Increased Amyloid Precursor Protein and Tau Expression Manifests as Key Secondary Cell Death in Chronic Traumatic Brain Injury

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In testing the hypothesis of Alzheimer’s disease (AD)-like pathology in late stage traumatic brain injury (TBI), we evaluated AD pathological markers in late stage TBI model. Sprague–Dawley male rats were subjected to moderate controlled cortical impact (CCI) injury, and 6 months later euthanized and brain tissues harvested. Results from H&E staining revealed significant 33% and 10% reduction in the ipsilateral and contralateral hippocampal CA3 interneurons, increased MHCII-activated inflammatory cells in many gray matter (8–20-fold increase) and white matter (6–30-fold increased) regions of both the ipsilateral and contralateral hemispheres, decreased cell cycle regulating protein marker by 1.6- and 1-fold in the SVZ and a 2.3- and 1.5-fold reductions in the ipsilateral and contralateral dentate gyrus, diminution of immature neuronal marker by two- and onefold in both the ipsilateral and contralateral SVZ and dentate gyrus, and amplified amyloid precursor protein (APP) distribution volumes in white matter including corpus callosum, fornix, and internal capsule (4–38-fold increase), as well as in the cortical gray matter, such as the striatum hilus, SVZ, and dentate gyrus (6–40-fold increase) in TBI animals compared to controls (P’s < 0.001). Surrogate AD-like phenotypic markers revealed a significant accumulation of phosphorylated tau (AT8) and oligomeric tau (T22) within the neuronal cell bodies in ipsilateral and contralateral cortex, and dentate gyrus relative to sham control, further supporting the rampant neurodegenerative pathology in TBI secondary cell death. These findings indicate that AD-like pathological features may prove to be valuable markers and therapeutic targets for late stage TBI.

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Materials and Methods

Subjects

All experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of South Florida, Morsani College of Medicine. Rats were housed two per cage in a temperature- and humidity-controlled room that was maintained on 12/12-h light/dark cycles. They had free access to food and water. All necessary steps were performed to minimize animal pain and stress throughout the study. Two-month-old Sprague–Dawley male rats (Harlan Laboratories, Indianapolis, IN) served as subjects and either exposed to sham or TBI surgery. All studies were performed by personnel blinded to the treatment condition.

Surgical procedures

Two-month-old Sprague–Dawley rats (n = 20) were subjected to either TBI using a controlled cortical impactor (CCI) (n = 14) or sham control (no TBI) (n = 6) (Pittsburgh Precision Instruments, Inc., Pittsburgh, PA). Deep anesthesia was achieved using 1–2% isoflurane, and it was maintained using a gas mask. All animals were fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). After exposing the skull, coordinates of −0.2 mm anterior and −0.2 mm lateral to the midline were used to impact the brain at the fronto-parietal cortex with a velocity of 6.0 m/sec reaching a depth of 1.0 mm below the Dura matter layer and remained in that position for 150 msec. The impactor rod was angled 15° vertically and 0.2 mm lateral to AP 3.8 mm in all 24 rats. Cell proliferation, differentiation into immature neurons, activated MHCII (OX6), respectively. Positive stains were analyzed with a Nikon Eclipse 600 microscope and quantified using the optical fractionator probe of unbiased stereological cell counting technique. The estimated volume of APP and MHCII-positive cells was examined using the Cavalieri estimator probe of unbiased stereological cell counting technique. The estimated volume of APP and MHCII-positive cells was examined using the Cavalieri estimator probe of unbiased stereological cell counting technique.
forming a virtual grid (125 × 125 μm), which was randomly generated and placed by the software within the outlined structure. The Cavalieri estimator is typically executed using a point grid spaced equally both across and down. The grid space used was of 100 μm in order to cover the entire region of interest (ROI) (Mayhew, 1991). Section thickness was measured in all counting sites.

