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Dissecting the Effects of Endogenous Brain IL-2 and Normal Versus Autoreactive T Lymphocytes on Microglial Responsiveness and T Cell Trafficking in Response to Axonal Injury

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Abstract

IL-2 is essential for T-helper regulatory (Treg) cell function and self-tolerance, and dysregulation of both endogenous brain and peripheral IL-2 gene expression may have important implications for neuronal injury and repair. We used an experimental approach combining mouse congenic breeding and immune reconstitution to test the hypothesis that the response of facial motoneurons to axotomy injury is modulated by the combined effects of IL2-mediated processes in the brain that modulate its endogenous neuroimmunological milieu, and IL2-mediated processes in the peripheral immune system that regulate T cell function (i.e., normal vs. autoreactive Treg-deficient T cells). This experimental strategy enabled us to test our hypothesis by disentangling the effect of normal versus autoreactive T lymphocytes from the effect of endogenous brain IL-2 on microglial responsiveness (microglial phagocytic clusters normally associated with dead motoneurons and MHC2⁺ activated microglia) and T cell trafficking, using the facial nerve axotomy model of injury. The results demonstrate that the loss of both brain and peripheral IL-2 had an additive effect on numbers of microglial phagocytic clusters at day 14 following injury, whereas the autoreactive status of peripheral T cells was the primary factor that determined the degree to which T cells entered the injured brain and contributed to increased microglial phagocytic clusters. Changes in activated MHC2⁺ microglia in the injured FMN were associated with loss of endogenous brain IL-2 and/or peripheral IL-2. This model may provide greater understanding of the mechanisms involved in determining if T cells entering the injured central nervous system (CNS) have damaging or proregenerative effects.

Keywords

IL-2; knockout; neuroimmunology; congenic mice; immune reconstitution; T cells; microglia; motoneuron; nerve injury; autoimmunity; brain

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Factors that determine whether T cells entering the central nervous system (CNS) have damaging autoimmune actions or proregenerative effects are not well understood. Autoreactive T cell mediated neuronal damage is a hallmark feature of multiple sclerosis and the rodent model of the disease, experimental autoimmune encephalomyelitis (EAE) [19, 33], whereas by contrast, T cell mediated neuroprotection may occur following peripheral axonal injury of central motoneurons [15]. IL-2 is indispensable for maintaining immunological homeostasis (e.g., self-tolerance, T regulatory cell development), essential for self-tolerance, and IL-2 gene deletion produces spontaneous generalized autoimmune disease elicited by autoreactive T cell infiltration [11, 16, 18, 27, 29, 32]. Brain cells also produced IL-2 [10, 17, 22]. Our research indicates that loss of brain IL-2 gene expression may play a pivotal role in altering the endogenous neuroimmunological milieu and the development of CNS autoimmunity, effects that mirror IL-2's actions in the peripheral immune system (e.g., regulatory T cell function, self-tolerance) [4, 6, 12, 14]. Thus, dysregulation of IL-2 expression in the peripheral immune system and the brain may have important implications for identifying injury-induced neuroimmunological processes that drive responses towards either CNS neurodegeneration or neuroregeneration.

The status of the immune system appears to be an important determinant of neuronal outcome measures. The facial nerve axotomy model has been used in neuroimmunology research to identify key factors and conditions that determine if T cell trafficking to axotomized FMN act in a proregenerative or neurodegenerative capacity [5, 8, 9, 26]. On the one hand, the T cell mediated proregenerative actions are seen when immunodeficient mice (which lack functionally mature T cells) are immune reconstituted with wild-type lymphocytes [2, 28], whereas, on the other hand, priming to activate the peripheral immune system concomitant with facial nerve axotomy may lead to significantly greater levels of neuronal loss [1]. Thus, the immune activation state of T cells trafficking into the injured FMN appears to be an important factor that may determine if the response to axonal injury is proregenerative or neurodegenerative.

