Systemic inflammation following traumatic injury and its impact on neuroinflammatory gene expression in the rodent brain

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Abstract

Background Trauma can result in systemic inflammation that leads to organ dysfunction, but the impact on the brain, particularly following extracranial insults, has been largely overlooked.

Methods Building upon our prior findings, we aimed to understand the impact of systemic inflammation on neuroinflammatory gene transcripts in eight brain regions in rats exposed to (1) blast overpressure exposure [BOP], (2) cutaneous thermal injury [BU], (3) complex extremity injury, 3 hours (h) of tourniquet-induced ischemia, and hind limb amputation [CEI+tl+HLA], (4) BOP+BU or (5) BOP+CEI and delayed HLA [BOP+CEI+dHLA] at 6, 24, and 168 h post-injury (hpi).

Results Globally, the number and magnitude of differentially expressed genes (DEGs) correlated with injury severity, systemic inflammation markers, and end-organ damage, driven by several chemokines/cytokines (*Csf3, Cxcr2, Il16, and Tgfb2*), neurosteroids/prostaglandins (*Cyp19a1, Ptger2, and Ptger3*), and markers of neurodegeneration (*Gfap, Grin2b, and Homer1*). Regional neuroinflammatory activity was least impacted following BOP. Non-blast trauma (in the BU and CEI+tl+HLA groups) contributed to an earlier, robust and diverse neuroinflammatory response across brain regions (up to 2–50-fold greater than that in the BOP group), while combined trauma (in the BOP+CEI+dHLA group) significantly advanced neuroinflammation in all regions except for the cerebellum. In contrast, BOP+BU resulted in differential activity of several critical neuroinflammatory-neurodegenerative markers compared to BU. t-SNE plots of DEGs demonstrated that the onset, extent, and duration of the inflammatory response are brain region dependent. Regardless of injury type, the thalamus and hypothalamus, which are critical for maintaining homeostasis, had the most DEGs. Our results indicate that neuroinflammation in all groups progressively increased or remained at peak levels over the study duration, while markers of end-organ dysfunction decreased or otherwise resolved.

Conclusions Collectively, these findings emphasize the brain's sensitivity to mediators of systemic inflammation and provide an example of immune-brain crosstalk. Follow-on molecular and behavioral investigations are warranted to understand the short- to long-term pathophysiological consequences on the brain, particularly the mechanism of blood–brain barrier breakdown, immune cell penetration–activation, and microglial activation.

Keywords Blast, Polysystem injury, Differential gene expression, Secondary brain injury, Neuroinflammation

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Introduction

Trauma is a major form of morbidity and mortality in both civilian and military populations [1–4]. Blast-induced trauma caused by exposure to explosive devices—a signature pathology of service members returning from recent wars and civilians of urban terrorist events [5, 6]—can result in primary brain injury and extracranial insults. Explosive blasts can cause injury via multiple mechanisms, including the transmission of shock waves through the body ("primary"), penetrating or blunt impact by projected objects ("secondary"), blast-induced acceleration/impact of the body and/or body parts ("tertiary"), and other mechanisms ("quaternary"), including toxic fumes, muscle crush, and burn injury" [7]. Traumatic brain injury induced by primary blast has been documented in a significant fraction of reported cases of deployment-related short- and long-term mental health sequelae [8, 9]. However, sustaining life-threatening heterogeneous blast-induced polysystem injuries, including comminuted open fractures, neurovascular injury, hemorrhage, soft tissue loss, burns, and traumatic amputations, complicate treatment options [7, 10–13]. Despite their severity, the impact of these polysystem injuries on brain health outcomes is less emphasized in research and clinical practice, underlining a significant gap in our understanding and management of blast-induced trauma.

Several recent studies highlight this issue. Following complex polytraumatic injuries, local tissue damage can induce exaggerated local and peripheral innate immune responses that lead to multifaceted systemic inflammatory response syndrome (SIRS), resulting in subsequent remote multiple organ dysfunction and failure (MOD/MOF) or even death [14–16]. In polytrauma patients that sustain head trauma, ample evidence indicates that pro-inflammatory mediators in the cerebral microenvironment can exit the brain and enter the bloodstream via an altered brain–blood barrier (BBB), thereby affecting remote organs [17–19]. These mediators can alter renal perfusion, increase alveolar hemorrhage, which contributes to acute lung injury, and change cytokine metabolism in the liver, leading to a prolonged inflammatory response [20–23]. Such damage initiates a cycle of reactivated immune mechanisms, both local and systemic, reinforcing the initial immune response. The cyclical processes and fluidity of the immune response underscore the importance of understanding the cellular and molecular mechanisms that drive neuroinflammation and subsequent brain tissue damage caused by various modes of injury.

Prolonged and exaggerated posttraumatic local and systemic immune responses following polytraumatic injury are clearly linked to poor outcomes in critically ill civilians and combat injured service members [24–28]. However, there is a paucity of research evaluating the early local and systemic cellular and molecular signaling pathways involved in the development and sequelae of trauma-induced neuroinflammation (e.g., heightened cellular metabolism, hypoxia, necrosis, inflammation), particularly after extracranial injury. Understanding the pathophysiology and clinical relevance of neurological complications following extracranial trauma is crucial for the proper treatment of patients with severe extremity injuries. Although immune response activation and inflammation are core features of virtually all neurodegenerative diseases [29–31], immunological research following severe traumatic injury from a non-blast insult has traditionally focused on SIRS, sepsis, and peripheral organ damage in the lung, kidney, liver, and gut. However, research has neglected the potential for cerebral injury and its chronic debilitating neurodegenerative outcomes after extracraial injury. Currently, physicians rely on conventional clinical serum biomarkers of end-organ function, such as transaminases (alanine transaminase [ALT], aspartate transaminase [AST]), blood urea nitrogen to creatinine ratio (BUN:Cr), and serum albumin (ALB) concentrations, to evaluate severity and recovery in response to severe trauma [32]. However, this approach often ignores the brain, a critical end-organ, as evidenced by its exclusion from most modern trauma scoring systems and biochemical assays to monitor its dysfunction and recovery.

Using various models of trauma, we recently demonstrated that neuroinflammatory gene signatures in the brain can be altered as a result of remote extracranial trauma [33]. By measuring early neuroinflammatory gene signatures in eight distinct regional brain compartments, we previously found that a complex musculoskeletal extremity injury (femoral fracture, soft-tissue crush over the zone of injury, tourniquet-induced ischemia followed by limb amputation) resulted in a rapid neuroinflammatory response at 6 h post-injury (hpi). Interestingly, whole-body blast exposure alone (~120 kPa), while causing changes in similar brain regions, did so to a much lesser degree (less diffuse) than extremity injury or mild burn trauma. However, blast injury coupled with complex extremity injury involving hind limb amputation significantly augmented the magnitude of the neuroinflammatory response across all structural brain regions [33]. To build upon these compelling findings, in this study, we assessed the extent of neuroinflammation over a seven-day (168 hpi) window and compared gene expression profiles with circulating cytokines/chemokines and classical clinical serum markers of end-organ damage. Additionally, we incorporated a second model of remote trauma, cutaneous thermal burn

injury ± blast overpressure exposure, as burns are a major injury experienced by combat-personnel and can result in a rapid and prolonged systemic inflammatory response [34]. Using different models of injury and severity, we hypothesized that the trajectory, extent, and duration of neuroinflammation post-trauma would correlate with the extent of tissue trauma/injury severity and the reported rise and fall of established clinical mediators of systemic inflammation and end-organ damage during the first 7 days post-trauma [34–37].

Materials and methods

Animals

Adult male Sprague–Dawley rats (478 ± 37.72 g; 11 to 14 weeks old; N = 91) were obtained from Taconic Biosciences (Derwood, MD, USA). Rats were pair-housed and maintained on a 12 h light–dark cycle in a vivarium accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC) with ad libitum access to food and water. All animal experiments involving rats were performed using approved institutional standard guidelines set forth by the Uniformed Service University Institutional Animal Care and Use Committee (IACUC) in compliance with the ARRIVE Guidelines [38] and all applicable federal regulations governing the use and protection of animals in research.

Experimental design and surgical procedures

Rats were randomly assigned to one of five trauma model groups (n = 5–7): (1) whole-body blast overpressure [BOP], (2) cutaneous thermal injury [BU], (3) BOP+BU, (4) complex extremity injury involving femur fracture and muscle crush injury plus tourniquet-induced ischemia and hind limb amputation [CEI+tI+HLA], or (5) BOP+CEI+delayed hind limb amputation [dHLA] (Fig. 1). Each injury occurred in a sequential fashion while under anesthesia, with the exception of BOP, in which the animals were recovered for a period of 30 min to 1 h prior to sedation for follow-on surgical procedures. Age- and sex-matched naïve rats (n = 7) served as controls.

Whole-body blast overpressure (BOP)

Rats received head-on non-shielded whole-body blast overpressure exposure (peak pressure: 125.76 ± 3.72 kPa) using the USUHS Advanced Blast Simulator (ABS) as previously described [33, 36]. Following the induction of anesthesia using isoflurane (4% in O_2 at 1 L/min) and the administration of a subcutaneous (SC) injection of prophylactic analgesia (Ethiqa XR, 0.65 mg/kg; Fidelis, North Brunswick, NJ, USA), a head wrap constructed from heavy duty cardboard and vet wrap was used to protect the mucus membranes prior to securing the rat

forward facing into a mesh pouch approximately 2.9 m distal to the driver membrane. Following the blast, the rats were immediately removed from the blast chamber and allowed to recover in the supine position.

Cutaneous thermal burn injury (BU)

Rats were anesthetized using isoflurane (2–5% in O_2 at 1 L/min), and the dorsum was shaved and depilated using a chemical depilator (Nair; Church & Dwight Co., Inc., Ewing, NJ). Anesthetized rats were placed on their dorsum in a custom polyvinyl chloride (PVC) cradle with an opening of 5 cm × 11.5 cm, corresponding to ~ 10% total body surface area (TBSA), based on the Meeh formula [39]. Immersion in a 96 °C hot water bath was performed for 8 s to achieve a deep partial thickness burn [34, 40]. The average calculated burn TBSA was 7.96 ± 3.33%.

