Sex-Specific Alterations in Inflammatory MicroRNAs in Mouse Brain and Bone Marrow CD11b+ Cells Following Traumatic Brain Injury

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Short Report

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Abstract

Sex is a key biological variable in traumatic brain injury (TBI) and plays a significant role in neuroinflammatory responses. However, the molecular mechanisms contributing to this sexually dimorphic neuroinflammatory response remain elusive. Here we describe a significant and previously unreported tissue enrichment and sex specific alteration of a set of inflammatory microRNAs (miRNAs) in CD11b + cells of brain and bone marrow isolated from naïve mice as well as mice subjected to TBI. Our data from naïve mice demonstrated that expression levels of miR-146a-5p and miR-150-5p were relatively higher in brain CD11b + cells, and that miR-155-5p and miR-223-3p were highly enriched in bone marrow CD11b + cells. Furthermore, while miR-150-5p and miR-155-5p levels were higher in male brain CD11b + cells, no significant sexual difference was observed for miR-146a-5p and miR-223-3p. However, TBI resulted in sex specific differential responses of these miRNAs in brain CD11b + cells. Specifically, miR-223 levels in brain CD11b + cells were markedly elevated in both sexes in response to TBI at 3 and 24 hr, with levels in females being significantly higher than males at 24 hr. We then focused on analyzing several miR-223-3p targets and inflammation-related marker genes following injury. Corresponding to the greater elevation of miR-223-3p in females, the miR-223-3p targets, *TRAF6* and *FBXW7* were significantly reduced in females compared to males. Interestingly, anti-inflammatory genes *ARG1* and *IL4* were higher in females after TBI than in males. These observations suggest miR-223-3p and other inflammatory responsive miRNAs may play a key role in sex-specific neuroinflammatory response following TBI.

Introduction

Neuroinflammation is recognized as a critical factor to acute and chronic secondary injury following traumatic brain injury (TBI). While the acute inflammatory stage is essential for removal of cellular debris and promoting cellular repair, dysregulation of this response leads to chronic microglia activation and pro-inflammatory signaling impacting long-term neurological function (Johnson et al. 2013; Loane et al. 2014; Pierce et al. 1998). This is characterized by the excessive release of pro-inflammatory factors by resident and recruited myeloid cells that differentiate along a continuum from cytotoxic to reparative phenotypes (Jin et al. 2012; Kumar et al. 2016; Morganti et al. 2016). This neuroinflammatory response also is sex-specific and characterized by a differential myeloid cell response in females relative to males (Acaz-Fonseca et al. 2015; Doran et al. 2019; Villapol et al. 2017).

MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression post-transcriptionally in almost all cellular events, including those controlling sex-specific responses (Kodama et al. 2020; Liu and Xu 2011; Sharma and Eghbali 2014). Numerous studies including ours have reported a strong correlation between altered miRNA expression levels and secondary brain injury events (Liu et al. 2014; Redell et al. 2009; Wang et al. 2015). Among these altered miRNAs, miR-146a, miR-223-3p, miR-155-5p, and miR-150 play key roles in inflammatory signaling (Cardoso et al. 2012; Zhao et al. 2011; Johnnidis et al. 2008; Shakerian et al. 2018). However, a mechanistic role for these inflammatory miRNAs in TBI-induced neuroinflammation is poorly understood. Moreover, no studies have revealed the involvement of these miRNAs to sex-specific neuroinflammatory responses.

Cluster of differentiation molecule 11b (CD11b, or Integrin alpha M (ITGAM)) is a leukocyte surface marker highly expressed on monocytes and macrophages of bone marrow as well as on resident CNS microglia (Gordon and Taylor 2005). Here we report a differential enrichment of these miRNAs in mouse CD11b + cells from bone marrow and brain, and a time-dependent alteration of these miRNAs following TBI. Our study suggests that the enrichment and alteration of miRNAs in CD11b + cells of a particular tissue origin may reflect their specific immunomodulatory role in response to TBI.

