Apolipoprotein A1 Modulates Teff/Treg Balance Through Scavenger Receptor Class B Type I-Dependent Mechanisms in Experimental Autoimmune Uveitis

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PURPOSE. Experimental autoimmune uveitis (EAU) is a representative animal model of human uveitis. In this study, we investigated whether apolipoprotein A1 (APOA1) can alleviate EAU and explored its underlying mechanism.

METHODS. Mice were immunized with interphotoreceptor retinoid-binding protein 1-20 and treated with APOA1 or vehicle. The retinas, draining lymph nodes (DLNs), and spleens were analyzed. Isolated T cells were used for proliferation, differentiation, and function assays in vitro. Selective inhibitors and pathway agonists were used to study signaling pathways. The effect of APOA1 on peripheral blood mononuclear cells (PBMCs) from uveitis patients was also examined.

RESULTS. Administration of APOA1 ameliorated EAU. APOA1 suppressed pathogenic CD4+ T cell expansion in DLNs and spleen, and decreased the infiltration of effector T (Teff) cells into retina. APOA1 also inhibited T cell proliferation and T helper 1 cell differentiation in vitro and promoted regulatory T (Treg) cell differentiation. APOA1 restricted inflammatory cytokine production from lipopolysaccharide-stimulated PBMCs. Mechanistic studies revealed that the effect of APOA1 was mediated by scavenger receptor class B type I (SR-BI) and downstream signals including phosphatidylinositol 3-kinase/Protein kinase B (PKB, or Akt), p38 mitogen-activated protein kinase, and nuclear factor–κB.

CONCLUSIONS. APOA1 ameliorates EAU by regulating the Teff/Treg partially through SR-BI. Our results suggest that APOA1 can be a therapeutic alternative for autoimmune uveitis.

Keywords: apolipoprotein A1, autoimmune uveitis, T cells, scavenger receptor class B type I

Autoimmune uveitis (AU) is an irreversible blinding ocular disorder *mediated by* chronic intraocular inflammation.[1] However, mechanisms of uveitis are complex and remain to be elucidated. Uveitis leads to 5∼10% blind worldwide,[2] and up to 35% of patients with uveitis have significant visual loss.[3] Overall, 70%∼90% uveitis patients present at age 20 to 60 years old, which will exert a significant socioeconomic burden.[4] Current treatments usually cannot be tolerated long term by patients because of unbearable adverse effects.[5] Hence, safe and effective alternatives are urgently needed.

Apolipoprotein A1 (APOA1) is a major structural protein of the high-density lipoprotein (HDL) family, which regulates cholesterol trafficking and protects against atherosclerosis.[6,7] In addition to its cardiovascular protective properties, APOA1 has multifunctional roles in immunity and inflammation.[8] Increasing evidence strongly supports the assertion that APOA1 is a "negative" acute-phase protein, and acts as a natural inhibitory factor in plasma during autoimmune diseases (AD).[9] Moreover, APOA1 has been reported to be a predictive plasma biomarker for a good response to biologics.[10] Taken together, these results suggest that therapies that increase APOA1 expression may potentially reduce inflammation in AD. However, the potential regulatory effects and mechanisms of APOA1 in AD have not yet been fully elucidated.

EAU is an organ-specific T lymphocyte–mediated AD that serves as a representative model of human noninfectious uveoretinitis.[11] The migration and expansion of effector T (Teff) cells, especially Th1 and Th17 cells in the eye, contribute to inflammation and retinal tissue damage in AU; meanwhile, decreased Treg cell number or compromised Treg function also participate in inflammation.[12–14] Thus strategies that restrict Th1/Th17 cells or promote Treg cells may suppress EAU. In this study, we investigated the therapeutic effect and underlying immune mechanism of APOA1 in EAU and found that APOA1 can alleviate EAU by regulating T cell responses.

METHODS

Mice

Female C57BL/6J mice, weighing 18~20 g and aged six to eight weeks were supplied by the Guangzhou Animal Experiment Center. Mice were maintained under specific pathogen-free conditions. Experiments were conducted strictly according to institutional guidelines (Sun Yat-sen University, Guangzhou, China). All procedures were performed in compliance with the ARVO Animal Statement for the Use of Animals.

EAU Model

Incomplete Freund's adjuvant (BD Difco, San Jose, CA, USA) containing 5 mg/mL *Mycobacterium tuberculosis* H37Ra was mixed with 2 mg/mL human interphotoreceptor retinoid-binding protein (IRBP)$_{1-20}$ (GL Biochem, Shanghai, China) in a 1:1 volume ratio. The emulsion was injected into the back of each mouse, near the tail and two flanks. In addition, 200 ng of Bordetella pertussis toxin (List Biological Laboratories, Campbell, CA, USA) was injected intraperitoneally into the immunized mice on days 0 and 2 after immunization.[15,16]

Evaluation of Retinal Inflammation

Fundus fluorescein angiograms (FFA) and fundus photographs were obtained to observe retinal lesions and vascular leakage. Clinical scores were graded from 0 to 4 according to previously reported criteria.[17] Eyes were fixed, dehydrated, sectioned, stained with hematoxylin and eosin (H&E), and photographed using a microscope to evaluate histopathological manifestations, which were then graded on a scale of 0 to 4.[17]

Collection of Retinal Infiltrated Cells

The eyeballs were removed from the orbital cavity of mice after cardiac perfusion and temporarily stored in RPMI-1640 culture medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) on ice. Retinas were dissected and incubated at 37°C for 20 minutes with collagenase D (1 mg/mL; Roche, Basel, Switzerland) and DNase I (100 mg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA).[18,19] Finally, the cells were washed with phosphate buffered saline solution (PBS), filtered through a 40 μm sieve, and resuspended for flow cytometry (FCM).

Treatment of Mice with APOA1

The APOA1 native protein (MyBioSource, Inc. San Diego, CA, USA) was dissolved in PBS and stored at −20°C. Mice were injected with APOA1 (1 mg/kg/d) via the tail vein in three different dosing regimens: from day 2 to day 14, day 2 to day 8, or day 8 to day 14 after immunization. Equal volume PBS was injected as vehicle.

Adoptive Transfer Experiments

T cells were separated from the DLNs and spleens of EAU mice on day 14 after immunization. The isolated T cells were stimulated with IRBP$_{1-20}$ (10 μg/mL) in the presence of APOA1 (10 μM) or vehicle for 72 hours. For some groups, blocking of lipid transport-1 (BLT-1, 2 μM, MedChemExpress, Monmouth Junction, NJ, USA) were performed. Next, cells were washed with PBS twice, and 2×10^7 cells were transferred back to each naive C57BL/6J mouse. All mice were evaluated for retinal inflammation two weeks later.

Treatment With T Cells From DLNs

Cells from DLNs of EAU mice were incubated with IRBP$_{1-20}$ (10 μg/ml) alone or with APOA1 (10 μM), BLT-1 (2 μM), 740Y-P (20 μM, Sellek Chemicals, Houston, TX), SC79 (10 μM, Selleck Chemicals, Houston, TX) or dehydrocorydaline (DEH, 5 μM, MedChemExpress, New Jersey, USA) for targeted regulation of T cells. After 72 hours, the cells were collected and analyzed using FCM.

