

Regulation of the Development of Asthmatic Inflammation by *In Situ* CD4⁺Foxp3⁺ T Cells in a Mouse Model of Late Allergic Asthma

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Abstract—CD4⁺Foxp3⁺T cells (Tregs) mediate homeostatic peripheral tolerance by suppressing helper T2 cells in allergy. However, the regulation of asthmatic inflammation by local (*in situ*) Tregs in asthma remains unclear. BALB/c mice sensitized and challenged with ovalbumin (OVA) (asthma group) developed asthmatic inflammation with eosinophils and lymphocytes, but not mast cells. The number of Tregs in the circulation, pulmonary lymph nodes (pLNs), and thymi significantly decreased in the asthma group compared to the control group without OVA sensitization and challenge in the effector phase. The development of asthmatic inflammation is inversely related to decreased Tregs with reduced mRNA expression such as interleukin (IL)-4, transforming growth factor- β 1, and IL-10, but not interferon- γ , in pLNs. Moreover, M2 macrophages increased in the local site. The present study suggests that Tregs, at least in part, may regulate the development of asthmatic inflammation by cell-cell contact and regional cytokine productions.

KEY WORDS: late allergic asthma; asthmatic inflammation; *in situ*; mouse model; Tregs; Th2 cell.

INTRODUCTION

Allergic asthma is a chronic inflammatory airway disease characterized by variable airflow obstruction, bronchial eosinophil infiltration, airway hyper-responsiveness, and mucus overproduction [1]. It has been widely accepted the idea that allergic asthma is associated with an imbalance from helper T (Th) 1 cells to Th2 cells, and Th2 cells orchestrate the recruitment and activation of mast cells and IgE in immediate type, and eosinophils and Th2 cells in late type of airway allergic reactions by releasing cytokines such as interleukin (IL)-4, IL-5, and IL-13 from Th2 cells in both types [2]. In addition, a late type of allergic asthma

was eosinophil-/Th2 cell-dependent [3] and IgE-/mast cell-independent [4].

Peripheral CD4⁺ CD25⁺Foxp3⁺ regulatory T cells (Tregs) have been shown to be critical in the maintenance of peripheral immune responses [5–9]. Tregs producing immunosuppressive cytokines such as transforming growth factor (TGF)- β , IL-10, and IL-35 have been shown to be responsible for the protection of immune reactions following exposure to allergens such as aero-allergens or food allergens [10–12]. In this regard, cell-cell contact through membrane-bound TGF- β 1 *via* suppressive molecules such as cytotoxic T lymphocyte-associated antigen (CTLA)-4 or released soluble immunosuppressive cytokines from Tregs have been shown to contribute to the maintenance of self-tolerance in peripheral tissues [6, 13]. Besides immunosuppressive cytokine production by Tregs, those cells are known to exert suppressive function in a number of ways, including the release of perforin [14] and granzyme B [15] and possibly through the release of cAMP [16]. Moreover, Tregs are capable of suppressing Th2 responses to allergens in health and attenuate allergic conditions [17]. Transfer of antigen-specific Tregs inhibited the development of asthma in mice [11], and the treatment

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with glucocorticosteroids in asthmatics might increase this Foxp3 protein expression within Tregs in human, [18] and fluorescence-activated cell sorter (FACS) analysis revealed decreased Tregs obtained from lung tissues in a model of asthma [19].

Asthmatic patients had lower Tregs ratio and Foxp3 messenger RNA (mRNA) expression with the decrease of Tregs ratio, and those functions in peripheral blood mononuclear cells may be associated with the pathogenesis of asthma in human [20]. Also, asthmatic patients have a decreased circulating Foxp3 protein expression in Tregs in patients with stable asthma [18]. The decreased number of peripheral blood Tregs in atopic patients to intrabronchial allergen provocation has been reported [21]. These suggest regulatory effects of Tregs in the development of asthmatic inflammation, but local (*in situ*) cell-cell interaction of Tregs such as at inflamed bronchus and regional lymph nodes (LNs) in human patients with asthma and animal models remains largely unclear.

The aim of the present study is to analyze the relationship between locally accumulated Tregs and the development of asthmatic inflammation in a mouse model of late allergic asthma.

MATERIALS AND METHODS

Animals

Specific pathogen-free, 8-week-old female BALB/c mice (Japan Charles River Co., Kanagawa, Japan) were used (total number of mice; $n=15$). The number of mice used in each experiment was shown in the parenthesis below. They were kept at 24 ± 2 °C room temperature, 45 ± 10 % humidity, and at a 12-h light/dark cycle (lightening time 08:00–20:00) in metal cages covered with woodtips sterilized by dry heating (180 °C, 30 min). Animals were fed with commercial pellets (CE-2; Clea Japan, Tokyo, Japan) and supplied water *ad libitum* sterilized by heating at 121 °C for 20 min. The number of mice used in each experimental group is given in parenthesis of experimental protocol. The animal experiments were approved by the Research Ethics Board of the Faculty of the Agriculture, Yamaguchi University.

Experimental Protocols and Sampling

A model of late allergic asthma was induced by a simple modification of our previous reports [22–25]. As shown in Fig. 1, mice were divided into two groups as

follows: sensitization and challenge with ovalbumin (OVA) (asthma group; $n=9$) and neither sensitization nor challenge with OVA (control group; $n=6$). Mice were sensitized by intraperitoneal injection (i.p.) of 10 µg of OVA (Grade V; Sigma-Aldrich Co., St. Louis, MO, USA) in 1.2 mg of aluminum hydroxide gel (Alum; SERVA, Heidelberg, Germany) adjuvant (OVA/Alum), which was suspended in 100 µl of phosphate-buffered saline (PBS; pH 7.4) on day 0 and boosted on day 10. On days 14, 15, 16, and 17, the mice were challenged intranasally (i.n.) with 200 µg of OVA in 50 µl of PBS (both right and left nasal cavities by a micropipette alternately) under anesthesia by injection with a 0.5-ml mixture of ketamin and xylazine i.p. The control group was treated as the same manner of the asthma group with PBS instead of OVA. Twenty-four hours after the last challenge (on day 18), blood, lungs, spleens, pulmonary LNs (pLNs), and thymi were obtained. For flow cytometry, peripheral blood leukocytes were obtained just before the time of sensitization (on day 0) and the challenge (on day 14) or the sampling (on day 18), respectively.

