Journal of Neuroimmunology 337 (2019) 577073

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Innate immune suppression after traumatic brain injury and hemorrhage in a juvenile rat model of polytrauma

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ARTICLE INFO

Keywords:
Traumatic brain injury
Hemorrhage
Rat
Juvenile
Immune suppression
Tumor necrosis factor alpha
Memory

ABSTRACT

Traumatic injury in children is known to cause immune suppression. Polytrauma involving a traumatic brain injury (TBI) may increase this degree of immune suppression, which increases the risk of developing nosocomial infections, potentially causing secondary brain injury and worsening patient outcomes. Despite the high prevalence of polytrauma with TBI in children, mechanisms of immune suppression following such injuries remain poorly understood. Here, we used a combined animal injury model of TBI and hemorrhage to assess immune function after polytrauma. Pre-pubescent rats were injured using a prefrontal controlled cortical impact method and a controlled hemorrhage by femoral arteriotomy. Immune function was measured by whole blood ex-vivo tumor necrosis factor alpha production capacity following incubation with lipopolysaccharide, measuring the percentage of monocytes by flow cytometry, and by examining concentrations of plasma cytokines. The degree of brain injury was sufficient to produce deficits in spatial memory testing (Barnes maze). Both hemorrhage and TBI with hemorrhage (combined injury) reduced several of the measured plasma cytokines, as compared with TBI alone. The combined injury correlated with reduced concentration of monocytes and reduced tumor necrosis factor alpha production capacity at post-injury day 1. These results demonstrate that this animal model can be used to study post-injury immune suppression.

1. Introduction

Traumatic brain injury (TBI) in children remains one of the most common causes of pediatric morbidity and mortality (Thurman, 2016). Despite comprehensive efforts, there is no therapeutic agent for the treatment of TBI, and improvements in outcome have largely been attributed to care following injury (Elf et al., 2002).

One known complication of severe TBI is nosocomial infection, and the incidence may be as high as 50% with mortality as high as 37% (Cardozo Junior and Silva, 2014; Dziedzic et al., 2004). Even in the absence of mortality, infection can lead to secondary brain injury and poor outcomes (Bronchard et al., 2004). TBI has been shown to lead to a post-traumatic immunosuppressive response in both clinical studies (Wolach et al., 2001) and in animal models of injury (Vermeij et al., 2013). When severe, this is termed immunoparalysis, a state in which the responsiveness of the innate immune system to a new challenge is significantly blunted (Muszynski et al., 2014). We have previously shown that early, severe reduction in innate immune function, within 48 h of injury, is associated with increased risks for nosocomial infection in critically injured children (Muszynski, Nofziger, 2014). Experimental evidence suggests that TBI combined with systemic injury may be particularly immunosuppressive (Shein et al., 2014), but this has not been evaluated in a juvenile animal model.

Despite the high prevalence of TBI and recognition of the importance of infectious complications in this population, mechanisms of TBI-induced immunosuppression in children remain uncertain. It is also unclear how systemic injuries accompanying TBI may modulate immune function following injury. Examining a model recapitulating TBI plus a systemic injury is clinically important as up to 50% of severe TBIs may include a significant extra-cranial injury (van Leeuwen et al.,

Abbreviations: TBI, traumatic brain injury; H, hemorrhage; EBV, estimated blood volume; TNFα, Tumor Necrosis Factor alpha; LPS, lipopolysaccharide; MHC, major histocompatibility complex; IL, interleukin; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; SSC, side scatter; FSC, forward scatter; FSC-A, forward scatter area; FSC-H, forward scatter height

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https://doi.org/10.1016/j.jneuroim.2019.577073
Received 6 May 2019; Received in revised form 16 September 2019; Accepted 22 September 2019

2012). Pre-clinical research assessing systemic immune changes following TBI has been limited to adult (Ritzel et al., 2018; Schwulst et al., 2013) or adolescent animal models (Chang et al., 2019). Here, we describe a juvenile rodent model to evaluate immunosuppression and cognition following TBI with the addition of systemic injury. Hemorrhage (either external or internal) is a common secondary insult that is strongly associated with mortality after TBI (Chesnut et al., 1993; Manley et al., 2001). Therefore, we utilized hemorrhage as a systemic injury to model pediatric polytrauma. To our knowledge, no prior studies have examined the effect of hemorrhage on immune function after TBI in a pre-clinical juvenile model. We hypothesized that circulating leukocyte numbers and cytokine production capacity would be reduced early after TBI in a juvenile rate model, and that the addition of hemorrhage to the model would amplify that immunosuppression.

