Molecular Immunology 101 (2018) 539–549

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

rhPLD2 inhibits airway inflammation in an asthmatic murine model through induction of stable CD25$^+$ Foxp3$^+$ Tregs

Chuan-Xing Yu[b,1], Ling-Yu Bai[a,1], Jun-Jin Lin[a], Song-Bo Li[a], Jun-Ying Chen[a], Wen-Juan He[a], Xiu-Ming Yu[a], Xi-Ping Cui[a], Hui-Li Wang[a], Yi-Zhong Chen[a], Ling Zhu[a,*]

[a] Immunology Dept. and Center of Neuroscience, Fujian Medical University, Fuzhou, Fujian 350004, PR China
[b] Internal medicine of Second People's Hospital of Fujian Province, Fuzhou, Fujian 350003, PR China

ARTICLE INFO

Keywords:
Recombinant human phospholipase D2 (rhPLD2)
Regulatory T cells
Asthma

ABSTRACT

Our previous studies have shown that recombinant human phospholipase D2 (rhPLD2) plays a modulator role on NF-κB and PKC signaling pathways. It also inhibits IL-5-induced inflammatory response in chronic asthmatic guinea pigs. Additionally, increasing evidence also has revealed that the adoptive transfer of induced regulatory T cells (Tregs) may be a therapeutic solution to airway allergic diseases. To investigate the epigenetic, transcriptomic and phenotypic variability of Treg population in an ovalbumin (OVA)-induced airway inflammation model derived from the induction of rhPLD2, OVA-induced asthmatic murine model is used in this study. The lung inflammation, eosinophil infiltration, the differentiation and proliferation of T helper cells and the amplification of Tregs were examined in this mouse model with and without rhPLD2 induction. Our data showed that rhPLD2 administration in asthmatic mice significantly increases CD4$^+$CD25$^+$ Foxp3$^+$ Treg cell numbers and alleviates lung inflammation. The addition of rhPLD2 *in vitro* enhanced the demethylation of Treg-specificdemethylated region (TSDR) in iTregs, suggesting that rhPLD2 protein may be involved in improving the quality and quantity of Treg cells that eventually significantly reduces lung inflammation in asthmatic murine model. These results suggest that rhPLD2 could have a clinical impact treating patients with allergic airway inflammation *via* promoting and stabilizing iTreg differentiation and function.

1. Introduction

Inflammatory cells including eosinophils, mast cells, T lymphocytes, neutrophils, and endothelial cells, are usually recruited to asthmatic airways by mammalian immune system. All of them are capable of synthesizing and releasing inflammatory mediators, like histamine, 5-hydroxytryptamine (5-HT), PAF, IL-4, IL-5, *etc.* (Su et al., 2012). The crosstalk between immune cells and airway epithelial cells causes breathlessness, wheezing, coughing, and hyperresponsiveness in asthmatic patients (Lloyd and Hessel, 2010). It has been generally recognized that allergic airway inflammation, airway hyperresponsiveness, the internal and external environmental factors, as well as genetic factors contribute to the symptoms of asthma (Cohn et al., 2004). However, the pathogenesis of asthma is still not fully understood. Many treatments have been proved successful in the alleviation of asthmatic symptoms, though asthma is yet to be completely cured.

An increasing numbers of research have indicated that phospholipase D (PLD), in particular, PLD2 is involved in proliferation, chemotaxis, and migration of lymphoctes (Adam et al., 2007; Hamdi et al., 2008; Gomez-Cambronero et al., 2007). The PLD2 enzymatic products, phosphatidic acid (PA) and diacylglycerol (DAG), as lipid second messengers locate at the intersection of several lipid metabolism and cell signaling events including membrane trafficking, survival, and proliferation. Additionally, PLD2 modulates MAPK signal transduction pathway and is involved in the regulation of lymphocyte proliferation, differentiation, cell secretion, and the expression of asthmatic inflammatory molecules (Chand et al., 2012; Altman and Villalba, 2002).

Our previous studies showed that the recombinant human PLD2

Abbreviations: DXM, dexamethasone; Foxp3, forkhead box protein 3; HBEpCs, human bronchial epithelial cells; TSDR, Treg cell specific demethylation region; NF-kB, nuclear factor kB; rhPLD2, recombinant human phosphplipase D2; Treg, CD4$^+$CD25$^+$ Foxp3$^+$ regulatory T; nTreg, naturally occurring, thymus-derived CD4$^+$CD25$^+$Foxp3$^+$ regulatory T; iTreg, induced CD4$^+$CD25$^+$Foxp3$^+$ regulatory T by TGF-β; P-iTreg, induced CD4$^+$CD25$^+$Foxp3$^+$ regulatory T by rhPLD2; PAF, platelet activating factor; FACS, fluorescence-activated cell sorting; MACS, magnetic activated cell sorting; 5-Aza, 5-aza-2′-deoxycytidine (5-aza-dC)

* Corresponding author.
E-mail address: lingzhu29@sina.com (L. Zhu).
[1] These author equally to this work.

https://doi.org/10.1016/j.molimm.2018.07.030
Received 9 July 2017; Received in revised form 28 January 2018; Accepted 26 July 2018

(rhPLD2) inhibits the secretion of platelet activating factor (PAF), a potent inflammatory mediator that promotes the expression and exocytosis of a group of inflammatory proteins if binding to PAF receptor (Ling et al., 2003). The down-regulation of PAF by rhPLD2 likely results in the suppression of PKC activation and the subsequent STAT signaling. rhPLD2 also inhibits the activation of p65 and the expression of IL-1β (Ling et al., 2006).

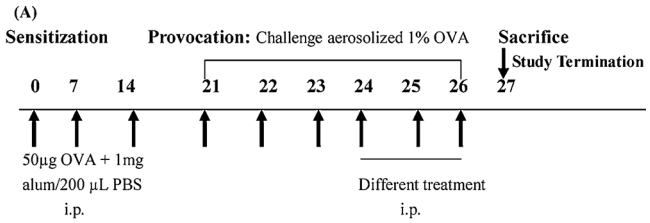
Increasing evidence has shown that asthmatic inflammation is related to aberrant cellular immunity, such as imbalanced Th1 to Th2 population, dysfunction of Th17 cells, or defective Treg cells (Dias and Banerjee, 2013; Ohkura et al., 2013; Kudo et al., 2012; Kearley et al., 2008; Kong et al., 2012). Th2 cytokines play a critical role in amplifying asthma, whereas Th1 cytokines prevent this allergic inflammation (Kim et al., 2010; Baraldo et al., 2007). Reducing Th2 cells in asthma model may significantly alleviate the eosinophilic inflammation (Gavett et al., 1994). Th17 are also important in mediating asthma (Morishima et al., 2013). Treg cells are essential in regulating the homeostasis and function of the immune system. Treg dysfunction leads to excessive immune responses in asthma patients. Suppression of Th2, Th17, as well as allergen-specific IgE production highly depends on the function of Treg cells (Afshar et al., 2008; Oukka, 2008; Sakaguchi, 2004; Xu et al., 2012; Lawless et al., 2018). *In vitro* induced Tregs has become a good source of adoptive therapy (Zheng et al., 2002, 2004). However, the transfer of nTreg cells in patients with established disease was not reliable (Zhou et al., 2010a,b; Zheng et al., 2008) as repeated amplification of nTregs *in vitro* may cause alterations in cell phenotype and functions. In addition, the ability of expanding Treg cells itself is limited for Treg cells have the characteristic of immunosuppressive, and perform no response to IL-2, so by this way of amplifying Treg cells is limited *in vitro*. Further research also demonstrated controversial results mainly due to the instability of iTregs. Due to the *in vivo* immune suppressive environment derived from IL-2, the amplification of Treg cells is usually performed *in vitro*. TGF-beta has been commonly used to induce Foxp3 expression *in vitro* (Zheng et al., 2002), thus further activating Smad3 signaling pathway (Meng et al., 2013), mTOR signaling pathway (Zeng and Chi, 2015) and CNS1 (Wu et al., 2014) *etc.* Among them, the enhancement of CNS1 by TGF-beta that promotes the expression of Foxp3 protein is the key. 5-Aza-dC is a kind of methyltransferase inhibitor, which induced Foxp3-TSDR hypomethylation and expression of the Treg cell specific genes Foxp3 (Singer et al., 2015). 5-azacytidine has been reported to augment Treg cell expansion in blood (Jan et al., 2014). Therefore, we used 5-azuridine as an inducement of regulatory T cell expansion in this experiment, namely, except with TGF-beta induction, another positive inducer control group.

In current study, we are interested in understanding the relationship between rhPLD2 and T cell differentiation, as well as how rhPLD2 affects asthmatic inflammation. We are particularly interested in evaluating the effects of rhPLD2 induction on lung inflammation and T cell population in ovalbumin (OVA)-induced asthmatic mouse model. Furthermore, we have investigated whether the immunomodulatory effects of rhPLD2 in asthmatic airway disease is mediated by the recruitment of regulatory T cells (Tregs) and how these rhPLD2-induced Tregs (P-iTregs) alleviate the asthmatic inflammation.

2. Materials and methods

2.1. Animals

BALB/c mice (SPF) were obtained from Silaike Laboratory Animal Co., Ltd. (SLAC), Shanghai, China. All animal care and experiments were performed under institutional protocols approved by Fujian Medical University Institutional Animal Care and Use Committee (Certificate Number: 2014-23). Female mice (aged 4–5 weeks) were used in the experiments and five to eight mice were assigned stochastically to each group (Fig. 1).



(B) Five-week-old female BALB/c mice (N=56)

Group	Sensitization	Challenge	treatment
Normal control (N)	PBS	NS	NS
Asthma (A)	OVA	OVA	NS
Saline group	OVA	OVA	NS
DXM	OVA	OVA	DXM
rhPLD2	OVA	OVA	rhPLD2
rhPLD2+Anti-PLD2	OVA	OVA	rhPLD2 + anti-PLD mAb
rhPLD2+cIgG	OVA	OVA	rhPLD2 + cIgG mAb

Fig. 1. The experimental protocol and group. **(A)** The mice were sensitized on days 0, 1, 7, and 14 by intraperitoneal injection of OVA and challenged with aerosolized 1% OVA on days 21–26. **(B)**The mice were stochasticlly divided into seven different groups in accordance with the different sensitization, challenge, and treatment.

