Deletion of NLRP3 gene blocks traumatic brain injury induced abnormal immune response in 3xTg AD mice

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Abstract

Traumatic brain injury (TBI) is a significant risk factor for the development of Alzheimer's disease (AD) and related dementia. In both TBI and AD, inflammation plays a pivotal role. Recent studies have found that TBI triggers activation of NLRP3 inflammasome in the brain whereas the NLRP3 inflammasome plays an important role in pathogenesis of AD. To evaluate the influence of TBI on the immune response and the importance of the NLRP3 inflammasome in mediating this process we have examined the immune profiles in the brain using a novel transgenic AD mouse line with the NLRP3 gene deleted. Briefly, a group of 3xTg and 3xTg/NLRP3$^{-/-}$ mice received a moderate TBI in the form of lateral fluid percusive injury FPI or sham surgery at the age of 4 months old, an age before the onset of AD. Injury-induced changes in immune profiling were assessed using flow cytometry, real-time quantitative PCR, or Western blot at the acute and subacute stages following TBI. We found that TBI altered immune profiles in 3xTg mice and NLRP3 gene knock out counteracts the injury effect with a significant sex-related difference. More specifically, at the acute stage after TBI in both male and female mice, TBI induced a significant infiltration of neutrophils, macrophages and γδ T-cells in the brains of 3xTg mice, however, NLRP3 knock down significantly blocked this injury effect in males and less so in females. We also found that NLRP3 gene knock down counteracted TBI-enhanced inflammatory cytokine IL1-β, TNF-α, and IL-17f expression. The abnormal immune profiling was not significant at 7 days post-injury, however, changes in cerebral vascular associated proteins including GFAP, AQP4, CD31, Occludin were observed in related to injury, NLRP3 and sex. In conclusion, our study has confirmed that TBI significantly alters immune profiles and vascular integrity in the context of predisposition of AD and the NLRP3 inflammasome is important in mediating these TBI-induced changes. Sex-related differences warrant further evaluation to define the role of NLRP3-related immune response and vascular pathology as a result to TBI and the predisposition to AD development.

Keywords: Traumatic brain injury, Alzheimer's disease, NLRP3 inflammasome, immune response, flow cytometry

Introduction

The intersection of traumatic brain injury (TBI) and Alzheimer's disease (AD) is a topic of much discussion due to the increased risk of developing AD after TBI.[1] TBI is the leading cause of death and disability for young people worldwide[1-3]. Following TBI, the primary injury induces irreversible and untreatable brain tissue damage. The subsequent secondary injury causes progressive neurodegeneration and delayed cell death[4,5]. Extensive studies have established that TBI leads to cognitive decline and dementia, and growing body of evidences have suggested that TBI accelerates AD development [6-12]. According to the United States Centers for Disease Control and Prevention (CDC), individuals with a history of moderate TBI have a 2-3 times higher risk of developing AD [2,3], and studies reported that TBI can lead to an earlier onset of AD pathology in as much as 4 to10 years earlier [5-12]. Studies have also shown that TBI accelerates beta-amyloid (Aβ) deposition and tau abnormalities, two pathological hallmarks of AD, and cognitive impairment [13,14].

Although those extensive epidemiological evidences have suggested that TBI accelerates the onset and severity of AD, the underlying mechanisms linking TBI to AD remain unclear. Among possible factors, inflammatory response is largely recognized as the major culprit as it is the common and essential player in both TBI and AD, and TBI-induced neuroinflammation persists for many years[15-19]. Following TBI, there is a robust inflammatory response characterized by infiltration of immune cells, activation of resident glial cells, and increase in the levels of inflammatory mediators including cytokines and chemokines, such as IL-1β, IL-18 and TNF-α[20-23]. TBI-induced inflammatory responses lasts for long time up to 47 years as reported in a recent study[23]. Accumulating evidence also suggests immune/inflammatory response as an essential driver for AD development. Pathologically, chronic glial activation and elevated levels of proinflammatory cytokines were observed in AD models and patients[24-27]. Such chronic inflammation can induce dysfunction of microglia cells causing inefficient clearance of Aβ species, as strong correlation of chronic inflammation to Aβ deposition and tau hyperphosphorylation is reported[28-31].

It is known that innate immunity plays a major role in inflammatory responses following TBI[32,33]. Inflammasomes are critical regulators of the innate immune response to pathogen-associated and danger-associated molecular patterns[34]. Recently the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome has been identified as a critical multiprotein platform regulating innate immune response and the production of pro-inflammatory cytokines [35]. Notably, emerging evidences suggest that TBI induces activation of NLRP3 inflammasome and

it is involved in pathological development of AD[36-48]. Activation of NLRP3 is induced by multiple stimuli, including reactive oxygen species (ROS), mitochondrial damage, ATP, and potassium ion efflux from injured cells following tissue damage such as TBI[49-51]. Following TBI, increased activation of NLRP3 inflammasome in the injured brain is observed in TBI models[52,53], and inflammasome-induced cell death contributes to secondary tissue damage[54]. This is supported by clinical evidences that proteins of NLRP3 inflammasome complex and downstream effectors including IL-1β and IL-18 are found in the CSF of TBI patients and the increased level is associated to poor prognosis[47,55]. Animal studies have also found that NLRP3$^{-/-}$ mice have preserved cognition with less brain damage and inflammation following TBI[45].

Studies have also supported the importance of NLRP3 inflammasome in AD. In both AD animal models and patients, the protein expression levels of NLRP3, ASC, caspase-1 and IL-1β are upregulated[37-41]. Both IL-1β and IL-18 have shown essential roles in AD pathological development including synaptic plasticity, amyloidogenesis, and tauopathy[56-61]. NLRP3$^{-/-}$ and Casp-1$^{-/-}$ mice carrying mutations associated with familial AD (APP/PS1) have exhibited reduced Aβ burden, preserved synaptic integrity, and improved spatial memory functions[39]. Studies in 5xFAD mice carrying the ASC$^{+/-}$ genotype also support this notion[37]. Conversely, NLRP3 over-activation could promote differentiation of T cells into pro-inflammatory Th1 and Th17 effector phenotypes contributing to sustained inflammation[62], while deletion of NLRP3 in aged mice provides protection from aging related cognitive decline[63]. Furthermore, studies have revealed that Aβ plaques induce activation of NLRP3 inflammasome, which in turn further exacerbates neuroinflammation and drives tau pathology[38,64].

Based on these findings we speculate that TBI-induced abnormal activation of NLRP3 inflammasome and downstream chronic inflammation acts as a mechanistic crosslink between TBI and AD accelerating the onset and/or severity of AD in patients with TBI history. In this study, utilizing a novel transgenic mouse model with NLRP3 gene knockdown at the base of a widely used triple transgenic AD mice (3xTg), we have investigated the importance of NLRP3 inflammasome in regulating post-TBI immune/inflammatory response and vascular changes in the context of AD predisposition at the acute and subacute stages following TBI in both male and female animals. We have found that TBI altered immune profiles in 3xTg mice and NLRP3 gene knock out counteracts the injury effect with a significant sex-related difference.

Materials and Methods

Experimental Animals

The triple transgenic AD (3xTg) mouse line carrying the APP695 gene with Swedish mutations (KM670/671NL/M596L), PSEN1 mutation (M146V), and tau mutation (P301L) (Jax. Stock No:) were used. To determine the importance of NLRP3 inflammasome in mediating post-TBI AD development, we have created a unique transgenic AD mice line with NLRP3 gene knock out (homozygous 3xTg with NLRP3 gene null). This line was derived by crossing homozygous 3xTg mice with NLRP3$^{-/-}$ mice (Jax. Stock: No). Double transgenic offspring were then crossed to establish the breeder line. The VCU Transgenic Core did breeding and genotyping of the 3xTg, 3xTg/NLRP3 null mouse lines. Animals were housed in a vivarium with controlled temperature, humidity, and were kept on a 12-hour light-dark cycle with food and water provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee.

