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Therapeutic Efficacy of Suppressing the JAK/STAT Pathway in Multiple Models of Experimental Autoimmune Encephalomyelitis

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Pathogenic Th cells and myeloid cells are involved in the pathogenesis of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. The JAK/STAT pathway is used by numerous cytokines for signaling and is critical for development, regulation, and termination of immune responses. Dysregulation of the JAK/STAT pathway has pathological implications in autoimmune and neuroinflammatory diseases. Many of the cytokines involved in MS/EAE, including IL-6, IL-12, IL-23, IFN-γ, and GM-CSF, use the JAK/STAT pathway to induce biological responses. Thus, targeting JAKs has implications for treating autoimmunity of the brain. We have used AZD1480, a JAK1/2 inhibitor, to investigate the therapeutic potential of inhibiting the JAK/STAT pathway in models of EAE. AZD1480 treatment inhibits disease severity in myelin oligodendrocyte glycoprotein-induced classical and atypical EAE models by preventing entry of immune cells into the brain, suppressing differentiation of Th1 and Th17 cells, deactivating myeloid cells, inhibiting STAT activation in the brain, and reducing expression of proinflammatory cytokines and chemokines. Treatment of SJL/J mice with AZD1480 delays disease onset of PLP-induced relapsing-remitting disease, reduces relapses and diminishes clinical severity. AZD1480 treatment was also effective in reducing ongoing paralysis induced by adoptive transfer of either pathogenic Th1 or Th17 cells. In vivo AZD1480 treatment impairs both the priming and expansion of T cells and attenuates Ag presentation functions of myeloid cells. Inhibition of the JAK/STAT pathway has clinical efficacy in multiple preclinical models of MS, suggesting the feasibility of the JAK/STAT pathway as a target for neuroinflammatory diseases. The Journal of Immunology, 2014, 192: 59–72.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EdU, 5-ethyl-2’-deoxyuridine; LFB, luxol fast blue; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; qRT-PCR, quantitative RT-PCR; RR, relapsing-remitting; SOCS, suppressor of cytokine signaling; UN, unimmunized.

Multiple sclerosis (MS) is an autoimmune disease of the CNS characterized by demyelination, inflammatory lesions, axonal damage, activation of IFN-γ-producing Th1 cells and IL-17–producing Th17 cells, inappropriate activation of innate immune cells (macrophages, dendritic cells [DCs], neutrophils, and microglia), and aberrant production of cytokines/chemokines (1, 2). Th1 cells, Th17 cells, and innate immune cells are also implicated in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (3, 4). The pathogenesis of MS and EAE is associated with the overexpression of cytokines including IL-12, IFN-γ, IL-6, IL-21, and IL-23, which function in part to promote differentiation of effect Th1 and Th17 cells (1, 3, 5, 6).

The JAK/STAT signaling pathway is used by numerous cytokines and is critical for initiating innate immunity, orchestrating adaptive immunity, and ultimately constraining immune responses (7). Cytokines are of paramount importance in regulating the development, differentiation, and function of myeloid cells and T cells (8, 9); thus, unrestrained activation of the JAK/STAT pathway has pathological implications for autoimmune diseases (7, 10, 11). In MS and EAE, there is evidence for aberrant functionality of the JAK/STAT pathway. T cells and monocytes from MS patients during relapse have elevated levels of activated STAT3 compared with cells from patients in remission (12), and high levels of activated STAT3 in T cells from patients with clinically isolated syndrome predict conversion to clinically defined MS (13). In EAE, IL-6 has a deleterious role by activation of STAT3, which is pivotal for induction of pathogenic Th17 cells (14–16). Loss of STAT3 in T cells renders mice resistant to EAE disease (17, 18). STAT target genes, including IL-23R, IL-6, IL-17A, and IL-17F, are implicated in contributing to MS and EAE. The JAK/STAT pathway has received attention as a therapeutic target in autoimmune diseases and cancers (7, 11). JAK inhibitors have demonstrated clinical efficacy in rheumatoid arthritis and other inflammatory diseases (19–21). Indeed, Bright et al. (22) previously demonstrated that tyrphostin B42, a JAK2 inhibitor, reduced severity of EAE. JAK inhibitors interrupt signaling downstream of multiple cytokines, a useful approach for EAE and MS, which are characterized by a “cytokine storm” in the periphery and CNS. Simultaneous inhibition of cytokine signaling by JAK inhibitors...
may break the cycle of inflammation characteristic of neuroinflammatory diseases.

AZD1480, an ATP competitive inhibitor of JAK1 and JAK2, has beneficial effects in cancer models by suppressing downstream activation of STATs, particularly STAT3 (23, 24). We demonstrate that AZD1480 is effective in suppressing clinical symptoms in five preclinical models of MS. AZD1480 treatment was associated with diminished STAT activation in the CNS, reduced pathogenic Th1 and Th17 cell responses, alterations in DC and macrophage functionality, decreased infiltration of immune cells into the CNS, reduced demyelination, and suppression of proinflammatory cytokine/chemokine expression in vivo. Using AZD1480 for proof-of-principle, we demonstrate that inhibiting the JAK/STAT pathway has striking clinical efficacy in multiple models of EAE.

Materials and Methods

Mice

C57BL/6 and myelin oligodendrocyte glycoprotein (MOG)35–55 TCR-transgenic 2D2 mice (25) were bred in the animal facility at the University of Alabama (Birmingham, AL). SJL/J mice were purchased from the National Cancer Institute-Frederick National Laboratory (Frederick, MD). Suppressor of cytokine signaling (SOCS)3-floxed transgenic (SOCS3fl/fl) mice (26) were gifts from Dr. W. Alexander (Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia) and were bred at the University of Alabama. SOCS3 conditional knockout (LysMCre-SOCS3fl/fl) mice were immunized s.c. with 200 mg MOG35–55 emulsified in CFA (supplemented with 2% DMSO, as described previously (23, 24).

