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Intrinsic STAT4 expression controls effector CD4 T cell migration and Th17 pathogenicity

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Abstract

Effector CD4 T cells are central to the development of autoimmune chronic inflammatory diseases, yet factors that mediate pathogenicity remain ill-defined. Single nucleotide polymorphisms in the human *STAT4* locus are associated with susceptibility to multiple autoimmune disorders and *Stat4* is linked to the pathogenic Th17 gene signature, however Th17 cells differentiate independently of STAT4. Hence, the interplay between STAT4 and CD4 T cell function, especially Th17 cells, during autoimmune disease is unclear. Herein, we demonstrate that CD4 T cell intrinsic STAT4 expression is essential for the induction of autoimmune CNS inflammation in mice, in part by regulating the migration of CD4 T cells to the inflamed CNS. Moreover, unbiased transcriptional profiling revealed that STAT4 controls the expression of >200 genes in Th17 cells and is important for the upregulation of genes associated with IL-23 stimulated, pathogenic Th17 cells. Importantly, we show that Th17 cells specifically require STAT4 to evoke autoimmune inflammation highlighting a novel function for STAT4 in Th17 pathogenicity.

Keywords

Autoimmunity; STAT4; Th17; Multiple Sclerosis

Introduction

CD4 T cells are implicated in the etiology of a number of autoimmune diseases, including Multiple Sclerosis (MS), Rheumatoid Arthritis, and Inflammatory Bowel Disease, yet the underlying mechanisms that potentiate T cell-mediated chronic inflammatory diseases remain ill-defined (1–7). Single nucleotide polymorphisms in the human *STAT4* locus are associated with susceptibility to multiple autoimmune disorders, including Multiple Sclerosis (MS) (8), and STAT4 is required for the induction of T cell mediated autoimmunity in animal models, including the mouse model of MS, experimental

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autoimmune encephalomyelitis (EAE) (9–11). IL-12 signaling in CD4 T cells via STAT4 promotes IFNγ production and Th1 differentiation (12–14), however for the induction of EAE neither IL-12 nor IFNγ are obligatory (15–19), indicating that STAT4 functions outside the Th1 lineage to elicit autoimmune inflammation. Therefore, understanding what cell types utilize STAT4 to mediate autoimmunity and how STAT4 directs pathogenicity in these cells is vital for devising new therapeutic interventions for MS and other autoimmune disorders.

Th17 effector CD4 T cells differentiate in response to TGFβ1 and IL-6 signaling, are dependent on the transcription factors RORγt and STAT3, and produce IL-17A, IL-17F, as well as other inflammatory cytokines (20–27). Studies using the EAE model have established that Th17 cells are critical for the induction of CNS inflammatory disease. Importantly, in the absence of IL-23 signaling, Th17 cells are deemed non-pathogenic and do not induce autoimmune inflammation, whereas exposure of Th17 cells to IL-23 promotes the acquisition of a pathogenic phenotype as exhibited by the ability to induce EAE in mice (28–32). The gain of pathogenic properties is associated with a unique transcriptional signature, however the exact mechanisms by which Th17 cells function to elicit autoimmune chronic inflammation are not fully resolved. Elucidating additional mechanisms that distinguish between pathogenic and non-pathogenic Th17 cells is vital to create therapeutic options for patients with autoimmune diseases.

Recent studies assessing pathogenic Th17 CD4 T cells during MS/EAE highlight an unexpected association with the transcription factor STAT4, which is a prototypic Th1 transcription factor (12, 13, 30, 33, 34). Notably, while IL-17A producing Th17 CD4 T cells develop independently of STAT4, the contribution of STAT4 to Th17 pathogenicity has not been established (21, 26). Therefore, we interrogated the importance of STAT4 to CD4 T cell mediated encephalogenicity, with a focus on the Th17 lineage. We find that STAT4 regulates multiple aspects of the CD4 T cell response during autoimmune inflammation, including the striking observation that STAT4 controls the migration of effector CD4 T cells to the inflamed CNS. Importantly, we show that IL-23-mediated CNS inflammation is dependent of CD4 T cell expression of STAT4 and that Th17 cells require STAT4 to induce EAE. Transcriptional profiling experiments revealed that STAT4 regulates the expression of numerous genes in Th17 cells that are associated with pathogenicity, however we find that *Il17a* mRNA and IL-17A protein are not controlled by STAT4, highlighting the function of STAT4 in Th17 cells is independent of IL-17A. These data highlight previously unrecognized, novel functions for STAT4 in mediating CD4 T cell autoimmune inflammation.

Materials and Methods

Mice.

The following mice were purchased from Jackson Laboratory: C57BL/6J, B6.SJL-*Ptprca Pep3*^{b}/BoyJ (WT CD45.1), C57BL/6-*Il17a*^{Tm1Bcgen}/J (WT IL17A-IRES-GFP-KI), *Il17a*$^{tm1.1(icre)Stck}$/J (Il17a^{cre}), B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J (ROSA26-YFP$^{fl/fl}$), and B6.Cg-Tg(Lck-icre)3779Nik/J (dLCK). B6.STAT4$^{fl/fl}$ mice were made by the Heflin Center for Genomic Sciences at The University of Alabama at Birmingham (see below).

B6.STAT4$^{-/-}$ (STAT4$^{-/-}$) mice were generously provided by Dr. Mark Kaplan (13). B6.IFN$\gamma^{-/-}$ mice were generously provided by Dr. Frances Lund at The University of Alabama at Birmingham. Mice were maintained and procedures approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Generation of mice with Stat4 flox allele.

STAT4 Flox CRISPR/sgRNA design and synthesis: CRISPR guides were designed using CRISPOR (crispor.tefor.org) to intron 5 and 6 of the Stat4 locus: 5' G1: 5'-CTAATCACAACTCCACTTAC-3'; 3' G1 5'-TAGGGGTGCACATGTTGCAG-3'. Modified synthetic sgRNAs (Synthego, Inc.) were allowed to complex with Alt-R® S.p. Cas9 Nuclease V3 (IDT, Inc.) at room temperature for 15 minutes before addition of the repair template (IDT, Inc.) and dilution with microinjection buffer to a final concentration of 50 ng/ul each of guide 1, guide 2, Cas9, and repair template, respectively. Gonadotropins: Female C57B6J embryo donors from 3–4 weeks of age were administered 5 IU of PMSG (Sigma, St. Louis, MO, USA) on day −3 followed by 5 IU of HCG (Sigma, St. Louis, MO, USA) on day −1 to induce superovulation. Donor and recipient females were mated to stud and vasectomized males, respectively, on day −1. Collection of embryos: At day 0.5 post-conception, superovulated donor females with copulatory plugs were humanely sacrificed using CO_2 followed by cervical dislocation. Oviducts were dissected into with sterile medium and nicked to expose the cumulus masses containing fertilized embryos. Embryos were cultured in KSOM (Millipore, Darmstadt, Germany) under 5% blood-gas prior to electroporation. Identification of founders: Founder animals were identified by PCR using primers flanking the target loci that amplified an 851 bp fragment in wild type animals (F1: 5'-ACTAGTACAGAGGGCAGCAGA-3'; R1: 5'-GCAATTACATGCACGTGCCA-3'). PCR samples were run on 6% polyacrylamide/TBE gels at 100 V for 45 minutes before staining with ethidium bromide. Positive samples were confirmed by modified Sanger sequencing.

EAE induction and clinical scoring.

EAE was induced and scored as previously described: classical EAE disease was scored as published (10), and atypical EAE disease was scored as published (35). Mice that reached a score of 4 were humanely euthanized. Scores were discontinued in the graph.

Littermate WT controls for Figure 1 were either STAT4$^{WT/Flox}$ mice or mice negative for the dLCKcre transgene. Similarly, WT littermate controls for Figure 4 were either STAT4$^{WT/Flox}$ or mice negative for the Il17a-cre transgene.

Th17 passive transfer EAE.

