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T cell-derived interleukin-10 is an important regulator of the Th17 response during lethal alphavirus encephalomyelitis

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Abstract

Neuroadapted Sindbis virus infection of mice causes T cell-mediated fatal encephalomyelitis. In the absence of IL-10, pathogenic Th17 cells are increased and disease is accelerated. Lymphoid and myeloid cell contributions to IL-10 production were determined using VertX IL-10 transcriptional eGFP reporter mice. Effector and regulatory CD4+ and CD8+ T cells in the brain, but not the cervical lymph nodes, were the primary producers of IL-10. Th17 and Th1/Th17 cells were increased in mice that lacked T cell IL-10 production, although less than in the absence of IL-10. Morbidity and mortality were not affected suggesting an IL-10 threshold for disease exacerbation.

1. Introduction

IL-10 is an important immunoregulatory cytokine with non-redundant functions that plays a critical role in determining the balance between inflammation and immune regulation by influencing antigen presentation, T cell differentiation, cytokine production, and intensity of inflammation (Couper et al., 2008; Moore et al., 2001). A variety of cell types, including macrophages, dendritic cells, monocytes, NK cells, B cells, and T cells, can produce IL-10 (Moore et al., 2001; Saraiva and O’Garra, 2010; Wack et al., 2011) and the timing of IL-10 production after infection is key: IL-10 production prior to pathogen clearance may facilitate persistent, chronic infection, while delayed IL-10 production may foster immune-mediated pathology (Wack et al., 2011). Most investigations examining the role of IL-10 during virus infections have focused on persistent infections, but IL-10 production is also important in the pathogenesis of acute virus infections and is produced at the height of the inflammatory response during acute influenza A virus, simian virus 5, and mouse hepatitis virus (MHV) infections (Palmer et al., 2010; Puntambekar et al., 2011; Sun et al., 2009; Trandem et al., 2011b).

In mice, regulatory T cells (Tregs) producing IL-10 provide protection against pathology later in disease (Cavaillon and O’Garra, 2002; Zychlinsky and Cavaillon, 2004), and IL-10 is important in determining the balance between Th1 and Th2 responses (Greene et al., 2008; Rowell and Griffin, 2002). Th17 and Th1/Th17 cells were increased in mice that lacked T cell IL-10 production, although less than in the absence of IL-10. Morbidity and mortality were not affected suggesting an IL-10 threshold for disease exacerbation. Il-10 provides protection against pathology later in disease (Wack et al., 2011). Although T cells are important producers of IL-10, intrinsic cells of the CNS can also produce IL-10 and early production at the site of infection by a virus engineered to express IL-10 provides protection against pathology later in disease (Wack et al., 2011)
of susceptible B6 mice in response to infection (Kulcsar et al., 2014). Tregs are implicated as a source of IL-10 because in resistant Balb/cj mice a robust IL-10-producing Treg response is associated with survival (Kulcsar et al., 2015). Therefore, IL-10 is an important immune modulator during NSV infection, but the cellular sources of this cytokine in the CNS and their relative importance during lethal infection are not known.

To determine what cell types produce IL-10 during NSV infection, we utilized VertX IL-10 mRNA reporter mice (Madan et al., 2009) to identify IL-10-expressing cells without the need for ex vivo stimulation. Analysis of these mice showed that IL-10 was expressed in the CNS after infection, but not in the draining cervical lymph nodes, and that the predominant IL-10-expressing cells were CD4+ and CD8+ T cells, with little contribution from myeloid cells. Within the CD4+ T cell compartment CD25+ and CD25− cells expressed IL-10. Examination of mice deficient in IL-10 production specifically in T cells (IL-10CD4KO) or in myeloid cells (IL-10Ly5MKO) identified T cells as the predominant source of IL-10 that restricts Th17 as well as Th1/Th17 cell development in the CNS. These data show that T cell-derived IL-10 is critical for regulation of the immune response during an acute lethal CNS alphavirus infection.