Surgical Procedure

At the age approximately 4 months old, animals were randomly divided into groups receiving either a moderate central fluid percussion injury (cFPI) or sham surgery. A total of 59 3xTg mice (male: n= 28; female: n=31) and 56 3xTgNLRP3-/- mice (male: n=27; female: n=29) were included. The cFPI produces a diffuse axonal injury with minimal focal tissue damage. The surgical instruments were sterilized and aseptic procedures were followed. Briefly, the mouse was first anesthetized in a plexiglass chamber with 4% isoflurane, then fixed to a stereotaxic frame and ventilated with 2.5% isoflurane mixed with 30% oxygen for the duration of the surgery. A continuously heated water pad was placed under the mouse during surgery to protect from hypothermia. After a midline incision on a shaved head to expose the skull, craniotomy was made at the left parietotemporal bone halfway between lambda and bregma using a 2.7mm trephine without breaching the dura mater. A Luer-lock hub made with a 20-gauge needle cap was affixed to the craniotomy site with cyanoacrylate and dental acrylic. Anesthesia was turned off and the animal was allowed to recover from surgery in a warm cage for 2 hours before being subjected to LFPI. For injury, the mouse was re-anesthetized with 4% isoflurane, hub filled with 0.9% saline and attached to a recalibrated FPI instrument. When the anesthesia was wear off, the mouse was injured at the moderate injury level with the fluid pulse at 1.75±0.05 ATM. Immediately after injury, the mouse was placed on a heated pad and righting time was recorded. After righted, the mouse was re-anesthetized and the Luer-lock syringe hub was removed, skin

incision was sutured. The animal was returned to a clean cage on a warm blanket until fully recovered before being returned to the animal care facility. Sham group animals went through the same surgical procedure without receiving the fluid pulse. All animals received post-operative care.

To examine the effect of TBI on immune cell response, animals were sacrificed 3 or 7 days post-injury (3 or 7 dpi). Mice were deeply anesthetized with an overdose of isoflurane inhalation followed by a transcardial perfusion with 30mL of ice-cold PBS. The brains were then quickly dissected on ice. Separate cohorts of animals for immune profiling study using flow cytometric analyses (FACs) and protein expression levels using Western blot. For FACs and real-time PCR, the whole brains were extracted (N=3-5/group/time point), a small piece (1x1 mm^2) of cerebral cortex close to the injury site was dissected for quantitative PCR, the rest of brain was dissociated as described below. For Western blotting (N=5/group/time point), cerebral cortex and hippocampus were dissected separately.

Flow cytometry and real-time PCR

The frequencies of myeloid cell populations and T lymphocyte subsets were examined using multi-color flow cytometric analyses (FACs) as we published previously. Briefly, the dissected individual brain was digested using the Neural Dissociation Kit (P) from Miltenyi Biotec to prepare single cell suspensions following the manufacturer's guidelines. The obtained single cells were washed in phosphate-buffered saline (PBS) and resuspended in 40% Percoll (Sigma-Aldrich). The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 400×g to enrich mononuclear cells (MNCs). MNCs from the brain were incubated in phorbol 12'-myristate-13'-acetate (PMA, 10 nM, Sigma Aldrich) plus ionomycin (0.5 µM, Sigma Aldrich) for 3 hours, followed by treatment with brefeldin A (BFA, 5 µg/mL, Biolegend) to block cytokine secretion for another 2 hours. Cells were then surface stained with a panel of surface antibodies including CD11b (M1/70, #101236), CD45 (30-F11, #103147), Ly6G (1A8, #127606), CD4 (GK1.5, #100412), gdTCR (UC7-13D5, #107508), after fixing on ice for 30 min, cells were permeabilized and stained with IL-17A (TC11-18H10.1, #506920). All antibodies were purchased from Biolegend (San Diego, CA). The the cells were analyzed using LSRFortessa™ Cell Analyzer (BD Biosciences).

Real-time quantitative PCR (RT-qPCR) analysis of inflammatory cytokines was performed as we previously described.[21] Briefly, total RNA was extracted from brains using TRIzol Reagent (ThermoFisher Scientific, Whaltham, MA). Reverse transcription and

quantitative PCR were conducted using carboxyfluorescein (FAM)-labeled Taqman probe sets from ThermoFisher Scientific. Gene transcription of *il1b* (#Mm01336189_m1), *tnfa* (#Mm00443258), and *IL-23p19* (Mm00518984_m1) was quantified relative to the expression of β-actin (#432933E), and normalized to that measured in control groups by standard 2(-ΔΔCt) calculation.

Western blotting

For protein analysis, the cortex and hippocampus were dissected separated and homogenized with RIPA buffer (Stock 10x RIPA, 20-188, EMD Millipore, MA), 0.1% SDS (Bio-Rad, 1610416), 1% Triton X-100 (Sigma-Aldrich, T8787), mini complete cocktail (Roche, 11836170001), 1mM EDTA (Quality Biological, 351-027-721). Homogenates were centrifuged at 16,000g for 25 minutes and supernatants were collected and stored at -80°C until use. The total protein concentration was determined using BCA method (Pierce, Rockford, IL). A 20µg of protein for each sample was boiled in Laemmli buffer (Bio-Rad, 1610747) and resolved using SDS-PAGE on a 4-20% Criterion TGX gel (Bio-Rad, 5671091). After SDS-PAGE, protein was transferred to a low-fluorescence PVDF (Bio-Rad) and blocked with either 5% BSA in tris-buffered saline with 0.1% Tween 20 (TBST) or 5% milk in TBST. The following primary and secondary antibodies were used: anti-GFAP (1:1000, DAKO, Z0334), anti-NF-κB (1:1000, Abcam, ab16502), anti-NLRP3 (1:800, Cell Signaling Techonology, 15101S), anti-CD31 (1:1000, BioRad, MCA1334G), anti-occludin (1:1000, Norvus, NBP1-77037), anti-AQP4 (1:200, Santa Cruz Biotechnology, sc-32739), anti-HMGB1 (1:3000, Novus, NB100-2322), anti-mouse-HRP (1:1000, Santa Cruz Biotechnology, sc-2005), anti-rabbit HRP (1:1000, Santa Cruz Biotechnology, sc-2357), anti-rat HRP (1:1000, Santa Cruz Biotechnology, sc-2065). The membranes were then washes and imaged using ChemiDoc MP imaging system (Bio-Rad, USA). The analysis of the images was done using the Image Lab 6.0 software (Bio-Rad, USA).

Statistical Analysis

All the data were expressed as means ± SEM when applicable. The differences among multiple groups were assessed using a 2-way analysis of variance (ANOVA). A Tukey's post hoc comparison between groups was used to determine specific group significance. Probability values less than 0.05 ($p<0.05$) were considered significant. Statistics were performed using JMP Pro 16 (JMP statistical software) and images prepared in GraphPad Prism 9.

Results

TBI enhances NLRP3 expression in 3xTg mice and this effect is absent in 3xTg/ NLRP3$^{-/-}$ mice

We have previously reported that TBI enhances expression of NLRP3 and downstream signaling elements at the acute and subacute stages following a focal brain injury in rats [53]. In this study using Western blotting, we first assessed the expression level of NLRP3 in cerebral cortex in a group of male 3xTg and 3xTg/ NLRP3$^{-/-}$ mice to confirm the injury effect and the knock out efficiency. At 3 days post injury (dpi), injured 3xTg mice had significantly higher expression of NLRP3 than the matched sham control, whereas NLRP3 was absent in 3xTg/ NLRP3$^{-/-}$ mice in both sham and TBI groups (**Fig. 1**).

TBI alters innate immune cell response in 3xTg mice and loss of NLRP3 counteracts the injury effect in a sex dependent manner

To evaluate the influence of TBI on the immune response and the importance of NLRP3 in mediating this process, using FACs, we have compared the immune cell profiles of both innate and adaptive immunities in the brains of 3xTg and 3xTg/ NLRP3$^{-/-}$ mice at 3 and 7 days following TBI. The 3xTg mice start to show cognitive deficits around 6 months old, extracellular Aβ plaques around 9-12 months old and tau pathology around 12 months old. We injured the animals at the age around 4 months old before the onset of AD to enable us examining injury-induced immune changes at the condition of AD predisposition. Both male and female mice were included in the study and comparison was made between the same sex 3xTg and 3xTg/ NLRP3$^{-/-}$ mice at the same post-injury time point.

For innate immunity, the myeloid cells include infiltrating neutrophils (CD11b^{+} Ly6G^{+}), macrophages (CD45^{high}CD11b^{high}), and the brain residential microglia (CD45^{low}CD11b^{low}) were assessed. Among all animal groups regardless of genotype or sex, the baseline level of all three types of cells was at the compatible level in the sham animals in both 3xTg and 3xTg/ NLRP3$^{-/-}$ mice in both sexes. Following TBI, genotype and sex related differences were found among neutrophils and macrophages, but not in microglia.

Specifically, at the acute stage following TBI (**3 dpi**), in female mice, a significant injury effect was observed in neutrophils and macrophages with significantly higher number of these cells infiltrating into the brain following TBI in both 3xTg and 3xTg/NLRP3$^{-/-}$ mice when compared to their genotype matched sham group (**Fig. 2A and B,** $p<0.0001$). When genotype

comparison was made to assess the effect of NLRP3 null in affecting injury on these cell profiles, only infiltrating neutrophils standing out with significant reduction in the injured 3xTg/NLRP3$^{-/-}$ group in comparison to the injured 3xTg mice (**Fig. 2A,** $p<0.0001$). In male mice, TBI induced higher number of neutrophils and macrophages infiltrating into the brain in 3xTg mice similar as 3xTg female mice (**Fig. 3A and B,** $p<0.0001$). This injury effect on these cells was diminished in male mice following NLRP3 gene knock down, as injured 3xTg/NLRP3$^{-/-}$ mice had significant reduction of both neutrophils and macrophages than injured 3xTg mice when genotype comparison was made (**Fig. 3A and C,** $p<0.0001$). At 3 dpi, in both male and female mice, microglia did not shown significant changes related to either injury or genotype (**Fig. 2C and Fig. 3C**).

