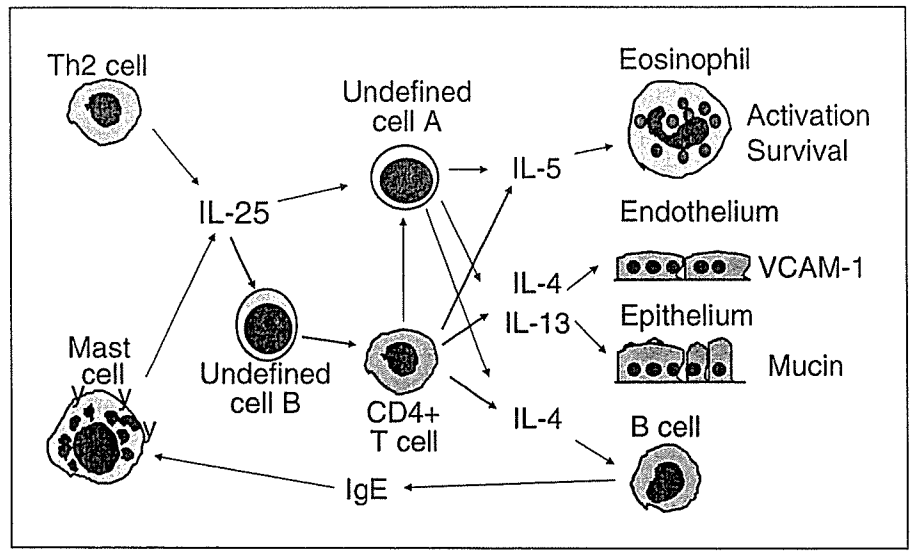

Fig. 1. Possible mechanisms for IL-25-mediated enhancement of Th2-type immune responses. Th2 cells and mast cells produce IL-25 upon activation. In a situation where IL-25 is abundant, IL-25 directly functions on a lineage-negative antigen-presenting cell (undefined cell A) to produce Th2 cytokines and then causes allergic inflammation [3, 4]. In contrast, in other situations where the amounts of IL-25 are limited, collaboration with CD4+ T cells is required for IL-25-mediated allergic inflammation. In this situation, IL-25 may induce the recruitment of antigen-specific CD4+ T cells to the site of allergic inflammation through the production of chemokines such as TARC by the undefined cell B and then may enhance CD4+ T-cell-mediated allergic inflammation. Because the amounts of the endogenously produced IL-25 in the lung of antigen-sensitized, antigen-inhaled mice seem to be limited, we speculate that the latter mechanism is mainly involved in the induction of allergic airway inflammation in a physiological setting. VCAM-1 = Vascular cell adhesion molecule 1.

and pathological changes in the multiple tissues [3–5]. Interestingly, previous studies have demonstrated that IL-25-induced Th2 cytokine production was observed even in mice lacking T cells [3, 5]. These findings suggest that once IL-25 is produced, IL-25 is capable of enhancing allergic inflammation even in the absence of Th2 cells.

Mast Cells Produce IL-25 upon Activation

Regarding IL-25-producing cells, a previous report has demonstrated that IL-25 mRNA is exclusively expressed in polarized Th2 cells [3]. However, quantitative RT-PCR analyses have revealed that IL-25 mRNA is detected in multiple tissues, including the colon, uterus, stomach, small intestine, kidney and lung [3, 4, 9]. Thus, it is suggested that, in addition to Th2 cells, other cell types would produce IL-25. In this regard, we have shown that when bone-marrow-derived mast cells are stimulated with IgE crosslinking, IL-25 mRNA is induced within 30 min and the levels of IL-25 mRNA are comparable with that of activated Th2 cells [10]. Therefore, mast cells are potent IL-25 producers, and mast-cell-derived IL-25 may be involved in the augmentation and/or prolongation of Th2-type immune response (fig. 1).

Role of IL-25 in Antigen-Induced Eosinophil Recruitment into the Airway

Antigen-induced allergic inflammation is induced by Th2 cells through the production of Th2 cytokines [11, 12]. In a murine model of allergic airway inflammation, we and others have provided evidence that IL-5-producing CD4+ T cells mediate antigen-induced eosinophil recruitment into the airways of sensitized mice [13, 14]. In addition, it has been shown that IL-13 is a key cytokine that induces goblet cell hyperplasia and airway hyperreactivity [15, 16]. Regarding the role of IL-25 in allergic inflammation, we have recently shown that IL-25 mRNA is detected in the airways of antigen-sensitized, antigen-inhaled mice [Tamachi et al., submitted], although we have not identified the IL-25-producing cells in the site of allergic airway inflammation. Furthermore, we have investigated the effect of soluble IL-25 receptor (sIL-

25R), which is able to neutralize the bioactivity of IL-25, on antigen-induced eosinophil and CD4+ T cell recruitment into the airways and have found that the injection of sIL-25R significantly inhibits antigen-induced eosinophil and CD4+ T cell recruitment into the airways [Tamachi et al., submitted]. Although we could not formally exclude the possibility that the other ligand IL-17B for IL-25R [8] is involved in the sIL-25R-mediated inhibition of antigen-induced eosinophil recruitment into the airways, it is suggested that IL-25 is the main target of sIL-25R, because the in vivo administration of IL-17B induces neutrophil but not eosinophil recruitment [8], whereas IL-25 induces eosinophil recruitment in many experimental systems including ours [3–5; Tamachi et al., submitted]. Further experiments using a neutralizing antibody specific for IL-25 or mice lacking IL-25 are needed to define the specific role of IL-25 in causing allergic airway inflammation.

Mechanisms Underlying IL-25-Mediated Eosinophil Recruitment into the Airways

To address the role of IL-25 in allergic airway inflammation, we generated CC10 IL-25 mice that express murine IL-25 specifically in the lung under the control of a rat CC10 promoter [Tamachi et al., submitted]. CC10 IL-25 mice exhibited no apparent abnormalities in cell populations in the thymus, spleen and peripheral blood. In addition, without the inhaled antigen challenge, no inflammatory cell infiltration was observed in the lung in CC10 IL-25 mice. However, antigen-induced eosinophil and CD4+ T cell recruitment into the airway was significantly enhanced in CC10 IL-25 mice as compared with that in wildtype mice. Th2 cytokine production in the airways was also increased in antigen-inhaled CC10 IL-25 mice. Importantly, depletion of CD4+ T cells by the in vivo administration of anti-CD4 antibody inhibited IL-25-mediated enhancement of eosinophil recruitment into the airways, indicating that CD4+ T cells are required for IL-25-mediated allergic inflammation. In contrast, previous studies have demonstrated that the intranasal administration of large amounts of recombinant IL-25 (5 μg/body) or the systemic expression of IL-25 induces Th2 cytokine production and eosinophil infiltration even in the absence of CD4+ T cells [3–5]. Together, these results suggest that IL-25 could induce allergic inflammation by two different mechanisms. In a situation where IL-25 is abundant, IL-25 itself is sufficient for causing allergic inflammation through the induction of IL-4, IL-5 and IL-13 from non-T/non-B cells. In contrast, in other situations where the amounts of IL-25 are limited, collaboration with CD4+ T cells is required for IL-25-mediated allergic inflammation. Because the levels of the endogenously produced IL-25 in the lung of antigen-sensitized, antigen-inhaled mice are lower than those of CC10 IL-25 mice, it is suggested that in a physiological setting, IL-25 needs antigen-activated CD4+ T cells to exert its function on allergic inflammation.

The mechanisms by which CD4+ T cells are required for IL-25-mediated allergic airway inflammation have not yet been elucidated. Because IL-25 does not directly enhance Th2 cell differentiation in vitro [3; our unpubl. data], it is unlikely that IL-25 enhances allergic inflammation through the induction of Th2 cell differentiation. On the other hand, it has been demonstrated that the expression of thymus and activation-regulated chemokine (TARC), a specific ligand for CC chemokine receptor 4 [17, 18], is enhanced in the lung upon IL-25 stimulation [4]. Because it has been shown that TARC induces chemotaxis of T cells, especially of Th2 cells [19, 20], and plays a significant role in the induction of Th2-cell-mediated eosinophil recruitment into the airways in a murine model of asthma [21], IL-25 may induce the recruitment of CD4+ T cells through TARC expression and may then enhance CD4+ T-cell-mediated allergic airway inflammation. However, it is also possible that some cytokines and/or chemokines produced by CD4+ T cells are required for the recruitment, differentiation and/or activation of undefined IL-25-responding cells (fig. 1). Future studies identifying IL-25-responding cells could help the understanding of the role of CD4+ T cells in IL-25-mediated enhancement of allergic airway inflammation.

In conclusion, although there has been no information available on the expression levels of IL-25 in allergic diseases in humans, accumulating evidence including ours raises the possibility that IL-25 may be involved in the enhancement and/or prolongation of Th2-cell-mediated allergic diseases such as asthma and allergic rhinitis and thus suggests that IL-25 could be a possible target of these diseases.

Acknowledgment

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■原著■

Paranasal sinus computed tomography (CT) images in patients with chronic sinusitis and bronchial asthma correlate with urinary leukotriene E4 level

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ABSTRACT

In bronchial asthma, leukotrienes (LT) are one of the factors exacerbating symptoms. Lately, it has been thought that the paranasal sinus is the source of LT. This study investigated the association between chronic sinusitis and LT level using images of paranasal sinus computed tomography (CT) in cases with bronchial asthma.

The type of bronchial asthma was classified into aspirin-tolerant asthma (ATA) and aspirin-intolerant asthma (AIA). Urinary leukotriene E4 (U-LTE4) was used as a marker of LT because it was a comparatively stable metabolic product of LT.

Quantity of shadow and shadow rate were measured using paranasal sinus CT, and these were correlated with U-LTE4 level.

In the ATA-group, there was no correlation between shadow quantity, shadow rate, and U-LTE4 level. On the other hand, in AIA-group, both shadow quantity and rate were correlated with U-LTE4 level. The relationship between aspirin asthma and LT suggested that surgery could lead to control of LT by reducing the amount of morbid mucous membrane.

