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ORIGINAL ARTICLE

Decreased CTLA4^{+} and Foxp3^{+} CD25^{high}CD4^{+} Cells in Induced Sputum from Patients with Mild Atopic Asthma

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ABSTRACT

Background: Details of the comparisons between airway and peripheral blood regulatory T cells (Tregs) in patients with atopic asthma are still unclear. The objective of this study is to investigate the profiles of both airway and circulating Tregs in atopic asthma.

Methods: We measured the numbers of Tregs and eosinophils in induced sputum and peripheral blood in 28 patients with mild atopic asthma and compared these with numbers in 18 healthy controls. The frequency (%) of Tregs (surface CTLA4^{+}, intracellular Foxp3^{+}, and CTLA4^{+}Foxp3^{+} on CD25^{high}CD4^{+} T cells) in sputum and blood was determined by intracellular 5-color flow cytometry. We also correlated the numbers with the level of airway hyperresponsiveness (AHR) in asthmatics.

Results: The mean frequencies of cells expressing CTLA4^{+} (19.4 ± 2.1%, p = 0.075), Foxp3^{+} (16.4 ± 3.3%, p = 0.001), and CTLA4^{+}Foxp3^{+} (7.0 ± 1.1%, p = 0.008) in induced sputum from asthmatics were significantly lower than controls (27.2 ± 3.7%, 37.4 ± 4.7%, and 18.2 ± 3.6%, respectively), whereas in peripheral blood, there was no inter-group difference in the frequencies of cells expressing CTLA4^{+} (7.1 ± 1.5% vs 5.7 ± 1.7%, p > 0.05), Foxp3^{+} (35.7 ± 3.2% vs 21.1 ± 3.9%, p > 0.05), and CTLA4^{+}Foxp3^{+} (6.6 ± 1.5% vs 4.2 ± 1.0%, p > 0.05). Moreover, the frequency of CD25^{high}CD4^{+} cells expressing CTLA4^{+}, but not Foxp3^{+}, in induced sputum was associated with AHR (r = 0.60, p = 0.009) and airway eosinophilic inflammation (r = -0.60, p = 0.008) in asthmatics.

Conclusions: Airway, but not circulating, Tregs are decreased in mild atopic asthmatics, and are negatively correlated to an increase of airway eosinophilic inflammation and AHR.

KEY WORDS

adult asthma, airway hyperresponsiveness, airway inflammation, atopic asthma, regulatory T cells

ABBREVIATIONS

AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; CTLA4, cytotoxic T-lymphocyte antigen 4; FEV_1, forced expiratory volume in 1 second; FSC, forward scatter; Foxp3, forkhead box P3; GINA, Global Initiative for Asthma; IgE, immunoglobulin E; MCh, methacholine; PBMC, peripheral blood mononuclear cells; PC_{20}, provocative concentration of methacholine causing a 20% fall in forced expiratory volume in 1 second; SEM, standard error of the mean; SSC, side scatter; Th, helper T cell; Tregs, regulatory T cells.

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INTRODUCTION

It is known that forkhead box P3 (Foxp3)-positive regulatory T cells (Tregs) are potent mediators of dominant self-tolerance.[1] Tregs have the potential to suppress both Th1- and Th2-induced inflammation.[2,3] Activation and expansion of Tregs may be exploited for treatment of immunologic diseases such as allergic asthma.[4] Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a homolog of CD28, which is expressed only on activated T cells, and binds to the accessory molecules B7-1 (CD80) and B7-2 (CD86).[5,6] $CTLA4^+$ $CD4^+CD25^+$ T cells also have the characteristics of Tregs that down-regulate Th1 and Th2 responses though CTLA4 signaling.[7] It is well known that CTLA4 express both surface and cytoplasm on cells. The surface CTLA4 expressing on T cells have immunosuppressive effects.[8,9] Surface CTLA4 also play as a sequester for B7 molecules without cytoplasmic CTLA4 and the sequestration is regulated by the levels of surface expression of CTLA-4.[10]

We have already demonstrated that in patients with atopic asthma, allergen inhalation challenge induces an immediate reduction in the frequency of surface, but not intracellular, expressed $CTLA4^+CD4^+CD25^+$ cells in induced sputum from subjects who develop allergen-induced isolated early responses, and that this returns to the baseline within 24 h. In contrast, subjects who develop allergen-induced dual responses have lower baseline levels of these cells, which do not change after inhaled allergen challenge,[11] and the number of such cells in induced sputum increases after treatment with inhaled corticosteroids.[12] Provoost and coworkers[13] have demonstrated that peripheral Tregs are decreased in asthmatics. In addition, a study of asthmatic children has demonstrated that Tregs in bronchoalveolar lavage fluid (BALF) are decreased in comparison with control children.[14] Conversely, it has been reported that Tregs in BALF are increased in adults with asthma, and even mild asthma, relative to healthy controls.[15] Also, a previous study has demonstrated that Tregs were more numerous in BALF after allergen provocation than before challenge.[16]

$CD25^+CD4^+$ cells have two phenotypes: $CD25^{high}CD4^+$ cells and $CD25^{low}CD4^+$ cells. $CD25^{high}CD4^+$ cells have immunosuppressive functions, whereas $CD25^{low}CD4^+$ cells are activated effector or memory T cells that lack the immunosuppressive functions as Tregs.[17,18] In this study, to investigate the profiles and roles of all three Treg phenotypes in atopic asthma, we have compared the relative frequencies of intracellular $Foxp3^+$ ($Foxp3^+$), surface $CTLA4^+$ ($CTLA4^+$), and $Foxp3^+CTLA4^+$ cells among $CD25^{high}CD4^+$ and $CD25^{low}CD4^+$ cells in induced sputum and peripheral blood, in steroid-naïve, non-smoking patients with mild atopic asthma, with the frequencies in non-atopic healthy subjects (controls), and examined the correlations between each phenotype and eosinophilic inflammation, lung function, and airway hyperresponsiveness (AHR) in asthmatics.

