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T cell memory in the injured facial motor nucleus: Relation to functional recovery following facial nerve crush

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Abstract

T cells have the ability to mount a memory response to a previously encountered antigen such that re-exposure to the antigen results in a response that is greater in magnitude and function. Following facial nerve transection, T cells have been shown to traffic to injured motor neurons in the facial motor nucleus (FMN) and may have the ability to promote neuronal survival and functional recovery. Previously, we demonstrated that early exposure to neuronal injury on one side of the brain during young adulthood elicited a T cell response that was greater in magnitude following exposure to the same form of injury on the contralateral side later in adulthood. Whether the T cell memory response to neuronal injury influenced functional recovery following nerve crush injury was unknown. In the current study, we tested the hypotheses that (1) transection of the right facial nerve in sensitized mice would result in faster recovery of the whisker response when the contralateral facial nerve is crushed 10 weeks later, and (2) the early recovery would be associated with an increase in the magnitude of the T cell response in the contralateral FMN following crush injury in sensitized mice. The onset of modest recovery in sensitized mice occurred between 3 and 5 days following crush injury of the contralateral facial nerve, approximately 1.5 days earlier than naïve mice, and was associated with more than a two-fold increase in the magnitude of the T cell response in the contralateral FMN following crush injury. There was no difference between groups in the number of days to full recovery. Further study of how T cell memory influences neuroregeneration may have important implications for translational research.

Keywords

T cell memory; Facial nerve crush; Functional recovery; Peripheral nerve regeneration

Peripheral nerve injury produces various cellular and molecular changes at the site of the damaged axons and at the level of the injured neuronal cell bodies that lead to successful nerve regeneration [12]. In addition to these changes, axonal injury has been associated with inflammatory events, which include site-specific T cell infiltration to and microglial activation around the injured neuronal cell bodies [22]. Although T cells have been known to cause or exacerbate pathology in certain neurodegenerative conditions, they can also promote neuronal survival and functional recovery following certain types of neuronal injury [3,13,18,25,26,29].

An important feature of T lymphocytes is their ability to mount a memory response to a previously encountered antigen such that re-exposure to the antigen elicits a T cell response that is greater in magnitude and effector function [23]. Previously, we showed that T cell memory can be elicited in response to neuronal injury [7]. Using the facial nerve axotomy model, we demonstrated that mice receiving prior exposure to neuronal injury in the right facial motor nucleus (FMN) exhibited nearly a two-fold increase in the number of T cells in the contralateral FMN following transection of the left facial nerve later in adulthood. Although the increased presence of T cells in the FMN did not impact measures of neuronal survival, the effect of T cell memory on functional recovery was not tested. Because the nerve transection model results in disconnection with variable levels of reconnection, it is not optimal for examining T cell memory effects on functional recovery. To address this issue in the current study, we used the facial nerve crush model, which produces an injury where damaged axons readily regenerate within an intact neural sheath [27]. We tested the hypothesis that prior transection injury to the right facial nerve in young adult mice would result in faster recovery of the whisker response following crush injury of the contralateral facial nerve later in adulthood and that the faster rate of recovery would be associated with an increase in the magnitude of the T cell response in the crushed FMN. We compared two groups of mice, referred to here as “sensitized” and “naïve.” Sensitized mice received a transection of the right facial nerve followed by crush injury of the contralateral nerve 10 weeks later, while naïve mice received sham surgery of the right facial nerve followed by crush injury of the contralateral nerve 10 weeks later. Facial nerve crush induces whisker paralysis with complete functional recovery occurring between 14 and 21 days post-injury, which coincides with the time when T cells have been shown to be at maximal levels in the FMN following crush injury [8,17]. Thus, we assessed the recovery of the whisker response, as described previously by Raivich et al. [21] on each day following crush injury for 14 days to compare the rates of recovery between naïve and sensitized mice. In mice that reached full functional recovery by 14 days post-crush, we assessed the injured FMN associated with the crush injury in both groups of mice for differences in the number of CD3⁺ T cells and CD11b⁺ perineuronal microglial phagocytic clusters, a measure of dead neurons.

Mice used in these experiments were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in microisolator cages under specific pathogen free conditions. Mice were 8-week-old at the time of the first surgery. Animals were anesthetized with 4% isoflurane. The experimental model is depicted in Fig. 1. For the first surgery, the right facial nerve was exposed and the main branch was transected in one group of mice, referred to as “sensitized.” In a second group of mice, referred to as “naïve,” the main branch of the facial nerve was exposed but not transected. Ten weeks later, the contralateral facial nerve was exposed in both groups of mice and the main branch was crushed for 10 s using a fine-tipped hemostat. The whisker response was assessed immediately after injury to ensure complete whisker paralysis.