2.2. Mouse model of asthma

To generate asthmatic murine model, BALB/c mice (n = 56) were intraperitoneally (i.p.) sensitized on days 0, 7 and 14 with 50 μg /0.2 mL chicken egg albumin (OVA, grade V, Sigma-Aldrich, St. Louis, MO, USA) emulsified in alum adjuvant. After the sensitization, mice were exposed to aerosolized 1% OVA for 30 min per day on days 21 through 26. On days 24, 25, and 26, the mice were intraperitoneally injected with saline, DXM (Dexamethasone) (2 mg/kg), rhPLD2 (8 mg/kg) with or without anti-PLD mAb (1:500) or mouse IgG (equivalent dose to anti-PLD mAb) 30 min before OVA challenge. The mice in the control group were administered (i.p.) with normal saline on days 0, 7, and 14, and challenged with PBS on days 21–26. In the *in vivo* blocking experiments, the mice were given 50 mg of neutralizing antibodies against rhPLD2, an isotype-matched irrelevant Ig was used as a negative control (Fig. 1). The mice were sacrificed 24 h after the final challenge. Bronchoalveolar lavage fluid (BALF), lungs, blood, and peripheral lymphocytes were obtained. Eosinophil cell counts in BALF, ELISA for BALF and serum cytokines, and flow cytometry analysis (BD FACSVerseTM System) were performed as described previously (Zheng et al., 2007). The right lungs were isolated and digested for cellular analysis as described above. The left lung was instilled with 0.4 ml of 4% paraformaldehyde for histology. Fixed lung sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) for mucus secretion.

2.3. Cell culture

Naive $CD4^+CD25^-CD62L^+$ cells from BALB/c spleen and lung draining lymph nodes were isolated by magnetic beads with negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (Ohkura et al., 2013). The purity of the selected cell populations was 96–98%. Purified $CD4^+CD25^-CD62L^+$ cells were stimulated with anti-CD3/CD28 beads (one bead to 1 cells [1:5]), IL-2 (300 U/mL), and rhPLD2 (0.5 ng/mL), with 5 ng/mL TGF-β (iTreg cells) or without TGF-β (T control cells) (PeproTech, Rocky Hill, NJ) for 4 days. The $CD4^+CD25^+$ cells in the spleen and lung lymph nodes were sorted by flow cytometry and expanded in RPMI-1640 with anti-CD3/CD28 beads (1:1) and IL-2 (300 U/mL) for 5 days. The expressions of CD25 and Foxp3 were determined by flow cytometry.

2.4. In vitro induction of rhPLD2-induced regulatory T (P-iTreg) cells

All naive $CD4^+CD62L^+$ T cells were isolated from spleens and lymph nodes by Miltenyi beads. The purity reached greater than 95%. iTreg cells were then prepared as described (Zhou et al., 2010a,b; Zhang et al., 2013). Briefly, BALB/c naive $CD4^+CD62L^+$ T cells were cultured alone for 5 days or along with mitomycin C–inactivated APCs (5×10^4 cells/well, used as antigen-presenting cells), anti-CD3 mAb (5 μg/mL; eBioscience, USA.), anti-CD28 mAb (1 μg /mL; eBioscience, USA), IL-2 (300 U/mL; eBioscience, USA) in RPMI 1640 with heat-inactivated 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L β-mercaptoethanol. P-iTreg cells were induced as above but with the addition of rhPLD2 (0.5 ng/mL) for 5 days. The rhPLD2-induced iTreg cells ($CD4^+CD25^+Foxp3^+$, P-iTreg) were then analyzed by BD FACSVerse™ System.

2.5. OVA-sensitized mouse asthmatic model and exogenous cell infusion

6 to 8-week-old female Balb/c mice weighing 20–25 g were used for the experiments. Mice were sensitized with OVA/alum i.p. on 0, 7, and 14 days. Twenty-four hours after the third sensitization (the 15th day), rhPLD2-induced Treg (P-iTreg) and TGF-β-induced Treg (iTreg) or control cells (with PBS) at 2×10^6 per mouse were intravenously injected into OVA-sensitized mice, one week after aerosol challenge with 1% OVA for 5 consecutive days, 30 min a day (Fig. 6A). Asthmatic mice and normal mice were used as experimental controls. The same experiments were performed four times.

2.6. Histopathological analysis

The lung samples were fixed in 10% formalin. They were then paraffin embedded, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). These samples were coded and read by a blinded investigator. To assess eosinophilic inflammation, Bronchoalveolar lavage fluid (BALF) was collected 24 h after the final challenge. Percent of eosinophils in BALF was evaluated under a light microscope.

2.7. Measurement of OVA-specific IgE

The level of OVA-specific IgE in sera was measured by Mouse anti-OVA-IgE ELISA kit (Cat#: ab157718, Abcam, USA) 24 h after the last challenge. All the samples, including the standards, were assayed in duplicate.

2.8. Reverse transcription and real-time PCR

Total RNA was extracted from flash-frozen spleen, lymph nodes, and lung tissue sections using the RNeasy Mini Kit. The RNAs were then reverse transcribed using the ReverTra Ace -α- Reverse Transcription Kit from TOYOBO. The PCR primers were: forward: 5′-CAGCTGCCTA CAGTGCCCCTAG-3′, reverse: 5′-CATTTGCCAGCAGTGGGTAG -3′. GAPDH was used to normalize equal addition of template cDNAs. All operations are strictly operated according to the kit instructions.

2.9. Western blotting analysis

Total protein from the lung tissue, spleen, and lymph nodes were extracted by RIPA protein extraction reagent. The lysates were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membrane. The membrane was then blocked with 5% BSA and incubated with primary antibodies overnight at 4 °C (Foxp3 1:1000 dilution, β-actin 1:5000 dilution). The membrane was incubated with HRP-conjugated IgG (1:5000) at 37 °C for 1 h and the blots were visualized and analyzed with E-Gel® Imager System.

2.10. Flow cytometry analyses

The single cell suspensions of spleen and lymph nodes were adjusted to 1×10^7/mL. One hundred microliters of cell suspension were used for the antibody staining. (1) Th1/Th2/Th17 analysis: cells from spleen and lymph nodes were stimulated with anti-CD3 and anti-CD28 antibodies for 6 h at 1×10^7/mL. suspended in 1 mL in T cell cultures, after addition of cytokines stimulants the cells were cultured at 37 °C and 5% CO_2 for 6 h. The cells were then stained with FITC-labeled anti-mouse CD4 antibody at 4 °C for 25 min. Fixed broken membrane liquid suspension, Anti-Mouse IFN-γ PE, Anti-Mouse IL-4 APC, Anti-Mouse IL-17A-APC, 4 °C in the dark, 25 min incubation on the ice. Dyeing buffer heavy suspension. (2) Treg analysis: $CD4^+CD25^+$ T cells were characterized by surface staining of the cells with FITC-labeled anti-mouse CD4 (GK1.5, eBioscience), PE-Cy7-conjugated anti-mouse CD25 (PC61.5, eBioscience) at 4 °C for 30 min in the dark. The cells were then washed with Flow Cytometry Staining Buffer (eBioscience) and permeabilized with Foxp3 Fixation/Permeabilization Buffer (eBioscience) for 30 min. The cells were stained with PE-conjugated anti-mouse/rat Foxp3 (FJK-16s, eBioscience) at 4 °C for 30 min. The cells were then washed twice and examined by flow cytometry (BD FACSVerse™ System). All FACS analysis was performed using FlowJo Software.

2.11. DNA isolation and TSDR methylation analysis

Methylation analysis was performed using Bisulfite Sequencing PCR (BSP). Genomic DNAs from iTregs were extracted using a Genomic DNA kit (Tiangen Biotech Co., Ltd., China). An EZ DNA Methylation kit (Zymo Research, U.S.A) was used to perform bisulfite conversion. This reaction converts all non methylated cytosine bases into uracil. The Treg cell specific demethylation region (TSDR) was amplified by PCR. The PCR products were cloned using a T Cloning Kit (Promega Biotech Co., Ltd., U.S.A). Each DNA from 10 individual clones was purified and sequenced (Invitrogen Corporation, Shanghai). The PCR primers were: forward: 5′-AGGAAGAGAAGGGGGTAGATA-3′, reverse: 5′-AAACTAAC ATTCCAAAACCAAC-3′.

2.12. Statistics

All data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS19.0. The statistical significance of the differences was evaluated by one-way analysis of variance (ANOVA) followed by the LSD test or Dunnett's test for multiple comparisons, with significance set at $p < 0.05$.

3. Results

3.1. rhPLD2 reduced eosinophil infiltration and IgE production in the lungs of asthmatic mice

To investigate the effects of rhPLD2 on airway inflammation, the BAL fluid was examined for eosinophil numbers. As shown in Fig. 2, the mice with OVA (asthmatic group, positive control) had significantly more eosinophils (57.5 ± 26.58%) than the mice with saline (saline group, negative control) (0.00 ± 0.00%), $t = 5.57, p = 0 < 0.05$. After treatment with rhPLD2, less eosinophil infiltration (5 ± 2%, $t = 5.08$, $p < 0.05$) was observed in the BAL fluid. Similar eosinophil counts were found between mice treated with rhPLD2 and with DXM ($p > 0.05$). The administration of anti-PLD antibody in the rhPLD2-treated mice significantly increased eosinophil lung infiltration (70 ± 15%) when compared to the group treated with rhPLD2 alone (5 ± 2%, $t = 5.89$, $p < 0.05$) or the one with rhPLD2 and the control antibody (11 ± 6.78%, $t = 5.71$, $p < 0.05$) (70 ± 15%). These results suggest that rhPLD2 specifically suppresses airway eosinophil infiltration (Fig. 2A).

