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INTERLEUKIN-2 DEFICIENCY-INDUCED T CELL AUTOIMMUNITY IN THE MOUSE BRAIN

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Abstract

Interleukin-2 (IL-2) has been implicated in the pathogenesis of neurodevelopmental and neurodegenerative disorders. Studies from our lab have shown that adult IL-2 knockout (KO) mice exhibit septohippocampal pathology and related behavioral deficits. Compared to IL-2 wild-type (WT) mice, IL-2 KO mice have a marked and selective loss of septal cholinergic neurons that occurs between the third postnatal week and adulthood. Given that the development of septal neurons is completed by embryonic day 17 and that IL-2 KO mice exhibit peripheral autoimmunity that develops progressively post-weaning, our data and others led us to postulate that the loss of septal neurons in adult IL-2 KO mice is due to selective autoimmune neurodegeneration that coincides with increasing levels of peripheral autoimmunity. Thus, the present study tested the hypotheses: 1) that T cells selectively target the septum, and; 2) that T lymphocyte infiltration to the septum would correlate with peripheral autoimmune disease. We quantified CD3⁺ T cells in the septum, hippocampus, and cerebellum of IL-2 KO and IL-2 WT mice at ages ranging from 2–14 weeks. T cells infiltrated the brains of IL-2 deficient mice, but were not selective for the septum. Brain T lymphocyte levels in IL-2 KO mice positively correlated with the degree of peripheral autoimmunity. We did not detect CD19⁺ B lymphocytes, IgG-positive lymphocytes or IgG deposition indicative of autoantibodies in the brains of IL-2 KO mice. Further study is needed to understand how IL-2 deficiency-induced autoimmune T lymphocytes interact with endogenous brain cells to alter function and promote disease.

Keywords

interleukin-2; T cells; autoimmunity; septum; hippocampus; cerebellum

Autoimmunity has been attributed to various clinical syndromes affecting the central nervous system (CNS). With the exception of multiple sclerosis and myasthenia gravis, however, little is known about specific factors and pathways that govern CNS autoimmunity. IL-2, the prototypical immunoregulatory cytokine, is also expressed by brain cells, and exerts potent effects on acetylcholine release from septohippocampal cholinergic neurons and trophic effects on fetal septal and hippocampal neurons [3, 9, 20, 24]. IL-2 has been implicated in the pathogenesis of CNS autoimmune disease, multiple sclerosis, as well

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as in schizophrenia and Alzheimer's disease [13, 17, 29]. To date, research examining the actions of IL-2 in the brain has focused almost exclusively on the cytokine's neuromodulatory and neurotrophic properties. In the immune system, IL-2 is indispensable for maintaining immunological homeostasis (e.g., self-tolerance, T regulatory cell development and function). It is now appreciated that IL-2 is essential for normal T regulatory cell function which is critical in self-tolerance [26]. Targeted gene deletion studies in mice have established that IL-2 deficiency produces the spontaneous development of autoimmune disease affecting several organ systems (e.g., intestines, heart) characterized by T cell infiltration and autoantibody deposition [10, 12, 21]. Using IL-2 KO mice, we have found that IL-2 gene deletion results in septohippocampal pathology and related behavioral deficits in adult mice [4–6, 16]. It was unknown, however, whether IL-2 deficiency results in the spontaneous development of CNS autoimmunity.

Previous data from our lab and others have led us to hypothesize that IL-2 deficient mice develop a unique form of autoimmunity that selectively targets septal cholinergic projection neurons. Autoimmune-mediated loss of brain septal cholinergic neurons has been found in animals immunized with septal cholinergic hybrid cells [11]. In a previous study, we compared the number of choline acetyltransferase (ChAT)-positive neurons in the medial septum/vertical diagonal band of Broca (MS/vDB) of IL-2 KO and IL-2 WT littermates at 3 weeks and 8–12 weeks of age [4, 5]. Whereas by 8–12 weeks of age IL-2 KO mice showed considerable evidence of peripheral autoimmunity (e.g., marked splenomegaly), 3-week-old IL-2 KO mice on the C57BL/6 background had not yet developed notable autoimmunity. We found that compared to WT littermates, adult IL-2 deficient mice had a marked reduction of MS/vDB ChAT-positive cell bodies. This loss of neurons was selective for septal cholinergic neurons, as adult WT and KO mice did not differ in the number of ChAT-positive neurons in the striatum or in GABAergic neurons in the MS/vDB. By contrast, 3-week-old IL-2 KO mice did not exhibit a similar reduction in cholinergic neuron number in the MS/vDB. Since the development of septal cholinergic neurons is essentially complete by embryonic day 17 [23], our working hypotheses were that the selective loss of septal neurons in IL-2 KO mice is due to neurodegeneration that occurs postnatally between weaning and early adulthood, and that the neurodegeneration is due principally to autoimmunity.