The number of Foxp3⁺ cells in pLNs and thymi ($n=3$ in each group) in immunohistochemically stained section was counted using an eyepiece micrometer (high view; $\times 400$, 0.25×0.25 mm²) morphologically 2 to 10 different fields of view at random in each section. Then the number of Tregs/mm² was expressed in each mouse. Each result was averaged and totaled in each mouse; and thereafter, the mean $\pm$ standard error of the mean (SEM) was calculated.

Also, the number of Tregs in bronchial-bronchiolar mucosa (the asthma group; $n=7$, the control group; $n=3$) was counted using an eyepiece micrometer (high view; $\times 400$, 0.1×0.25 to 0.25×0.25 mm²) morphologically in 3 to 6 different fields of view at random in each mouse section. Number of Tregs per bronchus-bronchiole was expressed (/µm²), and the relationship between the severity of bronchial-bronchiolar lesions (score index (S.I.); we will describe later) and accumulation of Tregs per one mouse was examined.

Leukocyte Number in Blood

Peripheral venous blood samples were obtained from orbital sinus under ether anesthesia on days 0 (just before the sensitization), 14 (just before the challenge), and 18 (asthma group; $n=6$, control group; $n=3$), and the absolute number of leukocytes (/µl) was counted. Percentage (%) of eosinophils, neutrophils, lymphocytes, and monocytes was calculated after a blood smear was stained with May-Grünwald-Giemsa. Then, the total number of each

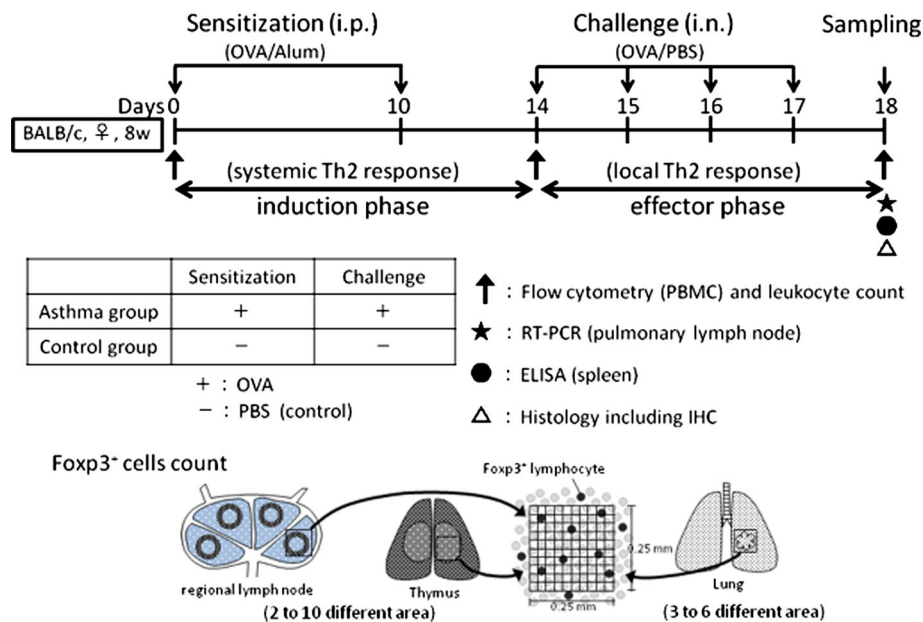


Fig. 1. Experimental protocol in a model of late allergic asthma. See detail in “Materials and methods”.

leukocyte was determined; and thereafter, the mean±SEM of each group was calculated.

Histological Evaluation of Lung Lesions

Lung samples (asthma group; $n=6$, control group; $n=3$) were fixed in 10 % neutral-buffered formalin solution (pH 7.0) and embedded in paraffin wax. Four-micrometer sections were stained with hematoxylin and eosin (H&E), methylgreen pyronin for plasma cells, periodic acid Schiff's (PAS), alcian blue (pH 2.5) for goblet cells and connective tissue-type mast cell, and methylene blue for mucosal-type mast cell [24]. The severity of lesions in each transversely sectioned bronchus-bronchiole was evaluated by light microscopy and scored as follows [23]: no lesions, 0; mucus or desquamated epithelial cells in the lumen, 1; a few goblet cells in the epithelial layer, 1; many such cells, 2; thickened basement membrane, 1; mild infiltration of eosinophils and lymphocytes in mucosa, 1; severe infiltration of those cells in the mucosa, 2; hypertrophy of smooth muscle cells in mucosa, 1. In each mouse, the S.I. was calculated by dividing the total number of bronchi-bronchioles examined. The average S.I. was then determined for each group. The number of eosinophils, lymphocytes, neutrophils, macrophages, plasma cells, and mast cells in the bronchial-bronchiolar mucosa was counted using an eyepiece micrometer morphologically on five different bronchi-bronchioles at random in each left lung of

H&E sections by two different observers. Each result was averaged and totaled in each mouse; and thereafter, the mean±SEM (/mm²) was calculated.

