AS101 ameliorates experimental autoimmune uveitis by regulating Th1 and Th17 responses and inducing Treg cells

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ABSTRACT

AS101 is an organotellurium compound with multifaceted immunoregulatory properties that is remarkable for its lack of toxicity. We tested the therapeutic effect of AS101 in experimental autoimmune uveitis (EAU), a model for human autoimmune uveitis. Unexpectedly, treatment with AS101 elicited Treg generation in vivo in otherwise unmanipulated mice. Mice immunized for EAU with the retinal antigen IRBP and treated with AS101 developed attenuated disease, as did AS101-treated recipients of retina-specific T cells activated *in vitro*. In both settings, eye-infiltrating effector T cells were decreased, whereas regulatory T (Treg) cells in the spleen were increased. Mechanistic studies *in vitro* revealed that AS101 restricted polarization of retina-specific T cells towards Th1 or Th17 lineage by repressing activation of their respective lineage-specific transcription factors and downstream signals. Retina-specific T cells polarized *in vitro* towards Th1 or Th17 in the presence of AS101 had impaired ability to induce EAU in naïve recipients. Finally, AS101 promoted differentiation of retina-specific T cells to Tregs *in vitro* independently of TGF-β. We conclude that AS101 modulates autoimmune T cells by inhibiting acquisition and expression of effector function and by promoting Treg generation, and suggest that AS101 could be useful as a therapeutic approach for autoimmune uveitis.

1. Introduction

The ammonium trichloro (dioxoethylene-O,O′) tellurate compound AS101 is a small non-toxic, immunomodulator that has multiple anti-cancer, anti-inflammatory and anti-apoptotic properties [1–4]. Because of its relative lack of toxicity, AS101 has been tested for its beneficial effects in diverse preclinical and clinical studies. Thus far it has demonstrated efficacy orally in AMD and topically in HPV and Psoriasis [5,6] and in several autoimmune disease models [4,7–12].

The diverse immunomodulatory functions of AS101 are mediated by its Te[IV] redox-modulating activities enabling, among other effects, the inactivation of cysteine proteases such as cathepsin B and inhibition of thiol-dependent integrins αvβ3, α4β1 (VLA4) and α4β7 [13–16]. Its diverse functional effects include sensitization of tumor cells to the effects of chemotherapy, inhibition of cell migration and entry into tissues, suppression of Th17 cells and enhancement of regulatory T cells

(Treg) [3,4]. The effects which predominate depend on the disease condition under study.

In experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, treatment with AS101 ameliorated disease by downregulating inflammatory cytokines in the central nervous system (CNS) [10,12]. In a model of inflammatory bowel disease (IBD), AS101 treatment not only downregulated inflammatory cytokines, but also increased Tregs in colonic lamina propria [8]. AS101 has also been shown to prevent type 2 diabetes (T2D) or a diabetic nephropathy in rat models [7,11] and our recent work indicates that it can also ameliorate T1D in NOD mice, in part through induction of Tregs (manuscript in revision). These experimental findings strongly support beneficial effects of AS101 in several inflammatory and autoimmune disease models. However, due to the multifaceted, and potentially opposing, biological effects of AS101 that involve cell activation, migration and survival, outcomes in specific disease situations are difficult to predict
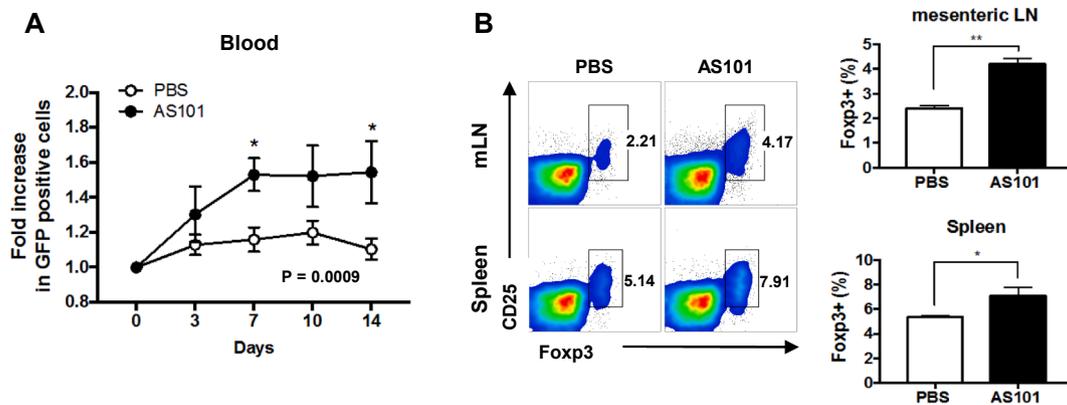
Fig. 1. Administration of AS101 increases Treg cells in naïve mice. Naïve Foxp3-GFP reporter mice on the B10.RIII background were injected AS101 (27 μg, i.p.) for 14 consecutive days. (A) On days 3, 7, 10 and 14, blood samples were obtained from lateral tail vein, and GFP-positive cells were detected by flow cytometry. Fold increase in Foxp3-GFP positive cells was calculated as [% of GFP positive at each time point]/[% of GFP positive at day 0]. (B) The percentages of Foxp3$^+$ CD4$^+$ cells in spleen, mesenteric LN (mLN) were assessed by flow cytometry on day 5. Representative data from 2 independent experiments. *p < 0.05, **p < 0.01 vs. PBS group.

and must be individually tested and optimized.

Autoimmune uveitis is an intraocular noninfectious inflammatory disease affecting the uvea and retina, which is sight-threatening if not successfully treated. The gradual irreversible damage of photoreceptors caused by autoreactive T cells and recruited leukocytes that secrete inflammatory cytokines eventually leads to visual deficit and blindness [17]. Various immunosuppressive drugs are now approved for the treatment of a wide spectrum of uveitis, but they potentially have serious systemic side effects during long-term treatment [18–20]. Thus, there is a need for development of novel and less toxic medications to treat autoimmune uveitis.

Experimental autoimmune uveitis (EAU) is an animal model of human uveitis and has been widely used as a template for preclinical studies [21]. EAU can be induced by immunization of mice with retinal proteins or their peptides, or by adoptive transfer of activated retina-specific T cells into naïve recipients [22,23]. Acute disease starts with infiltration of inflammatory cells into the retina [24], recruited by autoreactive retina-specific T cells secreting IFN-γ (Th1) or IL-17A (Th17) that penetrate the blood-retinal barrier and elicit an amplification loop that culminates in inflammation and tissue damage [25,26]. Th1 and Th17 cells are both pathogenic T cell subsets involved in disease progression [27–30]. The disease is controlled by Tregs which are induced in the periphery, and in part may also arise within the eye [31–33]. Thus, strategies that inhibit transcription factors and signaling pathways utilized by autoreactive Th1 and Th17 cells, or that promote the generation and expansion of cell types with immunosuppressive properties, such as Foxp3$^+$ Tregs would be expected to suppress disease.

In the present study, we observed that systemic administration of AS101 to naïve mice efficiently induced circulating Tregs in vivo. We therefore set out to examine the therapeutic efficacy of AS101 in autoimmune uveitis models and to study the mechanism. We report that treatment with AS101 attenuates EAU by reducing Th1 and Th17 effector cells and by increasing Tregs in the periphery. Mechanistic studies revealed that AS101 restricts polarization of retina-specific autoreactive T cells towards Th1 or Th17 lineages by downregulating lineage-specific transcription factors, T-bet or RORγt, respectively, and that Foxp3$^+$ Treg induction by AS101 is in part independent of, and synergizes with, TGF-β. These data suggest that AS101 directly modulates the immune responses in the setting of autoimmunity and has clinical potential as a therapeutic approach to human autoimmune uveitis.