Peptides, Abs, and cytokines

MOG35–55 peptide was synthesized from New England Peptide, and PLP39–51 peptide was from CPC Scientific. LPS was from Sigma-Aldrich. Neutralizing Abs to IL-4 and IFN-γ; Abs against mouse CD3, CD220, Gr-1, CD4, IFN-γ, and IL-17A; and human CD3, CD4, CD14, and HLA-DR used for flow cytometry are from eBioscience. Mouse IL-6, IFN-γ, IL-12, IL-23, and GM-CSF and human IFN-γ and TGF-β1 are from R&D Systems. Abs against mouse and human CD3 and CD28 are from BioLegend. Abs against phospho-STAT1 (Tyr701) and phospho-STAT3 (Tyr705) were used for flow cytometry from Cell Signaling Technology, and phospho-STAT4 (Tyr694), phospho-STAT6 (Tyr641), phospho-p65 (Ser536), phospho-JAK2 (Tyr221), phospho-STAT3 (Tyr705), phospho-STAT4 (Tyr694), phospho-STAT5 (Tyr617), phospho-Smad3 (Ser465/467), phospho-STAT5 (Tyr617), phospho-STAT5 (Tyr694), phospho-Smad3 (Ser465/467), phospho-JAK2 (Tyr221), STAT1, STAT3, STAT4, STAT5, STAT6, p65, and JAK2 used for immunoblots are from Cell Signaling Technology. Abs against GAPDH is from Abcam, and Abs against Ly-6C, CD11c, MHC class II, CD40, CD80, CD86, CD11b, and CD45 are from BD Pharmingen.

EAE induction, assessment and treatment with AZD1480

Active and adoptive transfer EAE was induced as described previously (27, 28). Eight- to 12-wk-old C57BL/6 or LysMCre-SOCS3fl/fl mice were immunized s.c. with 200 μg MOG35–55 emulsified in CFA (supplemented with 2 mg/ml Mycobacterium tuberculosis) and injected i.p. on days 0 and 2 with 500 ng pertussis toxin. For adoptive transfer EAE, C57BL/6 mice were immunized with MOG35–55 for 10 d, and then, splenic and axillary lymph node cells were isolated and restimulated with MOG35–55 (10 μg/ml) under Th1 cell differentiation conditions or with 20 ng/ml IL-6, 20 ng/ml IL-23, and 3 ng/ml TGF-β1 for Th17 cell differentiation for 3 d. Cells (30 × 106) were transferred into healthy recipients. The frequency of donor CD4+ T cells producing IL-17A or IFN-γ was assessed by FACs prior to transfer (data not shown). Relapsing–remitting (RR) EAE was induced as follows: female SJL/J mice (8–12 wk old) were immunized with MOG35–55 for 10 d, and then, splenic and axillary lymph nodes and spleen as previously described (27), and cell phenotype was determined by surface and intracellular staining by flow cytometry.

Cell preparation

Bone marrow cells were cultured with RPMI 1640 medium containing 10% FBS (v/v) and 20 ng/ml mouse M-CSF for 7 d to obtain bone marrow-derived macrophages (BMDMs) (30). Naive CD4+ T cells were obtained from spleen or lymph nodes of 8- to 12-wk-old mice and isolated by Dynabeads CD4 Positive Isolation Kit (Invitrogen). T cells were cultured in RPMI 1640 medium supplemented with 10% FBS (v/v), 2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and nonessential amino acids, and 2 μM 2-ME.

Human subjects

Cryopreserved PBMC obtained by standard Histopaque density centrifugation from healthy donors were used in this study. These subjects were recruited from the Alabama Vaccine Research Clinic at the University of Alabama. To obtain monocytes, PBMC (3 × 106 cells/ml) were incubated in serum-free medium (RPMI 1640) supplemented with 10% FBS (v/v), 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES for 3 h at 37°C. Adherent cells were obtained by washing four times with prewarmed serum-free medium. For T cells, nonadherent PBMC were collected after the 3-h period of plastic adherence and washed three times with serum-free medium. Written informed consent was obtained from all donors who participated in this study. The Institutional Review Board of the University of Alabama approved the study (IRB number X090708004).
RNA isolation, RT-PCR, and TaqMan gene expression assays

Total RNA was isolated from the cerebellum and spinal cord of mice, and reverse transcription reactions performed as described previously (27). RNA (500 ng) was used to reverse transcribed into cDNA and subjected to quantitative RT-PCR (qRT-PCR). The data were analyzed using the comparative threshold cycle method to obtain relative quantitation values.

Immunoblotting

Thirty micrograms of cell lysate or brain homogenate was separated by electrophoresis and probed with Abs as described previously (27). All immunoblots are representative of three individual experiments.

Cell proliferation assay in vitro and in vivo

For in vitro proliferation, CFSE-labeled CD4+CD25− T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) Abs, and cell proliferation was assessed by CFSE dilution detected by flow cytometric analysis 72 h poststimulation. For in vivo proliferation, C57BL/6 mice were immunized with MOG35–55 (200 μg) and then administrated vehicle or AZD1480 (25 mg/kg) at day 7 postimmunization. Mice were then injected with 5-ethyl-2'-deoxyuridine (EdU) on day 13. CNS-infiltrating mononuclear cells were isolated from the cerebellum and spinal cord 12 h later, and EdU incorporation was detected for proliferation.

RESULTS

AZD1480 inhibits Th1 and Th17 cell differentiation in vitro

AZD1480 potently inhibits the JAK/STAT pathway in tumor cells (23, 24) but has not been evaluated in primary immune cells. AZD1480 at 0.25 μM partially inhibits STAT1 and STAT4 tyrosine phosphorylation in T cells cultured in Th1 cell polarizing conditions (Fig. 1A). The differentiation of naïve T cells to Th1 cells was strongly inhibited by AZD1480 treatment, as assessed by decreased IFN-γ production, and decreased mRNA levels of IFN-γ and T-bet (Fig. 1B). As some of the key cytokines for Th17 differentiation, including IL-6 and IL-23, signal through JAK1/2, we investigated the influence of inhibiting the JAK/STAT pathway on Th17 cell polarization. Using the Th17 cell differentiation mixture of IL-6, IL-23, and TGF-β, we observed that treatment with AZD1480 partially inhibited STAT3 tyrosine phosphorylation (Fig. 1C), which led to reduced differentiation of naïve T cells to Th17 cells (Fig. 1D). AZD1480 treatment also inhibited mRNA levels of the STAT3 target genes IL-17A, RORγt, IL-22, and IL-23R.

