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IL-27 Limits Central Nervous System Viral Clearance by Promoting IL-10 and Enhances Demyelination

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IL-27 is a pleiotropic member of the IL-6 and IL-12 cytokine family composed of the IL-27p28 and the EBV-induced gene 3 (EBI3) subunits (1, 2). It is primarily produced by activated APC and exhibits critical immune-regulatory properties during both infection as well as autoimmunity (1, 2). IL-27 initially was considered a proinflammatory Th1-polarizing cytokine because of its ability to increase sensitivity of CD4+ T cells to IL-12 and promote T-bet expression (3, 4). However, in the initial stages of many infections, IFN-γ production by CD4+ T cells is independent of IL-27 signaling, suggesting that IL-27 does not regulate priming or differentiation of Th1 cells. Increased IFN-γ subsequent to T cell priming (5, 6) rather suggests that IL-27 regulates effector T cells. Similar to the pleiotropic proinflammatory and anti-inflammatory effects of IFN-γ and IL-6 (7, 8), IL-27 also exhibits broad anti-inflammatory activity via suppression of Th1, Th2, and Th17 effector CD4+ T cells as well as stimulation of IL-10 production by effector CD4+ and CD8+ T cells (2). IL-27 also antagonizes IL-2 production (9), inhibiting proliferation and survival of Ag-specific T cells. Recent data suggests that one effect of IL-27 is to limit the migration of effector T cells to the site of infection by suppressing chemokine secretion (2, 6, 10–12). Similarly, Th1-mediated intestinal immunopathology in the absence of IL-27 signaling has been linked to reduced recruitment of a unique T regulatory cell (Treg) population to the site of inflammation (10). Adding to the complexity of IL-27 regulation, Treg are not influenced by the absence of IL-27, although their generation in mice overexpressing IL-27 is severely impaired (13), indicating that IL-27 upregulation during inflammation may limit Treg at sites of infection. The role of IL-27 in the regulation of Treg remains unclear, because only a subset of Treg expresses the IL-27R (10, 14), and IL-27 negatively regulates Foxp3 expression (12) but conversely amplifies TGF-β–induced Foxp3 expression (15). These data suggest that IL-27 may be dispensable for Treg homeostasis under steady state conditions but may be necessary for optimal regulatory functions under inflammatory conditions. As indicated by its complex regulation of T cell immunity, the role of IL-27 in microbial infections is pathogen dependent. In contrast to inhibiting the clearance of intracellular parasites and bacteria and preventing CD4+ T cell–mediated immunopathology (2, 11, 16), IL-27 inhibits HIV-1 and hepatitis C virus replication by inducing antiviral genes (17–19). It is also required for both IFN-γ and IL-10 secretion by influenza and Sendai virus–specific CD8+ effector T cells (20–22), functions associated with increased antiviral activity (23, 24). By contrast, the absence of IL-27 secretion following mouse hepatitis virus y68 infection (25) suggests a defect in IL-10–secreting effector T cells. Although IL-27 is required for activation of IL-10–secreting CD8+ T cells during acute viral infection of the lung (21, 22), it is unable to induce IL-10 secretion following in vivo reactivation of virus-specific infections.
memory CD8+ T cells because of the loss of cell surface gp130 (22). Recent data also suggests that IL-27 signaling controls the level of acute lymphocytic choriomeningitis virus (LCMV) viremia, contributing to clearance of systemic persistent infection (26). These data suggest that during some, but not all viral infections, IL-27 plays a beneficial role in pathogenesis by enhancing viral control as well as restraining viral persistence and tissue damage. The balance between effective viral control and limited immune pathology is especially critical during viral CNS infections because of the potential loss of cellular functions essential for host survival. Furthermore, factors regulating T cell function in lymphoid tissue may have more severe or even distinct effects within the CNS because of highly specialized cells governing the microenvironment. Indeed, both autoimmune inflammation and Toxoplasma gondii infection of the CNS in the absence of IL-27 signaling are associated with unregulated Th17 immunopathology (27–29).

The complex roles of IFN-γ and IL-10, both potentially regulated by IL-27, are highlighted in the murine neurotropic coronaviruses model of encephalomyelitis. Infection of the CNS with the gliatropic JHM strain of mouse hepatitis virus (JHMV) results in an acute demyelinating encephalomyelitis that resolves into limited to the T cell compartment (34). CD25+IL-10+CD4+ JHMV clearance from the CNS but also limits tissue damage (23). The resulting viral persistence enhances the extent of demyelination in this viral model in contrast to regain an upright position; 3, complete hind limb paralysis; and 4, moribund or dead (34, 36, 38). All procedures were conducted in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation.

