

FK506 Aerosol Locally Inhibits Antigen-Induced Airway Inflammation in Guinea Pigs

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Key Words

Airways · Asthma · Eosinophils · FK506 · Guinea pigs · Interleukin-5

Abstract

Background: Eosinophilic airway inflammation is a common pathological feature of asthma. It has been shown that FK506 given systemically suppresses antigen-induced airway inflammation in animal models. However, it is unknown whether inhaled FK506 can suppress the airway allergic inflammation/immune response and whether it acts locally or systemically. **Methods:** We tested the effects of oral FK506 and inhaled FK506 on antigen-induced airway inflammation in guinea pigs. The tissue and blood concentrations of FK506 given via both routes were compared. The effect of inhaled FK506 on the expression of cytokine mRNA in lung and bronchoalveolar lavage fluid (BALF) cells was also tested. **Results:** Both routes of administration of FK506 suppressed the airway eosinophilia in egg albumin (EA)-sensitized and -challenged animals. The effect of three inhaled puffs was almost equal to that of 1 mg/kg administered by the oral route. Following inhalation of three puffs, FK506 concentration in blood (AUC_{0–24 h}) was approximately 1/21 of that following oral FK506 (1 mg/kg).

After EA challenge, mRNA expression of interleukin (IL)-5, eotaxin and IL-1β in BALF cells and IL-5 in the lung increased significantly. FK506 aerosol markedly inhibited IL-5 mRNA expression in the lung. In situ hybridization indicated that in the BALF IL-5 mRNA expression by lymphocyte-like cells was inhibited by FK506 aerosol. In addition, anti-IL-5 antibody injected intratracheally almost completely abolished eosinophilia in this model. **Conclusion:** Inhaled FK506 can suppress airway inflammation in guinea pigs, where the local action, presumably the inhibition of T-cell activation/function in the lung and airways, was primarily important.

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Introduction

Activated CD4⁺ T helper-2 cells and their cytokines, such as interleukin (IL)-4, granulocyte/macrophage colony-stimulating factor, IL-5, IL-9 and IL-13, are involved in airway inflammation in asthma [1]. Eosinophil infiltration into bronchial mucosa is a characteristic feature of this inflammation [2], with severity of asthma correlating with the degree of airway eosinophil infiltration [3]. Activated eosinophils release leukotrienes and cationic proteins, including major basic protein and eosinophil perox-

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idase protein, that may damage the airway epithelial cells, contributing to airway narrowing and the development of airway hyperresponsiveness in asthma [4]. Although the migration of eosinophils into the airways is regulated by various cytokines, chemokines and mediators, IL-5 has a distinct role, it is able to induce local eosinophilia, promote the terminal differentiation of the respective eosinophil precursors [5] and prolong survival [6]. As expected, anti-IL-5 antibody therapy dramatically reduces eosinophils in the circulating blood and/or sputum [7–9], but the clinical outcome of anti-IL-5 antibody therapy was rather discouraging. It may imply that the role of eosinophils might not be a critical one in the pathogenesis of asthma. However, the precise role is still subject to controversy, and their presence in the airways is at least a good marker of existing allergic inflammation.

FK506 (tacrolimus) is a macrolide immunosuppressant, which is used worldwide for liver and kidney transplantation. FK506 inhibits T-cell activation and T-cell-derived cytokine production by inhibiting responses to nuclear factor of activated T cells [10, 11]. Recently, FK506 ointment has been used for atopic dermatitis [12]. Also, FK506 eyedrops inhibit late and delayed-type responses in ocular allergy models [13], indicating that FK506 locally inhibits allergic responses. Although FK506 given systemically suppresses antigen-induced airway eosinophilia, hyperreactivity and late airway responses in animal models [14–16], it has not been determined whether inhaled FK506 acts locally or systemically to suppress airway eosinophilia. In this study, we addressed whether FK506 aerosol inhalation can inhibit antigen-induced eosinophilic airway inflammation in guinea pigs, and whether it acts locally or systemically.

Materials and Methods

Experimental Design

Male Hartley guinea pigs were purchased from Japan SLC (Hamamatsu, Japan). All experimental procedures were performed according to the guidelines of the Animal Experiment Committee of the Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). All animals were sensitized by intraperitoneal and subcutaneous injection of egg albumin (EA; Seikagaku Kogyo, Tokyo, Japan; 5 mg/head) on days 0 and 7. On day 14, animals were injected with pyrilamine (Sigma, St. Louis, Mo., USA), and 30 min later challenged with nebulized 1% EA for 3 min in a Pennock-type chamber (volume of nasal chamber approximately 1.5 liters; Buxco, Sharon, Conn., USA).