Immunohistochemistry

Deparaffinized sections of the lungs, pLNs, and thymus were treated with autoclaving in Dako Target Retrieval Solution (pH 6) at 120 °C for 5 min (Foxp3; the asthma group; $n=7$, the control group; $n=3$, iNOS, arginase-1, and CD4; $n=3$ in each group) or Dako Target Retrieval Solution (pH 9) at 121 °C for 1 min (CD3, CD20, and Iba1; $n=3$ in each group). Endogenous peroxidase activity was inactivated in 3 % hydrogen peroxide in distilled water for 10 min. Then, sections were treated with Protein Block Serum-Free (Dako Japan Inc., Tokyo, Japan) for 30 min to block the non-specific binding of antibody. Sections were treated with primary antibodies at 4 °C overnight as follows: rat anti-mouse Foxp3 monoclonal antibody (mAb) (eBioscience, San Diego, CA, USA) diluted in 1/20, rabbit anti-iNOS polyclonal antibody (pAb) (Abcam Japan, Tokyo, Japan) diluted in 1/1,000, rabbit anti-arginase-1 pAb (Fitzgerald, Acton, MA, USA) diluted in 1/1,000, rabbit anti-human CD3 pAb (Dako Japan, Tokyo, Japan) diluted in 1/400, mouse anti-human CD4 mAb (Abcam Japan) diluted in 1/10, rabbit anti-human CD20 pAb (Lab Vision Co., Fremont, CA, USA) diluted in 1/400, and rabbit anti-human Iba1 pAb (Wako, Osaka, Japan) diluted in 1/4,000, in which

cross-reactivity with mouse is reported of anti-human CD3 pAb, anti-human CD4 mAb, and anti-human Iba1 pAb, but not anti-human CD20 pAb. Thereafter, sections were treated with secondary antibodies, undiluted peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (Nichirei Bioscience, Tokyo, Japan), undiluted peroxidase-labeled goat anti-mouse IgG polyclonal antibody (Nichirei Bioscience), or undiluted peroxidase-labeled goat anti-rat IgG polyclonal antibody (Nichirei Bioscience) for 30 min depending on the immunized animals of primary antibodies. Positive reactions were visualized with 3',3'-diaminobenzidine tetrahydrochloride (Roch Diagnostic, GmbH, Penzberg, Germany) for 5 min, and sections were counterstained with Mayer's hematoxylin for 1 min. All incubations were done at room temperature except for those of primary antibodies (4 °C). Between each step, sections were washed three times with PBS. The reaction without primary antibody served as negative control.

Isolation of Splenocytes

Spleens (asthma group; $n=6$, control group; $n=3$) were aseptically removed and placed in phosphate-buffered-balanced salt solution (PBBS; pH 7.4) on day 18. Single-cell suspensions were made by teasing spleens apart with scissors and passing through a disposable syringe with a 23G needle (Terumo Co., Tokyo, Japan) as described previously [25, 26]. Cell suspensions were collected in sterile conical tubes (Fisher Sci., Pittsburgh, PA, USA); and thereafter, red blood cells were hemolyzed by Tris-buffered NH_4Cl solution and washed in PBS followed by centrifugation at 500g for 10 min at 4 °C. The total number of splenocytes was counted using a hemocytometer and diluted in minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) to a density of 1×10^6 cells/ml. Viability of cells was more than 98 % by trypan blue dye exclusion test. Splenocytes ($2 \times 10^5/200 \mu\text{l}$ of MEM with 5 % heat-inactivated fetal calf serum; Gibco., Grand Island, NY, USA) were cultured for 48 h at 37 °C under 5 % CO_2 in the presence of 5.5 $\mu\text{g/ml}$ of concanavalin A (Con A; ICN Biomedical, Aurora, OH, USA) using 96 well-plates (Coster, Cambridge, MA, USA), and supernatants were obtained and stored at -80 °C until use.

Flow Cytometry

Peripheral blood leukocytes were collected from blood and centrifuged at 1,000g for 30 min at 4 °C on days 0, 14, and 18 (asthma group; $n=9$, control group; $n=6$) in each time point. After that, lymphocytes from mice were separated by Ficoll-Hypaque density gradient centrifugation for

the isolation of mouse lymphocytes (Lymphocyte M; Cedarlane, Hornby, Ontario, Canada) and washed with FACS buffer (PBS containing 2 % FCS and 0.1 % NaN_3). After incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody (clone RM4-5; Beckton Dickinson, Tokyo, Japan) on ice for 30 min, the cells were washed with FACS buffer, followed by the intracellular staining of Foxp3 according to manufacturer's instruction. In brief, the fixed and permeabilized cells were stained with phosphatidylethanolamines (PE)-conjugated anti-mouse Foxp3 antibody (clone FJK016s; eBioscience) and stored at 4 °C until analysis. Appropriate isotype controls (rat IgG2a-PE antibody; eBioscience) were used for each sample. Flow cytometry analysis was performed on EPIX-XL (Beckman Coulter, Tokyo, Japan), and the results obtained were analyzed using the FlowJo software (Treestar, Inc., San Carlos, CA, USA).

After determining the percent of $\text{CD4}^+\text{Foxp3}^+$ cells in total CD4^+ cells (% of $\text{Foxp3}^+/\text{CD4}^+$) on day 0 given as point 1, ratio of $\text{Foxp3}^+/\text{CD4}^+$ (decrease in the number of Tregs) on days 14 or 18 was calculated based on that on day 0 in each individual; (% of $\text{Foxp3}^+/\text{CD4}^+$ on days 14 or 18)/(% of $\text{Foxp3}^+/\text{CD4}^+$ on day 0).

Production of OVA-Specific IgE in Plasma and IL-4, TGF- β 1, IL-10, or IFN- γ in Supernatants of Cultured Splenocytes by ELISA

Values for OVA-specific IgE (DS pharma Co., Ltd, Suita, Osaka, Japan) in plasma and concentrations of IL-4 (Bender MedSystems GmbH, Vienna, Austria), TGF- β 1 (R&D system, Inc.), IL-10 (GEN-PROBE, Inc., San Diego, CA, USA) (asthma group; $n=6$, control group; $n=3$), or IFN- γ (Bender MedSystems) (asthma group; $n=5$, control group; $n=3$), respectively, in supernatants from cultured splenocytes were measured by mouse ELISA kits according to the manufacturer's instructions. Detectable amounts of OVA-specific IgE, IL-4, TGF- β 1, IL-10, and IFN- γ are 2.7 ng/ml, 2.0 pg/ml, 1.7–15.4 pg/ml, 20 pg/ml, and 5.3 pg/ml, respectively. In case cytokines could not be detected, their concentration was considered as below sensibility.

