Regulation of autoreactive CD4 T cells by FoxO1 signaling in CNS autoimmunity

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Abstract

Background

Myelin-specific CD4 T effector cells (Teffs), Th1 and Th17 cells, are encephalitogenic in experimental autoimmune encephalomyelitis (EAE), a well-defined murine model of multiple sclerosis (MS) and implicated in MS pathogenesis. Forkhead box O 1 (FoxO1) is a conserved effector molecule in PI3K/Akt signaling and critical in the differentiation of CD4 T cells into T helper subsets. However, it is still unclear whether FoxO1 may be a target for redirecting CD4 T cell differentiation and benefit CNS autoimmunity.

Methods

Using a selective FoxO1 inhibitor AS1842856, we determined the effects of FoxO1 inhibition in regulating myelin-specific Th1 and Th17 cells, and the transcriptional balance of T-bet and Foxp3 in myelin-specific CD4 T cells from EAE mice. The effects of AS1842856 in regulating the encephalitogenicity of myelin-specific T cells and the expansion of human Th1 cells from MS patients were also characterized. Furthermore, we characterized the potential role of FoxO1 in mediating PD-1 signaling in CD4 T cells, critical for regulating Teff and Treg cells.

Results

Inhibition of FoxO1 suppressed the differentiation and expansion of Th1 cells. Moreover, the transdifferentiation of Th17 cells into encephalitogenic Th1-like cells was suppressed by FoxO1 inhibition upon reactivation of myelin-specific CD4 T cells from mice with EAE. When FoxO1 was inhibited in myelin-specific CD4 T cells, the transcriptional balance skewed from the Th1 transcription factor T-bet toward the Treg transcription factor Foxp3. Myelin-specific CD4 T cells treated with the FoxO1 inhibitor were less encephalitogenic in adoptive transfer EAE studies compared to control-treated cells. Inhibition of FoxO1 in T cells from MS patients significantly
suppressed the expansion of Th1 cells. Furthermore, the immune checkpoint programmed cell
death protein-1 (PD-1)-induced Foxp3 expression in CD4 T cells was impaired by FoxO1
inhibition, consistent with a bias toward Treg induction.

Conclusions:
These data illustrate an important role of FoxO1 signaling in CNS autoimmunity via regulating
autoreactive Teff and Treg balance.

**Keywords:** Multiple Sclerosis (MS), experimental autoimmune encephalomyelitis (EAE).
Forkhead box O 1 (FoxO1), AS1842856, T effector cells, T regulatory cells, Central Nervous
System (CNS)
Introduction

Multiple Sclerosis (MS) is an immune-mediated central nervous system (CNS) disease characterized by neuroinflammation, demyelination, and neuronal degeneration. MS is the leading cause of non-traumatic neurologic disability in young adults and affects over 1 million people in US (1). Myelin-specific CD4 T effector cells (Teff), Th1 and Th17 cells, drive the formation of acute inflammatory demyelinating lesions and clinical relapses in experimental autoimmune encephalomyelitis (EAE) model of MS and have been implicated in MS pathogenesis (2, 3). Early studies demonstrated Th1 cells that express key transcription factor T-bet and signature cytokine IFNγ to be highly encephalitogenic in EAE (4). Subsequent studies have identified Th17 cells as another encephalitogenic CD4 Teff population in EAE. Th17 cells display a high degree of plasticity upon antigen reencounter, depending on the inflammatory milieu in the microenvironment (5). TGFβ/IL-6 induces the development of Th17 cells that express the transcription factor RORγt and cytokine IL-17. However, they are not encephalitogenic in EAE adoptive transfer studies (6-8). In a microenvironment that is rich in IL-12 and/or IL-23, non-encephalitogenic RORγt+ Th17 cells may transdifferentiate into RORγt+ T-bet+ Th1-like cells and further convert into RORγt T-bet+ Th1 cells, both of which are highly pathogenic in MS/EAE and other autoimmune diseases (9-12).