METHODS

SUBJECTS

Twenty-eight non-smoking patients with stable, mild atopic asthma [female : male = 10 : 18, age range 24 to 45 yr] and 18 non-smoking, non-atopic, and age- and sex-matched controls [female : male = 6 : 12, age range 27 to 43 yr] were enrolled (Table 1). All of the asthmatics were steroid-naïve and had used only short-acting β_2-agonists (SABA) intermittently for treatment of their asthma. None of them had used SABA, inhaled or systemic corticosteroids, long-acting β_2-agonists (LABA), leukotriene receptor antagonists (LTRA), theophylline, anti-histamines, or anti-IgE antibody for at least 4 weeks prior to the study. None of the subjects had received allergen immunotherapy. Patients were excluded had suffered asthmatic exacerbations or airway infections during the previous 4 weeks. Diagnosis of asthma and its severity was based on the Global Initiative for Asthma (GINA) guidelines.[19]

STUDY PROTOCOL

Each subject underwent a medical interview and examination, chest radiography, electrocardiography, and spirometry training at the screening visit (visit 1). Blood tests, spirometry, AHR to methacholine (MCh), and sputum induction were conducted at between 8:00 and 9:00 am on visit 2 within 14 days after visit 1. Atopy was identified from the values of serum total IgE (cut-off value for atopy >250 IU/L) and levels of IgE against several specific allergens (*Dermatophagoides farina*, *Dermatophagoides pteronyssinus*, house dust mite, *Candida* spp., *Aspergillus* spp., and Japanese cedar pollen) determined using commercial kits (DPC-Immulyze Total IgE III® and Immulize 2000®, Mitsubishi Kagaku Iatron, Tokyo, Japan). Atopy was diagnosed from the patient's history of atopic diseases and symptoms, a total IgE level of >250 IU/L, and/or positive results for IgE against the specific allergens.

The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Approval (No. 10089) was obtained from the regulatory agency and human ethics committee of Kurume University, Japan, and all the patients provided written informed consent.

SPIROMETRY

Spirometry was performed as previously reported[20,21] and repeated 3 times, the best value being adopted. Subjects were required to have a forced expiratory volume in 1 second (FEV_1) of greater than 80% of the predicted value. AHR was expressed as PC_{20} MCh

Table 1 Characteristics of asthmatics and controls

	Mild asthma	Control	*p* value
Number of subjects	28	18	
Age, yr (Range)	33.3 ± 2.1 (24 to 45)	31.0 ± 1.9 (27 to 43)	NS
Female : Male†	10 : 18	6 : 12	NS
Atopy : Non-atopy†	29 : 0	0 : 18	<0.0001
% FEV_1 predicted (%)	93.7 ± 3.2	114.7 ± 4.3	0.0005
PC_{20} to MCh‡ (mg/mL)	2.4 ± 2.3	>16	<0.0001
No. of sputum Eos. (×10^3/mL)	210.7 ± 51.6	18.7 ± 8.4	0.0011
No. of blood Eos. (/mL)	293.3 ± 45.8	93.2 ± 10.9	0.0004
Total IgE levels (IU/L)	1,089 ± 314	31 ± 10	0.0004

Patients were excluded had suffered asthmatic exacerbations or airway infections during the previous 4 weeks. Diagnosis of asthma and its severity was based on the Global Initiative for Asthma guidelines.[16]

All data were expressed as mean ± SEM.

†Data were compared using Fisher's Exact test.

‡Geometric means ± GSEM.

FEV_1, forced expiratory volume in 1 second; PC_{20}, provocative concentration of MCh causing a 20% fall in FEV_1; MCh, methacholine; Eos, eosinophils; NS, not significant.

(the provocative concentration of MCh causing a 20% fall in FEV_1). The predicted values were calculated using the formula for Japanese.[21]

MCh INHALATION CHALLENGE

The MCh inhalation challenge was performed using the method described by Cockcroft and colleagues.[22] Briefly, subjects inhaled through a mouthpiece attached to an ultrasonic nebulizer. Normal saline, followed by doubling concentrations of MCh, were nebulized and supplied for 2 minutes each. FEV_1 was measured at 30, 90, 180, and 300 s after each inhalation using an electronic spirometer (Chestgraph Jr HI-101, CHEST, Tokyo, Japan). The test was terminated when FEV_1 had fallen to a level at least 20% below the baseline measurement. The concentration of MCh required to achieve a MCh PC_{20} was calculated through linear interpolation of the percentage fall in FEV_1 against the log-transformed MCh concentration.[22]

INDUCED SPUTUM

Sputum was induced using the method described by Pin and colleagues.[23] Briefly, after inhaling 200 μg of Salbutamol® (GlaxoSmithKline, Tokyo, Japan), subjects inhaled an aerosol of 3%, 4%, and 5% hypertonic saline for 7 min each from an ultrasonic nebulizer (NE-U07, Omron, Tokyo, Japan). After each inhalation period, the subjects expectorated sputum into a container.

The induced sputum was processed as described by Pizzichini and colleagues.[24] The sputum was accepted when total amount of 600 mg and over was obtained in each subject. Briefly, 4 parts 0.1% dithiothreitol (Sputolysin, Calbiochem, CA, USA) was added to 1 part sputum and incubated for 15 min, then 4 parts phosphate-buffered saline (PBS) was added. Total cell counts were determined using a hemocytometer and expressed as the number of cells per milliliter of sputum after trypan-blue staining. Sputum cells were pelleted by centrifugation and the supernatant was removed. The cell pellets were then resuspended in PBS at 0.75 to 1.0 × 10^6/ml, and cytospins were prepared using a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickly, PA, USA). Slides were stained using the May-Giemsa method, and a 1,000 differential cell counts was performed on each slide by a single observer blind to the clinical data; the mean count from two slides per subject was used for analysis. However, data for sputum samples containing >30% epithelial cells and >10% dead cells were not included. The mean frequency (range) of dead cells in asthmatics and controls was 4.1 (0.0 to 8.5%) and 5.2 (1.9 to 8.5%), respectively. Remaining cells were used for flow cytometry.

