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Short Communication

SOD1^{G93A} transgenic mouse CD4^{+} T cells mediate neuroprotection after facial nerve axotomy when removed from a suppressive peripheral microenvironment

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease involving motoneuron (MN) axonal withdrawal and cell death. Previously, we established that facial MN (FMN) survival levels in the SOD1^{G93A} transgenic mouse model of ALS are reduced and nerve regeneration is delayed, similar to immunodeficient RAG2$^{-/-}$ mice, after facial nerve axotomy. The objective of this study was to examine the functionality of SOD1^{G93A} splenic microenvironment, focusing on CD4^{+} T cells, with regard to defects in immune-mediated neuroprotection of injured MN. We utilized the RAG2$^{-/-}$ and SOD1^{G93A} mouse models, along with the facial nerve axotomy paradigm and a variety of cellular adoptive transfers, to assess immune-mediated neuroprotection of FMN survival levels. We determined that adoptively transferred SOD1^{G93A} unfractionated splenocytes into RAG2$^{-/-}$ mice were unable to support FMN survival after axotomy, but that adoptive transfer of isolated SOD1^{G93A} CD4^{+} T cells could. Although WT unfractionated splenocytes adoptively transferred into SOD1^{G93A} mice were able to maintain FMN survival levels, WT CD4^{+} T cells alone could not. Importantly, these results suggest that SOD1^{G93A} CD4^{+} T cells retain neuroprotective functionality when removed from a dysfunctional SOD1^{G93A} peripheral splenic microenvironment. These results also indicate that the SOD1^{G93A} central nervous system microenvironment is able to re-activate CD4^{+} T cells for immune-mediated neuroprotection when a permissive peripheral microenvironment exists. We hypothesize that a suppressive SOD1^{G93A} peripheral splenic microenvironment may compromise neuroprotective CD4^{+} T cell activation and/or differentiation, which, in turn, results in impaired immune-mediated neuroprotection for MN survival after peripheral axotomy in SOD1^{G93A} mice.

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1. Introduction

In previous studies, we have established an immune-mediated model of endogenous neuroprotection following facial nerve axotomy in wild-type (WT) and immunodeficient recombinase activating gene-2 knock-out (RAG2$^{-/-}$) mice lacking functionally mature B and T cells, but intrinsically maintaining antigen presentation by MHC class II-expressing peripheral antigen-presenting cells (APC; Serpe et al., 1999, 2003). Key to immune-mediated neuroprotection after axotomy is the generation of neuroprotective CD4^{+} T cells that are antigen-specific and require: (1) initial activation peripherally, *via* interaction with MHC class II-expressing APC, and (2) re-activation centrally, *via* interaction with MHC class II-expressing microglia (Byram et al., 2004).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease resulting in motoneuron degeneration and accompanied by neuroinflammation involving reactive microglia and astrocytes centrally and immune activation peripherally (Appel et al., 2010;

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Philips and Robberecht, 2011). The most widely used transgenic mouse model of ALS, involving the overexpression of human mutant superoxide dismutase-1 ($SOD1^{G93A}$), develops disease pathology similar to that in familial and sporadic ALS patients (Rosen et al., 1993; Gurney, 1994; Gurney et al., 1994). An axonal die-back response precedes MN cell death in SOD1 mice (Kennel et al., 1996; Fischer et al., 2004; Hegedus et al., 2007), resulting in a cascade of events similar to that observed in WT mice after peripheral nerve injury. Specifically, axonal degeneration, denervated neuromuscular junctions, afferent presynaptic stripping surrounding MN cell bodies in CNS, immune cell activation peripherally, and glial activation centrally are responses that occur both as a result of axonal die-back in ALS and peripheral nerve injury (Moran and Graeber, 2004; Jones et al., 2005; Zang et al., 2005; Chiu et al., 2009; Jinno and Yamada, 2011).

$SOD1^{G93A}$ mice demonstrate significantly increased FMN cell death following either a facial nerve transection or crush axotomy, relative to WT (Mesnard et al., 2011, 2013). Interestingly, while axotomized $SOD1^{G93A}$ FMN respond with a pro-regenerative response similar to WT, a dysregulated response to axotomy exists in the microenvironment surrounding the $SOD1^{G93A}$ FMN cell bodies (Mesnard et al., 2011). Target disconnection via disease or facial nerve axotomy in $SOD1^{G93A}$ mice results in comparable motoneuron- and glial-specific molecular changes within the facial nucleus (Haulcomb et al., 2014). Furthermore, $SOD1^{G93A}$ FMN exhibit a delayed functional recovery response to facial nerve crush axotomy, relative to WT mice (Mesnard et al., 2013), that resembles the delayed functional recovery response of FMN in immunodeficient mice following facial nerve crush (Serpe et al., 2002). Therefore, both peripheral and central immune cell irregularities appear to impact $SOD1^{G93A}$ FMN survival and functionality after facial nerve axotomy.