Key words: paranasal sinus computed tomography, chronic sinusitis, urinary leukotriene E4, aspirin-intolerant asthma

INTRODUCTION

In bronchial asthma, leukotrienes are one of the factors exacerbating symptoms. Leukotrienes are thought to participate particularly in aspirin-intolerant asthma (AIA). Urinary leukotriene E4 (U-LTE4) was adopted as a marker of leukotrienes because it was a comparatively stable metabolic product of LT. Many patients with bronchial asthma have chronic sinusitis. It is reported that patients with chronic sinusitis and bronchial asthma have high U-LTE4 levels[1].

After ESS (Endoscopic Sinus Surgery), U-LTE4 falls significantly below preoperative levels in both aspirin-tolerant asthma (ATA) and AIA patients[1]. It is guessed that sinusitis contributes to the rise in U-LTE4. Correlation between sinusitis and U-LTE4 level has not been previously reported.

In this study, we examined the association between chronic sinusitis and U-LTE4, using paranasal sinus computed tomography (CT).

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MATERIALS AND METHODS

ESS was performed in our hospital in 22 patients with chronic sinusitis and bronchial asthma between December 2005 and January 2002. The type of bronchial asthma in 12 cases was ATA, and in 10 cases, AIA (Table 1). The asthma diagnosis was made by a doctor of internal medicine in our hospital. The characteristics of the asthma and its management (stage of progression, treatment, and disease severity) were not determined by the examination.

Paranasal sinus computed tomography

The paranasal sinus CT was performed using a SIEMENS multi-slice CT system, SOMATOM PLUS Volume Zone. On the axis that is parallel with OM-Line, CT images were taken every 1 mm. Magnification was the same in all cases. The captured CT images were transferred to a computer via CD-R media.

Analysis of paranasal sinus CT

Taking a report of Miyazaki into account[2], image slices were taken at equal intervals in each paranasal sinus (maxillary sinus, ethmoid sinus, frontal sinus, and sphenoid sinus) (Fig. 1). There were 3 slices of the maxillary sinus, and 2 each of the ethmoid sinus, frontal sinus, and sphenoid sinus. As a result, the paranasal sinus was separated into 9 slices in every

Table 1 Object cases

	M	F	age
ATA-group	7	5	51.46 ± 13.98
AIA-group	1	9	46.73 ± 4.73

Table 2 The details of slices in paranasal sinus

Maxillary sinus	3 slices	upper slice
		middle slice
		lower slice
Ethmoid sinus, Frontal sinus, Sphenoid sinus	2 slices	upper slice
		lower slice
Total 9 slices of a patient		

case (Table 2). The quantity (area) of shadow and shadow rate were measured to show the area of sinusitis and to evaluate the quantity of morbid mucous membrane. The total area of shadow in each slice was measured and the quantity of total shadow (pixels) was calculated. Disease severity of sinusitis was estimated by shadow rate or ratio (%), which is the total area of 9 slices divided by the total area of shadow (Fig. 2). Image analysis software "Image J" was used for image measurement. A measurement was performed in triplicate by one doctor, and the mean was reported.

Method of LTE4 measurement

The urine was collected preoperatively during stable asthma. Fat soluble compounds were extracted by passing urine through an Empore C18 column.

The LTE4 fraction was collected after high performance liquid chromatography (HPLC) and passaged through an Empore C18 again.

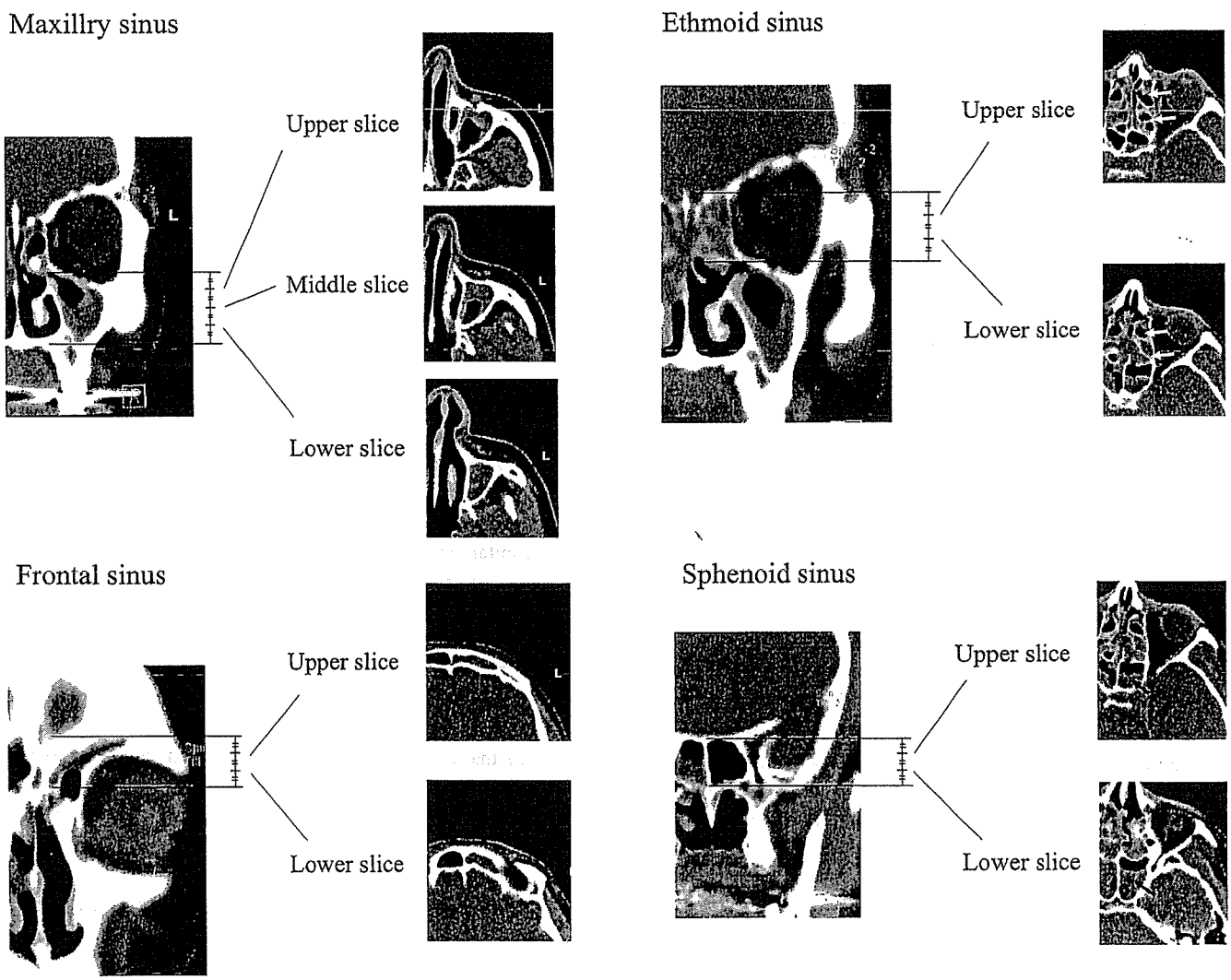
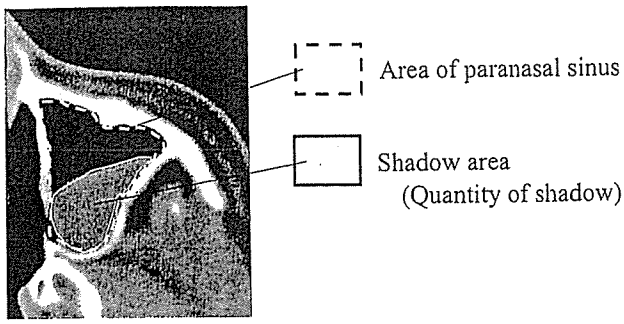


Fig. 1 Decision method of slice of images. Image slices were taken at equal intervals in each paranasal sinus. There were 3 slices of the maxillary sinus, and 2 each of the ethmoid sinus, frontal sinus, and sphenoid sinus. As an example, images of 53 years old man with ATA was shown. Through only left paranasal sinus were shown in Fig. 1, measurements were performed in both sides of paranasal sinus actually.



$$\text{Shadow rate} = \frac{\text{Total shadow area of 9 slices}}{\text{Total area of paranasal sinus in 9 slices}} \times 100(\%)$$

Fig. 2 Calculation method of shadow rate. Disease severity of sinusitis was estimated by shadow rate or ratio (%), which is the total area of 9 slices divided by the total area of shadow.

U-LTE4 was quantified by EIA and expressed on the basis of creatinine level (i.e., in picograms per milligram of creatinine). Differences in quantity of shadow, shadow rate, and U-LTE4 level between groups were evaluated by a t-test, and correlation between paranasal sinus CT and U-LTE4 level was evaluated by correlation analysis.

RESULT

In paranasal sinus CT, average amount of shadow in the ATA and AIA groups was 35,884.42±4579.06 (SD) pixels and 39,038.08±6288.78 (SD) pixels, respectively. Average shadow rate was 54.13±6.59 (SD)% and 62.05±6.48 (SD)%, respectively.

Though the amount of shadow and shadow rate was higher in the AIA group, the between-group difference was not significant (Fig. 3). The U-LTE4 value was 165.7±127.98 (SD) (pg/mg creatinine) in the ATA-group and 509.59±45.56 (SD) (pg/mg creatinine in the AIA-group. The between-group difference was not significant.

A correlation analysis was then performed for paranasal sinus CT and U-LTE4 value. No meaningful correlation between LTE4 value and quantity of shadow and shadow rate was found in the ATA group (Fig. 4). In contrast, the U-LTE4 value was significantly correlated with quantity of shadow and shadow rate in the AIA group (Fig. 5). In particular, the coefficient of correlation with quantity of shadow was large.

DISCUSSION

Many patients with bronchial asthma have chronic sinusitis[3]. Many of these patients have sense of smell disorder and resist treatment. The condition of a patient with sinusitis is consistent with bronchial asthma, and these clinical characteristics are remarkable in aspirin asthma. The mechanism of sinusitis coexisting with asthma is unknown, but systematic mechanism of a whole body is suggested.

It is suggested that the condition of a patient with simple sinusitis is different from one with both sinusitis and asthma. Leukotrienes participate in exacerbation of allergy-related inflammation and cause asthma and sinusitis symptoms to deteriorate[4][5]. In this investigation, the quantity and the rate of shadow due to sinusitis did not relate to U-LTE4 level in the ATA-group.