FK506 and its 0.1% aerosol were prepared at the Fujisawa Pharmaceutical Co., Ltd. Inhalation of FK506 aerosol (50 µg/puff) or oral administration of FK506 was carried out 1 h before antigen challenge. For aerosol inhalation, animals were placed in a two-chamber

box, where they were kept for 2 min following FK506 aerosol puffs. As treatment controls, appropriate placebos for oral and aerosol formula were used.

Anti-IL-5 antibody (TRFK5 clone; Pharmingen, San Diego, Calif., USA) or the corresponding control antibody (rat IgG1; Pharmingen) was injected intratracheally (100 µg/head) under anesthesia 1 h before antigen challenge. Bronchoalveolar lavage fluid (BALF) was collected 24 h after antigen challenge, and then smear samples of BALF cells were stained and differentially counted. FK506 concentrations were measured in the blood and lung by ELISA 0.25, 0.5, 1, 2, 6 and 24 h after FK506 administration [17]. Concentrations are expressed as nanograms per milliliter of blood and nanograms per milligram of tissue.

RT-PCR and in situ Hybridization

Messenger RNA expression levels of IL-5 and eotaxin in BALF cells and lung tissue were assessed by semiquantitative RT-PCR assay, using GAPDH mRNA as an internal control. Total RNA was extracted with TRIzol, according to the manufacturer's instructions. Complementary DNA was prepared by reverse transcriptase. Primers were as follows: IL-5 sense: gct ttc tac tca tgc aac tct gc, antisense: ctt gca ggt aat cca gga act gc; eotaxin sense: cac agt ctc tgc ttt cag cg, antisense: tga acc cac ttc ttc ttg gg; GAPDH sense: acc cag aag act gtg gat gg, antisense: agg tca gat cca caa ccg ac; IL-1β sense: ctg aac tca gca gcg aag tg, antisense: cat ggg tca gac aac acc ag. After an appropriate number of PCR cycles, amplified DNA was analyzed by gel electrophoresis and measured with Atto Densitograph software (Atto, Tokyo, Japan).

Cells expressing IL-5 mRNA in BALF cells, collected 2 h after antigen challenge, were identified by in situ hybridization (ISH). To detect guinea pig IL-5 mRNA, ISH using a digoxigenin-labeled complementary RNA (cRNA) probe (position 18–546) was used, as previously described [18]. BALF cell cytopins were permeabilized with protease K and then prehybridized with 50% formamide and 2 × standard saline citrate followed by hybridization at 42°C overnight. Nonspecific binding was removed by posthybridization washing under high-stringency conditions and subsequent treatment with ribonuclease. The cytopins were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase. Color development was performed by addition of substrate (X-phosphate-5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium). As a negative control, cytopins were hybridized with a sense probe.

Statistics

Data are presented as means ± SE. Differences between groups were tested by t test. Dunnett's multiple comparison test was used to assess the significance of differences between the EA treatment group and treatments with FK506. mRNA expression was analyzed using Wilcoxon's signed rank test. $p < 0.05$ was considered significant.

Results

Effect of FK506 on Antigen-Induced Airway Inflammation

First, the effect of inhaled or orally administered FK506 on antigen-induced airway inflammation in antigen-sensitized guinea pigs was evaluated. In antigen-chal-