Materials And Methods

Animal Studies: Controlled Cortical Impact Injury

All animal methodology was in full compliance with the National Institutes of Health (NIH) Guide and with the US *Public Health Service Policy on Humane Care and Use of Laboratory Animals* and were approved by the University of Kentucky's Institutional Animal Care and Use Committee. All procedures were conducted in a manner to minimize pain and discomfort of the animals. Young adult (9–10 week old) male and female C57BL/6J mice (RRID:IMSR_JAX:000664, Jackson Laboratories) were group-housed for one week prior to experimentation and kept in a 12/12-hr light/dark cycle in a temperature-controlled vivarium room with free access to food and water. For the surgical procedures, animals (n=5/group) were randomly assigned to either sham surgery or controlled cortical impact (TBI) groups. All surgical procedures were performed using techniques published previously including post-surgical recovery (Mbye et al. 2008)

Isolation of CD11b+ Cells from Bone Marrow and Brain

Animals were sacrificed at 3 hr, 24 hr, 7 days and 14 days following TBI. CD11b positive (CD11b+) cells were isolated from bone marrow and brain using EasySep™ Mouse CD11b Positive Selection Kit II (Catalog # 18970, STEMCELL Technologies) following the manufacturer's protocol. Briefly, bone marrow cells were harvested by flushing femurs and tibias with DMEM/F-12 with 10% FBS and 1% penicillin/streptomycin (Invitrogen Life Technologies) using syringe with a 25-gauge needle. A single cell suspension was prepared by passing harvested bone marrow cells through 18-gauge needle 4-6 times. After centrifugation at 300 Xg for 5 min, red blood cell (RBC) depletion was performed by adding 3 ml RBC lysis buffer (0.15 M NH_4Cl, 10 M $KHCO_3$, and 0.1 M Na_2EDTA, pH 7. 4) on ice for 3 min. RBC lysis was stopped by adding 15 ml of EasySep™ Buffer (Catalog #20144) followed by centrifugation at 300 Xg for 5 min. Cells were resuspended in 0.5 ml EasySep™ Buffer and subjected to CD11b+ cell isolation using EasySep™ Mouse CD11b Positive Selection Kit II.

For brain CD11b+ cells isolation, the injured or surgery hemisphere was minced in a petri dish containing 1 ml digestion media (DMEM/F-12, 15 mM HEPES, 2% FBS, 20 units/ml Papain (Catalog #07465) and 1 mg/ml DNase I). Minced brain pieces were then transferred to 50 ml conical tubes and incubated at 37°C for 20 min on a gently shaking platform. A single cell suspension was obtained by filtering the digested tissue through a 70 µm nylon mesh strainer and the strainer rinsed with sample preparation media

(DMEM/F-12, 15 mM HEPES and 2% FBS). The cell suspension was subjected to centrifugation at 300 Xg for 10 min at 4°C. After removing the supernatant, 6 ml of 30% isotonic percoll solution (Catalog #17-0891-01, GE Healthcare) was added to the suspended pellet followed by centrifugation at 700 Xg for 10 min at 4°C with the brake off. The upper myelin layer was carefully removed and the pellet subjected to RBC depletion and CD11b+ cell isolation as above.

RNA Isolation and Analysis

Isolation and analysis of total RNAs from CD11b+ cells followed the procedures described previously (Wang et al. 2015; Wang et al. 2020). MiRNA/gene expression were analyzed using TaqMan assays (ThermoFisher Scientific). Small nuclear U6 RNA and *Hprt* (hypoxanthine phosphoribosyltransferase 1) were served as endogenous controls for normalizing miRNA and gene expression in the same sample, respectively.

Statistical Analysis

GraphPad Prism (Version 8.0.2; San Diego, CA, United States; RRID:SCR_002798) was used for statistical analyses. All data in this report presented as means ± standard error of the mean (SEM). Repeated-measures two-way analysis of variance (ANOVA) was used for comparison among groups followed by Tukey's post hoc test to identify specific differences between groups. A value of $p < 0.05$ with 95% confidence interval is considered statistically significant. Number of animals used in each study was represented in figure legends section of each result.

