Yang J *et al* / Acta Pharmacol Sin 2004 Dec; 25 (12): 1666-1670

Interleukin-12 was not involved in promotion of T helper cell differentiation induced by theophylline

Jiong YANG[1], Wei GUO, Han-xiang NIE, Su-ping HU

Department of Respiratory Disease, Renmin Hospital, Wuhan University, Wuhan 430060, China

KEY WORDS asthma; theophylline; helper inducer T lymphocytes; Th1 cells; Th2 cells; interleukin-12

ABSTRACT

AIM: To investigate the effect of theophylline on the naive T cell differentiation and the probable role of interleukin-12 (IL-12). **METHODS**: Naive cord blood T cells were treated with theophylline 10 mg/L for 3 d after stimulation with PHA 100 mg/L. Differentiation of T cells was analyzed by flow cytometry. Theophylline 10 mg/L and IL-12-mAb 0.025 mg/L were added in cord blood mononuclear cell (CBMC) cultures primed with LPS 1 mg/L to detect the levels of IL-12 and IL-12P40. The whole blood cultures were obtained from twelve health volunteers with or without administration of theophylline (200 mg). Cytokines were measured by enzyme linked immuno-sorbent assay. **RESULTS**: Theophylline promoted T helper 1 (Th_1) cells differentiation from naive T cells (21.9 %±10.3 % *vs* 9.4 %±5.6 %, $P<0.05$), but had no significant effect on Th_2 deviation. But theophylline inhibited the production of IL-12 and IL-12P40 by CBMC *in vitro* (28±6 ng/L *vs* 57±14 ng/L and 88±34 ng/L *vs* 214±82 ng/L, $P<0.01$) and reduced IL-12 and IL-12P40 levels in whole blood cultures from healthy subjects (19±11 ng/L *vs* 31±15 ng/L and 92±13 ng/L *vs* 196±49 ng/L, $P<0.01$). **CONCLUSION:** Theophylline promoted the differentiation of Th_1 cells. IL-12 seemed not to be involved in this process.

INTRODUCTION

Theophylline has been a popular medication for asthma for over 50 years. Though it has traditionally been classified as a bronchodilator, which owes to its inhibiting phosphodiesterase (PDE) enzyme effect, theophylline is now believed to have more actions on asthma including anti-inflammatory actions[1,2]. In previous researches, theophylline down-regulated the function of inflammatory and immune cells *in vitro* and *in vivo* whether in animals with airway inflammation[3,4] or in human with asthma[5,6]. However the mechanism of its anti-inflammatory effect is not clear.

In asthma, more and more cells secreting T helper (Th) 2 cytokines in lungs and in blood was found. Th2 cytokines such as IL-4, IL-5, and IL-13 played an important roles in asthma[7]. The rise of IL-4/ IFN-γ ratio would lead to aberrant IgE production in response to both aeroallergens and sensitizations. Since previous researches found that theophylline reduced the eosinophils airway infiltration[5], decreased the numbers of epithelial T cells containing IL-4 and IL-5, and down-regulated serum and airway IL-4 and IL-5 production in asthma patients[8,9], it was hypothesized that regulation of the differentiation of $CD4^+$ T cells might underly the anti-inflammatory activities of theophylline. In this study, we investigated whether theophylline affected naive T cell differentiation *in vitro* and *in vivo* and

[1] Correspondence to Prof Jiong YANG.
Phn 86-27-8804-1919, ext 2215. Fax 86-27-8807-3047.
E-mail WLSCC@public.wh.hb.cn
Received 2004-03-10 Accepted 2004-06-29

whether IL-12 was involved in this process.

MATERIALS AND METHODS

Cell isolation Cord blood mononuclear cells (CBMC) were obtained by centrifugation over lymphocyte separation medium (Shanghai Second Reagent Plant, China), and then washed three times in Hanks' solution. Isolated CBMCs were either used immediately or preserved in RPMI-1640 (Gibco) with 1 % heat-inactivated fetal calf serum (FCS, Gibco) for the next isolation.