The present study sought to compare IL-2 KO and IL-2 WT littermates to test the specific hypotheses that: 1) T cells selectively target the septum, and that; 2) T cell infiltration in the septum correlates with the level of autoimmune lymphoproliferative disease that develops postnatally and increases through adulthood. Thus, we quantified brain CD3⁺ T lymphocytes and measured spleen weight in IL-2 KO and IL-2 WT mice ranging from 2 through 14 weeks of age. The substantial increase in lymphocyte proliferation seen in the spleen of various mouse models of autoimmunity leads to increased spleen weights that serve as a reliable measure to confirm the degree of autoimmune disease progression [10, 14]. Although we found alterations in several brain cytokines in the hippocampus of IL-2 KO mice in a previous study, we did not see evidence of T cell infiltration into the hippocampus of IL-2 KO. Those determinations of T cell infiltration were a qualitative assessment of hippocampal sections performed in a small number of animals, and the absence of T cells was consistent with our hypothesis that brain autoimmunity is selective for the septum of IL-2 KO mice [6]. In the present study, we used a more sensitive immunohistochemical detection method and performed systematic quantification across different ages and in 3 regions of the brain. We counted T cells in the septum and hippocampus to examine the septohippocampal system, and the cerebellum, which was selected as a non-specific region. In addition, we examined brain sections for the presence of CD19⁺ B cells, IgG-positive lymphocytes and IgG deposition to assess for autoantibodies *in vivo*.

All animals were housed under specific pathogen-free conditions in individual microisolate cages. C57BL/6-IL2^{-/+} heterozygote mice were purchased originally from Jackson Laboratories. C57BL/6-IL2^{-/-} knockout (IL-2 KO) and C57BL/6^{+/+} wild type (IL-2 WT) littermates were obtained from our breeding colony using IL-2 heterozygote by heterozygote crosses, and were genotyped as described previously [5,6]. Immunohistochemistry was performed as described previously [4,15] using 18 µm coronal sections. The primary antibodies (incubated overnight at 4°C) were rat anti-mouse CD3 (1:500; PharMingen), rat anti-mouse CD19 (1:400, PharMingen), and rabbit anti-mouse IgG primary antibody (1:400, Serotec). They were visualized using goat anti-rat secondary antibody (1:2000 for CD3 or 1:1500 for CD19, Vector Labs) or in goat anti-rabbit secondary antibody (1:1500, Vector Laboratories) for 1 h, followed by incubation in avidin-peroxidase conjugates (1:500, Sigma) for 1 h, both at room temperature. No signal was obtained with the primary or secondary antibodies alone. In the current study, we used 8 and 10 male IL-2 wild-type and IL-2 knockout mice, respectively, and 13 and 11 female IL-2 wild-type and IL-2 knockout mice, respectively. Seven groups of IL-2 KO and IL-2 WT mice (n=3/group at each age) were assessed at 2, 3, 5, 7, 9, 12 and 14 weeks of age.

The number of CD3⁺ T cells in each section was counted blindly. An average of 12 sections out of 40 were used to assess the cerebellum and hippocampus, while 8 sections out of 30 were used to assess the septum (approximately 30% of each region of interest). Attempts to limit our T cell counts within the boundary of the medial septum were hindered due to the inability to double immunostain for ChAT-positive neurons and CD3⁺ T cells (optimal staining for ChAT requires thick, free-floating sections, whereas CD3 requires thin, mounted sections). Thus, analysis of T cells in the septum included both the medial and lateral regions. To standardize comparisons between brain regions, the area of each region of interest was measured using the ImageJ software (National Institutes of Health). From this procedure, the average number of CD3⁺ T cells/area was calculated for each animal. Analysis of Variance (ANOVA) was used to make comparisons between IL-2 KO and IL-2 WT mice. Pearson correlation coefficient was used for correlational analysis between the number of T cells in each brain region of interest and spleen weight.

In Figure 1A, we assessed peripheral autoimmune status as reflected by spleen weight, in IL-2 KO and IL-2 WT mice. There was a marked increase in spleen weight in IL-2 KO mice beginning at 5 weeks of age that progressed through 14 weeks of age, with no apparent change in spleen weight observed in IL-2 WT mice. To determine whether there was an associated increase in T cell trafficking to the brain in IL-2 KO mice, we quantified the number of CD3⁺ T cells in the septum (S), hippocampus (HP), cerebellum (CB) of IL-2 KO and IL-2 WT mice. As shown in Figure 1B, the number of T cells in the septum and hippocampus was greater in IL-2 KO mice compared to IL-2 WT mice. The increase in the number of T cells in IL-2 KO mice was notable as early as 3 weeks of age and progressed through 14 weeks of age. Interestingly, a significant number of T cells in IL-2 KO mice also trafficked to the cerebellum, which served as a non-specific control region. With the data combined across all ages, we found no interaction between group and sex. At all ages examined, IL-2 WT mice exhibited few T cells, reflecting the low number of T cells that normally circulate under non-pathological conditions. Although IgG-positive and CD19-positive B cells were present in spleen sections, they could not be detected in brain parenchyma or surrounding blood vessels in the brain (data not shown).