**Immunofluorescent staining**

Phosphorylation of tau at Ser202/Thr205 and oligomeric tau stainings were carried out using the epitope AT8 and T22, respectively. Both tau species were conducted on every 1/3 sections, 40 μm thick, coronal brain sections. Coronal brain sections were washed three times for 10 min in 0.1 M TBS. Six sections were incubated with saline sodium citrate (SSC) solution at PH 6.0 for 40 min at 80°C for antigen retrieval. Then, samples were blocked for 60 min at room temperature with 8% normal goat serum (Invitrogen, CA) in 0.1 M TBS containing 0.1% Tween 20 (TBST) (Sigma, St. Louis, MO). Sections were then incubated overnight at 4°C with mouse monoclonal anti-AT8 (p-Ser202/Thr205; 1:100; MN1020 Thermo Scientific, Waltham, MA) or rabbit polyclonal anti-T22 (1:600; ABN454 millipore) with 10% normal goat serum. Then, the sections were washed five times for 10 min in 0.1 M TBST and soaked in 5% normal goat serum in 0.1 M TBST containing corresponding secondary antibodies, goat anti-mouse IgG-Alexa 488 (green) (1:1400; Invitrogen) or goat anti-rabbit IgG-Alexa 594 (red) (1:1500; Invitrogen) for 90 min. Finally, coronal sections were washed five times for 10 min in 0.1 M TBST and three times for 5 min in 0.1 M TBS, processed for 1:300 Hoechst 33258 (bisbenzimide H; 33258 trihydrochloride, Sigma) for 30 min, washed in 0.1 M TBS, and cover-slipped with Fluoromount (Aqueous Mounting Medium; sigma F4680). Coronal sections were examined using a confocal microscope (Olympus, Shinjuku, Tokyo, Japan). Control studies included exclusion of primary antibody substituted with 5% normal goat serum in 0.1 M TBS. No immunoreactivity was observed in these controls.

**Analysis of fluorescent staining**

From all sections, approximately 4–6 images of 20 μm magnification were taken from each coronal section using confocal microscopy (Olympus) and analyzed with imageJ (National Institutes of Health, Bethesda, MD). All photomicrographs were converted to gray scale. Background was selected from blank control images, and subsequently used to subtract the background from all images. The same threshold was used for all images. Thereafter, the staining intensity of each section was quantified as the average optical density readings of four randomly selected areas within that section. The final staining intensity of each group resulted as the average of each staining intensity per section.

**Statistical analysis**

For data analyses, contralateral and ipsilateral corresponding brain areas were used as raw data providing two sets of data per treatment condition (TBI vs. sham control), therefore one-way analysis of variance (ANOVA) was used for group comparisons, followed by subsequent pairwise comparisons; post hoc tests Bonferroni’s test. All data are represented as mean values with ± SEM. Statistically significance was set at P < 0.05 for all analyses.

**Results**

Preliminary analyses of these data, comparisons between sham control ipsilateral and sham control contralateral side, throughout all brain areas analyzed, demonstrated not significant differences (P > 0.05). Thus, the data from both sides of the sham group were combined.

