Anti-inflammatory protein TNF α -stimulated gene-6 (TSG-6) reduces inflammatory response after brain injury in mice

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Research article

Keywords: TSG-6, glial scar, astrocytes, glycosaminoglycans and inflammation

Posted Date: September 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-23682/v2>

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Version of Record: A version of this preprint was published at BMC Immunology on August 4th, 2021. See the published version at <https://doi.org/10.1186/s12865-021-00443-7>.

Abstract

Background: Current research suggests that the glial scar surrounding penetrating brain injuries is instrumental in preserving the surrounding uninjured tissue by limiting the inflammatory response to the injury site. We recently showed that tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6), a well-established anti-inflammatory molecule, is present within the glial scar. We hereby investigate the role of TSG-6 within the glial scar.

Methods: TSG-6 *null* and littermate control mice were subjected to penetrating brain injuries (2 mm puncture wound to the frontal cortex), after which both the injury site and remaining injured hemisphere were analyzed. The presence of activated astrocytes, inflammatory markers and glial scar components was evaluated by real-time PCR and immunofluorescence.

Results: Our findings show that mice lacking TSG-6 present a more severe inflammatory response after injury, which is correlated with an enlarged area of astrogliosis beyond the injury site.

Conclusion: Our data provides evidence that TSG-6 has an anti-inflammatory role within the glial scar.

Background

Traumatic brain injury (TBI) is a major medical concern that affects over 10 million people in the world each year (1,2). A variety of injuries can cause TBI leading to a variety of injury severities (3–6). With improved medical interventions over the years the mortality rate due to TBI has decreased, culminating in a significant population living with the long-term effects of TBI. It is well accepted that in addition to the immediate effects of TBI there are also multiple potential long-term gradually evolving sequelae that are influenced by the type of injury, severity of the injury and medical interventions at the time of injury (7,8). Additionally, a link between mild traumatic brain injuries and Alzheimer or chronic traumatic encephalopathy has long been suspected (9). At present, long-term effects of repeated TBI have been seen in multiple sports-related injuries, including post-traumatic parkinsonism, post-traumatic dementia and chronic post-concussion syndrome (10–13). Thus, studying the short and long-term consequences of TBI at a cellular and molecular level may lead to a new understanding and perhaps better long-term management of such injuries via new and/or refined treatment strategies.

Astrogliosis is a hallmark of TBI, which commences hours after injury and leads to an abnormal increase in the number of activated astrocytes in and around the injury site (14,15). Immediately after injury (acute phase), astrocytes become highly proliferative and up-regulate the production of extracellular proteins, culminating in the formation of a glial scar, which impedes axonal growth and therefore inhibits neuronal regeneration (14,16–19). The intensity of the reactive astrogliosis directly affects the immediate and long-term effects of TBI (6,15,20,21). Penetrating brain injuries (PBIs) cause direct parenchymal laceration, neuronal cell loss and hemorrhage, which lead to focal tissue damage at the injury site. Astrogliosis is triggered after PBIs and forms astrocyte-rich scar tissue surrounding the injury site (22–24). Mounting evidence shows that reactive astrocytes surrounding the injury site are instrumental in

preserving the surrounding uninjured tissue by forming scar borders, which separate damaged and inflamed tissue from adjacent viable neural tissue (14,15,20,25–29). Reactive astrocytes up-regulate intermediary filament proteins, Glial Fibrillary Acidic Protein (GFAP) and vimentin (VIM), and also increase the production of chondroitin sulfate proteoglycans (CSPGs), all of which have been implicated in glial scar formation (30–35). Indeed, deficiency of both GFAP and VIM (*Gfap*^{-/-}*Vim*^{-/-}) leads to defective and less organized glial scarring in mice (34–36). Moreover, Wilhelmsson et al. observed that *Gfap*^{P/-}*Vim*^{-/-} mice exhibited increased axonal regrowth post injury in comparison to the wild-type control, suggesting that the loss of these two proteins is enough to prevent glial scar formation and promote some neuronal regeneration (34). However, whether these mice present improved functional recovery compared to littermate control mice remains to be established. Recent work by the Sofroniew group has shown that scar tissue bordering the injury site is necessary for limiting inflammation and tissue damage to the injury site (26,37). Moreover, data from Hermann et al. show that GFAP-driven ablation of STAT3 in astrocytes leads to the loss of lesion demarcation and subsequent glial scar formation, and, in turn, results in increased invasion of inflammatory cells into adjacent viable tissue and further spread of inflammation (38). This suggests that early demarcation of the glial scar formed by astrocytes restricts movement of inflammatory cells located within the lesion site into adjacent healthy tissue, thereby restricting tissue damage to the injury site. There is currently a significant number of studies investigating how reactive astrocytes regulate and limit inflammation in the injury site and which cellular components and major pathways play a part in this process (16,37,39–41). We recently found that tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6) is secreted by astrocytes after injury and is a major constituent of the glial scar, but the role it plays within the glial scar remains to be established (42).

TSG-6 is a 35-kDa protein that is secreted by a wide range of cell types in response to inflammatory mediators and growth factors (43) and, was originally identified as a gene product induced in fibroblasts by TNF (12). TSG-6 contains a link module domain that mediates its interaction with the polysaccharide hyaluronan (HA), as well as other glycosaminoglycans (GAGs)(43–45). Interestingly, GAGs, in particular chondroitin sulfate (CS), have been shown to negatively impact nerve regeneration and have served as targets in various studies over the years in attempts to promote neuronal regeneration after CNS injury (44–47). Our recent study identified that TSG-6 is expressed in the central nervous system (CNS), where it catalyzes the transfer of heavy chains (HCs) from Inter- α -Inhibitor (I α I, also known as ITI) onto HA, forming a specialized HA/HC/TSG-6 matrix within the glial scar, but the role of this specialized matrix within the glial scar remains to be established (42,48–52). The HA/HC/TSG-6 matrix has been shown to be monocyte-adhesive and is found in most, if not all, inflammatory processes (53,54). These TSG-6 modified HA matrices bind inflammatory cells, and the interaction of these cells with the HA matrices modulates their responses, which are central to pathological inflammation (55–61). The main objective of this study was to investigate the role TSG-6 plays in the glial scar after a PBI. Given the well-characterized anti-inflammatory role of TSG-6 in other sites, the premise of this study was that TSG-6 could participate in the formation of an immunosuppressive environment within the glial scar. Our findings show that *TSG-6 null* mice present a more severe inflammatory response and increase glial scar

deposition after injury when compared to littermate control mice. This increased inflammatory response in the *TSG-6 null* mice was correlated with an enlarged area of astrogliosis beyond the injury site.

