Published in final edited form as:

J Trauma Acute Care Surg. 2013 November ; 75(5): 780–788. doi:10.1097/TA.0b013e318299616a.

Traumatic Brain Injury-Induced Alterations in Peripheral Immunity

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Abstract

Background—The complex alterations that occur in peripheral immunity after traumatic brain injury (TBI) have been poorly characterized to date. The purpose of this study was to determine the temporal changes in the peripheral immune response after TBI in a murine model of closed head injury.

Methods—C57Bl/6 mice underwent closed head injury via a weight drop technique ($n=5$) vs. sham injury ($n=3$) per time point. Blood, spleen, and thymus were collected and immune phenotype, cytokine expression, and antibody production were determined via flow cytometry and multiplex immunoassays at 1, 3, 7, 14, 30, and 60 days post injury.

Results—TBI results in acute and chronic changes in both the innate and adaptive immune response. TBI resulted in a striking loss of thymocytes as early as 3 days post-injury (2.1×10^7 TBI vs. 5.6×10^7 sham, $p=0.001$). Similarly, blood monocyte counts were markedly diminished as early as 24 hours post-TBI (372/dl TBI vs. 1359/dl sham, $p=0.002$) and remained suppressed throughout the first month post-injury. At 60 days post-injury monocytes were polarized towards an anti-inflammatory (M2) phenotype. TBI also resulted in diminished IL-12 expression from day 14 post-injury throughout the remainder of the observation period.

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Presentation at Meetings: This study will be presented at the 2013 AAST Annual Meeting per the requirements of the 2012 AAST Research and Education Foundation Scholarship.

Authorship

S.J.S. and H.P. contributed to the concept and design of the study. S.J.S., D.M.T., and R.S. acquired the data. S.J.S., D.M.T., R.S., and H.P. performed data analysis. S.J.S., D.M.T., and H.P. interpreted the data. S.J.S., D.M.T., and H.P. drafted the manuscript.

Disclosure: This study was supported by a grant from the American Association for the Surgery of Trauma Research and Education Foundation to S.J.S. and by grants from NIH grants (AR050250, AR054796, AI092490, and HL108795) and Funds provided by Solovy/Arthritis Research Society Professor to H.P.

Conclusion—TBI results in temporal changes in both the peripheral and central immune systems culminating in an overall immune suppressed phenotype and anti-inflammatory milieu.

Level of Evidence—Level 1 prospective randomized controlled animal experiment.

Keywords

traumatic brain injury; immunosuppression; anti-inflammatory; peripheral immunity; innate immunity

Background

Traumatic brain injury (TBI) afflicts nearly 2 million people each year, of whom over 300,000 require in-patient hospitalization and over 50,000 die (1). The leading cause of mortality after TBI is infection and its resultant multisystem organ failure (2). However, the mechanisms responsible for TBI-induced immune dysfunction have been poorly studied and are only partially understood (3). Other models of central nervous system injury, such as stroke or spinal cord injury, have studied limited aspects of peripheral immune function (4–7). To the best of our knowledge, to date, no study has characterized a murine model of TBI to understand the the relationship between TBI and peripheral immune function.

The ability to characterize TBI-induced immune suppression has important clinical implications for the treatment of brain-injured patients. Previously reported hypo-immune consequences of CNS injury include reduced leukocyte counts in peripheral blood, loss of delayed-type hypersensitivity, decreased MHC II expression, impaired phagocytic activity of macrophages, and a shift from a Th1 phenotype to a Th2 phenotype (8–14). In the present study, we determined the time-dependent changes in peripheral immunity after traumatic brain injury. Using a closed head injury model induced by a weight drop device (15), we assessed the immune phenotype, cytokine expression, and immunoglobulin expression in brain injured mice over time. The data contained herein provides an analysis of the peripheral immune response after TBI in vivo using a murine model of TBI. Taken together, our data suggest that TBI induces temporal alterations in peripheral immunity leading towards an overall immunosuppressive environment, thereby offering a potential means to identify those brain-injured hosts at greatest risk for infection.