Cord blood adherent mononuclear cells (CBAMC) were isolated by incubating CBMCs overnight in 75-cm^2 culture flask at 37 ºC, followed by removal of nonadherent cells by three washes in Hanks' solution[10]. CBAMCs were preserved in RPMI-1640/1 % FCS at 37 ºC in a 5 % CO_2 incubator until use, usually within 1 h of purification. These populations typically consisted of a mixture of about 80 % monocytes and a smaller number of B cells, few if any T cells remained in these preparations.

$CD4^+$ T cells were isolated from CBMCs by an immunomagnetic method according to the manufacture's instruction. In brief, CBMCs were incubated at 4 ºC for 30 min with mouse anti-human-CD4 antibody (PharMingen), and then cells were washed and incubated at 4 ºC for another 15 min with human anti-mouse IgG-coated magnetic beads (Dynal Biotech, Norway). Positive cells were separated in magnetic particle concentrator (Dynal MPC, Dynal, Norway) and washed thrice by RPMI-1640/1 % FCS. Naive T cells ($CD4^+CD45RA^+$ T cells) were obtained by secondly positive selection using mouse anti-human CD45RA antibody (PharMingen) as described above. After washing thrice by RPMI-1640 /1 % FCS, the purity of the naïve T cells were verified as over 98 % by FACS Calibur (Becton Dickinson, American).

***In vitro* differentiation of human naive T cells** The differentiation assay was designed according to the description[10,11]. Tissue culture 24-well plates were seeded with 1×10^5 CBAMCs and 1×10^6 purified naive T cells. The cultures were in the final volume of 1 mL of RPMI-1640/10 % FCS and were further supplemented with PHA (Alpha Biotech Company, China) at the concentration of 200 mg/L. Either theophylline 10 mg/L or culture medium of the same volume was added into the culture. The concentration of PHA was experimented in advance as the most proper one to assure T cell activation, and theophylline 10 mg/L was the most likely to prevent symptoms and decrease the need for rescue therapy[1]. Seventy-two hours later, cells were washed and put back in culture in the presence of IL-2 (2×10^5 U/L, Gene Drug LTD, Changchun). After being rested for another 72 h, T cells were collected and restimulated with PMA, ionomycin, and monensin of indicated concentrations under the manufacture's guide (CYTODETECT Kit, IQ company, Netherlands) . Then cells were fixed with paraformaldehyde and permeabilized with saponin (contained in the CYTODE-TECT Kit). Fixed T cells were then incubated at 4 ºC for 30 min with a pair of antibodies: FITC-conjugated anti-IFN-γ mAb and PE-conjugated anti-IL-4 mAb (IQ company). The proportion of 1 μL of antibody to 1×10^6 cells were chosen to optimize detection based on preliminary experiments. After washes twice with permeabilization buffer and wash once with staining buffer, the cells were resuspended in staining buffer and subjected to two-color FACSCalibur analysis.

Cell culture The neonatal cord blood of thirty donors was randomly designated into three groups. CBMCs were obtained as described above and resuspended at 1×10^9/L in RPMI-1640/10 % FCS. The cultures were stimulated with IFN-γ 1×10^6 U/L (Peprotech, American) and LPS 1 mg/L (Sigma). Then the cells were treated with theophylline 10 mg/L (Alexis, American), IL-12 mAb 0.025 mg/L (Peprotech, American), and culture medium of the same volume respectively. Cultures were incubated at 37 ºC for 24 h in a 5 % CO_2 incubator.

***In vivo* administration of theophylline** Twelve nonsmoking healthy volunteers had no history of asthma, allergic rhinitis, or atopic dermatitis. Six subjects (26.2± 2.4 a and 55.9±6.4 kg) received theophylline tablets (200 mg, Maite-Xinghua Pharmaceutical Factory, Guangzhou), and six subjects (25.8±2.1 a and 57.6±7.3 kg) received no treatment and served as controls. Blood samples were obtained before and at 6 h after treatment when theophylline concentration in plasma reached maximum of about 7.9 mg/L[8]. Whole blood was seeded in 24-well plates and stimulated with IFN-γ 1×10^6 U/L and LPS 1 mg/L for 24 h.

Cytokine detection After being incubated for 24 h, the supernatant of the *in vitro* culture and of the whole blood culture was harvested. IL-12 and IL-12-P40 were measured by enzyme linked immunosorbent assay (Gemyme, separated by Jingmei Biotech) according to the manufacture's instructions.