Expression of the IL-2 gene in the brain has important effects on the neuroimmunological milieu of the CNS. We have shown previously that IL-2 deficiency results in increased T cell trafficking and altered neuroimmunological homeostasis in the uninjured brain (e.g., hippocampus) by increasing chemokines and the proinflammatory T cell chemoattractant cytokine, IL-15, IL-2's cognate/partner cytokine that shares the same receptor subunits for signal transduction (IL-2/15 β and γ_c) as IL-2 [4, 13, 23]. Moreover, in the context of axonal injury, T cell trafficking into the axotomized FMN of mice following facial nerve axotomy is markedly increased in IL-2KO mice, but nearly absent in IL-15KO mice [13, 25]. Our lab has recently used a novel approach of coupling congenic mouse breeding and immune reconstitution by adoptive transfer to demonstrate that endogenous brain IL-2 gene expression and IL-2 expression by peripheral T cells (normal versus Treg-deficient IL-2 KO T cells) are both involved in regulating the spontaneous trafficking of T cells into the uninjured forebrain and cerebellum [14]. In the present study, we used this experimental approach (combining mouse congenic breeding and immune reconstitution) to test the hypothesis that the response of facial motoneurons to axotomy injury is modulated by the combined effects of IL2-mediated processes in the brain involved in regulating T cell trafficking and microglial responsiveness, and IL2-mediated processes in the peripheral immune system that regulate T cell activation and function (i.e., normal vs. autoreactive Treg-deficient T cells). This experimental strategy enabled us test our hypothesis by disentangling the effect of normal versus autoreactive T lymphocytes from the effect of endogenous brain IL-2 on T cell trafficking and measures of microglial status in response to axonal injury.

The experimental method of combining mouse congenic breeding with immune reconstitution by adoptive transfer was performed as described previously by our lab [14]. Mice used in these experiments were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were housed under specific pathogen-free conditions in individual microisolator cages. C57BL/6-IL2^{-/+} heterozygote mice used for breeding were generated in our colony (originally derived from Jackson Laboratories) and the RAG2^{-/-} (RAG2-KO) mice were from Taconic farms. The generation of these experimental mice using congenic breeding with mice of the C57BL/6 background with the RAG-2 gene deleted (RAG^{-/-}), mice that had either two wild-type IL-2 alleles (C57BL/6RAG2-IL-2^{+/+}, referred to here for clarity as brainIL2WT/RAG2KO) or both IL-2 alleles deleted (C57BL/6RAG2-IL-2^{-/-}, referred to here as brainIL2KO/RAG2KO). At the age of 4 weeks the peripheral immune system of these congenic immunodeficient RAG2-KO mice was reconstituted by adoptive transfer with either normal wild-type splenocytes or splenocytes from an autoimmune IL-2KO mouse. This resulted in 4 subject groups of mice that were compared to address the questions of interest in this study: 1) brainIL2WT/RAG2KO + IL2WTimmune (normal brain IL-2 gene expression with a wild-type immune system); 2) brainIL2WT/RAG2KO + IL2KOimmune (normal brain IL-2 gene expression with an IL-2 knockout immune system); 3) brainIL2KO/RAG2KO + IL2WTimmune (absence of brain IL-2 gene expression with a wild-type immune system), and; 4) brainIL2KO/RAG2KO + IL2KOimmune (absence of brain IL-2 gene expression with an IL-2 knockout immune system). In addition, we also used standard wild-type and IL-2KO mice as reference controls (littermates derived by breeding mice heterozygous for IL-2 and wild-type for the RAG2, to obtain C57BL/6IL-2^{+/+} RAG2^{+/+} and C57BL/6IL-2^{-/-} RAG2^{+/+} mice). On day 14 post-resection axotomy, the injured FMN of mice were assessed for the following dependent variables: 1) CD3⁺, CD4⁺ and CD8⁺ T cells; 2) MHC2⁺ activated microglia, and; 3) CD11b⁺ microglial phagocytic clusters, (normally associated with dead motoneurons in the injured FMN) [8, 26]. Spleen weights of the four subject groups were compared, as the substantial increase in T lymphocyte proliferation seen in the spleen of IL-2 knockout mice and other mouse models of autoimmunity results in increased spleen weight – a reliable measure to confirm if adoptive transfer resulted in normal splenic reconstitution (WT immune systems) or lymphoproliferative splenomegaly indicative of T cell autoimmunity [11, 14, 24].

