*Full Paper***Complete Dependence on CD4⁺ Cells in Late Asthmatic Response, but Limited Contribution of the Cells to Airway Remodeling in Sensitized Mice**

Takeshi Nabe^{1,*}, Toyoko Morishita¹, Kouki Matsuya¹, Ayumu Ikedo¹, Masanori Fujii¹, Nobuaki Mizutani², and Shin Yoshino²

¹Department of Pharmacology, Kyoto Pharmaceutical University,
5 Nakauchi, Misasagi, Yamashina, Kyoto 607-8414, Japan

²Department of Pharmacology, Kobe Pharmaceutical University,
4-19-1 Motoyamakita-machi, Higashinada, Kobe 658-8558, Japan

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Abstract. It is known that the late asthmatic response (LAR), a characteristic feature of asthma, is closely associated with CD4⁺ Th2 cell-mediated allergic inflammation. Airway remodeling is also a pathogenesis of asthma, but literature reporting roles of CD4⁺ cells in the remodeling is controversial. There has been no study that simultaneously assessed the roles of CD4⁺ cells in both LAR and airway remodeling. Sensitized mice were intratracheally challenged with ovalbumin 4 times. Treatment with an anti-CD4 monoclonal antibody (mAb) before the 1st challenge almost completely abolished increase in CD4⁺ cells in the tissues after the 4th challenge. The late phase increase in airway resistance after the 4th challenge was also completely inhibited by anti-CD4 mAb. Parameters of airway remodeling, subepithelial fibrosis and epithelial thickening were attenuated by treatment, whereas the inhibition was only 30% – 40%. Bronchial smooth muscle thickening was not affected. Because interleukin (IL)-5 production as well as eosinophilia was effectively suppressed by anti-CD4 mAb, the effect of anti-IL-5 mAb was also examined, resulting in no inhibition of airway remodeling. Collectively, although the LAR was completely dependent on CD4⁺ cell activation, airway remodeling was only partially dependent on the cell.

Keywords: airway remodeling, late asthmatic response, CD4⁺ cell, interleukin-5, fibrosis

Introduction

The late asthmatic response (LAR), one of the characteristic features of asthma pathogenesis, is a reversible airway obstructive response that occurs several hours after an antigen challenge and persists for at least several hours in asthma patients (1). In addition to the early asthmatic response that is induced immediately after an antigen challenge in asthma patients, more than 50% of patients reveal late phase bronchoconstriction (2, 3). The dual asthmatic response is associated with airway hyper-responsiveness, which is not seen in those individuals with an isolated early asthmatic response (4). It has been

suggested that the mechanisms underlying the LAR could be based on eosinophilic airway inflammation orchestrated by CD4⁺ Th2 lymphocyte activation through the production of interleukin (IL)-5, a cytokine playing roles in eosinophil differentiation and activation (5 – 7). Indeed, a murine study using an anti-CD4 monoclonal antibody (mAb) demonstrated that the depletion of CD4⁺ cells by anti-CD4 mAb completely suppressed LAR (8), indicating that CD4⁺ cells mainly consisting of Th2 lymphocytes play central roles in the induction of the late phase response.

In addition to the inflammatory process, another regular feature of asthma is airway remodeling, which leads to structural lung changes. These changes include fibroblast proliferation, airway smooth muscle hyperplasia and hypertrophy, and excess production of mucus (9, 10). All these modifications thicken airway walls in

*Corresponding author. nabe@mb.kyoto-phu.ac.jp
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asthmatic patients, leading to airway hyperresponsiveness to specific allergens and non-specific stimuli (9). Thus, the existence of airway remodeling may be closely associated with antigen-induced airway narrowing, including LAR (9, 10); however, the relationship between antigen-induced airway narrowing and airway remodeling has not been examined, even in experimental animals. Furthermore, studies using murine models have shown that airway remodeling was also mediated by activation of CD4⁺ cells because anti-CD4 mAb effectively suppressed the histological changes (11); however, another study demonstrated that CD4⁺ cells were not required for progressive airway remodeling (12).