Ten days post EAE induction, spleen cells were cultured for 3 days under Th17 polarizing conditions as previously described (36). Following culture, live CD4 T cells were isolated by centrifugation over Histopaque 1083 (Sigma) followed by Dynabeads FlowComp Mouse CD4 kit (Invitrogen). For bulk CD4 transfer, 2×10^6 cells were injected intraperitoneally into B6 (C57Bl/6 or B6.SJL-*Ptprca Pep3*^{b}/BoyJ) mice. For IL-17aGFP+ cell transfer, following CD4 purification, GFP+ CD4 T cells were sorted using Aria II and 5×10^5 cells were injected intraperitoneally into B6 (C57Bl/6 or B6.SJL-*Ptprca Pep3*^{b}/BoyJ) recipient mice.

Mixed bone marrow chimeric mice generation.

Mixed bone marrow chimeric mice were generated as previously described (10, 18). B6.Rag1$^{-/-}$ mice were lethally irradiated and reconstituted with a 1:1 ratio of CD45.1 WT and CD45.2 WT or STAT4−/− bone marrow (WT:WT and WT:STAT4−/− respectively). Ten weeks following reconstitution, mice were immunized for EAE.

Surface and intracellular staining.

Cell surface and intracellular staining was performed as described previously (10, 18). A viability dye (Life Technologies, Aqua) was applied to exclude dead cells. Intracellular staining was performed following restimulation with 50ng/ml PMA (Sigma) and 750ng/ml ionomycin (Calbiochem) and BFA for 4 hours using either the eBioscience Fixation/Permeabilization Diluent (eBioscience) or the Cytofix/Cytoperm plus Fixation/ Permeablization kit (BD Biosciences) according to the manufacturer's instructions.

Ex vivo stimulation and phosflow staining.

Single cell suspensions of pooled brain and spinal cord lymphocytes were incubated for 30 minutes in the presence of media only or 10ng/ml rmIL-23. Phosflow staining was performed using anti-pSTAT3 (Cell Signaling) and the BD Phosflow kit (BD Biosciences), according to the manufacturer's instructions.

cDNA synthesis and real-time PCR.

cDNA synthesis and real-time PCR was described previously (37). Relative gene expression was calculated according to the ΔΔ threshold cycle (Ct) method by utilizing β2-microglobulin as a housekeeping gene.

RNA purification, RNA sequencing, and analysis for RNA sequencing.

Ten days post EAE induction, splenocytes were cultured for 3 days under Th17 polarizing conditions as previously described (10, 38). Following culture, live cells were isolated by centrifugation over Histopaque 1083 (Sigma), and CD4 T cells were isolated using Dynabeads FlowComp Mouse CD4 kit (Invitrogen).

RNA isolation, sequencing, and alignment was completed as described previously (37). RNA was isolated from WT and STAT4−/− CD4 cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocols and submitted to GENEWIZ for sequencing. Total RNA was sequenced on illumine HiSeq 2500 (2 × 100 base pair, single-end reads) and aligned using mouse mm10 reference genome. The GI tools software along with DESeq2 software was used for differential expression analysis. RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) Repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227049).

For gene set enrichment analysis (GSEA), RNA sequencing data was pre-ranked according to an adjusted p value and the sign of differential expression. The normalized enrichment score (NES), nominal p value, and false detection rate (FDR) q-value were assessed using GSEA software from Broad Institute by running in pre-ranked list mode with 1,000 permutations. To perform GSEA with pathogenic Th17 cell gene signature (TGF

β+IL-6±IL-23), the microarray data (GSE43955) was used. GraphPad prism 9 was used to generate volcano plot.

In vitro Th1/Th17 differentiation.

In vitro Th1 and Th17 differentiation was completed as described previously (39). Naïve CD4 T cells were isolated from spleen and lymph nodes with the EasySep Mouse Naïve CD4+ T Cell Isolation Kit (StemCell Technologies) according to the manufacturer's instructions. For Th1 conditions, cultures were supplemented with IL-12 (10ng/mL; Peprotech) and αIL-4 (10 mg/ml; Bio X Cell). For Th17 conditions, cultures were supplemented with rmIL-6 (20 ng/ml; Biolegend), rhTGFb1 (2.5 ng/ml; Peprotech), anti-IL-4 (10 mg/ml; BioXCell, 11B11), and anti-IFNγ (10 mg/ml; BioXCell, XMG1.2).

ELISA.

Supernatants were removed from undisturbed Th1 cell cultures on day 3. IFNγ ELISA (eBioscience) was completed according to the manufacturers protocol.

Results

Generation of T cell specific STAT4 deficient mice

STAT4 regulates effector CD4 T cell differentiation and function and is required for the induction of CNS demyelination in mice (9–11), nevertheless the role T cell intrinsic STAT4 in the disease pathogenesis remains elusive. To determine if T cell intrinsic STAT4 is necessary for EAE, we established T cell specific STAT4 deficient mice by breeding newly generated mice that harbor a floxed *Stat4* allele with mice carrying the distal Lck (dLck) cre recombinase transgene (STAT4$^{\Delta dLck}$), which is known to delete in mature T cells (40). We verified the phenotype of newly developed STAT4$^{\Delta dLck}$ mice in vitro by comparing the expression and function of CD4 T cells derived from these mice with wild-type (WT) and global STAT4 deficient (STAT4−/−) mice. First, naïve CD4 T cells from the different strains of mice were analyzed directly ex vivo for *Stat4* mRNA levels by real-time PCR, and we did not detect a significant difference in the *Stat4* mRNA levels between the STAT4−/− and STAT4$^{\Delta dLck}$ CD4 T cells (Fig. 1A). Because STAT4 is activated and necessary during Th1 differentiation (12–14), we stimulated naïve CD4 T cells from the WT, STAT4−/−, and STAT4$^{\Delta dLck}$ mice under Th1 conditions for three days. As expected, the WT Th1 CD4 T cells exhibited robust pSTAT4 staining whereas the STAT4−/− and STAT4$^{\Delta dLck}$ CD4 T cells displayed negligible pSTAT4 staining (Fig. 1B–C). In addition, the production of the STAT4-dependent cytokine IFNγ by CD4 T cells was markedly reduced in the STAT4$^{\Delta dLck}$ CD4 T cells compared to the WT cells (Fig. 1D–G). It is worth noting that the STAT4$^{\Delta dLck}$ CD4 T cells did harbor a low level of pSTAT4 and IFNγ production after Th1 differentiation, likely due to incomplete cre deletion by the dLck transgene.

The development of EAE is associated with Th17 cells, and previous studies have shown that STAT4 is dispensable for the development of Th17 cells (10, 21, 26). Therefore, it was necessary to test the ability of STAT4$^{\Delta dLck}$ CD4 T cells to differentiate into Th17 cells. We isolated naïve WT, STAT4−/−, and STAT4$^{\Delta dLck}$ CD4 T cells and cultured these cells for three days under Th17 conditions. Consistent with published reports (10, 21, 26), there was

no difference in the production of IL-17A between WT and STAT4−/− CD4 T cells after Th17 differentiation, and the levels of IL-17A production by the STAT4$^{\Delta dLck}$ CD4 T cells were comparable (Fig. 1H–I). Together, these experiments demonstrate that the CD4 T cells in the STAT4$^{\Delta dLck}$ mice lack functional STAT4.

T cell intrinsic STAT4 is required for EAE

Confident that the STAT4$^{\Delta dLck}$ mice lack functional STAT4 in mature CD4 T cells, we set out to test the requirement of T cell intrinsic STAT4 for the induction of EAE. To do this, we immunized WT and STAT4$^{\Delta dLck}$ mice with MOG$_{35–55}$ peptide to induce EAE. As expected, the WT mice exhibited EAE symptoms, however the STAT4$^{\Delta dLck}$ mice did not show signs of disease (Fig. 2A). While these data do not rule out a role for STAT4 in non-T cells during EAE, it demonstrates T cell expression of STAT4 is indispensable to mediate CNS demyelination.

