doi: 10.1111/j.1365-2222.2012.04006.x
Clinical & Experimental Allergy, **42**, 946–957

ORIGINAL ARTICLE **Experimental Models of Allergic Disease**

IL-17 eliminates therapeutic effects of oral tolerance in murine airway allergic inflammation

H. Kawakami[1], T. Koya[1], H. Kagamu[1], Y. Kimura[1], H. Sakamoto[1], C. Yamabayashi[1], T. Furukawa[1], T. Sakagami[1], T. Miyabayashi[1], T. Hasegawa[2], E. Suzuki[2] and I. Narita[1]

[1]Division of Respiratory Medicine, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan and [2]Department of General Medicine, Niigata University Medical and Dental Hospital, Niigata, Japan

Clinical & Experimental Allergy

Summary

Background Oral tolerance is a classically used strategy for antigen-specific systemic immunotherapy. However, the roles of IL-17 in modification of oral tolerance are not yet understood.

Objective To define the effects of IL-17 on the modification of oral tolerance, the effects of transfer of Th17 cells, administration of IL-17 or anti-IL-17 antibody (αIL-17Ab) to a murine allergic airway inflammation model were investigated.

Methods Mice sensitized to and challenged with OVA, received OVA feeding, followed by OVA challenges. Transfer of Th17 cells, administration of IL-17 or αIL-17Ab were executed during OVA feeding. Airway hyperresponsiveness (AHR), airway inflammation, Th2 cytokine response and lung pathology were assessed.

Results Administration of IL-17 as well as transfer of Th17 cells aggravated AHR and airway allergic inflammation as compared with the findings in mice subjected to OVA feeding alone, whereas administration of αIL-17Ab ameliorated AHR and airway eosinophilia. The effects of Th17 transfer were presumably attributable to augmentation of endogenous IL-6 production in gut. The number of Foxp3-positive regulatory T (Treg) cells in lungs and Payer's patches was increased in the OVA fed mice, whereas the number of these cells was decreased in the mice subjected to OVA feeding + Th17 cell transfer. Neutralization of IL-6 by monoclonal antibody in the mice subjected to OVA feeding + transfer of Th17 cells restored the effects of oral tolerance.

Conclusions and Clinical Relevance These data suggest that IL-17 may inhibit the induction of tolerance to antigen through, at least in part augmenting IL-6 production, thereby suppressing the expansion of Treg cells.

Correspondence:
Toshiyuki Koya, Division of Respiratory Medicine, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.
E-mail: tkoya@med.niigata-u.ac.jp
Cite this as: H. Kawakami, T. Koya, H. Kagamu, Y. Kimura, H. Sakamoto, C. Yamabayashi, T. Furukawa, T. Sakagami, T. Miyabayashi, T. Hasegawa, E. Suzuki and I. Narita, *Clinical & Experimental Allergy*, 2012 (42) 946–957.

Keywords bronchial asthma, IL-6, immunotherapy, regulatory T cell, Th17
Submitted 24 September 2011; revised 18 February 2012; accepted 22 February 2012

Introduction

Eosinophilic airway inflammation, mucus hypersecretion and airway hyperresponsiveness (AHR) are characteristic features of allergic bronchial asthma (BA) [1, 2]. These pathognomonic pathophysiological features are mediated by antigen-specific Th2 cells and their cytokines, such as IL-4, IL-5 and IL-13 [1–3]. In addition to Th2 cells, the potential involvement of Th17 cells in the development of the clinical features of this disease has also been the focus of intense investigation. The expression of IL-17A in the airways or serum levels of IL-17 were augmented in the patients with BA and were correlated with the severity of BA [4, 5]. Recent studies in animal models have demonstrated that both IL-17 receptor-deficient and IL-17A-deficient mice exhibit reduced recruitment of eosinophils into the airways and lower levels of Th2 cytokines in the bronchoalveolar lavage (BAL fluid as a result of impaired priming of Th2 responses in IL-17R$^{-/-}$ mice [6, 7].