To determine whether T cells selectively target the septum in autoimmune IL-2 KO mice, we combined the data for the adult mice, 9 weeks of age and older (9, 12, and 14-week-old), and compared the average delta scores calculated for the septum, hippocampus, and cerebellum, as shown in Figure 1C (delta score = number of T cells in an IL-2 KO mouse – number of T cells in a randomly paired IL-2 WT mouse). The number of T cells/cm² did not

significantly differ between the three brain regions. Representative photomicrographs demonstrating the presence of CD3⁺ T cells in the septum, hippocampus, and cerebellum in IL-2 KO mice at various ages are shown in Figure 2. In Figures 2B–2C, representative T cells are shown in the medial septum, although T cells were also found distributed in the lateral septum (not shown). In Figures 2D and 2F, representative T cells are shown in the medial blade as well as the granule cell layer of the dentate gyrus, whereas in Figure 2E, T cells are shown in the apical dendritic field of the hippocampus. In Figures 2G–2H, T cells are depicted in the granule cell layer of the cerebellum. Although the majority of T cells appeared to be randomly distributed in the brain regions examined, there were some that were found clustered together, as shown in the molecular layer of the cerebellum in Figure 2I.

In Figure 3, we performed Pearson's correlation analysis to determine whether the number of T cells in each brain region of interest correlated with autoimmune-induced splenomegaly in IL-2 KO mice. As shown in Figures 3A–3C, the number of T cells in all brain regions of interest significantly correlated with spleen weight in IL-2 KO mice (septum $r=0.69$, $p<0.01$; hippocampus $r=0.72$, $p<0.01$; cerebellum $r=0.78$, $p<0.01$), with no significant correlation in the hippocampus and cerebellum of IL-2 WT mice (Figures 3E and 3F). There was, however, a significant correlation in the septum of IL-2 WT mice, as shown in Figure 3D ($r=0.58$, $p<0.01$), that did not appear to be attributable to age or sex.

These data are the first to our knowledge to demonstrate elevated numbers of T cells in the brains of IL-2 deficient mice. Additional comparisons with IL-2 heterozygote mice, would likely show levels of T cell infiltration to the CNS similar to WT mice since they also have normal levels of IL-2. Given previous findings in our lab demonstrating elevated levels of IL-15 in the brains of IL-2 KO mice and the potent effects of IL-15 on T cell chemoattraction, it is possible that changes in the levels of IL-15 may mediate altered T cell trafficking to the IL-2 KO brain [6]. Contrary to our hypothesis, however, T cell trafficking was not selective to the septum, but was also dispersed in a non-selective pattern throughout the hippocampus and cerebellum. This is consistent with the generalized peripheral autoimmunity affecting multiple organs in the periphery [10]. Visualization of other regions (e.g., amygdala, cortex, striatum) where T cells were not quantified for these analyses also appeared to exhibit the same non-specific pattern of T cell infiltration. Further study of various T cell subsets and activation markers (i.e., CD69, an early T cell activation marker and CD44, a marker for memory T cells) will be important in future studies. In a previous study, we did not detect an appreciable number of T cells in the hippocampus of IL-2 knockout mice [6]. That observation coupled with our previous neurobiological studies of IL-2 KO mice, led us to postulate that the hippocampal pathology we have found in IL-2 KO mice (e.g., fewer intrapyramidal granule cells, reductions in hippocampal infrapyramidal mossy fiber length; [4, 16]) was due to the loss of brain IL-2. Given the various neurotrophic effects on hippocampal neurons *in vitro* [3, 19, 20], our working hypothesis was that brain IL-2 may provide support for hippocampal neurons including the ongoing increase in dentate granule cells during the first year of life [1, 7] and maintain the integrity of axons in the dentate gyrus [22]. Our inability to detect T cells in the hippocampus previously was likely due to the methodology used (i.e., thick free-floating brain sections, T cells were not quantified, only a small number of sections from three adult mice were sampled and visualized qualitatively [6]), with the most likely factor due to tissue thickness. Here we used 18 μm slide mounted sections that were nearly one-third the thickness of the 50 μm free-floating sections we used in our previous study; such thick sections may not permit reliable penetration of the antibody for T cell immunohistochemistry [2]. Moreover, here we performed extensive analyses of the three areas of brain that were systematically quantified (counting T cells in approximately 30% across each of the three brain regions/animal/time point).