Results

Preferential Enrichment of Inflammatory MiRNAs in CD11b + Cells from Brain and Bone Marrow

TaqMan RT-qPCR was used to measure selected inflammatory miRNAs including miR-146a-5p, miR-223-3p, miR-155-5p, and miR-150-5p levels in brain and bone marrow CD11b + cells from female and male naïve mice. While miR-146a and miR-150 levels were detected in bone marrow CD11b + cells in both females and males, they were more than 25-fold higher in brain CD11b + cells compared to bone marrow in both sexes **(Fig. 1)**. Both miRNAs showed higher levels in brain CD11b + cells of male mice compared to females, but only miR-150 reached statistical significance. Conversely, miR-223-3p, and miR-155-5p were more than 25-fold higher in CD11b + cells isolated from bone marrow relative to brain in both sexes, with only miR-155-5p showing significant sex specific enrichment (males > females) in brain **(Fig. 1).** This pattern demonstrates that inflammatory miRNAs are differentially enriched in brain and bone marrow CD11b + cells.

Sex-specific Alterations of Inflammatory MiRNAs in CD11b + Cells Following Severe TBI

To assess sex-specific miRNA alterations following TBI, we measured levels of these miRNA in CD11b + cells from injured cortical tissue and bone marrow at 3 hr, 24 hr, 7 days, and 14 days after severe TBI. Levels of pro-inflammatory miR-155-5p in brain CD11b + cells were significantly increased in both female and male mice after TBI, although females showed a significantly greater induction at 3 hr compared to males **(Fig. 2a)**. The levels of anti-inflammatory miR-146a were altered marginally over time **(Fig. 2b)**, while miR-223-3p levels significantly increased by 24 hr after TBI in both females and males, with females having significantly higher levels compared to males **(Fig. 2c)**. MiR-150-5p was significantly increased in females but not males at 7 days and 14 days post-injury **(Fig. 2d).** Interestingly, we did not observe any TBI-related or sex differences in inflammatory miRNAs in bone marrow CD11b + cells **(Suppl Fig. 1)**.

Sex-specific Alterations of Brain MiR-223-3p Targets and Inflammatory Gene Expressions in CD11b + Cells Following Severe TBI

We then examined the expression profile of miR-223-3p specific targets and related inflammatory genes in brain CD11b + cells of female and male mice following TBI. The sham animals did not exhibit any sex-specific patterns in the expression levels of any of the inflammatory genes examined. Reverse-correlated with the increase in miR-223-3p reported above **(Fig. 2c)**, we found that the levels of two miR-223-3p targets, *FBXW7* and *TRAF6*, significantly decreased at 24 hr post-injury only in brain CD11b + cells from female mice **(Fig. 3a)**. Interestingly, we observed an increased expression of the anti-inflammatory gene, *ARG1*, in both female and male injured mice compare to sham animals at 24 hr post-injury, with females showing significantly higher expression than males **(Fig. 3b)**. At this same 24 hr time point, brain CD11b + cells from female, but not male, mice had significantly higher expression of another anti-inflammatory gene, *IL4* **(Fig. 3b)**.

Several pro-inflammatory gene markers also exhibited a sex-related differential expression in brain CD11b + cells following TBI. For example, *CCL2* was significantly increased in both female and male mice after TBI, although significantly greater increase was observed in males relative to females at 24 hr **(Fig. 3c)**. In addition, there was a significant increase in *COX2* expression only in male mice at 3 hr and 24 hr, which gradually decreased with time **(Fig. 3c)**. The levels of *TNF*α and *IL1b* showed different sex-related expression patterns over time following injury **(Fig. 3c)**. Specifically, both pro-inflammatory markers peaked at 3 hr after TBI in males and at 24 hr in females, with significantly higher levels in females compared to males at 24 hr **(Fig. 3c)**.