To assess the impact of intrinsic STAT4 deficiency on effector CD4 T cells during EAE, we analyzed the functional capacity of the brain and spinal cord infiltrating cells. Strikingly, in the absence of STAT4, the frequency and number of CD4 T cells in the spinal cord were significantly decreased (Fig. 2B–C). Functional cytokine analysis showed that the percentage of CD4 T cells in the brain and spinal cord producing IFNγ only or both IFNγ and IL-17A was reliant on STAT4, whereas the requirement for IL-17A production was less stringent (Fig. 2D–G), and this is consistent with previous reports utilizing global STAT4 deficient mice (9, 10). These data, combined with the diminished CD4 T cell recovery from the CNS, translated into significant reductions the absolute numbers of IFNγ+, IL-17A+ and IFNγ+IL-17A+ CD4 T cells in the spinal cords of the STAT4$^{\Delta dLck}$ mice, and similar trends were also observed in the cells recovered from the brains (Fig. 2D–G). Taken together, these experiments reveal that STAT4 not only instructs the functional properties of CD4 T cells but potentially impacts the ability of these cells to migrate to the CNS.

STAT4 functions to control effector CD4 T cell migration to the inflamed CNS.

Since differences in the inflammatory environment can influence the access of CD4 T cells to the CNS during EAE, we utilized mixed (50:50) bone marrow chimeric mice to alternatively test the T cell intrinsic role of STAT4 in T cell trafficking to the brain and spinal cord in the context of disease. Of note, we have previously published that WT:STAT4−/− mixed bone marrow chimeric mice develop EAE to the same extent as WT:WT chimeric mice (10), providing a system to study the role of CD4 T cell intrinsic STAT4 expression during active CNS inflammation. In the control WT:WT group, at the peak of EAE disease severity (day 17), the frequency of CD45.2+ CD4 T cells recovered from the brain and spinal cord was similar to the frequency detected in the spleen (Fig. 3A–B). However, in the experimental cohort, there was a significant reduction in the frequency of CD45.2+ STAT4−/− CD4 T cells in both the brain and spinal cord compared to the spleen at this timepoint. To test if the reduced accumulation of CD4 T cells in the CNS was due to a defect in cellular migration, we analyzed CD4 T cells in the brain and spinal cord at disease onset (day 11), a timepoint reflective of early T cell recruitment. There was a significant reduction in the migration efficiency of STAT4−/− CD4 T cells in the brain and spinal cord compared to WT CD4 T cells when normalized to the spleen at the initiation of disease (Fig.

3B), which is consistent with a role for STAT4 in entry of CD4 T cells into the inflamed CNS. Together, these findings highlight a previously unrecognized function of STAT4 in directing the migration of effector CD4 T cells to the sites of inflammation during EAE.

Interestingly, CD4 T cells which lack the ability to signal via the IL-23 receptor complex (IL-23R−/− or IL-12Rβ1−/− CD4 T cells) also exhibit a defect in CNS accumulation (28, 41, 42). However, IL-23 is published to support CD4 T cell proliferation in the CNS, not migration to the site; the defect in CNS accumulation of IL-23R−/− CD4 T cells was not present at the onset of disease but at the peak (42), which is distinct from the phenotype observed with STAT4−/− CD4 T cells. Nevertheless, we further probed the potential link between the IL-23 pathway and STAT4 in CD4 T cells during EAE using mixed bone marrow chimeric mice. Unlike IL-23R−/− CD4 T cells, CD4 T cells devoid of STAT4 expression did not exhibit reduced proliferation at the peak of disease as evidenced by Ki-67 staining (Fig. 3C–D). Moreover, IL-23 induction of STAT3 phosphorylation was unaffected by the lack of STAT4 in CD4 T cells (Fig. 3E–F). Together, these data indicate that notable downstream actions of IL-23 signaling are intact in the absence of STAT4 and that STAT4 modulates the accumulation of CD4 T cells in the inflamed CNS via a mechanism distinct from proliferation, potentially by promoting cell migration.

STAT4 is required for IL-23-mediated EAE by Th17 cells

IL-23 is a key pathogenic cytokine during EAE and is critical for the induction of pathogenic Th17 cells (28, 30, 33, 42). Upon IL-23 signaling, activated STAT3 dimerizes and translocates to the nucleus where it initiates transcription, and we demonstrate above that this signaling conduit is intact in the absence of STAT4. However it has been illustrated that IL-23 can also induce the phosphorylation of STAT4 (34, 43), potentially linking the IL-23 and STAT4 pathways. Hence, we hypothesized that STAT4 is critical for IL-23 mediated autoimmunity. Using a passive transfer model of EAE to test this, we find that transfer of IL-23-stimulated WT CD4 T cells elicited EAE whereas STAT4−/− CD4 T cells cultured in the presence of IL-23 did not induce disease (Fig. 4A). This is associated with a significant decrease in the number of donor STAT4−/− cells recovered from both the brain and spinal cord of recipient mice (Fig. 4B–D). Importantly, the donor WT and STAT4−/− CD4 T cells did not differ in proliferation during the in vitro culture (Fig. S1A), and there were equivalent numbers of both donor WT and STAT4−/− cells recovered in the spleen (Fig. 4C–D), indicating the migratory phenotype observed in the CNS is irrespective of cell proliferation and survival. Prior to transfer, the production of IL-17A or IFNγ was similar between the two genotypes (Fig. S1B–C), however after transfer, significant differences in the number of recovered donor WT and STAT4−/− cells producing the pro-inflammatory cytokines IL-17A and IFNγ were detected (Fig. 4E–H). In the recipient mice, the WT CD4 T cells predominantly produced IFNγ and minimal IL-17A, whereas STAT4−/− CD4 T cells produced high amounts of IL-17A and less IFNγ. Since production of IFNγ was one of the key differences in the donor cells, we performed the same experiment with IL-23-stimulated IFNγ−/− CD4 T cells. These cells did mediate EAE, indicating that IFNγ is dispensable for disease (Fig. S1D–F) and revealing that STAT4 functions independently of IFNγ to promote neuroinflammation.

STAT4 influences Th17 pathogenic genes and is required for IL-17A+ CD4 T cells to drive neuroinflammation.

To decipher the mechanistic role of STAT4 on Th17 cell pathogenicity, we performed RNA sequencing on CD4 T cells extracted from EAE immunized WT and STAT4−/− mice that were subsequently cultured under Th17 conditions. We found that STAT4 significantly influenced the expression of >200 genes, and importantly, expression of the Th17 lineage genes *Rorc, Stat3, Il22* and *Il17a* were not negatively impacted by STAT4 deficiency (Fig. 5A). We scrutinized this RNA sequencing data set for the expression of genes previously published to be associated with pathogenic and non-pathogenic Th17 cells by gene set enrichment analysis (GSEA). As expected, we show that WT EAE-Th17 cells were positively associated with genes upregulated in pathogenic, IL-23 stimulated Th17 cells (Fig. 5B) (32). In striking contrast, we find that the STAT4−/− EAE-Th17 cells were enriched for genes downregulated under these conditions (Fig. 5B). These data, together with the in vivo EAE experiments, indicate that STAT4 controls IL-23 mediated inflammation in part by regulating the balance between pathogenic and non-pathogenic Th17 cells.

IL-23 imprints pathogenic properties to Th17 cells, yet the above adoptive transfer experiments involved a heterogeneous population of IL-17A+ and IL-17A- CD4 T cells. To test if STAT4 is requisite in IL-17A+ CD4 T cells to elicit neuroinflammation, we utilized WT and STAT4−/− IL-17A-GFP knock-in reporter mice in conjunction with the IL-23-mediated passive transfer model of EAE. We performed the same IL-23-mediated passive transfer EAE model as above, but instead utilized FACS sorted GFP+ (IL-17A+) CD4 T cells as the donor population. After transfer, the WT GFP+ (IL-17A+) CD4 T cells rapidly induced EAE symptoms in all mice (100%; 11 out of 11), promoting classical EAE with ascending paralysis in the majority of the recipient mice (82%; 9 out of 11), while evoking ataxia or atypical EAE in almost half of the mice (45%; 5 out of 11) (Fig. 5C). In contrast, transfer of an equivalent number of STAT4−/− GFP+ (IL-17A+) CD4 T cells did not result in either classical or atypical EAE in the recipient mice (0 out of 10 mice) (Fig. 5C). The disparities in disease severity correlate with differences in the donor CD4 T cell populations after transfer. Consistent with our earlier data, lower numbers of donor STAT4−/− CD4 T cells were recovered from the CNS sites compared to the WT donor cells (Fig. 5D), again reaffirming a role for STAT4 in Th17 migration. Even though all donor cells were IL-17A+ pre-transfer, at the peak of disease the WT CD4 T cells had largely extinguished IL-17A production and exhibited robust manufacture of IFNγ (Fig. 5E–H). This functional profile was not observed in the donor STAT4−/− cells after transfer, which did downregulate IL-17A expression but did not increase IFNγ production to the extent the WT CD4 T cells did (Fig. 5E–H). These data further support the observation that STAT4 influences Th17 and CD4 T cell migration as well as the pro-inflammatory activities and demonstrate that STAT4 is essential for IL-23-mediated Th17 disease.