Complex extremity injury (CEI)

Rats received an intraperitoneal (IP) sedative injection containing ketamine (75 mg/kg; Henry Schein Animal Health, Dublin, OH, USA) and xylazine (10 mg/kg; Akorn Animal Health, Lake Forest, IL, USA) and were administered prophylactic analgesia (1.2 mg/kg; Buprenorphine—SR, ZooPharm, Laramie, Wyoming, USA) via subcutaneous injection (SQ). The injury involved a closed femoral fracture and soft tissue crush injury of the right hindlimb quadriceps. Briefly, a custom lateral long bone ballistic system [41] utilizing a drop weight of 581 g from a height of 88 cm was used to create a femoral diaphysis fracture, followed immediately after fracture by a crush injury with a force of 138 kPa (20 psi), which was calibrated against a digital force gauge (Ametek, Largo, FL, USA), for 1 min [35].

Tourniquet-induced ischemia (tI)

Immediately following CEI, the injured hind limb was elevated for ten minutes prior to tourniquet application to reduce venous pooling, reducing the likelihood of thrombotic complications following tourniquet release. Then, a pneumatic tourniquet (UDC1.6™, Hokanson, WA) connected to an aneroid sphygmomanometer (DS400, Hokanson, WA) was applied proximal to the fracture site [35, 42–44] and controlled using a segmental cuff selector enabling rapid and independent inflation and deflation (MV10, Hokanson, WA). The inflation pressures were maintained at 280–300 mmHg for a total duration of 3 h, which exceeded the 220 mmHg pressure required to stop vascular flow in a rat limb [42, 43].

Hind limb amputation (HLA/dHLA)

Either immediately after tI or one-hour post-injury (delayed, d), a transfemoral amputation (HLA) was made through the fracture site, with hemostasis, debridement

Injury Models

CEI+tI+HLA
| Femur fracture/ muscle crush | Tourniquet-induced ischemia | Hind limb amputation |

BOP+CEI+dHLA
| Whole-body blast exposure | Femur fracture/ Muscle crush | Delayed hind limb amputation |

BOP+BU
| Whole-body blast exposure | Cutaneous thermal injury |

BU
| Cutaneous thermal injury |

BOP
| Whole-body blast exposure |

Control

Naïve
| Age-sex matched rats |

Experimental Timeline

Onset of Injury

6h 24h 168h — Tail vein blood sampling

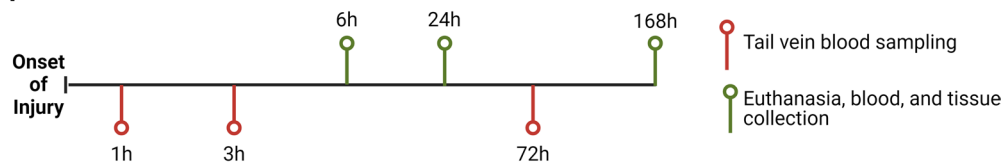1h 3h 72h — Euthanasia, blood, and tissue collection

Fig. 1 Injury models and experimental design schematic. Adult male Sprague-Dawley rats (400–500 g) either received (1) head-on whole-body blast overpressure exposure [120 kPa; BOP], (2) a partial-thickness cutaneous thermal burn [BU], (3) BOP+BU, (4) complex extremity trauma involving closed femoral fracture and soft-tissue crush injury, 3 h of prolonged tourniquet-induced limb ischemia and limb amputation through the zone of injury [CEI+tI+HLA], or (5) BOP+CEI plus delayed hind limb amputation [BOP+CEI+dHLA]. Age- and sex-matched naïve rats (n=7) served as controls. Following injury, whole blood obtained from tail vein venipuncture was obtained from each cohort (1, 3, or 72 hpi) at the timepoint that preceded euthanasia. At 6, 24, and 168 h postinjury (hpi), cohorts of rats (n=5–7 timepoint/injury paradigm) were euthanized, and eight anatomic regions of the brain were dissected and profiled for neuroinflammatory-neurodegeneration gene expression signatures using a custom low-density RT−qPCR microarray. The molecular heterogeneity of the gene profiles of the trauma-induced changes in the brain over time was compared with that of the naïve steady state of control animals. This scientific illustration was created in the *Biorender* web interface

of non-viable tissue, and amputation closure completed by hamstring and quadriceps myoplasty over the residual distal femur. Closure of the residuum was performed using 3–0 vicryl figure-of-eight suture in muscle tissue, continuous 4–0 monocryl subcuticular closure, and 5–0 simple interrupted suture wound closure. Wounds were observed twice daily. No debridement or surgical revisions were necessary for any animal in this study.

Blood collection and brain procurement anddissection
Whole blood (1.5 mL at 1, 3, and 72 hpi) was collected from the lateral tail vein of anesthetized rats using isoflurane (2–5% in O_2 at 1 L/min) or via cardiac puncture (~10 mL) following CO_2 euthanasia at 6, 24, and 168 hpi. Serum was obtained by centrifugation after coagulation and stored at − 80 ℃ for subsequent downstream

analysis. Intact brains were removed, flash frozen (liquid nitrogen; LN_2), and stored at − 80 ℃ prior to dissection. Thin coronal sections (~ 2 mm thick) from the rostral end of each brain specimen were made with a razor blade. Regional brain biopsy samples (1.5 mm) from each hemisphere were collected and pooled from eight anatomical sites (prefrontal cortex [PFCX], striatum [STRIA], neocortex [NCTX], hippocampus [HPCS], amygdala [AMGD], thalamus [THAL], hypothalamus [HYPT], and cerebellum [CEREB]) as previously described [33] and stored at − 80 ℃ until RNA isolation.

Serological analysis
Serum interleukin-beta (IL-β), interleukin-6 (IL-6), chemokine (C-X-C motif) ligand 1 (CXCL1), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant

protein-1 (MCP-1; CCL2) and interferon gamma (IFN-γ) levels were assayed at 1, 3, 6, 24, 72, and 168 hpi using multiplex protein arrays (n = 5–7/group; Meso Scale Diagnostics, Rockville, Maryland, USA). Individual protein levels were indexed to the total measured level of protein in the sample (pg/mL of protein) and compared to naïve baseline samples. The results were acquired and analyzed using Methodical Mind software (version MMPR 1.0.27; Meso Scale Diagnostics). To evaluate clinically relevant serum biomarkers of end-organ damage, the serum levels of AST, ALT, BUN, and ALB, which are indicators of liver injury, and the concentration of creatinine (Cr), which is an indicator of kidney injury and function, were determined at 6, 24, and 168 hpi by using a fully automated clinical chemistry analyzer (Element DC5X Veterinary Chemistry Analyzer; Heska, Loveland, Colorado, USA).

RNA isolation and quantification of gene expression

Snap-frozen biopsy samples (LN_2) were thawed on ice and homogenized in 500 μL Qiazol solution using a ceramic bead tissue homogenization system (VWR, Radnor, PA, USA) and Total mRNA was isolated from each sample using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA) and DNA contamination was removed using the RNase-free DNase Digestion kit (Qiagen, Valencia, CA, USA) solution applied to the miRNeasy column. cDNA was transcribed from brain biopsy specimens using iScript Advanced cDNA Reverse Transcriptase Kit (Bio-Rad, Hercules, CA, USA) [12, 44]. Total mRNA yields > 1 μg and an A260/280 ratio between 1.8 and 2.2 were considered sufficient in quantity and quality for further analysis. qPCR products were assessed by RT–qPCR SYBR-Green fluorescence (SsoAdvanced Universal SYBR Green Supermix; Bio-Rad, Hercules, CA, USA) on custom low-density PCR arrays comprising 86 genes known to be associated with neuroinflammation (cytokines-chemokines and their receptors), neurodegeneration, tissue damage, oxidative stress, apoptosis, and early transcriptional activators, in addition to five (*B2m, Gapdh, Hprt1, Rplp0, Rplp2*) suitable housekeeping genes, using a real-time qPCR machine (QuantStudio 7 Pro, Applied Biosystems, Foster City CA, USA).

RT–qPCR analysis

Analysis of gene expression patterns was carried out using the $2^{-\Delta\Delta Ct}$ method with normalization to a set of internal reference control genes and brain specimens from naïve animals. From the initial dataset, 12 genes were excluded because they had greater than 20% undetermined raw cycle threshold values. The $\Delta C(t)$ value for each gene was calculated by subtracting the $C(t)$ value of that gene from the geometric mean of the three most stable housekeeping genes across all experimental cohorts

(*B2m*, *Gapdh*, and *Hprt1*). Outliers in both injured and naïve data were identified and excluded using the R package *rstatix* [45], wherein any $\Delta C(t)$ values either above $Q3 + 3 \times IQR$ or below $Q1 - 3 \times IQR$ were removed. The $\Delta\Delta C(t)$ values were then calculated for each injured and naïve gene transcript value by subtracting the mean $\Delta C(t)$ value of a gene within that brain region in naïve rats from the individual $\Delta C(t)$ value. These data were converted to fold changes by expressing the values as $2^{-\Delta\Delta C(t)}$. Outliers were removed again at this step as noted above. Any missing naïve and injured transcript expression data (single outliers or exclusions) were imputed using the R package *MissForest* [46]. This final dataset was used for all downstream analyses.

