Cell type specific IL-27p28 (IL-30) deletion uncovers an unexpected pro-inflammatory property of IL-30 in autoimmune inflammation

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Abstract

IL-27 is an IL-12 family cytokine with potent immunoregulatory properties, capable of modulating inflammatory responses, including autoimmunity. While extensive studies have been performed to investigate the major target cells of IL-27 mediating its functions, the source of IL-27 especially during tissue specific autoimmune inflammation has not formally been tested. IL-27p28 subunit, also known as IL-30, was initially discovered as an IL-27-specific subunit, and its expression has thus been used as a surrogate for IL-27. However, there is emerging evidence that IL-27p28 can be secreted without Ebi3, a subunit that forms IL-27 with IL-27p28. Furthermore, IL-27p28 was also reported to act as a negative regulator antagonizing IL-27. In this study, we utilized various cell type specific IL-27p28-deficient mouse models and examined the major source of IL-27p28 in T cell mediated autoimmune neuroinflammation. We found that dendritic cell-derived IL-27p28 is dispensable for the disease development but that IL-27p28 expressed by infiltrating and CNS resident APC subsets, namely, infiltrating monocytes, microglia, and astrocytes, play an essential role in limiting inflammation. Unexpectedly, we observed that cell type specific IL-27p28 deficiency expressing severe disease phenotype is associated with dysregulated IL-27p28 expression in otherwise unaffected APC subsets, suggesting that disproportionate IL-27p28 expressed may increase disease susceptibility. Indeed, systemic recombinant IL-30 administration also induced severe disease. Taken together, our results uncover a pro-inflammatory property of IL-30 that supports encephalitogenic immunity in vivo.

1. Introduction

IL-27 is an IL-12 family heterodimeric cytokine composed of p28 (also known as IL-30) and Ebi3 subunits, and binds the IL-27 specific receptor, a heterodimeric surface receptor complex made of IL-27Rα and gp130 (1, 2). IL-27 mediates highly diverse, even opposing, pro- and anti-inflammatory functions by supporting Tbet/IFNγ expression in developing Th1 cells and by blocking Rorc expression and Th17 differentiation, respectively (3-5). Another well characterized anti-inflammatory function of IL-27 operates through IL-10 induction from activated CD4 T cells (6, 7). IL-10-producing helper T cells (known as Tr1 cells) are thought to play an important role in suppressing immune responses and maintaining tolerance in many conditions (8, 9). Indeed, mice deficient in IL-27Rα subunit are highly susceptible to Th17-mediated autoimmune inflammation, experimental autoimmune encephalomyelitis (EAE), which was partly attributed to the lack of IL-10-producing CD4 T cells (10). However, Tr1-independent anti-inflammatory roles of IL-27 have also been observed. We demonstrated that IL-27 directly acts on Foxp3$^+$ Treg cells to support their ability to suppress autoimmune inflammation via Lag3-dependent and Tr1-independent mechanisms (11). Therefore, IL-27 seems to control inflammatory responses by multiple mechanisms.

Being the IL-27-specific subunit, IL-30 was measured as a surrogate to assess IL-27 production. The primary source of IL-30 is cells of myeloid origin, including monocytes, macrophages, and dendritic cells (2). Signals triggering IL-30 secretion are mostly of innate immunity. TLR3 and TLR4 have previously been shown to trigger IL-30 expression in dendritic cells via an IRF3-dependent mechanism (12), and IFNγ signal can augment the expression (13, 14). IFNβ, a widely used immune suppressive cytokine for the treatment of autoimmunity, is another potent cytokine triggering IL-30 production and inhibiting Th17 differentiation (15). However, little is known regarding the precise source and immune regulatory functions of IL-30 during tissue specific autoimmune inflammation, such as EAE.

There is emerging evidence that IL-30 may exhibit immune regulatory function distinct from that of IL-27 (16, 17). Caspi and colleagues utilized IL-30-overexpressing

mouse model to show that IL-30 inhibits T cell differentiation to Th1 and Th17 lineage cells and the development of autoimmunity (18). IL-30 also negatively regulates humoral and cellular responses during parasite infection, independent of its role as an IL-27 subunit (19). In the context of tumorigenesis, IL-30 has been suggested to play an important pro-tumorigenic role, in part by supporting cancer stem-like cell survival, vascularization, and proliferation (20, 21). However, IL-30 may express regulatory property simply by inhibiting biological functions of IL-27 (22).

The current study aimed at identifying the key source of IL-30 and its potential role during autoimmune inflammation in the central nervous system (CNS). Utilizing cell type specific *Il27p28*$^{-/-}$ mouse models, we report that IL-30 expressed by myeloid cells, microglia, and astrocytes but not by dendritic cells plays a key role in limiting autoimmune inflammation. Unexpectedly, we noted that exacerbated inflammation seen in those cell type specific IL-27p28-mutant mice was associated with drastic overexpression of *Il27p28* mRNA in otherwise unaffected CNS-infiltrating and resident APC subsets, possibly resulting in the presence of excessive IL-30. In support, systemic administration of recombinant IL-30 alone into mice with ongoing EAE similarly aggravated the disease progression, suggesting that IL-30 appears to be able to enhance encephalitogenic immune activity. In vitro, we noted that IL-30 had no measurable biological activity on activated T cells as determined by its ability to phosphorylate Stat1 and Stat3 or to antagonize IL-27 activity. These results suggest pro-inflammatory roles of IL-30 in vivo, in part by antagonizing immune regulatory cytokines, such as IL-27, in order to drive inflammatory T cell responses.

2. Materials and Methods

2.1. Animals

C57BL/6, *Itgax* (CD11c)^{Cre} (strain #8068), *Gfap*^{Cre} (strain #24098), *Lyz2* (LysM)^{Cre} (strain #4781), and *Cx3cr1*^{Cre} (strain #25524) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Il27p28*$^{fl/fl}$ mice were previously reported (23). All mice were bred in a specific pathogen-free facility at Northwestern University Feinberg School of Medicine. All the animal experiments were approved by the institutional animal care and use committees (IACUC) of Northwestern University (protocol #IS00015862).

2.2. EAE induction

Mice were subcutaneously injected with 200 µL of an emulsion containing 300 µg of MOG_{35-55} peptide (BioSynthesis, Lewisville, TX) and equal volume of Complete Freund's adjuvant supplemented with 5 mg/mL of Mycobacterium tuberculosis strain H37Ra (Difco, Detroit, MI). Additionally, mice were intraperitoneally injected with 200 ng of pertussis toxin (Sigma, St. Louis, MO) at the day of immunization and 48 h later. Disease development was analyzed daily and scored on a 0-5 scale: 0, no clinical signs; 1, limp tail, 2, hind limb weakness, 3, hind limb paralysis, 4, hind limb paralysis and front limb weakness, 5, moribund or death.

2.3. Osmotic pump implantation

Mice anesthetized with Ketamine and Xylazine were subcutaneously implanted with a mini-osmotic pump (#1007D, Alzet Durect, Cupertino, CA) as previously described (11). This pump system has a reservoir volume of 100 µl and allows for the continuous delivery of the content for 7 days without the need for external connections or frequent handling of animals. Pumps containing 400 ng of rIL-27 or rIL-30 (R&D Systems, Minneapolis, MN) were implanted at day 12 post immunization. Mice with sham surgery were used as controls.

2.4. Flow Cytometry

Mononuclear cells from the CNS of EAE mice were isolated by Percoll gradient centrifugation as previously described (ref). The cells were then stained with anti-CD4 (RM4–5), anti-CD44 (IM7), anti-CD25 (PC61.5), anti-GITR (DTA-1) and anti-ICOS (C398.4A) antibodies. For intracellular staining, harvested cells were stimulated ex vivo with PMA (10ng/mL, Millipore-Sigma) and ionomycin (1 µM, Millipore-Sigma) for 4 h in the presence of 2 µM monensin (Calbiochem) during the last 2 h of stimulation. Cells were immediately fixed with 4% paraformaldehyde, permeabilized, and stained with anti-IL-17 (TC11-18H10), anti-IFNγ (XMG1.2), anti-TNFα (TN3-19), anti-GM-CSF (MP1-22E9) antibodies. All the antibodies were purchased from eBioscience (San Diego, CA), BD PharMingen (San Diego, CA), and Biolegend (San Diego, CA). Samples were acquired using a FACSCelesta (BD Bioscience) and analyzed using a FlowJo (Treestar, Ashland, OR). CNS APC subsets were sorted based on CD45 and CD11b expression using a FACSMelody cell sorter (BD Bioscience). Sorted cells were subjected to gene expression by qPCR as described below.