Forkhead box O1 (FoxO1) is a conserved effector molecule in PI3k/Akt signaling that regulates CD4 T cell development and function. The role of FoxO1 in regulating myelin-specific Th1 in CNS autoimmunity has not been well-characterized. Although FoxO1 has been shown to suppress TGFβ/IL-6-induced differentiation of naïve CD4 T cells into RORγt+Th17 cells (13), its role in regulating Th17 transdifferentiation is unclear. FoxO1 plays a critical role in maintaining naive T cell quiescence and survival. As several FoxO-targeted genes are important regulators of
naïve T cell trafficking and functions (14-16), FoxO1 deficiency may alter the quiescence of naïve CD4 T cells. Therefore, we took a pharmacological approach to characterize the potential role of FoxO1 signaling in regulating autoreactive CD4 Teff and Treg cells. AS1842856 is a small molecule compound that binds FoxO1 and inhibits FoxO1 transactivation (17). AS1842856 has been used extensively to determine the role of FoxO1 in regulating glucose metabolism, pulmonary hypertension, adipocyte differentiation and cancer development (17-21). To understand the potential role of FoxO1 signaling in CNS autoimmunity, we determined the effects of AS1842856 in regulating myelin-specific Th1 and Th17 cells, and the transcriptional balance of T-bet and Foxp3 in myelin-specific CD4 T cells from EAE mice. The effects of AS1842856 in regulating the encephalitogenicity of myelin-specific T cells and the expansion of human Th1 cells from MS patients were also characterized. Furthermore, we characterized the potential role of FoxO1 in mediating PD-1 signaling in CD4 T cells, critical for regulating Teff and Treg cells.

Materials and Methods

Animals

C57BL/6, SJL/J mice and TCR transgenic 2D2 mice that are specific for myelin oligodendrocyte glycoprotein (MOG) 35-55 were purchased from the Jackson Laboratory. B10.PL mice transgenic for the myelin basic protein (MBP) Ac1-11-specific TCR Vα2.3 or Vβ8.2 (22) were bred in a pathogen-free animal facility at Ohio State University. Age 8-12 wks male and female C57BL/6 and B10.PL mice and female SJL/J mice were used for EAE studies as male SJL/J mice are resistant to EAE induction. Animal protocols were approved by the OSU Institutional Animal Care and Use Committee.
**Human subjects**

All MS patients were treatment-naive for immunomodulatory drugs. Blood was obtained by leukapheresis from MS patients after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated over a Ficoll gradient and stored in liquid nitrogen until further use. The study was performed under OSU Internal Review Board protocol # 2015H0076 with written informed consent received from participants prior to inclusion in the study.

**EAE induction**

Immunization: EAE was induced in 8-10 week old female SJL/J mice by subcutaneously injection (s.c.) over four sites in the flank with 200 µg proteolipid protein (PLP) 139-151 respectively (CS bio) in an emulsion with CFA (Difco). 200 ng pertussis toxin (List) per mouse in PBS was injected intraperitoneally (i.p.) at the time of immunization.

Adoptive transfer: Splenocytes from naive 5-10 week-old B10.PL Vα2.3/Vβ8.2 TCR transgenic mice or immunized SJL/J mice (days 14–21 post-immunization) were activated with 10 µg/ml of MBP Ac1-11 or PLP 139-151 plus IL-12/IL-23 and AS1842856 (or DMSO) for 3 days. Then the cells were washed with PBS and 5-10 ×10^6 cells were injected i.p. into naive B10.PL mice or SJL/J mice.

Mice were scored on scale of 0 to 6: 0, no clinical disease; 1, limp/flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5 quadriplegia or premoribund state; and 6, death.

**In vitro culture of draining lymph node cells from EAE mice**
Draining lymph node cells (dLNs) were prepared from immunized SJL/J mice or C57BL/6 mice around day 9 post immunization and activated in 24-well plates at 4-8×10^6 cells/well with of PLP 139-151 (2 µg/ml) or MOG35-55 (10 µg/ml), plus IL-23 (25 ng/ml) or IL-12 (0.5 ng/ml), in the presence of AS1842856 (0.1 µM) (Selleckchem). DMSO was used as vehicle control.

**In vitro induction and expansion of Th1 and iTreg cells**

Naïve CD4 T cells were purified from splenocytes of WT mice using Miltenyi naïve CD4 isolation kit. For Th1 induction in Figure 1A, naïve CD4 T cells were cultured on 24-well plates coated with 1 µg/ml of αCD3/CD28 (Biolegend) plus IL-12 (0.5 ng/ml) and AS1842856 (or DMSO) for 72h. For iTreg induction in Figure 5, αCD3/CD28 (1 µg/ml) and recombinant mouse PD-L1-Ig chimera or human IgG1-Ig (10 µg/ml) (Biolegend) were used to coat plates. Naïve CD4 T cells were cultured on coated plates plus TGFβ (4 µg/ml) and AS1842856 (or DMSO) for 72h. For the expansion of human Th1 cells in Figure 4 C-D, PBMCs from treatment-naïve MS patients were plated in flasks for 2-4 hrs to remove adherent cells. The suspension cells were then collected and activated with plate-bound αCD3/CD28 plus IL-12 (0.5 ng/ml) and AS1842856 (0.1 µM) or DMSO for 3 days.