2. Materials and methods

2.1. Animal care

All experiments were performed in accordance with National Institutes of Health Guidelines, and protocols were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital. Juvenile Sprague Dawley rats (postnatal day 28; Charles River, Wilmington, MA) were used in all experiments and housed in separate cages with ad libitum access to water and chow. At this age, the rat equates roughly to a preadolescent youth (Sengupta, 2013). For all experiments, treatment groups consisted of at least 6 animals per group. For all operative procedures, rats were anesthetized using a mixture of isoflurane and oxygen. The isoflurane mixture was 5% for induction and 2–3% during the procedure for maintenance. Operative procedures required 30 min of anesthetic time, and this was kept consistent across the treatment groups. For the experiments, four treatment groups were used: 1) sham surgery animals (craniectomy without TBI and leg dissection without hemorrhage), 2) hemorrhage-alone (craniectomy without TBI and a femoral arteriotomy to induce hemorrhage), 3) TBI-alone (induced TBI and leg dissection without hemorrhage), and 4) TBI plus hemorrhage (induced TBI and a femoral arteriotomy to induce hemorrhage).

2.2. Controlled cortical impact

For the experimentally induced TBI, rats were placed in the prone position in a stereotactic device (Kopf, Tujunga, CA) with the head in a horizontal position. Standard sterile technique was used. A midline incision was made, and the scalp was retracted to expose the cranium. A circular bilateral craniectomy was made at midline (+2 mm to bregma). For TBI, injury was made to the frontal cortex using an Impact One Instrument (Leica, Buffalo Grove, IL). The impactor tip was 3 mm in diameter and delivered the injury at a velocity of 4 m/s with a dwell time of 500 m/s and a depth of 3 mm. For animals in the sham and hemorrhage-alone groups, animals were anesthetized using isoflurane for an equal duration, positioned in a similar fashion, and incision and craniectomy were made performed without performance of the induced brain injury.

2.3. Fixed-volume hemorrhage

Estimated blood volume (EBV) for each rat was calculated based on body weight (70 mL/kg) (Diehl et al., 2001). Hemorrhagic injury was performed following TBI by repositioning the animal from the prone position to the supine position. As with TBI, the procedure was performed under anesthesia, and standard sterile technique was used. A femoral arteriotomy was performed using a 25 gauge needle with removal of 25% of EBV. Shed blood was collected with sterile gauze pads, and shed blood volume calculated by weight (1 mL/g). For animals in the sham and TBI-alone groups, animals were anesthetized using isoflurane for an equal duration, and a similar leg incision with identification of the femoral artery was made without performance of the arteriotomy.

2.4. Measurement of whole blood Tumor Necrosis Factor alpha (TNFα) production capacity

For all immune function tests, whole blood was collected at pre-injury day 1 and on post-injury days 1 and 3 via saphenous vein puncture. Innate immune function was assessed by whole blood ex vivo lipopolysaccharide (LPS)-induced TNFα production capacity (Thurm and Halsey, 2005), with reduction in the TNFα response being characteristic of innate immune suppression. Whole blood samples were collected from the saphenous vein in heparin-lined Microtainer green top tubes (BD Biosciences, Franklin Lakes, NJ), and 50 μL of whole blood was added to 500 μL of prepared RPMI media containing 500 ng/mL *E. coli* LPS (055:B5, Sigma, St. Louis, MO) for 18 h at 37 °C. After 18 h, the supernatant was collected and stored at -80 °C for batch analysis of TNFα. TNFα was quantified in the stimulated supernatants via a Rat TNFα Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