Statistical analysis

Levels of significance for comparison between two groups were determined by one-sided two-sample Mann–Whitney rank-sum test and the Student t test distribution. A p value < 0.05 was considered statistically significant.
23R (Fig. 1D). Th17 cells generated in the absence of TGF-β (with IL-6 + IL-23 or IL-6 + IL-23 + IL-1β), so called Th17 (23) cells, are more pathogenic in vivo (31). The differentiation of naïve T cells to Th17 cells in the presence of IL-6 + IL-23 (Supplemental Fig. 2A) or IL-6 + IL-23 + IL-1β (Supplemental Fig. 2B) also was substantially inhibited by AZD1480 treatment. Furthermore, the expression of STAT3 target genes under those differentiation conditions was inhibited by AZD1480 (Supplemental Fig. 2). These results clearly indicate that inhibition of the JAK/STAT pathway in T cells suppresses differentiation of Th1 and Th17 cells.

To assess whether the inhibitory effect of AZD1480 was a direct or indirect effect, short-term experiments were performed. As shown in Supplemental Fig. 3A and 3B, AZD1480 inhibited the phosphorylation of STAT1, JAK2, STAT3, and STAT5 in naïve T cells, suggesting a direct inhibitory effect on the JAK/STAT pathway.

AZD1480 blocks JAK/STAT activation and gene expression in macrophages and DCs

Many cytokines important in activating macrophages and DCs, including IFN-γ and GM-CSF, use the JAK/STAT pathway for signaling (7, 30). BMDMs were stimulated with IFN-γ, a key cytokine in polarizing macrophages to the proinflammatory M1 phenotype (30, 32). IFN-γ stimulation led to strong STAT1 and STAT3 tyrosine phosphorylation, which was inhibited by AZD1480 treatment (Fig. 2A). GM-CSF, another important cytokine in polarization of M1 macrophages and inflammatory DCs (32, 33), induced STAT5 tyrosine phosphorylation, and AZD1480 inhibited this response (Fig. 2B). To examine the effect of AZD1480 on upstream JAK activation, BMDM were stimulated with IL-6/soluble IL-6R to activate JAK2 and STAT3. AZD1480 pretreatment inhibited phosphorylation of both JAK2 and STAT3 (Fig. 2C). We examined the influence of AZD1480 on IL-4 signaling, which through activation of STAT6, induces polarization of macrophages to the M2 anti-inflammatory phenotype (34). IL-4 activation of STAT6 was largely unaffected by AZD1480 (Supplemental Fig. 3C), indicating that there are selective effects of AZD1480 on different STATs, dependent on cytokine stimulation. Interestingly, when BMDM were stimulated with LPS, rapid activation of the NF-κB pathway was not affected, whereas delayed activation of STAT1 and STAT3 at 2–4 h (via IFN induction) was inhibited (Fig. 2D), indicating the specificity of AZD1480 treatment.

NO is typically produced by M1 macrophages upon triggering of IFN-γ and TLR pathways (30). The expression of NO and its reactive derivative peroxynitrite has been implicated in the pathogenesis of MS and EAE (35, 36). Pretreatment of BMDM with AZD1480 significantly inhibited LPS/IFN-γ–induced production of nitrite (Fig. 2E). IFN-γ–inducible MHC class II expression is

**p < 0.001.
mediated by activation of STAT1 (37). AZD1480, in a dose-dependent manner, inhibited MHC class II expression in BMDM (Fig. 2F) and DCs (Fig. 2G). IFN-γ-inducible CD40 costimulatory molecule expression on DCs was also inhibited by AZD1480 (Fig. 2H). This suggests that AZD1480 may regulate the Ag presentation capability of these cells. Taken together, these findings indicate that AZD1480 inhibits JAK/STAT signaling in macrophages and DCs.

**Inhibition of the JAK/STAT pathway suppresses classical, atypical, and RR EAE**

Given the striking effect of AZD1480 in inhibiting Th1 and Th17 cell differentiation, as well as blocking IFN-γ-inducible gene expression in myeloid cells, we assessed the therapeutic potential of AZD1480 in EAE. First, we evaluated the effect of AZD1480 in naïve mice by analyzing proliferation of naïve CD4+ T cells and hematological parameters. Dosage was determined based on previous studies (23, 24) and on preliminary dose-response experiments (data not shown). No significant differences were noted in the proliferation of CD4+ T cells stimulated with anti-CD3 and anti-CD28, as well as in any hematological parameters in mice administrated AZD1480 (Fig. 3A, 3B). These results demonstrate that in vivo AZD1480 treatment does not induce toxicity when assessing circulating populations of the major cell types, consistent with studies using other JAK inhibitors, which are well-tolerated in vivo (21, 38).

C57BL/6 mice were immunized with MOG35–55 peptide to induce classical EAE, and AZD1480 (25 mg/kg) or vehicle control was administrated i.p. daily for 10 d starting at the onset of disease on day 9. Animals were scored daily for signs of ascending motor paralysis graded on a scale of 0–5. Most strikingly, AZD1480 treatment resulted in a significant reduction in disease severity compared with vehicle treatment (Fig. 4A). Evaluation of the spinal cord revealed strong inhibition of STAT3 and STAT4 tyrosine phosphorylation, and a modest reduction in expression of STAT3 and STAT4 protein was also observed with AZD1480 (Fig. 4B). Concordant with disease attenuation, the absolute number of mononuclear cells in the spinal cord was markedly decreased in AZD1480-treated mice, and the absolute numbers of neutrophils, monocytes, macrophages, microglia, DCs, CD4+ T cells, and B cells were significantly reduced in the spinal cord (Fig. 4C). There was also a marked reduction of IFN-γ, T-bet, and IL-17A mRNA levels (Fig. 4D), indicating an inhibitory effect on Th1 and Th17 cell entry into the CNS. AZD1480 treatment also inhibited mRNA levels of numerous proinflammatory cytokines and chemokines in the spinal cord (Fig. 4D). Histological characterization of the spinal cord revealed less inflammatory infiltrates and less demyelination in the AZD1480-treated group (Fig. 4E).

To investigate whether AZD1480 treatment influenced mice with established active EAE, AZD1480 (25 mg/kg) or vehicle control was administrated to C57BL/6 mice immunized with MOG35–55 after they developed a clinical score of 1.5 (Fig. 4F). This therapeutic administration of AZD1480 resulted in significant reduction of ongoing disease severity (Fig. 4F).

