Prostaglandin E2 signal inhibits T regulatory cell differentiation during allergic rhinitis inflammation through EP4 receptor

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**ABSTRACT**

**Objective:** Allergic rhinitis (AR) is a common disease seriously affecting quality of life, and until now the effect of medical therapy is not satisfactory. It is essential to explore in depth the pathologic mechanism of AR to offer new ideas for developing novel treatment strategies. The defect of T regulatory (Treg) cells plays a critical role in the pathogenesis of AR, but the underlying mechanism remains to be elucidated. This study aims to determine the effect of Prostaglandin E2 (PGE2) on the differentiation of Treg cells in AR patients and the involvement of E prostanoid (EP) receptor signaling pathway.

**Methods:** The proportion of Treg cells and the level of PGE2 in the peripheral blood of AR patients and healthy controls were compared. Differentiation rate of Treg cells under the influence of various concentrations of PGE2 with or without diverse EP receptor agonists and antagonists were investigated through cell culture and flow cytometry in vitro. The cyclic AMP (cAMP) mimic or protein kinase B (Akt) inhibitor was also added to the culture to evaluate the downstream pathway of EP receptor signaling.

**Results:** The proportion of Treg cells decreased and PGE2 concentration increased in the peripheral blood of AR patients compared to healthy controls. PGE2 dose-dependently suppressed the differentiation of Treg cells from both human and mice naïve CD4\textsuperscript{+} T cells in vitro. This inhibitory effect was mediated through EP4 via a mechanism involving activation of cAMP-dependent protein kinase A (PKA) signaling pathway.

**Conclusion:** PGE2-EP4-cAMP signaling might mediate the development of AR by inhibiting the differentiation of Treg cells.

**Keywords:** Allergic rhinitis, T regulatory cell, Prostaglandin E2, EP receptor, Signaling pathway

**INTRODUCTION**

Allergic rhinitis (AR) is one of the most common allergic disorders of the airways and represents a global health problem. According to the results of telephone-based surveys, the prevalence of physician-confirmed AR was 14\% in American adults.\textsuperscript{1} Network data from a cross-sectional...
cohort of 333,200 children in the United States indicated an estimated AR prevalence rate of 19.9%. In China, 47,216 telephone interviews were conducted and the standardized prevalence of adult AR in 18 major cities was 9.8%-23% (mean rate = 17.6%) in 2011. Symptoms of allergic rhinitis include rhinorrhea, nasal obstruction, nasal itching, and sneezing, and 19%-38% of patients with AR have concomitant asthma. AR and asthma can cause a considerable burden to individuals leading to an impaired quality of life. Currently, the effect of medical treatment for AR is unsatisfactory. As shown by the phone-based survey in the United States, 14% of AR adult patients reported little to no relief by drug therapy. Symptom controlling medications induced common adverse effects including drowsiness, nosebleed, and feelings of irritation. Therefore, it is essential to explore the pathologic mechanism of AR to develop novel treatment strategies.

AR is classically considered to result from immunoglobulin E (IgE)-mediated allergy associated with nasal inflammation caused by complex interactions between B cells, T cells, mast cells, and basophils. In brief, allergen exposure at the nasal mucosal surface leads to activation of epithelial cells and local dendritic cells. Dendritic cells uptake and present antigens to naïve T cells to induce the response of type 2 subset of CD4+ helper T cells (Th2 cells). Allergen-specific IgE antibodies are produced, and the allergen re-exposure causes the cross-linking of IgE molecules on two or more receptors of mast cells or basophils, which initiates the process of cell activation and degranulation, releasing the cytokines causing AR symptoms.

Defects in Treg cells play a critical role in the establishment of allergic airway disease. In mice sensitized to ovalbumin, Treg cells inhibited allergen-specific IgE responses and affected allergen-specific Th2 cell priming both in terms of proliferative responses and cytokine secretion. In contrast, the depletion of Treg cells during the sensitization phase of allergy development resulted in a dramatic exacerbation of allergic inflammation. In patients with AR, circulating Treg cell numbers in the peripheral blood of patients were either lower than, or equivalent to, those in control patients. Furthermore, the suppressive capacity of CD4+CD25+ Treg cells from atopic allergic individuals was significantly reduced in allergen-stimulated cultures when compared with cells from non-atopic control subjects. These results highlight the importance of clarifying the specific mechanism of Treg cell dysfunction in AR inflammation, which is essential for the development of new treatments.