Materials and Methods

Animals

C57BL/6 (B6) male mice (12–14 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice were maintained in a pathogen free barrier facility within Northwestern University, s Center for Comparative Medicine. Animals were treated and cared for in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. The Northwestern University Institutional Animal Care and Use Committee approved the experimental protocol.

Traumatic Brain Injury (TBI)

Mice were anesthetized with 50 mg/kg Ketamine (Ketaset, Fort Dodge, IA) and 2.5 mg/kg Xylazine (Anased, Shenandoah IA) via intraperitoneal injection. After anesthesia induction, mice underwent a longitudinal 1cm scalp incision revealing both the sagittal and coronal sutures of the skull. TBI mice had the target area on the skull overlying the left parietal lobe marked— 2 mm left of the sagittal suture and 2 mm rostral to the coronal suture. Next, the TBI mice were positioned within the TBI weight drop apparatus and a 333gm impacting rod with a 3mm diameter tip was dropped from a height of 3 cm imparting 0.06 J to the closed skull (Fig. 1A). Immediately following injury, scalps were sealed with VetBond (3M). Sham injured mice received the anesthetic and a scalp incision only. All mice were recovered in separate cages over a warming pad. Severity of injury was determined via administration of the Neurological Severity Score (NSS) as described by Flierl *et al* (Fig. 1B–D) (15). Mice were euthanized at 1, 3, 7, 14, 30, and 60 days post injury. Blood, spleen, and thymus were harvested.

Cell isolation

Blood samples were divided into three aliquots: 40 μ L for a Complete Blood Count (CBC), 600–800 μ L for serum extraction and 100 μ L for cell surface staining. CBCs were performed on a Hemavet 950 (Drew Scientific, Oxford, CT). The remaining red cells were lysed with BD FACS Lysis Buffer (BD Falcon, Bedford, MA). The remaining white cells were washed and stained for flow cytometric analysis as below. Spleens and thymi were cut into fine pieces and digested with 10 mg of Collagenase D (Roche Diagnostics, Indianapolis, IN) and 1 mg DNase I (Roche Diagnostics). The spleen and thymus homogenates were then strained through a 40 μ m Nylon cell strainer (BD Falcon) and washed. Red blood cells within the spleen samples were lysed with BD Pharm Lyse Buffer (BD Biosciences, Sparks, MD). Total splenocyte and thymocyte counts were obtained with a Countess automated cell counter (Invitrogen, Carlsbad, CA). Nonviable cells were excluded using trypan blue staining (Life Technologies, Carlsbad, CA).

Flow Cytometry

Peripheral blood was incubated with Fc block followed by incubation with rat anti-mouse FITC CD4 (BD Biosciences), PE CD115 (eBiosciences, San Diego, CA), PerCP Cy5.5 Ly6C (eBiosciences), APC CD62L (BD Bioscience), eFluor 450 CD11b (eBiosciences), PE Texas Red B220 (BD Biosciences), APC Cy7 CD8 (BD Biosciences), PE Cy7 Ly6G (Biolegend, San Diego, CA), or mouse anti-mouse Alexa 700 NK1.1 (BD Biosciences). Red blood cells were lysed, and the remaining leukocytes were washed and fixed in paraformaldehyde.

Approximately 3 million splenocytes or thymocytes were separated for surface staining and resuspended in PBS. Cells were labeled as previously described (16). In brief, viable cells were labeled with LIVE/DEAD® Fixable Aqua Dead Cell Stain (Invitrogen). Samples were treated with Fc Block to prevent nonspecific binding (BD Biosciences). Cells were washed with PBS and labeled with rat anti-mouse FITC CD4 (BD Biosciences), PE CD8 (BD Biosciences), PerCP Cy5.5 Ly6G (BD Biosciences), APC F4/80 (eBiosciences), eFluor 450 CD11b (eBiosciences), PE Texas Red B220 (BD Biosciences), Alexa Fluor 700 NK1.1 (BD

Biosciences), APC Cy7 Ly6C (BD Biosciences), PerCP Cy5.5 CD45 (BD Biosciences), APC CD44 (BD Biosciences), eFluor 450 CD8 (eBiosciences), PE Texas Red CD4 (Invitrogen), Alexa Fluor 700 CD11b (BD Biosciences), Alexa Fluor 700 Gr-1 (BD Biosciences), Alexa Fluor 700 Nk1.1 (BD Biosciences), APC Cy7 CD25 (BD Biosciences), PE Cy7 CD19 (BD Biosciences), or hamster anti-mouse PE Cy7 CD11c (BD Biosciences) and PE Cy7 (BD Biosciences). After incubation all cells were washed with PBS and fixed with paraformaldehyde.