Methods

TSG6 null ($TSG6^{-/-}$) or heterozygous ($TSG6^{+/-}$) mice and animal maintenance

Transgenic *Tsg-6 null* mice (*Tnfr1^{0/0}*), hereafter referred to as *Tsg-6^{-/-}* mice, and heterozygous mice, hereafter referred to as *Tsg-6^{+/-}* mice, were maintained as previously described (52). Our previously published work demonstrated that *Tsg-6^{+/-}* mice present a similar distribution of astrocytes throughout the brain to wt mice (42). Moreover, *Tsg-6^{+/-}* mice have previously been shown not to display a phenotype and present similar TSG-6 expression levels as wt mice, and, therefore, were used as littermate controls in our study (52). Experimental procedures for handling the mice were approved by the Institutional Animal Care and Use Committee (IACUC), University of Houston under protocol 16-036.

Brain Injury

Mice (7 to 8 weeks old) were anesthetized with ketamine (80-100 mg/kg - Vedco INC, Catalog# 07-890-8598) and xylazine (5-10 mg/kg, Akorn INC, Catalog# 07-808-1947) by IP injection and allowed to go into full anesthetic state. A sterile surgical drill (Precision Tools, Model Craft PPV2237) was used to make a hole of approximately 1.5 mm in diameter in the skull over the right frontal cortex at the stereotaxic coordinates AP: 1.0mm, ML: 1.5mm, and DV: 1.5mm, according to Franklin and Paxinos (62). A 30-gauge needle (Exel, Catalog# 26437) was then used to make a puncture wound at a depth of 2 mm. After injury, the skin at the surgical site was closed with two sutures. The area was then cleaned with 70% ethanol, and mice were placed on a heating pad and monitored until they regained consciousness prior to being transferred to a clean cage. All surgeries were carried out at the same time of day to minimize bias. Mice were monitored daily and did not show any decrease in weight $\geq 15\%$ when compared to their pre-surgical weight. Mice were euthanized at 1, 3 and 5 days post injury to study the acute effects of brain injury, and at 10 and 14 days to study long-term/chronic effects. Five mice per experimental group were used for the real-time PCR analysis and at least seven mice per experimental group were used for immunofluorescence analysis.

Perfusion fixation and brain tissue processing

Brain samples were collected at 1, 3, 5, 10, and 14 days post injury for immunofluorescence analyses. Briefly, mice were initially injected with a lethal dose of combined anesthetics, ketamine and xylazine. Once mice were under deep anesthesia, abdominal excisions were performed to expose the heart, which was used to perfuse 2% formalin (Fisher Scientific, Catalog# SF100-4) throughout the whole body via a gravity-driven flow system for whole body fixation. Subsequently, the brain was isolated from the skull and further immerse fixed for 2 days in 2% paraformaldehyde (Electron Microscopy Sciences, Catalog# 15710). For cryosection processing, brains were immersed in 30% sucrose for 2 days, embedded in OCT

embedding medium (Fisher Healthcare, Catalog# 4585) and frozen. Sections 10 µm thick were obtained, mounted on superfrost slides (VWR, Catalog# 48311-703) and stored at -20°C until use.

Immunofluorescence

Upon use, the slides were heated at 65°C for 30 minutes and, subsequently, sections were washed with PBS to remove tissue freezing medium. Sections were then treated with 0.1% glycine (Fisher Chemical, Catalog# G46-500), blocked with 5% FBS (Seradigm, Catalog# 3100-500) and permeabilized with 0.1% saponin prepared in PBS. Sections were then incubated with the primary antibodies anti-Tenascin (Abcam, Ab108930), anti-GFAP (Abcam, Ab4647), anti-CD68 (Ab31630, Abcam) and anti-β III tubulin (Ab18207, Abcam). Sections were washed and incubated with appropriate secondary donkey antibodies conjugated with Alexa Fluor® 488 (Life Technologies) or Alexa Fluor® 555 (Life Technologies) for one hour at 18°C. For HA staining, tissues were incubated with biotinylated HA binding protein (385911, Millipore) followed by NeutrAvidin®Alexa 555 (Life Technologies). The tissues were then washed and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Sections were mounted in Prolong®Gold (Molecular Probes) and imaged using a ZEISS LSM 800 Confocal microscope with Airyscan. Secondary controls were done with a goat IgG isotype control (ab37388; Abcam) in place of the primary antibody and did not yield any significant staining (results not shown). For imaging, multiple z-stack tiles were captured of entire brain sections and frames were processed together into a single image (using the stitching mode followed by full orthogonal projection) using Zen Software (Zeiss). The number of GFAP⁺ and CD68⁺ cells in and around the injury site were counted by two independent investigators in a blinded manner and the relative Fluorescent intensity was measured using the Zen Software (Zeiss). At least 2 sections were scanned and analyzed from each animal for each set of antibodies and representative images shown in the figures.

RNA extraction from brains and real-time PCR analysis

Brains collected from injured mice at 1, 5 and 10 days post injury were used for RNA extraction. At least 5 mice were used per experimental group and each animal was analyzed separately. Briefly, mice were euthanized and brain tissue was immediately isolated from each mouse. Injury sites (A samples) were dissected from the rest of the injured right hemisphere, transferred into a labeled Eppendorf tube and immediately immersed in liquid nitrogen. The remaining right hemisphere brain tissue (B samples) from each animal was transferred into a different tube and also frozen as described. The samples were kept at -80°C until RNA extraction. Total RNA was isolated from these tissue samples using Trizol® Reagent (Invitrogen, Carlsbad, CA) and chloroform extraction (Sigma-Aldrich, Catalog# 650498). First strand cDNA was reverse transcribed using 1.5 to 2 µg of total RNA and the high capacity cDNA Reverse Transcription kit (Applied Biosystems, catalog# 4368814, lot 00593854) according to the manufacturer's instructions. Quantitative real-time PCR amplification was performed on 1 µl or 50 ng of the cDNA (1:5) using the PowerUp SYBR Green Master Mix kit (Applied Biosystems, Catalog# A25918) in a CFX Connect Real-time System from BIO-RAD, using an activation cycle of 95°C for 10 min, 40 cycles of 95°C for 15 seconds and

60°C for 1 min. A complete list of primers used in this study is shown in Table 1. Gene expression levels were normalized against *Actb* and *Gapdh* using the $2^{-\Delta Ct}$ and/or $2^{-\Delta\Delta Ct}$ methods.

