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Regulatory role of antigen-induced interleukin-10, produced by CD4$^+$ T cells, in airway neutrophilia in a murine model for asthma

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ABSTRACT

It has been suggested that interleukin (IL)-10 exerts immunosuppressive effects on allergic inflammation, including asthma, mainly through inhibition of Th2 cell-mediated eosinophilic airway inflammation. In a model of experimental asthma utilizing multiple intratracheal antigen challenges in sensitized mice, IL-10 production as well as eosinophilia and neutrophilia in the lung were induced by the multiple challenges. In this study, we set out to reveal the cellular source of endogenously produced IL-10, and the roles of IL-10 in airway leukocyte inflammation using an anti-IL-10 receptor monoclonal antibody. Balb/c mice were sensitized i.p. with ovalbumin + $Al(OH)_3$, and then challenged by intratracheal administration of ovalbumin 4 times. Flow cytometric analyses revealed that the cellular source of IL-10 was CD4$^+$ T cells lacking the transcription factor, forkhead box P3. Treatment with anti-IL-10 receptor monoclonal antibody prior to the 4th challenge significantly augmented airway neutrophilia as well as the production of IL-1β, and CXC chemokines, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, but not airway eosinophilia, Th2 cytokine (IL-4 and IL-5) production, or a late-phase increase in specific airway resistance. Approximately 40% of IL-10 receptor$^+$ cells expressed the macrophage marker F4/80, whereas only 3–4% of the IL-10 receptor$^+$ cells were granulocyte differentiation antigen (Gr)-1^{high} cells (neutrophils). In conclusion, multiple airway antigen challenges induced the proliferation of IL-10-expressing CD4$^+$ T cells in regulating airway neutrophilia. Systemic blockade of IL-10 function coincided with increases in IL-1β and CXC chemokines. Thus, IL-1β and CXC chemokines may be targets for development of novel pharmacotherapy for neutrophilic asthma.

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1. Introduction

Asthma is characterized by chronic airway inflammation (Djukanovic et al., 1990). CD4$^+$ T cells and their products, such as interleukin (IL)-4, IL-5 and IL-13, play central roles in orchestrating inflammation, especially through infiltration by eosinophils into the airway (Robinson, 2010). Additionally, other cell types are involved in asthma pathogenesis: for example, neutrophilic airway inflammation is associated with severe airflow obstruction. Neutrophilic airway inflammation is difficult to control with glucocorticoids (Foley and Hamid, 2007; Lamblin et al., 1998; Wenzel et al., 1997). Tumor necrosis factor (TNF)-α, IL-1β and IL-8 have been shown to play important roles in the induction of neutrophilic inflammation (Cowburn et al., 2008). The recently identified Th17 helper cell subset has also been implicated in neutrophil-dominated asthma through the production of IL-17 (Alcorn et al., 2010), and shown to be responsible for steroid-resistant asthma (McKinley et al., 2008).

IL-10 is an important cytokine with anti-inflammatory properties (Sabat et al., 2010). Although all types of leukocytes have the ability to produce IL-10 (Wolk et al., 2002), important sources of IL-10 in vivo are monocytes and macrophages as well as T cell subsets, including Th1, Th2, Tr1, and Treg cells (Sabat et al., 2010). IL-10 exerts its anti-inflammatory actions through activation of a transmembrane receptor complex consisting of IL-10 receptor 1 and IL-10 receptor 2 (Sabat et al., 2010). Activation of a variety of inflammatory cells was inhibited by IL-10, leading to a fall in the production of cytokines and chemokines (Lloyd and Hawrylowicz, 2009; Sabat et al., 2010). In asthma pathogenesis, it has been suggested that endogenous IL-10 acts as an attenuating regulator in both humans (O'Garra et al., 2008) and mice (Tournoy et al., 2000). Additionally, exogenously administered recombinant IL-10 or adenovirus-expressing IL-10 alleviated

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antigen-induced airway inflammation in mice (Fu et al., 2006; van Scott et al., 2000).

We showed that in an experimental model of asthma, late phase airway narrowing associated with both airway eosinophilia and neutrophilia was evoked after repeated intratracheal allergen challenges in sensitized mice (Mizutani et al., 2009; Nabe et al., 2005). We found that late-phase airway obstruction was dependent on neutrophils infiltrating the lung because selective reduction of neutrophilia by treatment with an anti-granulocyte differentiation antigen (Gr)-1 monoclonal antibody, RB6-8C5, effectively inhibited the late phase response (Nabe et al., 2011). Interestingly, multiple antigen challenges in sensitized mice induced not only the production of Th2 cytokines, but also induced the anti-inflammatory cytokine, IL-10, in the lung (Nabe et al., 2005). Altogether, these data led us to postulate that antigen-responsive IL-10-producing cells may be expanded by multiple challenges, and that endogenously produced IL-10 may inhibit these asthmatic responses.

In this study, we first tried to identify the cellular source of IL-10, which was induced by multiple antigen challenges in the lung. Secondly, we assessed whether endogenously produced IL-10 contributes to the regulation of allergic airway neutrophilia and eosinophilia in vivo by using an anti-IL-10 receptor monoclonal antibody, 1B1.3a (Kearley et al., 2005). Furthermore, the effects of the anti-IL-10 receptor monoclonal antibody on the production of cytokines and chemokines relating to cellular trafficking were assessed.

2. Materials and methods

2.1. Sensitization and challenge

Six-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). As shown in Fig. 1A, the mice were sensitized by i.p. injections of ovalbumin (Grade V; Sigma Chemical Co., St. Louis, MO, USA) adsorbed to $Al(OH)_3$, which was made according to our previously reported method (Nabe et al., 2005), at a dose of 50 μg ovalbumin adsorbed to 2 mg $Al(OH)_3$/0.5 ml phosphate buffered saline (PBS)/animal on days 0, 14 and 28. Sensitized mice were challenged on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane (Abbott Japan, Tokyo, Japan) with 2% ovalbumin at a volume of 25 μl by intratracheal administration, as reported previously (Ho and Furst, 1973).