We previously demonstrated that mice with conditional knockout of SOCS3 in cells of the myeloid lineage (LysMCre-SOCS3fl/fl) develop early onset of a severe and nonresolving disease with features of atypical EAE, which is associated with hyperactivation of the JAK/STAT signaling pathway in the CNS (27). Thus, we evaluated the effect of inhibiting the JAK/STAT pathway in this EAE model. AZD1480-treated mice had delayed onset of disease and significantly reduced severity of atypical EAE disease (Fig. 5A). In a separate experiment, after treatment was stopped at day 14, the protective effect of AZD1480 was observed until day 22, at which point clinical scores started to increase (Supplemental Fig. 4). AZD1480 treatment prevented activation of STAT1 and STAT3 in the cerebellum and decreased total levels of STAT1 (Fig. 5B). Decreased infiltration of neutrophils, monocytes, microglia, DCs, and B cells in the cerebellum was observed (Fig. 5C), with reduced expression of Th1 markers (IFN-γ and T-bet) and Th17 markers (IL-17A), and reduced expression of cytokines/chemokines in the cerebellum comparable to unimmunized (UN) mice (Fig. 5D). Decreased inflammation and demyelination was observed in the cerebellum of AZD1480-treated mice compared with control mice (Fig. 5E). Oral gavage of AZD1480 produced a comparable protective effect in both classical and atypical EAE as i.p. treatment (data not shown). Collectively, these results indicate that AZD1480 has the desired, selective

**FIGURE 3.** Effect of AZD1480 on CD4+ T cell proliferation and hematologic parameters. (A) C57BL/6 mice were injected i.p. with vehicle control (DMSO) or AZD1480 (25, 50, or 100 mg/kg) for 4 d. Naïve CD4+ T cells isolated from the spleen of vehicle control or AZD1480 treated mice were labeled with CFSE and stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) Abs for 4 d. T cell proliferation was analyzed by flow cytometry based on the dilution of CFSE intensity. (B) Peripheral blood from vehicle control or AZD1480 (25 or 50 mg/kg)-treated mice (n = 3) was analyzed with HEMAVET950. The number of RBCs, hemoglobin, WBCs, neutrophils, lymphocytes, and monocytes were calculated.
The JAK1/2 inhibitor AZD1480 suppresses classical EAE. (A) C57BL/6 mice were immunized with MOG35–55 peptide (200 μg) emulsified in CFA containing M. tuberculosis. The mice received i.p. injections of 250 ng pertussis toxin on days 0 and 2. Vehicle control (0.1% DMSO) (n = 14) or AZD1480 (25 mg/kg) (n = 15) was administered i.p. daily for 10 d starting at the onset of EAE (day 9). Mean ± SD of classical EAE clinical scores. (B) Protein extracts from the spinal cords of vehicle control or AZD1480-treated mice at day 16 were immunoblotted with the indicated Abs. (C) CNS-infiltrating mononuclear cells were isolated from the spinal cords of vehicle control or AZD1480-treated mice at day 16. Cells were stained with trypan blue and counted. Cells were stained with Abs to CD4, CD11b, Gr-1, CD45, CD11c, Ly-6C, and B220, and the percentage of CD11b+/Gr-1+ neutrophils, CD11b+/Ly-6C+ and CD11b+/Ly-6C− monocytes, CD11b+/CD45− macrophages, CD11b−/CD45+ microglia, CD11c+ DCs, CD4+ T cells, and B220+ B cells were gated. The absolute number of cells is shown. (D) Mice were perfused, the spinal cords were removed, and mRNA from the spinal cords of vehicle control or AZD1480-treated mice at day 16 was analyzed by qRT-PCR. (E) Sections from the spinal cords of vehicle control or AZD1480-treated mice at day 16 were stained with H&E for inflammatory infiltrates and LFB for demyelination. The mice received i.p. injections of 250 ng pertussis toxin on days 0 and 2. Vehicle control (0.1% DMSO) (n = 12) or AZD1480 (25 mg/kg) (n = 13) was administrated i.p. after mice reached a clinical score of ~1.5 for up to 12 d. *p < 0.05, **p < 0.001.

**Inhibition of the JAK/STAT pathway influences the priming and expansion phases of EAE**

Next, we examined the underlying cellular mechanisms leading to clinical improvement of actively induced EAE by AZD1480 treatment. Because MOG35–55-specific T cells are first primed to differentiate into specialized effector subsets in the periphery (3), the effect of AZD1480 on the priming phase of EAE was investigated. C57BL/6 mice were immunized with MOG35–55, and vehicle or AZD1480 (25 mg/kg) was administered at day 2 post-immunization for 3 d (Fig. 7A). Purified CD4+ T cells were isolated from the draining lymph nodes and spleen of these mice on day 10 postimmunization, restimulated with MOG35–55 peptide under Th1- and Th17-polarizing conditions, and the percentage of MOG35–55-specific T cells measured by flow cytometry. Treatment with AZD1480 led to a significant decrease of MOG35–55-specific Th1 and Th17 cells in the draining lymph nodes and spleen (Fig. 7B, 7C). Next, the effect of AZD1480 on expansion of MOG-specific T cells was examined. C57BL/6 mice were immunized with MOG35–55 and administrated vehicle or AZD1480 (25 mg/kg) at day 7 postimmunization for 3 d (Fig. 7D). Purified CD4+ T cells were isolated and stimulated as above. Decreased MOG35–55-specific Th1 cells in the draining lymph nodes were observed (Fig. 7E), and diminished percentages of MOG-specific Th17 cells were detected in draining lymph nodes and spleen (Fig. 7F). These results indicate that inhibition of the JAK/STAT pathway influences both the priming and expansion phases of EAE by suppressing MOG35–55-specific T cell responses in secondary lymphoid tissues.

