Microglial Inflammasome Activation in Penetrating Ballistic-Like Brain Injury

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Abstract

Penetrating traumatic brain injury (PTBI) is a significant cause of death and disability in the United States. Inflammasomes are one of the key regulators of the interleukin (IL)-1 β mediated inflammatory responses after traumatic brain injury. However, the contribution of inflammasome signaling after PTBI has not been determined. In this study, adult male Sprague-Dawley rats were subjected to sham procedures or penetrating ballistic-like brain injury (PBBI) and sacrificed at various time-points. Tissues were assessed by immunoblot analysis for expression of IL-1 β , IL-18, and components of the inflammasome: apoptosis-associated speck-like protein containing a caspase-activation and recruitment domain (ASC), caspase-1, X-linked inhibitor of apoptosis protein (XIAP), nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3), and gasdermin-D (GSDMD). Specific cell types expressing inflammasome proteins also were evaluated immunohistochemically and assessed quantitatively. After PBBI, expression of IL-1 β , IL-18, caspase-1, ASC, XIAP, and NLRP3 peaked around 48 h. Brain protein lysates from PTBI animals showed pyroptosome formation evidenced by ASC laddering, and also contained increased expression of GSDMD at 48 h after injury. ASC-positive immunoreactive neurons within the perilesional cortex were observed at 24 h. At 48 h, ASC expression was concentrated in morphologically activated cortical microglia. This expression of ASC in activated microglia persisted until 12 weeks following PBBI. This is the first report of inflammasome activation after PBBI. Our results demonstrate cell-specific patterns of inflammasome activation and pyroptosis predominantly in microglia, suggesting a sustained pro-inflammatory state following PBBI, thus offering a therapeutic target for this type of brain injury.

Keywords: inflammasome; microglia; penetrating ballistic-like brain injury; penetrating traumatic brain injury

Introduction

THE CENTERS FOR DISEASE CONTROL AND PREVENTION estimates that firearm-related injuries are the second leading cause of injury death in the United States.¹ Although improvements in early management and timely neurosurgical interventions have improved survival in penetrating traumatic brain injury (PTBI),^{2–4} individuals shot in the head are 3.3 times more likely to die, compared with those shot in other body parts.⁵ Also, PTBI survivors suffer more severe neurological outcomes, such as persistent vegetative state or severe disability,⁶ compared with other severe traumatic brain injury (TBI) patients.^{7,8} Thus, further research is required to clarify the pathomechanisms of PTBI, with the ultimate goal of developing novel treatments to help improve outcomes of these patients.

Similar to other forms of TBI, PTBI is associated with primary and secondary injury mechanisms.⁹ The primary injury results from acute mechanical tissue deformation from an external force leading to necrotic cell death, shearing, and tearing of blood vessels, neurons, and glia.¹⁰ The mechanical tissue deformation in PTBI originates

from a slow- or fast-moving foreign object such as a knife or bullet breaching the skull and dura. The slow-moving knife leads to slicing, shearing, and compression of brain along the travel path. A fast-moving bullet will cause similar injury and create a shock wave of high pressure prior to penetration. As the bullet travels through the brain, it pushes the brain matter away from the track, creating a conical cavity. The cavity size commonly ranges from two to fifteen times the diameter of the bullet. Tissue deformation from expansion and contraction of the cavity in high-speed penetrating injuries plays an important role in the continued disruption of the brain tissue and vascular structures.^{11,12} In addition, secondary injury mechanisms include the release of cell products such as glutamate and aspartate,¹³ leading to glutamate excitotoxicity,¹⁴ mitochondrial dysfunction,¹⁵ oxidative stress,¹⁶ inflammation,¹⁷ and apoptosis.¹⁸ The initial injury is therefore magnified by secondary injury mechanisms leading to progressive brain injury.^{19–21}

The increased production and release of pro-inflammatory molecules after TBI, including interleukin (IL)-1 β , tumor necrosis factor, and IL-6 has been implicated in the pathophysiology of

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secondary brain injury.^{20,22} More recently, the innate immune response, regulated through the inflammasome, has been shown to play a role in secondary injury mechanisms after experimental TBI.^{23–29} In humans, previous studies have reported increased levels of the inflammasome proteins, such as the adaptor protein apoptosis speck-like protein containing a caspase-activating recruitment domain protein (ASC), caspase-1, and nucleotide-binding oligomerization domain (NOD)-like receptor protein 1 (NLRP1), and NOD-like receptor protein 3 (NLRP3), in both cerebrospinal fluid (CSF) and purified CSF exosomes correlate with poor outcomes in severe adult and pediatric TBI patients.^{30–32} In brain injury, danger/danger-associated molecular patterns (DAMPs) such as adenosine triphosphate and high extracellular potassium³³ are released from dead or dying cells from the primary injury. These signals are recognized by the NOD-like receptor family of pattern recognition receptors, NLRP1, NLRP2, and NLRP3 found predominately in neurons,³⁴ astrocytes,³⁵ and microglia,³⁶ and lead to inflammasome activation.²⁶ Once activated, the inflammasome protein complex polymerizes and leads to the cleavage of pro-caspase-1 into proteolytically active subunits. Active caspase-1 cleaves IL-1 β and IL-18 into its active forms, which are then secreted into the extracellular space³⁷ mediating pyroptosis, a form of inflammatory cell death.^{38,39} Recently, gasdermin-D (GSDMD) cleavage and pore formation have been reported to be necessary components of pyroptosis in human cells,^{40,41} with cells not expressing GSDMD demonstrating apoptotic cell death. Although implicated in the pathophysiology of other forms of TBI,^{23–29} the cell origins and importance of inflammasome activation has yet to be investigated in PTBI.

