Distinct Polarization Dynamics of Microglia and Infiltrating Macrophages: A Novel Mechanism of Spinal Cord Ischemia/reperfusion Injury

Lin Yang
Department of Anesthesiology, The Second Xiangya Hospital, Central South University, Changsha, China
https://orcid.org/0000-0002-9318-9812

Lin Tang
Department of Anesthesiology, The Second Xiangya Hospital, Central South University, Changsha, China

Jingjing Sun
Department of Anesthesiology, The Second Xiangya Hospital, Central South University, Changsha, China

Wei Luo
Department of Anesthesiology, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Pengfei Wang
Department of anesthesiology, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Junmei Xu
Department of Anesthesiology, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Ruping Dai
Department of Anesthesiology, The Second Xiangya Hospital, Central South University, Changsha, China

Hui Li (lihui_1166@csu.edu.cn)
Department of Anesthesiology, The Second Xiangya Hospital, Central South University, Changsha, China

Research

Keywords: spinal cord, ischemic-reperfusion injury, microglia, macrophage, polarization

DOI: https://doi.org/10.21203/rs.3.rs-154344/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background: Macrophages arising from microglia and monocyte-derived macrophages (MDMs) have extensively studied and characterized in spinal cord injury. However, the infiltration of MDMs and the precise phenotypes of the two different populations during spinal cord ischemia and reperfusion injury (SCIRI), remains ambiguous.

Methods: The SCIRI model was established by transient aortic occlusion followed by reperfusion. The Basso mouse scale (BMS) was used to quantify hind limb locomotion over the following three weeks. The histopathology of the spinal cord was evaluated by hematoxylin-eosin (HE) staining and NF-200 histochemistry. In addition, the expression of macrophage polarization phenotype were observed by double immunofluorescences, real-time PCR, and flow cytometry.

Results: Compared with the Sham group, all mice in the SCIRI group developed acute paraplegia after reperfusion, gradually recovering neurological function by day 21. HE staining revealed that SCIRI induced evident pathological changes in the spinal cord. M1-type genes (iNOS, TNF-α, CD86, and CD16) were dramatically upregulated, mainly during the 1st week of SCIRI, whereas the M2 genes, CD206, and CD204, were elevated at a later stage. In addition, double-immunofluorescence confirmed in these activated microglia/macrophages phenotypes that CD86 and CD206 were co-localized. Finally, flow cytometry further demonstrated the infiltration of MDMs (CD11b⁺ CD45⁰ cells) that were principally a pro-inflammatory M1 type, which peaked at day 3 and decreased by day 7 post-injury. Conversely, microglia (CD11b⁺CD45⁺ cells), rather than invading MDMs, was the principal source of M2 polarized cells.

Conclusions: SCIRI induced a transient influx of MDMs characterized by a M1 pro-inflammatory type, whereas resident microglia essentially maintained an M2 anti-inflammatory phenotype at later stage. These findings suggest that there is a differential regulatory role in the two cell populations following SCIRI.

Background

Spinal cord ischemic/reperfusion injury (SCIRI) remains a devastating complication after thoracoabdominal aortic surgery [1, 2]. Conventional strategies, including hypothermic circulatory arrest and cerebrospinal fluid drainage, do not provide sufficient protection for the prevention of SCIRI-induced paraplegia [3]. Once the injury has manifested clinically, no pharmacological or surgical intervention is currently available. Therefore, new treatment strategies targeting different stages of SCIRI are urgently required.

Macrophage activation after ischemic injury of the central nervous system (CNS) has been widely targeted to improve outcomes [4, 5]. Recent studies have revealed that macrophages at the site of CNS injury originate from two ontogenetically distinct populations: resident microglia and monocyte-derived macrophages (MDMs) [6]. Despite sharing the same cell markers and similar morphology, microglia and
MDMs display distinct functions in response to special environmental stimuli [7]. For example, MDMs associate with nodes of Ranvier and initiate demyelination, whereas microglia appear to clear debris [8, 9]. Gene expression profiles further confirm that MDMs are highly phagocytic and inflammatory, whereas those arising from the microglia demonstrate an unexpected signature of globally suppressed cellular metabolism at disease onset [10, 11]. Thus, distinguishing tissue-resident macrophages from infiltrating monocytes represents a novel strategy for the treatment of disease and promotion of repair in diverse inflammatory pathologies in various organs.

