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Important role of neutrophils in the late asthmatic response in mice

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Abstract

Aims—Neutrophils have been found increasingly in the lungs of patients with severe asthma; however, it is unclear whether the neutrophils contribute to the induction of the airway obstruction. We determined using a murine model whether neutrophils are involved in the late asthmatic response (LAR), and analyzed mechanisms underlying the antigen-induced airway neutrophilia.

Main methods—BALB/c mice sensitized by ovalbumin (OVA)+Al(OH)₃ were challenged 4 times by intratracheal administration of OVA. Airway mechanics were measured as specific airway resistance.

Key findings—Induction of the LAR after the 4th challenge coincided with airway neutrophilia. In contrast, eosinophil infiltration was established prior to the 4th challenge. A treatment with an anti-Gr-1 monoclonal antibody (mAb) before the 4th challenge selectively suppressed increases in the neutrophil number and myeloperoxidase (MPO) level in bronchoalveolar lavage fluid (BALF), and attenuated the magnitude of LAR by 60–70%. Selective suppression of eosinophilia by anti-IL-5 mAb had little effect on the LAR. The increases in neutrophil number and MPO level were partially inhibited by an anti-CD4 mAb treatment. The CD4⁺ cell depletion also significantly inhibited increases in neutrophil chemoattractants, IL-17A, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 in BALF. However, blockade of FcγRII/III failed to suppress the neutrophilia.

Significance—These data suggest that neutrophils are key inducers of the LAR, and that the antigen-induced neutrophilia is partially dependent on activated CD4⁺ cells that are involved in the production of IL-17A, KC and MIP-2.

Keywords

asthma; late phase response; neutrophil; eosinophil; CD4⁺ T cell; IL-17; keratinocyte-derived chemokine; macrophage inflammatory protein-2

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Introduction

Asthma is characterized by an airway obstructive response that is based on chronic airway inflammation (Djukanovic et al. 1990). Eosinophils as well as CD4⁺ T cells are major inflammatory cells that migrate into the lungs of asthmatic patients (Djukanovic et al. 1990), while neutrophils have been regarded as minor effector cells in the pathogenesis of asthma. There has been mounting evidence suggesting that neutrophils are increased in the lungs of some patients with chronic asthma and in some patients who have died after exacerbation of asthma (Sur et al. 1993; Hahy et al. 1995; Lamblin et al. 1998; Wenzel et al. 1997; Foley and Hamid 2007). However, the functional role of neutrophils in the pathogenesis of asthma is still unclear and remains controversial.

The late asthmatic response (LAR), which is induced several hours after an allergen challenge, is regarded as one of the characteristic phenotypes of asthma (Varner and Lemanske Jr 2000). We have established a murine asthma model in which there is a late phase increase in airway resistance (Nabe et al. 2005). In this model, ovalbumin (OVA)-sensitized BALB/c mice were intratracheally challenged four times with the allergen. The LAR was induced after the 4th but not the 1st through 3rd challenges. Accumulation of eosinophils and CD4⁺ T cells had been induced in the lungs by the time before the 4th challenge (Nabe et al. 2005). In contrast, neutrophilia was induced during the 2nd and 3rd challenges, but then disappeared prior to the 4th challenge. The 4th challenge induced a recurrence of neutrophilia (Nabe et al. 2005). Based on these findings, we hypothesize that the cellular mechanism for the development of the LAR is as follows. The 1st through 3rd challenges are responsible for establishing the airway inflammation that is characterized by infiltrations of eosinophils and CD4⁺ T cells. Subsequently, the 4th challenge induces neutrophil infiltration into the airway, which contributes importantly to the induction of the LAR.

In this study, we examined the association of neutrophils with the induction of the LAR. Since Gr-1 is known to be predominantly expressed on neutrophils, we used RB6-8C5, an antibody against Gr-1 (Egan et al. 2008), to deplete neutrophils. However, because it is known that Gr-1 is also expressed on other leukocytes such as eosinophils and mononuclear cells (MNC) in mice (Lopez 1984; Fleming et al. 1993; Nagendra and Schlueter 2004), we used a flow cytometer to examine the composition of the Gr-1⁺ cells that infiltrated the lung. Since our initial results indicated that eosinophils were the other Gr-1⁺ cells, we assessed their role by examining the selective suppression of eosinophilia using an anti-IL-5 monoclonal antibody (mAb). Additionally, whether the airway neutrophilia and production of neutrophil chemoattractive cytokines and chemokines are dependent on antigen-induced CD4⁺ cell activation was investigated using an anti-CD4 mAb. Also, whether FcγRII/III activation by antigen-antibody immune complexes was involved in the neutrophilia was assessed by using an anti-FcγRII/III mAb.