**Decreased cell proliferation, impaired neurogenesis, and rampant hippocampal cell loss in late stage TBI**

ANOVA revealed significant treatment effects on cell proliferation (Ki67+) in SVZ and DG (F2,15 = 39.51, P < 0.0001; F2,15 = 45.35, P < 0.0001), as well as on neurogenesis (DCX+) in SVZ and DG (F2,15 = 17.60, P < 0.0001; F2,15 = 16.47, P < 0.0001) (Fig. 1). Post hoc test revealed significant decrements in cell proliferation (Ki67+) in the ipsilateral SVZ and DG of TBI animals relative to the contralateral SVZ and DG respectively of TBI animals (P’s < 0.0001). Similarly, there were significant reductions in cell proliferation (Ki67+) in the ipsilateral (P’s < 0.001), and the contralateral (P’s < 0.05) SVZ and DG of TBI animals compared to sham animals. In addition, there were significant diminution of neurogenesis (DCX+) in the ipsilateral SVZ and DG of TBI animals compared to the contralateral SVZ and DG respectively of TBI animals (P’s < 0.0001). Likewise, there were significant decrements in neurogenesis (DCX+) in the ipsilateral SVZ and DG (P’s < 0.0001) of TBI animals compared to sham animals. DCX+ cells were significantly reduced on the contralateral (P’s < 0.001) DG of TBI animals, but not the contralateral (P > 0.05) SVZ of TBI animals compared to sham animals. Furthermore, ANOVA revealed significant treatment effects on hippocampal CA3 cells loss (F2,15 = 54.27, P < 0.0001). Post hoc test revealed significant hippocampal CA3 cells loss in the ipsilateral side compared to the contralateral side of TBI animals (P < 0.05). There were 33% and 10% reduction of hippocampal CA3 cells in the ipsilateral and contralateral side, respectively of hippocampal CA3 interneurons of TBI animals compared to sham animals (P’s < 0.0001) (Fig. 1).

**Region-specific exacerbation of MHCI+ activated cells in gray and white matter areas in late stage TBI**

The estimated volume of MHCI+ activated cells was quantified using stereological techniques. ANOVA revealed overall significant treatment effect on inflammation in cortical and subcortical regions as evidenced by MHCI+ immunostaining in all gray matter regions examined here as follows: cortex (F2,15 = 47.78, P < 0.0001); striatum (F2,15 = 40.54, P < 0.0001); DG (F2,15 = 43.16, P < 0.0001); hilus (F2,15 = 35.44, P < 0.0001); SVZ (F2,15 = 80.04, P < 0.0001); and thalamus (F2,15 = 6.484, P < 0.001) (Fig. 2). Posthoc test revealed significant upregulations of activated MHCI+ cells in the ipsilateral side of TBI animals compared to their contralateral side across all gray matter areas analyzed (P’s < 0.0001), except hilus and thalamus (P > 0.05). There were significant upregulations of activated MHCI+ cells in both ipsilateral and contralateral gray matter areas of TBI animals in all gray matter regions examined compared to sham animals (P’s < 0.0001) (Fig. 2).

Similarly, ANOVA demonstrated significant treatment effects on inflammation in several white matter subcortical regions as evidenced by MHCI+ immunostaining in all regions examined here as follows: corpus callosum (F2,15 = 786.2, P < 0.0001); fornix (F2,15 = 21.92, P < 0.0001); and internal capsule (F2,15 = 198.9, P < 0.0001) (Fig. 3). Posthoc test revealed no significant difference of activated MHCI+ cells in the ipsilateral side of TBI animals compared to their contralateral side across all white matter areas analyzed (P > 0.05). However, there were significant upregulations of activated MHCI+ cells in both ipsilateral and contralateral side of TBI animals across all white matter areas analyzed compared to sham animals (P’s < 0.0001) (Fig. 3).
Fig. 1. Reduced cell proliferation, impaired neurogenesis, and increased hippocampal cell loss in late stage TBI. Part A, quantitative stereological analysis revealed significant decrements in cell proliferation and neurogenesis in the SVZ and DG after late stage TBI compared to sham animals ("C3" < 0.05). Part A, bottom right, quantitative analyses of total number of CA3 neurons revealed a significant increase in neuronal cell loss after TBI compared to sham animals ("C3" < 0.05). Part B, top, photomicrographs are representative coronal brain sections of the corresponding ipsilateral and contralateral side of the SVZ, and DG regions stained with a cell proliferation marker (Ki67), an immature neuronal marker (DCX) at 6 months post TBI of sham, and TBI animals. Arrows denote positive staining. Part B, bottom, photomicrographs are representative coronal brain sections staining with H&E from ipsilateral and contralateral CA3 area of the hippocampus of sham, and TBI animals. Arrows denote dark pink/purple cells characteristic of shrunken and condensed nuclei and hypereosinophilic cytoplasm, and neuronal cell loss within the CA3 region to the contralateral and ipsilateral side respectively in TBI animals. Scale bar: 50 μm. "P < 0.05; "P < 0.05, ""P < 0.01, """"P < 0.001. ns, not significant. Ki67 and DCX data are expressed as estimated # of positive cells. H&E data are expressed as total # of cells. Data are expressed as mean ± SEM.
Fig. 2. Increased MHCII⁺ activated cells in proximal and remote gray matter areas in late stage TBI. Part A, stereological analysis of MHCII⁺ cells estimated volume in cortex, striatum, DG, and SVZ revealed significant upregulation of activated MHCII⁺ cells in the ipsilateral side of TBI animals compared to their contralateral side across all gray matter areas analyzed ($P_s < 0.0001$), except hilus and thalamus ($P > 0.05$). There were significant upregulations of activated MHCII⁺ cells in both ipsilateral and contralateral gray matter areas of TBI animals ($P_s < 0.0001$) compared to sham animals ($P_s < 0.05$). Part B, photomicrographs are representative coronal brain sections showing gray matter areas ipsilateral to injury stained with M1 activated immune cells marker (MHCII) 6 months post TBI injury. Arrows indicate positive staining for activated MHCII⁺ cells in cortex, striatum, DG, hilus, SVZ, and thalamus. Scale bar = 50 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant. MHCII data are expressed as estimated volume of positive cells. Data are expressed as mean ± SEM.
Overexpression of APP+ cells in gray and white matter areas in late stage TBI