mRNA Expression for IL-4, TGF- β 1, IL-10, and IFN- γ in pLNs by Real-time PCR

Total RNAs used for real-time PCR analysis were isolated from the frozen pLNs ($n=3$ in each group except for IFN- γ ; $n=2$) with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. One microgram of total RNA was treated with

Turbo DNA-free (Applied Biosystems Japan, Tokyo, Japan) and transcribed into complementary DNA (cDNA) using Superscript III (Invitrogen Life Technologies, Inc., Tokyo, Japan) according to the manufacturer's instructions. Oligo dT primers were used to prime the first-strand synthesis for each reaction. After single-strand cDNA was generated from each sample, it was subjected to real-time PCR amplification with a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The primers used for assaying IL-4 [27], TGF- β 1 [28], IL-10 [29], IFN- γ [30], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31] are as follows: IL-4; forward, 5'-ACAGGAGAAGGGACGCCAT-3' and reverse 5'-GAAGCCCTACAGACGAGCTCA-3', TGF- β 1; forward, 5'-ACCGGGTGGCAGGCGAGAG-3' and reverse 5'-CGGGACAGCAATGGGGGTTCT-3', IL-10; forward, 5'-GGACAACATACTGCTAACCG-3' and reverse 5'-TTCATGGCCTTGTAGACACC-3', IFN- γ ; forward, 5'-TCAAGTGGCATAGATGTGGAAGAA-3' and reverse 5'-TGGCTCTGCAGGATTTTCATG-3', and GAPDH; forward, 5'-TCACCACCATGGAGAAGGC-3' and reverse 5'-GCTAAGCAGTTGGTGGTGCA-3', respectively. Each assay was performed in duplicate. Predenaturation at 95 °C for 15 min was followed by 45 cycles of PCR amplification consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR and fluorescence intensity detection were performed with the StepOne PCR system (Perkin-Elmer, Waltham, MA, USA). The data were analyzed using StepOne software v.2.0. Briefly, the PCR cycle number at the threshold was represented as C_T . The difference between C_{TS} for the target and internal control, *i.e.*, Delta C_T was calculated. The value of 2Delta C_T was considered to represent the amount of target mRNA relative to the amount of internal control.

Statistical Analysis

The means $\pm$ SEM were calculated and analyzed by F test and then by student's t test (one-tailed or two-tailed). Correlation and regression analysis were performed using Microsoft Excel 2007 (Microsoft Corp., Redmond, Washington, USA). The accepted level of significance was $P < 0.05$.

RESULTS

Number of Leukocyte in Blood

The number of total leukocytes, neutrophils, lymphocytes, and monocytes in the asthma group increased on

days 14 and 18 compared to the control group (Fig. 2a, c–e; $P < 0.05$ and $P < 0.01$, two-tailed t test). On day 18, but not on day 0, there was a significant increase in the number of eosinophils ($P < 0.05$, two-tailed t test) in the asthma group compared to the control group (Fig. 2b). The number of eosinophils on day 14 in the asthma group increased when compared to that in the control group ($P < 0.01$, two-tailed t test).

Asthmatic Inflammation and Characterization of Inflammatory Cell

The asthma group developed asthmatic inflammation (Fig. 3a–c, j), and infiltrated cells were summarized in Fig. 3k. There were goblet cell metaplasia and/or hyperplasia with mucous hypersecretion in the asthma group (Fig. 3b, c), although those changes varied within the same lung and among mice. There was a severe infiltration of eosinophils with degranulation and lymphocytes including some neutrophils, macrophages, and plasma cells, but not mast cells (Fig. 3i). At the site of asthmatic inflammation, edematous lamina propria was recognized, and in some cases destructed and desquamated epithelial cells into the bronchial-bronchiolar lumen, and hypertrophic smooth muscle cells were observed. There was no asthmatic inflammation in the control group (Fig. 3d, j). In addition, eosinophilic vasculitis in venules and small-sized arterioles was often observed throughout the lung tissue.

The number of total cells in the asthma group increased when compared to that in the controls (asthma group $3,622.1 \pm 263.4/\mu\text{m}^2$; control group $0/\mu\text{m}^2$). Inflammatory cell consisted of lymphocytes expressing CD3⁺ (Fig. 3e), CD4⁺ (Fig. 3f), and CD20⁺ (Fig. 3g), including Iba1⁺ macrophages (Fig. 3h), plasma cells (Fig. 3i), and mucosal-type, but not connective tissue-type, mast cells (Fig. 3j). Compared to other inflammatory cells, mast cell infiltration was few (Fig. 3l).

Relationship Between the Severity of Asthmatic inflammation (S.I.) and Number of Tregs

S.I. of each bronchus varied within the same lung in the asthma group. In the control, there were no or a few Tregs at bronchial walls (0 to $9.6 \times 10^{-5}/\mu\text{m}^2$) (Fig. 4a, b). On the other hand, there were mild to moderate infiltrations of Tregs at bronchial walls (Fig. 4c, d). The number of Tregs was inversely correlated with S.I. per bronchus-bronchiole in one mouse of the asthma group (regression analysis) (Fig. 4a).