The influx of T cells in the brain coincided with the progression of the development of peripheral autoimmunity assessed by splenomegaly. Correlational analyses among the adult IL-2 KO animals (9–14 wks) confirmed that there were significant positive correlations between spleen weight and T cell levels for each of the three brain regions. Among the wild-type animals, as expected, spleen weight and T cells in the hippocampus and cerebellum were not well correlated. Surprisingly, however, there was a significant positive correlation between these variables in the septum that was not attributable to age or sex. It will require further study to determine if this is a spurious association given the truncated range of scores for both variables in the wild-type mice or a real biological relationship. As early as 3 weeks of age, there were more T cells in the brains of IL-2 KO mice than in the brains IL-2 WT mice. The numbers of T cells in the brain increased with age throughout adulthood, and appeared to continue to rise up to 14 weeks of age (the oldest age tested). It remains to be determined if T cell numbers continue to rise in the brain as the animals progress in age. By contrast to our findings here in the brain, numbers of T cells isolated from colonic tissue of IL-2 KO mice were found to peak and plateau between 5 and 8 weeks in age, and decrease substantially out to 19 weeks of age [18]. On the other hand, whereas B cells, anti-colon antibodies and IgG-positive cells were found in the colon in that study, we could not find any evidence of CD19⁺ B cells, IgG deposition, or IgG-positive cells in either perivascular regions or in the brain parenchyma under the conditions tested.

Since the clinical observation that treatment with this pivotal immunoregulatory cytokine in cancer patients induced prominent, untoward neuropsychiatric side effects including cognitive dysfunction and psychosis [8, 28], IL-2 has been implicated in several brain diseases. Interest has focused on both neurodevelopmental and neurodegenerative processes involving the septohippocampal system in neurological disorders, in particular, schizophrenia and Alzheimer's disease, respectively [16, 17, 29]. With the exception of multiple sclerosis [13], less attention has been given to IL-2 related autoimmune effects on the brain. We have found that IL-2 deficiency results in the elevation of several pro-inflammatory cytokines in the hippocampus [6]. Given the present findings of T cells in the brains of IL-2 KO mice, it will be important to determine if these cytokines changes are secondary to the loss of endogenous IL-2 in the brain, activated T cells entering the brain, or both factors. This knockout model could provide important new insights into how changes in immunological homeostasis and T regulatory function seen in IL-2 KO mice modifies neuroimmunological processes involved in neurodevelopment and neurodegeneration. In autism, for example, neurodevelopmental and autoimmune processes involving the cerebellum have been speculated to be involved in the pathogenesis of cerebellar circuitry [25, 27]. Moreover, as models of spontaneous autoimmune disease affecting the brain are lacking, understanding how IL-2 deficiency-induced T cells interact with endogenous brain cells to alter brain function and promote disease could help to advance the field of brain autoimmunity.

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Figure 1.

Peripheral immune status in IL-2 KO and IL-2 WT mice is associated with the number of CD3⁺ T cells found in the brain. In Figure 1A, spleen weight was compared in IL-2 KO and IL-2 WT mice ranging from 2–14 weeks of age. In Figure 1B, the number of T cells in the septum (S), hippocampus (HP), and cerebellum (CB) is shown in IL-2 KO and IL-2 WT mice at different ages. Each data point in Figures 1A–1B represents the mean $\pm$ S.E.M. of 3 mice per group. In Figure 1C, the delta scores were calculated by taking the difference of the number of CD3⁺ T cells between randomly paired IL-2 KO and IL-2 WT mice for each brain region. Data from adult mice aged 9 weeks of age and older (9, 12, and 14-weeks-old) were combined. Each bar represents the mean $\pm$ S.E.M. of 9 delta scores/brain region.

Figure 2.

Immunohistochemistry for CD3⁺ T cells in the septum (A–C), hippocampus (D–F), and cerebellum (G–I) of 2, 3, and 14-week-old IL-2 KO mice. High power magnification of a T cell is shown in the inset of C. As viewed in the online version of the manuscript, CD3⁺ T cells were immunostained brown and are indicated by arrowheads, while neuronal and glial cell bodies were counterstained with cresyl violet and are shown in blue. Scale bar=40 μm. Inset scale bar=10 μm.

Figure 3. Pearson's correlation between spleen weight and number of CD3⁺ T cells in the septum (A,D), hippocampus (B,E), and cerebellum (C,F) of IL-2 KO and IL-2 WT mice. Pearson correlation coefficient, r , is indicated for each region. * $p < 0.01$.