Intramuscular insulin-like growth factor-1 gene therapy modulates reactive microglia after traumatic brain injury

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ABSTRACT

Reactive gliosis is a key feature and an important pathophysiological mechanism underlying chronic neurodegeneration following traumatic brain injury (TBI). In this study, we have explored the effects of intramuscular IGF-1 gene therapy on reactive gliosis and functional outcome after an injury of the cerebral cortex. Young adult male rats were intramuscularly injected with a recombinant adenoviral construct harboring the cDNA of human IGF-1 (RAD-IGF1), with a control vector expressing green fluorescent protein (RAD-GFP) or PBS as control. Three weeks after the intramuscular injections of adenoviral vectors, animals were subjected to a unilateral penetrating brain injury. The data revealed that RAD-IGF1 gene therapy significantly increased serum IGF1 levels and improved working memory performance after one week of TBI as compared to PBS or RAD-GFP lesioned animals. At the same time, when we analyzed the effects of therapy on glial scar formation, the treatment with RAD-IGF1 did not modify the number of glial fibrillary acidic protein (GFAP) positive cells, but we observed a decrease in vimentin immunoreactive astrocytes at 7 days post-lesion in the injured hemisphere compared to RAD-GFP group. Moreover, IGF-1 gene therapy reduced the number of Iba1+ cells with reactive phenotype and the number of MHCII+ cells in the injured hemisphere. These results suggest that intramuscular IGF-1 gene therapy may represent a new approach to prevent traumatic brain injury outcomes in rats.

1. Introduction

Traumatic Brain Injury (TBI) is one of the principal causes of disability around the world (Rashno et al., 2019; Stein et al., 2018d; Mangiola et al., 2015). TBI is mainly caused by impacts with conspicuous objects (Taylor et al., 2017). The pathophysiology of TBI is classified into primary and secondary phases. Mechanical disruption of brain tissue causes a primary injury, which often results in axonal shearing, neuronal loss and hemorrhage (Werner and Engelhard, 2007), and with time, will lead to secondary cell damage and death (Larphaveesarp et al., 2015). These mechanisms include molecular and cellular events in glial cells that evolve over several weeks (Burda and Sofroniew, 2014). In this context, quiescent glial cells of multiple types become rapidly activated via a process termed reactive gliosis. This process involves activated microglia initiating and sustaining astrocytic

glia activation via the generation and release of inflammatory mediators that act on surrounding glia and neurons (Stein et al., 2015; Crupi et al., 2013).

Although the activation of glial cells after TBI is initiated as a homeostatic reparative response, an excessive production of pro-inflammatory cytokines by astrocytes and microglia becomes an important driving force for the pathological progression of the secondary injury (Woodcock and Morganti-Kossmann, 2013; Loane and Kumar, 2016). Thus, sustained neuroinflammation induces morphological and functional alterations that impair neuronal–glial and glial–glial interactions (Acáz-Fonseca et al., 2015) and may cause dysfunction of synaptic connections, imbalance of neurotransmitter homeostasis, axonal degeneration and secondary neuronal death (Chiu et al., 2016).

The levels of several neuroprotective factors are increased in the brain after injury (Carlson et al., 2014; Schober et al., 2010). One of

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these is insulin-like growth factor-1 (IGF-1), which plays an active role in normal brain function (Fernandez and Torres-alemán, 2012) and is a promising therapeutic molecule for neurodegenerative diseases (Santi et al., 2018a; Bryan and Bowman, 2017; Mazucanti et al., 2015; Bassil et al., 2014). IGF-1 has critical implications in regulating brain plasticity mechanisms and acts as a pleiotropic molecule with both neuroprotective and regenerative potential (Carlson et al., 2014; Bassil et al., 2014; Bianchi et al., 2017) especially for neuroinflammatory processes. TBI induces a temporal increase in IGF-1 and its signaling molecules in the brain (Santi et al., 2018b). However, this increase is not sufficient to provide the necessary neuroprotection or repair after large brain injuries (Sama et al., 2018; Carlson and Saatman, 2018). Therefore, exogenous administration of IGF-1 may potentiate and extend the actions of endogenous IGF-1. Indeed, subcutaneous administration of IGF-1 exerts neuroprotective actions after TBI (Santi et al., 2018b) and intramuscular administration of IGF-1 gene therapy has implication in sex frailty differences in ageing mice (Herrera et al., 2020a).