Allergen (antigen)-specific immunotherapy is an immune-modifying therapy that has been recommended for the treatment of allergic rhinitis, venom hypersensitivity, some drug allergies and mild BA [8]. Oral adminis-

tration of antigen is a classically used method of inducing antigen-specific systemic immunotherapy and is termed oral tolerance [9, 10]. It is now widely accepted that the mechanisms of oral tolerance include not only anergy or apoptosis (deletion) of antigen-specific T cells in the gut but also active suppression through the induction of antigen-specific regulatory T (Treg) cells [11, 12]. Most of the induced Treg cells are characterized by abundant production of immunosuppressive cytokines, such as IL-10 and TGF-β, and expression of the transcription factor, forkhead box P3 (Foxp3) [13].

An intriguing connexion between IL-6 and Th17 has now been reported: CD4 T cells activated by antigen in the presence of TGF-β have been shown to differentiate into either Th17 or Treg, depending on the dominance of IL-6 or retinoic acid, respectively [14]. IL-6 secreted from parenchymal cells such as fibroblasts, keratinocytes, epithelial cells and endothelial cells in response to IL-17 [15] have also been shown to have the potential to inhibit the differentiation of Treg [16]. Administration of IL-17 has been shown to eliminate the therapeutic effects of myelin basic protein-induced nasal tolerance in experimental autoimmune encephalomyelitis (EAE) by upregulating IL-6 [17]. However, it is still uncertain whether IL-17 has the potential to modify the oral tolerance to antigens in a Th2 cytokine milieu like allergic BA.

In spite of the theory of immunotherapy, the key therapy for BA is inhaled corticosteroids. Immunotherapy is merely an optional therapy according to current guidelines and is less effective in severe asthma [18, 19]. Additionally, several reports have indicated that levels of IL-17 in the serum or BAL fluid are higher in severe asthma than in mild or moderate asthma [20, 21]. These severe states also induce Treg activity to a lesser extent in various situations [22, 23]. On the basis of this evidence, we speculated that IL-17/Th17 may have an influence on the efficacy of immunotherapy.

In this study, we investigated the role of IL-17/Th17 in modulating the induction of oral tolerance in a murine airway allergy model. After challenges with OVA, we evaluated the AHR and the allergic inflammation in the airways. We also investigated the modulating effects of transfer of Th17 cells, administration of IL-17 or anti-IL-17 antibody on oral tolerance, and we assessed whether the effects of IL-17 were mediated by IL-6, by using anti-IL-6 antibody after the Th17 cell transfer.

Materials and methods

Animals

Female C57BL/6 (B6) mice and OT-II TCR transgenic (OT-II) mice were purchased at 6–8 weeks of age from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan), and enhanced green fluorescent protein transgenic mice maintained on a C57BL/6J background were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Animals were housed under specific-pathogen-free conditions and maintained on an OVA-free diet. All animal experiments were conducted with the approval of the Niigata University Ethics Committee for animal experiments.

Generation of OVA-specific Th2 cells and Th17 cells

OVA-specific Th2 or Th17 cells were generated from the splenocytes of OT-II mice according to a method described previously, with minor alterations [24]. In brief, CD4 T cells (1×10^6/mL) were stimulated with OVA 323–339 peptide (10 μg/mL) in the presence of irradiated C57BL/6 spleen cells (2×10^5/mL) as antigen-presenting cells at 37°C for 3 days, and incubated with recombinant human IL-2 (20 U/mL; eBiosciences, Camarillo, CA, USA). Incubated CD4 T cells were cultured with IL-4 (1 ng/mL; R&D Systems, Minneapolis, MN, USA), anti-IL-12/IL-23 p40 mAb (5 μg/mL, C17.8; R&D Systems) and anti-IFN-γ mAb (5 μg/mL, XMG1.2; eBiosource) in Th2 cell generation or with IL-6 (20 ng/mL; R&D Systems) and TGF-β (3 ng/mL; R&D Systems) in Th17 cell generation. Cells were then washed with PBS and cultured for another 3 days in the presence of cytokines and antibodies without antigen stimulation.

