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Functional recovery and facial motoneuron survival are influenced by immunodeficiency in crush-axotomized mice

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Abstract

Facial nerve axotomy is a well-described injury paradigm for peripheral nerve regeneration and facial motoneuron (FMN) survival. We have previously shown that CD4⁺ T helper (Th) 1 and 2 effector subsets develop in the draining cervical lymph node, and that the IL-4/STAT-6 pathway of Th2 development is critical for FMN survival after transection axotomy. In addition, delayed behavioral recovery time in immunodeficient mice may be due to the absence of T and B cells. This study utilized a crush axotomy paradigm to evaluate FMN survival and functional recovery in WT, STAT-6 KO (impaired Th2 response), T-Bet KO (impaired Th1 response), and RAG-2 KO (lacking mature T and B cells) mice to elucidate the contributions of specific CD4⁺ T cell subsets in motoneuron survival and recovery mechanisms. STAT-6 KO and RAG-2 KO mice exhibited decreased FMN survival after crush axotomy compared to WT, supporting a critical role for the Th2 effector cell in motoneuron survival before target reconnection. Long term FMN survival was sustained through 10 wpo after crush axotomy in both WT and RAG-2 KO mice, indicating that target derived neurotrophic support maintains FMN survival after target reconnection. In addition, RAG-2 KO mice exhibited delayed functional recovery compared to WT mice. Both STAT-6 and T-Bet KO mice exhibited partially delayed functional recovery compared to WT, though not to the extent of RAG-2 KO mice. Collectively, our findings indicate that both pro- and anti-inflammatory CD4⁺ T cell responses contribute to optimal functional recovery from axotomy-induced facial paralysis, while FMN survival is supported by the anti-inflammatory Th2 response alone.

Keywords

T helper cells; Facial nerve axotomy; Motoneuron survival; Functional recovery

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Introduction

Two types of peripheral nerve injuries include transection (cut) and crush axotomies. With regard to facial nerve injury in rodents, both types of axotomy result in loss of vibrissae movement, eye blink reflex, and drooping of the mouth because the facial motor axons are fully disconnected. In contrast to transection, crush axotomy does not separate the nerve sheath and therefore maintains a guiding channel for regenerating axons (Kujawa et al., 1989; Serpe et al., 2002; Moran and Graeber, 2004). Consequently, recovery of facial paralysis in adult WT mice generally occurs within several weeks after crush axotomy, and this model provides a valuable tool to study functional recovery (Serpe et al., 2002; Moran and Graeber, 2004; Hetzler et al., 2008).

Recently, the immune system has been shown facilitate neuroprotective mechanisms following facial nerve transection (reviewed in Jones et al., 2005). Specifically, we have demonstrated that both Th1 and Th2 effector CD4⁺ immune responses are generated in the draining cervical lymph node following axotomy, and that the IL-4/STAT-6-mediated Th2 response is critical for WT levels of FMN survival (DeBoy et al., 2006; Xin et al., 2008). Other reports have shown T cell infiltration into the axotomized facial nucleus, neurotrophic factor (NTF) production by CD4⁺ T cells, and a dual compartment model of antigen presentation that requires both peripheral and central activation steps (Raivich et al., 1998; Byram et al., 2004; Serpe et al., 2005; Ha et al., 2008b). Taken together, these studies suggest that activated Th2 cells may provide NTF support to injured motoneuron cell bodies, and/or direct an anti-inflammatory environment around motoneurons that is conducive for survival. However, the contribution of CD4⁺ T cell responses to FMN survival and functional recovery after facial nerve crush is currently unknown. We hypothesize that similar Th2-mediated neuroprotective mechanisms contribute to FMN survival after crush axotomy; especially in the early stages of recovery before target reconnection is complete. In addition, the peripheral Th1 response develops after axotomy possibly to assist macrophage activation and Wallerian degeneration at the site of injury. Interestingly, the literature demonstrates that full recovery of vibrissae movement and eye blink reflex is delayed after a facial nerve crush in severely combined immunodeficient (*scid*) mice compared to WT and reconstituted (with whole splenocytes) *scid* mice (Serpe et al., 2002). These data imply that delayed functional recovery in *scid* mice is attributed to the absence of T and B lymphocytes.