Atopic allergies, including atopic (allergic) asthma and atopic

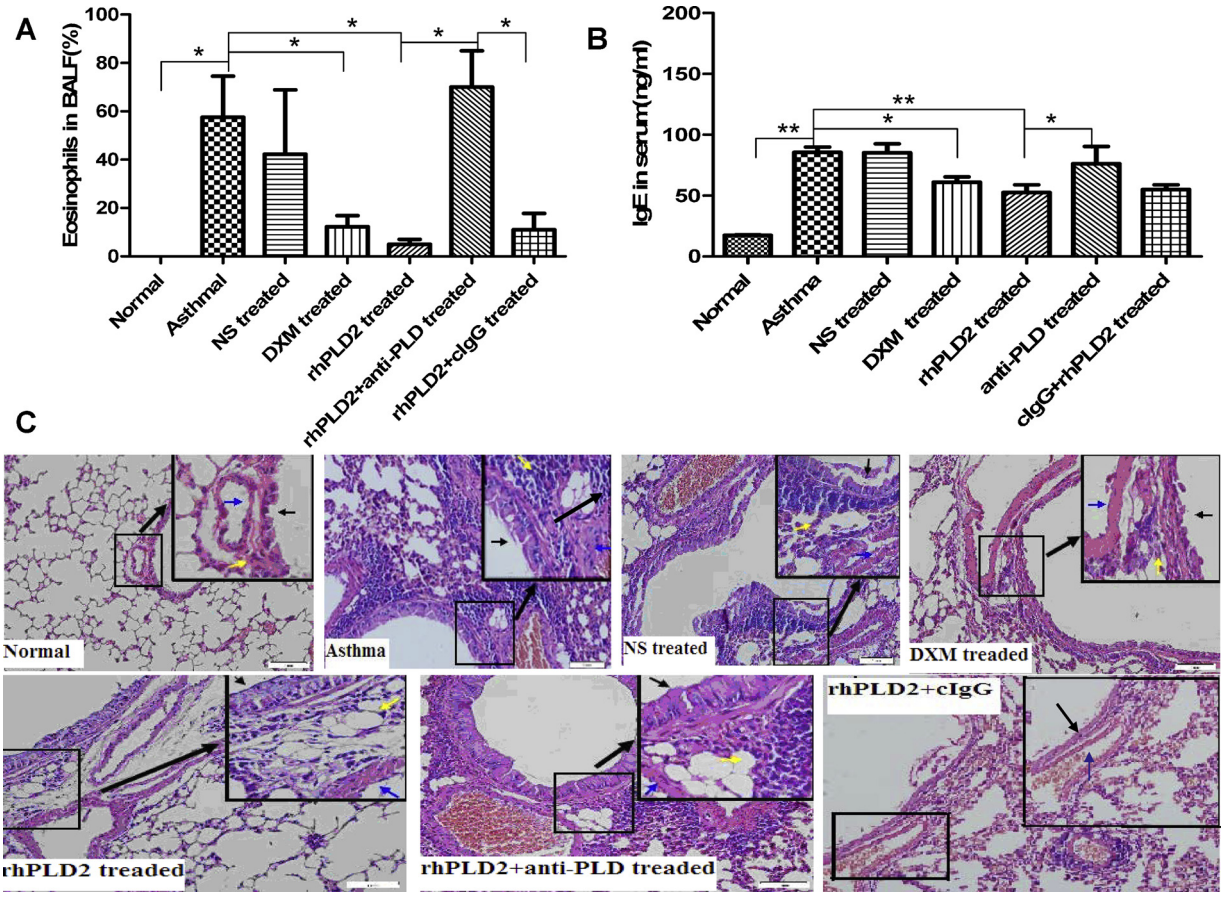


Fig. 2. rhPLD2 negatively regulates allergic asthma responses. **(A)** Quantification of eosinophils in bronchoalveolar lavage fluid from different treated mice in which allergic asthma was induced. **(B)** IgE production in serum was measured by ELISA. All data were expressed as means ± SEMs (n = 5–8 mice/group). $*p < 0.05$(LSD-t) and $**p < 0.01$ *vs* OVA control. **(C)** The right hilum was tied off, and right lungs were isolated and digested for cellular analysis by RT-PCR and Western bloting. The left lung was instilled with 0.4 ml of 4% paraformaldehyde for histology. Fixed lung sections and peribronchial inflammatory cell infiltration was visualized by H&E staining. Insets show (400×) magnified views of the respective panels.

dermatitis, are characterized by increased local and systemic production of IgE which can mediate AHR (Amit et al., 2014; Hizawa et al., 2001). The high IgE production is the result of high Th2 polarization in T cell responses. We thus investigated if rhPLD2 could reduce the level of OVA-specific IgE in serum. Compared to saline-treated asthmatic mice (85.570 ± 4.373 ng/mL) ($t = 3.653$, $p = 0.001$), the concentration of OVA-specific IgE in mice treated with rhPLD2 was significantly lower (52.572 ± 6.315 ng/mL). Similar levels of IgE were found in groups treated with rhPLD2 and DXM (61.157 ± 4.263 ng/mL, $p > 0.05$). The levels of OVA-specific IgE in the group treated with rhPLD2 + anti-PLD (76.199 ± 14.283 ng/mL) were significantly higher than those in the groups treated with rhPLD2 alone ($t = 2.217$, $p = 0.035 < 0.05$) or with rhPLD2 + control IgG (55.053 ± 3.842 ng/mL). These data suggest that rhPLD2 could suppress IgE production in asthmatic mice (Fig. 2B).

The lung tissues at 24 h post final OVA challenge were examined by histological staining. Massive inflammation at the peribronchiolar region (as indicated by hematoxylin and eosin, H&E staining) was revealed in the asthmatic group treated with saline (positive control) or rhPLD2 + anti-PLD antibody. In contrast, the extent of cell infiltration and mucus secretion were markedly reduced in the mice treated with rhPLD2 or DXM (Fig. 2C). Collectively, the results indicate that rhPLD2 inhibits airway inflammation in mice.

3.2. rhPLD2 enhanced Treg expansion in the lungs of asthmatic mice

It is well known that $CD4^+CD25^+Foxp3^+$ Treg cells are crucial immunoregulatory cells that suppress immune responses, including hypersensitive responses such as asthma (Takashi et al., 2014). To study the mechanism underlying the immunomodulatory effects of rhPLD2 in asthmatic mice, $CD4^+Foxp3^+$ Tregs were investigated. As shown in Fig. 3, the percentages of Tregs in the spleen and lung draining lymph nodes were markedly increased in asthmatic mice administered with rhPLD2 when compared to the asthmatic mice treated with PBS or rhPLD2 plus anti-PLD2 antibody. 4.0383 ± 1.3880% in the rhPLD2 treated asthmatic group, 2.2850 ± 0.13868%, $|t| = 2.891763$, $p = 0.008 < 0.001$ in asthmatic group, and 2.2067 ± 0.24583%, $|t| = 2.757755$, $p = 0.011 < 0.05$ in the group treated with rhPLD2 plus anti-PLD mAb). The Treg percentage in the rhPLD2 treated asthmatic group was very close to normal mice without asthma induction (4.4575 ± 1.14211%, $t = 0.691$, $p = 0.496 > 0.05$) (Fig. 3A and C). A similar increase in the percentage of circulating $CD4^+Foxp3^+$ Treg cells was found in rhPLD2 treated asthmatic group but not in the group treated with rhPLD2 plus anti-PLD mAb (Wilcoxon $W = 10$, $p = 0.014 < 0.05$) (Fig. 3B and D). Therefore, rhPLD2 mediated increase in $CD4^+Foxp3^+$ Treg cell numbers is likely associated with the reduction of lung inflammation in these asthmatic mice.

The protein and mRNA levels of Foxp3 in lung tissue, spleen and lung draining lymph nodes were further determined by Western blotting and RT-PCR (Fig. 4A and B). As expected, the levels of Foxp3 protein in lung tissues of rhPLD2 administered asthmatic group were significantly higher than that of the controls (1.481 ± 0.339 *vs* 1.004 ± 0.276, $t = 2.780$, $p = 0.008 < 0.01$) (Fig. 4C). The Foxp3 mRNA levels in the spleen and lung draining lymph nodes

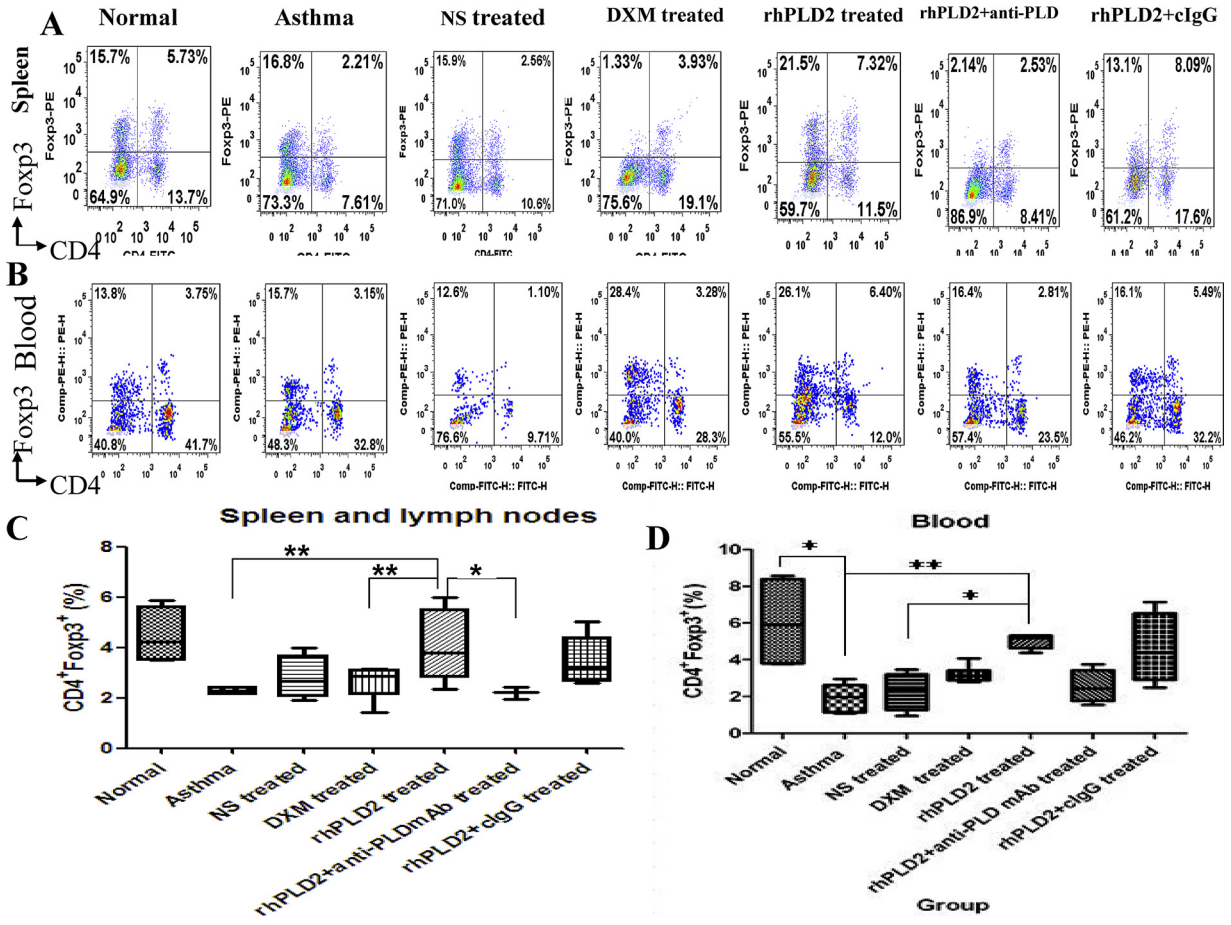


Fig. 3. Effects of recombinant human phospholipase D2 (rhPLD2) on Tregs *in vivo*. The similar results were seen in the analysis of the effects of rhPLD2 on $CD4^+$ $Foxp3^+$ T cells in blood **(A)**, spleen and LLNs **(B)**. rhPLD2 treatment significantly increased the frequency of Tregs in asthmatic mice, with Foxp3 expression determined by flow cytometry **(C, D)**. n = 5–8 mice/group, $*p < 0.05$, $**p < 0.01$.