2.5. Cytometric Beads Array

Serum cytokines were determined using Cytometric Beads Array (BD Biosciences) according to the manufacturer's instructions. The data were analyzed using the CBA software, and the standard curve for each cytokine was generated using the mixed cytokine standard.

2.6. Real-Time Quantitative PCR

Mice with EAE were euthanized and perfused with PBS. The brain and spinal cords were isolated and total RNA was extracted using a TRIzol reagent according to the manufacturer’s instructions (Invitrogen). cDNA was then obtained using a MMLV reverse transcriptase (Promega, location). qPCR analysis was performed using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Waltham, MA) using a Radiant qPCR mastermix (Alkali Scientific, Fort Lauderdale, FL) or SYBR green mastermix (Applied Biosystems). The data were normalized by housekeeping *Gapdh* gene and then compared to the control group. Primers used for the study are listed in Supplementary Table 1.

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165 **2.7. Statistical Analysis**

166 Statistical significance was determined by the Mann-Whitney test using Prism software

167 (GraphPad, San Diego, CA). $p<0.05$ was considered statistically significant.

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3. Results

3.1. Il27p28 mRNA expression in EAE

We first performed time course analysis to measure cytokine gene expression within the inflamed brain and spinal cords during EAE. Three time points were chosen: days 8, 14, and 21 post induction, representing the disease onset, peak, and remission, respectively (Fig 1A). *Il27p28* mRNA expression mirrored the disease activity, and it peaked at day 14, showing >10-fold increase in both tissue sites compared to those of naïve mice (Fig 1B). The *Ebi3* subunit mRNA expression displayed similar pattern as the *Il27p28* (Fig 1B). *Il12p40* mRNA expression in the spinal cord was markedly increased with the similar kinetics, reaching ~100-fold increase over naïve tissue, although its expression in the brain was not observed (Fig 1B). On the other hand, *Il12p35* or *Il23p19* mRNA expression only slightly increased (Fig 1B and data not shown). Expression of inflammatory cytokines, namely, *Tnfa*, *Ifng*, *Il17a*, *Il1b,* and *Il6,* as well as of key transcription factors, *Tbx21* and *Rorc*, followed the similar pattern (Fig 1C and 1D). Foxp3 mRNA expression substantially increased at the peak of the disease and thereafter, demonstrating Treg accumulation in the tissue (24). We also measured inflammatory chemokines, including *Ccl2*, *Ccl3*, *Ccl7*, *Cxcl1*, *Cxcl9*, and *Cxcl10* mRNA expression. The expression pattern showed similar kinetics, with the greater magnitude in the spinal cord (Supp Fig 1). Myeloid cells capable of presenting antigens, including macrophages and dendritic cells, are the primary source of IL-12 family cytokines including IL-27. To examine relative sources of each cytokine during autoimmune inflammation in the CNS, we FACS sorted different APC subsets from the inflamed CNS tissues; $CD45^{high}$ $CD11b^{high}$ infiltrating myeloid cells, $CD45^{int}$ $CD11b^{high}$ microglia, and $CD45^{low}$ cells including astrocytes and oligodendrocytes, at the peak of the disease, and cytokine gene expression was measured. While both infiltrating myeloid cells and microglia similarly expressed all the tested IL-12 family cytokines, the level of *Il27p28* and *Ebi3* mRNA expression was particularly greater than any other subunits tested (Fig 1E). These results prompted us to investigate the central source of IL-27, especially IL-27p28 subunit which has been considered an IL-27-specific subunit (1), during the development of autoimmune neuroinflammation.

3.2. DC-derived Il27p28 is dispensable.

IL-27 produced by DCs promotes the generation of IL-10^{+} CD4 T cells capable of attenuating autoimmune inflammation (25). Utilizing the IL-27p28-floxed mouse model, it was reported that DC-derived IL-27 plays a role in antitumor immunity by regulating NK and NKT cell recruitment and activation (23, 26). To test the role of DC-derived IL-27 in EAE, DC-specific IL-27p28$^{-/-}$ (*Itgax*^{Cre} *Il27p28*$^{fl/fl}$) mice were induced for EAE. qPCR analysis validated DC-specific IL-27p28 deficiency in these mice (Supp. Fig 2). Despite the involvement of DC-derived IL-27 in EAE, we found that DC-specific IL-27p28 deletion did not affect EAE pathogenesis, as both disease onset and the clinical severity remained analogous to those of wild type control mice (Fig 2A). CD4 T cells infiltrating the CNS tissues were similar in both proportions and absolute numbers (Fig 2B). Likewise, Foxp3^{+} Treg cell accumulation in the CNS was also comparable (Fig 2C). Treg cell expression of surface markers associated with the suppressive functions, such as ICOS, GITR, and CD25, remained unchanged regardless of DC-derived IL-27p28 (Fig 2D). We then measured CD4 T cell expression of encephalitogenic cytokines by flow cytometry. As shown in Fig 2E, intracellular expression of GM-CSF, IFNγ, IL-17, and TNFα in CNS infiltrating CD4 T cells was similar in both proportions and absolute numbers (Fig 2E). Furthermore, expression of the *Ifng*, *Il17a*, and *Il1b* mRNA in the CNS tissues was also similar between WT and DC-specific IL-27p28$^{-/-}$ groups (Fig 2F). Consistent with cytokine expression, serum cytokine levels measured by CBA analysis were comparable between the groups (Fig 2G). Inflammatory chemokine and IL-12 family cytokine expression in the tissues was found similar between the groups (Supp Fig 3A and 3B). Lastly, CNS APC subsets were FACS sorted as above, and IL-12 family cytokine gene expression was determined. Consistent with the EAE severity and overall immune responses, we found no differences in cytokine gene expression between the groups (Fig 2H). Taken together, these results demonstrate that DC-derived IL-27p28 plays little role in regulating encephalitogenic immune responses.

3.3. Myeloid cell-derived IL-27p28 regulates encephalitogenic immune responses

Cells of myeloid origin, especially monocytes and macrophages, are another key source of the IL-27p28 subunit (1, 27). To test the role of myeloid cell-derived IL-27 in EAE

pathogenesis, we utilized myeloid cell-specific IL-27p28$^{-/-}$ (*Lyz2*^{Cre} *Il27p28*$^{fl/fl}$) mice. The lack of *Il27p28* mRNA expression in macrophages in *Lyz2*^{Cre} *Il27p28*$^{fl/fl}$ mice validated myeloid cell-specific IL-27p28 deficiency (Supp Fig 2). Unlike DC-specific IL-27p28$^{-/-}$ mice shown above, myeloid cell-specific IL-27p28$^{-/-}$ mice rapidly developed severe EAE (Fig 3A), which was further reflected by increased CD4 T cells infiltrating the CNS (Fig 3B). We also noted that the accumulation of Foxp3^{+} Treg cells was significantly greater in these mice, although Treg cell proportion or Foxp3 expression were comparable (Fig 3C). Treg cell expression of ICOS, GITR, and CD25 remained unchanged regardless of myeloid cell-derived IL-27p28 (Fig 3D). CD4 T cells expressing inflammatory cytokines were substantially increased in the CNS tissues of *Lyz2*^{Cre} *Il27p28*$^{fl/fl}$ mice (Fig 3E and 3F), consistent with severe EAE phenotypes in these mice. In support, we found that serum cytokine levels, especially IL-2, IL-6, IFNγ, IL-17A, and TNFα, but not IL-4, were markedly increased in myeloid cell specific IL-27p28$^{-/-}$ mice (Fig 3G). Inflammatory chemokine expression in the CNS tissues was similarly elevated in mice with myeloid cell specific IL-27p28 deletion (Supp Fig 4). Moreover, CNS expression of IL-27p28 and IL-12p40 subunits was substantially greater in the absence of myeloid cell-derived IL-27p28 (Fig 3H). IL-23p19 subunit expression was slightly increased in the KO mice, while IL-12p35 expression was similar between the groups (Fig 3H). We further measured cytokine gene expression in FACS sorted CNS APC subsets and found that *Il27p28* mRNA expression was dramatically increased in microglia and CD45^{low} cells in the absence of myeloid cell-derived IL-27p28 (Fig 3I). Of note, *Il27p28* mRNA expression in infiltrating monocytes was absent in *Lyz2*^{Cre} *Il27p28*$^{fl/fl}$ mice, validating IL-27p28 deficiency of myeloid cells (Fig 3I). Although *Il12p40* and *Ebi3* mRNA expression was also increased in microglia and CD45^{low} cells (Fig 3I), relative expression of *Il27p28* mRNA was greater than other subunits. Therefore, these results demonstrate that IL-27p28 produced by myeloid lineage cells may play a key regulatory role in EAE.