Statistical analysis

All values are presented as the mean $\pm$ standard deviation of the mean. The difference between the two groups was compared by means of the Student's t-test. $P \leq 0.05$ was considered to be statistically significant. Statistical analysis was performed using the GraphPad Prism version 7 software package (GraphPad Software, San Diego, CA, USA). * was used to indicate the statistical differences of ≤ 0.05 . Unless indicated otherwise, * indicates the statistical difference of *Tsg-6*^{-/-} mice compared to *Tsg-6*^{+/-} mice.

Results

TSG-6 expression after PBI

In order to investigate whether TSG-6 is present in the glial scar after brain injury we analyzed the expression profile of *Tsg6* in the injury site and injured hemisphere before and after a PBI in *Tsg-6*^{+/-} mice (Figure 1A). There was a 2-fold increase in *Tsg-6* expression 5 days after injury when compared to uninjured mice. There was a further increase in *Tsg-6* expression over time after injury, with expression increasing 2 fold from 5 to 10 days after injury (Figure 1A). Interestingly, we did not find a difference in the expression levels of *Tsg-6* between the injury site and the remaining hemisphere, indicating that *Tsg-6* expression is not contained solely to the injury site (Figure 1A). Therefore, there is also an increase in *Tsg-6* expression in the surrounding tissue after injury. No *Tsg-6* expression was identified in any of the samples from *Tsg-6*^{-/-} mice confirming that these mice are indeed *null* for *Tsg-6*.

Analysis of astrocyte recruitment after PBI

We assessed the level of astrogliosis in the injury site and in the remaining injured hemisphere by quantifying the levels of GFAP⁺ astrocytes using real-time PCR (Figure 1B and C). For such, we isolated mRNA from the injury site and remaining injured hemisphere 1 and 5 days after injury of *Tsg-6*^{-/-} and *Tsg-6*^{+/-} mice. Both *Tsg-6*^{-/-} and *Tsg-6*^{+/-} mice presented an increase in the levels of GFAP expression in the injury site when compared to the remaining injured hemisphere, which corroborates literature (36,63,64). *Tsg-6*^{-/-} mice showed a significant increase in GFAP levels within the injury site at both 1 and 5 days post-injury when compared to *Tsg-6*^{+/-} mice (Figure 1B and C). This data indicates that *Tsg-6*^{-/-} mice have more astrocytes in the injury site when compared to *Tsg-6*^{+/-} mice. At 5 days after injury there was a significant increase in GFAP expression in the injured hemisphere of *Tsg-6*^{-/-} mice compared to *Tsg-6*^{+/-} mice, indicating that *Tsg-6*^{-/-} mice present astrogliosis beyond the injury site at 5 days post-injury.

The effect of TSG-6 on the secretion of inflammatory markers after PBI

The inflammatory response was also assessed in *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice 1, 5 and 10 days post-injury by quantifying the expression levels of *NfκB*, *Rantes* and *IL 1b* (Figure 2). Higher expression levels of *NfκB*, *Rantes* and *IL 1b* were detected in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice during the acute phase after injury. Specifically, a ~2.5-fold and 3-fold increase in *NfκB* expression was found in the injury site and remaining injured hemisphere, respectively, in *Tsg-6^{-/-}* mice compared to *Tsg-6^{+/-}* mice 5 days after injury (Figure 2B). 10 days after injury there was still a significant increase in *NfκB* expression in the surrounding hemisphere of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 2C). No significant differences were found in the expression of *NfκB* between *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice 1 day after injury (Figure 1A). The levels of *Ccl5* (*Rantes*) were also assessed 1, 5 and 10 days post-injury. There was a significant increase in the expression of *Rantes* in the injured hemisphere of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (a 4-fold increase) 5 days after injury; however, no difference was found between *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice 1 and 10 days after injury (Figure 2D-F). *IL 1b* levels were increased in the injury site of *TSG-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice at 1-day post-injury (Figure 2G). At 5 days post-injury a 3-fold and 4-fold increase in the expression of *IL 1b* were noted in the injury site and remaining injured hemisphere, respectively, of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 2H). At 10 days post-injury a 2.5-fold increase in the expression of *IL 1b* was noted in the injury site of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 2I).

The effect of TSG-6 on the activation of microglia and infiltration of macrophages into the injury site after PBI

In order to assess the inflammatory response in *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice, we also evaluated the number of CD68⁺ cells present within the injury site at 3 days post-injury (Figure 3). CD68 is routinely used as a marker for macrophages and activated microglia. There was a significant increase in the number of CD68⁺ cells in and surrounding the injury site of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 3A panels i and ii). Importantly, even when analyzing deeper regions of the injury site of *Tsg-6^{+/-}* mice, the level of CD68⁺ cell infiltration was not as intense as that observed in *Tsg-6^{-/-}* mice (Figure 3A panel iii). The combined number of CD68⁺ cells in the injury site and within a range of 100 mm from the wound edge was counted from images obtained from 2 different sections from at least 5 mice from each experimental point. A 2-fold increase in CD68⁺ cells was found in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice.

Correlation between increased inflammatory response and neuronal damage

In order to verify whether the increased inflammatory response observed in *Tsg-6^{-/-}* mice correlates with neuronal loss, the distribution of neurons in and around the injury site was analyzed in *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice 14 days post-injury (Figure 3B). For such, b III tubulin was used as a tissue-specific marker for identifying neurons within injured and non-injured brains. The distribution of b III tubulin can be seen in the equivalent region of uninjured *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice (Figure 3 B panels i and ii). A significant

increase in the area devoid of β III tubulin staining can be observed in and around the injury site of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice 14 days post-injury (Figure 3B iii and iv). The relative fluorescence units (RLU) were quantified from an image of the injury site captured from at least 3 mice per experimental point. There was a 4-fold decrease in β III tubulin staining in and around the injury site of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice 14 days post-injury (Figure 3D).