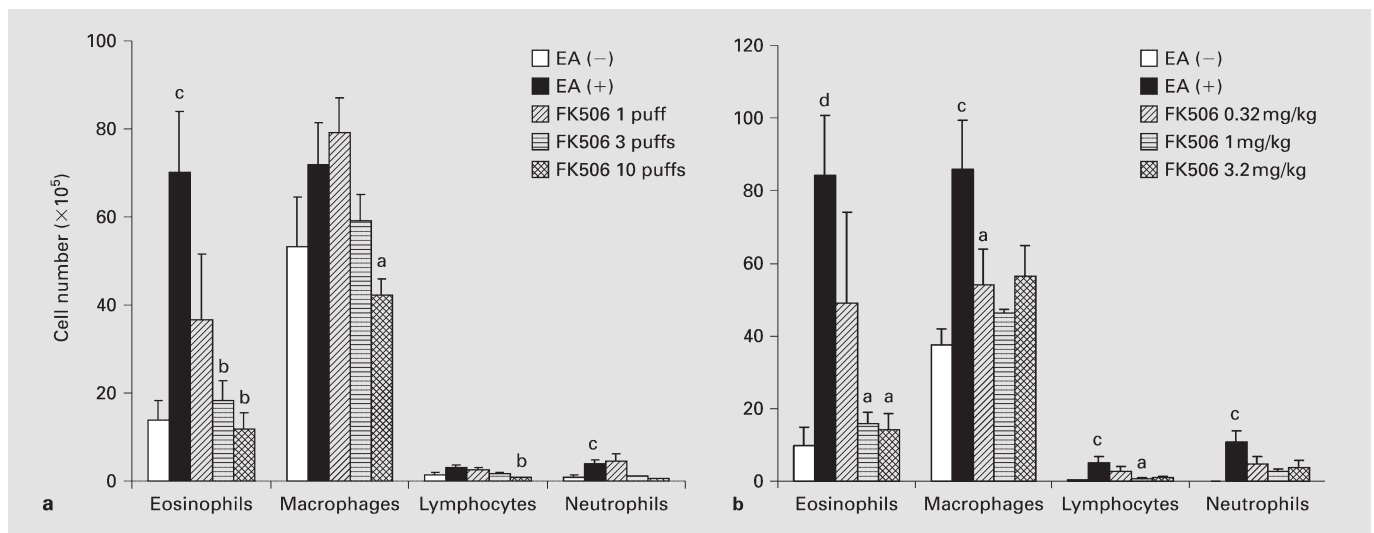


Fig. 1. Effect of FK506 on inflammatory cell numbers in BALF in antigen-challenged guinea pigs. Guinea pigs were actively immunized by subcutaneous and intraperitoneal injection of EA on days 0 and 7. On day 14, they were exposed to 1% EA (EA+ and FK506 groups) or saline (EA- group) for 3 min. Drugs were administered by inhalation (a) or perorally (b) 1 h before antigen challenge. 24 h after antigen challenge, BALF was collected. Values are means $\pm$ SE of 5 animals in each group. ^a $p < 0.05$, ^b $p < 0.01$, vs. EA+, ^c $p < 0.05$, ^d $p < 0.01$, vs. EA-.

Table 1. FK506 concentrations in blood and lung tissue

Admin- istration	AUC ₀₋₂₄ ng·h/ml or ng·h/g tissue	C _{max} ng/ml or ng/g tissue
Inhalation: 3 puffs		
Blood	44.03	17.62
Lung	617.7	168.8
Oral: 1 mg/kg		
Blood	919.3	137.3
Lung	7,304	1,160

lenged animals, inflammatory cells, predominantly eosinophils, increased in BALF collected 24 h after antigen challenge. Inhalation of three (or more) puffs of FK506 aerosol significantly inhibited the increase in eosinophils (fig. 1a). Other inflammatory cells (neutrophils, macrophages and lymphocytes) also tended to increase after antigen challenge, and three (or more) puffs of FK506 restored the normal levels. Regarding oral FK506, ≥ 1 mg/kg of FK506 significantly inhibited airway eosinophilia (fig. 1b). The effect of three puffs of FK506 aerosol was almost equal to that of 1 mg/kg FK506 given orally.

Effect of FK506 on Blood Concentration

To determine whether FK506 induced systemic or local inhibition of the airways, we measured FK506 concentrations in blood and lung tissue following the corresponding doses, i.e. three puffs or 1 mg/kg (table 1). Following three puffs of FK506, C_{max} and AUC_{0-24 h} in blood were approximately 1/16 and 1/21 of those following oral administration of 1 mg/kg. In addition, in the lung C_{max} and AUC_{0-24 h} were lower following three puffs compared to the oral administration of 1 mg/kg.

Cytokine mRNA Expression

To characterize cytokine and chemokine mRNA expression in this model, we performed PCR analysis with total RNA isolated from BALF cells and lung tissue (fig. 2). Increased mRNA expression for IL-5, eotaxin and IL-1 β was observed in BALF cells 2 h after antigen challenge, which peaked at 4–6 h, and then returned to normal levels at 24 h. IL-5 mRNA expression also increased in lung tissue, but the time course was quite different from that in BALF cells, peaking 2 h after antigen challenge, then dramatically decreasing at 4 h. In the lung, a consistent increase in the expression of eotaxin and IL-1 β was not observed (data not shown).