PERIPHERAL BLOOD

Total white blood cell counts were calculated using a hemocytometer, and differential cell counts were obtained from blood smears using the same method as that for the sputum slides. Cell populations were expressed as the number per milliliter. Peripheral blood mononuclear cells (PBMC) were isolated for flow cytometry on an Accuprep™ (Accurate Chemical and Scientific, Westbury, NY, USA) density gradient. There were >99.9% of live cells of PBMC in both of asthmatics and controls by trypan-blue staining.

FLOW CYTOMETRY

Cells were incubated in 5% normal mouse serum (Sigma) for 10 min, then washed in PBS. This was followed by 5-color and intracellular staining with anti-

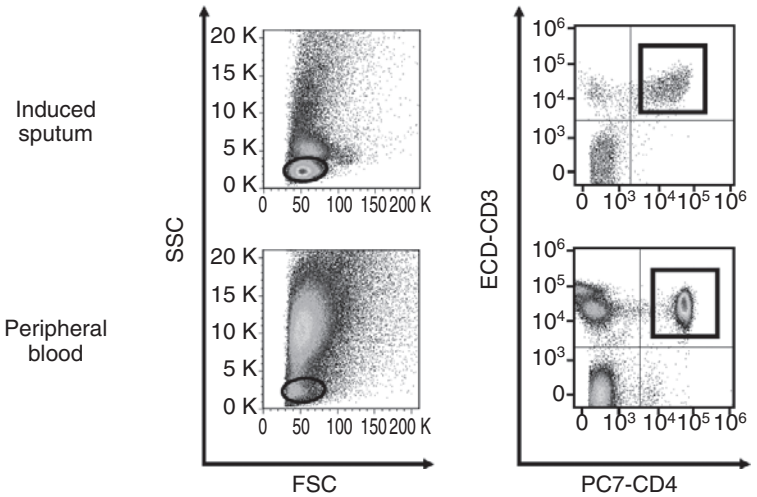

Fig. 1a Detection of Tregs in induced sputum by flow cytometry. Panels show the areas of lymphocytes plotted between the SSC and FSC axes, and the areas of $CD4^+CD3^+$ ($CD4^+$) cells plotted between the ECD-CD3 and PC7-CD4 axes for sputum and peripheral blood (PBMC). SSC, side scatter; FSC, forward scatter; PBMC, peripheral blood mononuclear cells.

human R phycoerythrin-Texas Red®-X (ECD, Energy Coupled Dye)-conjugated CD3 (Beckman Coulter, CA, USA), phycoerythrin-Cy7 (PC7)-conjugated CD4 (Beckman Coulter), phycoerythrocyanin 5.1 (PC5)-conjugated CD25 (Beckman Coulter), fluorescent isothiocyanate (FITC)-conjugated Foxp3 (eBioscience, CA, USA), phycoerythrin (PE)-conjugated CD152 (CTLA4) (PharMingen, CA, USA) antibodies. Briefly, isolated cells were stained with PE-CD152, ECD-CD3, PC5-CD25, PC7-CD4 antibodies, and/or control isotype-matched antibodies. The cells were treated with 1% paraformaldehyde and 0.1% saponin. Then, the cells were further stained with FITC-Foxp3 antibodies, and/or control isotype matched antibodies. A flow cytometer (Cytomics 500, Beckman Coulter) equipped with a 15-mA argon ion laser and filter settings for FITC (525 nm) (FL-1), PE (575 nm) (FL-2), ECD (620 nm) (FL-3), PC5 (675 nm) (FL-4), and PC7 (650 nm) (FL-5) was used. All events were acquired in list mode with debris excluded by the forward scatter threshold.

The area of lymphocytes and $CD4^+$ ($CD3^+CD4^+$) cells in both induced sputum and peripheral blood after preparation of thresholds to remove the area of debris or dead cells was gated with the forward and side scatter described by Yoshida and coworkers (Fig. 1a).[25] The area of $CD25^+$ cells among $CD4^+$ cells was recognized by control isotype matched antibodies. The borderline between $CD25^{high}$ and $CD25^{low}$ cells was defined as lying within the area of $CD25^+$ $CD4^-CD3^+$ cells that represented the 1% of the area of $CD3^+$ cells gated along the CD4 and CD25 axis, as $CD25^+CD4^-CD3^+$ cells were low positive for $CD25^+$ ($CD25^{low}$), but not $CD25^{high}CD4^+$ antigen, in accordance with previous reports.[17,26,27] The frequency of each subpopulation such as $CD25^{high}$ and $CD25^{low}$ cells on $CD4^+$ cells was determined (Fig. 1b). The frequency of $CD25^+$ cells among $CD4^+$ cells was determined as both the areas of $CD25^{high}$ and $CD25^{low}$ cells. Figure 1c shows the area of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ cells on panels of PE and FITC axes. The frequency of $CTLA4^+$ cells was calculated as the frequency of $CTLA4^+Foxp3^-$ plus $CTLA4^+$ $Foxp3^+$ cells, and the frequency of $Foxp3^+$ cells was also calculated as the frequency of $CTLA4^-Foxp3^+$ plus $CTLA4^+Foxp3^+$ cells. (Fig. 1d). Three hundred and over events recognized the $CD25^+CD4^+$ cells were acquired from each sample. The cutoff level for definition of positive cells was less than 2% of cells positively stained with mouse or rat isotype control antibodies (Beckman Coulter). The data were analyzed using the Flow-Jo® software package for Windows (Tomy Digital Biology, Tokyo, Japan).

