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

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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)

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(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).

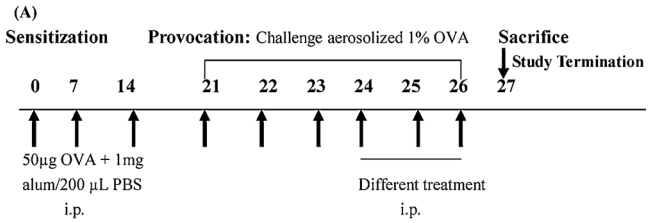
Increasing evidence has shown that asthmatic inflammation is related to aberrant cellular immunity, such as imbalanced Th1 to Th2 population, dysfunction of Th17 cells, or defective Treg cells (Dias and Banerjee, 2013; Ohkura et al., 2013; Kudo et al., 2012; Kearley et al., 2008; Kong et al., 2012). 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.