On the other hand, we have reported a murine model of asthma that showed LAR by 4 intratracheal administrations of an antigen, ovalbumin (OVA), to sensitized BALB/c mice (13). A few studies have focused on LAR as a parameter of an asthma phenotype (8, 13–17). Most studies of murine asthma have examined airway hyperresponsiveness to a cholinergic agonist as a pulmonary functional change, probably because inhalation of antigen mists through the nasal cavity or intranasal instillation of antigen solution could not evoke an airway obstructive response in sensitized animals. Since we have used an intratracheal technique for antigen challenge, both early and late phase airway narrowing could be evoked (13). It is important to analyze not only airway obstruction to a non-specific stimulus but also that to a specific allergen. In addition, our previous study found that airway remodeling including subepithelial fibrosis, epithelial thickening, and mucus accumulation in the epithelium was also developed in the LAR model (18).

In this study, in order to examine the relationship between LAR and airway remodeling in experimental asthma, the effects of anti-CD4 mAb on the development of both LAR and airway remodeling were simultaneously examined in the murine model of LAR.

Materials and Methods

Sensitization and challenge

Six-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu). The mice were sensitized by intraperitoneal (i.p.) injections with OVA (Grade V; Sigma Chem., St. Louis, MO, USA) adsorbed to Al(OH)₃, which was made according to our previously reported method (13), at a dose of 50 µg OVA adsorbed to 2 mg Al(OH)₃/0.5 ml PBS per animal on days 0, 14, and 28. The sensitized mice were challenged on days 35, 36, 37, and 40 under inhalation anesthesia with isoflurane (Abbott Japan, Tokyo) with 2% OVA at a volume of 25 µl by intratracheal administration, as reported previously (19). As a negative control group, mice were sensitized

with OVA + Al(OH)₃, i.p. without any airway challenges with OVA.

This animal study was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Treatment with anti-CD4 mAb, dexamethasone or anti-IL-5 mAb

A cell line producing rat IgG2b mAbs that recognize the murine CD4 molecule (YTS191.1.2) was kindly provided by Prof. David D. Chaplin (University of Alabama at Birmingham, Birmingham, AL, USA). The hybridoma cells were cultured and grown, and then i.p. injected into severe combined immunodeficiency mice (Clea Japan, Tokyo) that had been treated with pristane (Sigma Chem.). Ascites was collected 10–14 days after the injection. IgG was purified by protein G affinity chromatography (GE Healthcare, Uppsala, Sweden), followed by desalting using PD-10 column chromatography (GE Healthcare). The purified anti-CD4 mAb, rat IgG (Sigma Chem.) or PBS was i.p. administered once 18 h prior to the 1st antigen challenge at a dose of 600 µg/animal.

A cell line producing rat IgG2b mAbs that recognize the murine IL-5 molecule (TRFK-5) was kindly provided by Prof. Kohtaro Fujihashi (University of Alabama at Birmingham). The anti-IL-5 mAb was produced and purified as described above. The purified anti-IL-5 mAb or rat IgG was i.p. administered once 3 h prior to the 1st antigen challenge at a dose of 50 µg/animal.

Dexamethasone 21-phosphate disodium salt (Sigma Chem.) (3 mg/kg as dexamethasone) or the vehicle PBS was i.p. administered once daily for 6 days (day 35–40). When antigen challenge was performed, dexamethasone was given 3 h before the respective challenges.

Measurement of pulmonary function

As an indicator of airway resistance, specific airway resistance [sRaw: cmH₂O × ml/(ml/s)] was measured by double-flow plethysmography (20, 21) (Pulmos-I. II. III; M.I.P.S., Osaka). The parameter was measured 1 h before and at various times (10 min and 1, 2, 3, and 4 h) after the 4th OVA challenge. The system is a non-invasive technique that makes it possible to assess pulmonary functions longitudinally over a prolonged time course.