Additionally, we hypothesize that if a Th1 immune response enhances debris-clearing processes at the site of peripheral nerve crush axotomy, then functional recovery will be delayed in Th1-deficient mice. Furthermore, if Th2 cells support FMN survival before target reconnection, then we expect FMN survival to be decreased in Th2-deficient mice. To test these hypotheses, we analyzed functional recovery after facial nerve crush in several mouse models with specific immune cell deficiencies. STAT-6 KO mice have been shown to have significantly decreased Th2 cell development and defective IL-5 and IL-13 production after *in vivo* immune challenge (Shimoda et al., 1996; Kaplan et al., 1998; Takeda and Akira, 2000; Wurster et al., 2000; Zhou and Ouyang, 2003). Due to impaired Th2 development, STAT-6 KO mice more readily develop pro-inflammatory immune responses that are dominated by Th1 effector cells and the cytokines they produce (IL-2, IFN- γ ; Takeda and Akira, 2000; Zhou and Ouyang, 2003). Conversely, T-Bet KO mice lack the transcription factor T-Bet, which is a necessary for Th1 cell development. T cell responses in T-Bet KO mice are therefore primarily anti-inflammatory and dominated by Th2 effector cells and the cytokines they produce (IL-4, -5, -10, and -13; Szabo et al., 2002). The overall goal of this study was to compare functional recovery and FMN survival profiles in T-Bet (Th1-deficient), STAT-6 KO (Th2-deficient), and RAG-2 KO (lacking mature T and B cells) to strain-matched WT in order to elucidate the contributions of specific CD4⁺ T cell subsets in motoneuron survival and recovery mechanisms.

Materials and methods

Animals and surgical procedures

All surgical procedures were completed in accordance with National Institutes of Health guidelines on the care and use of laboratory animals for research purposes. All WT and immunodeficient mice used in this study were females (6–8 weeks old) on a C57BL/6 background, purchased from either Jackson Laboratory (Bar Harbor, ME) or Taconic labs (Germantown, NY). Eight wk-old T-Bet KO, STAT-6 KO, RAG-2 KO, and WT laboratory mice were allowed 1 week to acclimate to their environment before surgical manipulation. Each experimental group contained 6 mice. All mice were housed under a 12-hr light/dark cycle in microisolator cages contained within a laminar flow system designed to maintain a pathogen-free environment.

Experimental manipulations were performed under aseptic conditions approximately 4 hr into the light cycle. Prior to all surgical procedures, mice were anesthetized with 2.5% isoflurane. The skin behind the right ear was shaved and cleansed with 70% ethanol and betadine. A small incision was made behind the right ear and the muscle was gently separated with forceps to expose the facial nerve. The right facial nerve was exposed at the level of the stylomastoid foramen and crushed two times with fine jewelers forceps for 30 second intervals, with the forceps held at 180 degree opposing orientations for each interval (Semba and Egger, 1986; Kujawa et al., 1989). To ensure that the entire nerve fiber was crushed, the axotomy was performed proximal to the bifurcation of the posterior and anterior auricular branches. Following the surgery, separated muscles were reapposed, the skin was sealed with a wound clip, and a single injection of buprenorphine (0.3 mg/kg) was given to ameliorate post-operative pain. Completeness of the injury was determined by an immediate post-surgical assessment for loss of vibrissae orientation, vibrissae movement, and mouth droop on the axotomized (right) side. Animals that did not meet these requirements were excluded from the study (n=1).

Behavioral analysis

Functional recovery was measured daily beginning immediately after the surgery until full recovery was achieved. Animal groups were coded by one investigator and analyzed under “blind” conditions by a second investigator. Functional recovery of vibrissae movement, eye blink reflex, and vibrissae orientation was measured on a 3-point scale (1 = no recovery; 2 = partial recovery; 3 = complete recovery) during the recovery process (Hetzler et al., 2008).