Materials and Methods

Sensitization and challenge

Six-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were sensitized by intraperitoneal (i.p.) injections with OVA (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed to Al(OH)₃, which was made according to our previously reported method (Nabe et al. 2005), at a dose of 50 μg OVA adsorbed to 2 mg Al(OH)₃/0.5 ml of PBS/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, Japan) with 2% OVA at a volume of 25 μl by intratracheal administration, as reported previously (Ho and Furst 1973). As a negative control group, mice that had been sensitized

with OVA i.p. were treated with 4 mock airway challenges with PBS alone (Sensitized-Non-Challenged, S-NC).

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

Measurement of pulmonary function

Two different indicators of airway resistance, specific airway resistance (sRaw: $\text{cmH}_2\text{O} \times \text{ml}/(\text{ml}/\text{sec})$) and/or enhanced pause (Penh). “Penh” has been known to be a unit-less index, which can be obtained in laboratory animals using whole body plethysmography (Hamelmann et al. 1997) (Buxco Electronics, Troy, NY). It is the unique indicator of airway responsiveness and resistance that can be obtained in conscious unrestrained animals and therefore is freed from the bias imposed by anesthesia or restraining stress. However, it was also suggested that Penh could not be a reliable parameter of airway resistance (Bates et al. 2004). Thus, we used not only Penh but also “specific airway resistance (sRaw: $\text{cmH}_2\text{O} \times \text{ml}/(\text{ml}/\text{sec})$)”, which can be obtained by double-flow plethysmography (Pennock et al. 1979; Flandre et al. 2003) (Pulmos-I. II. III, M.I.P.S., Osaka, Japan). These parameters were measured 1 h before and at various times (10 and/or 30 min, and 1, 2, 3 and 4 h) after the 4th OVA challenge. Both systems are non-invasive technologies that make it possible to assess pulmonary function longitudinally over a prolonged time course.

To increase the reliability and reproducibility of the plethysmography data collected, all measurements were made in an environment controlled for both temperature (21–24°C) and humidity (45–65%).

Treatment with mAbs

In accordance with a previously reported method, a single intraperitoneally administered dose (150 $\mu\text{g}/\text{animal}$) of either an anti-Gr-1 mAb (RB6–8C5, eBioscience, San Diego, CA), an isotype-matched control Ab, rat IgG2b (eBioscience) or rat IgG (Sigma, St. Louis, MO), or PBS was given 18 h prior to the 4th antigen intratracheal challenge (Czuprynski et al. 1994).

The anti-IL-5 mAb-producing hybridoma cells, TRFK-5, were kindly donated by Prof. Kohtaro Fujihashi (University of Alabama at Birmingham, Birmingham, AL). The hybridoma cells were cultured and grown, and then intraperitoneally injected in severe combined immunodeficiency mice (Clea Japan, Tokyo, Japan) that had been treated with pristane (Sigma). 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-IL-5 mAb or rat IgG was intraperitoneally administered a single time at 3 h prior to the 1st antigen challenge at doses of 50, 100 and 200 $\mu\text{g}/\text{animal}$.

A cell line producing rat IgG2b mAbs that recognize the murine CD4 (YTS191.1.2) molecule were kindly provided by Prof. Osami Kanagawa (Washington University, St. Louis, MO). The anti-CD4 mAb was produced and purified as described above. The purified anti-CD4 mAb or rat IgG was intraperitoneally administered a single time at 18 h prior to the 4th antigen challenge at a dose of 0.6 mg/animal .

Anti-Fc γ RII/III mAb-producing hybridoma cells, 2.4G2, were purchased from American Type Culture Collection (Manassas, VA). The anti-Fc γ RII/III mAb was produced and purified as described above. The purified anti-Fc γ RII/III mAb or rat IgG was intraperitoneally administered a single time at 1.5 h prior to the 4th antigen challenge at a dose of 1 mg/animal .

Analysis of cells recovered by bronchoalveolar lavage (BAL)

Mice were sacrificed by lethal intramuscular injection using 50 μ l of a mixture of ketamine (25 mg/ml) and xylazine (10 mg/ml). The pulmonary circulation was perfused using 5 ml PBS, with the lungs then lavaged via a tracheal catheter using two aliquots of 0.8 ml PBS containing 2% FBS. Total leukocyte numbers were determined by a particle counter and size analyzer (Z2, Beckman Coulter, Brea, CA) after treatment with ACK lysis buffer to remove any contaminating erythrocytes. For the differential cell counts, cells were transferred onto a glass slide by centrifugation in a cell settling chamber (Neuro Probe, Gaithersburg, MD). Subsequently, cells were then stained with Diff-Quik solution (Sysmex International Reagent, Kobe, Japan).

The numbers of CD4⁺ and CD8⁺ cells in BALF were measured by a flow cytometry as previously reported (Nabe et al. 2005). In brief, after incubation with anti-mouse Fc γ RII/III mAb (clone 2.4G2, BD Biosciences, San Diego, CA) to block binding of subsequent antibodies to Fc γ RII/III, cells were incubated with PE-conjugated anti-mouse CD8 α mAb (clone 53-6.7) and Cy-Chrome-conjugated anti-mouse CD4 mAb (clone H129.19) (both from BD Biosciences). After washing, the stained cells were fixed with 4% paraformaldehyde, and then analyzed using a FACSCalibur (BD Biosciences) and Cell Quest software (version 3.3, BD Biosciences).