The rodent model of penetrating ballistic-like brain injury (PBBI) recapitulates cranial gunshot wound pathology and is associated with progressive tissue loss.⁴² The purpose of this study was to characterize regional patterns of increased inflammasome protein expression and determine the role of pyroptotic cell death

in the pathophysiology after PBBI. We show with immunohistochemistry that inflammasome protein expression after PBBI begins in perilesional neurons and subsequently peaks in microglia, spreading to areas remote from the primary injury site. Taken together, we show that the neuro-inflammatory response to PBBI includes pyroptotic cell death mediated by inflammasome activation, leading to continued tissue loss in surrounding brain regions.

Methods

Animals

Animal procedures were approved by the University of Miami's Institution of Animal Care and Use Committee and adhered to the Animal Research: Reporting of In Vivo Experiments guidelines and those established by the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (280–350 g) age 3–5 months were used for all experiments. Male rats were used because males are at higher risk of TBI than females, with the highest male-to-female ratio occurring in adolescence and young adulthood.⁴³ Rats were randomly assigned to each experimental group (sham and PBBI 4 h, 24 h, 48 h, and 72 h after injury). An $n=5$ was established for biochemical analyses based on power analysis and previous studies in this model to determine sample size calculation.^{44–47} All rats were housed in our viral antigen-free animal facility on site at the Lois Pope Life Center on 12-h light/dark cycles. The veterinary staff at the Lois Pope Life Center conducted routine husbandry procedures twice per week and checked on the condition of all animals daily. Animals were monitored after surgery to ensure they did not develop post-operative infections or experience discomfort.

Unilateral PBBI surgery

Rats were anesthetized with 2–5% isoflurane delivered in a mixture of 70% nitrous oxide and 30% oxygen. The body temperature was maintained at normothermia ($37 \pm 1^\circ\text{C}$) throughout

FIG. 1. Rat ipsilateral cortical lysates were resolved in 4–20% TGX precast gels and blotted for interleukin (IL)-1 β and IL-18. Total protein was used as a loading control. The cleaved, mature bioactive form of IL-1 β was found to be significantly increased 4–48 h after penetrating ballistic-like brain injury (PBBI) injury. The cleaved, mature form of IL-18 was found to be significantly increased 48–72 h after injury. Data are presented as mean $\pm$ standard error of the mean. Statistical significance was determined with one-way analysis of variance followed by Dunnett's *post hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. sham. $n = 5$ per group.

the entire surgery by a homeothermic heating pad. The head of the anesthetized rat was secured in the stereotaxic device for insertion of the PBBI probe. The PBBI apparatus consists of a penetrating probe (Kadence Science, Lake Success, NY), a stereotaxic frame (Kopf, Tujunga, CA) equipped with a specially designed probe holder, and a hydraulic pressure-pulse generator (4B080; MITRE, MA). The penetrating probe is made of a 20-gauge stainless steel tube with fixed perforations along one end that are sealed by a piece of airtight elastic tubing. The probe is secured on the probe holder with the unperforated end attached to the pulse generator angled at 50° from the vertical axis and 25° counter clockwise from the midline. After a midline scalp incision, a burr hole (diameter=4 mm) was created using a dental drill to expose the right frontal pole at +4.5 mm anterior-posterior, +2 mm medial-lateral to

bregma, as previously described.⁴² The PBBI probe was then advanced through the cranial window into the right hemisphere to a depth of 12 mm from the surface of the brain. Once the probe was in place, the pulse generator was activated by a computer to release a pressure pulse calibrated to produce a rapid expansion of the water-filled elastic tubing to induce an elliptical-shaped balloon (diameter=0.633 mm, duration=40 msec) to a volume equal to 10% of the total brain volume. This rapid inflation/deflation mimics the generation of a ballistic force shock wave, thereby creating a temporary unilateral cavity in the brain. After deflation, the probe was immediately retracted from the brain and the skin incision closed with wound clips. Sham surgeries consisted of the midline scalp incision, the right frontal burr hole, and the skin incision closed with wound clips.