2.5. Flow cytometry

Heparin-lined tubes (BD Biosciences) were used for collection, and red blood cells were lysed using lysing solution (BD Biosciences). Fc receptors were blocked with anti-CD32 antibody (BD Biosciences) for 10 min at room temperature. Cells were washed and incubated with the following antibodies for 15 min at room temperature: CD3 (PE, BioLegend, San Diego, CA), CD45RA (PE/Cy7, BioLegend), His48 (FITC, BD Biosciences), major histocompatibility complex (MHC)II RT1B (BV421, BD Biosciences), CD4 (BV786, BD Biosciences), and CD11b/c (APC, BioLegend). Cells were washed and re-suspended in stain buffer (BD Biosciences) for analysis. Compensation was set using single-stain control beads (Thermo Fisher, Waltham, MA). Live cells were discriminated using side scatter (SSC) and forward scatter (FSC) gating properties and singlets were isolated by forward scatter area (FSC-A) and forward scatter height (FSC-H) properties. To quantify specific cell populations, cells were quantified as a percentage of live cells, and gating was determined based on appropriate isotype stained controls. Data were acquired using a cytometer (LSR II, Becton Dickinson) and analyzed using FlowJo software (Tree Star, Ashland, OR). For additional details on the gating strategy, see Supplementary Fig. 1.

2.6. Measurement of plasma cytokines

Plasma from unstimulated blood samples was collected via cardiac puncture in EDTA-lined Microtainer purple top tubes (BD Biosciences) after centrifugation of whole blood at 2000 x*g* for 15 min. Plasma was stored at -80 °C for subsequent quantification. Plasma cytokines were quantified using the Bio-Plex Rat Cytokine Th1/Th2 12-Plex immunoassay (Bio-Rad, Hercules, CA). Cytokines measured included interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), and TNFα.

2.7. Behavioral studies

A Barnes maze was used to assess hippocampal-dependent spatial memory (Barnes, 1979). Prior to surgery, naïve rats were acclimated to the Barnes maze. This included two trials: (1) rats were guided to the escape hole and were allowed 2 min in the escape box, and (2) rats were guided to an incorrect hole then guided to the escape hole for 2 min under a bright light as an aversive stimulus. Following the acclimation date, the position of the escape hole was shifted 180° to its testing

location. The acquisition phase consisted of four trials for four consecutive days. Rats were placed in the center of the maze under a semi-opaque container. Recording began when the container was lifted. Each trial lasted 180 s or until the rat entered the escape box. If the rat did not enter the escape box, then it was guided to the hole. Both acclimation and acquisition occurred in the days immediately prior to injury. To evaluate memory recall, rats were subjected to a probe trial 2 days after injury. The probe trial consisted of one 60 s trial with the escape hole replaced with a dummy hole. Rats were tracked and recorded using a CCD camera, and video was analyzed using Smart V3.0 tracking software (Panlab Harvard Apparatus, Holliston Massachusetts) to determine the escape latency.

2.8. Motor function

The Rotarod performance test was used to assess motor function after injury with a previously established protocol (Geddes et al., 2014) using an accelerating Economex Rotarod (Columbus Instruments, Columbus, OH). Rats were acclimated to the Rotarod 3 days prior to injury. Training consisted of 3 trials: the Rotarod was set at 10 rpm for the first 2 trials. During the last trial, the Rotarod velocity increased from 10 to 40 rpm. Testing occurred 3 days post injury and consisted of 3 trials with the Rotarod gradually increasing from 4 to 40 rpm over 5 min with a 5 min break between each trial. The average time spent on the rod before falling is reported.

2.9. Statistical analysis

Data were analyzed by two-way ANOVA using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA). Population variance was assessed using the Brown-Forsythe test and Bartlett's test. Individual data points more than two standard deviations above or below the mean were counted as outliers and excluded in analysis. Samples from two animals met this criteria and were removed: one from the hemorrhage-alone group and one from the TBI-alone group. The threshold for significance was set at $p < .05$. Main effects of experimental treatment or treatment interaction effects were evaluated by an F-protected post hoc test using Fisher's Least-Significant Difference procedure of GraphPad Prism. All data are expressed as treatment means ± standard error of the mean. As this study outlines novel model development, power analysis was not able to be performed in the study design.

3. Results

3.1. Whole blood TNFα production capacity

The results of the whole blood TNFα production capacity following ex-vivo LPS-stimulation are shown in Fig. 1. No differences between the four treatment groups were noted at the pre-injury time point. On post-injury day 1, there was a significant decrease in LPS-induced TNFα production capacity measured in the TBI plus hemorrhage (TBI/H) animals, as compared with sham animals. No intergroup differences were noted on post-injury day 3.