On the other hand, in the AIA-group, this correlation was significant and involved a larger amount of shadow than shadow rate. This result shows that production of leukotrienes is closely related to the amount of morbid mucous membrane in the paranasal sinus of AIA-group patients. In addition, it is considered that this relationship proves that the origin of these leukotrienes is the paranasal sinus. In the ATA-group, why leukotriene level did not correlate with shadow amount or rate is unknown, though the difference in paranasal sinus CT shadow between the ATA-group and AIA-group was not significant. We suggest that the dependence on leukotrienes may be higher in the AIA-group than the ATA-group.

If the origin of the leukotrienes is the paranasal sinus, treatment of sinusitis should lead to control of LT and be very important for treatment of systematic allergic disease. In other

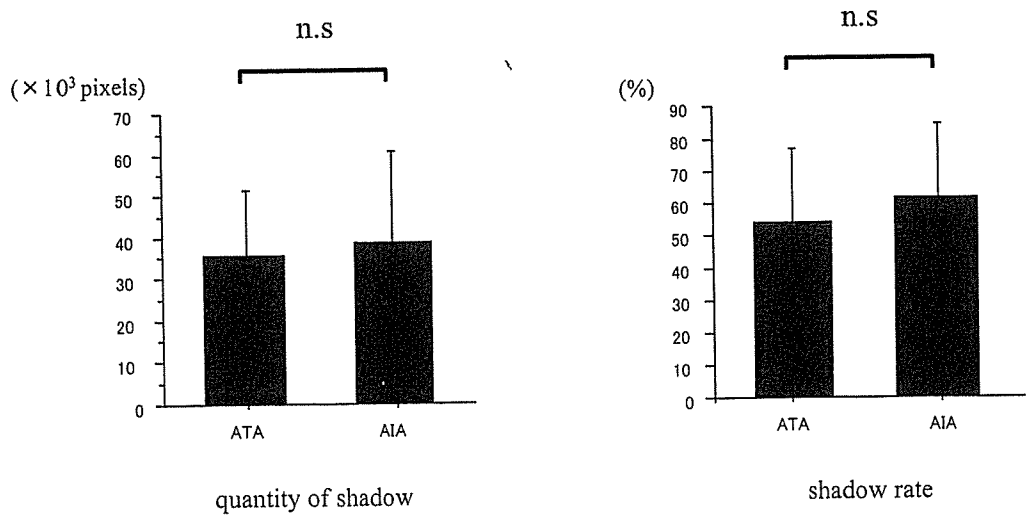


Fig. 3 Comparison of quantity of shadow and shadow rate. Though the amount of shadow and shadow rate was higher in the AIA group, the between-group difference was not significant.

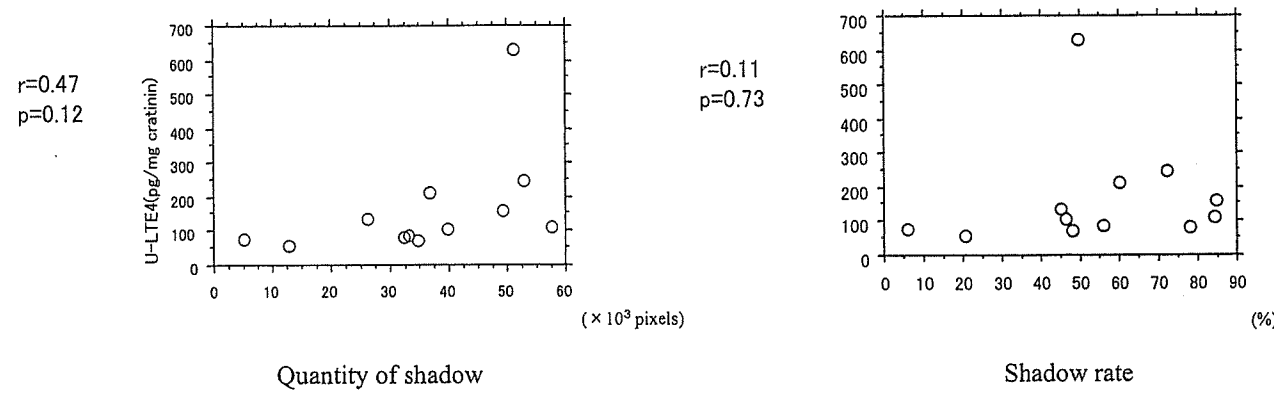


Fig. 4 Chronic sinusitis and U-LTE4 in ATA-group (N=12). No meaningful correlation between LTE4 value and quantity of shadow and shadow rate was found in the ATA group.

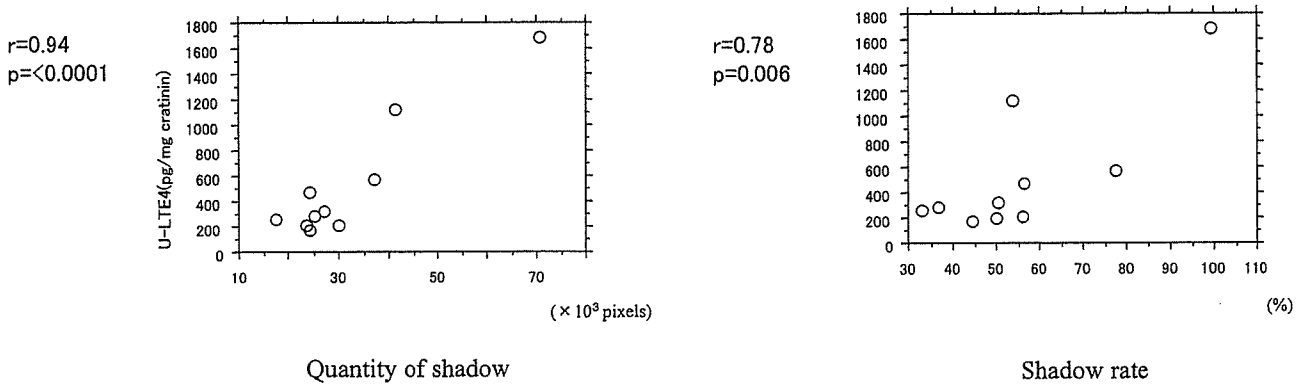


Fig. 5 Chronic sinusitis and U-LTE4 in ATA-group (N=10). The U-LTE4 value was significantly correlated with quantity of shadow and shadow rate in the AIA group.

words, in cases of sinusitis with aspirin asthma, we suggest that target treatment to control U-LTE4 level would be needed to reduce the weight of the morbid paranasal sinus mucous membrane.

The purpose of operative treatment for chronic sinusitis is to improve ventilation by opening the natural meatus. One author insists that this can be accomplished only by opening the middle nasal meatus enough in patients with AIA[6], but we do not agree. Rhinostenosis may be temporarily improved by reduction surgery. But without weight loss of the morbid mucous membrane, we suggest that control of leukotrienes is impossible. We consider that sinusitis with asthma should have been treated from a systematic standpoint.

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T-bet inhibits both T_H2 cell–mediated eosinophil recruitment and T_H17 cell–mediated neutrophil recruitment into the airways

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Background: Previous studies have shown that mice lacking T-bet, a critical transcription factor for T_H1 cell differentiation, spontaneously develop airway inflammation with intense eosinophil infiltrates. However, the mechanism underlying T-bet–mediated inhibition of allergic airway inflammation is still unknown.

Objective: To determine the regulatory role of T-bet in antigen-induced allergic airway inflammation.

Methods: We examined the role of T-bet in antigen-induced allergic airway inflammation using T-bet$^{-/-}$ mice on a BALB/c background that did not develop spontaneous airway inflammation. We also examined the role of T-bet expression of $CD4^+$ T cells in airway inflammation by adoptive transfer experiments.

Results: We found that antigen-induced eosinophil recruitment, goblet cell hyperplasia, and T_H2 cytokine production in the airways were enhanced in T-bet$^{-/-}$ mice. However, in the absence of signal transducer and activator of transcription 6 (STAT6), T-bet deficiency could not induce the antigen-induced eosinophilic airway inflammation. Adoptive transfer of T-bet$^{-/-}$ or T-bet$^{+/+}$ $CD4^+$ T cells to T-bet$^{-/-}$Rag-2$^{-/-}$ mice revealed that the expression of T-bet in $CD4^+$ T cells was vital for the inhibition of antigen-induced eosinophilic airway inflammation. Interestingly, antigen-induced neutrophil recruitment in the airways was also enhanced in T-bet$^{-/-}$ mice. Moreover, T-bet$^{-/-}$ $CD4^+$ T cells preferentially differentiated into IL-17–producing cells that mediated neutrophilic airway inflammation.

Conclusion: T-bet inhibits both T_H2 cell–mediated eosinophilic inflammation and T_H17 cell–mediated neutrophilic inflammation in the airways.

Clinical implications: The dysfunction of T-bet may be involved in the pathogenesis of severe asthma, in which accumulation of neutrophils as well as eosinophils in the airways is a hallmark of disease. (J Allergy Clin Immunol 2007;119:662-70.)

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Key words: Allergic inflammation, T-bet, Eosinophils, T_H2 cells, T_H17 cells

Asthma is characterized by allergic airway inflammation with intense eosinophil and $CD4^+$ T-cell infiltrates.[1-3] It has been demonstrated that T_H2 cell–derived cytokines such as IL-4, IL-5, IL-9, and IL-13 play critical roles in orchestrating and amplifying allergic inflammation in asthma.[1-3] On the other hand, IFN-γ, the principal T_H1 cell–derived cytokine, has been shown to prevent the development of antigen-induced allergic airway inflammation.[4,5] Thus, allergic airway inflammation is regulated by the balance between T_H2 cells and T_H1 cells.