STATISTICAL ANALYSIS

Unless otherwise stated, all results were expressed as the mean ± standard error of the mean (SEM). Values of MCh PC_{20} were log-transformed prior to analysis and were reported as geometric means ± SEM (GSEM).[22] Paired *t*-tests were used with correction by Wilcoxon matched-pairs single rank test. Correlations were determined by Spearman's rank correlation test. All comparisons were two-tailed, and p values of <0.05 were considered significant. JMP version 9 for Windows (SAS Institute, Cary, NC, USA) was used for data analysis.

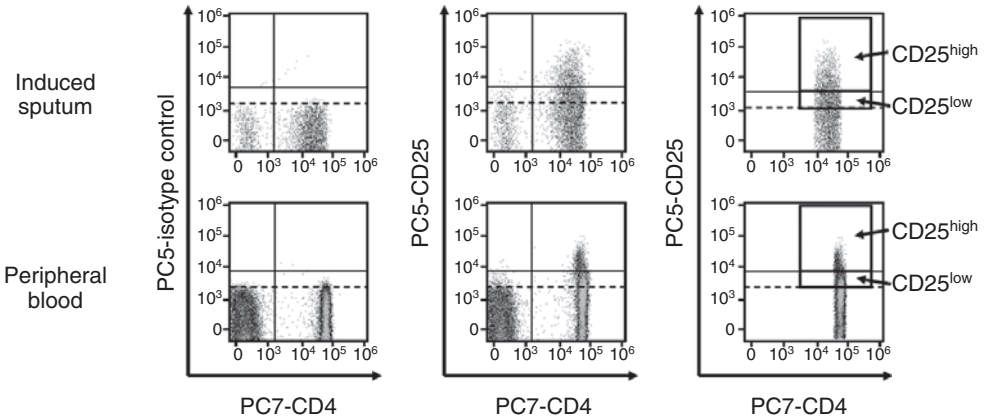

Fig. 1b Detection of Tregs in induced sputum by flow cytometry. Areas of $CD25^{high}$ and $CD25^{low}$ $CD4^+$ cells defined in accordance with the isotype control and $CD25^+CD3^+$ cells in induced sputum and PBMC from asthmatics and controls. SSC, side scatter; FSC, forward scatter; PBMC, peripheral blood mononuclear cells.

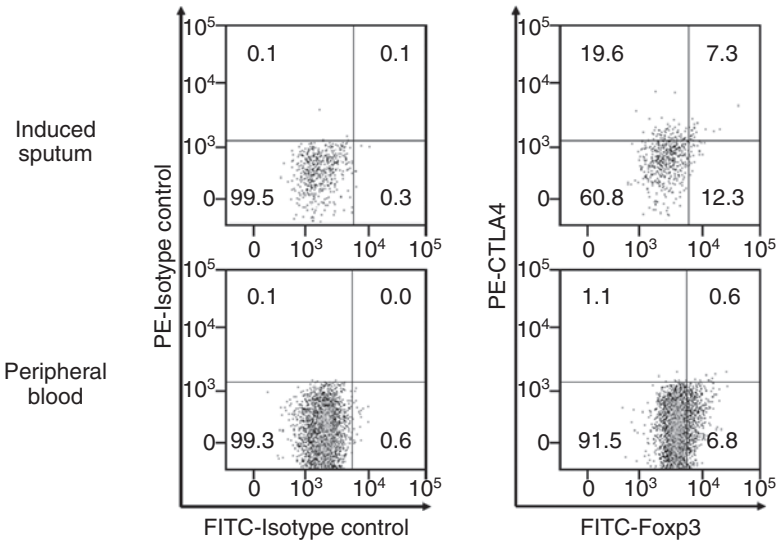

Fig. 1c Detection of Tregs in induced sputum by flow cytometry. Tregs were divided into three phenotypes, namely $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ cells among $CD25^{high}$, $CD25^{low}$ or $CD25^+CD4^+$ cells in induced sputum and PBMC from asthmatics and controls. SSC, side scatter; FSC, forward scatter; PBMC, peripheral blood mononuclear cells.

RESULTS

COMPARISON OF CHARACTERISTICS BETWEEN ASTHMATICS AND CONTROLS

All of the asthmatics had atopy and mild asthma (%FEV_1 pred. >80%), whereas all of the controls had no respiratory symptoms. As expected by the selection of the subjects, the %FEV_1 in asthmatics was significantly lower than that in controls ($p = 0.001$), and the MCh PC_{20} values in asthmatics was significantly lower than in controls ($p < 0.0001$). The absolute number of eosinophils in asthmatics was significantly higher than in controls in both induced sputum and peripheral blood ($p < 0.005$ and $p = 0.0004$, respectively) (Table 1). However, there was no significant inter-group difference in the total cell counts or other differential cells counts (data not shown).