3.2. Flow cytometry

Prior to injury, all immune cell populations (relative to total leukocytes) were similar across treatment groups. Moreover, no significant differences in lymphocyte populations were observed post injury (Supplementary Fig. 1). At post-injury day 1, animals who received TBI/H showed a significant decrease in circulating monocytes (relative to total leukocytes), as compared with sham animals (Fig. 2a and b). At post-injury day 1, there was also a significant decrease in $MHCII^{hi}$ and $MHCII^{lo}$ expressing monocytes (relative to total leukocytes), reflecting reduced antigen presenting capacity in animals that received TBI/H, as compared with sham animals (Fig. 2c and d). Additionally, there was a main effect of hemorrhage to increase granulocytes at post-injury day 1 in animals that received hemorrhage-alone (H) or TBI/H (Fig. 2a and b). No intergroup differences were noted on post-injury day 3.

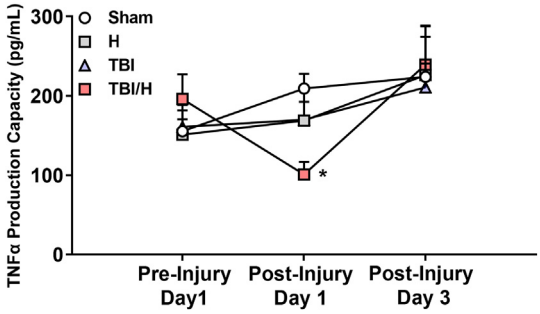

Fig. 1. Measurement of whole blood ex vivo LPS-induced TNFα production capacity at 1 day prior to injury and at post-injury day 1. There was a main effect of TBI on LPS-induced TNFα production capacity at post-injury day 1. The symbol * indicates $p < .05$, as compared to sham ($n = 8$ per group). Abbreviations include: hemorrhage-alone (H), traumatic brain injury (TBI), and combined traumatic brain injury and hemorrhage (TBI/H).

3.3. Plasma cytokine levels

Unstimulated plasma levels of several pro-inflammatory cytokines were lower in hemorrhage-alone (H) and TBI/H groups, as compared with TBI alone, including IL-1β, IL-6, GM-CSF, IFNγ and TNFα (Fig. 3). Levels of these cytokines were not significantly different between H, TBI/H, and sham injured animals. Animals who received TBI/H showed significantly lower plasma levels of IL-4, as compared with animals treated with TBI alone. There were no significant differences in plasma cytokine levels between the four treatment groups for IL-1α, IL-2, IL-5, IL-10, IL-12, or IL-13.

3.4. Behavioral testing

Following a 1 day period of acclimation (pre-injury day 5), rats were trained on the Barnes maze over four days (period of acquisition), and animals were not tested on the day of surgery or post-injury day 1 (Fig. 4a). All animals showed similar patterns of decreasing escape latency over the period of acquisition (Fig. 4b). On post-injury day 2, a probe test was performed, and escape latency was recorded for all treatment groups. No significant differences were noted between sham animals and H animals (Fig. 4c). Both TBI-alone and TBI/H animals demonstrated a significant 200% increase in escape latency, as compared with sham animals (Fig. 4c).

Reduced motor activity following surgery is a potential confounder for Barnes maze performance. To assess motor coordination, rats were tested using the Rotarod performance test on post-injury day 3. Rotarod testing showed no significant difference between the four treatment groups (Fig. 4d).

4. Discussion

Our results describe the successful development of a pre-clinical model of pediatric polytrauma-induced innate immune suppression. We demonstrate a transient but significant reduction in innate immune function early after a combined injury with TBI and hemorrhage using a juvenile rat model that can be used to test interventions designed to prevent or reverse immune suppression in this setting. While similar results were observed in adult animal models (Schwulst, Trahanas, 2013, Shein, Shellington, 2014), this is the first observation of a post-injury immunosuppressive effect from combined injury in a juvenile animal model.