Through polarization to different phenotypes, macrophages display a spectrum of stages of activation and heterogeneous functions depending on the stimulus, its duration, and the environment. “Classically activated” M1 macrophages express highly pro-inflammatory cytokines, including interleukin-1 beta (IL-1β) and tumor necrosis factor-α and exacerbate neurological dysfunction via an increase in the demise and impairment of axonal regrowth in nerve cells [12]. By contrast, the “alternatively activated” M2 phenotype, characterized by high levels of arginase-1 (Arg-1), IL-10, and transforming growth factor-beta (TGF-β) expression, prolongs neuronal survival and restricts the expansion of damage after injury [13]. Importantly, previous studies have shown that microglia produce high levels of IL-10 and display greater myelin phagocytosis capability compared with MDMs, indicating that microglia are more likely to be the source of this anti-inflammatory cytokine in inflammatory conditions [14, 15]. A number of studies support a beneficial role for MDMs and have established that infiltrating MDMs are vital cells that play an anti-inflammatory role in recovery from spinal cord injury in mice [16]. In contrast, during the pathological process of status epilepticus, infiltrating MDMs exacerbate both brain inflammation and neuronal damage [17], partly accounting for the failure of anti-inflammatory drugs for the treatment of victims of CNS injury in clinic. Therefore, the dynamics of infiltration and the polarization of MDMs and microglia appear to critically important for determining neurological outcome. However, evidence in relation to their roles and phenotypes in the pathological processes occurring in SCIRI remain lacking.

In the present study, a well-established murine model of SCIRI was used to determine the precise phenotype and activation status of microglia and MDMs over time following injury. We demonstrated for the first time that resident microglia and infiltrating macrophages initially exhibit an M1 phenotype in the early stages of ischemia-reperfusion, gradually switching to an M2 phenotype and remaining for a later period of time. Furthermore, we also determined that the role of MDMs, recruited from the peripheral circulation, is primarily characterized by the M1 type.

**Methods**

**Mice**

Young adult male C57BL/6 mice, aged 12-weeks, were purchased from Silaike Jingda Experimental Animal Co., Ltd (Changsha, Hunan, China) and provided with food and water *ad libitum*. All procedures were performed using aseptic techniques and approved by the Institutional Animal Care and Use Committee of Central South University.
Animal Procedures

In total, sixty-three mice were randomly divided into 2 groups: Sham group (n = 9 at day 21 after surgical intervention) and SCIRI group (n = 9 at days 1, 3, 5, 7, 14, and 21 after aortic clamping). A mouse SCIRI model (representing the SCIRI group), was established by descending aorta clamping (DAC), as described previously [18]. Briefly, all animals were anesthetized (pentobarbital, 50mg/kg, intraperitoneal injection (i.p.)) after which tracheal intubation was performed using a 1.5-inch, 20-gauge IV catheter (BD Insylte, Sparks, MD), as described in detail in a previous study [19]. Ventilation (SAR-1000, CWE Inc, PA) was conducted using the following parameters: concentration of oxygen: 50%, tidal volume = 400µL; rate = 230bpm. Thereafter, a small transverse (dorsal to ventral, beneath the 2nd rib) incision was created below the left forelimb and shoulder, and the descending aorta, beginning 1 mm distal to the left subclavian artery, was exposed and completely cross-clamped for 9 min. Finally, the chest was closed and the animals ventilated until recovery had been achieved. In contrast, mice in the Sham group only had the aortic arch exposed without DAC. During the surgical procedure, the core temperature of the animals was maintained at 37°C using a temperature-controlled operating bed, while left femoral systolic blood pressure (FSBP) was monitored and blood sampled 5 min prior to ischemia and 10 min after reperfusion, and samples collected for blood-gas analysis.

Functional Locomotor Scores

As performed in a previous study [20], Basso mouse scale (BMS) score and sub-scores, ranging from 0 to 10, were assessed to quantify hind limb function in mice for both groups at each time point. Assessments were completed by two researchers blinded to the study groupings, and mean scores used in the final results.

Histological Analysis

After BMS scores and sub-score were evaluated, the mice were placed into deep anesthesia then transcardiac perfusion of 150 ml of cold PBS containing 0.1% heparin was performed, followed by 500 ml of ice-cold 4% paraformaldehyde. The Enlarged lumbar tissue was harvested, fixed in 4% paraformaldehyde for 8 hours, then cryoprotected in 30% (w/v) sucrose. Serial frozen, 10µm-thick sections were created then stained with hematoxylin-eosin (HE) using a standard protocol in order to record morphological changes after SCIRI.

Immunohistochemistry and Immunofluorescence

The procedures for immunohistochemistry and double immunofluorescence have been described in detail previously [21]. Anti-rabbit NF-200 (1:500, Proteintech, IL, US) was used as the primary antibody for immunohistochemistry, while Biotin-conjugated Affinipure goat anti-rabbit IgG (H + L) (1:100, Proteintech, IL, US) was used as the secondary antibody. Primary antibodies for double-labeled immunofluorescence included anti-rabbit CD11b (1:100, Affinity, OH, US), anti-rat CD86 (1:100, R&D system, MN, US), and anti-mouse CD206 (1:100, Abcam, Cambridge, UK), while the secondary antibodies included donkey anti-rabbit, donkey anti-mouse and donkey anti-rat IgG H+L (1:500, Jackson Immuno-Research, PA, US). All
sections were imaged microscopically (Olympus U-HGLGPS, Japan) and analyzed using ImageJ software (National Institutes of Health) for positive cell counting (n = 4 to 5 animals per group).