It has recently been shown that mice lacking T-bet, a T-box transcription factor required for T_H1 cell differentiation and IFN-γ production,[6,7] spontaneously develop airway inflammation with intense eosinophil infiltrates and airway hyperreactivity.[8] It has also been shown that the number of $CD4^+$ T cells expressing T-bet is decreased in the airways of patients with asthma.[8] Moreover, polymorphisms of the T-bet gene have been reported to be associated with asthma and airway hyperresponsiveness.[9] These findings suggest that T-bet may be a negative regulator for allergic airway inflammation and airway hyperreactivity in both human beings and mice. However, the mechanism underlying T-bet–mediated inhibition of allergic airway inflammation is still largely unknown. Particularly, the spontaneous airway inflammation in T-bet–deficient (T-bet$^{-/-}$) mice makes it difficult to address the role of T-bet in the regulation of allergic airway inflammation provoked by the specific antigen.

Therefore, we examined the regulatory role of T-bet in antigen-induced allergic airway inflammation using T-bet$^{-/-}$ mice on a BALB/c background in which we unexpectedly found that spontaneous airway inflammation did not develop. We show here that the expression of T-bet in $CD4^+$ T cells is crucial not only for the inhibition of T_H2 cell–mediated eosinophil recruitment but also for the inhibition of IL-17–mediated neutrophil recruitment into the airways.

METHODS

Mice

T-bet$^{-/-}$ mice[7,8] on a BALB/c background (BALB/c T-bet$^{-/-}$ mice) and on a C57BL/6 background (C57BL/6 T-bet$^{-/-}$ mice) were

Abbreviations used
BALF: Bronchoalveolar lavage fluid
FACS: Fluorescence-activated cell sorting
HE: Hematoxylin-eosin
mEAR2: Mouse eosinophil-associated ribonuclease 2
OVA: Ovalbumin
PAS: Periodic acid-Schiff
WT: Wild-type

obtained from Jackson Laboratory (Bar Harbor, Me). BALB/c T-bet$^{-/-}$ mice were crossed with STAT6$^{-/-}$ mice[10] or Rag-2$^{-/-}$ mice to obtain T-bet$^{-/-}$ STAT6$^{-/-}$ mice or T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, respectively. Ovalbumin (OVA)-specific DO11.10^{+} T-cell receptor transgenic mice were backcrossed over 10 generations onto BALB/c mice and then crossed with BALB/c T-bet$^{-/-}$ mice to obtain DO11.10^{+} T-bet$^{-/-}$ mice. All mice were housed in microisolator cages under pathogen-free conditions, and all experiments were performed according to the guidelines of Chiba University.

Antigen-induced allergic inflammation in the airways

Mice (age 7-8 weeks) were immunized intraperitoneally twice with 6 μg OVA (Sigma Chemical Co, St Louis, Mo) in 4 mg aluminum hydroxide (alum) at 2-week intervals. Two weeks after the second immunization, the sensitized mice were given with aerosolized OVA (50 mg/mL) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer (DeVilbiss Corp, Somerset, Pa) for 20 minutes. As a control, 0.9% saline alone was administered by the nebulizer. Thirty-six hours after the inhalation, a sagittal block of left lung and trachea was excised, fixed in 10% buffered formalin, and embedded in paraffin. Sections of lung and trachea (3 μm thick) were stained with hematoxylin-eosin (HE), Luna, and periodic acid-Schiff (PAS) according to standard protocols. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per the length of the basement membrane of trachea as described previously.[11] The number of lymphocytes, eosinophils, and neutrophils recovered in the bronchoalveolar lavage fluid (BALF) was evaluated as described previously.[12] A fraction of the cells was subjected to a flow cytometric analysis for the lymphocyte surface phenotyping.

Cytokine levels in BALF

The amounts of IL-4, IL-5, and IFN-γ in the BALF were determined by ELISA kits from BD PharMingen (San Diego, Calif). The amounts of IL-13 and IL-17 in the BALF were determined by ELISA kits from R&D Systems Inc (Minneapolis, Minn). The detection limits of these assays were 15 pg/mL of IL-4 and IL-5, 1.5 pg/mL of IL-13, 5 pg/mL of IL-17, and 50 pg/mL of IFN-γ.

Flow-cytometric analysis

Cells from the BALF were stained and analyzed on a FACS Calibur (Becton Dickinson, San Jose, Calif) by using BD CellQuest Pro software (Becton Dickinson) as described previously.[12]

RT-PCR

Total cellular RNA was prepared from lung and RT-PCR for CD3ε and eosinophil-associated RNase 2 (mEAR2) was performed.[13] Primer pairs for mEAR2 were described previously.[14] Primer pairs for CD3ε were 5′caggacgatgccgagaacattgaa3′ and 5′tcatagtctgggttgggaacaggt3′. RT-PCR for β-actin was performed to control the sample-to-sample variation in RNA isolation and integrity, RNA input, and reverse transcription. All PCR amplifications were performed at least 3 times with multiple sets of experimental RNAs.

Antigen-induced T_H cell differentiation

Splenocytes (2 × 10^6/mL) from DO11.10^{+} T-bet$^{-/-}$ mice and the littermate DO11.10^{+} mice were stimulated with OVA323-339 peptide (50 μmol/L) in a 24-well microtiter plate at 37°C for 48 hours. Where indicated, IL-12 (20 ng/mL; R&D Systems Inc) was added to polarize toward T_H1 cells (T_H1 condition), and IL-4 (20 ng/mL; R&D Systems Inc) and anti–IFN-γ antibody (20 μg/mL; XMG1.2) was added to polarize toward T_H2 cells (T_H2 condition). Cells were washed with PBS and cultured for another 3 days in the same condition except that IL-2 (5 ng/mL; R&D Systems Inc) was added in nonpolarizing T_H0 condition and T_H1 condition. Intracellular cytokine analyses for IL-4, IL-17, and IFN-γ were performed by using anti–IL-4 phycoerythrin (BVD4-1D11; BD PharMingen), anti–IL-17 phycoerythrin (TC11-18H10.1; BD PharMingen), and anti–IFN-γ allophycocyanin (APC) (XMG1.2; BD PharMingen) according to the manufacturer's instructions. Detection of intracellular IL-13 was performed with biotinylated anti–IL-13 antibody (R&D Systems Inc) and streptavidin-APC (BD PharMingen).

Adoptive transfer experiments for antigen-induced airway inflammation

Splenic CD4^{+} T cells were purified (>95% pure by flow cytometry) using T cell enrichment columns (R&D Systems Inc) from DO11.10^{+} T-bet$^{-/-}$ mice and DO11.10^{+} mice as described previously.[13] CD4^{+} T cells (2 × 10^6 cells/mouse) were transferred intravenously to T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, and these mice were then immunized twice with 6 μg OVA in 4 mg alum at 2-week intervals. Two weeks after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/mL) in saline. Where indicated, anti-murine IL-17 antibody (150 μg/mouse; clone 50104; R&D Systems Inc) or control rat IgG_{2a} (150 μg/mouse) was administered intraperitoneally at 12 hours before the inhaled OVA challenge. At 36 hours after the OVA inhalation, the number of lymphocytes, eosinophils, and neutrophils in the BALF and cytokine levels in the BALF was evaluated.

Data analysis

Data are summarized as means ± SDs. The statistical analysis of the results was performed by the unpaired *t* test. *P* values < .05 were considered significant.

RESULTS

Spontaneous airway inflammation does not develop in BALB/c T-bet$^{-/-}$ mice

It has been shown that T-bet$^{-/-}$ mice develop peribronchial inflammation with eosinophil and lymphocyte infiltrates in the absence of inhaled antigen challenge.[8] To determine the role of T-bet in the regulation of allergic airway inflammation in more detail, we first examined airway inflammation in T-bet$^{-/-}$ mice on a T_H2-biased BALB/c background (BALB/c T-bet$^{-/-}$ mice) in parallel with T-bet$^{-/-}$ mice on a T_H1-biased C57BL/6 background (C57BL/6 T-bet$^{-/-}$ mice). Consistent with the previous report,[8] C57BL/6 T-bet$^{-/-}$ mice developed peribronchial inflammation with eosinophil and lymphocyte infiltrates in the absence of antigen challenge (Fig 1, *A*). However, unexpectedly, BALB/c T-bet$^{-/-}$ mice did not develop peribronchial inflammation in the absence of

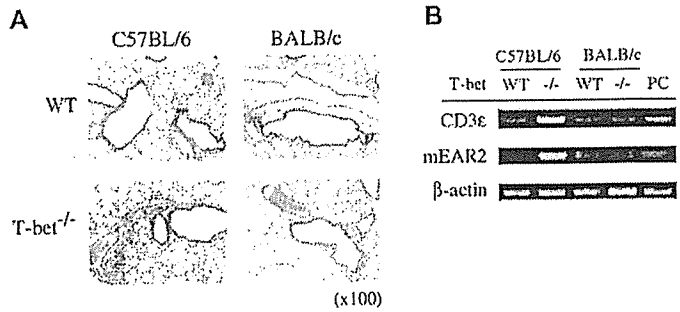

FIG 1. Spontaneous airway inflammation develops in T-bet$^{-/-}$ mice on a C57BL/6 background but not on a BALB/c background. A, Representative photomicrographs of HE-stained lung sections from 12-week-old T-bet$^{-/-}$ mice and littermate WT mice on either C57BL/6 or BALB/c background (x100; n = 5 mice in each group). B, RT-PCR analyses for CD3ε and mEAR2 mRNA were performed on lung tissues of T-bet$^{-/-}$ mice and WT mice on either C57BL/6 or BALB/c background (n = 5). A positive control *(PC)* is lung tissues of OVA-sensitized, OVA-inhaled BALB/c mice.

antigen challenge (Fig 1, *A*). RT-PCR analysis for CD3ε and mEAR2, one of orthologues of human eosinophil–derived neurotoxin,[15] of the lung tissue confirmed the presence of T cells and eosinophils in C57BL/6 T-bet$^{-/-}$ mice but not in BALB/c T-bet$^{-/-}$ mice (Fig 1, *B*). These results indicate that the induction of allergic airway inflammation by the absence of T-bet depends on the genetic backgrounds of mice.