**JAK/STAT pathway inhibition suppresses proliferation of CD4+ T cells and CD11b+ cells**

The decreased number of CNS-infiltrating cells in AZD1480-treated mice may be due to reduced cell proliferation; thus, the effect of AZD1480 on T cell and myeloid cell proliferation during EAE development was assessed. C57BL/6 mice were immunized...
with MOG<sub>35-55</sub> and then administrated vehicle or AZD1480 (25 mg/kg) at day 7 postimmunization. Mice were then perfused, the cerebellums were removed, and protein extracts from UN or MOG<sub>35-55</sub>-immunized vehicle control or AZD1480-treated mice at day 14 were immunoblotted with the indicated Abs. (C) CNS-infiltrating mononuclear cells were isolated from the cerebellum of vehicle control or AZD1480-treated mice at day 14 after MOG immunization. Cells were stained with Abs to CD4, CD11b, Gr-1, CD45, CD11c, Ly-6C, and B220, and the percentages of CD11b<sup>+</sup>/Gr-1<sup>+</sup> neutrophils, CD11b<sup>+</sup>/Ly-6C<sup>hi</sup> and CD11b<sup>+</sup>/Ly-6C<sup>lo</sup> monocytes, CD11b<sup>+</sup>/CD45<sup>hi</sup> macrophages, CD11b<sup>+</sup>/CD45<sup>int</sup> microglia, CD11c<sup>+</sup> DCs, CD4<sup>+</sup> T cells, and B220<sup>+</sup> B cells were gated. The absolute number of cells is shown. (D) Mice were perfused, the cerebellums were removed, and mRNA was analyzed by qRT-PCR. (E) Sections from the cerebellums of vehicle control or AZD1480-treated mice at day 14 were stained with H&E for inflammatory infiltrates and LFB for demyelination. Original magnification ×60. *p < 0.05, **p < 0.001.

The transfer of myelin-specific CD4<sup>+</sup> T cells induces EAE in naive recipients; thus, we tested whether AZD1480 treatment would be effective in treating EAE induced by adoptive transfer of pathogenic CD4<sup>+</sup> Th1 or Th17 cells. C57BL/6 mice were adoptively transferred with MOG<sub>35-55</sub>-specific Th1 or Th17 cells to induce EAE, and AZD1480 was administrated i.p. daily for 10 d starting at day 7 for Th1-induced EAE (Fig. 8A) or on day 8 for Th17-induced EAE (Fig. 8D). Notably, we observed a delay in onset of disease from days 10 to 14 and diminished disease severity in Th1-induced EAE (Fig. 8A). When AZD1480 treatment was discontinued, a protective effect was still noted at days 17 and 18 but was lost at days 19 and 20. A substantial reduction of STAT1 and STAT3 tyrosine phosphorylation in the spinal cord of AZD1480-treated mice was observed (Fig. 8B), and mRNA levels of proinflammatory cytokines were markedly decreased (Fig. 8C). AZD1480 treatment suppressed mRNA levels of IFN-γ and T-bet in Th1-induced EAE at the site of disease in the spinal cords of treated mice (Fig. 8C). It should be noted that IL-17A and RORγt mRNA expression was not detected in mice with Th1-induced EAE (data not shown). In Th17-induced EAE, disease onset and severity were significantly inhibited by AZD1480 treatment (Fig. 8D), although the mice developed clinical signs of EAE after AZD1480 withdrawal. STAT1 and STAT3 tyrosine phosphorylation was inhibited in Th17-induced EAE by AZD1480 as were total levels of STAT1 and STAT3 protein (Fig. 8E). In addition, markers of Th1 and Th17 cells and proinflammatory cytokine mRNA levels were inhibited by AZD1480 treatment (Fig. 8F). These data indicate that AZD1480, by inhibiting the JAK/STAT pathway, can diminish peripheral T cell–mediated damage against the CNS in vivo.

AZD1480 treatment in vivo affects the function of myeloid cells and Th1 cells

Myeloid cells are pivotal for the development of EAE by modulating T cell responses because of the production of proinflammatory
cytokines and presentation of Ag. To assess the functional influence of JAK/STAT inhibition in myeloid cells, C57BL/6 mice were injected with vehicle or AZD1480 (25 mg/kg) for 4 d, and BMDM isolated from these mice were examined for JAK/STAT pathway activation. IL-6–induced STAT3 tyrosine phosphorylation was strongly inhibited in BMDM obtained from AZD1480-treated mice (Fig. 9A), as was IFN-γ–induced STAT1 tyrosine phosphorylation (Fig. 9B). In addition, IFN-γ–inducible MHC class II expression was suppressed by in vivo AZD1480 treatment (Fig. 9C). Consistent with the in vitro data, LPS activation of the NF-κB pathway was not affected (Fig. 9D).

To address whether inhibiting the JAK/STAT pathway modulates the APC functions of myeloid cells, purified CD11b+/CD11c+ cells from vehicle- or AZD1480-treated C57BL/6 mice were used as APCs for stimulation of naïve MOG35–55–specific 2D2 CD4+ T cells under Th1- or Th17-polarizing conditions. Notably, we found that APCs from AZD1480-treated mice (AZD-APC) were less efficient than those from vehicle-treated mice (V-APC) in promoting MOG35–55–specific Th1 cell differentiation (Fig. 9E, compare panels 1, 2) and Th17 cell differentiation (Fig. 9F, compare panels 1, 2). These results indicate that CD11b+/CD11c+ APCs from AZD1480-treated mice are defective in their ability to induce MOG35–55–specific Th1 and Th17 cell differentiation.

Next, the response of purified naïve MOG35–55–specific CD4+ T cells from vehicle- or AZD1480-treated 2D2 cells to CD11b+/CD11c+ APCs from vehicle-treated C57BL/6 mice was examined. CD4+ MOG-specific T cells from AZD1480-treated 2D2 mice (AZD-T cells) were unable to differentiate into Th1 cells when provided with vehicle-treated APCs (Fig. 9E, compare panels 1, 3), and there was a further decrease when MOG-specific T-cells from AZD1480-treated 2D2 mice were cultured with AZD1480-treated APCs (Fig. 9E, panel 4). However, CD4+ T cells from AZD1480-treated 2D2 mice were capable of differentiating into Th17 cells in the presence of APCs (Fig. 9F, compare panels 1, 3). We next investigated whether the inhibitory effect of AZD1480 on Th1 cell differentiation was Ag specific. Purified naïve CD4+ T cells from vehicle- or AZD1480-treated C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 Abs under Th1 or Th17 differentiation conditions. Consistent with the results from MOG35–55–specific T cells, in vivo treatment with AZD1480 resulted in partial inhibition of Th1 cell polarization (Fig. 9G), whereas Th17 cell polarization was not affected (Fig. 9H). These findings indicate that in vivo inhibition of the JAK/STAT pathway has an inhibitory effect on the ability of CD4+ T cells to become polarized to the Th1 phenotype but does not appear to affect the Th17 polarization program.