**Real-time PCR**

Total RNA was extracted using Trizol™ reagent (Thermo Fisher Scientific, MA, US). cDNA for each group was obtained by reverse transcription of the RNA (Thermo Fisher Scientific, Waltham, MA, US). Real-time PCR was performed using a CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, US) and SYBR® Green Real-Time PCR Master Mix (Roche, Germany). The primers are listed in Table 1. Quantitative PCR was performed as described in a previous study [22] and repeated in triplicate. Data were processed using the $2^{-\Delta\Delta Ct}$ method.

| Primer | Forward          | Reverse                      |
|--------|------------------|------------------------------|
| iNOS   | GAACGGAGAAACGTTGGATTTG | TCAGGTCACTTTTGTTAGGATTTT     |
| TNF-α  | TTGCTCTGTGAAGGAATGG | GGCTCTGAGGAGTAGACAAATAAAG    |
| CD16   | CATCAGCTCTGTCTGTTT  | CTCTCTGCAGCCTGTGTATTT        |
| CD86   | TGTTTCTGTAGAGAGCTTTT | GTAGAGTCCAGTTGTTCCTGTC       |
| Arg-1  | TGTTGTGGTGCGAGGATTC | ACTGCCAGACTGTGCTCCACC        |
| IL-4   | TGTCATCTGCTCTTTCTTTC | TCTGTGGTGTCTTCCGTC          |
| CD204  | CTGATGTCAGAGCCGTTGA | CAGACTGGACTTCTGCTGATAC       |
| CD206  | GTCCTCTCTGTGATTGATGAG | CACTTGTTCTGGACTAGATTA        |
| GAPDH  | GCATCCTGGAGGACTAAG   | TGGTCCTCAGTGAGCCAAG          |

**Isolation of Spinal Cord Cells and Flow Cytometric Analysis**

Mice were euthanized using an overdose of sevoflurane, and their spinal cords harvested for flow cytometric analysis following perfusion with PBS. The cells from the spinal cord were then filtered through a 40 µm nylon cell strainer (BD Biosciences) and separated from myelin and debris through Percoll density gradient medium (GE Healthcare; 70% and 30%). The cells were carefully collected from the interface of the gradient, washed, then stained for flow cytometric analysis using the following antibodies or the respective isotype controls for 20 min on ice: PE-cy7-labeled rat anti-mouse CD11b (BioLegend, 1:100), FITC-labeled rat anti-mouse CD45 (BioLegend, 1:100), APC-labeled rat anti-mouse CD369 (BioLegend, 1:100) or BV421-labeled rat anti-mouse CD86 (BioLegend, 1:100). FACS analysis was performed on a FACSCalibur with corresponding Cell Quest software (BD Biosciences).

**Statistical analysis**
All data were analyzed using SPSS v19.0 software (IBM, NY, US). Continuous variables are presented as means ± SEM. Data describing the effects of ACC on behavioral testing were analyzed using a two-way ANOVA, followed by Sidak’s multiple comparisons test for significance. Gene expression levels and fluorescence intensity were evaluated using paired sample t-tests. All results were plotted using Prism software (GraphPad Inc., CA, USA). Results were considered statistically significant where \( P < 0.05 \).

**Results**

**SCIRI causes immediate acute paraplegia**

Physiological parameters of the animals are presented in Table 2. Rectal temperature was maintained at 37°C ± 0.5°C during surgery in all mice. Femoral arterial mean blood pressure (FMBP) fell dramatically after aortic clamping (35.8 ± 2.5 vs 15.8 ± 5.6 mmHg, \( P < 0.01 \)) and returned to pre-ischemic levels following reperfusion (35.8 ± 2.5 vs 32.8 ± 2.9 mmHg, \( P > 0.05 \)). In addition, reductions in pH (7.51 ± 0.04 vs 7.21 ± 0.08, \( P < 0.01 \)), \( \text{PaO}_2 \) (91.6 ± 3.4 vs 82.6 ± 4.2 mmHg, \( P < 0.05 \)), and BE (-2.4 ± 3.3 vs -11.6 ± 3.8, \( P < 0.05 \)) were observed 10 min after reperfusion compared with 5 min prior to DAC (as displayed in Table 2).

| Distal hemodynamics and blood-gas analysis before and after DAC |
|---------------------------------------------------------------|
| **Pre-ischemia** | **Ischemia** | **Reperfusion** |
|------------------|-------------|----------------|
| Femoral SBP (mmHg) | 35.8 ± 2.5 | 15.8 ± 5.6** | 32.8 ± 2.9 |
| pH | 7.51 ± 0.04 | Nd | 7.21 ± 0.08** |
| \( \text{PaO}_2 \) (mmHg) | 91.6 ± 3.4 | Nd | 82.6 ± 4.2* |
| \( \text{PaCO}_2 \) (mmHg) | 33.2 ± 5.6 | Nd | 35 ± 5.0 |
| Base Excess | -2.4 ± 3.3 | Nd | -11.6 ± 3.8* |

* and ** separately represent \( P < 0.05 \) and \( P < 0.01 \) compared with the pre-ischemic values.