Proliferation Assay

Cells from DLNs and spleens of C57BL/6J mice were labeled with CFSE (Biolegend, San Diego, CA, USA), after which CD3$^+$ T cells were isolated with the Dynabeads Untouched Mouse T Cells Kit (Invitrogen). The labeled CD3$^+$ T cells were stimulated with anti-CD3/CD28 beads (mice) with or without APOA1 (0, 5, 10, 20 or 40 μM) for 72 hours.

Differentiation Assay

Naïve CD4+ T cells were isolated using the EasySepMouse Naïve CD4+ T Cell Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada) and stimulated with anti-CD3/CD28 beads (mice) with or without APOA1 (0, 5, and 10 μM) for 72 hours. The cultures were also supplemented with Th1 or Treg induction condition (ImmunoCult mouse Th1 or Treg differentiation supplement, Stemcell Technologies) for respective conversion according to the manufacturer's instructions.

PBMCs Assay

PBMCs and plasma were isolated from the peripheral venous blood (10 mL) of seven patients with uveitis and seven healthy age- and sex-matched individuals. Immunoturbidimetric assay kit (Shanghai Kehua Biological Engineering Co. Shanghai, China) for the detection of APOA1 plasma levels. PBMCs (2×10^5 cells/well) were incubated with lipopolysaccharide (LPS) (100 ng/mL) or anti-CD3/CD28 beads (human) and APOA1 (10 μM) or BLT-1 (2 μM) for 72 hours and analyzed by FCM. Consistent with the Declaration of Helsinki, written informed consent was obtained from each participant.

Flow Cytometry

The cells were first labeled with LIVE/DEAD (Thermo Fisher Scientific) to distinguish the living cells. The following surface markers from BioLegend were used in this study: anti-CD45 (BV510), anti-CD3 (BV421), anti-CD4 (Percp-cy5.5), anti-CD8 (BV786), anti-CD25 (PE-cy7), anti-CD44 (APC), and anti-CD62L (FITC). The cells were stimulated with phorbol-12-myristate-13-acetate (PMA, 5 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma), and brefeldin A (1 μg/ml; Sigma) for intracellular of interferon (IFN)-γ (m-BV786 or h-PE), IL-17A (BV650), tumor necrosis factor (TNF)-α (BV421), Foxp3 (FITC) and IL-10 (BV421) staining. For p-PI3K (PE), p-AKT (APC), p-p38 (PE), and p-nuclear factor (NF)–κB

(PE) staining, cells were fixed with 4% paraformaldehyde at 37°C and permeabilized with methanol at −20°C. For SR-BI staining, cells were treated with the 1:200 diluted anti-human/mice SR-BI antibody (Thermo Fisher Scientific) and anti-CD4 (Percp-cy5.5) at 4°C, fixed with 1% fixative solution and then incubated with 1:500 diluted Alexa Fluor 488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific). Labeled cells were assayed using LSR Fortessa (BD Biosciences). Finally, the data were analyzed using Flow Jo software 10.0 (TreeStar, Ashland, OR, USA).

Western Blotting

Proteins from the DLNs were prepared according to the manufacturer's instructions. Equal amounts of protein were separated by gel electrophoresis and transferred onto membranes. Membranes were incubated with SR-BI (Abcam, Cambridge, MA, USA) or GAPDH (Abcam) antibodies after blocking, and incubated with secondary antibody after washing. Blots were examined using enhanced chemiluminescence kit (Millipore) with a molecular imaging system (Bio-Rad). Grayscale values were determined using ImageJ software (NIH, Bethesda, MD, USA) and normalized to GAPDH levels.

Quantitative RT-PCR

Total RNA from retinas, DLNs, PBMCs and HRVECs (ScienCell, Carlsbad, CA, USA) was extracted using the RNA-Quick Purification Kit (ESscience, Shanghai Yishan Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions and measured using NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA was reverse-transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa Bio. Co., Shiga, Japan). The relative amounts of IFN-γ, IL-17A, IL-1β, IL-6, IL-2, SR-BI, APOA1, ICAM-1, VCAM-1, IL-6, TNF-α, VEGF, NF-κB, fibronectin (FN) 1 and transforming growth factor–β genes were detected by quantitative real-time PCR using SYBR Premix ExTaq III (TaKaRa Bio. Co.). Primer sequences were designed using Primer-Bank (Boston, MA, USA). The relative mRNA expression levels were calculated using the $2-\Delta\Delta Ct$ method.

Statistical Analysis

Either Student's *t*-test (for parametric data) or the Mann-Whitney U test (for nonparametric data) was used for two-group comparisons. Statistical significance was set at $P < 0.05$. Data are displayed as mean±SD and analyzed using GraphPad Prism 7.04 software (GraphPad, Inc., San Diego, CA, USA). All experiments were repeated at least thrice.

RESULTS

APOA1 Treatment Alleviates Intraocular Inflammation in EAU

To assess the therapeutic effects of APOA1, C57BL/6J mice were immunized with IRBP$_{1-20}$ and treated with either APOA1 (1 mg/kg) or PBS (vehicle) daily. The severity of EAU was significantly ameliorated in mice treated with APOA1 on day 14 after immunization, as determined by fundus examination, FFA, and histopathological analysis. EAU mice treated with the vehicle developed severe uveitis manifesting as retinal edema, severe vasculitis, or large confluent chori-

oretinal lesions. FFA in the vehicle group showed severe optic disc hyperfluorescence and tortuous dilation of the retinal vessels with leakage. HE in the vehicle group showed retinal folds, extensive photoreceptor cell damage, and granulomatous lesions, consistent with clinical changes. EAU mice treated with APOA1 exhibited minimal retinal inflammation, characterized by an almost normal retinal structure (Figs. 1A–D). Furthermore, post-onset APOA1 administration or early cease of APOA1 led to compromised control of ocular inflammation in EAU (Supplementary Figs. S1 and S3).

To address how APOA1 treatment alleviates EAU, we analyzed effector T cells in the eye using FCM. In APOA1-treated mice, there was a significant decrease in the number of CD4+ T cells in the eyes. Furthermore, APOA1 reduced the frequency of eye-infiltrating Th1 and Th17 cells during EAU (Figs. 1E–J). Inflammatory gene expression in the eyes was measured using quantitative PCR. Significantly decreased mRNA expression of IL-17A, IFN-γ, IL-1β, IL-6, and IL-2 was observed in the APOA1-treated group (Figs. 1K–O). Our results suggest that systemic APOA1 treatment protects EAU mice by reducing the infiltration of effector T cells and the secretion of inflammatory cytokines, resulting in the suppression of ocular inflammation. In addition, APOA1 decreased the mRNA expression of ICAM-1, VCAM-1, IL-6, TNF-α, VEGF and NF-κB, and elevated FN1 and transforming growth factor–β in HRVECs stimulated with TNF-α (Supplementary Fig. S4). It is suggested that APOA1 may have a direct effect on the retinas.