At the subacute stage following TBI (**7dpi**), in female mice, no difference was found between injured and sham groups in the number of all three type of cells in both 3xTg and 3xTg/NLRP3$^{-/-}$ mice (**Fig. 4**). In male mice, injury-induced infiltrating macrophages remained high in the 3xTg mice (**Fig. 5B,** $p<0.0149$), whereas no difference was found between injured and sham groups in the number of neutrophils and microglia in both 3xTg and 3xTg/NLRP3$^{-/-}$ mice (**Fig. 5**).

TBI induces changes in adaptive immunity in 3xTg mice at the acute stage following injury and loss of NLRP3 counteracts the injury effect

For adaptive immunity, we assessed the effect of TBI and NLRP3 gene knock down on responses of CD4, CD8 and gamma delta T cells (γδ T cells, γδTCR^{+}IL-17A^{+}). Gamma delta T cells (γδ T cells) are considered as regulatory T cells bridging innate and adaptive immune responses, and their accumulation in the brain indicates that they act as crucial regulators of brain inflammation. In our study groups at both 3 and 7 dpi, no difference was found in CD4 and CD8 cell profiles regardless of injury or NLRP3 knock down. However, injury and NLRP3 null induced γδ T cells response was observed at the acute stage following TBI. Specifically, at 3 dpi, higher number of γδ T cells was observed in the brains of injured 3xTg mice in both males and females when compared to the matched sham (**Fig. 6 A and B**, $p<0.0001$), while this injury effect was not observed following NLRP3 gene knock down in both sexes (**Fig. 6A and B**). When genotype comparison was made, injured 3xTg/NLRP3$^{-/-}$ mice had significantly lower frequency of γδ T cells in the brain compared to injured 3xTg mice in both sexes (**Fig. 6A and B,** male: $p=0.0002$; female: $p=0031$). At the 7dpi, no difference was observed in the frequency of γδ T cells among all groups studied.

Knockdown of NLRP3 gene reduces TBI-enhanced NLRP3 downstream cytokine gene expression only in male AD mice with opposite effect in female mice

Using real-time PCR, we assessed the influence of TBI and NLRP3 gene knock down on gene expression levels of NLRP3 downstream target including IL1-β, TNF-α, IL-18 and IL-17f. A differential expression was found related to NLRP3 knockdown and sex.

At the acute stage following TBI (**3 dpi**), in male mice, TBI induced a significant increase of IL1-β and TNF-α gene expression in the 3xTg mice (**Fig. 7A**, $p<0.0001$) but not in the 3xTg/ NLRP3$^{-/-}$ mice when compared to the matched sham (**Fig. 7A**). When comparison was made between genotypes, injured 3xTg/ NLRP3$^{-/-}$ mice had significantly lower level of these genes compared to injured 3xTg mice (**Fig. 7A**, IL1-β: $p=0.0001$; TNF-α: $p=0.0016$). In contrary, the opposite response was found in female mice, with 3xTg/ NLRP3$^{-/-}$ mice showing higher expression of both IL1-β and TNF-α gene in compared to matched sham (**Fig. 7B**, IL1-β: $p=0.0013$; TNF-α: $p=0.0005$), and injured 3xTg mice (**Fig. 7B,** IL1-β: $p=0.0018$; TNF-α: $p=0.0003$). No group difference was found in the expression level of IL-18 and IL-17f at this time point.

At the subacute stage following TBI (**7 dpi**), injury induced elevation of IL-1β and TNFα genes remained high in the injured male 3xTg mice when compared to the matched sham (**Fig. 8A**). In the injured 3xTg/ NLRP3$^{-/-}$ mice, IL-1β gene was higher than the matched sham (**Fig. 8A**, $p=0.0063$), but was significantly lower than the injured 3xTg mice (**Fig. 8A**, $p=0.0128$), while TNFα was not significantly affected (**Fig. 8A**). In female mice, injury-enhanced elevation of IL-1β and TNFα genes remained similarly high in both 3xTg and 3xTg/ NLRP3$^{-/-}$ mice when compared to the matched sham (**Fig. 8B,** IL-1β: 3xTg, $p=0.0004$; 3xTg/ NLRP3$^{-/-}$, $p=0.0002$. TNFα: 3xTg, $p=0.0027$; 3xTg/ NLRP3$^{-/-}$, $p=0.0011$). Similar as 3dpi, no injury effect was found in the expression level of IL-18 and IL-17f in 3xTg mice in both sexes. However, a slightly reduction of IL-18 level was found in the male injured 3xTg/ NLRP3$^{-/-}$ mice when compared to matched sham (Fig. 8A, $p=0.0325$), and the female injured 3xTg/ NLRP3$^{-/-}$ mice when compared to female injured 3xTg mice (Fig. 8B, $p=0.0333$).

The PCR data suggested a sustained injury-effect on neuroinflammation in the 3xTg mice unrelated to the immune cell profiling. Furthermore, the female mice exhibited a NLRP3 independent inflammatory response following TBI.

TBI induced response of astrocytes and brain endothelial cells in 3xTg mice, and NLRP3 knock further augments this injury effect

We also assessed post-injury astrocyte and endothelial cell response in the ipsilateral cortex and hippocampus at the subacute stage post-TBI as NLRP3 has shown affecting the function of these cells (ref.). Protein expression level of glial fibrillary acidic protein (GFAP), aquaporin 4 (AQP4), CD31 and Occludin were measured using Western blotting.

The baseline level of all these proteins was not effected by NLRP3 gene knock down in both male and female mice. Injury effect was observed in both sexes while NLRP3 gene knock down effect was mostly affecting females. Specifically, in male animals, TBI elevated GFAP expression in both 3xTg and 3xTg/ NLRP3$^{-/-}$ mice in ipsilateral cortex (**Fig.9A**, 3xTg: p=0.0092; 3xTg/ NLRP3$^{-/-;,}$ p=0.0036) and hippocampus (**Fig.9C**, 3xTg: p=0.0059; 3xTg/ NLRP3$^{-/-;,}$ p=0.0034), NLRP3 knock down did not affect the change level. In the female mice, no significant injury effect was found in the 3xTg mice, however, injured 3xTg/ NLRP3$^{-/-}$ mice showed significantly higher GFAP expression in the cortex (**Fig 9B**, 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0004; injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.0062) and hippocampus (**Fig. 9D**, injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.05). For AQP4 expression, in males, injury-induced elevation was observed in the cortex in 3xTg mice (**Fig. 10A**, p=0.0189), while the elevation in 3xTg/ NLRP3$^{-/-}$ mice did not research statistical significance in the cortex (**Fig.10A**, p=0.0882) but was significant in the hippocampus (**Fig.10C**, p=0.0498). Injury-induced changes of AQP4 was more striking in the females showing significant elevation in both genotypes in both cortex (**Fig.10B**, 3xTg injured vs sham: p=0.0436; 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0004; injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.0187) and hippocampus (**Fig. 10D,** 3xTg injured vs sham: p=0.021; 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0062).

Brain endothelial cell response to TBI and NLRP3 knockout showed similar trend as astrocyte response, with increased expression following TBI and sex-related difference in expression levels. More specifically, in males, CD31 expression in ipsilateral cortex was significantly increased in 3xTg mice (**Fig.11A**, p=0.0243) but not in the hippocampus. Whereas the injury-induced elevation of CD31 in 3xTg/ NLRP3$^{-/-}$ mice did not research statistical significance (**Fig.11A**, p=0.069) in the cortex but was significant in the hippocampus (**Fig.11C**, p=0.0166). Overall NLRP3 knockdown did not affect the change level. In the female mice, no significant injury effect was found in the 3xTg mice. However, injured 3xTg/ NLRP3$^{-/-}$ mice showed significantly higher CD31 expression in the cortex (**Fig. 11B**, 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0011; injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.0067) and hippocampus (**Fig. 11D**, 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0001; injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.0004). Occludin levels were similarly affected as CD31 with increased expression in males only in the

cortex of injured 3xTg mice (**Fig. 12A**, p=0.0243). In females, higher level of expression was found in the injured groups in the cortex compared to matched sham (**Fig. 12B**, 3xTg: p=0.0223; 3xTg NLRP3$^{-/-}$: p=0.001), while in the hippocampus only 3xTg NLRP3$^{-/-}$ group had significant elevation (**Fig. 12D**, 3xTg NLRP3$^{-/-}$ injured vs sham, p=0.0002; injured 3xTg NLRP3$^{-/-}$ vs injured 3xTg, p=0.0005).