### Tissue processing, virus titration, and isolation of CNS cells

Brains were homogenized in Dulbecco’s PBS using ice cold Tenbroeck tissue homogenizers (Wheaton Science Products, Millville, NJ). After centrifugation at 450 × g for 7 min at 4°C, cell free supernatants were collected and stored at −80°C for analysis of virus replication and cytokine content. CNS cells were resuspended in RPMI 1640 medium containing 25 mM HEPES (pH 7.2), adjusted to 30% Percoll (Pharmacia, Piscataway, NJ), and separated from myelin debris by centrifugation at 850 × g for 30 min at 4°C onto a 70% Percoll cushion. Cells were collected from the 30/70% interface, washed, and analyzed phenotypically by flow cytometry and functionally for cytolytic activity (see below). Supernatants were analyzed for virus by plaque assay on monolayers of the continuous Delayed Brain Tumor astrocytoma cell line as described (36, 38). Single cell suspensions from the cervical lymph nodes (CLN) were prepared from infected individual mice as described previously (36, 38).

### Flow cytometric analysis

Cells were incubated at 4°C with 1% mouse serum and 1% rat anti-mouse CD16/CD32 mAb to prevent nonspecific staining. Surface marker expression was examined using mAb purchased from BD Biosciences (San Diego, CA) or eBioScience (San Diego, CA). Staining used FITC, PE, and allophycocyanin-conjugated mAb specific for CD45 (30-F11), CD4 (L3T4), CD8 (53-6.7), F4/80 (Serotec, Raleigh, NC), and MHC class II (2C9). Virus-specific CD8+ T cells were identified using D5′510 MHC class I tetramers (Beckton-Dickinson, Franklin, CA) and IL-27Rα-deficient type 1 regulatory T (Tr1) cells. A recent report examining the role of EBI3 indeed suggested that IL-27 and/or IL-35 are critical in regulating immunity during JHMV infection (37). JHMV-infected EBI3−/− mice exhibit increased mortality and CNS inflammation unrelated to viral load (37), suggesting detrimental immune damage independent of the anti-viral response. The highly polarized Th1 response with essentially no Th17 cells elicited by JHMV infection (38) provides a unique model to determine the role of IL-27 in Th1-mediated CNS viral pathogenesis. Using JHMV-infected IL-27Ra−/− mice, the current study examines the role of IL-27 in the absence of potentially confounding effects of IL-35 depletion. In contrast to other viral infections (20–23, 37), the data indicate that IL-27 dampens antiviral function within the CNS by promoting IL-10 secretion by virus-specific CD4+ but not CD8+ T cells. In addition, IL-27 enhances the extent of demyelination in this viral model in contrast to its protective role in other models of CNS inflammation (2, 27–29).

### Materials and Methods

#### Mice and virus

Homozygous IL-27Ra−/− (WSX-1) mice on the C57BL/6 background (provided by C. Saris, Amgen, Thousand Oaks, CA) were bred locally under pathogen-free conditions. C57BL/6 (wild-type [wt]) control mice were purchased from the National Cancer Institute (Frederick, MD). Female and male mice at 6 wk of age were infected intracranially with 1000 PFU of the sublethal glial tropic mAb-selected J2,2v-1 variant of JHMV (39). Mice were scored daily for clinical signs of encephalitis as follows: 0, healthy; 1, hunched back; 2, partial hind limb paralysis or reduced ability to regain an upright position; 3, complete hind limb paralysis; and 4, moribund or dead (34, 36, 38). All procedures were conducted in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation.
ELISA
IFN-γ in cell free CNS supernatants was measured by ELISA. Briefly, plates were coated with 1 μg IFN-γ mAb (R-4-6A2; BD Biosciences) and incubated overnight at 4°C. After washing with Dulbecco’s PBS containing 0.5% Tween 20, plates were blocked with 10% FBS in Dulbecco’s PBS for 1 h at room temperature and washed, and supernatants were added in triplicate and incubated overnight at 4°C. IFN-γ was detected using biotinylated anti-IFN-γ mAb (XMG1.2; BD Biosciences) and avidin-HRP, followed by 3,3′,5′,tetramethylbenzidine substrate reagent set (BD Biosciences). Optical densities were read at 450 nm in a Bio-Rad Model 680 microplate reader and analyzed using Microplate Manager 5.2 software (Bio-Rad, Hercules, CA).