APOA1 Treatment Alters the Teff/Treg Balance in Systemic Immunity

Th1 and Th17 cells are pathogenic T cell subsets involved in disease progression, whereas Tregs contribute to the amelioration of EAU.[11,20] We investigated the effect of APOA1 on the systemic immune response and focused on naïve T cells, Teffs, and Tregs in the DLNs and spleen. In APOA1-treated mice, there was an increase in naive CD4+ T cells (CD4+CD62L+CD44−) and a decrease in memory CD4+ T cells (CD4+CD62L−CD44+) in the DLNs and spleen (Figs. 2A–C; a–c). The frequencies of Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17A+) cells, as well as TNF-α, were decreased, whereas that of IL-10 (CD4+IL-10+) and Treg (CD4+CD25+Foxp3+) cells was increased in APOA1-treated mice (Figs. 2D–L; d–l). The results for cell numbers are consistent with the percentages (Supplementary Fig. S5). These results indicate that APOA1 suppresses the systemic immunological response by downregulating CD4+ T cell activation, decreasing Th1 and Th17 cells, and potentiating Tregs in EAU.

APOA1 Modulates T Cell Proliferation and Differentiation

T cells regulate the pathogenesis and progression of EAU.[21] To further investigate whether APOA1 affects the state and function of T cells, we performed proliferation and differentiation assays in vitro. Viable cells were not greatly reduced by APOA1 at a concentration of 5 μM and 10 μM, mildly reduced at 20 μM, whereas significant cell death occurred at 40 μM (Figs. 3A, 3B). Thus 5 μM or 10 μM concentrations were chosen for subsequent experiments. The proliferation of CD3+ T cells was markedly inhibited
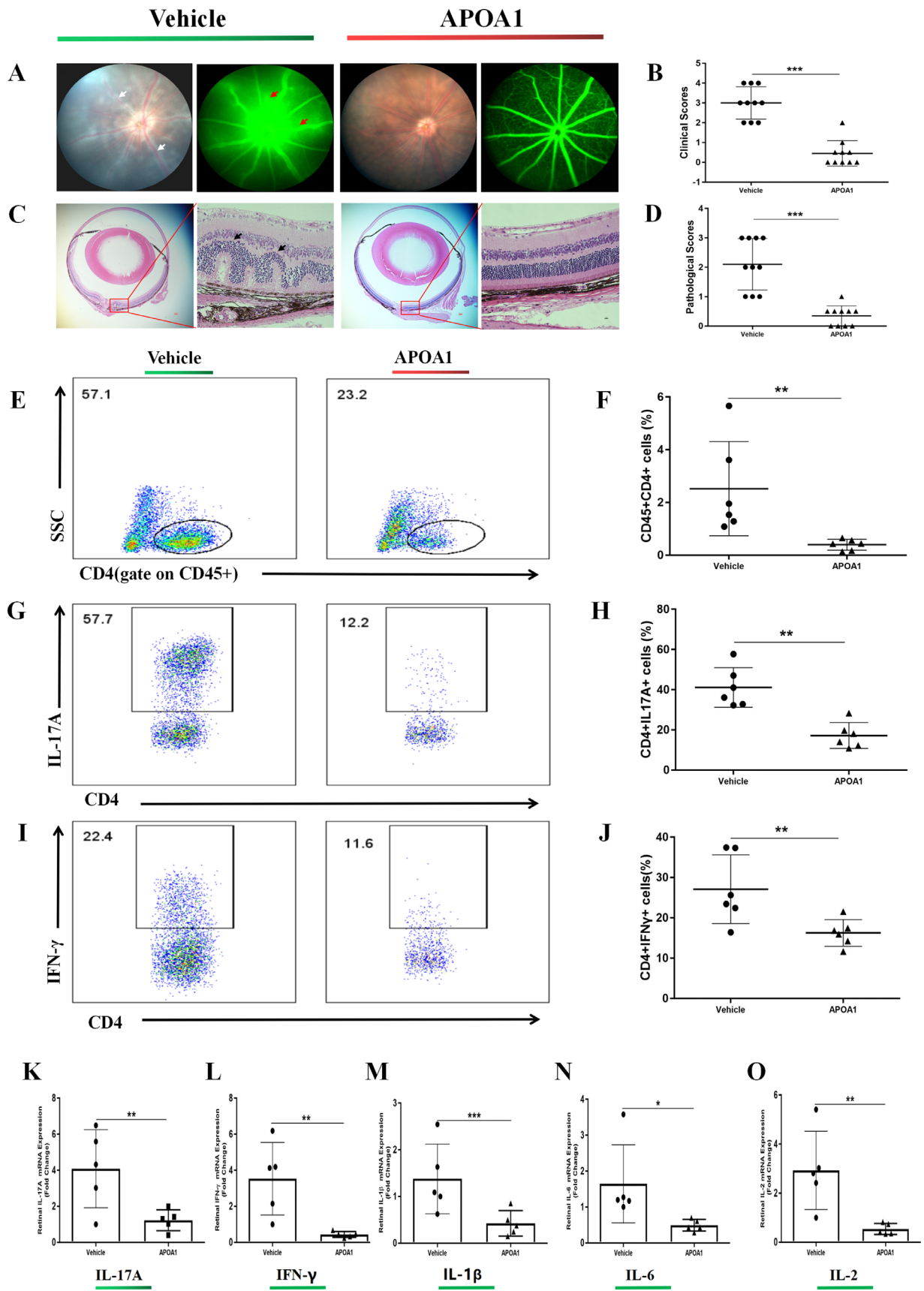
FIGURE 1. APOA1 treatment alleviates intraocular inflammation in EAU. **(A, B)** Representative images of fundus and FFA examination of eyes in EAU mice and APOA1-treated mice with clinical scores on day 14 after immunization (n = 10). APOA1 decreased retinal inflammation, characterized by less retinal edema, vasculitis and infiltrations. The *white arrows* indicate inflammatory infiltrations and vascular deformation.