Th17 intrinsic STAT4 expression is critical for CNS demyelinating disease

In the above experiments, STAT4 is deleted in the CD4 T cell prior to T cell receptor stimulation, Th17 differentiation, and the initiation of EAE disease. Therefore, we sought to determine if STAT4 functions early in CD4 T cells to direct pathogenic potential or

after commitment to the Th17 lineage. To test this, we created Th17 specific STAT4 deficient mice by crossing the $STAT4^{fl/fl}$ mice and the IL-17A-Cre x $ROSA26\text{-}YFP^{fl/fl}$ fate-mapping mice ($STAT4^{\Delta IL17A}$), and we immunized control and $STAT4^{\Delta IL17A}$ mice to induce EAE. As expected, the control group of animals developed EAE symptoms, however the $STAT4^{\Delta IL17A}$ mice displayed significantly lower disease scores (Fig. 6A). This was associated with dramatically decreased numbers of CD4 T cells in the brain and spinal cord of the $STAT4^{\Delta IL17A}$ mice compared to the control mice (Fig. 6B) at the peak of disease. While these data do not rule out a role for STAT4 early in the programming of pathogenic CD4 T cells, it does show that STAT4 does function after the commitment to the Th17 lineage to promote disease.

Our earlier data shows that STAT4 is critical for Th1 differentiation and IFNγ production by CD4 T cells but does not impact IL-17A production by Th17 cells, hence it was necessary to assess how STAT4 deletion in Th17 cells specifically influences functionality. The availability of the ROSA26-YFP fate-mapping allele provided us with the opportunity to track IL-17A+ CD4 T cells during disease; in both the brains and spinal cords of $STAT4^{\Delta IL17A}$ mice the frequency and number of YFP+ cells was significantly decreased (Fig. 6C–E and Fig. S2A–C). Furthermore, intracellular staining for IFNγ and IL-17A in the CNS infiltrating CD4 T cells showed marked reductions in the numbers of IFNγ+, IL-17A+ and IFNγ+IL-17A+ CD4 T cells in the $STAT4^{\Delta IL17A}$ mice (Fig. 6F–I). Taken together, these experiments complement the results obtained with the global and T cell-specific STAT4 deficient mice and further corroborate a definitive role for STAT4 in Th17 pathogenesis.

Discussion

Herein, we demonstrate that STAT4 functions in IL-23 mediated autoimmunity by influencing migration and the pathogenic potential of Th17 cells. Using a novel strain of T cell-specific STAT4 deficient mice, we show that T cell intrinsic expression of STAT4 is required to elicit autoimmune CNS inflammation, and this is in part by regulating the migration of CD4 T cells to the CNS. In addition, we employed multiple animal models, including STAT4 deficient IL-17A reporter mice and Th17-specific STAT4 deficient mice, to illustrate an indispensable role for STAT4 in Th17 mediated disease. Importantly, STAT4 does not control the differentiation of Th17 cells or the production of IL-17A, but instead governs the expression of genes associated with pathogenicity.

One of the major findings from this study is that STAT4 impacts the migration of effector CD4 T cells, including Th17 cells, to the inflamed CNS during EAE. The importance of T cell trafficking during MS and EAE is exemplified by the successful clinical use of Natalizumab, a monoclonal antibody specific for the integrin α4, and Fingolimod, a sphingosine 1-phosphate receptor agonist, to treat MS patients (44–47). Interestingly, using Th1 versus Th17 mediated adoptive transfer EAE models, Rothhammer et al. demonstrated that integrin α4 is necessary for Th1 cells to infiltrate the CNS and mediate disease, but that Th17 cells utilize CD11a (*Itgal*), not integrin α4, to induce EAE (48). We previously published that LFA-1 expression by CD4 T cells is not regulated by STAT4 following acute viral infection (49), suggesting that STAT4 controls T cell migration via an alternative mechanism. Chemokine:chemokine receptor interactions are also known to be important

for the recruitment of T cells to inflammatory sites. The chemokine receptor CXCR3 is preferentially expressed by Th1 cells, and notably, Ghoreschi et al. published that *Cxcr3* mRNA levels are elevated in IL-23 stimulated, pathogenic Th17 cells that induce EAE (33). Nevertheless, the CXCR3 deficient mice do not phenocopy the STAT4−/− mice in regards to the EAE disease course (50, 51), thus indicating that this is not the likely pathway by which STAT4 regulates CD4 T cell dependent CNS demyelination. Overall, we find that STAT4 influences the expression of numerous molecules in Th17 cells, hence we favor the hypothesis that STAT4 controls CD4 T cell encephalitogenicity in part by regulating a constellation of molecules that function cooperatively to facilitate entrance to the CNS during inflammation and demyelinating disease.

STAT4 is an integral component of the Th1 differentiation pathway; IL-12 induced STAT4 phosphorylation functions to promote the epigenetic remodeling of Th1-associated genes and acts to directly activate *Ifng* gene transcription (13, 52). In the EAE model of CNS inflammation, STAT4 is required for disease induction whereas both IL-12 and IFNγ signaling are dispensable (9, 15, 28) (53), highlighting a role for STAT4 independent of the classic Th1 lineage. While the role of Tbet, the Th1 master transcription factor, in the development of EAE is debatable, our laboratory does not find an essential role for Tbet in EAE (38, 54–56). Additionally, we published that Tbet is not important for the migration of CD4 T cells to the inflamed CNS during EAE, using the same mixed bone marrow chimera approach employed in this study (38). Together, these findings describe a novel function for STAT4 in promoting effector CD4 T cell encephalitogenicity, unique to its function in Th1 differentiation and IFNγ production.

Published studies have documented an essential role for the cytokine GM-CSF in the development of EAE (57–59). Both Th1 and Th17 effector CD4 T cell lineages produce GM-CSF and the encephalitogenicity of both cell types is dependent on GM-CSF. El-Behi et al, showed that adoptive transfer of *Csf2*−/− Th1 cells or Th17 cells was not able to induce EAE in recipient mice, even though production of the lineage signature cytokines IFNγ and IL-17A, respectively, was not corrupted (58). Moreover, Codarri et al, published that adoptive transfer of lymphocytes from *Ifng*−/−*Il17a*−/− mice induced EAE in WT recipient mice, and this correlated with GM-CSF production by CD4 T cells (57). Interestingly, IL-23 stimulation was important for the upregulation of GM-CSF in CD4 T cells (57, 58), and in this study we show that STAT4 is required for IL-23 mediated EAE. Importantly, we previously reported that GM-CSF production by effector CD4 T cells during EAE, including Th17 cells, is dependent on STAT4; STAT4-deficient CD4 T cells secreted lower levels of GM-CSF and STAT4 directly bound to the *Csf2* promoter (10). Taken together, these data are consistent with the data presented herein that STAT4-deficient Th17 cells are able to produce ample amounts of IL-17A yet not cause demyelination in IL-23 mediated CNS inflammation.