The time course of neurological function as assessed by BMS and sub-BMS scoring ranged from 0 (paraplegia) to 9 (normal) over the 21 days following reperfusion. In detail, the BMS and sub-BMS scores of the mice in the Sham group remained at the baseline of 9, while achieved lower BMS scores and sub-scores immediately at day 1 (\( P < 0.01 \)), and lasted until day 7 (\( P < 0.01 \)), and gradually improved since day 14 (still lower than the Sham group, \( P < 0.01 \)) indicating that DAC for 9 min while maintaining a central temperature of 37°C produced acute and moderate paraplegia.

No mice died in the Sham group, while 9 died in the SCIRI group due to refractory seizures immediately following surgery, 2 died of prolonged anesthesia, and a further 11 died of other unrelated reasons. Bowel
infarction was not detected by macroscopic examination.

**SCIRI causes significant morphological change and motor neuron loss**

HE staining of spinal tissue was performed to examine morphological changes following SCIRI. As displayed in Fig. 2A and B, subtle changes are evident on day 1 after DAC, characterized by isolated vacuolar necrosis predominantly in the region of gray matter of the lumbar spinal cord, coincident with the time point at which mice began to display locomotor deficit in their hind limbs. Necrosis then progressed and peaked at day 7 following SCIRI, accompanied with increasing infiltration of inflammatory cells. Then, tissue injury began to alleviate since day 14, together with gradual dissipation of inflammatory cells infiltration.

Consistently, compared with the Sham group, DAC resulted in progressive loss of anterior horn motor neurons as visible by NF-200 staining during the 1st week after SCIRI. The cell nuclei appeared darker and pyknosis was apparent especially at day 3 and day 7 post-injury. Then, the neuron death became alleviated at day 14 and day 21, as increasing nucleolus had been re-appeared at the lesion site (Fig. 2C and D).

**Time Course of Messenger RNA Expression of M1 and M2 Polarization Markers Following SCIRI**

Polarized microglial cells and macrophages are commonly distinguished by the expression of surface markers and cytokine/chemokine secretion. To measure the dynamics of the polarization, gene expression analysis of markers of M1 and M2-like polarization was performed of the microglial cells and macrophages in the spinal cord after 1, 3, 7, 14, and 21 days following SCIRI. M1-like markers included iNOS, TNF-α, CD16, and CD86 while M2-like markers included Arg-1, IL-4, CD204, and CD206. Compared with the Sham group, expression levels of the M1-type genes after SCIRI increased markedly as early as day 1 (iNOS: 2.24-fold, \( P < 0.05 \); TNF-α: 3.16-fold, \( P < 0.05 \); CD16: 2.87-fold, \( P < 0.01 \), and CD86: 3.85-fold, \( P < 0.01 \)) and were maintained at a high level, peaking only after the first week (iNOS day 5: 2.74-fold; TNF-α day 7: 4.61-fold; CD16 day 5: 3.83-fold, and CD86 day 7: 5.09-fold, \( P < 0.01 \)). The majority of the M1 markers decreased sharply and returned to pre-clamped levels except for CD86, the expression of which downregulated but was still 1.70-fold greater compared to the Sham group at day 21 (\( P < 0.05 \)). In contrast to M1 markers, the mRNA expression levels of M2 markers, including CD204 and CD206, were higher at day 7 (CD204: 2.99-fold, \( P < 0.01 \); CD206: 3.47-fold, \( P < 0.05 \)), and remained at a relatively high level for the subsequent 2 week period. However, Arg-1 and IL-4 began to increase between days 1 to 3 after SCIRI (Arg-1 day 1: 2.79-fold, \( P < 0.001 \); IL-4 day 3: 4.30-fold, \( P < 0.01 \)), and had returned to preinjury levels by day 14 (as shown in Fig. 3).

**Dynamic Changes in Microglial cells and Macrophages with M1 or M2 Phenotypes Following SCIRI**
We then confirmed the presence of the M1-type and M2-type microglia/macrophages by immunohistochemistry using confocal microscopy. As shown in Fig. 4, expression of the M1 marker CD86 in the microglial cells and macrophages increased dramatically from day 1 after SCIRI, remaining relatively high for a whole week, similar to that of CD11b expression. In contrast, the M2 marker CD206 was expressed at a low level in microglial cells and macrophages in the early stages but increased significantly over control levels 7 days after injury. Taken together, these results suggest that during the early stages of SCI, M1-type microglia/macrophages are in the majority, whereas the M2-type increased at the site of spinal cord injury mainly during the later stages.