The estimated volume of APP+ expression was quantified using stereological techniques. The whole brain was examined to reveal TBI-induced APP+ overexpression in both gray and white matter areas (Fig. 4). ANOVA revealed overall significant treatment effect on APP+ cells in both cortical and subcortical regions as evidenced by APP+ immunostaining in all gray matter regions examined here as follows: cortex (F2,15 = 256.3, P < 0.0001); stratum (F2,15 = 31.47, P < 0.0001); DG (F2,15 = 21.37, P < 0.0001); hilus (F2,15 = 18.68, P < 0.0001); internal capsule (F2,15 = 4.435, P < 0.05), except thalamus (F2,15 = 0.222, P > 0.05). Posthoc test revealed significant overexpression of APP+ cells in the ipsilateral side of TBI animals compared to their contralateral side across all gray matter areas analyzed (P's < 0.0001), except SVZ (P > 0.05). There were significant overexpressions of APP+ cells in both ipsilateral and contralateral in all gray matter areas analyzed of TBI animals compared to sham animals (P's < 0.0001) (Fig. 4).

Fig. 3. Increased MHCII+ activated cells in proximal and remote white matter areas in late stage TBI. Part A, stereological analysis of MHCII+ cells estimated volume in corpus callosum, fornix, and internal capsule revealed no significant differences of activated MHCII+ cells in the ipsilateral side of TBI animals compared to their contralateral side across all white matter areas analyzed (P's > 0.05). There were significant upregulation of activated MHCII+ cells in both ipsilateral and contralateral side of TBI animals (P's < 0.0001) across all white matter areas analyzed compared to sham animals (P's < 0.05). Part B, photomicrographs are representative coronal brain sections showing white matter areas ipsilateral to injury stained with M1 activated immune cells marker (MHCII) 6 months post TBI injury. Arrows indicate positive staining for activated MHCII+ cells in corpus callosum, fornix, and internal capsule. Scale bar = 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. MHCII data are expressed as estimated volume of positive cells. Data are expressed as mean ± SEM. Scale bar = 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. Data are expressed as mean ± SEM.
Fig. 4. Overexpression of intra-neuronal APP in proximal and remote gray matter areas in late stage TBI. Part A, stereological analysis of APP+ neurons estimated volume in cortex, striatum, DG, and hilus revealed significant increase of APP+ neurons in the ipsilateral side of TBI animals compared to their contralateral side across all gray matter areas analyzed (P s < 0.0001), except SVZ and thalamus (P > 0.05). There was significant overexpression of APP+ expressing neurons in both ipsilateral and contralateral gray matter areas of TBI animals (P s < 0.0001) compared to sham animals (P s < 0.05), except DG, hilus, SVZ, and thalamus (P s > 0.05). Part B, photomicrographs are representative coronal brain sections showing gray matter areas ipsilateral to injury stained with APP marker post TBI injury. Arrows indicate overexpression of intra-neuronal APP staining in cortex, striatum, DG, hilus, and SVZ. Scale bar = 50 µm. * P < 0.05, ** P < 0.01, *** P < 0.001; ns, not significant. APP data are expressed as estimated volume of positive cells. Data are expressed as mean ± SEM.
