APJAI
Asian Pacific Journal of
Allergy and Immunology

ORIGINAL ARTICLE

Prostaglandin E2 inhibits the differentiation of T regulatory cells by Peroxisome Proliferator-Activated Receptor– Gamma during allergic rhinitis

Yurong Ju,[1] Lisha Li,[2] Ye Zhao,[1] Zhifeng Yang,[1] Ziheng Zhao,[1] Zhaofei Wu,[1] Xuewen Pang,[1] Wei Wang[1]

Abstract

Background: Allergic rhinitis (AR) represents a significant global health concern that can give rise to numerous diseases and result in labor productivity. T regulatory (Treg) cells are pivotal players in the pathogenesis of AR, and their deficiencies are closely related to Prostaglandin E2 (PGE2). However, the downstream mechanisms of this relationship remain poorly understood.

Objective: This study aims to investigate the inhibitory mechanisms through which PGE2 impacts the differentiation of Treg cells.

Methods: We compared the differentiation of Treg cells from naïve $CD4^+$ T cells of AR patients and healthy controls, with or without the presence of PGE2 by flow cytometry. Intracellular cAMP concentration, mRNA and protein levels of cyclic-AMP dependent protein kinase A (PKA), as well as their downstream target, Peroxisome Proliferator-Activated Receptor–γ (PPAR-γ) were examined in Treg cells from AR and healthy donors. AR mouse model was established by pollen administration.

Results: PGE2 suppressed the differentiation of Treg cells from human naïve $CD4^+$ T cells through the EP4 receptor. Furthermore, in AR patients and AR mouse, the expression of EP4 receptor were observed enhanced. The PGE2-EP4 signal was carried out by activating cAMP-PKA signaling pathway. Subsequently, phospholated PKA would suppress PPAR-γ expression. Treatment of Pioglitazone, a PPAR-γ agonist, was demonstrated to rescue the differentiation of Treg and help alleviate inflammation in the AR mouse model.

Conclusion: In AR disease, the PGE2-EP4 signaling exerts an inhibitory effect on Treg differentiation by influencing the cAMP-PKA pathway and its downstream target PPAR-γ.

Key words: Allergic rhinitis, Treg cells, PGE2, EP4, PPAR-γ

Citation:
Ju, Y., Li, L., Zhao, Y., Yang, Z., Zhao, Z., Wu, Z., Pang, X., Wang, W. (0000). Prostaglandin E2 inhibits the differentiation of T regulatory cells by Peroxisome Proliferator-Activated Receptor– Gamma during allergic rhinitis.
Asian Pac J Allergy Immunol, 00(0), 000-000.
https://doi.org/10.12932/ap-210923-1695

Affiliations:
[1] Department of Immunology, School of Basic Medical Sciences, Peking University. NHC Key Laboratory of Medical Immunology (Peking University), Beijing, China
[2] Department of Allergy, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing Key Laboratory of Precision Medicine for Diagnosis and Treatment on Allergic Diseases, National Clinical Research Center for Immunologic Diseases, Beijing, China

Corresponding author:
Wei Wang
Department of Immunology, Peking University Health Science Center, 38 Xue Yuan Road, Beijing, 100191, China
E-mail: wangwei83427@bjmu.edu.cn

Introduction

Allergic rhinitis (AR) stands as one of the most prevalent chronic conditions in numerous countries, maked by symptoms such as sneezing, nasal congestion, nasal itching and rhinorrhoea (nasal discharge).[1] The reported prevalence of AR varies, ranging from approximately 2% to 25% in children[2] and 1% to greater than 40% in adults.[3,4] As of now, pharmacological treatments for AR encompass oral, intranasal or ocular H1-antihistamines,

intranasal corticosteroids, leukotriene receptor antagonists, anticholinergics and IgE neutralizing antibody. Regrettably, none of them have demonstrated sufficient effectiveness. Therefore, the development of more suitable treatment aimed at relieving symptoms or reducing the occurrence of AR holds great value in enhancing the quality of life and work or school performance.

AR involves a cascade of allergen induced reaction across various immune cells.[1,5] Among these, Treg cells play a pivotal role in balancing the immune response to allergens.[6] A study conducted by Meng et al.[7] revealed that the occurrence and development of AR may be attributed to Treg cells, due to their reduced numbers or dysfunction, resulting in weakened inhibitory effects on T helper cells, mast cells and other effector cells. In another research, when ovalbumin (OVA) peptide–specific $CD4^+CD25^+$ Treg cells were transferred to OVA-sensitized mice, there was a significant reduction in airway hyperreactivity (AHR) and Th2 cell numbers in the bronchoalveolar levage (BAL) and lung tissue, compared to mice that were only OVA-sensitized and -challenged.[8] However, the factors initiating the abnormal suppressive function of Treg cells in AR, as well as the upstream regulatory targets, remain unclear.

PGE2, a member of Prostaglandins (PGs) family, stands as the most abundant product of cyclooxygenase. Its role in allergic disease has garnered attention since 1969.[9] PGE2 transmits signals through four EP receptors (EP1-4), each varying in affinity, signal duration and signaling pathways.[10,11] Among these receptors, $CD4^+$ T cells predominantly express receptor EP2 and EP4.[12,13] In a rheumatoid arthritis (RA) study, RA patients exhibited excessive PGE2 production, which inhibited the differentiation of Treg cells via the EP2-cAMP/PKA signaling pathway.[14] Additionally, EP4 has been identified as a major contributor to the differentiation and expansion of Th1 and Th17 both in vitro and in various autoimmune/inflammatory models, such as autoimmune encephalomyelitis.[13,15,16] Notably, data from a mouse model involving the administration of a nonselective COX inhibitor, indomethacin, or co-administration with a selective EP4 agonists, L-902,688, indicated that indomethacin could induce intestinal Treg cell frequencies, whereas L-902,688 inhibited them.[17] In our previous study on AR, we also observed that PGE2 could inhibit Treg cell differentiation via the EP4-cAMP signaling pathway. However, the precise mechanism remains unclear.[18]

Peroxisome-proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily.[19,20] To date, three subtypes of PPARs (PPAR-α, β/δ, and γ) have been identified. Originally, PPAR-γ was primarily known for its role in regulating adipocyte differentiation and lipid metabolism. However, more and more recent evidences have emerged suggesting that PPAR-γ also has the capacity to modulate inflammation.[21] For instance, one study demonstrated that treating obese rats with PPAR-γ agonists like rosiglitazone reduced TNF-α expression in the retroperitoneal and mesenteric white adipose tissues, ultimately leading to weight loss.[22] Another research reported that treatment of monocytes with natural PPAR-γ ligand 15-deoxy-PGJ2 or PPAR-γ synthetic agonists inhibits the production of TNF-α, interleukin (IL)-1β and IL-6.[23] In the context of airway allergic disease, increased PPAR-γ expression has been detected in the airway epithelium of patients with asthma.[20] Furthermore, when 3T3-L1 pre-adipocytes were exposed rosiglitazone and Abscisic acid (ABA), PPAR-γ activity significantly increased. It's worth noting that the ABA induced PPAR-γ activity could be abolished by addition of either cAMP or PKA specific inhibitor.[24] Notably, most of the previously reported immunoregulatory effect of PPAR-γ have been associated with adipocytes or innate immune cells. A few of studies have yet established a connection between PPAR-γ and adaptive immune cells, particularly specific T cells subsets.[25,26] However, given the naturally high expression of PPAR-γ on Tregs cells,[27] we hypothesize that PPAR-γ may be linked to the differentiation or function of Treg cells in AR disease.

In this study, our objective was to delve into the mechanism of PGE2 signal on Treg cell differentiation in the context of AR disease. Additionally, we aimed to investigate whether PPAR-γ play a role in modulating PGE2 signal and whether it contributes to reduce Treg cell numbers in AR patients and in pollen-induced AR mice. We also assessed therapeutic effect of an activator of PPAR-γ, Pioglitazone, in this AR mouse model.

Materials and Methods

Patients

Fourty-two adult patients with AR (20 males and 22 females) aged 23–54 years (median age, 34 years) were recruited consecutively into the study. To enhance the homogeneity of AR patients in this study, we only recruited subjects allergic to mugwort pollens.

Inclusion criteria were: (1) a history of moderate-to-severe allergic rhinitis, which was defined as troublesome symptoms with a visual analogue scale (VAS) score (ranging from 0 to 10) of ≥ 4; (2) rhinitis symptoms that manifested between March and April for at least the previous 2 years; (3) positive intradermal skin test (a wheal 10 mm) to mugwort pollen with histamine dihydrochloride as the positive control and normal saline as the negative control, and serum-specific immunoglobulin E against cypress pollen of ≥ class 2 (ImmunoCAP, Phadia, Sweden) were required. Patients allergic to other aeroallergens or having coexisting asthma were excluded. Ten age- and sex-matched, non-atopic healthy adults were recruited as normal control subjects. Blood samples were collected while the AR patients had typical rhinitis symptoms without taking any medicine.

Approval of the Peking Union Medical College Hospital Ethics Committee for the experimental procedures had been obtained, and all the subjects had given written informed consent (LA2020375).

Mice and AR mice model

Female BALB/c mice, 8 to 10 weeks of age and free of murine-specific pathogens were obtained in a standard laboratory under a specific pathogen-free state.

To construct AR model, mice were sensitized on days 0 and 7 and 14 by intraperitoneal injection of 50 μg pollen (Stallergenes Greer, Texas, USA) dissolved in 400 μL of sterile phosphate-buffered saline (PBS). For pulverization challenge, 1% solution of pollen was instilled lasts 1 hour on day 21. Mice received Pioglitazone (30 μg/kg body weight) or PBS by intranasal challenge after pulverization challenge.

The experimental procedures on use and care of animals had been approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center No. IRB00001052-21083).

Reagents

PGE2, PGE1-Alchohol, ONO-AE3208, Butaprost and AH68-09 were bought from Cayman Chemical. Dibutyryl cAMP (db-cAMP) was purchased from SigmaAldrich. H-89 was purchased from Selleck Chemicals. Pioglitazone, T0070907 and SQ22536 were purchased from MedChemExpress.

Cell purification and Treg cell polarization

Mononuclear cells were isolated from the peripheral blood of AR patients and healthy donors by Ficoll-Hypaque density gradient (Solarbio, Beijing, China). Then human naïve $CD4^+$ T cells were isolated by human naïve $CD4^+$ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. Mouse naïve $CD4^+$ T cells were obtained from lymph nodes and spleens and purified by mouse naïve $CD4^+$ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the sorted cells was determined by flow cytometric analysis (>95% for $CD4^+$ cells).