Inhaled FK506 showed a trend towards the inhibition of the increase in IL-1 β and eotaxin mRNA expression in

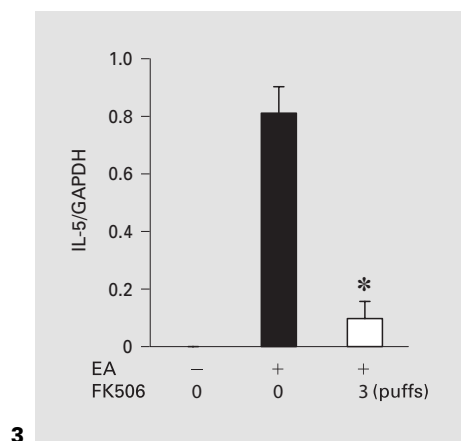
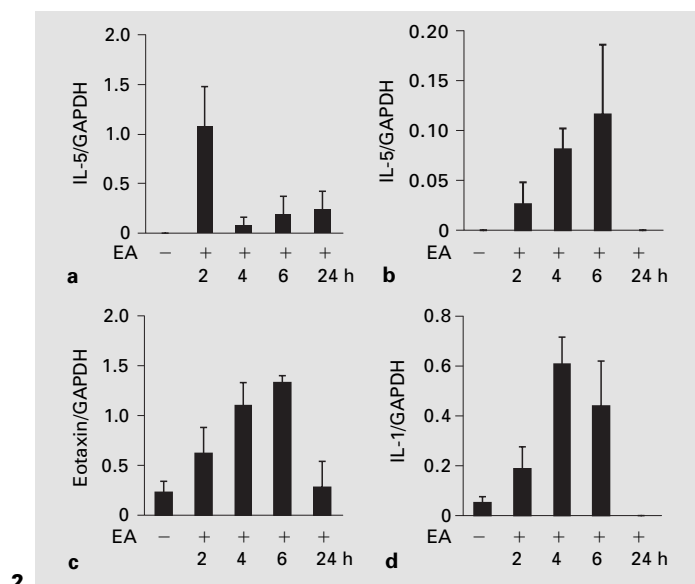


Fig. 2. Time course of mRNA expression in BALF cells and lung tissue. Guinea pigs were challenged with 1% EA. Messenger RNA expression was determined using RT-PCR. The expression levels were normalized using GAPDH expression. Data represent IL-5 in lung tissue (**a**), and IL-5 (**b**), eotaxin (**c**) and IL-1 β (**d**) in BALF cells, respectively.

Fig. 3. Effect of FK506 inhalation on IL-5 mRNA expression. 2 h after antigen challenge, IL-5 mRNA expression levels were determined in lung tissue using RT-PCR. Values are means $\pm$ SE of 5 animals in each group. * $p < 0.05$, FK506 treatment vs. EA-challenged, placebo group.

BALF cells, but it was not statistically significant (data not shown). In contrast, the increase in IL-5 mRNA expression was markedly inhibited by FK506 inhalation in the lung (fig. 3).

We also performed ISH with IL-5 mRNA detection probe to identify the cell type expressing IL-5 mRNA and