Discussion

TBI is a major risk factor for AD, however, the mechanistic link between the two is unclear. Extensive studies have implicated that NLRP3 inflammasome plays an important role in AD, whereas TBI activates NLRP3 inflammasome. In this study, we investigated the importance of NLRP3 inflammasome in regulating post-TBI immune/inflammatory response in the context of AD predisposition using a novel 3xTg/NLRP3 null mice. We also compared sex-related difference in relating to TBI and AD. We found that TBI altered immune profiles in 3xTg mice and NLRP3 gene knock out counteracts the injury effect with a significant sex-related difference. More specifically, at the acute stage after TBI in both male and female mice, TBI induced a significant infiltration of neutrophils, macrophages and γδ T-cells in the brains of 3xTg mice, whereas NLRP3 knock down significantly blocked this injury effect in males but less so in females. We also found that NLRP3 gene knock down counteracted TBI-enhanced inflammatory cytokine IL1-β, TNF-α, and IL-17f expression. While injured induced immune cell phenotype change was not significant at 7 days post-injury, changes in cerebral vascular associated proteins including GFAP, AQP4, CD31, Occludin were observed in related to injury, NLRP3 and sex. In summary, this study has confirmed that TBI significantly alters immune profiles and vascular integrity in the context of predisposition of AD and the NLRP3 inflammasome is important in mediating these TBI-induced changes, there is a sex-related difference in both immune and vascular changes.

Neuroinflammation is an essential player driving disease development in many neurological diseases including TBI and AD. In AD pathogenesis, inflammation is an important player, and TBI significantly exacerbates this response. Studies have reported that even a single mild injury induces abnormal inflammatory response exacerbating AD pathology and cognitive deficits in APP/PS1 mice[65]. The effects of the immune response as a result of injury are well documented with many contributing proteins and signaling molecules identified and evaluated. NLRP3 is a more recent protein with an integral role in the inflammatory and immune response to injury or infection. In Alzheimer's disease, NLRP3 is known to play an integral role in the propagation of the disease and promote tau pathology[66]. Additionally, in the brain, NLRP3 is one of the sources of inflammation the originates directly from the microglia[67,68]. In TBI, NLRP3 is a major contributor to neuroinflammation via the release of IL-1β and pyroptosis in neurons[69-73]. In the present study, we assessed the direct effects of NLRP3 in mediating TBI-induced innate and adaptive immune responses in the condition of predisposition of AD via a genetic knockout of NLRP3 gene in 3xTg mice. By assessment of immune cell profiles with

FACs and PCR measurement of inflammatory cytokine gene expression at acute and subacute stages following TBI, we have found that in the context of predisposition of AD, there is a significant immune profile changes including both innate (neutrophils and macrophages) and adaptive immune (γδ T cells) systems in the brain following a moderate TBI. Neutrophils are an abundant immune cell found in the blood that are able to penetrate into the brain upon injury. In TBI, neutrophils peak in abundance around day 2 and decrease in number until about day 7 post injury[74]. In the context of TBI, neutrophils are believed to influence blood flow, cerebrospinal fluid levels, BBB integrity, hypoxia, neuroinflammation, neurodegeneration, and neuronal recovery[74]. Our results confirm the timing of injury-induced neutrophil infiltration into the brain. Our study also suggests a regulatory role on NLRP3 in this process as both male and female 3xTg/NLRP3$^{-/-}$ mice have reduced level of neutrophil infiltration following TBI compared to 3xTg mice (**Fig 2A** and **3A**). Macrophages and microglia play a similar critical role in the immune response to TBI. However, they differ in the release damage-associated molecular patterns (DAMPs) and other innate immune responses to promote immune cell recruitment and inflammation via NLRP3. Our results have found injury-enhanced infiltration of macrophages into the brain at the acute stage following TBI in 3xTg mice in both male and females (**Fig. 2B and 3B**), and it persists to the subacute stage in males but not in females (**Fig. 4B and 5B**). NLRP3 knock down blocked the injury effect only in males not in females (**Fig. 2B and 3B).** Our data suggests that the TBI-induced infiltration of macrophages is transient at more heightened level in females, whereas in males this response lasts longer. At both acute and subacute stages in both sexes regardless NLRP3 present or absent, microglia profiles have no significant injury-induced changes (**Fig. 2-5 C**) suggesting at this stages microglial cell number is not the major factor contributing to injury-induced neuroinflammation.

In the current study, we have also examined the response of γδ T cells to injury and NLRP3 knock down. As the bridge of innate and adaptive immunities, γδ T cells enriched in brain are crucial regulators of CNS inflammation[75-77]. Production of IL-17A by γδT cells in the absence of αβT cells is critical for the initiation of CNS inflammation. However, the role of γδT cells in TBI and AD remains to be elucidated. It is known that IL-1β, IL-18, and IL-23 can promote the secretion of IL-17A from γδT cells in the absence of primary (TCR) and secondary (co-stimulator) signals[78.79]. It is demonstrated that IL-17A can further recruit IL-1β-secreting myeloid cells amplifying neuroinflammation[80]. Thus, the cooperative actions of resident and infiltrating immune cells (microglial/macrophages/monocytes, γδ T cells) promote pathogenic neuroinflammation in CNS diseases, including TBI and AD[76,77,81]. In the current study, we have

found an injury-induced significant activation of γδ T cells in 3xTg mice but not 3xTg/NLRP3$^{-/-}$ mice in both males and females the acute stage following TBI (**Fig. 6A and 6B**). This observation for the first time establishes the potential association between NLRP3 inflammasome activity and activation of IL-17-producing γδ T cells following TBI.

In the current study, we have examined the influence of injury and NLRP3 on immune response in both males and females, as there is a significant sex-related difference in both TBI and AD. Thus far published studies examining NLRP3 in the context of TBI have primarily been performed in males[71]. Here we report notable changes in innate immune responses to TBI in males and females. Apart from injury-induced difference in the degree of infiltration of neutrophils and macrophages, the expression level of pro-inflammatory cytokine genes of IL-1β, TNF-α and IL-18 also showed significant sex-related differences. In males, IL-1β and TNF-α were found to be increased post TBI in 3xTg males while the 3xTg/NLRP3$^{-/-}$ males showed no change (**Fig. 7A**). Females showed completely difference response with no injury-induced change in 3xTg mice in the expression of IL-1β, TNF-α and IL-18 genes, whereas injury-increased expression of IL-1β, TNF-α was observed in the 3xTg/NLRP3$^{-/-}$ females. In both male and females, IL-18 had no change following TBI in 3xTg mice but decreased in 3xTg/NLRP3$^{-/-}$ mice (**Fig. 1A and 7B**).

In the link between TBI and AD, apart from neuroinflammation, TBI-induced cerebrovascular damage and clearance impairment also play important role in AD pathologenesis[82]. NLRP3 is also likely involved as it is expressed in brain endothelial cells. Following the primary sources of injury in TBI, cerebrovascular damage occurs in part due to Aβ and tau accumulation and subsequent aggregation[82]. The resulting microbleeds and microvascular damage lead into the promotion of inflammation via macrophages and activated microglia[82,83]. Additionally, another aspect that has garnered recent attention has been the impairment of the cerebrovascular clearance mechanism[82]. With the inability to clear Aβ from the surrounding tissues, extracellular deposits of Aβ accumulate and eventually lead to the development of the hallmark Aβ plaques of AD[83]. In the current study, using Western blotting analysis, we quantified the protein expression levels of several vascular related proteins in including GFAP, AQP4, CD31 and Occludin. We assessed the influence of TBI and NLRP3 on their expression in both the cerebral cortex and hippocampus, and sex-related difference at the subacute stage following injury, the time when TBI-induced blood-brain barrier breakdown is sealed and new vasculature forming is observed.

GFAP and AQP4 are expressed specifically by astrocytes. Post-TBI GFAP expression is elevated and is a key feature of activated astrocytes responding to the injury. In our study, we found TBI-increased expression of GFAP in the injured cerebral cortex in both 3xTg and 3xTg/NLRP3$^{-/-}$ mice in both males and females (**Fig. 9A and 9C**). In the hippocampus, higher expression of GFAP was only observed 3xTg/NLRP3$^{-/-}$ group (**Fig. 9B and 9D).** AQP4 is densely expressed on the astrocyte end-foot, increased expression is generally related to cerebrovascular edema[84-87]. AQP4 is known to regulate the transfer of water, interstitial fluid playing a role in blood flow regulation in the brain beyond a maintenance of intracranial pressure[87]. Our results have shown a significant injury-induced increase of AQP4 expression particularly in the injured hippocampus in both 3xTg and 3xTg/NLRP3$^{-/-}$ mice in both sexes (**Fig. 10**). This findings are consistent with previous studies[85,86]. Our data of GFAP and AQP4 evaluation have shown injury-induced astrocytes response is similarly present in males and females, and NLRP3 has no significant impact on this.