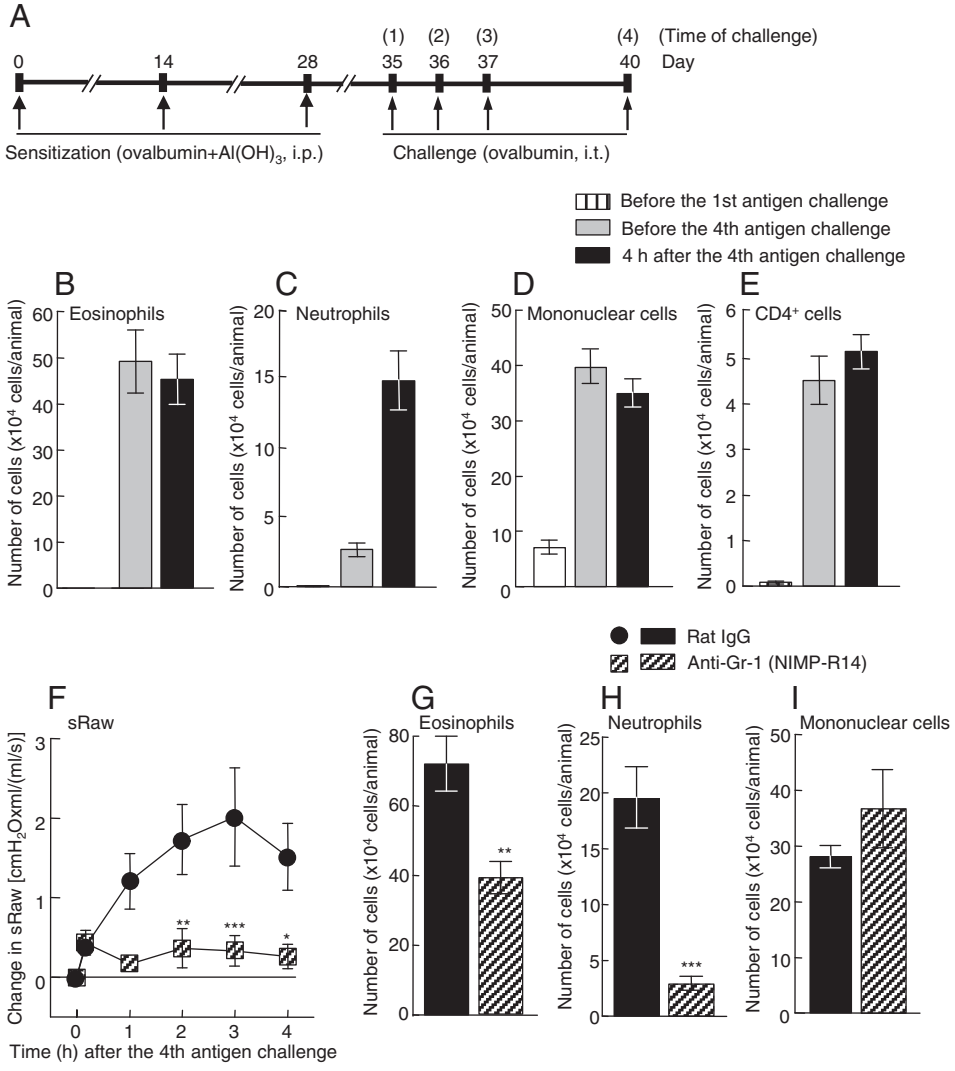

Fig. 1. (A) Schedule for i.p. sensitization and intratracheal (i.t.) antigen challenges to develop an asthmatic model in mice, (B–E) time-course changes in numbers of eosinophils (B), neutrophils (C), mononuclear cells (D) and $CD4^+$ cells (E) in bronchoalveolar lavage fluid before and/or after the 1st and 4th antigen challenges, and (F–I) effects of an anti-granulocyte differentiation antigen (Gr)-1 monoclonal antibody, NIMP-R14, on the increase in specific airway resistance (sRaw, F), and the increased numbers of eosinophils (G), neutrophils (H) and mononuclear cells (I). Anti-Gr-1 monoclonal antibody (300 μg/animal) or rat IgG (300 μg/animal) was i.p. administered 5 h before the 4th challenge. Each column in panels B–E represents the mean ± S.E.M. of 4–10 animals. Each point and column in panels F–I represent the mean ± S.E.M. of 14 animals. *, ** and ***: $P<0.05$, $P<0.01$ and $P<0.001$, vs rat IgG-treated group.

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

2.2. Treatment with anti-Gr-1 monoclonal antibody or anti-IL-10 receptor monoclonal antibody

A cell line producing rat immunoglobulin (Ig) G2b monoclonal antibody, which recognizes the murine Gr-1 (Ly6C/Ly6G) molecule (clone NIMP-R14), was cultured and grown, and then i.p. injected into severe combined immunodeficient mice (Clea Japan, Tokyo, Japan) that had been treated with pristane (Sigma). Ascites was collected 10–14 days after injection. IgG was purified by protein G affinity chromatography (GE Healthcare, Uppsala, Sweden), followed by desalting using PD-10 column chromatography (GE Healthcare). Purified anti-Gr-1 monoclonal antibody or rat IgG (Sigma) was administered i.p. once, 5 h prior to the 4th antigen challenge at a dose of 300 μg/animal.

A purified rat IgG1 monoclonal antibody, which recognizes the murine IL-10 receptor 1 molecule (clone 1B1.3a) (Kearley et al., 2005), was kindly provided by Prof. Casey T. Weaver (Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA). The purified anti-IL-10R monoclonal antibody or rat IgG was administered i.p. once, 30 min prior to the 4th antigen challenge at a dose of 500 μg/animal.

2.3. Measurement of pulmonary function

As an indicator of airway resistance, specific airway resistance (sRaw: $cmH_2O \times ml/(ml/s)$) was measured by double-flow plethysmography (Pulmos-I, II, III; M.I.P.S., Osaka, Japan) (Flandre et al., 2003; Pennock et al., 1979). The parameter was measured 1 h before and at various times (10 min and 1, 2, 3 and 4 h) after the 4th ovalbumin challenge. The system is a non-invasive technology that makes it possible to assess pulmonary function longitudinally over a prolonged time course.