**Microglia and Invading MDMs Display Distinct Polarization Patterns during SCIRI**

Differential CD45 expression was evaluated to determine whether the accumulated macrophages were resident or invading, and extended the analysis to observe the dynamics of polarization of microglia and macrophages using fluorescence-activated cell sorting (FACS) analysis. As displayed in Fig. 5A and B, gating of the CD45high populations revealed that peripheral infiltrated MDMs, characterized with CD11b+ CD45high increased rapidly and robustly since day 3 post-injury (P < 0.01), peaked at day 7 (P < 0.01) and fell to the Sham levels since day 14. Furthermore, MDM expressing CD86 increased consistently and sharply from day 3 to day 7 post SCIRI injury (P < 0.01, Fig. 5C), whereas MDM expressing Dectin remained unchanged at each timepoint (Fig. 5D). In contrast, resident microglia expressing CD86 increased at day 3 post-injury (P < 0.05, Fig. 5E), while microglia with Dectin positive increased at day 7 after SCIRI (P < 0.05, Fig. 5F).

**Discussion**

Recent studies in the field of SCIRI pathology, in addition to many other neurological diseases, point to the dual role of microglial cells and macrophages in neuronal injury and recovery [23, 24]. In support of the hypothesis for a protective role for microglia and macrophages, it has been reported that microglial depletion by using the colony-stimulating factor-1 receptor (CSF-1R) inhibitor PLX5622 resulted in increased vascular leaking in both white and gray matter in the spinal cord when hypoxic [25]. However, mounting evidence has revealed that robust activation of microglial cells and macrophages contribute to a deterioration of neurological outcomes [26, 27]. Activated microglia/macrophages have been reported to exacerbate damage by interacting with multiple pathological processes other than inflammation during injury, including crosstalk with astrocytes that affects proliferation [28], oligodendrocyte apoptosis, and demyelination [29]. However, the infiltration of MDMs and the precise inflammatory profile of MDMs and microglia during SCIRI remain largely unresolved. Therefore, a more clear understanding of the dynamic alterations of M1 and M2 phenotypes of microglia and macrophages will advance our understanding of SCIRI and inform future treatment options.
The relative secretion levels of pro- and anti-inflammatory factors provide an indication of the activation state of microglia and macrophages. Thus, we studied the expression of inflammatory markers at the transcriptional level. Firstly, similar with traumatic brain injury [30], a sharp upregulation of M1-type genes, such as iNOS, TNF-α, CD86, and CD16, was observed in the present study, which appeared mostly at an early stage (almost within the 1st week). M1-type cells are believed to release devastating levels of pro-inflammatory mediators (e.g. TNF-α, IL-1β, and IL-6) while oxidative metabolites impair axonal regrowth [5]. In contrast, M2 biomarkers, including CD204 and CD206, became elevated at later stages, mainly after the 1st week. The recruitment of M2 microglia and macrophages may represent an endogenous process aimed at restricting ischemic damage by releasing protective and neurotrophic factors, scavenging cell debris, and resolving local inflammation [5]. Secondly, the pattern of M1 to M2 conversion occurred 7 days post-injury, coincident with improved BMS scores, alleviation of morphological damage, and loss of motor neurons in the spinal cord at a later stage. Finally, we also found that the expression of some M2 biomarkers (such as Arg-1 and IL-4) altered in an uncoordinated manner, as reported in other studies [31–33], suggesting that inflammatory microenvironments may favor a particular biomarker but not others. Taken together, these data suggest a distinct pattern of microglial and macrophage phenotypic change from M2 to M1 in spinal cord injury, indicating that different pathological mechanisms underlie the migration of the two cell types to the spinal cord.

Peripheral monocytes are known to enter the CNS following injury and contribute to traumatic injury in mouse models of multiple sclerosis [34, 35]. Monocyte infiltration occurs 1 and 3 d after the onset of status epilepticus, whereas in the majority of CNS disorders, such as stroke and experimental autoimmune encephalomyelitis (EAE), a significant influx of MDMs is initiated into the injured area on day 3 [36]. In the present study, we also found that monocyte infiltration was not immediately evident after injury until day 3. The underlying mechanisms that limit monocyte transmigration into acutely reperfused spinal cord tissue are yet to be fully understood. As reported in previous studies [37], the migration of leukocytes into the CNS is highly regulated, requiring coordinated activation of the leukocytes and endothelium, and the presence of appropriate chemoattractant gradients between the blood and brain. Furthermore, we have demonstrated that invading MDMs decrease from day 7 after SCIRI. Such changes were similar to the infiltration of MDMs in mice in which spinal cord injuries occurred [10, 13, 16], suggesting that the ablation of MDMs from the second week onward may have no effect on functional recovery. Taken together, these two observations indicate that the role of MDMs is essentially transiently restricted to the first week after SCIRI, and probably only between days 3 and 7 post-injury.