To assess TBI/NLRP3 influence on vascular, we also assessed the protein expression level of CD31 and Occludin in ipsilateral cerebral cortex and hippocampus. CD31, or platelet/endothelial cell adhesion molecule 1 (PECAM-1), is a common endothelial cell marker used to evaluate the degree of angiogenesis, and plays a role in trans-endothelial migration and inflammation[88,89]. We found injury-enhanced expression of CD31 is most significantly in the 3xTg/NLRP3$^{-/-}$ mice the injured hippocampus in both sexes (**Fig. 11**). Occludin is a tight-junction protein typically expressed in decreased amounts immediately after injury and increases over time and as part of the tight junction protein class contributing to BBB maintenance and closure after injury. In our study, the expression level of Occludin showed injured-enhanced expression most significantly in males, while in females injury-enhanced expression was only significant 3xTg/NLRP3$^{-/-}$ mice in the hippocampus. Our data of CD31 and Occludin expression suggest the involvement of NLRP3 in post-TBI angiogenesis, and sex-related difference in this process. Published studies have shown the connection of neuroinflammation to Occludin expression as anti-inflammatory compounds or inhibition of NLRP3 inflammasome expression improved occludin expression significantly the acute stage post injury, respectively[90-92]. Further study regarding the role of NLRP3 in regulating angiogenesis is needed.

While our results clearly show a connection between NLRP3 and the markers discussed above, we did not analyze the inflammasome components directly nor what specifically might be causing the changes we have discussed so far. Further studies would need to look into expanding the reasons for the sex related differences we observed as well as how NLRP3 is

specifically affected by sex hormonal signaling. Additionally, we have only reported results analyzing the acute and subacute stages following injury. Studying the chronic effects of the injury and if that leads to a further predisposition to AD development warrant further examination. Investigating the effects of TBI on advanced aged mice likewise warrant further investigation as older individuals clinically account for a significant portion of all TBI patients.

Declarations

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Availability of data and materials: Data generated or analyzed for this study are available from the corresponding author on reasonable request.

Authors' contribution: DS conceived the conceptual idea, and designed the study. JG performed IHC WB analysis, statistical analysis, and wrote the manuscript. ZL and CG performed FACs and PCR experiments and statistical analysis. ST and CN performed TBI. CS performed WB analysis. XW assisted FACs and PCR data interpretation and manuscript editing.

Ethical approval and Consent to participate: No human tissue was involved in this study. Animal study was performed strictly following guidelines of IACUC, all procedures were approved by IACUC.

Consent for publication: The submission was approved by all authors.

Competing interests: No competing interests.

Figures

Fig. 1. Protein expression level of NLRP3 is increased following TBI in 3xTg mice and is absent in 3xTg/NLRP3$^{-/-}$ mice. Western blotting image shows expression of NLRP3 protein (120KDa) in the cerebral cortex in male mice at 3 days post-TBI. Increased NLRP3 expression was observed in the injured 3xTg mice compared to sham while it was absent in 3xTg/NLRP3 $^{-/-}$ mice.

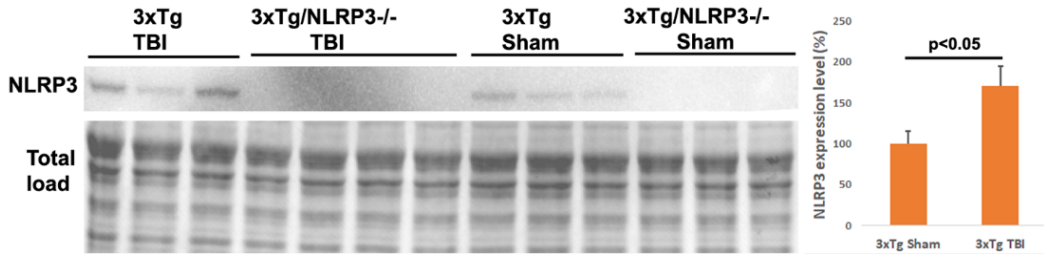


Fig. 2. TBI-induced innate immune cell profiling changes in female 3xTg or 3xTg/NLRP3-/- mice at acute stage (3dpi) following TBI. A-C). Graphs show quantification analysis of frequencies of neutrophils ($CD11b^{+}Ly6G^{+}$), microglia ($CD45^{low}CD11b^{low}$) and infiltrating macrophages ($CD45^{high}CD11b^{high}$) infiltrating into the brain at 3dpi by FACs. TBI significantly increased the number of neutrophils (**A**, p<0.0001) and macrophages (**B**, p<0.0001) in both 3xTg and 3xTg/ $NLRP3^{-/-}$ mice compared to their matched sham. Among the injured animals, 3xTg/$NLRP3^{-/-}$ mice had significant less infiltrating neutrophils compared to injured 3xTg group (A, p<0.0001). Microglia did not show injury or genotype related changes (**C**). **E-F**). Representative gating images of infiltrating neutrophils ($CD11b^{+}Ly6G^{+}$) and macrophages ($CD45^{high}CD11b^{high}$) in the brain. Gating image of microglial ($CD45^{low}CD11b^{high}$) is not included as difference was not observed at this study time point.

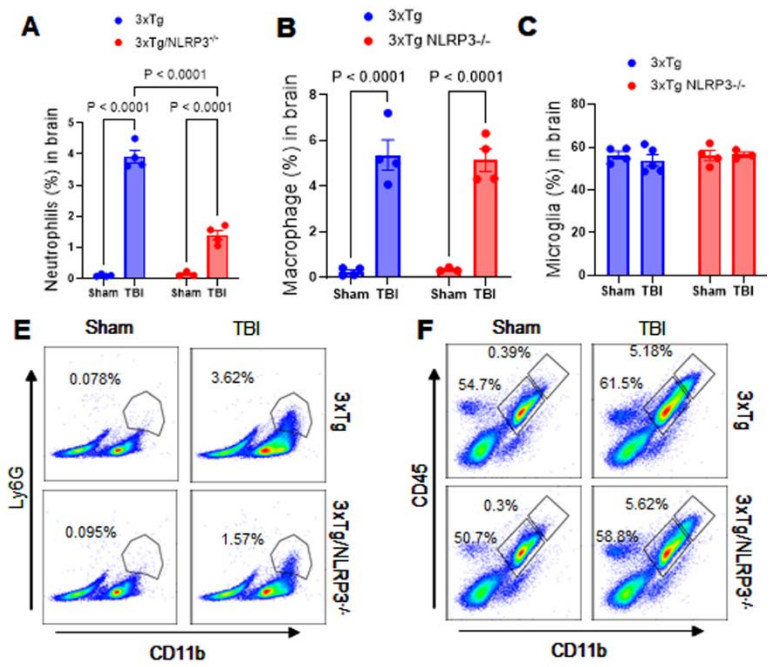


Fig. 3. TBI-induced innate immune cell profiling changes in male 3xTg or 3xTg/NLRP3-/- mice at acute stage (3dpi) following TBI. A-C). Graphs show quantification analysis of frequencies of neutrophils ($CD11b^{+}Ly6G^{+}$), microglia ($CD45^{low}CD11b^{low}$) and infiltrating macrophages ($CD45^{high}CD11b^{high}$) in the brain at 3dpi by FACs. In male 3xTg mice, TBI significantly increased the number of neutrophils (**A**, p<0.0001) and macrophages (**B**, p<0.0001) compared to sham, while in male 3xTg/ $NLRP3^{-/-}$ mice, TBI only induced a moderate increase of neutrophils (**A**, p=0.0005) compared to matched sham. Among the injured animals, 3xTg/$NLRP3^{-/-}$ mice had significant less infiltrating neutrophils (**A**, p<0.0001) and macrophages (**B**, p<0.0001) compared to injured 3xTg mice. Microglia did not show injury or genotype related changes (**C**). **E-F**). Representative gating images of infiltrating neutrophils ($CD11b^{+}Ly6G^{+}$) and macrophages ($CD45^{high}CD11b^{high}$) in the brain. Gating image of microglial ($CD45^{low}CD11b^{high}$) is not included as difference was not observed at this study time point.