Flow cytometry data were acquired on a BD LSR II cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with 405 nm, 488 nm, 561 nm, and 640 nm excitation lasers as previously described (16). All data collection was performed using BD FACS Diva software (BD Biosciences) and data analyses were performed using FlowJo software (Tree Star, Ashland, OR). Fluorescence minus one (FMO) controls were used for gating analysis to distinguish positively from negatively staining cell populations. Compensation was performed using single color controls prepared from BD Comp Beads (BD Biosciences) for cell surface staining or Arc Beads (Invitrogen) for Aqua live/dead discrimination. Compensation matrices were calculated and applied using FlowJo software (Tree Star). Bioexponential transformation was adjusted manually when necessary.

Luminex Assays

Whole blood was centrifuged at 15,000 RPM for 10 minutes at 4°C. The supernatant (serum) was collected in aliquots and stored at -80°C. The cytokine levels of G-CSF/CSF-3, GM-CSF/CSF-2, GROalpha/KC/CINC1/CXCL1, IFN-gamma, IL-10/CSIF, IL-12/IL-23p40, IL12p70, IL-13, IL-15, IL-17A/CTLA-8, IL-17F/ML-1, IL-1alpha/IL-1F1, IL-1beta/IL-1F2, IL-2, IL-23, IL-3, IL-4, IL-6, IL-7, IP-10/CXCL10, LIX/GCP2/CXCL5, M-CSF/CSF-1, MCP-1/JE/CCL2, MCP-3/MARC/CCL7, MIP-1alpha/CCL3, MIP-2/GRO beta/CINC3/CXCL2, RANKL/TNFSF11, RANTES/CCL5, and TNF-alpha were measured using the Procarta Cytokine Immunoassay Kit (Affymetrix, Santa Clara, CA) following the manufacturer's instructions. Immunoglobulin (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM) quantities were also measured using the Procarta Immunoglobulin Assay Kit (Affymetrix) according to the manufacturer's instructions.

Statistics

The data are reported as the mean $\pm$ standard error of the mean (SEM) using the statistical software program PRISM (GraphPad Software, Inc., La Jolla, CA). Data involving two groups was analyzed by a Student t test, whereas data involving more than 2 groups was analyzed using 1-way ANOVA with Tukey multiple comparison posttest. Significance was accepted at $p < 0.05$. $N=5$ brain-injured mice and 3 sham-injured mice per time point. There was no significant difference between sham-injured animals at any time point and they are illustrated as a single column to simplify representation of the data.

Results

TBI Decreases Mean Hemoglobin Concentration and Platelet Counts

Alterations in hematopoiesis after TBI were assessed with complete blood cell counts in TBI and sham-injured mice at the time of euthanasia. No difference in white blood cell or red blood cell counts was observed (Fig. 2A–B). However, additional parameters such as the mean corpuscular hemoglobin, platelet count, and mean platelet volume (MCH, PLT, MPV) demonstrated a significant decrease in TBI mice as compared to sham-injured mice at 1 month post-injury (Fig. 2C–D)—MCH ($14.5 \text{ pg/cell} \pm 0.24$ vs. $15.4 \text{ pg/cell} \pm 0.14$; $p=0.004$), PLT ($953 \text{ k/vl} \pm 85 \text{ k/vl}$ vs. $1369 \text{ k/vl} \pm 109 \text{ k}$; $p=0.03$), MPV ($5.26 \text{ fL} \pm 0.12 \text{ fL}$ vs. $5.77 \text{ fL} \pm 0.09 \text{ fL}$; $p=0.01$).