3.4. Microglia expression of IL-27p28 plays a similar regulatory function in EAE

Microglia are the resident CNS glial cells capable of producing IL-27 (28, 29). While *Lyz2*^{Cre}-mediated gene targeting can occur in microglia as well (30), it is important to test whether microglia expression of IL-27p28 alone is sufficient for the immune

regulatory function. To target microglia expression of IL-27p28, we crossed *Il27p28*$^{fl/f}$ mice with *Cx3cr1*^{Cre} mice (31). Interestingly, microglia specific IL-27p28$^{-/-}$ mice also exhibited severe EAE analogous to myeloid cell specific IL-27p28$^{-/-}$ mice (Fig 4A). CNS infiltration of total CD4 and Foxp3^{+} Treg cells was significantly increased in these mice, supporting severe EAE phenotypes (Fig 4B and 4C). Treg cell associated surface marker and Foxp3 expression remained unchanged (Fig 4C and 4D). CNS infiltrating CD4 T cell expression of inflammatory cytokines was markedly increased in microglia-specific IL-27p28$^{-/-}$ mice, further supporting greater susceptibility of these mice (Fig 4E). We measured inflammatory cytokine and chemokine gene expression by qPCR and confirmed that microglia-specific IL-27p28 deficiency results in greater increase of the expression (Fig 4F and Supp Fig 5). Analogous to other mouse models tested above, *Il27p28* mRNA expression in the CNS of microglia-specific IL-27p28$^{-/-}$ mice was significantly greater than that of wild type mice (data not shown). Also observed is that *Il27p28* and *Il12p40* mRNA expression in CNS APC subsets, namely infiltrating monocytes and CD45^{low} cells, was significantly increased in microglia-specific IL-27p28$^{-/-}$ mice (Fig 4G). *Ebi3* mRNA expression was also observed in infiltrating monocytes and CD45^{low} cells but not in microglia (Fig 4G). The lack of *Il27p28* mRNA expression in sorted microglia further validated microglia-specific IL-27p28 deficiency (Fig 4G). Taken together, these results demonstrate that microglia expression of IL-27p28 may also play a regulatory role in EAE pathogenesis.

3.5. Astrocyte-derived IL-27p28 plays a regulatory role in EAE

IFNγ-dependent IL-27 secretion by astrocytes was previously shown to be important in EAE pathogenesis (32). Similarly, IL-27 expression is found in active multiple sclerosis plaques by astrocytes of MS patients (33). Therefore, we next examined if astrocyte-derived IL-27p28 plays a role in EAE pathogenesis. EAE was induced in astrocyte-specific IL-27p28$^{-/-}$ (*Gfap*^{Cre} *Il27p28*$^{fl/fl}$) mice. Analogous to other CNS APC-targeted models tested above, astrocyte specific deletion of IL-27p28 resulted in severe EAE (Fig 5A). Consistent with the severity, the accumulation of CD4 T cells and Treg cells in the CNS tissue was markedly increased (Fig 5B and 5C). As we have observed in earlier model systems, Treg cell expression of surface molecules and Foxp3 was

comparable regardless of astrocyte expression of IL-27p28 (Fig 5C and 5D). Likewise, T cell expression of inflammatory cytokines was also significantly increased in astrocyte-specific IL-27p28$^{-/-}$ mice (Fig 5E). Serum cytokine levels were similarly elevated in those mutant mice, especially IL-2, IFNγ, TNFα, and IL-17A (Fig 5F). CNS expression of inflammatory cytokine and chemokine mRNAs was elevated in astrocyte-specific IL-27p28$^{-/-}$ mice (Fig 5G and Supp Fig 6). Analogous to myeloid cell- and microglia-specific IL-27p28$^{-/-}$ mice, expression of the *Il27p28* mRNA in CNS APC subsets, both infiltrating monocytes and microglia, was drastically increased in astrocyte-specific IL-27p28$^{-/-}$ mice (Fig 5H). *Il12p40* and *Ebi3* mRNA expression was also increased in the absence of astrocyte-derived IL-27p28; however, the extent of the increase was lower than that of *Il27p28* (Fig 5H). Therefore, like other CNS-infiltrating monocytes or resident microglia, astrocyte-derived IL-27p28 appears to be able to limit encephalitogenic immunity.

3.6. In vivo administration of IL-30 exacerbates encephalitogenic inflammation

Although the results that IL-27p28 deficiency in one of the inflammatory or CNS resident APC subsets results in exacerbated autoimmune inflammation support the IL-27's ability to modulate inflammation, we were especially intrigued by the unexpected observations that IL-27p28 expression in the remaining CNS APC subsets was dysregulated in these conditions. Notably, IL-27p28, also known as IL-30, can be secreted independent of the Ebi3 subunit and may function as a natural antagonist of gp130-mediated signaling (34). Indeed, it was previously reported that the secreted IL-27p28 subunit inhibits the biological functions of IL-27 (22). Therefore, IL-27p28 subunits abundantly present in those conditions may antagonize anti-inflammatory roles of IL-27, driving encephalitogenic inflammation. To test this possibility, we induced EAE in B6 mice and then administered recombinant IL-27 or IL-30 (IL-27p28) via a mini-osmotic pump once the mice develop noticeable clinical score. As shown in Fig 6A, IL-27 administered substantially dampened the EAE severity, as previously reported (35). We found that IL-30 administered exacerbated the clinical severity of the recipient mice (Fig 6A). In support of the disease severity, CD4 T cells infiltrating the CNS were diminished by IL-27 administration, while IL-30 administration substantially increased the infiltration (Fig

6B). Consistent with T cell infiltration, Treg cell accumulation in the CNS also increased in IL-30 treated mice (Fig 6C). Treg cell expression of Foxp3 and other surface markers remained unchanged by IL-27 or IL-30 treatment (Fig 6C and 6D). Inflammatory cytokine expression by infiltrating CD4 T cells was similarly affected by IL-27 and IL-30 administered in vivo (Fig 6E). TNFα-, IL-17-, GM-CSF-, and IFNγ-expressing CD4 T cell accumulation in the CNS was markedly diminished by IL-27. By contrast, the accumulation was dramatically increased following IL-30 administration (Fig 6E). Inflammatory chemokine mRNA expression in the CNS followed similar pattern and was significantly diminished by IL-27 but increased by IL-30 (Supp Fig 7). Interestingly, *Il27p28* mRNA expression itself was decreased by IL-27 and increased by IL-30 administered (Fig 6F), which is likely attributed to the degree of inflammation. Therefore, these results suggest that IL-30 appears to express pro-inflammatory functions to promote encephalitogenic immune responses.

3.7. IL-30 antagonizes IL-27 functions in vivo but not in vitro

Unlike IL-27, IL-30 fails to support T cell proliferation or IFNγ production (36). However, IL-30 can inhibit the production of IL-17 and IL-10 triggered by IL-27 or IL-6 stimulation in activated T cells (34, 37). It was reported that IL-30 also induces LPS-induced TNFα and IP-10 production in monocytes (38). Since we observed increased *Il27p28* mRNA expression in conditional knockout mice tested above, we revisited whether increased IL-30 may directly or indirectly modulate T cell cytokine expression. Naïve CD4 and CD8 T cells were stimulated in the presence of recombinant IL-27 or IL-30. As expected, IL-27 rapidly phosphorylated both Stat1 and Stat3 in CD4 and CD8 T cells (Fig 7A and data not shown). On the other hand, IL-30 stimulation had little effects on Stat phosphorylation (Fig 7A). The lack of Stat phosphorylation by IL-30 led us to further test its ability to induce IFNγ or IL-10 expression in activated T cells. Indeed, CD4 T cells stimulated under Th1 or Th17 polarization condition substantially upregulated *Ifng* mRNA in response to IL-27 but not to IL-30 (Fig 7B and data not shown). Likewise, IL-27 induced robust *Il10* mRNA expression in both developing Th1 and Th17 type CD4 T cells, while IL-30 failed to do so (Fig 7C and data not shown). Pre-stimulation with IL-27 effectively inhibited IL-27-induced Stat phosphorylation, whereas IL-30 pre-stimulation

had no ability to interfere with IL-27-induced Stat phosphorylation (Fig 7D). Therefore, IL-30 alone does not appear to alter cytokine expression in activated T cells in vitro. We previously showed that IL-27 stimulation upregulates Lag3 expression in CNS infiltrating Treg cells in vivo (11). We thus compared Treg cell expression of Lag3 in mice administered with IL-27 or IL-30 in the system described in Fig 6. We found that IL-27 significantly increased Lag3 expression in Treg cells as expected (Fig 7E). On the other hand, IL-30 treatment significantly downregulated the expression in Treg cells (Fig 7E), suggesting that IL-30 administered may antagonize Lag3 expression possibly induced by endogenous IL-27 in vivo. To further confirm if endogenously produced IL-30 also antagonizes IL-27 in vivo, we decided to measure Lag3 expression in CNS infiltrating Treg cells in the aforementioned models, where IL-30 expression is enhanced. We compared Treg cell expression of Lag3 in microglia-specific IL-27p28$^{-/-}$ mice where IL-27p28 expression of infiltrating monocytes and CD45^{low} cells is drastically increased. Indeed, Treg cell expression of Lag3 was significantly downregulated in microglia-specific IL-27p28$^{-/-}$ mice compared to that in control mice, suggesting that abundant IL-30 may antagonize IL-27's function to upregulate Lag3 expression in Treg cells in vivo.