OVA-induced allergic airway inflammation and oral tolerance

Mice were sensitized on days 0 and 14 by intraperitoneal injection of 20 μg of OVA premixed with 2.25 mg of $Al(OH)_3$ in 100 μL of PBS. After sensitization, the animals were exposed to aerosolized OVA (10 mg/mL in 0.9% saline) for 20 min on days 28, 29 and 30. From days 37 to 41, the mice were administered OVA (100 mg/day) by gavage once a day for induction of oral tolerance, following aerosolized exposure to OVA using same procedure on days 48, 49 and 50. As control, some mice received same volume of PBS by gavage. In some experiments, recombinant mouse IL-17 (60 ng/mouse, Invitrogen, Carsbad, CA, USA) was administered intraperitoneally from days 37 to 41. OVA-specific-Th2 cells or -Th17 cells (1×10^7 cells/mouse) were transferred via the tail vein on day 37. The administration of anti-mouse IL-17 antibody (50 μg/mouse, clone 50104; R&D Systems) or anti-mouse IL-6 antibody (50μg/mouse, clone MP5-20F3; R&D Systems) was performed from days 37 to 41 in accordance with tolerance induction. According to the manufacturer, this anti-IL-17 antibody is specific for IL-17A, although approximately 40% reactivity with

the recombinant mouse IL-17A/IL-17F heterodimer is observed in direct ELISAs, but not with IL-25 (IL-17E). Twenty-four hours after the last OVA challenge, the severity of the AHR was assessed, and specimens of BAL fluid, serum and lungs were obtained for further analysis. Figure 1 shows a summary of the experimental protocols used in this study (Fig. 1). In some mice, specimens of Peyer's patches (PP) were obtained 24 h after the last OVA feeding.

Airway responsiveness

Airway hyperresponsiveness was assessed by measuring the changes in the respiratory resistance using the Flexivent system (SCIREQ, Montreal, Quebec, Canada) in response to increasing doses of inhaled methacholine, as previously reported [25].