Flow cytometric analyses of Gr-1⁺ cells

Composition of Gr-1⁺ cells in the BAL fluid (BALF) was analyzed using flow cytometry and morphological observation. In brief, BAL cells were first incubated with the anti-mouse Fc γ RII/III antibody for 20 min at 4°C. The cells were then incubated with FITC-labeled anti-Gr-1 mAb (RB6-8C5, eBioscience) or FITC-labeled rat IgG2b (eBioscience) at 5 μ g/ml for 20 min at 4°C. After washing three times with PBS supplemented with 2% FBS, the stained cells were fixed with 4% paraformaldehyde for 12–18 h, and then analyzed using FACSCalibur and Cell Quest software. Dot plot for Gr-1⁺ cells was expressed by FL1 and FL2 after precise compensation.

For analysis of the Gr-1⁺ cell morphology, the cells were sorted using a FACSCalibur, centrifuged onto a glass slide, and then stained with Diff-Quik.

Histological studies

In separate experiments from BAL study, the lung was isolated before or 4 h after the 1st or 4th challenge. The isolated lung was fixed with 10% formalin, and then the tissues were embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin for light microscopic examination.

Assays of levels of myeloperoxidase (MPO), IL-17A, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2 and tumor necrosis factor (TNF)- α and IL-1 β in BALF

Levels of MPO, IL-17A, KC, MIP-2, TNF- α and IL-1 β in the BALF supernatant were measured using commercially available enzyme-linked immunosorbent assay kits for mouse MPO (Cell Sciences, Canton, MA), and mouse IL-17A, KC, MIP-2, TNF- α and IL-1 β (R&D Systems, Minneapolis, MN).

Statistical analyses

Statistical analyses were performed using one-way analysis of variance. If significant differences were detected, individual group differences were determined by a Bonferroni-Dunn test. Comparison between the pre- and post-challenge values was performed by a

paired *t*-test. A *P* value less than 0.05 was considered statistically significant. These analyses were performed by using the software package Prism 4 for Macintosh (version 4.0c, GraphPad Software Inc., San Diego, CA).

Results

Time-course changes for the leukocyte numbers and MPO levels in BALF

As previously reported (Nabe et al. 2005), mononuclear cells (MNC) and eosinophils have infiltrated into the airway tissue by the time before the 4th challenge, after which no further increases in those leukocyte numbers were observed (Figs. 1A and 1B).

We have previously reported that the 2nd and 3rd OVA challenges increased the number of neutrophils, which was then followed by an almost complete disappearance of the neutrophil infiltration prior to the 4th challenge (Nabe et al. 2005). In contrast to the eosinophil and MNC results, there was marked induction of neutrophilia in the lungs after the 4th challenge (Fig. 1C). This migration appeared to coincide with the induction of LAR. We also measured the MPO levels in BALF, as these levels are markers for neutrophil activation. Time-course changes in the MPO levels were very similar to those seen for the number of neutrophils (Figs. 1C and 1D).

Effect of an anti-Gr-1 mAb on the LAR and neutrophilic airway inflammation

In order to assess involvement of neutrophils in the induction of LAR, we attempted to evaluate the effects of an anti-Gr-1 mAb on LAR. Although it has been reported that Gr-1 is highly expressed on neutrophils (Egan et al. 2008), it has also been suggested that other cells express Gr-1 on their cell membrane (Lopez 1984; Fleming et al. 1993; Nagendra and Schlueter 2004). Thus, we evaluated the composition of the Gr-1⁺ cells within the cells that infiltrated into the lung. Our findings indicated that the Gr-1⁺ cells consist of two populations, Gr-1^{low} and Gr-1^{high} cells (Fig. 2). After using a flow cytometer to separate these two populations, we then observed these cells morphologically. As shown in Table 1, more than 90% of Gr-1^{low} and Gr-1^{high} cells were eosinophils and neutrophils, respectively.

A single treatment with the anti-Gr-1 mAb prior to the 4th challenge strongly suppressed the development of the neutrophilia that was assessed at 4 h after the 4th challenge (Fig. 3C). This coincided with the time that LAR was evoked. In addition, the increase in the MPO amount in the BALF was also markedly inhibited by the anti-Gr-1 mAb (Fig. 3D). In contrast, the MNC and eosinophil numbers that had accumulated in the lung after the 3rd challenge were not significantly affected by this anti-Gr-1 mAb treatment (Figs. 3A and 3B).

Figures 3E and 3F show the effect of the anti-Gr-1 mAb treatment on the induction of LAR. When Penh was used as the parameter to measure airway resistance, the magnitude of LAR was found to be significantly suppressed (60–70%) by anti-Gr-1 mAb treatment but not by rat IgG treatment (Fig. 3E). Similarly, when sRaw was used as another different parameter, the late phase increase in sRaw clearly indicated LAR suppression by anti-Gr-1 mAb treatment (Fig. 3F). Additionally, we measured Penh and sRaw over the entire time course in a negative control group of sensitized animals that received mock airway challenges with PBS alone. These controls demonstrated that baseline Penh and sRaw values were stable over this time course (Figs. 3E and 3F).