Naive $CD4^+$ T cells (2×10^5/well) were cultured for 3 days in 10% heat-inactivated FCS/RPMI 1640 containing soluble anti-CD28 Ab (1 μg/mL, Biolegend) in 96-well plates pre-coated with anti-CD3 (2 μg/mL, Biolegend). The cultures were generated in the presence of TGF-β1 (10 ng/ml for human and 5 ng/ml for mouse, Bio-Techne, R&D systems) and IL-2 (20 ng/mL, PeproTech) for Treg differentiation.

Flow cytometry

Fresh peripheral blood mononuclear cells from AR patients and health donors, cells from mouse lymph nodes and spleens, as well as the cultured cells under Treg-priming condition, were collected and analyzed. For surface staining, cells were incubated for 30 minutes at 4°C with fluorescent labeled monoclonal antibodies specific for human and mouse CD4, CD25 (eBiosciences). For intracellular staining, cells were first fixed and permeabilized with Cytofix/Cytoperm (Invitrogen) and then incubated with PE-conjugated anti-Foxp3 (Biolegend). Appropriate isotype-matched antibodies were utilized for compensation adjustment. Flow cytometric analysis was performed on BD FACSCanto. Data were analyzed with FlowJo software (TreeStar, Becton, Dickinson & Company).

Enzyme-linked immuno sorbent assay (ELISA)

To determine cAMP in Intracellular, ELISA development kits (Elabscience, Beijing, China) were used according to the manufacturer's directions.

Real-Time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) from Treg cells, and the first strand was reverse-transcribed using random primer (TaKaRa). The levels of *Ptger2*, *Ptger4*, *Prkaca*, *Pparg* and *Actb* mRNA expression were determined by real-time polymerase chain reaction (PCR) using the ABI PRISM 7300 Real-Time PCR System and SYBR Green PCR master mix (TaKaRa). Specific primer sequences were displayed in **Table 1**. The levels of these mRNA expressions were analyzed by the ABI 7300 Sequence Detection System. The results were normalized by *Actb* expression and shown as fold increase over the expression of the control group.

Table 1. Primer Sequences.

Ptger2	Forward: 5′- GCTCCTTGCCTTTCACGATTT -3′
	Reverse: 5′AGGATGGCAAAGACCCAAGG -3′
Ptger4	Forward: 5′- AACTCTGACCTCGGTGTCCA -3′
	Reverse: 5′CAAGGCTGGGTCTGTAGCG -3′
Prkaca	Forward: 5′- GAGCAGGAGAGCGTGAAAGA -3′
	Reverse: 5′GCCGAGGGTCTTGATTCGTT -3′
Pparg	Forward: 5′- CTGGGGCGCTTGGGTC -3′
	Reverse: 5′GTGTCAACCATGGTCATTTCGTTA -3′
Actb	Forward: 5′- GCCGCCAGCTCACCAT -3′
	Reverse: 5′TCGTCGCCCACATAGGAATC -3′

Western blot analysis

Cell lysate were lysed by RIPA lysis buffer, size-fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto intracellular membranes. The blots were incubated with appropriate antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using ECL western blotting substrate (Thermo Fisher) according to the manufacturer's recommendation.

Dual-Luciferase reporter assay

HEK293T cells were seeded in 48-well plates and when confluency came to 70%, *Foxp3* luciferase reporter and Flag-PPAR-γ with HA-RXRα plasmids were transfected. Cells were lysed in 1 × Passive Lysis Buffer (Promega) and then firefly luminescence and Renilla luminescence were measured using a luminometer. Data are presented as fold activation of the reporter alone and represent means ± SD of at least three independent experiments.

Statistical analysis

Results were reported as mean ± s.e. or mean ± s.d. for independent experiments with or without triplicates. Statistical analysis was performed with GraphPad Prism 9. Two-tailed *Student's t* tests were used for single comparison.

Results

The regulation of Treg cell differentiation by PGE2 was mediated through prostanoid receptor EP4.

In our previous article, we observed that the proportion of $CD4^+Foxp3^+$ Treg cells in the peripheral blood of AR patients was significantly lower compared to healthy donors, while the PGE2 concentration was higher.[23] To delve deeper into the mechanism through which PGE2 inhibits Treg cells, we purified naïve $CD4^+$ T cells from AR patients and healthy donors and cultured them under Treg-polarization conditions with or without the presence of PGE2. Although the differentiation capacity of Tregs from naïve $CD4^+$ T cells was comparable between healthy donors and AR patients (**Figure 1A**), we found that $CD4^+$ T cells from AR patients were more sensitive to PGE2 (**Figure 1B**). The percentage of $Foxp3^+$ Treg cells decreased significantly from 62.6% to 35.8% in response to 100nM PGE2, leading to a more pronounced inhibition compared to healthy donors $CD4^+$ T cells.

Furthermore, when we explored the relationship between PGE2 concentration and the ratio of Treg cells on naïve $CD4^+$ T cells in peripheral blood, the result revealed that higher PGE2 levels were associated with a lower the proportion of Treg cells (P = 0.0002, **Figure 1C**). This observation could be attributed to alterations in the expression of EP receptors, particularly the EP4 receptor (**Figure 1D**). Later, we analyzed the correlation between the expression levels of EP4 and Treg ratios on naïve $CD4^+$ T cells, and indeed, the two variables were found to be negatively correlated (P = 0.0036, **Figure 1E**). In line with these findings from human cells, PGE2 also significantly suppressed the differentiation of mouse Treg cells from naïve $CD4^+$ T cells isolated from lymph nodes. Notably, EP4-selective antagonist ONO-AE3208 could reverse the inhibitory effect of exogenous PGE2 on Treg differentiation (**Figure 1F**). In contrast, EP2 agonist Butaprost or antagonist AH68-09 didn't have a clear regulatory effect on mouse Tregs (**Figure S1C**). These results collectively suggested that PGE2 influenced the differentiation but not survival of Treg cells (**Figure S1B**), and this inhibitory signal is mediated through the EP4 receptor.

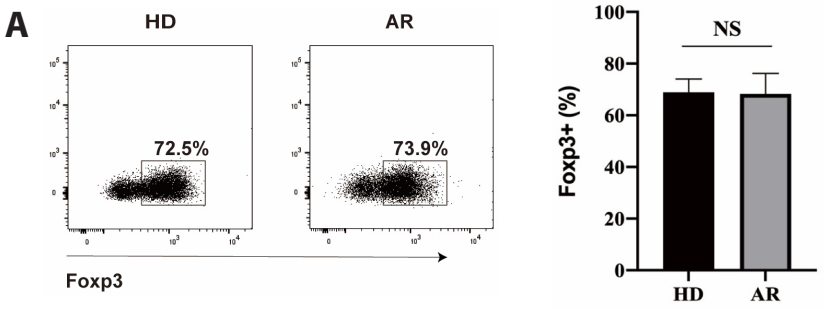

Figure 1. The effects of PGE2 and diverse EP4 antagonist on the differentiation of human Treg cells *in vitro*. Naïve $CD4^+$ T cells isolated from human peripheral blood were stimulated with TGF-β1 (10 ng/ml) and IL-2 (20 ng/ml) in the presence of anti-CD28 (1 μg/ml) and pre-coated anti-CD3 (2 μg/ml) for 3 days. The proportion of induced $Foxp3^+$ Treg cells was measured by flow cytometry. (A) Naïve $CD4^+$ T cells were purified from the PBMC of AR patients (n = 3) and health donors (n = 3) and cultured under Treg polarization condition. (B) Naïve $CD4^+$ T cells were purified from the PBMC of AR patients (n = 3) and health donors (n = 3) and cultured under Treg polarization in the presence of 100 nm of PGE2. (C) The correlation of PGE2 concentration in peripheral blood and $Foxp3^+$ Treg cells ratios on naïve $CD4^+$ T cells in PBMC. (D) Transcription levels of EP receptors Ptger2 and Ptger4 in the induced Tregs from AR patients (n = 3) and healthy donors (n = 3). (E) The correlation of protein expression levels of EP4 by flow cytometry and $Foxp3^+$ Treg cells ratios on naïve $CD4^+$ T cells in PBMC. (F) Human naïve $CD4^+$ T cells from PBMC were treated with 5μm and 10 μm EP4 antagonist ONO-AE3-208 in the presence of PGE2 (100 nM) (n = 3). Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, NS ≥ 0.05.

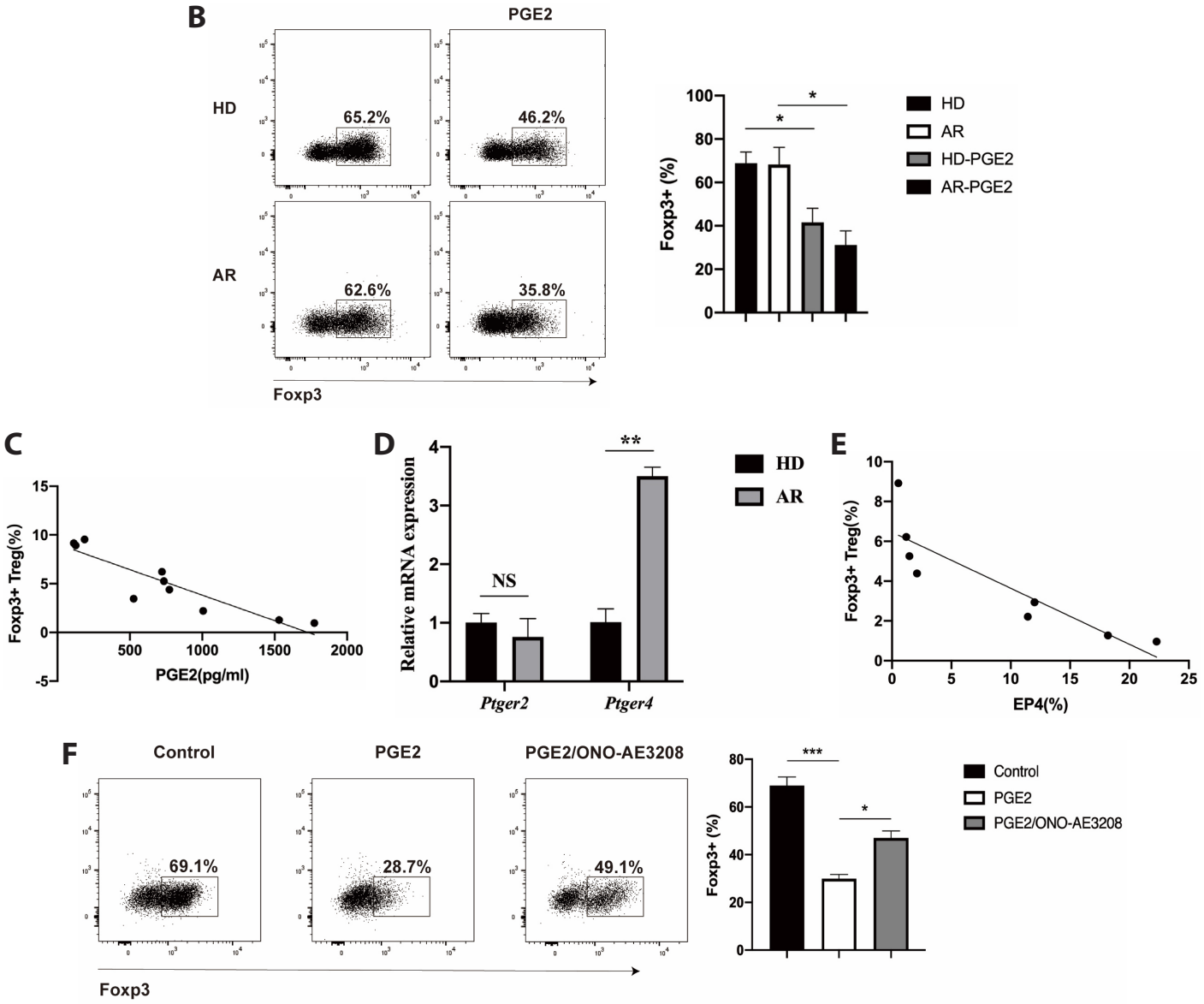

Figure 1. (Continued)

The suppression signal downstream of EP4 is transmitted via cyclic AMP (cAMP)-PKA.