Briefly, mice were weaned, and received adoptive transfer at 28 days of age. One animal (recipient) to one animal (donor) adoptive transfer was performed; a total of 20×10^6 splenocytes obtained from young adult donor mice (8 weeks of age) were adoptively transferred by intraperitoneal injection in a total volume of 0.2–0.5 ml of 1x PBS. Adoptive transfer was performed as described previously, post-weaning at week 4, and axotomy surgery was performed at 8 weeks of age. Animals were anesthetized with 4% isoflurane. The right facial nerve was exposed, and a portion of the main branch resected to prevent nerve reconnection as described previously [8, 20]. The whisker response was assessed after surgery to ensure complete whisker paralysis. All groups were matched for age and balanced for sex. At 10 weeks of age (6 weeks after immune reconstitution by adoptive transfer and 2 weeks after surgery), animals were euthanized by intraperitoneal injection of a 0.5 mg/ml ketamine cocktail (ketamine/xylazine/acepromazine) in a 3:3:1 ratio, and subsequently perfused with 1 x phosphate buffered saline [23]. Brains were removed and snap frozen in isopentane (–80°C) and stored at –80°C. 15 µM coronal sections were cut throughout the FMN. Sections were collected on Superfrost/Plus slides (Fisher Scientific) and stored at –80°C until staining was performed. Eight sections (approximately 1/5 of the sections collected from the FMN) were used to assess each of the number of CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, MHC2⁺ microglia and CD11b⁺ perineuronal microglial phagocytic clusters. Tissue slides from –80°C storage were dried briefly at room temperature and postfixed overnight in 4% paraformaldehyde at 4°C for CD3, CD11b and MHC2 staining,

and in IHC Zinc Fixative (BD Pharmingen) at 4°C for CD4 and CD8 staining, followed by incubation in normal goat serum (NGS) (Vector, 1:30 NGS/PBS) for 1–2 h at room temperature. Sections were in overnight immersion with anti-mouse CD3, CD4, CD8 and MHC2 (1:500; PharMingen) or CD11b (1:500; Serotec) primary antibody at 4°C and followed by washes in 1 x PBS. 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. The chromagen reaction was revealed by incubation in 3, 3'-diaminobenzidine (DAB)-H₂O₂ solution (Sigma; 0.07% DAB/0.004% H₂O₂). Sections were counterstained with cresyl violet, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped. No signal was obtained with each of the primary or secondary antibodies alone.