Discussion

Our present study demonstrates the existence of a significant and previously unreported preferential enrichment and sex-specific response of inflammatory miRNAs in brain and bone marrow CD11b + cells. The inflammatory miRNAs examined in this study play key roles in inflammatory signaling in myeloid cells and are all implicated in TBI (Liu et al. 2014; Redell et al. 2009; Wang et al. 2015): miR-146a is a negative regulator of NF-κB pathway (Taganov et al. 2006); miR-223-3p plays a key role in limiting

myeloid cell pro-inflammatory signaling (Jiao et al. 2021); miR-155-5p is a highly inducible, pro-inflammatory miRNA (Jablonski et al. 2016; Sun et al. 2012); and miR-150-5p is an important modulator for differentiation and activation of immune cells (Zhou et al. 2007). Our results revealed that miR-146a-5p and miR-150-5p are relatively enriched in brain CD11b + resident microglia and that miR-223-3p and miR-155-5p are relatively enriched in bone marrow CD11b + monocytes and macrophages. Although we did not detect significant changes of these miRNAs in bone marrow CD11b + cells following TBI, intriguingly, the response of brain CD11b + miRNAs to TBI are vastly different with regards to sex and time following injury. For example, an early and significant elevation of pro-inflammatory miR-155-5p in brain CD11b + cells was observed in both female and male mice compared to shams, which returned to sham levels by 7 days post-injury but again increased significantly at 14 days. It is interesting to point out that the levels of miR-155-5p were higher in brain CD11b + cells from naïve males, while the levels of miR155-5p in CD11b + cells from injured female brain were significantly higher than males at the earliest time point examined (3 hr). Anti-inflammatory miR-223-3p was significantly increased in brain CD11b + cells from males at 3 hr, and in both sexes at 24 hr, with significant higher levels in females compared to males. However, this increase diminished at 7 and 14 days following TBI in both females and males. The dynamic changes of pro- and anti-inflammatory miRNAs is consistent with the experimental and clinical observations that while reparative anti-inflammatory signaling peak shortly following brain injury, pro-inflammatory effectors persist for weeks to months to years (Loane et al. 2014; Pierce et al. 1998).

We further examined the expression of targets of miR-223-3p and several inflammatory marker genes. MiR-223-3p is directly associated with reparative inflammatory response via regulation of key inflammatory signaling pathways such as NF-κB and the inflammasome (Bauernfeind et al. 2012; Kumar et al. 2014; Zhou et al. 2018). Our data showed a significant down regulation of the miR-223-3p validated targets *FBXW7* and *TRAF6*, which are key modulators of the NF-κB pathway, in females compared to males at 24 hr following TBI. Interestingly, the expression of the anti-inflammatory genes, *ARG1* and *IL4* were significantly increased in female relative to male mice and sham at the same 24 hr time point. Whether the increased expression of anti-inflammatory genes is a direct result of miR-223-3p regulation will need to be further investigated. The pro-inflammatory marker gene profiles were more diverse. There is a clear increase in the levels of the pro-inflammatory genes *CCL2*, *COX2*, *TNFa*, and *IL1b* in both male and female injured brains compared to sham. However, *COX2* and *CCL2* were higher in males at 3 and 24 hr post-injury, whereas greater levels of *TNFa* and *IL1b* were observed in females TBI at 24 hr, although an initial higher level were seen in males at 3 hr. These complicated sex-bias inflammatory responses could be a consequence of multiple signaling events that involve the dynamic changes of inflammatory miRNAs. Regardless, these results are in line with the findings that inflammatory factors display sexual dimorphic responses following TBI (Spani et al. 2018; Villapol et al. 2017).

We realize there are limitations and caveats of the current study. For example, there are many other inflammatory responsive miRNAs that may participate in cell/tissue- and sexual-specific modulation of inflammatory response, but our studies were limited to only four well-studied inflammatory responsive miRNAs. Moreover, we only examined two acute time points (3 and 24 hr) and two chronic time points (7 and 14 d). We believe there could be additional significant sex-related changes at other time intervals. In

addition, although CD11b + cells contain major monocytes, such as macrophages and microglia, the isolation procedure used cannot distinguish subtypes of these monocytes. Future investigations will be required to fully address the tissue/cell specific and sexual dimorphic miRNA inflammatory response following TBI.