The effect of TSG-6 on the secretion of glial scar components after PBI

We also evaluated glial scar secretion within the injury site and injured hemisphere by evaluating the expression levels of the biosynthetic enzymes responsible for HA and CS chain elongation, specifically hyaluronan synthase 2 (*Has2*), carbohydrate (chondroitin 4) sulfotransferase (*chst 11*) and carbohydrate (chondroitin 4) sulfotransferase 12 (*chst 12*) (Figure 4). *Has2* expression increased in the injury site when compared to the remaining injured hemisphere 5 days post injury in both *Tsg-6^{+/-}* and *Tsg-6^{-/-}* mice, confirming the numerous previously published reports showing that HA is an integral component of the glial scar. Interestingly, there was a 2-fold increase in *Has2* expression in the injury site of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice 5 days after injury, indicating that there is a higher rate of glial scar production in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 4A). At 10 days post-injury, *Has2* expression was still increased by 2-fold in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice, but at this time point there was also an increase in *Has2* expression in the remaining injured hemisphere of *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 4B). Thus, at 10 days after injury, in *Tsg-6^{-/-}* mice, the expression of glial scar components was no longer limited to the injury site, but was also present within the remaining injured hemisphere. Interestingly, this is also true for the expression of *Chst11* and *Chst12*, which show a 5-fold and 4-fold increase, respectively, within the injured hemisphere of *Tsg-6^{-/-}* mice at 5 days post injury when compared to *Tsg-6^{+/-}* mice (Figure 4C and E). The increase in *Chst11* and *Chst12*, in both the injury site and injured hemisphere, was maintained through to 10 days post-injury (Figure 4D and F).

The effect of TSG-6 on astrocyte activation and recruitment after PBI

In order to further investigate the process of astrogliosis in *Tsg-6^{+/-}* and *Tsg-6^{-/-}* mice, injured brains were harvested and processed for histology. Sections were stained for GFAP in order to assess the distribution of astrocytes in and around the injury site and also throughout the remaining brain tissue. The number of astrocytes (GFAP⁺ cells) was counted within the injury site, throughout the injured hemisphere and also throughout the contralateral hemisphere 3 and 14 days post-injury (Figure 5A and B). At 3 and 14 days post-injury there was a significant increase in the number of astrocytes within the injury site when compared to the injured hemisphere and contralateral hemisphere in both *Tsg-6^{+/-}* and *Tsg-6^{-/-}* mice. At 3 days post-injury there was no significant difference between the number of astrocytes within the injury site between *Tsg-6^{+/-}* and *Tsg-6^{-/-}* mice; however, there was a significant increase in the number of astrocytes within the injured hemisphere in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 5A). At 14 days post-injury there was a significant increase in the number of astrocytes within the injury site and

injured hemisphere in *Tsg-6^{-/-}* mice when compared to *Tsg-6^{+/-}* mice (Figure 5B and D). The increase in astrocytes can be seen beyond the injury site in *Tsg-6^{-/-}* mice (Figure 5C and D panel iv).

Discussion

Chondroitin sulfate proteoglycans (CSPGs) are well established as major extracellular matrix components in the central nervous system (65). Over a decade ago, Silver et al. identified that CSPGs within the glial scar inhibit axonal growth, and this triggered a great deal of interest in targeting CS within the scar tissue as a means to promote axonal regeneration (32,66–68). Over the years, strategies utilizing the enzymes chondroitinase ABC (ChABC) and ChAC have been used to remove the CS component of the glial scar as a means to promote axonal growth and regeneration (44,46,69–72). Many studies have shown that specifically removing CS within the glial scar is enough for axons to grow across the injury site (66,67,73,74). However, significant regeneration was never observed in these studies, and many groups found limited or no improvement after targeting CS within the glial scar (66). One known unique characteristic of TSG-6 is its known ability to bind to a number of ligands including HA, CS and core proteins of proteoglycans (i.e. versican and aggrecan), forming specific HA/HC/TSG-6 matrices with immunosuppressive characteristics (57,75–79). Our previous study suggests these HA/HC/TSG-6 matrices are also present within the glial scar (42). Therefore, given that TSG-6 directly binds to both CS to form specific anti-inflammatory HA/HC/TSG-6 matrices, the ChABC and ChAC treatments used over the years to target the glial scar as a means to promote regeneration would also have removed TSG-6, a known anti-inflammatory molecule that is also a component of the glial scar (77). This could, in part, explain why significant functional recovery was never obtained after ChABC and/or ChAC treatments, since these therapies were also removing TSG-6.

To explore the role of TSG-6 in TBI, specifically astrogliosis, we compared the differences in injury outcomes in *Tsg-6^{-/-}* and *Tsg-6^{+/-}* mice after PBIs. Our data show an increase in TSG-6 expression in the injured hemisphere of *Tsg-6^{+/-}* mice after TBI. This increase in expression after CNS insults supports our earlier findings in a rat model that astrocytes secrete high levels of TSG-6 upon injury, which aids in the formation of a specialized HA/HC/TSG-6 matrix during an inflammatory response (42). As TSG-6 is known for having anti-inflammatory properties, to further study whether high levels of TSG-6 served a purpose of rapidly suppressing inflammation after injury, we performed similar penetrating injuries in *Tsg-6^{-/-}* mice. We used immunofluorescence and RNA expression analyses of inflammatory and glial scar markers to elucidate the outcome during the acute phase as well as chronic phase of TBI. During the acute phase of injury, the observed increased in astrocyte activation, inflammatory cell infiltration and expression of inflammatory cytokines in *Tsg-6^{-/-}* mice, indicate that the loss of TSG-6 results in a greater inflammatory response. Moreover, during the chronic phase of injury, unrestricted inflammatory response is observed throughout the injured hemisphere and does not seem to be limited to the injury site as is common with glial scar formation. Thus, injured *Tsg-6^{-/-}* mice appear to experience more severe tissue damage than their *Tsg-6^{+/-}* counterpart, both within and surrounding the injury site. Thus, the loss of TSG-6 allows the damage to spread from the injury site to neighboring healthy tissues. We postulate that the