## 2. Materials and methods

2.1. Mice

B10.RIII mice (Stock No. 000457) were obtained from Jackson Laboratories (Bar Harbor, ME). Foxp3-GFP reporter mice obtained from Dr. A. Rudensky (Sloan-Kettering Cancer Center, New York, NY) and CD90.1 congenic mice (Jax) were backcrossed onto the B10.RIII background for > 10 generations. IRBP-specific TCR transgenic (R161H) mice were generated on the B10.RIII background in our laboratory as previously described [34] and backcrossed onto CD90.1 congenic background for > 10 generations. All mice were kept under a specific pathogen free facility. Animal care and the animal study protocols were approved by the Animal Care and Use Committees of the National Eye Institute (protocol # NEI-581).

2.2. Flow cytometry

Cells were incubated with Fc block (clone 2.4G2, BioXcell) and stained with following antibodies from BioLegend, eBioscience or BD Biosciences: anti-CD3 (clone 145-2C11), anti-CD4 (RM4.5), anti-CD25 (PC61.5), anti-CD45 (30-F11), anti-CD90.1 (OX-7) and anti-CD90.2 (53-2.1). For intracellular cytokine staining, cells were pulsed for 4 h with PMA (50 ng/ml), ionomycin (500 ng/ml), and Brefeldin A (GolgiPlug, BD), followed by 4% paraformaldehyde fixation and 0.05% Triton X-100 permeabilization before intracellular cytokine staining for T-bet (clone 4B10), RORγt (AFKJS-9), IFN-γ (XMG1.2) and IL-17A (TC11-18H10.1). For Foxp3 (FJK-16s) staining, eBioscience Foxp3 transcription factor staining kit was used according to the manufacturer's protocol. For pSTAT3 (pY705), pSTAT4 (pY693), pAKT (pS473) staining, cells were fixed with Cytofix and permeabilized with Perm III buffer (BD Biosciences) according to manufacturer's instructions. Stained cells were collected on a MACSQuant analyzer (Miltenyi Biotec). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.3. AS101 treatment and detection of Foxp3-GFP positive cells

Naïve Foxp3-GFP reporter B10.RIII mice were injected with AS101 (27 μg in 200 μl PBS) intraperitoneally (i.p.) for 14 consecutive days. On day 3, 7, 10 and 14, blood samples were obtained from lateral tail vein, and red blood cells were lysed by ACK buffer (Thermo Fisher Scientific). After washing with PBS + 2% FBS buffer, GFP-positive CD4$^+$ T cells were detected by flow cytometry. Fold increase in Foxp3-GFP expression of CD4$^+$ in blood at each time point was calculated as [% of GFP
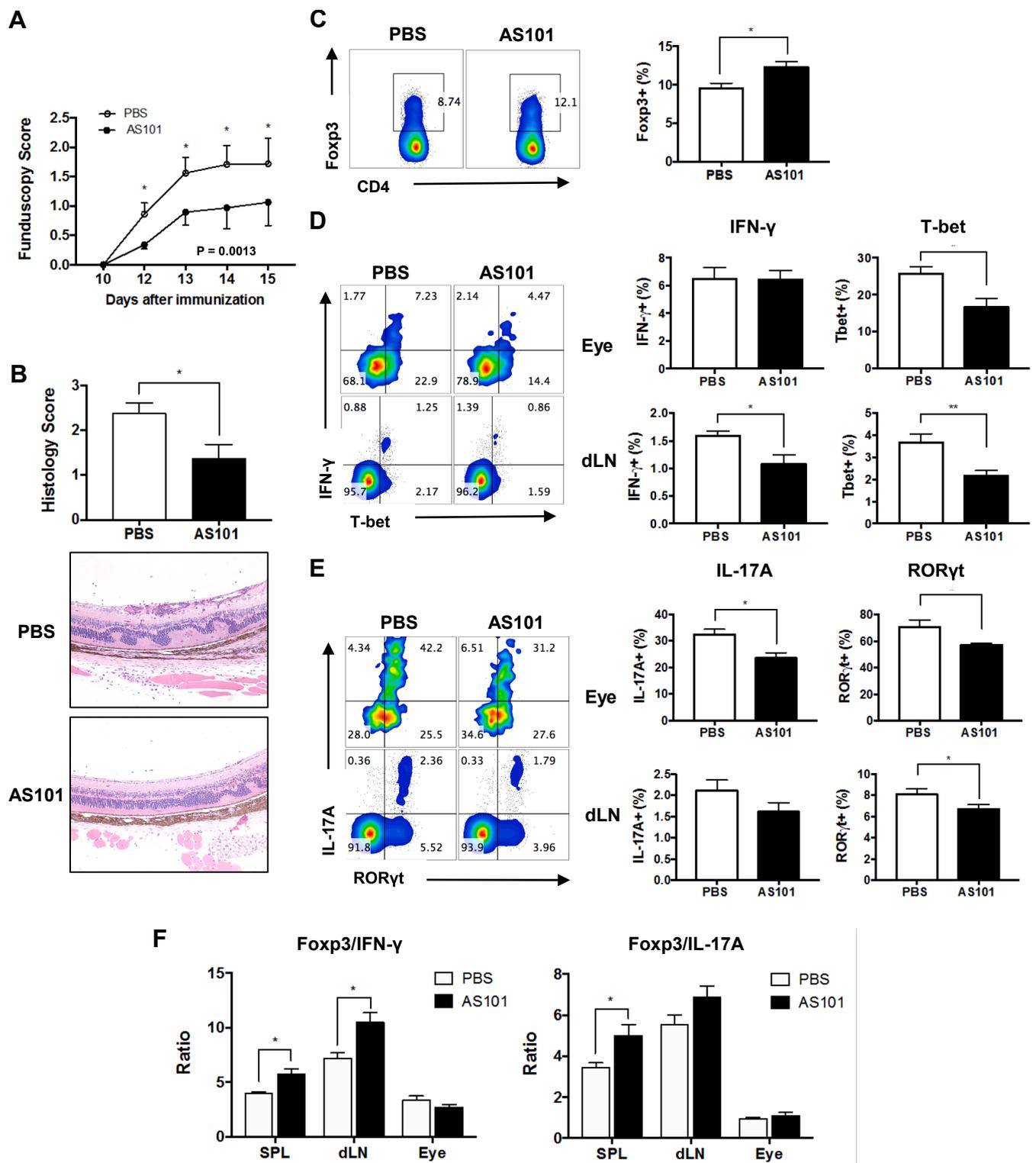
Fig. 2. AS101 treatment attenuates severity of EAU by reducing effector T cells and increasing Tregs. EAU was induced in B10.RIII mice by active immunization with 5 μg of IRBP$_{161-180}$ emulsified in CFA. AS101 (27 μg/mouse, i.p.) was administered daily from 2 days before immunization and treatment continued for the duration of the study. (A) Fundoscopy scores of immunized mice were recorded every 1–3 days after immunization. (B) Histology was performed on eyes collected on day 14. Magnification; X100. (C) The percentage of Foxp3$^+$CD4$^+$ cells in the spleen of immunized mice. Representative data from 3 to 4 independent experiments, and each group contains at least 5 mice. (D, E) Intracellular cytokine staining for Th1 (IFN-γ and T-bet, D) or Th17 (IL-17A and RORγt. E) markers in eye-infiltrating cells and draining LN (dLN) collected on day 14 and gated on live CD4$^+$ cells after ex vivo stimulation with PMA/ionomycin and brefeldin A for 4 h. (F) Ratios of Tregs (Foxp3$^+$) to Th1 (IFN-γ$^+$) or Th17 (IL-17A$^+$) were calculated. Data shown as mean ± SEM. Significance was determined using Mann-Whitney test (A, B), unpaired t-test (C–E), or two-way ANOVA (F). *p < 0.05, **p < 0.01 vs. PBS group.