2. Materials and methods

2.1. Mice and infection

C57Bl/6J (B6), B6.129P2-II10tm1Cre/J (C57Bl/6 IL-10−/−) (Kuhn et al., 1993), and B6.129P2-Ly5Mtm1Jfs/J (C57Bl/6 Ly5M−/−) (Clausen et al., 1999) mice were purchased from Jackson Laboratories (Bar Harbor, ME). IL-10eGFP reporter (VertX) mice on a B6 background, were kindly provided by Dr. Christopher Karp (Cincinnati Children’s Hospital) (Madan et al., 2009). IL11+Glo−/− × CD4Cre−/− (IL-10−/−CD4-KO) mice on a B6 background were kindly provided by Dr. Werner Muller (University of Manchester) (Roers et al., 2004). IL11+Glo−/− × Ly5MCre−/− (IL-10−/−Ly5M-KO) mice were generated in house (Siewe et al., 2006). Mice were sex-matched and intranasally infected at 4–6 weeks of age with 105 PFU NSV (Jackson et al., 1988) diluted in 20 μL HBSS. For assessment of morbidity and mortality, mice were monitored daily using the following scoring system: 0) no clinical signs, 1) abnormal hind-limb and tail posture, ruffled fur, and/or hunched back, 2) unilateral hind-limb paralysis, 3) bilateral hind-limb paralysis or full-body paralysis, and 4) dead. For tissue collection, mice were anesthetized with isoflurane and bled via cardiac puncture. Mice were perfused with ice-cold PBS and brains and spinal cords were collected and used fresh or snap frozen and stored at −80 °C. All experiments were performed according to protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

2.2. Gene expression analysis using quantitative real-time RT-PCR

RNA was isolated from frozen tissue using the RNeasy Lipid Mini RNA Isolation Kit (Qiagen). RNA was quantified using a nanodrop spectrophotometer and cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Life Technologies) using 2.5 μg of input RNA. Quantitative real-time PCR was performed using 2.5 μL cDNA, the PrimeTime Mouse IL-10 assay (Integrated DNA Technologies), and 2 × Universal PCR Mastermix (Applied Biosystems). Gapdh mRNA levels were determined using the rodent primer and probe set (Applied Biosystems). All reactions were run on an Applied Biosystems 7500 Real-time PCR machine with the following conditions: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min for 50 cycles. Transcript levels were determined by normalizing the target gene Ct value to the Ct value of the endogenous housekeeping gene gapdh. This normalized value was used to calculate the fold-change relative to the average of the uninfected control (∆∆Ct method).

2.3. Mononuclear cell isolation

Single-cell suspensions from brain and spinal cord tissues were prepared as previously described (Kulcsar et al., 2014). Briefly, tissues were homogenized using the GentleMACS system (Miltenyi) with enzymatic digestion (RPMI + 1% FBS, 1 mg/mL collagenase and 0.1 mg/mL DNase [Roche]). The homogenate was filtered through a 70 μm filter and myelin debris and red blood cells were removed by centrifuging the single-cell suspension on a 30/70% discontinuous percoll gradient for 30 min at 4 °C. Mononuclear cells at the interface were collected, resuspended in PBS + 2 mM EDTA, and live cells were identified using trypan blue exclusion and counted.

2.4. Flow cytometry

Approximately 1–2 × 106 cells were used for immunophenotyping by flow cytometry. Cells were stained with the violet Live/Dead Fixable Cell Stain kit (Invitrogen) in PBS + 2 mM EDTA, blocked using rat anti-mouse CD16/CD32 (BD Pharmingen), diluted in PBS + 2 mM EDTA + 0.5% BSA, surface stained for 25 min on ice, fixed, and resuspended in 200 μL of PBS + 2 mM EDTA + 0.5% BSA. All antibodies were from BD Pharmingen or ebioscience: CD45 (clone 30-F11), CD11b (clone M1–70), Ly6G (clone 1A8), Ly6C (clone HK1.4), CD3 (clone 17A2), CD4 (clone RM4–5), CD8 (clone 53-6.7), and CD25 (clone CP61.5). Cell types were defined as follows: microglia (CD45−CD11b+Ly6G−Ly6C−), macrophages/monocytes (CD45+CD11b+Ly6G−Ly6C+), neutrophils (CD45+CD11b+Ly6G−Ly6Cint), T cells (CD3+), CD4+ T cells (CD3+CD4+), and CD8+ T cells (CD3+CD8+).