Histopathological study

Before the 1st OVA challenge and 4 h after the 4th OVA challenge, the left lobe of the lung was isolated under anesthetization with ketamine and xylazine and then fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 4-µm sections were stained with hematoxylin and eosin, Masson trichrome, and pe-

riodic acid–Schiff (PAS) for light microscopical observation.

Masson trichrome–stained sections were used for assessment of subepithelial fibrosis by scoring the extension of the blue fibrotic area by a 4-point graded scale from 0 to 3: 0, no blue area existed underneath the basement membrane; 1, 2, and 3 blue fibrotic area existed underneath the basement membrane by approximately 30, 60 μm , and more than 60 μm , respectively. All specimens were analyzed in a blind fashion.

The degree of epithelial thickening was quantified using PAS-stained sections as follows: the bronchus, the diameter of which was 200 – 300 μm , was photographed, and then the epithelial area (μm^2) and the length of the basement membrane (μm) were measured using Adobe Photoshop CS2 (version 9.0.2; Adobe Systems, San Jose, CA, USA) and Image J (version 1.42; NIH, Bethesda, MD, USA). The degree of epithelial thickening was expressed by dividing the epithelial area by the length of the basement membrane.

Mucus accumulation in the epithelial layer was also quantified using PAS-stained sections by dividing the PAS⁺ area in the epithelium (μm^2) by the length of the basement membrane (μm).

Immunohistochemistry

α -Smooth muscle actin (α -SMA) in the smooth muscle layer was detected by immunohistochemistry. Antigenicity in the 4- μm tissue sections prepared as described above was generally activated by immersion of the sections on slides in Histo VT one (Nacalai Tesque, Kyoto). After washing, the sections were treated with 3% hydrogen peroxide–methanol solution followed by washing and stained with biotinylated anti-smooth muscle actin mAb (clone 1A4; LifeSpan BioSciences, Seattle, WA, USA) or biotinylated mouse IgG2a antibody (Cedarlane Labs., Burlington, NC, USA). After washing, the sections were stained with streptavidin–horseradish peroxidase. After washing, the color was developed using a 3,3'-diaminobenzidine substrate kit (BD Biosciences, San Diego, CA, USA), followed by counterstaining with hematoxylin solution.

The bronchus, the diameter of which was 100 – 200 μm , was photographed and the α -SMA⁺ area and the length of the basement membrane were measured as described above. The degree of thickening of the smooth muscle layer was quantified by dividing the α -SMA⁺ area by the length of the basement membrane.

Analysis of cells in bronchoalveolar lavage fluid (BALF), peripheral blood, and spleen

Four hours after the 4th challenge, before isolation of the left lobe of the lung for histological studies, peripheral

blood was drawn from the vena cava in the thoracic cavity and mixed with heparin immediately after drawing. Total leukocyte number was counted by hemacytometer following Türk staining. After treatment with ACK lysis buffer (0.15 M NH_4Cl , 1 mM KHCO_3 , 0.1 mM ethylenediaminetetraacetic acid disodium salt, pH 7.2) to remove erythrocytes, the ratio of CD4⁺ cells was determined by a flow cytometer as described below.

After isolation of the left lobe of the lung for histological studies, the right lobes were lavaged under ligation of the bronchial tree that had been connected to the left lobe. Lavage was performed using two aliquots of 0.6 ml PBS containing 2% FBS. Total leukocyte number was determined by a particle counter and size analyzer (Z2; Beckman Coulter, Brea, CA, USA) after treatment with ACK lysis buffer. For the differential cell counts, cells were transferred onto a glass slide by centrifugation in a cell settling chamber (Neuro Probe, Gaithersburg, MD, USA). Subsequently, cells were then stained with Diff-Quik solution (Sysmex International Reagent, Kobe).

Finally, the spleen was isolated, physically disrupted in PBS supplemented with 2% FBS, and forced through a 40- μm pore size nylon screen. Single cell suspensions were treated with ACK lysis buffer. The total leukocyte number was determined by counting with a hemacytometer.