TBI Results in a Bimodal Loss of Thymocytes

Alterations in thymocyte counts after TBI and sham injury were assessed with flow cytometry at 1, 3, 7, 14, 30, and 60 days post injury. TBI resulted in a striking loss of thymocytes over a bimodal distribution—characterized by a marked 2.7 fold reduction in total thymocyte counts at 3 days post-injury ($p=0.001$). This was followed by thymic reconstitution at 14 days and a second, 2.2 fold, reduction between 30 and 60 days post-injury (Fig. 3A, $p=0.0004$). This bimodal loss of total thymocytes was further delineated into mature T cell populations or early lineage-negative populations. All thymic subpopulations followed the same bimodal pattern of cell loss: Initial cell loss at 3 days post-TBI, recovery back to sham levels between 7 and 14 days post-TBI, and a second depression between 30 and 60 days post-TBI. Further breakdown of the lineage negative population into $\text{CD}25^{+/-}$ and $\text{CD}44^{+/-}$ demonstrated identical results (data not shown). Further evidence of thymic cell loss was detected via diminished thymic mass in TBI mice at 3 days and 60 days post-injury (Fig. 3B–C).

TBI Results in a Late Depletion of Splenic Macrophages

Alteration in splenocyte counts after TBI and sham injury were assessed via flowcytometry at 1, 3, 7, 14, 30, and 60 days post-injury. No difference in splenic B cell, T cell (data not shown), NK cell (data not shown), or neutrophil counts was noted at any time point post-TBI (Fig. 4A–B). However, there was a significant 2.3-fold decrease ($p=0.02$) in red pulp macrophage (F4/80^{+}) counts as well as a 1.3-fold ($p=0.04$) reduction in tissue macrophage ($\text{Ly}6\text{c}^{\text{low}}$) counts between 30 and 60 days post-TBI (Fig. 4C–D). This late depression in red pulp macrophage counts corresponds to the decrease seen in MCH and PLT counts.

TBI Results in Reduced Blood Monocyte Counts and M2 Polarization

Alterations in peripheral blood mononuclear cell counts after TBI and sham-injury were assessed via flow cytometry for B cells, T cells, NK cells, neutrophils, and monocytes at 1, 3, 7, 14, 30, and 60 days post-injury. Our gating strategy is shown in figure 5A. There were no differences in B cell, T cell, NK cell, or neutrophil counts after TBI (data not shown). However, the monocyte population exhibited an early and sustained suppression after TBI (Fig. 5B). At 24 hours post-injury there was a 3.7-fold reduction ($p=0.002$) in blood monocyte counts in TBI mice compared to sham. Although there was a partial recovery

shortly after the initial loss, the monocyte population remained suppressed throughout the remainder of the first month post-injury (2.3-fold reduction ($p=0.01$) at 7 days, 1.8-fold reduction ($p=0.01$) at 14 days, and 2.0 fold reduction ($p=0.04$) at 30 days.

Monocyte populations, which are delineated into classical (inflammatory) or non-classical (patrolling) monocytes, were determined post-TBI. Since recent evidence suggests that these subpopulations ultimately differentiate into M1 and M2 macrophages (17–19), monocyte populations will be designated as inflammatory M1 ($CD115^{+}CD62L^{++}$) monocytes and anti-inflammatory M2 ($CD115^{+}Ly6C^{-}CD62L^{-}$) monocytes. The inflammatory M1 population remained suppressed throughout the post-injury period whereas the anti-inflammatory M2 population increased in total cell count. The elevated numbers of M2 monocytes accounted for the apparent rebound in overall monocyte counts at 60 days post injury (Fig. 5C, $p<0.04$). These data suggest that TBI induces a significant polarization of blood monocytes towards an anti-inflammatory phenotype by 60 days post injury.