$CD25^{high}$, $CD25^{low}$ AND $CD25^+$ CELLS ON $CD4^+$ CELLS IN INDUCED SPUTUM AND PERIPHERAL BLOOD IN ASTHMATICS AND CONTROLS

In both asthmatics and controls, the frequency of $CD25^{high}$, $CD25^{low}$ and $CD25^+$ cells among $CD4^+$ cells in induced sputum was significantly higher than in peripheral blood ($p < 0.0001$) (Table 2). In peripheral blood, the frequency of $CD25^{high}$ ($p < 0.05$), but not

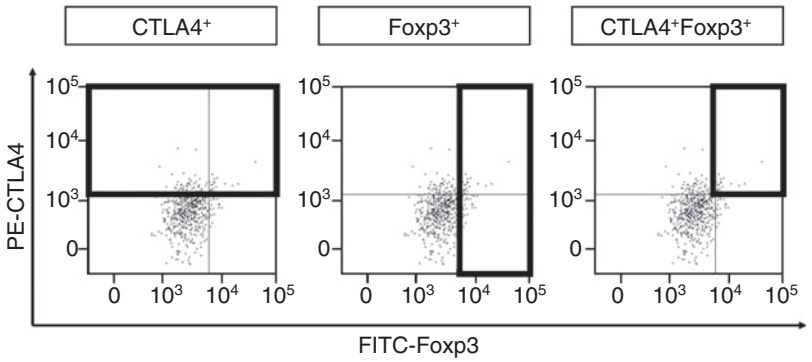

Fig. 1d Detection of Tregs in induced sputum by flow cytometry. Each phenotype of Tregs was recognized the percentages of events in bold boxes as the frequency of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ cells, respectively. SSC, side scatter; FSC, forward scatter; PBMC, peripheral blood mononuclear cells.

Table 2 The frequency of $CD25^{high}$, $CD25^{low}$, and $CD25^+$ cells among $CD4^+$ cells in induced sputum and peripheral blood from asthmatics and controls

	Mild asthma (n = 28)		Control (n = 18)	
Specimens	Sputum	Blood	Sputum	Blood
$\%CD25^{high}$ cells	19.4 ± 1.8*	9.1 ± 1.2**	22.8 ± 2.0*	4.4 ± 0.5
$\%CD25^{low}$ cells	16.5 ± 1.1*	3.6 ± 0.3	14.2 ± 0.6*	3.7 ± 0.3
$\%CD25^+$ cells	36.8 ± 2.5*	12.7 ± 1.5	36.5 ± 2.5*	8.1 ± 0.7

All data were expressed as mean ± SEM.

*$p < 0.001$ induced sputum vs peripheral blood from asthmatics or controls.

**$p < 0.05$ asthmatics vs controls in peripheral blood.

$CD25^{low}$ and $CD25^+$ cells among $CD4^+$ cells, was higher in asthmatics than in controls, whereas there was no significant inter-group difference in the frequency of $CD25^{high}$, $CD25^{low}$ and $CD25^+$ cells among $CD4^+$ cells in induced sputum (Table 2).

TREGS AMONG $CD25^{high}$ AND $CD25^{low}CD4^+$ CELLS IN INDUCED SPUTUM AND PERIPHERAL BLOOD FROM ASTHMATICS AND CONTROLS

Among $CD25^{high}CD4^+$ cells in induced sputum, the frequency of $CTLA4^+$ (19.4 ± 2.1%, p = 0.075), $Foxp3^+$ (16.4 ± 3.3%, p = 0.001), and $CTLA4^+Foxp3^+$ (7.0 ± 1.1%, p = 0.008) cells in asthmatics were lower than those in controls (27.2 ± 3.7%, 37.4 ± 4.7%, and 18.2 ± 3.6%, respectively), whereas there was no significant inter-group difference in the frequencies of $CTLA4^+$ (7.1 ± 1.5% vs 5.7 ± 1.7%, $p > 0.05$), $Foxp3^+$ (35.7 ± 3.2% vs 21.1 ± 3.9%, $p > 0.05$), and $CTLA4^+Foxp3^+$ (6.6 ± 1.5% vs 4.2 ± 1.0%, $p > 0.05$) in peripheral blood (Fig. 2 a). Asthmatics had a higher frequency of $CTLA4^+$ (p = 0.002) cells and a lower frequency of $Foxp3^+$ (p = 0.011) cells, whereas controls had higher frequencies of $CTLA4^+$ (p = 0.001), $Foxp3^+$ (p = 0.024) and $CTLA4^+Foxp3^+$ (p = 0.004) cells in induced sputum than in peripheral blood (Fig. 2a).

Among $CD25^{low}CD4^+$ cells in induced sputum, there were no significant differences in the frequencies of $CTLA4^+$ cells (8.3 ± 2.1% vs 10.5 ± 1.9, $p > 0.05$), $Foxp3^+$ (13.8 ± 4.8% vs 21.0 ± 4.3, $p > 0.05$), and $CTLA4^+Foxp3^+$ (2.3 ± 0.8% vs 5.1 ± 1.6, $p > 0.05$) between asthmatics and controls. There were also no significant inter-group differences in the frequencies of $CTLA4^+$ cells (3.1 ± 2.1% vs 1.2 ± 0.2%, $p > 0.05$), $Foxp3^+$ cells (16.0 ± 4.5% vs 10.4 ± 2.7%, $p > 0.05$), and $CTLA4^+Foxp3^+$ cells (2.7 ± 1.2% vs 0.6 ± 0.1%, $p > 0.05$) in peripheral blood (Fig. 2b). Controls had higher frequencies of $CTLA4^+$ cells (p = 0.003), $Foxp3^+$ (p = 0.087) and $CTLA4^+Foxp3^+$ cells (p = 0.022) in induced sputum than in peripheral blood, whereas asthmatics showed no significant differences in the frequencies of $CTLA4^+$ cells ($p > 0.05$), $Foxp3^+$ cells ($p > 0.05$) and $CTLA4^+Foxp3^+$ cells ($p > 0.05$) between induced sputum and peripheral blood (Fig. 2b).

The frequency of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ cells on $CD25^{high}CD4^+$ cells were significantly higher than that of each cells on $CD25^{low}CD4^+$ cells in both induced sputum and peripheral blood in asthmatics and controls (all $p < 0.05$, respectively) (Fig. 2 a, b).