Similarly, ANOVA demonstrated significant treatment effects on overexpression of APP in white matter subcortical regions as evidenced by APP+ immunostaining in all regions examined here as follows: corpus callosum ($F_{2,15} = 297.34$, $P < 0.0001$); fornix ($F_{2,15} = 121.4$, $P < 0.0001$); and internal capsule ($F_{2,15} = 190.2$, $P < 0.0001$) (Fig. 5). Posthoc test revealed significant overexpressions of APP+ cells in the ipsilateral side of TBI animals compared to their contralateral side across all white matter areas analyzed ($P's < 0.0001$). There were significant overexpressions of APP+ cells in both ipsilateral and contralateral white matter areas of TBI animals compared to sham animals ($P's < 0.0001$) (Fig. 5).

**Increased expression of phosphorylated tau (AT8) in the cortex and in the dentate gyrus of the hippocampus in late TBI**

The expression of phosphorylated tau, AT8+ cells, was quantified using immunofluorescent techniques and analyzed with Image J software. ANOVA revealed significant treatment effect on phosphorylated tau as evidenced by AT8+

![Image of graphs and photomicrographs showing overexpression of APP in different brain regions](image-url)
immuno
fluorescent staining in the frontal cortex ($F_{2,15} = 17.35, P < 0.0001$) and DG ($F_{2,15} = 6.895, P < 0.05$) (Fig. 6). Posthoc test revealed significant increased expression of AT8+ staining in the soma of ipsilateral cortex of TBI animals compared to their contralateral cortex ($P < 0.0001$). There were significant upregulations of AT8+ staining within neurons of both ipsilateral and contralateral cortex of TBI animals compared to sham animals ($P's < 0.05$) (Fig. 6).

Evidence of oligomeric tau (T22) accumulation in the cortex and in the dentate gyrus of the hippocampus in late TBI

The expression of oligomeric tau, T22+ cells was quantified using immunofluorescent techniques and analyzed with Image J software. ANOVA revealed significant treatment effect on oligomeric tau as evidenced by T22+ immunofluorescent staining in the frontal cortex ($F_{2,15} = 8.933, P < 0.05$) and DG ($F_{2,15} = 23.43, P < 0.001$) of TBI animals (Fig. 7). Posthoc test revealed evidence of significant accumulation of T22+ tau species within the cytosol of cortical neurons and granular neurons in the DG of ipsilateral cortex of TBI animals compared to their contralateral cortex ($P < 0.05$). There were significant accumulation of T22+ tau within neurons of both ipsilateral and contralateral cortex of TBI animals compared to sham animals ($P's < 0.001$) (Fig. 7).

Discussion

The present study demonstrated the propagation of secondary injury in brain regions proximal and remote to the primary core impact of injury in late stage TBI, characterized by AD-like pathology. That a neurogenerative process involving aberrant APP+, AT8, and T22 accumulations, reminiscent of AD, plagues TBI suggests overlapping cell death mechanisms between acute and late stage brain disorders. This progression of APP overexpression in advanced TBI was accompanied by a persistent cell loss in hippocampal CA3 neurons, reduced number of proliferating precursor cells and immature neurons, in neurogenic niches (i.e., SVZ and DG), and closely associated with exacerbation of MHCII inflammatory cells in specific brain regions displaying APP+ overexpression. The significant accumulations of additional AD-like phenotypic markers including amyloid precursor protein (APP) and tau (phosphorylated tau AT8 and oligomeric tau T22) in ipsilateral and contralateral cortex, and dentate gyrus relative to sham control further support the neurodegenerative feature of the secondary cell death in TBI.