4. Discussion

In this study, we took advantage of various cell type specific IL-27p28 (IL-30)-deficient mouse models to identify the key sources of IL-30 to gain insights into the immune regulatory functions of IL-27. Consistent with the previous studies, we found that myeloid cell (i.e., CNS infiltrating monocytes)-derived IL-30 is critical to limit autoimmune inflammation in the CNS, whereas DC-derived IL-30 was unexpectedly dispensable. Other CNS resident APC subsets, including microglia and astrocytes, which have also been shown capable of producing IL-30, were equally important sources of IL-30 in modulating inflammatory responses within the CNS. Unexpectedly, in mice deficient in IL-30 in one of the APC subsets (i.e., myeloid cells, microglia, or astrocytes) that develop severe EAE, we observed markedly elevated *Il27p28* mRNA expression in the remaining cellular sources of IL-30 within the CNS. For example, when IL-30 is absent in microglia, *Il27p28* expression in infiltrating monocytes or in $CD45^{low}$ CNS cells (including astrocytes) was significantly increased. Such dysregulation did not occur when DCs lack IL-30, where the disease severity remained unchanged. Moreover, the extent of *Il27p28* upregulation is substantially greater than that of *Ebi3*, suggesting a disproportionate expression of IL-30. We thus posit that escalated IL-30 expression may be related to exacerbated disease. In support, systemic administration of recombinant IL-30 caused severe EAE development. Therefore, IL-30 may express pro-inflammatory property exacerbating the encephalitogenic immune responses.

IL-30 was initially thought as an IL-27-specific subunit, and mice deficient in or overexpressing the IL-30 (*Il27p28*) gene have been used to investigate the immune regulatory functions of IL-27. Mice overexpressing IL-30 are resistant to autoimmune inflammation and such resistance appears to be originated from IL-30's ability to antagonize inflammatory T cell responses, particularly Th1 and Th17 immunity (18). Likewise, mice deficient in IL-30 are highly susceptible to EAE and express heightened Th17 immunity (5). Therefore, it was naturally concluded that IL-30's ability to modulate inflammatory responses is through its role as an IL-27 subunit.

It was then discovered that IL-30 can be secreted in the absence of the Ebi3 subunit and modulate immune responses (34). Tagawa and colleagues reported that IL-30 alone can mediate immune regulatory functions, suppressing allogenic T cell responses (22). More recently, Park et al. reported that IL-30 can act as a negative regulator of both B and T cell responses during *T. gondii* infection, independent of IL-27 (19). The mechanism by which IL-30 exerts immune regulatory functions has been examined. IL-30 was shown to antagonize other cytokines, such as IL-27 or IL-6 that utilizes the gp130 for the signaling (34). Although IL-30 stimulation itself does not trigger any detectable Stat phosphorylation, its presence was sufficient to hinder Stat1 and Stat3 phosphorylation induced by IL-27 or IL-6 (34). Consistent with these results, we made similar observation that IL-30 stimulation does not induce Stat phosphorylation in T cells. Unexpectedly, however, we also found that IL-30 does not interfere with IL-27-induced Stat1/3 phosphorylation nor IL-27-stimulated gene expression in vitro. The reason underlying the discrepancy is not clear. Concentrations used may account for the difference, as the previous study used higher IL-30 concentration. Indeed, IL-30 may be able to signal through gp130 homodimers without soluble IL-6Rα only at high concentrations (39). Alternatively, IL-30 may be able to enhance APC functions, as *Il12p40* mRNA expression was similarly increased in those CNS infiltrating and resident APCs when *Il27p28* expression was higher. Therefore, IL-30-mediated control of APC functions may impact T cell activation and the subsequent inflammatory responses.

Finding dysregulated *Il27p28* mRNA expression in one of the cell type specific IL-27p28$^{-/-}$ mice with severe disease is of particular interest. What does trigger such dysregulated expression? IFNγ has previously been reported to induce IL-27p28 expression in myeloid cells (40), and we did find elevated IFNγ expression in infiltrating CD4 T cells. IL-30 expression may be directly correlated with the disease severity and inflammation. In support, disturbed *Il27p28* mRNA expression was observed in all the conditional knockout mice with exacerbated disease (IL-30 targeted in myeloid cell, microglia, and astrocytes but not in DCs). Notably, *Ebi3* mRNA expression was also increased when IL-30 expression increased. More interestingly, Ebi3 upregulation was not found in *Il27p28*$^{-/-}$ cells, suggesting that IL-30 expression may be linked to that of

Ebi3. However, the magnitude of Ebi3 upregulation was substantially lower than that of IL-30. Therefore, disproportionate expression of *Il27p28* mRNA may result in excessive presence of IL-30, which may then be capable of antagonizing IL-27's function to inhibit the inflammation. We previously demonstrated that IL-27 signaling in Treg cells is critical for Treg control of inflammation and that Lag3 expression is one of the key downstream molecules crucial for proper Treg cell functions (41). From the cytokine administration experiments performed, we confirmed that Treg cell expression of Lag3 seems directly modulated by IL-27 and IL-30. In particular, IL-30 significantly dampens Lag3 expression in Treg cells, suggesting that it may interfere with Lag3 expression possibly induced by endogenous IL-27. Moreover, Treg cell Lag3 expression was significantly diminished in microglia-specific IL-27p28$^{-/-}$ mice, in which IL-27p28 expression of CNS APC subsets was dysregulated. Although we found no evidence that IL-30 alone induces Stat phosphorylation and gene expression in T cells in vitro, our findings suggests that IL-30 may modulate inflammatory responses in vivo, in part, based on its ability to antagonize IL-27. Expression of other Treg cell markers, ICOS, GITR, CD25, and Foxp3 remained unchanged during IL-27 or IL-30 treatment. Lag3 expression in Treg cells is critical in mediating Treg cell control of inflammatory responses (11, 42). Therefore, IL-30-induced effects may be in part mediated via Treg cells, although we cannot totally exclude the possibility that IL-30 itself poses other immune regulatory functions independent of Ebi3. For example, IL-30 increases TNFα and IP-10 expression in monocytes (38). Alternatively, IL-30 may form a complex with other subunits, such as cytokine-like factor 1 (CLF) or soluble IL-6Rα (43). Whether these complexes mediate differential functions remains to be determined. Neutralizing or blocking antibody against IL-30 will affect both IL-30 and IL-27; therefore, it is not appropriate to test these possibilities.

DCs, especially XCR1^{+} cDC1 type subsets, express IL-30 when immunized with a combination adjuvant, poly IC and agonistic anti-CD40 Ab (44). Our finding that DC-derived IL-30 plays little role in limiting EAE pathogenesis and encephalitogenic immune responses suggests that DCs may not be the primary source of IL-30 during autoimmune inflammation in the CNS. DC-derived IL-30 may be important in the

secondary lymphoid tissues during priming event as seen in the spleen following intravenous immunization with adjuvants (44), and IL-30-derived from inflammatory monocytes/macrophages or tissue resident APC subsets may be more crucial in limiting immune responses in the target tissues.

In summary, the current study reports that IL-30 expresses a pro-inflammatory property in chronic autoimmune inflammation which could partially be mediated by interfering regulatory features of IL-27 in the absence of IL-30's ability to directly stimulate T cells.

Ethics Statement

The animal study was reviewed and approved by Northwestern University IACUC.

Author Contributions

DK designed and performed most of the experiments, analyzed the data, and wrote the manuscript. SK helped with experiments. ZY provided key reagents. BM designed the experiments, analyzed the data, and wrote the manuscript.