Epidemiological research has shown that children are more likely

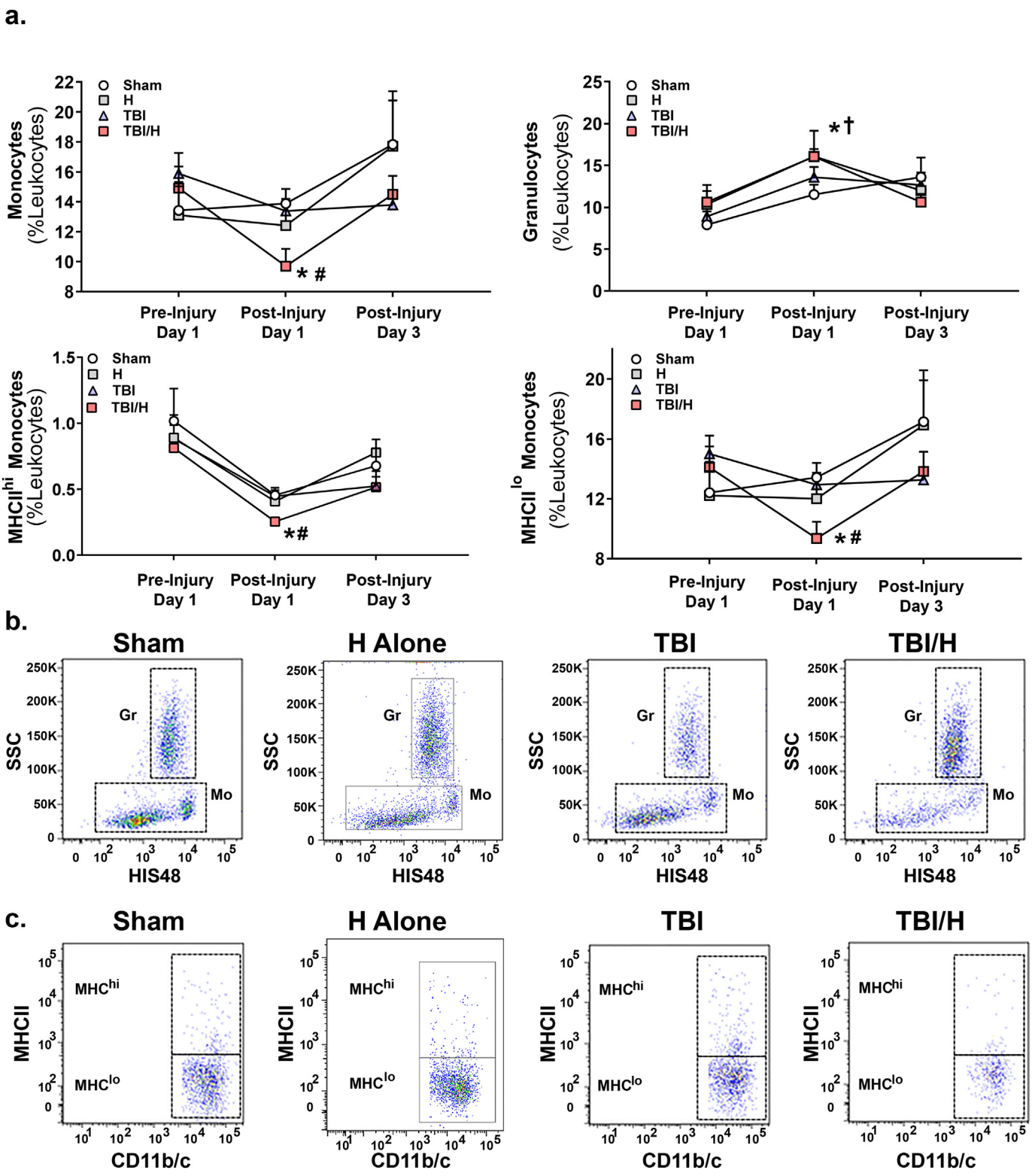

Fig. 2. Measurement of circulating myeloid cells using flow cytometry. Myeloid cells were gated by CD11b/c (supplemental Fig. 1). All measurements are given as percentage of total leukocytes. (a) Monocytes, MHCII^{lo} monocytes, MHCII^{hi} monocytes, and granulocytes were compared between the four treatment groups at 1 day pre-injury and at post-injury day 1. At post-injury day 1, there was a main effect of hemorrhage (H) to reduce monocytes, MHCII^{lo} monocytes, and MHCII^{hi} monocytes. Post hoc analysis revealed that TBI/H significantly reduced total monocytes, MHCII^{lo} monocytes, and MHCII^{hi} monocytes compared with sham. At post-injury day 1, there was a main effect of hemorrhage-alone to increase granulocytes. Post hoc analysis revealed that granulocytes were increased in hemorrhage-alone and TBI plus hemorrhage, as compared with sham. (b) Representative flow Bi-variate dot plots of His48 vs SSC labeling from CD11b/c$^+$ cells at 1 day post-injury. (c) Representative flow Bi-variate dot plots of MHCII and CD11b/c labeling on monocytes at 1 day post-injury. The symbol # indicates $p < .05$, as compared with TBI/H vs TBI; * indicates $p < .05$, as compared with TBI/H vs sham; and † indicates $p < .05$, as compared with hemorrhage-alone vs sham ($n \geq 6$ per group). Abbreviations include: hemorrhage-alone (H), traumatic brain injury (TBI), and combined traumatic brain injury and hemorrhage (TBI/H).