For determination of T cell cytokine production and foxp3 expression, 2–3 × 106 cells in RPMI + 1% FBS were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin in the presence of GolgiPlug-brefeldin A (BD Pharmingen) for 4 h. Cells were washed and live/dead staining, blocking, and surface staining were performed as described above. After surface staining, cells were fixed and permeabilized using either the CytoFix/Cytoperm kit (BD Pharmingen) and stained for intracellular proteins 25 min on ice, and resuspended in 200 μL of PBS + 2 mM EDTA + 0.5% BSA. Antibodies used were: IFNγ (clone XMG1.2) and IL-17a (clone eBio17B7). Data were acquired using a BD FACS Canto II with FACS Diva software (version 6.0), and analyzed using FlowJo 8.8.7 (TreeStar Inc.).

2.5. Statistical analysis

Data from 2 to 4 independent experiments or at least 3 mice per group were used for analysis. Survival was compared using Kaplan–Meier survival curves (log rank test). Multiple groups were compared over time using a one-way ANOVA with a Dunn post-test at each time point. Two groups over time were compared using a two-way ANOVA with a Bonferroni post-test or at a single time point using an unpaired, two-tailed Student’s t test with a 95% confidence interval. All statistical analyses were done using Graph Prism 5 (GraphPad).

3. Results

3.1. Expression of IL-10eGFP in NSV-infected mice occurs primarily in the brain, not the draining cervical lymph nodes

IL-10 is an important immunoregulatory molecule during a variety of infections (Couper et al., 2008; Wack et al., 2011). During virus infections of the CNS IL-10 plays an important role in limiting inflammation and protecting against death during acute infections and protecting against demyelination during chronic infections (Lin et al., 1998; Trandem et al., 2011a; Zhao et al., 2014). Previously, we showed that IL-10 was an important cytokine for immune modulation and protection during NSV infection (Kulcsar et al., 2015; Kulcsar et al., 2014). To determine the cell type(s) responsible for IL-10 production, we utilized
VertX IL-10 mRNA reporter mice (Madan et al., 2009). VertX mice express eGFP under control of an internal ribosomal entry site placed immediately downstream of the stop codon and before the poly-A site for IL-10 and do not affect IL-10 protein production (Madan et al., 2009).

The cervical lymph nodes (CLNs) and brains of VertX mice infected intranasally with $1 \times 10^5$ pfu NSV were evaluated for cells expressing IL-10eGFP. There were few IL-10eGFP$^+$ cells in the CLNs at any time after infection. Three days after infection, 0.44% of cells were IL-10eGFP$^+$ and this increased to only 1.41% at 5 d and 1.58% at 7 d after infection (Fig. 1A, B). In the brain, the number and frequency of IL-10eGFP$^+$ cells increased substantially during the course of infection. Few IL-10eGFP$^+$ cells were present 3 d after infection (0.1%, 430 cells/brain). However, by 5 d the number of IL-10eGFP$^+$ cells began to increase (0.61%, $8 \times 10^3$ cells) and the increase continued through 7 d with 8.5% of cells and approximately $4 \times 10^5$ IL-10eGFP$^+$ cells in the brain of each animal (Fig. 1A, C). Therefore, IL-10-producing cells were found infrequently in the CLNs, but often in the brains of NSV-infected mice and were most abundant 7 d after infection, a time of peak inflammation.

3.2. T cells are the primary cells expressing IL-10eGFP in the brains of NSV-infected mice

To determine the cell type-specific expression of IL-10 during NSV infection, we used flow cytometry to assess IL-10eGFP expression in monocytes/macrophages, microglia, neutrophils, and CD3$^+$ T cells 5 and 7 d after infection. Evaluation of myeloid cells was difficult due to autofluorescence. To compensate for this, we subtracted the FITC