Consistent with a Th1-independent function for STAT4, we demonstrate in this study a vital role for STAT4 in IL-23 dependent, Th17-mediated EAE. IL-23 signaling is essential for the development of EAE in mice and members of the IL-23 pathway are associated with MS susceptibility (28, 29, 42). Early findings documenting the discovery of IL-23 indicated that this cytokine not only induced by the phosphorylation of STAT3, but that it also activated

STAT4 (60). Moreover, a recent study by Lee et al, detailed the induction of STAT3/STAT4 heterodimers by IL-23 in CD4 T cells if the cells were previously primed in the presence of certain inflammatory cytokines or antigen presenting cell populations (34). This link between IL-23 and STAT4 is further supported by the data in this study as well as others, which show that IL-23R deficient and STAT4 deficient CD4 T cells both exhibit a defect in accumulation in the inflamed CNS (42). However, we observed that STAT4 was essential for the early recruitment of CD4 T cells to the CNS and it did not impact proliferation or survival, whereas the impaired accumulation of IL-23R deficient CD4 T cells in the inflamed CNS is the result of reduced cellular proliferation. These data beg to question if there are differences in IL-23 signals dependent on STAT3:STAT3 homodimers versus STAT3:STAT4 heterodimers. To this end, Poholek et al, published that deletion of STAT3 in Th17 cells abrogated EAE disease, and many of the IL-23 dependent cell cycle genes in CD4 T cells were similarly affected (61). Strikingly though, certain genes previously identified to be regulated by IL-23 were not affected by STAT3 deficiency, highlighting the possibility that distinct IL-23 functions are mediated by distinct signaling modalities, potentially involving STAT4.

In conclusion, in this study we document novel functions for STAT4 in IL-23 mediated autoimmune disease. We demonstrate that STAT4 directs the migration of effector CD4 T cells into the inflamed CNS during EAE. We also show that Th17 intrinsic expression of STAT4 is required for the encephalitogenic capacity of the cells during CNS demyelination, in part by controlling the expression of the Th17 pathogenic gene signature. These data, together with previous work from others as well as our group, highlight that STAT4 operates at multiple levels in effector CD4 T cells to regulate autoimmune disease (10–12, 30, 34, 43, 62). Further understanding of the mechanisms by which STAT4 coordinates pathogenic gene expression in Th17 cells and other effector CD4 T cell populations will yield therapeutic options for individuals with chronic inflammation and autoimmunity, including MS, Rheumatoid Arthritis, and Inflammatory Bowel Disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

CD4 T cell intrinsic STAT4 expression controls migration to the CNS during EAE.

STAT4 is required for Th17 pathogenicity independent of IL-17A production.

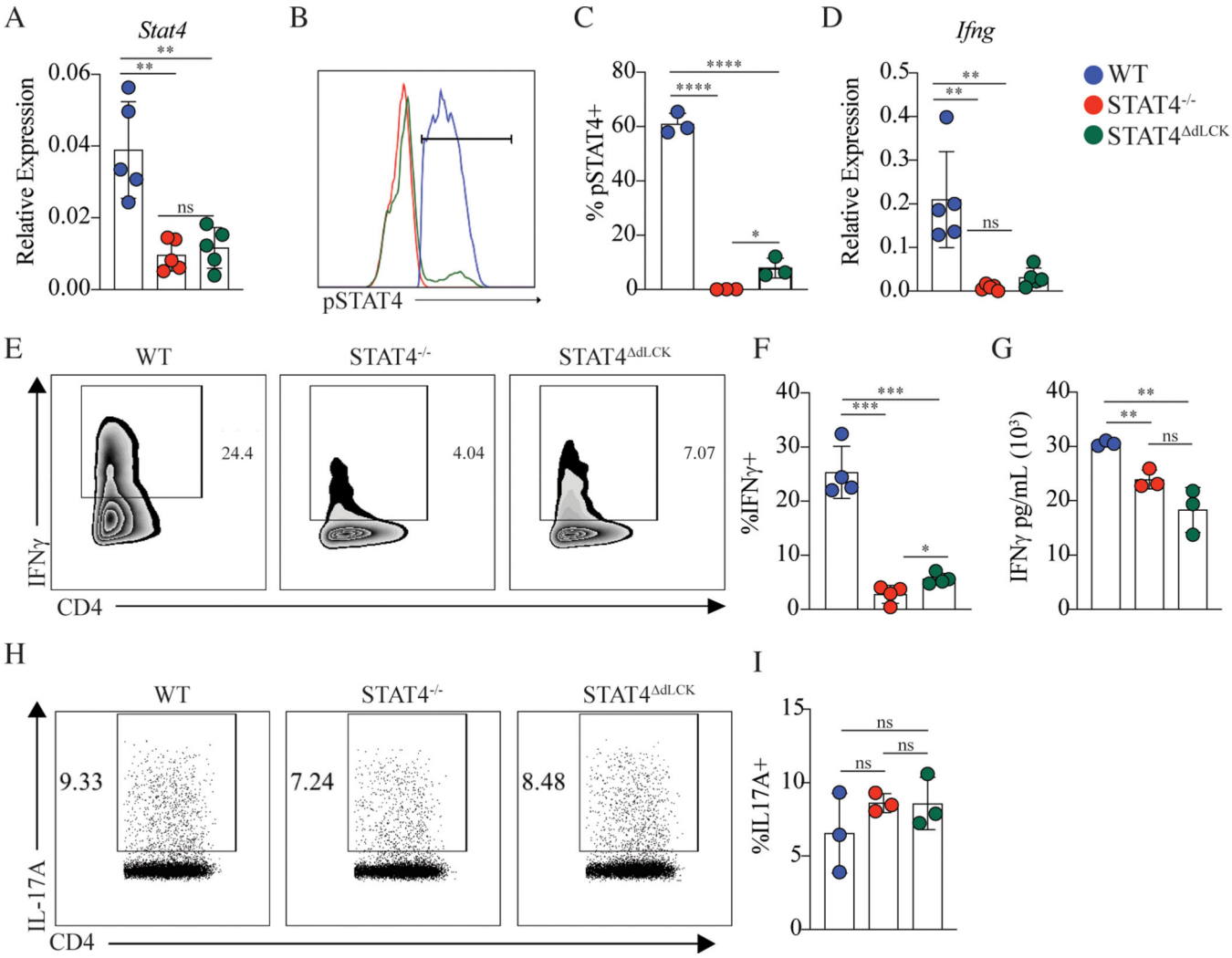

Figure 1. Establishment of T cell specific STAT4−/− mice.

(A) *Stat4* mRNA expression from isolated WT, STAT4−/−, or STAT4$^{\Delta dLck}$ CD4 T cells. (B-G) Naïve CD4 T cells were isolated from the spleens of WT, STAT4−/−, or STAT4$^{\Delta dLck}$ mice and polarized under Th1 cell conditions. Cells were analyzed on day 3 of differentiation. (B) Representative plots of pSTAT4 staining. (C) Frequency of pSTAT4+ cells. (D) Relative expression of *Ifng* mRNA. (E) Representative plots of IFN**γ** staining. (F) Frequency of IFN**γ**+ cells. (G) ELISA of IFN**γ** production. (H-I) Naïve CD4 T cells were isolated from the spleens of WT, STAT4−/−, or STAT4$^{\Delta dLck}$ mice and polarized under Th17 cell conditions. Cells were analyzed on day 3 of differentiation. (H) Representative plots of IL-17A staining. (I) Frequency of IL-17+ cells. All representative plots are gated on live CD4+ cells. Data represent 3–5 independent experiments. Unpaired T test; ns = not significant, *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