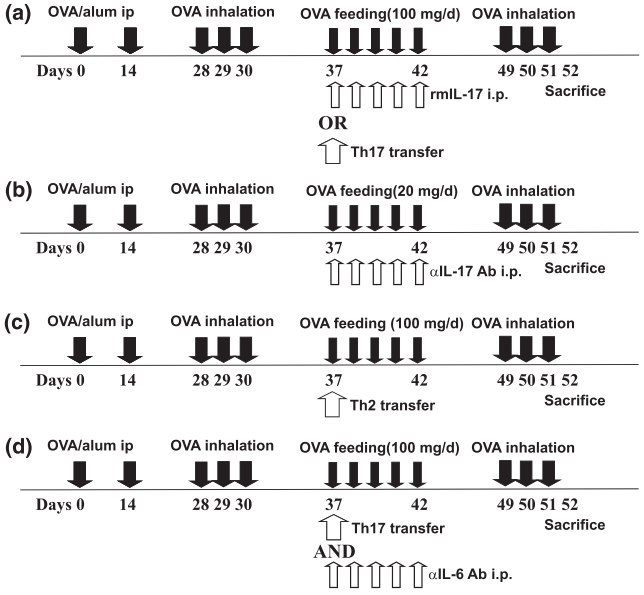

Fig. 1. Experimental protocol for antigen sensitization and challenge with antigen feeding, cell transfer, IL-17 and anti-IL-6 antibody administration as described in Materials and methods in the repository text. (a) Mice treated with OVA feeding (100 mg/day) for 5 days after first OVA challenges received either recombinant murine IL-17 (60 ng/day/mouse administered by intraperitoneal injection for 5 days) or Th17 cell transfer (1×10^7 cells from the tail vein), followed by secondary OVA challenges. (b) Mice treated with suboptimal OVA feeding (20 mg/day) for 5 days after first OVA challenges received anti-IL-17 antibodies (50 μg/day/mouse administered by intraperitoneal injection for 5 days), followed by secondary OVA challenges. (c) Mice treated with OVA feeding (100 mg/day) for 5 days after first OVA challenges received Th2 cell transfer (1×10^7 cells from the tail vein), followed by secondary OVA challenges. (d) Mice treated with OVA feeding (100 mg/day) for 5 days after first OVA challenges received Th17 cell transfer and anti-IL-6 antibodies (50 μg/day/mouse administered by intraperitoneal injection for 5 days), followed by secondary OVA challenges. Protocol a, b, c and d correspond to the data shown in Fig. 2, Fig. 3, Fig. 4 and Fig. 7, respectively.

BAL fluid and lung histology

Immediately after measurement of AHR, BAL was performed via a tracheal tube, as previously described [26].

Lungs were fixed in 10% formalin and processed for paraffin embedding. Mucus-containing goblet cells were detected by staining the paraffin sections (5 mm thick) with periodic acid-Schiff. Histological analyses were performed as previously described [27].

For tracing the transferred Th17 cells generated from the OT-II/GFP double-transgenic mice, PP were fixed for 4 h at 4°C in periodatelysine-paraformaldehyde and embedded in OCT compound (Miles, Elkhart, Ind.), frozen in dry ice-acetone and cut on a cryostat (Bright, Huntington, UK) into 6-μm-thick sections. The fluorescence from the transferred Th17 cells was analysed by fluorescence microscopy.

Cell preparations of lung cells and cultures with anti-CD3 stimulation

Lung cells were isolated as previously described [28] using collagenase digestion. Lung CD4 T cells were purified from lung cells using the Dynabeads™ mouse CD4 (Invitrogen) (purity > 98%). CD4 T cells were cultured on BD Biocoat™ T cell activation assay plates (BD Biosciences, San Jose, CA, USA) for 72 h, at which time supernatants were recovered for the cytokine assays.

Cell preparations and protein isolation from Peyer's patches

The small intestine was removed and flushed with PBS to eliminate any luminal contents. PP were then resected, passed through a steel mesh to remove any aggregates and then washed twice with PBS containing 0.2% BSA and 0.02% NaN_3 before use for flow cytometry. For some experiments, freshly resected PPs were rapid-frozen in liquid nitrogen and stored at −80°C until further analysis. To isolate proteins from the PP specimens, the specimens were homogenized in RIPA buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and centrifuged at 17530 **g** for 15 min at 4°C, and the supernatants were obtained. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from the PP with RNAiso plus™ (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. The cDNA was generated by reverse transcription with Transcription High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Tokyo, Japan).

For real-time polymerase chain reaction (PCR), the following primers were used to detect mouse IL-6 mRNA: forward primer, 5′- ttccatccagttgccttctggg-3′ and reverse primer, 5′- ttctcatttccacgatttcccag-3′. Expression of IL-6 mRNA were analysed using SYBR Premix Ex Taq (Takara Bio Inc.). GAPDH (forward primer, 5′-accacagtccatgaaatcac-3′ and reverse primer, 5′-tccaccaccctgttgctgta-3′) was used as the endogenous control. The amplification was performed at 95–60°C for 40 cycles in Thermal Cycler Dice™ Real Time System (Takara Bio Inc.). All samples were assayed in duplicate. The results were determined by the relative expression of IL-6 mRNA to GAPDH mRNA in each treatment group.

Flow cytometry

The surface phenotypes of the lung and PP CD4 T cells were analysed by flow cytometry with a three-colour immunofluorescence test, using monoclonal antibodies. The monoclonal antibodies included anti-CD4 (RM4-5), anti-CD25 (PC61) (all obtained from BD Biosciences) and anti-Foxp3 (FJK-16S) (eBiosciences). After washing, the staining was analysed by flow cytometry on a FACS caliber using the CellQuest software (BD Biosciences).