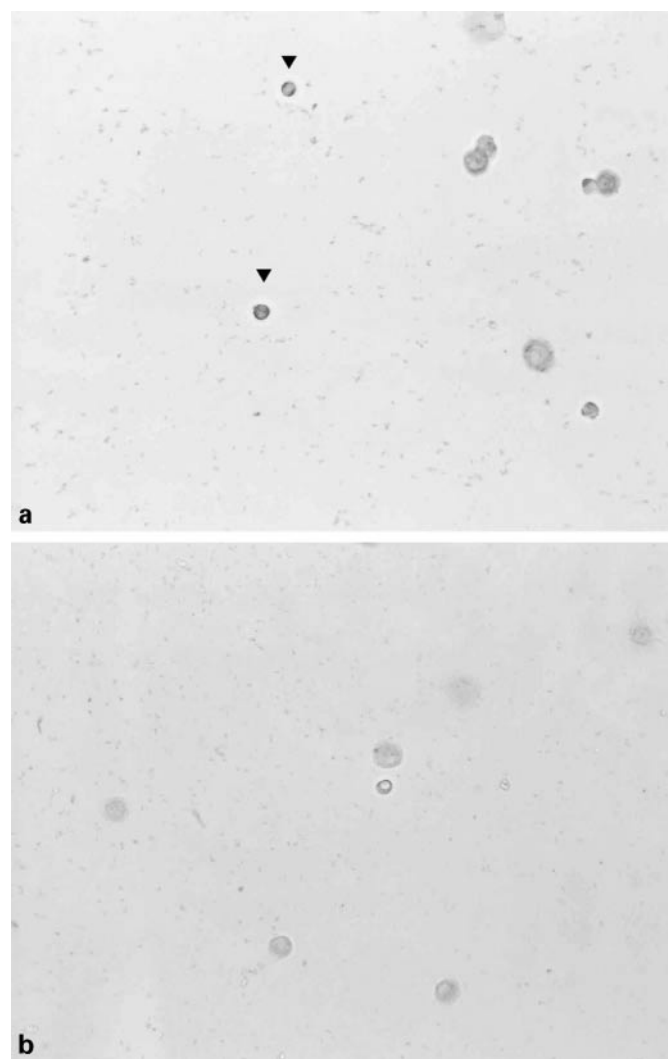


Fig. 4. Reduction in IL-5 mRNA in BALF after FK506 inhalation by ISH. 2 h after antigen challenge, IL-5 mRNA expression was determined in BALF using ISH. Data represent antigen-challenged BALF cells (**a**) and FK506 treatment (**b**). Arrowheads indicate IL-5-mRNA-positive cells. IL-5-mRNA-positive cells detected were lymphocyte-like cells. Signal-positive cells could be hardly found in BALF cells of animals receiving 3 puffs of FK506. No signal was observed with the corresponding sense probe (data not shown). $\times 400$.

test the effect of inhaled FK506 on the mRNA expression. In BALF cells 2 h after antigen challenge, only lymphocyte-like small round cells, but no other type of cells, showed strong IL-5 mRNA expression. IL-5 mRNA expression was reduced following inhalation of FK506 aerosol (fig. 4).

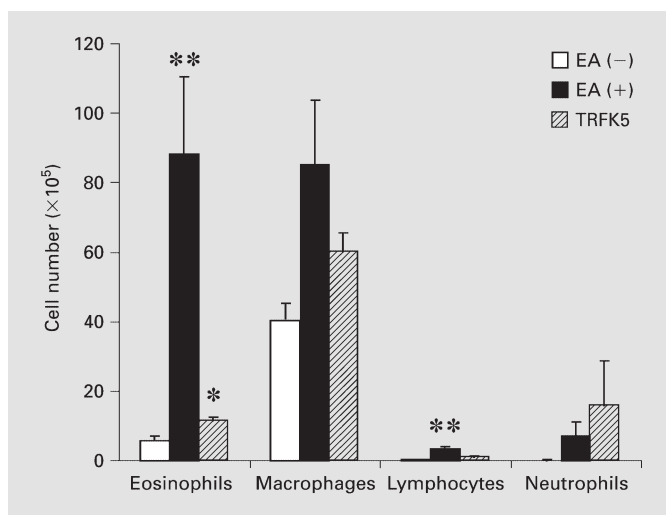


Fig. 5. Effect of anti-IL-5 antibody on antigen-induced airway inflammation. Anti-IL-5 antibody (TRFK5 group) or control antibody (EA+) was administered intratracheally 1 h before antigen challenge. BALF was collected 24 h after antigen challenge. Values are means $\pm$ SE of 5 animals per group. * $p < 0.05$, TRFK5 vs. control antibody group; ** $p < 0.05$, EA-challenged (EA+) vs. non-challenged group (EA-).

Effect of Intratracheally Injected Anti-IL-5 Antibody on Airway Inflammation

In order to test whether or not airway inflammation depended on IL-5 in our model, sensitized animals received intratracheal injection of anti-IL-5 antibody before antigen challenge. Administration of anti-IL-5 antibody almost completely inhibited airway eosinophilia in this model (fig. 5). Antibody administration also tended to inhibit the increase in macrophages and lymphocytes, but the suppressive effect was only partial.