To determine the overall similarity of each sample using the expression of all genes, the data were visualized using t-distributed stochastic neighbor embedding (t-SNE) with the R package *Rtsne* [47]. This allows for visualizing multidimensional data in two-dimensional space with ellipses highlighting a confidence interval of 85% for each brain region. Overall, the expression of each gene in each region and at each time point after injury was visualized by calculating the mean $2^{-\Delta\Delta C(t)}$ value. To determine differences between the treatment timepoints and the naïve expression levels, one-way ANOVA with Tukey's HSD to correct for multiple comparisons was performed for each brain region/injury using the R package *stats* [48]. Trends for each gene in each injury/brain region were investigated by determining first whether the values for each timepoint were significantly different from those of the naïve group (p < 0.05). Visualizations were generated using the R packages *ggplot2* and *ggpubr* [49, 50]. To gain an understanding of cellular functions and biological processes at play involving the 86 genes evaluated, protein–protein interactive (PPI) networks of differentially expressed genes (DEGs) were constructed using the Search Tool for the Retrieval of Interacting Genes (STRING; Version 12.0) [51]. A combined score of > 0.4 (medium confidence) was set as the cut-off criterion. Gene signature hubs were defined via functional enrichment analysis and as having ≥ 3 connections.

Data analysis and statistics

Data analysis and illustration were performed using R and GraphPad Prism (version 10.0.3; GraphPad Software, San Diego, CA, USA). Parametric data are presented as the mean ± standard error of the mean unless otherwise noted and were analyzed using analysis of variance (ANOVA) or Welch's t test. Noncontinuous data, such as gene expression data, were assessed using the Mann–Whitney U test or Kruskal–Wallis one-way analysis. The criterion for statistical significance was set to *p* < 0.05.

Results

Survival and systemic markers of injury

There were no animal deaths in any of the injury models. In general, the extent and magnitude of serum IL-6 correlated with markers of kidney and liver injury and injury model severity (Fig. 2). With the exception of BU, a marked increase in serum IL-1β was detected at 6 hpi in all injury groups post-trauma (Fig. 2a). Compared with those in naïve animals, baseline or near-undetectable levels of proinflammatory cytokines were detected in BOP-treated animals, albeit transient increases in the levels of IL-1β, CXCL1, and IFN-γ were detected at 6 hpi. BU injury resulted in significantly (p < 0.05) elevated and persistent IFN-γ concentrations for the duration of the study, whereas all other systemic markers of inflammation remained at normal/baseline or near-undetectable levels. BOP+BU injury resulted in significant elevations in IFN-γ concentrations early (6 hpi) and late (24–168 hpi), but otherwise resulted in normal/baseline systemic measurements. CEI+tI+HLA injury resulted in significantly elevated levels of IL-1β, IL-6, TNF-α, and

IFN-γ, which peaked at 6 hpi, and increased MCP-1 concentrations from 24 through 168 hpi. BOP+CEI+dHLA resulted in the most severe systemic inflammatory response, in which IL-1β and IL-6 levels peaked at significant concentrations at 6 hpi, TNF-α levels transiently increased throughout the experimental window, CXCL1 levels significantly increased as early as 1 hpi through 24 hpi, and MCP-1 and IFN-γ concentrations remained elevated through 168 hpi.

The levels of serum clinical markers of kidney and liver injury were significantly increased with regard to the levels of ALT, AST, and BUN:Cr and were strongly associated with injury severity (Fig. 2b). The BUN:Cr ratio was heterogeneous among the study groups, with BOP+BU injury resulting in the greatest increase in concentrations at 6 and 168 hpi. All injury models resulted in a transient period of transaminitis between 6 and 24 h; however, the concentrations normalized by 168 hpi. Significant hypoalbuminemia was observed in the polytrauma models for the duration of the study. By 168 h, all groups had renormalized to the naïve group.
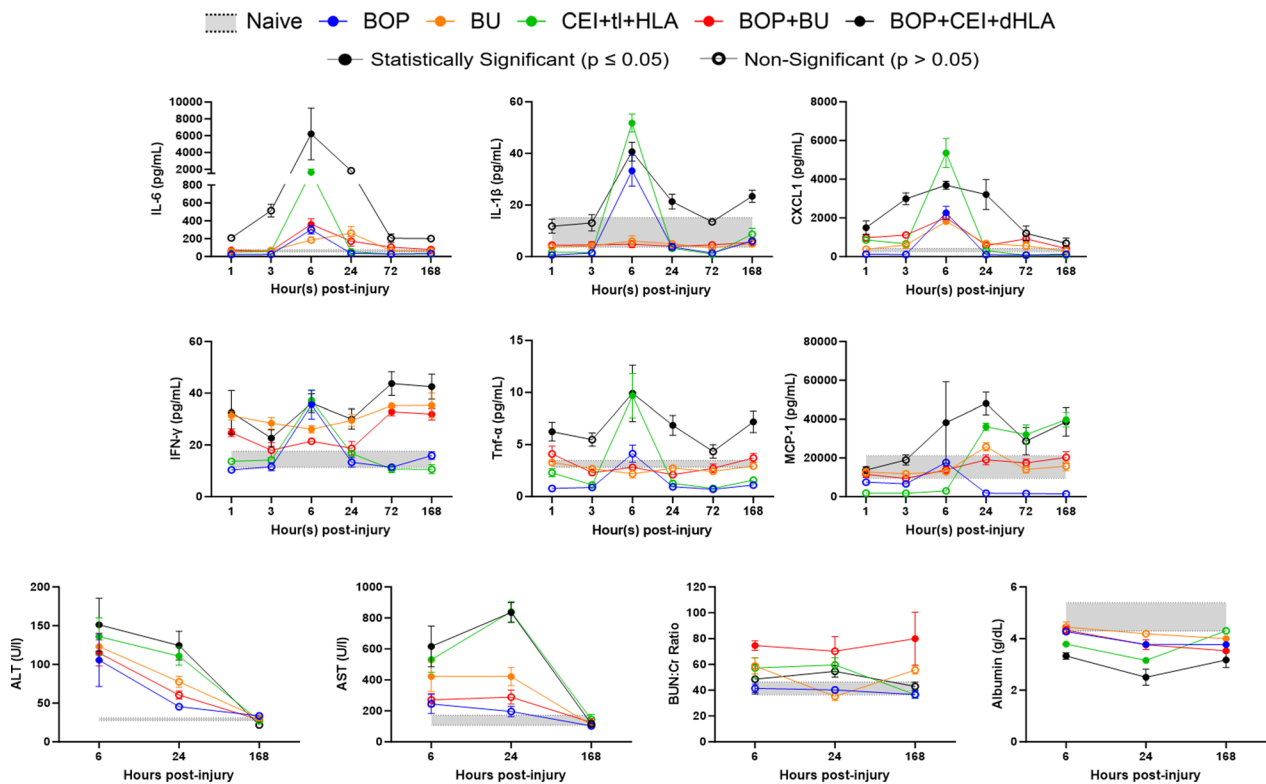
Fig. 2 Clinical indicators of systemic inflammation and remote organ dysfunction. **a** Changes in serum cytokine/chemokine levels were profiled in the naïve and trauma cohorts using a multianalyte MesoScale diagnostic profiling platform. **b** Serum chemical markers of end-organ damage were measured using a Heska Element DCX chemistry analyzer. Serum values from naïve animals (n=7) were used to construct the 95% confidence intervals (gray shading). All the graphs show the mean values (n=6–7 samples/timepoint/injury model) ± SEMs; significant differences (p < .05) in naïve animals are denoted with closed circles, and nonsignificant values are denoted with open circles at the indicated timepoints postinjury. The values were compared using one-way ANOVA and Tukey post-hoc analysis. Abbreviations: alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (Cr)

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Global changes in neuroinflammatory gene signatures

First, we constructed a heatmap to depict the differential fold changes in the expression of all 74 target genes organized into eight different gene classes for each sample consisting of five injury models, eight brain regions, and three timepoints compared to those of naïve controls. As shown in Fig. 3, the differentially expressed genes were organized by brain region over time within each injury pattern, whereas Supplemental Fig. 1 shows the same differential fold changes in expression between the injury model and brain region over time; additionally, region-specific samples obtained from naïve control animals were compared. BOP-alone resulted in a progressive augmentation of neuroinflammatory-neurodegenerative gene signatures that ramped up over the course of the 168 hpi experimental window, whereas all other injury models exhibited an early (within 6 hpi) neuroinflammatory expression response that remained elevated over the study duration. Compared with BOP, non-BOP trauma (BU and CEI+tI+HLA) primarily results in an earlier and more pronounced inflammatory response. Compared with BU alone, CEI+dHLA combined with BOP resulted in the most profound and heightened response, whereas BU combined with BOP resulted in a subtle downregulatory neuroinflammatory response. Globally, prostaglandins and prostaglandin receptors, which suppress acute inflammatory mediators [52], were highly activated (>25-fold) earlier in all models except for BOP. Additionally, early and more prolonged responses of neuronal activity markers (*Gfap*, *Grin2b*, *Homer1*) were detected in more complex, severe injury models (CEI+tI+HLA and BOP+CEI+dHLA) across all brain regions.

To determine trends and classify samples into groups with similar transcriptional profiles independent of the mode of injury, brain region and time, we first applied a t-distributed stochastic neighbor embedding (t-SNE) visualization and analysis technique. This technique was used to analyze relative changes in gene expression ($2^{-\Delta\Delta C(t)}$) measurements of 74 gene transcripts across 660 samples collected from eight anatomically distinct brain regions, three timepoints, and five injury paradigms, including naïve paradigms (Fig. 4). Regardless of injury (mode/severity) or time, the gene expression profiles of the eight brain regions tended to cluster based on brain region, suggesting that anatomically and functionally collocated brain regions presented similar neuroinflammatory expression profiles. In general, the magnitude and profile of the neuroinflammatory response to trauma in the brain are region dependent and independent of the time and injury model. For instance, the neuroinflammatory gene expression signatures of THAL and HYPT, as well as those of PFCX and NCTX, overlapped with one another. The expression profile of the STRIA was the most distinct and was isolated from all other brain regions across every injury pattern and timepoint (Fig. 4).