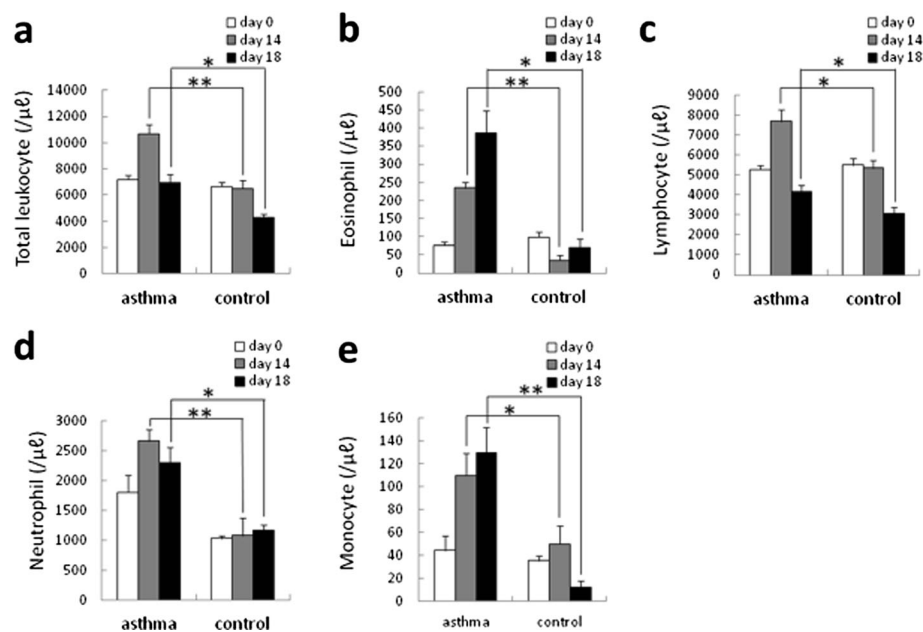


Fig. 2. Number of each leukocyte (a–e) in blood (μl) on days 0, 14, and 18. Each value represents the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

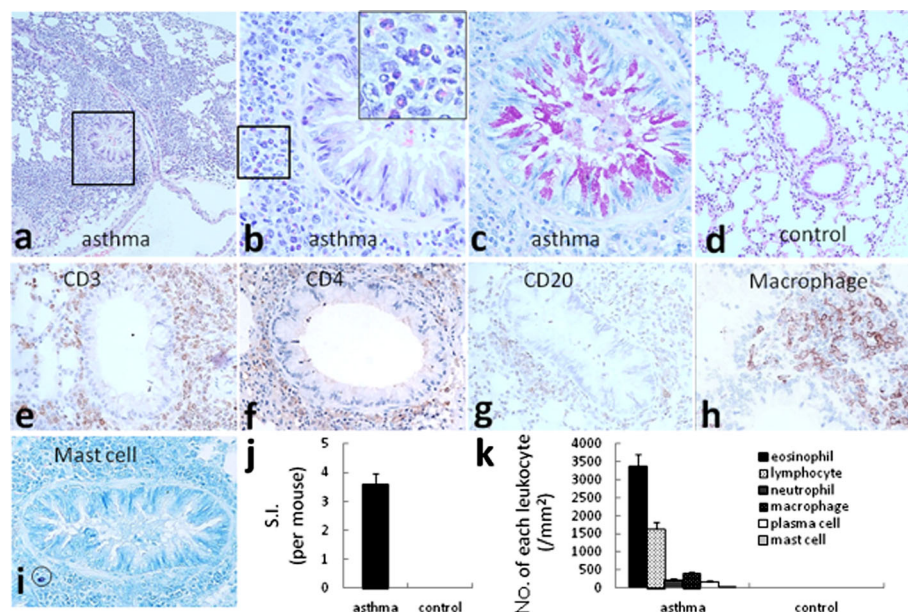


Fig. 3. Histopathology of asthmatic inflammation on day 18. Severe asthmatic inflammation with desquamated epithelial cells in the bronchial lumen, and hypertrophic smooth muscle cells are seen in the asthma group (a and b; enlargement of rectangle in a). Densely infiltrated eosinophils and lymphocytes (b and insert; enlargement of rectangle in b) and metaplasia and hyperplasia of goblet cells (c) with mucous hypersecretion are visible. There are no changes in the control group (d). Characterization of infiltrated cells. CD3⁺ (e), CD4⁺ (f) and CD20⁺ (g) lymphocytes, Iba1⁺ (h) macrophages, and mast cells (i; circle indicates mast cell). S.I. (severity of lesions) of asthmatic inflammation per one mouse (j). Number of inflammatory cells ($/\text{mm}^2$) at the site of bronchi-bronchioles is shown (k). Each value represents the mean $\pm$ SEM. Asthma group (a–c, e–j) and control group (d). a, b, and d H&E; c PAS; (e–h) immunohistochemistry (brown color positive cells) and counterstained with Mayer's hematoxylin, and i methylene blue. a $\times 100$; d $\times 200$; e–i $\times 400$; b, c $\times 600$, and insert of b $\times 1,200$.

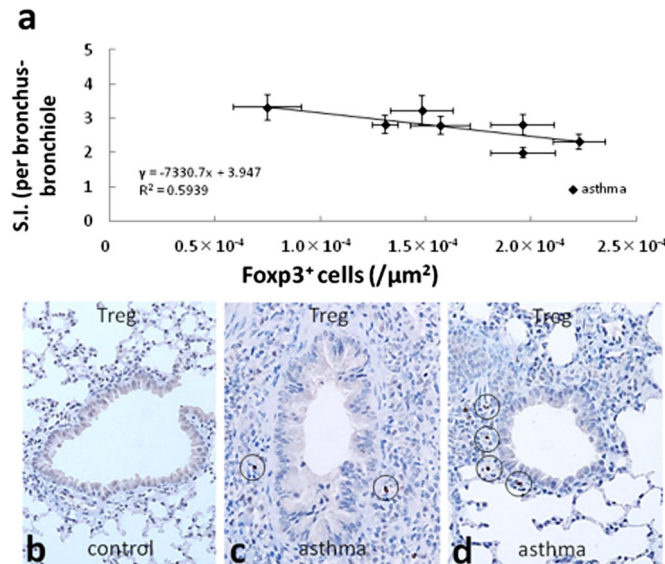


Fig. 4. Relationship between Tregs number and S.I. of asthmatic inflammation per bronchus-bronchiole in each mouse (a). The control shows no Tregs (b). High S.I. with no increase of Tregs (c) and low S.I. with increased Tregs (d) can be recognizable. Immunohistochemistry (Foxp3; brown color positive cells including circles). The presence of Tregs in asthmatic inflammation in the control group b and the asthma group c, d. There is no S.I. in the control. Counterstained with Mayer's hematoxylin. Number of Tregs per asthmatic inflammation is expressed per $1 \mu\text{m}^2 \times 200$.

M1/M2 Macrophages in Bronchi-Bronchioles Lesions

Compared to the control, there were severe infiltrations of arginase-1⁺ macrophages (alternative activated M2 macrophages) at the site of asthmatic inflammation in the asthma group (Fig. 5a). On the other hand, iNOS⁺ macrophages (classically activated M1 macrophages) were very few at the same inflamed bronchi-bronchioles in the asthma group (Fig. 5b).