Fig. 1. IL-10eGFP$^+$ cells increased in the brain, but not the cervical lymph nodes, during NSV infection. VertX and WT mice were infected intranasally with $10^5$ pfu of NSV and assessed at 3, 5, and 7 d after infection. (A) Flow cytometric analysis of IL-10eGFP expression in cells isolated and pooled from the cervical lymph nodes (n = 4–6) and brains (n = 4–12) of VertX mice. The IL-10eGFP$^+$ gate was set using cells from WT mice as the negative control at each time point. The contour plots are representative of 3 independent experiments. (B–C) The absolute number of IL-10eGFP$^+$ cells per animal (closed circle, solid line) and frequency of IL-10eGFP$^+$ cells (÷ of live cells) (open squares, dashed line) were determined at each time point in the cervical lymph nodes (B) and brains (C) of mice by subtracting the background signal observed in B6 mice in each tissue at each time point. The data represent the mean ± SEM from 3 independent experiments. *P < 0.05.
channel signal from WT mice for all cell types at the same time points to determine the level of eGFP expression above background. Five days after infection, 7.14% of T cells expressed IL-10eGFP, which was higher than the percentage of microglia expressing IL-10eGFP (0.66%, \(P = 0.0051\)) (Fig. 2A, B). Although the percentage of IL-10eGFP+ T cells was greater than that of neutrophils (1.96%) and monocyte/macrophages (2.87%), the differences were not significant (Fig. 2A, B). By 7 d after infection, the frequency of IL-10eGFP+ T cells increased to 55.1% and was significantly greater than the percentage of IL-10eGFP+ microglia (2.0%, \(P = 0.0007\)), monocyte/macrophages (13.5%, \(P = 0.0447\)) or neutrophils (5.29%, \(P = 0.0024\)) (Fig. 2A, C). These data suggest that T cells serve as the primary source of IL-10 during NSV infection.

We next evaluated the relative contributions of myeloid cells and T cells to overall IL-10 production in the brain using mice selectively deficient for IL-10 production in either T cells or myeloid cells. IL-10 flox/lox mice (IL-10\(^{fl/\text{lox}}\)) were crossed to CD4-Cre and to LysM-Cre mice to produce mice that were deficient in IL-10 production from CD4+ and CD8+ T cells (IL-10CD4-KO) or myeloid cells (IL-10LysM-KO) (Roers et al., 2004; Siewe et al., 2006). IL-10 gene expression in the brains of WT, IL-10CD4-KO, and IL-10LysM-KO mice was then evaluated during NSV infection. Levels of \(\text{Il10}\) mRNA were similar in the brains of WT mice and IL-10LysM-KO mice throughout infection (Fig. 3). In contrast, \(\text{Il10}\) mRNA levels in the brains of IL-10CD4-KO mice was significantly lower during the course of infection (\(P = 0.0009\)), particularly at 7 d (\(P < 0.001\)) (Fig. 3). Together these data suggest that T cells are the primary producers of IL-10 in the brain during NSV infection and in the absence of T cell-derived IL-10 there was very little \(\text{Il10}\) mRNA expressed.

### 3.3. Both CD4+ and CD8+ T cells contribute to IL-10 production in the CNS during NSV infection

CD4+ and CD8+ T cells can produce IL-10 in the context of virus infection and lead to dampening of the immune response as well as virus persistence (Couper et al., 2008). Previous studies have shown that individual T cell subsets produce IL-10 with different kinetics during acute and persistent CNS viral infections (Puntambekar et al., 2011; Trandem et al., 2011b; Zhao et al., 2014). IL-10 production selectively knocked-out in CD4+ and CD8+ T cells significantly reduced the amount of \(\text{Il10}\) mRNA expressed (Fig. 3). To determine the dynamics of IL-10 production by CD4+ and CD8+ T cells, T cell subsets were evaluated in VertX mice for expression of IL-10eGFP in the brain 5 d and 7 d after infection and in the spinal cord at 7 d when there were enough T cells to reliably detect IL-10eGFP expression (Fig. 4). Both CD4+ and CD8+ T cells in the brains and spinal cords of NSV-infected mice expressed IL-10eGFP. The numbers of CD4+ IL-10eGFP+ cells increased from \(1.87 \times 10^3\) cells per brain 5 d after infection to \(3.63 \times 10^4\) cells per brain 7 d after infection (\(P = 0.0382\), Fig. 4A, C). The numbers of

![Fig. 2](image-url)