IGF-1 is also described as an anti-inflammatory molecule in the nervous system and is known to reduce reactive gliosis after brain injury (Labandeira-Garcia et al., 2017; Serhan et al., 2020; Bake et al., 2014). Therefore, gliosis is a relevant phenomenon to analyze, in order to assess the efficacy of IGF-1 therapy. Previous studies have shown that the intracerebroventricular (ICV) administration of recombinant adenoviral vectors to deliver the IGF-1 gene is a successful strategy to increase IGF-1 levels in the cerebrospinal fluid (Pardo et al., 2016, 2018) and to modulate astrocyte morphology (Pardo et al., 2016) and microglia reactivity (Falomir-Lockhart et al., 2019) in the aging brain. In this study we have assessed whether a peripheral route of administration of the adenoviral vectors, more suitable for human therapy, may prevent and/or regulate reactive gliosis caused after TBI. With this aim, recombinant adenoviral vectors harboring the cDNA of IGF-1 were intramuscularly injected. Our findings indicate that intramuscular IGF-1 gene therapy is an effective approach that reduces regional cortical gliosis and prevent post-traumatic cognitive function after TBI.

2. Materials and methods

2.1. Animals

Male Wistar rats, of 3–4 months old, obtained from the Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP) facility (Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Argentina), were

used in this study. Animals were maintained in a temperature-controlled room, with 12:12 h light/dark cycle and received food and water *ad libitum* through all the experiment. Animal care and procedures were in accordance with the guidelines published in the NIH. Experimental procedures were approved by our institutional animal use and care committee CICUAL. Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

2.2. Adenoviral vector injections

Three-month-old male rats (250–330 g) were intramuscularly injected in the right hindlimb with: i) 10^{10} plaque forming units (pfu) of a recombinant adenoviral construct harboring the cDNA of human IGF-1 as a therapeutic vector (RAD-IGF1), ii) a control vector expressing green fluorescent protein (RAD-GFP) or iii) PBS, as vehicle solution. We used a set of rats without lesion and intramuscularly injection as SHAM group (Fig. 1). For description of viral construction and characterization see Hereñú et al. (2007). Previous studies have shown that the intramuscular administration of RAD-IGF1 to old mice, results in a significant increase in IGF-1 serum levels over the values detected in mice at 3 weeks after adenoviral injection (Herrera et al., 2020a, 2019a).

2.3. Cortical injury

Three weeks after intramuscular injections of adenoviral vectors, animals were anesthetized with halothane (Fluothane, AstraZeneca Pharmaceuticals, Madrid, Spain) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The cranium was exposed by making an incision of the scalp. Then, a unilateral opening of the skull was made with a dental drill. A 2 mm solid stainless blade was introduced in the right hemisphere through the neocortex. The coordinates were fixed by positioning the most rostral end of the blade at the following coordinates from bregma: anteroposterior, -3.5 mm; medio-lateral, -2 mm and dorsoventral, -4.3 mm. Coordinates were established according to the stereotaxic atlas of Paxinos and Watson (2004). To relieve post surgical pain, the rats received, shortly after anesthesia induction, an intraperitoneal (i.p.) injection of buprenorphine (Buprex, Schering Plough, Madrid, Spain).

Fig. 1. Experimental protocol design. Schematic representation of the experimental protocol. Male Wistar rats were injected intramuscularly with PBS, RAD-GFP and RAD-IGF1 in the right paw. Three weeks after administration, a cortical stab injury was performed in the right hemisphere through the neocortex. Seven days after trauma, animals performed behavioral tests and then sacrificed for RIA and immunohistochemistry analyses for different gliosis markers: Iba1, GFAP, Vimentin and MHCII.

2.4. Body-weight analysis

In order to assess possible effects of trauma and previous treatment, we performed daily weight controls, since the day after stereotaxic lesion.