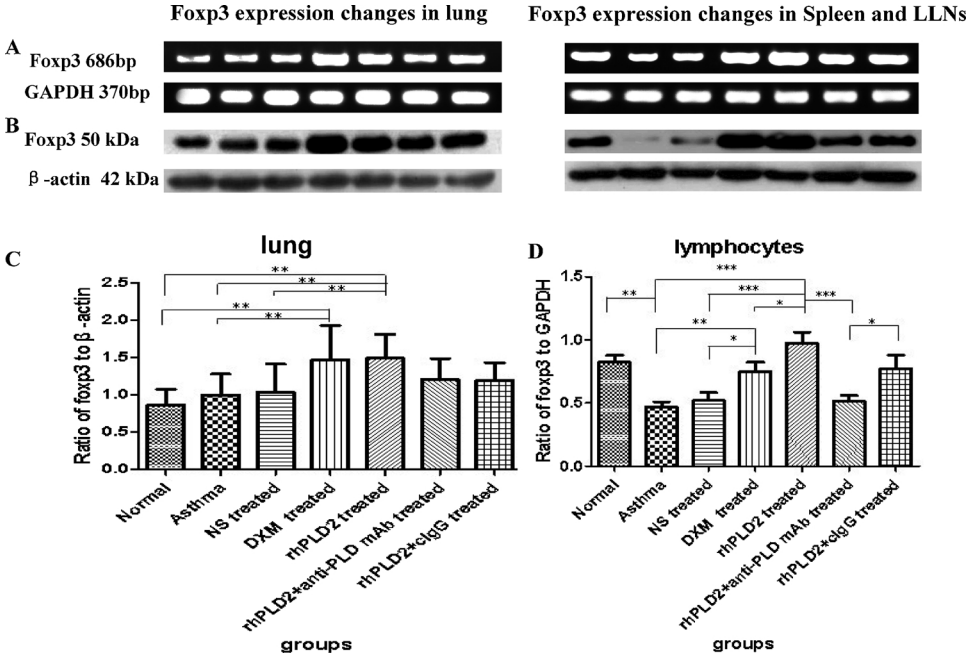


Fig. 4. Effects of rhPLD2 on expression of Foxp3 protein and mRNA. **(A)** RT-PCR pictures of the mRNA expression of Foxp3 in mice lungs, spleen and lymph nodes. **(B)** Western blotting results of the expression of protein changes of Foxp3 and β-actin in the lungs or spleen and lymph nodes of mce. **(C)** Western blotting results, statistics, the levels of Foxp3 protein changes relative to control β-actin in lungs of mice. Data are expressed as means ± SEMs of the levels of each type of transcription factor relative to control β-actin (n = 5–8 per group). $*p < 0.05$, $**p < 0.01$. **(D)** RT-PCR results statistics, the levels of Foxp3 mRNA transcripts relative to control GAPDH in spleen and lymph nodes. Data are expressed as means ± SEMs of the levels of each type of transcription factor relative to control GAPDH (n = 5–8 per group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

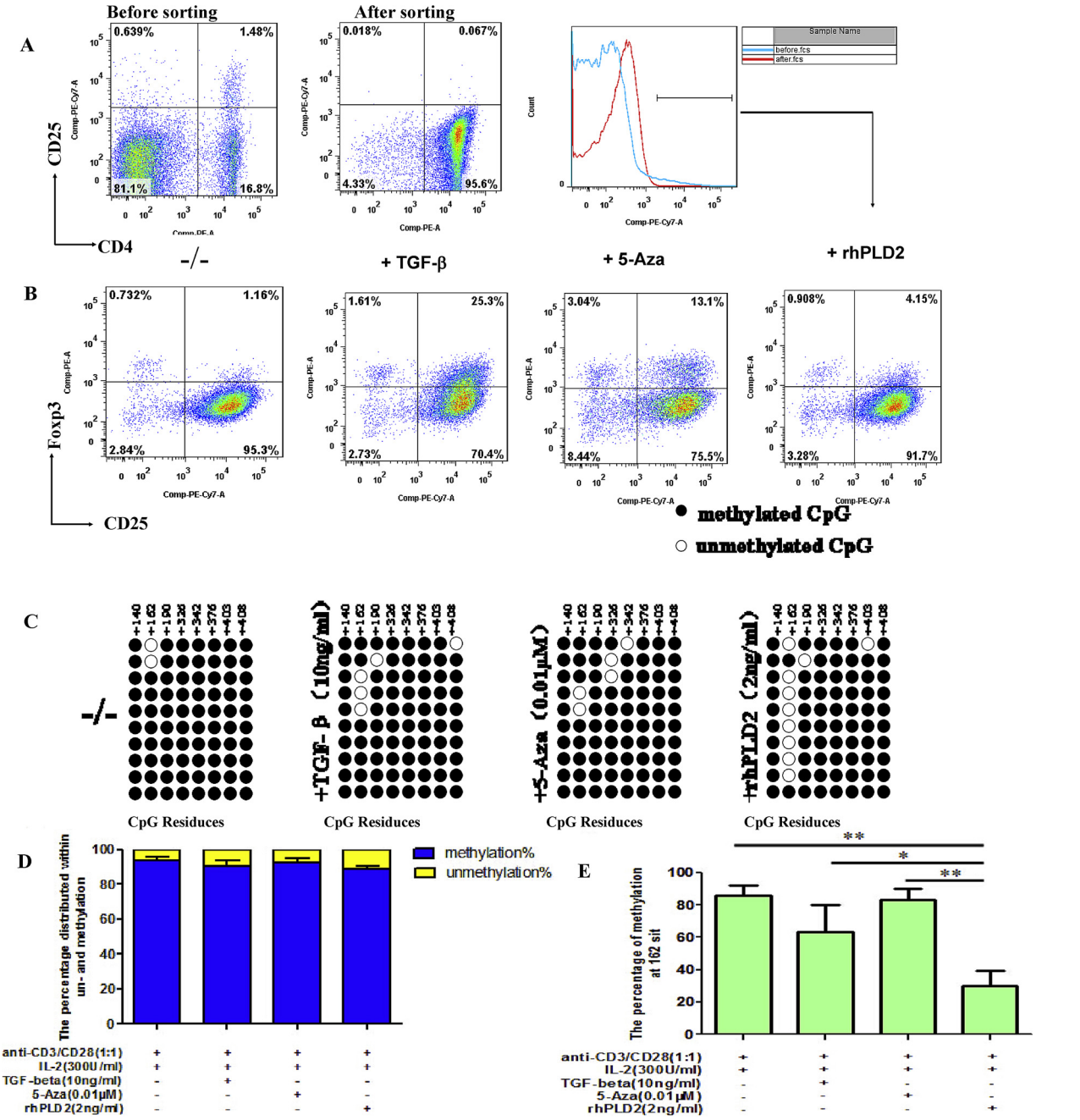


Fig. 5. The DNA methylation status of CpG positions in the TSDR region of the Foxp3 gene. Methylation status was analyzed by bisulfite sequencing PCR (BSP). After sorting by MACS, the $CD4^+CD25^-$Treg cells (1×10^5/well) were cultured with Nil, TGF-β (10 ng/ml), 5-Aza(0.01μM) and rhPLD2(2 ng/ml) in the presence of anti-CD3/CD28(1:1) and IL-2(300U/ml) for 4 days. Four days later, the cells were harvested, and genomic DNAs were prepared. **(A)** FACS analysis showed the purity of $CD4^+CD25^-$T before and after sorting, numbers display frequency of cells within indicated populations. **(B)**The percentage of $CD4^+CD25^+Foxp3^+$T cells cultured under different conditions was analysed by flow cytometry, respectively. **(C)**The methylation status of individual CpG position was detected by BSP. **(D)**The graph shows the percentage of the methylation status analyzed by BSP. **(E)** The DNA methylation status of CpG positions at the site 162 in the TSDR region of the Foxp3 gene. Methylation status was analyzed by bisulfite sequencing PCR. The graph shows the percentage of the methylation status analyzed by BSP at the site 162. n = 4, $*p < 0.05$; $** p < 0.01$. **Note:** 1) rhPLD2 induction percentage lower than TGF-beta, consider the possible reasons is that $CD4^+CD25^+$Treg cells were amplified by only 2 ng/mL rhPLD2, whereas 10 ng/mL TGF-β induced the production of $CD4^+CD25^+Foxp3^+$T cell were (44.27 ± 3.4)%. So, the optimal rhPLD2 induced concentration has not yet been found. 2) Although rhPLD2 induced percentage is lower than the TGF-beta, but this experiment has proved that the Treg induced by rhPLD2 was more stable. We also detected IL-10 and other cytokines, due to the limitation of the paper length, these data does not show.

(0.470 ± 0.088) were low in PBS treated of asthmatic mice (0.824 ± 0.108), $t = 3.200$, $p = 0.003 < 0.01$, whereas the asthmatic mice treated with DXM and rhPLD2 revealed a significant increase in Foxp3 mRNA levels: 0.749 ± 0.180 and 0.974 ± 0.212, respectively (0.470 ± 0.088, $t = 5.055$, $p = 0.000 < 0.001$; 0.519 ± 0.126, $t = 4.2074$, $p = 0.000 < 0.001$; 0.749 ± 0.180, $t = 2.371$, $p = 0.025 < 0.05$) (Fig. 4D). As rhPLD2 enhances the levels of Foxp3 mRNA in asthmatic mice, it is likely that rhPLD2 promotes iTreg cell differentiation *in vivo*. The percentages of Tr1 cells in the blood, spleen, and lung draining lymph nodes had no significant differences among the experimental groups (data not shown).