2.4. Analysis of cells recovered by bronchoalveolar lavage (BAL)

Mice were sacrificed by lethal intramuscular injection using 50 μl of a mixture of ketamine (25 mg/ml) and xylazine (10 mg/ml). The pulmonary circulation was perfused using 5 ml PBS, and then the lung was lavaged via a tracheal catheter using two aliquots of 0.8 ml PBS containing 2% fetal bovine serum (FBS). In experiments evaluating the effects of anti-IL-10 receptor monoclonal antibody, only the right lobes were lavaged using two aliquots of 0.6 ml PBS containing 2% FBS after ligation of the left mainstem bronchus. Total leukocyte numbers were determined by a particle counter and size analyzer (Z2; Beckman Coulter, Brea, CA, USA) 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 any contaminating erythrocytes. For 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 stained with Diff-Quik solution (Sysmex International Reagent, Kobe, Japan).

The numbers of $CD4^+$ cells in BAL fluid (BALF) were measured by flow cytometry as previously reported (Nabe et al., 2005). In brief, after incubation with anti-mouse Fcγ receptor II/III monoclonal antibody (clone 2.4G2; BD Biosciences, San Diego, CA, USA) to block binding of subsequent antibodies to Fcγ receptor II/III, cells were incubated with Cy-Chrome-conjugated anti-mouse CD4 monoclonal antibody (clone H129.19) (both from 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).

2.5. Cellular source of IL-10 produced from lung cells by antigen challenge in vitro

The cellular source of IL-10 after the 4th antigen challenge was assessed in vitro. The left lobe of the lung was isolated prior to the 4th challenge (3 days after the 3rd challenge). The lung tissue was digested by incubation with 150 U/ml collagenase (Iwaki Glass, Tokyo, Japan) and 30 μg/ml deoxyribonuclease I (from bovine pancreas; Calbiochem, San Diego, CA, USA) at 37 °C for 1 h. The single cell suspension obtained was suspended in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at a concentration of 5×10^6 cells/ml, and then distributed into the wells of a 12-well plate at 2.5 ml/well. The cells were incubated with PBS or ovalbumin at a final concentration of 10^{-4} or 10^{-3} g/ml at 37 °C in 5% CO_2 for 2 h. A protein transport inhibitor solution containing monensin (BD Golgistop™; BD Biosciences) was added at a volume of 3.5 μl/well followed by further incubation for 4 h.

After incubation, the cells were washed twice with PBS supplemented with 2% FBS and were then incubated with anti-mouse Fcγ receptor II/III monoclonal antibody for 15 min at 4 °C in order to block Fcγ receptors. After washing once with PBS supplemented with 2% FBS, the cells were incubated at 4 °C with PerCP-labeled anti-mouse CD4 monoclonal antibody (clone GK1.5; BioLegend, San Diego, CA, USA) at 1 μg/ml and/or eFluor® 660-labeled anti-mouse CD3 monoclonal antibody (clone 17A2; eBioscience, San Diego, CA, USA) at 1 μg/ml for 20 min. After washing three times with PBS supplemented with 2% FBS, the cells were incubated with fixation/permeabilization solution (BD Cytofix/Cytoperm™; BD Biosciences) at 4 °C for 12–18 h. After fixation and permeabilization, the cells were washed twice with permeabilization/wash buffer (BD Perm/Wash™; BD Biosciences), and were then incubated with 10 μg/ml fluorescein isothiocyanate (FITC)-labeled anti-IL-10 monoclonal antibody (clone JES5-16E3; BD Biosciences) and/or 2 μg/ml Alexa Fluor® 647-labeled anti-forkhead box P3 (Foxp3) monoclonal antibody (Clone PJK-16S, eBioscience) at 4 °C for 40 min. Finally, the cells were washed three times with PBS supplemented with 2% FBS. Each sample was analyzed using a FACSCalibur and Cell Quest software.

2.6. Detection of IL-10 receptor$^+$ cells in lung cells

IL-10 receptor$^+$ cells in the lung were detected using tissue isolated prior to the 1st and 4th challenges. Leukocytes recovered from collagenase/deoxyribonuclease I-digested lung tissue were suspended in PBS supplemented with 2% FBS. Cells were counted using a hematocytometer after staining the dead cells with 0.4% trypan blue. Cells were then incubated with anti-mouse Fcγ receptor II/III monoclonal antibody for 15 min at 4 °C. After washing once with PBS supplemented with 2% FBS, the cells were incubated at 4 °C with phycoerythrin (PE)-labeled anti-mouse CD210 (IL-10 receptor) monoclonal antibody (clone 1B1.3a; BD Biosciences) at 2 μg/ml, and PE-Cy5-labeled anti-mouse F4/80 monoclonal antibody (clone BM8; eBioscience) at 2 μg/ml or FITC-labeled anti-mouse Ly6G (Gr-1) monoclonal antibody (clone RB6-8 C5; eBioscience) at 5 μg/ml for 25 min. After washing three times with PBS supplemented with 2% FBS, the cells were fixed with 4% paraformaldehyde. Each sample was analyzed using a FACSCalibur and Cell Quest software.

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

Levels of TNF-α, IL-1β, IL-17, KC and MIP-2 in the BALF supernatant were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). These assay kits provided reagents for determining standard curves

covering ranges of 10.9–700 pg/ml (TNF-α), 12.8–800 pg/ml (IL-1β), 10.9–700 pg/ml (IL-17), 15.6–1000 pg/ml (KC) and 7.8–500 pg/ml (MIP-2). When the OD value of a sample was below or equal to that with no added cytokine/chemokine standard, the amount was regarded as 0 pg/ml. When the OD value of a sample was between 0 pg/ml and the lowest value of the standard curve, the amount was calculated from the equation of the standard curve regression line.