The *red arrows* indicate leakage of sodium fluorescein. **(C, D)** The H&E staining of EAU mice and APOA1-treated mice with histological scores on day14 after immunization (n = 10). The *black arrows* indicate retinal folding. *Scale bars:* 100 μm (Front) and 10um (μost). **(E, F)** The intraocular infiltrated cells were isolated and measured by FCM, APOA1 decreased CD4$^+$ T cell infiltration. **(G, H)** APOA1 decreased Th17 cells (CD4$^+$IL-17A$^+$) and **(I, J)** Th1 cells (CD4$^+$IFN-γ^+) (n = 6). **(K-O)** The mRNA in retinas from EAU mice and APOA1-treated mice on day14 after immunization were assessed by real-time quantitative PCR. APOA1 suppressed the gene expression of IL-17A, IFN-γ, IL-1β, IL-6 and IL-2 (n = 5). The values represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
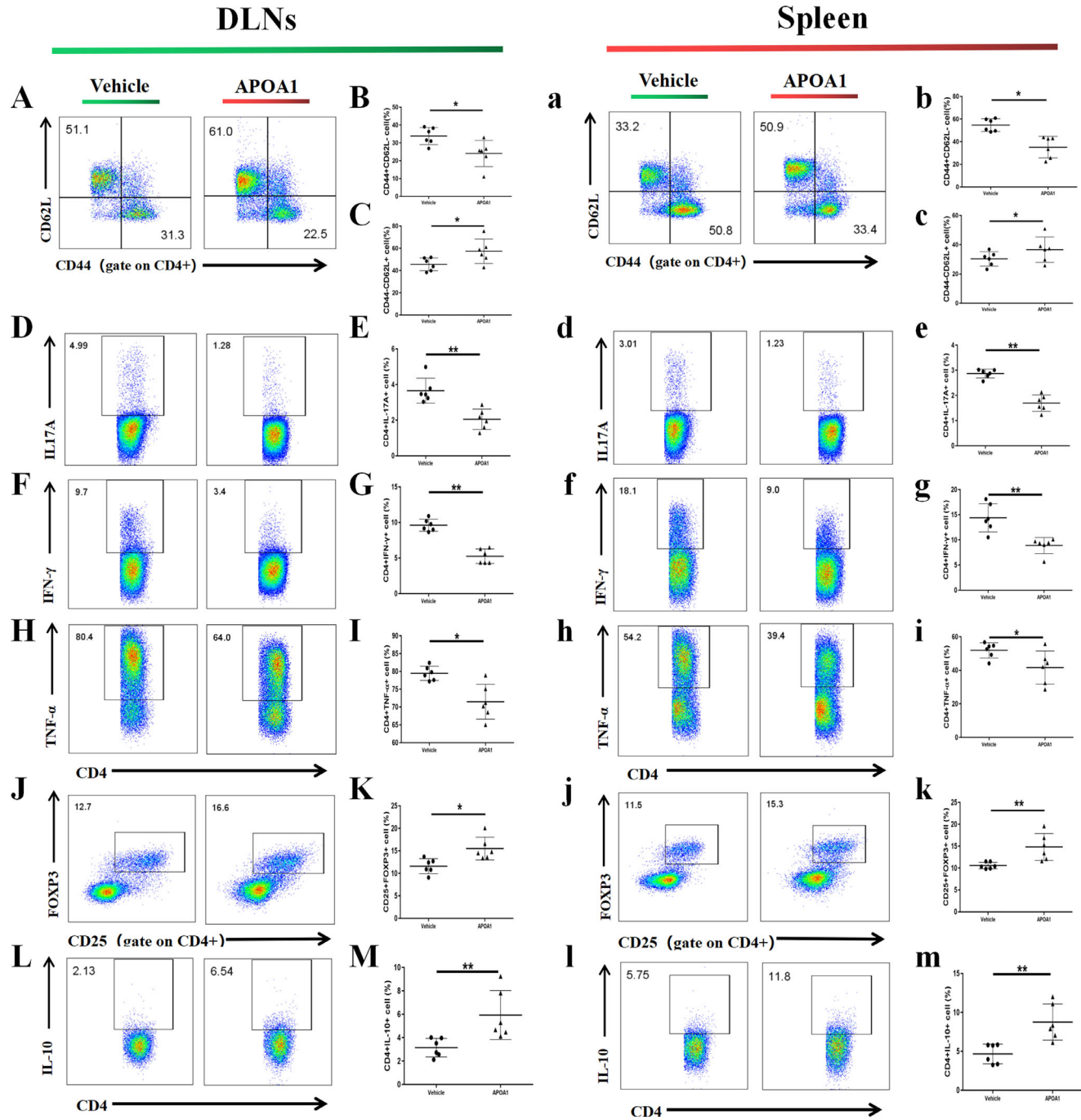

FIGURE 2. APOA1 treatment alters Teffs/Tregs balance in systemic immune. **(A-C; a-c)** APOA1 inhibited the naive CD4$^+$ T cells (CD44$^-$CD62L$^+$) differentiation into memory CD4$^+$ T cells (CD44$^+$CD62L$^-$) in DLNs and spleen compared to vehicle. **(D–H; d–h)** APOA1 decreased the proportion of Th17 cells (CD4$^+$IL-17A$^+$), Th1 cells (CD4$^+$IFN-γ^+) and the secretion of TNF-α in CD4$^+$ T cells (CD4$^+$TNF-α^+) in DLNs and spleen compared to vehicle. **(J–M; j–m)** APOA1 increased Treg (CD4$^+$CD25$^+$Foxp3$^+$) and the secretion of IL-10 in CD4$^+$ T cells (CD4$^+$IL-10$^+$) in DLNs and spleen compared to vehicle (n = 6). The values represent the mean ± SD. *$P < 0.05$, **$P < 0.01$.
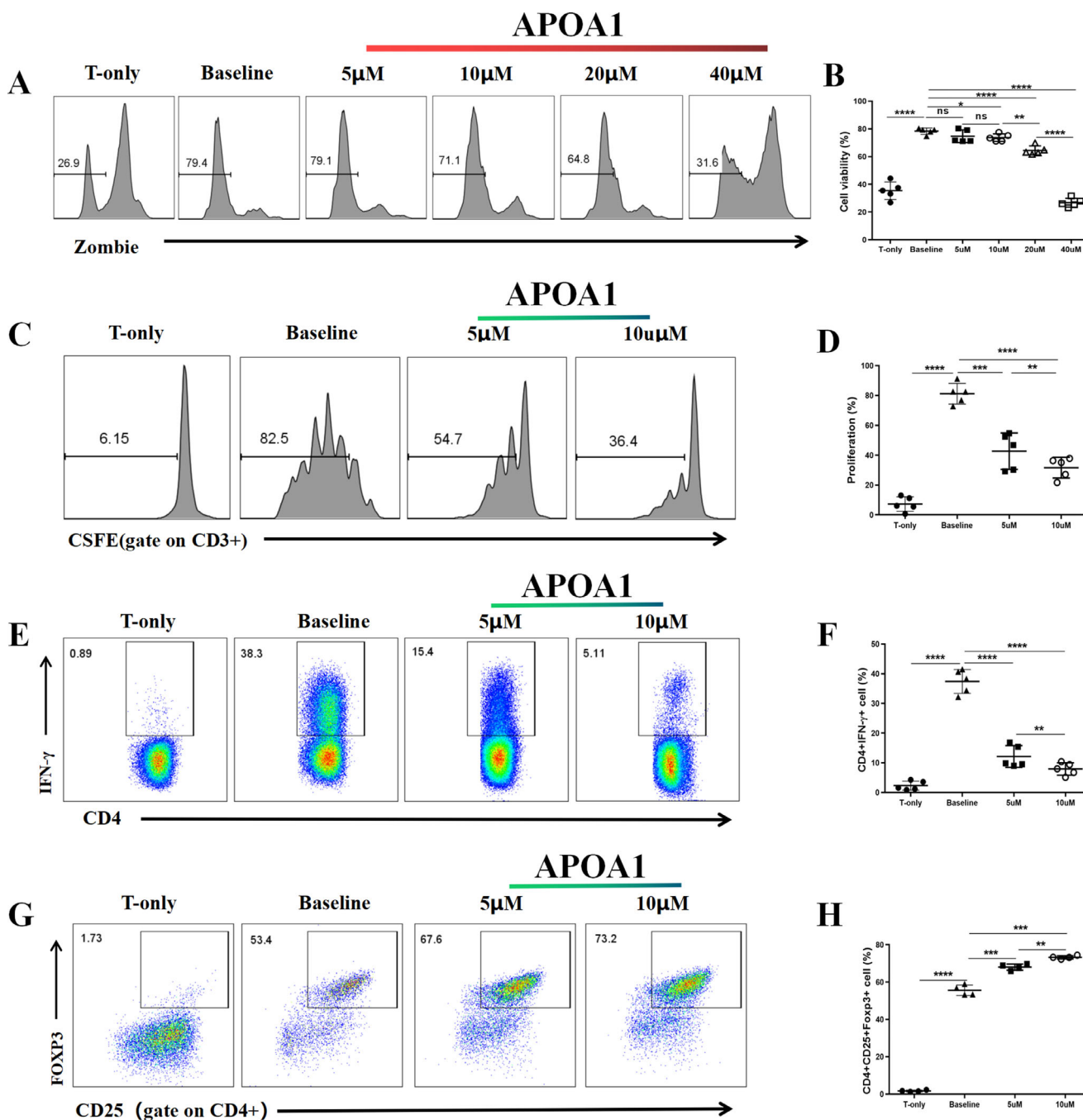
FIGURE 3. APOA1 modulates proliferation and differentiation of T cells. **(A, B)** The viability of T cells was affected by APOA1 inconspicuously at 5 μM and mildly at 10 μM, but significantly at 20 μM and 40 μM (n = 5). **(C, D)** The proliferation of CD3$^+$ T cells was markedly inhibited by APOA1 at concentrations of 5 μM and 10 μM (n = 5). **(E, F)** The induction of Th1 differentiation (CD4$^+$IFN-$\gamma$$^+$) was reduced by APOA1 (n = 5). **(G, H)** The induction of Treg differentiation (CD4$^+$CD25$^+$Foxp3$^+$) was expedited by APOA1 (n = 4). The values represent the mean ± SD. nsp>0.05, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