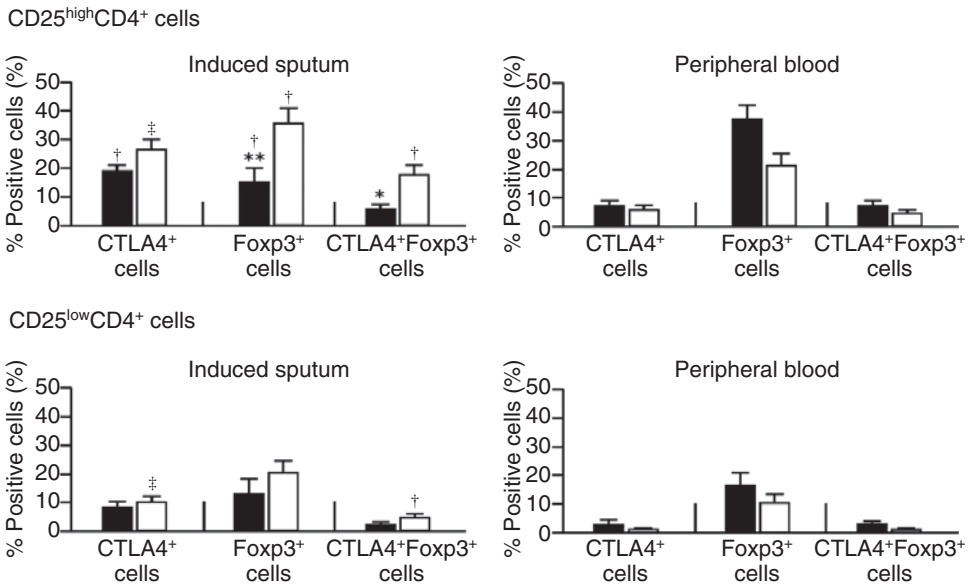

Fig. 2 $CD25^{high}$ and $CD25^{low}CD4^+$ Tregs in induced sputum and peripheral blood of asthmatics and controls. **a**) The mean (error bar = SEM) frequencies of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$cells among $CD25^{high}CD4^+$ cells in induced sputum and peripheral blood from asthmatics (black bars) and controls (white bars). **b**) The mean (error bar = SEM) frequencies of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ cells among $CD25^{low}CD4^+$ cells in induced sputum and peripheral blood from asthmatics (black bars) and controls (white bars). $^*p < 0.05$, $^{**}p < 0.01$ asthmatics vs controls for each phenotype in induced sputum or peripheral blood. $^\dagger p < 0.05$, $^\ddagger p < 0.01$ induced sputum vs peripheral blood for each phenotype in asthmatics or controls.

CORRELATIONS BETWEEN TREGS, EOSINOPHILIC INFLAMMATION, LUNG FUNCTION, AND AHR IN ASTHMATICS

In the asthmatic subjects, the absolute number of sputum eosinophils was significantly correlated with MCh PC_{20} ($r = -0.69$, $p = 0.007$), but not with $\%FEV_1$ pred. ($r = -0.25$, $p > 0.05$), whereas there was no correlation between peripheral blood eosinophils and MCh PC_{20} ($r = 0.21$, $p > 0.05$) or $\%FEV_1$ pred. ($r = 0.15$, $p > 0.05$). Among the Treg phenotypes, only $CTLA4^+$ $Foxp3^+$ cells in induced sputum were significantly associated with lung function ($\%FEV_1$ pred.) ($r = 0.56$, $p = 0.02$) (Table 3).

The frequency of $CTAL4^+$ cells ($r = -0.60$, $p = 0.008$), but not $Foxp3^+$ cells ($r = -0.25$, $p > 0.05$), in induced sputum among $CD25^{high}CD4^+$ cells was negatively correlated with the absolute number of sputum eosinophils (Fig. 3). The frequency of $CTLA4^+$ cells ($r = 0.60$, $p = 0.009$), but not $Foxp3^+$ cells ($r = -0.00$, $p > 0.05$), among $CD25^{high}CD4^+$ cells in induced sputum was also correlated with MCh PC_{20}.

The frequencies of $CTLA4^+$, $Foxp3^+$ and $CTLA4^+$ $Foxp3^+$ cells among $CD25^{low}CD4^+$ cells in induced sputum were not associated with $\%FEV_1$, sputum eosinophils, or MCh PC_{20}. The frequency of $CTLA4^+$ cells among $CD25^+CD4^+$ cells in induced sputum was significantly correlated with MCh PC_{20} ($r = 0.47$, $p = 0.031$), but not with the absolute number of eosinophils in induced sputum ($r = -0.36$, $p > 0.05$) (Table 3). There was no significant correlation between the frequency of Tregs in peripheral blood, the absolute number of eosinophils in induced sputum, and the $\%FEV_1$ pred. and MCh PC_{20} (Table 3).

DISCUSSION

This study has identified that the profiles of three phenotypes of Tregs, namely $CTLA4^+CD25^{high}CD4^+$, $Foxp3^+CD25^{high}CD4^+$, and $CTLA4^+Foxp3^+$ $CD25^{high}CD4^+$ T cells, were significantly decreased in induced sputum from steroid-naïve adult patients with stable, mild atopic asthma in comparison with those in non-atopic healthy subjects.