2.5. Behavioral tests

At 7 days after TBI, we performed two behavioral tests to assess cognitive and motor function. All tests were carried out between 10:00 to 17:00 in the same low-intensity light room and analyzed by the same experimenter. Arenas were cleaned with 70 % ethanol to remove odor cues between each tested animal. Rats were left inside the experimental room 40 min previous the beginning of the tests for habituation and to avoid stressors. Trials were monitored through a video camera positioned above each arena. Videos were later analyzed with Tracker® software by a trained observer.

2.5.1. Y-maze test

We used a protocol from Herrera et al. (2020b) to test animals for working memory by Y-maze test to explore areas that have not been previously explored. Y-maze apparatus is made of three arms (50 cm long, 10 cm wide and 20 cm high) of wood covered with impermeable Formica elevated to a height of 50 cm above the floor (Fig. 2A). During the 5 min-test, rats were placed at the end of arm #1 facing the center and they could choose between arm #2 and arm #3. Entry into an arm was defined as placement of the four paws into the arm and an alternation was defined as triplet of consecutive entries to different arms. We considered two selection criteria: i) minimum of entries 5 and ii) 2 min without a new movement or entry were excluded.

2.5.2. Locomotor activity test

Specific motor function was assessed with an open field arena. Rats were allowed to freely explore the arena for 10 min in an arena of 100cm × 100cm. Videos were converted to 1 frame per second (FPS) with a total of 600 FPS for Tracker analyses. We analyzed the total distance in meters walked in the arena.

2.6. Histological procedures

2.6.1. Cresyl violet staining

At seven days after brain injury, animals were deeply anaesthetized with pentobarbital (100 mg/kg body weight, Normon Veterinary Division, Madrid, Spain). Subsequently, they were perfused through the left cardiac ventricle, first with prewarmed (37 °C) 0.9 % NaCl and then for 5 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post fixed for 5 h in the same fixative and washed three times with 0.1 M phosphate buffer, pH 7.4 at room temperature. Coronal sections of the brain, 50 µm thick, were obtained using a Vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany) in 8

sets of sections for each animal.

One set of sections per rat were mounted on gelatinized slides an air-dried overnight. To rehydrate, the mounted sections were dipped in distilled water for 10 min. Samples were stained with cresyl violet (Sigma-Aldrich, MO, USA) for 2 min and dipped in distilled water (Occhieppo et al., 2017). After staining, slides were sequentially dipped into 70 % alcohol (2 min), acetic acid - 96 % alcohol mixture (2 min), 96 % alcohol (1 min) and 100 % alcohol (1 min). Finally, the mounted sections were air-dried overnight, dehydrated and cleared in xylene, and placed under a cover slip with DPX mountant for histology (Sigma).

An observer blinded to the experimental condition verified injury placement through the neocortex in the right hemisphere under light microscope. Animals with inaccurate injury placement or extensive damage throughout dorsal hippocampus were excluded from data analysis (Supplementary Fig. 1).

2.6.2. Tissue fixation and immunohistochemistry

Immunohistochemistry was carried out in free floating sections under moderate shaking. All washes and incubations were done in 0.1 M phosphate buffer pH 7.4, with 0.3 % bovine serum albumin and 0.3 % triton X-100. Tissue sections were first incubated for 10 min at room temperature in a solution of 3% hydrogen peroxide in 30 % methanol to quench the endogenous peroxidase activity. After three washes in buffer, sections were incubated overnight at 4 °C with one of the following primary antibodies: (i), a mouse monoclonal antibody for GFAP (diluted 1:1000; Sigma-Aldrich), marker of reactive and resting astrocytes; (ii), a monoclonal antibody for vimentin (diluted 1:500, clone V9; Dako, Barcelona, Spain), marker of reactive astrocytes; (iii), a rabbit polyclonal antibody for Iba1 (Ionized calcium binding adaptor molecule 1) (diluted 1:2000; Wako Chemical Industries, Japan), marker of microglia and macrophages and (iv), a monoclonal antibody for major histocompatibility complex II (MHCII; diluted 1:300; MCA46 G, Serotec, Bicester, United Kingdom), marker of reactive microglia/macrophages. Sections were rinsed in buffer and incubated for 2 h at room temperature with the corresponding anti-mouse or anti-rabbit biotinylated secondary antibody (diluted 1:300; Pierce, Rockford, IL, USA). Then, after several washes in buffer, sections were incubated for 90 min at room temperature with avidin-biotin-peroxidase complex (diluted 1:250; Immuno-Pure ABC peroxidase staining kit; Pierce, Rockford, IL, USA). For the immunostaining reaction we incubated the sections with 2 mg/ml 3,3'-diaminobenzidine (Sigma-Aldrich, Tres Cantos, Spain) and 0.01 % hydrogen peroxide in 0.1 m phosphate buffer. Sections were dehydrated, mounted on gelatinized slides, coverslipped and examined with a Leica DM 4000B microscope equipped with a DFC Leica digital camera attached to a contrast enhancement device.