**Flow cytometric analysis**

Antibody (Ab) staining was performed to evaluate the expression of surface markers, transcription factors (T-bet, RORγt and Foxp3) and cytokines (IFNγ and IL-17) in CD4 T cells as described previously (6). Briefly, the cells were first incubated with Abs to the cell-surface markers for 30’ at 4°C, followed by treatment with Cytofix/Cytoperm solution from ebioscience (for T-bet, RORγt and Foxp3) for 1h or BD Bioscience (for IFNγ and IL-17) for 20’-40’. Then cells were stained for
transcription factors (T-bet, RORγt and Foxp3) or intracellular cytokines (IFNγ or IL-17) for 30’. Approximately 100,000 live cell events were acquired on a FACSCantoII and analyzed using FlowJo software (Tree Star, Inc.). PE-αIL-17, APC-αIFNγ, Pacific Blue-αT-bet, AF647-αFoxp3, FITC-αCD4, APC-αCD4, Pacific Blue-αCD44 and PE-Cy7-αCD25 were purchased from Biolegend. Fixable Viability Dye eFluor™ 780 and PE-αRORγt were purchased from eBioscience.

Statistics

GraphPad software (GraphPad Prism Software, Inc., San Diego, CA, USA) was utilized for statistical analysis. Quantitated flow data comparisons were performed using two-tailed unpaired Student’s t-test with two groups, one-way ANOVA with three or more groups or two-way ANOVA in Figure 2 G-H and Figure 3D. Mann-Whitney U-test was used to compare EAE data in Figure 4 A-B. Wilcoxon matched-pairs signed rank test was used to compare flow data in Figure 4 D. Differences with p<0.05 were considered significant.

Results:

FoxO1 inhibition with AS1842856 suppresses Th1 differentiation and expansion

Th1 cells are highly encephalitogenic in EAE and implicated in MS pathogenesis. To understand the potential role of FoxO1 signaling in CNS autoimmunity, we first determined whether FoxO1 inhibition with AS1842856 affects the differentiation of naïve CD4 T cells into Th1 cells. AS1842856 potently blocks FoxO1 activity at 0.05-1 µM without showing significant cellular cytotoxicity (17). Thus, AS1842856 was used at 0.05 and 0.1 µM during Th1 differentiation. Naive CD4 T cells were activated with αCD3/CD28 plus IL-12, in the presence of AS1842856 or vehicle control DMSO (Figure 1A-B). Th1 key transcription factor T-bet and
signature cytokine IFNγ were analyzed. T-bet⁺ (Figure 1A) and IFNγ⁺ CD4 T cells (Figure 1B) were significantly lower in AS1842856-treated groups compared to the control group, suggesting FoxO1 inhibition with AS1842856 suppresses the differentiation of naïve CD4 T cells into Th1 cells. To determine whether FoxO1 inhibition alters the expansion of myelin-specific Th1 cells from EAE mice, dLNs from immunized SJL/J mice were activated in vitro with PLP 139-151 alone or plus IL-12 for 3-6 days, in the presence of AS1842856 or vehicle control DMSO (Figure 1C-D). There was no significant difference of IFNγ⁺ CD4 T cells between AS1842856 and the control group when activated with myelin antigen alone (Figure 1C-D upper panels). However, in the presence of IL-12, IFNγ⁺ Th1 cells were significantly lower in AS1842856 groups compared to control groups at both day 3 and day 6 post reactivation (Figure 1C-D lower panels), suggesting FoxO1 inhibition significantly suppresses IL-12-induced IFNγ expression of effector/memory myelin-specific Th1 cells from EAE mice. These data illustrate the role of FoxO1 inhibition in suppressing the differentiation and expansion of myelin-specific Th1 cells.