**Fig. 2.** IL-10eGFP expression is most prevalent in CD3+ T cells in the brains of NSV-infected mice 5 and 7 d after infection. VertX and WT mice were infected intranasally with \(10^5\) pfu of NSV and assessed 5 and 7 d after infection for cell type-specific expression of IL-10eGFP. (A) Microglia, monocytes, neutrophils, and CD3+ T cells isolated from the brains of VertX mice (pooled, \(n = 4\)–\(12\)) were assessed for IL-10eGFP expression using flow cytometry. The IL-10eGFP+ populations (black line) are shown relative to WT cells (gray, filled) for each cell population. The histograms are representative of 2–3 independent experiments. The values in parentheses are percent of positive cells after removing background. (B, C) The frequency of IL-10eGFP+ cells (% of live cells) was determined at 5 (B) and 7 (C) d after infection by subtracting the background signal observed in WT mice in each cell type at each time point. The data represent the mean ± SEM from 2 to 3 independent experiments. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
CD8+ IL-10eGFP+ cells also increased from 1.74 × 10³ cells per brain 5 d after infection to 1.56 × 10⁵ cells per brain 7 d after infection (P = 0.0281, Fig. 4A, C). The frequency of CD4+ and CD8+ T cells expressing IL-10eGFP also significantly increased from 5 d to 7 d after infection (CD4: 5 d 10.2% vs. 7 d 33.8%, P = 0.0369; CD8: 5 d 8.8% vs. 7 d 57.1%, P = 0.0470) (Fig. 4A, B). Not only were more CD4+ and CD8+ T cells present 7 d after infection, but more cells within each population were expressing IL-10 than at 5 d.

We also compared IL-10eGFP expression between CD4+ and CD8+ T cells. Five days after infection there were similar numbers as well as similar percentages of CD4+ and CD8+ T cells that expressed IL-10eGFP (Fig. 4A–C). By 7 d after infection, however, there was a higher frequency as well as a greater number of CD8+ T cells that expressed IL-10eGFP compared to CD4+ T cells, although this did not reach statistical significance (Fig. 4A–C). A similar trend was present in the spinal cord 7 d after infection where more CD8+ T cells expressed IL-10eGFP+ compared to CD4+ T cells (Fig. 4A–C).

These data suggest that during the initial stages of T cell infiltration into the CNS, both CD4+ and CD8+ T cells contribute similarly to IL-10 production; however, as inflammation increases, CD8+ T cells become the dominant source of IL-10. Furthermore, both CD4+ and CD8+ T cells also contribute to IL-10 production in the spinal cord.

3.4. Tregs and IL-10 expression

To determine if Tregs contribute to IL-10 production, we characterized these cells and their IL-10 expression 5 d and 7 d after infection (Fig. 5). Our previous studies showed that both CD4+ CD25+ foxp3− and CD4+ CD25− foxp3+ cells express IL-10 during NSV infection in both B6 and Balb/c mice (Kulcsar et al., 2015). In VertX mice, both CD4+ CD25− and CD4+ CD25+ T cells expressed IL-10eGFP. The number of CD4+ CD25− cells increased from 1.37 × 10³ cells 5 d after infection to 2.5 × 10⁴ cells 7 d after infection (P = 0.0433, Fig. 5A, C). Similarly, the number of CD4+ CD25+ cells increased from 637 cells 5 d after infection to 1.24 × 10³ cells 7 d after infection (P = 0.0157, Fig. 5A, C). The percentage of CD4+ CD25− cells that expressed IL-10eGFP increased from 8.4% at 5 d to 26.0% at 7 d after infection (P = 0.0145) while the percentage of CD4+ CD25+ cells expressing IL-10eGFP increased from 19.2% at 5 d to 36.0% 7 d after infection (Fig. 5A, B). Within the CD4+ CD25+ population there was a higher frequency of IL-10eGFP+ cells than in the CD4+ CD25− population 5 and 7 d after infection, although this did not reach statistical significance (Fig. 5B). There were, however, significantly more CD4+ CD25− IL10eGFP+ cells than CD4+ CD25+– IL10eGFP− cells 5 d after infection (P = 0.0064), a trend that was maintained at 7 d (Fig. 5C). These data show that within the CD4+ T cell compartment, there were more CD25– than CD25+ cells expressing...
IL-10 during NSV infection, but a similar proportion within each population were IL-10eGFP⁺.