Gene expression

Brains, spinal cords, and CLN were homogenized in TRizol (Invitrogen, Carlsbad, CA) using a Tissuemizer and stainless steel beads (Qiagen, Valencia, CA). Briefly, following chloroform extraction RNA was isolated by centrifugation at 12,000 × g for 15 min at 4°C. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and suspended in RNase-free water (Invitrogen). DNA was eliminated using a DNA-free kit (Ambion, Austin, TX), according to the manufacturer’s instructions. The cDNA was obtained by reverse transcription using 2 μg RNA. Moloney murine leukemia virus reverse transcriptase (Invitrogen), 10 mM deoxy nucleoside triphosphate mix, and 250 ng random hexamer primers (Invitrogen) for 1 h at 37°C. Real-time quantitative PCR used SYBR Green or TaqMan master mixes in a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA). Primers specific for RNA encoding the JHMV nucleocapsid (N) protein, IL-10, GAPDH, IL-12p35, and Foxp3 were as described previously (34, 36, 38, 41). Ct values were normalized to GAPDH mRNA levels using the following formula: 2(Ct [GAPDH] – Ct [gene of interest]) × 10,000 where Ct is the threshold cycle.

For purification of brain-derived macrophages and microglia for RNA analysis, brains were finely minced using razor blades, and cells were released for 1 h at room temperature and washed, and supernatants were added in 0.5% Tween 20, plates were blocked with 10% FBS in Dulbecco’s PBS and incubated overnight at 4°C. After washing with Dulbecco’s PBS containing 0.5% Tween 20, plates were blocked with 10% FBS in Dulbecco’s PBS and incubated overnight at 4°C. IFN-γ was detected using biotinylated anti-IFN-γ mAb (XMG1.2; BD Biosciences) and avidin-HRP, followed by 3,3′,5′-tetramethylbenzidine substrate reagent set (BD Biosciences). Optical densities were read at 450 nm in a Bio-Rad Model 680 microplate reader and analyzed using Microplate Manager 5.2 software (Bio-Rad, Hercules, CA).

Histopathological analysis

Spinal cords were fixed in 10% zinc formalin, divided into six sections corresponding to cervical, thoracic, and lumbar regions, and embedded in paraffin as described previously (36). Sections were stained with H&E to visualize inflammation or Luxol fast blue (LFB) to visualize myelin. For spinal cord analysis, brains were finely minced using razor blades, and cells were released for 1 h at room temperature and washed, and supernatants were added in 0.5% Tween 20, plates were blocked with 10% FBS in Dulbecco’s PBS and incubated overnight at 4°C. After washing with Dulbecco’s PBS containing 0.5% Tween 20, plates were blocked with 10% FBS in Dulbecco’s PBS and incubated overnight at 4°C. IFN-γ was detected using biotinylated anti-IFN-γ mAb (XMG1.2; BD Biosciences) and avidin-HRP, followed by 3,3′,5′-tetramethylbenzidine substrate reagent set (BD Biosciences). Optical densities were read at 450 nm in a Bio-Rad Model 680 microplate reader and analyzed using Microplate Manager 5.2 software (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical significance was determined using a two-tailed Student t test, assessed by GraphPad Prism 5.0 software (La Jolla, CA). Results are shown as either mean ± SEM or mean ± SD for each group. A p value < 0.05 was considered statistically significant.

Results

IL-27 expression during acute encephalomyelitis

The fatal outcome of JHMV infection in mice deficient in EBI3 (37), the common chain shared by IL-27 and IL-35, implicated IL-27 and/or IL-35 as a critical regulator of an otherwise sublethal encephalomyelitis. However, the relative expression and kinetics of IL-27 in CLN, the site of lymphocyte activation following CNS infection, or the CNS during JHMV infection are unknown. In the CLN, IL-27p28 mRNA expression increased by ~10-fold above basal levels at day 3 postinfection (p.i.) and rapidly declined thereafter (Fig. 1A). In the CNS, IL-27p28 mRNA was significantly lower at basal levels compared with the CLN but also was upregulated during the innate phase of infection between days 3 and 5 p.i. (Fig. 1B). However, contrasting the transient increase in CLN, IL-27p28 mRNA expression levels peaked in the CNS at day 7 p.i. coincident with peak cellular inflammation and IFN-γ levels (30, 34, 36). Furthermore, although IL-27p28 mRNA subsequently declined, it remained above basal levels throughout viral persistence (Fig. 1B). The mRNA encoding EBI3 was also maximal at the peak of inflammation, declined more slowly than IL-27p28 mRNA, and also remained elevated throughout viral persistence (Fig. 1C). By contrast, expression of the IL-12p35 component of IL-35 in the JHMV infected CNS remained essentially unchanged during infection (Fig. 1D), consistent with previous data (38). Expression of IL-27Rα mRNA in brain was maximal coincident with peak cellular inflammation and was sustained throughout persistence, similar to the components of IL-27 (Fig. 1E). These results suggested that IL-27 expression within the CNS coincides with the acute inflammatory response. Furthermore, consistent with primary IL-27Rα expression on lymphoid cells (1, 2), the data imply that IL-27 can exert regulatory function in both the CLN and the CNS during JHMV-induced encephalomyelitis.