Compartmentalization differences and key neuroinflammatory gene signatures

To compare region-specific changes in neuroinflammatory gene expression across brain regions over time, we summed the number of significant DEGs above naïve gene expression values at each timepoint for each injury model (Fig. 5 and Supplemental Fig. 2). The BOP alone group had the least number of DEGs, with the STRIA and NCTX groups displaying the greatest number of DEGs early (6 hpi) and the CEREB, HPCS, PFCX, THAL and HYPT groups at later timepoints (24–168 hpi). In contrast, the other four trauma models exhibited significantly more DEGs across all brain regions, reflecting more immediate, diverse, and long-term sensitivity/impact, particularly in the PFCX, NCTX, THAL, and HYPT models in the BU, BOP+BU, CEI+tI+HLA, and BOP+CEI-dHLA injury groups. Among all the genes, *Ccl2*, a potent chemokine associated with microglia and macrophage recruitment [53], was the most commonly downregulated gene across all injury cohorts and in nearly all brain regions (Fig. 3 and Supplemental Fig. 2). Interestingly, *Bdnf*, a protein important for neuronal plasticity [54], was highly upregulated (>50-fold) in all STRIA samples irrespective of injury and timepoint compared to that in naïve controls (Fig. 3 and Supplemental Fig. 1).

Based on the heatmaps (Fig. 3, Supplemental Figs. 1 and 2), we plotted key DEGs at the individual level that remained significantly upregulated throughout the experiment or were highly expressed in various/multiple brain regions at 168 hpi (Fig. 6). The significant

(See figure on next page.)

Fig. 3 Heatmap depicting the expression of 74 neuroinflammatory genes in eight brain regions according to the type of trauma. The values reported were calculated based on the $2^{-\Delta\Delta C(t)}$ method and normalized to the geometric mean of three specific, constitutively active, and stable housekeeping genes (*B2m, Gapdh, Hprt1*) and regional brain biopsy samples from naïve uninjured animals. Blue shading represents a decrease in expression compared to that in the naïve group, red shading represents an increase in expression compared to that in the naïve group, and white shading represents nonsignificant changes (− 2 to 2 fold-change). The color intensity correlates with the magnitude of gene expression relative to that in naïve, uninjured rats

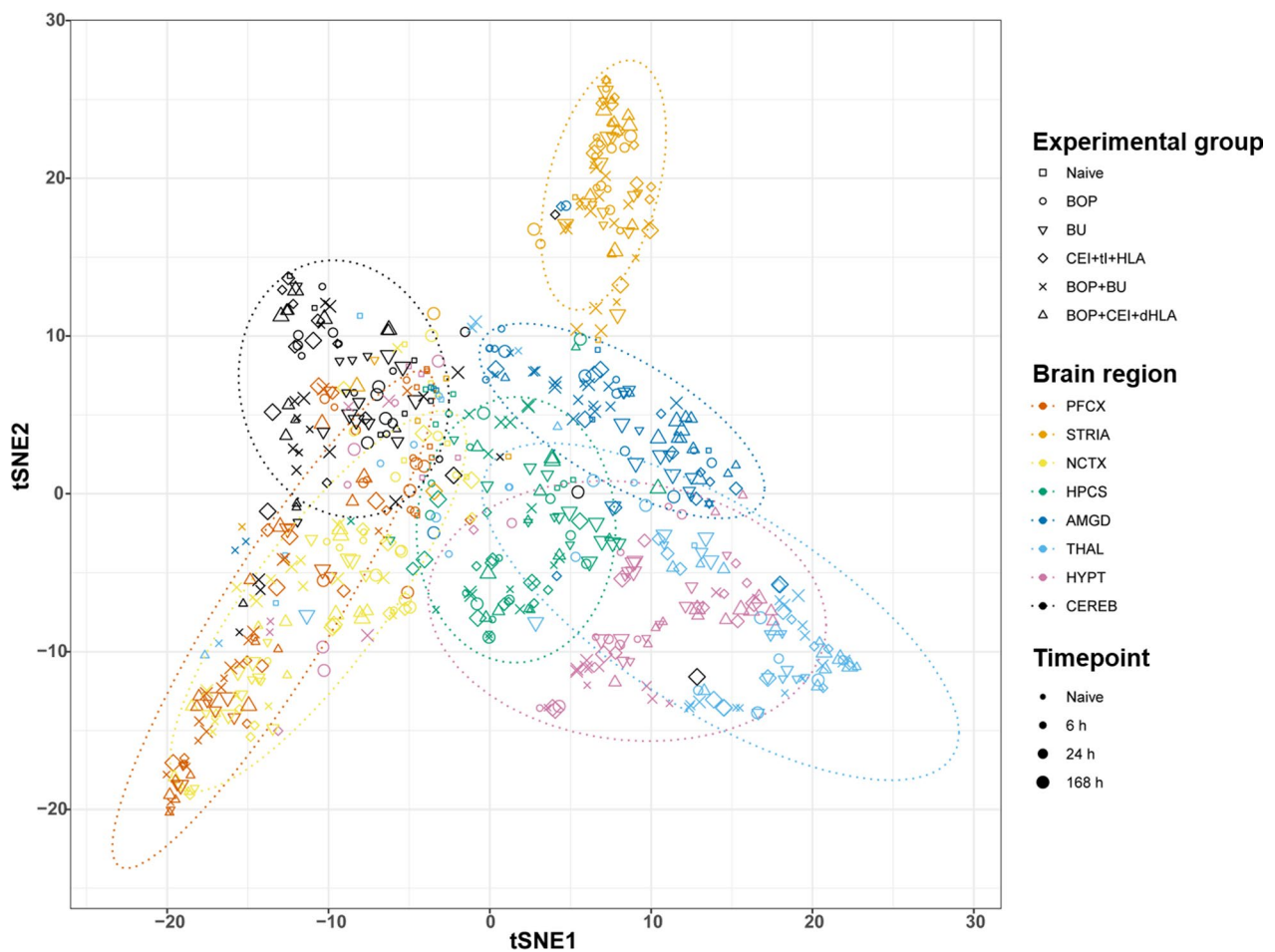**Fig. 3** (See legend on previous page.)

Fig. 4 The magnitude and neuroinflammatory gene expression response to trauma are brain region dependent, and the mode of injury is independent. t-distributed stochastic neighbor embedding (tSNE) data visualization and analysis were used to plot the $2^{-\Delta\Delta C(t)}$ profiles of 74 genes for each regional brain biopsy sample, and the results demonstrated that the gene expression profiles of individual samples tended to cluster by region as opposed to timepoint or injury model. The dotted area around the clusters represents the circle of best fit at an 85% confidence interval for gene expression

DEGs within the injury models sorted by gene class and time postinjury are presented in Table 1. *Casp8*, an initiator protease for apoptotic cell death [55], is primarily expressed following severe injury at 168 hpi in a number of brain regions. The levels of *Il16*, a chemoattractant involved in microglial M1 polarization [56], were relatively elevated in the AMGD, THAL, and HYPT groups, often exceeding 50-fold. *Cxcr2*, a neutrophil recruitment factor [57], was most frequently upregulated across all brain regions within all model groups at all timepoints. *Cd40*, a receptor on antigen-presenting cells that mediates cellular activation of the adaptive immune system and inflammation [58],

was upregulated in all brain regions, with the exception of PFCX and NCTX, across injury paradigms and timepoints. *Vegf*, a potent angiogenic factor, was substantially activated (10–25-fold) in non-blast trauma models (BU and CEI+tI+HLA) but less so in animal models subjected to BOP+CEI+dHLA. Protein–protein Interaction (PPI) analysis (Fig. 7) of significant DEGs within injury models from Table 1, indicated increasing complexity of gene networks correlated to injury severity (with the exception of BOP+BU) with the majority of clusters related to early innate inflammatory molecules (chemokines/cytokines) and neuronal activity.
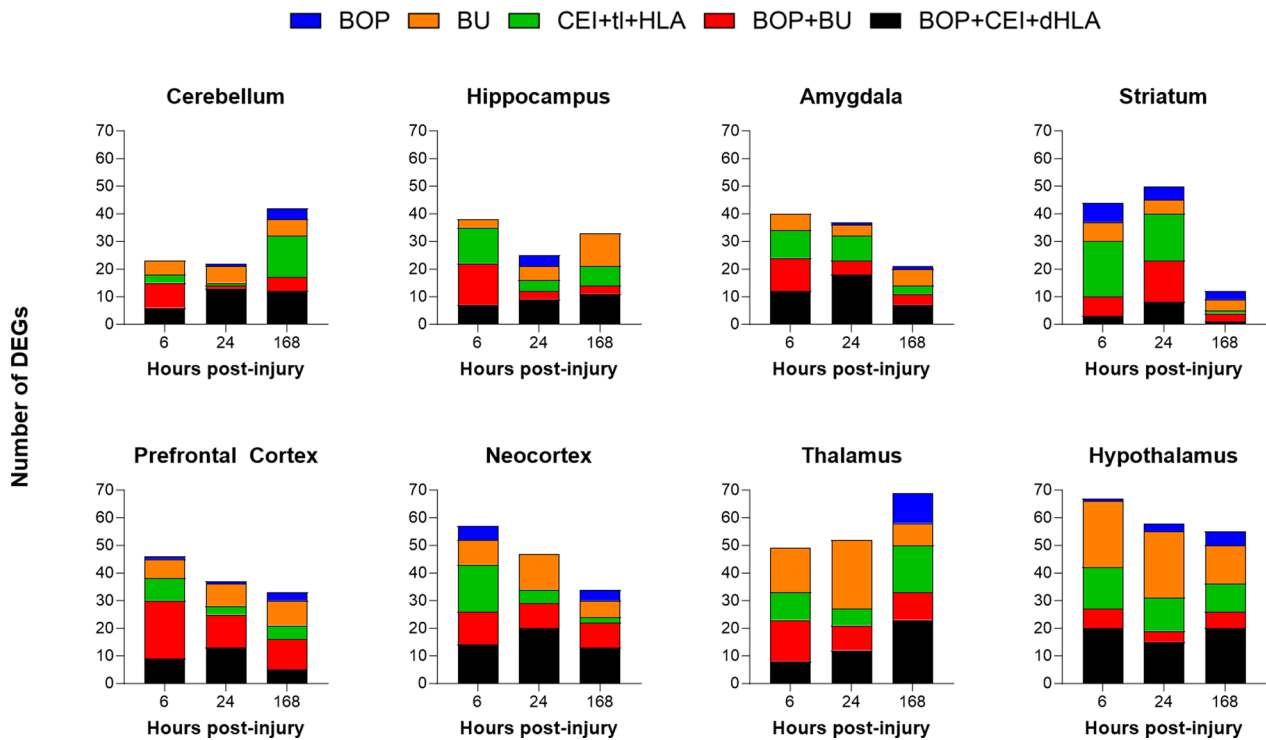
Fig. 5 Brain region-specific changes in neuroinflammatory gene transcript levels over time correlate with injury severity. Stacked bar charts are used to illustrate the number of differentially expressed genes (DEGs; up- or downregulated) in eight anatomical brain regions for each injury pattern. The data quantitatively reflect the number of genes with significant expression changes compared to those in naïve, uninjured control animals. The brain regions are displayed in ascending order based on the aggregate number of significantly altered genes (DEG counts irrespective of injury pattern and timepoint: cerebellum: 87, hippocampus: 96, amygdala: 98, striatum: 106, prefrontal cortex: 116, neocortex: 138, thalamus: 170, and hypothalamus: 180)