3.5 T cell-derived IL-10 contributes to the regulation of the pathogenic Th17 response

A primary role for IL-10 during NSV infection is regulation of the pathogenic Th17 response. In the absence of IL-10, more pathogenic Th17 cells, as well as Th1/Th17 cells, are present in the CNS at the onset of paralysis and contribute to accelerated morbidity and mortality (Kulcsár et al., 2014). In WT mice, the primary source of IL-10 is T cells, not myeloid cells (Fig. 3). To verify that IL-10 produced by T cells is responsible for regulating the Th17 response during viral encephalomyelitis, we compared the CD4⁺ T cell skewing of IL-10CD4-KO and IL-10LysM-KO mice infected with NSV, and found no significant differences in the Th1 responses as measured by numbers of IFN-γ-producing CD4⁺ T cells. However, a recent study showed that with vivo stimulation virus-specific Tregs in the draining CLNs do produce IL-10 (McKinstry et al., 2009). These data suggest that a threshold for an increase in disease severity was not reached. Together these data show that T cell-derived IL-10 is important in dampening pathogenic Th17 responses, but either that other mechanisms also contribute or that small amounts of locally produced IL-10 by myeloid cells are sufficient to modulate disease.

4. Discussion

IL-10 restricts the pathogenic Th17 response in the CNS and is an important determinant of survival from alphavirus encephalomyelitis (Kulcsár et al., 2015; Kulcsár et al., 2014). These studies of VertX IL-10-reporter mice infected with NSV showed that IL-10-producing cells were increasingly abundant in the brain and spinal cord after infection, but infrequent in the draining CLN. The numbers of IL-10eGFP⁺ cells in the CNS were primarily CD4⁺ and CD8⁺ T cells and within the CD4⁺ T cell population, more IL-10eGFP⁺ cells were CD25⁺ than CD25⁻ at the height of inflammation. In the absence of T cell-derived IL-10 in IL-10⁻/⁻ mice, numbers of Th1 and Th1/Th17 cells increased, although not as much as in IL-10 KO mice, and the course of disease was not altered. Therefore, effector, as well as regulatory CD4⁺ and CD8⁺ T cells differentiated in the CNS to become important sources of IL-10 that modulated the inflammatory response and development of Th1 and Th1/Th17 cells.

Cells in the brain and spinal cord produced IL-10, whereas few IL-10-expressing cells were found in the draining CLN indicating acquisition of this function after entry into the CNS. Tissue-specific expression of IL-10 also occurs during CNS infection with the glatiretic JM strain of MHV (Puntambekar et al., 2011; Trandem et al., 2011b). These data suggest that although virus-specific T cells are generated in the draining CLN, they do not begin producing IL-10 until they reach the site of infection. Similarly, during influenza virus infection the majority of IL-10⁺ T cells are in the lung, not in the draining LN or spleen (McKinstry et al., 2009). However, a recent study showed that with vivo stimulation virus-specific Tregs in the draining CLNs do produce IL-10 (Zhai et al., 2014) suggesting that some T cell subsets are programmed to produce this regulatory cytokine prior to encountering the milieu of the inflamed CNS. Together, these data suggest that T cell production of IL-10 is
dependent on the environment and the level of stimulation (Saraiva and O’Garra, 2010). Contributing factors may include the local presence of cytokines that stimulate IL-10 production such as IL-6 and TGF-β (Li and Flavell, 2008, Lee et al., 2013).

Once in the CNS, T cells were the primary producers of IL-10 during NSV infection (Puntambekar et al., 2011; Trandem et al., 2011b) with similar contributions from CD4+ and CD8+ T cells initially. However, by 7 d after infection the proportion of CD8+ T cells that expressed IL-10 was higher than that of CD4+ T cells so that at the peak of inflammation CD8+ T cells were the primary producers of IL-10. This may correlate with an increase in CD8+ T cell cytolytic activity as seen during MHV infection (Trandem et al., 2011b).