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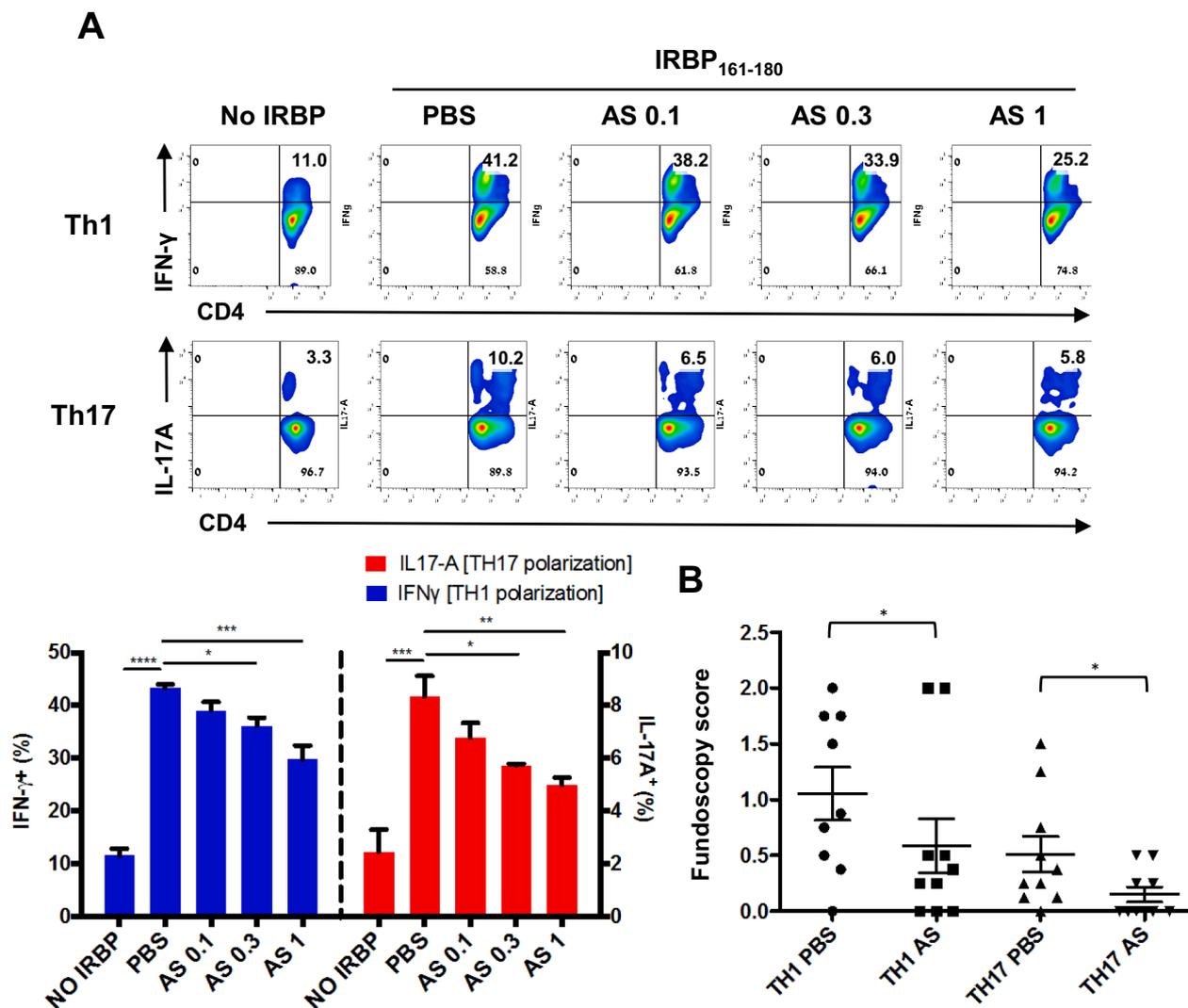
Fig. 3. AS101 inhibits effector T cell polarization and acquisition of pathogenicity in vitro. R161H LN cells were stimulated with 2 μg/ml IRBP$_{161-180}$ under Th1 or Th17 polarizing conditions with PBS or various concentrations of AS101 (0.1, 0.3, or 1 μg/ml) for 72 h. (A) After polarization towards Th1 or Th17, the CD3$^+$CD4$^+$ population was assessed for IFN-γ and IL-17A expressing cells, respectively, by flow cytometry. Data shown as mean ± SEM from 3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 vs. PBS. (B) After Th1 or Th17 polarization with PBS or 1 μg/ml of AS101, viable cells were harvested in Lympholyte M, and Th1 cells (1 million cells/mouse) or Th17 cells (3 million cells/mouse) were adoptively transferred i.p. into naïve B10.RIII recipient mice. Graph shows fundoscopy scores of eyes of B10.RIII recipient mice on day 10. Number of animals: Th1 PBS, n = 9; Th1 AS, n = 10; Th17 PBS, n = 10; Th17 AS, n = 10. Data shown as mean ± SEM from 3 independent experiments *p < 0.05 vs. Th17 PBS group **p < 0.01 vs. Th1 PBS group.

positive at each time point]/[% of GFP positive at day 0].

2.4. T cell polarization

Cells isolated from lymph nodes (LN) of R161H mice were cultured (2 million cells/ml) in RPMI + 10% fetal bovine serum (FBS) + IRBP$_{161-180}$ (2 μg/ml). Some cultures contained graded doses of AS101, as specified (0.1–3 μg/ml). Cytokines and neutralizing antibodies for the desired polarization conditions were as follows. Th17: *anti*-IFN-γ (10 μg/ml; R4-6A2), *anti*-IL-4 (10 μg/ml; 11B11), TGF-β (2.5 ng/ml), IL-6 (25 ng/ml), and IL-23 (10 ng/ml); Th1: IFN-γ (10 ng/ml) and IL-12 (10 ng/ml); Treg: TGF-β (5 ng/ml). For testing TGF-β-independent Treg induction, exogenous TGF-β was omitted, and instead, cultures were supplemented with *anti*-TGF-β (1D11, 10 μg/ml) to neutralize any TGF-β present in FBS. For serum-free condition, CD hybridoma medium (Thermo Fisher Scientific) supplemented with ʟ-glutamine (2 mM) and penicillin/streptomycin (100 U/ml), was used.

2.5. Induction of EAU and treatment with AS101

B10.RIII WT mice were immunized for EAU by subcutaneous injections with 5 μg of IRBP$_{161-180}$ peptide (SGIPYIISYLHPGNTILHVD) (Anaspec, Fremont, CA) emulsified in an equal volume of Complete Freud's Adjuvant containing 2.5 mg/ml *Mycobacterium tuberculosis* strain 37RA (Sigma-Aldrich, St. Louis, MO). Mice were treated with 27 μg of AS101 (or PBS as control) in 200 μl as i.p. injections starting two days before immunization. Experiments were harvested and tissues collected for analysis at the peak of disease (between day 14 and 16).