Tregs in pLNs and Thymi

Tregs distributed in the cortex and medulla of pLNs (Fig. 6a) and thymus medulla (Fig. 6b) in the asthma and control groups (Fig. 6c, d). Tregs at pLNs and thymi in the

asthma group decreased compared to the control group (Fig. 6e, f; $P < 0.01$, two-tailed t test).

Proportion of Tregs in PBMCs

To clarify the Tregs change in the asthma group, quantitative analysis was performed with the flow cytometry (Fig. 7a). On day 18, there was a significant difference in the percent of Foxp3⁺/CD4⁺ and the ratio of Foxp3⁺/CD4⁺ between the asthma and control groups (Fig. 7b, c; $P < 0.05$, two-tailed t test). In addition, on day 18 compared on day 14, the ratio of Foxp3⁺/CD4⁺ at the effector phase, but not the induction phase, was significantly decreased in the asthma group, but not in the control group (Fig. 7c; $P < 0.05$, one-tailed t test but not two-tailed test). Moreover,

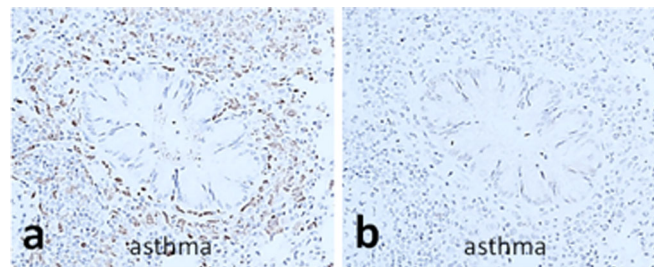


Fig. 5. M1/M2 macrophages in asthmatic inflammation. Compared to the control, numerous arginase-1⁺ macrophages (M2 macrophages) are observed at asthmatic inflammation in the asthma group (a). There are very few iNOS⁺ macrophages (M1 macrophages) in the asthma and control groups (b the asthma group). Representative immunohistochemistry (brown color positive cells) and counterstained with Mayer's hematoxylin. a, b $\times 200$.

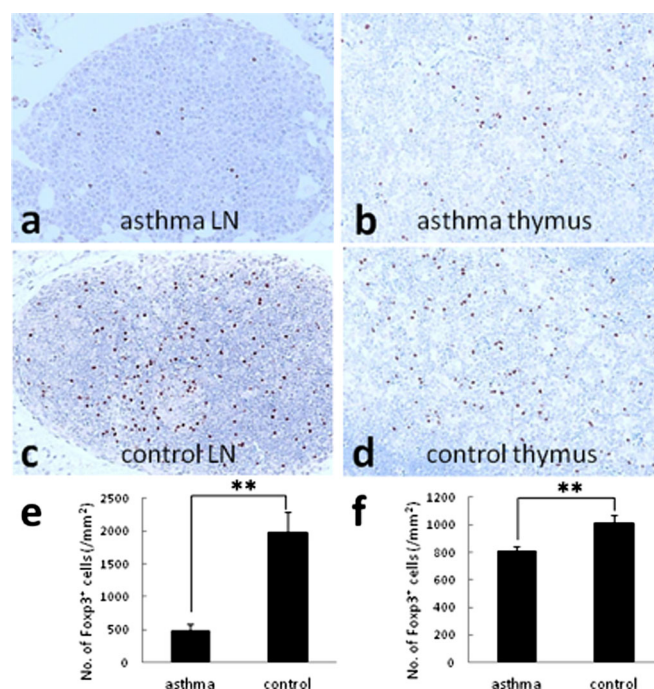


Fig. 6. Tregs in pLNs and thymi. Asthma group (a, b) and control group (c, d). Immunohistochemistry (Foxp3; brown color positive cells). Counterstained with Mayer's hematoxylin. $\times 200$. The presence of Tregs in pulmonary LN (e) and thymus (f) in the asthma and control groups. Numbers of Tregs per bronchus are expressed per 1 mm². Each value represents the mean $\pm$ SEM. $**P < 0.01$.

the percent of Foxp3⁺/CD4⁺ and the ratio of Foxp3⁺/CD4⁺ on day 18 compared on day 0 significantly increased in the control group (Fig. 7b, c; $P < 0.01$, two-tailed t test). The proportion of Tregs was inversely correlated with S.I. in each mouse (regression analysis) (Fig. 7d, e).

Systemic Production of OVA-Specific IgE in Plasma, IL-4, TGF- β 1, IL-10, and IFN- γ from Supernatants of Cultured Splenocytes with Concanavalin A and Local mRNA Expression for IL-4, TGF- β 1, IL-10, and IFN- γ in pLNs

OVA-specific IgE (Fig. 8a) and the production of IL-4 (Fig. 8b), but not IFN- γ (Fig. 8e), of the asthma group increased statistically ($P < 0.05$, two-tailed t test) compared to the control group. In addition, there was no significant difference in TGF- β 1 (Fig. 8c) and IL-10 (Fig. 8d) values between the asthma and control groups.

IL-4 mRNA expression of the asthma group increased statistically ($P < 0.05$, two-tailed t test) compared to the control group (Fig. 8f). In the asthma group, mRNA expression of TGF- β 1 (Fig. 8g; $P < 0.05$, one-tailed t test) and IL-10 (Fig. 8h; $P < 0.05$, one-tailed t test) decreased compared to the control group. There was no difference in

mRNA expression for IFN- γ between the asthma and control groups (Fig. 8i).

Relationship Between Production and mRNA Expression of Cytokines and Tregs Number or Relationship Between those of Cytokines and S.I.

As shown in Fig. 9a–d, there was no relationship between cytokine production and Tregs number in blood. There was no relationship between S.I. per mouse and IL-4, but not IFN- γ , including TGF- β 1 and IL-10 production (Fig. 9e–h).