**FoxO1 inhibition suppresses the transdifferentiation of myelin-specific Th17 cells**

Th17 cells are also a subset of CD4 Teff cells that can be encephalitogenic in EAE and implicated in MS pathogenesis (4). Although TGFβ/IL-6 induces the differentiation of naïve CD4 T cells into Th17 cells that express transcription factor RORγt and cytokine IL-17, those Th17 cells are not encephalitogenic in adoptively transferred EAE studies (6-8). Upon antigen reencounter, non-encephalitogenic RORγt⁺ Th17 cells may transdifferentiate into Th1-like Th17 cells that co-express RORγt and the Th1 key transcription factor T-bet (RORγt⁺ T-bet⁺), which are highly encephalitogenic in adoptive transfer, and may further convert into Th1 cells that do not express RORγt (RORγt⁻ T-bet⁺) (5, 9-12). While Th17 transdifferentiation may occur naturally,
IL-12 and IL-23 promote Th17 transdifferentiation. mTORC1 has been shown to be important for Th17 transdifferentiation (23). As mTORC1 and FoxO1 are two conserved effector molecules of PI3K/Akt signaling, we determined whether FoxO1 plays a role in regulating the transdifferentiation of myelin-specific Th17 cells. dLN cells from EAE mice were reactivated in vitro with myelin antigen, or plus IL-12 or IL-23 for 3-6 days, in the presence of AS1842856 or vehicle control DMSO (Figure 2). Subpopulations of CD4 T cells differentially expressing RORγt and T-bet were analyzed to determine whether FoxO1 regulates specific subsets of Th1 or Th17 cells. CD4 T cells expressing RORγt, but not T-bet (RORγt⁺T-bet⁻), represent non-encephalitogenic Th17 cells, while encephalitogenic Th1-like Th17 cells are RORγt⁺ T-bet⁺. Th1 cells are RORγt⁻ T-bet⁺. As shown in Figure 2, both Th1-like Th17 cells (RORγt⁺ T-bet⁺) and Th1 cells (RORγt⁻ T-bet⁺) were significantly lower in AS1842856-treated groups compared to the control groups at day 3 and day 6 post reactivation (Figure 2A, D), suggesting FoxO1 inhibition suppresses the transdifferentiation of Th17 cells and the expansion of Th1 cells. Addition of IL-12 significantly increased the percentage of Th1 cells (RORγt⁻ T-bet⁺) at day 3 and 6 post reactivation (Figure 2G-H). Addition of IL-23 significantly increased the percentage of Th1-like Th17 cells (RORγt⁺ T-bet⁺) and Th1 cells (RORγt⁻ T-bet⁺) at day 3 and 6 post reactivation, compared to the group treated with myelin antigen alone (Figure 2 G-H). These data confirm that IL-12 and IL-23 promote Th1 expansion and Th17 transdifferentiation. In the presence of IL-12 or IL-23, both Th1-like Th17 cells (RORγt⁺ T-bet⁺) and Th1 cells (RORγt⁻ T-bet⁺) were significantly lower in AS1842856-treated groups compared to the control groups at day 3 and day 6 post reactivation (Figure 2B, C, E, F). Non-encephalitogenic Th17 cells (RORγt⁺ T-bet⁻) were at similar levels in AS1842856-treated groups and the control groups at both time points. These
data suggest that FoxO1 inhibition significantly suppresses Th17 transdifferentiation and Th1 expansion of autoreactive CD4 T cells from EAE mice.