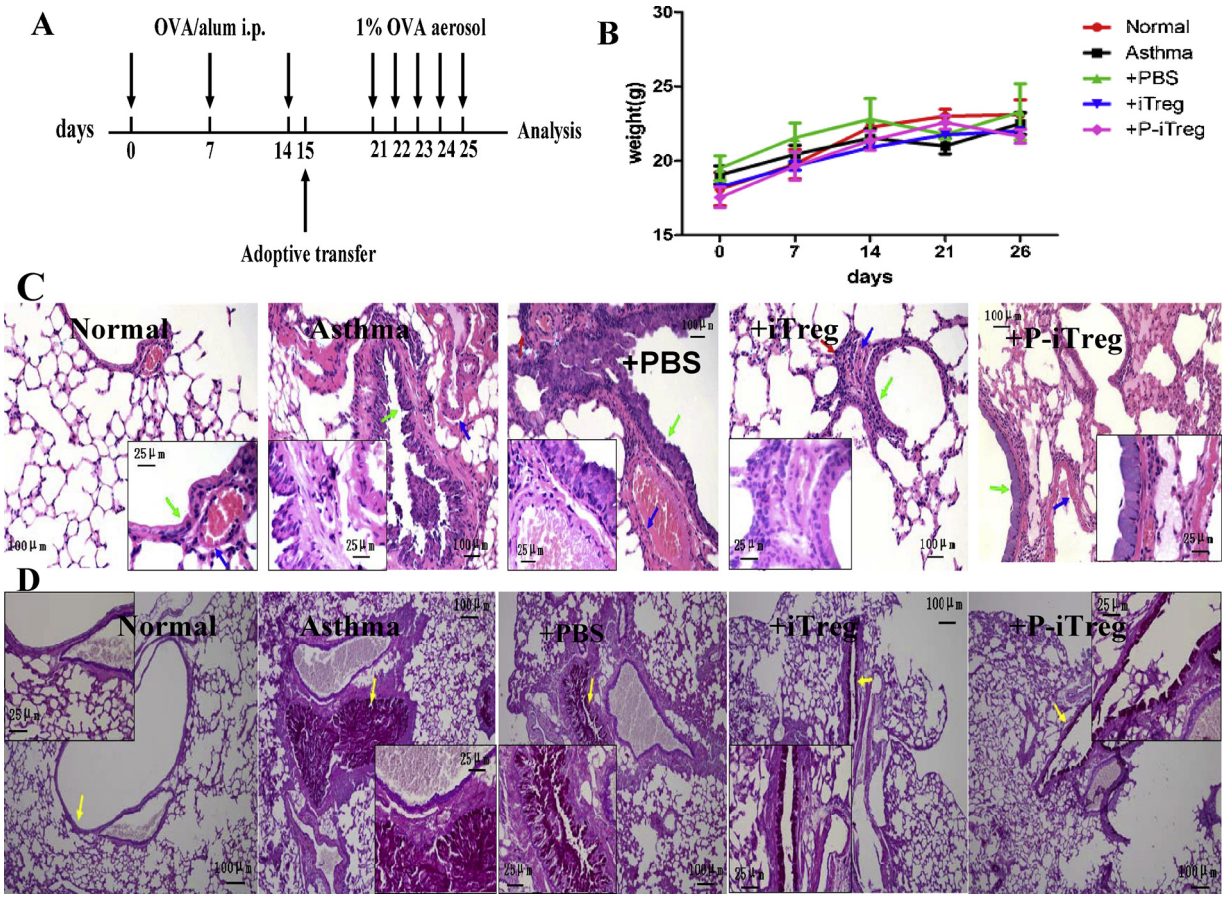


Fig. 6. Adoptive transfer of P-iTregs can relieve airway inflammation in a murine asthma model. **(A)** Experimental protocol. Mice were sensitized with OVA/alum i.p. on 0,7 and 14 days. 24 h after the third sensitization (the 15th day), 2×10^6 OVA-specific $CD4^+CD25^+Foxp3^+$T cells were adoptively transferred into OVA-sensitized mice. The transferred cells were cultured for 4 days *in vitro*.7 days later, aerosol challenge with 1%OVA for 5 consecutive days, 30 min a day. The mice were sensitized and challenged with PBS served as normal controls. The mice that were sensitized and challenged with OVA and received no transfer were included as asthma. **(B)** Groups of mice at different stages of weight change. At every time on the day of sensitization and atomization, the weights of mice were recorded. The weight of rhPLD2 induced Treg (P-iTreg) adoption group and TGF-beta induced Treg (iTreg) adoption group is consistent with the normal group. **(C)** The number of total inflammatory cells and eosinophils was significantly decreased in the P-iTreg adoption group compared to the OVA group. The arrows indicate areas of peribronchial cellular infiltration (H&E). **(D)** As detected by Periodic acid-Schiff (PAS) staining of lung tissue, P-iTregs blocked epithelial cell mucin production in airways, and PAS-positive cells around the airway and blood. whereas control group did not CD4 T cells. The yellow arrow points to goblet cells secreting mucus (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Effect of rhPLD2 on the stability of Foxp3 expression

To investigate whether rhPLD2 directly promotes Treg differentiation, an *in vitro* induced Treg (iTreg) differentiation model was applied. Compared to the medium control, more $Foxp3^+$ cells were found in the wells with rhPLD2. As similar cell proliferation and survival were found between rhPLD2-treated and control T cells, the increase in $Foxp3^+$ cells may not be due to the effect of rhPLD2 on T cell activation and survival.

Foxp3 is the dominant transcription factor in Treg differentiation. The Treg-specific demethylated region (TSDR) in Foxp3 gene is highly conserved and is fully demethylated in natural Tregs (nTregs) but is methylate in effector T cells. However, the differentiation of iTreg *in vitro* with TGF-β did not result in highly demethylated TSDR in Foxp3 gene. This indicates that the switch-on of Foxp3 expression is not affected by TSDR methylation status. Instead, the methylated TSDR in iTreg has been correlated with the instability of Foxp3 expression and loss of inhibitory function under certain conditions (Stefan et al., 2007). This suggests that TSDR methylation status is a useful tool in monitoring Treg stability. To investigate whether rhPLD2 affects the stability of Foxp3 expression in iTregs, we performed methylation-specific PCR (MSP) of TSDR in $CD4^+CD25^+$ cells derived from medium control, TGF-β alone, TGF-β with rhPLD2, and TGF-β with demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC).

$CD4^+CD25^-$ T cells with purity of 95.6% were sorted by magnetic activated cell sorting (MACS) and cultured with TGF-β, 5-aza-dC and rhPLD2 for 4 days, respectively. As can be seen from Fig. 5A and B, $CD4^+CD25^+$ Treg cells were amplified by only 2 ng/mL rhPLD2 compared with Nil group, whereas 10 ng/mL TGF-β induced the production of $CD4^+CD25^+Foxp3^+$T cell were (44.27 ± 3.4)%. And as shown in Fig. 5C–E, the overall levels of DNA methylation in Nil group, TGF-β treated, and 5-aza-dC-treated group were 93.6 ± 1.87%, 90.95 ± 2.86%, and 92.5 ± 2.59%, respectively. However, DNA methylation in rhPLD2 treated group was significantly lower than those in the other three groups. In particular, the methylation level at 162 loci of CpG sites was (30 ± 7.79%) in rhPLD2 treated cells whereas (85.83 ± 6.29%) in Nil group ($p = 0.002$), (63.3 ± 16.67%) in TGF-β treated group ($p = 0.04$), and (83.3 ± 6.67%) in 5-aza-dC treated group ($p = 0.004$). These results intimate that rhPLD2 facilitates the differentiation and stability of iTreg cells by promoting the demethylation of Foxp3 gene promoter.

3.4. Adoptive transfer of rhPLD2-treated iTreg (P-iTreg) cells attenuates asthma

To confirm that rhPLD2-treated iTreg (P-iTreg) reduced

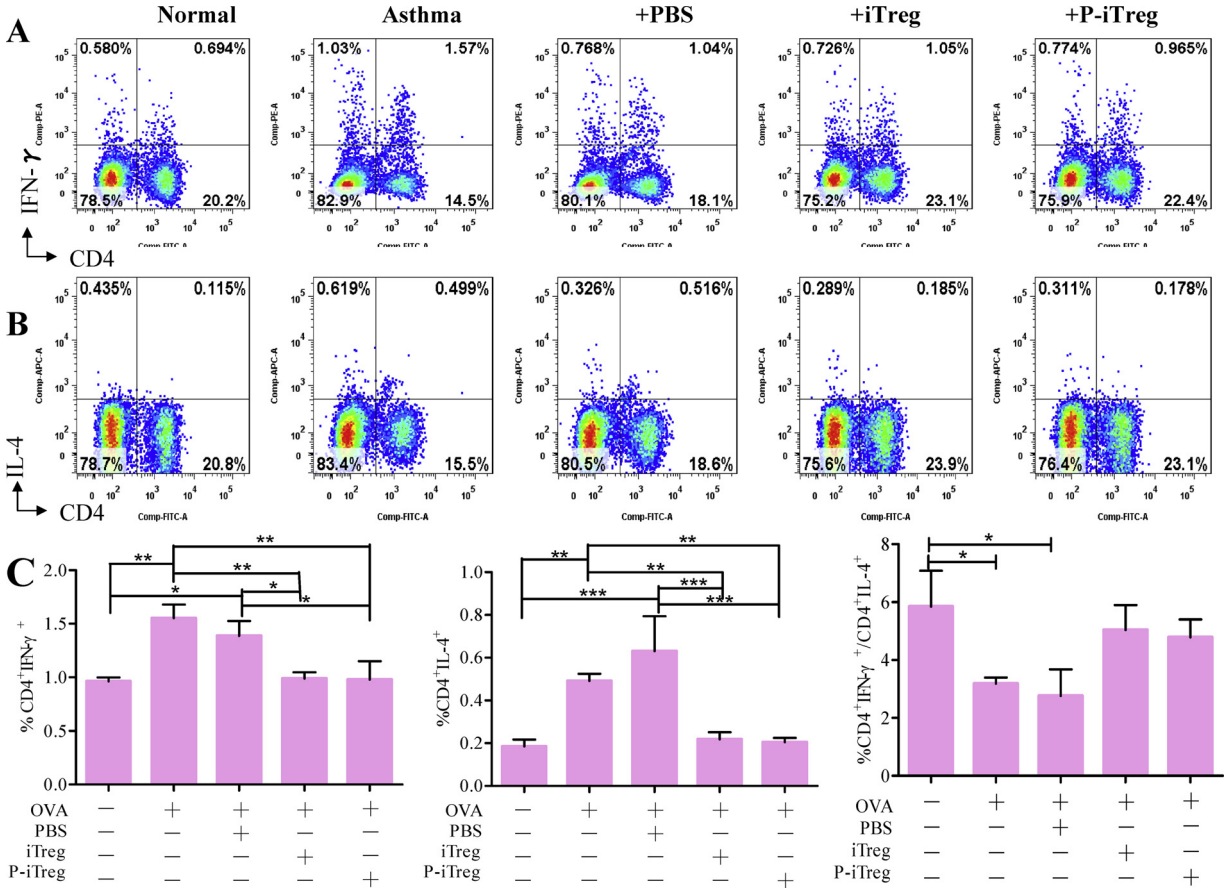


Fig. 7. P-iTreg adoption treatment improved Th1/Th2 balance. Mouse peripheral lymphocytes were cultured with 1 × Cell Stimulation Cocktail for 6–10 h. Cultures were harvested, anti-CD4 FITC surface stained, fixed and permeabilized with IC Fixation Buffer and Permeabilization Buffer, anti-IFN-γ PE and anti-IL-4 APC intracellular staining. **(A)** Th1 cells ($CD4^+IFN\text{-}\gamma^+$) and **(B)** Th2 cells ($CD4^+IL\text{-}4^+$) in the lymphocyte gate were determined by flow cytometry. **(C)** Statistical analysis (n = 5–7), iTreg and P-iTreg adoption treatment can significantly reduce Th1 and Th2 cells in asthma mice. In particular, Th2 cells effectively improve the Th1/Th2 balance. Data are means ± SEMs for 2 mice per group for each of 4 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 1
Percentage of CD4 subsets in different treatment groups (n = 5–7).