Antigen-induced allergic airway inflammation is enhanced in BALB/c T-bet$^{-/-}$ mice

We then addressed the role of T-bet in antigen-induced airway inflammation in BALB/c T-bet$^{-/-}$ mice. OVA-sensitized BALB/c T-bet$^{-/-}$ mice and littermate wild-type (WT) mice were challenged with inhaled OVA, and histologic analysis of the lung was performed at 36 hours after the OVA inhalation. As shown in Fig 2, *A*, antigen-induced inflammatory cell infiltration in the peribronchial and perivascular areas was significantly enhanced in T-bet$^{-/-}$ mice compared with WT mice, and the majority of cells infiltrating into the lung were eosinophils (Fig 2, *A, a vs b*). Consequently, the number of eosinophils infiltrating into the submucosal tissue of trachea was significantly increased by 88% in T-bet$^{-/-}$ mice (n = 8 mice in each group; $P < .01$; Fig 2, *A, c vs d*). Antigen-induced eosinophil recruitment into the trachea at 72 hours after the OVA inhalation was also significantly increased in T-bet$^{-/-}$ mice (data not shown). In addition, antigen-induced epithelial goblet cell hyperplasia was increased in T-bet$^{-/-}$ mice compared with that in WT mice (Fig 2, *A, e vs f*).

Consistent with the histologic analysis, the number of eosinophils recovered in BALF at 36 hours after the OVA inhalation was significantly increased in T-bet$^{-/-}$ mice (n = 8 mice in each group; $P < .01$; Fig 2, *B*). The number of neutrophils recovered in the BALF at 36 hours was also increased in T-bet$^{-/-}$ mice (n = 8; $P < .05$; Fig 2, *B*). On the other hand, the number of lymphocytes in the BALF was not significantly increased in T-bet$^{-/-}$ mice (Fig 2, *B*), and fluorescence-activated cell sorting (FACS) analysis of lymphocytes in the BALF revealed that the majority of lymphocytes were T-cell receptor (TCR)$\alpha\beta^+$CD4$^+$ T cells in both T-bet$^{-/-}$ mice and WT mice (data not shown).

The levels of IL-4, IL-5, and IL-13 in the BALF at 36 hours after the OVA challenge were significantly increased in T-bet$^{-/-}$ mice (n = 5; $P < .01$, $P < .05$, and $P < .05$, respectively; Fig 2, *C*). On the other hand, IFN-γ was undetectable in both WT mice and T-bet$^{-/-}$ mice at 36 hours after the OVA challenge (data not shown). Taken together, these results suggest that T-bet is crucial for the inhibition of antigen-induced T_H2 cytokine production and eosinophil recruitment in the airways.

The enhanced antigen-induced eosinophil recruitment but not neutrophil recruitment into the airways of T-bet$^{-/-}$ mice depends on STAT6

We next examined whether STAT6, a critical transcription factor for T_H2 cell differentiation under IL-4 signaling,[16] was involved in the enhanced antigen-induced allergic airway inflammation in T-bet$^{-/-}$ mice by generating mice lacking both T-bet and STAT6 (T-bet$^{-/-}$ STAT6$^{-/-}$ mice). Again, the number of eosinophils and neutrophils recovered in the BALF was significantly increased in T-bet$^{-/-}$ mice as compared with that in littermate WT mice (n = 5 each; $P < .01$ and $P < .05$, respectively; Fig 3, *A*). Antigen-induced eosinophil recruitment into the airways in T-bet$^{-/-}$STAT6$^{-/-}$ mice was significantly decreased compared with that in T-bet$^{-/-}$ mice (n = 5 each; $P < .001$; Fig 3, *A*), suggesting that STAT6 is essential for the enhanced antigen-induced eosinophil recruitment in T-bet$^{-/-}$ mice. Histological analysis of the lung confirmed that STAT6 was essential not only for the enhancement of antigen-induced peribronchial inflammation with eosinophil infiltrates (Fig 3, *B, upper panels*) but also for the goblet cell hyperplasia (Fig 3, *B, bottom panels*) in T-bet$^{-/-}$ mice. These results indicate that STAT6 plays a dominant role over T-bet–mediated inhibition in the induction of T_H2 cell–mediated airway inflammation. In contrast, antigen-induced neutrophil recruitment into the airways of T-bet$^{-/-}$ mice was not

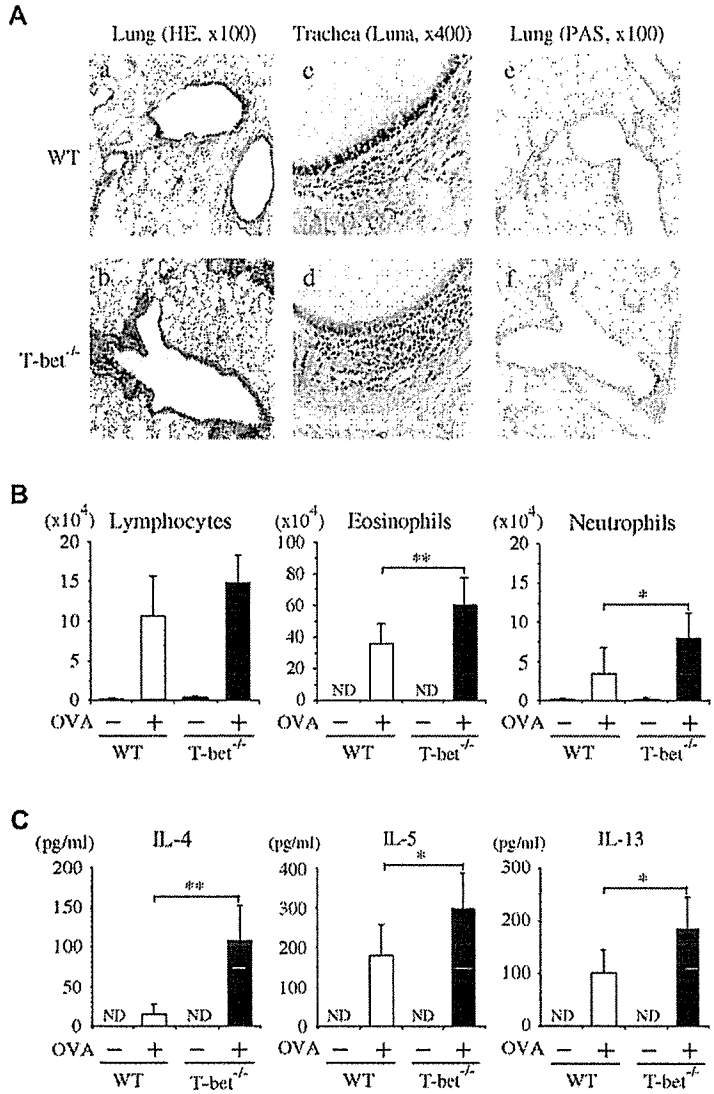

FIG 2. Antigen-induced eosinophil and neutrophil recruitment into the airways is enhanced in T-bet$^{-/-}$ mice. **A-C,** OVA-sensitized T-bet$^{-/-}$ mice and WT mice on a BALB/c background were challenged with the inhalation of OVA. *A,* At 36 hours after the OVA inhalation, lung and trachea were removed and sections were stained with HE, PAS, or Luna (n = 5). The number of lymphocytes, eosinophils, and neutrophils *(B)* and the levels of IL-4, IL-5, and IL-13 *(C)* in the BALF were evaluated at 36 hours after the OVA inhalation. Data are means ± SDs (n = 8 for *B* and 5 for *C,* respectively). *$P < .05$; **$P < .01$. *ND,* Not detectable.

significantly affected by the absence of STAT6 (Fig 3, *A*), suggesting that the mechanism underlying the neutrophil recruitment enhanced by the absence of T-bet is different from that underlying the enhanced eosinophil recruitment.

Differentiation of IL-13–producing CD4$^+$ T cells is increased in T-bet$^{-/-}$ CD4$^+$ T cells

To address the role of T-bet in antigen-induced T_H cell differentiation, antigen-induced T_H cell differentiation in DO11.10$^+$T-bet$^{-/-}$CD4$^+$ T cells was compared with that in DO11.10$^+$CD4$^+$ T cells. As shown in Fig 4, *A*, when splenocytes from DO11.10$^+$T-bet$^{-/-}$ mice were stimulated with antigenic peptide (OVA323-339) in nonpolarizing T_H0 condition, T_H1 cells (IL-4$^-$IFN-γ^+ cells) were decreased but T_H2 cells (IL-4$^+$IFN-γ^- cells) were increased compared with those in DO11.10$^+$ mice (n = 5 mice in each group). T_H1 cell differentiation was still significantly reduced in DO11.10$^+$ T-bet$^{-/-}$CD4$^+$ T cells even when IL-12 was added to induce T_H1 cell differentiation (T_H1 condition; n = 5; Fig 4, *A*). On the other hand, in T_H2 polarizing condition (in the presence of IL-4 and anti-IFN-γ antibody), the number of T_H2 cells (IL-4$^+$ IFN-γ^- cells) was similar between DO11.10$^+$T-bet$^{-/-}$ CD4$^+$ T cells and DO11.10$^+$CD4$^+$ T cells (Fig 4, *A*).