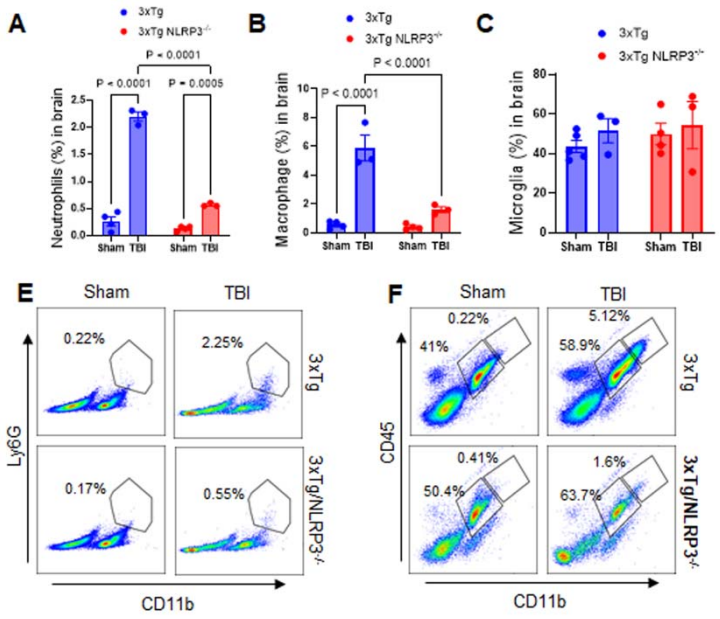


Fig. 4. TBI-induced innate immune cell profiling changes in female 3xTg or 3xTg/NLRP3-/- mice at acute stage (7dpi) following TBI. A-C). Graphs show quantification analysis of frequencies of neutrophils ($CD11b^{+}Ly6G^{+}$), microglia ($CD45^{low}CD11b^{low}$) and infiltrating macrophages ($CD45^{high}CD11b^{high}$) infiltrating into the brain at 3dpi by FACs. TBI significantly increased the number of neutrophils (**A**, $p<0.0001$) and macrophages (**B**, $p<0.0001$) in both 3xTg and 3xTg/ $NLRP3^{-/-}$ mice compared to their matched sham. Among the injured animals, 3xTg/$NLRP3^{-/-}$ mice had significant less infiltrating neutrophils compared to injured 3xTg group (A, $p<0.0001$). Microglia did not show injury or genotype related changes (**C**). **E-F**). Representative gating images of infiltrating neutrophils ($CD11b^{+}Ly6G^{+}$) and macrophages ($CD45^{high}CD11b^{high}$) in the brain. Gating image of microglial ($CD45^{low}CD11b^{high}$) is not included as difference was not observed at this study time point.

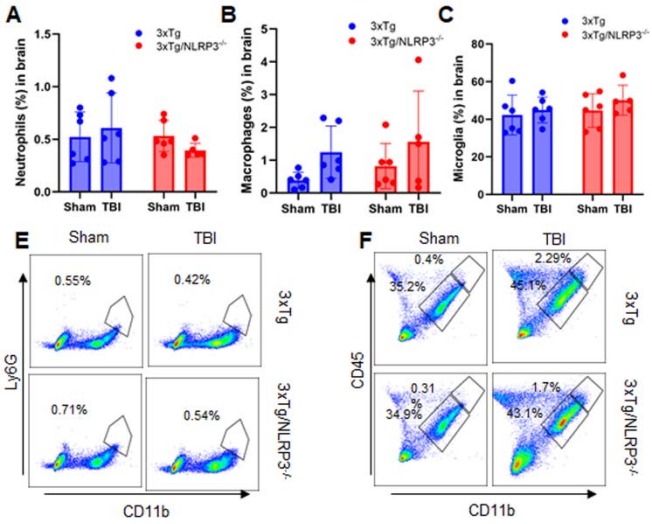


Fig. 5. TBI-induced innate immune cell profiling changes in male 3xTg or 3xTg/NLRP3-/- mice at acute stage (7dpi) following TBI. A-C). Graphs show quantification analysis of frequencies of neutrophils ($CD11b^{+}Ly6G^{+}$), microglia ($CD45^{low}CD11b^{low}$) and infiltrating macrophages ($CD45^{high}CD11b^{high}$) infiltrating into the brain at 3dpi by FACs. TBI significantly increased the number of neutrophils (**A**, $p<0.0001$) and macrophages (**B**, $p<0.0001$) in both 3xTg and 3xTg/ $NLRP3^{-/-}$ mice compared to their matched sham. Among the injured animals, 3xTg/$NLRP3^{-/-}$ mice had significant less infiltrating neutrophils compared to injured 3xTg group (A, $p<0.0001$). Microglia did not show injury or genotype related changes (**C**). **E-F**). Representative gating images of infiltrating neutrophils ($CD11b^{+}Ly6G^{+}$) and macrophages ($CD45^{high}CD11b^{high}$) in the brain. Gating image of microglial ($CD45^{low}CD11b^{high}$) is not included as difference was not observed at this study time point.

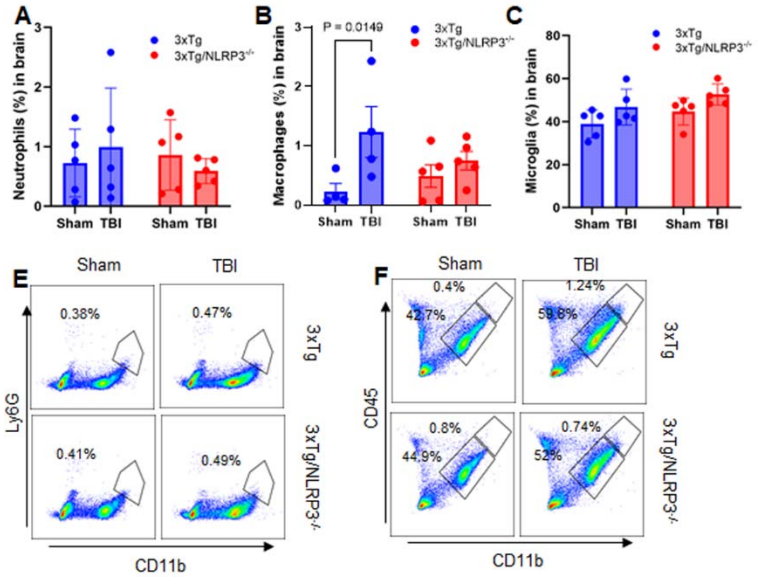


Fig. 6. TBI induced activation of γδT cells in the brain and absence of NLRP3 neutralizes the injury effect in male and female mice 3 dpi. Frequencies of activated γδT cells (γδTCR^{+}IL-17A^{+}) infiltrating into the brain were analyzed by flow cytometry. Higher number of cells was observed in the injured groups in 3xTg mice as compared to their matched sham, with 3xTg mice most significant. This injury effect was not observed in 3xTg/NLRP3$^{-/-}$ mice. **A**) Male mice, **B**) Females mice N=3-5/group.

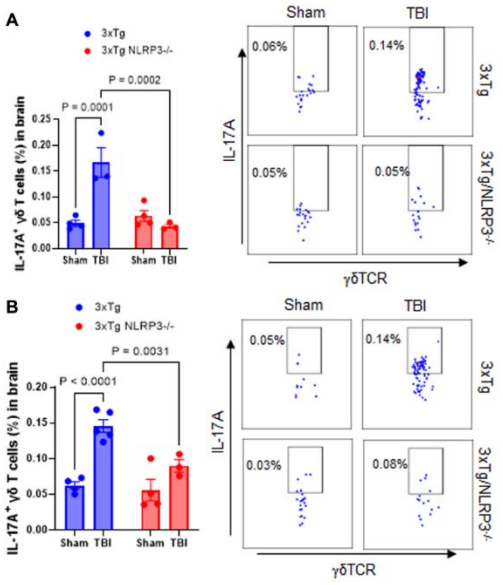


Fig. 7. TBI-induced inflammatory gene expression changes in male and female mice 3 dpi. **A**) Gene transcription of *il1b* and *tnfa* in the brain was determined by real-time PCR and revealed that TBI induced increased gene expression in the 3xTg mice groups but not in the NLRP3 null group compared to their matched sham. N=3-5/group. **B**) Female 3xTg and 3xTg/NLRP3 null mice (n=3-5/group, 5 months old) received a moderate central fluid percussive injury. Brain tissue were dissected 2 days post. Transcription of *il1b* and *tnfa* in the brain was determined by realtime PCR and found significance in the NLRP3 null group. *, $p<0.05$; ***, $p < 0.001$; NS, not significant.