cause of such widespread damage is due to the lack of the specialized HA-TSG6 or HA/HC/TSG-6 matrix that could possibly stabilize the glial scar thereby protecting adjacent tissues from further damage. This hypothesis is further supported by the increase in CSPG and HA biosynthesis, glial scar components, in *Tsg-6*^{-/-} mice. Specifically, these mice show increased *Has2*, *Chst11* and *Chst12* expression levels in tissues collected after the onset of glial scarring and also during the chronic phase of astrogliosis indicating an increase in scar tissue formation. This increase in expression was not only observed at the injury site but throughout the whole hemisphere, suggesting that the tissue damage spreads beyond the injury site in the absence of TSG-6. Collectively, these results demonstrate that the loss of TSG-6 leads to a more severe inflammatory response and consequently increased scarring after TBI. Thus our results support the hypothesis put forward by many groups over the past decade that preventing the formation of the glial scar leads to inflammation and damage beyond the injury site. We also provide experimental evidence that shows that the glial scar functions to restrict the damage to the injury site. Importantly, these findings should be taken into account when attempts are made to disrupt the glial scar as a means to promote neuronal regeneration, since preventing formation of the glial scar may not have the beneficial outcomes as previously presumed.

Conclusion

Our results show that TSG-6 has an anti-inflammatory role in the glial scar. Our study further supports the hypothesis that the glial scar forms a protective border surrounding the injury site thereby preventing the spread of inflammation and damage beyond the injury site.

Abbreviations

TBI: Traumatic brain injury

TNF: Tumor necrosis factor

TSG-6: TNF-stimulated gene-6

GFAP: Glial Fibrillary Acidic Protein

VIM: Vimentin

CSPGs: Chondroitin sulfate proteoglycans

STAT3: Signal transducer and activator of Transcription 3

HA: Hyaluronan

GAGs: Glycosaminoglycans

CNS: Central nervous system

HC: Heavy Chain

ITI or I α I: Inter-alpha inhibitor

hUMSCs: Human umbilical cord mesenchymal stem cells

PTX3: Pentraxin-3

NFKB: Nuclear factor kappa-light-chain-enhancer

IL1 β : Interleukin 1 beta

Ccl5: Chemokine (C-C motif) ligand 1 or Rantes

HAS: Hyaluronan synthase

Chst: Carbohydrate (chondroitin 4) sulfotransferase

Declarations

Ethics approval

Experimental procedures for handling the mice and animal care were in accordance to regulations of the National Institute of Health and were approved by the Institutional Animal Care and Use Committee, University of Houston.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by start-up funds from the University of Houston to VJCT, The Mizutani Foundation grant to VJCT and the National Institute of Health/National Eye Institute R01 EY029289 to VJCT and Core grant P30 EY07551.

Authors' contributions

VH and VCT generated the hypothesis and experimental design. VCT, KNM and MS contributed to the experimental design. KNM, MS, AN and VCT conducted the experiments and helped with the data analysis. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank Denise Lerma and Nicole Grimmes who participated in the study. The authors are in debt to Tarsis F. Gesteira and Yvette May Coulson- Thomas for their invaluable discussions and suggestions throughout the study. Transgenic *Tsg-6 null* mice (*Tnfrp6^{D/D}*) were kindly provided by Dr. Mark Lauer.

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References

1. Hyder AA, Wunderlich CA, Puvanachandra P, Gururaj G, Kobusingye OC. The impact of traumatic brain injuries: a global perspective. *NeuroRehabilitation*. 2007;
2. Bose P, Hou J, Thompson FJ. Traumatic Brain Injury (TBI)-Induced Spasticity: Neurobiology, Treatment, and Rehabilitation. *Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects*. 2015.
3. Blennow K, Brody DL, Kochanek PM, Levin H, McKee A, Ribbers GM, et al. Traumatic brain injuries. *Nat Rev Dis Prim*. 2016;
4. Graham DI, McIntosh TK, Maxwell WL, Nicoll JAR. Recent advances in neurotrauma. *Journal of Neuropathology and Experimental Neurology*. 2000.
5. Zaninotto ALC, Costa BT, Ferreira IS, French M, Paiva WS, Fregni F. Traumatic brain injury. In: *Neuromethods*. 2018.
6. Burda JE, Bernstein AM, Sofroniew M V. Astrocyte roles in traumatic brain injury. *Experimental Neurology*. 2016.
7. Kovacs SK, Leonessa F, Ling GSF. Blast TBI models, neuropathology, and implications for seizure risk. *Frontiers in Neurology*. 2014.
8. Sharp DJ, Scott G, Leech R. Network dysfunction after traumatic brain injury. *Nature Reviews Neurology*. 2014.
9. Fakhran S, Alhilali L. Neurodegenerative changes after mild traumatic brain injury. In: *Concussion*. 2012.
10. NAGAHIRO S, MIZOBUCHI Y. Current Topics in Sports-related Head Injuries: A Review. *Neurol Med Chir (Tokyo)*. 2014;