For induction of EAU by adoptive transfer of AS101-treated retina-specific T cells, LN cells from R161H mice were activated with 2 μg/ml IRBP$_{161-180}$ under the polarization conditions towards Th1 or Th17 cells, as described above, for 3 days in the presence of PBS or AS101 (1 μg/ml) in RPMI + 10% FBS media. β-Mercaptoethanol (ME) was omitted from these cultures because it reacts with AS101 and results in toxicity. After purification of live cells by centrifugation over Lympholyte-M (Cedarlane, NC), donor Th1 (1 million/mouse) or Th17
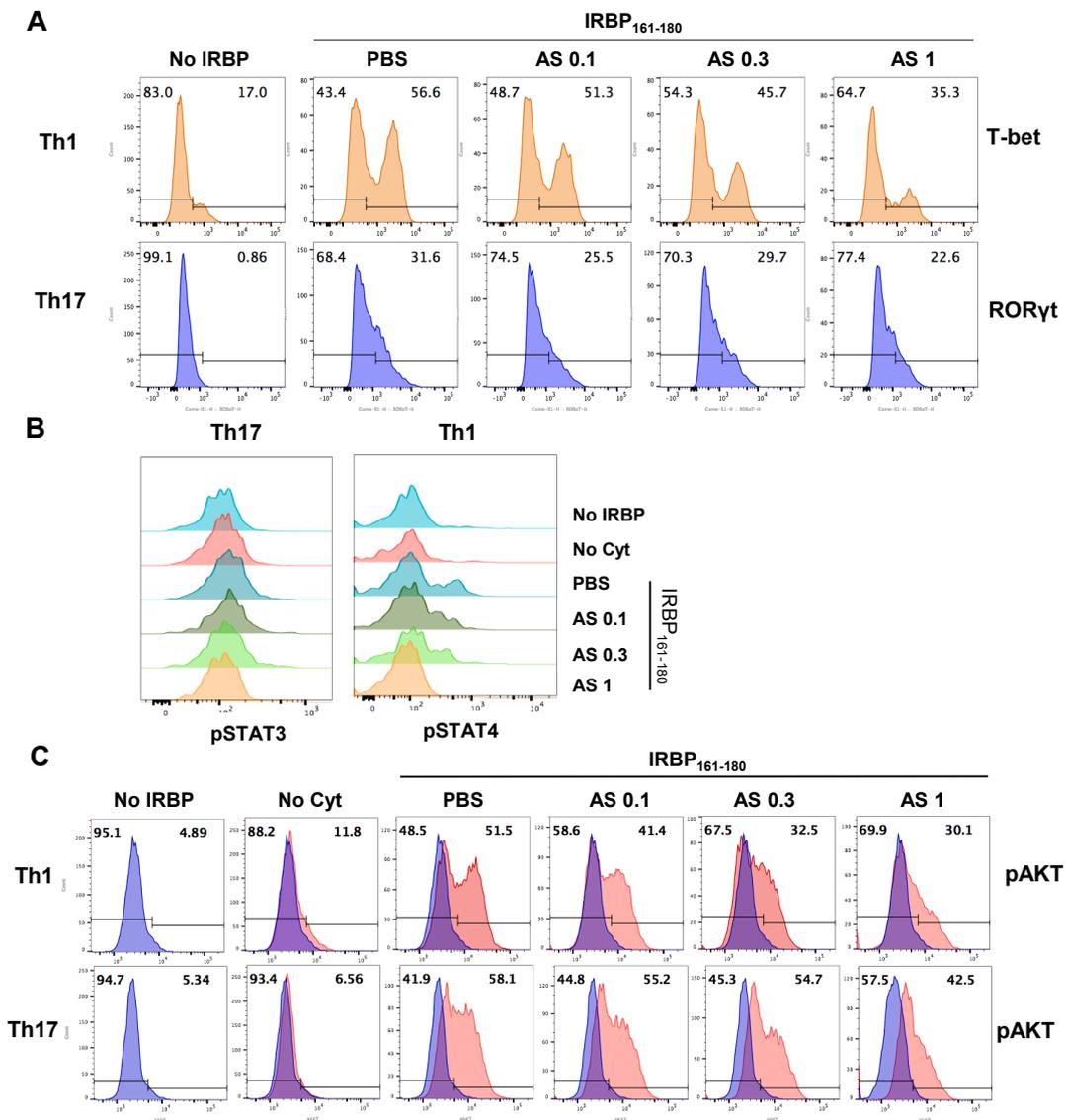
Fig. 4. AS101 downregulates the expression of Th1 or Th17 lineage-specific transcription factors and the phosphorylation of downstream signaling molecules. R161H LN cells were stimulated for 72 h with 2 μg/ml IRBP$_{161-180}$ (IRBP) under Th1 or Th17 polarizing conditions in the absence (PBS) or presence of AS101 (0.1, 0.3, or 1 μg/ml). (A) After Th1 or Th17 polarization, CD3$^+$CD4$^+$ population was assessed for T-bet or RORγt expression, respectively, by flow cytometry. Results represent one of 3 independent experiments. (B) Th1- or Th17-polarized CD3$^+$CD4$^+$ population was assessed for pSTAT4 or pSTAT3, respectively. Results represent one of 2 independent experiments. (C) Th1- or Th17-polarized CD3$^+$CD4$^+$ population was assessed for pAKT expressing cells. No cytokine (Cyt) condition was used as a negative control. Results represent one of 2 independent experiments.

(3 million/mouse) cells were adoptively transferred into congenic B10.RIII recipient mice. For the adoptive transfer experiments with in vivo AS101 treatment, LN cells from R161H mice were activated with 2 μg/ml IRBP$_{161-180}$ under Th0, Th1 or Th17 conditions for 3 days in DMEM (high glucose) +10% FBS media. Purified live Th0 (10 million/ mouse, i.v.), Th1 (2 million/mouse, i.p.) or Th17 (5 million/mouse, i.p.) cells were transferred into B10.RIII congenic mice. The optimal cell numbers to induce disease with 100% incidence were determined by preliminary experiments. Starting from the day of adoptive transfer, the

recipient mice were given daily i.p. injections of either AS101 (27 μg/ mouse) or PBS for control.