Ethnics All *in vivo* experiments were permitted by Renmin Hospital, Wuhan University and consented by all volunteers.

Statistic analysis Data were expressed as mean±SD. The paired *t*-test was used for the comparison of the naive $CD4^+$ T cell differentiation. The one-way ANOVA was applied for the comparison of IL-12 and IL-12P40 levels in *in vitro* culture. The classic *t*-test was used for the comparison of cytokines levels of whole blood culture. $P<0.05$ was considered significant.

RESULTS

Theophylline promoted the differentiation of Th_1 cells from naive T cells Neonatal $CD4^+$ T cells preferentially developed into IFN-γ-secreting cells after stimulation with PHA and a small population of Th_2 cells that produced only IL-4 not IFN-γ. After the addition of theophylline to the cell culture, a marked promotion of the development of IFN-γ producing cells was observed ($P<0.05$). On the other hand, there was no significant change of the population of Th_2 cells in the presence of theophylline (Tab 1, Fig 1).

Tab 1. T cell differentiation *in vitro* after stimulation with PHA 100 mg/L in the presence of theophylline 10 mg/L. *n*=4. Mean±SD. $^bP<0.05$ *vs* control group.

	Control group	Theophylline group
Th1 (IFN-γ)/%	9.4±5.6	21.9±10.3[b]
Th2 (IL-4)/%	1.0±0.8	0.8±0.9

Theophylline down-regulated IL-12 and IL-12P40 production in CBMCs *in vitro* Theophylline inhibited production of IL-12 and IL-12P40 compared with control group (both $P<0.01$, Tab 2). In contrast, IL-12 level in theophlline group was even significantly lower than that in IL-12 mAb group ($P<0.05$, Tab 2).

Tab 2. Effect of IL-12 mAb 0.025 mg/L and theophylline 10 mg/L on IL-12 and IL-12P40 production in CBMC cultures after incubation with IFN-γ 1×10^6 U/L and LPS 1 mg/L. Mean±SD. $^cP<0.01$ *vs* control group. $^eP<0.05$, $^fP<0.01$ *vs* IL-12 mAb group.

	Control group (*n*=10)	IL-12 mAb group (*n*=9)	Theophylline group (*n*=11)
IL-12 level/ng·L^{-1}	57±14	30±8[c]	28±6[ce]
IL-12P40 level/ng·L^{-1}	214±82	27±6[c]	88±34[cf]

Effect of theophylline on IL-12 production of whole blood culture after stimulation with LPS *in vivo* There was no significant difference in IL-12 and IL-12P40 levels of whole blood cultures between control group and pre-theophylline-treated group. Theophylline decreased IL-12 and IL-12P40 production compared with control and pre-administration group (both $P<0.01$, Tab 3).

DISCUSSION

Our results showed that stimulation with PHA, a

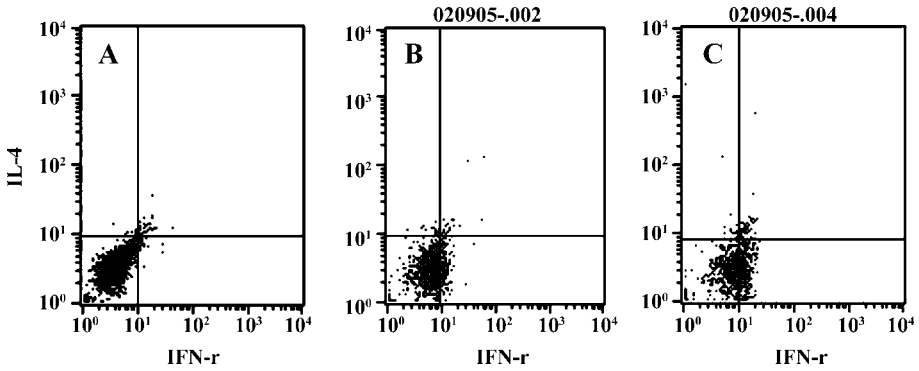

Fig 1. FACS analysis of cell differentiation. (A) Operational control. (B) Cord blood- purified $CD4^+CD45RA^+$ T cells after stimulation with PHA 200 mg/L in control medium. The population of cells producing IFN-γ was 16.6 %, while the population of cells producing IL-4 was 1.58 %. (C) Cord blood-purified $CD4^+CD45RA^+$ T cells in the presence of theophylline 10 mg/L after stimulation with PHA 100 mg/L. The proportion of IFN-γ producing cells was promoted to 33.4 %, while the proportion of IL-4 producing cells was 2.01 %. One experiment out of four was present.