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Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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641 **Figure Legends**

642 **Figure 1. Cytokine gene expression in the CNS during EAE**

643 (A-D) EAE was induced in C57BL/6 mice as described in the Methods. RNA was
644 isolated from the brain and spinal cords at disease on set (day 8 post immunization),
645 acute phase (day 14 post immunization) and remission phase (day 21 post
646 immunization). n = 3 per group. (A) EAE severity. (B-D) mRNA expression of IL-12
647 family genes, cytokines and transcription factors were measured by qRT-PCR. Data
648 were normalized by *Gapdh* gene expression and compared to that of naive mice. (E)
649 $CD45^{high}$ $CD11b^{high}$ (infiltrating monocyte), $CD45^{int}$ $CD11b^{high}$ (microglia) and $CD45^{low}$
650 (including astrocyte and oligodendrocyte) cells sorted from the CNS at the peak of
651 disease (day 17 post immunization) and expression of the indicated genes was
652 measured by qPCR. Gene expression was normalized by *Gapdh* and compared to that
653 of naïve mice. *p < 0.05; **p < 0.01; ***p < 0.001; as determined by Mann-Whitney
654 nonparametric test.

655

656 **Figure 2. EAE in DC-specific IL27p28$^{-/-}$ mice**.

657 $CD11c^{WT}$ (n = 9) and $CD11c^{\Delta Il27p28}$ (n = 10) were induced for EAE. (A) Time course of
658 the development of EAE. (B-D) The numbers of CNS-infiltrating $CD4^+$ and $CD4^+Foxp3^+$
659 Treg cells, and the mean fluorescence intensity (MFI) of Foxp3, CD44, ICOS, GITR and
660 CD25 were determined by flow cytometry at day 17 post immunization. (E) Flow
661 cytometry analysis of GM-CSF, IFN-γ, IL-17, and TNFα $CD4^+$ T cells from the CNS of
662 EAE mice (day 17 post immunization). (F) RNAs isolated from the brain and spinal
663 cords at day 17 post immunization were analyzed for the expression of *Ifng, Il17, Il1b,*
664 *Il27p28, Il12p40, Il23p19* and *Il12p35*. n = 3 per group. Gene expression was
665 normalized by *Gapdh* and compared to that of naïve mice. (G) The levels of IL-2, IL-4,
666 IL-6, IFNγ, TNFα and IL-17A in the serum of EAE mice (day 17 post immunization) were
667 measured using the Cytometric Bead Array. Each serum sample was analyzed in
668 duplicates. (H) qPCR analysis of the indicated mRNAs in freshly sorted $CD45^{high}$
669 $CD11b^{high}$ (infiltrating monocyte), $CD45^{int}$ $CD11b^{high}$ (microglia) and $CD45^{low}$ (astrocyte
670 and oligodendrocyte) cells from $CD11c^{WT}$ and $CD11c^{\Delta Il27p28}$ mice with EAE. *p < 0.05;
671 **p < 0.01; ***p < 0.001; as determined by Mann-Whitney nonparametric test.

Figure 3. EAE in LysM$^{\Delta Il27p28}$ mice.

LysM^{WT} (n = 7) and LysM$^{\Delta Il27p28}$ (n = 11) mice were induced for EAE. (A) EAE clinical score. (B-D) The numbers of CNS infiltrating CD4$^+$ and CD4$^+$Foxp3$^+$ Treg cells, and the mean fluorescence intensity (MFI) of Foxp3, CD44, ICOS, GITR and CD25 were determined by flow cytometry at day 17 post immunization. (E) Flow cytometry analysis of GM-CSF, IFNγ, IL-17, and TNFα CD4$^+$ T cells from the CNS of EAE mice (day 17 post immunization). (F, H) qPCR analysis of the indicated mRNAs in the brain and spinal cords from naïve, LysM^{WT}, and LysM$^{\Delta Il27p28}$ mice 17 days post immunization. Gene expression was normalized by *Gapdh* and compared to that of naïve mice. n = 3 per group. (G) The levels of IL-2, IL-4, IL-6, IFNγ, TNFα and IL-17A in the serum of EAE mice (day 17 post immunization) were measured using Cytometric Bead Array. Each serum sample was analyzed in duplicates. (I) qPCR analysis of the indicated mRNAs in freshly sorted CD45^{high} CD11b^{high} (infiltrating monocyte), CD45^{int} CD11b^{high} (microglia) and CD45^{low} (astrocyte and oligodendrocyte) cells from LysM^{WT} or LysM$^{\Delta Il27p28}$ mice 17 days post immunization. n = 3 per group. *p < 0.05; **p < 0.01; ***p < 0.001; as determined by Mann-Whitney nonparametric test.

Figure 4. EAE in *Cx3cr1*$^{\Delta Il27p28}$ mice.

Cx3cr1^{WT} (n = 9) and *Cx3cr1*$^{\Delta Il27p28}$ (n = 10) were induced for EAE. (A) EAE clinical scores. (B-D) Total numbers of CNS-infiltrating CD4$^+$ and CD4$^+$Foxp3$^+$ Treg cells, and the mean fluorescence intensity (MFI) of Foxp3, CD44, ICOS, GITR and CD25 were determined by flow cytometry at day 17 post immunization. (E) Flow cytometry analysis of GM-CSF, IFNγ, IL-17, and TNFα CD4$^+$ T cells from the CNS of EAE mice (day 17 post immunization). (F) qPCR analysis of the indicated mRNAs in the brain and spinal cords from naïve, *Cx3cr1*^{WT}, and *Cx3cr1*$^{\Delta Il27p28}$ mice 17 days post immunization. Gene expression was normalized by *Gapdh* and compared to that of naïve mice. n = 3-5 per group. (G) qPCR analysis of the indicated mRNAs in freshly sorted CD45^{high} CD11b^{high} (infiltrating monocyte), CD45^{int} CD11b^{high} (microglia) and CD45^{low} (including astrocyte and oligodendrocyte) cells from *Cx3cr1*^{WT} or *Cx3cr1*$^{\Delta Il27p28}$ mice 17 days post

immunization. n = 3 per group. *p < 0.05; **p < 0.01; ***p < 0.001; as determined by Mann-Whitney nonparametric test.

Figure 5. EAE in *Gfap*$^{\Delta Il27p28}$ mice.

Gfap^{WT} (n = 9) and *Gfap*$^{\Delta Il27p28}$ (n = 12) mice were induced for EAE. (A) EAE clinical score. (B-D) Absolute cell number of CNS infiltrating CD4$^+$ and CD4$^+$Foxp3$^+$ cells, and the mean fluorescence intensity (MFI) of Foxp3, CD44, ICOS, GITR and CD25 were determined by flow cytometry at day 17 post immunization. (E) Flow cytometry analysis of GM-CSF, IFNγ, IL-17, and TNFα CD4$^+$ T cells from the CNS of EAE mice (day 17 post immunization). (F) The levels of IL-2, IL-4, IL-6, IFNγ, TNFα and IL-17A in the serum of EAE mice (day 17 post immunization) were measured using Cytometric Bead Array. Each serum sample was analyzed in duplicates. (G) qPCR analysis of indicated mRNAs in brain and spinal cords from naïve, *Gfap*^{WT}, *Gfap*$^{\Delta Il27p28}$ mice 17 days post immunization. Gene expression was normalized by *Gapdh* and compared to that of naïve mice. n = 3 per group. (H) qPCR analysis of the indicated mRNAs in freshly sorted CD45^{high} CD11b^{high} (infiltrating monocyte), CD45^{int} CD11b^{high} (microglia) and CD45^{low} (including astrocyte and oligodendrocyte) cells from *Gfap*^{WT} or *Gfap*$^{\Delta Il27p28}$ mice 17 days post immunization. n = 3 per group. *p < 0.05; **p < 0.01; ***p < 0.001; as determined by Mann-Whitney nonparametric test.

Figure 6. In vivo IL-30 administration develops significantly exacerbated EAE.