To investigate the mechanism through which the EP4 signal modulated Treg cell differentiation, we examined one of the reported downstreams of the EP4 signal. Our data revealed that the intracellular cAMP content in induced Treg from the peripheral blood of AR patients was significantly higher than that in the healthy donor group (**Figure 2A**). In the meanwhile, Akt, the other known important downstream of EP4 signal, seemed to have remained unchanged, since the phosphorylation of Akt did not have big alteration in AR Tregs (**Figure S2A**). To further ascertain whether the enhanced cAMP signal was the primary factor responsible for the inhibition of Treg cell differentiation by PGE2, we cultured naïve $CD4^+$ T cells isolated from human peripheral blood under Treg polarization conditions. As shown in **Figure 2B**, db-cAMP, a mimic of cAMP, exerted a similar inhibitory effect. Furthermore, db-cAMP also significantly suppressed the differentiation of Treg cells from mouse naïve $CD4^+$ T cells in a dose-dependent manner (**Figure 2C**). By contrary, the addition of SQ22536, a cAMP production inhibitor, could hamper the production of cAMP and promote Tregs differentiation (**Figure S2B**). It's worth noting that the effective concentration of db-cAMP on mouse $CD4^+$ T cells was much higher than that on human $CD4^+$ T cells, possibly due to the resistance of C57/B6J strain to allergy. Additionally, db-cAMP exerted a synergic effect with PGE2, amplifying the inhibitory signal of PGE2 (**Figure 2D**). In order to demonstrate that PGE2 regulates Tregs through EP4-cAMP signaling, we also measured intracellular cAMP levels by collecting cells at different time points with and without PGE2 treatment, and found that the addition of PGE2 promoted intracellular cAMP production in a time-dependent manner (**Figure S2C**).

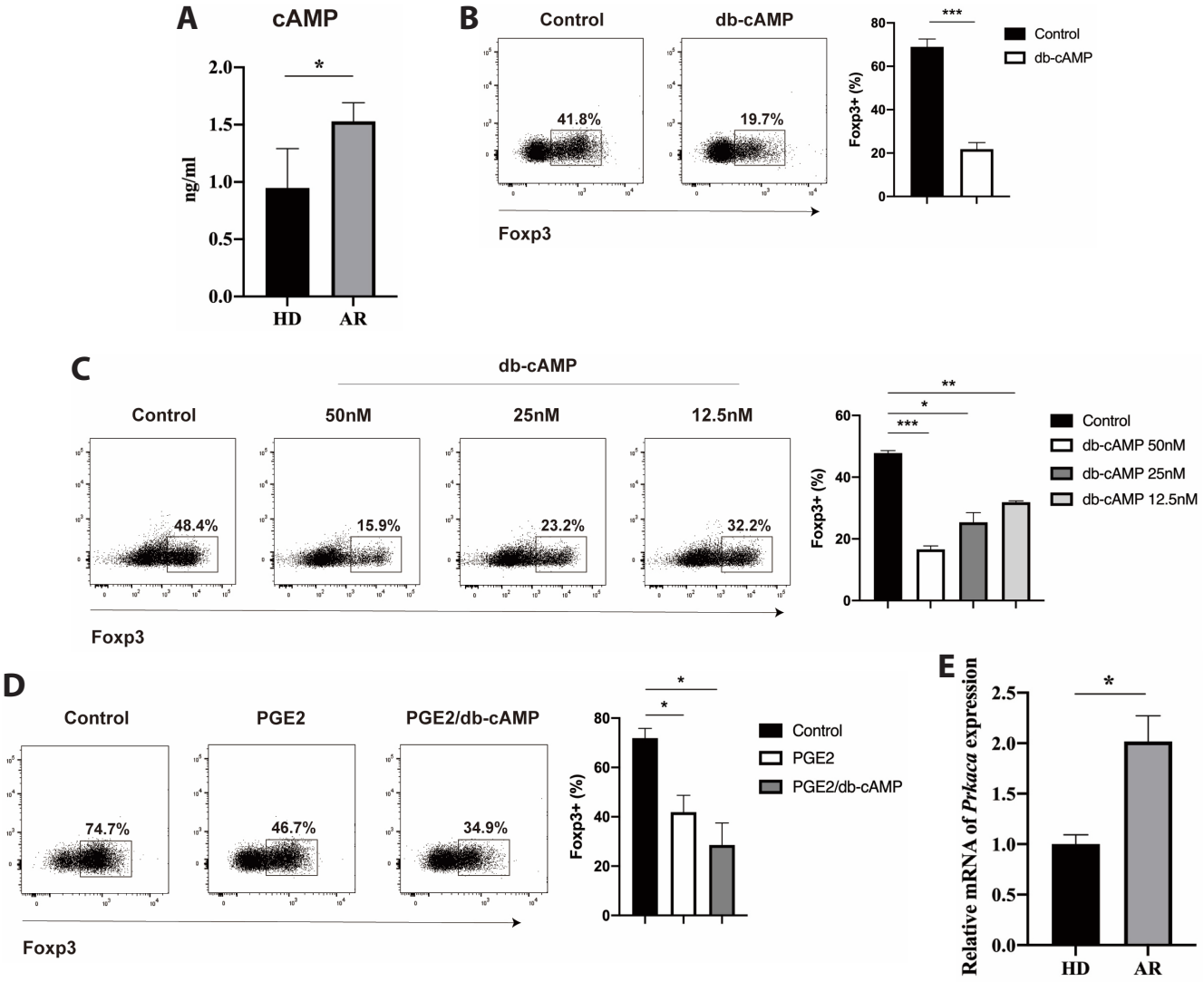

Figure 2. The effect of cAMP-PKA pathway on the differentiation of Treg cells from naïve CD4⁺ T cells. (A) Naïve CD4⁺ T cells were purified from the PBMC of AR patients (n = 3) and health donors (n = 3) and cultured under Treg polarization condition and intracellular cAMP levels were detected by ELISA. (B) Naïve CD4⁺ T cells were purified from the PBMC of health donors (n = 3) and cultured under Treg polarization condition in the presence of 1 nm cAMP mimic db-cAMP. (C) Naïve CD4⁺ T cells were isolated from mouse lymph nodes (n = 3) and cultured under Treg polarization condition in the presence of 12.5 nm, 25 nm and 50 nm cAMP mimic db-cAMP. (D) Naïve CD4⁺ T cells were purified from the PBMC of health donors (n = 3) and cultured under Treg polarization condition in the presence of 100 nm PGE2 with or without 1 nm db-cAMP. (E) Transcription levels of PKAα subunit *Prkaca* in the induced Tregs from AR patients (n = 3) and healthy donors (n = 3). (F) Protein levels of p-PKA and PKA in differentiated Treg cells from AR patients (n = 3) and healthy donors (n = 3) by western blot. (G) Naïve CD4⁺ T cells from mouse lymph nodes were cultured under Treg polarization condition in the presence of 10 nm PKA inhibitor H-89. (H) Naïve CD4⁺ T cells from mouse lymph nodes were cultured under Treg polarization condition with 10 nm PGE1-Alchohol in the presence or absence of 10 nm H-89. Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS ≥ 0.05.

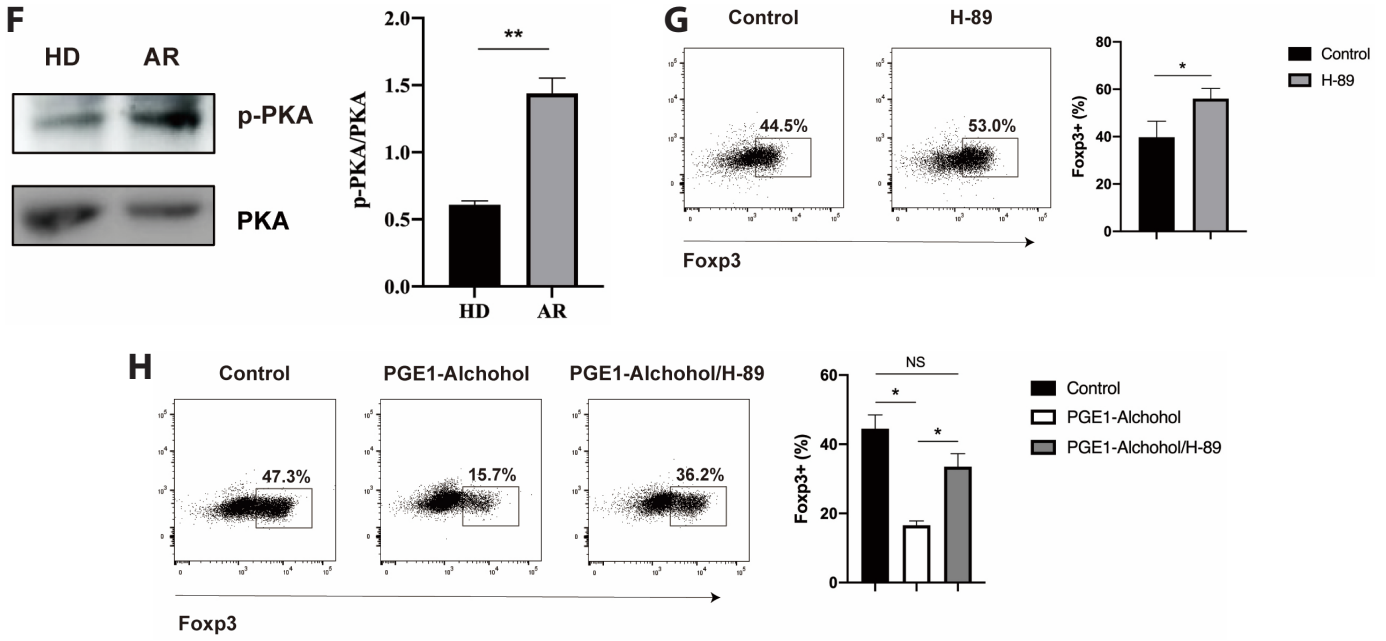

Figure 2. (Continued)