Importantly, the present study highlights the fact that infiltrating MDMs and resident microglia differ in phenotype following SCIRI. Although indistinguishable by standard immunohistochemical techniques, these two macrophage populations are commonly viewed as functionally homogenous [38]. Previous SCIRI-related studies have generally suppressed or eliminated these cells at the site of the lesion. However, multiple other studies have reported distinct developmental origins of MDMs in other CNS diseases, suggesting that they may exert functions different from those of microglia in pathological processes [15]. For example, previous studies have established that invading monocytes induce axonal
damage by initiating demyelination, whereas microglia clear debris [14]. Furthermore, the effect of monocyte infiltration is dependent on disease context. In EAE mice, infiltrating MDMs are highly inflammatory compared with microglia, and their depletion results in enhanced recovery [39]. Conversely, MDMs provide neuroprotection and promote recovery following spinal cord injury [40]. The results of the present study, however, attribute a pro-inflammatory function to MDMs, primarily at an early stage following SCIRI. In addition, resident microglia play a pro-inflammatory role in the early response to SCIRI, followed by an increased proportion of M2 phenotype that promotes endogenous repair at a later stage. These findings indicate that damage and repair during SCIRI are elegantly modulated via endogenous mechanisms involving coordinated phenotype modulation of microglia and MDMs that are dependent on timing.

Together, the study brings new insight into the long-standing unsolved problems regarding the differential contributions of MDMs and microglia to recovery from SCIRI. We have revealed a pro-inflammatory effect of infiltrating MDMs restricted probably to between day 3 and 7 post-injury, while later resident microglia increased their conversion to an M2 phenotype that enhanced endogenous repair. Importantly, in a previous study [41] we reported an increased ratio of M1/M2-like monocytes in the peripheral circulation in patients with Stanford type-A aortic dissection (AAD), indicating that once AAD patients are subjected to the process of deep hypothermic circulatory arrest, increased numbers of M1 polarized macrophages migrate to the re-perfused spinal cord, aggravating the severity of inflammation in the micro-environment of the site of the lesion. Thus, a better understanding of the differential phenotypes of activated resident microglia and infiltrating MDMs following CNS might enable the development of novel approaches to attenuate SCIRI, for example, by timely targeting infiltrating monocytes through regulation of monocyte migration and intracellular signaling may help for the recovery.

Several limitations of this study merit comment. First, the mice only underwent moderate paresis after clamping across the descending aorta for 9 min. In this regard, the number of invading MDMs in spinal cord may differ according to the degree of paresis. More severe paresis should be also observed. However, most mice would die within several days after clamping more than 9 min to cause severe paresis, which did not fulfil the requirement of a survival time as long as 21 days to explore the dynamic polarization of macrophage/microglia. Indeed, most clinical patients undergoing moderate paresis instead of severe paresis, which makes this model closer to clinical setting. Second, although the polarization of MDMs was determined in the present study, further studies to intervene these cells are needed.

**Conclusions**

In conclusion, we recorded the dynamic alteration in phenotypes of both microglia and MDMs during SCIRI. The results indicate that during SCIRI, the effect of MDMs is essentially transiently restricted to the first week after SCIRI, and probably only between days 3 and 7 post-injury. Importantly, infiltrating macrophages were primarily the pro-inflammatory M1 type, whereas microglia, in contrast, may have been the principal source of M2 polarized cells.
List Of Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| MDMs         | monocyte-derived macrophages |
| SCIRI        | spinal cord ischemia and reperfusion injury |
| BMS          | Basso mouse scale |
| HE           | hematoxylin-eosin |
| CNS          | central nervous system |
| IL           | interleukin |
| TNF-α        | tumor necrosis factor-α |
| Arg-1        | arginase-1 |
| TGF-β        | transforming growth factor-beta |
| DAC          | descending aorta clamping |
| FMBP         | Femoral arterial mean blood pressure |
| FACS         | fluorescence-activated cell sorting |
| CSF-1R       | colony-stimulating factor-1 receptor |
| EAE          | encephalomyelitis |
| AAD          | Stanford type-A aortic dissection |

Declarations

1 Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee of Central South University.

2 Consent for publication

All authors have read and approved the content, and agree to submit for consideration for publication in the journal.

3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

4 Competing interests
No competing interests exist in the present manuscript.

5 Funding

This research was supported by the National Natural Science Foundation of China (NSFC 82071347 and 81771354 to Ruping Dai, 81873770 to Hui Li).

6 Authors’ contributions

Hui Li and Ruping Dai designed the study and wrote the manuscript; Lin Yang performed the experiment and helped to draft the manuscript; Lin Tang was in charge of the animal model accomplishment and feeding; Jingjing Sun was in charge of the flow cytometry, Pengfei Wang, Wei Luo and Junmei Xu participated in the data analysis.

7 Acknowledgement

not applicable

References

1. Greenberg RK, Lu Q, Roselli EE, et al. Contemporary Analysis of Descending Thoracic and Thoracoabdominal Aneurysm Repair A Comparison of Endovascular and Open Techniques[J]. Circulation. 2008;118(8):808–17.

2. Coselli JS, LeMaire, et al. Mortality and Paraplegia After Thoracoabdominal Aortic Aneurysm Repair: A Risk Factor Analysis[J]. ANNALS OF THORACIC SURGERY. 2000. 69(2): p. 409 – 14.