Prostaglandins exert diverse and complex modulatory roles in physiological and pathophysiological conditions including cancer, inflammation, and arthritis. Prostaglandin E2 (PGE2), the most abundant prostanoid in the human body, alters the characteristics and functionality of T helper cells, dendritic cells, and other immune cells. PGE2 regulated CD4+ T cells toward Th2 cell development by suppressing the secretion of Th1 cytokines while increasing Th2 cytokines. PGE2 also promoted the development of IL-17-producing T cells (Th17) in multiple models of infection and autoimmunity. However, there are conflicting results regarding the effects of PGE2 on the development of Treg cells and the balance between Treg and Th17 cells, and the regulatory mechanisms involved have not been fully clarified. PGE2 is essential for the E prostanoid receptor EP2 and EP4 dependent induction of murine Treg cells in cancer and following skin ultraviolet irradiation with an analogous role demonstrated in human tumor tissues. However, in rheumatoid arthritis patients, PGE2 negatively regulated the differentiation of human naïve CD4+ T cells into Treg cells. Also there is scant literatures reports regarding the effects of PGE2 on the differentiation and function of Treg cells in AR patients.

Considering the important role of Treg cells in the pathogenesis of AR, it is important to determine the modulatory impact of PGE2 on Treg cells, especially in AR patients. In this study, we investigated the contribution of PGE2 to the differentiation of Treg cells in AR patients and the involvement of EP receptor signaling as well as its downstream pathway in the regulatory process.
MATERIALS AND METHODS

Patients

Thirty-seven adult patients with AR (21 males and 38 females) aged 18–50 years (median age, 36 years) were recruited consecutively into the study in the outpatient allergy clinic of Peking Union Medical College Hospital. All the patients lived in Beijing. To enhance the homogeneity of AR patients in this study, we only recruited subjects allergic to cypress pollens, which is a leading cause of AR during springtime in Beijing.

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 10th and April 10th (the cypress pollen season in Beijing) for at least the previous 2 years; (3) positive intradermal skin test (a wheal ≥10 mm) to cypress pollen (XieheXinhualian Pharmacy, Beijing, China) 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. Sixteen 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 was obtained, and all the subjects provided written informed consent.

Mice

Pathogen-free C57BL/6 mice (8–10 weeks) were brought from Vital River Laboratories. The mice were kept in a specific pathogen-free facility at Peking University Health Science Center (Beijing, China). Mice were used at 8-10 weeks of age and were age matched for experiments. 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. This study was carried out in accordance with these approved guidelines.

Reagents

PGE2, PGE1-Alcohol (EP4 agonist), ONO-AE3-208 (EP4 antagonist), Butaprost (EP2 agonist) and AH68-09 (EP2 antagonist) were bought from Cayman Chemical (Ann Arbor, MI, USA). Dibutryl cAMP (db-cAMP) was purchased from Sigma-Aldrich (Merck KGaA, Germany). MK2206 was purchased from Selleck (Selleck Chemicals, USA).

Cell culture and Treg cell polarization

Human naïve CD4⁺ T cells were isolated from the peripheral blood of the AR patients and healthy controls using the human naïve CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

For naïve CD4⁺ T cell isolation in mice, CD4⁺CD62L⁺ T cells were isolated from splenic single cell suspension using a CD4⁺CD62L⁺ T cell Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic cells sorting system (MACS) selection according to the manufacturer's protocol. Cell purity assessed by flow-cytometry was ≥92%. Purified naïve CD4⁺ T cells were cultured in RPMI-1640 at 1 x 10⁶ cell/ml with supplementary of 1% fetal calf serum (FCS, Biochrom Ag, Berlin), 100 U/ml glutamine and 1% penicillin/streptomycin (GibCo), 50 μM 2-mercaptoethanol. Naïve CD4⁺ T cells were cultured in 24-well plates stimulated with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml) for 3 days in the presence of transforming growth factor (TGF)-β1 (5 ng/ml) and IL-2 (500 IU/ml) for Treg cell differentiation. Cytokines were purchased from R&D systems (R&D Systems Inc., Minneapolis, USA). In certain experiments, proper PGE2, EP2 agonist/antagonist, EP4 agonist/antagonist, db-cAMP or MK2206 were added in the medium.

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 25 minutes at 4 °C with fluorescent-labeled monoclonal antibodies specific for human and mouse CD4, CD8, CD25, CD62L, and CD44.
(eBiosciences, California, USA), EP2 and EP4. For intracellular staining, cells were first fixed and permeabilized with Cytotox/Cytoperm (BD Biosciences, New Jersey, USA) and then incubated with PE-conjugated anti-Foxp3 (Biolegend, San Diego, CA). Appropriate isotype-matched antibodies were utilized for compensation adjustment. Flow cytometric analysis was performed using a fluorescence activated cell sorter (FACS) Gallios (Beckman Coulter, Indianapolis, IN, USA). Data were analyzed with FlowJo software (TreeStar, Becton, Dickinson & Company, USA).