To verify that EP4 receptors modulated Treg cell differentiation through the activation of the cAMP-dependent pathway, we examined the activation of PKA, downstream of cAMP signal. PKA in Tregs from AR patients had an elevated transcript level (**Figure 2E**), and the phosphorylation of PKA protein in induced Treg cells from AR patients was also higher than that from healthy donors (**Figure 2F**). As expect, the phospholylation of PKA could also be elevated by PGE2 treatment in a time course(**Figure S2D**). More importantly, the PKA inhibitor H-89 could directly enhance the percentage of differentiated mouse Tregs (**Figure 2G**). It could also capable of reversing the inhibitory effect of the EP4 agonist (**Figure 2H**). And SQ22536 can clearly inhibit the effect of PGE2 on the phosphorylation of PKA (**Figure S2E**). Collectively, these data provided a clue that PGE2 regulated Treg cell differentiation through the EP4-cAMP-PKA signaling pathway.

The cAMP-PKA pathway effects Treg cell differentiation by modulating PPAR-γ.

The cAMP-PKA pathway does not typically play a significant role in influencing Treg cells differentiation. Therefore, we employed the protein interaction prediction tool STRING to identify proteins that PKA may interact with. The prediction results revealed that both α and β subunits of PKA can interact with PPAR-γ through CREB protein (**Figure 3A**). To verify the involvement of PPAR-γ in the downstream regulation of Treg differentiation in AR, we compared the mRNA and protein levels of PPAR-γ after Treg cells differentiation in the presence of PGE2 from AR patients and healthy donors. As expect, both mRNA (**Figure 3B**) and protein expression (**Figure 3C**) of PPAR-γ were found to be lower in AR patients. In addition, the PGE2 treatment can inhibit PPAR-γ protein expression in a time-dependent manner (**Figure S3A**). This effect was through cAMP-PKA signaling, since SQ22536 significantly recovered the lower expression of PPAR-γ caused by PGE2 (**Figure S3B**).

To investigate whether PPAR-γ affected Treg cell differentiation, we treated mouse naïve $CD4^+$ T cells with PPAR-γ antagonist T0070907 under Treg-polarization conditions. As illustrated in **Figure 3D**, $Foxp3^+$ Treg cell ratio decreased from 44.0% to 28.2%, in a dose-dependent manner. Meanwhile, the PPAR-γ agonist pioglitazone (PIO) was able to restore the suppressed proportions of human $Foxp3^+$ Treg cells (**Figure 3E**) as well as mouse $Foxp3^+$ Treg cells (**Figure S3C**) induced by exogenous PGE2. For AR patients, PIO was also highly effective in rescuing Treg differentiation (**Figure 3F**). Later, we conducted a comprehensive analysis of the correlation between EP4 and PPAR-γ across various diseases, including Adrenocortical carcinoma, Rectum adenocarcinoma, Cholangiocarcinoma, Testicular Germ Cell Tumors, Uterine Corpus Endometrial Carcinoma and Uterine Carcinosarcoma. The result demonstrated a strong correlation between these two proteins (**Figure 3G**). Furthermore, we assessed the regulatory role of transcription factors PPAR-γ and its heterodimer RXR-α in *Foxp3* transcription through a Dual-Luciferase reporter assay. The results indicated that the presence of PPAR-γ and RXR-α could enhance the transcription activity of *Foxp3* by approximately 1.7 times (**Figure 3H**). In summary, it appeared that PPAR-γ may be the target of cAMP-PKA signaling, which modulated the reduced differentiation of Treg cells in AR disease.

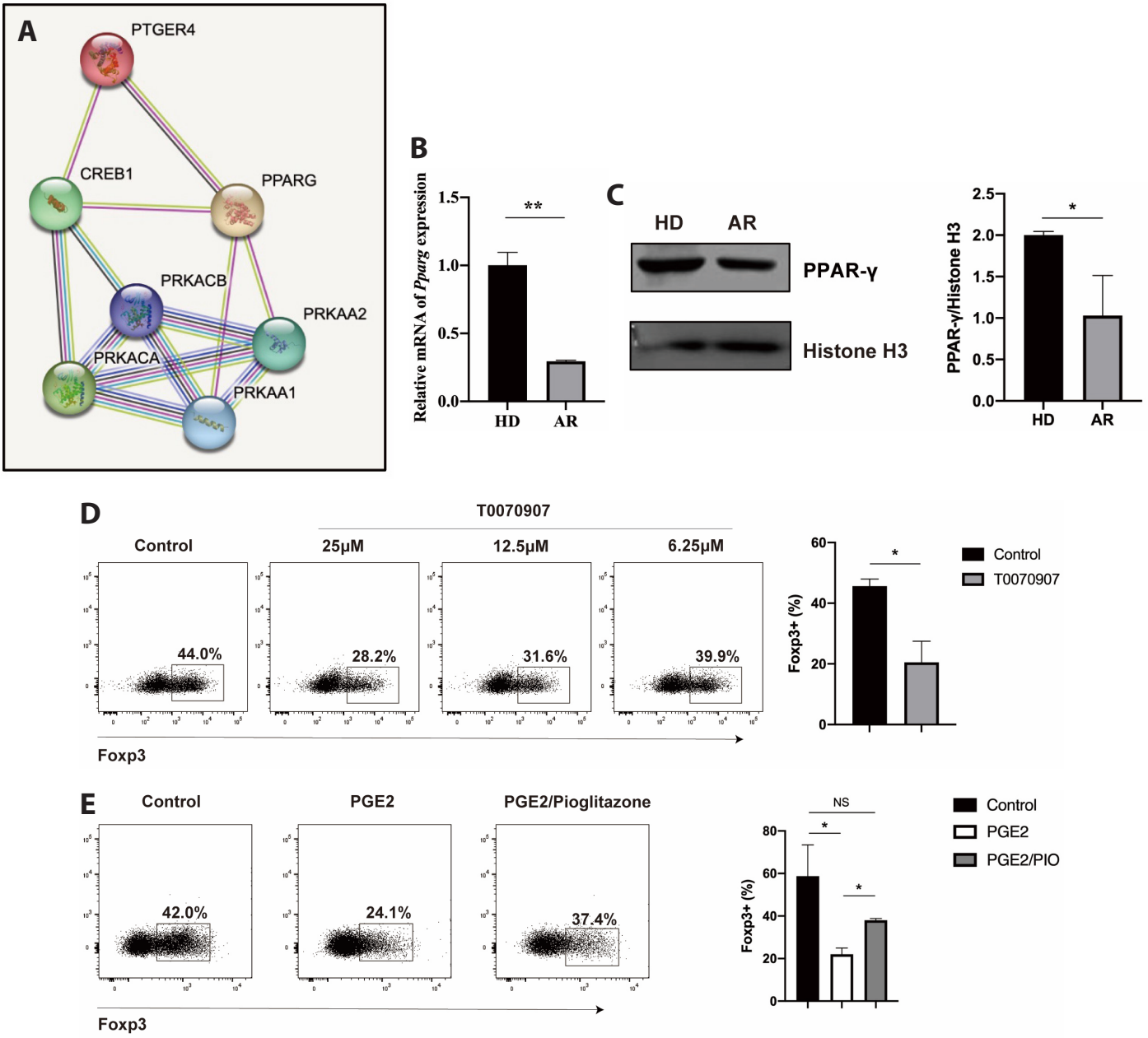

Figure 3. The effect of PPAR-γ on the differentiation of Treg cells. (A) the protein-protein interaction network (PPI) of Ptger4, CREB and PPARγ, PPI enrichment, *p*-value: 0.00000411. (B) Transcription levels of *Pparg* in the induced Tregs from AR patients (n = 3) and healthy donors (n = 3). (C) Protein levels of PPAR-γ in differentiated Treg cells from AR patients (n = 3) and healthy donors (n = 3). (D) Naïve CD4⁺ T cells from mouse lymph nodes (n = 3) were cultured under Treg polarization condition in the presence of 6.25 μm, 12.5 μm and 25 μm PPAR-γ antagonist T0070907. (E) Naïve CD4⁺ T cells from health donors (n = 3) were cultured under Treg polarization condition in the presence of 100 nm PGE2 with or without 100 nm pioglitazone. (F) Naïve CD4⁺ T cells were isolated from AR patients peripheral blood and cultured under Tregs-induced conditions (5 ng/ml human TGF-β1 and 20 ng/mL human IL-2) with or without 100 nm pioglitazone (n = 3). (G) The correlation of EP4 and PPAR-γ in varies of diseases including Adrenocortical carcinoma, Rectum adenocarcinoma, Cholangiocarcinoma, Testicular Germ Cell Tumors, Uterine Corpus Endometrial Carcinoma and Uterine Carcinosarcoma. (H) The combination of transcription factors PPAR-γ and its heterodimer RXR-α with Foxp3 transcription by Dual-Luciferase reporter assay. Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS ≥ 0.05.

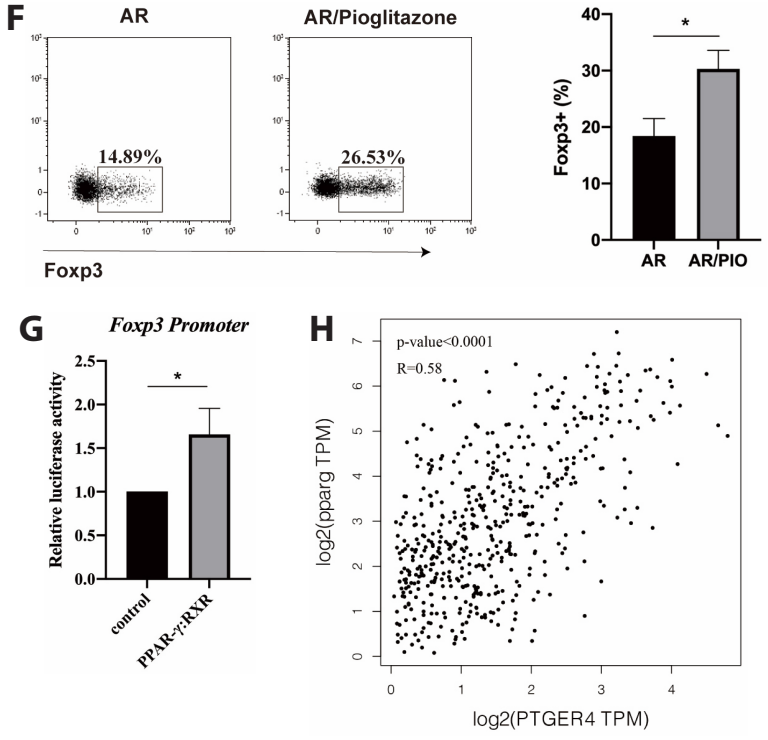

Figure 3. (Continued)

Treatment targeting PPAR-γ activation rescued Treg cells in an AR murine model.