by APOA1 at concentrations of 5 μM and 10 μM (Figs. 3C, 3D).

Naïve CD4$^+$ T cells were isolated and polarized for Th1 or Treg differentiation. The expression of IFN-γ or CD25/Foxp3 was assayed by FCM after 72 hours of culture. Treatment of cells with APOA1 resulted in a significant decrease in IFN-γ expressing CD4$^+$ T-cells (Figs. 3E, F). In contrast, APOA1 increased CD25$^+$Foxp3$^+$ expression in CD4$^+$ T cells (Figs. 3G, H). Thus, APOA1 inhibits T cell proliferation and Th1 differentiation, while promoting Treg induction.

APOA1-Mediated Immunoregulation Partially Depends on SR-BI

SR-BI, first identified as a physiological HDL receptor, mainly binds to APOA1 for the intracellular uptake of cholesterol and has been confirmed as a novel potential target for mediating the development and function of T cells.[22,23] Therefore we focused on the effect of SR-BI on APOA1-mediated T cell immunoregulation. Elevated gene and protein expression of SR-BI were detected in DLNs of EAU compared with normal
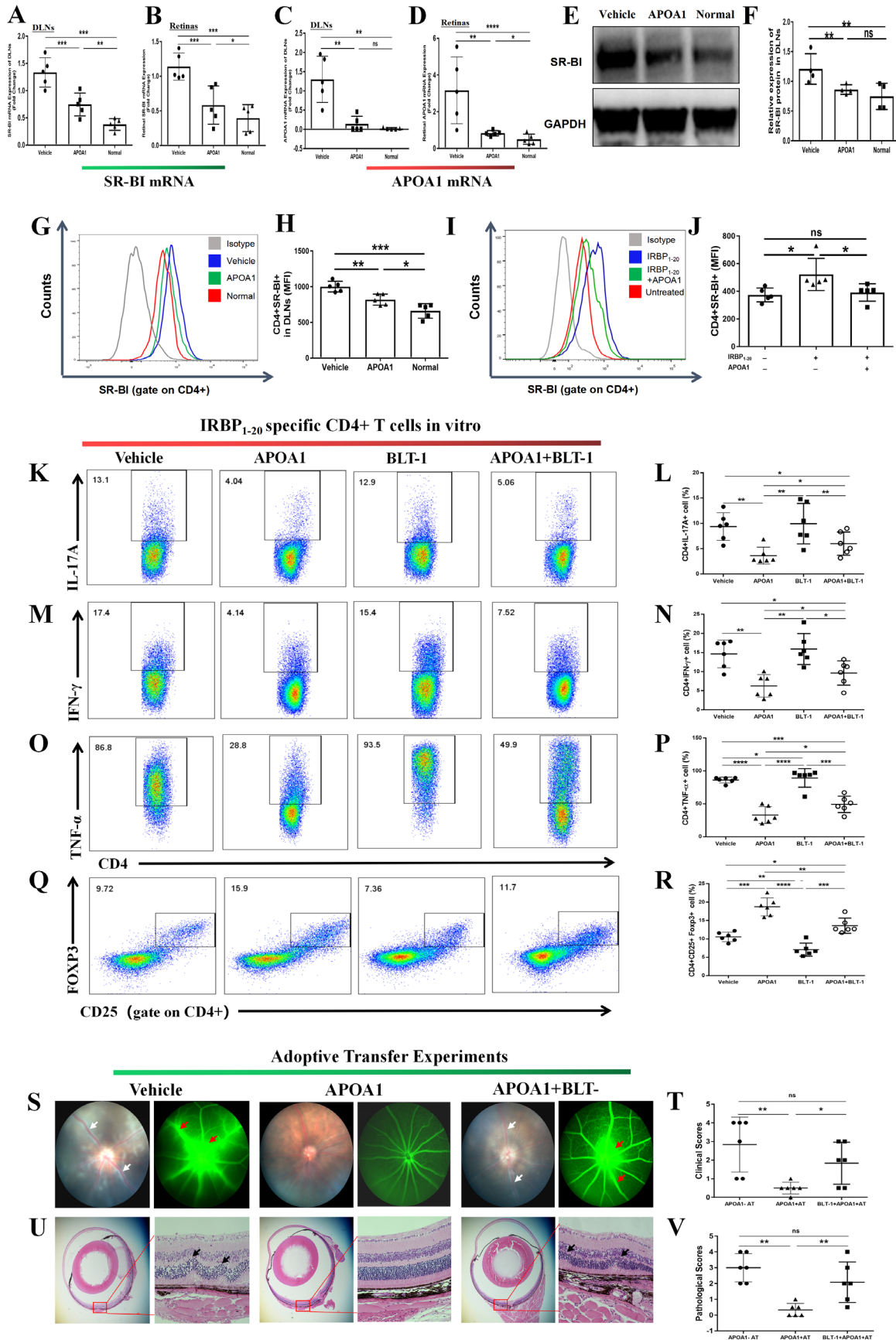
FIGURE 4. APOA1-mediated immunoregulation Partially Depend on SR-BI. **(A–D)** Elevated gene expression of SR-BI**(A, B)** and APOA1 **(C, D)** were detected in DLNs and retinas of EAU compared with normal mice, but decreased in APOA1-treated mice (n = 5). **(E, F)** Elevated protein levels of SR-BI were detected in DLNs of EAU compared with normal mice, but decreased in APOA1-treated mice (n = 4).