2.7. Morphometric analysis

Morphometric analysis was performed by an investigator that was unaware of the identity of the experimental groups. The number of

Fig. 2. Behavioral tests. Working memory in the Y-maze task. (A) Total number of arm entries for the different experimental groups. (B) Percentage of alternations in this task. Locomotor activity. (C) Total distance walked expressed in meters. Data are represented as Mean ± SEM.

GFAP + and Iba1+ cells was estimated with a modified version of the optical dissector method (Pardo et al., 2016; West, n.d.), using total section thickness for dissector height (Hatton and von Bartheld, 1999), a counting frame of $84 \times 84 \mu\text{m}$ and a x40 objective. GFAP and Iba1 cells were evaluated within a distance of 0–1250 μm from the wound border. In addition, some counts were performed in function of the distance to the wound, using five consecutive rectangles (frames F1–F5) of $250 \mu\text{m}$ (horizontal side) $\times$ $835 \mu\text{m}$ (vertical side), starting from the lateral wound edge and moving laterally. Furthermore, the number of Iba1 immunoreactive cells with different morphologies was assessed. Thus, Iba1+ cells were classified according to previous reports (Diz-Chaves et al., 2012) as Non-reactive, corresponding to cells with a ramified morphology with long and thin processes, or as Reactive, corresponding to cells with amoeboid morphology or with enlarged cell body and cell processes. For this purpose, 50 cells from each frame in the region of analysis were classified according to their reactive or non-reactive phenotypes. Vimentin and MHCII cells were evaluated within a distance of 5 counting frames - $420 \mu\text{m}$ (horizontal side) $\times$ $835 \mu\text{m}$ (vertical side)-, leaving two frames from the wound border.

2.8. RIA analysis

At 7 days after TBI, we evaluated IGF-1 serum levels. For IGF-1 RIA, serum samples were assayed in duplicate. Samples were subjected to acid-ethanol extraction as described by Breier et al. (1991). IGF-1 was determined as previously described (Díaz-Torga et al., 2002) using antibody (UB2-495) provided by Drs L Underwood and JJ Van Wyk, and distributed by the Hormone Distribution Program of the NIDDK and Dr AF Parlow. Recombinant human IGF-I (Chiron Corp., Emeryville, CA, USA) was used as radioligand and unlabelled ligand.

2.9. Statistical analysis

Data were analyzed with the GraphPad Prism v.6 software (GraphPad Software, Inc, San Diego, CA, USA). Comparisons between groups were assessed using one-way or two-way analysis of variance of repeated measures, assuming a data normal distribution. When the ANOVA was significant, Tukey's or Bonferroni's multiple comparisons test was chosen as a post hoc test. Differences were considered statistically significant at $P < 0.05$. In this study, we used an experimental N of six male rats for each group at 7 days. Another set of four rats in each experimental group for RIA analysis. For behavioral test we used an experimental N of 7–8 male rats for each group. Data are represented as Mean $\pm$ SEM.

3. Results

3.1. Body weight analysis

In order to assess possible effects of trauma and previous treatment, we performed daily weight controls, since the day after stereotaxic lesion. We observed a significant difference between time and treatment ($F_{(21, 168)} = 8.035$; $P < 0.0001$), specifically between SHAM group and those who received the cortical injury (Supplementary Fig. 2).