FIG. 2. Rat ipsilateral cortical lysates were resolved in 4–10% or 10–20% TGX precast gels and blotted for caspase-1, apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC), X-linked inhibitor of apoptosis protein (XIAP), and nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3). Total protein was used as a loading control. The cleaved form of caspase-1 was found to be significantly increased 24–48 h after penetrating ballistic-like brain injury (PBBI) injury. The monomeric form of ASC was found to be significantly increased 4–48 h after injury. The cleaved form of XIAP (cleavage of XIAP disinhibits caspase-1) was found to be significantly increased 24–72 h after injury. NLRP3 was found to be significantly increased 24–48 h after injury. Data are presented as mean $\pm$ standard error of the mean. Statistical significance was determined with one-way analysis of variance followed by Dunnett's *post hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. sham. $n = 5$ per group.

FIG. 3. (A) Rat ipsilateral cortical lysates from a sham and 48 h penetrating ballistic-like brain injury (PBBI) animal were partially purified for the pyroptosome, chemically cross-linked, resolved by gel electrophoresis, and reacted with anti-apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC; mouse). Pyroptosome formation or ASC oligomerization is indicated by ASC laddering at 25 kDa, 50 kDa, and 75 kDa. (B) Rat ipsilateral cortical lysates were resolved in 10–20% TGX precast gels and blotted for gasdermin-D (GSDMD). Total protein was used as a loading control. GSDMD was found to be significantly increased 24–48 h after injury. Data are presented as mean $\pm$ standard error of the mean. Statistical significance was determined with one-way analysis of variance followed by Dunnett's *post hoc* test. * $p < 0.05$ vs. sham. $n = 5$ per group.

Immunoblotting

Sham animals were sacrificed 24 h post-surgery, while PBBI injured animals were sacrificed 4 h, 24 h, 48 h, and 72 h after injury. Rats were anesthetized with high dose 2–5% isoflurane for 5 min and decapitated in a guillotine. Ipsilateral and contralateral (to the injury) cortical tissue was dissected and flash-frozen in liquid nitrogen and stored at -80°C . Cortical tissues were lysed in 1 mL of buffer and the supernatants were resolved by gel electrophoresis. To control for protein loading, tissue homogenates were evaluated by bicinchoninic acid assay and loaded samples were normalized to 25 μg of protein. Protein was resolved in either 4–20% or 10–20% Criterion TGX Stain-Free Precast gels (BioRad) and transferred to either 0.2- μm or 0.45- μm pore polyvinylidene difluoride (PVDF) membranes for low molecular weight and high molecular weight proteins, respectively. Membranes were then blotted using antibodies (1:1000) for IL-1 β (CST), IL-18, caspase-1 (Novus), ASC (Santa Cruz), X-linked inhibitor of apoptosis protein (XIAP; R&D Systems), NLRP3 (Abcam) and GSDMD (Novus). Membranes were imaged using the ChemiDoc Touch Imaging System (BioRad). Total protein was resolved by activating the stain-free gels with ultraviolet light, resulting in the binding of a tri-halo compound to the tryptophan residues of all proteins in the gel. The tri-halo compound then migrates to the membrane during the protein transfer. Prior to applying enhanced chemiluminescence substrate, the membrane is imaged in the ChemiDoc system. Quantification

of band density was performed with Image Lab (BioRad) and data were normalized to total protein. Recent studies have shown that total protein, particularly total protein using stain-free technology, is superior to high abundance proteins such as β -actin and glyceraldehyde 3 phosphate dehydrogenase as loading control.^{48–50} High abundance proteins are easily overloaded, with studies showing that less than 2 μg of total protein should be loaded when using β -actin.⁵¹ A representative membrane with total protein is shown in Supplementary Figure 1 (see online supplementary material at www.liebertpub.com).

Partial purification of pyroptosome

ASC oligomers were partially purified from rat cortical lysates as previously described.⁵² Briefly, Sham and 48 h homogenates (equal to 50 μg of total protein) were filtered through 5 μm PVDF centrifugal filter tubes at $200 \times g$ for 5 min. The flowthrough was then centrifuged at $2700 \times g$ for 8 min. The supernatant was then discarded and the pellet was resuspended in 40 μL of 3[(3-chloromidopropyl) dimehtylammonio]-propanesulfonic acid (CHAPS) buffer (20 mmol/L HEPES-KOH, pH7.5, 5 mmol/L MgCl_2 , 0.5 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl-fluoride, protease inhibit cocktail, and 0.1% CHAPS). The samples were then spun at $10,000 \times g$ for 8 min and the pellet resuspended in 27.8 μL CHAPS; 2.2 μL of 2 mM disuccinimidyl suberate (Thermo