Tab 3. Effect of theophylline 10 mg/L on IL-12 and IL-12P40 production of whole blood cultures after stimulation with IFN-γ 1×10^6 U/L and LPS 1 mg/L for 24 h. *n*=6. Mean±SD. ^cP <0.01 *vs* control group. ^rP<0.01 *vs* pre-administration group.

	Control group	Theophylline group	
		Before	After
IL-12/ng·L^{-1}	32±15	32±16	19±11^{cf}
IL-12P40/ng·L^{-1}	196±49	204±25	92±13^{cf}

substitute of antigen, could drive naive T cells to differentiate to Th_1 cells, which was consistent with the previous report[11]. Promoted Th_1 differentiation was observed but no changes in Th_2 cells. It suggested that theophylline had no apparent effect on Th_2 cell differentiation when it was added in naive T cell culture. However, in our study the mitogen PHA itself initiated very small population of Th_2 cells which could be the explanation for the lower differentiation of Th_2 population in theophylline-treated group.

It is now clear that the imbalance of Th_1/Th_2 subgroups in airway and in blood is closely associated with the inflammation in asthmatic patients. In patients with asthma, more T cells from bronchoalveolar-lavage fluid (BALF) contained m-RNA for IL-3, IL-4, and IL-5 than that from normal subjects[12]. And the same result was obtained in bronchial-biopsy. Th_2 cells up-regulated IgE production, recruited eosinophils, and were prominent in the pathogenesis of allergic diseases, whilst the other subset Th_1 cells secreted IFN-γ and inhibited the synthesis of IgE and the differentiation of precursor cells to Th_2 cells[13]. Just because there was a reciprocal inhibition, that Th1-type cytokines inhibited the production of Th_2-type cytokines and *vice versa*, the predominance of Th_1 cells might inhibit the function of Th_2 cells. So we conferred that theophylline might exert its anti-inflammatory effect by regulating naive T cells to develop toward Th_1 cells and thus inhibiting the function of Th_2 cells though the specific mechanism was unknown.

In vitro studies in both human[14] and murine[15] systems and *in vivo* studies in mice showed that IL-12 promoted Th_1 responses. In this study, theophylline was given *in vivo* at the dose of 200 mg, which was demonstrated to be related with the plasma concentration of nearly 10 mg/L[5] which we choose for the *in vitro* experiment. Theophylline was a potent inhibitor of IL-12 production by mononuclear cells when it was either administered *in vivo* or added into *in vitro* cultures. Theophylline and other PDE inhibitors could inhibit the hydrolysis of cyclic AMP phosphodiesterases and thus elevate intracellular cAMP concentration[16]. And the increased intracellular cAMP level, was correlated with the decrease of IL-12[17]. So this might be the explanation of down-regulation of IL-12 *in vivo* and *in vitro* by theophylline.

In our study theophylline promoted the differentiation of Th_1 cells though it seemed to be the IL-12 inhibitor. We arranged the same condition for control and theophylline group, and engaged autologous adherent cells-CBAMC as antigen presenting cells for providing the second signal[10]. So we suspected that theophylline promoted Th_1 cell differentiation directly or indirectly. There might exist some other pathway to affect the Th cell differentiation independent of IL-12. Th_1 cell differentiation was regulated in several pathways. For instance in transcription level there were T-bet, Stat1, and Stat4, as well as in cytokine levels, there were IL-12, IL-23, and IL-27[18]. Being secreted by myeloid cells and composed of the cross subunit of IL-12, IL-23, and IL-27 are both belonged to IL-12 family and are demonstrated to have the same action of Th_1 polarization[19, 20].

As there was no anti-IL-12 group involved in our differentiation experiment, we could not be sure that theophylline promoted Th_1 differentiation in IL-12-independent pathway.