3.2. Behavioral tests

Regarding working memory evaluation with Y-Maze test, treatment and injury had no differences in the number of arm entries in the four experimental groups analyzed ($F_{(3,44)} = 2.310$; $P = 0.0894$; Fig. 2A). Furthermore, there was significant changes in the percentage of alternations in the different treated groups ($F_{(3, 44)} = 11.08$; $P < 0.0001$; Fig. 2B). SHAM rats made a major percentage of alternations respect to PBS rats ($P = 0.0003$) and GFP rats ($P = 0.0026$). Moreover, there was an increase in percentage of alternations in RAD-IGF1 respect to PBS group ($P = 0.0005$) and GFP group ($P = 0.0052$) but no differences respect to

SHAM group ($P = 0.9624$). Regarding motor function, there were no significant differences in the distances recorded between the four experimental groups ($F_{(3, 24)} = 0.2826$; $P = 0.8374$; Fig. 2C).

3.3. IGF-1 levels

To assess IGF-1 intramuscular gene therapy efficacy, we performed RIA assays in serum levels. Data showed significant increase of IGF-1 serum levels ($F_{(2, 9)} = 7.052$; $P = 0.0144$, Fig. 3) in Rad-IGF1 group as compared to RAD-GFP group ($P = 0.0368$) or PBS group ($P = 0.0176$).

3.4. Effect of IGF-1 gene therapy on astrocytes

The number of GFAP + cells and the number of Vimentin + cells were counted 7 days after brain injury. A representative example of GFAP immunoreactivity in the injured cerebral cortex is shown in Figs. 4A at 2.5X, 4B at 10X and 4C at 40X magnifications. Total number of GFAP immunoreactive cells was assessed within an area $1250 \mu\text{m} \times 835 \mu\text{m}$ from the injury border at 7 days after stab wound injury. Firstly, when we used an equivalent region without injury (SHAM group) as control group we observed significant differences respect to PBS, RAD-GFP and RAD-IGF1 groups at 7 days ($F_{(3, 16)} = 31.81$; $P < 0.0001$; Fig. 4D). Similar results were obtained in the analysis of the number of GFAP + cells in the injured hemisphere in function of the distance from the wound ($F_{(12, 60)} = 5.89$; $P < 0.0001$; Fig. 4F). We observed significant differences between SHAM group and the rest of experimental groups in the five frames, respectively. Although we did not observe significant differences between PBS, RAD-GFP and RAD-IGF1 groups, we observe intragroup differences along the frames.

The number of Vimentin immunoreactive cells in the injured hemisphere had significant changes according treatment at 7 days ($F_{(2, 11)} = 11.20$; $P = 0.0022$; Fig. 5B). Furthermore, RAD-IGF1 decreased the number of Vimentin + cells respect to the GFP group ($P = 0.0017$).

3.5. Effect of IGF-1 gene therapy on microglia/macrophages

The number of Iba1+ cells and the number of MHCII + cells were counted 7 days after brain injury. Representative examples of Iba1 immunoreactivity in the injured cerebral cortex is shown in Fig. 6A–C. Data shown a significant increase in the total number of Iba1+ cells in the injured hemisphere in PBS, RAD-GFP and RAD-IGF1 groups respect to SHAM group at 7 days after stab wound injury ($F_{(3, 15)} = 15.19$; $P < 0.0001$; Fig. 6D).

Also, at this time point we analyzed the number of Iba1+ cells, in the injured hemisphere, in function of the distance from the wound and treatment ($F_{(12, 60)} = 9.62$; $P < 0.0001$; Fig. 6E). We observed significant

Fig. 3. Serum IGF-1 levels. Quantification of RIA analysis at 7 days post-lesion. Data are represented as Mean $\pm$ SEM.