Figure 4 represents histological changes in the lung tissue isolated before the 1st challenge, and before and 4 h after the 4th challenge. As has been reported (Nabe et al. 2005), compared to the histology of the lung tissue isolated before the 1st challenge (Fig. 4A), epithelial metaplasia in the bronchus and massive infiltration of inflammatory cells mainly consisting of eosinophils and MNC were evidently observed in the lung tissue that was

isolated prior to the 4th challenge (Fig. 4B). We have reported that the epithelial metaplasia was closely associated with mucus deposition as assessed by periodic acid-Schiff staining (Nabe et al. 2005). Under the existence of the airway inflammation, the 4th challenge apparently induced infiltration of neutrophils (Fig. 4C). This histological finding became a strong confirmation of the result in BAL study (Fig. 1C). The treatment with anti-Gr-1 mAb before the 4th challenge did not improve the established epithelial metaplasia and infiltration of eosinophils and MNC, whereas the recruitment of neutrophils induced by the 4th challenge was abolished by the treatment (Fig. 4D). This effect of anti-Gr-1 mAb was also consistent with that in the BAL study shown in Fig. 3C.

Effect of a selective suppression of eosinophils on the LAR

To determine whether eosinophils play a role in the induction of LAR, we assessed the selective suppression of the development of eosinophilia. After a single treatment with anti-IL-5 mAb (50 µg/animal) prior to the 1st challenge, the development of eosinophilia in the airway was strongly suppressed by approximately 70% (Fig. 5B). However, when the dose of the anti-IL-5 mAb was increased to 100 and 200 µg/animal, there was no further inhibition of the airway eosinophilia noted (data not shown). In contrast, anti-IL-5 mAb caused no changes or decreases in any of the other cells, such as the neutrophils and the MNC in BALF (Figs. 5A and 5C).

It should be noted, however that even though the development of eosinophilia was selectively and strongly suppressed, the anti-IL-5 mAb treatment did not inhibit the late phase increase in sRaw after the 4th challenge (Fig. 5D). The magnitude of LAR in the control group was smaller than that in Fig. 3F. Although reasons for the differences are not clear, we suspect that this is due to batch to batch differences in animals. On the other hand, histological changes were not affected by the anti-IL-5 mAb treatment (data not shown).

Effect of an anti-CD4 mAb on the LAR and neutrophilic airway inflammation

We next assessed whether the 4th challenge-induced neutrophilia was related to CD4⁺ cell activation. A treatment with an anti-CD4 mAb (0.6 mg/animal) 18 h prior to the 4th challenge almost completely depleted CD4⁺ cells not only in BALF (Table 2) but also in the spleen and peripheral blood (data not shown). In contrast, numbers of neither total cells nor CD8⁺ cells were affected by the anti-CD4 mAb treatment (Table 2).

Table 3 shows effects of the anti-CD4 mAb treatment on increases in Th2 cytokine levels in BALF collected 4 h after the 4th challenge. The increases in IL-5 and IL-13 levels were almost completely suppressed by the anti-CD4 mAb (Table 3). The IL-4 level was also reduced by the treatment, whereas the inhibitory rate was approximately 50% (Table 3). We have data indicating that combined treatment with the anti-CD4 mAb and an anti-IgE neutralizing mAb completely abolished the increase in IL-4 level (unpublished data). These data indicate that basophils, together with CD4⁺ T cells, contribute as important cellular sources of IL-4. These findings are consistent with the observations of Luccioli et al. (2002), using a different murine model of asthma.

The anti-CD4 mAb did not affect the eosinophilia, which had been established by the first three challenges (Fig. 6A), whereas the increase in the number of neutrophils in BALF tended to be suppressed by the anti-CD4 mAb (Fig. 6B). Thus, we measured the amount of MPO as a marker of neutrophil activation, resulting that the anti-CD4 mAb treatment significantly suppressed the increase in MPO level by more than 50% (Fig. 6C). The late increase in sRaw at 2–4 h was significantly inhibited by the anti-CD4 mAb by approximately 70% (Fig. 6D).

From the finding that the infiltration and/or activation of neutrophils was decreased by the anti-CD4 mAb, we next examined whether the anti-CD4 mAb affected production of cytokines and chemokines that have been reported to function as neutrophil chemoattractants. We assessed concentrations of IL-17A, KC, MIP-2, TNF- α and IL-1 β in BALF before and after the 4th challenge. Consequently, all those neutrophil chemoattractants were increased in BALF by the 4th challenge during the occurrence of LAR, although increased levels of respective molecules were considerably different from each other (Figs. 7A, 7B, 7C, 7D and 7E). Increase in IL-17A (Fig. 7A), KC (Fig. 7B) and MIP-2 (Fig. 7C) were partially but significantly suppressed by the anti-CD4 mAb treatment, although that in neither TNF- α (Fig. 7D) nor IL-1 β (Fig. 7E) was affected by the treatment.