Discussion

Severe trauma triggers extensive local and systemic inflammatory responses, leading to secondary end-organ damage and even death [59–61]. However, the ramifications of posttraumatic immune responses in the brain, particularly following extracranial injury, have been largely overlooked. Building upon our prior findings, using well-established military-relevant trauma models with increasing severity [33, 35–37], we aimed to understand the impact of trauma-induced systemic inflammation on the evolution of neuroinflammatory gene expression in eight distinct brain regions over a seven-day study window. We then analyzed the gene expression profiles in the context of clinical parameters such as systemic inflammatory mediators and clinical biomarkers of

end-organ damage. Reflecting the profound and systemic and neuroinflammatory responses previously reported at 6 hpi [33], we reasoned that the extent and duration of neuroinflammatory-neurodegenerative expression changes would peak at/near 6 hpi and wane/return to near-baseline levels by the 168 hpi evaluation window. In this study, we investigated the extent of trauma-induced systemic inflammation (evidenced by increased inflammatory mediators and conventional biomarkers of end-organ dysfunction), and the kinetics of neuroinflammation across brain regions correlated with tissue trauma/injury severity (Fig. 8). Collectively, these unexpected and insightful findings demonstrate that remote, extracranial insults such as extremity polytrauma or burn injury caused a more robust neuroinflammatory response

(See figure on next page.)

Fig. 6 Neuroinflammatory gene signatures remain elevated at 168 hpi following various forms of trauma. The mean relative expression of individual genes across brain regions at 168 hpi, depicted by injury mode, was normalized to the geometric mean of three specific, constitutively active, and stable housekeeping genes (*B2m, Gapdh, Hprt1*) and regional brain biopsies from naïve uninjured animals. Two-way ANOVA was used to assess the main effect of injury on each of the brain regions. Tukey–Kramer post hoc analyses were used to assess differences between injured and naïve expression changes. Asterisks (*) indicate significant differences from naïve uninjured controls. *$p < .05$, **$p < .01$, and ***$p < .001$

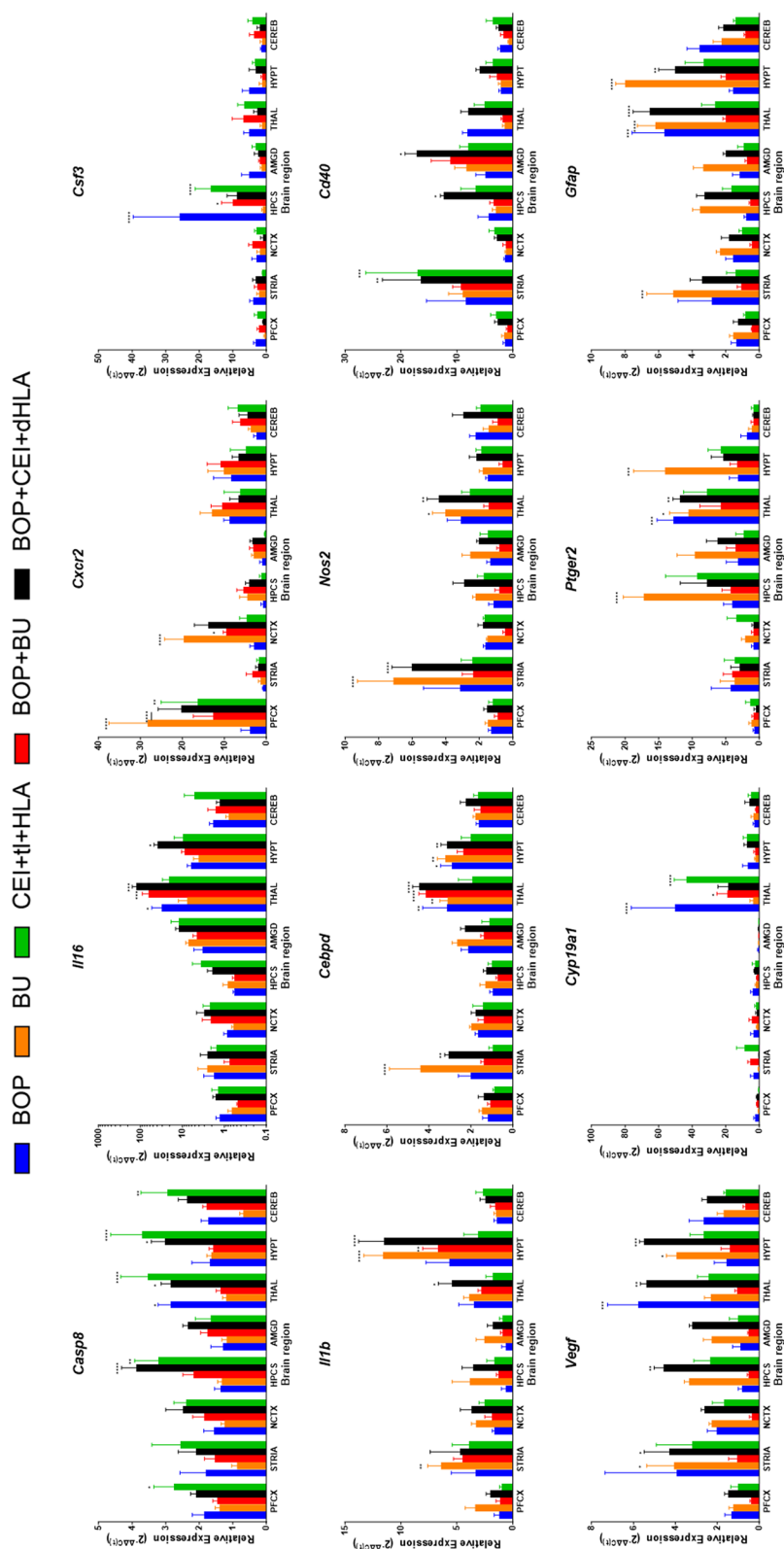**Fig. 6** (See legend on previous page.)

Table 1 Functional group analyses of differentially expressed genes (DEGs) determined in different brain regions for each injury group

Functional group	6 h					24 h					168 h				
	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA
Apoptosis	Pycard Tnf	Casp7 Casp8 Pycard Tnf Tp53	Bcl2 Tp53	Bcl2 Casp7 Casp8 Casp9 Tp53	Bcl2 Casp3 Casp7 Casp8 Pycard		Bcl2 Casp1 Casp7 Casp8 Pycard	Bcl2 Casp1 Pycard Tp53	Casp1 Tnf	Bcl2 Casp1 Casp3 Casp7 Casp8 Casp9	Bcl2 Casp8 Pycard Tp53	Casp1 Casp3 Casp8 Casp9	Bcl2 Casp1 Casp7 Casp8 Pycard Tnf	Pycard	Bcl2 Casp3 Casp7 Casp8 Casp9 Pycard Tp53
Chemoattractant		Ccl3 Ccl5 Cxcl10 Il16	Ccl3 Ccl5 Cxcl10 Il16	Cxcl10	Ccl5 Cxcl10	Ccl5 Cxcl10 Il16	Ccl3 Ccl5 Cxcl10	Ccl3 Cxcl10	Ccl5 Cxcl10	Ccl3 Ccl5 Cxcl10 Il16	Il16	Ccl3 Ccl5		Cxcl10 Il16	Ccl5 Cxcl10 Il16
Chemokine/ Chemokine Receptors	Ccl2 Ccl4 Cxcl9 Cxcr2	Ccl19 Ccl2 Ccl4 Cxcl9 Cxcr2	Ccl2 Cxcr2	Ccl19 Ccl4 Cx3Cl1 Cxcl5 Cxcl9 Cxcr2	Ccl19 Ccl2 Ccl5 Cxcl5 Cxcl9 Cxcr2	Ccl19 Cxcl9	Ccl19 Ccl2 Ccl20 Ccl4 Cxcr2	Ccl19 Ccl2 Ccl4 Cxcl5 Cxcl9	Ccl19 Ccl4 Cx3Cl1 Cxcl9	Ccl2 Cxcl1 Cxcl5	Ccl4 Cxcl1 Cxcl12 Cxcl9	Ccl19 Ccl4 Cxcl5 Cxcr2	Cxcl12 Cxcl5 Cxcl9	Cx3Cl1 Cxcl5 Cxcl9 Cxcr2	Ccl19 Cxcl1 Cxcl12 Cxcl5 Cxcl9
Cytokines	Il2	Il12a Il15 Il1b Il2 Il6 Tgfb2	Csf3 Il12a Il1a Il1b Il2 Il6 Tgfb1 Tgfb2	Csf1 Csf3 Il12a Il2 Il6 Tgfb2	Csf1 Il1b Il2 Il6 Tgfb1	Il15 Il1b Il2	Il12a Il15 Il1b Il2 Tgfb1 Tgfb2	Il12a Il15 Il1b Il2 Tgfb1 Tgfb2	Il12a Il1b Il2 Tgfb1 Tgfb2	Csf1 Il12a Il15 Il6 Tgfb1	Il12a Tgfb1	Il12a Il15 Il1a Il1b	Csf1 Csf3 Il12a Il15 Il1a Il1b Il2 Tgfb1 Tgfb2	Il12a Il2 Tgfb1 Tgfb2	Csf1 Il12a Il15 Il1b Tgfb1
Early Transcriptional Activators/ TLRs	Fas Nos2	Cebpd Nfkb1 Tlr2 Tlr3	Cebpd Nfkb1 Nos2 Prkcq Tlr3 Tlr9	Cebpd Mapk10 Nfkb1 Rela Tlr2	Cebpd Mapk3 Nfkb1	Cebpd Fas	Cebpd Fas Mapk3 Nfkb1 Prkcq Rela Tlr3	Cebpd Nfkb1 Tlr3	Cebpd Fas Rela Tlr9	Cebpd Fas Lif Mapk10 Mapk3 Nfkb1 Tlr9	Cebpd Rela Tlr3	Nos2 Tlr2 Tlr3	Lif Nfkb1 Nos2 Tlr3	Nfkb1 Rela	Fas Lif Mapk10 Mapk3 Nfkb1 Nos2 Tlr9
Immune Cell/Adhesion/Vascular Markers	Cd68	Cd14 Cd40 Cd86 Vcam1 Vegfa	Cd14 Itgam Vegfa	Cd14 Cd86 Icam1 Vegfa	Cd14 Cd86 Icam1 Itgam Vcam1	Cd68	Cd14 Cd40 Cd86 Icam1 Vcam1 Vegfa	Cd68 Vegfa	Cd86 Icam1 Vegfa	Cd14 Cd40 Icam1 Vegfa	Cd40 Cd68	Cd68 Vcam1 Vegfa	Icam1 Vcam1 Vegfa	Vegfa	Cd40 Icam1 Vcam1 Vegfa
Neurosteroids/Prostaglandins		Ptger2 Ptger3 Ptgs2	Cyp19a1 Gper1 Ptger3 Ptges2 Ptgs2	Cyp19a1 Gper1 Ptger2 Ptger3 Ptger4	Ptgs2	Ptger2	Ptger2 Ptger3 Ptgs2	Ptger3	Cyp19a1 Gper1 Ptger3	Cyp19a1 Gper1 Ptger2 Ptgs2	Cyp19a1 Ptger4 Ptges2 Ptgs2	Ptger2 Ptger3 Ptger4 Ptges2	Cyp19a1 Gper1	Cyp19a1 Ptger3	Gper1 Ptgs2