FIG. 4. Confocal images of free-floating rat brain sections stained with 2-(4-amidnophenyl)-1H-indole-6-carboximide (DAPI; blue), neuronal nuclei (NeuN; green), apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC; red), and glial fibrillary acidic protein (GFAP; white). (A) Immunoreactive cells from a representative sham animal and 10% penetrating ballistic-like brain injury (PBBI) animal 24 h after injury at $100 \times$ magnification. ASC co-localizes with NeuN+ cells predominantly in the perilesional cortex (white arrows). Further from the lesion there are fewer ASC/NeuN double positive cells. (B) Whole-brain sections from a representative sham animal and 10% PBBI animal 48 h after injury. Sections show that reactive GFAP+ astrocytes are more prominent in the ipsilateral hemisphere, particularly around the PBBI lesion. White boxes in the whole-brain images are shown at $100 \times$ magnification. (C) Numerous ASC immunoreactive cells are present in the PBBI cortical perilesional area and PBBI intact cortex ipsilateral to lesion. Fewer cells are present in contralateral cortex and cells are absent in Sham. The ASC staining pattern in the 48 h perilesional and intact cortex is similar to that seen in the intact cortex at 24 h (A). GFAP+ astrocytes in the ipsilateral cortex appear ramified with thicker processes (rows 2 and 3), compared with the GFAP+ astrocytes, in the sham and contralateral cortices (rows 1 and 4). However, GFAP+ astrocytes do not express ASC. ASC is present in neurons at 24 h but also in cells with non-neuronal morphologies at 24 h and 48 h. Scale bars on whole-brain images are 1000 μm and $100 \times$ images are 20 μm .

Scientific, Waltham, MA) for 10 min at room temperature to cross-link ASC oligomers. An equal volume of 2×Laemli buffer was added, and samples were boiled for 7 min and subjected to gel electrophoresis on 10–20% Criterion TGX Stain-Free Precast gels (BioRad) and immunoblot analysis.

Histopathology and immunohistochemistry

Rats were anesthetized with high-dose 2–5% isoflurane for 5 min and perfused transcardially with 0.9% saline, followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were dissected out and post-fixed in 4% PFA for 12 h.

Hematoxylin and eosin (H&E). Animals were sacrificed 1 week post-injury. After post-fixation, brains were then transferred to phosphate-buffered saline (pH 7.4), then paraffin embedded. Serial coronal sections (20 μ m) were cut from 4.0 to –7.0 mm from bregma. Sections were mounted on Superfrost plus microscopic slides and H&E stained.

Immunostaining. Animals were sacrificed 48 h and 12 weeks post-injury. After post-fixation, brains were then transferred to a 30% sucrose solution for 24 h and then frozen in M1 embedding matrix or GFM tissue freezing medium using dry ice and stored at –80°C before cryosectioning. Serial coronal sections (40 μ m) were cut from 4.0 to –7.0 mm from bregma. A group of 24 serial sections were collected at 960 μ m intervals and stored free-floating in cryopreserve prior to immunostaining. Samples were assessed with the following primary antibodies with the indicated dilutions: neuronal nuclei (NeuN) 1:100; glial fibrillary acidic protein (GFAP) 1:800; ionized calcium-binding adapter molecule 1 (Iba-1) 1:400; IL-1 β 1:250; and ASC 1:1000. Appropriate goat anti-mouse 488, goat anti-chicken 488, goat anti-rabbit 594, and goat anti-guinea pig 647 (Life Technologies; 1:250) secondary antibodies also were used. Sections were incubated with 2-(4-amidnophenyl)-1H-indole-6-carboximide (DAPI) prior to mounting and imaging. High-resolution fluorescent images were generated on a confocal microscope and post-processed using Imaris software (version 8.0.1).

Stereological counts of microglia subtypes

Unbiased stereological counts were conducted on serial sections immunostained with Iba-1 and ASC using StereoInvestigator. A 500×500 μ m counting grid and 75×75 μ m counting frame was used to estimate the number of microglia in the sham cortex and contralateral and ipsilateral cortices of each animal ($n=5$). Microglia were counted into four morphological subtypes related to activation state as described by Torres-Platas and colleagues.⁵³ Each microglia phenotype count was further divided into cells expressing ASC (double-labeled) or cells not expressing ASC. Therefore, eight total markers were used to quantify ASC expression within the different microglia morphology.

Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA). All measures were expressed as mean $\pm$ standard error of the mean (SEM), with $p \leq 0.05$ considered significant in all statistical tests. Immunoblot results were compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons. Stereological results were compared using both one-way and two-way ANOVA followed by Tukey's multiple comparisons.

Results

PBBI induces the expression of inflammatory cytokines and inflammasome proteins

To test for the production of inflammatory cytokines after PBBI, the temporal profile of IL-1 β and IL-18 was assessed for the ipsilateral cortex between 4 and 72 h after injury. Cortical expression of both IL-1 β and IL-18 was significantly upregulated after PBBI, compared with sham (Fig. 1). The mature bioactive form of IL-1 β was significantly increased 4 to 48 h post injury, with peak expression levels occurring at 24 h and subsiding by 72 h. Expression of mature IL-18 significantly increased after IL-1 β between 48–72 h post-injury, peaking at 72 h. Consistent with the biological activity of IL-1 β , H&E-stained PBBI brain sections showed clearing of necrotic tissue and lining of the lesion cavity with inflammatory cells by 1 week post-injury (Supplementary Fig. 2; see online supplementary material at www.liebertpub.com).