We established a murine AR model using mugwort pollen and aluminum hydroxide to assess the pharmacological effect of PIO in AR disease (**Figure 4A**). Control group mice received PBS, AR group mice were administrated mugwort as described, and the PIO group mice were additionally given pioglitazone (30 μg/kg body weight by intranasal challenge after pulverization challenge on day 21). Typical allergic symptoms, such as the frequency of nasal rubbing and sneezing, were recorded and scored for 10 minutes following the final mugword pollen aeroso nasally challenge. A score greater than five points indicated the successful induction of the disease model. In pollen-exposed mice, we observed that their scores were all above 5, and notably, their scores were significantly higher than those in the control group. Conversely, mice gained PIO treatment exhibited much lower scores, suggesting milder allergic symptoms (**Figure 4B**). Histologic staining (HE) estimating the local inflammation of nasal mucosa yielded similar result. The pollen-exposed group displayed typical pathologic features of AR in the submucosa, with numerous infiltrating inflammatory cells. However, in mice treated with PIO, the infiltration of inflammatory cells was obviously reduced (**Figure 4C**). Using immunofluorescence, we observed lower expression of PPAR-γ in AR group, consistent with findings in AR patients, and higher PPAR-γ expression in $CD4^+Foxp3^+$ cells in the PIO group (**Figure 4D**). Furthermore, we examined

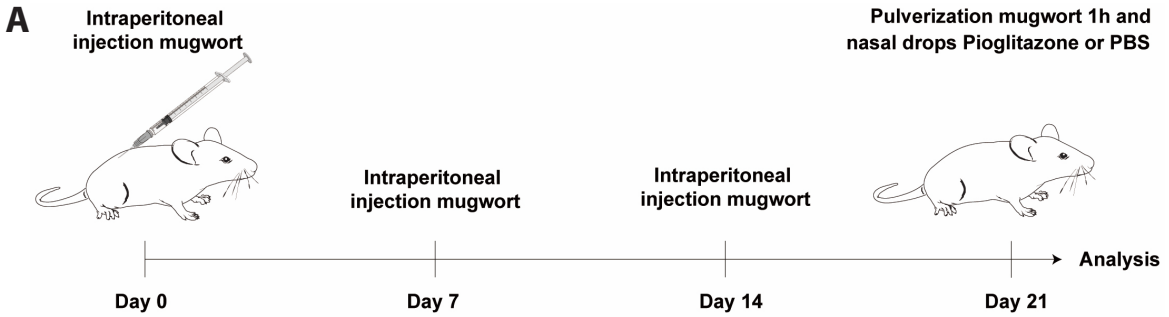

Figure 4. The therapeutic effect of PPAR-γ in AR murine model. (A) Schematic diagram of AR mouse model. (B) Score of pollen-exposed AR mouse model including control (n = 4), AR (n = 4) and AR + Pioglitazone groups (n = 4). (C) Representative sections of the nasal tissues from AR mouse model at an original magnification of ×40 after hematoxylin and eosin staining. (D) PPAR-γ expression in $CD4^+Foxp3^+$ cells in three group through immunofluorescence. (E) The proportion of $CD4^+CD25_{hi}$ Treg cells in facial lymph nodes from AR mouse models. Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, NS ≥ 0.05.

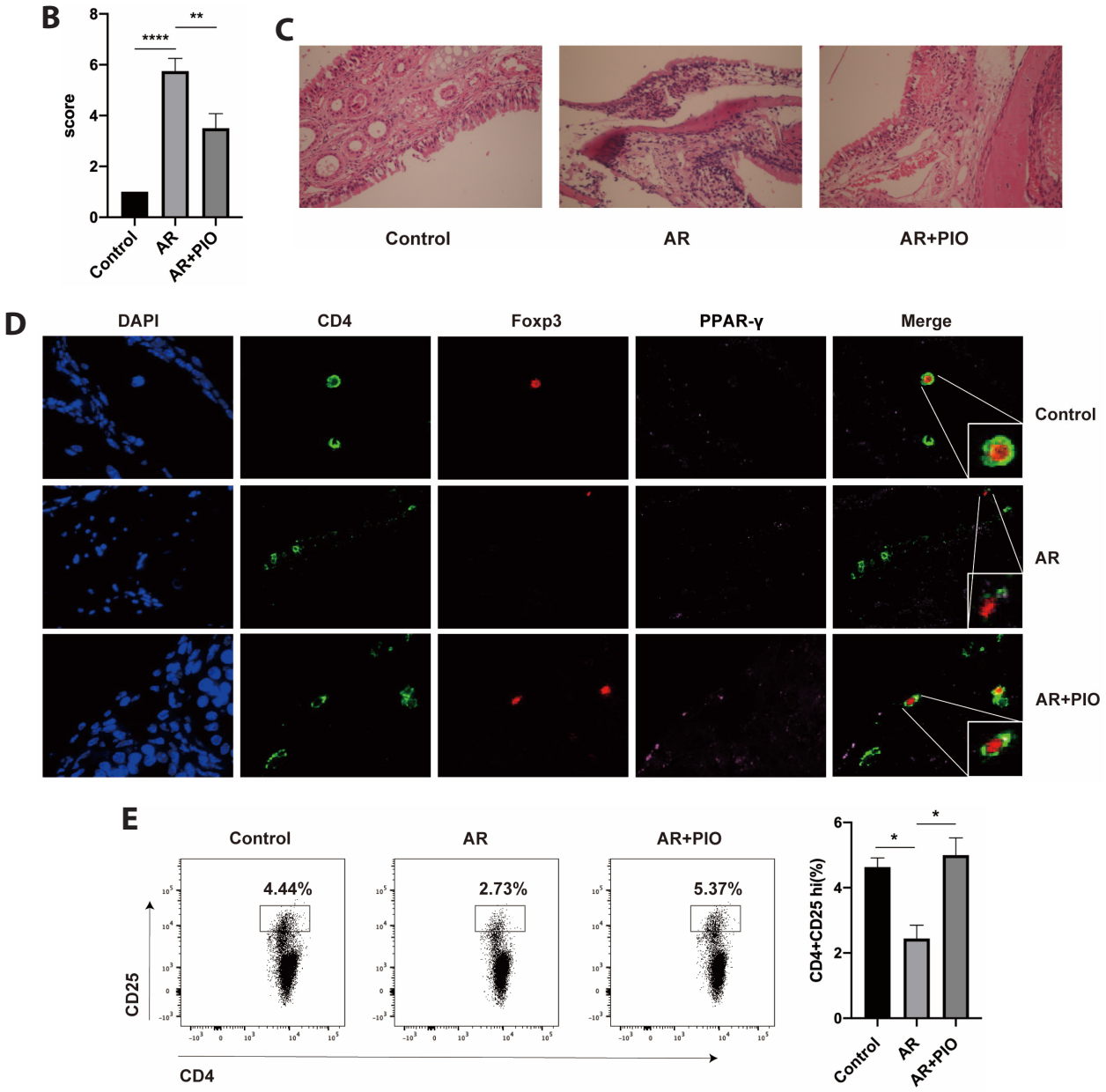

Figure 4. (Continued)

the Treg population in the AR group and the PIO group. Flow cytometry results illustrated an increased number of Treg cells in the driven lymph nodes (facial lymph nodes) (**Figure 4E**) following PIO treatment, with their percentage nearly returning to that of non-AR mice. In brief, the PPAR-γ agonist pioglitazone effectively promoted the presence of more Treg cells in local nasal mucosa and contributed a lot to the alleviation of clinical AR symptoms in our mouse model.

Discussion

In this study, we have provided evidence that elevated level of PGE2 transmit signal through the cAMP-PKA pathway in Treg cells from the peripheral blood of both AR patients and AR mouse model. The heightened PKA signaling indirectly hinders the expression of PPAR-γ by acting through CREB. Consequently, the diminished PPAR-γ ultimately leads to the loss of its capacity to promote Treg cell differentiation, thereby exacerbating inflammation in AR disease (**Figure 5**).

Tregs play a crucial role in allergic diseases, as they have the ability to dampen both innate and adaptive immune response, thereby reducing the overall inflammatory effect. Several lines of evidence have demonstrated that regulatory function of Treg cells in immediate hypersensitivity reactions, primarily by influencing mast cells and inhibiting their degranulation.[28,29,30] Takasato *et al.*[31] found that oral immunotherapy enhanced the expansion of regulatory T cells and suppressed mast cell activation, leading to the alleviation of allergic reactions in food allergies. Furthermore, Tregs are known to inhibit Th2 polarization and limit the production of cytokines such IL-4 and IL-5 and IL-13 through mechanisms involving CTLA-4, IL-10 and TGF-β.[32] In allergic diseases, it's often observed that the functions or numbers of Treg cells were deficient, yet the underlying mechanisms remain unclear. In our previous study, we discovered that PGE2 could inhibit Treg cell differentiation via PGE2-EP4 signal.[18] Another study by Li H's[14] demonstrated that PGE2 could suppressed Treg cell

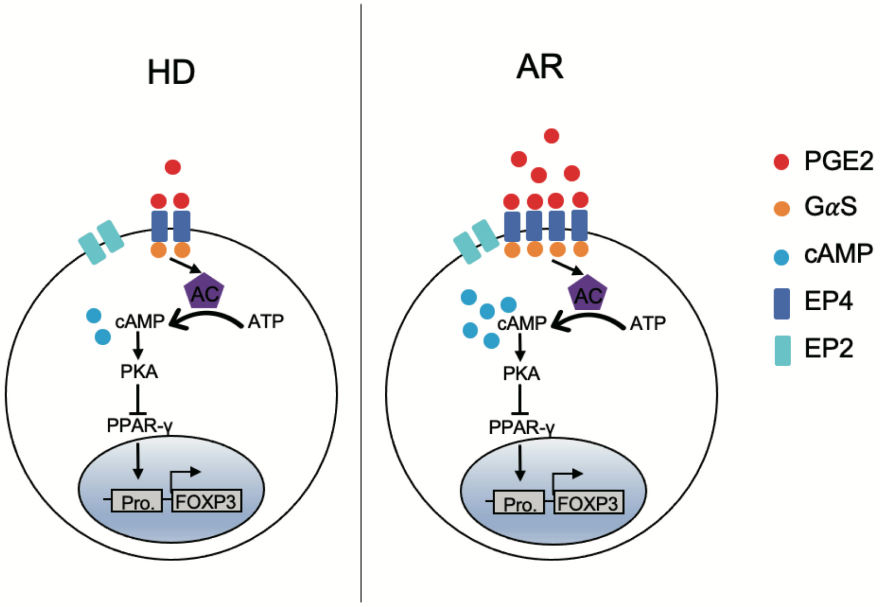

Figure 5. Working model figure of PGE2-EP4-cAMP-PKA- PPAR-γ pathway in health and AR Treg cells.

differentiation via EP2 receptor in patients with rheumatoid arthritis. Since the expression of EP2 and EP4 is inducible, these results hinted that PGE2 might exert its effects through different receptors in distinct inflammation disease.