The rationale for measuring Ki67+ cells was to determine the influence of late stage secondary injuries on the overall proliferative capacity of cells within specific regions regardless of fate of differentiation. Further image analysis of the location of the positive Ki67 cells did not correlate with the location of
the MHCII+ cells, thus we discarded the possibility that MHCII+ cells were proliferating specifically at the neurogenic niches. Since the analysis showed a decreased on cell proliferation, then the next question was whether secondary injury also altered neurogenic capacity of the neurogenic niches. This required lineage specific characterization, which we pursued in this study using DCX as a marker of immature neurons. Clearly, additional lineage markers will be needed to fully assess the cell fate, but this will require a large study that is best addressed in the future. Collectively, these observations support our hypothesis that neurodegeneration in TBI progresses via an AD pathological mechanism involving APP+ overexpression.

The onset of cognitive deficits has been documented in TBI patients presenting with AD-like pathology (Fleminger et al., 2003; Vasterling et al., 2012; Aungst et al., 2014; Sharp et al., 2014). The detection of increased APP+ expression, in tandem with impaired neurogenesis, in the hippocampus may lead to dysfunctions in hippocampal-mediated cognitive performance (Reddy et al., 2010; Zhao et al., 2011). Of note, robust hippocampal plasticity likely participates in learning and memory consolidation, a crucial process in cognitive function (Scoville, 1954; Amaral and Witter, 1989; Nakashiba et al., 2008). That such hippocampal neurodegeneration, seen to underlie many of cognitive impairments of AD, similarly manifests in TBI may explain the evolution of learning and memory deficits in TBI. Indeed, cognitively impaired experimental TBI animals present with APP+ deposition with coincident apoptosis and reduced neurogenesis in the hippocampus (Murakami et al., 1998; Kempermann, 2002; Rola et al., 2006; Park et al., 2014). To date, these published reports document the progressive amyloidosis in acute and subacute TBI; the present findings of APP+ in late stage TBI support the notion that AD hippocampal neurodegeneration, including likely the accompanying cognitive impairment, is not transient, but permanent sequelae of the TBI event (Strittmatter and Roses, 1995; Gottlieb, 2000; Hernandez-Ontiveros et al., 2013; Soldatovic-Stajic et al., 2014; Lozano et al., 2015).