**FoxO1 inhibition shifts transcriptional balance of T-bet and Foxp3 in autoreactive CD4 T cells**

As Foxp3 expressing CD4 Tregs have the potential to suppress encephalitogenic Teff cells (24), the balance between Tregs and encephalitogenic Teff cells may decide the outcome of inflammation and be critical for the progression of autoimmunity. As our data show that FoxO1 inhibition suppresses T-bet+ Th1 cells as well as the transdifferentiation of Th17 cells into encephalitogenic RORγt+ T-bet+ Th1-like cells (Figure 1-2), we determined whether the transcriptional balance of T-bet: Foxp3 of myelin-specific CD4 T cells from EAE mice may be shifted by AS1842856. The expression of T-bet and Foxp3 were analyzed in myelin-specific CD4 T cells from EAE mice that were reactivated ex vivo with myelin antigen, in the presence of AS1842856 or vehicle control DMSO (Figure 3). Foxp3+ Tregs show functional plasticity and may adopt Teff phenotype under inflammatory conditions (25-27). IFNγ expressing Foxp3+ Tregs have been identified in MS patients with reduced suppressive function (28). Thus, Foxp3+ T-bet+ cells may represent Tregs with reduced suppressive function while Foxp3+ T-bet− cells represent functional Tregs. T-bet+ Foxp3− cells represent encephalitogenic CD4 Teff cells, including Th1 and/or Th1-like Th17 cells. As shown in Figure 3, encephalitogenic Teff cells (T-bet+ Foxp3+) were significantly lower in the group treated with AS1842856 compared to the control group (Figure 4A), confirming FoxO1 inhibition suppresses encephalitogenic T-bet+ Teff cells. Meanwhile, functional Treg cells (Foxp3+ T-bet−) were significantly higher, while Tregs with reduced suppressive function (Foxp3+ T-bet+) were significantly lower in the group treated with
AS1842856 compared to the control group (Figure 4A), suggesting AS1842856 may stabilize functional Tregs. Addition of IL-12 or IL-23 led to a significant increase of T-bet+ CD4 T cells, especially encephalitogenic T-bet+ Foxp3+ Teff cells (Figure 4D), confirming the critical role of IL-12 and IL-23 in promoting T cell encephalitogenicity. In the presence of IL-12 or IL-23, AS1842856 treatment led to a significant decrease of encephalitogenic Teff (T-bet+ Foxp3+) cells and Tregs with reduced suppressive function (Foxp3+ T-bet+), while functional Treg (Foxp3+ T-bet-) cells were significantly higher in the groups treated with AS1842856 compared to the control groups (Figure 4B-C). These data indicate that FoxO1 inhibition shifts the transcriptional balance between Tregs and encephalitogenic Teff cells toward Tregs, potentially favoring the resolution of inflammation.

**FoxO1 inhibition suppresses T cell encephalitogenicity and the expansion of Th1 cells of MS patients**

To understand the impact of FoxO1 in CNS autoimmunity, we determined whether AS1842856 may affect T cell encephalitogenicity in EAE and the expansion of pathogenic Teff cells from MS patients. Although AS1842856 has been administered *in vivo* to diabetic mice for a short period of time, most studies were limited to *in vitro* studies as the plasma concentration of AS1842856 could not be detected 2 hrs after oral administration (17), making it unsuitable for EAE studies *in vivo*. Therefore, we determined whether AS1842856 treatment *in vitro* altered the encephalitogenic potential of myelin-specific CD4 T cells following adoptive transfer. Splenocytes from MBP Ac1-11-specific TCR transgenic mice were activated with MBP Ac1-11 and IL-12/IL-23 for 3 days in the presence of 0.1 μM of AS1842856 or vehicle control DMSO, and injected into naive B10PL recipient mice (Figure 4A). EAE severity in the mice receiving
AS1842856-treated myelin-specific CD4 T cells was significantly lower compared to the mice receiving control-treated cells (Figure 4A), indicating that FoxO1 inhibition suppresses the encephalitogenic potential of myelin-specific CD4 T cells. To make certain that the suppression of T cell encephalitogenicity by AS1842856 is not specific to this EAE model [MBP Ac1-11 or the major histocompatibility complex (MHC) (H-2^d)], we performed a similar experiment by adoptively transferring splenocytes from immunized SJL/J mice (H-2^s) that were activated with PLP 139–151 plus IL-12/IL-23 for 3 days ex vivo in the presence of AS1842856 or DMSO (Figure 4B). While the mice receiving vehicle control-treated cells developed EAE, none of the mice receiving AS1842856-treated cells developed EAE (Figure 4B), confirming that FoxO1 inhibition suppresses the encephalitogenicity of myelin-specific CD4 T cells.

To understand the potential role of FoxO1 in regulating human Th1 cells, we determined whether AS1842856 may regulate the expansion of Th1 cells from MS patients. PBMCs from treatment-naïve MS patients were activated with αCD3/CD28 plus IL-12 for 3 days, in the presence of AS1842856 or vehicle control DMSO (Figure 4 C-D). The percentage of IFNγ+CD4 T cells were significantly lower in AS1842856-treated group compared to the control group (Figure 4E). The percentage of CD45RA− effector/memory CD4 T cells that express IFNγ (Figure 4F) and the percentage of T-bet+ IFNγ+ CD4 T cells (Figure 4G) were also significantly lower in AS1842856-treated group compared to the control group, demonstrating that AS1842856 suppresses the expansion of Th1 cells from MS patients. These data suggest that FoxO1 inhibition may limit pathogenic Th1 cells and provide therapeutic benefits in MS.