We have developed a 5-color flow cytometry technique that allowed identification of $CTLA4^+$, $Foxp3^+$, and $CTLA4^+Foxp3^+$ on $CD25^+CD4^+$ cells in both induced sputum and peripheral blood. The technique was also able to detect not only $CD25^+CD4^+$ cells, but also $CD25^{high}CD4^+$ and $CD25^{low}CD4^+$ cells. $CD25^{high}CD4^+$ cells were recognized as Tregs, but $CD25^{low}CD4^+$ cells were activated effector or memory T cells that lack Treg function.[17,18] In our present study, we found that asthmatics had significantly higher frequencies of $CD25^{high}$ cells among $CD4^+$ cells in peripheral blood, but not in induced sputum, relative to controls, although there was no significant inter-group difference in the frequency of $CD25^{low}$

Table 3 Correlations between Tregs, eosinophilic inflammation, lung function, and AHR in asthmatics (n = 18)

	%FEV_1 pred, (%)	PC_{20} to MCh, (mg/mL)	No. of sputum Eos., (×10^3/mL, log)
Induced sputum			
$CD25^{high}CD4^+$cells			
%$CTLA4^+$ cells (%)	0.27	0.60*	-0.60*
%$Foxp3^+$ cells (%)	0.31	-0.00	-0.25
%$CTLA4^+Foxp3^+$ cells (%)	0.56*	0.53	-0.15
$CD25^{low}CD4^+$ cells			
%$CTLA4^+$ cells (%)	-0.14	-0.02	-0.00
%$Foxp3^+$ cells (%)	0.07	0.04	-0.30
%$CTLA4^+Foxp3^+$ cells (%)	0.16	0.09	-0.22
$CD25^+CD4^+$ cells			
%$CTLA4^+$ cells (%)	-0.24	0.47*	-0.36
%$Foxp3^+$ cells (%)	-0.02	0.19	-0.08
%$CTLA4^+Foxp3^+$ cells (%)	0.40	0.30	-0.21
Peripheral blood			
$CD25^{high}CD4^+$cells			
%$CTLA4^+$ cells (%)	0.13	0.36	-0.30
%$Foxp3^+$ cells (%)	-0.39	-0.26	-0.26
%$CTLA4^+Foxp3^+$ cells (%)	-0.05	0.12	-0.31
$CD25^{low}CD4^+$ cells			
%$CTLA4^+$ cells (%)	0.21	-0.13	-0.26
%$Foxp3^+$ cells (%)	0.05	-0.08	-0.02
%$CTLA4^+Foxp3^+$ cells (%)	0.20	-0.10	-0.15
$CD25^+CD4^+$ cells			
%$CTLA4^+$ cells (%)	0.21	-0.20	-0.22
%$Foxp3^+$ cells (%)	-0.10	-0.12	-0.10
%$CTLA4^+Foxp3^+$ cells (%)	0.15	-0.17	-0.13

All data are expressed as correlation r determined by parametric Spearman's test.

* $p < 0.05$.

and $CD25^+$ cells among $CD4^+$ cells in induced sputum or peripheral blood. However, the frequencies of $CD25^{high}$, $CD25^{low}$, and $CD25^+$ cells among $CD4^+$ cells in induced sputum were significantly higher than in peripheral blood in both asthmatics and controls. In asthmatics, there was a significantly negative correlation between the frequency of $CTLA4^+$ cells among $CD25^{high}CD4^+$ cells in induced sputum and AHR (Fig. 3). The coefficient of the correlation between $CTLA4^+$ among $CD25^{high}CD4^+$ cells in induced sputum and AHR was higher than that for $CD25^+$ $CD4^+$ cells and $CD25^{low}CD4^+$ cells (Table 3).

Although the present study should ideally have examined both the frequency and absolute number of Tregs (e.g. $Foxp3^+CD25^{high}CD4^+$ cells) in the induced sputum and peripheral blood of asthmatic patients, $CD25^+CD4^+$ cells were a minor population in induced sputum, as were peripheral $CD4^+$ T cells. The total cell counts in induced sputum obtained from asthmatics varied widely. The absolute numbers of Tregs and $CD25^+CD4^+$ cells were dependent on the total cell counts in induced sputum. Therefore, we determined the frequency of Tregs (e.g. $Foxp3^+$ $CD25^{high}$ $CD4^+$ cells) in both the induced sputum and peripheral blood of patients with mild asthma. However, we did not evaluate the absolute number of Tregs in the induced sputum and peripheral blood of these patients. Further analysis will be needed to verify this issue.

Expression of Foxp3 molecules by helper T cells is a feature of Tregs.[3,28] However, the roles of airway $Foxp3^+$ Tregs are still unclear. Smyth and coworkers[15] demonstrated that the frequency of $Foxp3^+CD4^+$ cells in BAL was increased in patients with mild to severe asthma, relative to healthy subjects. However, in the present study, we recognized $CTLA4^+$ and $Foxp3^+$ on $CD25^{high}CD4^+$ cells as Tregs, and examined induced sputum, as well as selecting patients with mild atopic asthma, not on any treatment. We found that asthmatics had a significantly lower frequency of $Foxp3^+$ and $CTLA4^+Foxp3^+$ cells among $CD25^{high}CD4^+$ cells in induced sputum than the controls, although there were no significant inter-group differences in the profiles of $CTLA4^+$, $Foxp3^+$, or $CTLA4^+Foxp3^+$ cells among $CD25^{low}CD4^+$ cells in induced sputum and peripheral blood. In asthmatics, the coefficient of correlation between $CTLA4^+$ among $CD25^{high}CD4^+$ cells in induced sputum and AHR was