Mice were scored for the intensity of the whisker response each day following crush injury for 14 days, using a scale from 0–3, as previously described [21]. A score of 0 represents complete paralysis while a score of 3 represents complete recovery. As we previously showed that the peak of the T cell response occurs at 14 days post-crush, it was decided a priori that only mice that reached full recovery by day 14 post-crush would be included in the functional and neuroimmune analyses [8]. For euthanasia, mice were anesthetized by intraperitoneal injection of a 0.5 mg/ml ketamine cocktail (ketamine/xylazine/acepromazine given in a 3:3:1 ratio) and were perfused with 4% paraformaldehyde. Brains were collected, post-fixed in 4% PF for 2 h at room temperature, and cryoprotected in 30% sucrose overnight at 4 °C. Using the ambiguous nucleus and the facial nerve root as the starting and ending points, respectively, 15 µm coronal sections were cut throughout the caudal-rostral

extent of the facial motor nucleus. Sections were collected on Superfrost/Plus slides (Fisher Scientific) and stored at -80°C . For immunohistochemistry, tissue sections were incubated in normal goat serum (NGS) (Vector) for 1 h at room temperature followed by overnight incubation with anti-mouse CD3 (17A2; PharMingen) or anti-mouse CD11b (5C6; 1:500, Serotec) at 4°C . Sections were washed in $1 \times \text{PBS}$ after each incubation step. Visualization of the primary antibodies was performed by incubation of sections in goat anti-rat secondary antibody (1:2000, Vector Labs) for 1 h at room temperature followed by incubation in avidin–peroxidase conjugates (1:500, Sigma) for 1 h. No signal was obtained with each of the primary or secondary antibodies alone. The chromagen reaction was revealed by incubation in 3,3'-diaminobenzidine (DAB)– H_2O_2 solution (Sigma; 0.07% DAB/0.004% H_2O_2). Sections were counterstained with cresyl violet, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped.

For analysis of functional recovery, groups were compared for days to reach each score. The number of CD3^+ T cells or CD11b^+ microglial phagocytic clusters was quantified in sections throughout the FMN by an experimenter under blind conditions. Eight sections per mouse (approximately 1/5 of the entire FMN) were used to assess each variable. Analysis of variance (ANOVA) was used to make statistical comparisons.

As shown in Fig. 2A, sensitized mice showed an earlier onset of recovery and reached a score of 1 by 3.28 ± 0.18 days compared to 4.63 ± 0.32 days in naïve mice ($F(1, 13) = 11.92$, $p < 0.01$). There was no significant difference in the number of days for both groups to reach a score of 2 or 3. In Fig. 2B, the overall rates of recovery for sensitized and naïve mice are depicted with differences in the rates of recovery occurring early between days 3 and 5 post-crush (arrow). Between 6 and 14 days post-crush, the rates of recovery between groups were comparable.

In Fig. 3, we compared the T cell and neuronal death response in naïve and sensitized mice that reached full recovery by day 14 post-crush. As shown in Fig. 3A, the number of T cells in the injured FMN following crush injury of naïve and sensitized was 3.75 ± 0.90 and 8.30 ± 1.88 T cells per section, respectively ($F(1,13) = 5.20$, $p < 0.05$). The number of microglial phagocytic clusters in the injured FMN was negligible in both subject groups. Because microglial cell cluster counts in the sensitized group were at zero, accurate variance estimates were not possible. Thus, cluster count data from these two subject groups were not subjected to parametric or nonparametric statistical analysis to avoid violating mathematical assumptions used in these inferential statistical models (Fig. 3B). Representative photomicrographs of the T cell response in the FMN of naïve and sensitized at 14 days post-crush are shown in Fig. 4. Note the increased number of T cells in the injured FMN of sensitized compared to naïve mice.

In the current study, we demonstrated that sensitized mice receiving prior exposure to neuronal injury exhibited an early onset of modest functional improvement that was associated with a T cell memory response in the FMN following crush injury. Contrary to our hypothesis, however, the time to full recovery did not differ between groups. Although the appearance of weak whisker movement in sensitized mice occurred between 3 and 5 days post-crush, approximately 1.5 days earlier than was seen in naïve mice, the modest recovery effect was unlikely due to axonal regeneration. A previous study showed that whisker reinnervation is not apparent until 9 days post-crush, which is consistent with the second phase of recovery that we observe in sensitized and naïve mice between 8 and 9 days post-crush [28]. Moreover, by 4 days post-crush, the fastest growing axons extended approximately 6–7 mm, a distance that is likely insufficient to bridge the gap between the proximal and distal ends of the crushed nerve. It is possible that the spontaneous recovery effect was due to the degeneration-induced release of neurotransmitters at the neuromuscular

junction. In studies where fluorogold was injected distal to the site of injury following facial nerve crush, however, few fluorogold-positive neurons were observed as early as 3 days following nerve crush [9]. It was suggested that nerve compressions of different duration and magnitude can produce crush injuries of varying severity, which result in a mixed population of damaged and spared axons [11]. While the regeneration of damaged axons may be unlikely at early post-injury time points, the early sprouting of spared axons to their target in the injured FMN is plausible. Future study is needed to examine this intriguing hypothesis. With the whisker response being crucial for sensory processing in the mouse, the early onset of recovery that we observed in sensitized mice may, for example, be beneficial to their survival in the wild. Moreover, the association between T cell memory following neuronal injury and functional recovery may be more significant in severe models of CNS injury, in which spontaneous recovery occurs despite the failure of damaged axons to regenerate [1,2,4,5].