The upper panel of Figure 1 is comprised of bar graphs illustrating the comparison of the 4 subject groups for the T cell variables, and Figure 2 is a series of representative photomicrographs depicting differences between the subject groups. For CD3⁺ T cell counts, ANOVA confirmed that there was a significant effect of subject group [$F(3,27)=11.48$, $p < .001$]. As seen in Figure 1 A, the two subject groups that were immune reconstituted with splenocytes from WT mice had significantly lower levels of CD3⁺ T cell counts in the injured FMN than the two groups that were immune reconstituted with splenocytes from IL-2KO mice. Post-hoc analysis (Fisher's Least Significant Difference Test) confirmed that, compared to brainIL2WT/RAG2KO + IL2WTimmune mice, CD3⁺ T cell counts were significantly higher in brainIL2KO/RAG2KO + IL2KOimmune mice ($p=.003$) and brainIL2WT/RAG2KO + IL2KOimmune mice ($p<.001$), whereas CD3⁺ T cell counts were not different between brainIL2WT/RAG2KO + IL2WTimmune mice and brainIL2KO/RAG2KO + IL2WTimmune mice. As depicted in Figure 1 B and C, there were similar subject group differences for CD4⁺ and CD8⁺ T cell subsets. For CD4⁺ T cell counts, ANOVA confirmed that there was a significant effect of subject group [$F(3,27)=5.35$, $p=.005$]. Post-hoc analysis confirmed that, compared to brainIL2WT/RAG2KO + IL2WTimmune mice, CD4⁺ T cell counts were significantly higher in brainIL2KO/RAG2KO + IL2KOimmune mice ($p<.05$) and brainIL2WT/RAG2KO + IL2KOimmune mice ($p<.005$), and that CD4⁺ T cell counts were not different between brainIL2WT/RAG2KO + IL2WTimmune mice and brainIL2KO/RAG2KO + IL2WTimmune mice. For CD8⁺ T cell counts, ANOVA confirmed that there was a significant effect of subject group [$F(3,27)=4.61$, $p=.01$]. Post-hoc analysis confirmed that, compared to brainIL2WT/RAG2KO + IL2WTimmune mice, CD8⁺ T cell counts were significantly higher in brainIL2KO/RAG2KO + IL2KOimmune mice ($p<.05$) and brainIL2WT/RAG2KO + IL2KOimmune mice ($p<.01$), and that CD8⁺ T cell counts were not different between brainIL2WT/RAG2KO + IL2WTimmune mice and brainIL2KO/RAG2KO + IL2WTimmune mice. As seen in the Figure 1 D, comparison of spleen weights of the four subject groups confirmed that the subject groups that were immune reconstituted with IL2-deficient splenocytes from IL-2KO donor mice exhibited the expected, markedly enlarged spleens consistent with T cell lymphoproliferative autoimmunity.

Figure 3 shows the comparison of CD11b⁺ microglial phagocytic clusters and MHC2⁺ activated microglia for the 4 subject groups. For CD11b⁺ microglial phagocytic clusters, there was a significant effect of subject group [$F(3,27)=38.70$, $p<.001$]. Post-hoc analyses confirmed that the highest levels of microglial phagocytic clusters were in the brainIL2KO/RAG2KO + IL2KOimmune mice and that those levels were higher than each of the other subject groups ($p<.001$). The brainIL2WT/RAG2KO + IL2WTimmune mice had the lowest levels of microglial phagocytic clusters when compared to the other three subject groups, whereas the brainIL2WT/RAG2KO + IL2KOimmune mice and brainIL2KO/RAG2KO + IL2WTimmune mice showed intermediate levels of phagocytic clusters that did not differ

from each other ($p < .05$). For MHC2⁺ activated microglia, ANOVA revealed that main the effect of subject group did not quite reach statistical significance [$F(3,25)=2.84$, $p=.058$], although post-hoc analysis revealed that brainIL2WT/RAG2KO + IL2WTimmune mice had lower levels of MHC2⁺ microglia than the other subject groups ($p < .05$), the latter groups did not differ from one another. Finally, comparison of immunodeficient mice with and without brain IL-2 gene expression that were not immune reconstituted with peripheral lymphocytes - brainIL2WT/RAG2KO and brainIL2KO/RAG2KO mice – did not differ in numbers of either microglial phagocytic clusters or MHC2⁺ activated microglia.

Together, the findings of this study support our hypothesis that the response of facial motoneurons to axotomy injury is modulated by the combined effects of endogenous IL2-mediated processes in the brain and peripheral IL-2's effects on T cell function. Although T cell homing to the site of axotomized motoneurons was largely dependent on T cell IL-2 status (normal vs. autoreactive/IL2-deficient), our data indicate that levels of microglial activating and microglial phagocytic clusters resulting from axotomy were a combination of the both peripheral and endogenous brain IL-2 deficiency.