There was a relationship between mRNA expressions for TGF- β 1, but not for IL-4 and IFN- γ , and Tregs number in blood (Fig. 9i–l). mRNA expression of IL-4, TGF- β 1, and IL-10, but not IFN- γ , was related with the degree of S.I. per mouse (Fig. 9m–o).

DISCUSSION

The present study clearly demonstrated the evidence of the development of Th2-dominant late allergic asthmatic inflammation [19] with decreased Tregs at the local sites such as pLNs and asthmatic inflammation with a systemic reduction of circulating Tregs during the effector phase, but

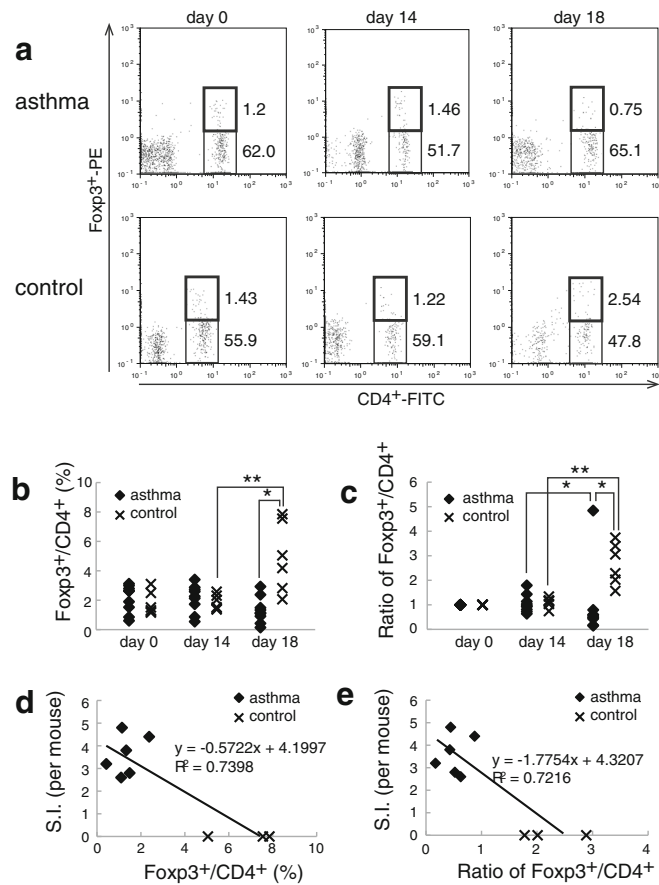


Fig. 7. Representative FACS analysis of Tregs in the asthma and control groups on day 0, 14, and 18, respectively (a). Percent of Foxp3⁺/CD4⁺ (b) and ratio of Foxp3⁺/CD4⁺ (c) based on those on day 0 given as the point 1 by flow cytometry analysis. Correlation analysis between d percent of Foxp3⁺/CD4⁺ and e ratio of Foxp3⁺/CD4⁺ and S.I. of asthmatic inflammation per one mouse. Trendline and correlation coefficient were shown. * $P<0.05$, ** $P<0.01$.

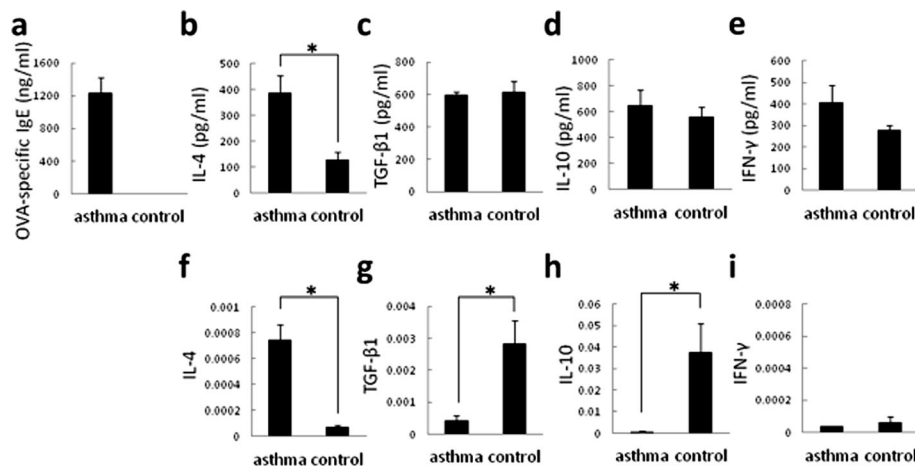


Fig. 8. Concentration of OVA-specific IgE in plasma (a) and values of IL-4 (b), TGF-β1 (c), IL-10 (d), and IFN-γ (e) in supernatants of cultured splenocyte cells stimulated with Con A detected by ELISAs. IL-4 (f), TGF-β1 (g), IL-10 (h), and IFN-γ (i) mRNA expression in pLNs detected by real-time PCR. Each value represents the mean±SEM. * $P<0.05$.