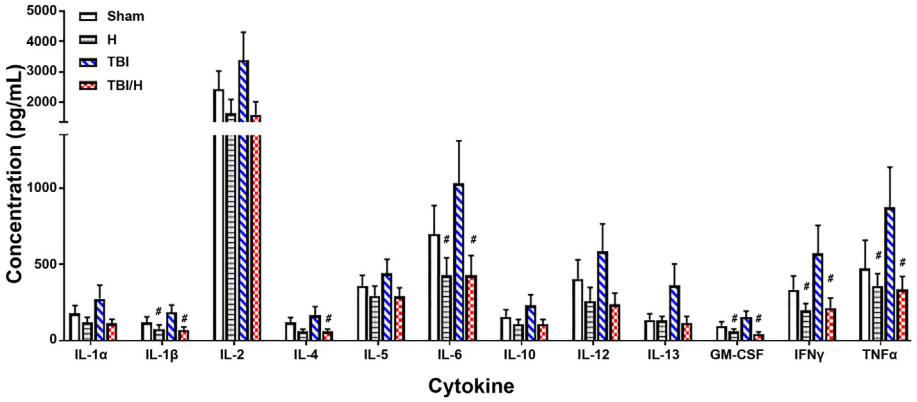

Fig. 3. Measurement of plasma cytokine concentration at post-injury day 1. Plasma cytokines were measured using Bio-Plex ELISA immunoassay. There was a main effect of TBI/H and hemorrhage-alone to significantly reduce plasma levels of IL-1β, IL-6, GM-CSF, IFNγ and TNFα, as compared with TBI. There was a main effect of TBI/H to significantly reduce plasma levels of IL-4, as compared with TBI. The symbol # indicates $P < .05$, as compared with TBI alone ($n \geq 7$ per group). Abbreviations include: hemorrhage-alone (H), traumatic brain injury (TBI), and combined traumatic brain injury and hemorrhage (TBI/H).

than adults to have cranial involvement in a traumatic injury but despite this, relatively little work has been done to create a juvenile animal model of polytrauma with TBI (Wyen et al., 2010). For example, juvenile piglets have been used in a combined injury model, however, the primary focus was on resuscitation techniques rather than behavioral outcomes or systemic immune responses (Fritz et al., 2005; Glass et al., 2001). We and others have shown that immune function is impaired following brain or spinal injury in adults and children (Dziedzic, Slowik, 2004, Muszynski, Nofziger, 2014, Riegger et al., 2009). Expression of the antigen-presenting molecule human leukocyte antigen (HLA)-DR is known to be reduced on monocytes from critically injured adults, with severe reduction in monocyte HLA-DR expression being associated with adverse clinical outcomes (Cheron et al., 2010; Ditschkowski et al., 1999; Flohe et al., 1999; Gouel-Cheron et al., 2012; Kampalath et al., 2003). Wang et al. reported increased monocyte apoptosis at 24 h post-injury in blood samples taken from adult TBI patients, and the degree of monocyte apoptosis correlated with poor outcome (Wang et al., 2014). Muszynski et al. (Muszynski, Nofziger, 2014) reported marked reduction in the ex vivo TNFα response in critically injured children using blood samples obtained within the first 48 h of injury. This was strongly associated with the subsequent development of nosocomial infection, with severe reduction in the TNFα response and severe TBI both being independent predictors of nosocomial infection risk. However, the immunologic impact of concurrent extra-cranial injury along with TBI was not evaluated in that study (Muszynski, Nofziger, 2014).

