Long-Term Kinetics of Immunologic Components and Neurological Deficits in Rats Following Repetitive Mild Traumatic Brain Injury

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Background: Despite growing awareness of repetitive mild traumatic brain injury (rmTBI), understanding of the involvement of long-term kinetics of immunologic components in the central and peripheral immune system took part remains incomplete. The present study aimed to provide a quantitative assay for certain immune system parameters in rmTBI rats.

Material/Methods: Neurological functions were assessed by modified Neurological Severity Score (mNSS) and Morris Water Maze (MWM), immunologic components from brain and peripheral blood were analyzed by flow cytometry (FCM), and concentrations of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA).

Results: Neurological functions of rmTBI rats were seriously impaired. In the brain, T cells were up-regulated and peaked at week 1. The percentage of CD4<sup>+</sup> T cells decreased from week 1 to week 4, while CD8<sup>+</sup> T cells notably decreased at week 1, then increased until week 4. The infiltration proportion of Treg cells was reduced at week 1 and peaked at week 2. CD86<sup>+</sup>/CD11b<sup>+</sup> M1 peaked at week 4 and CD206<sup>+</sup>/CD11b<sup>+</sup> M2 rose at week 1. IL-6/IL-10 showed a similar pattern, whose rise corresponded to the decrease in TNF-α at week 2 after rmTBI. FCM demonstrated peripheral immune dysfunction after rmTBI.

Conclusions: mNSS and MWM demonstrated neuronal deficits in rmTBI rats, and central and peripheral immune systems were implicated in the pathophysiological processes of rmTBI. Long-term immune response may play dual roles in injury and repair of rmTBI.

MeSH Keywords: Brain Injuries • Immunity, Cellular • Nervous System Diseases

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Background

TBI, a leading cause of death and disability worldwide [1], presents major social, economic, and health problems globally [2]. TBI is present in 85% of traumatically injured children [3], and occurs mainly in adults aged 15–24 years [4,5].

TBI is classified as primary injury or secondary injury. Primary injury occurs at the moment of trauma, while secondary injury occurs in the minutes to days after trauma, with complicated cellular processes and biochemical cascades [6]. Unfortunately, secondary events (e.g., excessive release of inflammatory factors and glutamate) further deteriorate primary injury-generated damage [7].

Under multiple circumstances, the brain communicates with the immune system in a bidirectional manner [8], and a lymphatic system inside the dural sinuses has been recently described [9]. Despite the recent surge of interest in TBI and its impact on CNS and peripheral immunity [10,11], the changes during the subacute/chronic phase of TBI remain elusive.

The present study focuses on rmTBI-induced changes, including neurological deficits, CD3+/CD8+ cell levels and IL-6/IL-10/TNF-α concentrations, and proposes a potential therapeutic strategy for TBI by targeting the brain or peripheral immune system.

Material and Methods

Animals

We used adult male Sprague-Dawley (SD) rats weighing about 200 g. They were purchased from the Chinese Academy of Military Science (Beijing, China) and housed at the Experimental Animal Laboratories of Tianjin Neurological Institute, with controlled temperature of 25°C, 12-h light/dark cycle, and fed ad libitum. Rats were randomly divided into 4 groups: the sham group and 1/2/4-week post-injury groups, with 6 rats in each group. All procedures were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

rmTBI

Briefly, rats were anesthetized with 10% chloral hydrate (3.5 ml/kg, i.p.), followed by placing on a delicate task wiper (Kimwipe, Kimberly-Clark, Irving, TX). Thereafter, the head was put under a hollow guide tube. An impact was delivered to the dorsal skull by dropping a 54-gram metal bolt from the height of 28 inches with a guide pipe, and head rotational acceleration was generated by the delicate task wiper. Rats in the sham group underwent anesthesia and were not subjected to injury.

mNSS

mNSS, including motor, sensory, reflex, and balance, was assessed as previously reported [12]. Rats were examined at weeks 1, 2, and 4 after rmTBI to display effects at different post-injury time points on neurological function. Neurological function was graded on a scale from 0 to 18, with a score of 0 for normal, and a score of for inability to conduct the test or lack of test reflex. Therefore, the higher score, the more severe the injury. Potential motor impairments were quantified by mNSS at 24 h after rmTBI to ensure relatively comparable injuries in rats.

MWM

Spatial learning and memory were measured by use of the MWM at 2 and 4 weeks after injury, respectively. In brief, a maze (150 cm diameter, 50 cm deep) was filled with water at the temperature of approximately 22°C. A target platform (10 cm diameter) was placed 2 cm below the surface of the water.