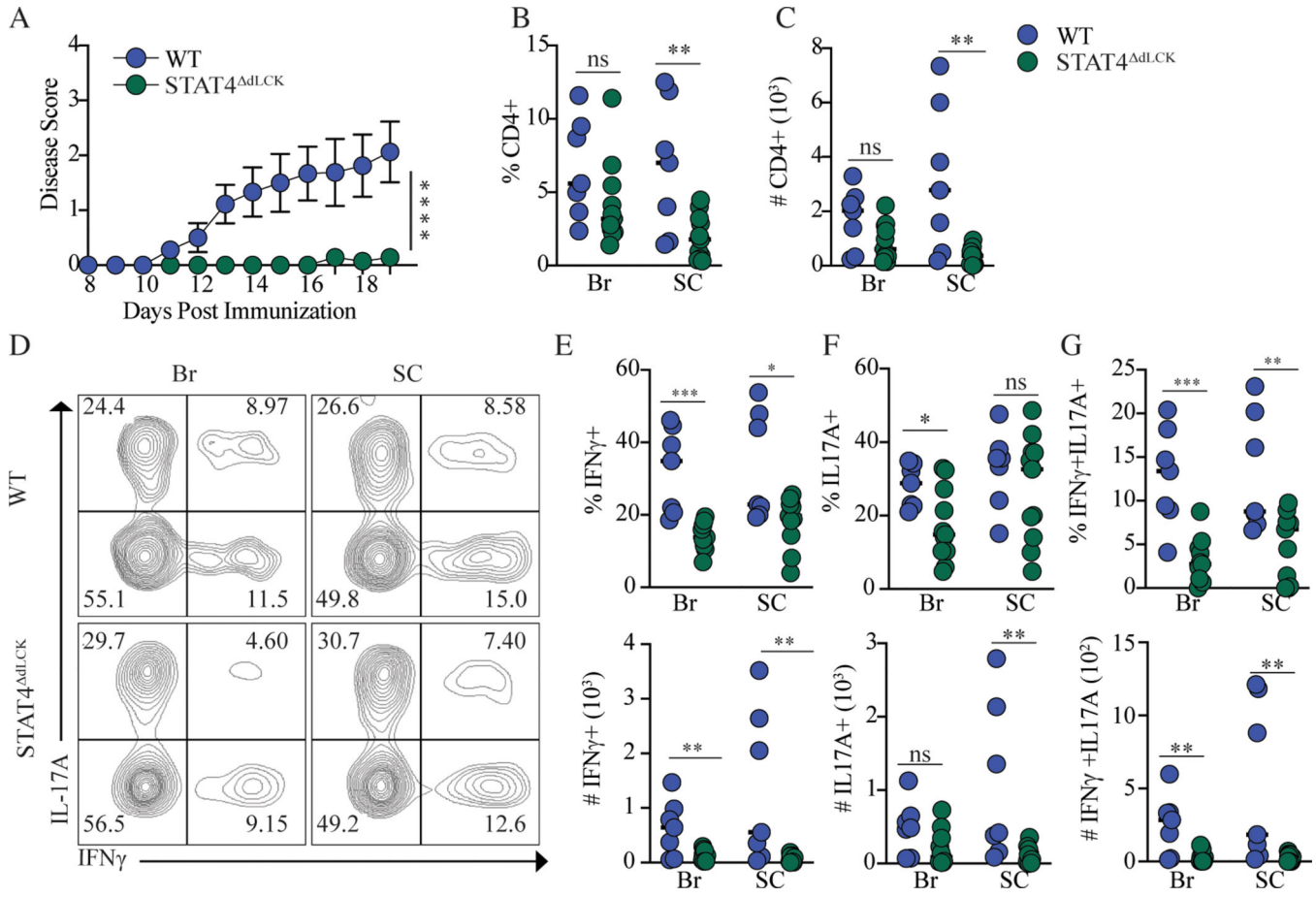

Figure 2. T cell intrinsic STAT4 is required for EAE.

(A) STAT4$^{\Delta dLCK}$ and littermate WT controls were immunized for EAE. Disease symptoms were monitored daily (n=9–14, 5 independent experiments). (B) Frequency and (C) number of CD4 T cells infiltrating to the CNS at the peak of disease (n=7–11, 3 independent experiments). (D) Representative flow plot of the expression of IL-17A+ and IFN γ+ cells (gated on CD4+ T cells). (E-G) Frequency (top) and number (bottom) of CD4+ T cells in the CNS producing (E) IFNγ (F) IL-17A and (G) IFNγ and IL17A. (n=7–11, 3 independent experiments). (A) Two- way ANOVA and (E, F, G) unpaired T test; ns = not significant, *=p<0.05. **=p<0.01; ***=p<0.001; ****=p<0.0001.

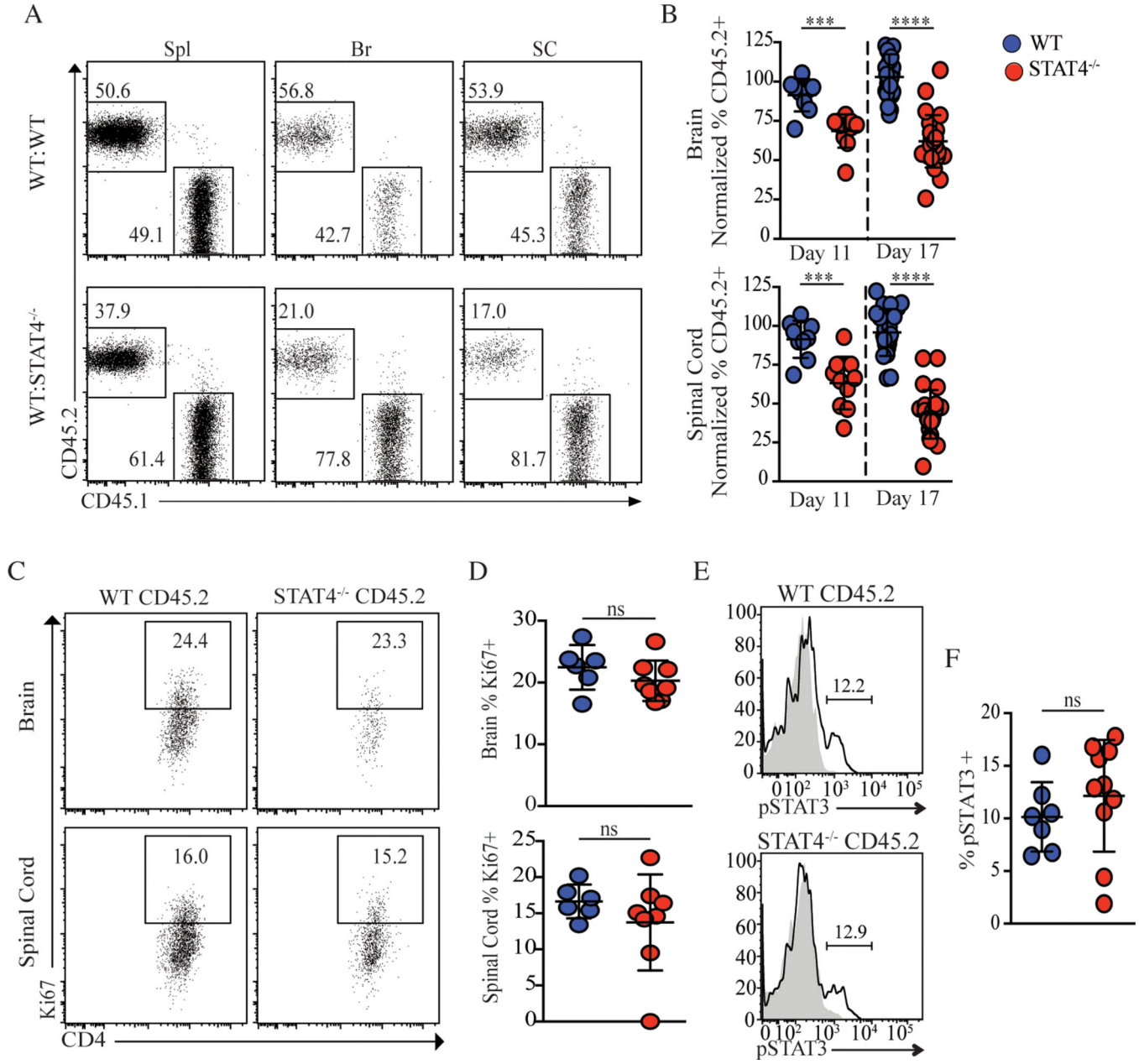

Figure 3: CD4 T cells require intrinsic STAT4 expression for accumulation in the CNS during EAE.