Mechanisms underlying impaired immune function following trauma are poorly understood in adults (Corps et al., 2015) and are unknown in children. These likely include, in the case of TBI, a combination of circulating and neurally-mediated factors that promote immune suppression (Meisel et al., 2005). The concurrent presence of hemorrhagic shock, as a surrogate for polytrauma, has been shown to promote an immunosuppressive systemic cytokine profile in an adult murine model of TBI (Shein, Shellington, 2014), though leukocyte function was not assessed in that study. The development of a juvenile animal model of post-injury immune suppression is essential for the study of mechanisms of disease as well as the development of novel immunomodulatory therapies. The latter is particularly urgent, given that evidence suggests that critical illness- and injury-induced innate immune suppression may be reversible in vivo with immunostimulants such as recombinant IFNγ or GM-CSF (Docke et al., 1997; Hall et al., 2011; Livingston et al., 1994; Meisel et al., 2009). The relationships between the systemic immune effects of immunostimulatory therapies, modulation of immune response within the central nervous system, and neurodevelopmental outcomes are unknown and merit investigation in pre-clinical models such the one we describe here.

Our behavioral studies following injury included both Barnes Maze and Rotarod performance testing. Both TBI and TBI/H caused similar cognitive deficits revealed in the post-injury Barnes Maze probe trials. These results indicate that the induced brain injury leads to a reproducible neurological change which is neither exacerbated by the hemorrhage nor influenced by the degree of systemic immune suppression. No differences between the post-injury Rotarod testing were noted, suggesting that the Barnes Maze results are due to deficits in memory rather than deficits in motor function. Given the location of our injury (prefrontal cortex), we would not expect to see a motor deficit. One concern in using femoral arteriotomy to induce hemorrhage in the combination injury was the possibility that either the lower limb dissection for arterial exposure or arteriotomy itself would influence behavioral test performance, but our results suggest otherwise. Our combined injury model utilizes a TBI and hemorrhage; however, several other models of combined injury exist including TBI and a femoral fracture (Probst et al., 2012), TBI and a tibial fracture (Shultz et al., 2015), or TBI plus injection of a toxin to induce peripheral myopathy (Sun et al., 2018). We chose the TBI plus hemorrhage model as hemorrhage (internal or external) is commonly associated with TBI and appears to have a clear impact on clinical outcome (McHugh et al., 2007). Additionally, in our hands, the systemic injury delivered (25%

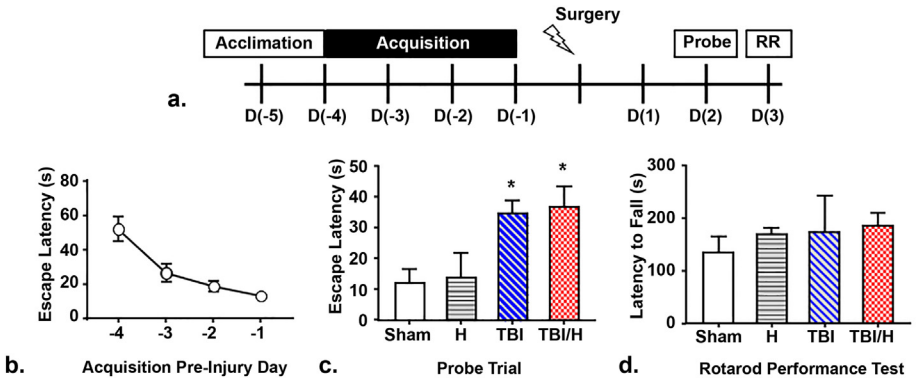

Fig. 4. Behavioral testing. The protocol for behavioral testing is shown (a). The mean escape latencies during the four day acquisition period are shown (b). A probe test was performed two days after surgery, and mean escape latencies for the four treatment groups are shown (c). The latency to fall from the Rotarod (RR) was also tested (d). The symbol * indicates $p < .05$, as compared to sham ($n \geq 7$ per group). Abbreviations include: hemorrhage-alone (H), traumatic brain injury (TBI), and combined traumatic brain injury and hemorrhage (TBI/H).

EBV hemorrhage) is reproducible and does not appear to independently influence Barnes maze testing results. Going forward, the TBI and TBI/H models can be used in experiments designed to manipulate the immune response, and behavioral testing can be used to determine if such treatments positively or negatively influence post-injury cognition. Behavioral testing was performed and reported as this type of combined injury model is rarely used in adult animals and even more rarely in juvenile animals. The emphasis of the injury design was to perform a reproducible injury that would be robust enough to be clinically relevant but not so severe as to be lethal.