**Enzyme-linked immuno sorbent assay (ELISA)**

To determine PGE2 in culture medium, ELISA development kits (Enzo Biochem, New York, USA) were used according to the manufacturer’s directions.

**Statistical analysis**

The results were expressed as the mean ± standard deviation (SD). The student T-test was used to compare the data conforming to normal distribution, and analysis of Mann-Whitney U test was used to verify the difference of data that did not comply with normal distribution. A P level < 0.05 was considered significant. All the statistical analyses have been computed using Statistical Package for Social Science (SPSS) statistical software, version 23 (SPSS Inc., USA).

**RESULTS**

Decreased proportions of Treg cells and increased PGE2 concentrations in the peripheral blood of AR patients compared with healthy controls

To understand the relation between PGE2 and Treg cells in AR disease, we examine the concentration of PGE2 and the percentage of Treg cells in the peripheral blood of AR patients and healthy donors. The study participants in the AR and control groups had comparable anthropometric data, including age and gender. In the peripheral blood of 37 AR patients and 16 healthy controls, Treg cells were examined by flow cytometry. We defined Treg cells as...
CD4⁺CD25hi cells (Fig. 1A CD25hi) or CD4⁺Foxp3⁺ cells (Fig. 1A Foxp3+), since the CD25⁺ population highly overlapped with the Foxp3⁺ population (Fig. 1A Overlap). PGE2 levels were measured by ELISA. The proportion of CD4⁺CD25hi (p = 0.039) or CD4⁺Foxp3⁺ (p = 0.016) cells in AR patients was significantly reduced compared with the control group (Fig. 1B). The PGE2 concentration in the peripheral blood of AR patients was significantly higher than in that of controls (p = 0.0003; Fig. 1C).

Decreased expression of EP2 and increased expression of EP4 on CD4⁺ T cells in the peripheral blood of AR patients compared with healthy subjects

PGE2 produces physical or pathological effects by binding to E prostanoid (EP) receptors, including EP1, EP2, EP3, and EP4. To identify which EP receptor has a major role in the pathogenesis of AR, the expressions of different EP receptors on the surface of CD4⁺ T cells were measured by flow cytometry. Naïve T cells and Treg cells from AR patients had higher EP4 and lower EP2 expressions compared with controls indicating a shift from EP2 to EP4 in AR patients. Fig. 1D showed the results from naïve T cells.

PGE2 dose-dependently suppressed the differentiation of Treg cells from AR patients in vitro

To verify that the elevated concentration of PGE2 had a direct relationship with the reduced population of Treg cells, naïve CD4⁺ T cells purified from healthy people were cultured under Treg-polarization conditions with different concentrations of PGE2. In the presence of 50 μM PGE2, there...
was less than 10% Foxp3+ T cells after Treg cell induction compared with more than 30% Treg cells in the absence of PGE2, suggesting the Treg cell differentiation was blocked with an inhibitory rate >70% (Fig. 2A and B). Furthermore, the suppressive effect of PGE2 on the differentiation of Treg cells was dose-dependent (1.2- to 3.9-fold; P < 0.01; Fig. 2A and B). Notably, the decreased percentage of induced Treg cells was not related to PGE2 toxicity. The highest concentration of 50 μM PGE2 did not induce a great rate of apoptosis in human naïve CD4+ T cells. The Th1 polarization results from healthy donors with or without PGE2 were similar (Fig. 2C). Therefore, we used 10 μM PGE2 in subsequent experiments. When naïve CD4+ T cells purified from AR patients were cultured under Treg-polarization conditions with PGE2, we found that PGE2 could also inhibit the differentiation of Treg cells from AR patients dose-dependently (Fig. 2D). Consistent with the results using human cells, PGE2 significantly suppressed the differentiation of Treg cells from naïve CD4+ T cells isolated from the lymph nodes of mice in a dose-dependent manner (1.2- to 2.0-fold, Fig. 3A and B). Taken together, these data suggested that PGE2 influenced the differentiation of Treg cells from healthy donors and AR patients, and its inhibitory effect was specific for Treg cell induction.