2.6. Assessment of EAU by fundoscopy and histology

For clinical scoring EAU, mice were anesthetized by i.p. injections of ketamine/xylazine and their eyes were evaluated by fundoscopy on a scale of 0–4 according to the following criteria: 0 = no inflammation; 0.5 = trace disease with minimal vasculitis; 1 = mild and active disease
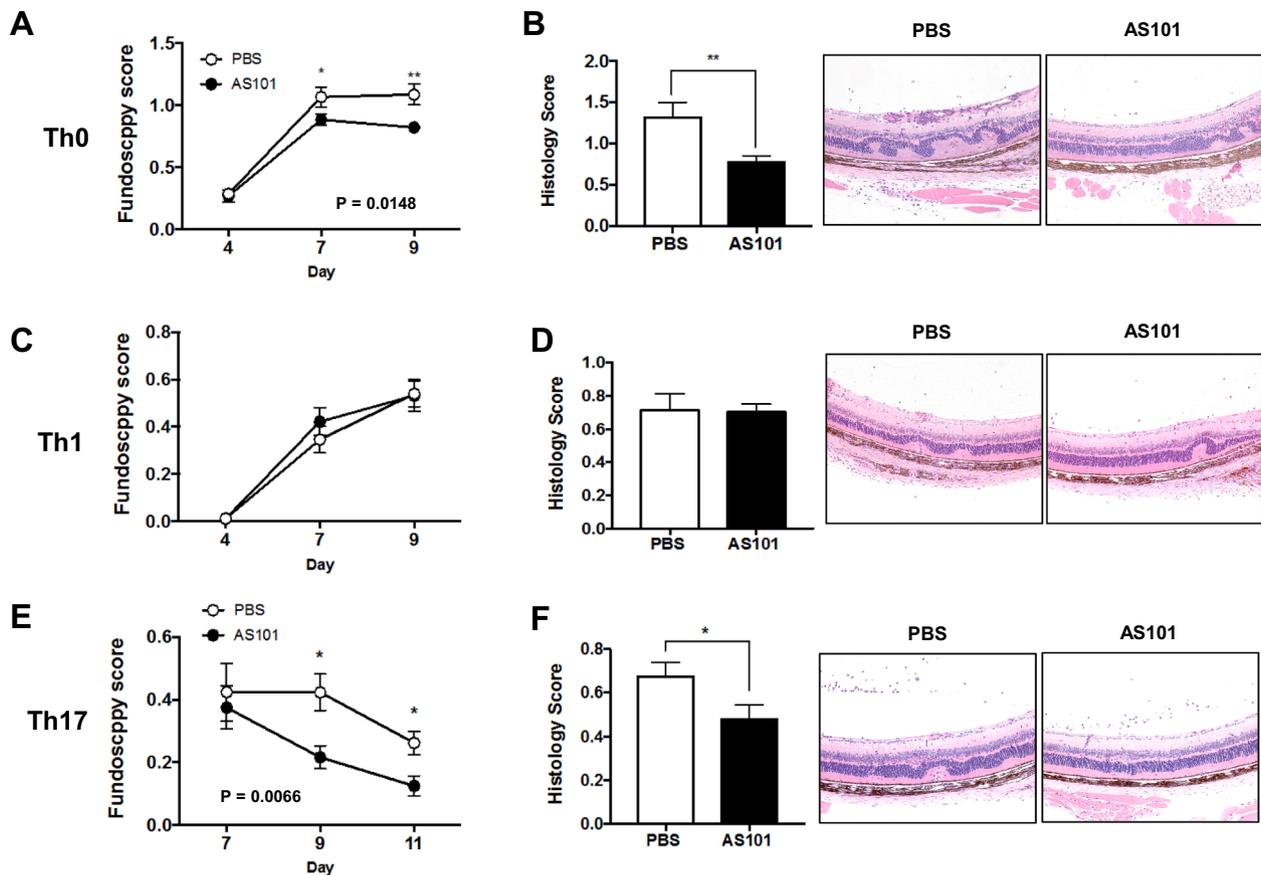
Fig. 5. AS101 suppresses EAU induced by IRBP-specific Th0 or Th17, but not Th1, effector T cells. Cells were isolated from LN of CD90.1 R161H mice and stimulated with IRBP$_{161-180}$ peptide under Th0, Th1 or Th17 conditions. After 3 days, viable cells were collected in Lympholyte M and adoptively transferred into CD90.2 B10.RIII WT mice. AS101 (27 μg, i.p.) was injected to the animals daily. (A) Disease was monitored by fundoscopy at days 4, 7 and 9 post-transfer of Th0 cells. Data pooled from 3 experiments. (B) Histology scores and representative images of H&E stained histology slides on day 10. (C) Fundoscopy scores of Th1 cell transferred mice. Data pooled from 6 experiments. (D) Histology scores and representative images of histology slides (H&E) on day 10. (E) Fundoscopy scores of Th17 cell transferred mice. Data pooled from 3 experiments. (F) Histology scores and representative images of histology slides (H&E) on day 11. Magnification; X100. Data shown as mean ± SEM. Significance was determined using Mann-Whitney test. *p < 0.05, **p < 0.01 vs. PBS group.

with a few retinal folds; 2 = moderately active disease with multiple lesions; 3 = active disease with multiple diffuse lesions and hemorrhages; and 4 = very active inflammation, often with hemorrhage, retinal detachment or atrophy. The precise criteria for scoring and example photographs can be found elsewhere [22]. For histology, eyes were enucleated and immediately fixed for 1 h in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processing. Fixed and dehydrated tissue was embedded in methacrylate. Sections (4–7 μm) were cut through the pupillary-optic nerve plane and were stained with hematoxylin and eosin. Severity of EAU was scored on a scale of 0–4 using previously published criteria, based on the number, type, and size of lesions [23]. Both fundoscopy and histology score were evaluated by masked observers who were not given information about experimental design or group identity. The same highly experienced investigators score experiments of the same type to keep consistency and comparability among consecutive experiments.

2.7. Isolation of eye-infiltrating cells

Eyes were enucleated and external tissues and lenses were removed. The remaining tissue was minced in HL-1 media (Lonza, Walkersville, MD) and incubated with 1 mg/ml collagenase D treatment (Roche, Indianapolis, IN) for 45 min at 37 °C. Cells were then dispersed by trituration, washed, filtered, and resuspended in RPMI + 10% FBS.

2.8. Statistical analysis

Student's *t*-test (parametric data) or Mann-Whitney *U* test (non-parametric data) was used for two-group comparisons. A *p*-value of < 0.05 was considered statistically significant. Data are displayed as mean ± SEM. Experiments were repeated at least twice, and usually three or more times.