Effect of an anti-Fc γ RII/III mAb on the neutrophilia and LAR

In order to determine whether immune complexes of the antigen and antigen-specific IgG Ab are also involved in the induction of LAR through activation of Fc γ Rs on inflammatory cells such as macrophages, effects of an anti-Fc γ RII/III mAb, 2.4G2 on neutrophilia and LAR were assessed. According to other reports (Kurlander et al. 1984; Strait et al. 2006), the anti-Fc γ RII/III mAb was intraperitoneally administered 1.5 h before the 4th challenge at a dose of 1 mg/animal. However, this receptor blockade suppressed neither increases in numbers of leukocytes (Figs. 8A, 8B and 8C) nor the LAR (Fig. 8D).

Discussion

In this intratracheal antigen challenge-induced asthma model, the LAR was induced several hours after the 4th challenge but not after the 1st, 2nd, or 3rd challenge. Infiltration of inflammatory cells such as eosinophils and CD4⁺ T cells along with epithelial metaplasia associated with mucus deposition were established in the lung prior to the 4th challenge (Nabe et al. 2005). Once the airway inflammation was present, the attack on the airway tissue due to the 4th antigen challenge then led to LAR. Neutrophils were not observed just prior to the 4th challenge, whereas the 4th challenge induced neutrophilia that coincided with the LAR. Our data reported here suggest that after the 4th airway allergen challenge, the murine LAR is mediated by neutrophils at the effector phase of the response.

When examining the roles of neutrophils in various disease models, the anti-Gr-1 mAb, RB6–8C5, has been extensively used as a tool for depleting these cells in mice (Kurlander et al. 1984; Chen et al. 2001; Mednick et al. 2003). It has been previously shown that Gr-1 is expressed on the Ly6G and Ly6C glycoproteins, while neutrophils, in particular, express high levels of Gr-1 associated with Ly6G expression (Egan et al. 2008). It has also been shown that leukocytes such as other granulocytes, eosinophils, and MNC, including plasmacytoid dendritic cells, can also express Gr-1 (Lopez 1984; Fleming et al. 1993; Nagendra and Schlueter 2004). Thus, we analyzed the composition of the Gr-1⁺ cells that infiltrated into the lungs. Two populations of Gr-1⁺ cells existed in the inflamed lung: GR-1^{high} and Gr-1^{low} cells. More than 90% of these GR-1^{high} and Gr-1^{low} cells were morphologically determined to be neutrophils and eosinophils, respectively. Gr-1⁺ MNC made up only a minor part of the overall population.

RB6–8C5 treatment strongly suppressed both the 4th challenge-induced neutrophilia and the increase in the MPO level, which is a marker of neutrophil activation. The neutrophils in the circulation were also selectively and almost completely depleted by the anti-Gr-1 treatment (data not shown). Interestingly, the airway eosinophilia, which had been established by the 1st-3rd challenges, was not reduced by the mAb treatment. These results suggest that neutrophils but not eosinophils play an important role in the induction of LAR. There is also the possibility that the activation of eosinophils due to the release of toxic proteins or mediators is induced by the 4th challenge but then inhibited by the anti-Gr-1 mAb treatment.

Since no tools exist that can be utilized to specifically assess eosinophil activation in mice, the roles of eosinophils in the development of LAR could only be tested by selectively reducing the eosinophil infiltration into the lung via the use of the anti-IL-5 mAb, TRFK-5. Our findings indicated that even though the lung eosinophilia was reduced by approximately 70%, the LAR was not inhibited. Collectively, these results further support the possibility that neutrophils and not eosinophils are key effector cells in the induction of LAR.

A previous study using a murine model of the LAR reported that the anti-IL-5 mAb, TRFK-5, inhibited not only airway eosinophilia but also the LAR that is detected by Penh (Cieslewicz et al. 1999). Reasons for the difference in the effect of the anti-IL-5 mAb on the LAR between our results and this previous report are not clear at this time. It can be speculated that this difference could be due to the magnitudes of the airway obstructive responses. In other words, when comparing the increases in the Penh values, the magnitude of the LAR in our model might have been considerably more severe than that seen in the previous report (Cieslewicz et al. 1999). On the other hand, it has been reported that a humanized anti-IL-5 mAb, mepolizumab, failed to improve airway hyperresponsiveness and the LAR in asthmatic patients even though there was a marked reduction of the eosinophilia in the circulation and airway tissue (Leckie et al. 2000). This clinical ineffectiveness on changes in the airway physiology was further confirmed by an additional study using mepolizumab (Flood-Page et al. 2007) and another study that used a different anti-IL-5 mAb, SCH 55700 (Kips et al. 2003). Our present results for anti-IL-5 mAb are very similar to those clinical studies (Leckie et al. 2000; Kips et al. 2003; Flood-Page et al. 2007), which suggest that the pathophysiological mechanisms of the LAR in our mouse model may indeed be associated with those for the human LAR. In addition, consistent with a recent review by Foley and Hamid (2007) that described the particularly important role of neutrophils in asthma, our findings suggest that neutrophils rather than eosinophils contribute to the pathogenesis of the LAR in this asthmatic model.

