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Granulocyte-Macrophage Colony Stimulating Factor Exerts Protective and Immunomodulatory Effects in Cortical Trauma

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ABSTRACT

Neurodegeneration after traumatic brain injury is facilitated by innate and adaptive immunity and can be harnessed to affect brain repair. In mice subjected to controlled cortical impact (CCI), we show that treatment with granulocyte macrophage colony stimulating factor (GM-CSF) affects regulatory T cell numbers in the cervical lymph nodes coincident with decreased lesion volumes and increased cortical tissue sparing. This paralleled increases in neurofilament and diminished reactive microglial staining. Transcriptomic analysis showed that GM-CSF induces robust immune neuroprotective responses seven days following CCI. Together, these results support the therapeutic potential of GM-CSF for TBI.

1. Introduction

Traumatic brain injury (TBI) leads to chronic neurodegeneration driven, in part, by innate and adaptive immune responses. Both influence neuronal injury (Jin et al., 2012). Brain shearing forces affect mechanical trauma causing release of purinergic signaling molecules (Davalos et al., 2005), excitatory neurotransmitters (Faden et al., 1989; Palmer et al., 1993), and other damage-associated factors (Laird et al., 2014). These affect resident glial (astrocytes and microglia) function leading to the production of pro-inflammatory mediators. These include reactive oxygen species, cytokines and pro-apoptotic proteins; all known to perpetuate neural injury (Burda and Sofroniew, 2014). Notably, the traumatic event can damage the neurovasculature and attract immunocytes, such as neutrophils, lymphocytes and blood-borne macrophages, to sites of brain injury (Soares et al., 1995; Carlos et al., 1997; Schwarzmaier et al., 2010). Once in the parenchyma, the cells can become immune competent, affecting glial responses and accelerating ongoing neural damage. Such changes can affect microglial responses, where a pro-inflammatory, classically activated state (M1) and an anti-inflammatory, alternatively activated state (M2) evolve in a temporally ordered fashion over prolonged time periods (Loane et al., 2014). Indeed, long-term immune response changes were observed in human brains up to 17 years after brain injury (Ramlackhansingh et al., 2011).

The role of the adaptive immunity in the pathobiology of TBI is heralded by a steady increase in the number of lymphocytes that enter the brain, but not in draining lymph nodes or spleen (Clausen et al., 2009; Jin et al., 2012). Such cellular shifts occur as early as one day following injury and can continue for up to 28 days after the traumatic event. Whether the cells serve to accelerate damage or perform homeostatic functions is not understood. Previous work by our group and others demonstrated that regulatory T cells (Tregs) can attenuate microglial pro-inflammatory activities leading to robust neuroprotective responses in models of stroke, HIV-1 encephalitis, amyotrophic lateral sclerosis, myasthenia gravis and Parkinson's disease (PD) (Reynolds et al., 2007; Sheng et al., 2008; Liesz et al., 2009; Reynolds et al., 2009; Reynolds et al., 2010; Gendelman and Appel, 2011; Kosloski et al., 2013; Li et al., 2013).

As neurodegenerative and neuroinflammatory disorders, immune activity changes with time following TBI (Kox et al., 2008; Jin et al., 2012; Loane et al., 2014). The dynamics of immune mediated injuries versus neuroprotection are also controlled by environmental events and immune regulatory activities (Burda and Sofroniew, 2014). The transformation of innate microglial and astrocyte activities together with the emergence of an altered adaptive immune response can certainly aggravate neural damage (Jin et al., 2012). Such events are operative immediately following injuries but, interestingly, evolve over time leading to compensatory neuroprotective outcomes (Ziebell et al., 2014). On the cellular level, astrocytes contribute to these complex immunoregulatory events by affecting blood–brain barrier integrity and microglial secretory responses that include the production of neuroregulatory and neuroprotective factors to speed neuronal repair (Segev-Amzaleg et al., 2013). The degree and timing of cell-based cross-talk are nonetheless limited for TBI, leaving open the notion that

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pharmacological measures that strengthen neurotrophic immune responses could significantly affect disease outcomes. To these ends, we investigated the use of granulocyte macrophage colony-stimulating factor (GM-CSF), an approved adjunctive therapy for chemotherapy-induced granulocytopenia (Buchsel et al., 2002). GM-CSF, a hematopoietic cell growth and differentiation factor, is produced by macrophages, T cells, mast cells, endothelial cells, and fibroblasts. GM-CSF, in turn, stimulates the mobilization of hematopoietic progenitor cells, including Tregs (Schabitz et al., 2008; Sheng et al., 2011; Zou et al., 2011), which affect neuroprotective responses as we recently demonstrated in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease (Kosloski et al., 2013). We now demonstrate that GM-CSF can have broad neuroprotective responses following a controlled cortical impact (CCI) injury. The CCI model was used to investigate potential GM-CSF neuroprotective activities as it elicits similar pro-inflammatory responses, neuronal loss and long-term behavioral deficits seen in humans and may be modulated through immune regulation (Xiong et al., 2013). When injured, mice develop a lesion in the injured cortex that is identified as early as 6 h following trauma and evolves over time to frank neurodegeneration (Hall et al., 2008). Lesion size remains relatively unchanged at 48 h with peak area of degenerative neurons observed at this time point as determined by silver staining. By seven days, lesion volume has decreased slightly from a peak at 24 h post-injury. Herein, neuroprotection was readily seen in mice following CCI and treatment with GM-CSF. This, in part, was linked to the induction of Treg populations that serve to transform innate immune responses (Liesz et al., 2013). Supporting these findings were a combination of histology, immunohistochemistry, flow cytometry, and molecular transcriptomic tests to evaluate broad immune regulatory responses of GM-CSF that serve to facilitate neural repair following CCI.