We also investigated IL-13 production in DO11.10$^+$ T-bet$^{-/-}$ CD4$^+$ T cells at single cell levels. Interestingly, in nonpolarizing T_H0 condition, CD4$^+$ T cells that produce IL-13 but not IL-4 were significantly

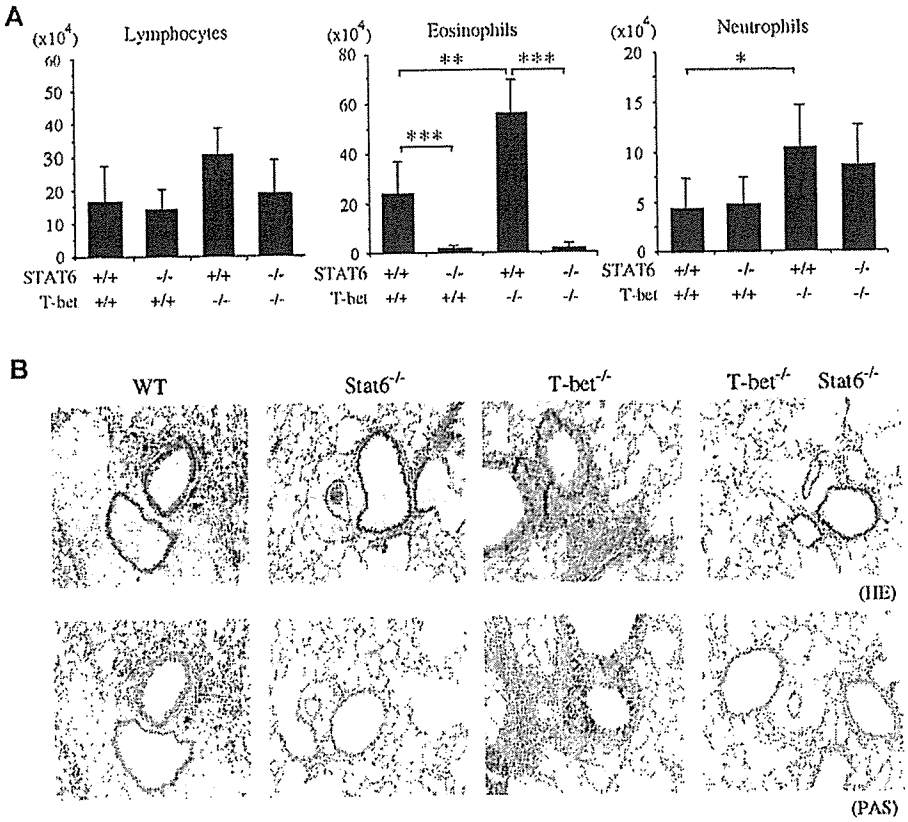

FIG 3. The enhanced antigen-induced eosinophil recruitment but not neutrophil recruitment into the airways of T-bet$^{-/-}$ mice depends on STAT6. **A and B,** OVA-sensitized T-bet$^{-/-}$ mice, STAT6$^{-/-}$ mice, T-bet$^{-/-}$STAT6$^{-/-}$ mice, and littermate WT mice were challenged with the inhalation of OVA. *A,* The number of lymphocytes, eosinophils, and neutrophils in the BALF was evaluated at 36 hours after the OVA inhalation. Data are means ± SDs for 5 mice in each group. *$P < .05$; **$P < .01$; ***$P < .001$. *B,* Representative photomicrographs of HE staining *(upper panels)* or PAS staining *(bottom panels)* of lung sections are shown (n = 5).

increased in DO11.10^{+}T-bet$^{-/-}$CD4^{+} T cells compared with those in DO11.10^{+}CD4^{+} T cells (Fig 4, *B*). The measurement of IL-13 levels in the supernatant of antigen-stimulated CD4^{+} T cells confirmed the enhanced IL-13 production in DO11.10^{+}T-bet$^{-/-}$CD4^{+} T cells (data not shown). These results suggest that in addition to the T-bet's role in inducing T_H1 cell differentiation, T-bet may be required for the downregulation of IL-13 production during antigen-specific CD4^{+} T-cell differentiation.

T-bet expression in CD4^{+} T cells is vital for the inhibition of antigen-induced eosinophil recruitment into the airways

It has been shown that T-bet is expressed and exhibits its function not only in CD4^{+} T cells but also in CD8^{+} T cells, B cells, and nonlymphoid cells.[17-19] To determine whether the expression of T-bet in CD4^{+} T cells is vital for the inhibition of antigen-induced allergic airway inflammation, we performed the adoptive transfer experiments of T-bet$^{-/-}$CD4^{+} T cells. To eliminate the influence of endogenous immune response during the sensitization and the inhaled antigen challenge as well as the influence of T-bet expression in nonlymphoid cells in recipient mice, we used T-bet$^{-/-}$Rag-2$^{-/-}$ mice as recipient mice. CD4^{+} T cells from DO11.10^{+}T-bet$^{-/-}$ mice or littermate DO11.10^{+} mice were transferred to T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, and these mice were immunized twice with OVA/alum and then challenged with the inhaled OVA. As shown in Fig 5, *A*, when CD4^{+} T cells from DO11.10^{+} mice were transferred to T-bet$^{-/-}$Rag-2$^{-/-}$ mice, eosinophil recruitment into the airways was induced at 36 hours after OVA inhalation. As expected, in the absence of the CD4^{+} T-cell transfer or the OVA inhalation, few eosinophils were detected in the BALF (data not shown), indicating that both the transferred CD4^{+} T cells and the OVA challenge are required for the eosinophil recruitment in this system. When T-bet$^{-/-}$Rag-2$^{-/-}$ mice were transferred with CD4^{+} T cells from DO11.10^{+} T-bet$^{-/-}$ mice, antigen-induced eosinophil recruitment into the airways was significantly increased compared with those transferred with CD4^{+} T cells from DO11.10^{+} mice (n = 5; $P < .005$; Fig 5, *A*). Moreover, IL-13 was increased but IFN-γ was decreased in the BALF in the mice transferred with DO11.10^{+}T-bet$^{-/-}$ CD4^{+} T cells (n = 5; $P < .005$; Fig 5, *B*). These results suggest that the expression of T-bet in CD4^{+} T cells is

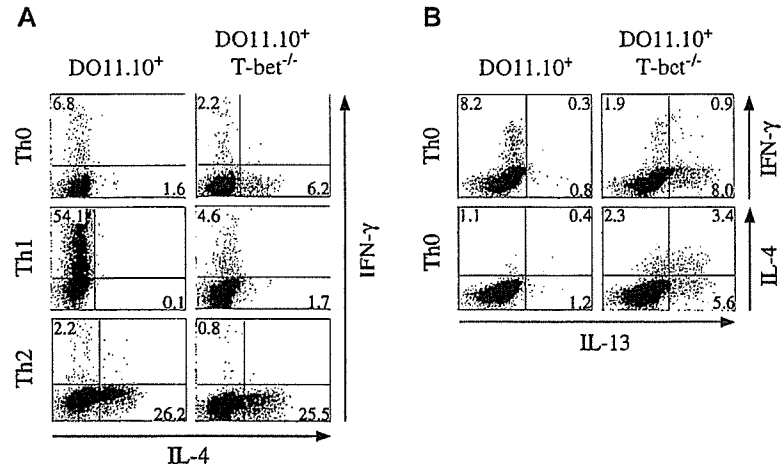

FIG 4. Differentiation of IL-13-producing $CD4^+$ T cells is increased in T-$bet^{-/-}$ mice. A, Splenocytes from DO11.10^+ T-$bet^{-/-}$ mice or littermate DO11.10^+ mice were stimulated with OVA323-339 peptide in T_H0, T_H1, or T_H2 condition and intracellular cytokine profiles (IL-4 vs IFN-γ) of $CD4^+$ T cells were determined. Shown are representative FACS profiles (n = 5). B, Similar to *A*, intracellular cytokine profiles (IL-13 vs IFN-γ and IL-13 vs IL-4) were determined on $CD4^+$ T cells in T_H0 condition (n = 5).

vital for the inhibition of antigen-induced T_H2 cytokine production and eosinophil recruitment in the airways.

T-bet expression in $CD4^+$ T cells is vital for the downregulation of antigen-induced IL-17 production and neutrophil recruitment in the airways

Interestingly, in addition to antigen-induced eosinophil recruitment, antigen-induced neutrophil recruitment was significantly enhanced in the mice transferred with DO11.10^+T-$bet^{-/-}CD4^+$ T cells (Fig 5, *A*). Moreover, the levels of IL-17, a representative cytokine that induces neutrophil-rich inflammation,[20] in the BALF were profoundly increased in mice transferred with DO11.10^+ T-$bet^{-/-}CD4^+$ T cells (n = 5; $P < .005$; Fig 5, *B*). To examine whether IL-17 is involved in the enhanced neutrophil recruitment in the mice transferred with DO11.10^+T-$bet^{-/-}CD4^+$ T cells, a neutralizing antibody against IL-17 was administered to the mice at 12 hours before the inhaled OVA challenge. As shown in Fig 5, *C*, antigen-induced neutrophil recruitment but not eosinophil recruitment in the airways was significantly inhibited by the administration of anti–IL-17 antibody (n = 4; $P < .05$). These results suggest that the expression of T-bet in $CD4^+$ T cells is involved in the suppression of IL-17 production and subsequent neutrophil recruitment in the airways on antigen inhalation.

T-bet inhibits the differentiation of IL-17–producing $CD4^+$ T cells

It has recently been suggested that IL-17–producing $CD4^+$ T cells (T_H17 cells) are a distinct lineage from T_H1 cells and T_H2 cells,[21] and that IFN-γ and T-bet can repress the differentiation of T_H17 cells.[22-24] Therefore, we finally examined the role of T-bet in the regulation of IL-17–producing $CD4^+$ T cells by using DO11.10^+CD4^+ T cells. As shown in Fig 6, when splenocytes from DO11.10^+ T-$bet^{-/-}$ mice were stimulated with OVA323-339 in nonpolarizing T_H0 condition, T_H17 cells (IL-17^+IFN-γ^- cells) were consistently increased compared with those in DO11.10^+ mice (n = 5 mice in each group). In T_H1 polarizing condition, the number of T_H17 cells in DO11.10^+T-$bet^{-/-}CD4^+$ T cells was also significantly higher than that in DO11.10^+CD4^+ T cells. These results suggest that T-bet is involved in the inhibition of antigen-induced T_H17 cell differentiation.