11. Jordan BD. The clinical spectrum of sport-related traumatic brain injury. *Nature Reviews Neurology*. 2013.
12. Costanza A, Weber K, Gandy S, Bouras C, Hof PR, Giannakopoulos P, et al. Review: Contact sport-related chronic traumatic encephalopathy in the elderly: Clinical expression and structural substrates. *Neuropathology and Applied Neurobiology*. 2011.
13. McAllister T, McCrea M. Long-Term Cognitive and Neuropsychiatric Consequences of Repetitive Concussion and Head-Impact Exposure. *J Athl Train*. 2017;
14. Sofroniew M V., Vinters H V. Astrocytes: Biology and pathology. *Acta Neuropathologica*. 2010.
15. Sofroniew M V. Astrogliosis. *Cold Spring Harb Perspect Biol*. 2015;
16. Sofroniew M V. Reactive astrocytes in neural repair and protection. *Neuroscientist*. 2005.
17. Smith PD, Coulson-Thomas VJ, Foscari S, Kwok JCF, Fawcett JW. "GAG-ing with the neuron": The role of glycosaminoglycan patterning in the central nervous system. *Experimental Neurology*. 2015.
18. McGraw J, Hiebert GW, Steeves JD. Modulating astrogliosis after neurotrauma. *Journal of Neuroscience Research*. 2001.
19. Kawano H, Kimura-Kuroda J, Komuta Y, Yoshioka N, Li HP, Kawamura K, et al. Role of the lesion scar in the response to damage and repair of the central nervous system. *Cell and Tissue Research*. 2012.
20. Burda JE, Sofroniew M V. Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron*. 2014.
21. Sofroniew M V. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in Neurosciences*. 2009.
22. Davies SJA, Goucher DR, Doller C, Silver J. Robust Regeneration of Adult Sensory Axons in Degenerating White Matter of the Adult Rat Spinal Cord. *J Neurosci*. 2018;
23. McKeon RJ, Jurynek MJ, Buck CR. The Chondroitin Sulfate Proteoglycans Neurocan and Phosphacan Are Expressed by Reactive Astrocytes in the Chronic CNS Glial Scar. *J Neurosci*. 2018;
24. Okada S, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, et al. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*. 2006;
25. Voskuhl RR, Peterson RS, Song B, Ao Y, Morales LBJ, Tiwari-Woodruff S, et al. Reactive Astrocytes Form Scar-Like Perivascular Barriers to Leukocytes during Adaptive Immune Inflammation of the CNS. *J Neurosci*. 2009;
26. Wanner IB, Anderson MA, Song B, Levine J, Fernandez A, Gray-Thompson Z, et al. Glial Scar Borders Are Formed by Newly Proliferated, Elongated Astrocytes That Interact to Corral Inflammatory and Fibrotic Cells via STAT3-Dependent Mechanisms after Spinal Cord Injury. *J Neurosci*. 2013;
27. Faulkner JR. Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury. *J Neurosci*. 2004;
28. Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew M V. Essential protective roles of reactive astrocytes in traumatic brain injury. *Brain*. 2006;

29. Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Experimental Neurology*. 2008.
30. Massey JM, Amps J, Viapiano MS, Matthews RT, Wagoner MR, Whitaker CM, et al. Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3. *Exp Neurol*. 2008;
31. Tan AM. Antibodies against the NG2 Proteoglycan Promote the Regeneration of Sensory Axons within the Dorsal Columns of the Spinal Cord. *J Neurosci*. 2006;
32. Bradbury EJ, Carter LM. Manipulating the glial scar: Chondroitinase ABC as a therapy for spinal cord injury. *Brain Research Bulletin*. 2011.
33. Pekny M, Wilhelmsson U, Pekna M. The dual role of astrocyte activation and reactive gliosis. *Neuroscience Letters*. 2014.
34. Wilhelmsson U. Absence of Glial Fibrillary Acidic Protein and Vimentin Prevents Hypertrophy of Astrocytic Processes and Improves Post-Traumatic Regeneration. *J Neurosci*. 2004;
35. Pekny M. Astrocytic intermediate filaments: Lessons from GFAP and vimentin knock-out mice. *Prog Brain Res*. 2001;
36. Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallén Å, Perlmann T, et al. Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. *J Cell Biol*. 1999;
37. Bush TG, Puvanachandra N, Horner CH, Polito A, Ostensfeld T, Svendsen CN, et al. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron*. 1999;
38. Herrmann JE, Imura T, Song B, Qi J, Ao Y, Nguyen TK, et al. STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *J Neurosci*. 2008;
39. Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew M V. Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury. *J Neurosci*. 2004;
40. Fan H, Zhang K, Shan L, Kuang F, Chen K, Zhu K, et al. Reactive astrocytes undergo M1 microglia/macrophages-induced necroptosis in spinal cord injury. *Mol Neurodegener*. 2016;
41. Gesteira TF, Coulson-Thomas YM, Coulson-Thomas VJ. Anti-inflammatory properties of the glial scar. *Neural Regeneration Research*. 2016.
42. Coulson- Thomas VJ, Lauer ME, Soleman S, Zhao C, Hascall VC, Day AJ, et al. TSG-6 is constitutively expressed in adult CNS and associated with astrocyte-mediated glial scar formation following spinal cord injury. *J Biol Chem*. 2016;
43. Milner CM, Day AJ. TSG-6: A multifunctional protein associated with inflammation. *J Cell Sci*. 2003;116(10):1863–73.
44. Bradbury EJ, Moon LDF, Popat RJ, King VR, Bennett GS, Patel PN, et al. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature*. 2002;