2.8. Statistical analyses

Two groups were compared by the unpaired t-test. To compare 3 groups, one-way analysis of variance was performed, followed by Dunnett's multiple comparison test. For the results of time-course changes in sRaw, two-way analysis of variance was performed, and then individual group differences were determined by Bonferroni's multiple test. $P \leq 0.05$ was considered significant. These analyses were performed using the software package Prism 4 for Macintosh (version 4.0c; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Airway leukocyte inflammation after multiple antigen challenges, and importance of neutrophils in the induction of the late asthmatic response

When OVA-sensitized animals received daily intratracheal OVA challenge for 3 days followed by 3 days without challenge (see Fig. 1A), the numbers of airway eosinophils, mononuclear cells, and $CD4^+$ T cells were maximal, and not increased by a 4th OVA challenge (Fig. 1B, D and E). In contrast, the numbers of neutrophils, which increased before and after the 2nd and 3rd challenges (Nabe et al., 2005), returned towards baseline prior to the 4th challenge, but showed recurrent airway neutrophilia after the 4th challenge (Fig. 1C).

The Gr-1 (Ly6G/Ly6C) antigen is known to be preferentially expressed on neutrophils. We have reported that Gr-1^+ cells consisted of Gr-1^{high} and Gr-1^{low} cells, which consist mainly of neutrophils and eosinophils, respectively (Nabe et al., 2011). Systemic treatment with the anti-Gr-1 monoclonal antibody, NIMP-R14, markedly suppressed 4th challenge-induced airway neutrophilia (Fig. 1H). The increased eosinophils were also reduced by approximately 50% (Fig. 1G). The induction of a late-phase increase in sRaw was strongly suppressed by anti-Gr-1 monoclonal antibody treatment (Fig. 1F). However, the increase in the number of mononuclear cells was not affected by the antibody treatment (Fig. 1I).

In our previous paper, another clone of anti-Gr-1 monoclonal antibody selectively suppressed airway neutrophilia as well as LAR, whereas selective suppression of eosinophilia without suppression of neutrophilia by an anti-IL-5 monoclonal antibody exerted no inhibition on the LAR (Nabe et al., 2011). Based on these observations, we have considered that the suppression of LAR by anti-Gr-1 monoclonal antibody was due to the antibody's ability to suppress airway neutrophilia.

3.2. Time-course changes in cytokine production during multiple antigen challenges

Consistent with our previous paper (Nabe et al., 2005), levels of Th2 cytokines, IL-4 and IL-5, increased in BALF before and/or 4 h after the 2nd–4th challenges (Fig. 2A and B). In contrast, IL-10 was not produced at easily measurable levels after the 1st–3rd challenges, whereas the 4th challenge caused a marked increase in IL-10 in BALF (Fig. 2C).

These cytokine profiles suggested that IL-10-producing cells accumulated in the lung until the time prior to the 4th challenge, and are not identical to IL-4- and IL-5-producing cells. Thus, we examined which cells are responsible for IL-10 production.

3.3. Cellular source of IL-10 produced by antigen challenge in vitro

In order to elucidate the cellular source of IL-10 produced after the 4th antigen challenge, a single cell suspension was prepared using lung tissue harvested prior to the 4th challenge, and these cells were stimulated with OVA for 6 h in vitro. There was a small population of intracellular IL-10^+ cells in cells that had been stimulated in vitro with 10^{-3} g/ml antigen (Fig. 3A). The ratio of IL-10^+ cells increased in a concentration-dependent manner (Fig. 3B). We assessed whether IL-10^+ cells expressed the CD3 and/or CD4 molecules. In vitro, most IL-10^+ cells were either $CD3^-$ $CD4^-$ or $CD3^+$ $CD4^+$ (Fig. 3A). The number of $CD3^-$ $CD4^-$ cells was not increased by ovalbumin stimulation (Fig. 3C), whereas that of $CD3^+$ $CD4^+$ cells was markedly and significantly elevated by the antigen challenge (Fig. 3C), indicating that the main cellular source of IL-10 produced by the antigen challenge was $CD4^+$ T cells.

Prior studies have shown that, different from naturally occurring T regulatory cells, antigen-induced Tr1 cells, which produce a large amount of IL-10, lack expression of the transcription factor Foxp3 (Huber et al., 2011; Vieira et al., 2004). Thus, we next examined whether the IL-10-producing $CD4^+$ $CD3^+$ cells express intracellular Foxp3. Although there was a population of Foxp3$^+$

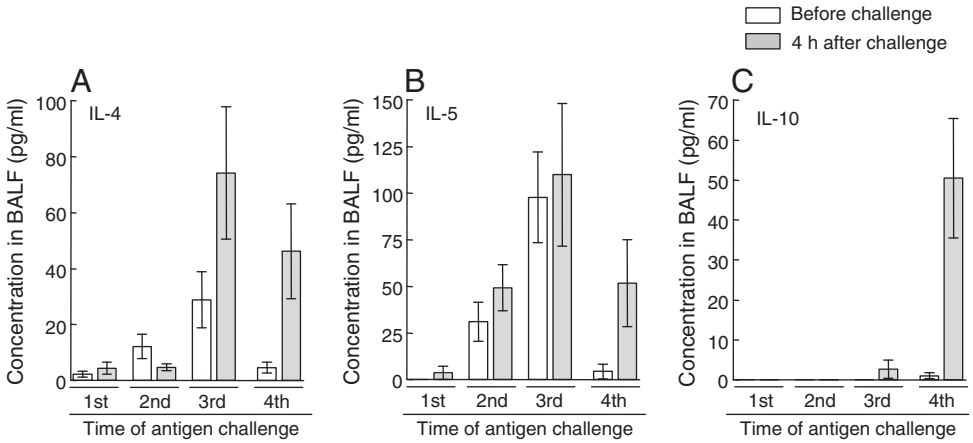

Fig. 2. Time-course changes in amounts of interleukin (IL)-4 (A), IL-5 (B) and IL-10 (C) in bronchoalveolar lavage fluid before and 4 h after the 1st–4th challenges. Each column represents the mean ± S.E.M. of 4–10 animals.