Group	Normal	Asthma	+PBS	+iTreg	+P-iTreg
Th1	0.96 ± 0.07**	1.55 ± 0.31	1.39 ± 0.28	0.99 ± 0.13**	0.98 ± 0.39**
Th2	0.18 ± 0.07**	0.49 ± 0.08	0.63 ± 0.33	0.22 ± 0.07**	0.20 ± 0.04**
Th1/Th2	5.84 ± 2.48*	3.19 ± 0.51	2.77 ± 1.82	5.04 ± 1.93	4.79 ± 1.37
Th17	0.21 ± 0.08**	0.49 ± 0.18	0.54 ± 0.21	0.21 ± 0.08**	0.21 ± 0.11**
Treg	1.52 ± 0.33	1.30 ± 0.25	1.33 ± 0.65	1.77 ± 0.53*	1.75 ± 0.26*
Th17/Treg	0.14 ± 0.06	0.40 ± 0.21#	0.56 ± 0.53#	0.12 ± 0.04	0.13 ± 0.08

Note: Data are presented as means ± SEM (n = 5–7 mice/per group). *p < 0.05 and **p < 0.01 compared to the asthma group or PBS adoptive group. #p < 0.05 compared to the iTreg adoptive group or P-iTreg adoptive transfer group.

inflammatory cell infiltration and IgE production in asthmatic mice, iTregs with or without rhPLD2 treatment (2×10^6 per mouse) were adoptively transferred into asthmatic mice one week after aerosol challenge with 1% OVA. Compared to asthmatic mice without adoptive transfer or with PBS injection, the mice receiving iTreg and P-iTreg cells revealed diminished clinical symptoms. In 14 days after sensitization, different groups mice bodyweight began to appear in difference, asthma group and PBS adoptive group appeared weight loss, while in iTreg adoptive group and P-iTreg adoptive transfer group their body weight changes were consistent with normal group that showed a slight rise. Especially the P-iTreg adoption group and normal group to maintain consistent trend. Although after some fluctuations, the overall difference is not significant (Fig. 6B). Histological analysis of lung tissues revealed a decrease in the infiltration of inflammatory cells and thickness of the airway wall in mice receiving iTreg and P-iTreg cells (Fig. 6C). The PAS staining also revealed a marked reduction of airway epithelial cell hyperplasia and mucus secretion in mice adoptively transferred with iTreg and P-iTreg cells (Fig. 6D).

3.5. The P-iTreg adoptive transfer corrects the imbalance of Th1/Th2 and Th17/Treg

The abnormal cellular immunity, including an enhanced Th2 and Th17 response, has been found in asthmatic inflammation and has been considered as a key mediator in the development of asthma (Awasthi and Kuchroo, 2009). In particular, Th17 cells enhance both neutrophilic and eosinophilic airway inflammation in the mouse model of asthma (Wilson et al., 2009). We thus examined whether the adoptive transfer of iTregs or P-iTregs attenuated asthmatic inflammation *via* reducing Th2 and/or Th17 cell numbers. As shown in Fig. 7, the percentages of

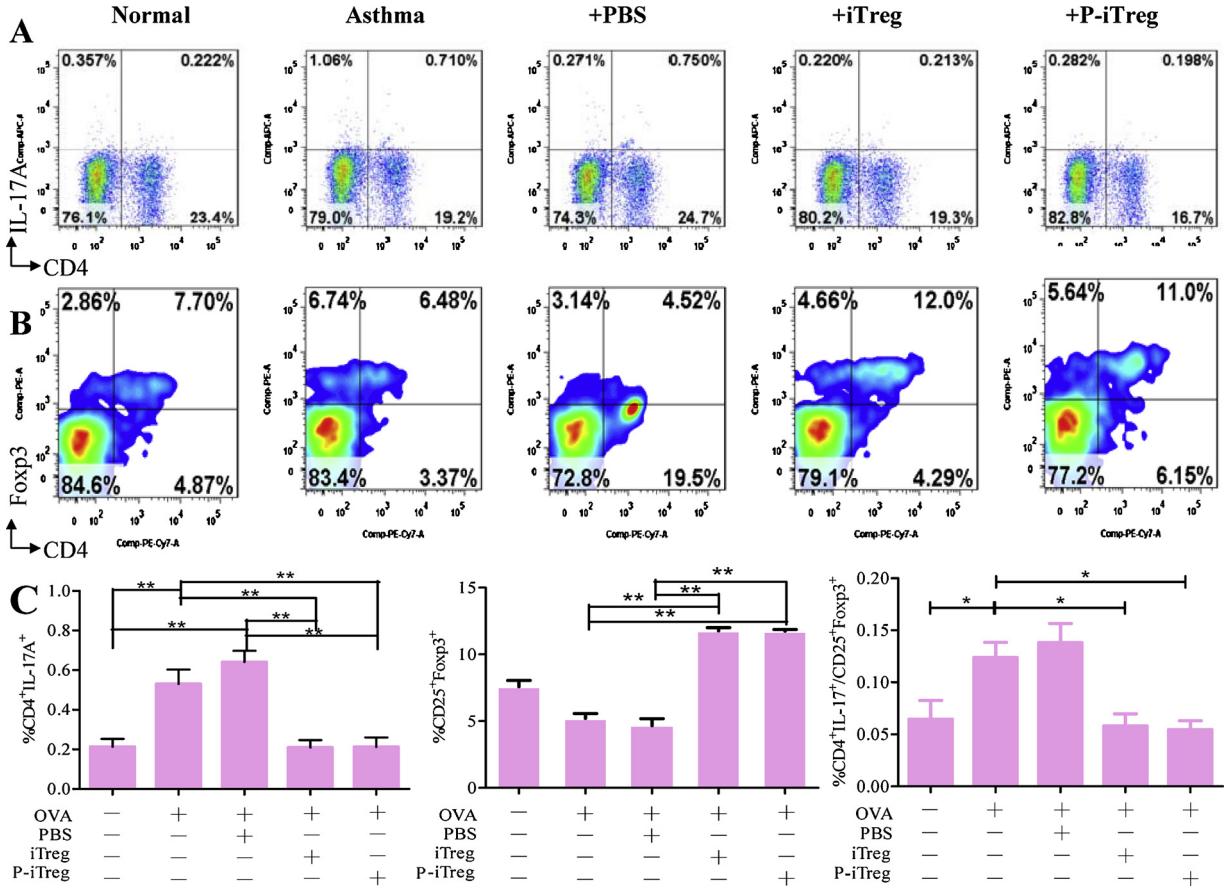


Fig. 8. Effects of adoptive transfer of P-iTregs on Th17/Treg balances. 1×10^6/mLOVA-specific $CD4^+CD25^+Foxp3^+$T cells from *in vitro* cultures were adoptively transferred into OVA-sensitized mice after the day of the third sensitization.7 days later, aerosol challenge with 1% OVA for 5 consecutive days, 30 min a day. In preparations of single-cell suspensions of spleen and lymph node, we detected the percentage of Th17 and $CD25^+Foxp3^+$Treg in every group by flow cytometry (n = 5–7). Adoptive transfer of P-iTregs can significantly reduce the proportion of Th17and increase Treg cells in the peripheral lymphoid organs of asmatic mice correcting Th17/Treg imbalances. **(A)** Flow cytometry analysis of the percentage of Th17 ($CD4^+IL\text{-}17A^+$) and **(B)** Treg ($CD4^+CD25^+Foxp3^+$) cells. **(C)** ANOVA analysis showed that the P-iTreg treated group as with the iTreg treated group were significantly different compared to the with the untreated asthma group ($p < 0.05$); but PBS treated group had no significant difference with asthma group. Statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$.

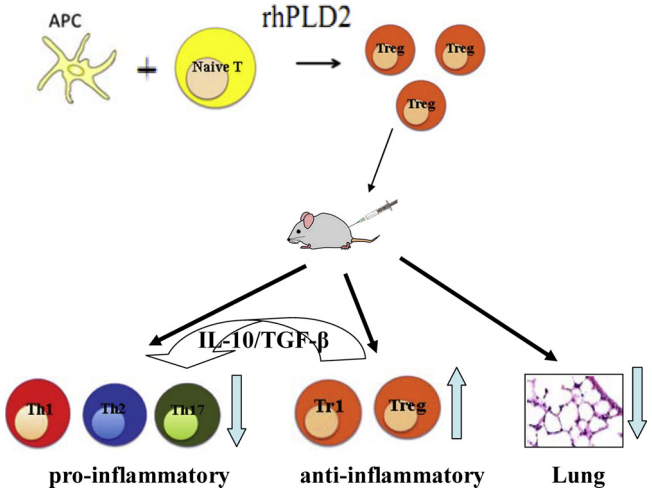


Fig. 9. Adoptive transfer of P-iTreg cells improved the imbalance of immunity. In the last 24 h murines were atomized excited, collected mouse spleen lymph nodes, and detected expression of $CD4^+$T cell subsets in peripheral lymphoid tissues. Th1/Th2/Th17 cell expression was increased in asthmatic mice, which is associated with Th1 and Th17 mediated inflammatory response. These results are consistent with asthmatic mice in which the typical Th2 cells response is reflected in eosinophilic inflammation. P-iTreg adoptive therapy restored the normal balance of immunity is in asmatic mice.

both Th1 ($CD4^+IFN\text{-}\gamma^+$) and Th2 ($CD4^+IL\text{-}4^+$) cells were increased in the spleen and lung draining lymph nodes of asthmatic mice when compared to PBS controls. The adoptive transfer of iTregs and P-iTregs significantly decreased the ratio of Th2 and Th1 cells in asthmatic mice to the levels similar to that in control mice without asthmatic induction. The ratio of Th1/Th2 in iTreg-treated asthmatic mice was similar to the control mice without asthma that was higher than non-treated asthmatic mice (Table 1). Moreover, the mice that received iTreg and P-iTreg cells had significantly less Th17 ($CD4^+IL\text{-}17^+$) cells in the spleen and lung draining lymph nodes when compared to non-treated asthmatic mice ($p < 0.01$) (Fig. 8A). In addition, in asthmatic mice that received iTreg or P-iTreg cells, about 2-fold increase in Treg cells ($CD4^+CD25^+Foxp3^+$) was detected in the draining lymph nodes and the spleen ($p < 0.01$, Fig. 8B). Moreover, Th17/Treg ratio was found in Treg-treated asthmatic mice lower than asthmatic mice and PBS-treated asthmatic mice, near to non-asthmatic control mice (Fig. 8C).