The numbers of CD4⁺ cells in the BALF and leukocyte suspensions derived from the peripheral blood and spleen were measured by flow cytometry, as previously reported (13). In brief, after incubation with anti-mouse Fc γ RII/III mAb (clone 2.4G2, BD Biosciences) to block the binding of subsequent antibodies to Fc γ RII/III, cells were incubated with Cy-Chrome–conjugated anti-mouse CD4 mAb (clone H129.19, BD Biosciences). After washing, the stained cells were fixed with 4% paraformaldehyde and then analyzed using FACSCalibur (BD Biosciences) and Cell Quest software (version 3.3, BD Biosciences).

Assays of levels of Th2 cytokines in BALF

Levels of Th2 cytokines, IL-4, IL-5, and IL-13, in the BALF supernatant were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

Statistical analyses

One-way analysis of variance (ANOVA) was performed. For the results of time-course changes in sRaw, two-way ANOVA was applied. If significant differences were detected, individual group differences were determined by Bonferroni's multiple tests. A probability value (*P*) below 0.05 was considered significant.

Results

Development of airway remodeling and effects of dexamethasone

Histological changes in the lung tissue after the 4th challenge were compared with the histology of the lung isolated before the 1st challenge. Histological images obtained by various staining techniques revealed that the 4 antigen challenges markedly induced epithelial thickening and the accumulation of inflammatory cells around the bronchi and alveoli (Fig. 1: B, E, H, K and N), in contrast to the lung histological features of the non-challenged mice, as shown in Fig. 1: A, D, G, and J. Masson trichrome staining demonstrated that fibrosis was also markedly induced around the subepithelial area (Fig. 1: E and M). Furthermore, PAS staining clearly revealed mucus in the thickened epithelium (Fig. 1: H and O). Interestingly, immunohistochemical analysis using an anti- α -SMA antibody showed that the smooth muscle bundle around the bronchi was thickened (Fig. 1: K and P) compared with before the 1st challenge (Fig. 1J).

Daily treatment with dexamethasone markedly inhibited the histological changes, subepithelial fibrosis, epithelial thickening, mucus accumulation in the epithelium, and smooth muscle thickening by more than 70% with statistical significance (Fig. 1).

Development of airway cellular infiltration and LAR, and effects of dexamethasone on them

As we have reported previously (13), mononuclear cells including CD4⁺ T cells, and eosinophils were increased at 4 h after the 4th challenge (Fig. 2: A, B, and C). We reported that CD4⁺ cells and eosinophils were already infiltrating the lung before the 4th challenge (13). In other words, CD4⁺ cells and eosinophils had accumulated in the lung by the 1st – 3rd challenges. In contrast, neutrophils increased before and after the 2nd – 3rd challenges, returning to the circulation until before the 4th challenge (13, 22). The 4th challenge caused the recurrence of neutrophilia at 4 h after the 4th challenge (Fig. 2D).

Daily treatment with dexamethasone significantly abolished the increases in the numbers of mononuclear cells, CD4⁺ cells, and eosinophils (Fig. 2: A, B, and C), whereas neutrophilia was not affected by dexamethasone (Fig. 2D), in agreement with the literature that neutrophilia is resistant to glucocorticoid treatment (23, 24).

In addition, as has been reported that sRaw was increased after the 4th OVA challenge in a biphasic fashion (13, 22). Both early (10 min) and late phase (2 – 4 h) increases in sRaw were almost completely inhibited by daily treatment with dexamethasone (Fig. 2E).

Effects of anti-CD4 mAb on cellular inflammation and LAR

A single treatment with anti-CD4 mAb at the time before the 1st challenge completely suppressed the increase in the number of CD4⁺ cells in BALF (Fig. 3B). In addition, anti-CD4 treatment markedly reduced the numbers of CD4⁺ cells in the peripheral blood and the spleen that were isolated 4 h after the 4th challenge (Fig. 3: E and F). Airway eosinophilia was also significantly decreased, although the inhibition was approximately 60% compared with rat IgG-treated animals (Fig. 3C). In contrast, neutrophilia was significantly augmented to approximately 200%.