(A, B) WT:WT and WT:STAT4$^{-/-}$ mixed bone marrow chimeric mice were immunized for EAE. (A) Representative plots show CD45.1 and CD45.2 staining gated on CD4 T cells from the spleen, brain, and spinal cord on day 17 post immunization. (B) Normalized accumulation frequencies of CD45.2+ WT and STAT4$^{-/-}$ CD4 T cells in the brain and spinal cord were determined on day 11 and day 17 of disease. Normalization frequency = (%CD45.2+CD4+ brain or spinal cord ÷ %CD45.2+CD4+ spleen) x 100. (C-D) Ki67 staining in CNS infiltrating CD45.2+ WT or STAT4−/− CD4 T cells from mixed bone marrow chimeric mice on day 17 post immunization. (C) Representative plots are gated

on CD45.2+ CD4 T cells. (D) Cumulative frequencies of Ki67+ CD45.2+ WT or STAT4$^{-/-}$ CD4 T cells in the CNS. (E-F) Phosphorylated STAT3 (pSTAT3) staining in CD45.2+ WT or STAT4$^{-/-}$ CD4 T cells following media or IL-23 stimulation of pooled CNS cells. (E) Representative plots gated on CD45.2+ CD4 T cells illustrate media (grey filled) and IL-23 (black line) induced pSTAT3. (F) Cumulative frequencies of IL-23 induced pSTAT3+ CD45.2+ WT or STAT4$^{-/-}$ CD4 T cells from pooled CNS tissue. Data represent 2–6 independent experiments with (A-B) 2–5, (C-D) 3–4, or (E-F) 2–5 mice in each group (mean ± SD). ns=not significant; ***=p<0.001; ****=p<0.0001.

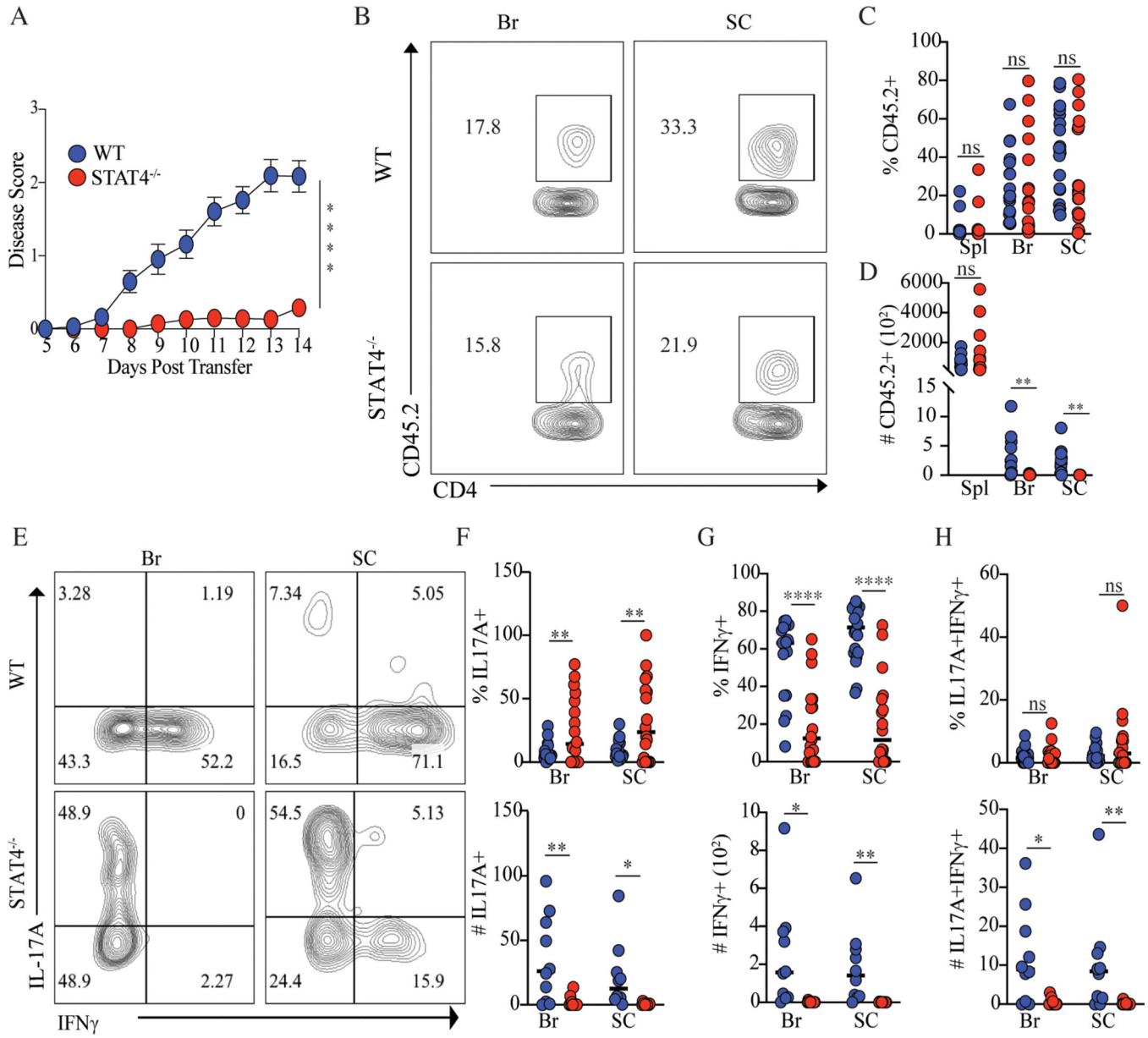

Figure 4: STAT4 is required for IL-23 mediated EAE.

(A) Splenocytes were isolated from previously immunized mice and cultured under Th17 conditions for three days. CD4 T cells were isolated and transferred into CD45.1 WT recipient mice. Disease symptoms were monitored daily (n=30–36, 10 independent experiments). (B) Representative flow diagram of CD45.2 donor cells in the brain and spinal cord (gated on CD4+ T cells). (C) Frequency and (D) number of CD45.2 donor cells in the spleen, brain, and spinal cord (gated on CD4+ T cells) (n=18–20, 3 independent experiments). (E) Representative flow diagram of donor cell cytokine profile. (F-H) Frequency (top) and number (bottom) of (F) IL-17A+, (G) IFNγ+ (H) IL-17A+IFNγ+ T cells Gated on (B-D) CD4+ and (E-H) CD4+CD45.2+ cells (n=18–20, 3 independent experiments). (A) Two- way ANOVA and (C, D, F-H). Unpaired T test; ns = not significant, *=p<0.05. **=p<0.01; ***=p<0.001; ****=p<0.0001.