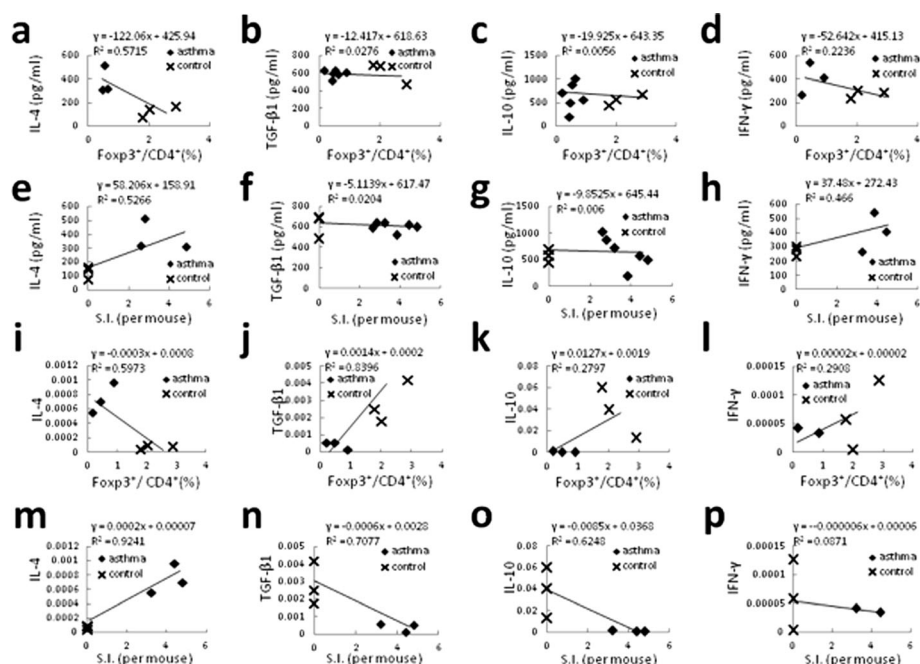


Fig. 9. Correlation analysis between systemic cytokine production and the ratio of Foxp3⁺/CD4⁺ (a–d) and S.I. of asthmatic inflammation (e–h) and between local (pLNs) mRNA expression and the ratio of Foxp3⁺/CD4⁺ (i–l) and S.I. (m–p) per one mouse. Trendlines and correlation coefficients are shown in each graph.

not at the phase of induction. These suggest that the systemic sensitization and the following exposure of OVA may evoke the reduction of Tregs at inflamed site resulting in the promotion of asthmatic inflammation. The parallel association of the reduced Tregs in the circulation and *in situ* accumulation of Tregs seems likely, since bronchial allergen inhalation in sensitized human patients (predominantly in those who did not develop significant bronchoconstriction) was associated with the decreased proportion of Tregs in the circulation [21]. In addition, exposure to allergen (house dust mite) in sensitized patients led to the decrease in circulating Tregs in an early response (6 h) in immediate reaction [21].

In each bronchus showed a different relationship between the S.I. of asthmatic inflammation and Tregs number within the same lung, although the development of asthmatic lesions and decrease in peripheral Tregs in asthma was inversely correlated. The less accumulation of Tregs led to severe bronchial lesions (Fig. 4c), whereas increased Tregs reduced the severity of bronchial lesions (Fig. 4d), suggesting the idea that Tregs may determine the outcome of the development of asthmatic inflammation by their cell-to-cell contact suppression with productions of immunosuppressive cytokines locally and systemically. Induction of inducible Tregs (iTregs) and naturally occurring Tregs

(nTregs) [32, 33], including allergen-specific Tregs [34] in animal models and human patients with allergic asthma have been reported. Also, Treg cells suppress allergen-specific Th1 and Th2 cell responses playing an important role in the physiological immune response to allergens [35]. Moreover, it has been reported recently that suppressor of cytokine signaling (SOCS) proteins especially SOCS2 expressed in both nTregs and iTregs and SOCS2 inhibited the development of Th2 cells and local allergic immune responses *in vivo* and *in vitro* by flow cytometry analysis [36].

The process of diseases may be determined by other suppressive mechanisms other than Tregs. For example, the pathogenesis of allergic asthma is orchestrated by a complex interplay of several cells such as immature dendritic cells, which maintain tolerance [37], suggesting that the direction of the development of asthmatic inflammation may be determined by the cooperation of different mechanisms. Taken together, dynamic changes may occur within each bronchus in terms of the development of asthmatic inflammation. Further study is needed to clarify those points. Decreased local mRNA expression, but not systemic production, of immunosuppressive cytokines such as TGF-β1 and IL-10 [10–12] was associated with the reduced number of Tregs, suggesting that decreased mRNA expression of those

cytokines may be due to the decreased number of Tregs [38–40]. Also, the decrease in Tregs, at least in part, may reflect the decrease in circulating Tregs with impairment of the quality and quantity of Tregs function. Alternatively, a decreased local IL-10 mRNA expression may decrease Tregs number, since it has been reported that IL-10 may differentiate from naïve helper T (Th0) to Tregs [41, 42] and that IL-10 mRNA expression was decreased in pLNs. In addition, another possibility is that the appearance of M2 macrophages may induce Tregs [43, 44]. If so, there are some contradictions since there were many M2 macrophages at inflamed bronchi. Further study is needed to clarify the relation between the role of M2 macrophages and the decrease in Tregs. Alternatively, the dissociation between systemic productions and local mRNA expression in TGF- β 1 and IL-10 was found, whereas the systemic productions of Th2 cytokines (e.g., IL-4 and IL-5) were paralleled with those local mRNA expression in our mouse model of late allergic asthma [22, 23, 45]. On the other hand, systemic TGF- β 1 and IL-10 responses do not mean local response. It seems likely, since TGF- β 1 or IL-10 will be produced by several cells (e.g., fibroblasts, endothelial cells, and Th3 cells other than Tregs [10].

We reported previously that the expression of vascular cell adhesion molecule (VCAM)-1 on endothelium and very late antigen (VLA)-4 on lymphocytes and eosinophils may contribute to the accumulation and infiltration of those cells at inflamed bronchi-bronchioles in a model of late allergic asthma [25]. On the other hand, accumulation and/or recruitment of Tregs were impaired at the site of severe lesions, but not less severe lesions, nevertheless those adhesion molecules were expressed in this model. It has been reported that tumor cells and microenvironmental macrophages in human produce the C-C chemokine ligand (CCL)-22, which mediate trafficking of Tregs to the tumor [46]. Normal blood Tregs express C-C chemokine receptor (CCR)4 and CCR8 and migrate in response to CCL1, CCL17, or CCL22 on the basis of *in vitro* assay [47], suggesting that the recruitment of Tregs and Th2 cells and eosinophils may use different adhesion molecules. Moreover, relatively many Tregs accumulated at the site of severe asthmatic inflammation. These suggest that the accumulation of Tregs may be determined by not only adhesion molecules but also other unknown factors.

In conclusion, the present study demonstrated the local presence of Tregs morphologically and suggests that *in situ* Tregs may, at least in part, regulate the development of asthmatic inflammation by cell-cell interaction and cytokine productions.

Conflict of Interest. We declare that we have no conflict of interest.

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