3. Results

3.1. AS101 treatment increases the frequency of regulatory T cells in naïve mice

Tregs play a pivotal role in maintaining immune homeostasis and preventing autoimmunity. To examine whether AS101 treatment in naïve mice alters circulating Treg populations *in vivo*, we intraperitoneally (i.p.) injected AS101 into naïve Foxp3-GFP reporter mice for 14 consecutive days and measured GFP-expressing cells in blood every 3–4 days. As shown in Fig. 1A, significant increases of GFP-expressing cells in the blood were detected in AS101-treated Foxp3-GFP reporter mice on day 7 and 14. The increase of Treg cells by AS101 treatment was also confirmed as early as day 5 in the spleen and mesenteric LN, which are the organs immediately exposed to AS101 upon injection (Fig. 1B). However, such increase of Treg cells by AS101 was not observed in the peripheral LN or even in the blood on day 5 (data
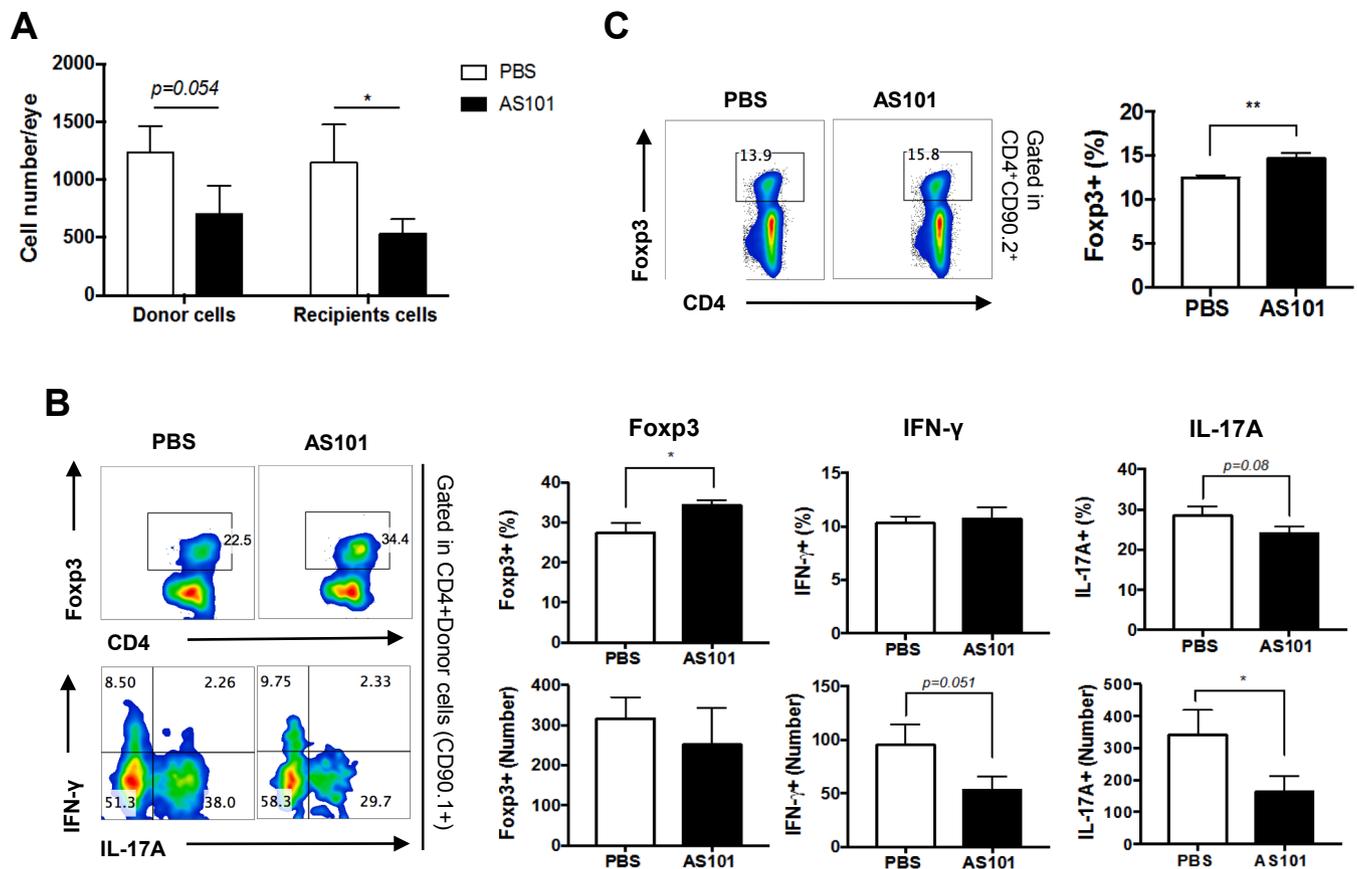
Fig. 6. AS101 treatment induces development and/or expansion of regulatory T cells and inhibits migration of IRBP-specific Th17 cells. Cells were isolated from LNs of CD90.1 R161H mice and stimulated with IRBP$_{161-180}$ peptide for 3 days (Th0 condition). After the removal of dead cells, 10 million cells were adoptively transferred into CD90.2 B10.RIII WT mice. (A) On day 10, the numbers of CD4$^+$CD90.1$^+$ (donor) and CD4$^+$CD90.2$^+$ (recipient) cells in infiltrating in the eyes of recipient mice were measured. (B) Foxp3, IFN-γ, and IL-17A producing donor cells (CD4$^+$CD90.1$^+$) in the eyes of recipient mice were assessed by flow cytometry after *ex vivo* stimulation with PMA/ionomycin and brefeldin A for 4 h. (C) The percentage of Foxp3$^+$ cells among CD4$^+$CD90.2$^+$ gated T cells in the spleen of recipient mice. The means and S.E.M. from individual mice from one of four experiments (n = 6/group) are shown. *p < 0.05, **p < 0.01 Student's *t*-test.

not shown). These results indicate that prolonged treatment with AS101 increases frequencies of Tregs in nearby lymphoid organs and in circulation.

### 3.2. AS101 treatment alters effector and regulatory T cell balance and suppresses EAU

The above finding that higher proportions of Tregs were induced systemically in AS101-treated naïve mice prompted us to investigate the therapeutic ability of AS101 to prevent autoimmune uveitis. We immunized groups of B10.RIII mice with IRBP$_{161-180}$ and treated them daily with either AS101 or PBS, as the vehicle control. Disease was first observed on day 12 after immunization in both groups, but the progression of EAU was significantly reduced in mice treated with AS101 from day 12 to day 15, as determined by fundus examination (Fig. 2A). Histopathological analysis confirmed that AS101-treated mice had milder disease compared to PBS-treated mice with fewer cellular infiltrates and photoreceptor lesions (Fig. 2B). To address how AS101 treatment may have shaped T cell immune responses and ameliorated EAU, we analyzed Tregs and effector T cells in the eye and LN (inguinal and iliac) draining the immunization sites for lineage-specific markers by intracellular flow cytometry. In AS101-treated mice, there was an increase of CD4$^+$Foxp3$^+$ Treg cells in the spleen (Fig. 2C). T-bet and RORγt are the key transcription factors for the differentiation of IFN-γ-producing Th1 or IL-17A-producing Th17 cells, respectively, and were used to identify the corresponding lineages. Treatment of mice with AS101 reduced the frequency of eye-infiltrating CD4$^+$ Th1 and

Th17 cells during EAU (Fig. 2D and E). The frequencies of Th1 and Th17 cells were also decreased in the draining LNs of AS101-treated mice (Fig. 2D and E), whereas the ratio between Treg to Th1 or Treg to Th17 was upregulated (Fig. 2F). These results suggest that systemic AS101 treatment changes the balance of effector and regulatory T cells in the periphery, resulting in suppression of ocular inflammation.

3.3. AS101 inhibits pathways of Th1 and Th17 effector cell differentiation

To address the mechanism(s) by which AS101 regulates Th1 and Th17 differentiation of retina-specific T cells, we examined the effects of AS101 on Th1 and Th17 cell differentiation *in vitro*. IRBP-specific CD4$^+$ T cells were purified from LN of IRBP TCR transgenic (R161H) mice and stimulated with IRBP$_{161-180}$ peptide under Th1- or Th17-polarizing conditions. Presence of AS101 in the culture inhibited generation of IFN-γ- or IL-17A-producing T cells under Th1 or Th17 conditions, respectively, in a dose-dependent manner (Fig. 3A). We then asked whether AS101 treatment during activation/polarization of R161H T cells affects the pathogenic potential of these autoreactive T cells when they are adoptively transferred into naïve recipient mice. R161H Th1 or Th17 effector cells activated with antigen under the respective polarization condition in the presence of AS101 were less pathogenic than parallel effector cells activated in the absence of AS101 (Fig. 3B). These results indicate that AS101 prevents antigen-specific T cells from becoming autopathogenic effector T cells.