IL-27 is primarily produced by APC (1, 2). CNS resident microglia and infiltrating monocytes constitute the vast majority of APC within the CNS following JHMV infection (30). Therefore, expression of IL-27p28 mRNA was compared in microglia and macrophages purified from the inflamed CNS by FACS at various times p.i. IL-27p28 mRNA was readily detected in microglia of naive mice at levels exceeding basal levels in total CNS derived RNA (Fig. 1B, 1F). Following the kinetics of total CNS IL-27p28 mRNA, IL-27p28 mRNA in microglia was upregulated at days 3 and 5 p.i. and peaked at day 7 p.i. (Fig. 1B, 1F). In contrast to microglia, IL-27p28 mRNA expression in CNS infiltrating macrophages was already maximal at day 3 p.i. and progressively declined thereafter (Fig. 1F). Peak early expression of IL-27 in CNS-infilarting monocytes resembled the overall kinetics in CLN, and suggested IL-27 rapidly was activated in the periphery prior to CNS infiltration. Alternatively, infiltrating monocytes express higher basal levels of IL-27p28 than microglia, and expression is downregulated as monocytes differentiate into resident tissue macrophages. However, analysis of IL-27p28 mRNA in bone marrow–derived macrophages suggested basal levels were similar or even lower compared with microglia (Supplemental Fig. 1). Furthermore, infiltration of bone marrow–derived macrophages with the related MHV strain A59, which exhibits increased infection efficiency in vitro, upregulated IL-27p28 mRNA (Supplemental Fig. 1). Overall, these data suggest that early and transient IL-27 upregulation in the periphery is potentially mediated by IFN-α/β (30, 40, 43, 44) and imprints T cells during initial activation in the CLN. However, these data do not exclude the possibility that T cells may be further influenced by local IL-27 expression within the CNS.

IL-27 increases morbidity independent of CNS cellular inflammation or IFN-γ

JHMV infection causes a sublethal encephalomyelitis in which clinical symptoms correlate with antiviral T cell responses but not necessarily viral load (30, 36–38). To specifically evaluate the role of IL-27 signaling, JHMV pathogenesis in IL-27Rα−/− mice was compared with syngeneic wt mice. Similar survival rates of ~95%
in IL-27R<sup>−/−</sup> and wt mice (Supplemental Fig. 2) contrasted with the mortality evident following JHMV infection of EBI3<sup>−/−</sup> mice (37). Moreover, clinical symptoms were even reduced compared with wt mice and declined more rapidly in the absence of IL-27 signaling (Fig. 2A), suggesting IL-27 and IL-35 play distinct roles during JHMV-induced encephalomyelitis. The initiation of clinical symptoms during JHMV infection correlates with a vigorous inflammatory response, which peaks within the CNS at approximately day 7 p.i (30, 34, 36). We therefore determined whether reduced morbidity of infected IL-27R<sup>−/−</sup> mice reflected an alteration in CNS cellular inflammation. CD45<sup>hi</sup> inflammatory cells were increased within the CNS of infected IL-27R<sup>−/−</sup> compared with wt mice throughout infection (Fig. 2B); however, the increase was only statistically significant at day 10 p.i. Similar to wt mice, CNS inflammatory cells declined in IL-27R<sup>−/−</sup> mice after the acute phase but were sustained during viral persistence (Fig. 2B). As reduced clinical symptoms did not correlate with decreased inflammation, alterations in inflammatory cell composition were assessed. Reflecting increased numbers of CD45<sup>hi</sup> cells, the numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were increased at day 10 p.i. (Fig. 2C, 2D); however, the relative frequencies of both T cell subsets within the infiltrating population were not altered in the absence of IL-27 signaling (Supplemental Fig. 3A, 3B). Similarly, there was no difference in the frequency of CD45<sup>hi</sup>CD11b<sup>+</sup> macrophages (Supplemental Fig. 3C), indicating that IL-27 signaling partially limits CNS inflammation, but does not alter the overall composition of inflammatory cells recruited into the CNS.