Table 1 (continued)

	6 h					24 h					168 h				
	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA	BOP	BU	CEI+tI+HLA	BOP+BU	BOP+CEI+dHLA
Neuronal Activity/ Neuroinflammation/ Neurodegeneration	Bdnf Grin1 Homer1	Dner Drd1a Gfap Homer1 Hpca Htr1a	Bdnf Dner Gabra1 Gfap Grin2b Hpca Htr1a Ntrk2	Bdnf Chi3l1 Drd1a Gfap Grin1 Grin2b Homer1 Htr1a	Bdnf Dner Gabra1 Gfap Grin1 Grin2a Grin2b Homer1 Ntrk2	Htr1a	Dner Gfap Homer1 Hpca Htr1a Ntrk2	Bdnf Dner Drd1a Gabra1 Gfap Grin2b Htr1a Htrk2	Bdnf Chi3l1 Dner Drd1a Gabra1 Bfap Grin1 Grin2b Homer1 Hpca Htr1a Ntrk2	Bdnf Dner Grin1 Grin2b Homer1 Hpca Ntrk2	Gfap Grin1 Homer1 Htr1a	Gfap Homer1 Htr1a	Grin1 Grin2b Homer1 Ntrk2	Gabra1G- fap Homer1 Htr1a	Bdnf Dner Grin1 Grin2b Homer1 Hpca Htr1a Ntrk2
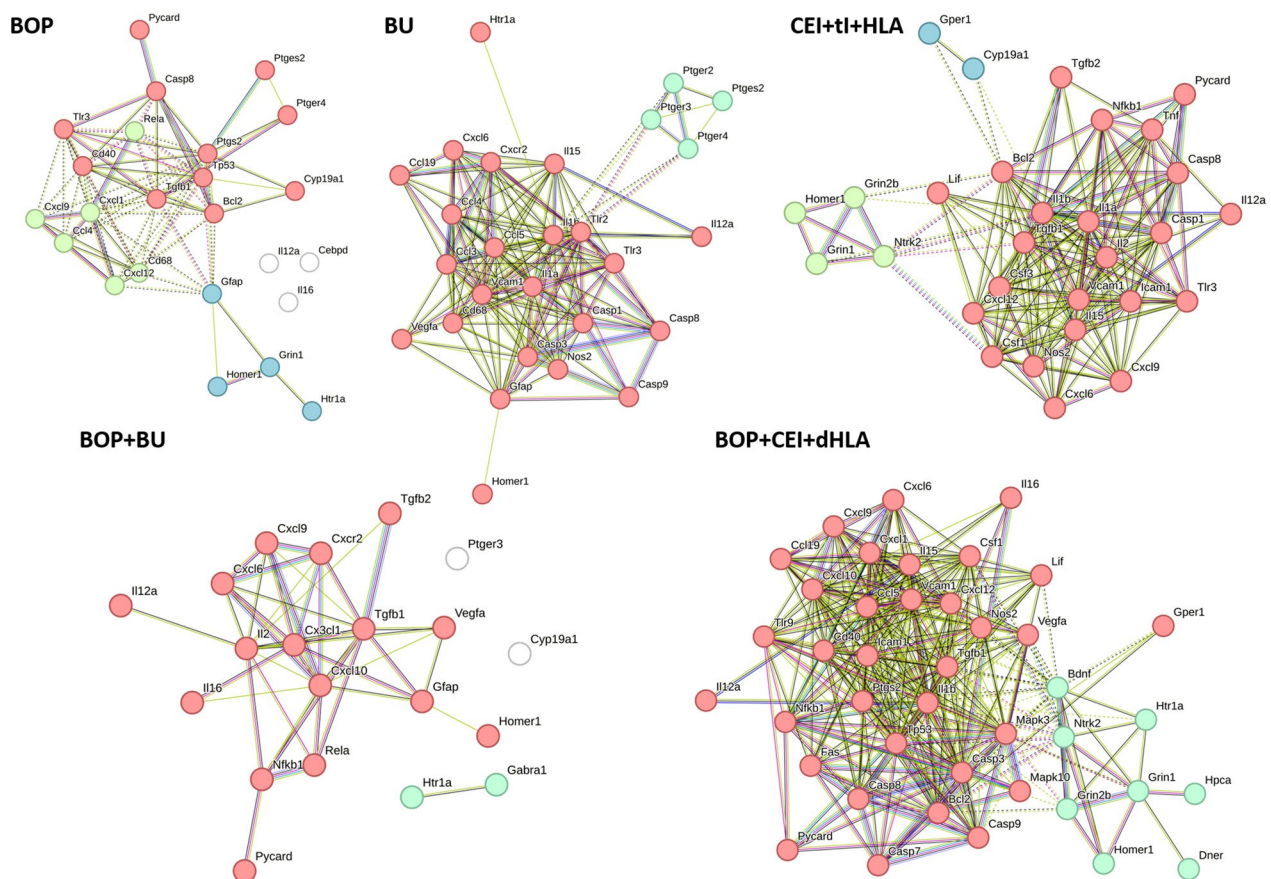
Fig. 7 Protein–protein interaction networks of the differentially expressed genes in each trauma model. STRING protein–protein interaction (PPI) software was used to construct the gene networks of the differentially expressed genes (DEGs) determined in different brain regions for each injury group to highlight gene–gene interactions. Each color represents a different gene cluster

across more brain regions than did BOP (Fig. 8a). Furthermore, following all forms of trauma, the neuroinflammatory-neurodegenerative gene signatures we profiled either increased or remained elevated over the study duration, while classical clinical measurements of systemic inflammation and end-organ damage decreased or resolved (Fig. 8b-c). These findings underscore the brain's sensitivity to mediators of systemic inflammation derived from extracranial forms of trauma, hence portraying a critical link between systemic inflammation and trauma-induced neuroinflammation.

The trauma models used in this study result in graded levels of systemic inflammation and end-organ dysfunction [35–37]. The degree of trauma-induced neuroinflammation appears to correlate with injury severity and systemic inflammation levels—with more robust injuries triggering a stronger systemic inflammatory response early postinjury, as observed in the CEI+tI+HLA and BOP+CEI+dHLA models—and resulting in prolonged and heightened global neuroinflammation in the brain. Others have demonstrated connections between the