45. Massey JM, Hubscher CH, Wagoner MR, Decker JA, Amps J, Silver J, et al. Chondroitinase ABC digestion of the perineuronal net promotes functional collateral sprouting in the cuneate nucleus after cervical spinal cord injury. *J Neurosci*. 2006;
46. Elkin BS, Shaik MA, Morrison B. Chondroitinase ABC reduces brain tissue swelling in vitro. *J Neurotrauma*. 2011;28(11):2277–85.
47. Moon LDF, Asher RA, Fawcett JW. Limited growth of severed CNS axons after treatment of adult rat brain with hyaluronidase. *J Neurosci Res*. 2003;71(1):23–37.
48. Carrette O, Nemade R V., Day AJ, Brickner A, Larsen WJ. TSG-6 Is Concentrated in the Extracellular Matrix of Mouse Cumulus Oocyte Complexes Through Hyaluronan and Inter-Alpha-Inhibitor Binding1. *Biol Reprod*. 2001;
49. Salustri A, Yanagishita M, Hascall VC. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. *J Biol Chem*. 1989;
50. Salustri A, Yanagishita M, Underhill CB, Laurent TC, Hascall VC. Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle. *Dev Biol*. 1992;
51. Camaioni A, Hascall VC, Yanagishita M, Salustri A. Effects of exogenous hyaluronic acid and serum on matrix organization and stability in the mouse cumulus cell-oocyte complex. *J Biol Chem*. 1993;
52. Fulop C. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development*. 2003;
53. Stober VP, Johnson CG, Majors A, Lauer ME, Cali V, Midura RJ, et al. TNF-stimulated gene 6 promotes formation of hyaluronan-inter- α -inhibitor heavy chain complexes necessary for ozoneinduced airway hyperresponsiveness. *J Biol Chem*. 2017;
54. Lauer ME, Glant TT, Mikecz K, DeAngelis PL, Haller FM, Husni ME, et al. Irreversible heavy chain transfer to hyaluronan oligosaccharides by tumor necrosis factor-stimulated gene-6. *J Biol Chem*. 2013;
55. Petrey AC, De La Motte CA. Thrombin Cleavage of Inter- α -inhibitor Heavy Chain 1 Regulates Leukocyte Binding to an Inflammatory Hyaluronan Matrix. *J Biol Chem*. 2016;
56. Hill DR, Rho HK, Kessler SP, Amin R, Homer CR, McDonald C, et al. Human milk hyaluronan enhances innate defense of the intestinal epithelium. *J Biol Chem*. 2013;
57. Baranova NS, Foulcer SJ, Briggs DC, Tilakaratna V, Enghild JJ, Milner CM, et al. Inter- α -inhibitor impairs TSG-6-induced hyaluronan cross-linking. *J Biol Chem*. 2013;
58. Kessler SP, Obery DR, De La Motte C. Hyaluronan Synthase 3 Null Mice Exhibit Decreased Intestinal Inflammation and Tissue Damage in the DSS-Induced Colitis Model. *Int J Cell Biol*. 2015;
59. Lim Y, Bendelja K, Opal SM, Siryaporn E, Hixson DC, Palardy JE. Correlation between Mortality and the Levels of Inter-Alpha Inhibitors in the Plasma of Patients with Severe Sepsis. *J Infect Dis*. 2003;

60. Schmidt EP, Overdier KH, Sun X, Lin L, Liu X, Yang Y, et al. Urinary glycosaminoglycans predict outcomes in septic shock and acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2016;
61. Coulson-Thomas VJ, Gesteira TF, Hascall V, Kao W. Umbilical cord mesenchymal stem cells suppress host rejection: The role of the glycocalyx. *J Biol Chem*. 2014;
62. Franklin KBJ, Paxinos G. *The Mouse Brain in Stereotaxic Coordinates (map)*. Academic Press. 2007.
63. Pelinka LE, Kroepfl A, Schmidhammer R, Krenn M, Buchinger W, Redl H, et al. Glial fibrillary acidic protein in serum after traumatic brain injury and multiple trauma. *J Trauma - Inj Infect Crit Care*. 2004;
64. Eng LF, Ghimikar RS, Lee YL. Glial Fibrillary Acidic Protein: GFAP-Thirty-One Years (1969-2000). *Neurochem Res*. 2000;
65. S.M. D, S. K-A. Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. *Exp Neurol*. 2015;
66. McKeon RJ, Höke A, Silver J. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp Neurol*. 1995;
67. Silver J, Miller JH. Regeneration beyond the glial scar. *Nature Reviews Neuroscience*. 2004.
68. McKeon RJ, Schreiber RC, Rudge JS, Silver J. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci*. 1991;
69. Foerster AP. Spontaneous regeneration of cut axons in adult rat brain. *J Comp Neurol*. 1982;210(4):335–56.
70. Brückner G, Bringmann A, Härtig W, Köppe G, Delpech B, Brauer K. Acute and long-lasting changes in extracellular-matrix chondroitin-sulphate proteoglycans induced by injection of chondroitinase ABC in the adult rat brain. *Exp Brain Res*. 1998;121(3):300–10.
71. Filous AR, Miller JH, Coulson-Thomas YM, Horn KP, Alilain WJ, Silver J. Immature astrocytes promote CNS axonal regeneration when combined with chondroitinase ABC. *Dev Neurobiol*. 2010;70(12):826–41.
72. Moon LDF, Asher RA, Rhodes KE, Fawcett JW. Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nat Neurosci*. 2001;
73. Busch SA, Silver J. The role of extracellular matrix in CNS regeneration. *Current Opinion in Neurobiology*. 2007.
74. Klapka N, Hermanns S, Straten G, Masannek C, Duis S, Hamers FPT, et al. Suppression of fibrous scarring in spinal cord injury of rat promotes long-distance regeneration of corticospinal tract axons, rescue of primary motoneurons in somatosensory cortex and significant functional recovery. *Eur J Neurosci*. 2005;
75. Milner CM, Tongsoongnoen W, Rugg MS, Day AJ. The molecular basis of inter- α -inhibitor heavy chain transfer on to hyaluronan: Figure 1. *Biochem Soc Trans*. 2007;

76. Getting SJ, Mahoney DJ, Cao T, Rugg MS, Fries E, Milner CM, et al. The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter- α -inhibitor-independent manner. J Biol Chem. 2002;

77. Baranova NS, Nilebäck E, Haller FM, Briggs DC, Svedhem S, Day AJ, et al. The inflammation-associated protein TSG-6 cross-links hyaluronan via hyaluronan-induced TSG-6 oligomers. J Biol Chem. 2011;

78. Higman VA, Briggs DC, Mahoney DJ, Blundell CD, Sattelle BM, Dyer DP, et al. A refined model for the TSG-6 link module in complex with hyaluronan: Use of defined oligosaccharides to probe structure and function. J Biol Chem. 2014;

79. Martin J, Midgley A, Meran S, Woods E, Bowen T, Phillips AO, et al. Tumor necrosis factor-stimulated gene 6 (TSG-6)-mediated interactions with the inter- α -inhibitor heavy chain 5 facilitate tumor growth factor β 1 (TGF β 1)-dependent fibroblast to myofibroblast differentiation. J Biol Chem. 2016;