The main objective of the current study was to begin to define whether an immune defect in $SOD1^{G93A}$ $CD4^+$ T cell development, activation, or re-activation is associated with the increased axotomy-induced cell death of SOD1 FMN or the defect lies within the previously identified central glial response (Mesnard et al., 2011). Through a variety of adoptive transfer experiments utilizing $SOD1^{G93A}$ and $RAG2^{-/-}$ mice, our results suggest that a defective $SOD1^{G93A}$ peripheral microenvironment and/or response, rather than a defect in the $CD4^+$ T cell itself, may underscore the impaired immune-mediated neuroprotection required for motoneuron survival and regeneration.

2. Materials and methods

2.1. Animals

Female, C57Bl/6 wild-type (WT) and transgenic SOD1 ($SOD1^{G93A}$) were obtained from Jackson, and recombination activating-2 gene knock-out ($RAG2^{-/-}$) from Taconic, at 6 weeks of age and permitted 1 week to acclimate prior to experimental manipulation. The mice were provided autoclaved pellets and water ad libitum, and housed under a 12 h light/dark cycle in microisolater cages contained within a laminar flow system to maintain a pathogen-free environment.

2.2. Cellular adoptive transfers

Cellular adoptive transfers were completed at 7 weeks of age and 1 week prior to undergoing facial nerve axotomy. Spleens were removed from WT or $SOD1^{G93A}$ mice, and the splenocytes were isolated as previously described by our laboratory (Serpe et al., 1999, 2003; Byram et al., 2003). Specifically, WT or $SOD1^{G93A}$ splenocytes were collected for adoptive transfer at a concentration of 50×10^6 splenocytes/100 μL PBS *via* tail vein injection per animal. Naïve WT or $SOD1^{G93A}$ $CD4^+$ T cells were isolated from the splenocyte samples *via* autoMACS magnetic cell sorting for adoptive transfer at a concentration of 5×10^6 $CD4^+$ T cells/100 μL PBS per animal. Axotomy-activated $CD4^+$ T cells (Byram et al., 2004) were isolated from WT or $SOD1^{G93A}$ spleens 3 days after facial nerve axotomy *via* autoMACS. Ten specific groups ($N = 3$–6/group) were utilized for FMN survival analyses, including WT, $SOD1^{G93A}$, $RAG2^{-/-}$, $SOD1^{G93A}$ + WT splenocytes, $RAG2^{-/-}$ + $SOD1^{G93A}$ splenocytes, $RAG2^{-/-}$ + WT $CD4^+$ T cells, $RAG2^{-/-}$ + $SOD1^{G93A}$ $CD4^+$ T cells, $SOD1^{G93A}$ + WT $CD4^+$ T cells, $SOD1^{G93A}$ + WT CD4-depleted splenocytes, and $SOD1^{G93A}$ + Axot WT $CD4^+$ T cells. All $SOD1^{G93A}$ mice were utilized by 12 weeks of age, and pre-symptomatic.

2.3. Facial nerve axotomy

A right facial nerve transection axotomy was performed in mice at 8 weeks of age. All surgical procedures were completed in accordance with National Institutes of Health guidelines on the care and use of laboratory animals for research purposes. Using aseptic techniques, mice were anesthetized with 3% isoflurane and maintained at 1.5%, and the right facial nerve was exposed and completely transected at its exit from the stylomastoid foramen (Jones and LaVelle, 1985; Serpe et al., 1999). The proximal and distal facial nerve stumps were manually pushed away from each other in order to prevent reconnection. The left facial nerve remained intact, leaving the left facial nucleus to serve as an internal control for comparison purposes.

2.4. Facial motoneuron (FMN) counts

For each experiment, FMN survival levels were assessed at 4 weeks following facial nerve transection axotomy, where surviving FMN were counted in the ipsilateral facial nucleus and compared to the contralateral uninjured (control) facial nucleus. At 4 weeks post-axotomy, 25 μm cryosections were collected throughout the rostrocaudal extent of the facial motor nucleus (Mesnard et al., 2011). FMN were counted in thionin-stained tissue sections, and after application of the Abercrombie correction factor, results were presented as the average percent of FMN survival ± the standard error of the mean (SEM), as described in detail (Serpe et al., 1999; Mesnard et al., 2011). Representative photomicrographs of facial motor nuclei were obtained with an Olympus microscope and Image-Pro software. Statistical analysis was accomplished using a one-way ANOVA, followed by the Student–Newman–Keuls post hoc multiple comparison test, with significance at $p < 0.05$.

3. Results

3.1. Significant FMN cell loss in $SOD1^{G93A}$ and $RAG2^{-/-}$ mice after axotomy

Compared to the uninjured facial nucleus in WT mice (92 neurons ± 2; Fig. 1A), baseline FMN counts were not altered in transgenic mice containing overexpression of the human mutant $SOD1^{G93A}$ gene (90 neurons ± 2; Fig. 1B) or deletion of the *RAG2* gene (90 neurons ± 3; Fig. 1C) at 12 weeks of age (quantitative data not shown). However, at 4 weeks post-axotomy, FMN survival levels in the axotomized facial nucleus of WT mice (84% ± 2.0; Figs. 1D and 2) were significantly higher compared to both $SOD1^{G93A}$ (68% ± 1.0; Figs. 1E and 2) and $RAG2^{-/-}$ mice in (57% ± 2.5; Figs. 1F and 2), relative to the respective uninjured facial nuclei.

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