Declarations

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Conflicts of interest/Competing interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Code availability: Not applicable

Authors' contributions: Study concept and design: JES, WXW, PP; Experimental implementations: PP, WXW, SP, UG; Data acquisition and analysis: PP, WXW; Statistical analysis: PP, WXW; Manuscript preparation: PP, WXW, JES; Manuscript editing and review: JES, WXW, PP, SP, UG

Compliance with Ethical Standards: All animal procedures used in this study conformed to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the University of Kentucky's Institutional Animal Care and Use Committee (IACUC protocol no.: 2019-3223).

Ethics approval (include appropriate approvals or waivers): Not applicable

Consent to participate (include appropriate statements): Not applicable

Consent for publication (include appropriate statements): Not applicable

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Figures

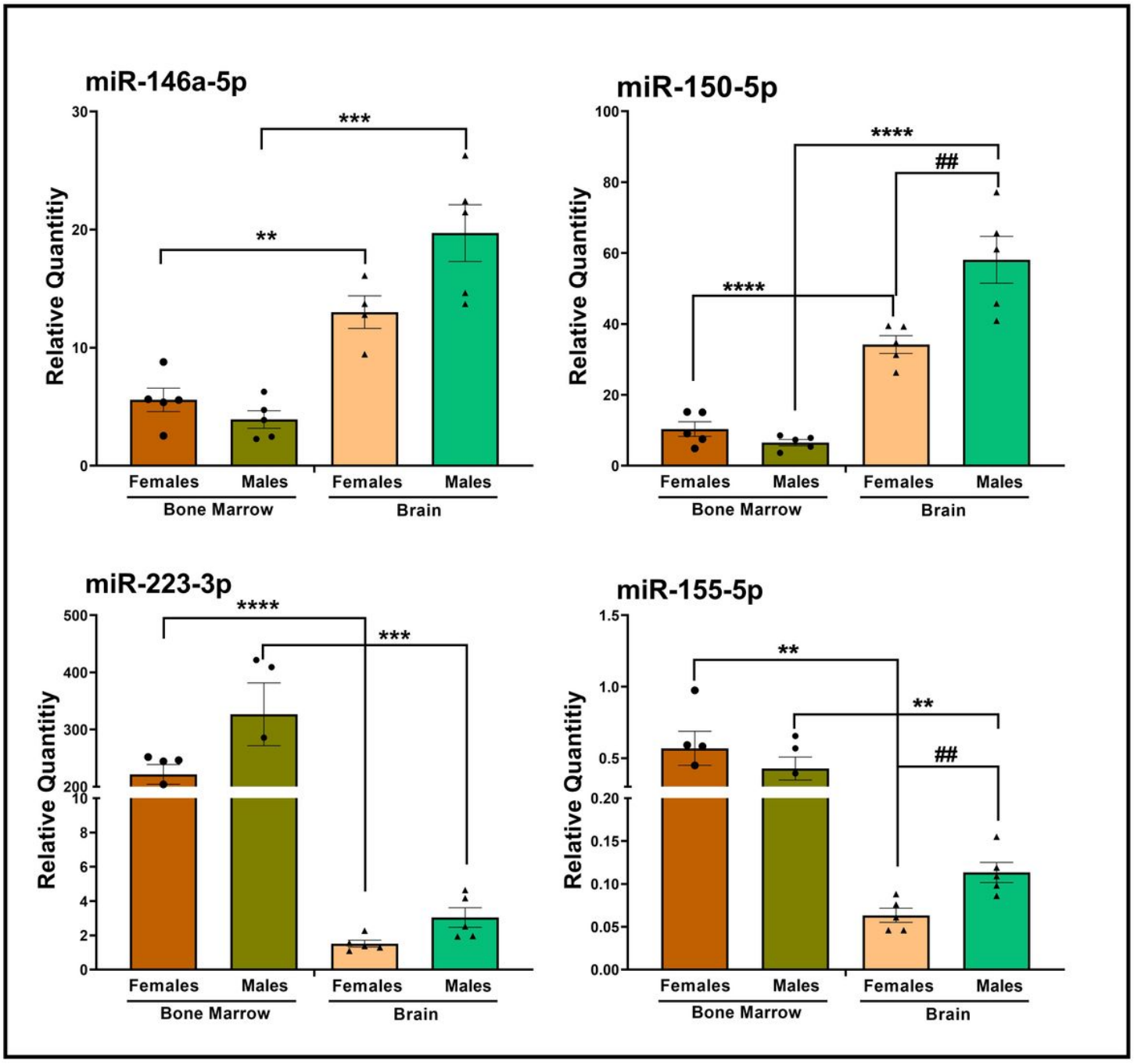

Figure 1

Enrichment of miRNAs in CD11b+ cells of naïve mice. Levels of inflammatory miRNAs miR-146a-5p and miR-150-5p, miR-223-3p, and miR-155-5p in brain and bone marrow CD11b+ cells of male and female