**FoxO1 inhibition impairs PD-1-induced Foxp3 expression**
Programmed cell death protein 1 (PD-1) is an important immune checkpoint that regulates immune tolerance and autoimmunity (29-36). PD-1 ligation enhances the development of TGFβ-induced Foxp3+ Treg cells (iTregs) (37). As PD-1 ligation generally regulates PI3K/Akt signaling in CD4 T cells (37-39), we determined whether FoxO1 signaling plays a role in PD-1-induced Foxp3 expression in CD4 T cells. Naïve CD4 T cells were activated with plate-bound αCD3/CD28/PD-L1-Ig (or control-Ig) plus TGFβ for 3 days, in the presence of AS1842856 or vehicle control DMSO (Figure 5). Foxp3+ iTregs were significantly higher in the group activated with PD-L1-Ig compared to the group activated with control-Ig (Figure 5 A left two panels), confirming PD-1 ligation promotes Foxp3 expression in CD4 T cells. AS1842856 appeared to promote the induction of Foxp3+ iTregs (Figure 5A, top row). However, the percentage of Foxp3+ iTregs in the groups activated with PD-L1-Ig and AS1842856 is similar to the group activated with PD-L1-Ig and vehicle control DMSO (Figure 5, lower row). The observation that FoxO1 inhibition enhances iTreg induction in the absence of PD-1 engagement, but fails to enhance iTreg induction when PD-1 is engaged, suggests that FoxO1 signaling may play a role in PD-1-induced Foxp3 expression in CD4 T cells.

**Discussion**

Using a selective FoxO1 inhibitor AS1842856, we report that FoxO1 inhibition suppresses the differentiation and expansion of Th1 cells as well as the transdifferentiation of myelin-specific Th17 cells into Th1-like cells upon reactivation with myelin antigen. FoxO1 inhibition shifts the transcriptional balance of T-bet and Foxp3 of myelin-specific CD4 T cells from EAE mice toward Foxp3 upon reactivation with myelin antigen. FoxO1 inhibition also suppresses T cell encephalitogenicity in adoptively transferred EAE studies and the expansion of human Th1 cells.
from MS patients. In addition, inhibition of FoxO1 signaling impairs PD-1-induced Foxp3 expression.

As a conserved effector molecule of PI3K/Akt signaling pathway, FoxO1 plays a critical role in maintaining naïve T cell quiescence and survival. As a result, some phenotypes observed in FoxO1 deficient mice or naïve T cells from FoxO1 deficient mice may reflect indirect effects of FoxO1 deficiency in T cells. Thus, it is important to use pharmacological approaches to determine the role of FoxO1 in regulating T cells and CNS autoimmunity. AS1842856 is a small-molecule compound that binds FoxO1 and potently inhibits its transactivation of target gene expression (17). AS1842856 has been widely used to characterize the potential roles of FoxO1 signaling in various physiological and pathological conditions. AS1842856 reduces glucose production in hepatic cells (17), reproduces features of pulmonary hypertension in pulmonary artery smooth muscle cells (18) and suppresses adipocyte differentiation (19). AS1842856 also reduces leukemia load and prolongs survival in a preclinical model of BCP-ALL (21), inhibits progestin-induced p21 expression and blocks progestin-induced senescence in ovarian cancer (20). IC50 of AS1842856 to inhibit FoxO1 is 0.033 µM (17). Although FoxO1, FoxO3 and FoxO4 share a high degree of sequence homology, FoxO1-mediated promoter activity was decreased by more than 70% while FoxO3- and FoxO4-mediated promoter activity by 3 and 20% respectively when AS1842856 was used at 0.1 μM (17). FoxO3 deficiency in T cells does not alter Th17 development (13). FoxO4 deficient mice have no known phenotypic differences from WT mice (15). Therefore, the effects that we observed with AS1842856 appear due to inhibition of FoxO1. The plasma concentration of AS1842856 could not be detected 2 hours after orally administration (17), which is not suitable for in vivo EAE studies. Further investigation is needed to improve the
pharmacokinetic properties of AS1842856 for evaluating the potential role of FoxO1 signaling in CNS autoimmunity in vivo.