As shown in Fig. 3G, the increase in sRaw did not seem to be biphasic in this experiment even though increases in sRaw at 2 – 4 h were apparent. Although the reasons for the difference from the time course shown in Fig. 2E are not clear, we suspect that this is due to batch differences in animals. The marked increase in sRaw during the late phase (2 – 4 h) was completely abolished by anti-CD4 mAb treatment, although the early phase increase at 10 min was not significantly affected.

Effects of anti-CD4 mAb on the development of airway remodeling

The depletion of CD4⁺ cells throughout the 1st – 4th challenges by anti-CD4 mAb treatment significantly reduced the development of subepithelial fibrosis by approximately 40% (Fig. 4: D, E, F, and M). Epithelial thickening was also partially inhibited by CD4⁺ cell depletion, although it was not statistically significant (Fig. 4: G, H, I, and N). On the other hand, neither mucus accumulation (Fig. 4: G, H, I, and O) nor smooth muscle thickening (Fig. 4: J, K, L, and P) was affected by anti-CD4 mAb treatment compared with the rat IgG-treated group.

Effects of anti-CD4 mAb on Th2 cytokine production

As we have reported previously (13), levels of Th2 cytokines, IL-4, IL-5, and IL-13 were increased at 4 h after the 4th challenge (Fig. 5). Treatment with anti-CD4 mAb augmented the increase in the level of IL-4 (Fig. 5A); however, the increase in IL-5 was effectively inhibited by anti-CD4 mAb (Fig. 5B). The increase in IL-13 also tended to be reduced by anti-CD4 mAb treatment (Fig. 5C).

Effects of anti-IL-5 mAb on the development of airway remodeling

Because anti-CD4 mAb treatment suppressed IL-5 production, we next examined the roles of IL-5 in the development of airway remodeling. We have reported that treatment with anti-IL-5 once before the 1st chal-

Fig. 1. Development of airway remodeling by multiple antigen challenges in sensitized mice and effects of dexamethasone. Isolated lung tissue was fixed with 10% formalin, embedded in paraffin, and cut into 4- μ m sections, followed by staining with hematoxylin and eosin (H&E: A, B, and C), Masson trichrome (D, E, and F), periodic acid-Schiff (PAS: G, H and I), or anti- α -smooth muscle actin (α -SMA) antibody (J, K, and L). The degree of subepithelial fibrosis was scored using Masson trichrome-stained sections (M). Degree of epithelial thickening was quantified using PAS-stained sections by dividing epithelial area (μ m²) in the bronchus (diameter: 200–300 μ m) by the length (μ m) of basement membrane (N). Degree of mucus accumulation was calculated by dividing PAS⁺ area (μ m²) in the bronchus by the length (μ m) of basement membrane (O). Degree of smooth muscle thickening was calculated by dividing α -SMA⁺ area (μ m²) by the length (μ m) of the BM (P). Dexamethasone (3 mg/kg) or PBS was i.p. administered once daily from the day of the 1st challenge until the day of the 4th challenge. On the days of antigen challenge, dexamethasone was administered 3 h before the respective challenges. Each column represents the mean $\pm$ S.E.M. of 5–8 animals. ** P < 0.01 and *** P < 0.001 vs. each value before the 1st challenge. † P < 0.05 and †† P < 0.01 vs. PBS-treated.

lenge caused marked inhibition of the increase in eosinophils by approximately 70% (22). Consequently, the development of airway remodeling, subepithelial fibrosis, epithelial thickening, mucus accumulation, and

smooth muscle thickening was not affected by treatment with anti-IL-5 mAb (Fig. 6). The induction of the late phase increase in sRaw was also not inhibited by anti-IL5 mAb (22).

Fig. 2. Effect of dexamethasone on increases in the numbers of mononuclear cells (A), CD4⁺ cells (B), eosinophils (C), and neutrophils (D) in bronchoalveolar lavage fluid collected 4 h after the 4th challenge and an increase in specific airway resistance (sRaw: E) after the 4th challenge in sensitized mice. Dexamethasone (3 mg/kg) or PBS was i.p. administered once daily from the day of the 1st challenge until the day of the 4th challenge, as described in the legend of Fig. 1. Each point and column represents the mean $\pm$ S.E.M. of 4–8 animals. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. each value before the 1st challenge. † P < 0.05 and †† P < 0.01 vs. PBS-treated.