Rats were allowed to adapt to the maze for 2 min before training. Training continued for 5 days with 4 trials/day (each for 2 min, with an interval of 15 s) from 4 different random positions in the north, east, southeast, and northwest quadrants of the maze. Rats that failed to find the platform within 2 min were guided by an experimenter and the maximum latency of 120 s was recorded. Latency (time to platform), head angle, swimming speed, and time spent in the goal quadrant were recorded with a tracking software system (Ethovision 3.0, Noldus Information Technology, Wageningen, the Netherlands).

ELISA

For ELISA, traumatic sections were isolated from brains on ice as soon as possible, followed by flash freezing and storage in liquid nitrogen. Concentrations of IL-6, TNF-α, and IL-10 were evaluated by ELISA assay kits (Tianjin Lianxing Biotechnology) according to the manufacturer's instructions.

FCM

For FCM, rats were deeply anesthetized with 10% chloral hydrate at corresponding post-injury time points (1, 2, and 4 weeks).

Brains were homogenized with a 70-μM cell strainer to acquire cell suspension, then centrifugation was carried out and cell pellets were re-suspended in 30% Percoll and 70% Percoll. CNS mononuclear cells were at the mid-layer, which was the interface of 30% and 70% Percoll.
After obtaining peripheral blood samples, cold PBS was immediately perfused transcardially. Peripheral blood mononuclear cells (PBMCs) were separated from fresh blood samples, then subjected to density gradient centrifugation at 300 g for 20 min at room temperature and purification with PBS 3 times. Mononuclear cells were stained with antibodies for CD4-FITC, CD4-PE, CD4-APC, Rat T Lymphocyte Cocktail, CD25-APC, Froxp-PE, CD11b-FITC, CD86-PE, and CD206-APC (BD Biosciences) following the manufacturer’s instructions. Data were obtained using a FACSCalibur (BD Biosciences) device and analyzed with Flow Jo software.

Statistics analysis

All tests are carried out 3 independent times. Data are expressed as mean ± standard deviation (SD) and were analyzed with SPSS 22.0 and GraphPad Prism 5. P values were calculated with the single-factorial analysis of variance (ANOVA) and Student’s t-test. P values <0.05 presented as statistical significance.

Results

Neural deficiency within post-injury 4 weeks

The highest mNSS was indicated in rats at day 1, and was clearly lower at 4 weeks post-injury (Figure 1A). In rmTBI rats, MWM demonstrated that latency increased at 2 weeks (Figure 1B) and latency at 4 weeks were consistent with that at 2 weeks (Figure 1C). There was less time spent in goal quadrant (Figure 1D), no significant difference was observed in swimming velocity among different groups (Figure 1E), and there was higher head angle (Figure 1F) at 14 and 28 days compared with the sham group.

Number of T cells in the brain within 4 weeks post-injury

The time course of immune cell accumulation was analyzed using isolated cells from the cerebral cortex. Analysis on gating strategy of live cells is shown in Figure 2A. The number of T cells was significantly increased at 1 week, but was decreased at 2 and 4 weeks, which were all higher than that in the sham group (Figure 2B, 2C).
Percentages of T cell subsets in the brain within 4 weeks post-injury

As acknowledged, T cell subsets were characterized by cell surface markers, including CD3⁺, CD3⁺CD4⁺, or CD3⁺CD8⁺ [13]. The percentage of CD3⁺CD4⁺ T cells gradually decreased from 1 week to 4 weeks (Figure 3A). CD3⁺CD8⁺ T cells had decreased at 1 week, then rose by 4 weeks (Figure 3B). Corresponding statistical data are shown in Figure 3C, 3D.

Infiltration proportion of Treg cells in the brain within 4 weeks post-injury

CD4, CD2,5 and Foxp3 were markers for Treg cells. The infiltration proportion of Treg cells was clearly reduced at 1 week, then remarkably increased and peaked at 2 weeks, and finally declined by 4 weeks. Figure 4A shows dot plots of isolated live immune cells in the brain, Figure 4B shows representative FCM for Treg cells in injured brain, and Figure 4C shows quantitative data for accumulated Treg cells.

The portion of microglia in the brain within 4 weeks post-injury

Two distinct populations of cells were observed: CD45high/CD11b⁺ cells (macrophages) and CD45low/CD11b⁺ cells (microglia) [14]. Microglia is classified into pro-inflammatory M1 and anti-inflammatory M2, which may contribute to pathophysiology of rmTBI [15]. CD86 (M1 macrophage marker) and CD206 (M2 macrophage marker) [16] were used to test alterations between M1 and M2.
FCM showed that microglia cells account for 90% of the isolated cells (Figure 5A). The portion of CD86+/CD11b+ M1-like microglia had significantly peaked at 4 weeks (Figure 5B), while CD206+/CD11b+ M2-like microglia peaked at 1 week but gradually decreased from 1 week to 4 weeks (Figure 5C). Quantitative data for CD86+/CD11b+ cells are shown in Figure 5D and statistical data for CD206+/CD11b+ cells are shown in Figure 5E.