Next, to gain insights into activation of these cytokines, expression of upstream inflammasome proteins involved in their proteolytic cleavage was assessed. The cleaved active form of caspase 1, ASC, cleaved XIAP, and NLRP3 were significantly increased 24–48 h after injury in the ipsilateral cortex, compared with sham (Fig. 2). In contrast, there was no significant increase in expression of inflammasome proteins compared with sham in the contralateral cortical tissue (data not shown). These findings indicate that the inflammasome proteins and inflammasome-regulated cytokines are upregulated in the ipsilateral cortex after unilateral PBBI.

PBBI induces pyroptosis in the cortex 48 h after injury

To determine if pyroptosis was induced by the increased expression of inflammasome proteins and cytokines in the ipsilateral cortex, ASC oligomerization and GSDMD were assayed. Formation of the pyroptosome, indicated by ASC oligomerization and presence of GSDMD (a protein implicated in pyroptotic pore formation), are key features of pyroptosis. Oligomers of ASC were present only in the ipsilateral cortical lysates at 48 h but not in lysates from sham animals (Fig. 3). ASC monomers and oligomers were observed at 25 kDa, 50 kDa, and 75 kDa. These results indicate the

FIG. 5. Confocal images of free-floating rat brain sections stained with 2-(4-amidnophenyl)-1H-indole-6-carboximide (DAPI; blue), ionized calcium-binding adapter molecule 1 (Iba-1; green) and apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC) or interleukin (IL)-1 β (red). Top panels (A) show whole-brain sections from a representative 10% penetrating ballistic-like brain injury (PBBI) animal 48 h after injury. Sections show Iba-1+ microglia widely dispersed throughout the brain. White boxes in the whole-brain images are shown at 100× magnification. Panels (B) and (C) show numerous immunoreactive cells around the sham cortex, PBBI perilesional area, PBBI intact ipsilateral dorsal cortex, and contralateral cortex. (B) Iba-1 and ASC double positive cells are present in the ipsilateral hemisphere. The Iba-1 and ASC co-labeled cells in the cortex are either large and round with few thin, wispy processes (row 2) or hypertrophied with stubby and swollen short branches in the intact dorsal cortex (row 3). Also present in the perilesional cortex are Iba+ and ASC+ cells that appear monocytic with a small round cell body and no processes (row 2). The sham and contralateral cortices contain microglia with thin elongated and highly branched processes, with few Iba+ cells expressing low levels of ASC, compared with injured cortices (row 1 and 4). Similar to the Iba-1/ASC staining, in (C) Iba-1 and IL-1 β co-labeled cells are predominantly present in the ipsilateral cortex. Double positive cells are morphologically large with round/hypertrophied cell bodies and short processes. Scale bars on whole brain images are 1000 μ m and 100× images are 20 μ m.

supramolecular assembly of the pyroptosome at 48 h after PBBI. Immunoblot analysis of GSDMD showed a significant increase in expression of GSDMD 24 h and 48 h after PBBI. Taken together with evidence for pyroptosome formation, the increase in GSDMD expression demonstrates a role for pyroptosis in secondary cell death mechanisms after PBBI.

Different cell types produce inflammasome protein ASC at 24 h, compared with 48 h after PBBI

To determine the cellular source of the mediators of inflammation, systematic double-immunostaining of tissue sections for ASC or IL-1 β with cell type specific markers were used to investigate their contribution in neurons, astrocytes, and microglia as potential ASC sources at 24 and 48 h post-PBBI. Brain sections were co-stained for ASC, NeuN, GFAP, and Iba-1. As shown in Figure 4A, ASC was expressed predominantly in the ipsilateral hemisphere of the PBBI brain. At 24 h post-PBBI, NeuN-positive cells in the perilesional cortex strongly co-expressed ASC when compared with the NeuN positive cells in the intact cortex. NeuN-positive cells in the contralateral cortex and sham cortex exhibited a speckled baseline pattern of ASC staining (Fig. 4A). By 48 h post-PBBI, NeuN positive cells in the perilesional cortex, the intact cortex, contralateral cortex, and sham cortex all showed baseline ASC speckled patterning (Fig. 4C).

GFAP immunoreactivity was more prominent in the ipsilateral hemisphere, compared with both the contralateral hemisphere and the sham brain, at 48 h after injury (Fig. 4B). The morphology of astrocytes in the ipsilateral and contralateral hemispheres also differed. In the ipsilateral side, the astrocytic somas appeared hypertrophic and processes appeared to be thicker, indicative of highly ramified reactive astrocytes (Fig. 4C). In the perilesional cortex, GFAP staining was more punctate and less intense, possibly indicating astrocytic cell death (Fig. 4C, row 2). Although we did not observe cells co-staining with ASC and GFAP at 48 h, other cells in the injured cortex appeared to stain positively for ASC. These results indicate that the major cellular source of ASC may vary between 24 h and 48 h after PBBI.