mice. “*” indicate significant difference between bone marrow and brain and “#” indicate significant difference between male and female (n= 5 mice/per group)

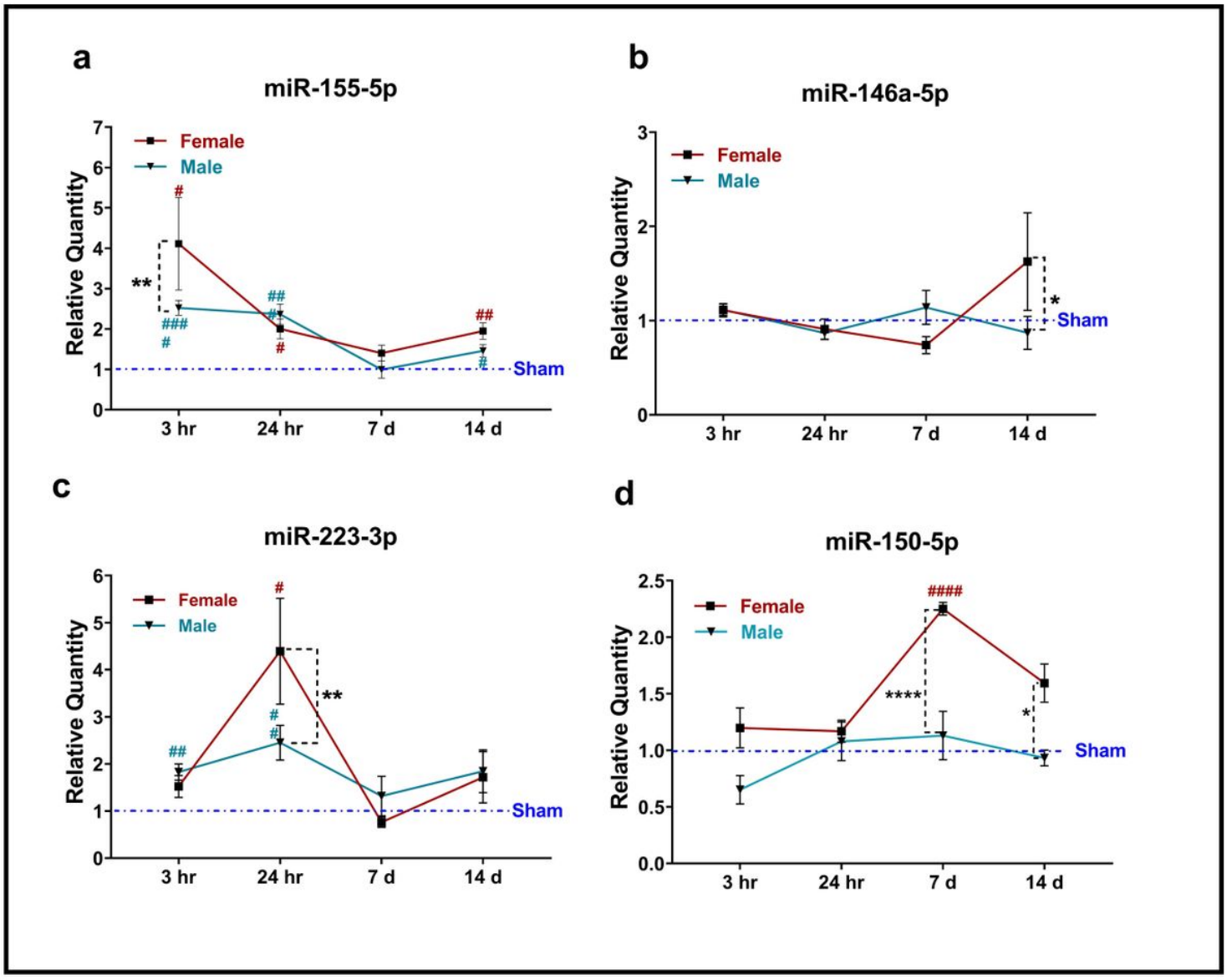

Figure 2

Response of inflammatory miRNAs to TBI over time. Levels of inflammatory miRNAs (a) miR-155, (b) miR-146a, (c) miR-223a, and (d) miR-150 in brain CD11b+ cells of male and female mice at different time points following TBI. Levels of sham group were set as 1. “#” indicate significant difference between sham and TBI group and “*” indicate significant difference between females and males (n= 4-8 mice/time point)