Measurement of cytokines in supernatants from cell culture, tissue extracts or BAL fluid

Supernatants from the cell cultures, tissue extracts or BAL fluid were stored at −80°C until use for the cytokine measurements. ELISA kits for the detection of IL-4, IL-5, IL-6, IFN-γ and TGF-β were obtained from eBioscience (San Diego, CA, USA). The IL-10 and IL-13 ELISA kits were purchased from R&D Systems.

Statistical analysis

Mann–Whitney's *U*-test was used to determine the significance of the differences among the groups. The data were basically pooled from three independent experiments with four mice/group in each experiment ($n = 12$). Comparisons for all pairs were performed using the Kruskal–Wallis test. Significance was assumed at *P*-values of < 0.05 for all tests. Values for all measurements were expressed as means ± SEM.

Results

Combination treatment of IL-17 or Th17 + OVA feeding abolished the effect of oral tolerance on the AHR and airway allergic inflammation

We initially examined the effects of oral administration of OVA in a murine airway allergy model. As shown in Fig. 1a, OVA-sensitized mice received OVA by inhalation for consecutive 3 days. After the challenges, the mice were fed OVA 100 mg per day for consecutive 5 days, followed by OVA challenges for 3 days from the week after last OVA feeding. Mice fed the OVA showed reduced AHR to methacholine, airway eosinophilia, the levels of Th2 cytokines and IL-17 in BAL fluid, and mucus production in the bronchi as compared with the mice treated with PBS alone (Fig. 2). Elevated levels of IL-10 and TGF-β, which represent inhibitory cytokines, were observed in BAL fluid. To determine the effects of IL-17 on the induction of oral tolerance, mice were administered recombinant mouse IL-17 intraperitoneally for 5 days in combination with OVA feeding (Fig. 1a). Combined treatment with IL-17 + OVA feeding completely abolished the attenuating effects of oral tolerance on the AHR and airway allergic inflammation. In brief, administration of IL-17 was associated with significant elevation of the AHR, airway eosinophilia, levels of Th2 cytokine and IL-17 in BAL fluid, and bronchial mucus production (Fig. 2). Similar to the effects of IL-17, transfer of OVA-specific Th17 cells (1×10^7 cells/mouse) on the first day of OVA feeding also abolished the effects of oral tolerance on the degree of AHR and airway allergic inflammation (Fig. 2). The levels of IL-10 and TGF-β in the BAL fluid were also decreased (Fig. 2c).

Administration of anti-IL-17 antibody on the induction phase of oral tolerance augmented the effects of oral tolerance on the AHR and airway eosinophilia

We examined the effects of murine anti-IL-17 antibody on the modulation of oral tolerance in a murine airway allergy model. As shown in Fig. 1b, the mice were fed OVA at a dose of 20 mg per day for consecutive 5 days, followed by OVA challenges for 3 days from the week after last OVA feeding. Mice fed the OVA showed little change of AHR to methacholine and airway eosinophilia as compared with the mice treated with PBS alone (Fig. 3). The levels of IL-5, IL-13 and IL-17 in the BAL fluid of mice fed OVA at a dose of 20 mg were significantly higher than those of mice fed OVA at a dose of 100 mg (data not shown). To determine the effects of anti-IL-17 antibody on the induction of oral tolerance, mice were administered anti-IL-17 antibody intraperitoneally for 5 days in combination with OVA feeding (Fig. 1b). Combined treatment with anti-IL-17 antibody + OVA feeding (20 mg/day) showed reduced AHR to methacholine and airway eosinophilia with similar levels to the mice fed large amount of OVA (100 mg/day) (Fig. 3). Administration of anti-IL-17 antibody to mice without OVA feeding as control showed no effect on AHR or airway eosinophilia (Fig. 3).