In conclusion theophylline could regulate naive T cell developing toward Th_1 cells. It may be a useful reference in the treatment of other allergic diseases such as allergic rhinitis and dermatitis. Whether theophylline reverses the imbalance of Th_1/Th_2 in asthmatic patients still needs further study.

ACKNOWLEDGEMENT We are so grateful for the technical help of director Hua-ming JIN in Laboratory of Urinary Department, Renmin Hospital, Wuhan University.

REFERENCES

1 Weinberger M, Hendeles L. Theophylline in asthma. N Engl J Med 1996; 334: 1380-8.

2 Liu XS, Xu YJ, Zhang ZX, Li CQ, Yang DL, Zhang N, *et al*. Isoprenaline and aminophylline relax bronchial smooth muscle by cAMP-induced stimulation of large-conductance Ca^{2+}-activated K^+ channel. Acta Pharmacol Sin 2003; 24: 408-14.

3 Scordamaglia A, Ciprandi G, Ruffoni S, Caria M, Paolieri F, Venuti D, *et al*. Theophylline and the immune response: *in vitro* and *in vivo* effects. Clin Immunol Immunopathol 1988; 48: 238-46.
4 Lagente V, Pruniaux MP, Junien JL, Moodley I. Modulation of cytokine-induced eosinophil infiltration by phosphodiesterase inhibitors. Am J Respir Crit Care Med 1995; 151: 1720-4.
5 Yang J, Tu HH, Li QQ. Effect of theophylline on airway inflammation in asthma. Acta Pharmacol Sin 2001; 22: 475-80.
6 Essayan DM, Kage Y. Modulation of allergen-induced cytokine gene expression and proliferation by phosphodiesterase inhibitors *in vitro*. J Allergy Clin Immunol 1993; 91: 254-9.
7 Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM, *et al*. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Am J Respir Crit Care Med 2000; 161: 1790-6.
8 Nie HX, Yang J, Hu SP, Wu XJ. Effect of theophylline on CD^{4+}T lymphocytes, interleukin-5, and interferon gamma in induced sputum of asthmatic subjects. Acta Pharmacol Sin 2002; 23: 267-72.
9 Kosmas EN, Michaelides SA, Polychronaki A, Roussou T, Toukmatzi S, Polychronopoulos V, *et al*. Theophylline induces a reduction in circulating interleukin-4 and interleukin-5 in atopic asthmatics. Eur Respir J 1999; 13: 53-8.
10 Ma W, Pober JS. Human endothelial cells effectively costimulate cytokine production by, but not differentiation of, naïve $CD4^+$ T cells. J Immunol 1998; 161: 2158-67.
11 Panina-Bordignon P, Mazzeo D, Lucia PD, D'Ambrosio D, Lang R, Fabbri L, *et al*. β_2-Agonists prevent Th_1 development by selective inhibition of interleukin-12. J Clin Invest 1997; 100: 1513-9.
12 Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. N Engl J Med 1989; 320: 271-7.
13 Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001; 344: 350-62.
14 Zaki MH, Shane RB, Geng Y, Showe LC, Everetts SE, Presky DH, *et al*. Dysregulation of lymphocyte interleukin-12 receptor expression in Sezary syndrome. J Invest Dermatol 2001; 117: 119-27.
15 Stober D, Schirmbeck R, Reimann J. IL-12/IL-18-Dependent IFN-γ release by murine dendritic cells. J Immunol 2001; 167: 957-65.
16 Wang K, Chen JQ, Chen Z, Chen JC. Inhibition of human phosphodiesterase 4A expressed in yeast cell GL62 by theophylline, rolipram, and acetamide-451. Acta Pharmacol Sin 2002; 23: 1013-7.
17 Link AA, Kino T, Worth JA, McGuire JL, Crane ML, Chrousos GP, *et al*. Ligand-activation of the adenosine A2a receptors inhibits IL-12 production by human monocytes. J Immunol 2000; 164: 436-42.
18 Sinigaglia F, D'Ambrosio D. Regulation of helper T cell differentiation and recruitment in airway inflammation. Am J Respir Crit Care Med 2000; 162; S157-60.
19 Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, *et al*. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. J Clin Immunol 2003; 23: 147-61.
20 Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, *et al*. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000; 13: 715-25.