IL-27 influences cytokine secretion by CD4<sup>+</sup> T cells (1, 2), which are primary producers of IFN-γ during JHMV infection (45). Moreover, JHMV morbidity coincides with the level of IFN-γ within the CNS (38, 45). Less severe clinical disease was therefore anticipated to correlate with reduced IFN-γ production in the absence of IL-27 signaling. However, the frequency of CNS-derived virus-specific CD8<sup>+</sup> T cells secreting IFN-γ was moderately increased in the absence of IL-27 signaling rather than decreased compared with wt mice throughout days 10–21 p.i. (Fig. 3A, 3B). To assess whether the capacity of CNS-derived CD8<sup>+</sup> T cells to secrete IFN-γ also was enhanced, virus-specific CD8<sup>+</sup> T cells were examined for IFN-γ production following stimulation with the H-2<sup>b</sup> immunodominant class I–restricted viral peptide (46, 47). Similar to CD4<sup>+</sup> T cells, the frequency of CD8<sup>+</sup> T cells secreting IFN-γ was increased in the absence of IL-27 signaling (Fig. 3C). Elevated frequencies of IFN-γ–producing T cells following ex vivo stimulation supported enhanced differentiation, expansion, and/or survival of virus-specific T cells in infected IL-27R<sup>−/−</sup> mice. To reveal whether T cell function also was increased in vivo, IFN-γ levels were directly measured in CNS supernatants. IFN-γ was maximal in both groups of mice at day 7 p.i. and rapidly declined by day 10 p.i. (Fig. 3D), consistent with previous results in wt mice (38, 45). However, there were no statistically significant differences in infected IL-27R<sup>−/−</sup> relative to wt mice at any time point postinfection. These results demonstrate that IFN-γ production by JHMV-specific T cells within the CNS is independent of IL-27 signaling and may even be inhibited to a modest extent by IL-27. Decreased clinical disease during JHMV-induced encephalomyelitis in the absence of IL-27 signaling therefore does not correlate with reduced T cell–dependent IFN-γ production.

**IL-27 delays virus control**

Morbidity is not only associated with CNS IFN-γ, a major effector of viral clearance, but also with the extent of virus replication (30, 34).
31). In the absence of IL-27 signaling, virus replication initially was similar to wt mice (days 5 and 7 p.i.); however, by day 10 p.i., infectious virus was cleared from the CNS of 50% of IL-27Rα−/− mice (Fig. 4A). Although some wt mice still harbored infectious virus at day 14 p.i., infectious virus was eliminated from the CNS of all IL-27Rα−/− mice, indicating more rapid virus clearance. This apparently enhanced control of infectious virus at days 10–14 p.i. coincided with increased numbers of both virus-specific CD4+ and CD8+ T cells within the CNS at day 10 p.i. (Fig. 3). To determine whether increased cytolytic activity contributed to accelerated virus control, we examined ex vivo cytolytic activity of CNS-derived virus-specific CD8+ T cells. However, no difference in cytolytic activity was evident at a single-cell level comparing cells derived from the CNS of infected IL-27Rα−/− and wt mice (Fig. 4B). Therefore, although IL-27 signaling did not alter the capacity of CD8+ T cells to lyse-infected cells, increased overall numbers of virus-specific CD8+ T cells implied that increased cytolytic capacity at the population level contributed to accelerated virus control.

**IL-27 induces IL-10 in virus-specific CD4+ but not CD8+ T cells**

In addition to promoting IFN-γ during some infections, IL-27 also induces subsets of T cells to secrete IL-10 (21), including Tr1-like cells that secrete both IFN-γ and IL-10 (48, 49). IL-10 is critical during JHMV infection in that it counteracts both viral clearance and tissue damage (23). IL-10 is primarily produced by CNS-infiltrating CD4+ T cells during JHMV infection (34), and IL-10 mRNA peaks in the CNS of infected wt mice at day 7 p.i. (Fig 5A) concomitant with CD4+ T cell infiltration. By contrast, infected IL-27Rα−/− mice expressed significantly less IL-10 mRNA in the CNS during acute infection (Fig 5A). Nevertheless, IL-10 mRNA expression reached similar low levels in both groups by day 10 p.i. (Fig 5A) when the majority of infectious virus is cleared from the CNS of IL-27Rα−/− mice and both groups maintained low IL-10 mRNA levels throughout viral persistence (Fig 5A). As only a proportion of the CD4+ T cells secreting IL-10 within the CNS are Treg (34–36), we assessed whether IL-27-dependent Tr1-like cells are induced during JHMV infection (Fig 5B). Virus-specific IFN-γ+IL-10+CD4+ T cells, indicative of Tr1 cells, were indeed present in the CNS of infected wt mice by day 7 p.i. and reached a maximal frequency of ~10% within the CD4+ T cell population at day 10 p.i., which was sustained following viral clearance (Fig. 5B, 5C). In the absence of IL-27 signaling, this population was reduced >80% at all times postinfection (Fig. 5B, 5C). By contrast, although virus-specific CD8+ T cells also secrete IL-10 (21–24), which can be promoted by IL-27 (21, 22), there were no differences in the frequency of IL-10–producing JHMV-specific CD8+ T cells within the CNS at any time postinfection (Fig. 5D).