Independent of brain IL-2 status, here we found that mice immune reconstituted with autoreactive Treg-deficient T cells from IL-2KO mice - which produced the expected peripheral autoimmunity (splenomegaly) - showed approximately double the number of T cells entering the injured FMN compared with mice that were immune reconstituted with WT splenocytes. As IL-2 is critical for self-tolerance and Treg development and function, deficiency of this pivotal immunoregulatory cytokine leads to generalized autoimmunity characterized by autoreactive Treg-deficient T infiltration in different organs [11, 18, 24, 30, 32]. Here we found that for T cell trafficking it did not matter whether the IL-2 gene was present or deleted from the brain, as the groups of mice immune reconstituted with splenocytes from IL-2KO mice had markedly higher CD3⁺ T cells entering the axotomized FMN. Those CD3⁺ T cell numbers in the injured FMN were approximately double the numbers found in the injured FMN of mice immune reconstituted with WT splenocytes. Similar subject group differences were also found for CD4⁺ and CD8⁺ T cells homing to the injured FMN. The approximately 2-fold increase in CD3⁺ T cells entering the axotomized FMN in the two groups of mice adoptively transferred with IL-2KO T cells was comparable to levels we found previously in standard IL-2KO mice [25]. This observation that levels of T cell trafficking into the injured FMN were independent of brain IL-2 gene status, is similar to our previous finding examining spontaneous basal T cell entry into cerebellum (which was also dependent on the autoreactive status of T cells) using this same experimental strategy [14]. Conversely, T cell trafficking seen in the present study contrasted with basal T cell trafficking into the septum and hippocampus in our previous study where we found that the effect was dependent on a combination of both brain and peripheral IL-2 gene status.

The microglial and neuronal changes elicited by axotomy injury observed here (unlike the T cell trafficking response) was due to a combination of both endogenous brain IL-2 and peripheral IL-2 deficiency. Adult facial motoneurons die by necrosis or a necrosis-like process of degeneration [21, 31]. Microglial phagocytic clusters are a group of microglia that normally surround and clear each motoneuron that is dead, and thus represent a cross-sectional snapshot of the number of dead motoneurons/section at a given point in time (here, at day 14 post-axotomy), and has been used as a marker of dead neurons in sections of the injured rodent FMN [26, 31]. Figure 3 illustrates that, compared to mice with brain IL-2 (two wild-type alleles) that were immune reconstituted with a wild-type immune system, congenic mice without brain IL-2 (two IL-2 KO alleles) that were reconstituted with a normal wild-type immune system and congenic mice with brain IL-2 (two wild-type alleles) that were immune reconstituted with an IL-2KO immune system, exhibited on average

approximately twice the level of microglial phagocytic clusters. Moreover, congenic mice without brain IL-2 (two IL-2 KO alleles) that were reconstituted with an IL-2KO immune system had approximately double again the level of microglial phagocytic clusters than the two aforementioned subject groups. Thus, both the loss of IL-2 in the brain and the peripheral immune system had additive effects on injury-induced levels of microglial phagocytic clusters at this cross-section in time (day 14) post-axotomy. It is important to note, however, that microglial phagocytic clusters do not reflect the status of cumulative neuronal loss over time, and this measure could potentially be affected by changes in the rate of clearance of dead neurons by microglia (e.g., reduced phagocytic efficiency). The status of surviving neurons is also further complicated by the fact that a subset of facial motoneurons atrophy significantly over time following axotomy (8, 20). Thus, it will be important to assess neuronal survival and account for such factors in future studies (8, 20, 26, 28). For activated microglia, loss of either endogenous brain IL-2 and/or peripheral IL-2 led to an approximate doubling of activate MHC2⁺ microglia.