3. Sa HJ, Miller CC, Huynh TTT, et al. Distal Aortic Perfusion and Cerebrospinal Fluid Drainage for Thoracoabdominal and DescendingThoracic Aortic Repair: Ten Years of Organ Protection[J]. Ann Surg. 2003;238(3):372–80.

4. Zhao X, Sun G, Zhang J, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: Role for peroxisome proliferator-activated receptor in microglia/macrophages[J]. Ann Neurol. 2007;61(4):352–62.

5. Wan S, Cheng Y, Jin H, et al. Microglia Activation and Polarization After Intracerebral Hemorrhage in Mice: The Role of Protease-Activated Receptor-1[J]. Translational Stroke Research. 2016;7(6):1–10.

6. Xiong XY, Liu L, Yang QW. Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke.[J]. Prog Neurobiol. 2016;142:23–44.

7. Durafourt BA, Moore CS, Zammit DA, et al. Comparison of polarization properties of human adult microglia and blood-derived macrophages.[J]. Glia. 2012;60(5):717–27.

8. Girard S, Brough D, Lopez-Castejon G, et al. Microglia and macrophages differentially modulate cell death after brain injury caused by oxygen-glucose deprivation in organotypic brain slices.[J]. Glia. 2013;61(5):813–24.
9. Hu X, Leak RK, Shi Y, et al. Microglial and macrophage polarization - New prospects for brain repair[J]. Nature Reviews Neurology. 2014;11(1):56–64.

10. Teresa AE, Deborah SB, Jay TM, et al. High-resolution intravital imaging reveals that blood-derived macrophages but not resident microglia facilitate secondary axonal dieback in traumatic spinal cord injury[J]. Exp Neurol. 2014;254(4):109–20.

11. Yamasaki R, Lu H, Butovsky O, et al. Differential roles of microglia and monocytes in the inflamed central nervous system[J]. J Exp Med. 2014;211(8):1533–49.

12. Bell MT, Puskas F, Agoston VA, et al. Toll-like receptor 4-dependent microglial activation mediates spinal cord ischemia-reperfusion injury[J]. Circulation. 2013;128(11_suppl_1):152–6.

13. Isaac F-Q, Jesús, et al. IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury[J]. Glia. 2016;64(12):2079–92.

14. Girard S, Brough D, Lopez-Castejon G, et al. Microglia and macrophages differentially modulate cell death after brain injury caused by oxygen-glucose deprivation in organotypic brain slices[J]. Glia. 2013;61(5):813–24.

15. Durafourt BA, Moore CS, Zammit DA, et al. Comparison of polarization properties of human adult microglia and blood-derived macrophages[J]. Glia. 2012;60(5):717–27.

16. Kong X, Gao J. Macrophage polarization: a key event in the secondary phase of acute spinal cord injury[J]. J Cell Mol Med. 2017;21(5):941–54.

17. Lijie F. Madhuvika, et al. Microglial proliferation and monocyte infiltration contribute to microgliosis following status epilepticus[J]. Glia. 2019;67(8):1434–48.

18. Awad H, Ankeny DP, Guan Z, et al. A mouse model of ischemic spinal cord injury with delayed paralysis caused by aortic cross-clamping.[J]. Anesthesiology. 2010;113(4):880–91.

19. Das S, Macdonald K, Chang HYS, et al. A Simple Method of Mouse Lung Intubation[J]. Journal of Visualized Experiments. 2013;73(73):e50318.

20. Basso DM, Fisher LC, Anderson AJ, et al. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains.[J]. J Neurotrauma. 2006;23(5):635–59.

21. He L, Xu JM, Li H, et al. Moderate hypothermia increased the incidence of delayed paralysis through activation of the spinal microglia in an aortic cross-clamping rat model[J]. Int J Cardiol. 2016;220:454–61.

22. Wang Z, Wu JL, Zhong F, et al. Upregulation of proBDNF in the Mesenteric Lymph Nodes in Septic Mice[J]. Neurotox Res. 2019;36(3):540–50.

23. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils[J]. Nat Immunol. 2011;12(11):1035–44.

24. Smith PD, Puskas F, Meng X, et al. The Evolution of Chemokine Release Supports a Bimodal Mechanism of Spinal Cord Ischemia and Reperfusion Injury[J]. Circulation. 2012;126(11-suppl-1):110–7.
25. Halder SK, Milner R. A critical role for microglia in maintaining vascular integrity in the hypoxic spinal cord[J]. Proceedings of the National Academy of Sciences, 2019, 116: 26029–26037.

26. Smith PD, Bell MT, Puskas F, et al. Preservation of motor function after spinal cord ischemia and reperfusion injury through microglial inhibition.[J]. The Annals of thoracic surgery, 2013, 95(5):1647–1653.

27. Mantovani A, Biswas SK, Galdiero MR, et al. Macrophage plasticity and polarization in tissue repair and remodelling[J]. J Pathol. 2013;229(2):176–85.