Earlier and enhanced upregulation of IL-27 mRNA in CLN relative to the CNS after JHMV infection suggested that IL-27 may exert effects on T cells during both the priming and effector phases. Activation of JHMV-specific T cells in CLN (45, 50) and subsequent reactivation in the infected CNS suggests that IL-27–dependent induction/differentiation of a Tr1-like population is likely more prominent in the CLN, because activated T cells down-regulate IL-27Rα (14). Responsiveness to IL-27 was therefore examined by the ability of IL-27 to activate STAT-1 phosphorylation in activated CD4+ T cells derived from the infected CNS. In contrast to naïve splenic CD4+ T cells, the ability of IL-27 to induce STAT-1 phosphorylation in activated CNS-derived CD4+ T cells was reduced (Fig. 5E). Although CNS-derived CD4+ T cells isolated at days 7 and 10 p.i. still exhibited a detectable response to IL-27, they had essentially lost all ability to respond to IL-27 by day 14 p.i. (Fig. 5E). Interestingly, by day 21 p.i., a modest responsiveness was recovered. These data support the notion that the Tr1 phenotype was already acquired during initial activation in the CLN when IL-27 was transiently increased and potentially further promoted in the CNS as T cells exert maximal effector function between days 7 and 10 p.i. Unfortunately, the frequency of virus-specific IFN-γ+IL-10+CD4+ T cells in the CLN is too low to measure (45, 50). IL-27R signaling was also examined in CD8+ T cells to determine whether the absence of an influence of IL-27 on IL-10 secretion by CNS-derived CD8+ T cells was due to altered regulation of IL-27Rα expression. However, the IL-27–induced STAT-1 phosphorylation profile was similar to CD4+ T cells, with naïve CD8+ T cells being most responsive and CNS-derived CD8+ T cells losing responsiveness by day 14 p.i. (Fig. 5E). The differential ability of IL-27 to influence CD4+, but not CD8+ T cells, was thus independent of overall IL-27 responsiveness. These data indicate that IL-27 signaling accounts for the majority of virus specific IL-10+IFN-γ+CD4+ T cells, characteristic of Tr1 cells (48, 49), thereby contributing the majority of IL-10 present in the inflamed CNS.

**FIGURE 2.** IL-27 increases disease severity and limits inflammation. (A) Progression of clinical symptoms in wt and IL-27Rα−/− mice infected with JHMV. Representative of three independent experiments (wt n = 30 and IL-27Rα−/− n = 27 mice). Total numbers of CD45hi bone marrow–derived inflammatory cells (B), CD4+ (C), and CD8+ (D) T cells per brain of infected wt and IL-27Rα−/− mice analyzed by flow cytometry. (B–D) represent mean ± SEM of three combined experiments. Statistical differences determined by two tailed unpaired t test. *p < 0.05.
expression by CNS-derived CD4+ T cells from wt and IL-27R−/− mice per group. Data represent mean ± SEM. Statistical differences determined by two tailed unpaired t test. *p < 0.05.

**FIGURE 3.** IL-27 reduces IFN-γ production within CNS. (A) IFN-γ expression by CNS-derived CD4+ T cells from wt and IL-27R−/− mice. Representative of two to three independent experiments. Frequencies of CNS-derived virus-specific IFN-γ+CD4+ T cells (B) and virus-specific IFN-γ+CD8+ T cells (C) following peptide stimulation. Data represent mean ± SD of two to three combined experiments. (D) Concentration of IFN-γ within the infected CNS of infected wt and IL-27R−/− mice analyzed by ELISA. Representative of two experiments with at least three mice per group. Data represent mean ± SEM. Statistical differences determined by two tailed unpaired t test. *p < 0.05.