4. Discussion

It is found the TSDR methylation level of the induced Treg cells by rhPLD2 is significantly lower than by TGF-beta or by 5-Aza. The demethylation on the CpG162 site by rhPLD2 ranks the top, which has never been previously reported. Even though, the expression of Foxp3 protein in $CD4^+CD25^+Foxp3^+$Treg cells induced by rhPLD2 shows higher stability than by TGF-β, which is likely due to stable epigenetic change. Besides, our data shows that adoptive transfer of P-iTregs into

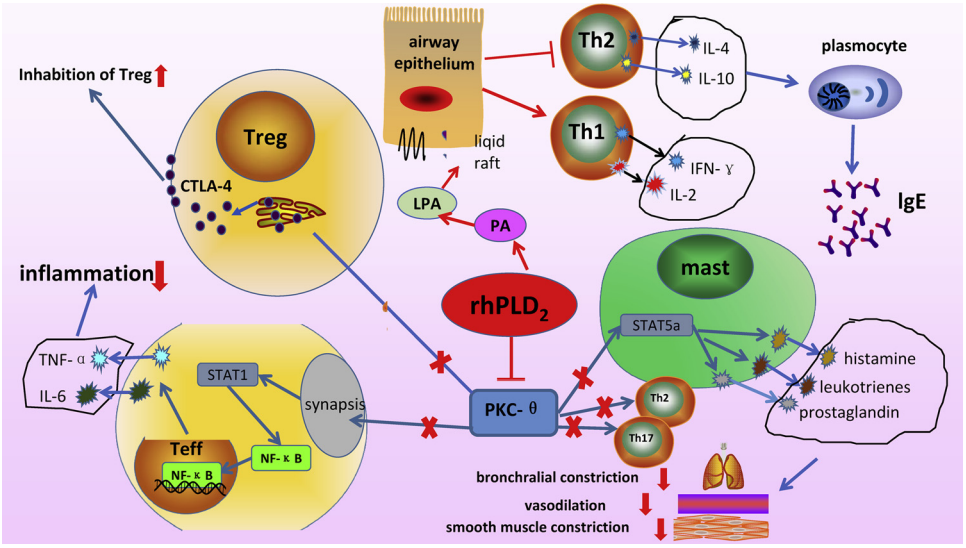


Fig. 10. Possible mechanisms by which rhPLD2 suppresses inflammatory immune-mediated responses in asthmatic airway inflammation. These possibilities include: In the first proposed pathway rhPLD2 corrects immune deviation of Th1/Th2 through its downstream metabolites PLA. Whereas the second pathway may involve regulatory T cells activation of phospholipase D sufficient to trigger release of CTLA-4 to the plasma membrane (PM). In this case it is possible that rhPLD2 may control the CTLA-4 trafficking. The third possibility is that rhPLD2 inhibits the NFκB or PKC signaling pathways. rhPLD2 could block release of inflammatory mediators *via* inhibiting NF-κB or PKC signaling and as a result inhibit STAT signaling, and block recruitment of PKC-θ into the Treg immunological synapse. Thus, PKC-θ would be sequestered away from the Treg immunological synapse and this blockade PKC-θ by rhPLD2 would cause enhanced Treg function.

the asthmatic mice alleviate mucus secretion and inflammation in lung tissue; down-regulated the ratio of Th2/Th1 as well as promotion of Treg function and suppression of Th17 function. These findings demonstrate that rhPLD2 mediates therapeutic intervention of the inflammation in asthmatic mice through rebalancing the $CD4^+CD25^+Foxp3^+$Tregs (Fig. 9). Our data suggest that P-iTreg adoptive therapy can correct the immune imbalance of the effector T cells and promote the inhibitory effect of Treg cells *in vivo*.

There are potentially several mechanisms by which rhPLD2 mediates its immunosuppressive effect on inflammation in asthma. First, PLD generates PA, which is converted to LPA by the action of PLA1/PLA2. Enhanced COX-2 and PGE2 expression by LPA which exhibit anti-inflammatory properties in the airway may have a protective role in airway inflammation and remodeling (Scott et al., 2013; Toews et al., 2002). These results imply that LPA may play an anti-inflammatory role in innate immunity through the secretion of Th-1 type cytokines, while in the airway may induce adaptive immunity by reducing Th-2 cytokine function. A second possible mechanism is that PLD controls CTLA-4 trafficking that influences T cell anergy. More and more evidences indicate that CTLA-4 is critical for maintaining tolerance to self-tissues and Treg development (Chambers et al., 1997; Mead et al., 2005; Zheng et al., 2006). In regulatory T cells, activation of phospholipase D is sufficient to trigger release of CTLA-4 to the plasma membrane (PM) but does not inhibit endocytosis. Taken together, these data suggest that CTLA-4 may be stored in a specialized compartment in regulatory T cells that can be triggered rapidly for deployment to the PM in a phospholipase D and ADP ribosylation factor-1-dependent manner. Thus our future investigations will need to determine the mechanisms of the role of rhPLD2 on the control of CTLA-4 trafficking. A third possibility is that rhPLD2 reduces the ability of effector T cells to produce inflammatory factors by inhibiting NFκB or PKC signaling pathways.

Because protein kinase C-θ (PKC-θ) recruitment to the immunological synapse is required for full effector T cell (Teff) activation, such as Th2 and Th17 cells both in *vitro* and in *vivo* (Kwon et al., 2010). In contrast, PKC-θ was sequestered away from the Treg immunological synapse. Furthermore, PKC-θ blockade enhanced Treg function. Thus, inhibition of PKC-θ in Tregs may be a valuable component in Treg adoptive immunotherapy to treat autoimmunity and graft *versus* host disease (Alexandra et al., 2010). And our early research data clearly demonstrated that intraperitoneal administration of rhPLD2 can suppress the p65 activity [9]. At the same time, we found that inflammation increased PKC in airway smooth muscle, increased STAT1 and STAT5a activation in the lung tissue. rhPLD2 could block those changes *via* inhibit STAT signaling by inhibiting PKC signaling. The results also indicate a relationship among PKC, STAT1, STAT5a, and rhPLD2 in airway inflammation in asthma (Ling et al., 2008) (Fig. 10).

5. Conclusions

In summary, this study describes the initial immunological characterization of rhPLD2 which is a fusion protein. we show that rhPLD2 intervention in asthmatic mice can significantly increase the $CD4^+CD25^+Foxp3^+$Treg expression *in vitro*, and induced $CD4^+CD25^+Foxp3^+$ regulatory T by rhPLD2 (P-iTreg) adoptive therapy can reduce asthma inflammation *in vivo*. As yet, no single model of rhPLD2 function has completely emerged, and it is possible that more than one mechanism exists. The data shown here underlies that rhPLD2 can mediate therapeutic intervention of the inflammation associated with asthma in mice through the induction of stable $CD25^+Foxp3^+$ Tregs, suggesting that rhPLD2 is a promising alternative therapeutic approach for treating allergic asthma inflammation.

Conflict of interest

None.

Acknowledgments

*Project supported by National Natural Science Foundation of China, No. 81671562; Fujian Science and Technology Foundation, No. 2009I0008; and supported by Natural Science Foundation of Fujian, No. 2010J01170; and supported by Fujian Medical University Professor Foundation, No. JS06006.

References

Adam, Mor, Campi, Gabriele, Du, Guangwei, Zheng, Yang, Foster, David A., Dustin, Michael L., Philips, Mark R., 2007. The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane ras via phospholipase D2. Nat. Cell Biol. 9, 713–719.

Afshar, R., Medoff, B.D., Luster, A.D., 2008. Allergic asthma: a tale of many T cells. Clin. Exp. Allergy 38, 1847–1857.

Alexandra, Zanin-Zhorov, Ding, Yi, Kumari, Sudha, Attur, Mukundan, Hippen, Keli L., Brown, Maryanne, Blazar, Bruce R., Abramson, Steven B., Lafaille, Juan J., Dustin, MichaelL., 2010. Protein kinase C-θ mediates negative feedback on regulatory T cell function. Science 328, 372–376.

Altman, A., Villalba, M., 2002. Protein kinase C-theta (PKC theta): a key enzyme in T cell life and death. J. Biochem. 132, 841–846.