immune peripheral systemic inflammatory response and neuroinflammation in the brain. The production of a variety of cellular and systemic proinflammatory mediators that regulate inflammation-related signaling pathways appears to be a key requirement for the development of immune-neuroinflammatory activity and neurological dysfunction [62, 63] following trauma [64, 65], infection (pneumonia, LPS challenge and sepsis) [66, 67] and a number of systemic autoimmune disorders [30, 68]. Within a few hours of trauma, evidence from studies involving primary brain injury has demonstrated that peripheral immune–brain crosstalk can contribute to a "leaky-disturbed" blood–brain barrier (BBB), immune cell penetration–activation, and activation–recruitment of microglia–astrocytes, leading to multifaceted neuroinflammation, cell death and exacerbated brain injury [69–71]. These induced inflammatory cascades in the brain reportedly result in demyelination and neuronal and axonal cell loss, ultimately contributing to impaired brain function and culminating in long-term behavioral perturbations, psychological conditions and irreversible
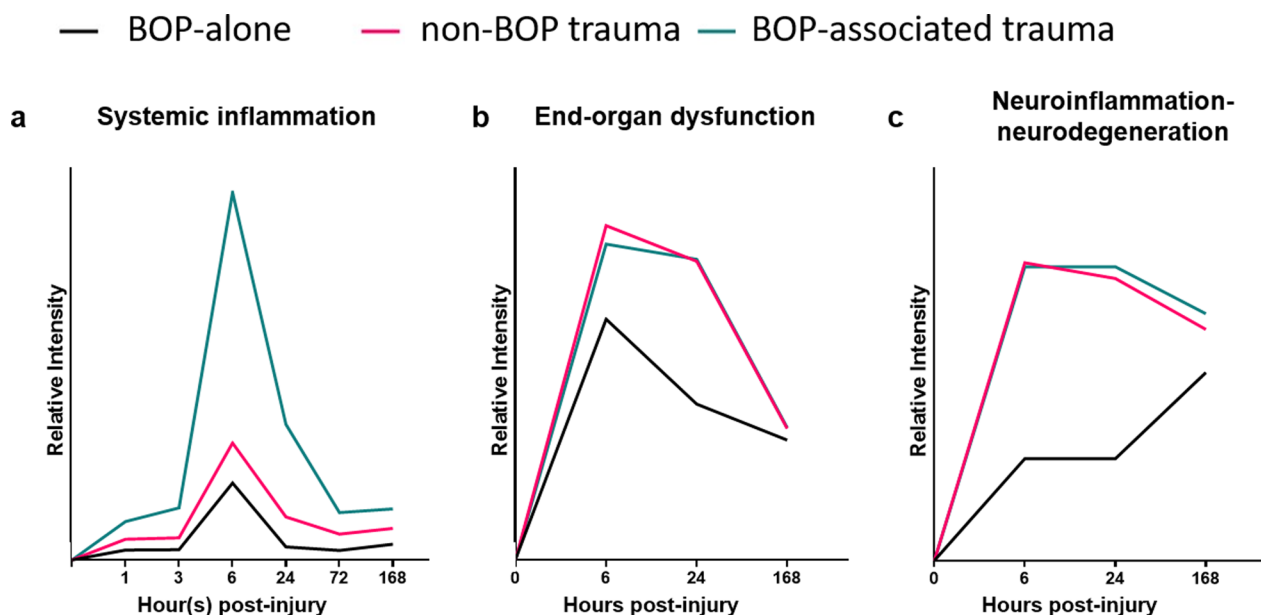
Fig. 8 Systemic inflammation elicited persistent acute neuroinflammation following non-blast or blast-associated trauma. The mean levels of serum cytokines (IL-1β, IL-6, CXCL1, TNF-α, MCP-1, and IFN-γ) and clinical chemistry markers of end organ damage (BUN:Cr ratio, AST, ALT, and Albumin) normalized to those of naïve uninjured controls of individual animals were used to compute the relative intensity of (**a**) systemic inflammation and (**b**) level of end organ damage following three types of injury: cranial trauma (as represented by BOP), noncranial trauma (as represented by BU and CEI+tI+HLA), or combined cranial/noncranial trauma (as represented by BOP+BU and BOP+CEI+dHLA) over time. The number of significantly differentially expressed genes was utilized to compute the (**c**) relative intensity of trauma-induced neuroinflammation gene expression profile for each type of trauma over time. Serum systemic inflammation and circulating clinical biomarkers of end-organ damage peak at 6 h postinjury (hpi) and wane or return to baseline/naïve levels by 168 h postinjury, whereas the neuroinflammatory gene expression profiles in the context of various forms of trauma remain elevated by 168 hpi compared to those in the naïve steady state

neurocognitive side effects [72]. We speculate from our findings that the level of extracranial-induced neuroinflammation is primarily regulated by the time of onset, scale, complexity and persistence of the acute inflammatory response at the injury site in conjunction with the provoked peripheral systemic inflammatory response and its impact on secondary organ pathology. Furthermore, we believe that some undefined threshold of persistent low-grade chronic inflammation is necessary to initiate and sustain neuroinflammation in the brain. However, the duration and impact on functional/behavioral outcomes remain to be determined. These findings are consistent with studies showing that neuroinflammation may be caused by inflammation (acute or chronic) and injury to peripheral organs [73].

Despite the resolution of classical markers of systemic inflammation and end-organ dysfunction (Fig. 8a, b), we detected near peak levels of neuroinflammatory gene expression across the majority of brain regions at 168 hpi (Fig. 8c). These results are consistent with other studies suggesting that persistent neuroinflammation is not due simply to a prolonged acute systemic proinflammatory state [74]. We suspect that early immune regulatory processes that prevent exaggerated adaptive response-based compensatory mechanisms [75] that exist in the periphery may be missing in the brain or have reduced potency in regulating the activity of resident immune cells (microglia, perivascular macrophages, Tregs) and repairing cell processes [76, 77]. Tregs have been reported to play opposing dichotomous immunoregulatory functions (neuroinflammatory and neuroprotective) in modulating local and systemic innate and adaptive immune responses as well as pathologies in the periphery and tissues/organs, including the brain [76, 78]. The depletion of Tregs has been shown to enhance neuroinflammation and microglial activation, while the adoptive transfer of ex vivo-expanded Tregs suppresses proinflammatory cascades and attenuates neuroinflammatory pathologies [78]. There is no doubt that crosstalk between the nervous system and the immune system plays an important role in regulating acute and chronic neuroinflammation, albeit via regulatory mechanism(s) that at this point remain unclear.

Applicable to this line of research, acute brain injury and cognitive dysfunction have been documented following acute infections and prolonged sedation [17, 79]. The degree of injury and nature of neuroinflammation appear to lie along a spectrum, as our findings as well as others

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on acute inflammatory markers of chronic activation of microglia in the CNS indicate. In intensive care unit (ICU) settings, where prolonged sedation is often needed, the phenomenon of ICU-induced delirium is attributed to multiple factors, including neurotransmitter toxicity, an aberrant stress response, and neuroinflammation [79]. Delirium, usually described as "acute partial brain failure," is often reversible through the avoidance of triggering events and treatment of the underlying critical illness but can have long-lasting sequelae such as mild cognitive impairment or dementia, particularly in the elderly population [17]. Reports of neurological and psychiatric symptoms associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus infectious disease 2019 (COVID-19), have become a pertinent example of immune-brain crosstalk. COVID-19 is initially characterized by severe systemic infection initiated by a systemic inflammatory "cytokine storm" producing immunopathogenic damage, primarily in the lungs, which presents as acute respiratory distress syndrome followed by MODs, MOF, sepsis, and even death. Among survivors, "long-COVID" manifests as profound fatigue and gastrointestinal, cardiovascular and neuropsychiatric symptoms [80–83]. Approximately one-third of hospitalized COVID-19 patients develop persistent neurological sequalae [84], an array of symptoms ranging from headache to depression, fatigue, seizures, delirium, ischemia or hemorrhage [85]. Although there is no current clinical evidence that the COVID-19 virus impacts the brain directly, the general consensus is that serum cytokines, triggered by systemic inflammation from the initial viral infection, can influence BBB function, leading to its disruption and the onset of neurological disease [85]. For example, early innate proinflammatory mediators such as IL-6 or IL-1β disrupt the BBB via endothelial cell activation and may also play key roles in modulating sickness behaviors and CNS function post-acute phase recovery [86, 87]. In one Dutch study, nearly one-third of adults with bacterial meningitis suffered cognitive impairment following resolution of infection. In addition to the cytotoxic impacts of bacterial meningitis, there is also disruption of the BBB and potentiation of resultant neuroinflammation as a result of peripheral leukocyte infiltration into the CNS [65, 88]. Collectively, these findings demonstrate a direct relationship between immune and brain crosstalk and reveal a potential role for systemic inflammation following non-BOP trauma and its impact on the brain.

Although nervous system susceptibility to systemic inflammation-induced damage is well documented [89], research on region-specific brain changes following various traumatic injuries have been limited given the heterogeneity of injury and the tendency of investigators to specialize in a single brain area rather than adopting a holistic brain approach. In contrast, we provide multidimensional insights into the gene expression changes in numerous brain regions following various forms of trauma over the course of seven days. Specifically, our data demonstrated that HYPT and THAL are highly vulnerable to inflammation after trauma, irrespective of the trauma mode, although the response following BOP was delayed at 168 hpi. The THAL is critical for relaying motor and sensory signals, circadian sleep/wake awareness, and cognition to the cortex and subcortical nuclei and is implicated in several neurodegenerative disorders, such as multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, and PTSD [90]. BOP-associated trauma can induce damage to the THAL, which is mediated by heightened and prolonged microglial activity in the white matter, with symptoms sometimes manifesting up to 17 years after the initial injury [90]. The HYPT is essential for regulating homeostasis, acts as a surveillance center for crucial changes in the body, and responds to stress by stimulating various glands and organs to release hormones [91]. BOP-associated trauma and systemic inflammation have been shown to provoke stress-mediated release of cortisol and catecholamines via the hypothalamus–pituitary–adrenal (HPA) axis. In turn, the release of stress hormones can promote further activation of chemokines and adhesion molecules via a feed-forward mechanism, ultimately worsening the neuroinflammatory environment [92–94]. Therefore, a multiregional assessment of the brain following injury is invaluable for understanding clinical outcomes because it helps identify the specific regulatory systems of the brain involved in various aberrant and compensatory processes and thereby guides further molecular and physiologic studies.