Fig. 4. GFAP immunoreactivity. (A) Representative microphotographs of GFAP immunoreactivity in the injured cerebral cortex of rats at $2.5 \times$. Bar scale at 500um. (B) Representative microphotograph of stab wound injury at $10 \times$. Bar scale at 100um. (C) Representative microphotograph of GFAP cells at $40 \times$. Bar scale at 100um. (D) Number of immunoreactive GFAP cells in the injured hemisphere in SHAM, PBS, RAd-GFP and RAd-IGF1 groups. Data are expressed as Mean $\pm$ SEM. (E) Schematic representation of the five counting frames at 20X magnification. Bar scale at 50um (F) Number of GFAP + cells depending on the distance to the wound analyzed in the five consecutive frames at 7 days after lesion. Data are expressed as Mean $\pm$ SEM. * Comparisons between SHAM and PBS, RAd-GFP and RAd-IGF1 groups (* = $P < 0.05$).

Fig. 5. Vimentin immunoreactivity. (A) Representative microphotograph of Vimentin immunoreactivity in the injured cerebral cortex of rats, at 40X magnification. Bar scale at 100um. (B) Number of immunoreactive Vimentin cells in PBS, RAd-GFP and RAd-IGF1 groups, at 7 after lesion. Data are expressed as Mean $\pm$ SEM.

differences between SHAM group and the rest of experimental groups in the five frames, respectively. Although we did not observe significant differences between PBS, RAd-GFP and RAd-IGF1 groups, we observe intragroup differences along the frames. Animals treated with RAd-IGF1 showed a significant decrease in the number of Iba1+ cells with a

reactive phenotype in the injured hemisphere compared to animals treated with RAd-GFP ($F_{(8, 56)} = 4.241$; $P = 0.0005$; Fig. 6F), at 7 days. This decrease was observed in frames 1, 2, 3 and 4 (i.e., from 0 to 1000 μ m from the border of the wound). A representative example of Iba1 phenotypes in the injured cerebral cortex is shown in Fig. 6G.

Fig. 6. Iba1 immunoreactivity. (A) Representative reconstruction of a microphotograph of Iba1 immunoreactivity in the injured cerebral cortex at $2.5 \times$. Bar scale at 500μm. (B) Representative microphotograph of stab wound injury at $10 \times$. Bar scale at 100μm. (C) Representative microphotograph of Iba1+ cells at $40 \times$. Bar scale at 100μm. (D) Number of immunoreactive Iba1 cells in the injured hemisphere in SHAM, PBS, RAD-GFP and RAD-IGF1 groups. Data are expressed as Mean $\pm$ SEM. (E) Number of Iba1+ cells depending on the distance to the wound analyzed in the five consecutive frames at 7 days after lesion. Data are expressed as Mean $\pm$ SEM. * Comparisons between SHAM and PBS, RAD-GFP and RAD-IGF1 groups (* = $P < 0.05$). (F) Number of Iba1+ cells with reactive phenotype with respect to the distance to the wound in the injured hemisphere in a total of 50 Iba1+ cells per frame at 7 days after lesion. Data are expressed as Mean $\pm$ SEM. * Comparisons between SHAM and PBS, RAD-GFP and RAD-IGF1 groups (* = $P < 0.05$). ** = $P < 0.01$, *** = $P < 0.001$. # Comparisons between RAD-GFP and RAD-IGF1 (### = $P < 0.0001$, ## = $P < 0.01$, # = $P < 0.05$). (G) Representative micrographs of reactive and non-reactive phenotypes at 100X magnification.

Furthermore, the decrease in the number of Iba1+ cells with reactive phenotype in the injured hemisphere of the RAD-IGF1 group was in agreement with the observation of a significant decrease in the number of MHCII + cells, a marker of reactive microglia at 7 days (F (2, 13) = 14.57; $P = 0.0005$, Fig. 7B).

4. Discussion

The present results indicate that systemic RAD-IGF1 treatment modulates glia responses to cortical stab wound injury and prevent the concomitant cognitive impairment. We assessed reactivity signs at 7 days after TBI since certain glial responses reach their highest levels after one week (Acáz-Fonseca et al., 2015; Robel et al., 2011). As a protective therapeutical tool, we administered intramuscular IGF-1 gene

therapy, three weeks before trauma. It has been previously reported (Herrera et al., 2019b) that intramuscular IGF-1 gene therapy has a neuromodulatory effect in sex differences observed in aging mice after 21 days of treatment, with an increase of IGF-1 serum levels (Herrera et al., 2020a). Here, we also observed an increment of this neurotrophic factor serum levels. All the above evidence confirms that systemic RAD-IGF1 administration results in an increase of IGF-1 serum levels that could prevent TBI associated damage by modulating glia reactivity at brain level.