In addition to CD4 T cells, FoxO1 also plays a role in regulating antigen presenting cells (40, 41). FoxO1 deficiency in DCs decreases IL-12 produced by DCs in mucosal surfaces. Therefore, suppression of FoxO1 in DCs may contribute to the suppressive effects of AS1842856 in suppressing IFNγ and T-bet expression in myelin-specific CD4 T cells from EAE mice upon reactivation. However, the effects of AS1842856 in suppressing the differentiation of naïve CD4 T cells into Th1 cells (Figure 1A) and mediating PD-1 signaling in CD4 T cells (Figure 5) were analyzed using purified naïve CD4 T cells activated with αCD3/CD28, which excludes the involvement of antigen presenting cells and reflects the effects of inhibition of FoxO1 in CD4 T cells.

mTORC1 and FoxO1 are two conserved effector molecules in PI3K/Akt signaling pathway. They may regulate each other’s expression directly or indirectly via feedback of the upper signaling molecules of PI3K/Akt pathway. pAkt positively regulates mTORC1 while mTORC1 suppresses pAkt activity through a negative feedback loop. Foxo1 is phosphorylated by pAkt, which leads to its nuclear exclusion and inactivation. Thus it is possible that inhibition of mTORC1 may activate pAkt through the removal of negative feedback, leading to increased Foxo1 phosphorylation and inactivation. Moreover, mTORC1 inhibition does not significantly alter IFNγ expression during T cell activation (42), making it unclear about the detailed mechanisms governing the expression of T-bet/IFNγ in Th1 like cells during Th17 transdifferentiation. Further investigation is needed to dissect the detailed molecule mechanisms of PI3K/Akt signaling pathway in regulating Th17 transdifferentiation and T cell encephalitogenicity.
The role of FoxO1 in regulating Tregs is still controversial. FoxO1 deficiency in T cells impairs Treg development and leads to a mild autoimmune phenotype (14, 43), suggesting FoxO1 promotes iTreg development. However, unprimed FoxO1−/−CD4 T cells express relatively lower levels of TGFβRII compared to naïve FoxO1+/- CD4 T cells from WT mice (14), which could impair iTreg development since it is dependent on TGFβ signaling. Conversely, forced expression of constitutively active FoxO1 in T cells also impairs Treg development and leads to severe autoimmunity (44), suggesting FoxO1 suppresses Treg development. Our data show that FoxO1 inhibition with AS1842856 led to increased Foxp3 expression (Figure 5), which appears to be consistent with the notion that FoxO1 suppresses Treg development. Further investigation is needed to further characterize the role of FoxO1 in regulating Tregs.

PD-1 ligation signals through PI3K/Akt signaling (38, 39). But little is known about the major signaling nodes of PI3K/Akt pathway that mediate PD-1 signaling in T cells. Our data show that AS1842856 impairs PD-1-induced Foxp3 expression (Figure 5), suggesting FoxO1 may play a role in mediating PD-1 signaling in Treg cells. Although PD-1 ligation promotes iTreg induction, PD-1 can be an activation or exhaustion marker, making it unclear whether PD-1 activation enhances or suppresses the suppressive function of Treg cells. PD-L1 has been shown to enhance suppressive function of iTregs (37) while PD-1−/− Tregs lack suppressive function (45), suggesting PD-1 ligation may promote Treg suppressive function. However, a recent publication shows that PD-1 deficiency in Treg cells leads to an activated phenotype and enhanced suppressive function, which involves reduced signaling through PI3K/Akt pathway (46). Further investigation is needed to dissect the precise molecular mechanisms of FoxO1 signaling in PD-1 regulation of Treg suppressive function, which may reveal innovative therapeutic targets for autoimmunity and cancer.
Conclusions

Our data illustrated an important role of FoxO1 signaling in regulating autoreactive CD4 cells in CNS autoimmunity via suppressing encephalitogenic Teff cells and shifting Teff: Treg balance.

List of abbreviations

MS: Multiple Sclerosis; CNS: central nervous system; EAE: experimental autoimmune encephalomyelitis; Teff: T effector cells; FoxO1: Forkhead box O 1; MBP: myelin basic protein; PLP: proteolipid protein; MOG: myelin oligodendrocyte glycoprotein; PD-1: immune checkpoint programmed cell death protein-1; PBMCs: Peripheral blood mononuclear cells.

Ethics Statement

The protocols used for these experiments received prior approval by the OSU Institutional Animal Care and Use Committee and were conducted in accordance with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. The study on human cells was performed under OSU Internal Review Board protocol # 2015H0076 with written informed consent received from participants prior to inclusion in the study.

Availability of data and materials

The data from this manuscript are available from the corresponding author upon reasonable request.
Authors’ contributions

Y.Y. designed research, analyzed the results and wrote the paper. E.E.K., L.K.A., M.F.F. and M.J. performed the experiments. A.L.R. helped with data analysis and manuscript review. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare that they have no competing interests with the contents in this paper.