Fig. 3. Effect of anti-CD4 monoclonal antibody (mAb) on increases in the numbers of mononuclear cells (A), CD4⁺ cells (B), eosinophils (C), and neutrophils (D) in bronchoalveolar lavage fluid and CD4⁺ cells in the peripheral blood (E) and spleen (F) collected 4 h after the 4th challenge, and the increase in specific airway resistance (sRaw: G) after the 4th challenge in sensitized mice. Anti-CD4 mAb (YTS191.1.2, 600 $\mu\text{g}/\text{animal}$), rat IgG (600 $\mu\text{g}/\text{animal}$), or PBS was i.p. administered once 18 h before the 1st challenge. Each point and column represents the mean $\pm$ S.E.M. of 4–16 animals. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. rat IgG-treated.

Fig. 4. Effects of anti-CD4 monoclonal antibody (mAb) on the development of airway remodeling by multiple antigen challenges in sensitized mice. Sections of isolated lung tissue were stained with hematoxylin and eosin (H&E: A, B, and C), Masson trichrome (D, E and F), periodic acid-Schiff (PAS: G, H, and I), or anti- α -smooth muscle actin (α -SMA) antibody (J, K, and L). Degrees of subepithelial fibrosis (M), epithelial thickening (N), mucus accumulation (O), and smooth muscle thickening (P) were quantified as described in the legend of Fig. 1. Anti-CD4 mAb (YTS191.1.2, 600 μ g/animal), rat IgG (600 μ g/animal), or PBS was i.p. administered once 18 h before the 1st challenge. Dotted lines in panels represent the respective mean values before the 1st challenge as shown in Fig. 1. Each column represents the mean $\pm$ S.E.M. of 4–8 animals. * P < 0.05 vs. rat IgG-treated.

Discussion

In this study, it was demonstrated that the development of LAR was totally dependent on CD4⁺ cell activation, whereas the role of CD4⁺ cells in that of airway remodel-

ing was limited in the murine model of asthma. An important question in this context is whether there is a pathogenic relationship between the marked inhibition of the LAR and the limited suppression of airway remodeling by treatment with anti-CD4 mAb in this model. Al-

Fig. 5. Effects of anti-CD4 monoclonal antibody (mAb) on increases in the levels of Th2 cytokines, interleukin (IL)-4 (A), IL-5 (B), and IL-13 (C) in bronchoalveolar lavage fluid (BALF) collected 4 h after the 4th challenge in sensitized mice. Anti-CD4 mAb (YTS191.1.2, 600 μ g/animal), rat IgG (600 μ g/animal), or PBS was i.p. administered once 18 h before the 1st challenge. Each column represents the mean $\pm$ S.E.M. of 6 – 18 animals.

Fig. 6. Effects of anti-interleukin (IL)-5 monoclonal antibody (mAb) on the development of airway remodeling by multiple antigen challenges in sensitized mice. Sections of isolated lung tissue were stained with Masson trichrome, periodic acid–Schiff (PAS), or anti- α -smooth muscle actin (α -SMA) antibody. Degrees of subepithelial fibrosis (A), epithelial thickening (B), mucus accumulation (C), and smooth muscle thickening (D) were quantified as described in the legend of Fig. 1. Anti-IL-5 mAb (TRFK-5, 50 μ g/animal) or rat IgG (50 μ g/animal) was i.p. administered once 3 h before the 1st challenge. Dotted lines in panels represent respective mean values before the 1st challenge, as shown in Fig. 1. Each column represents the mean $\pm$ S.E.M. of 4 – 6 animals.

though there is no direct evidence demonstrating this relationship, we cannot deny that the two phenomena are dissociated. As demonstrated in this study and our previous literature (13), the 1st – 3rd challenges caused not only cellular inflammation but also airway remodeling before the 4th challenge. Thus, we consider that against a background of airway pathological changes, the 4th OVA challenge could produce further acute airway inflammation, leading to the occurrence of the LAR. In contrast to the 4th challenge, LAR was not induced after the respective 1st – 3rd challenges (13), probably because no or immature airway pathological changes had been induced at the time of the former challenges; therefore, the reduction of subepithelial fibrosis and epithelial thickening may be causes of the inhibition of late phase airway narrowing, as has been suggested in review articles (9, 10).