Concentrations of inflammatory cytokines at 4 weeks post-injury

The IL-6 concentration was obviously increased after rmTBI, peaked at 2 weeks, and had dramatically decrease at 4 weeks (Figure 6A).

The peak of TNF-α concentration was at 1 week, followed by a decrease to the baseline at 2 weeks, which again rose significantly at 4 weeks (Figure 6B).

The dynamic pattern of IL-10 concentration was similar to that of IL-6 (Figure 6C).

T cell number in the peripheral blood within 4 weeks post-injury

The number of T cells increased at 1 week and 2 weeks, followed by a clear decrease at 4 weeks (Figure 7).

Dot plots of isolated immune cells in the peripheral blood are provided in Figure 7A, FCM for CD3+ cells in the peripheral

Figure 3. Changes in T cell subsets in the brain during 4 weeks post-injury. Representative FCM for CD3+CD4+ cells (A) and CD3+CD8+ cells (B) in injured brain after rmTBI. Statistical data for CD3+CD4+ cells (C) and CD3+CD8+ cells (D). * indicates P<0.05, ** indicates P<0.01 when compared CD3+CD4+ cells or CD3+CD8+ cells with cells in the sham group.
blood are shown in Figure 7B, and statistical data for accumulated T cells are provided in Figure 7C.

**Percentages of T cell subsets in the peripheral blood within 4 weeks post-injury**

The percentage of CD3⁺CD4⁺ T cells continuously decreased from 1 week to 4 weeks after rmTBI (Figure 8A), while the percentage of CD3⁺CD8⁺ T cells increased from 1 week to 4 weeks (Figure 8B). Quantitative data for the percentages of T cell subsets are shown in Figure 8C and 8D.

**Treg cell level in the peripheral blood within 4 weeks post-injury**

The level of Treg cells in the peripheral blood had significantly increased by 4 weeks (Figure 9). Dot plots of isolated immune cells in the peripheral blood are shown in Figure 9A), FCM for Treg cells is showed in Figure 9B, and quantitative data for accumulated Treg cells is provided in Figure 9C.

**Discussion**

mNSS and MWM demonstrated serious neuronal deficits of rmTBI rats, and central and peripheral immune systems were implicated in the pathophysiological processes of rmTBI.
In the present study, rmTBI rats showed improved mNSS over time and persistent behavioral deficits were observed. MWM demonstrated serious memory deficits in rmTBI rats: increased latency, bigger rotation angle, slightly changed swimming velocity, and less time spent in the goal quadrant, consistent with results of previous studies [17]. We further investigated whether long-term kinetics of immunologic components in the CNS and peripheral immune system took part in this process.

Ischemia/reperfusion injury models demonstrated deleterious effects of B cells [18]. Conversely, a murine stroke model showed that B cells serve a protective rather than pathogenic effect.

Figure 5. Changes in specific subsets of microglia in the brain during 4 weeks post-injury. Dot plots of isolated specific subsets of microglia in the brain (A). Representative FCM for CD86+/CD11b+ M1-like microglia (B). Representative FCM for CD206+/CD11b+ M2-like microglia (C). Quantitative data for CD86+/CD11b+ cells (D). Statistical data for CD206+/CD11b+ cells (E). * P<0.05, rmTBI group vs. sham group.
**Figure 6.** Inflammatory cytokine levels during 4 weeks post-injury. Expression levels of inflammatory cytokines IL-6 (A), TNF-α (B), and IL-10 (C) during 4 weeks post-injury. * P<0.05, ** P<0.01, ### P<0.001, rmTBI group vs. sham group.

**Figure 7.** T cell number in the peripheral blood during 4 weeks post-injury. Dot plots of isolated immune cells in the peripheral blood (A). Representative FCM for CD3+ cells in the peripheral blood (B). Statistical data for accumulated T cells in the peripheral blood (C). * P<0.05 at 1 week, ** P<0.01 at 2 and 4 weeks compared with sham.
The role of B cells in brain injury is multifaceted; therefore, we did not investigate B cells in the present study. T cells have been shown to infiltrate the brain to protect neurons from injury after nerve crushing or contusion [20]. We discovered that the number of T cells was significantly increased at 1 week, decreased at 2 weeks, and slightly elevated at 4 weeks. Further studies are needed to confirm our hypothesis that increased T cells in acute rmTBI are protective, while in chronic rmTBI they are destructive.