**IL-27 enhances viral induced pathology without altering viral persistence**

IL-27 limits CNS damage by controlling inflammatory responses during both parasite infections and autoimmune disease (2). Following JHMV infection, virus replication is controlled, but sterile immunity is not achieved resulting in viral persistence associated with chronic tissue damage (30, 31). To evaluate whether accelerated viral control correlates with decreased viral persistence within spinal cord, expression of viral RNA encoding the N protein was compared in IL-27R−/− and wt mice because of the absence of detectable infectious virus during persistence (30–32). Although viral gene expression in the IL-27R−/− mice was decreased at days 10–14 p.i., consistent with the increased efficiency of viral clearance, expression during persistence at day 21 p.i. was independent of IL-27 signaling (Fig. 6A). This suggested that IL-27 did not alter the set point of viral persistence. Furthermore, although IL-10 mRNA expression also was reduced in spinal cords of infected mice lacking IL-27 signaling during the early chronic phase of infection, levels were only marginally different by day 21 p.i. (Fig. 6B). To determine whether decreased IL-10 mRNA reflected a decrease in Treg, Foxp3 mRNA levels also were analyzed in spinal cords. No differences in the expression of Foxp3 mRNA comparing IL-27R−/− and wt mice (Fig. 6C) supported the notion that IL-27 does not alter Foxp3+ Treg during viral persistence.

Viral load and the antiviral immune response both contribute to pathological changes, principally prominent spinal cord demyelination, during JHMV encephalomyelitis (30, 36, 38). JHMV infection results in the foci of both infection and tissue damage scattered throughout the spinal cord. However, consistent with equivalent inflammatory cells detected by flow cytometry in brain at day 14 p.i. (Fig. 2), no differences in either the extent of cellular CNS inflammation or anatomical distribution of inflammatory foci were detected comparing both groups at day 14 p.i. (Fig. 7A). The number of foci of infected cells was reduced in the infected IL-27R−/− mice (Fig. 7A), consistent with the reduced viral mRNA expression (Fig. 6). Nevertheless, in both groups, the predominant foci were within white matter tracks, and the infected cells exhibited morphology consistent with oligodendroglia (Fig. 7, inset). Too few virus-infected cells were present at day 21 p.i. to confirm the viral mRNA analysis. Control of infectious JHMV is dependent on cellular immunity and independent of humoral immunity (30–32). However, control of persistent virus is dependent on neutralizing Ab (31). Although IL-27 has been implicated in regulating humoral immunity via IL-21 (51), it is not required for the generation of Ab in other models of infectious disease (52). Consistent with the absence of viral recrudescence in the CNS of IL-27R−/− mice, no differences in neutralizing Ab were detected when comparing the two groups (Supplemental Fig. 4). IL-27 signaling is associated with limiting pathological changes within the CNS following both parasite infection and autoimmune-mediated demyelination (2, 27–29). Furthermore, because JHMV-induced demyelination is increased in the global absence of IL-10 (23), the extent of demyelination was quantified to determine whether the reduced IL-10 associated with the absence of...
IL-27 signaling altered tissue damage during JHMV persistence. In contrast to the total absence of IL-10 (23) and the role of IL-27 in limiting CNS inflammation and autoimmunity (2, 27–29), the absence of IL-27 signaling during JHMV-induced encephalomyelitis limited tissue damage throughout viral persistence (Fig. 7B).

Discussion

IL-27 mediated strategies to limit pathology are evident during infections associated with predominant IFN-γ responses as well as CNS autoimmune diseases associated with IFN-γ and IL-17 (2, 52). The present results demonstrate that IL-27 does not play a proinflammatory role during JHMV-induced encephalomyelitis. The data rather imply that IL-27 impairs viral control in the CNS by induction of IL-10 in virus-specific CD4+ T cells, thereby promoting Tr1-like T cells and limiting overall T cell CNS infiltration. The distinct and even opposing effects of IL-27 signaling on T cell function in different infections may reflect differences in the T cell priming milieu or target tissue hosting viral replication. During JHMV infection, the prominent anatomical effector site of IL-27 remains unclear. However, earlier and increased upregulation of IL-27 mRNA in CLN relative to the CNS supports an early influence of IL-27 on T cells at the site of priming (30, 45, 50) prior to trafficking into the CNS. This is consistent with the limited ability of activated T cells within the CNS to respond to IL-27 compared with peripheral naive T cells. T cell imprinting in the CLN also is supported by the rapid emergence of IL-10+CD4+ T cells in the CNS of wt-infected mice (34), which is dramatically reduced in the absence of IL-27 signaling. Nevertheless, additional effects on early infiltrating T cells, still exhibiting modest responsiveness to IL-27 compared with T cells retained after virus control, cannot be excluded, especially as IL-27 mRNA is prominently elevated throughout acute infection. In this respect, it is interesting to note that IL-27 mRNA was expressed by both infiltrating monocytes and microglia, which can act as potential APC allowing proximal IL-27 exposure to T cells. Although frequencies of virus-specific IFN-γ-secreting CD4+ and CD8+ T cells were elevated, total IFN-γ levels in the CNS were not significantly different in the absence of IL-27 signaling. This suggested that down regulatory effects of IL-27 apparent after in vitro restimulation are constrained in vivo by other factors regulating T cell activity.