(G, H) Elevated expression of SR-BI on CD4+ T cells were detected by FCM in DLNs of EAU compared with normal mice, but decreased in APOA1-treated mice (n = 5). **(I, J)** Lymphocytes from DLNs of EAU mice were obtained after immunization. Increased expression of SR-BI on CD4$^+$ T cells after stimulated by IRBP$_{1-20}$ for 72 hours compared to untreated group, but decreased in APOA1-treated group (n = 5). **(K-R)** Lymphocytes from DLNs of EAU mice stimulated by IRBP$_{1-20}$ with or without APOA1 and with or without BLT-1 for 72 hours. Cells were measured by FCM on the gate of CD4$^+$ T cells. APOA1 decreased the expression of IL-17A **(K, L)**, IFN-γ **(M, N)** and TNF-α **(O, P)**, but elevated Treg **(Q, R)**, whereas, BLT-1 partially blocked the APOA1-mediated effects (n = 6). **(S, T)** Representative images of fundus and FFA examination of eyes on day 14 after injection of IRBP-specific CD4+ T cells in three different conditions: vehicle, APOA1 and APOA1 plus BLT-1 (n = 6). BLT-1 partially reversed the beneficial effects of APOA1 in decreasing of the clinical scores and retinal inflammation. The *white arrows* indicate inflammatory infiltrations and vascular deformation. The *red arrows* indicate leakage of sodium fluorescein. **(U, V)** The H&E staining results correspond to the three different conditions: vehicle, APOA1 and APOA1 plus BLT-1(n = 6). BLT-1 partially reversed the effects of APOA1 in decreasing of the histological scores on day14 after immunization. The *black arrows* indicate retinal folding. *Scale bars:* 100 μm (Front) and 10 μm (Post). The values represent the mean ± SD. nsp>0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

mice, but decreased in APOA1-treated mice, as well as gene expression in retinas (Figs. 4A, 4B, 4E, 4F), suggesting that the effects of APOA1 on T cells may be related to SR-BI. Elevated expression of SR-BI on CD4$^+$ T cells were detected by FCM in DLNs of EAU compared with normal mice but decreased in APOA1-treated mice (Figs. 4G, 4H). Increased expression of SR-BI on CD4$^+$ T cells after stimulated by IRBP$_{1-20}$ compared to untreated group, but decreased in APOA1-treated group (Figs. 4I, 4J). Increased gene expression of APOA1 were found in DLNs and retinas of EAU but lowered after APOA1 treated (Figs. 4C, D).

To confirm whether the therapeutic effects of APOA1 are related to SR-BI, IRBP$_{1-20}$ stimulated CD4$^+$ T cells assay and adoptive transfer experiments were conducted. BLT-1, a selective SR-BI inhibitor, is a thiosemicarbazone copper chelator that inhibits the combination of APOA1 with SR-BI. BLT-1 (2 μM) treatment partially reversed the APOA1-mediated effects on the secretion of IL-17A, IFN-γ, and TNF-α in CD4$^+$ T cells, together with the effects on the expression of CD25$^+$Foxp3$^+$ Tregs (Figs. 4K-R).

In the adoptive transfer experiment, APOA1 treatment of IRBP-specific CD4$^+$ T cells failed to induce EAU, manifesting as no vasculitis or confluent chorioretinal lesions, which verified that a therapeutic effect of APOA1 on EAU was mediated by decreasing CD4$^+$ T cell pathogenicity. However, BLT-1-induced suppression of APOA1's effects on IRBP-specific CD4$^+$ T cells, which was characterized by mild retinal inflammation (Figs. 4S–V). These results confirm that SR-BI exerts a pivotal effect on APOA1-mediated T cell immunoregulation.

Downstream APOA1 Signaling Involves the PI3K/AKT, p38 MAPK and NF-κB Pathways

PI3K/AKT and p38 mitogen-activated protein kinase (p38-MAPK) play critical roles in the immune response to EAU, which was previously reported by our group.[19,24] NF-κB activation was also enhanced after EAU induction.[25] To elucidate the downstream signaling pathways of APOA1-SR-BI binding interaction, we examined whether the activation of these signaling pathways was modulated by APOA1. T cells from the DLNs of EAU mice were stimulated with IRBP$_{1-20}$, both with APOA1 (10 μM) and/or BLT-1 (2 μM) or not. APOA1 treatment downregulated the phosphorylation of phosphatidylinositol 3-kinase (PI3K)/AKT, p38, and NF-κB signaling, whereas BLT-1 reversed the function of APOA1 in IRBP-specific CD4$^+$ T cells (Figs. 5A–H).

To further determine whether PI3K/AKT and p38 MAPK signaling pathways are involved in the effect of APOA1 on the downregulation of inflammatory cytokines, we used 740Y-P (PI3K activator), SC79 (Akt activator), or DEH (p38 MAPK activation) to treat IRBP-specific CD4$^+$ T cells under

APOA1 treatment. The 740Y-P, SC79, and DEH promoted the expression of inflammatory mediators, including IL-17A, IFN-γ, and TNF-α, while inhibiting Foxp3 against APOA1 in IRBP-specific CD4$^+$ T cells (Figs. 5I–P). Collectively, these findings suggest that APOA1 regulates T cell function by targeting the PI3K-Akt, MAPK, and NF-κB pathways.

APOA1 Restricted Inflammatory Cytokine Production From LPS-Stimulated PBMCs

We found that APOA1 and SR-BI gene expression in human PBMCs was significantly lower in patients with uveitis than in healthy individuals (Figs. 6A, 6B). The plasma levels of APOA1 and expression of SR-BI on CD4$^+$ T cells were much lower in uveitis patients (Figs. 6C–E). To further investigate whether APOA1 restricts inflammatory cytokine production in human PBMCs, we isolated PBMCs from patients with uveitis and stimulated them with LPS (100 ng/mL) in the presence or absence of APOA1. The expression of IL-17A, IFN-γ, and TNF-α in CD4$^+$ T cells was significantly suppressed by APOA1, whereas Treg cells were potentiated by APOA1 treatment. BLT-1 treatment partially reversed the effects of APOA1 (Figs. 6F–M). Meanwhile, we also found APOA1 suppressed anti-human CD3/CD28 beads-stimulated immunological responses of PBMCs (Supplementary Fig. S2). Therefore treatment of PBMCs with APOA1 regulates human T cell pathogenicity and suppresses the production of proinflammatory cytokines that involve SR-BI.

Discussion

APOA1, the most abundant component of HDL, has been known for anti-inflammatory and antioxidant properties that play a major role in atherosclerosis.[26,27] However, its potential role in the pathogenesis of AD has not been fully investigated. The present study revealed a new therapeutic effect elicited by APOA1 in EAU. We found that APOA1 relieved ocular inflammation and reduced the proportion of pathogenic T cells in EAU mice. We determined that APOA1 modulates the Teff/Treg balance by suppressing the proliferation and differentiation of T cells. Furthermore, blocking SR-BI with BLT-1 partially reversed the suppressive effect of APOA1 on T cells. Subsequently, we demonstrated that APOA1 regulated the PI3K/AKT, p38 MAPK, and NF-κB pathways. Moreover, our results showed that APOA1 inhibited Teffs and promoted Tregs in PBMCs. We believe that the immunomodulatory effects of APOA1 may offer a new therapeutic approach for AD.