Tables

Table 1

Gene (Mus musculus)	accession number	Forward (5' 3')	Reverse (5' 3')
<i>Tenascin C (tnc)</i>	NM_011607.3	CCAGGGTTGCCACCTATTT	GTCTAGAGGATCCCACTCTACTT
<i>Gfap</i>	NM_001131020.1	AACAACCTGGCTGCGTATAG	TCTCGAACTTCCTCCTCATAGAT
<i>Tsg-6 /Tnfaip6</i>	NM_009398.2	CCCACATGCAAAGGAGTGTG	TGAGCCGAATGTGCCAGTAG
<i>Chst1</i>	NM_021439.2	CACCCAGTCATGCGGAGGAA	GCAGGATGGCAGTGTTGGAT
<i>Chst12</i>	NM_021528.3	GAGCTGGAGAACGAAGAGTTT	CAGGAGGTACTGGATGAAGTTG
<i>IL1b</i>	NM_008361.4	GTGCAAGTGTCTGAAGCAGC	CTCATCACTGTCAAAAGGTGGC
<i>Cspg4</i>	NM_139001.2	TCTACAGCTCCTGCCTCCTT	ATGTGGAGAACTGGAGCAGC
<i>Ccl5 (Rantes)</i>	NM_013653.3	CCTCACCATATGGCTCGGAC	ACGACTGCAAGATTGGAGCA
<i>Nfkb1</i>	NM_008689.2	GTCACCCATGGCACCATAAA	CCTTCACCTTCAGTTTCCTTCTC
<i>Has1</i>	NM_008215.2	CTA TGC TAC CAA GTA TAC CTC G	TCT CGG AAG TAA GAT TTG GAC
<i>Has2</i>	NM_008216.3	CGG TCG TCT CAA ATT CAT CTG	ACA ATG CAT CTT GTT CAG CTC
<i>Has 3</i>	NM_008217.4	GAT GTC CAA ATC CTC AAC AAG	CCC ACT AAT ACA TTG CAC AC
<i>Itih1</i>		CCA CCC CAT CGG TTT TGA AGT GTC T	TGC CAC GGG TCC TTG CTG TAG TCT
<i>Itih2</i>		ATG AAA AGA CTC ACG TGC TTT TTC	ATT TGC CTG GGG CCA GT
<i>Itih3</i>		TGA GGA GGT GGC CAA CCC ACT	CGC TTC TCC AGC AGC TGC TC
<i>Actb</i>	NM_007393.5	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG
<i>Gapdh</i>	NM_001289726.1	AACAGCAACTCCCACTCTTC	CCTGTTGCTGTAGCCGTATT

Figures

Figure 1

TSG-6 and GFAP expression after PBI. TSG-6 and GFAP mRNA expressions were quantified in the injury site and the injured hemisphere after PBI. (A) TSG^{+/-} mice were submitted to PBI, and the injury site and remaining injured hemisphere were collected 5 and 10 days after injury for analysis of TSG-6 expression. (B and C) TSG^{+/-} and TSG^{-/-} mice were subjected to PBI, and the injury site and remaining injured

hemisphere were collected 1 day (B) and 5 days (C) after injury. * = $p \leq 0.05$ comparing TSG-6^{+/-} and TSG-6^{-/-} mice.

Figure 2

Analysis of inflammatory markers after PBI. NFκB, RANTES and IL1β mRNA expressions were quantified in the injury site and the injured hemisphere after PBI. TSG^{+/-} mice and TSG-6^{-/-} mice were subjected to PBI and the injury site and remaining injured hemisphere were collected 1, 5 and 10 days after injury.

mRNA was extracted and subjected to real-time PCR analysis for NF κ B (A, B and C), RANTES (D, E and F) and IL1 β (G, H and I) mRNA expression. * = $p \leq 0.05$ comparing TSG-6 $^{+/-}$ and TSG-6 $^{-/-}$ mice.

Figure 3

Analysis of inflammatory cell infiltration and neuronal cell loss after PBI. The distribution of macrophages and activated microglia was evaluated within the injury site of TSG $^{+/-}$ and TSG-6 $^{-/-}$ mice 3 days post-injury (dpi) by anti-CD68 immunostaining (red) (A). Neuronal cells were immunostained with anti- β III tubulin (green) in the equivalent area of uninjured TSG $^{+/-}$ (i) and TSG-6 $^{-/-}$ (ii) mice and within the injury site of TSG-6 $^{+/-}$ (iii) and TSG-6 $^{-/-}$ (iv) mice 14 days post-injury (dpi). The number of CD68 $^{+}$ cells was counted in the injury site and within 100 μ m of the wound edge of TSG-6 $^{+/-}$ and TSG-6 $^{-/-}$ mice 3 days post-injury (C). The relative fluorescent units (RFU) of anti- β III tubulin staining were quantified in

and around the injury site of TSG-6/+ and TSG-6/- mice 14 days post-injury (D). Nuclei were counterstained with DAPI. Scale bar represents 100 μ m. * = $p \leq 0.05$ comparing TSG-6/+ and TSG-6/- mice.

Figure 4

Analysis of glial scar ECM components after PBI. HAS2, Chst 11 and Chst 12 mRNA expression levels were quantified in the injury site and the injured hemisphere after PBI. TSG^{+/+} and TSG-6^{-/-} mice were

subjected to PBI, and the injury site and remaining injured hemisphere were collected 5 and 10 days after injury. mRNA was extracted and subjected to real-time PCR analysis for HAS2 (A and B), Chst11 (C and D) and Chst 12 (E and F) mRNA expression. * = $p \leq 0.05$ comparing TSG-6^{-/+} and TSG-6^{-/-} mice.

Figure 5

Analysis of astrocyte activation and recruitment after PBI. Brain sections from TSG-6^{+/-} and TSG-6^{-/-} mice were analyzed by immunofluorescence. Astrocytes were identified with anti-GFAP (green) and the

glial scar with HABP (red). Nuclei were counterstained with DAPI (blue). Z-stacks were captured of the entire brain section using the tiling mode, and images were stitched together using Zen software. Thereafter, the number of astrocytes was counted within the injury site, within the injured hemisphere and in the contralateral hemisphere of brains 3 (A) and 14 dpi (B) in a double blinded manner. The distribution of astrocytes throughout the brain sections shows that in TSG-6^{-/-} mice the increase in astrocytes is not restricted to the injury site (C). Magnified images of the areas demarcated in (C) can be seen in (D). At least 3 mice were analyzed per genotype for each time point. * = $p \leq 0.05$ comparing TSG^{+/-} and TSG-6^{-/-} mice.

Supplementary Files

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