TBI Results in Decreased Inflammatory Cytokine Expression

Alterations in cytokine and antibody expression in TBI and sham injured mice were analyzed via Luminex based assays at 1, 3, 7, 14, 30, and 60 days post-injury. IL-12 expression was decreased by 4.6 fold at 14 days post injury as compared to sham ($p=0.025$). This depression persisted throughout the duration of the 60-day study period (Fig. 6A). INF- γ was also decreased at the same time points although it did not reach statistical significance (Fig. 6B, $p=0.08$). CCL5, the chemotactic cytokine responsible for recruiting leukocytes to inflammatory sites, was decreased 3.8-fold ($p=0.024$) compared to sham injury at 14 and 30 days post injury (Fig 6C). IL-6 was elevated immediately post injury, but returned to sham levels after 72 hours (Fig. 6D). No significant difference was noted in any of the other cytokines or antibodies assayed (data not shown). Taken together, the results of this study are the first to fully characterize the temporal changes in peripheral immunity after TBI and point towards the generation of an overall anti-inflammatory milieu in the post-TBI host.

Discussion

There is large body of evidence demonstrating that infection is a highly relevant and lethal complication of TBI (1, 3, 20–25). This predisposition to infection is driven by a poorly understood, brain-specific, suppression of the immune system. Although prior models of trauma have shown variable immunosuppressive effects, recent data suggest that different injury patterns create a tissue-specific environment thereby priming the immune response in an injury and tissue-specific manner (26, 27). The current study utilizes a clinically relevant murine model of TBI to specifically characterize the temporal changes in peripheral immunity following traumatic brain injury (7, 28). The capacity to characterize the peripheral immune response to brain injury has potentially important clinical implications—the ability to prospectively identify patients with suspicious alterations in immune phenotype may allow for preventative therapy to be instituted prior to the onset of secondary infection (29). The present study did not directly examine infection or other markers associated with infection. Rather, we showed a time-dependent pattern of immune suppression in both the peripheral and central immune compartments following

experimentally induced brain injury. This is important as it implies the host may be susceptible secondary infection at different time points throughout the course of brain injury. For example, Prass and colleagues (6) demonstrated that stroke results in a rapid loss and deactivation of T cells as early as 3 days poststroke. Subsequently, they showed that these mice developed spontaneous bacteremia and pneumonia within 3 days. The same group later identified that prophylactic antibacterial treatment during this immunosuppressed period led to improved clinical outcomes (29, 30).

In our study, we demonstrated a striking temporal loss of cells from both the peripheral and central immune system after TBI. Specifically, we observed an early and sustained loss of circulating blood monocytes—an essential component of the innate response to pathogens. It is interesting to speculate on both the cause and effect of this marked loss of circulating monocytes. Jin and colleagues (7) demonstrated a rapid infiltration of macrophages into the injured brain in a controlled cortical impact model of brain injury. This may explain the initial decrease in monocytes at 24 hours post injury, but the authors also noted a rapid clearance of brain macrophages after the initial insult. Our data show that the loss of monocytes is sustained throughout the first two months post-injury. There is mounting evidence that these cells play an important role in the both the direction and activation of the adaptive immune response (31). In fact, in a recent study the monocytic arm of the innate immune system was implicated in the suppression of the adaptive immune system resulting in T cell exhaustion—a process resulting in protracted immunosuppression (32). Our data may be consistent with this observation. When further delineated into inflammatory M1 vs. anti-inflammatory M2 subpopulations, we observed that the M1 population remained suppressed even at 2 months post injury whereas the M2 population increased in total cell counts. These data suggest that TBI results in a shift towards on overall anti-inflammatory immune phenotype. Additional evidence supporting this conclusion is the markedly decreased level of IL-12, INF- γ , and CCL5 in the serum of brain-injured mice. IL-12 is a potent inducer of the Th1 response and plays a key role in the induction and maintenance of inflammatory responses. The striking reduction in IL-12 observed in our study may be one of the early mechanisms by which brain injury produces a sustained hypo-immune phenotype.