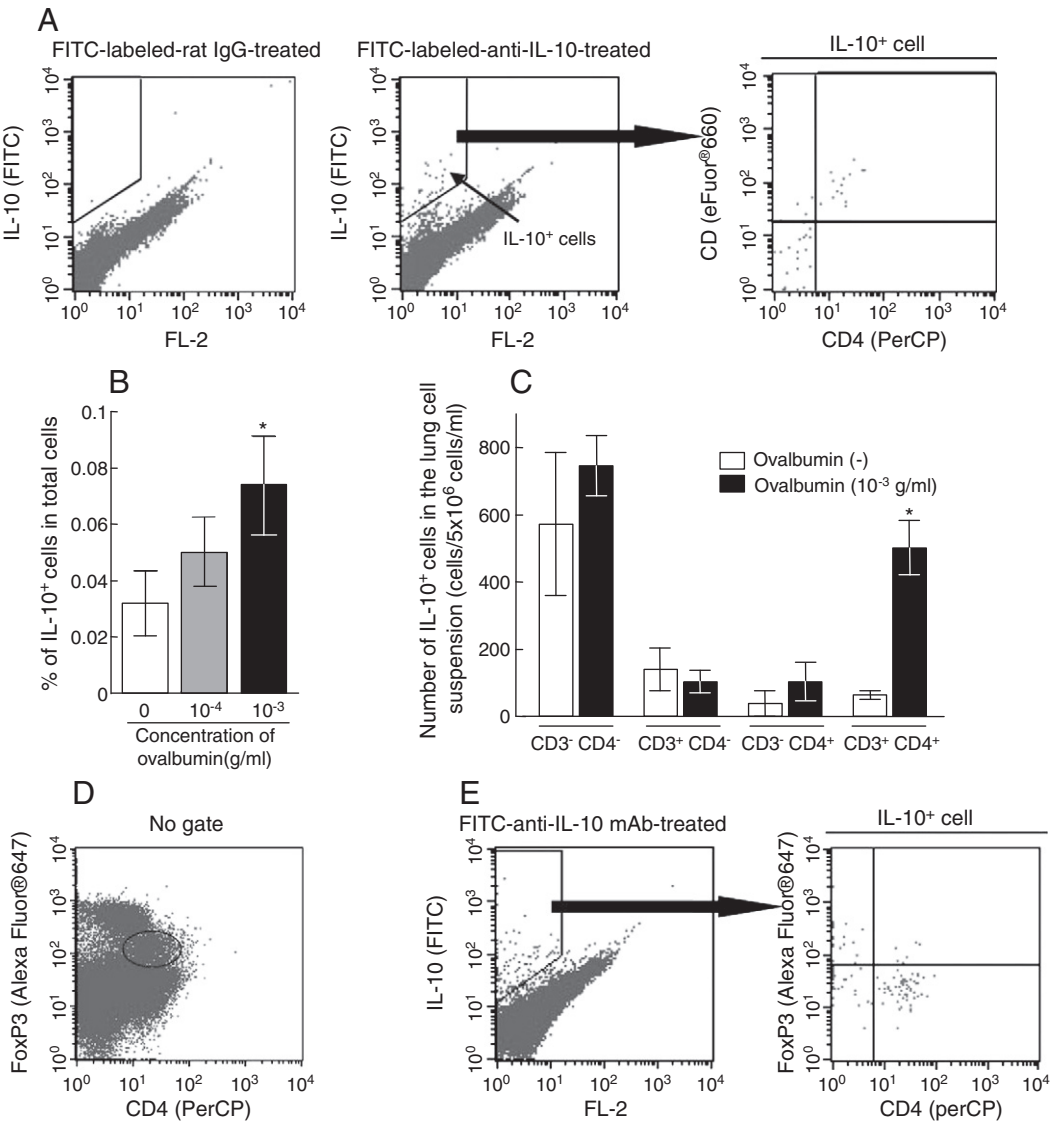

Fig. 3. (A) Flow cytometric analysis for classification of intracellular IL-10$^+$ cells in a lung cell suspension after stimulation with ovalbumin (10^{-3} g/ml) in vitro by cell surface expression of CD3 and CD4 molecules, (B) increase in intracellular IL-10$^+$ cells in the lung cell suspension after stimulation with 10^{-4} and 10^{-3} g/ml ovalbumin, (C) ovalbumin (10^{-3} g/ml)-induced increase in intracellular IL-10$^+$ cells in the CD3$^+$ CD4$^+$ cells, (D) CD4$^+$ Foxp3$^+$ cells in the lung cell suspension, and (E) analysis of IL-10-producing CD4$^+$ cells by intracellular Foxp3. The lung was isolated prior to the 4th challenge, and then enzymatically digested to obtain the single cell suspension. Antigen challenge with ovalbumin was performed in vitro. Each column in panels B and C represents the mean ± S.E.M. of 5 and 3 experiments, respectively. *: $P<0.05$, vs ovalbumin (−). FITC: fluorescein isothiocyanate.

cells in the CD4$^+$ cell fraction (Fig. 3D), most of IL-10$^+$ cells lack Foxp3 (Fig. 3E).

3.4. Effects of anti-IL-10 receptor monoclonal antibody on airway infiltration of neutrophils and eosinophils, and the late asthmatic response

Whether the anti-inflammatory cytokine, IL-10, produced after the 4th antigen challenge, exerts suppressive effects on airway eosinophilia and neutrophilia after challenge was examined by blocking IL-10 receptors with an anti-IL-10 receptor monoclonal antibody. Although neither the increased number of eosinophils in the lung (Fig. 4A) (% of total cells: PBS, 48.8 ± 8.9%; rat IgG, 53.9 ± 4.7%; anti-IL-10 receptor, 47.0 ± 3.8%) nor the increased amounts of Th2 cytokines, IL-4 and IL-5, in BALF (Table 1) were affected by the neutralization of IL-10 receptors, 4th challenge-induced neutrophilia was significantly augmented (Fig. 4B) (% of total cells: PBS, 8.3 ± 1.5%; rat IgG, 11.2 ± 0.9%; anti-IL-10 receptor, 17.2 ± 1.9%). On the other hand, the late-phase increase in sRaw was not affected by antibody treatment (Fig. 4C).