It remains to be determined which neuroimmunological factors are responsible for regulating different axotomy induced responses – T cell trafficking, microglial activation, and neuronal changes. Cytokines, chemokines (e.g., IL-15, CCL-2, CXCL10) and other neuroimmunological factors can affect microglial and T cell function in neuronal injury [3, 7, 13, 25, 26, 34]. In summary, these data show that loss of both brain and peripheral IL-2 have an additive effect on microglial status following injury in this model, whereas the activation/autoreactive status of peripheral T cells may be the primary factor that determines the degree to which T cells enter the injured brain and contribute to neurodegeneration [1]. Use of this model could provide greater understanding into the mechanisms involved in determining whether T cells entering the injured CNS have damaging or proregenerative effects, and could ultimately lead to the development of novel treatment strategies for brain trauma and neurological diseases.

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Highlights

1. The loss of both brain and peripheral IL-2 had an additive effect on neuronal damage.
2. The status of peripheral T cells may determines the degree to which T cells enter the injured brain.
3. The status of peripheral T cells may contribute to neuronal loss in the injured brain.
4. Loss of either brain IL-2 and/or peripheral IL-2 led to an approximate doubling of activate MHC2⁺ microglia.

Fig. 1.

A, B and C. Comparison of CD3⁺, CD4⁺ and CD8⁺ cell counts in the axotomized FMN between subject groups. Bars represent the mean $\pm$ S.E.M for the IL-2WT/RAG2KO+WT (brainIL2WT/RAG2KO + IL2WTimmune n=8), IL-2KO/RAG2KO+WT (brainIL2KO/RAG2KO + IL2WTimmune n=8), IL-2WT/RAG2KO+KO (brainIL2WT/RAG2KO + IL2KOimmune n=7) and IL-2KO/RAG2KO+KO (brainIL2KO/RAG2KO + IL2KOimmune n=8) mice. **Fig. 1. D.** Assessment of lymphoproliferative autoimmunity using spleen weight for subject groups. Each bar represents mean $\pm$ S.E.M of 8 mice per group except for 7 animals/group for IL-2WT/RAG2+KO mice. Mice were immune reconstituted at age of 4 weeks and FMN T cell assessments were made at 10 weeks of age, 14 days after facial nerve axotomy. * $p < .05$, ** $p < .01$ compared to IL-2WT/RAG2KO+WT.

Fig. 2.

Immunohistochemistry for CD3⁺ T cells (A–D), CD4⁺ T cells (E–H) and CD8⁺ T cells (I–L) in the FMN 14 days after facial nerve axotomy for the subject groups: IL-2WT/RAG2KO+WT, IL-2KO/RAG2KO+WT, IL-2WT/RAG2KO+KO and IL-2KO/RAG2KO+KO mice. High power magnification of T cells are shown in the insert of A, E and I. As viewed in the online version of the manuscript, T cells were immunostained brown and are indicated by arrows, while neuronal and glial cell bodies were counterstained with cresyl violet and are shown in blue. Scale bar = 20µm.

Fig. 3.

Quantification of CD11b⁺ microglial phagocytic clusters (**A**) and MHC2⁺ activated microglial cells (**B**) in the injured FMN of subject groups at 14 days post-injury. Bars represent the mean $\pm$ S.E.M for the IL-2WT/RAG2KO+WT (brainIL2WT/RAG2KO + IL2WTimmune n=8), IL-2KO/RAG2KO+WT (brainIL2KO/RAG2KO + IL2WTimmune n=8), IL-2WT/RAG2KO+KO (brainIL2WT/RAG2KO + IL2KOimmune n=7) and IL-2KO/RAG2KO+KO (brainIL2KO/RAG2KO + IL2KOimmune n=8) mice. Mice were immune reconstituted at age of 4 weeks and, CD11b⁺ microglial phagocytic clusters and MHC2⁺ activated microglial cells assessments were made at 10 weeks of age. * p .05, ** p< .01 compared to IL-2WT/RAG2KO+WT.