We previously characterized chronic neuroinflammation in brain regions remote from the primary impacted area at 2 months after TBI (Acosta et al., 2013). Although some studies have reported that MHCII is not a specific marker for Th1/M1 pro-inflammatory cells, in that MHCII is also expressed in some M2 anti-inflammatory cells, our study used a clone of OX-6 which is exclusively expressed as an MHCII antigen when immune cells are fully activated during inflammation responses (McLaurin et al., 1995; Marshall et al., 2013). Comparing our previous study (Acosta et al., 2013) and the present one based on MHCII analyses revealed that at 2 months post-TBI significant exacerbation of OX-6 positive activated MCHII cells were relegated to regions ipsilateral to injury including gray and white matter regions, whereas at 6 months post-TBI, there was a clear exacerbated inflammatory pathology characterized by increased OX-6 positive activated MCHII cells in the ipsilateral
area that trespassed into the contralateral side including anterior-proximal and remote-posterior to the original injury. Moreover, in the 2 months post-TBI model we demonstrated significant downregulation of Ki67-positive proliferating cells in ipsilateral SVZ and ipsilateral SGZ relative to sham control. Analysis of neurogenic niches, after 2 months post-TBI, revealed that only trends of reduced neurogenesis (DCX-positive immature neuronal cells) in ipsilateral SVZ and ipsilateral SGZ were detected relative to contralateral and sham control. However, in the 6 months post-TBI model, Ki67-positive proliferating cells were downregulated in both ipsilateral and contralateral SVZ and SGZ relative to sham control. In addition, there was a significant diminution of neurogenesis in both the ipsilateral and contralateral SVZ and SGZ relative to sham controls. After 2 months post-TBI, results from H&E staining demonstrated a significant decrease of hippocampal pyramidal neurons in the CA3 region ipsilateral to injury compared to sham control. There was no cell loss found in the contralateral side in the CA3 region at this 2-month TBI time point. In contrast, after 6 months post-TBI, a significant 33% and 10% cell loss was found in both the ipsilateral and contralateral side, respectively in the CA3 of the hippocampus. It is important to highlight that in the present 6 months post-TBI period, delayed secondary injury maintained a significant MHCI cells recruitment and activation in the ipsilateral hemisphere and such inflammatory response could now be seen trespassing or invading the contralateral side. Finally, laboratory evidence from acute and long-term studies suggests that many of the TBI models can lead to detrimental effect of secondary injuries across different time points after TBI (Scholzen and Gerdes, 2000; Hayward et al., 2010). In-depth investigations into long-term brain remodeling may reveal discreet inflammatory processes within the neurovascular unit, allowing a better understanding of TBI pathology and its treatment.

Gray and white matter degeneration in the present study manifested as increased volume of activated MHCI+ neuroinflammatory cells detected within and adjacent to areas exhibiting APP+ overexpression in proximal and distal areas from the mechanical injury. This localized neuroinflammation and APP+ overexpression following TBI remains a poorly understood cell death mechanism. The present study focused on characterizing the neuroinflammatory response at 6 months post TBI using OX-6 antibody against MHCI-expressing cells (Raivich et al., 1999; O’Keefe et al., 2002). Neuroinflammatory processes, characterized by activated immune cells including microglia, dendritic cells, macrophages and monocytes, play crucial roles in the development and progression of neurodegenerative diseases such as dementia and the accumulation of senile plaques in AD, but the mechanism of action, especially for TBI, is yet to be elucidated (Povlishock and Cristman, 1995; Cho et al., 2011; Mizuno et al., 2011). Notwithstanding, the inflammatory response has both pro-inflammatory (or classical) response and anti-inflammatory (Th2/M2 or alternative) response. MHCI is a marker for inflammation, but it is not a specific marker for pro-inflammatory response because it is also expressed in anti-inflammatory cells (Marshall et al., 2013). Furthermore, T helper (Th) cells-mediated immune response requires the expression of MHCI in antigen presenting cells (microglia, macrophages, dendritic cells) to activate both M1/Th1 and M2/Th2 responses. Our present study implicates a potential pathologial link between late stage TBI and AD as evidenced by activated neuroinflammatory cells and accumulation of APP+ in late stage TBI. Although traditionally shown to be expressed in neurons, APP+ cells are also detected in microglia and astrocytes (Haass et al., 1991). TBI as a risk factor for AD pathology may involve a dysfunctional immune clearance of APP+ and Aβ by activated MHCI+ cells and an aberrant overexpression of APP and Aβ (Selkoe, 1997; Sheng et al., 2003; Hickman et al., 2008). In support of the dysfunctional immune system hypothesis, deleting the TNFR1 in transgenic mice prevents learning and memory impairments (He et al., 2007). Additionally, injecting APPswe transgenic mice with lipopolysaccharide leads to an overexpression of APP and Aβ (Sheng et al., 2003). An aberrant overexpression of APP may precipitate during sub-acute stages and persists through late stage stages of TBI (Iwata et al., 2002; Uryu et al., 2007; Johnson et al., 2012; Mannix and Whalen, 2012).