Discussion

In this study, the administration of aerosolized FK506 resulted in the inhibition of the increase in inflammatory cells, especially eosinophils, in BALF recovered from EA-sensitized and -challenged animals. Maximum efficacy of aerosol administration was almost equal to that of oral FK506 administration. FK506 administered via both routes abolished airway inflammation at the highest doses tested. However, in the blood the C_{max} and AUC of FK506 were much higher after oral administration compared to inhaled administration. In the lung, C_{max} and AUC were also higher after oral administration than after inhaled administration, suggesting that most of the

FK506 given orally was distributed to the blood and parenchymal tissue in the lung. Thus, these results indicated that local administration of FK506 into the airways was effective in inhibiting antigen-induced airway inflammation, and strongly suggested that the local action was primarily important for the anti-inflammatory effect of inhaled FK506. This does not exclude the possibility that the local effect of inhaled FK506 results in subsequent systemic effects, such as changes in the number/activity of peripheral blood leukocytes or cytokine levels, for example, which were not monitored in the present study.

Allergic inflammation is characterized by a prominent eosinophilic inflammation in which Th2 cells orchestrate the response, and various cytokines are involved. In this study, we tested the mRNA expression of IL-5, eotaxin and IL-1 β , putative major cytokines involved in airway inflammation [19–22]. As expected, expression of these mRNAs was clearly upregulated in BALF cells, with IL-5 expression also increasing in the lung. We also tested the expression of RANTES and TNF- α , other candidate cytokines thought to be involved in airway inflammation [23, 24], but neither changed significantly in experiments using antigen inhalation (data not shown), suggesting that these cytokines were less important in our model. Expression of IL-5, eotaxin and IL-1 β in BALF cells increased from 2 h after antigen inhalation, remained elevated for a few hours and then returned to normal levels after 24 h. Interestingly, the time course of IL-5 mRNA expression in the lung was distinct from that in BALF cells, which peaked already 2 h after antigen challenge. This difference in the time course of mRNA expression may be explained by the different cell types expressing IL-5 mRNA in lung tissue and airways. Alternatively, cells expressing IL-5 mRNA might move from submucosal areas or parenchymal tissue into airway lumina, thus IL-5-expressing cells might accumulate in the airways.

Among the cytokines tested, IL-5 appeared to be the most important cytokine for eosinophil infiltration, since inhalation of FK506 inhibited most of the expression of IL-5 mRNA in the lung tissue, whereas it exerted only slight inhibition of eotaxin and IL-1 β . The expression of IL-5 mRNA in BALF cells was also reduced by FK506. Furthermore, anti-IL-5 antibody abolished eosinophil infiltration, consistent with a previous report [25, 26], directly indicating that the airway eosinophilia in our model was IL-5 dependent. These results strongly suggested that the inhibitory effects of inhaled FK506 on eosinophil infiltration were primarily due to inhibition of IL-5 expression.

ISH indicated that lymphocyte-like cells expressed IL-5 mRNA in BALF. Matsuo et al. [27] reported that FK506 inhibited IL-5 production, histamine and leukotriene 4 release following antigen stimulation of passively sensitized human lung tissue, suggesting that mast cells were most likely the cells producing cytokine and chemical mediators in the lung in the in vitro model used. We cannot entirely exclude the possibility that the quick expression of IL-5 mRNA in the lung after antigen challenge might be due to mast cell activation. However, we conducted ISH with BALF cells 2 h after antigen challenge and could not find any other type of cell expressing IL-5 mRNA. Considering FK506 forms a complex with FKBP12 and calcineurin, resulting in inhibition of T-cell receptor signaling and subsequent inhibition of cytokine production by T cells [11], it is most likely that T cells are the predominant cells expressing IL-5 mRNA in our model.

Taken together, our results suggest that inhaled FK506 inhibits local IL-5 expression, presumably from T cells

after antigen challenge in airways, leading to the suppression of subsequent eosinophil infiltration in this guinea pig model. Since CD4⁺ T cells isolated from BALF also express increased levels of IL-5 mRNA in asthmatic subjects [28], inhaled FK506 is expected to ameliorate the eosinophilic inflammation. Since FK506 is a very potent immunosuppressant, FK506 aerosol should be superior to oral administration in avoiding possible undesirable effects, such as systemic immune suppression. Recently, the role of IL-5 in asthma has been quite controversial. Inhibition of IL-5 only and consequent inhibition of eosinophilia may not be sufficient to control asthma. FK506, however, can inhibit the production of various cytokines such as IL-2, IL-4, interferon- γ as well as IL-5 [29–31], and is very likely to exert additional effects compared to anti-IL-5 antibody therapy alone. Anti-IL-5 antibody showed only partial inhibition of neutrophil and T-cell infiltration in the present study, whereas FK506 restored it to a normal level. Thus, inhaled FK506 could be a good candidate of anti-asthmatic drug therapy.

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