DISCUSSION

In the current study, we show that the expression of T-bet in $CD4^+$ T cells is crucial not only for the inhibition of T_H2 cell–mediated eosinophilic inflammation in the airways but also for the inhibition of T_H17 cell–mediated neutrophilic inflammation in the airways. We found that antigen-induced eosinophil recruitment, goblet cell hyperplasia, and T_H2 cytokine production in the airways were enhanced in BALB/c T-$bet^{-/-}$ mice (Fig 2). However, in the absence of STAT6, T-bet deficiency could not induce the antigen-induced eosinophilic airway inflammation (Fig 3). Adoptive transfer of T-$bet^{-/-}$ or T-$bet^{+/+}$ $CD4^+$ T cells to T-$bet^{-/-}$Rag-$2^{-/-}$ mice revealed that the expression of T-bet in $CD4^+$ T cells was vital for the inhibition of antigen-induced eosinophil recruitment into the airways (Fig 5). Interestingly, we also found that antigen-induced, IL-17–mediated neutrophil recruitment into the airways was enhanced in T-$bet^{-/-}$ mice (Figs 2, 3, and 5) and that T-$bet^{-/-}CD4^+$ T cells preferentially differentiated into T_H17 cells that mediated neutrophilic inflammation in the airways (Figs 5 and 6). Taken together, these results indicate that the expression of T-bet in $CD4^+$ T cells is crucial for the inhibition of the differentiation of both T_H2 cells and T_H17 cells and thereby for the downregulation of T_H2 cell–mediated eosinophilic

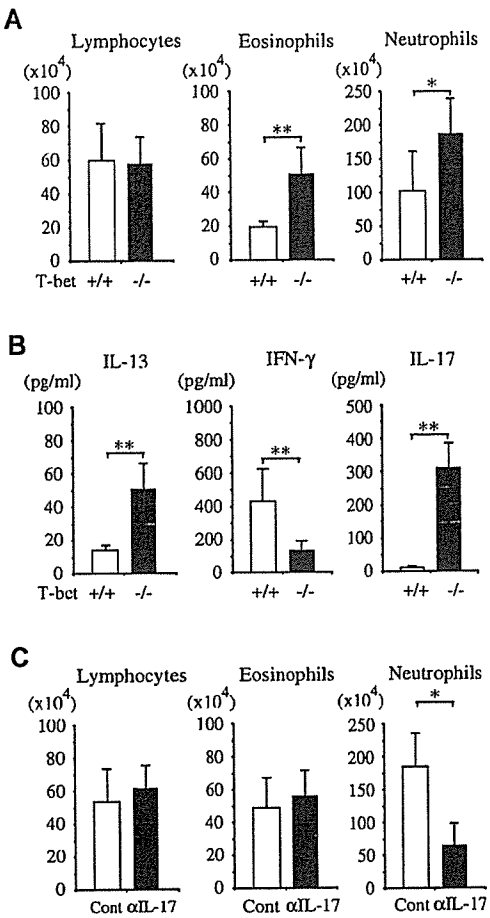

FIG 5. Expression of T-bet in $CD4^+$ T cells is vital in the inhibition of antigen-induced eosinophil and neutrophil recruitment into the airways. **A and B,** Purified $CD4^+$ T cells from $DO11.10^+$ $T\text{-}bet^{-/-}$ mice or $DO11.10^+$ mice were transferred intravenously to $Rag\text{-}2^{-/-}$ $T\text{-}bet^{-/-}$ mice. These mice were immunized with OVA/alum and then challenged with the inhaled OVA. Thirty-six hours later, the number of lymphocytes, eosinophils, and neutrophils *(A)* and the levels of IL-13, IL-17, and IFN-γ *(B)* in the BALF were evaluated. Data are means ± SDs (n = 5). $*P < .05$; $**P < .005$. IL-4 and IL-5 were not detected in both groups. **C,** Similar to *A*, $DO11.10^+$ $T\text{-}bet^{-/-}CD4^+$ T cells were transferred to $Rag\text{-}2^{-/-}T\text{-}bet^{-/-}$ mice, and these mice were immunized with OVA/alum. At 12 hours before the inhaled OVA challenge, these mice were injected intraperitoneally with antimurine IL-17 antibody or control rat IgG_{2a}. The number of lymphocytes, eosinophils, and neutrophils in the BALF was evaluated at 36 hours after the OVA inhalation. Data are means ± SDs (n = 4). $*P < .05$. *Cont*, Control.

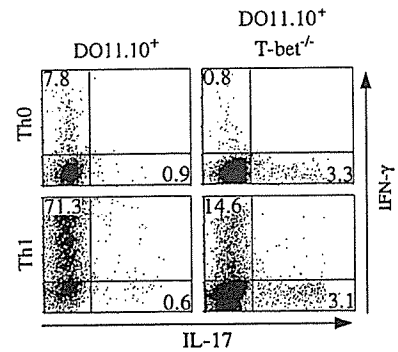

FIG 6. Differentiation of IL-17–producing $CD4^+$ T cells is increased in $T\text{-}bet^{-/-}CD4^+$ T cells. Splenocytes from $DO11.10^+T\text{-}bet^{-/-}$ mice or $DO11.10^+$ mice were stimulated with OVA323-339 peptide in T_H0 or T_H1 polarizing condition. Intracellular cytokine profiles (IL-17 vs IFN-γ) were determined on $CD4^+$ T cells. Shown are representative FACS profiles (n = 5).

inflammation and T_H17 cell–mediated neutrophilic inflammation in the airways.

We demonstrate that T-bet inhibits antigen-induced T_H2 cell–mediated allergic airway inflammation, as evidenced by enhanced antigen-induced eosinophilic airway inflammation in BALB/c $T\text{-}bet^{-/-}$ mice (Figs 2 and 5). It has been shown that allergic eosinophilic airway inflammation is mediated by T_H2 cells and also that the T_H1/T_H2 cell balance is important for regulating allergic airway inflammation.[1,2,25] STAT6 is clearly a key transcription factor that induces antigen-induced eosinophilic airway inflammation[1,2,25] (Fig 3). T-bet is a T_H1-specific transcription factor that controls T_H1 cell differentiation through the transcriptional activation of the IFN-γ gene and upregulation of IL-12Rβ2 chain expression.[6,7,26] Moreover, T-bet represses T_H2 cytokine genes independently of IFN-γR signaling.[6] Thus, our results obtained by generating $T\text{-}bet^{-/-}STAT6^{-/-}$ mice indicate that Stat6 is essential for T_H2 cell–mediated allergic airway inflammation and dominates over T-bet–mediated suppression of antigen-induced allergic airway inflammation (Fig 3).

We also show that in addition to conventional T_H2 cells, $CD4^+$ T cells that produce IL-13 but not IL-4 are increased by the absence of T-bet (Fig 4). By using $DO11.10^+$ T-cell receptor transgenic system, we found that the frequency of $CD4^+$ T cells that produce IL-13 but not IL-4 was significantly increased in $DO11.10^+$ $T\text{-}bet^{-/-}CD4^+$ T cells compared with that in $DO11.10^+$ $CD4^+$ T cells (Fig 4). Recently, a substantial body of evidence points to a nonredundant role for IL-13 in the regulation of allergic airway inflammation and goblet cell hyperplasia.[25] More recently, it has been demonstrated that neutralization of IL-13 but not IL-4 results in the suppression of spontaneous eosinophilic airway inflammation in C57BL/6 $T\text{-}bet^{-/-}$ mice.[27] Further studies uncovering the mechanism by which T-bet suppresses IL-4 and IL-13 differently may provide a possible target for bronchial asthma.

We unexpectedly found that BALB/c $T\text{-}bet^{-/-}$ mice did not develop airway inflammation spontaneously (Fig 1), whereas we confirmed that C57BL/6 $T\text{-}bet^{-/-}$ mice, which were bred in the same conditions as BALB/c $T\text{-}bet^{-/-}$ mice, spontaneously developed airway inflammation with intense eosinophil and lymphocyte infiltrates[8] (Fig 1). Therefore, the difference in the environmental factors could not account for the absence of spontaneous airway inflammation in BALB/c $T\text{-}bet^{-/-}$ mice. BALB/c mice have been reported to exhibit predominantly T_H2 responses to exogenous antigens, whereas C57BL/6 mice exhibit T_H1 dominant responses to the same

stimuli.[28] Indeed, BALB/c mice exhibit significantly higher levels of antigen-induced eosinophil recruitment in the airways than C57BL/6 mice.[29] In addition, antigen-induced eosinophil recruitment into the airways was high in BALB/b (H-2^b) mice, like BALB/c mice, but low in B10.D2 (H-2^d) mice,[29] suggesting that the BALB background rather than the H-2 haplotype is vital for the enhanced antigen-induced eosinophil recruitment in the airways. Currently, the exact reason why C57BL/6 T-bet$^{-/-}$ mice but not BALB/c T-bet$^{-/-}$ mice develop spontaneous airway inflammation is unknown. We speculate that the inhibitory effect by T-bet on T_H2 cells may be stronger in T_H1-biased C57BL/6 mice than in T_H2-biased BALB/c mice, and thus the absence of T-bet may result in uncontrolled T_H2 cell activation even by the environmental antigens to induce spontaneous allergic airway inflammation in C57BL/6 T-bet$^{-/-}$ mice.

Second, we clearly show that T-bet regulates antigen-induced neutrophilic inflammation in the airways by inhibiting T_H17 cell development. We showed that antigen-induced neutrophil infiltration in the airways was significantly enhanced in T-bet$^{-/-}$ mice compared with that in WT mice (Figs 2 and 3). The neutrophil response was STAT6 independent as shown by the results of T-bet$^{-/-}$ STAT6$^{-/-}$ mice (Fig 3). We also showed that T-bet$^{-/-}$ CD4$^+$ T cells mediated the enhanced antigen-induced neutrophil infiltration and IL-17 production in the airways, and the enhanced response was canceled by anti–IL-17 antibody treatment (Fig 5). Moreover, the *in vitro* study revealed that antigen-stimulated T-bet$^{-/-}$CD4$^+$ T cells preferentially differentiated into IL-17–producing cells (Fig 6). Thus, T-bet potently inhibits not only T_H2 cell–mediated eosinophilic inflammation in the airways but also T_H17 cell–mediated neutrophilic inflammation in the airways.