3.5. Effects of anti-IL-10 receptor monoclonal antibody on airway production of cytokines and chemokines relating to neutrophil recruitment

Cytokines and chemokines that are related to neutrophilia, TNF-α, IL-1β and IL-17, KC and MIP-2, were all increased in BALF 4 h after the 4th challenge when neutrophilia was clearly evoked (Fig. 5A, B, C, D and E). The increases of TNF-α, KC and MIP-2 were especially marked (Fig. 5A, D and E). Compared with the rat IgG-treated group, the increased IL-1β, KC and MIP-2 were significantly augmented by treatment with anti-IL-10 receptor monoclonal antibody (Fig. 5B, D and E). The production of IL-17 also tended to be enhanced by the monoclonal antibody (Fig. 5C) although TNF-α production was not affected (Fig. 5A).

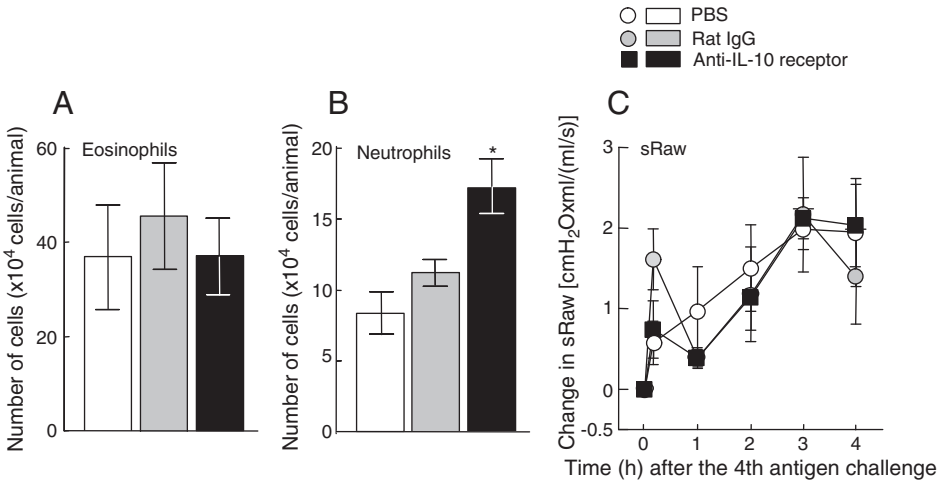

Fig. 4. Effects of an anti-interleukin (IL)-10 receptor monoclonal antibody, 1B1.3a, on the increased numbers of eosinophils (A) and neutrophils (B) in bronchoalveolar lavage fluid, and the increase of specific airway resistance (sRaw, C) after the 4th antigen challenge. Anti-IL-10 receptor monoclonal antibody (500 μg/animal) or rat IgG (500 μg/animal) was i.p. administered 30 min before the 4th challenge, followed by bronchoalveolar lavage 4 h after the challenge. Each point and column represent the mean ± S.E.M. of 7 or 8 animals. *: $P<0.05$, vs rat IgG-treated group.

3.6. Composition of IL-10 receptor$^+$ cells in lung tissue

Compared with the 1st challenge, the number of IL-10 receptor$^+$ cells in the left lobe of the lung increased 7-fold at the 4th challenge (Table 2). The percentage of F4/80$^+$ cells in IL-10 receptor$^+$ cells was also increased by the multiple antigen challenged from 18.5% to 36.6% (Table 2), meaning that the number of IL-10 receptor$^+$ F4/80$^+$ cells was increased 14-fold by the multiple antigen challenges. On the other hand, we have reported that Gr-1^{high} cells are neutrophils (Nabe et al., 2011). Gr-1^{high} cells existed at only 3% in the IL-10 receptor$^+$ cell population (Table 2).

4. Discussion

In intratracheal antigen challenge-induced asthma, not only late-phase airway obstruction (late asthmatic response) but also IL-10 production was evoked after the 4th challenge, but not after the 1st, 2nd or 3rd challenge (Nabe et al., 2005), suggesting that antigen-responsive IL-10-producing cells are induced by multiple antigen challenges, and that IL-10 may affect the induction of a late asthmatic response. Because the late asthmatic response was substantially inhibited by selectively reducing neutrophilia, this response should be induced by neutrophil-dependent mechanisms (Nabe et al., 2011). Thus, we could hypothesize that there is a link between IL-10 and neutrophilia: IL-10 produced by the 4th antigen challenge may negatively regulate neutrophilia.

We analyzed the cellular sources of IL-10. It is generally known that IL-10 is produced from not only various types of T cells but also monocytes/macrophages (Sabat et al., 2010). In this study, spontaneous production of IL-10 was derived mainly from CD3$^-$ CD4$^-$ cells, which may be monocytes/macrophages, whereas most IL-10 produced by the antigen challenge was from CD4$^+$ T cells. IL-10-producing T cells include Th1, Th2, natural thymic-derived CD4$^+$ Treg cells and antigen-induced adaptive CD4$^+$ Treg cells (Lloyd and Hawrylowicz, 2009; Sabat et al., 2010). Because we could not detect any interferon-γ in the lung (Nabe et al., 2005), Th1 cells may not be developed in this model. As shown in the time-course changes in IL-4, IL-5 and IL-10, both IL-4 and IL-5 were produced in the lung even at 2nd and 3rd challenges, whereas IL-10 production was induced only at the 4th challenge, suggesting that IL-10-producing cells are not identical to IL-4- and IL-5-producing Th2 cells. On the other hand, naturally occurring Treg cells do not have allergen-responsive features (Lloyd and Hawrylowicz, 2009). In addition, it has been demonstrated that IL-10-producing Tr1 cells are negative for a transcription factor, Foxp3 (Huber et al., 2011; Vieira et al., 2004), which is a critical factor for the naturally occurring Treg cells (Kim et al., 2007). Identical to the literatures (Huber et al., 2011; Vieira et al., 2004), the IL-10-producing CD4$^+$ T cells were Foxp3-negative in this study. Collectively, it was strongly suggested that the cellular source of IL-10 produced after the 4th challenge is antigen-induced adaptive CD4$^+$ Tr1 cells.