In addition to the marked decreases in peripheral and central monocytic populations, we observed loss from the adaptive immune system as well. Specifically, we noted a significant bimodal loss of thymocytes. Similar to monocytic cell types, it is also known that T cells infiltrate acutely injured brain tissue. However, the observed degree of cell loss in the thymus cannot be explained solely by increased trafficking of T cells into the injured brain. Evidence from the stroke literature suggests brain injury leads to an apoptotic loss of lymphocytes from the central immune compartment (6). It is speculated that this apoptotic loss is driven by the hyperadrenergic response occurring after an acute insult to the brain (33). Data supporting this concept demonstrates that adrenergic blockade attenuates the degree of lymphocyte apoptosis seen after stroke (6, 34). Additionally, several observational studies in brain injured human patients have shown improved outcomes in patients receiving adrenergic blockade(35–37). Taken collectively, these data suggest a contributing role of the sympathetic nervous system in the TBI-induced apoptosis of T cells. Moreover, evolving evidence suggests uptake of these apoptotic cells by monocytes/macrophages further impairs

immune function by shifting the overall inflammatory milieu towards an anti-inflammatory M2 phenotype (38, 39).

In conclusion, our experimental model of TBI resulted in a rapid and profound, time dependent, depletion of cells from the peripheral and central immune system. The mechanism behind this loss remains elusive, but likely consists of a combination of recruitment to areas of injury, apoptotic cell loss, and an overall shift towards an anti-inflammatory milieu. Future studies are needed to determine the mechanism behind the immune cell loss, whether this loss can be prevented, and whether infectious morbidity and mortality after brain injury can be improved.

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Figure 1. Clinically relevant murine model of traumatic brain injury

A, The impact point is identified 2 mm left of the sagittal suture and 2 mm rostral to the coronal suture and a 333gm impacting rod imparts 0.06 J to the impact area. B–C, Injured and sham mice undergo a closed circle exit, a series of beam walks, and balancing assessment. D, A score is generated on the modified neurological severity scoring (NSS) system (*, $p=0.0001$).

Figure 2. TBI results in late evidence of bone marrow suppression

A–B, No significant difference in WBC or RBC counts was observed at any time point post injury as compared to sham-injured mice. C–D, Mean corpuscular hemoglobin concentration (MCH) and overall platelet count (PLT) were all depressed at 30 days status post TBI as compared to sham-injured mice (* $p < 0.03$).

Figure 3. TBI results in a bimodal loss of thymocytes

A, Bimodal loss of thymocytes at 3 days and 60 days post injury (*, $p < 0.001$). B–C, Gross examination of thymi reveals a 1.7 fold reduction in thymic mass at 3 days post TBI and a 1.8 fold reduction in thymic mass at 60 days post TBI ($p < 0.001$).

Figure 4. TBI-induces a late depression of macrophages in the spleen

A–B, There was no statistical difference in lymphocyte or neutrophil counts in the spleen at any time point post injury. *C*, Significant depression in red pulp (F4/80⁺) macrophage counts at 30 and 60 days post injury (*, $p=0.02$). *D*, Significant depression in differentiated tissue (Ly6c^{low}) macrophage counts at 60 days post injury (*, $p=0.04$).

Figure 5. TBI results in monocyte depression and M2 Polarization

A, Flow cytometry gating strategy for identification of monocytes (B220⁻ CD4⁻ CD8⁻ Nk1.1⁻ Ly6G⁻ CD11b⁺ CD115⁺). M1 and M2 subsets are identified as Ly6C⁺ CD62L⁺ or Ly6C⁻ CD62L⁻, respectively. B, Brain-injured mice demonstrate a 3.7 fold reduction in blood monocyte counts over the first month post injury (* $p=0.002$). C, Reconstitution of blood monocyte counts at 60 days post injury is predominated by anti-inflammatory M2 monocytes (*, $p<0.04$).

Figure 6. TBI results in a late depression of pro-inflammatory cytokines

A, IL-12 expression is markedly decreased 14 days post TBI and remained suppressed throughout the 60-day observation period (*, $p < 0.025$). B, INF- γ expression is induced by IL-12 and is similarly decreased at 14 days, although nonsignificant (#, $p = 0.08$). C, Expression of CCL5 is significantly reduced at 14 days post TBI and remained suppressed throughout the first month post injury (*, $p < 0.025$). D, IL-6 expression is elevated after injury returns to baseline expression by 14 days post TBI (*, $p < 0.004$).