Although eosinophilic inflammation can be well controlled by treatment with glucocorticoids, neutrophilic airway inflammation is resistant to glucocorticoid therapy (Fukakusa et al. 2005). The LAR in our model was completely suppressed when eosinophil accumulation was inhibited by daily treatments of the strong glucocorticoid dexamethasone during the 1st through the 4th challenges (Nabe et al. 2005). In contrast, only partial attenuation was achieved when dexamethasone was only administered a single time prior to the 4th challenge (Nabe et al. 2005). However, it should be noted that although only a single dose was given, the dose used was a high dose (3 mg/kg). Thus, since mice are known to be high responders to glucocorticoids, doses of 0.1–1 mg/kg should be pharmacologically sufficient to suppress glucocorticoid-sensitive inflammatory responses.

Regarding mechanisms underlying allergic neutrophil recruitment into inflammatory sites, various pathways have been reported to date. It has been recently established that CD4⁺ Th17 cells activated by antigen stimulation play important roles in neutrophil infiltration into the lung through IL-17 production (McKinley et al. 2008; Wilson et al. 2009). In order to analyze mechanisms that underlie the antigen-induced lung neutrophilia associated with the LAR, we first examined whether CD4⁺ cell activation was involved in the response. The CD4⁺ cell depletion by the anti-CD4 mAb partially suppressed the increased numbers of neutrophils, MPO levels and quantity of IL-17A in BALF of challenged mice, suggesting that CD4⁺ Th17 cells could play significant roles in the antigen-induced airway neutrophilia through IL-17 production. On the other hand, because IL-8, TNF- α and IL-1 β have also been well known as neutrophil chemoattractants that orchestrates their activation and recruitment from the circulation into sites of inflammation (Kobayashi 2006; Yu and Gaffen 2008), the effects of depleting CD4⁺ cells on the production of those molecules were also investigated. Elevation of the functional murine homologues of human IL-8 (Kobayashi

2006), KC and MIP-2, but neither TNF- α nor IL-1 β , were also partially but significantly inhibited by treatment with the anti-CD4 mAb, suggesting that the production of KC and MIP-2 was mediated by CD4⁺ cell activation. Because KC and MIP-2 are produced from macrophages, epithelial cells, etc. in the lung (Smit and Lukacs 2006), it can be speculated that activated CD4⁺ cells directly or indirectly induced production of the CXC chemokines by activating macrophages and/or epithelial cells in the lung, leading to the airway neutrophilia.

As another mechanism relating to neutrophilia, the roles of immune complexes of antigen and antigen-specific IgG were analyzed, because 1) immune complexes can induce neutrophil-mediated lung injury through activation of the complement system (Guo et al. 2005), 2) a large amount of antigen-specific IgG1 was produced in the serum of this model (Nabe et al. 2005), and 3) we have reported that a C3a receptor antagonist significantly reduced not only eosinophilia but also neutrophilia in sensitized/challenged mice (Mizutani et al. 2009). It has also been reported that the immune complex-induced response is mediated by activation of Fc γ RII/III on macrophages (Strait et al. 2006). Therefore, we also assessed the effects of an anti-Fc γ RII/III mAb, 2.4G2 on neutrophilia and LAR. However, this receptor blockade failed to suppress the inflammatory changes. Thus, immune complexes of antigen and antigen-specific IgG could not be involved in the neutrophilia in this model.

In contrast to the mobilization of eosinophils, the trafficking of neutrophils from the circulation into the airway tissue is known to be rapid, as demonstrated further in our present study. During the infiltration process, activated neutrophils are capable of producing a variety of mediators such as reactive oxygen species (ROS) and proteases (Beeh and Beier 2006; Louis and Djukanovic 2006; Cowburn et al. 2008). Although detailed mechanisms underlying the neutrophil-mediated airway obstruction during the late phase are unclear, prior studies have suggested that activated neutrophils could contribute to the development of airway obstruction through induction of mucus hypersecretion (Louis and Djukanovic 2006). Because mediators released by neutrophils, such as elastase and proteinase 3 are potent stimuli for glandular secretion in vitro (Witko-Sarsat et al. 1999), these proteases may play roles in the development of the LAR via inducing mucus hypersecretion. Data suggesting a relationship between ROS and airway narrowing have also been reported: ROS can increase the contractile response of airway smooth muscle (Samb et al. 2002), and lead to endothelial barrier dysfunction with subsequent plasma leakage (Lee et al. 2006). On the other hand, neutrophils have also been suggested to contribute to the development of airway remodeling (Beeh and Beier 2006; Louis and Djukanovic 2006; Cowburn et al. 2008). In this study, the anti-Gr-1 mAb was administered once before the 4th challenge, at which time airway remodeling, including epithelial metaplasia, had already been established. In order to assess the roles of neutrophils in the remodeling process, it would be necessary to treat with the anti-Gr-1 mAb repeatedly, beginning at the time of the first airway antigen challenge. However, probably because of the remarkable ability of the activated bone marrow to replenish neutrophil numbers, it would be difficult to sustain neutropenia for the time required by such an experiment.