T cells are classified as CD3\(^+\), CD4\(^+\), and CD8\(^+\) cells [13]. CD3\(^+\) cells reflect cellular immune response to exogenous antigens. Regarded as T helper/inducible cells, CD4\(^+\) cells assist B cells in promoting the function of diverse antibodies and IL-2. Defined as T suppression/cytotoxic cells, CD8\(^+\) cells help B cells to restrain various antibodies and exert a cytotoxic effect on MHC-I antigen of target cells. We found that the CD4\(^+\) cell percentage first increased and then decreased, while the percentage of CD8\(^+\) cells first decreased and then increased, which resulted in an obvious vibration of CD4/CD8 ratio.

Treg cells ensure correct measure of adaptive immune responses [21]. Unfortunately, controversy on the role of Treg cells in CNS injury exists. Reduction of Treg cells in the brain was observed 1 week after rmTBI, followed by a strong increase at 2 weeks and a steady decline at 4 weeks in the present study, suggesting that Treg cells might play distinct roles at the beginning vs. the end of rmTBI. We propose that Treg cell deactivation at the initial stage permit autoimmune T cells to promote

**Figure 8.** T cell subsets in the peripheral blood during 4 weeks post-injury. Representative FCM for CD3\(^+\)CD4\(^+\) cells in the peripheral blood (A). Representative FCM data for CD3\(^+\)CD8\(^+\) cells in the peripheral blood (B). Quantitative data for the percentages of T cell subsets (C, D). * P<0.05, ** P<0.01 compared with sham.
a beneficial autoimmune response, which is later terminated by the restoration of their suppressive activity. Therefore, understanding these mechanisms have been the focus of clinicians and researchers for the purpose of maintaining appropriate Treg cell levels and providing effective therapeutic intervention for rmTBI.

Microglia, resident immune cells in the brain, maintain a chemical/physical environment for proper CNS functioning [22]. M1 cells are competent in promoting inflammation and cytotoxicity [23], while M2 cells are characterized by pro-angiogenesis, anti-inflammation, and tissue remodeling [24]. M1-like microglia are closely involved in neurobehavioral sequelae after rmTBI [25]. A recent study reported that nuclear factor erythroid 2-related factor 2 (Nrf2) mediates neuroprotection in TBI, partially by inactivating microglia [26]. We carried out FCM to show that microglia strongly infiltrated the brain in the entire phase, with an initial peak of CD206⁺/CD11b⁺ M2-like microglia at 1 week and a secondary peak of CD86⁺/CD11b⁺ M1-like microglia at 4 weeks, which is consistent with results of a previous study [27]. Our findings corroborate that M1 microglia are associated with cognitive function.

Extended microglia processes determine the production of cytokines [28]. We performed ELISA in the present study and found that IL-6 and IL-10 showed similar patterns in the brain and that the increase in IL-6 and IL-10 corresponded with the decrease in TNF-α at 2 weeks, which is consistent with a previous study [29]. Thus, our study corroborated that there were cytokine cascades following rmTBI. Taken together, the central

Figure 9. Treg cells in the peripheral blood during 4 weeks post-injury. Dot plots of isolated immune cells in the peripheral blood (A). Representative FCM for Treg cells (CD4⁺, CD25⁺, and Foxp3⁺) in the peripheral blood (B). Quantitative data for accumulated Treg cells in the peripheral blood (C). * P<0.05 at 4 weeks compared with no injury.
immune system is implicated in pathophysiological processes of rmTBI, so we further studied whether the peripheral immune system was involved.

T cell frequency in the peripheral blood increased until 2 weeks, then had sharply decreased by 4 weeks, which was lower than baseline. Taken together, our data indicate immunosuppression during the chronic phase of rmTBI, which is consistent with a previous view that T cells in the peripheral blood respond to activated microglia in transiently injured brains [30]. Disruptions in percent of peripheral T cell subsets were found, CD4⁺ T cells sustained to a sharp down-regulation from 1 week to 4 weeks after rmTBI, and CD8⁺ T cells increased from 1 week to 4 weeks. Elevated Treg cells within 4 weeks of injury were discovered. Thus, our findings prove that rmTBI affects the circulating peripheral immune system, as elaborated by a previous study [31].

In summary, further studies with time-frames longer than 4 weeks after rmTBI should be carried out to ascertain whether other changes in immunologic components and neurological deficits exist and when they return to baseline. Although multiple studies have reported the effect of TBI on peripheral immunity [32], few reported immunity to CNS [33], with 1 study providing a mechanism [34]. There is a need for further research to elucidate the detailed mechanisms underlying TBI-induced changes in the immunity dialogue between the CNS and peripheral immune system, requiring the development of novel immune-based therapeutic strategies with the principle of adjusting the well-balanced interplay between protective and detrimental responses.

Conclusions

mNSS and MWM verified neuronal deficits in rmTBI rats, showing that the central and peripheral immune systems participate in pathophysiological processes in rmTBI.

Conflict of interest

The authors declare that they have no conflicts of interest.

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