Accelerated JHMV control in the CNS in the absence of IL-27 signaling contrasts with recent data demonstrating that IL-27 enhances viral control during acute systemic LCMV infection, without altering the activation of virus-specific CD4+ T cells (26). Similar levels of persisting JHMV, despite accelerated control of infectious virus, also contrast with the suggestion that the diminished capacity of IL-27Rα−/− mice to control acute LCMV reduces virus-specific T cells during chronic infection (26). The suggestion that decreased CD4+ T cells during chronic LCMV reflects an IL-27 interaction with APC (26) also does not apply to JHMV CNS infection as virus prominently persists in MHC class II+ oligodendrocytes (30). IL-27 has further been associated with enhanced pathology (23, 24). Interestingly, during JHMV infection, the absence of IL-27 signaling alone only reduced IL-10 secretion by virus-specific CD4+ T cells (26).

Reduced clinical signs of encephalitis and demyelination in JHMV-infected IL-27Rα−/− mice in contrast to the increased mortality of EBI3−/− mice following JHMV infection (37) sug-
gests IL-27 and IL-35 play distinct roles during JHMV encephalomyelitis. The basis for this discrepancy in pathogenesis is unclear but may reflect subtle differences in viruses or a critical anti-inflammatory activity of IL-35 during acute viral infection. The observation that EBI3 deficiency prevents induction of IL-10+ CD8+ T cells following influenza virus infection (24), yet the IL-10+CD8+ T cell population within the JHMV infected CNS is not affected by IL-27 signaling deficiency alone, suggests that this population may be uniquely regulated by IL-35. In support of this concept, the anti-inflammatory activity of IL-35 is critical in the tumor microenvironment (53) and in both collagen-induced arthritis and asthma (54, 55). In addition, IL-35+CD4+ T cells have been described in patients with chronic hepatitis (56), suggesting IL-35 may be an important regulator of established immune responses.

Hallmarks of JHMV infection are viral persistence associated with ongoing demyelination (30–32). The precise mechanisms of JHMV-induced demyelination are unclear, yet both infection of oligodendrocytes and T cell responses are absolute requirements (30). Importantly, demyelination is exacerbated in the absence of IL-10 (23), the major cytokine dysregulated by IL-27Rα deficiency in this model. However, despite impaired IL-10 production by the majority of CD4+ T cells, tissue damage was not increased in suggesting ongoing control of detrimental responses. This contrasted with uncontrolled CD4+ T cell mediated pathology within the CNS during parasite and autoimmune-mediated inflammation in the absence of IL-27 signaling (2, 27–29). Consistent with an adverse role for IL-27 in viral-induced demyelination, IL-27 expression during infection with a HSV type 1 vector expressing IL-2 enhanced demyelination (57). It remains unclear whether IL-10 secreted by virus-specific CD8+ T cells, Treg, or the more efficient control of viral load contributed to limited tissue damage. However, reactivation of virus-specific memory CD8+ T cells in the presence of IL-27 does not induce IL-10 secretion because of the loss of receptor signaling (22). Similarly, limited IL-27 responsiveness during JHMV persistence is predicted by downregulated
IL-27R expression on both CD4+ and CD8+ T cells and low levels of IL-27 mRNA within the CNS. In addition to IL-27–induced effector T cells producing IL-10, Foxp3+ Treg are implicated in CNS viral persistence and pathology (58). IL-27 also induces a subset of IL-27Rα−/− mice, and preliminary experiments indicate that the frequency of Treg expressing CXCR3 within the CNS also was not decreased, in contrast to Toxoplasma gondii infection (10). Although Treg infiltrate the CNS during JHMV infection (34–36), Treg depletion in immune suppression at the site of infection (10). These data are consistent with a minimal role for Treg in regulating JHMV persistence (36).

In summary, our data demonstrate that IL-27 plays a unique role in regulating the immune response within the CNS during JHMV-induced encephalomyelitis. Rapid induction of IL-27 specifically promotes virus-specific Tr1-like CD4+ T cells, which limit both the overall inflammatory response and antiviral T cell activity during acute infection. By contrast, IL-27 does not influence viral persistence, suggesting that the Tr1-like population secreting both IFN-γ and IL-10 in the CNS plays a minor role in regulating persistent infection. Nevertheless, although virus clearance was accelerated in the absence of IL-27, immunopathology manifested by demyelination was not enhanced.

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Disclosures

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References