28. Niels H, Bangfu Z, Jian W, et al. Crosstalk between macrophages and astrocytes affects proliferation, reactive phenotype and inflammatory response, suggesting a role during reactive gliosis following spinal cord injury.[J]. Journal of neuroinflammation, 2015; 30;12:109.

29. Zhou X, He X, Ren Y. Function of microglia and macrophages in secondary damage after spinal cord injury.[J]. Neural Regeneration Research. 2014;9(20):1787–95.

30. Hsieh CL, Kim CC, Ryba BE, et al. Traumatic brain injury induces macrophage subsets in the brain[J]. Eur J Immunol. 2013;43(8):2010–22.

31. Kigerl KA, Gensel JC, Ankeny DP, et al. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J Neuroscience. 2009;29:13435–44.

32. Ma SF, Chen YJ, Zhang JX, et al. Adoptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury[J]. Brain Behav Immun. 2015;45:157–70.

33. David S, Kroner A. Repertoire of microglial and macrophage responses after spinal cord injury[J]. NATURE REVIEWS NEUROSCIENCE. 2011;12(7):388–99.

34. Orr MB, Gensel JC. Spinal Cord Injury Scarring and Inflammation: Therapies Targeting Glial and Inflammatory Responses[J]. Neurotherapeutics. 2018;15(3):541–53.

35. Akaishi T, Takahashi T, Nakashima I. Peripheral blood monocyte count at onset may affect the prognosis in multiple sclerosis[J]. journal of neuroimmunology. 2018;319:37–40.

36. Zarruk JG, Greenhalgh AD, David S. Microglia and macrophages differ in their inflammatory profile after permanent brain ischemia [J]. Exp Neurol. 2017;301(Pt B):120–32.

37. Ransohoff RM, Kivisakk P, Kidd G. Three or more routes for leukocyte migration into the central nervous system. Nat Rev Immunol. 2003;3(7):569–81.

38. Fernandez-Suarez, Diana F, et al. Alternatively activated microglia and macrophages in the central nervous system[J]. Progress in Neurobiology An International Review Journal. 2015;131:65–86.

39. Ajami B, Bennett JL, Krieger C, et al. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool.[J]. Nat Neurosci. 2011;14(9):1142–9.

40. Shechter R, London A, Varol C, et al. Infiltrating Blood-Derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. PLoS Med. 2009;6(7):e1000113.

41. Weiyun Shen, Luo C, Hurtado PR, et al. The regulatory role of ProBDNF in monocyte function: Implications in Stanford type II aortic dissection disease[J]. FASEB J. 2020;34(2):2541–53.
Figures

Figure 1

SCIRI causes immediate acute paraplegia following DAC. Motor function is evaluated by BMS score and subscore. **, compared with the Sham group, P<0.01. Each time point represents means ± standard error for nine rats.

Figure 2

SCIRI causes morphological changes and loss of motor neurons in the spinal cord. HE staining results shown in A (scale bar=100μm) and B (scale bar=25μm) indicate that DAC results in significant vacuolar necrosis and inflammatory cell infiltration into the grey matter of the spinal cord. C and D.
Immunohistochemistry for NF-200 (scale bar, C=100μm, D=25μm) demonstrate that DAC causes progressive motor neuron loss in the anterior horn of the spinal cord (n=3 for both groups at each time point).

**Figure 3**

Time course of gene expression of M1 and M2 polarization markers following SCIRI. Quantitative PCR was used to assess the expression levels of M1- and M2-like microglial/macrophage activation genes in spinal cord of sham, 1d, 3d, 5d, 7d, 14d and 21d SCIRI mice. (A) M1-like genes included iNOS, TNFα, CD16, and CD86. (B) M2a-like genes included Arg-1, IL-4, CD204 and CD206. *, ** and *** separately represent P<0.05, P<0.01, and P<0.001 compared with the Sham group. Each column represents 3 rats at each time point.
Figure 4

Dynamic changes in M1 and M2 phenotypes of microglia/macrophages following SCIRI. A. The immunofluorescence staining of CD11b+ (green), CD86 (red) and CD206 (red) at day 1, day 3 and day 7 after SCIRI was shown. B. Quantification of CD11b+, CD86 and CD206 at at day 1, day 3 and day 7 after SCIRI. C. Double immunofluorescence further proved that both CD86 and CD206 were mainly accumulated in
Microglia and invading MDMs display distinct patterns of polarization during SCIRI.A. Examples of flow cytometry analysis of the spinal cord are shown, identifying monocyte-derived macrophages (MDMs) and microglia as CD45+CD11bhigh and CD45+CD11b low, respectively. B. Spontaneous infiltration of
circulating monocytes to the spinal cord increases after SCIRI. C-F. Quantification of expression of CD86 (C and E), Dectin (D and F) in MDMs and microglia in spinal cord after SCIRI. Cells were pooled from 3 mice per group for a total of 3 separate experiments for each time point. *, significant difference compared to sham group (*, P<0.05; **, P<0.01)