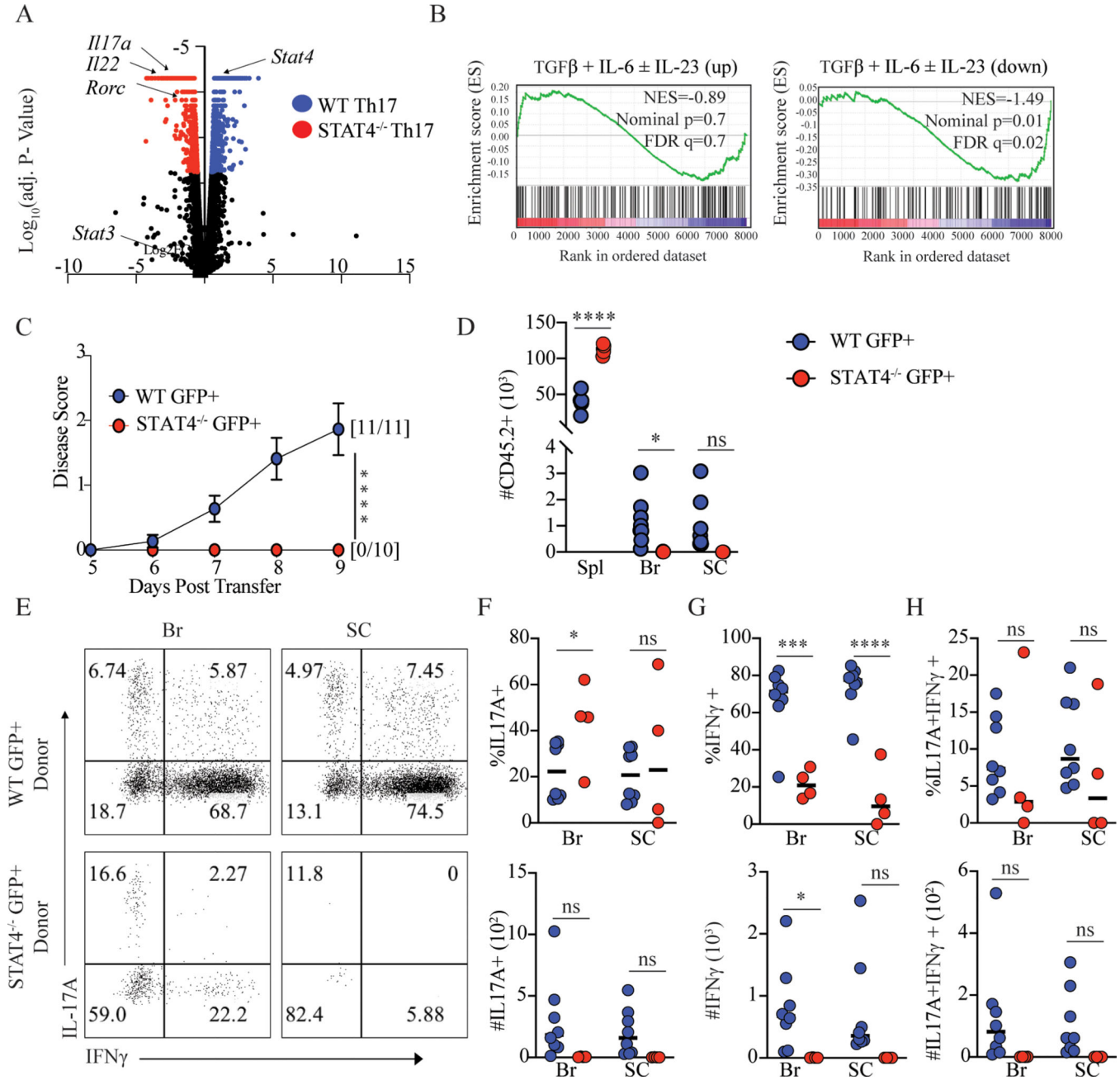

Figure 5. STAT4 influences Th17 pathogenic genes and is required for IL-17A+ to drive neuroinflammation.

(A-B) Splenocytes from previously immunized mice were cultured under Th17 conditions. CD4 T cells from these cultures were isolated and RNA sequencing was performed. (A) Volcano plot of differentially expressed genes between WT and STAT4$^{-/-}$ Th17 cells (red) genes significantly elevated in STAT4−/− Th17 cells and (blue) genes significantly elevated in the WT Th17 cells. (B) GSEA plots show enrichment of genes elevated in WT Th17 cells → elevated in STAT4−/− Th17 cells (left → right) when compared with pathogenic and non-pathogenic Th17 cells (GSE43955), (C-H) Splenocytes were isolated from previously immunized WT IL17AGFP+ or STAT4−/− IL17AGFP+ mice and cultured under Th17

conditions for three days. GFP+CD4+ T cells were isolated and transferred into CD45.1 WT recipient mice. Disease symptoms were monitored daily (n=10–11, 3 independent). (D) Number of recovered donor cells in the CNS (n=4–8, 3 independent experiments). (E) Representative flow diagram of IFNγ and IL-17A expression (gated on CD4+CD45.2+). (F-H) Frequency (top) and number (bottom) of (F) IL-17A+ (G) IFNγ and (H) IL-17A+IFNγ+ producing cells (gated on CD4+CD45.2+) (n-4–8, 3 independent experiments). (C) Two-way ANOVA and (D,F-H). Unpaired T test; ns = not significant, *=p<0.05. **=p<0.01; ***=p<0.001; ****=p<0.0001.

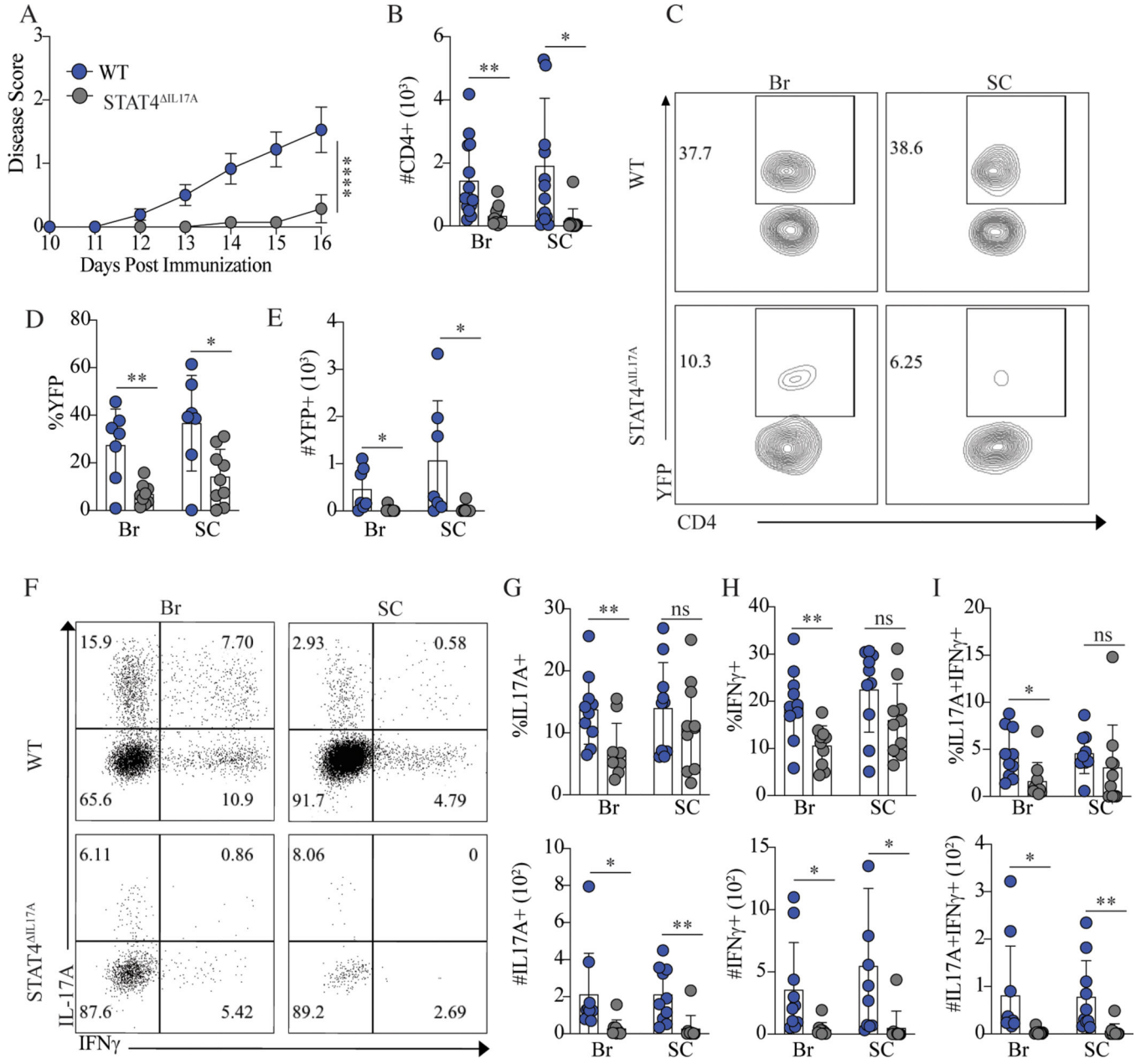

Figure 6. Th17 intrinsic STAT4 expression is critical for CNS demyelinating disease.

(A) STAT4$^{\Delta IL17A}$ and littermate WT controls were immunized for EAE, and disease was scored daily (n=14–20, 6 independent experiments). (B) Number of CD4 T cells infiltrating to the CNS at the peak of disease. (C) Representative flow diagram YFP+ cells (gated on CD4+). (D) Frequency and (E) number of YFP+ cells (gated on CD4+) (n=7–9, 6 independent experiments). (F) Representative flow diagram of IL17A+ and IFNγ+ cells (gated on CD4+). (G-I) Frequency (top) and number (bottom) of (G) IL-17A+ (H) IFNγ+ and (I) IFNγ+IL17A+ cells (gated on CD4+) (n=10, 5 independent experiments). (A) Two-way ANOVA and (B, D, E, G-I) unpaired T test; ns = not significant, *=p<0.05. **=p<0.01; ***=p<0.001; ****=p<0.0001.