Regulation of Treg cell differentiation by PGE2 was mediated through prostanoid receptor EP4

We examined the effects of EP receptor agonists and antagonists separately in vitro to determine their involvement in the effect of PGE2 on Treg cell differentiation. The EP4 receptor agonist PGE1-alcohol significantly suppressed Treg cell differentiation from human naïve CD4+ T cells, whereas the EP2-selective agonist Butaprost or the EP1/3 receptor agonist Sulprostone had no significant effect (Fig. 4A). An EP2 receptor antagonist AH68-09 and EP4-selective antagonist ONO-AE3-208 were also used to verify these results. Because the amount of endogenous PGE2 secreted by cultured T cells was too small, we examined the antagonistic effects of EP2 and EP4 antagonists on exogenous PGE2. Fig. 4B showed that the EP2 receptor antagonist did not reverse the negative function of exogenous PGE2 on Treg cell differentiation, whereas EP4-selective antagonist strongly abrogated the inhibitory activity of PGE2 in vitro. Experiments in mice showed similar
results: the EP4 receptor agonist significantly inhibited Treg cell differentiation, whereas the EP2 or EP1/3 agonist did not (Fig. 5A). Consistent with the findings in humans, the EP4 antagonist reversed the inhibitory effect of PGE2 on the differentiation of Treg cells in mice, whereas the EP2 antagonist had little effect on the activity of PGE2 (Fig. 5B). Therefore, PGE2 might suppress Treg cell differentiation mainly via the EP4 receptor.

Fig. 4 The effects of diverse EP receptor agonists and antagonists on the differentiation of human Treg cells. (A) Naïve CD4⁺ T cells isolated from peripheral blood were stimulated with TGF-β1 and IL-2, and cultured in the presence of EP4, EP2 or EP1/3 agonist separately. The EP4 receptor agonist significantly suppressed Treg cell differentiation, while the EP2-selective agonist or the EP1/3 agonist had no significant inhibitory effect. (B) Naïve CD4⁺ T cells were treated with EP4 or EP2 antagonist separately in addition to incubation with exogenous PGE2. EP2 antagonist did not reverse the negative function of exogenous PGE2 on Treg cell differentiation, whereas the EP4 antagonist abrogated the inhibitory activity of PGE2.

Fig. 5 The effects of diverse EP receptor agonists and antagonists on the differentiation of mouse Treg cells. (A) Naïve mouse CD4⁺ T cells were stimulated with TGF-β1 and IL-2, and cultured in the presence of EP4, EP2 or EP1/3 agonist separately. EP4 agonist significantly inhibited Treg cell differentiation, whereas the EP2 or EP1/3 agonist did not. (B) Naïve mouse CD4⁺ T cells were treated with EP4 or EP2 antagonist separately in addition to incubation with exogenous PGE2. EP4 antagonist reversed the inhibitory effect of PGE2 on the differentiation of Treg cells, whereas the EP2 antagonist did not.
PGE2 activated the EP4 receptor to regulate Treg cell differentiation via activation of the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway

To further explore the mechanism through which PGE2 regulated the differentiation of Treg cells, its downstream signaling pathway was investigated using T cells from mouse lymph nodes. It was known that activation of EP2 or EP4 receptors increased cAMP and promoted the activation of cAMP-dependent PKA. In addition, EP4 receptor stimulation activated the phosphatidylinositol 3 kinase (PI3K) - protein kinase B (Akt) signaling pathway. In our culture experiments, the cAMP mimic db-cAMP simulated the inhibitory activity of PGE2 on Treg cell differentiation and the ratio of Treg cells decreased from >50% to <10% (Fig. 6A), indicating that cAMP was responsible for the downstream signaling of PGE2-EP4. However, the addition of MK2206, an inhibitor of Akt, did not rescue the suppressing effect of PGE2 on Treg cells. In the presence of TGF-β and IL-2, a similar Treg cell differentiation ratio was observed in the PGE2+Akt inhibitor group and PGE2 only group (Fig. 6B), suggesting Akt signals delivered by the EP4 receptor may not be essential for the inhibitory effect of PGE2. Taken together, these results indicated that PGE2 activated the EP4 receptor to regulate Treg cell differentiation via a mechanism that involved activation of cAMP-dependent PKA, but not PI3K-Akt, signaling pathway.

DISCUSSION

AR is a common disease seriously affecting quality of life, and so far the efficacy of medical treatment is unsatisfactory. It is essential to explore in depth the pathologic mechanism of AR to offer new ideas for developing novel treatment strategies.

AR is characterized by polarization towards Th2 cells, raised serum IgE, and eosinophilic cell infiltration. CD4+CD25+Foxp3+ Treg cells play a critical role in the regulation of Th2-induced AR.
through inhibiting activation of Th2 cells and suppressing the production of IgE.\textsuperscript{24,25} We already know that PGE2 inhibits the secretion of interferon-\(\alpha\) by Th1 cells while promoting Th2 responses,\textsuperscript{18} but the modulatory effect of PGE2 on Treg cells has not been fully clarified, especially in AR subjects.