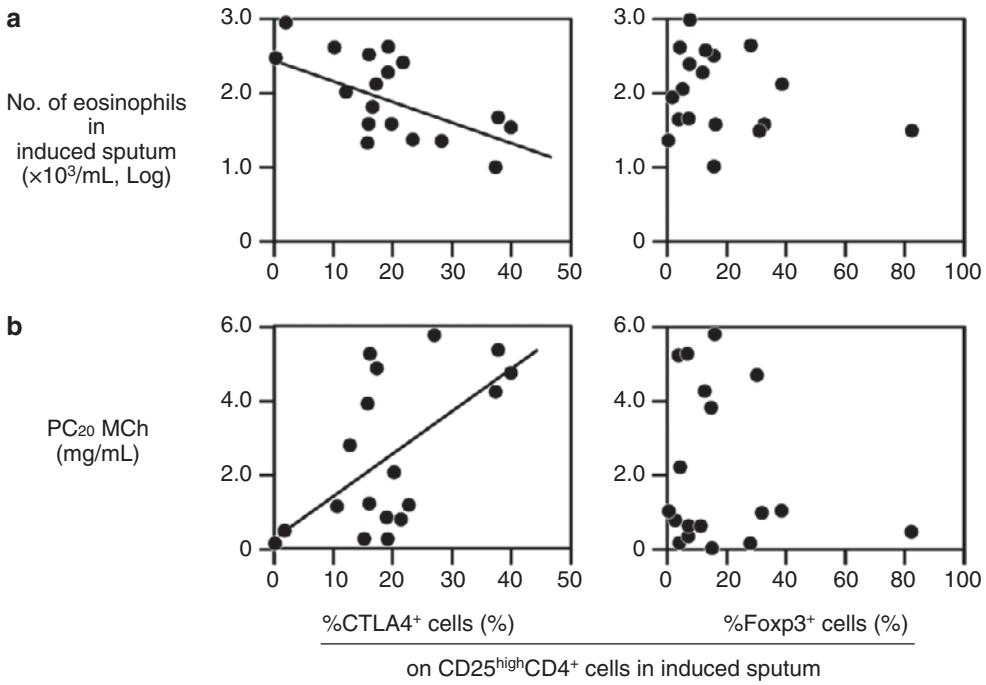

Fig. 3 Correlation between CTLA4$^+$ and Foxp3$^+$ cells among CD25^{high}CD4$^+$ cells in induced sputum, eosinophils in induced sputum and AHR in asthmatics. Panels show the correlation between the frequency (%) of CTLA4$^+$ and Foxp3$^+$ cells among CD25^{high}CD4$^+$ cells in induced sputum, and the absolute number (×10^3/mL, log) of eosinophils in induced sputum (**a**) and PC_{20} MCh (mg/mL) (**b**) in asthmatics (n = 18). The frequency of CTAL4$^+$ (r = -0.60, p = 0.008), but not Foxp3$^+$ (r = -0.25, p > 0.05) cells, among CD25^{high}CD4$^+$ cells in induced sputum was negatively associated with the absolute number of eosinophils in induced sputum. The frequency of CTLA4$^+$ (r = 0.60, p = 0.009), but not Foxp3$^+$ (r = -0.00, p > 0.05) cells, among CD25^{high}CD4$^+$ cells in induced sputum was positively associated with AHR.

higher than that of CD25$^+$CD4$^+$ cells and CD25^{low}CD4$^+$ cells. In addition, there were obvious differences in the profiles of these phenotypes in induced sputum and peripheral blood between asthmatics and controls. In particular, asthmatics had low and high frequencies of Foxp3$^+$CD25^{high}CD4$^+$ cells in the airway and peripheral blood, respectively; whereas in controls, an opposite tendency was observed. Information on the numbers of Tregs in the airway (induced sputum) in asthmatics may be more important than that in the circulating peripheral blood. Our results suggest that airway Foxp3$^+$CD25^{high}CD4$^+$ cells, but not CTLA4$^+$CD25^{high}CD4$^+$, cells may be associated with the pathogenesis or development of atopic asthma.

Surprisingly, the frequency of CTLA4$^+$, but not Foxp3$^+$ and CTLA4$^+$Foxp3$^+$ cells, among CD25^{high}CD4$^+$ cells in induced sputum was associated with AHR in asthmatics. A low frequency of CTLA4$^+$ cells among CD25^{high}CD4$^+$ cells was also associated with airway eosinophilic inflammation. CTLA4 is a potent T-cell down-regulatory molecule expressed by naturally occurring Tregs during peripheral immune responses. Polymorphism of the CTLA4 gene may be associated with AHR and disease severity in asthma.[24,29] Thus, these results suggest that airway CTLA4$^+$CD25^{high}CD4$^+$ cells may play a role in atopic asthma, modifying features such as airway eosinophilic inflammation and AHR.

CTLA4 is required for the function of Foxp3-expressing Tregs.[25,30] The CTLA4 and Foxp3 molecules of Tregs mutually control the function of the other.[26,31] CTLA4 expression on natural occurring Foxp3$^+$ Tregs may reveal opposing effects of costimulation modulators on the steady-state level and quality of Tregs.[27,32] Interaction between airway CTLA4 and Foxp3 protein expression on CD25^{high}CD4$^+$ cells is also marked in asthmatics. Here we found that airway CTLA4$^+$Foxp3$^+$ cells, but not CTLA4$^+$ and Foxp3$^+$, cells were associated with %FEV$_1$ pred. in asthmatics.

It is known that glucocorticosteroids enhance the expression of Foxp3 protein on CD25^{high}CD4$^+$ cells in asthmatics.[10,13,28,33] We previously demonstrated that inhaled corticosteroids increased sputum CTLA4$^+$ CD25$^+$CD4$^+$ cells, along with an increase in the level of IL-10 in patients with mild asthma.[9,14] CD80/CD86 molecules enhance the expression of CTLA4.[29,30,34,35] Immunotherapy against specific allergens has also

been shown to significantly increase Foxp3$^+$ Tregs cells, with the presence of IL-10 and TGF-beta, leading to improvement of asthma control and quality of life in allergic subjects.[31,36] Enhancement of airway CTLA4 and Foxp3 molecules on T cells may contribute to asthma control.[32,37] On the basis of the known data, therefore, in the present study we carefully excluded asthmatics that were using inhaled corticosteroids.

In summary, the present findings suggest that it would be better to define CD25^{high}CD4$^+$ cells as Tregs, rather than CD25^{low} or CD25$^+$CD4$^+$ cells, in patients with atopic asthma. Information on airway Tregs in asthmatics is more relevant than that for circulating Tregs, not only. Airway CTLA4$^+$ Tregs appear to contribute to airway eosinophilic inflammation and AHR.

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