More importantly, we speculated that a shared signaling pathway involving adenylate cyclase-triggered cAMP-PKA-CREB,[33] rather than the PI3K-Akt pathway,[34] made major contribution to Treg cell differentiation in allergic disease.[18] In this study, we have validated this hypothesis. Our data showed that the protein level and intracellular cAMP content of Tregs from AR patients were higher than those from healthy donors. The effect of PGE2 on Treg cell differentiation could be mimicked by a cAMP agonist (db-cAMP) but attenuated by a PKA inhibitor H-89. Similarly, another study also indicated that db-cAMP could impair the percentage of $CD25^{+}Foxp3^{+}$ T cells and the expression of Foxp3 mRNA under Treg-skewing conditions in human naive $CD4^{+}$ T cells.[35] Given that low intracellular cAMP is typically required for the activation of conventional $CD4^{+}$ T cells,[36] it is plausible that high levels of cAMP induced by the PGE2-EP4 signal act as barrier to the activation of naïve $CD4^{+}$ T cells in allergic tissue, thereby inhibiting their differentiation into Treg cells.

PPAR-γ was initially discovered to play an essential role in regulating lipid metabolism in adipose cells. Recently, PPAR-γ has been reported to have multiple functions in innate immune cells. For example, specific deletion of PPAR-γ has been shown to disrupt the maturation of alternative activated macrophages by reducing arginase 1 messenger RNA and activity.[37] Besides, PPAR-γ could restrain dendritic cells maturation and retinoic acid production from them.[38] In the context of Treg cells, Li *et al*. had reported the elevation of PPAR-γ expression on Treg cells in visceral adipose tissue, which expression plays a critical role in insulin-sensitizing activity.[27] In allergic rhinitis, PPAR-γ was found to be expressed on Treg cells from nasal mucosa of patients.[39] Experimentally, one study involving a mouse allergic model induced by ovalbumin (OVA)[40] demonstrated that OVA could downregulate *foxp3* transcription, while Pioglitazone treatment could rescue the inhibition slightly. Nevertheless, there has been a noticeable scarcity of evidence on the correlation between PPAR-γ and Treg cells in human data. In the current study, we have taken the lead to demonstrating that the cAMP-PKA signaling inhibited PPAR-γ expression in Treg cells of AR patients. Furthermore, our preliminary findings suggested that PPAR-γ could somehow promote *foxp3* transcription activity. The precise mechanism on how PPAR-γ may enhance on this transcription need be determined in future.

Nowadays, pioglitazone, an activator of PPAR-γ, has been widely utilized as an insulin sensitizer in type 2 diabetes mellitus.[41,42] It is worth noting that several clinical studies have reported that type 2 diabetes patients have lower risks to develop allergic diseases, compare to those type 1 diabetes patients. When treating with thiazolidinediones, which belong to a class of pioglitazone medications, diabetic patients with asthma also had lower hazard to experience asthma exacerbation.[43] Here, we firstly provided evidence that PIO treatment could rescue the inhibitory effect of cAMP and promote Treg cell differentiation in both human and mouse. Our results presented a novel potential application for PPAR-γ in AR treatment.

In summary, our findings have unveiled a previously unknown cellular mechanism detailing how PGE2-EP4-cAMP signaling influences Treg cell differentiation in AR development. The inhibition brought about by the elevated PGE2 concentration in AR mice could be partially alleviated by regulation on PPAR-γ. This discovery establishes a proof-of principle that PIO holds the potential therapeutical use in allergic diseases.

Acknowledgments

We thank all members in our laboratory for insightful discussion and technical assistance.

Funding

This work was supported by grants from Beijing Municipal Natural Science Foundation (7222136, 7212058), the National Natural Sciences Foundation of China (31872735) and Clinical Medicine Plus X - Young Scholars Project, Peking University, the Fundamental Research Funds for the Central Universities (71006Y3025).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' contributions

- YJ, LL and WW designed the project.
- YJ and WW did the experiment and wrote the manuscript.
- YZ established AR tumor model.
- LL collected the human samples and clinical information.
- ZY and ZW help HE staining.
- XP and ZZ help analyze data.

Ethics Statement

This study was carried out in accordance with the recommendations of Ethics Committee of Peking University Health Science Center. The protocol (No. IRB00001052-21083 and LA2020375) was approved by the Ethics Committee of Peking University Health Science Center.

References

1. Bousquet PJ, Leynaert B, Neukirch F, Sunyer J, Janson CM, Anto J, et al. Geographical distribution of atopic rhinitis in the European Community Respiratory Health Survey I. Allergy. 2008;63(10):1301-9.
2. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet. 2006;368(9537):733-43.
3. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy. 2008;63 Suppl 86:8-160.
4. Katelaris CH, Lee BW, Potter PC, Maspero JF, Cingi C, Lopatin A, et al. Prevalence and diversity of allergic rhinitis in regions of the world beyond Europe and North America. Clin Exp Allergy. 2012;42(2):186-207.
5. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. Lancet. 2011;378(9809):2112-22.
6. Xu G, Mou ZL, Jiang HY, Cheng L, Shi JB, Xu R, et al. A possible role of CD4(+)CD25(+) T cells as well as transcription factor Foxp3 in the dysregulation of allergic rhinitis. Laryngoscope. 2007;117(5):876-80.
7. Meng SS, Gao R, Yan BD, Ren J, Wu F, Chen P, et al. Maternal allergic disease history affects childhood allergy development through impairment of neonatal regulatory T-cells (vol 17, 114, 2016). Respir Res. 2016;17.
8. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4(+) CD25(+) regulatory T cells is interleukin 10 dependent. J Exp Med. 2005;202(11):1539-47.
9. Lee K, Lee SH, Kim TH. The Biology of Prostaglandins and Their Role as a Target for Allergic Airway Disease Therapy. Int J Mol Sci. 2020;21(5).
10. Harizi H. The immunobiology of prostanoid receptor signaling in connecting innate and adaptive immunity. Biomed Res Int. 2013;2013:683405.
11. Rodriguez M, Domingo E, Municio C, Alvarez Y, Hugo E, Fernandez N, et al. Polarization of the innate immune response by prostaglandin E2: a puzzle of receptors and signals. Mol Pharmacol. 2014;85(1):187-97.
12. Konya V, Marsche G, Schuligoi R, Heinemann A. E-type prostanoid receptor 4 (EP4) in disease and therapy. Pharmacol Ther. 2013;138(3):485-502.
13. Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. J Exp Med. 2009;206(3):535-48.
14. Li H, Chen HY, Liu WX, Jia XX, Zhang JG, Ma CL, et al. Prostaglandin E-2 restrains human Treg cell differentiation via E prostanoid receptor 2-protein kinase A signaling. Immunol Lett. 2017;191:63-72.
15. Esaki Y, Li Y, Sakata D, Yao C, Segi-Nishida E, Matsuoka T, et al. Dual roles of PGE2-EP4 signaling in mouse experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2010;107(27):12233-8.
16. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, et al. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009;15(6):633-40.
17. Crittenden S, Goepp M, Pollock J, Robb CT, Smyth DJ, Zhou Y, et al. Prostaglandin E2 promotes intestinal inflammation via inhibiting microbiota-dependent regulatory T cells. Sci Adv. 2021;7(7).
18. Li LS, Guan K, Zhou Y, Wu J, Wang Y, Wang W. Prostaglandin E2 signal inhibits T regulatory cell differentiation during allergic rhinitis inflammation through EP4 receptor. World Allergy Organ J. 2019;12(12).
19. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83(6):835-9.
20. Benayoun L, Letuve S, Druilhe A, Boczkowski J, Dombret MC, Mechighel P, et al. Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways - Relationship with proliferation, apoptosis, and airway remodeling. Am J Respir Crit Care Med. 2001;164(8):1487-94.
21. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem. 1998;273(40):25573-80.
22. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, et al. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J Clin Invest. 1998;101(6):1354-61.
23. Jiang CY, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391(6662):82-6.
24. Guri AJ, Hontecillas R, Bassaganya-Riera J. Abscisic acid synergizes with rosiglitazone to improve glucose tolerance and down-modulate macrophage accumulation in adipose tissue: possible action of the cAMP/PKA/PPAR gamma axis. Clin Nutr. 2010;29(5):646-53.
25. Miao Y, Zhang C, Yang L, Zeng X, Hu Y, Xue X, et al. The activation of PPARγ enhances Treg responses through up-regulating CD36/CPT1-mediated fatty acid oxidation and subsequent N-glycan branching of TβRII/IL-2Rα. Cell Commun Signal. 2022;20(1):48.
26. Klotz L, Burgdorf S, Dani I, Saijo K, Flossdorf J, Hucke S, et al. The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity. J Exp Med. 2009;206(10):2079-89.
27. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, et al. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. Nature. 2012;486(7404):549-53.
28. Noval Rivas M, Burton OT, Wise P, Charbonnier LM, Georgiev P, Oettgen HC, et al. Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. Immunity. 2015;42(3):512-23.
29. Burton OT, Noval Rivas M, Zhou JS, Logsdon SL, Darling AR, Koleoglou KJ, et al. Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. Immunity. 2014;41(1):141-51.
30. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, et al. CD4(+)CD25(+) Regulatory T Cells Suppress Mast Cell Degranulation and Allergic Responses through OX40-OX40L Interaction. Immunity. 2008;29(5):771-81.