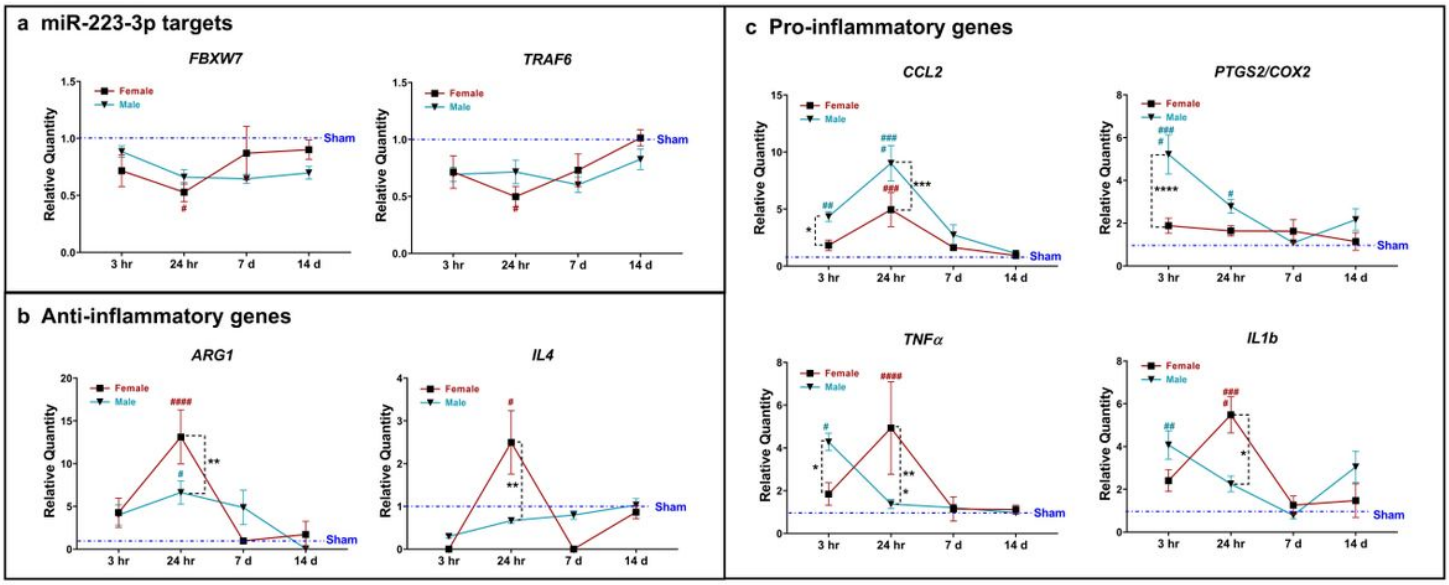

Figure 3

Changes of inflammatory marker genes and targets of miR-223-3p following TBI. Targets of miR-223-3p (a), anti-inflammatory marker genes (b), and pro-inflammatory marker genes (c) in brain CD11b+ cells of male and female mice at different time points following TBI. Levels of sham group were set as 1. “#” indicate significant difference between sham and TBI group and “*” indicate significant difference between females and males (n= 4-8 mice/time point)

Supplementary Files

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