IL-10 production and a robust regulatory T cell response are both associated with protection from lethal disease induced by NSV infection (Kulcsar et al., 2015). In the brain 7 d after NSV infection there were more IL-10GFP+ CD4+ cells in the CD25− compartment than the CD25+ compartment indicating that effector, as well as regulatory, CD4+ T cells contributed to IL-10 production in the CNS during NSV infection. However, during MHV infection of the CNS more CD25+ than CD25− cells were IL-10GFP+ 7 d after infection (Puntambekar et al., 2011). The numbers of both CD4+ Foxp3+ and Foxp3− T cells producing IL-10 increased as inflammation increased and both can inhibit pathogenic Th17 responses (Huber et al., 2011). Therefore, multiple CD4+ T cell subsets contribute to IL-10 production in the CNS during NSV infection.

Complete absence of IL-10 increases the pathogenic Th17 and Th1/Th17 response to NSV infection and results in more rapid disease onset and death (Kulcsar et al., 2014). A significant increase in the Th17 and Th1/Th17 response in mice lacking T cell-derived IL-10, but not in mice lacking myeloid cell-derived IL-10, indicated that T cells were the primary source of IL-10 regulating the CNS development of Th17 cells. However, the increase of Th17 cells in IL-10−/− mice was less than that observed in mice completely deficient in IL-10 and did not have a significant impact on the course of disease. These data suggest that although T cell-derived IL-10 modulates the Th17 and Th1/Th17 responses, other sources of IL-10 also participate in regulation.

Fig. 6. IL-10 produced by T cells, but not myeloid cells, contributes to controlling the Th17 and Th1/Th17 response in the brain during NSV infection. WT, IL-10KO, IL-10fl/fl, IL-10CD4-KO and IL-10LysM-KO mice were infected intranasally with 10^5 pfu of NSV. Cells were isolated from the brain 5 days after infection and pooled (n = 2–4). The mononuclear cells were stimulated ex vivo with PMA and ionomycin and subsequently stained for the surface markers CD3 and CD4 followed by intracellular cytokine staining for IFNγ and IL-17a to identify Th1 and Th17 cells, respectively. (A) Contour plots showing CD4+ IFNγ+ and CD4+ IL-17a+ cells are representative of 3–6 independent experiments. The percentages of CD4+ T cells that produced IFNγ (B), IL-17a (C) and IFNγ and IL-17a (D) were determined for all groups. The absolute numbers of CD4+ IFNγ+ (E), CD4+ IL-17a+ (F) and CD4+ IFNγ+ IL-17a+ (G) T cells were determined for all groups. The data are pooled from 3 to 6 independent experiments and are presented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 7. Lack of T cell or myeloid IL-10 production does not affect the disease outcome. IL-10fl/fl, IL-10CD4-KO, and IL-10LysM-KO mice were infected intranasally with 10^5 pfu of NSV and monitored daily for clinical signs. (A, C) Clinical score scale: 0, no signs; 1, abnormal hind limb and tail posture, ruffled fur; hunched back; 2, unilateral hind-limb paralysis; 3, bilateral hind limb paralysis and/or moribund; and 4, dead. Data were pooled from 3 independent experiments and are presented as the mean ± SEM. (B, D) Survival was assessed using a Kaplan–Meier analysis and log-rank test. Survival was 0% with a mean day of death of 9 d after infection for all groups. Data were pooled from 3 independent experiments. IL-10−/− and IL-10CD4-KO n = 19–20, IL-10fl/fl and IL-10LysM-KO n = 12.
Most microglia and dendritic cells from lymphoid tissues do not express LysM, thus these cells will maintain the ability to produce IL-10 in the IL-10Vim KO mice (Claussen et al., 1999; Goldmann et al., 2013; Siewe et al., 2006) even if it is at low levels. These cells may modulate the Th17 response by locally producing IL-10 and affecting Th cell differentiation.

In summary, CD4+ and CD8+ T cells were the primary producers of IL-10 in the CNS during NSV infection. Within the CD4+ T cell population, both effector and regulatory T cells contribute to IL-10 production. Therefore, T cell-derived IL-10 is an important source of IL-10 for the regulation of the Th17 response during NSV infection.

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