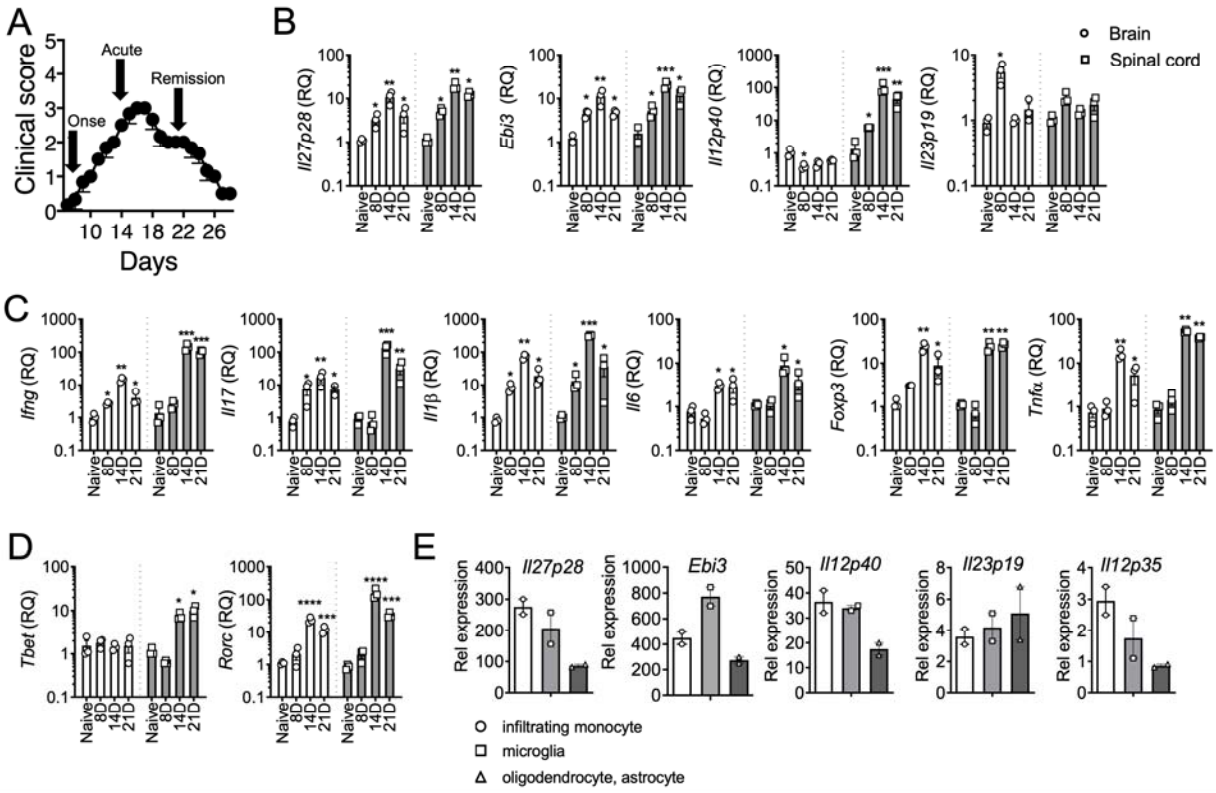
C57BL/6 mice were induced EAE. Osmotic pump containing IL-27 (400ng, n = 5), IL-30 (400ng, n = 5) were subcutaneously implanted or sham surgery (n = 4) was performed at 12 d post induction. (A) EAE score. (B) Total CD4$^+$ T cell numbers of the CNS at 22 d post induction. (C-D) Total CD4$^+$Foxp3$^+$ Treg cell numbers in the CNS and the mean fluorescence intensity (MFI) of Foxp3, CD44, ICOS, GITR, and CD25 were determined by flow cytometry at day 22 post immunization. (E) The numbers of GM-CSF, IFNγ, IL-17, and TNFα$^+$ CD4 T cells were determined by intracellular cytokine staining at day 22 post immunization. (F) qPCR analysis of the *Il27p28* and *Il12p40* mRNA expression in the brain from sham, IL-27-pump and IL-30-pump group. Gene expression was

normalized by *Gapdh* and compared to that of sham surgery group. N = 4-5 per group. *p < 0.05; **p < 0.01; ***p < 0.001; as determined by Mann-Whitney nonparametric test.

Figure 7. IL-30 stimulation in CD4 T cell activation in vitro.

(A) FACS sorted $CD4^+$ naïve cells were stimulated with recombinant IL-27, IL-30. Phosphorylated STAT1 and STAT3 expression was determined by flow cytometry at 10- and 30-minutes following stimulation. (B-C) Naive CD4 T cells were stimulated under Th1 polarization conditions in the presence of IL-27 or IL-30 (0-50 ng/ml) for 3 days. *Ifng and Il10* mRNA expression was determined by qPCR. (D) Naïve CD4 T cells were incubated with media (Nil), 50ng IL-27, or 50ng IL-30 for one hour. The cells were then washed and restimulated with IL-27. Stat1 and Stat3 phosphorylation was determined by flow cytometry at 15 and 30 minute following stimulation. The data shown are representative of two independent experiments. (E) C57BL/6 mice induced for EAE were administered with IL-27 or IL-30 via a mini-osmotic pump, and Lag3 expression of CNS infiltrating Treg cells was determined by flow cytometry as described in Fig 6. (F) $Cx3cr1^{WT}$ and $Cx3cr1^{\Delta Il27p28}$ mice induced for EAE as described in Fig 4 were used to measure CNS infiltrating Treg cell expression of Lag3.