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Consent for publication

Not applicable.
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Figure Legends:

Figure 1. FoxO1 inhibition with AS1842856 suppresses Th1 differentiation and expansion. (A-B) Naïve CD4 T cells were activated with αCD3/CD28 plus IL-12 for 3 days, in the presence of different concentrations of AS1842856 or vehicle control DMSO. T-bet (A) and IFNγ (B) were analyzed by intracellular staining, gating on CD44+CD4 T cells, and compared with one-way ANOVA. (C-D) dLNs from immunized SJL/J mice were isolated on day 9 post immunization and activated with PLP 139-151 alone or plus IL-12 for 3 (C) or 6 days (D), in the presence of AS1842856 (0.1 μM) or vehicle control DMSO. IFNγ was analyzed by intracellular staining, gating on CD44+CD4 T cells, and compared with unpaired student’s t test (A, C: n=5, B, D: n=6). Plots represent mean ± SEM of 3 independent experiments. **P<0.01; ***p<0.001.

Figure 2. FoxO1 inhibition suppresses the transdifferentiation of myelin-specific Th17 cells. dLNs from immunized SJL/J mice were isolated on day 9 post immunization and activated with PLP 139-151 (A, D), or plus IL-12 (B, E) or IL-23 (C, F), in the presence of AS1842856 (0.1 μM) or vehicle control DMSO for 3-6 days. T-bet and RORγt were analyzed by intracellular staining on day 3 (A-C) and day 6 (D-F), gating on CD44+CD4+ T cells. Unpaired student’s t test was used to compare the difference between the groups treated with AS1842856 (Squares) and vehicle control (Dots). % of CD4 T cells differentially express RORγt and T-bet in the groups treated with IL-12/DMSO or IL-23/DMSO was compared to the group treated with PLP/DMSO with 2-way ANOVA on day 3 (G) and day 6 (H). Plots represent mean ± SEM of 3 independent experiments. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.
Figure 3. FoxO1 inhibition shifts transcriptional balance of T-bet and Foxp3 in autoreactive CD4 T cells. dLNs from immunized SJL/J mice were activated with PLP 139-151 (A), plus IL-12 (B) or IL-23 (C), in the presence of AS1842856 (0.1 μM) or vehicle control DMSO for 3 days. Foxp3 and T-bet were analyzed by intracellular staining, gating on CD44+CD4 T cells. Unpaired student’s t test was used to compare the difference between the group treated with AS1842856 and vehicle control. % of CD4 T cells differentially express Foxp3 and T-bet in the group treated with IL-12/DMSO or IL-23/DMSO was compared to the group treated with PLP/DMSO with 2-way ANOVA (D). Plots represent mean ± SEM of 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 4. FoxO1 inhibition suppresses T cell encephalitogenicity and the expansion of Th1 cells of MS patients. (A) Splenocytes from naive TCRαβ transgenic mice that are specific for MBP Ac1-11 were activated with MBP Ac1-11 and IL-12/IL-23 for 3 days in the presence of AS1842856 (0.1 μM). DMSO was used as vehicle control. At the end of culture, the cells were injected i.p. into naive B10 PL recipient mice. EAE was monitored in recipient mice. Disease incidence (sick mice/total mice) is indicated in parentheses. A statistically significant difference was considered to be P < 0.05, as determined by Mann-Whitney U-test. (B) dLNs from SJL/J mice that were immunized with PLP 139-151 were activated with PLP 139–151 plus IL-12/IL-23 for 3 days in the presence of AS1842856 or DMSO (0.1 μM). Then the cells were injected i.p. into naive SJL/J recipient mice. EAE development was monitored and compared by Mann-Whitney U-test. (C-G) PBMCs from treatment-naïve MS patients (n=6) were activated with plate-bound αCD3/CD28 plus IL-12 for 3 days, in the presence of AS1842856 (0.1 μM) or DMSO. CD45RA,
IFNγ and T-bet expression was determined by flow cytometry, gating on CD4 cells, and compared with Wilcoxon matched-pairs signed rank test (E-G). *P<0.05. ****P<0.0001.

Figure 5. FoxO1 inhibition impairs PD-1-induced Foxp3 expression. Naïve CD4 T cells were activated with plate-bound αCD3/CD28/PD-L1-Ig (or control-Ig) plus TGFβ for 3 day, in the presence of AS1842856 (0.1 μM) or DMSO. Foxp3 expression was measured by intracellular staining, gating on CD4 cells (A), and compared with 2-way ANOVA (B). Plots represent mean ± SEM of 5 independent experiments. ***P<0.001.
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