4.1. IGF-1 has a positive effect on working memory after TBI

Multiple studies provide insights into the neural mechanism that may underlie cognitive deficits after TBI (Palermo et al., 2020; Sebastian

Fig. 7. MHCII immunoreactivity. (A) Representative microphotograph of MHCII immunoreactivity in the injured cerebral cortex at $40\times$. Bar scale at 100μm. (B) Number of total immunoreactive MHCII cells in the injured hemisphere at 7 days after lesion. Data are represented as Mean $\pm$ SEM.

et al., 2013; Lawton and Huang, 2019). It has been suggested that TBI reflect disruptions of the neural networks for attention and working memory and spatial reference (Anderson and Knight (2010); Ozdemir et al. (2012)). Consistent with these observations, we observed serious impairments in the performance of PBS and RAd-GFP groups respect to SHAM group. Moreover, there has been important concerns about treatments and therapeutics regarding this cognitive task, for example dynamic cognitive remediation (Lawton and Huang, 2019) and a correlation between training and recovery (Sebastian et al., 2013) at a behavioral level. In this study, adenoviral IGF-1 gene therapy improves memory impairment caused by cortical injury in lesioned animals compared to PBS or Rad-GFP groups, showing no differences respect to the SHAM group (no lesioned animals). Similar results were found in spatial memory in aging rats (Pardo et al., 2016; Uriarte et al., 2017), these authors also found that this therapy modulates hippocampal genes relevant to memory function (Pardo et al., 2018). Although, it was reported that IGF-1 gene therapy improves motor function (Falomir-Lockhart et al., 2019; Hereñú et al., 2009), neither treatment nor trauma had effect on motor performance under our experimental conditions.

Other authors postulate that astrocyte-derived IGF-1 reduces regional hippocampal neurodegeneration and improves post-traumatic cognitive and motor function in a mice model of brain injury (Madathil et al., 2013). Is beyond the purpose of this work to evaluate neuronal damage, but we hypothesize that IGF1 effects on gliosis could exert positive functions on neuronal populations, this is an aspect to evaluate in further works.

4.2. IGF-1 modulates microglial response after TBI

TBI is a complex injury with a broad spectrum of symptoms and disabilities. A crucial aspect to diminish the progression of TBI symptoms is to modulate the glial response that is characteristic of the secondary injury (Younger et al., 2019). One approach was to increase the levels of anti-inflammatory neurotrophic factors, such as IGF-1 (Santi et al., 2018a,c). Previous studies have shown that IGF-1 decreases the inflammatory response of astrocytes and microglia *in vitro* and *in vivo* (Acáz-Fonseca et al., 2015; Santi et al., 2018a, c; Fernandez et al., 2017; Bellini et al., 2011). It has been also described that astrocyte-specific overexpression of IGF-1 protects hippocampal neurons and reduces behavioral deficits following cortical controlled injury (Madathil et al., 2013). In addition, intracerebral IGF-1 gene therapy probed to effectively regulate microglia reactivity in the aging brain (Falomir-Lockhart et al., 2019). In the present study we assessed the effect of intramuscular IGF-1 gene therapy to modulate the glial response induced in the cerebral cortex by a stab wound injury. To evaluate reactive gliosis, we quantified the density of immunoreactive cells near the lesion using antibodies against GFAP, vimentin, Iba1 and MHCII, as markers of total

astrocytes, reactive astrocytes, total microglia/macrophages and reactive microglia/macrophages, respectively. We observed a significant increase in the number of astrocytes and microglia in the injured cerebral cortex in comparison to the SHAM group, which is in concordance with previous findings (Acáz-Fonseca et al., 2014; Anderson et al., 2016). Also, in agreement with previous studies, we observed a gradual decrease in the number of GFAP and Iba1 immunoreactive cells with increasing distance from the wound. This gradient in astrocytes and microglia is considered a healing sign in mature focal lesions (Anderson et al., 2016; Sofroniew, 2009).