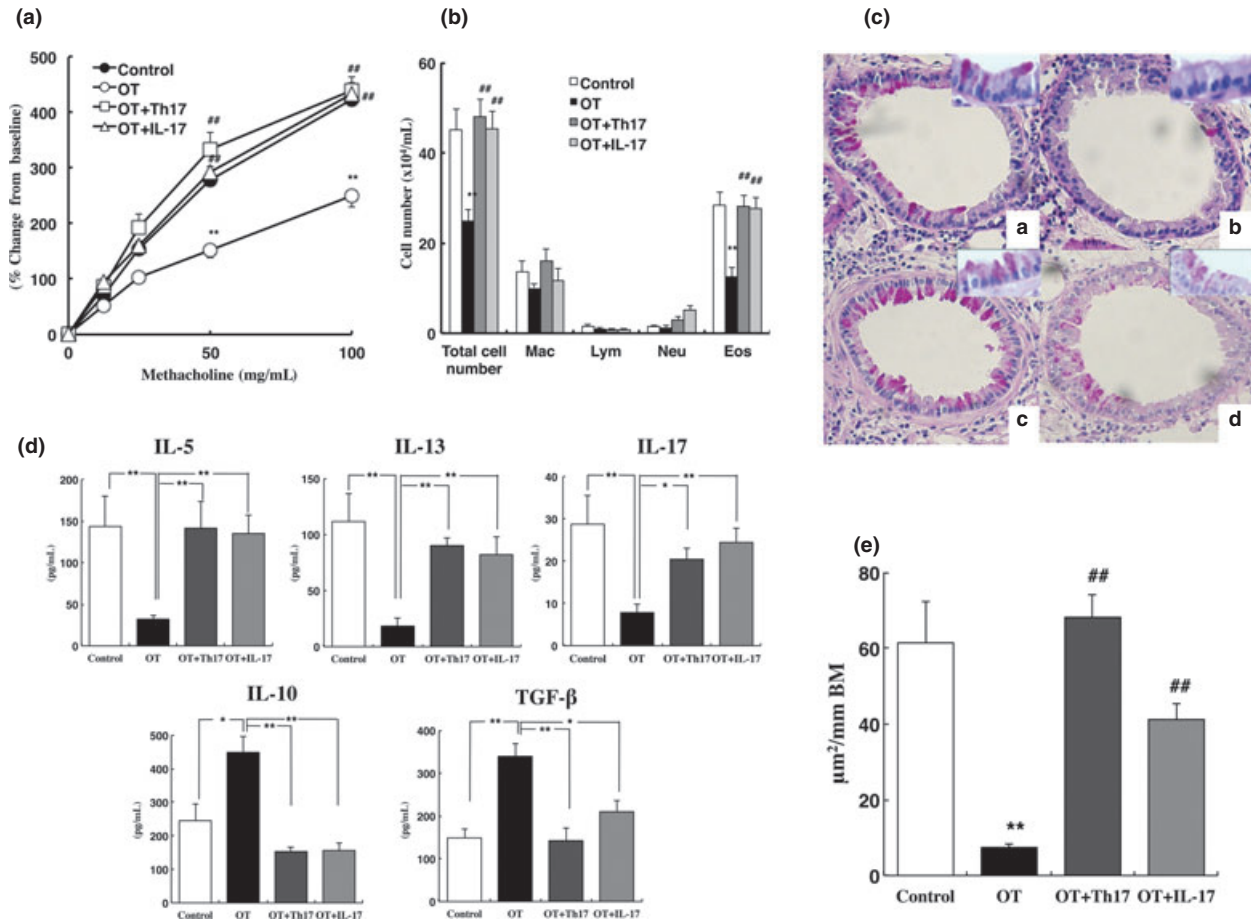

Fig. 2. Effects of OVA feeding (oral tolerance to OVA) and IL-17 administration or transfer of Th17 cells on lung allergic responses. (a) Changes in respiratory resistance following OVA challenges. Increasing concentrations of nebulized methacholine were administered through the tracheal cannula 24 h after the last OVA challenge in C57BL/6 mice. (b) Bronchoalveolar lavage (BAL) fluid cellular composition. (c) Cytokine levels in BAL fluid. (d) Representative periodic acid-Schiff (PAS)-stained histological sections of lung tissues (original magnification: ×100, insets: ×400) obtained 24 h after the last challenge in a) control, b) OT, c) OT mice + Th17 d) OT mice + IL-17. (e) Quantitative analysis of PAS-positive cells. Data represent means ± SEM from three independent experiments (n = 12). *P < 0.05 or **P < 0.01 compared with control group or as indicated. #P < 0.05 or ##P < 0.01 compared with OT group. Control, secondarily challenged control mice (with PBS feeding); OT, secondarily challenged + OVA-fed mice; OT + Th17, OT mice with transfer of Th17 cells; OT + IL-17, OT mice with administration of IL-17; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils; BM, basement membrane.

Transfer of Th2 cells on the induction phase of oral tolerance did not abolish the effects of oral tolerance on the AHR and severity of airway allergic inflammation

To investigate whether the Th2 cells might have similar effects on Th17 cells in attenuating the effects of oral tolerance on the degree of AHR and airway allergic inflammation, OVA-specific Th2 cells (1×10^7 cells/mouse) were transferred via the tail vein on the first day of oral administration of OVA (Fig. 1c). Interestingly, the transfer of Th2 cells did not change the degree of AHR or airway eosinophilia as compared with the mice subjected to OVA feeding alone (Fig. 4). From these results, it was concluded that transfer of Th2 cells did not have the potential to modulate the induction of oral tolerance.