Lastly, in these experiments we chose not to use endotoxin-free antigen because natural exposure generally includes both antigenic proteins and environmental endotoxin together. Thus, endotoxin that contaminates most preparations of OVA may contribute to the neutrophilia we have observed here. It has been suggested that endotoxin has the potential to contribute to the development of asthma in several different ways; studies indicate endotoxin's potential either to suppress the development of asthma through induction of counterregulatory Th1 cells, or to exacerbate asthma severity presumably through its pro-inflammatory activities (Schwartz 2001; Liu 2002; Reed and Milton 2003; Jung et al. 2006).

Jung et al. (2006) demonstrated by experiments using TLR4 mutant mice that antigen and endotoxin act in a synergistic manner in the development of airway inflammation. In order to assess the contributions of contaminating endotoxin in the induction of neutrophilia and LAR, future studies will be required to investigate the magnitudes of asthmatic responses induced by endotoxin-free OVA or by conventional OVA in TLR4-deficient mice. Underscoring the potential importance of these studies, bacterial infections are known to modulate asthmatic responses (Chu et al. 2003). Furthermore, viral infections have been reported to exacerbate allergen-induced airway neutrophilia and hyperresponsiveness (Nagarkar et al. 2010). The neutrophil-associated LAR in this model may be a tool for analyzing the relationship between innate and acquired immunity in asthma pathogenesis.

In conclusion, our findings suggest that intratracheal antigen challenge-induced LAR is mediated in part by the infiltration of neutrophils but not eosinophils. The antigen-induced neutrophilia was partially dependent on activated CD4⁺ cells that were involved in the production of IL-17A, KC and MIP-2, whereas FcγRII/III activation was not related to the neutrophilia. This model provides a valuable tool to elucidate further the mechanisms that underlie neutrophilic airway inflammation associated with LAR.

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Fig. 1. Time-course changes in the number of mononuclear cells (MNC, A), eosinophils (B), neutrophils (C), and in the amount of myeloperoxidase (MPO, D) in bronchoalveolar lavage fluid after the 4th antigen challenge in sensitized mice. Each point represents the mean $\pm$ S.E. of 5 or 6 animals.

Fig. 2. Typical flow cytometric profiles of Gr-1⁺ cells in cell suspensions from bronchoalveolar lavage fluid collected 4 h after the antigen challenge in sensitized mice. Cells were incubated with FITC-labeled rat IgG2b (A), or stained with FITC-labeled anti-Gr-1 mAb (B).

Fig. 3.

Effect of the anti-Gr-1 mAb, RB6-8C5, on the increase in the number of mononuclear cells (MNC, A), eosinophils (B), neutrophils (C), and in the amount of myeloperoxidase (MPO, D) in bronchoalveolar lavage fluid collected 4 h after the 4th antigen challenge, and on the increases in the enhanced pause (Penh, E) and specific airway resistance (sRaw, F) by the 4th challenge in sensitized mice. The anti-Gr-1 mAb (150 μ g/animal, black triangles), rat IgG2b (150 μ g/animal, closed squares), or PBS (open squares) was administered intraperitoneally 18 h before the 4th challenge. S-NC (open circles): Sensitized animals that received mock airway challenges with PBS. Each column or point represents the mean $\pm$ S.E. of 12–19 animals. ††, †††: $P < 0.01$, $P < 0.001$ vs. rat IgG2b-treated.

(A) Before the 1st challenge

(B) Before the 4th challenge

(C) Four h after the 4th challenge
(Rat IgG-treated) (D) Four h after the 4th challenge
(Anti-Gr-1 mAb-treated) **Fig. 4.**

Histological changes in the lung tissue isolated before the 1st challenge (A), and before (B) and 4 h after the 4th challenge (C), and effect of the anti-Gr-1 mAb, RB6-8C5, on the histological changes (D). The anti-Gr-1 mAb (150 μ g/animal, D) or rat IgG (150 μ g/animal, C) was administered intraperitoneally 18 h before the 4th challenge. Arrows indicate neutrophils.

Fig. 5.

Effects of the anti-IL-5 mAb, TRFK-5, on the increase in the number of mononuclear cells (MNC, A), eosinophils (B) and neutrophils (C) in bronchoalveolar lavage fluid collected 4 h after the 4th antigen challenge, and on the increases in the specific airway resistance (sRaw, D) by the 4th challenge in sensitized mice. The anti-IL-5 mAb (50 $\mu\text{g}/\text{animal}$) or rat IgG (50 $\mu\text{g}/\text{animal}$) was administered intraperitoneally 3 h before the 1st challenge. Each column or point represents the mean $\pm$ S.E. of 14 animals. $\dagger\dagger$: $P < 0.01$ vs. rat IgG-treated.