750 Figure 1



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753 Figure 2

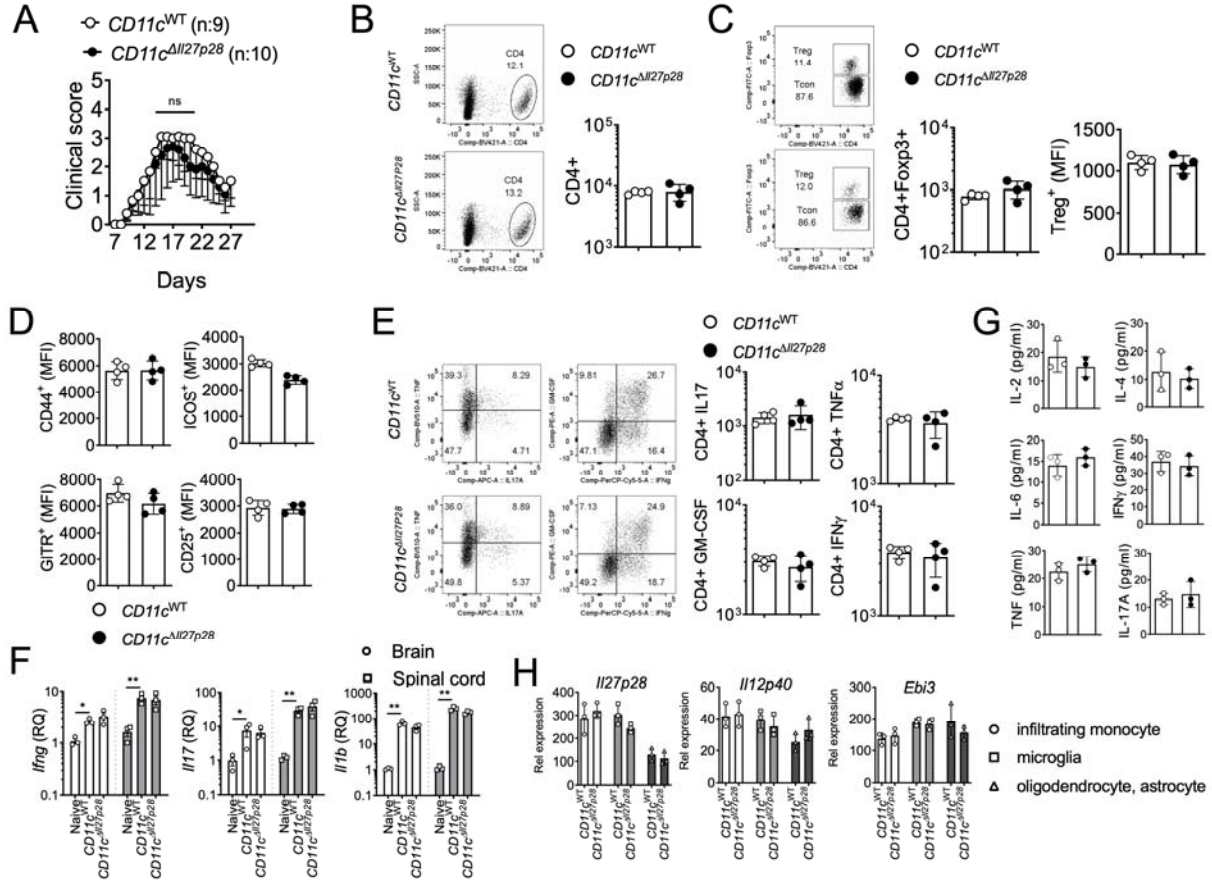

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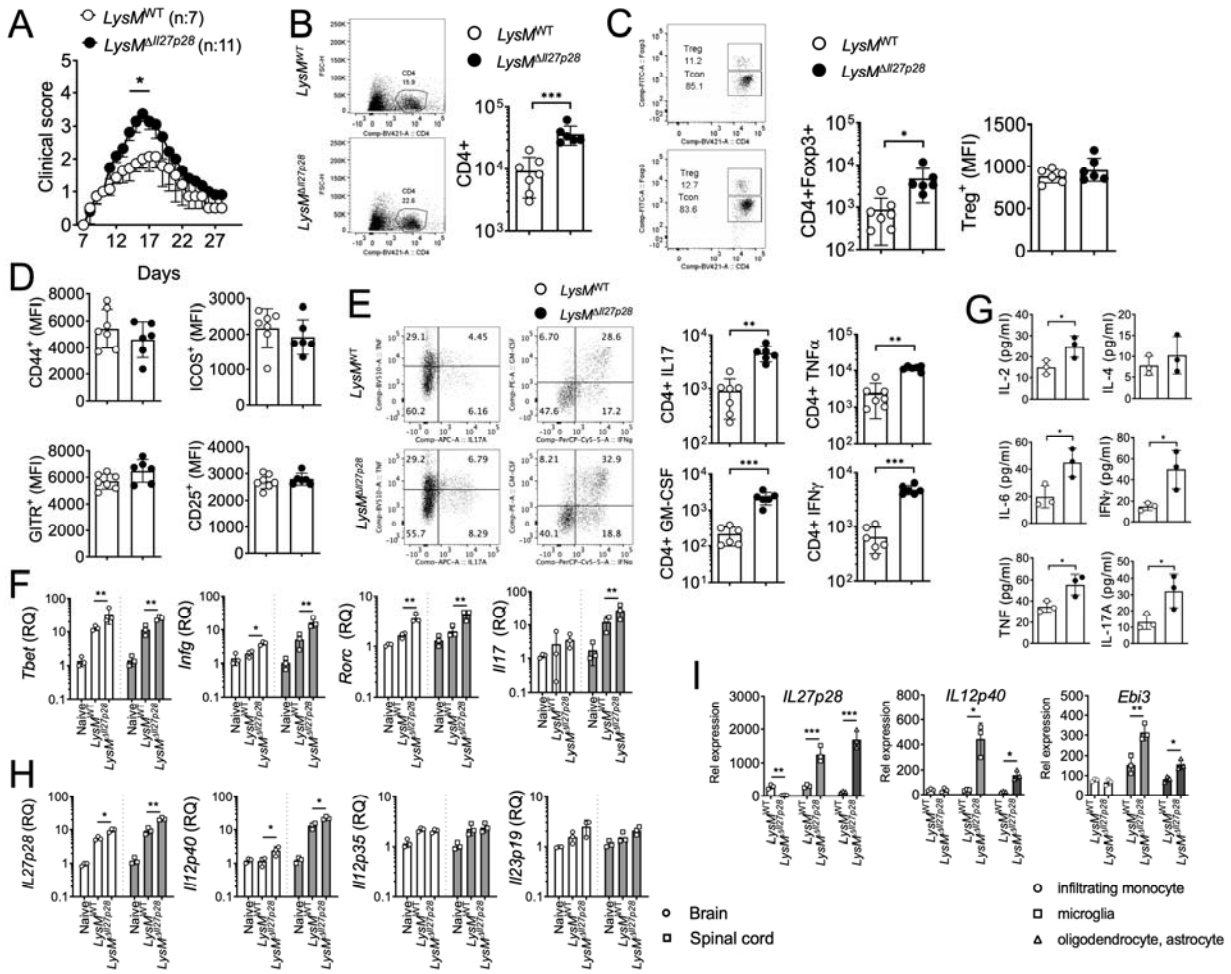
Figure 3



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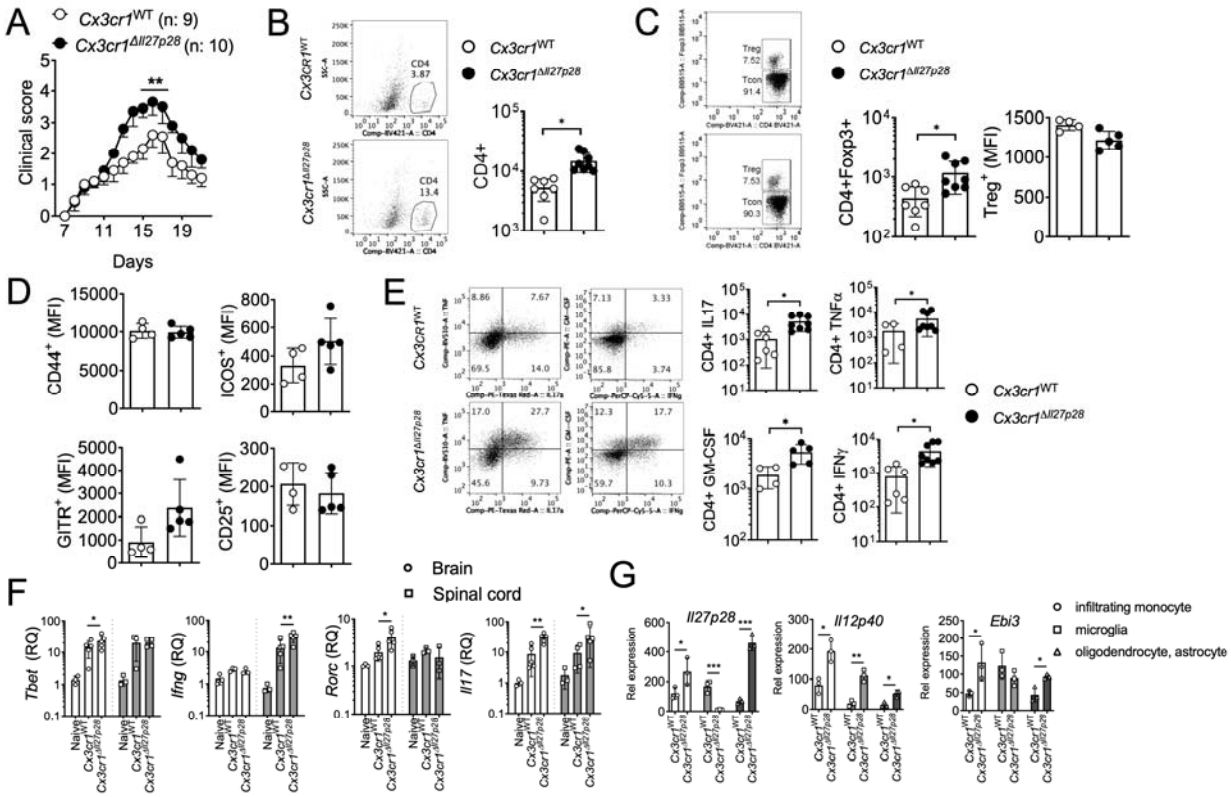