To our knowledge, this is the first publication to demonstrate an early decrease in TNFα production capacity following TBI/H in juvenile animals. These animals also showed reduced MHC class II expression on monocytes, consistent with the reduction in HLA-DR expression seen in critically injured patients. Levels of pro-inflammatory cytokines in the plasma were lower in the TBI/H group, as compared to TBI alone, though were not different between sham, hemorrhage, and TBI/H groups. Our findings are in agreement with the work of Shein et al. (Shein, Shellington, 2014), done in an adult murine model, though we did not observe elevations in the plasma levels of the anti-inflammatory cytokine IL-10 in our model. It is unclear if this is related to species differences or other elements of the model, but the use of the rat allowed for successful surgical manipulation at a younger age than would be possible using a murine model. Comparing the plasma cytokine levels in the four treatment groups, cytokine levels were highest (on post-injury day 1) in the isolated TBI group, further highlighting the disconnect between plasma cytokine levels and systemic immune function. That is, the plasma cytokine data did not match what was seen when we examined TNFα response to LPS. It should be noted that other research utilizing a combined injury model has shown an increased release of systemic cytokines (Weckbach et al., 2012) or no effect on neuroinflammation (Sun, Brady, 2018), as compared to isolated TBI. It has been shown that elevations in circulating cytokines can be seen with systemic immune cell hyporesponsiveness (Leijte et al., 2018; Timmermans et al., 2015). As such, measuring circulating levels of cytokines without measuring immune cell function may fail to identify systemic immune hyporesponsiveness, which is why we chose to measure ex-vivo TNF α response to LPS.

There were several limitations to this research. As volatile anesthetic agents can influence immune function (Stollings et al., 2016), all animals (sham and injury) were operated on and anesthetized in a similar fashion to eliminate this effect as variable; however, future studies may include a naive group (that receives no sham surgery or anesthetic agent) to quantify the effect of surgery/anesthesia on immune function. Additionally, the immune suppression seen in our model was transient, with immune recovery typically occurring by post-injury day 3, and the data presented in this manuscript, which is our initial characterization of this model, did not evaluate whether the combined injury would lead to diminished immune response capable of resulting in increased infection risk. Monocytes were measured as a percentage of leukocytes, so a change may also be due to a shift in leukocyte composition. While there was variability in the basal levels of cytokines (e.g. plasma levels of TNFα were reduced in both the TBI-alone and TBI/H groups), cytokine levels alone may not be sufficient to measure a reduced immune response, so immune competency was measured by TNFα response to ex-vivo LPS stimulation. Although TNFα response is a commonly used assay (Dillingh et al., 2014) to examine immunologic hyporesponsiveness, it is not typically used in the clinical setting. However, the degree of decrease in TNFα response seen in the presented model (approximately 50%) has been shown to be strongly associated with nosocomial infection risk in trauma patients in the pediatric ICU (Muszynski, Nofziger, 2014).

All of the animals survived the experimental intervention with minimal post-injury support. Accordingly, the model is not immediately generalizable to critically injured child who require treatment in an intensive care unit and may have more prolonged immune suppression. The fact that the data were collected using a juvenile animal model that was designed to reflect a pediatric injury is, in our view, a strength of the work but it limits is applicability to the study of traumatized adults. In addition, the focus of the current work is on innate immune function. It is likely, based on other pre-clinical models of TBI (Ritzel, Doran, 2018) and stroke (Prass et al., 2003), that adaptive immune function may also be reduced after acute brain injury. This is also under investigation in our laboratory.

In summary, our results demonstrate a significant reduction in innate immune function characterized by reduced TNFα production capacity, reduced monocyte antigen presenting capacity, a relative reduction in circulating monocytes (relative to total leukocytes), and lower levels of plasma pro-inflammatory cytokines in a juvenile rat model of TBI with hemorrhage. We believe that this model will be useful for the investigation of mechanisms of pediatric critical injury-induced immune suppression and for the development and evaluation of immunomodulatory therapies designed to prevent or reverse immune suppression in this setting.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.577073.

Funding source

The Research Institute at Nationwide Children's Hospital.

Financial disclosure statement

The authors have no financial relationships to disclose regarding this manuscript.

Declaration of Competing Interest

The authors have no conflicts of interests to report regarding this manuscript.

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