ASC and IL-1 β proteins are expressed in Iba-1+ cells 48 h after PBBI

Morphologically distinct Iba-1 positive cells were present in the ipsilateral and contralateral cortices 48 h after PBBI and sham cortices. Resting microglia demonstrated two different morphologies including either narrow and elongated or small and round cell bodies. Both resting cell body types exhibited thin and highly branched long processes and were found throughout the contralateral hemisphere and in the sham cortex (Fig. 5). Few of these resting microglia expressed ASC in the microglial cell bodies. The “primed” microglia had larger cell bodies, compared with resting microglia, but highly branched processes. Primed microglia were present in both the sham and ipsilateral and contralateral injured cortices. Compared with primed microglia, activated and ameboid microglia displayed more hypertrophied cell bodies with stubby and swollen short processes. These cells were present throughout the intact dorsal cortex ipsilateral to the lesion and the perilesional cortex. Also present in the perilesional cortex were small round cells expressing Iba-1, indicative of infiltrating monocytes. These perilesional cells highly co-expressed ASC in both the cytoplasm and nucleus of the cells (Fig. 5B, second row). In the intact, dorsal regions of the cortex, the Iba⁺ ameboid and activated microglia co-expressed ASC throughout the hypertrophied cell body and in the

stubby short microglial processes. The pattern of IL-1 β was similar to that of ASC. These observations indicate that reactive and active microglia upregulate the expression of inflammasome protein ASC and IL-1 β 48 h after PBBI.

The number of activated microglia expressing ASC significantly increase in injured cortex after PBBI

We conducted unbiased stereology procedures to estimate the number of microglia subtypes expressing ASC 48 h after injury. The microglia subtypes were categorized as resting, primed, activated and ameboid (Fig. 6A). There was a significant increase in the total number of microglia in the ipsilateral cortex, compared with both the contralateral and sham cortices and no significant increase in the number of microglia in the contralateral cortex, compared with sham (Fig. 6B). There also was a 5-fold increase in the number of microglia expressing ASC in the ipsilateral cortex 48 h after injury, compared with sham and the contralateral cortex (Fig. 6C). The number of activated and ameboid microglia significantly increased in the ipsilateral cortex, compared with sham and the contralateral cortex (Fig. 6D). The number of activated and ameboid microglia expressing ASC also significantly increased in the ipsilateral cortex (Fig. 6E). Therefore, PBBI induces a significant increase in activated and ameboid microglia expressing ASC.

Elevated ASC staining in activated microglia in the internal capsule and cortex persists until 12 weeks after PBBI

PBBI recapitulates the remote neuronal and axonal degeneration commonly seen in TBI patients. To determine if activated microglia continue to express ASC at longer survival periods and potentially produce secondary injury, PBBI brain sections 12 weeks after injury were stained for ASC and Iba-1. Iba-1 positive cells were present throughout the brain, but microglia in the ipsilateral cortex and underlying internal capsule, a remote region away from the initial injury site with well-documented persistent axonal degeneration, showed more intense Iba-1 staining. At higher magnification, the microglia exhibited an activated and ameboid morphology with ASC co-localization (Fig. 7A, 7B). These results indicate that activated microglia express inflammasome markers up to 12 weeks post-injury.

Discussion

This study provides several new findings that clarify the underlying pathophysiology of secondary injury mechanisms after severe TBI. First, PBBI induces inflammasome protein expression in neurons and microglia that is associated with inflammatory cell death. Second, time course studies revealed that the predominant cell types expressing inflammasome proteins switch from neurons to microglia between 24 and 48 h after injury. Third, the number of activated and ameboid-type microglia expressing inflammasome proteins significantly increases at 48 h after PBBI, compared with earlier post-traumatic periods. Finally, inflammasome protein expression in activated and ameboid microglia persists in the injured brain up to 12 weeks after injury. These findings indicate that activation of the inflammasome and pyroptosis participates in the acute and more chronic secondary inflammatory responses to PBBI and that therapeutic interventions targeting microglial derived pro-inflammatory processes may have a broader therapeutic window for treatment than initially assumed.