APOA1 participates in AD, significant decrease in APOA1 plasma levels are observed in patients with AD, including systemic lupus erythematosus and rheumatoid arthritis
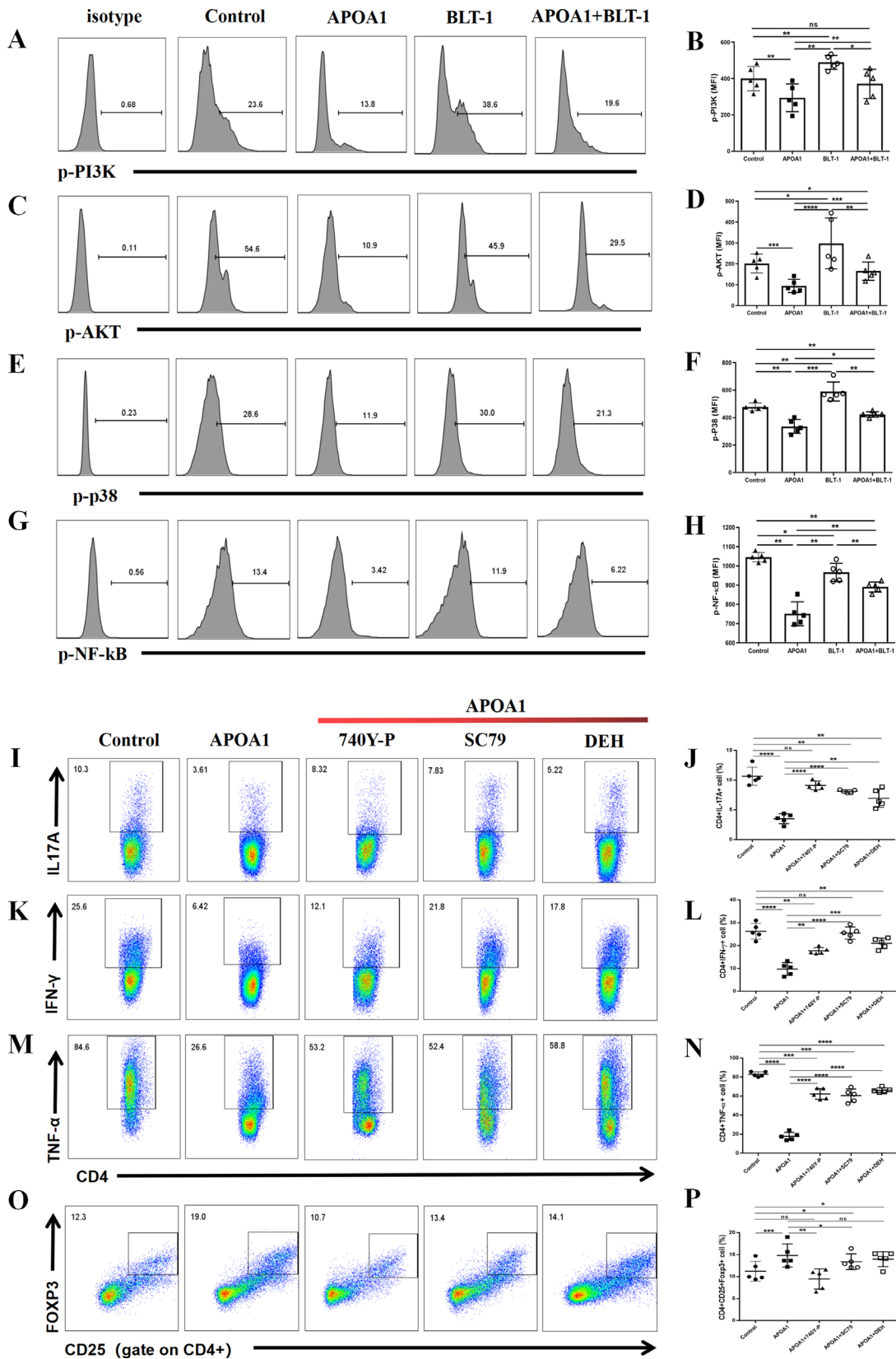
FIGURE 5. Downstream APOA1 signaling involves the PI3K/AKT, p38 MAPK and NF-κB pathways. **(A–H)** APOA1 treatment downregulated the p-PI3K **(A, B)**, p-AKT **(C, D)**, p-p38 **(E, F)** and p-NF-κB **(G, H)** expression significantly in the lymphocytes from DLNs of EAU mice, which were stimulated with IRBP$_{1-20}$. BLT-1 partially reversed the effects of APOA1. **(I–P)** Lymphocytes from DLNs of EAU mice were obtained and stimulated with IRBP$_{1-20}$ under different conditions: untreated (control), APOA1, APOA1 plus 740Y-P, APOA1 plus SC79, APOA1 plus DEH for 72 hours. Cells was measured with FCM on the gate of CD4$^+$ T cells. APOA1 decreased the expression of IL-17A **(I, J)**, IFN-γ **(K, L)** and TNF-α **(M, N)**, but elevated Treg **(O, P)** compared with control. Whereas, the pathway agonists, 740Y-P, SC79 and DEH partially suppressed the APOA1-mediated effects (n = 5). The values represent the mean ± SD. nsp>0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
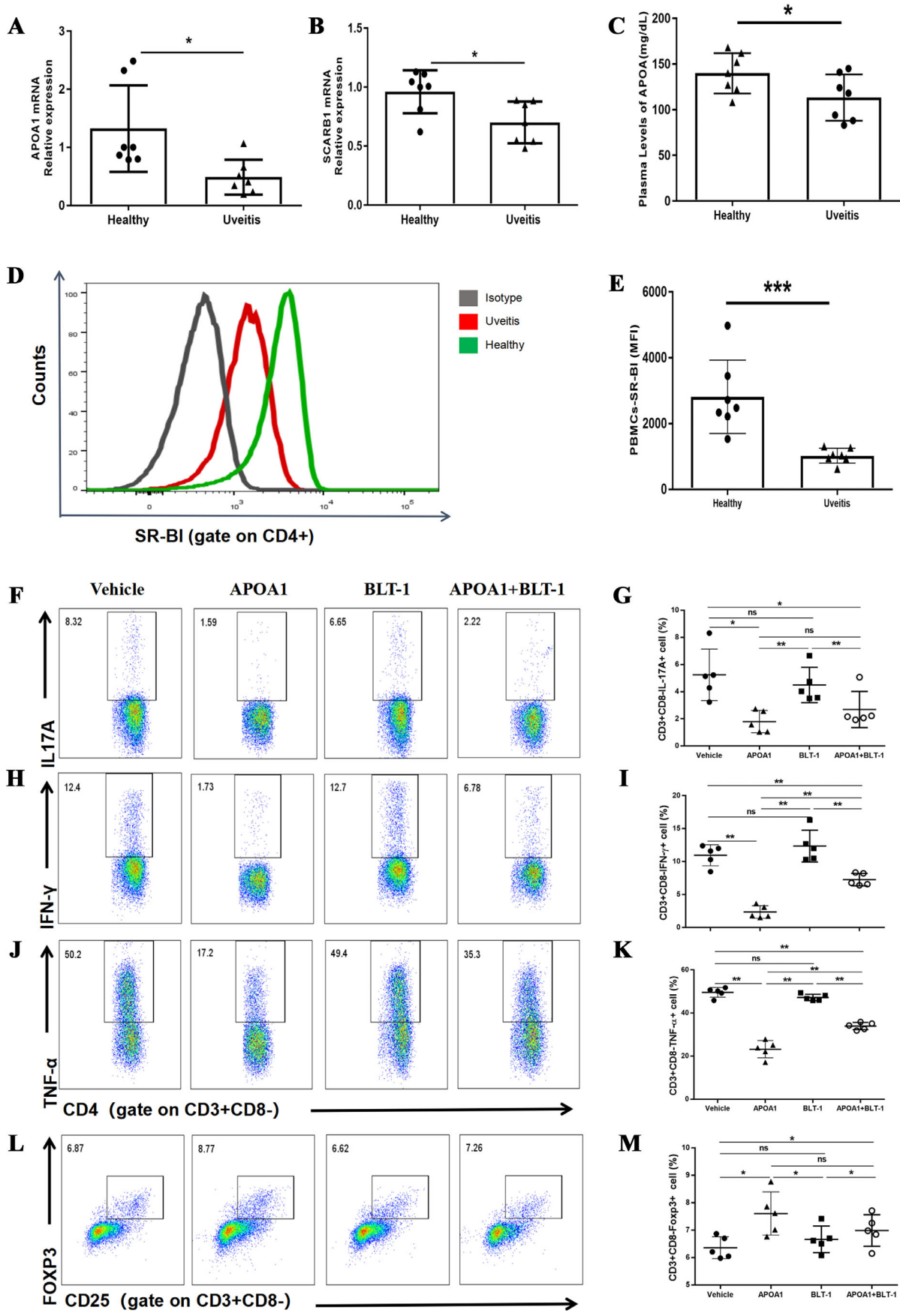
FIGURE 6. APOA1 restricted inflammatory cytokine production from LPS-stimulated PBMCs. **(A, B)** The mRNA extraction from PBMCs of uveitis patients and healthy people. APOA1 and SR-BI transcription levels were significantly decreased in uveitis patients (n = 7). **(C)** Compared to healthy individuals, the plasma levels of APOA1 measured by immunoturbidimetric assay were lower in uveitis patients