To further investigate the mechanism by which Th differentiation into pathogenic effectors was inhibited by AS101, we examined T-bet
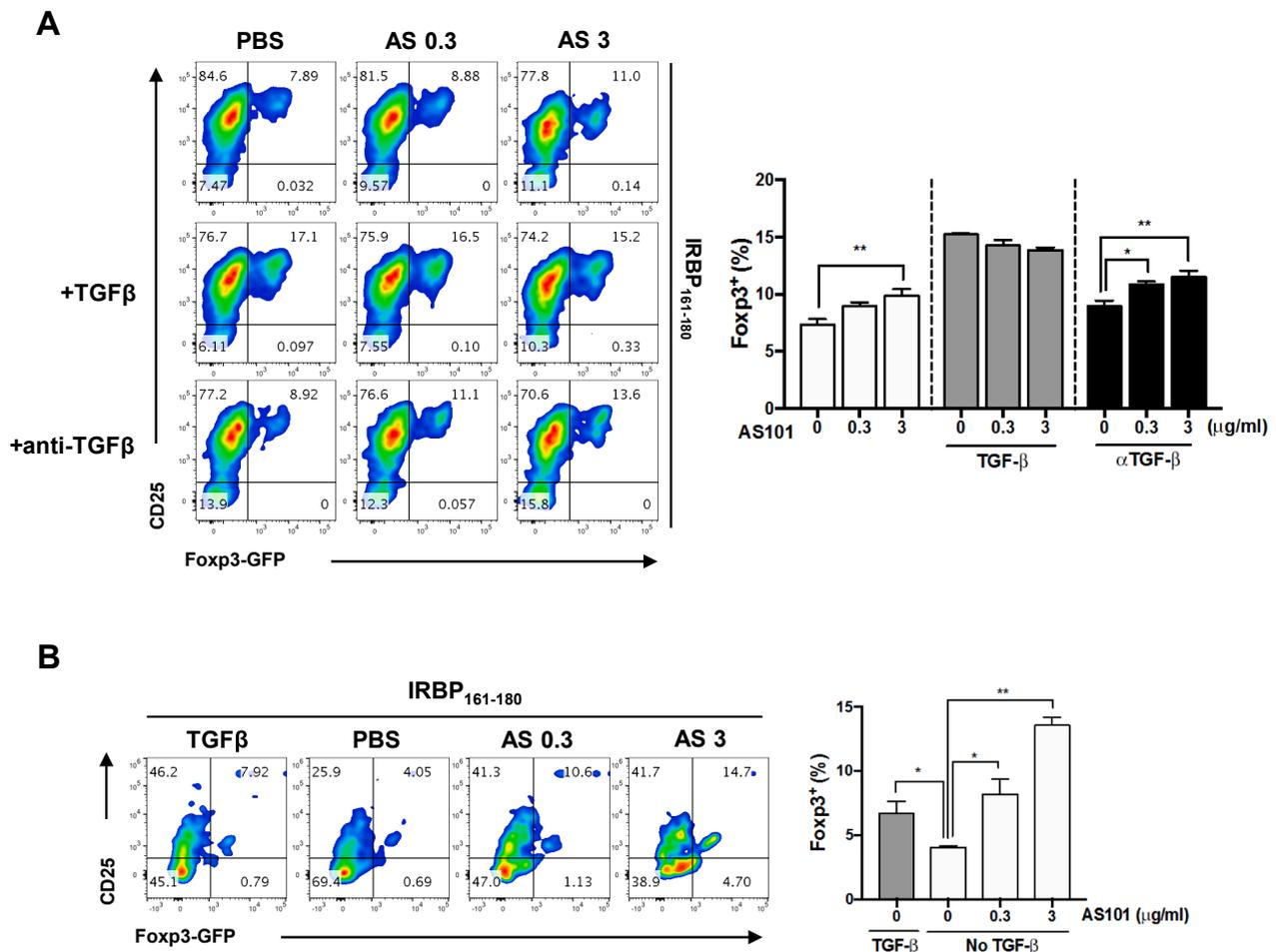
Fig. 7. AS101 promotes Treg differentiation independently of TGF-β. LN cells from R161H-Foxp3-GFP mice were stimulated for 96 h with 2 μg/ml IRBP$_{161-180}$ in presence of TGFβ$_1$ (5 ng/ml), or in presence of *anti*-TGFβ (1D11, 10 μg/ml) and graded doses of AS101 in serum-containing (A) or serum-free (B) medium. After polarization, the CD3$^+$CD4$^+$ population was assessed for Foxp3-GFP and CD25 expression by flow cytometry. Results represent one of 2 independent experiments. Significance was determined using ANOVA test. *p < 0.05, **p < 0.01 vs. PBS group.

and RORγt expression levels in R161H T cells cultured under Th1- or Th17-polarizing conditions in the presence or absence of AS101. Expression levels of these lineage-specific transcription factors were decreased in R161H T cells when AS101 was present in culture (Fig. 4A). Since members of the STAT transcription factor family play a crucial role in the differentiation of Th cells, we examined whether the Jak/STAT intracellular signaling pathway was modulated by AS101. LN cells isolated from R161H mice were stimulated with IRBP$_{161-180}$ in the presence or absence of AS101 for 72 h. Phosphorylation of STAT3 and STAT4 proteins was induced by antigen stimulation but attenuated in a dose-dependent fashion when AS101 was present in culture (Fig. 4B). Because phosphatidylinositol 3-kinase (PI3K)/Akt signaling has been shown to be important for Th17 differentiation [35,36], we examined whether AS101 affects PI3K/Akt signaling. The Akt protein was markedly activated by antigen stimulation in R161H T cells under both Th1 and Th17 polarizing conditions, and this activation was effectively inhibited in the presence of AS101, again in a dose-dependent manner (Fig. 4C). Together, these results support the notion that AS101 regulates differentiation of pathogenic T cells by suppressing the expression of master lineage transcription factors and the phosphorylation of downstream signaling molecules.

3.4. AS101 treatment in vivo suppresses EAU induction by Th0 or Th17, but not by Th1 cells

As demonstrated above, *in vitro* treatment with AS101 of retina

specific T cells inhibited their lineage commitment and acquisition of pathogenicity (Fig. 3). Next, we explored whether AS101 treatment in vivo can dampen the pathology caused by already activated effector T cells polarized to Th1 or Th17, or non-polarized. To study effects on the effector phase of disease we used an adoptive transfer model, in which disease depends on already activated uveitogenic T cells, but before irreversible tissue destruction occurs that would obscure treatment effects. Recipients of non-polarized Th0 cells treated with vehicle developed severe EAU. In contrast, recipients treated daily with AS101 developed attenuated disease (Fig. 5A and B). Histological analysis of eyes harvested at the end of the experiment confirmed the clinical observations (Fig. 5B). We next examined the effect of AS101 treatment on the disease induced by adoptively transferred Th1 or Th17 cells (Fig. 5C–F). AS101-treated recipient mice that had received polarized uveitogenic Th1 effector cells developed similar EAU to PBS-treated controls (Fig. 5C and D), whereas disease in AS101-treated recipients of Th17 effector cells was significantly ameliorated compared to corresponding PBS-treated controls (Fig. 5E and F). These results suggest that AS101 regulates the effector functions of Th1 and Th17 cells differently.