FIG. 6. (A) Representative images of microglia that were used for stereological counts of microglia subtypes based on morphology: resting, primed, activated, and ameboid as previously defined by Torres-Platas and colleagues.⁵³ (B) The number of ionized calcium-binding adapter molecule 1 (Iba-1)+ cells significantly increased in the ipsilateral cortex 48 h after injury when compared with both contralateral and sham. There was no significant increase in the number of Iba-1+ cells in the contralateral cortex compared with sham. (C) The total number of Iba and apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC) double-positive cells was significantly increased (5-fold) in the ipsilateral cortex, compared with sham cortex. (D) After injury, the microglia took on a significantly more activated and ameboid morphology in the ipsilateral cortex compared with both sham and contralateral cortex. (E) The morphologically activated and ameboid Iba+ cells had a significant increase in ASC expression when compared with the Iba+ cells in the sham or contralateral cortex. In contrast, morphologically resting or primed Iba+ cells had lower amounts of ASC expression. With an increase in microglial activation, there is an increase in expression of ASC. Data are presented as mean $\pm$ standard error of the mean. Statistical significance was determined with one-way analysis of variance followed by Tukey's *post hoc* test (B and C) and two-way analysis of variance followed by Tukey's *post hoc* test (D and E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 5$ per group.

Neuroinflammation contributes to secondary injury responses to TBI, which aggravates the primary injury.⁵⁴ Inflammatory cytokine expression after TBI has been shown to increase immediately after injury and remain elevated for days to weeks thereafter.^{23,55} Our study shows that inflammasome cytokine expression increases acutely after PBBI and supports previous findings that IL-1 β messenger RNA and protein increase immediately after injury with the expression of IL-1 β protein remaining elevated through 7 days after injury. Therefore, the time course of IL-1 β and IL-18 expression described in the ipsilateral cerebral cortex (Fig. 1) is consistent with published findings.^{44,46}

The inflammasome acts as the master-regulator of IL-1 β and facilitates the innate immune response after CNS injury.^{26,56–58} Our results show increased levels of NLRP3 inflammasome proteins (NLRP3, caspase-1, and ASC) acutely after injury, indicating that activation of the NLRP3 inflammasome plays a significant role inducing the innate immune response after PTBI. In addition to NLRP3, other inflammasomes such as the NLRP1 also may activate after injury. XIAP is a protein that has been demonstrated to be associated with the NLRP1 inflammasome and we found a signif-

icant increase in cleaved XIAP in the PBBI cortical tissue (Fig. 2). Studies in other rodent models of TBI have reported model-dependent time courses of inflammasome expression. For example, in the moderate fluid-percussion injury model, inflammasome protein expression in the injured cortex was elevated as early as 15 minutes after injury and remained elevated through 24 h.²³ In the controlled cortical impact model, inflammasome protein expression in the injured cortex was significantly elevated 4, 7, and 14 days after injury.²⁹ By comparison, in the more severe PBBI model, caspase-1, ASC, cleaved XIAP, and NLRP3 expression in the cortex was increased by 4 h after injury and remains elevated through 72 h. TBI is a heterogeneous condition and may result from many different types of insults and severities, leading to variable inflammatory responses.⁵⁹ The observed differences in the temporal profile of inflammasome expression between the various TBI models are likely to correspond to model-specific responses and tissue vulnerability patterns produced by the different models.

The biochemical techniques employed in this study to evaluate cell death are specific to pyroptosis (Fig. 3; i.e. ASC oligomerization and increased levels of gasdermin D).^{38–41} These endpoints

FIG. 7. Adjacent free-floating 12 week post-penetrating ballistic-like brain injury (PBBI) rat brain sections stained with immunofluorescence. **(A)** Numerous immunoreactive cells around the sham cortex, PBBI perilesional area, PBBI intact ipsilateral dorsal cortex, and contralateral cortex. Ionized calcium-binding adapter molecule 1 (Iba-1) and apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC) double positive cells are found in the ipsilateral cortex near the lesion (row 2). Similar to the morphology found 48 h after injury (i.e., panel **B**), the Iba-1 and ASC co-labeled cells are large and round in morphology with thin, wispy processes. Cells in the more intact dorsal cortex (row 3) are morphologically primed or active. **(B)** Iba-1 and ASC immunoreactive cells in the sham internal capsule (IC), ipsilateral IC, and contralateral IC. The ipsilateral IC contain more Iba-1+ cells when compared with both sham IC and contralateral IC. The morphologically activated microglia in the ipsilateral IC appear to co-express high levels of ASC. Cells in the sham IC and the contralateral IC appear to be resting or primed in morphology and express low levels of ASC. Scale bars on are 20 μ m.

are felt to be more selective for pyroptosis than non-specific assays such as terminal deoxynucleotidyl transferase dUTP nick end labeling staining or lactate dehydrogenase release. Nonetheless, crosstalk between the different cell death mechanisms (i.e., pyroptosis, apoptosis, oncosis) is a consideration when interpreting our findings.⁶⁰ While conventionally classified as separate injury processes,⁶¹ the limits of each cell death mechanism are increasingly less discrete.⁶⁰ It is more likely that *in vivo*, pyroptosis is not an

isolated process since a significant increase in caspase 8, 9, and 3 activity has been reported 4–24 h after PBBI and in the bax/bcl2 ratio at 72 h, indicating a role for both death receptor-mediated and mitochondrial-mediated apoptosis.^{62,63}