**AZD1480 inhibits the JAK/STAT pathway in human T cells and monocytes**

The findings thus far demonstrate that AZD1480 suppresses the function of murine T cells and macrophages. To extend these findings to humans, PBMCs were stimulated in vitro with anti-CD3 and anti-CD28 Abs in the absence or presence of AZD1480, and phosphorylation of STAT1, STAT3, and STAT4 was examined in gated CD3+CD4+ T cells. Treatment with AZD1480 led to substantially reduced levels of STAT1 and STAT3 tyrosine phosphorylation and partial inhibition of STAT4 tyrosine phosphorylation (Fig. 10A). In addition, mRNA levels of IFN-γ and IL-17A were significantly reduced by AZD1480 (Fig. 10B). We also assessed the effect of AZD1480 on the JAK/STAT signaling pathway in human monocytes. CD3+CD14+ monocytes were stimulated with IFN-γ in the absence or presence of AZD1480, and phosphorylation of STAT1 and STAT3 was examined. IFN-γ induced strong phosphorylation of STAT1 and moderate phosphorylation of STAT3, both of which were inhibited by AZD1480 (Fig. 10C). Furthermore, consistent with the inhibition of IFN-γ–induced STAT1 phosphorylation, the expression of IFN-γ–inducible HLA-DR expression in AZD1480-treated monocytes was strongly suppressed (Fig. 10D). These in vitro experiments demonstrate that activated human T cells and monocytes are direct targets of AZD1480.

**Discussion**

Genome-wide association studies have shown that cytokines, their receptors, JAKs, STATs, and SOCS proteins are associated with human autoimmune diseases, especially pathways leading to STAT3...
and STAT4 activation (39, 40). STAT3 has been identified as an MS susceptibility gene (41, 42), and independent replication supports the association between STAT3 and increased MS risk (43). We demonstrate that inhibition of the JAK/STAT pathway, specifically inhibition of STAT1, STAT3, and STAT4 activation, has broad actions on cells of the innate and adaptive immune systems, leading to amelioration of clinical disease in EAE models. Treatment with AZD1480, an inhibitor of JAK1 and JAK2, effectively prevents infiltration of immune cells into the CNS, inhibits STAT activation in the CNS, suppresses differentiation and proliferation of CD4+ cells, inhibits proliferation and deactivates myeloid cells, and reduces expression of proinflammatory cytokines and chemokines.

In MS and EAE, the pivotal role of pathogenic Th1 and Th17 cells, as well as myeloid cells, has been well documented (44–46). Inhibition of the JAK/STAT pathway by AZD1480 suppressed the in vitro differentiation of naive T cells to Th1 cells and also suppressed in vitro Th17 cell differentiation induced by three different polarization conditions: IL-6 + IL-23 + TGF-β; IL-6 + IL-23; and IL-6 + IL-23 + IL-1β. Because STAT3 signaling is a central component of Th17 cell differentiation and Th17-dependent autoimmune processes (17, 47), using a JAK inhibitor that suppresses STAT3 activation is a promising strategy for therapeutic intervention. Disease-supporting functions of macrophages, DCs, and microglia have been described for CNS autoimmune diseases (45, 48, 49). In vitro results indicate that AZD1480 suppresses IFN-γ induction of genes that mediate some of the detrimental effects of macrophages and DCs, including MHC class II, CD40, and NO. IFN-γ promotes the polarization of macrophages to the classically activated, M1 phenotype, characterized by high levels of IL-6, IL-1, IL-12, and IL-23; increased levels of reactive oxygen species, MHC class II and CD40, and low levels of IL-10 (50). M1 macrophages have detrimental effects in a number of CNS diseases, including EAE/MS, Alzheimer’s disease, and spinal cord injury (51–55). We demonstrated that AZD1480 inhibited IFN-γ-induced NO release, which contributes in a significant manner to EAE pathogenesis and axonal damage (35). AZD1480 suppressed IFN-γ induction of MHC
For 10 d, splenocytes and lymph node cells were isolated, and then, MOG-specific T cells were cultured with MOG35–55 peptide (10 μg/ml) under Th1 differentiation conditions for 3 d. Cells (30 × 10^6) were injected i.v. into C57BL/6 mice. Vehicle control (n = 10) or AZD1480 (25 mg/kg) (n = 11) was administrated daily by i.p. starting at day 7 for 10 d. Mean ± SD of classical EAE clinical scores. (B) Mice were perfused, the spinal cords were removed, and mRNA from vehicle control or AZD1480-treated mice at day 17 was analyzed by qRT-PCR. (C) Mice were perfused, the spinal cords were removed, and protein extracts from spinal cord of vehicle control or AZD1480-treated mice at day 17 was analyzed by qRT-PCR. (D) C57BL/6 mice were immunized with MOG35–55 peptide (200 μg) for 10 d, splenocytes and lymph node cells were isolated, and then, MOG-specific T cells were cultured with MOG35–55 peptide (10 μg/ml) under Th1 differentiation conditions for 3 d. Cells (30 × 10^6) were injected i.v. into C57BL/6 mice. Vehicle control (n = 11) or AZD1480 (25 mg/kg) (n = 10) was administrated daily by i.p. starting at day 8 for 10 d. Mean ± SD of classical EAE clinical scores. (E) Mice were perfused, the spinal cords were removed, and protein extracts from spinal cord of vehicle control or AZD1480-treated mice at day 18 was analyzed by qRT-PCR. *p < 0.05, **p < 0.001.