Amit, Agarwal, Singh, Meenu, Chatterjee, B.P., Chauhan, Anil, Chakraborti, Anuradha, 2014. Interplay of T helper 17 cells with $CD4^+CD25^{high}$ $FOXP3^+$Tregs in regulation of allergic asthma in pediatric patients. Int. J. Pediatr. 1–8.
Awasthi, A., Kuchroo, V.K., 2009. Th17 cells: from precursors to players in inflammation and infection. Int. Immunol. 21, 489–498.
Baraldo, S., Oliani, K.L., Turato, G., Zuin, R., Saetta, M., 2007. The role of lymphocytes in the pathogenesis of asthma and COPD. Curr. Med. Chem. 14, 2250–2256.
Chambers, C.A., Sullivan, T.J., Allison, J.P., 1997. Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of CD4 cells. Immunity 7, 885.
Chand, S., Mehta, N., Bahia, M.S., Dixit, A., Silakari, O., 2012. Protein kinase C-theta inhibitors: a novel therapy for inflammatory disorders. Curr. Pharm. Des. 18, 4725–4746.
Cohn, L., Elias, J.A., Chupp, G.L., 2004. Asthma: mechanisms of disease persistence and progression. Annu. Rev. Immunol. 22, 789–815.
Dias, P.M., Banerjee, G., 2013. The role of Th17/IL-17 on eosinophilic inflammation. J. Autoimmun. 40, 9–20.
Gavett, S.H., Chen, X., Finkelman, F., Wills-Karp, M., 1994. Depletion of murine CD4+T lymphocytes prevents antigen-induced airway hyper reactivity and pulmonary eosinophilia. Am. J. Respir. Cell Mol. Biol. 10, 587–593.
Gomez-Cambronero, J., Di Fulvio, M., Knapek, K., 2007. Understanding phospholipase D (PLD) using leukocytes: PLD involvement in cell adhesion and chemotaxis. J. Leukoc. Biol. 82, 272–281.
Hamdi, SafouaneM., Cariven, Clotilde, Coronas, Sophie, Malet, Nicole, Chap, Hugues, Perret, Bertrand, Salles, Jean-Pierre, Record, Michel, 2008. Potential role of phospholipase D2 in increasing interleukin-2 production by T-lymphocytes through activation of mitogen-activated protein kinases ERK1/ERK2. Biochim. Biophys. Acta 1781, 263–269.
Hizawa, N., Yamaguchi, E., Jinushi, E., Konno, S., Kawakami, Y., Nishimura, Masaharu, 2001. Increased total serum IgE levels in patients with asthma and promoter polymorphisms at CTLA4 and FCER1B. J. Allergy Clin. Immunol. 108, 74–79.
Jan, Kehrmann, Tatura, Roman, Zeschnigk, Michael, Probst-Kepper, Michael, Geffers, Robert, Steinmann, Joerg, Buer, Jan, 2014. Impact of 5-aza-2′-deoxycytidine and epigallocatechin-3-gallate for induction of human regulatory T cells. Immunology 3 (142), 384–395.
Kearley, J., Robinson, D.S., Lloyd, C.M., 2008. $CD4^+CD25^+$ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. J. Allergy Clin. Immunol. 122 (e6), 617–624.
Kim, H.Y., Dekruyff, R.H., Umetsu, D.T., 2010. The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat. Immunol. 11, 577–584.
Kong, N., Lan, Q., Chen, M., Wang, J., Shi, W., Horwitz, D.A., Quesniaux, V., Ryffel, B., Liu, Z., Brand, D., Zou, H., Zheng, S.G., 2012. Antigen-specific TGF-β-induced regulatory T cells but not natural Tregs ameliorate autoimmune arthritis by shifting the balance of Th17 toward Treg cells. Arthritis Rheum. 64 (8), 2548–2558.
Kudo, M., Melton, A.C., Chen, C., Engler, M.B., Huang, K.E., Ren, X., Wang, Y., Bernstein, X., Li, J.T., Atabai, K., Huang, X., Sheppard, D., 2012. IL-17A produced by alpha beta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat. Med. 18, 547–554.
Kwon, Myung-Ja, Wang, Ruiqing, Ma, Jian, Sun, Zuoming, 2010. PKC-θ is a drug target for prevention of T cell-mediated autoimmunity and allograft rejection. Endocr. Metab. Immune Disord. Drug Targets 10 (2010), 367–372.
Lawless, Oliver J., Bellanti, Joseph A., Brown, Milton L., Sandberg, Kathryn, Umans, Jason G., Zhou, Li, Chen, Weiqian, Wang, Julie, Wang, Kan, Zheng, SongGuo, 2018. In vitro induction of T regulatory cells by a methylated CpG DNA sequence in humans: potential therapeutic applications in allergic and autoimmune diseases. Allergy Asthma Proc. 39 (2), 143–152.
Ling, Z., Jian-Feng, X., Chuan-Xing, Y., Don-Hui, S., Wei-Da, H., Huang, Ru., 2003. Analysis of cDNA and protein sequence of rhPLD2 mutation form. J. Chin. Biotechnol. 23, 80–84.
Ling, Zhu, Zou, Weibin, Yu, Chuanxing, Lin, Junjin, He, Xiaoli, 2006. Human recombinant PLD2(rhPLD2) *in vivo* can significantly repress the p65 activity in chronic asthma of guinea pigs. Cell. Mol. Immunol. 3, 307–310.
Ling, Zhu, Wang, Ya-Xiong, Yu, Chuan-Xing, 2008. Effects of recombinant human PLD2 on proliferation and apoptosis of HL-60 cells. Cell Biol. Int. 32, 253–263.
Lloyd, C.M., Hessel, E.M., 2010. Functions of T cells in asthma: more than just T(H)2 cells. Nat. Rev. Immunol. 10, 838–848.
Mead, K.I., Zheng, Y., Manzotti, C.N., Perry, L.C., Liu, M.K., Burke, F., Powner, D.J., Wakelam, M.J., Sansom, D.M., 2005. Exocytosis of CTLA-4 is dependent on phospholipase D and ADP ribosylation factor-1 and stimulated during activation of regulatory T cells. J. Immunol. 15, 4803–4811.
Meng, X., Zhang, Y., Jia, Z., Huo, X., He, X., Tian, G., Wu, M., Wang, Z., Zhou, X., Xiong, S., Gao, X., Wu, Z., Han, J., Zhao, L., Wang, P., Hong, Z., Wang, Q., Yin, Z., 2013. A novel tylophorine analog w-8 up-regulates forkhead boxp3 expression and ameliorates murine colitis. J. Leukoc. Biol. 93, 83–93.
Morishima, Y., Ano, S., Ishii, Y., Ohtsuka, S., Matsuyama, M., Kawaguchi, M., Hizawa, N., 2013. Th17 associated cytokines as a therapeutic target for steroid insensitive asthma. Clin. Dev. Immunol. 2013, 6093–6095.
Ohkura, N., Kitagawa, Y., Sakaguchi, S., 2013. Development and maintenance of regulatory T cells. Immunity 38, 414–423.
Oukka, M., 2008. Th17 cells in immunity and autoimmunity. Ann. Rheum. Dis. 67, 26–29.
Sakaguchi, S., 2004. Naturally arising $CD4^+$regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol. 22, 531–562.
Scott, Sarah A., Xiang, Yun, Mathews, Thomas P., Cho, HyekyungP., Myers, David S., Armstrong, Michelle D., Tallman, Keri A., O'Reilly, Matthew C., Lindsley, Craig W., Brown, H.Alex, 2013. Regulation of phospholipase D activity and phosphatidic acid production after purinergic (P2Y6) receptor stimulation. J. Biol. Chem. 12, 20477–20487.
Singer, B.D., Mock, J.R., Aggarwal, N.R., Garibaldi, B.T., Sidhaye, V.K., Florez, M.A., Chau, E., Gibbs, K.W., Mandke, P., Tripathi, A., Yegnasubramanian, S., King, L.S., D'Alessio, F.R., 2015. Regulatory T cell DNA methyltransferase inhibition accelerates resolution of lung inflammation. Am. J. Respir. Cell Mol. Biol. 52 (5), 641–652.
Stefan, Floess, Freyer, Jennifer, Siewert, Christiane, Baron, Udo, Olek, Sven, Polansky, Julia, Schlawe, Kerstin, Chang, Hyun-Dong, Bopp, Tobias, Schmitt, Edgar, Klein-Hessling, Stefan, Serfling, Edgar, Hamann, Alf, Huehn, Jochen, 2007. Epigenetic control of the Foxp3 locus in regulatory T cells. PLoS Biol. 5, e38.
Su, W., Fan, H.M., Chen, M.G., Wang, J., Brand, D.D., He, X., Quesniaux, V., Ryffel, B., Zhu, L., Liang, D., Zheng, S.G., 2012. Induced CD4 forkhead box protein–positive T cells inhibit mast cell function and established contact hypersensitivity through TGF-b1. J. Allergy Clin. Immunol. 130 (2), 444–452.
Takashi, Kinoshita, Baatjes, Adrian, Smith, Steven G., Dua, Benny, Watson, Richard, Kawayama, Tomotaka, Larche, Mark, Gauvreau, Gail M., O'Byrne, Paul M., FRCPI, 2014. Natural regulatory T cells in isolated early responders compared with dual responders with allergic asthma. J. Allergy Clin. Immunol. 133, 696–703.
Toews, M.L., Ediger, T.L., Romberger, D.J., Rennard, S.I., 2002. Lysophosphatidic acid in airway function and disease. Biochim. Biophys. Acta 1582, 240–250.
Wilson, R.H., Whitehead, G.S., Nakano, H., Free, M.E., Kolls, J.K., Cook, D.N., 2009. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. Am. J. Respir. Crit. Care Med. 180, 720–730.
Wu, C., Thalhamer, T., Franca, R.F., Xiao, S., Wang, C., Hotta, C., Zhu, C., Hirashima, M., Anderson, A.C., Kuchroo, V.K., 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. Immunity 41, 270–282.
Xu, Wei, Lan, Qin, Chen, Maogen, Chen, Hui, Zhu, Ning, Zhou, Xiaohui, Wang, Julie, Fan, Huimin, Yan, Chun-Song, Kuang, Jiu-Long, Warburton, David, Togbe, Dieudonnée, Ryffel, Bernhard, Zheng, Song-Guo, Shi, Wei, 2012. Adoptive transfer of induced-Treg cells effectively attenuates murine airway allergic inflammation. PLoS One 7 (7), e40314.
Zeng, H., Chi, H., 2015. Metabolic control of regulatory T cell development and function. Trends Immunol. 36, 3–12.
Zhang, Xiaolong, Chang Li, Xian, Xiao, Xiang, Sun, Rui, Tian, Zhigang, Wei, Haiming, 2013. $CD4^+CD62L^+$ central memory T cells can be converted to $Foxp3^+$ T cells. PLoS One 8, 1–12 e77322.
Zheng, S.G., Gray, J.D., Ohtsuka, K., Yamagiwa, S., Horwitz, D.A., 2002. Generation ex vivo of TGF-β producing regulatory T cells from $CD4^+CD25^-$ precursors. J. Immunol. 169 (8), 4183–4189.
Zheng, S.G., Wang, J.H., Gray, J.D., Soucier, H., Horwitz, D.A., 2004. Natural and induced $CD4^+CD25^+$ cells educate $CD4^+CD25^-$ cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J. Immunol. 172 (9), 5213–5221.
Zheng, S.G., Wang, J.H., Stohl, W., Kim, K.S., Gray, J.D., Horwitz, D.A., 2006. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive $CD4^+CD25^+$ regulatory cells. J. Immunol. 176 (March (6)), 3321–3329.
Zheng, S.G., Wang, J., Wang, P., Gray, J.D., Horwitz, D.A., 2007. IL-2 is essential for TGF-beta to convert naive $CD4^+CD25^-$ cells to $CD25^+Foxp3^+$ regulatory T cells and for expansion of these cells. J. Immunol. 178, 2018–2027.
Zheng, S.G., Wang, J., Horwitz, D.A., 2008. Cutting edge: $Foxp3^+CD4^+CD25^+$ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. J. Immunol. 180 (11), 7112–7116.
Zhou, X., Kong, N., Wang, J., Fan, H., Zou, H., Horwitz, D., Brand, D., Liu, Z., Zheng, S.G., 2010a. Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. J. Immunol. 185, 2675–2679.
Zhou, X., Wang, J., Shi, W., Brand, D.D., Liu, Z., Fan, H., Zheng, S.G., 2010b. Isolation of purified and live $Foxp3^+$ regulatory T cells using FACS sorting on scatter plot. J. Mol. Cell Biol. 2, 164–169.