Complete recovery of vibrissae orientation and movement was acknowledged when the investigators determined that the parameters, measured on the injured side, were equal in appearance and magnitude to that of the uninjured side. To determine functional recovery of the eye blink reflex, the investigator provided a small (500 μ l) air puff stimulus to each eye with a pipette tip. Complete recovery was acknowledged when all three facial functions had been fully restored. It should be noted that two functional recovery experiments were performed (Figs. 1, 2), and statistical comparisons cannot be made between them because of differing methodologies and personnel changes. To increase the power of observation, behavioral assessments were made twice daily (8–9am and 4–5pm) in the second experiment, compared to once daily in the first. The second trial included RAG-2 KO (n=6) and WT (n=6) experimental groups, which are compared to the original trial for qualitative purposes only.

Tissue sectioning and cell counting procedures

At 4 and 10 weeks postoperative (wpo), mice were euthanized by CO₂ asphyxiation and the brains were removed and immediately flash frozen. Mouse brains were stored at -20°C and embedded in optimal cutting temperature (OCT) compound prior to cryostat sectioning at

25 μ m. Coronal sections were collected throughout the caudal-rostral extent of the facial motor nucleus followed by fixation in 4% paraformaldehyde and staining with thionin.

One investigator coded all slides and animal groups and a second investigator determined surviving FMN counts in each section using light microscopy. The nucleus ambiguus and genu of the facial nerve were used as anatomical landmarks to accurately locate the caudal and rostral extent of the facial nucleus, respectively. FMNs were counted based on their morphological recognizability as nucleated multipolar cells ~25–40 μ m in size. Only motoneuron profiles with clear nuclei were counted. The percent loss of motoneurons between the right (ipsilateral) injured side and left (contralateral) uninjured control side was calculated and compared between experimental groups. To compensate for double counting neurons in adjacent sections, the Abercrombie correction factor ($N = n \times T/T + D$) was used where N is the actual number of cells, n is the number of nuclear profiles, T is the section thickness, and D is the mean diameter of nuclei (5.0 μ m; Abercrombie, 1946). Statistical analysis was measured using a one-way ANOVA test for statistical significance. The post-hoc (Tukey) test was employed for pair-wise comparisons of multiple groups. When only two groups were present, the two-tailed Student's t -test with unequal variances was used.

Results

Functional recovery is delayed in RAG-2KO mice

To determine the effects of immunodeficiency on functional recovery after facial nerve crush, recovery times measured in days post crush injury (dpi) $\pm$ SEM of RAG-2 KO, STAT-6 KO, and T-Bet KO mice were compared to WT mice ($n=6, 5, 6$, and 6 , respectively). Compared to WT mice, RAG-2 KO mice exhibited significantly delayed functional recovery in 5 of the 7 measured parameters: vibrissae orientation, onset of and complete vibrissae movement, onset of eye blink reflex, and complete recovery (Fig. 1, A-D). Onset of vibrissae orientation recovery (Fig. 1A) was the same in both RAG-2 KO and WT (3.4 ± 0.2 and 3.4 ± 0.1 dpi, respectively), as was complete eye blink reflex (Fig. 1C; 10.4 ± 0.2 dpi and 9.7 ± 0.3 dpi, respectively). The largest delay in a single parameter was complete recovery of vibrissae orientation (Fig. 1A), which required 8.1 ± 0.2 dpi in RAG-2 KO mice compared to only 5.3 ± 0.2 dpi in WT mice ($p<0.01$). Onset and complete recovery of vibrissae movement (Fig. 1B) required 7.3 ± 0.3 dpi and 12.2 ± 0.2 dpi, respectively, in RAG-2 KO mice compared to only 6.0 ± 0.3 dpi and 10.8 ± 0.2 dpi in WT mice ($p<0.05$ for onset, and $p<0.01$ for complete recovery). The onset of partial eye blink reflex was significantly delayed in RAG-2 KO mice (6.8 ± 0.2 dpi) compared to WT mice (5.4 ± 0.3 dpi, $p<0.05$). Finally, complete overall recovery (Fig. 1D) occurred by 12.2 ± 0.2 dpi in RAG-2 KO mice and 10.8 ± 0.2 dpi in WT mice ($p<0.01$).