FIGURE 8. Effect of AZD1480 on Th1- and Th17-induced adoptive transfer EAE. (A) C57BL/6 mice were immunized with MOG35–55 peptide (200 μg) for 10 d, splenocytes and lymph node cells were isolated, and then, MOG-specific T cells were cultured with MOG35–55 peptide (10 μg/ml) under Th1 differentiation conditions for 3 d. Cells (30 × 10^6) were injected i.v. into C57BL/6 mice. Vehicle control (n = 10) or AZD1480 (25 mg/kg) (n = 11) was administrated daily by i.p. starting at day 7 for 10 d. Mean ± SD of classical EAE clinical scores. (B) Mice were perfused, the spinal cords were removed, and protein extracts from spinal cord of vehicle control or AZD1480-treated mice at day 17 was immunoblotted with the indicated Abs. (C) Mice were perfused, the spinal cords were removed, and mRNA from spinal cord of vehicle control or AZD1480-treated mice at day 17 was analyzed by qRT-PCR. (D) C57BL/6 mice were immunized with MOG35–55 peptide (200 μg) for 10 d, splenocytes and lymph node cells were isolated, and then, MOG-specific T cells were cultured with MOG35–55 peptide (10 μg/ml) under Th1 differentiation conditions for 3 d. Cells (30 × 10^6) were injected i.v. into C57BL/6 mice. Vehicle control (n = 11) or AZD1480 (25 mg/kg) (n = 10) was administrated daily by i.p. starting at day 8 for 10 d. Mean ± SD of classical EAE clinical scores. (E) Mice were perfused, the spinal cords were removed, and protein extracts from spinal cord of vehicle control or AZD1480-treated mice at day 18 were immunoblotted with the indicated Abs. (F) Mice were perfused, the spinal cords were removed, and mRNA from spinal cord of vehicle control or AZD1480-treated mice at day 18 was analyzed by qRT-PCR. *p < 0.05, **p < 0.001.

class II and CD40, which are critical for Ag presentation functions of macrophages/DCs. IFN-γ induction of MHC class II and CD40 expression is STAT1 dependent (56, 57), indicating that inhibition of the JAK/STAT pathway is an effective means of suppressing these genes. In macrophages, AZD1480 inhibited GM-CSF activation of STAT5, and IL-6 activation of STAT3, while not influencing IL-4 activation of STAT6. IFN-γ, GM-CSF, and IL-6 signaling use JAK1 and/or JAK2, which explains the inhibitory effect of AZD1480. IL-4 signaling involves both JAK1 and JAK3; thus, inhibition of JAK1 by AZD1480 may not suffice to inhibit this pathway because of the kinase activity of JAK3.

Inhibition of the JAK/STAT pathway by AZD1480 has beneficial effects in mitigating clinical symptoms in five EAE models: chronic progressive disease in C57BL/6 mice; atypical disease with involvement of the cerebellum in LysMCre-SOCS3fl/fl mice; RR disease in SJL/J mice; adoptive Th1 cell EAE; and adoptive Th17 cell EAE. Improvement in clinical scores was associated with suppression of inflammatory responses in lymph node and spleen, indicating that inhibition of the JAK/STAT pathway affects the pathogenic potential of T cells outside the CNS. The most reliable parameter of JAK inhibition is that of downstream inhibition of STAT activation (23, 24). AZD1480 treatment inhibited STAT1, STAT3, and/or STAT4 activation within the CNS. These STATs are activated downstream of JAK1 and/or JAK2 and are essential for development and differentiation of Th1 and Th17 cells and maturation of myeloid cells. Thus, inhibition of STAT activation may be responsible for the reduction of inflammatory lesions and diminished expression of proinflammatory mediators in the CNS. Total levels of STAT proteins were also inhibited by AZD1480 treatment, most notably that of STAT1. STAT1 is itself a STAT1-inducible gene (37); therefore, inhibiting STAT activity will function in a negative feedback loop to inhibit STAT expression. Mice deficient in STAT3 in CD4+ T cells are resistant to EAE (17, 18), as are mice deficient in STAT4 (58), whereas mice lacking STAT1 are highly susceptible to EAE (25). It is possible that in the absence of STAT1, STAT3, and/or STAT4 signaling may compensate to drive Th1 cell responses, because these mice were characterized by IFN-γ-producing Th1 cells (25). Nonetheless, our results demonstrate that pharmacologic inhibition of STAT1, STAT3, and STAT4 signaling contributes to significant lessening of EAE disease severity.

The beneficial effects of inhibiting the JAK/STAT pathway on EAE pathogenesis appear to involve a number of mechanisms, including effects on both APCs and T cells. First, both the primary and expansion phases of EAE were inhibited by AZD1480 treatment, as reflected by suppressed Ag-specific Th1 and Th17 responses in secondary lymphoid tissues, indicating that inhibition of the JAK/STAT pathway affects the early stages of the immunological cascade leading to EAE. Second, this, in turn, leads to diminished infiltration of immune cells into the CNS. Third, of the T cells and myeloid cells that did extravasate into the CNS, AZD1480 treatment diminished their proliferative capacity, which
we believe contributes to the therapeutic effectiveness of AZD1480 treatment. Fourth, in vivo AZD1480 treatment inhibits both the innate and adaptive arms of the immune system that are associated with EAE. AZD1480 treatment in vivo was effective in inhibiting the ability of T cells to differentiate into Th1 cells, in both Ag-specific and non–Ag-specific manners. This intriguing finding needs to be examined in more detail but may relate to the effectiveness of AZD1480 in inhibiting IFN-γ and IL-12 signaling, which are critical for Th1 cell differentiation. This suggests a direct effect of AZD1480 on Th1 differentiation. In contrast, although our in vitro data documented an inhibitory effect of AZD1480 on Th17 cell differentiation, we did not observe a direct inhibitory effect on Th17 cell polarization in vivo. This may reflect the complexities of promoting Th17 cell differentiation by diverse cytokines including TGF-β1, TGF-β3, IL-1, IL-6, and IL-23 and inhibition by IL-2 and IL-27 (59, 60). We do know that AZD1480 is a strong inhibitor of IL-6 and IL-23 signaling and suppresses expression of the IL-23R on T cells, a critical determinant of Th17 cell polarization. However, the influence of inhibiting the JAK/STAT pathway on TGF-β1, TGF-β3, and IL-27 signaling is unknown, and IL-1β signaling is unaffected (data not shown). Inhibition of the JAK/STAT pathway has potent sup-
pressive effects on myeloid cell functions in vivo. AZD1480 treatment renders myeloid cells nonresponsive to IFN-γ and IL-6 signal transduction, resulting in inhibition of STAT1 and STAT3 activation, and downstream inhibition of gene expression such as MHC class II. Furthermore, AZD1480 treatment has a direct effect on APC functions of myeloid cells as demonstrated by the findings that CD11b+/CD11c+ APCs from AZD1480-treated mice inhibited the development of MOG35–55-specific Th1 and Th17 cells. This is in line with observations on the in vivo treatment of mice with glatiramer acetate, an approved therapy for RRMS (61), fumarates (62), which were approved by the U.S. Food and Drug Administration on March 27, 2013, for treatment of RRMS, and laquinimod, which has been successfully evaluated in phase II/III studies of RRMS (63, 64). All of these agents had direct effects on cells of the innate immune system and, importantly, skewed macrophages and DCs toward a regulatory phenotype. Characteristics of the regulatory, anti-inflammatory phenotype included reduced STAT1 phosphorylation, decreased production of IL-6, IL-12, and IL-23, and elevated secretion of IL-10 (61–64). At this point, we do not know whether in vivo treatment with JAK inhibitors skews monocytes/macrophages and DCs toward a regulatory phenotype, but we do demonstrate a suppression of the M1 proinflammatory phenotype by AZD1480. Thus, collectively, AZD1480 functions to promote immune modulation by a direct effect on myeloid APCs, which impacts the differentiation of Th1 and Th17 cells in vivo, as well as a direct effect of AZD1480 in vivo on Th1 cell differentiation.