Interestingly, the enhanced T cell response in sensitized mice at 14 days post-crush did not coincide with the recovery effect observed at 3 days post-crush. Previously, we reported a peak in the T cell response occurring at 14 days post-crush [8]. In addition to responding in greater numbers, another characteristic of a T cell memory response is the ability of T cells to elicit a faster response to a previously encountered antigen [23]. It is possible that the time course of the T cell response following facial nerve crush was shifted, such that the peak of the response occurred earlier in sensitized mice. Alternatively, T cells may have entered the injured FMN sooner as a result of sensitization but still exhibited a peak at 14 days post-crush. Although Raivich et al. demonstrated that the T cell response in the FMN at 3 days post-injury was not affected by previous injury to the contralateral side, the spacing between injuries in their experiment was 11 days. This finding is in contrast to the current study, where the injuries were separated by a period of 10 weeks, allowing for a greater potential for T cell sensitization to occur. Finally, inflammatory events occurring at the proximal end of the damaged axons could also influence the rate of functional recovery [10,16].

That we observed more than a two-fold increase in the number of T cells that traffic to the crushed FMN is consistent with a previous finding in our lab [7]. We showed that prior exposure to neuronal injury in the right FMN resulted in greater T cell trafficking to the contralateral FMN when the left facial nerve was transected later in adulthood. Here, we found a similar increase in the T cell response of sensitized mice receiving crush instead of transection for the second injury. It is noteworthy that in both studies, we observed a greater T cell response in the FMN that was not due to an increase in the level of neuronal death, as sensitized and naïve mice exhibited comparable numbers of per-injury microglial phagocytic clusters. By contrast, in a different double injury model where the same facial nerve was re-injured, we did not detect a prominent T cell response in the previously injured FMN following re-injury [6]. Moreover, facial nerve re-injury was associated with a regenerative response in the FMN where the atrophic status of neurons was shown to be reversed by re-injury [6,14,15]. Altogether, the data suggest that T cell memory responses are elicited under conditions of neurodegeneration and not regeneration. A previous study by Yoles et al. showed that the survival of retinal ganglion cells was increased when optic crush injury was preceded by spinal cord contusion injury [29]. It is noteworthy, however, that experimentally-induced injuries initiated within the CNS, such as those described in the Yoles study, result in significant blood-brain-barrier (BBB) breakdown, allowing non-specific cells from the periphery to traffic to the CNS [19,20,24]. In the current study, we used a peripheral nerve model of injury where T cells have been shown to traffic across an intact BBB [22].

In summary, we showed that the presence of T cell memory in the injured FMN was associated with an early onset of recovery following facial nerve crush. Although the

presence of T cell memory did not affect overall functional recovery following nerve crush injury, the role of T cell memory in the injured CNS may impact other aspects of axonal regeneration and functional recovery and warrants future investigation.

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Fig. 1.

Schematic of double injury paradigm. At day 0, naïve and sensitized mice received a sham and transection injury, respectively, of the right facial nerve. At week 10, both groups received a crush injury of the left facial nerve. Following facial nerve crush, mice were assessed for functional recovery of the whisker response each day for 14 days. In mice that reached full recovery (score of 3) by 14 days post-crush, the number of CD3⁺ T cells and CD11b⁺ microglial phagocytic clusters were quantified in the left FMN (black oval) 14 days after crush injury, at week 12.

Fig. 2.

Comparison of functional recovery in naive and sensitized mice. The number of days required for each group to reach each behavioral score is depicted in Fig. 2A, where each bar represents the mean S.E.M. of 8 naive and 7 sensitized mice. In 2B, the overall rates of recovery are shown for naive (solid) and sensitized (dashed) mice, with differences between groups indicated by the arrow.

Fig. 3. Quantification of CD3⁺ T cells and CD11b⁺ microglial phagocytic clusters in the injured FMN of naïve and sensitized mice at 14 days post-crush. For 3A, each bar represents the mean S.E.M. of 7 naïve and 8 sensitized mice. For 3B, each bar represents the mean S.E.M. of 5 naïve and 3 sensitized mice.

Fig. 4. Representative photomicrographs of CD3⁺ T cells in the FMN of naïve (A) and sensitized (B) mice 14 days following crush injury. Nissl-stained motor neuronal cell bodies are stained blue while arrows indicate T cells. Scale bar = 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)