Functional recovery is partially delayed in T-Bet and STAT-6 KO mice

T-Bet and STAT-6 deficiencies negatively impacted functional recovery in mice. As displayed in Fig. 2, STAT-6 KO mice showed significant delays in complete recovery of vibrissae orientation (10.6 ± 0.4 dpi) and onset of eye blink reflex (8.0 ± 0.5 dpi) compared to WT mice (4.0 ± 0.4 dpi, $p<0.01$, and 4.7 ± 0.8 dpi, $p<0.01$, respectively) while T-Bet KO mice showed delays in complete recovery of vibrissae orientation alone (8.2 ± 1.1 dpi, $p<0.05$). Only onset of eye blink reflex was statistically delayed in STAT-6 KO compared to T-Bet KO mice. No significant differences were observed in the other parameters of functional recovery. These data indicate that, compared to WT, T-Bet and STAT-6 KO mice exhibited partially delayed functional recovery, though not to the extent of RAG-2 KO mice.

RAG-2 and STAT-6 KO mice exhibit decreased FMN survival

The mean percent FMN survival after nerve crush axotomy $\pm$ SEM was measured at 4 and 10 wpo to determine the kinetics of FMN survival in WT and immunodeficient mice. Figure 3a

shows representative sections from the uninjured and crush-axotomized facial motor nucleus of WT, RAG-2 KO, STAT-6 KO, and T-bet KO mice at 4 wpo. In agreement with the literature, WT FMN survival levels in the crush-axotomized (right) facial nucleus were $97.4\% \pm 1.4\%$ of the uninjured control (left) side at 4 wpo, indicating no significant decrease in FMN survival following crush axotomy in adult WT mice (Kuzis et al., 1999; Moran and Graeber, 2004; Ha et al., 2008a). In contrast, the injured FMN survival levels at 4 wpo in both RAG-2 KO and STAT-6 KO mice were significantly decreased relative to WT ($82.5\% \pm 1.4\%$ and $79.9\% \pm 4.0\%$ respectively, both at $p < 0.01$; Fig. 3B). T-Bet KO mice exhibited FMN survival levels that were not significantly different than WT mice ($96.5\% \pm 1.2\%$; Fig. 3B). Importantly, mean constitutive FMN levels in the uninjured (left) facial nuclei of all experimental groups (2211 ± 73) were comparable to previous findings in the literature (Ashwell, 1992), and no statistically significant differences were found between groups (data not shown). Given the aforementioned immunodeficiency descriptions of RAG-2 KO, T-Bet KO, and STAT-6 KO mice, these results suggest that the adaptive immune response is essential for supporting WT levels of FMN survival after crush axotomy.

Unlike the trend observed after cut axotomy (Serpe et al., 2000), relative % FMN survival levels did not decrease between 4 and 10 wpo in crush-axotomized WT or RAG-2 KO mice. Figure 4a shows representative sections from the uninjured and crush-axotomized facial motor nucleus of WT and RAG-2 KO mice at 10 wpo. At this timepoint, % FMN survival levels in RAG-2 KO and WT mice were $83.8\% \pm 2.2\%$ and $97.7\% \pm 0.8\%$ respectively, which indicates statistical significance compared to each other ($p < 0.01$; Fig. 4B), but not compared levels observed in RAG-2 KO and WT mice at 4 wpo ($82.5\% \pm 1.4\%$ and $97.4\% \pm 1.4\%$ respectively; Fig. 3B). These results suggest that gradual and progressive motoneuron degeneration is not a hallmark of crush axotomy.

Discussion

The current study demonstrates that rate of functional recovery and level of FMN survival are influenced by immunodeficiency in crush-axotomized mice. Importantly, the results support published findings that functional recovery is delayed in immunodeficient mice lacking mature T and B lymphocytes (Serpe et al., 2002). This report is also the first to observe 7 specific parameters of functional recovery and identify statistical significance in 5 parameters. In addition, decreased levels of FMN survival were observed in crush-axotomized RAG-2 KO and STAT-6 KO mice compared to WT. Interestingly, FMN loss in STAT-6 KO mice (with impaired Th2 development), but not T-Bet KO mice (with impaired Th1 development), mirrors previously published findings using STAT-6 KO and STAT-4 KO (Th1 deficient) mice in the facial nerve transection paradigm (DeBoy et al., 2006). While the relative trends of FMN loss were similar, the magnitude of cell loss was greater after transection compared to crush axotomy. This suggests that similar neuroprotective immune mechanisms, particularly the IL-4/STAT-6-mediated development of CD4⁺ Th2 effector cells, are critical for FMN survival after both transection and crush axotomy.