Next, we examined whether there is a relationship between IL-10 and neutrophilia. Treatment with anti-IL-10 receptor monoclonal antibody prior to the 4th challenge significantly augmented the 4th challenge-induced airway neutrophilia. Interestingly, antigen-induced airway production of IL-1β and CXC chemokines, KC and MIP-2, which have been considered to be murine functional homologues of human IL-8 and growth-related oncogene (GRO) chemokines (Oquendo et al., 1989; Tekamp-Olson et al., 1990), was also significantly enhanced by anti-IL-10 receptor monoclonal antibody treatment. Because it has been reported that cellular sources of not only IL-1β but also KC and MIP-2 in mice include macrophages (Smit and Lukacs, 2006), we next examined whether macrophages infiltrating the lung possess IL-10 receptors on the cell membrane. Consequently, approximately 40% of IL-10 receptor$^+$ cells in the lung were positive for a macrophage marker, F4/80, whereas the percentage of neutrophils in the IL-10 receptor$^+$ cell population was only 3–4%. Thus, it could be concluded that endogenously produced IL-10 after the 4th antigen stimulation attenuated antigen-induced airway neutrophil infiltration indirectly through reducing the production of IL-1β, KC and MIP-2 from IL-10 receptor$^+$ macrophages. However, because many other cell types, including epithelial cells, fibroblasts, T cells etc.; also produce IL-1β, KC and MIP-2

Table 1
Influence of anti-IL-10 receptor monoclonal antibody on increased amounts of IL-4 and IL-5 in bronchoalveolar lavage fluid (BALF) 4 h after the 4th antigen challenge in sensitized mice.

	Concentration in BALF (pg/ml)	
	IL-4	IL-5
PBS	59 ± 16	82 ± 58
Rat IgG	102 ± 35	205 ± 99
Anti-IL-10 receptor	106 ± 18	117 ± 39

Anti-IL-10 receptor monoclonal antibody (0.5 mg/animal) was i.p. administered 30 min prior to the 4th challenge. Each value is the mean ± S.E.M. of 7 or 8 animals.

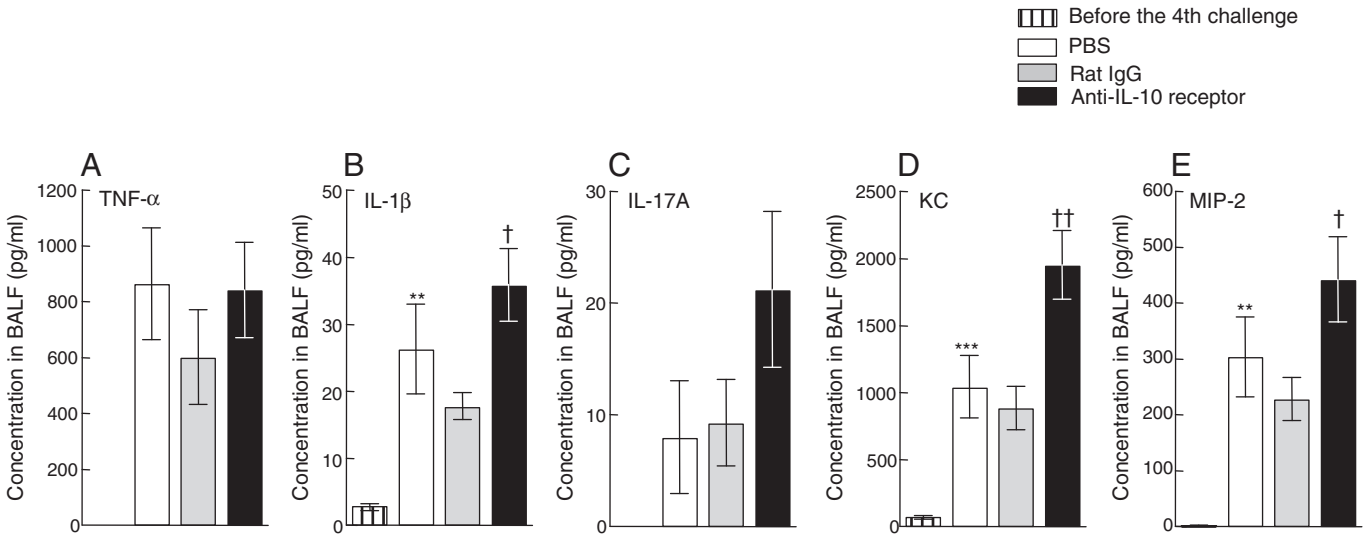

Fig. 5. Effects of an anti-interleukin (IL)-10 receptor monoclonal antibody, 1B1.3a, on increased amounts of tumor necrosis factor (TNF)-α (A), IL-1β (B), IL-17 (C), keratinocyte-derived chemokine (KC, D) and macrophage inflammatory protein (MIP)-2 (E) in bronchoalveolar lavage fluid after the 4th antigen challenge. Anti-IL-10 receptor monoclonal antibody (500 μg/animal) or rat IgG (500 μg/animal) was i.p. administered 30 min before the 4th challenge, followed by bronchoalveolar lavage 4 h after the challenge. Each point and column represent the mean ± S.E.M. of 7 or 8 animals. ** and ***: $P<0.01$ and $P<0.001$, vs before the 4th challenge. † and ††: $P<0.05$ and $P<0.01$, vs. rat IgG-treated group.

(Armstrong et al., 2004; Lappalainen et al., 2005), the involvement of such cells, which can be target cells of IL-10, should also be examined in the future.

The amount of IL-17 also increased in BALF after the 4th challenge, although the level was low and its production was relatively variable. Treatment with anti-IL-10 receptor monoclonal antibody also showed a tendency to augment the IL-17 level. It has been recently reported that IL-10 signaling in Treg cells is required for suppression of Th17 cell-mediated inflammation: ablation of IL-10 receptors in Treg cells resulted in selective dysregulation of Th17 cell responses and colitis (Chaudhry et al., 2011; Huber et al., 2011). Although we have not examined whether IL-17 is involved in allergic airway neutrophilia in our study, there may also be a regulatory mechanism that IL-10 negatively modulates Th17 cell activation in neutrophilia.