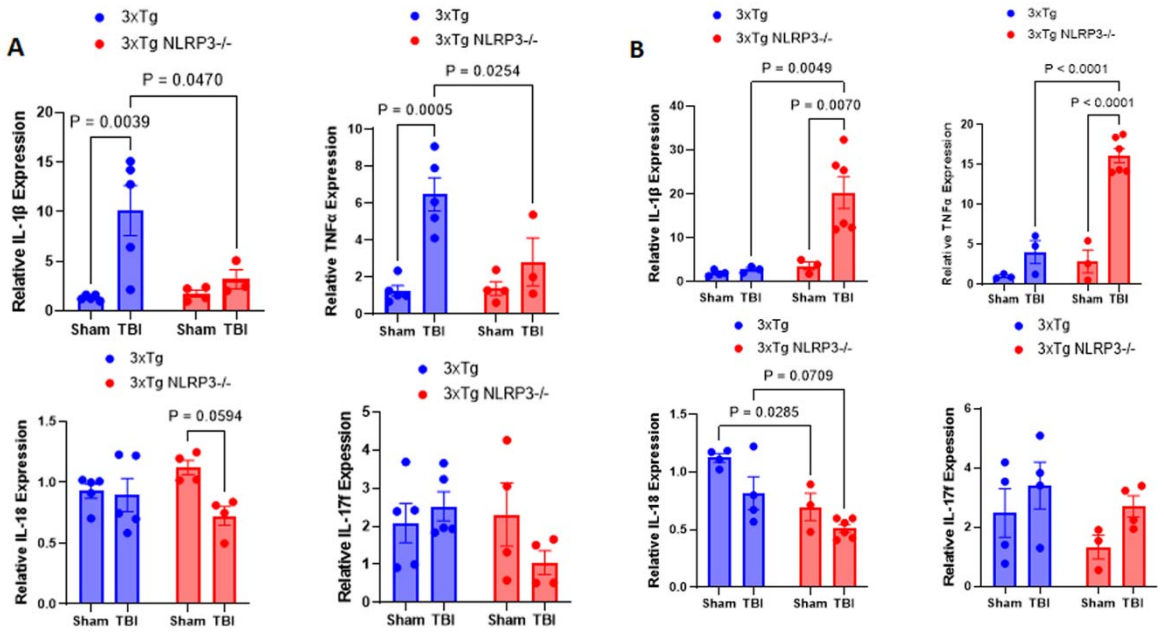


Fig. 8. TBI-induced inflammatory gene expression changes in male and female mice 7 dpi. **A**) Gene transcription of *il1b* and *tnfa* in the brain was determined by real-time PCR and revealed that TBI induced increased gene expression in the 3xTg mice groups but not in the NLRP3 null group compared to their matched sham. N=3-5/group. **B**) Female 3xTg and 3xTg/NLRP3 null mice (n=3-5/group, 5 months old) received a moderate central fluid percussive injury. Brain tissue were dissected 2 days post. Transcription of *il1b* and *tnfa* in the brain was determined by realtime PCR and found significance in the NLRP3 null group. *, $p<0.05$; ***, $p < 0.001$; NS, not significant.

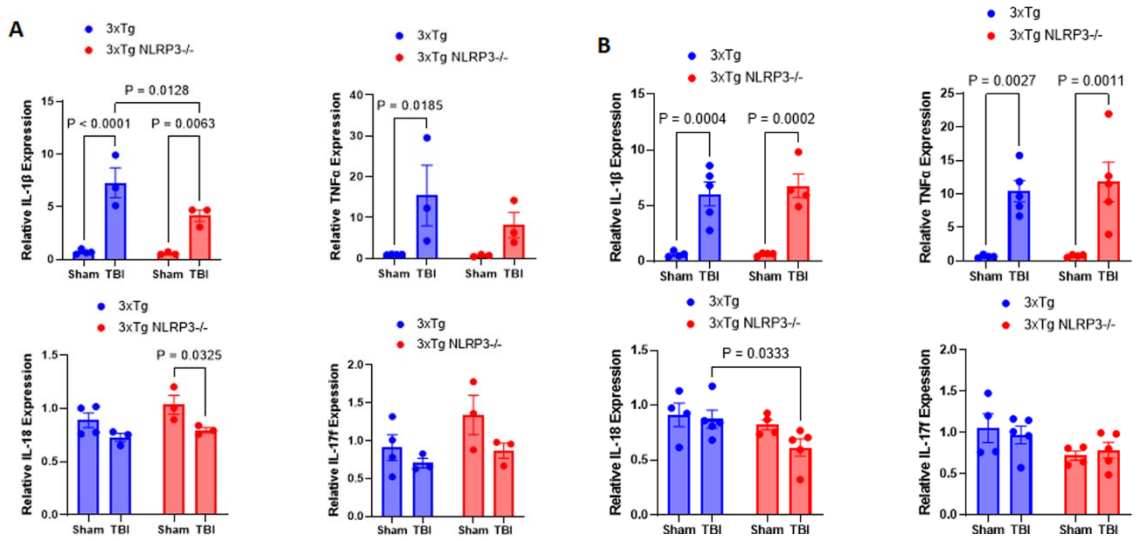


Figure 9: TBI upregulates GFAP expression in 3xTg and 3xTg/NLRP3$^{-/-}$ mice at 7 dpi. GFAP expression in the cerebral hemisphere and hippocampus was measured with Western blotting analysis. The baseline level of GFAP was not effected by NLRP3 gene knock down in both male and female mice. In male animals, TBI elevated GFAP expression in both 3xTg and 3xTg/ NLRP3$^{-/-}$ mice in ipsilateral cortex (**Fig.9A**) and hippocampus (**Fig.9C)**. In the female mice, no significant injury effect was found in the 3xTg mice, however, injured 3xTg/ NLRP3$^{-/-}$ mice showed significantly higher GFAP expression in the cortex (**Fig 9B**) and hippocampus (**Fig. 9D**). *indicating outliers by Grub test.

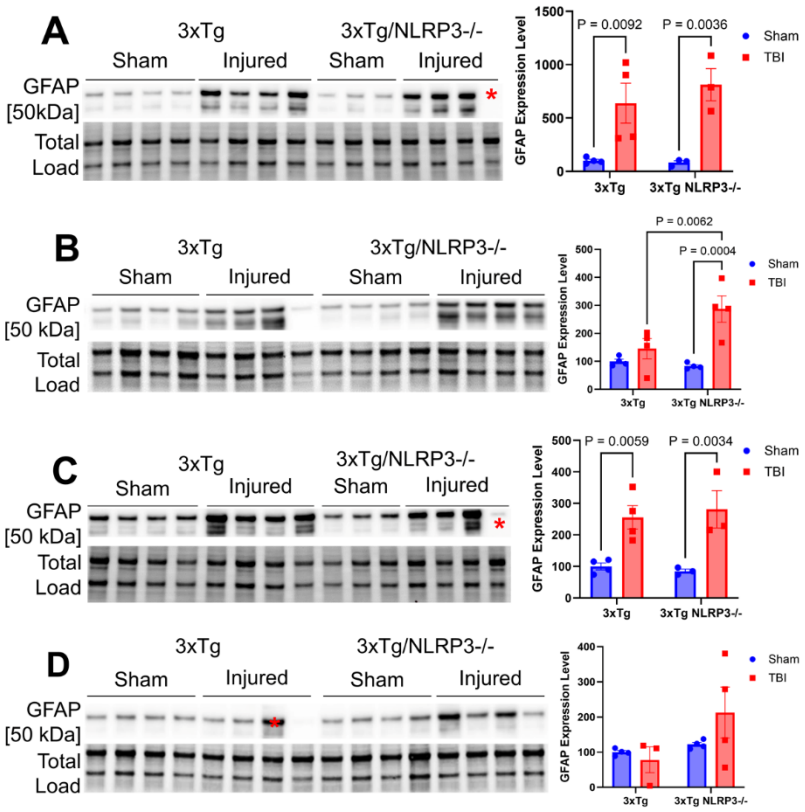


Figure 10. TBI upregulates AQP4 in 3xTg and 3xTg/NLRP3$^{-/-}$ mice at 7 dpi. At subacute stage following TBI, the effect of TBI on brain vasculature was evaluated by AQP4 protein expression in the cerebral hemisphere and hippocampus with Western blotting analysis. The baseline level of AQP4 was not effected by NLRP3 gene knock down in both male and female mice. In males, TBI-induced elevation was significant in the cortex in 3xTg mice (**Fig. 10A**), while the elevation in 3xTg/ NLRP3$^{-/-}$ mice did not research statistical significance in the cortex (**Fig.10A**,) but was significant in the hippocampus (**Fig.10C**). In females, injury-induced changes of AQP4 was more striking with significant elevation in both genotypes in both cortex (**Fig.10B**) and hippocampus (**Fig. 10D**). *indicating outliers by Grub test.