31. Takasato Y, Kurashima Y, Kiuchi M, Hirahara K, Murasaki S, Arai F, et al. Orally desensitized mast cells form a regulatory network with Treg cells for the control of food allergy. Mucosal Immunol. 2021;14(3):640-51.
32. Lim HW, Hillsamer P, Banham HH, Kim CH. Cutting edge: Direct suppression of B cells by CD4(+)CD25(+) regulatory T cells. J Immunol. 2005;175(7):4180-3.
33. Breyer RM, Bagdassarian CK, Myers SA, Breyer MD. Prostanoid receptors: subtypes and signaling. Annu Rev Pharmacol Toxicol. 2001; 41:661-90.
34. Fujino H, Xu W, Regan JW. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. J Biol Chem. 2003;278(14):12151-6.
35. Liu W, Li H, Zhang X, Wen D, Yu F, Yang S, et al. Prostaglandin I2-IP signalling regulates human Th17 and Treg cell differentiation. Prostaglandins Leukot Essent Fatty Acids. 2013;89(5):335-44.
36. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell mediated suppression. J Exp Med. 2007;204(6):1303-10.
37. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. Nature. 2007;447(7148):1116.
38. Szatmari I, Töröcsik D, Agostini M, Nagy T, Gurnell M, Barta E, et al. PPARgamma regulates the function of human dendritic cells primarily by altering lipid metabolism. Blood. 2007;110(9):3271-80.
39. Kang HJ, Cinn YG, Hwang SJ, Won CS, Woo JS, Lee SH, et al. Up-regulation of peroxisome proliferator-activated receptor gamma in perennial allergic rhinitis. Arch Otolaryngol Head Neck Surg. 2006;132(11):1196-200.
40. Wang WH, Zhu ZH, Zhu BW, Ma ZX. Peroxisome Proliferator-Activated Receptor-gamma Agonist Induces Regulatory T Cells in a Murine Model of Allergic Rhinitis. Otolaryngol Head Neck Surg. 2011;144(4):506-13.
41. Karásek D. Pioglitazone. Vnitr Lek. 2020;66:121-5.
42. Dubois M, Vantyghem MC, Schoonjans K, Pattou F. [Thiazolidinediones in type 2 diabetes. Role of peroxisome proliferator-activated receptor gamma (PPARgamma)]. Ann Endocrinol (Paris). 2002;63:511-23.
43. Rinne ST, Feemster LC, Collins BF, Au DH, Perkins M, Bryson CL, et al. Thiazolidinediones and the risk of asthma exacerbation among patients with diabetes: a cohort study. Allergy Asthma Clin Immunol. 2014;10:34.

Supplementary Materials

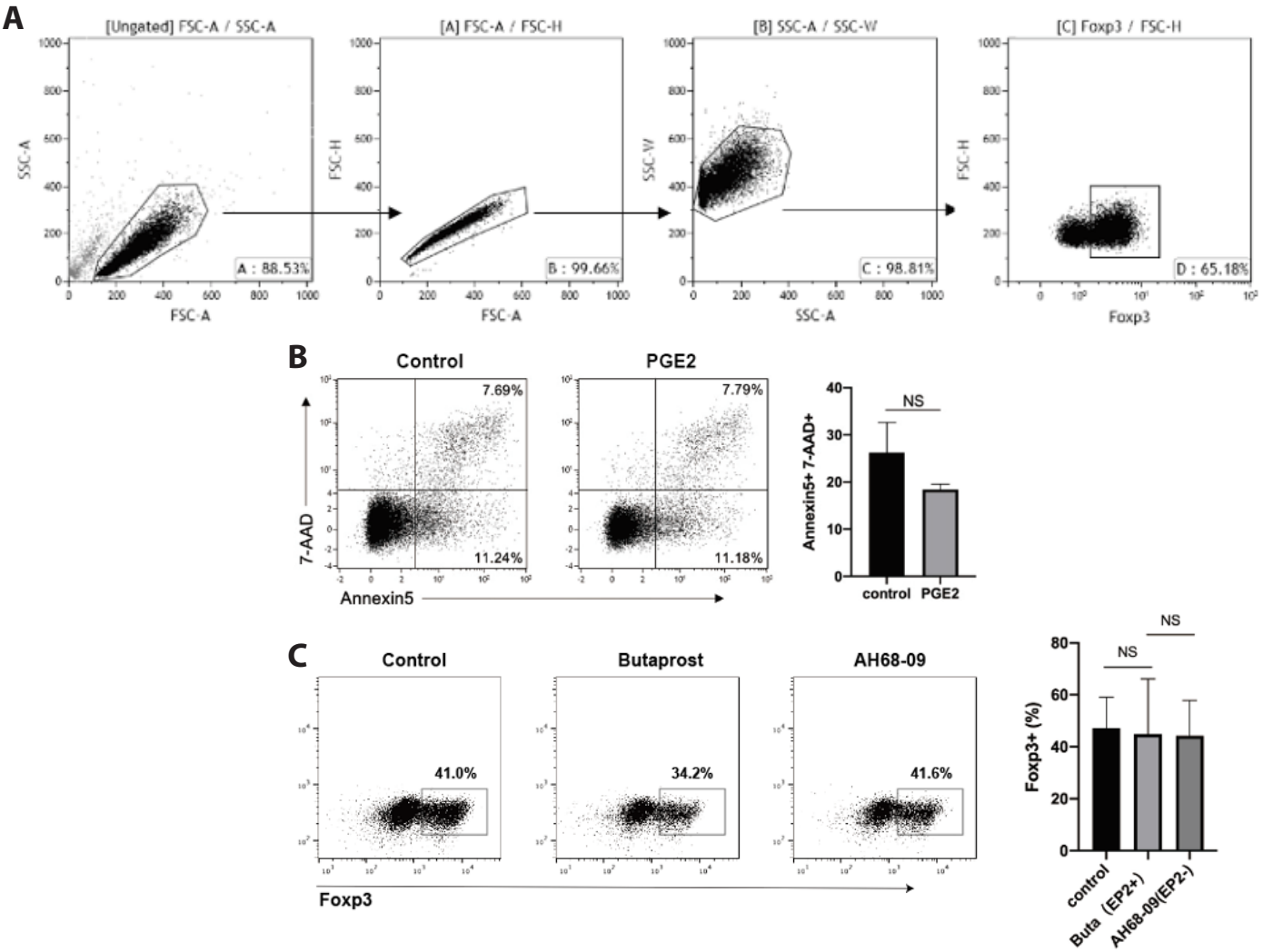

Figure S1. PGE2 affects Tregs differentiation via EP4 pathway. A. Gating strategy. The arrow points to indicate the Gating process. B. Naïve CD4⁺ T cells were isolated from human peripheral blood (n = 3) and cultured under Tregs-induced conditions in the presence or absence of PGE2, the effect of PGE2 to cell survival was detected in day 3. C. Naïve CD4⁺ T cells, isolated from mouse lymph nodes and spleens (n = 3) were treated with 10 nm EP2 agonist Butaprost and 10 nm EP2 antagonist AH68-09 under Treg-polarization conditions for 3 days. Foxp3⁺CD4⁺ T cells were analyzed by flow cytometry. Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, NS ≥ 0.05.

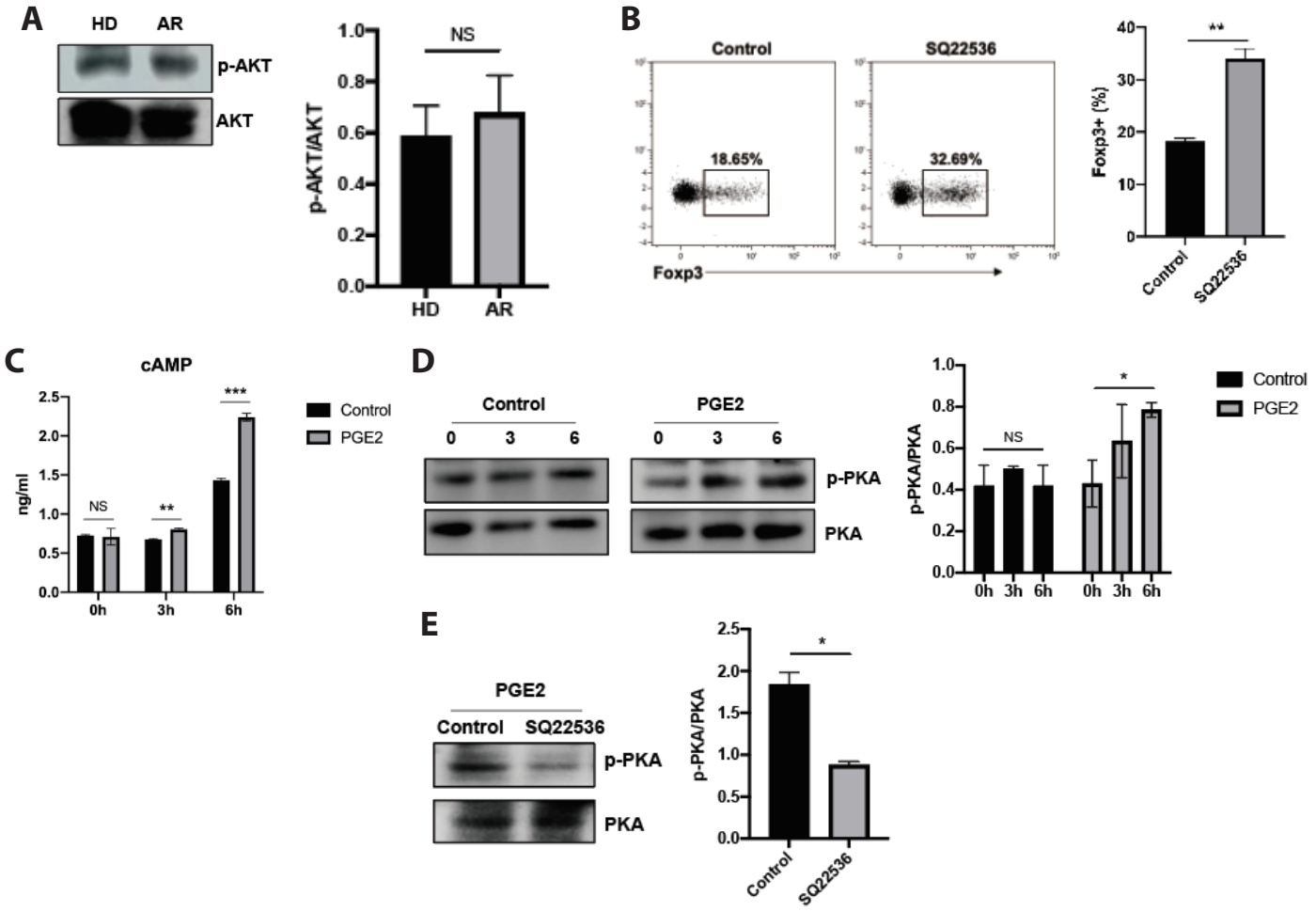

Figure S2. PGE2-EP4-PKA inhibitory didn't through Akt-mediated signal pathway. A.Protein levels of p-AKT and AKT in differentiated Treg cells from AR patients (n = 3) and healthy donors (n = 3). B. Naïve $CD4^+$ T cells were isolated from human peripheral blood (n = 3) and cultured under Tregs-induced conditions in the presence or absence of SQ22536. C. Naïve $CD4^+$ T cells were isolated from human peripheral blood and cultured under Tregs-induced conditions in the precense or absence of PGE2, cells were collected at different time points and cAMP content was detected (n = 3). D. Naïve $CD4^+$ T cells were isolated from human peripheral blood and cultured under Tregs-induced conditions with or without PGE2. Cells were collected at different time points and their intracellular protein expression were detected (n = 3). E. Naïve $CD4^+$ T cells were isolated from human peripheral blood and cultured under Tregs-induced conditions with or without 10 μm SQ22536 in the precense of PGE2. Cells were collected at different time points and their intracellular protein expression were detected (n = 3). Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, NS ≥ 0.05.