Organ-specific biomarkers are utilized to monitor the onset, progression, and resolution of end-organ dysfunction/damage, making their sensitivity and specificity crucial for understanding a patient's clinical status. Although serum biomarkers have traditionally been used to characterize ongoing inflammatory responses in extracranial organs, our findings show that the brain undergoes a profound inflammatory shift after remote trauma, suggesting that the brain should be assessed as an end-organ. Clinically, functional recovery after BOP-associated injury is heterogeneous and unpredictable, with prolonged recovery resulting in significant patient morbidity and substantial healthcare burdens. At present, the scarcity of reliable brain-specific fluidic biomarkers and the typically silent imaging effects of brain injury complicate the accurate determination of recovery timelines. Drieu et al. [95] reported that rats that sustained mild TBI exhibited signs of ongoing neuroinflammation 3 weeks postinjury, as indicated by significantly elevated GFAP and TPSO levels

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on imaging, suggesting the presence of active microgliosis and astrogliosis. Similarly, increased microglial activation has been observed in patients with moderate to severe brain injury up to 17 years after initial injury, suggesting a transition from an acute to chronic inflammatory response [15]. Although organ-specific biomarkers are effective in characterizing the inflammatory state, the absence of neuroinflammatory biomarkers creates an incomplete inflammatory picture that potentially contributes to poor outcomes. Therefore, the identification and/or potential linkage of systemic biomarkers to local brain mediators would be transformative to clinical care for patients presenting with potential for overt trauma-induced neuroinflammation, mitigating behavioral perturbations, and central nervous system manifestations. Furthermore, understanding the molecular mechanisms and key cell types is important for identifying and devising targeted therapies for the short- and long-term sequelae that follow trauma-induced brain injury [96].

This exploratory study is not without limitations. First, we acknowledge that a study with a relatively small sample size, with only 5–7 replicates per injury/timepoint cohort, may not have the statistical power to detect small effects. Despite this limitation, the regional and temporal effects observed in rats subjected to various injury modes alone or in combination were reproducible. Although a larger sample size would increase the internal and external validity of our study, our power analysis indicated that five subjects per injury group would be sufficient for understanding differences between cohorts. Second, this study included only adult male rats; therefore, our analysis does not account for potential sex differences in gene expression in either naïve steady-state or posttraumatic injury. A plethora of studies implicate sex as a biological variable in brain pathophysiology, neurological recovery, and protection [97–100]. Current epidemiologic data suggest that males are approximately forty percent more likely to suffer from head trauma [100]. This pattern is similarly replicated in the military [101], likely due to the overwhelming male predominance in combat roles. Currently, women constitute less than three percent of combat arms and subsequently represent only 2.5% and 2.9% of primary brain injury diagnoses in Afghanistan and Iraq, respectively [102]. We hypothesize that over the next decade, we may observe a shift in these patterns as more women enter combat roles. Given the growing number of women in the armed forces, the sex differences that exist in neurologic sequelae, and the sexually dimorphic expression of brain injury, it is crucial to include sex differences in future studies [103]. Third, while our study models include blast injuries and the post-injury immune sequelae, they do not perfectly

recapitulate the simultaneous nature of these injuries, as observed in real-world scenarios. Instead, the rats were injured in a sequential manner. Fourth, brain specimens were not perfused with DPBS at necropsy prior to tissue collection. As such, it is probable that some of the gene expression changes noted could be due to the presence of blood cells present in the specimen. Last, the animals randomized to the CEI+tI+HLA group were subjected to a sequence of soft-tissue crush injury, mid-shaft femur fracture, and hind limb amputation, followed by 3 h of tourniquet-induced ischemia prior to immediate amputation. This contrasts with the concomitant BOP+CEI+dHLA group, which did not undergo tourniquet application, as those samples were unfortunately lost during the storage process and unable to be salvaged. Furthermore, each injury model utilized a different anesthetic condition to include drug type/route, timing and dosage, which were not individually controlled for. However, both ketamine and isoflurane have been shown to have downregulatory properties to reduce inflammation following trauma in animal model systems [104, 105].

Modeling acute trauma in a research setting using *in vivo* systems is complex, highlighting the need for better tools to appropriately score and quantitatively assess the posttraumatic injury state, predict outcomes, and compare the results against human traumatic injuries. Currently, in the preclinical setting, no such scoring system exists to quantify the severity of injury post-trauma in animal models, with only a few reports extrapolating Injury Severity Scores (ISS) to animal models [106]. The limitations of current clinical injury severity scoring systems have become increasingly apparent when considering their inability to assess the immediate and long-term impacts of trauma, specifically regarding the posttraumatic systemic inflammatory response and potential end-organ dysfunction. However, prevailing evidence has demonstrated a positive relationship between injury severity, worsening neurologic injury, and mortality [107]. Martin et al. demonstrated that patients who sustained combined brain and burn injury have significantly increased mortality compared to burn patients alone and even burn with trauma but without neurologic injury [108]. As such, inadequate diagnostic tools may contribute to the consistently poor outcomes observed in patients with SIRS and MODS [109–112], particularly the reliance on nonspecific macroparameters such as heart rate, respiration rate, Glasgow coma scale, and serum clinical measurements (transaminases, creatinine) in the diagnosis of cell-mediated syndromes. Moreover, the present scoring systems largely overlook the brain, either as an initial organ of traumatic insult or as an end-organ susceptible to remote inflammatory processes and

modulating systemic inflammation. In light of these limitations, a future direction of preclinical modeling studies should include the development of new diagnostic and prognostic measurements that could be modeled to create a more comprehensive and accurate scoring system.

Conclusion

This study demonstrated the profound effect of extracranial trauma plus/minus BOP exposure on neuroinflammatory processes in eight anatomical regions of the brain. In conjunction with our prior study [33], these data emphasize the vulnerability of the brain to remote humoral and/or cellular immune responses, normally considered to be an "immune privileged" environment. Independent of the mode of trauma, neuroinflammation steadily increases over 7 days, while classical clinical measurements of systemic inflammation and secondary organ damage are worsening or have resolved. These findings further emphasize the vulnerability of the brain to systemic immune responses, as blast exposure exacerbates the neuroinflammatory response to remote extremity injury. Future molecular and behavioral investigations are warranted to understand the short- to long-term pathophysiological consequences on the brain, with a particular focus on the mechanism(s) of blood–brain barrier breakdown, immune cell penetration–activation, and microglial activation.

Abbreviations

AAALAC	American Association for the Accreditation of Laboratory Animal Care
ABS	Advanced blast simulator
ALB	Albumin
AMGD	Amygdala
BBB	Blood–brain barrier
BOP	Blast overpressure
BOP+BU	Blast overpressure plus cutaneous thermal injury
BOP+CEI+dHLA	Blast overpressure, complex extremity injury, and delayed hind limb amputation
BU	Cutaneous thermal injury
BUN:Cr	Blood urea nitrogen to creatinine ratio
CEI+tI+HLA	Complex extremity injury, three hours of tourniquet-induced ischemia, and hind limb amputation
CEREB	Cerebellum
CO_2	carbon dioxide
DEG(s)	Differentially expressed gene(s)
HPA	Hypothalamus–pituitary–adrenal axis
HPCS	Hippocampus
hpi	Hours post-injury
HYPT	Hypothalamus
IACUC	Institutional Animal Care and Use Committee
IP	Intraperitoneal
ISS	Injury severity score
LN_2	Liquid nitrogen
MOD	Multiple organ dysfunction
MOF	Multiple organ failure
NCTX	Neocortex
O2	oxygen
PFCX	Prefrontal cortex
PTSD	Posttraumatic stress disorder
PVC	Polyvinyl chloride
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SC	Subcutaneous
SIRS	Systemic inflammatory response syndrome
SEM	Standard error of the mean
STRIA	Striatum
STRING	Search tool for the Retrieval of Interacting Genes
TBSA	Total body surface area
THAL	Thalamus
t-SNE	T-distributed stochastic neighbor embedding
USU	Uniformed Services University

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-024-03205-5.

Additional file 1: Fig. 1 Heatmap depicting the expression of 74 neuroinflammatory-neurodegenerative genes in eight brain regions following cranial, noncranial or combined trauma. At three timepoints postinjury (6, 24, and 168 h), intact brains were harvested from blast (BOP)-, cutaneous burn (BU)-, extremity (CEI)-, BOP+BU-, and blast plus concomitant extremity (BOP+CEI+dHLA)-injured animals. RNA was isolated, and cDNA was transcribed using standard methods. The transcriptomic profiles of 74 genes were determined using a low-density custom microarray. The values reported were calculated based on the $2^{-\Delta\Delta C(t)}$ method and normalized to the geometric mean of three specific, constitutively active, and stable housekeeping genes (*B2m, Gapdh, Hprt1*) and regional brain biopsies from naïve uninjured animals. Blue shading represents a decrease in expression compared to that in the naïve group, red shading represents an increase in expression compared to that in the naïve group, and white shading represents nonsignificant changes (− 2- to 2-fold change). The color intensity correlates with the magnitude of gene expression relative to that in naïve, uninjured rats. Fig. 2 Heatmap/dot plot of significantly differentially expressed neuroinflammatory-related genes (DEGs) induced by trauma over the experimental window. Dot plot illustrating gene expression relative to the injury paradigm, brain region, and time after injury. One-way ANOVA was used to determine gene expression differences between the injury models and naïve animals at each study timepoint, and Tukey's post hoc multiple comparison test was used to determine the source of significance, with α = .05. Each dot color represents gene expression across three measured time points (6, 24, and 168 h postinjury), with the above denoting those significantly upregulated, the lower denoting those significantly downregulated, or ND indicating no difference between naïve controls within each brain region and time point. The combined differential expression profile over the course of the 3 days is color-coded as depicted in the legend. Additional File 2: ANOVA multiple comparison table for injury model, timepoint, and brain region, as compared to naïve control samples.

Disclaimer

The contents of this publication are the sole responsibility of the author(s) and do not necessarily reflect the views, opinions or policies of Uniformed Services University of the Health Sciences (USUHS), The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., the Department of Defense (DoD), or the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

Author contributions

C.J.R. and T.A.D. acquired funding, designed the study, conducted analysis, constructed figures, and wrote the manuscript. C.J.R., U.N., L.M., B.H., J.M. and D.S. conducted experiments. C.S., S.G., and B.K.P provided data interpretation and critical review. J.L.L., E.R.G., and S.A.S. conducted data analysis/visualizations using R. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
All procedures and experiments were performed in an AAALAC accredited facility, in strict accordance with all applicable Federal, State and institutional regulations and policies governing the protection of animals in research, and the ARRIVE guidelines [38] with the permission and oversight of the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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