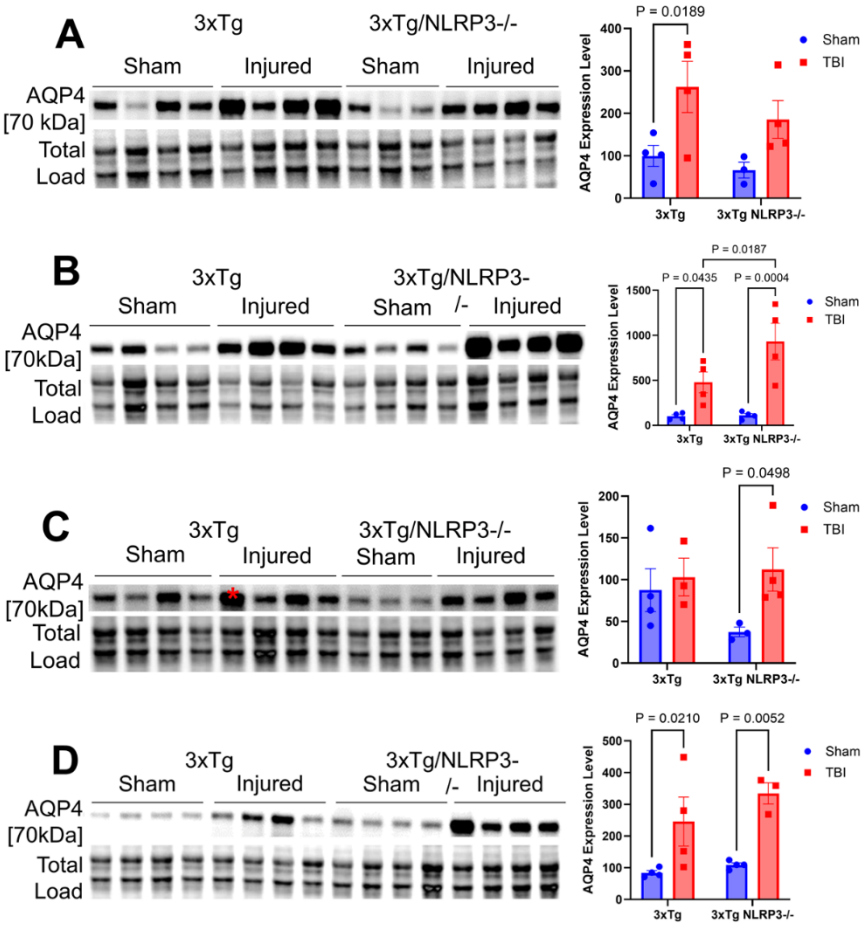


Figure 11. TBI upregulates CD31 expression in 3xTg and 3xTg/NLRP3$^{-/-}$ mice at 7 dpi. At subacute stage following TBI, the effect of TBI on brain vasculature was evaluated by expression level of endothelial cell marker CD31with Western blotting analysis. In males, CD31 expression in ipsilateral cortex was significantly increased in 3xTg mice (**Fig.11A**) but not in the hippocampus. Whereas the injury-induced elevation of CD31 in 3xTg/ NLRP3$^{-/-}$ mice did not research statistical significance (**Fig.11A**) in the cortex but was significant in the hippocampus (**Fig.11C**). Overall NLRP3 knockdown did not affect the change level. In the female mice, no significant injury effect was found in the 3xTg mice. However, injured 3xTg/ NLRP3$^{-/-}$ mice showed significantly higher CD31 expression in the cortex (**Fig. 11B**) and hippocampus (**Fig. 11D**). *indicating outliers by Grub test.

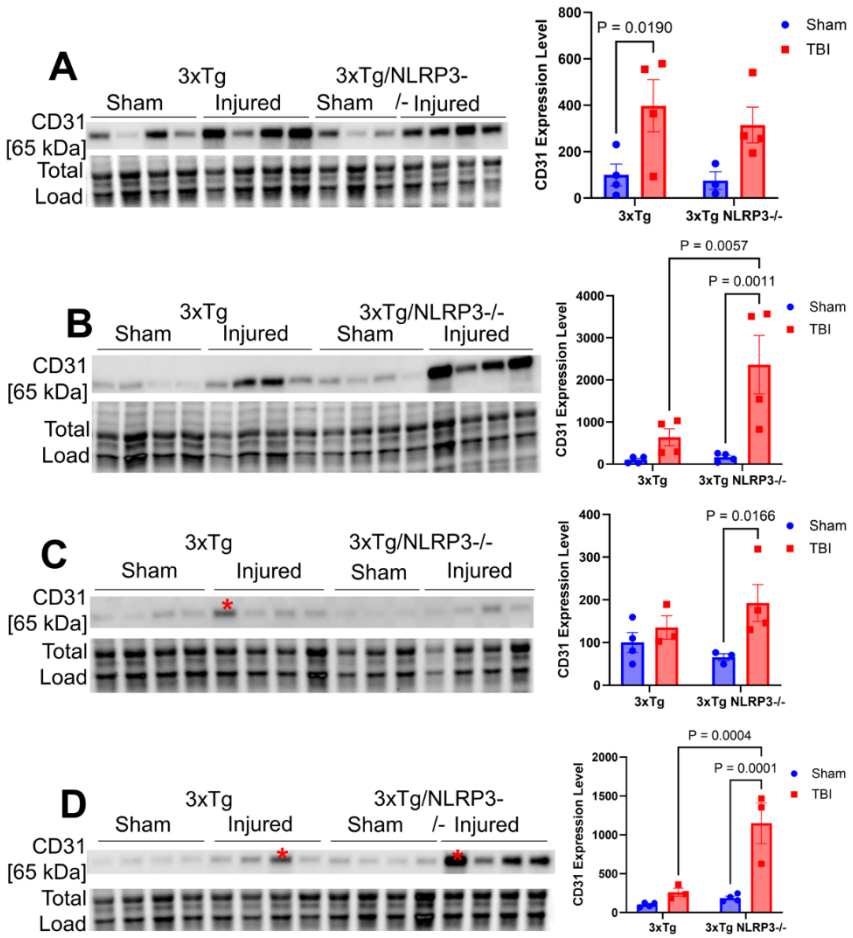
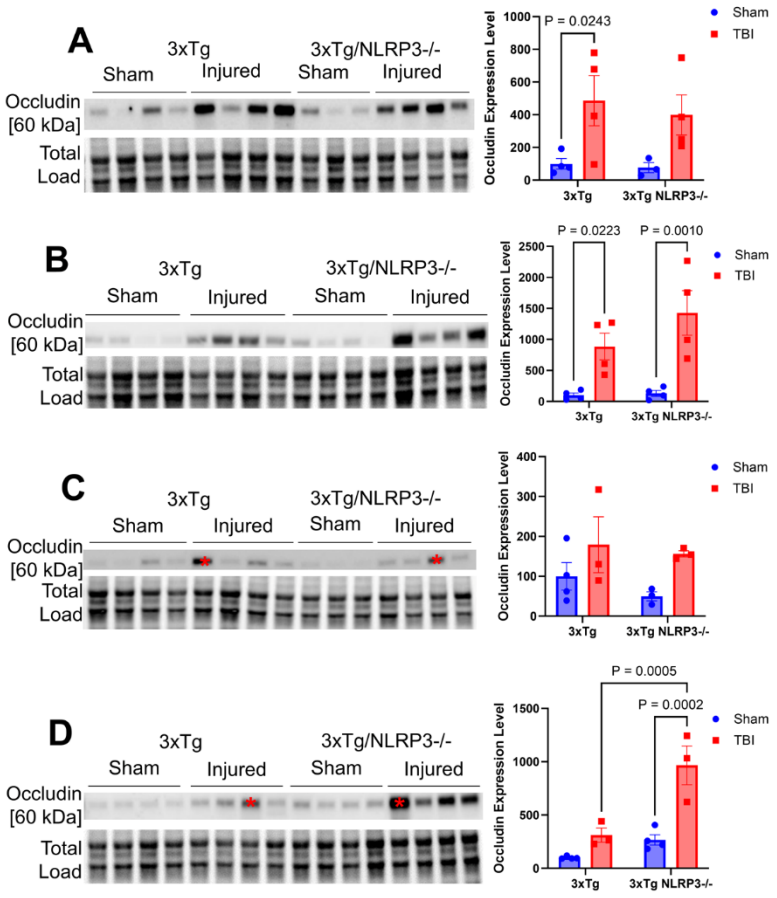


Figure 12. ***TBI upregulates Occludin expression in 3xTg and 3xTg/NLRP3$^{-/-}$ mice at 7 dpi.*** At subacute stage following TBI, the effect of TBI on brain vasculature was also evaluated by expression level of Occludin with Western blotting analysis. A similar changes as CD31 was found in male animals with increased Occludin levels in males only in the cortex of injured 3xTg mice (**Fig. 12A**). In females, higher level of expression was found in the injured groups in the cortex compared to matched sham (**Fig. 12B**), while in the hippocampus only 3xTg NLRP3$^{-/-}$ group had significant elevation (**Fig. 12D**). *indicating outliers by Grub test.



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