DeBoy et al. demonstrated that reconstitution of STAT-6 KO mice with CD4⁺ T cells, or reconstitution of RAG-2 KO mice with CD4⁺ T cells purified from STAT-4 KO mice, but not STAT-6 KO mice, was able to restore FMN survival back to WT levels (DeBoy et al., 2006). This argues that the transgenic impairment in STAT-6 KO mice, which leads to decreased FMN survival, relates directly to CD4⁺ T cells and that the Th2 cell contributes to FMN survival. Therefore, FMN survival levels in crush-axotomized STAT-6 KO mice support an important immune mechanism involving a Th1-Th2 shift before 4 wpo. Based on the literature, this shift appears largely mediated by injured motoneurons themselves, as well as the surrounding microenvironment. Upregulation of PACAP, IL-10, and NTFs from multiple central nervous system (CNS) sources may directly protect motoneurons and support an anti-

inflammatory, Th2 driven environment within the injured CNS (Fu and Gordon, 1997; Asadullah et al., 2003; Wainwright et al., 2008). A Th1-Th2 shift cannot take place in RAG-2 or STAT-6 KO mice, which could lead to exacerbated FMN loss after both crush and cut axotomy. Furthermore, sustained motoneuron survival observed at 10 wpo in WT and RAG-2 KO mice (Fig. 4) contrasts with the gradual and progressive FMN loss observed in immunodeficient (*scid*) mice after facial nerve transection (Serpe et al., 1999). These differences support an important role for target derived NTF support in FMN survival after target reconnection has been achieved and the neuroprotective immune infiltration to the facial nucleus has diminished (Raivich et al., 1998).

Regarding functional recovery, the hypothesis predicted that Th1 deficient (T-Bet KO and/or RAG-2 KO) mice would exhibit delayed functional recovery compared to WT mice. This theory was developed based on the known immune functions of Th1 cells, observations that both Th1- and Th2-associated cytokines develop in the draining cervical lymph node after axotomy (Xin et al., 2008), and literature suggesting that Th1 development is not necessary for the central immune-mediated rescue of FMN survival (DeBoy et al., 2006). Possible mechanisms related to functional recovery could include CD4⁺ Th1 cell-mediated activation of debris-clearing processes at the injury site, and/or CD4⁺ Th2 cell-mediated release of NTFs and IL-10 to increase motoneuron survival within the CNS (Fu and Gordon, 1997; Jones et al., 2005; Serpe et al., 2005). However, since RAG-2 KO mice exhibited the highest degree of delayed functional recovery, while T-Bet KO animals did not, it becomes difficult to implicate specific immune cells involved in mechanisms of functional recovery based on these data alone.

Compared to WT, complete vibrissae orientation was delayed in both STAT-6 KO and T-Bet KO mice, whereas onset of eye blink reflex was delayed in STAT-6 KO mice alone. Comparison between the two immunodeficient models revealed that only onset of eye blink reflex was delayed in STAT-6 KO compared to T-Bet KO mice. These results indicate that both Th1- and Th2- deficient mice exhibit partially delayed functional recovery compared to WT mice. The recovery time frame in T-Bet KO mice implies that lack of a normal Th1 response does not critically delay the rate of functional recovery. While this result does not eliminate the possible contributions of Th1 recovery mechanisms, especially since a small delay was observed, it suggests that Th1 effector cells are only partial mediators of functional recovery. If STAT-6 KO mice had shown delayed functional recovery similar to RAG-2 KO mice, it would be in correlation with impaired Th2 cell development and significant motoneuron death in the CNS (Fig. 3). However, because STAT-6 KO mice showed only partial delays in functional recovery, the central rescue effects of Th2 cells on motoneuron survival is not the only mediator of WT functional recovery.