(n = 7). **(D, E)** Decreased expression of SR-BI on CD4+ T cells were detected by FCM in PBMCs of uveitis patients compared to healthy individuals (n = 7). **(F-M) The** PBMCs from uveitis patients were obtained and stimulated by LPS with or without APOA1 and with or without BLT-1 for 72 hours. Cells were measured by FCM on the gate of CD4$^+$ T cells (CD3$^+$CD8$^-$). APOA1 decreased the expression of IL-17A **(F, G)**, IFN-γ **(H, I)** and TNF-α **(J, K)**, but elevated Treg **(L, M)**, whereas, BLT-1 partially reversed the APOA1-mediated effects (n = 5). The values represent the mean ± SD. nsp>0.05, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(RA),[28,29] consistent with our finding that APOA1 expression was decreased in the plasma and PBMCs of patients with uveitis. APOA1 infiltration was found in inflamed synovial tissue of active RA with T lymphocyte and macrophage accumulation, while faint or no APOA1 was observed in apparent remission or negative controls.[30] Our results identified increased APOA1 expression in the DLNs and retinas of EAU mice, whereas, decreased in the mild or disease-free EAU mice after APOA1 treatment. The differential APOA1 expression was recognized in the lesion tissues and plasma of AU, which may represent physiological mechanisms associated with APOA1 inhibition of inflammation. Moreover, APOA1 plasma levels were higher in patients who responded well to immunotherapy than in non-responders.[31] Thus APOA1 may play an important role in the development and progression of AD and may be a promising therapeutic candidate.

Activated pathogenic T cells and disruption of the Teff/Treg balance are critical to the pathogenesis of both EAU and human uveitis.[32] APOA1 is a specific HDL-binding site in human lymphocytes.[33,34] However, its effect on T cells has not been fully investigated. In our study, investigation of T cell subsets revealed increased activated CD4$^+$ T cells and Teffs but decreased Tregs in DLNs and the spleen of EAU mice, and this change was inhibited by APOA1-treated. Pathogenic T cells contribute to ocular inflammation and retinal tissue damage in EAU.[14,20] We found that that APOA1 treatment suppressed autoreactive Th1/Th17 cells, reduced retinal vasculitis and infiltration, as well as inflammatory cytokines in the eye. APOA1$^{-/-}$ mice exhibit enlarged lymph nodes with increased T cell activation and proliferation.[35] APOA1 injection prevented Treg conversion into pro-atherogenic T follicular helper cells in APOE$^{-/-}$ mice.[36] Our data are consistent with those of previous studies. Therefore the beneficial modulation of the immune response by APOA1 via regulating the Teff/Treg balance is a pivotal mechanism for the therapeutic effect on EAU.

SR-BI is a binding receptor for APOA1 in macrophages, antigen-presenting cells, endothelial cells, and liver cells.[27,37–39] However, whether APOA1 can modulate T cells via SR-BI remains unknown. SR-BI deficiency impaired T-cell development and enhanced lymphocyte proliferation,[23,40] which elucidated a previously unrecognized link between SR-BI and adaptive immunity. Indeed, we found that the expression of SR-BI in DLNs and retinas was increased in EAU but was inhibited by APOA1. Furthermore, our in vitro study suggests that BLT-1, a selective SR-BI inhibitor, partially reversed the effect of APOA1 on regulating the Teff/Treg balance. Moreover, APOA1 treatment significantly attenuated EAU induced by IRBP-specific CD4$^+$ T cells, BLT-1 treatment reversed this beneficial effect. Therefore the present study confirms that APOA1 lowering the pathogenicity of CD4$^+$ T cell partially depends on SR-BI.

There is convincing evidence for the involvement and potential therapeutic effect of PI3K/Akt, p38 MAPK, and NF-κB signaling pathways in AD.[41–45] Upregulation of these pathways in IRBP-specific CD4$^+$ T cells has been reported in our previous studies,[19,24] as well as in the present study.

Furthermore, APOA1 treatment inhibited IRBP-induced upregulation of phosphorylation in these pathways, and agonists reversed the inhibitory effect. APOA1/SR-BI inhibits NF-κB translocation via the PI3K/Akt pathway in vascular endothelial cells.[38] Whether APOA1/SR-BI has similar mechanisms in T cells remains unknown. Indeed, our study revealed that BLT-1 treatment resulted in the reversal of APOA1. Therefore our data indicate that the immunomodulatory effect of APOA1 on T cells may account for SR-BI–dependent mechanisms via the PI3K/Akt, p38 MAPK, and NF-κB signaling pathways. Our study enriched the understanding of the mechanism of APOA1 in T cells, but further research is needed to clarify the mechanisms of APOA1.

To the best of our knowledge, this study reveals the therapeutic effect of exogenous APOA1 on EAU for the first time. Our investigation demonstrated that APOA1 greatly reduced autoreactive T cell responses and significantly attenuated retinal inflammation in EAU. These findings reveal a previously unknown but pivotal role of APOA1 in autoimmune diseases and suggests that APOA1 could be used as new therapeutic alternative for AU. We also found that APOA1 can weaken CD4$^+$ T cell pathogenicity and proposed a potential underlying SR-BI-dependent mechanism for its molecular regulation. Therefore this study broadens our understanding of the immune regulatory mechanisms underlying APOA1 and suggests that T cells are critical targets of APOA1 in EAU.

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