The profile of lung CD4 T cells after transfer of Th17 cells

To investigate the phenotype of the CD4 T cells infiltrating the lungs, *ex vivo* culture of lung CD4 T cells was performed. The lung CD4 T cells were cultured with CD3 stimulation for 72 h and the culture supernatants were harvested for ELISA. The levels of Th2 cytokines and IL-17 in the supernatants were decreased in the oral tolerance groups, whereas complete recovery of the Th2 cytokine and IL-17 levels was observed in the oral tolerance + Th17 groups (Fig. 5a). With regard to the inhibitory cytokines, e.g. IL-10 and TGF-β, elevation of the levels of these cytokines was observed in the oral tolerance groups, whereas the levels were significantly decreased in the oral tolerance + Th17 groups. The

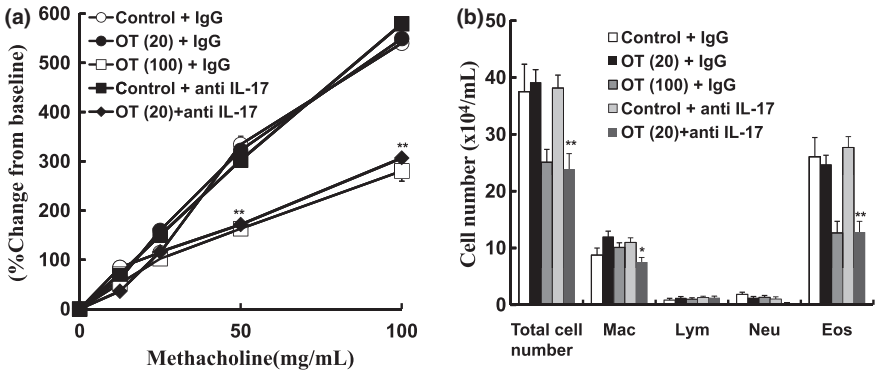

Fig. 3. Effects of OVA feeding (oral tolerance to OVA) and anti-IL-17 antibody administration on lung allergic responses. (a) Changes in respiratory resistance following OVA challenges. (b) Bronchoalveolar lavage cellular composition. Data represent means ± SEM from two independent experiments ($n = 8$). $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with OT (20) + IgG group. Control + IgG: Secondarily challenged control mice with administration of control rat IgG (with PBS feeding). OT (20) + IgG: Secondarily challenged + OVA (20 mg/day) fed mice with administration of control rat IgG. OT (100) + IgG: Secondarily challenged + OVA (100 mg/day) fed mice with administration of control rat IgG. Control + anti-IL-17: Secondarily challenged control mice with administration of anti-IL-17 antibody (with PBS feeding). OT (20) + anti-IL-17: OT (20) mice with administration of anti-IL-17 antibody. Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