As expected, anti-CD4 mAb treatment suppressed the production of a Th2 cytokine, IL-5, whereas IL-4 pro-

duction was augmented by the treatment. We have demonstrated that when anti-CD4 mAb was administered once before the 4th challenge, both Th2 cytokines were reduced (22). In this study, the antibody was administered before the 1st challenge to deplete CD4⁺ cells throughout the 1st – 4th challenges, so the effect on IL-4 was found to be different from that when administered once before the 4th challenge. In our preliminary study, basophils (IgE⁺ c-kit⁺ CD49b⁺ cells) were increased in the lung by multiple challenges and anti-CD4 mAb treatment augmented the increase in basophils (T. Nabe et al., unpublished data). Because basophils as well as Th2 cells are potential cellular sources of IL-4 several hours after an antigen challenge in sensitized mice (25), the increase in IL-4 level could be closely linked to the increase in basophils; however, the mechanisms underlying the increase in basophils in the lung by CD4⁺ cell depletion are not clear.

Apart from the results of IL-4, the suppression of IL-5

production by anti-CD4 mAb should be a cause of the inhibition of lung eosinophilia by the antibody. From these results, we next examined the effects of anti-IL-5 mAb on the development of airway remodeling. Consequently, airway remodeling was not inhibited by the neutralization of IL-5, although anti-IL-5 mAb treatment markedly suppressed eosinophilia (22). However, because the magnitude of inhibition was only 70% (22), the dose (50 μ g/animal) of anti-IL-5 mAb was increased to 200 μ g/animal, whereas the inhibition was not amplified (22). In another experimental asthma model exhibiting airway remodeling reported by Tanaka et al. (26), complete inhibition of airway eosinophilia was achieved by anti-IL-5 treatment, leading to marked reduction of the development of peribronchial fibrosis. Furthermore, the reduction of eosinophilia and fibrosis was closely associated with a decrease in transforming growth factor (TGF)- β 1 production in the lung (26). Thus, they suggested that the infiltrated eosinophils could be involved in fibrosis via the production of TGF- β 1 (26). From our present results, it was not likely that IL-5 was an essential factor for the development of subepithelial fibrosis; however, we could not reject the possibility that eosinophils play a role in histopathological change because the inhibition of eosinophilia by anti-IL-5 mAb was incomplete. IL-5-independent mechanisms should exist in the airway eosinophilia in our model. Other eosinophil chemotactic factors such as eotaxin are also closely involved in eosinophilia. It was recently demonstrated that both airway eosinophilia and remodeling were reduced in phosphoinositide 3 kinase γ (PI3K γ)-deficient mice challenged with OVA (27). Because PI3K γ is known to be expressed particularly in leukocytes (28) and is known to be activated by G protein-coupled chemokine receptors to induce leukocyte migration (28), it was suggested that chemokines such as eotaxin play key roles in not only airway eosinophilia but also airway remodeling. Whether the infiltrated eosinophils are involved in remodeling should be further examined under the condition in which eosinophilia completely disappeared.

As demonstrated in this study, the increase in another Th2 cytokine IL-13 in BALF was also suppressed by the anti-CD4 mAb treatment. IL-13 proved to be a potent stimulator of eosinophilic inflammation and airway remodeling (29). Lee et al. (30) demonstrated that IL-13-induced lung fibrosis was ameliorated by treatment with a TGF- β 1 antagonist, soluble TGF- β R2-Fc, indicating that IL-13 is an activator of TGF- β 1 in vivo. Thus, the inhibition of subepithelial fibrosis by the anti-CD4 mAb in our present study suggests that CD4⁺ cells may induce the fibrosis partly through IL-13 production.