Our findings establish a role for the NLRP3 inflammasome and pyroptosis in the pathophysiology of PTBI, providing additional evidence that functional pattern recognition receptors recognize and respond to DAMPs. While the NLRP1 inflammasome has been

reported in TBI,^{23,30,31} NLRP3 has emerged as a relevant target after injury. It has recently been shown that NLRP3 is elevated in CSF after severe TBI in infants and children³² and both an NLRP3 blockade and NLRP3^{-/-} mice showed improved recovery after TBI.⁶⁴ The increased NLRP3 expression after PBBI is consistent with previous reports of microglial activation and involvement of the NLRP3 inflammasome in neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease.^{65–67} In the present study, ASC and IL-1 β cell-type specific expression indicates a role for the inflammasome in microglial activation as early as 48 h after PBBI. In the normal brain, microglia maintain brain homeostasis by continuously surveying for immunogens.^{68–71} Upon recognition of DAMPs released by dead or dying cells, microglia become activated.^{71–73} In response to injury, activated microglia produce pro-inflammatory cytokines and undergo a morphological change from resting to amoeboid-phagocytic microglia.^{53,74} The Iba-1 expressing microglia at 48 h after PBBI (Fig. 5) resemble microglia in the brain 24 h after an *in vivo* injection of lipopolysaccharide.⁷⁵ Therefore, the observed increase in the number of activated and amoeboid Iba-1/ASC co-expressing cells (Fig. 6) suggests that they are pro-inflammatory cells either differentiating from infiltrating monocytes⁷⁶ or activating from resident resting microglia.⁵³ Importantly, previous reports have concluded that amoeboid microglia confer neurotoxicity by expressing pro-inflammatory cytokines.^{71,74}

Our data also show that the inflammasome is activated predominantly in neurons 24 h after injury (Fig. 4). Previous studies in the PBBI model have shown a wave of acute neuronal death, indicated by Fluoro-Jade B-positive cells that peak between 6 and 24 h after injury.^{77,78} The expression of ASC positive and NeuN positive cells at the 24 h time-point is consistent with the temporal sequence of early neuronal cell death in this model and indicates that these ASC/NeuN positive cells are dying by pyroptosis. The change of cell-type expression of the inflammasome from neurons to microglia between 24 and 48 h (Fig. 4 and Fig. 5) could suggest that pyroptosis of neurons may lead to inflammasome activation in adjacent microglial populations. Although astrocytes did not express ASC 48 h after injury, it is possible that astrocytic inflammasome activation may occur in a more delayed fashion following PBBI, compared with other injury models.²⁵ The present observations also show that ASC expressing activated microglia remain in the PBBI brain up to 12 weeks post-injury in the cortex and internal capsule, an area distinct from the initial injury (Fig. 7). This result is interesting because microglia are mobile and may migrate to more remote regions, through white matter tracts.^{21,79} Recently it has been shown that microglia ASC specks contribute to the aggregation and spreading of amyloid- β , a hallmark of neurodegeneration in (AD).⁸⁰ It is possible that the amoeboid and activated microglia-expressing ASC and IL-1 β 48 h post-PBBI maintain a chronic pro-inflammatory state in the injured brain, which confers continued neurodegeneration.^{79,81} The state of chronic inflammation has previously been advanced in other models of TBI⁷⁹ and neurodegenerative disorders⁸¹ and likely participates in the long-term consequences of PTBI.

In summary, this study demonstrates that PBBI induces significant increases in inflammasome protein expression in the cerebral cortex of the brain that leads to inflammatory cell death. Based on time course studies, the predominant cell types expressing inflammasome proteins change from neurons to microglia between 24 and 48 h after injury. At 48 h after PBBI, there is a significant increase in the number of activated and amoeboid-type microglia expressing inflammasome proteins. These activated and amoeboid

microglia expressing inflammasome proteins persist in the injured brain up to 12 weeks post-injury. Our data suggest a scheme for microglial inflammasome activation and pyroptosis after PBBI as illustrated in Supplementary Fig. 3 (see online supplementary material at www.liebertpub.com). PBBI results in inflammasome activation and ASC speck formation leading to cleavage of pro-IL-1 β and pro-IL-18 to their mature bioactive forms. Additionally, caspase-1 cleaves GSDMD, leading to GSDMD pore formation and subsequent pyroptotic cell death. Our characterization of the NLRP3 inflammasome and pyroptosis provides new information that could be used to develop strategies to protect against the lesion environment and prevent progressive loss of tissue. Collectively, the data illustrate that resident brain cells actively participate in the innate immune response after PBBI and their persistence and mobility may help spread the injury effects to remote areas not initially damaged by the primary injury.

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Author Disclosure Statement

JPdRV, WDD, and RWK are managing members in InflamaCORE, LLC, a company dedicated to discovering novel diagnostic and therapeutic strategies targeting abnormal inflammasome activation in clinical conditions. For the other authors, no competing financial interests exist.

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