3.5. AS101 treatment enhances regulatory T cell generation and/or expansion during EAU

In our adoptive transfer experiments (Fig. 5), we used congenic mice that allow to distinguish donor cells (CD90.1$^+$) from recipient

cells (CD90.2$^+$). To understand how AS101 modulates the phenotype of the donor-derived effector T cells, as well as recipient-derived immune cells, we examined the numbers and frequencies of eye-infiltrating Th1/Th17/Treg cells on day 10 in the congenic recipient mice that had received Th0 effector cells (Fig. 5A). The absolute numbers of both donor and recipient cells were reduced in uveitic eyes of AS101-treated recipient mice (Fig. 6A). More importantly, AS101 treatment resulted in increased frequency of Foxp3$^+$ Treg cells as well as fewer IFN-γ- and IL-17A-producing donor cells in the eye (Fig. 6B). Of note, frequencies and numbers of Foxp3$^+$ Tregs, IFN-γ- or IL-17A-producing CD4$^+$ T cells of recipient origin in the eyes were not affected by AS101 treatment (data not shown), but splenocytes of AS101-treated recipient mice contained higher proportions of recipient origin Foxp3$^+$ Treg cells than those from PBS-treated recipient mice (Fig. 6C). These results are in line with the findings in naïve AS101 treated mice (Fig. 1) as well as in immunization-induced EAU where AS101 treatment enhanced Foxp3$^+$ Treg cells in the spleen (Fig. 2).

3.6. AS101 treatment promotes induction of antigen-specific Tregs independently of TGF-β$_1$

Our findings thus far point suggests that the therapeutic action of AS101 may depend, at least in part, on promoting the generation of polyclonal Tregs *in vivo*. To examine whether AS101 can support induction of retina-specific Tregs directly, and whether retina-specific T cells can also be differentiated to Tregs, LN cells from R161H mice were subjected to a Treg inducing protocol *in vitro* in the presence or absence of antigen IRBP$_{161-180}$ and/or TGF-β$_1$ and AS101 for 96 h. As might be expected TGF-β$_1$ substantially enhanced the number of Foxp3$^+$ T cells. Importantly, however, AS101 could substitute for TGF-β$_1$ in the Treg polarization cultures. If TGF-β$_1$ was omitted, Foxp3$^+$ T cells were still enhanced by graded doses of AS101 in a dose dependent fashion, even when *anti*-TGF-β antibodies (10 μg/ml) were added to the polarization cultures to neutralize TGF-β that might be present in the serum supplement (Fig. 7A). To address the possibility that TGF-β might not have been neutralized entirely, we repeated the experiment in serum-free medium. Although activation of T cells cultured without serum was not as robust, the results confirmed that AS101 was able to promote T cell to Treg conversion in the absence of TGF-β (Fig. 7B). These results indicate that AS101, similarly to and independently of, TGF-β$_1$, can enhance the differentiation of antigen-specific T cells to Tregs.

4. Discussion

In this study, we investigated the efficacy of a tellurium-based compound, AS101, as a treatment for EAU, a well-established model of human uveitis. Systemic administration of AS101 induced Tregs and downregulated the infiltration of inflammatory cells in the neuroretina in both immunization-induced EAU and adoptive transfer models by affecting differentiation and function of uveitogenic effector and of regulatory T cells, which are central players in elicitation and in control of uveitis. From a mechanistic point of view, much of the biological activity of AS101 is directly related to its chemical redox interactions with vicinal thiols in the exofacial domain of integrins causing their inactivation, and it has also been reported to inactivate cysteine proteases including Cathepsin B [13–16]. It is likely that the complex effects described here on effector and regulatory T cell activation and lineage commitment, combined with effects on integrin-dependent cell extravasation and migration (as reported by others), would reflect an integration of multiple activities, which together translate to attenuation of the disease process.

Lineage commitment to Th1 and Th17 cells involves phosphorylation of STAT4 and STAT3, respectively [37] and for Th17 also PI3K/Akt signaling [35,36]. From *in vitro* culture data (Figs. 3 and 4), AS101 was clearly able to inhibit commitment of T cells to the Th1 as well as Th17 lineage. This involved suppression of the expression of the master

transcription factors for the respective lineages, T-bet for Th1 and RORγt for Th17, and the phosphorylation of downstream signaling molecules, STAT4 for Th1 and STAT3 and PI3K/Akt for Th17. On the other hand, AS101 promoted commitment to the Treg lineage not only by enhancing the effect of TGF-β$_1$ on the process, but also by promoting conversion of T cells to Foxp3$^+$ Tregs even in the absence of added TGF-β$_1$. The downstream signaling events elicited by AS101 in the process of Treg induction remain to be elucidated.

Cytotoxic CD8$^+$ T cells are also reported to play major roles as effectors of autoimmunity. In the EAU model, CD8$^+$ T cells were thought to be either "suppressive" [38,39] or "dispensable" because their depletion did not affect disease development [40]. However, more recent data suggested that CD8$^+$ T cells can also be pathogenic effectors in EAU [33,41,42]. It has also been reported that there are few of CD8$^+$ Tregs that produce anti-inflammatory cytokines and have regulatory roles in the LN during EAU [43]. Although the role of CD8$^+$ T cells was not examined in this study, our data do not exclude the possibility that AS101 can induce or upregulate CD8$^+$ Tregs in the spleen (or periphery), similarly to its effect on CD4$^+$ Tregs.

In vivo adoptive transfer studies and analysis of the cells isolated from the treated hosts demonstrated that AS101 is also able to affect lineage stability and function of Th0 and Th17 cells, but not of polarized Th1 cells (Fig. 5). Th0 cells are still plastic and can transition to Th17 or Th1 cells. Th17 cells also have been described as having the plasticity to differentiate further, e.g., to Th1-like cells or to Tregs [44–46]. In fact, Hirota et al. postulated that Th17 cells must differentiate to Th1-like cells to effect CNS pathology [45]. However, the Th1 phenotype appears to be more stable [44]. Together with the data discussed above, this raises the possibility that AS101 can affect T cells that are still in the process of differentiation and phenotype change, but not stably committed effectors such as Th1. Our data also do not exclude the possibility that AS101, through its action on integrins, also acts by affecting migration to the retina of effector T cells, and of recruited inflammatory leukocytes. This possibility is supported by the recent report in the EAE model where AS101 decreased VLA-4 (α4β1 integrin) activity on immune cells and prevented their migration into the CNS [10], resulting in reduction of inflammation associated IL-6, IL-1β and TNF-α levels and enhancement of anti-inflammatory IL-10 [10]. Other researchers showed that AS101 reduced the IL-17, IFN-γ, GM-CSF, and IL-6 mRNA expression in inflammatory cells from spinal cords in the EAE model [12]. Systemic treatment of AS101 consistently increased Treg in the spleen (Figs. 1B, 2C and 6C), but not in the eye or the eye-draining LN (data not shown). The reason for this compartmentalization of the Treg response under AS101 treatment is unclear and will require further investigation. Treg induction by AS101 in vivo was also seen in an IBD model [8 and Yossipof et al., submitted for publication]. It appears from our results that AS101 drives a Treg response not only in disease but also in steady state in naïve mice and can promote Tregs also without dependence on TGF-β. Taken together, our current study expands and sheds new light on data from these previous reports and provides a mechanistic explanation for some of these observations.

Th17 cells have been implicated in the pathogenesis of human uveitis [47,48]. The ability of AS101 to modulate disease induced by polarized Th17 cells (a disease reversal paradigm) coupled with the remarkable lack of toxicity of this compound, make this substance a promising therapeutic candidate. Notably, we recently found that a closely related tellurium compound, SAS, can mitigate inflammatory responses of human RPE cells by inhibiting αvβ3 and downregulating inflammatory cytokines [6]. Based on that, AS101 is currently being examined in clinical trials for AMD [49]. We believe that the activity if AS101 in reducing pathogenic Th1 and Th17 immune response and promoting Tregs differentiation during EAU suggest that should also be examined for clinical treatment of uveitis.

Acknowledgements

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