Studies have implicated the JAK/STAT axis in regulating clinical manifestations of EAE. Peroxisome proliferator activated receptor γ and cyclooxygenase 2 inhibitors suppress EAE severity, in part, by inhibiting IL-12–induced activation of the JAK/STAT pathway and subsequent suppression of Th1 cell differentiation (65, 66). The protective effect of glatiramer acetate in EAE is in part due to inhibition of STAT4 and STAT3 phosphorylation in T cells, altering Th1 and Th17 cell differentiation, respectively (67). Two herbal compounds, plumbagin and berberine, exert protective effects in EAE models by inhibiting STAT activation and Th1 and Th17 cell differentiation (68, 69). In this study, we have used a specific inhibitor of the JAK/STAT pathway, AZD1480, and documented a striking beneficial immunomodulatory effect in five different models of EAE. Importantly, AZD1480 treatment was administered at the onset of disease and in a therapeutic manner after the appearance of clinical symptoms, with potent clinical efficacy. These findings collectively suggest the JAK/STAT axis may serve as a therapeutic target for intervention in MS, as well as other neuroinflammatory conditions such as Parkinson’s disease, spinal cord injury, and Alzheimer’s disease.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Figure Legends of Supplemental Figures:

Figure S1. Chemical Structure of the JAK1/JAK2 Inhibitor AZD1480.

Figure S2. AZD1480 Inhibition of Th17 Cell Differentiation Under Different Polarization Conditions In Vitro. Naïve CD4+ T-cells were isolated from the spleen of C57BL/6 mice. Dendritic cells (DCs) were used as antigen-presenting cells at a 1:5 ratio to CD4+ T-cells in co-culture. Vehicle control or AZD1480 (0.25 μM) was added simultaneously with IL-6 + IL-23 Th17 differentiation cocktail (A) or with IL-6 + IL-1b + IL-23 Th17 differentiation cocktail (B). At day 4, cells were stimulated with PMA/Ionomycin plus GolgiStop for 4 h, stained for the surface marker CD4 and by intracellular flow for IL-17A. For mRNA expression, cells were collected at day 4, and mRNA was analyzed by qRT-PCR for RORgt, IL-17A, IL-17F, IL-22 and IL-23R. *p<0.05 and **p<0.001.

Figure S3. Inhibitory Effect of AZD1480 in Naïve CD4+ T-cells and Macrophages. (A). Naïve CD4+ T-cells were isolated from the spleen of C57BL/6 mice, and pretreated with Vehicle or AZD1480 (0.25 μM) for 2 h. Cells were then stimulated with medium (-) or IFN-γ (10 ng/ml) for up to 60 min. STAT1 tyrosine phosphorylation was examined by flow cytometry. (B). Naïve CD4+ T-cells were isolated from the spleen of C57BL/6 mice, and pretreated with Vehicle or AZD1480 (0.25 μM) for 2 h. Cells were then stimulated with medium (-) or IL-6 (10 ng/ml) plus sIL-6R (25 ng/ml) for up to 2 h. Cell lysates were immunoblotted with the indicated antibodies (left panel). The fold activation of phosphorylated JAK2, STAT3 and STAT5, and the inhibitory effect of AZD1480 is shown in the right panel. Mean ± S.D. of three independent experiments. **p<0.001. (C). BMDMs were treated with Vehicle Control or AZD1480 (0.25 μM) for 2 h, stimulated with medium (-) or IL-4 (10 ng/ml) for 0.5 h, then cell lysates were subjected to immunoblotting with the indicated antibodies (left panel). The fold activation of phosphorylated STAT6 in the absence or presence of AZD1480 is shown in the right panel. Mean ± S.D. of three independent experiments. **p<0.001.

Figure S4. The JAK1/2 Inhibitor AZD1480 Suppresses EAE Disease after Termination of Treatment. LysMCre-SOCS3fl/fl mice were immunized with MOG35-55peptide (200 μg)
emulsified in CFA containing *M. tuberculosis*. The mice received intra-peritoneal injections of 250 ng of pertussis toxin at days 0 and 2. Vehicle Control (n=5) or AZD1480 (25 mg/kg) (n=5) was administrated i.p. daily for 10 days starting at day 5 post-immunization. Mean of atypical EAE clinical scores was monitored up to day 30 in AZD1480 treated mice, and up to day 15 in vehicle-control treated mice.
Liu, Y. et al., Figure S1

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Mol. Wt.: 348.77
Liu, Y. et al., Figure S2

A.

Vehicle  AZD 0.25 µM

IL-6 + IL-23

IL-17A

Fold of mRNA expression

RORγt  IL-17A  IL-17F  IL-22  IL-23R

Vehicle  AZD 0.25 µM

B.

Vehicle  AZD 0.25 µM

IL-6 + IL-1β + IL-23

IL-17A

Fold of mRNA expression

RORγt  IL-17A  IL-17F  IL-22  IL-23R
Liu, Y. et al., Figure S3

A. 15 min  30 min  60 min

B. -  0.5  1  2  0.5  1  2

IL-6/sIL-6R (h)

P-JAK2
JAK2
P-STAT3
STAT3
P-STAT5
STAT5
GAPDH

Vehicle  AZD1480

CD4+ T-Cells

C. 0  0.5  0  0.5

IL-4 (h)

P-STAT6
STAT6
GAPDH

BMDM
Liu, Y. et al., Figure S4

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Atypical EAE Clinical Score

0
1
2
3
4