Our study found that the number of peripheral Foxp3\,\textsuperscript{+}/CD25\,\textsuperscript{hi} Treg cells in AR patients was significantly lower while PGE2 concentration was higher than that in healthy controls. These results were consistent with literature reports. Both the researches of Huang X et al and Genc S et al. showed that the proportion of Foxp3\,\textsuperscript{+} Treg cells in peripheral blood mononuclear cells in the AR group was dramatically reduced when compared with the control group.\textsuperscript{12,14} Another study also found the expression of Foxp3 in the nasal biopsy specimens of AR patients was significantly lower than controls.\textsuperscript{26} About PGE2, one study conducted in children reported similar results with our findings, which showed that oral and nasal PGE2 concentrations were higher in the exhaled breath condensate of children with AR than healthy controls.\textsuperscript{27} The increase of PGE2 and decrease of Treg cells in AR patients suggested that there might be some relationship between these two factors.

Next our results showed that PGE2 consistently suppressed the differentiation of Treg cells from naïve CD4\,\textsuperscript{+} T cells of healthy subjects, AR patients and mice, and its inhibitory effect was dose-dependent. These results were consistent with some published data.\textsuperscript{16,28} Li H et al. reported that PGE2 decreased the quantity of Treg cells and the expression of Foxp3 mRNA, and also suppressed the production of IL-10 in rheumatoid arthritis patients.\textsuperscript{16} But some other studies had different findings. Baratelli et al. showed that PGE2 induced Foxp3 gene expression and enhanced the CD4\,\textsuperscript{+}CD25\,\textsuperscript{+} Treg cell function in human CD4\,\textsuperscript{+}T cells.\textsuperscript{29} Another study found PGE2-EP4 signaling facilitated increase of Treg cells in regional lymph nodes after ultraviolet irradiation.\textsuperscript{22} Herein, we suggested that due to the diverse expression of PGE2 and its receptors in various disease or by various manipulated method, PGE2 may make totally different effort to the surrounding T cells, especially CD4\,\textsuperscript{+}CD25\,\textsuperscript{+} Treg cells. Our study focused on AR and we demonstrated during AR process, PGE2 had inhibitory effect on the differentiation of Treg cells from naïve CD4\,\textsuperscript{+} T cells.

Combining with the results mentioned above, it is suspected that abundant PGE2 provided by AR microenvironment might play a potential role in suppressing Treg cells’ restraint on the other immune cells and therefore aggravated the pathogenesis of AR. This modulating effect of PGE2 needs to be further investigated and replicated in vivo in the nasal mucosa of AR patients and also using AR animal models. Once it is confirmed, novel medicine targeting PGE2 pathway and suppressing its action would bring new answers for the challenge of AR treatment.

PGE2 functions by acting on one of the four EP receptors, EP1-4, and the major receptors expressed on T cells are EP2 and EP4.\textsuperscript{30,31} In our study, both analysis on human and mouse T cells showed that suppression of Treg cells differentiation by PGE2 was mediated through EP4, but not EP2. However, Li H’s study had a different result,\textsuperscript{16} which showed that PGE2 suppressed human Treg cell differentiation via EP2. This discrepancy might be caused by different PGE2 concentration. Our data were collected after the human naïve CD4\,\textsuperscript{+} T cells were stimulated with 10 M PGE2, while they selected 1 M PGE2 for the experiment investigating the involvement of EP2 receptors in the actions of PGE2. Under different PGE2 abundance, the receptors may deliver diverse signals. Also we observed a shift from EP2 to EP4 on naïve CD4\,\textsuperscript{+} T cells in AR patients, supporting the critical role of EP4.

Both EP2 and EP4 receptors signal through the adenylate cyclase-triggered cAMP-PKA-CREB (cAMP-response element binding protein) pathway,\textsuperscript{31} and EP4 is featured by the exclusive ability to activate PI3K-Akt signaling pathways.\textsuperscript{32} Our results indicated that PGE2 activated EP4 receptor to regulate Treg cell differentiation via cAMP-PKA signaling, and this result is consistent with the finding of Li H’s team.\textsuperscript{16}

Thus PGE2-EP4-cAMP signaling might mediate the development of AR by inhibiting the differentiation of Treg cells. New therapy could be developed to inhibit EP4 and its downstream signaling molecules and contribute to the recovery of active...
Treg cells, which are pivotal for controlling Th2 inflammation in AR.

Conflict of interests
The authors report no competing interests.

Consent for publication
All contributing authors consent to this publication.

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