We are cognizant that the present results are limited to a single time point whereby we evaluated neuroinflammation, altered neurogenesis and APP+ overexpression in an experimental model of late stage TBI. In addition, based on our present data, we recognize that our hypothesis warrants further investigations separate from the current study in order to further elucidate the mechanism of action by which inflammation influences the propagation and overexpression of APP in white and gray matter regions. Evaluation of the detrimental effect of secondary injuries across different time points after TBI is an important area of future research.
points (i.e., acute, sub-acute, and delayed) coupled with anti-inflammatory treatments to assess the modulatory role of the immune system in neurogenesis and APP expression in TBI will further provide information about possible mechanisms. Another limitation of the study is that the model of TBI here is unilateral injury. Accordingly, the subsequent pathology arising from this unilateral injury, including the observed AD-like histological alteration (i.e., APP overexpression within the ipsilateral TBI hemisphere), cannot be claimed as a laterality event, but rather a consequence of the model. A series of studies is now underway to fully capture the MHCII and APP interaction after TBI, in particular detailing the progression of APP overexpression on proximal and remote white and gray matter regions and the delayed exacerbation of MCHII inflammatory cells in specific brain regions displaying APP overexpression.

As seen in this study, exacerbation of activated immune cells was detected not only on ipsilateral to injury but also throughout the brain after 6 months from the initial injury. Treatments targeting the secondary cell death characterized by neuroinflammation post TBI have been proposed to manage the multifactorial nature of the disease progression after TBI (Tajiri et al., 2013; Acosta et al., 2014; Lozano et al., 2015). For instance, we have previously shown that combined therapy of human umbilical cord blood cells (hUCB) and granulocyte colony stimulating factor (G-CSF) optimally abrogated exacerbation of active inflammatory cells rescuing neurogenesis and ameliorating motor function deterioration in chronic TBI animals (Acosta et al., 2014). In addition, we have reported that selective inhibitors of nuclear export (SINE) sequenced TBI-induced neuroinflammation-related proteins (i.e., NF-kB, AKT, FOXO1), and it decreased TBI-induced cell death in the core impact area and improved motor and neurological outcomes (Tajiri et al., 2013).

Interestingly, in relation to APP and inflammation, it has been shown that hyperactivity of GSK-3, a promoter of inflammation, causes dysregulation on the APP cleavage and instigates its abnormal accumulation. Lithium, a GSK-3 inhibitor have been shown to have anti-amyloid properties and to have neuroprotective effects on hippocampal tissue (Piel et al., 2003). In addition, Curcumin, the spice from turmeric and curry has been shown to be highly antioxidant and anti-inflammatory and decrease the Aβ levels and plaque load in aged transgenic APP mice (tg2576) (Lim et al., 2001). Accordingly, an anti-inflammatory therapy could be a promising treatment to decrease inflammation and APP in diseases whereby primary and secondary damage are followed by neuroinflammatory responses. Previous studies have also demonstrated that attenuation of microglial activation after TBI is associated with decreases on Aβ precursor and amelioration of behavioral outcome after TBI. Minocycline, simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have all been shown to have anti-inflammatory effects, to mitigate increases in Aβ and to ameliorate TBI-induce behavioral deficits (Abrahamsson et al., 2009; Homsi et al., 2010). These studies support our hypothesis that manipulating the inflammatory response after TBI might reduce APP overexpression and other AD-like pathologic markers.

The present study highlights the pivotal role of APP in chronic neuroinflammation, in that the same brain regions with exacerbated inflammatory cells displayed aberrant APP accumulation, altogether suggesting a close pathological interaction between TBI and AD, at least in the long-term inflammatory response associated with the disease progression. Recognizing that APP closely accompanies late stage TBI suggests that treatments designed to target unloading aberrant APP accumulation may be therapeutic for TBI.

Authors' Contributions
S.A.A. and C.V.B. conceived the study, participated in its coordination, and wrote the manuscript. S.A.A., C.V.B., Y.K., and N.T. performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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