and B cells as well as an increase of monocytes and neutrophil in the peripheral blood in the acute phase of SCI. In contrast, only a minor (not statistically different) decrease of T cells was observed in the SCI rat spleen. T and B lymphocytes in the spleen are activated upon SCI and infiltrated the spinal cord injury epicenter. It would be possible that the cells in the spleen migrated to the spinal cord. As a result, the spleen index in the SCI rats declined significantly,

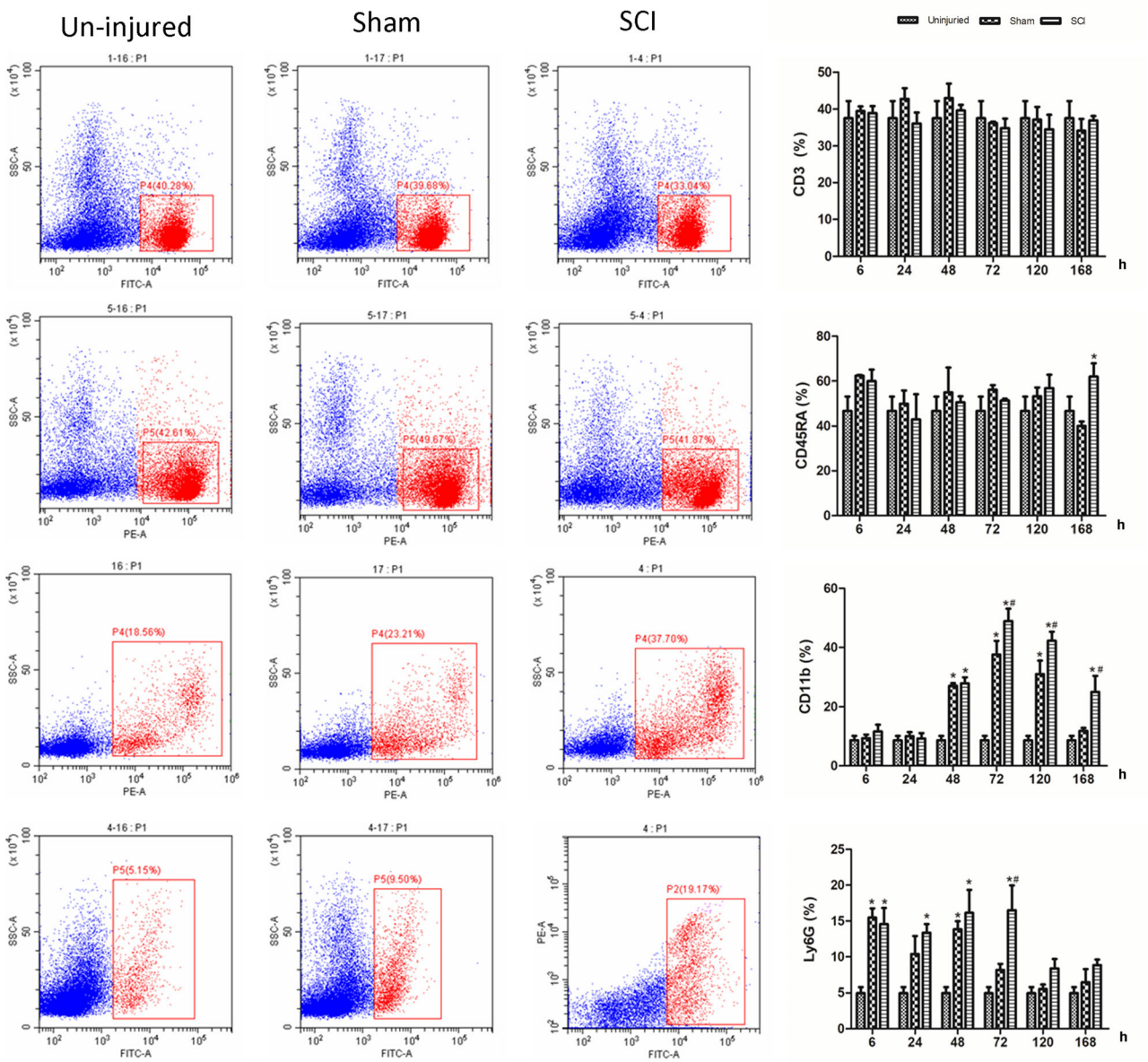

Fig. 7. Time course of changes of the four most prevalent inflammatory cells recorded in the spleen. $^{*}p < 0.05$ *versus* un-injured, $^{\#}p < 0.05$ *versus* sham.

indicating that SCI might have caused a decline in the immune function. The splenic atrophy after severe SCI represents a physiological adaptation.

The monocyte and neutrophil have received the most attention as mediators of immune reactivity in the acute phase of SCI [33]. These inflammatory cells are present at the injury site in far greater numbers than at the spleen; the accumulation of them in the spinal cord leads to neural damage. Interestingly, the magnitude of monocyte and neutrophil in the primary injury site of the spinal cord is similar to that in the spleen.

Our results indicate a cellular inflammatory response of the spleen exhibited a complex pattern in a time-related way after SCI, encompassing different phases. There are similarities with results of the global histone H4 acetylation obtained by previous study [30]. In addition, they demonstrated that glial fibrillary acidic protein (GFAP), but not S100 calcium-binding protein B (S100B)—two classical damage-related markers—was associated with global histone H4 acetylation levels [30]. Their results reinforce that the imbalance of these epigenetic markers

is a key factor for the development and progression of SCI. The change of these biomarkers is time-related and provides insights into new therapeutic targets, which develop neuroprotective strategies.

These findings might lead to the identification of potential therapeutic windows for further interventions after acute SCI. Some evidence revealed a molecular signal could activate axon outgrowth mechanisms at specific stages. De Menezes and colleagues [30] found histone-modifying enzymes could modulate spinal cord plasticity and improve the potential for axonal growth and expression of regeneration-associated genes during the first week post SCI. In the present study, though the rapidity of cellular changes is noted in the blood relative to that in the spleen, the spleen still shows immunoregulation to SCI in the acute phase of SCI. After injury, the spleen plays an important role in initiating systemic inflammation.

SCI-induced immune response is characterized by activation of neutrophil, macrophages, and lymphocytes. The response may ultimately affect functional recovery. Therefore, the spleen contributes to the pathophysiology of SCI, which suggest that the spleen represents a potential therapeutic approach in acute SCI. Thus, early intervention is likely to be important. Splenectomy-related timing, such as splenectomy within 6 h after SCI, seems to be more susceptible to the modulation of inflammation after SCI, in which splenectomy reduces the inflammatory response to SCI.

We show cellular inflammatory response of the spleen in a high thoracic SCI model during the first week following SCI. But, we do not observe inflammatory response of the spleen during the chronic SCI. Thus, further investigation is needed to resolve the issues surrounding the detection of inflammatory cells.

This study had the following limitations: First, the rats developed complete paralysis of the hindlimbs and did not recover the ability to walk, so motor assessment was not conducted, and second, the time point chosen was not optimal for demonstrating the changes in the spleen, so further research is needed to determine the duration of splenic atrophy.

In conclusion, our experimental data demonstrate that cell inflammation in the spleen is pronounced at 48–72 h post SCI. So, the spleen plays a detrimental role in the development of SCI at acute stage. The study is of potential clinical significance and could considerably influence the design of prophylactic practices, such as splenectomy.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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