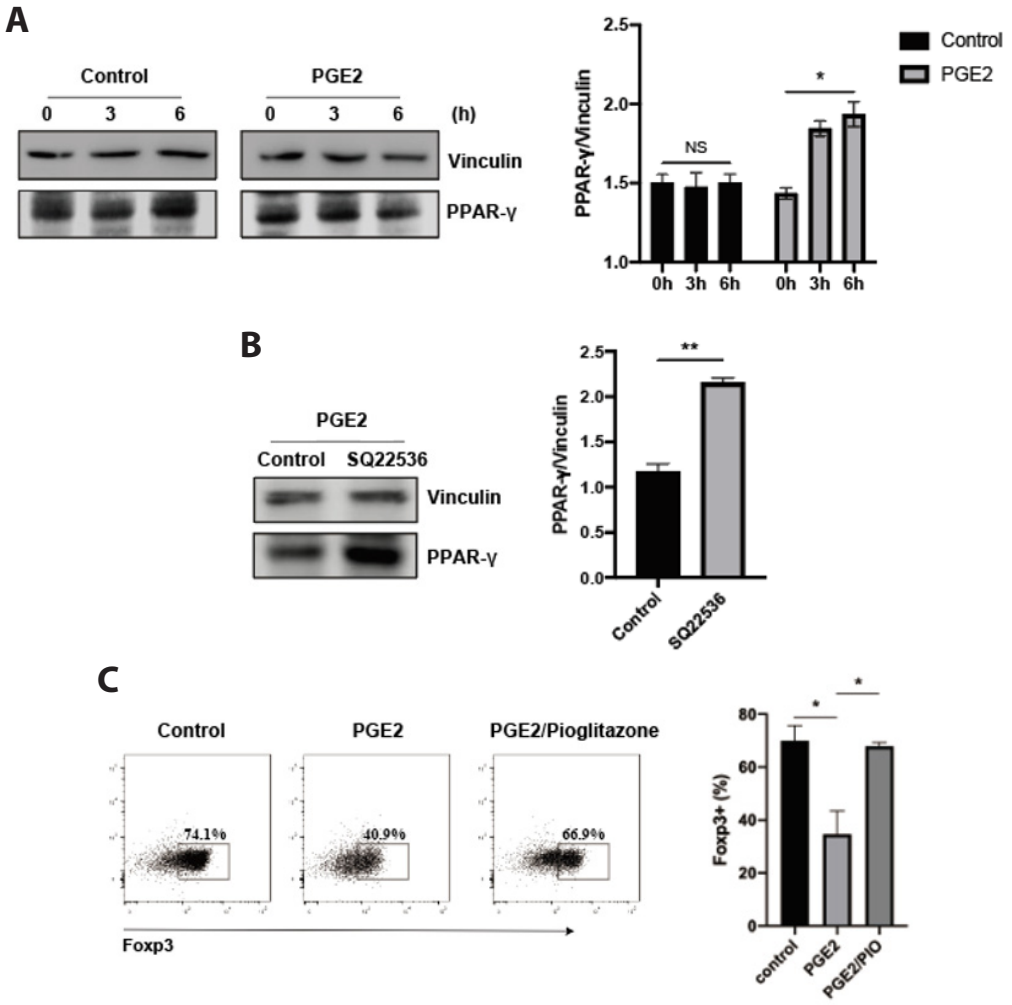

Figure S3. The rescue effect of PPAR-γ on differentiation of mouse Treg cells and AR patients. A. Naïve CD4⁺ T cells were isolated from human peripheral blood and cultured under Tregs-induced conditions with or without PGE2. Cells were collected at different time points and their intracellular protein expression were detected (n = 3). B. Naïve CD4⁺ T cells were isolated from human peripheral blood and cultured under Tregs-induced conditions with or without 10 μm SQ22536 in the precense of PGE2. Cells were collected at different time points and their intracellular protein expression were detected (n = 3). C. Naïve CD4⁺ T cells were isolated from mouse lymph nodes and cultured under Treg polarization condition in the presence of 5 μm pioglitazone with or without 100 nm PGE2 for 3 days (n = 3). Data are presented as mean ± SD or representing one of at least three independent experiments. Statistical differences were determined by unpaired two-tailed *Student's t*-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS ≥ 0.05.

Figure 2F

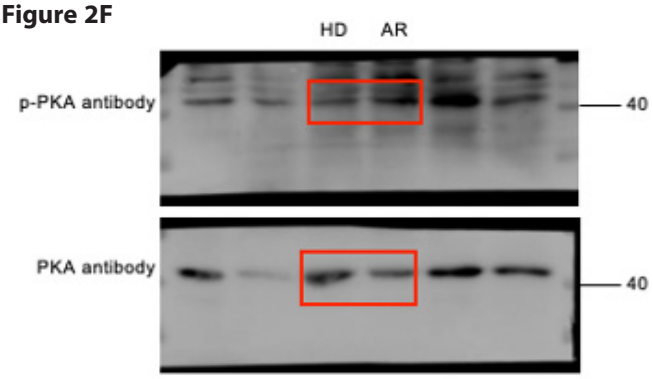

Figure 3C

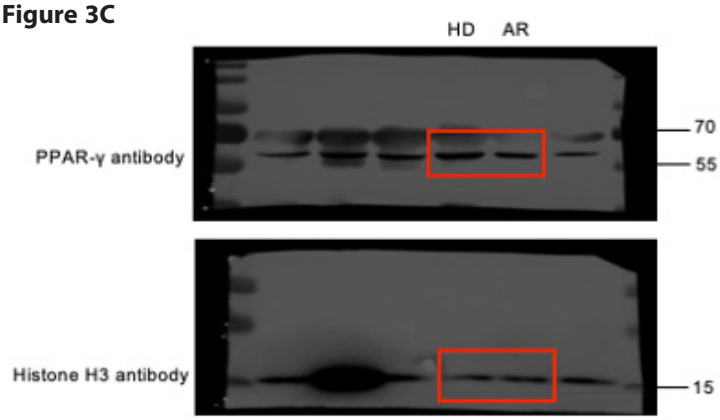

Figure S2D

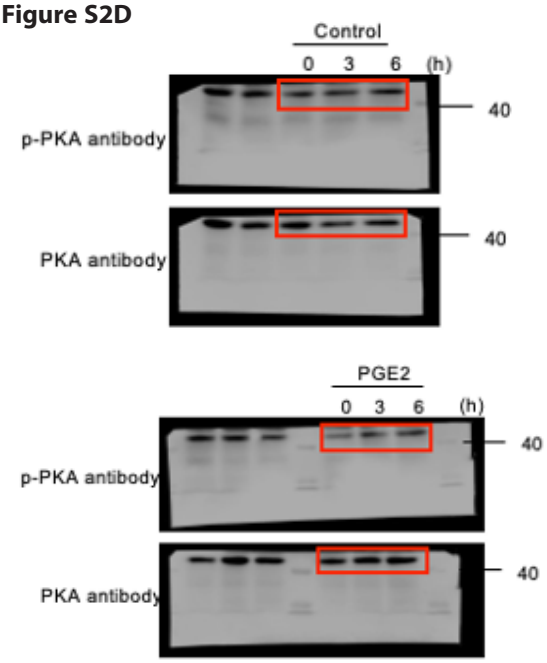

Figure S2E

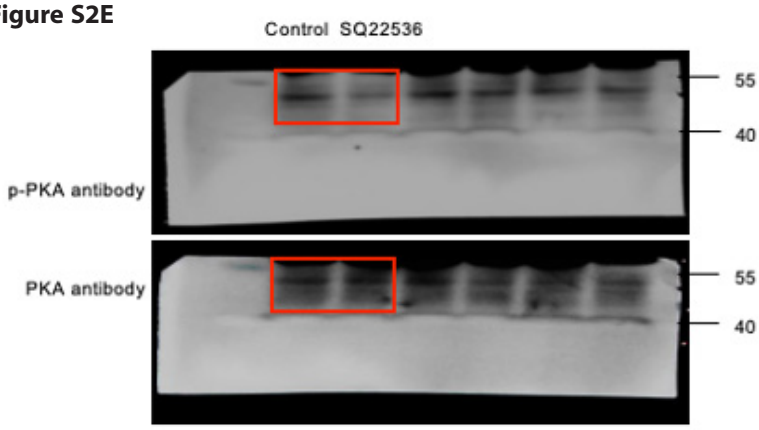

Figure S3B

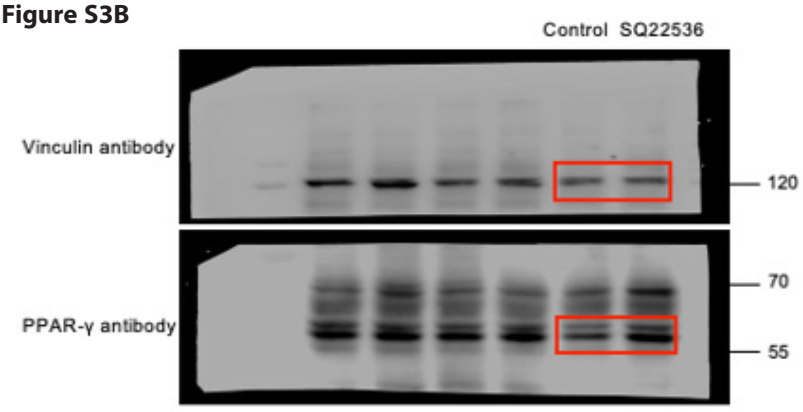

Figure S4. Uncropped and unmanipulated images of all Western blot results.