In spite of the fact that IGF-1 gene therapy has been shown to reduce the inflammatory response of astrocytes exposed to lipopolysaccharide *in vitro* (Bellini et al., 2011), in the present study intramuscular IGF-1 gene therapy did not significantly affect the number of GFAP + cells but reduces the number of vimentin immunoreactive astrocytes in the injured hemisphere. This data suggests that attenuated astrocytic reactivity, has a beneficial consequence on the functional outcome after TBI. This observation is an important finding of our work and is also illustrated in other lesion models (Madathil et al., 2013). Our results prompt us to postulate that IGF-1 gene therapy is modifying astroglial response to injury, leading to a less harmful glial scar in the cortex and to an improvement in working memory. Considering the multifaceted effects of reactive astrocytes, and to fully understand how IGF-1 could modulate inflammatory parameters it will be interesting to perform further studies.

Emerging evidence indicates that TBI causes prolonged activation of microglia and the development of a primed profile (Norden et al., 2016). Indeed, the higher expression of several inflammatory mediators including MHC II, CD68 and NADPH oxidase (NOX2) in the cortex persisted 12 months after injury (Loane et al., 2014) and due to their close link to neuronal function, changes in microglia morphology had serious effects on neuronal and circuit function (Morrison et al., 2017). Consequences of exaggerated inflammatory responses by microglia include the development of cognitive deficits, impaired synaptic plasticity and accelerated neurodegeneration (Norden et al., 2016). According to this, Caplan et al., reported that TBI results in a spatiotemporal increase of amoeboid microglia in the hippocampus (Caplan et al., 2020). In the present work we found that IGF-1 gene therapy decreased the number of Iba 1+ microglia/macrophage cells with a reactive phenotype in the injured hemisphere, suggesting that microglia profile is committed by the therapy. This finding is in agreement with a previous study *in vivo*, in which intracerebral IGF-1 gene therapy modified the number and morphology of microglia in the brain of older rats (Falomir-Lockhart et al., 2019). We postulate that IGF-1 gene therapy could be modifying the pro-inflammatory environment and/or pro-inflammatory microglial/macrophages state, thus establishing a favourable scenario and an improvement in behavior outcome. However, we could not elucidate the mechanism that underlie this

modulation and its exact correlation with behavioral improvements. It is well established that microglia express IGF-1 receptors and maybe regional and sensitivity differences in the expression of these receptors could explain these effects (Falomir-Lockhart et al., 2019). Also, Song et al., reported a neuroprotective effect of exogenous IGF-1 through an enhancement of the activation of mTOR cascade after a few days of TBI (Song et al., 2015).

In summary, our results indicate that intramuscular administration of an adenoviral vector harboring the IGF-1 gene is effective in modulate glial response, reducing reactive microglia in the cerebral cortex after TBI and preventing behavioral outcomes. Our findings are in concordance with several studies that propose that high serum IGF-1 levels have the potential to improve behavioral performance in elderly and neuronal protection in TBI models (Stein et al., 2018d; Mangiola et al., 2015). Some of this works suggest that IGF1 short half-life in the systemic circulation makes it difficult to maintain a therapeutic concentration. Therefore, we suggest the potential utility of this feasible and controllable route of IGF-1 gene delivery, that improved stability and half-life while retaining its ability to enter the brain from the periphery, increasing its viability as a translational approach for preventing brain injuries.

Author contributions

MJB designed the study. MLH, CBH and MJB performed adenoviral injections, MLH and MJB performed cortical surgeries. MLH and LGC performed immunohistochemistry experiments. MLH, SB and MJB performed the quantifications and the analysis, interpretation and designed the different plots and graphs. MLH and MJB wrote different sections of the manuscript. MJB and MLH assembled the final version of the paper.

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article and its online resource files.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the guidelines published in the NIH. Experimental procedures and were approved by our institutional animal use and care committee IACUC (Protocol #T09-01-2013). Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.brainresbull.2021.07.023>.

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