2. Material and methods

2.1. Animals

Male C57BL/6 mice (20–24 g; Charles River Laboratories, Wilmington, MA) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at the University of Nebraska Medical Center. The animal housing facility was on a 12-hour light/dark cycle held at constant temperature (21–25 °C) and humidity (45–50%). Animals were allowed free access to food and water. All experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

2.2. Controlled cortical impact injury and treatment paradigm

Mice were randomized and subjected to CCI injury using a Precision Systems and Instrumentation (Fairfax, VA) TBI-0310 Impactor (Kelso et al., 2006; Kelso et al., 2009; Boulet et al., 2013). Briefly, anesthesia was induced in a Plexiglass chamber with 5% inhaled isoflurane on an O_2 carrier and was maintained at 2–2.5% via a nose cone during the procedure. The head was shaved and the animal was secured in a stereotactic frame (David Kopf, Tujunga, CA). After disinfecting the skin, the skull was exposed by a midline scalp incision. A left parietal craniotomy was performed midway between the bregma and lambda and lateral to the midline using a high-speed dental drill so as to expose the somatosensory cortex. Care was taken not to disturb the underlying dura. The stereotaxic frame was positioned so that the exposed brain was placed directly under an electronically controlled, air-driven piston that delivered the impact. This compressed the cortical surface by 0.5 mm (3.0 mm tip diameter, 3.5 m/s velocity, 200 ms dwell time). After impact, Surgicel (Johnson & Johnson, Dallas, TX) was applied to the dura, the skullcap was replaced and affixed with dental adhesive and the incision was closed with wound clips. Mice were then placed back into the home cage to recover from anesthesia. By 6 h after injury, recovered animals were administered either GM-CSF (50 μg/kg; Peprotech, Rocky Hill, NJ) or an equal volume of vehicle (sterile phosphate buffered saline with 0.1% bovine serum albumin) by intraperitoneal (ip) injection as previously reported (Kosloski et al., 2013). Injections were administered daily until the time of sacrifice at either seven or fourteen days post-injury, except for the PCR array when animals were sacrificed at two, seven, or fourteen days post-injury. For sham treatment, animals were treated as CCI-injured animals, but received no CCI injury.

2.3. Flow cytometric analysis of peripheral lymphocyte populations

To determine T cell phenotypes, we harvested and prepared single cell suspensions from cervical lymph nodes and spleens of sham mice (mice that underwent an identical surgical procedure except that an impact was not performed; $n = 10$), and animals treated with GM-CSF ($n = 5$ treated for 7 and 14 days), or vehicle ($n = 5$ for 7 and 14 days). Monoclonal antibodies (eBiosciences, San Diego, CA, USA) for CD4 [fluorescein isothiocyanate (FITC)], CD25 [phycoerythrin (PE)] and FoxP3 [allophycocyanin (APC)] were used to distinguish T cell subsets (Hori et al., 2003). Briefly, lymphoid organs were minced and suspended in Hanks' Balanced Saline Solution (HBSS). Erythrocytes were lysed from the samples using an ammonium–chloride–potassium (ACK) lysis solution (0.155 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). The erythrocyte-free samples were then double labeled with 1 μg antibody (PE-anti-CD25 and FITC-anti-CD4)/1 million cells for 30 min. One set of lymphocytes was permeabilized using a FoxP3 T regulatory cell staining kit (BioLegend, San Diego, CA) and incubated with APC-anti-FoxP3 for 30 min at 4 °C. Stained cell suspensions were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson ImmunoCytometry Systems, San Jose, CA). Data analysis was performed with FACSDiva software.

2.4. Tissue preparation and measurements of cortical tissue sparing

Animals were terminally-anesthetized with a lethal overdose of sodium pentobarbital (150 mg/kg body weight) by intraperitoneal injection. Anesthetized mice were transcardially perfused with 50 mL of chilled (4 °C) 0.1 M PBS, pH 7.4, followed by 75 mL of chilled 4% paraformaldehyde in 0.1 M PBS. The brains were rapidly removed and cryoprotected in 30% sucrose in PBS for an additional 24 h. Brains were sectioned by the use of a Hacker–Bright cryostat into 20 μm and 12 μm thick coronal slices. Every 6th 20 μm section was mounted onto a glass slide and stained with 0.1% cresyl violet. The slides were blinded and cresyl violet-stained cortical areas were measured with Image J software (NIH, Bethesda, MD). The area of ipsilateral cortical tissue was compared with the area of cortical tissue contralateral to the injury; results are expressed as percentage of cortical tissue spared and inversely reflect the extent of the lesion (Kelso et al., 2006; Kelso et al., 2009). The values from each group were then averaged and compared using Student's t-test.

2.5. Immunohistochemistry, image acquisition and analyses

Four equally-spaced cryosections (12 μm) taken throughout the lesion area were sequentially treated with blocking solution (10% normal goat serum in Tris-buffered saline/Tween 20) for 1 h, incubated with polyclonal antibodies against anti-ionized calcium binding adaptor molecule 1 (Iba1, 1:1000; Wako, Richmond, VA), glial fibrillary acidic protein (GFAP, 1:1000; Dako, Carpenteria, CA), microtubule associated protein-2 (MAP-2, 1:500; Millipore, Billerica, MA), or with monoclonal antibodies against axonal cytoskeleton neurofilament protein 68 kDa (NF68, 1:500; Dako) and were reacted with secondary anti-rat or anti-rabbit conjugated to fluorescent probes (Alexa Fluor 488 or Alexa Fluor 568). To ensure uniform staining for each cell type, tissues were reacted en masse with the same antibody dilution for the same time period. Slides were cover slipped with ProLong Gold anti-fade reagent with 4′,6-diamidino-2-