One explanation for the functional recovery data is that both a central Th2 deficiency and a peripheral Th1 deficiency are required for mice to suffer maximal delays in functional recovery after facial nerve crush. Using this interpretation, partially delayed recovery in STAT-6 KO mice can be explained by the beneficial effects of a functional Th1 response on debris-clearing and axonal regeneration processes at the site of injury. Conversely, the recovery in T-Bet KO mice could be explained by an increased number of surviving motoneurons capable of regeneration (Fig. 3). Without either the Th1 and Th2 responses, compensation events fail to occur in RAG-2 KO mice leading to the largest delays in functional recovery.

Through utilization of the facial nerve crush axotomy paradigm, this work confirms that functional recovery of vibrissae orientation, vibrissae movement, and onset of eye-blink reflex is delayed in RAG-2 KO compared to WT mice. It is speculated that both Th1 and Th2 effector responses are involved in functional recovery mechanisms after crush axotomy. Although mice with either specific Th1 or Th2 effector cell deficiencies did not exhibit delayed functional recovery comparable to RAG-2 KO mice, Th2 cell-deficient mice suffered decreased FMN

survival after crush axotomy. These data support previous literature findings that Th2, but not Th1, effector cells play a distinct role in the central mechanisms of immune-mediated FMN survival.

For the first time, data presented in this study shows that healthy immune function is important for motoneuron survival and functional recovery after peripheral nerve injury where the nerve sheath is left intact and target reconnection can occur. The immunodeficient mouse models used provide insight into the cells involved in survival and recovery mechanisms. It is possible that similar correlations between immune status, injury severity, and recovery from common nerve injuries could be identified in human patients, particularly when clinical nerve injuries resemble the crush axotomy. These data, along with future clinical studies, may therefore have implications for medical treatment strategies and recovery expectations, especially when patients with peripheral nerve trauma have been prescribed immunosuppressive drugs.

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Figure 1.

Mean recovery times measured in days post crush injury (dpi), $\pm$ SEM of onset and complete (A) vibrissae orientation, (B) vibrissae movement, (C) eye blink reflex, and (D) complete recovery following facial nerve crush. Two-tailed t-tests were used to analyze differences between WT (C57BL/6) (n=6) and RAG-2 KO mice (n=6). Complete vibrissae orientation, onset and complete vibrissae movement, onset of eye blink reflex, and complete recovery were significantly delayed in RAG-2 KO mice compared to WT mice. * represents statistical significance at $p < 0.05$, ** represents statistical significance at $p < 0.01$.

Figure 2.

Mean recovery times measured in days post crush injury (dpi), $\pm$ SEM of onset and complete (A) vibrissae orientation, (B) vibrissae movement, (C) eye blink reflex, and (D) complete recovery following facial nerve crush. One-way ANOVA was used to analyze differences between WT (C57BL/6), STAT-6 KO, and T-Bet KO mice (n=6, 5, and 6, respectively). Complete vibrissae orientation was delayed in both STAT-6 KO and T-Bet KO mice compared to WT. STAT-6 KO mice exhibited delayed onset of eye blink reflex compared to WT. * represents statistical significance at $p < 0.05$, ** represents statistical significance at $p < 0.01$.

Figure 3.

Immune deficiency potentiates crush axotomy-induced FMN death. (A) Micrographs show thionin-stained FMNs in the facial motor nucleus after crush axotomy at 4 wpo (40× magnification, 200× for inset). (B) Mean percentage survival ± SEM of FMN from the crushed (right) side of WT (C57BL/6), RAG-2 KO, STAT-6 KO, and T-Bet KO mice (n= 6, 6, 5, and 6, respectively) relative to the uninjured (left) control side at 4 wpo. Using one-way ANOVA, RAG-2 KO and STAT-6 KO mice exhibit significantly decreased FMN survival compared to WT and T-Bet KO mice (** represents statistical significance at $p < 0.01$).

Figure 4.

Long-term FMN survival after facial nerve crush. (A) Micrographs show thionin-stained FMNs in the facial motor nucleus after crush axotomy at 10 wpo (40× magnification, 200× for inset). (B) Mean percentage survival $\pm$ SEM of FMN from the crushed (right) side of WT (C57BL/6) and RAG-2 KO mice relative to the uninjured (left) control side at 10 wpo (n=6 for both groups). Using two-tailed t-test, RAG-2 KO mice showed a significant decrease in FMN survival compared to WT (** represents statistical significance at $p < 0.01$).