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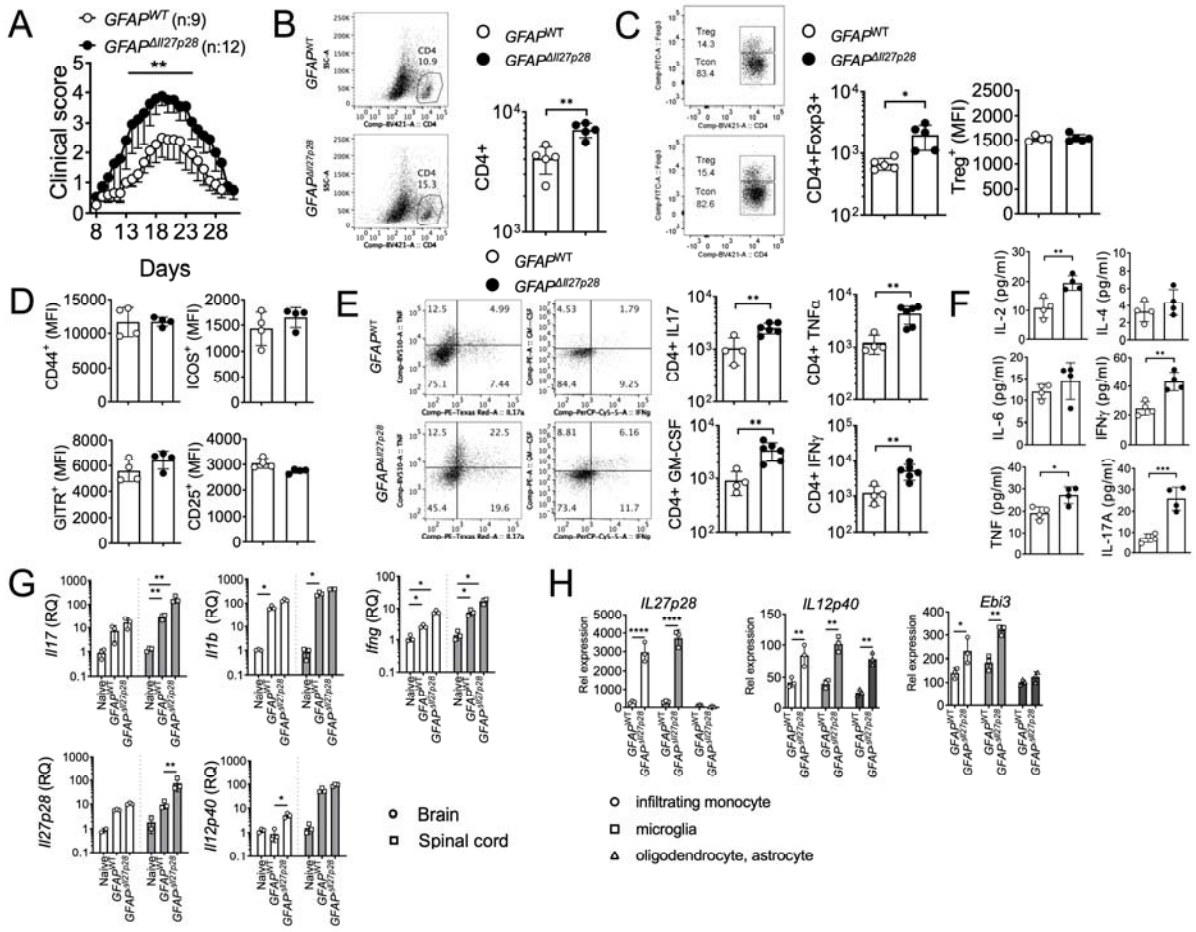
762 Figure 5



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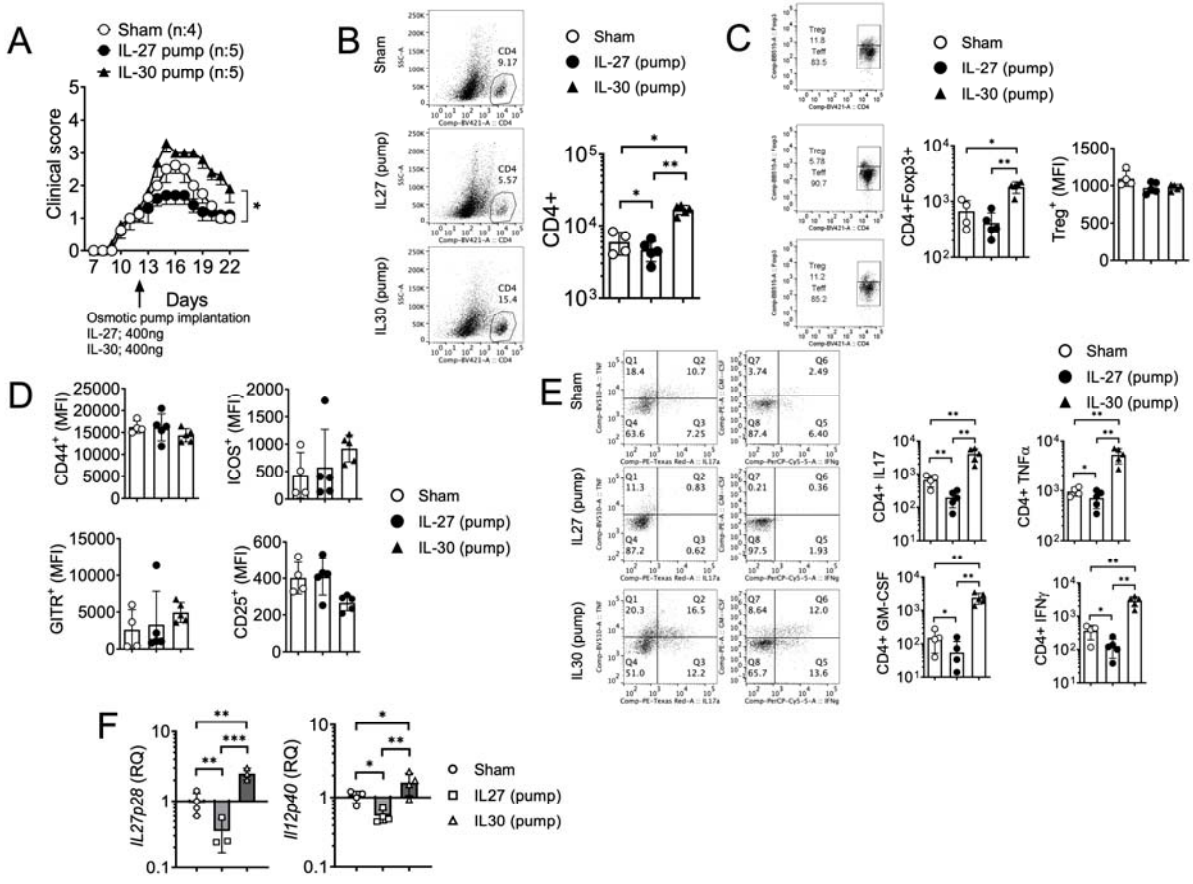
765 Figure 6



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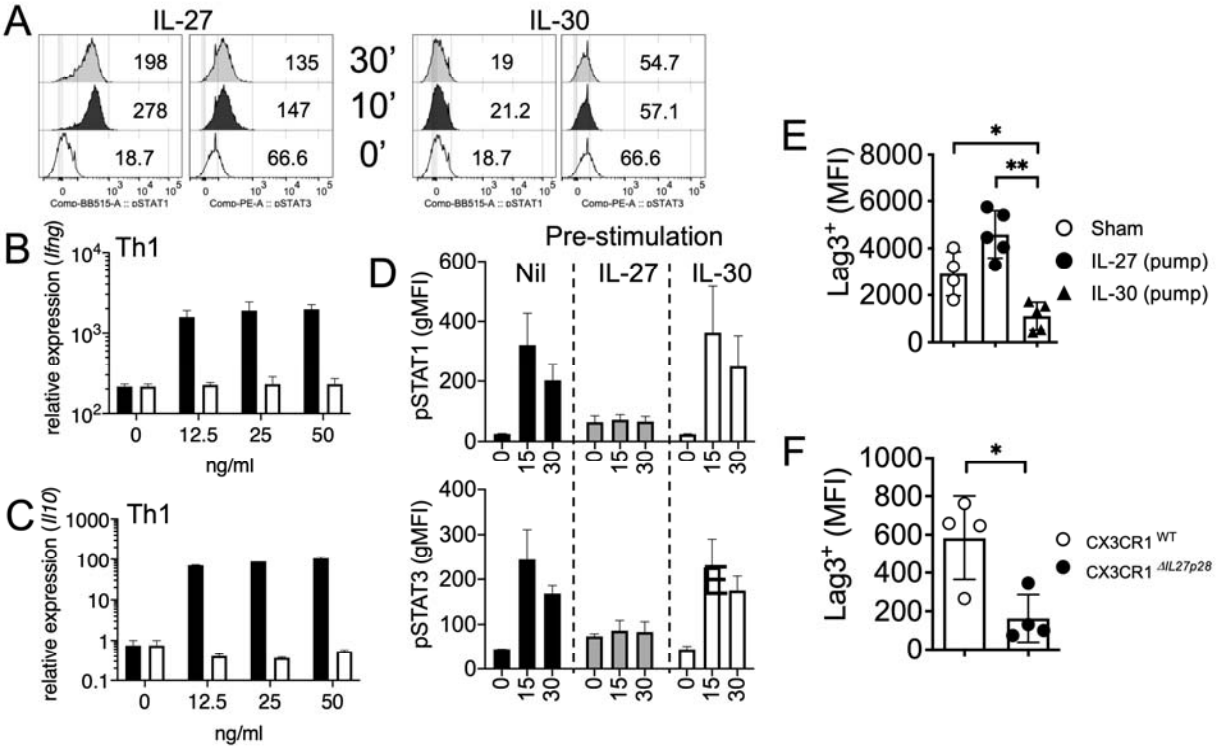
768 Figure 7



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