A study using human neutrophils revealed little or no expression of IL-10 receptor 1 on circulating neutrophils (Crepaldi et al., 2001); however, peripheral blood neutrophils collected from septic patients, and thus presumably exposed in vivo to lipopolysaccharide and IL-4, were found to display elevated surface levels of IL-10 receptors (Tamassia et al., 2008). Thus, the neutrophils could respond promptly to IL-10 ex vivo. In this study, neutrophils infiltrating the lung may have been exposed to various pro-inflammatory cytokines during the recruitment process. We cannot explain why the neutrophils in the lung expressed only low levels of IL-10 receptors on their cell surface in the present study, although it may have been due to just a species difference. The low expression of IL-10 receptors on the neutrophil cell surface suggests that IL-10 may not inhibit neutrophil function directly by acting on the granulocyte itself. On the other hand, the number of IL-10 receptor$^+$ F4/80$^+$ cells was markedly increased by the multiple antigen challenges, although composition of IL-10 receptor$^+$ cells in the lung has not been defined at present. Responsiveness of macrophages to IL-10 for suppression of their own activity could also be augmented by the multiple antigen challenges.

Table 2
Composition of IL-10 receptor$^+$ cells in single cell suspensions of the lung isolated prior to the 1st and 4th antigen challenges in sensitized mice.

	IL-10 receptor$^+$ cells	% of IL-10 receptor$^+$ cells	
	Total number ($\times 10^4$ cells/left lobe)	F4/80$^+$ cells (%)	Gr-1^{high} cells (%)
1st challenge	1.5 ± 0.3	18.5	3.9
4th challenge	10.5 ± 5.3	36.6	3.0

Number of IL-10 receptor$^+$ cells is the mean ± S.E.M. of 3 animals. % of F4/80$^+$ cells and Gr-1^{high} cells were analyzed using combined cell suspensions of 3 animals.

It has been extensively suggested that Treg cell-derived IL-10 inhibits Th2 cell activation in the pathogenesis of allergic disorders (Lloyd and Hawrylowicz, 2009). Kearley et al. (2005) demonstrated that airway eosinophilic inflammation and hyperreactivity were resolved by in vivo transfer of CD4$^+$ CD25$^+$ Treg cells via IL-10 production from the cells. Fu et al. (2006) reported that intratracheal delivery of adenovirus-expressing IL-10 alleviated eosinophilic airway inflammation and hyperresponsiveness. To our knowledge, this is the first report to indicate that IL-10 produced by antigen challenge inhibited allergic neutrophilia; however, the consideration that IL-10 inhibits airway neutrophilia is not surprising because various studies have revealed that neutrophil functions were impaired by IL-10 (Bazzoni et al., 2010). In in vivo various inflammatory models, exogenous IL-10 or *IL-10* gene transfer effectively reduced the severity of inflammation, mainly by blocking neutrophil trafficking (Bazzoni et al., 2010). Among them, consistent with our present findings, reduced accumulation of neutrophils in inflamed organs was due to IL-10-mediated inhibition of the production of neutrophil chemoattractants, which were derived from macrophages etc. (Inoue, 2000; Murakami et al., 2002; Shanley et al., 1995).

In contrast to the augmentation of allergic airway neutrophilia by anti-IL-10 receptor monoclonal antibody, the magnitude of late-phase airway narrowing was not influenced by treatment. This lack of augmentation seems to conflict with our findings that the reduction of airway neutrophilia significantly suppressed the late asthmatic response (Nabe et al., 2011). Although the reasons for this discrepancy are unclear, we speculate that the contribution of neutrophils to the induction of the late asthmatic response may have already hit the ceiling, meaning that further potentiation of neutrophil infiltration into the lung may not further augment the late asthmatic response. Alternatively, as shown in the present finding, the anti-Gr-1 monoclonal antibody, NIMP-R14, strongly inhibited neutrophilia, and eosinophilia was also partially reduced by the antibody. This inhibitory tendency on eosinophilia was also recognized in our previous experiment using another clone of anti-Gr-1 monoclonal antibody, RB6-8C5 (Nabe et al., 2011), although the inhibitory degree was

smaller than that recognized in the NIMP-R14-treated group. Thus, partial inhibition of eosinophilia may contribute to inhibition of the late asthmatic response by anti-Gr-1 monoclonal antibodies. If anti-IL-10 receptor monoclonal antibody potentiated the activation of Th2 cells and eosinophils together with the augmentation of neutrophil infiltration into the lung, the late asthmatic response might be also potentiated.

Lastly, in the present 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 ovalbumin may contribute to the neutrophilia 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 the induction of counterregulatory Th1 cells, or to exacerbate asthma severity, presumably through its pro-inflammatory activities (Liu, 2002; Reed and Milton, 2001; Schwartz, 2001). Jung et al. (2006) demonstrated by experiments using toll-like receptor 4 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 the late asthmatic response, future studies will be required to investigate the magnitude of asthmatic responses induced by endotoxin-free ovalbumin or by conventional ovalbumin in toll-like receptor 4-deficient mice. Alternatively, a recently developed toll-like receptor 4 signal inhibitor could be utilized (Seki et al., 2010). 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 allergic airway neutrophilia and modulation of the response by IL-10 observed in this model may be a tool for analyzing the relationship between innate and acquired immunity in asthma pathogenesis.

In conclusion, multiple antigen challenges induced allergen-responsive $CD4^+$ Treg cells, which produced IL-10 following the 4th antigen challenge in vivo. Airway neutrophilia induced by the 4th antigen challenge was negatively regulated by endogenously produced IL-10 indirectly by reducing the production of IL-1β, KC and MIP-2 from IL-10 $receptor^+$ macrophages and others. This is the first report to show the regulatory mechanisms of endogenous IL-10 in allergic neutrophilia in sensitized mice. Potentiation of IL-10 and suppression of IL-1β and IL-8 (KC and MIP-2) can be expected to be strategies for novel pharmacotherapy for neutrophilic asthma.

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