Fig. 6.

Effect of the anti-CD4 mAb on the increase in the number of eosinophils (A) and neutrophils (B), and myeloperoxidase (MPO, C) amount in bronchoalveolar lavage fluid collected 4 h after the 4th antigen challenge, and on the increases in the specific airway resistance (sRaw, D) by the 4th challenge in sensitized mice. The anti-CD4 mAb (0.6 mg/animal) or rat IgG (0.6 mg/animal) was administered intraperitoneally 18 h before the 4th challenge. Each column and point represents the mean $\pm$ S.E. of 10–20 animals. ***: $P < 0.001$ vs each cell number before the challenge, †, ††, †††: $P < 0.05$, $P < 0.01$, $P < 0.001$ vs rat IgG-treated.

Fig. 7.

Effect of the anti-CD4 mAb on increases in amounts of IL-17A (A), keratinocyte-derived chemokine (KC, B), MIP-2 (C), TNF- α (D) and IL-1 β (E) in bronchoalveolar lavage fluid collected 4 h after the 4th antigen challenge in sensitized mice. The anti-CD4 mAb (0.6 mg/animal) or rat IgG (0.6 mg/animal) was administered intraperitoneally 18 h before the 4th challenge. Each column represents the mean $\pm$ S.E. of 10–20 animals. ***: $P < 0.001$ vs each value before the challenge, ††, †††: $P < 0.01$, $P < 0.001$ vs rat IgG-treated.

Fig. 8.

Effects of the anti-FcγRII/III mAb, 2.4G2, on the increase in the number of mononuclear cells (MNC, A), eosinophils (B) and neutrophils (C) in bronchoalveolar lavage fluid collected 4 h after the 4th antigen challenge, and on the increases in the specific airway resistance (sRaw, D) by the 4th challenge in sensitized mice. The anti-FcγRII/III mAb (1 mg/animal) or rat IgG (1 mg/animal) was administered intraperitoneally 1.5 h before the 4th challenge. Each column or point represents the mean ± S.E. of 7 or 8 animals.

Table 1

Morphological classification of Gr-1^{high} and Gr-1^{low} cells in bronchoalveolar lavage fluid collected 4 h after the 4th challenge in sensitized mice.

	% of gated cells		
	MNC	Eosinophils	Neutrophils
Gr-1 ^{high} cells	3.1±0.4	5.7±0.9	91.2±0.8
Gr-1 ^{low} cells	1.9±0.3	96.2±0.4	1.9±0.7

Gr-1^{high} and Gr-1^{low} cells were fractionated using a flow cytometer as shown in Fig. 2. MNC: mononuclear cells. Each value represents the mean±S.E. of 3 experiments. Each experiment was performed using bronchoalveolar lavage cells collected from 6 animals.

Table 2

Effect of the anti-CD4 mAb on the increased number of total cells, CD4⁺ cells and CD8⁺ cells in bronchoalveolar lavage fluid collected 4 h after the 4th challenge in sensitized mice.

	Number of cells ($\times 10^4$ cells/animal)		
	Total cells	CD4 ⁺ cells	CD8 ⁺ cells
Before the 4th challenge	90.3 $\pm$ 10.7	4.9 $\pm$ 0.8	2.2 $\pm$ 0.4
Four h after the 4th challenge			
Rat IgG-treated	109.6 $\pm$ 8.0	6.0 $\pm$ 0.6	1.7 $\pm$ 1.2
Anti-CD4 mAb-treated	116.3 $\pm$ 10.7	0.03 $\pm$ 0.01 ^{†††}	1.8 $\pm$ 0.4

The anti-CD4 mAb (0.6 mg/animal) or rat IgG (0.6 mg/animal) was administered intraperitoneally 18 h before the 4th challenge. Each value represents the mean $\pm$ S.E. of 10–20 animals.

^{†††}P<0.001 vs rat IgG-treated.

Table 3

Effect of the anti-CD4 mAb on increases in amounts of Th2 cytokines, IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid collected 4 h after the 4th challenge in sensitized mice.

	Amount (pg/ml)		
	IL-4	IL-5	IL-13
Before the 4th challenge	5±1	4±3	32±10
Four h after the 4th challenge			
Rat IgG-treated	182±25 ^{***}	108±20 ^{***}	246±43 ^{***}
Anti-CD4 mAb-treated	83±15 ^{†††}	13±5 ^{†††}	55±12 ^{†††}

The anti-CD4 mAb (0.6 mg/animal) or rat IgG (0.6 mg/animal) was administered intraperitoneally 18 h before the 4th challenge. Each value represents the mean±S.E. of 10–20 animals.

^{***} P<0.001 vs each value before the challenge,

^{†††} P<0.001 vs rat IgG-treated.