Our results indicate that T-bet, but not Stat6, is an important regulator for T_H17 cell–mediated neutrophilic inflammation *in vivo*. It has been shown that T_H17 cell development is potently inhibited by a T_H1 cytokine, IFN-γ, and a T_H2 cytokine, IL-4.[22,23] Indeed, the blockade of IFN-γ and IL-4 is required for the development of a substantial number of T_H17 cells.[22,23] It has also been shown that the downstream signaling of STAT1/T-bet and STAT6, respectively, can inhibit IL-23–induced T_H17 cell differentiation from naive CD4$^+$ T cells.[22] However, we found that STAT6 had no significant inhibitory effect on antigen-induced neutrophil infiltration in the airways in the presence or absence of T-bet (Fig 3). This is consistent with the findings that in STAT6-deficient T cells, there was no difference in IL-17 production compared with that of WT T cells.[23] Regarding the mechanisms for T-bet–mediated inhibition of T_H17 cell development, it has been suggested that IFN-γ inhibits T_H17 cell differentiation by downregulating the IL-23 receptor expression.[22] IFN-γ has also previously been shown to inhibit TGF-β signaling, which is critical for T_H17 cell development,[21] by a STAT1-induced synthesis of the inhibitory Smad7.[30] Taken together, it is suggested that in addition to the inhibition of T_H2 cell differentiation in allergic inflammation, the inhibition of T_H17 cell development may be one of nonredundant functions of T-bet to prevent the development of neutrophilic inflammation as well as autoimmune pathology.[21]

Regarding the possible involvement of IL-17 in asthma, the ability of IL-17 to evoke migration of neutrophils[20] but not of eosinophils makes it likely that this cytokine is involved in the pathogenesis of severe asthma, in which accumulation of neutrophils in the airways is a hallmark of disease.[3] Indeed, it has been shown that IL-17 is expressed in the airways of patients with asthma[31] and that its expression is increased in patients with moderate-to-severe asthma compared with patients with mild asthma and normal controls.[32] In addition, we have shown here that T-bet is crucial for the suppression of antigen-induced IL-17 production and subsequent neutrophil recruitment in the airways (Fig 5). Taken together, it is suggested that the dysfunction of T-bet may be involved in the pathogenesis of severe asthma.

In conclusion, from the analysis of T-bet$^{-/-}$ mice, we have established 2 important points. First, the expression of T-bet in CD4$^+$ T cells is vital for the inhibition of antigen-induced T_H2 cell differentiation and thereby regulates antigen-induced, T_H2 cytokine-mediated eosinophil recruitment into the airways. Second, T-bet is also crucial for the inhibition of antigen-induced T_H17 cell differentiation and thereby regulates antigen-induced, IL-17–mediated neutrophil recruitment into the airways. These results provide new insights into the pathophysiology of allergic airway inflammation, especially in severe asthma.

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BTLA Inhibits Antigen-induced Eosinophil Recruitment into the Airways

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ABSTRACT

BACKGROUND: Signaling through CD28 family co-receptors regulates activation of $CD4^+$ T cells positively and negatively. It has been shown that stimulatory co-receptors such as CD28 and ICOS play critical roles in the induction of allergic airway inflammation. However, the role of B and T lymphocyte attenuator (BTLA), an inhibitory co-receptor expressed preferentially in Th1 cells, in the regulation of allergic airway inflammation remains to be determined.

METHODS: We examined antigen-induced eosinophil recruitment and cytokine production in the airways in antigen-sensitized BTLA-deficient ($BTLA^{-/-}$) mice. We also examined antigen-induced cytokine production and cell proliferation of splenic T cells in antigen-sensitized $BTLA^{-/-}$ mice.

RESULTS: Antigen-induced eosinophil recruitment and IL-5 production in the airways was enhanced in antigen-sensitized $BTLA^{-/-}$ mice. On the other hand, antigen-induced Th1 and Th2 cytokine production as well as T cell proliferation of splenocytes was normal in $BTLA^{-/-}$ mice.

CONCLUSION: BTLA inhibits antigen-induced eosinophil recruitment into the airways by preventing IL-5 production from Th2 cells.

INTRODUCTION

Allergic airway inflammation is associated with intense eosinophil and T cell infiltration into the submucosal tissue of airways and the inflammatory cells cause epithelial damage and then airway

hyperreactivity (1, 2). In a murine model of allergic airway inflammation, it has been demonstrated that Th2 cells and their cytokines such as IL-4, IL-5, and IL-13 orchestrate the recruitment of eosinophils into the airways and airway hyperreactivity upon antigen exposure (3).

Activation and subsequent differentiation of naïve $CD4^+$ T cells is regulated not only by the strength of signals through T cell receptor but also by the signals derived from stimulatory and/or inhibitory co-receptors (4). Stimulatory co-receptors expressed on T cells include CD28 and ICOS and transduce signals necessary to fully activate T cells (4-6). Inhibitory co-receptors include CTLA-4, programmed death receptor-1 (PD-1), and B and T lymphocyte attenuator (BTLA) and transduce signals that inhibit T cell activation (4-6). The balance between positive and negative co-receptor signaling is thought to be critical not only for the maintenance of immune tolerance to self-antigens but also for the appropriate immune responses to exogenous antigens.

Recently, some of co-receptors have been demonstrated to be involved in the regulation of Th2 cell-mediated allergic airway inflammation. It has been shown that the inhibition of signaling through CD28 by CTLA-4 Ig results in the suppression of Th2 cell-mediated allergic airway inflammation and airway hyperreactivity (7, 8). B7-2 blocking exhibits similar effects (7, 9, 10), suggesting that interaction between B7-2 and CD28 is important in the induction of Th2 cell-mediated allergic airway inflammation. Moreover, it has been shown that ICOS is also involved in the initiation and maintenance of allergic airway inflammation (11). However, the role of BTLA, an inhibitory co-receptor expressed preferentially in Th1 cells and B cells (12), in the regulation of allergic airway inflammation is still largely unknown.

In this study, we examined the regulatory role of BTLA in allergic airway inflammation using BTLA-deficient ($BTLA^{-/-}$) mice. We found that antigen-induced eosinophil recruitment and IL-5 production in the airways was enhanced in $BTLA^{-/-}$ mice. We also found that antigen-induced Th1 and Th2 cytokine production of splenic T cells was normal in $BTLA^{-/-}$ mice. Our results indicate that BTLA negatively regulates antigen-induced eosinophil recruitment into the airways by inhibiting IL-5 production from Th2 cells.

MATERIALS and METHODS

Mice and genetic analysis

BTLA-deficient ($BTLA^{-/-}$) mice (12) were backcrossed to BALB/c mice (Charles River Laboratories, Kanagawa,

Japan) for at least 8 generations and littermate wild-type (WT) mice were used as controls. Mice were housed in microisolator cages under pathogen-free conditions. All experiments were performed according to the guidelines of Chiba University.

Antigen-induced eosinophil and T cell recruitment into the airways of sensitized mice

Antigen-induced allergic airway inflammation was induced as described previously (13, 14). In brief, $BTLA^{-/-}$ mice (age 7-8 weeks) and littermate WT mice were immunized intraperitoneally twice with 4 μg of ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) in 4 mg of aluminum hydroxide (alum) at a two-week interval. Twelve to 14 days after the second immunization, the sensitized mice were challenged with aerosolized OVA dissolved in 0.9% saline by a nebulizer for 20 minutes. As a control, 0.9% saline alone was administered by the nebulizer. Forty-eight hours after the OVA inhalation, bronchoalveolar lavage (BAL) was performed with 2 ml of phosphate-buffered saline containing 1% bovine serum albumin (Sigma Chemical Co.). Bronchoalveolar lavage fluid (BALF) was centrifuged at 400 g for 5 minutes at 4°C and differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution. A fraction of the cells were subjected to a flow cytometric analysis for the lymphocyte surface phenotyping of CD4 and CD8 using a FACScalibur (Becton Dickinson, Mountain View, CA) and CELLQuest software.

Antigen-induced goblet cell hyperplasia in sensitized mice

OVA-sensitized $BTLA^{-/-}$ mice and littermate WT mice were challenged with the aerosolized OVA by a nebulizer as described above. Forty-eight hours after the OVA inhalation, a sagittal block of left lung was excised, fixed in 10% buffered-formalin, and embedded in paraffin. Lung sections (3 μm thick) were stained with periodic acid-Schiff (PAS) according to standard protocols. The number of goblet cells was counted on PAS-stained lung sections as described elsewhere (15).

Antigen-induced proliferation and cytokine production of splenocytes

Spleen was removed from OVA-sensitized $BTLA^{-/-}$ mice and littermate WT mice and a single cell suspension of splenocytes was prepared. Splenocytes (2×10^5) were then suspended in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (MBL, Nagoya, Japan), 10 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were cultured in triplicate in the

absence or presence of OVA (100-400 μg/ml) in a 96-well microtiter plate for 72 hours at 37°C. In some experiments, splenocytes (2 x 10^5) were cultured for 72 hours in a microtiter plate coated with 5 μg/ml of anti-CD3ε mAb (145-2C11, BD PharMingen, San Diego, CA). The culture supernatant was collected and the amounts of IL-4, IL-5, and IFN-γ were determined as described below. Splenocytes (2 x 10^5) were also cultured in the same conditions and the proliferation of cells was evaluated using CellTiter-Glo reagent (Promega, Madison, WI) as described previously (16).

ELISA for cytokines

The amounts of IL-4, IL-5, and IFN-γ in the BALF and the culture supernatant were determined by the enzyme immunoassay using murine IL-4, IL-5, and IFN-γ ELISA kits from BD PharMingen. The assays were performed in duplicate according to the manufacturer's instruction. The amounts of IL-13 in the BALF were determined by an ELISA kit from R&D systems (Minneapolis, MN). The detection limits of these assays were 15 pg/ml of IL-4 and IL-13, 30 pg/ml of IL-5, and 50 pg/ml of IFN-γ.

Data Analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t-test. P values <0.05 were considered significant.

RESULTS

Antigen-induced eosinophil recruitment into the airways is enhanced in BTLA$^{-/-}$ mice

Given that CD4$^+$ T cells and their cytokines play an important role in regulating antigen-induced allergic airway inflammation (1-3) and that BTLA is expressed on CD4$^+$ T cells and attenuates cytokine production and proliferation of CD4$^+$ T cells (12), we examined whether BTLA is involved in the regulation of antigen-induced allergic airway inflammation using BTLA$^{-/-}$ mice. OVA-immunized BTLA$^{-/-}$ mice and littermate WT mice were challenged with aerosolized OVA and the number of eosinophils and CD4$^+$ T cells recovered in bronchoalveolar lavage fluids (BALF) was evaluated. As shown in Fig. 1A, antigen-induced eosinophil recruitment into the BALF at 48 hours after the inhalation was significantly enhanced in BTLA$^{-/-}$ mice by 47% as compared with WT mice (WT mice: 20.7 ± 6.6 vs. BTLA$^{-/-}$ mice: 30.4 ± 12.1, x10^4/mice, mean ± SD, n = 10 mice in each group, $p<0.05$). Antigen-induced eosinophil recruitment into the BALF at 72 hours after antigen inhalation was also enhanced in BTLA$^{-/-}$ mice (data not shown).