The almost complete dependence on CD4⁺ cells in the LAR was not surprising because Myets et al. (8) previ-

ously demonstrated that a late phase increase in enhanced Pause (Penh) was completely abolished by treatment with anti-CD4 mAb, although researchers have indicated that Penh measured by a whole-body plethysmography is not a reliable parameter of airway resistance (31, 32). On the other hand, it was also reported that airway remodeling was considerably dependent on CD4⁺ cells. In a murine model of asthma, in which sensitized BALB/c mice were exposed to a low dose of OVA for 30 min/day on 3 days/week for 6 weeks, not only airway hyperresponsiveness to methacholine but also airway remodeling was markedly suppressed by anti-CD4 mAb (11). As suggested by Kumar and Foster (33), the roles of cells and molecules in airway remodeling and hyperresponsiveness were altered by changing the protocols (dose and duration) for antigen challenge. Although our present protocol is a short-term schedule, in which a relatively high dose of antigen was used, this intratracheal challenge method has made it possible to detect specific antigen-induced airway obstruction in the late phase. It may be necessary to develop another late asthmatic model that can be developed by intratracheal administration of an antigen at a low dose for the long term. In addition, in order to further mimic the pathogenesis, natural antigen contaminated with endotoxin is also indispensable for an ideal model of experimental asthma, as indicated by the literature (34, 35).

The magnitude of inhibition by anti-CD4 mAb on airway remodeling was partial, even though CD4⁺ cells were almost completely depleted, indicating that CD4⁺ cells are required for the development of airway remodeling, especially for mucin accumulation and smooth muscle hyperplasia/hypertrophy. Doherty et al. (12) reported that when anti-CD4 mAb was administered beginning on a certain day after the initial antigen challenge, airway remodeling was not inhibited, indicating that ongoing progressive airway remodeling was not dependent on CD4⁺ cells. Because eosinophilia was also reduced by anti-CD4 mAb treatment in the study (12), they speculated that macrophages and activated epithelial cells may be involved in the formation of remodeling through the production of growth factors, such as TGF- β and vascular endothelial growth factor (VEGF) (12). Additionally, it has been suggested by human studies that neutrophils may also be involved in airway remodeling through the production of proteolytic enzymes, elastase and matrix metalloproteinase-9 (36, 37). In the present murine model, neutrophils infiltrated the lung after the respective 2nd–4th antigen challenges (13, 22), suggesting that neutrophils may play a role in the development of airway remodeling.

Because Th2 cytokines were markedly increased in the lung in this study, most CD4⁺ cells increased in the lung

by multiple antigen challenges should be CD4⁺ Th2 cells; however, it is well known that some dendritic cells and iNKT cells are also CD4⁺ (38, 39). In addition, we have to consider CD4⁺ regulatory T cells, which generally consist of two types, naturally occurring CD4⁺ CD25⁺ FoxP3⁺ Treg cells and antigen-induced CD4⁺ Tr1 cells (40, 41). We have detected antigen-induced Tr1-type IL-10-producing cells in the lung (T. Nabe et al., unpublished data). The proliferation of both CD4⁺ Tr1 cells and CD4⁺ Th2 cells could be simultaneously induced by multiple antigen challenges (T. Nabe et al., unpublished data). Because the induction of both types of CD4⁺ T cells could be abolished by anti-CD4 mAb treatment, airway remodeling might be augmented, resulting in only partial inhibition of the histopathological change by antibody treatment. The roles of IL-10-producing Tr1 cells in the development of airway remodeling have to be examined in the future.

In conclusion, the development of LAR was completely dependent on CD4⁺ cell activation, whereas the contribution of CD4⁺ cells to the development of airway remodeling was only partial and limited. Thus, this murine model of asthma may be useful for analysis of the mechanisms underlying not only CD4⁺ cell-mediated airway remodeling but also the pathogenesis mediated by CD4⁺ cells such as macrophages, epithelial cells, and neutrophils.

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