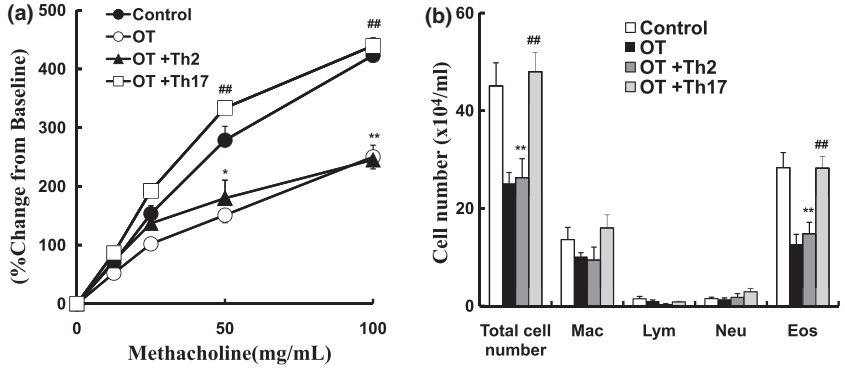

Fig. 4. Effects of Th2 or Th17 cell transfer on lung allergic responses. (a) Changes in respiratory resistance following OVA challenge. (b) Bronchoalveolar lavage cellular composition. Data represent means ± SEM from three independent experiments ($n = 9$). $^{*}P < 0.05$ or $^{**}P < 0.01$ compared with the control group. $\#P < 0.05$ or $\#\#P < 0.01$ compared with OT group. Control: Secondarily challenged control mice (with PBS feeding). OT: Secondarily challenged + OVA-fed mice. OT + Th17: OT mice with transfer of Th17 cells. OT + Th2: OT mice with transfer of Th2 cells. Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

levels of IFN-γ in lung CD4 T cells in the OT group were higher than those in the control group, but similar to those in the OT + Th17 groups.

Since IL-10 and TGF-β are known to be produced abundantly from Treg cells, the proportion of lung Treg cells expressing Foxp3 as a master transcription factor was checked by flow cytometry. As shown in Fig. 5b, c, the ratio of $CD4^{+}CD25^{+}$Foxp3 T cells to the total count of CD4 T cells was increased in the oral tolerance groups as compared with that in the control groups, whereas significant decrease of this ratio was observed in the oral tolerance + Th17 group as compared with that in the oral tolerance groups (Fig. 5b, c). From these results, it was concluded that Th17 abolished the attenuating effects of oral tolerance on the AHR and allergic airway inflammation by suppressing the expansion of Treg cells in the lung.

Modulation of IL-6 production in Peyer's patches after Th17 transfer

According to previous reports, the principal mechanism of induction of oral tolerance is expansion of Tregs in the gut-associated lymphoid tissue (GALT), such as PP and mesenteric lymph nodes (MLN) [29, 30]. To investigate the direct effects of Th17 cells in the gut, we isolated PP 24 h after the last OVA feeding and analysed whether the transferred cells had truly immigrated to the PP by flow cytometry and fluorescence microscopy. As shown in Fig. 6a, the transferred Th17 cells generated from the OT-II/GFP double-transgenic mice were able to detect the $GFP^{+}CD4^{+}$ fraction in the PP. Transferred cells were also detected by fluorescence microscopy, being localized in the perifollicular lesion of the PP (Fig. 6b). The levels of IL-17 in the PP corresponded

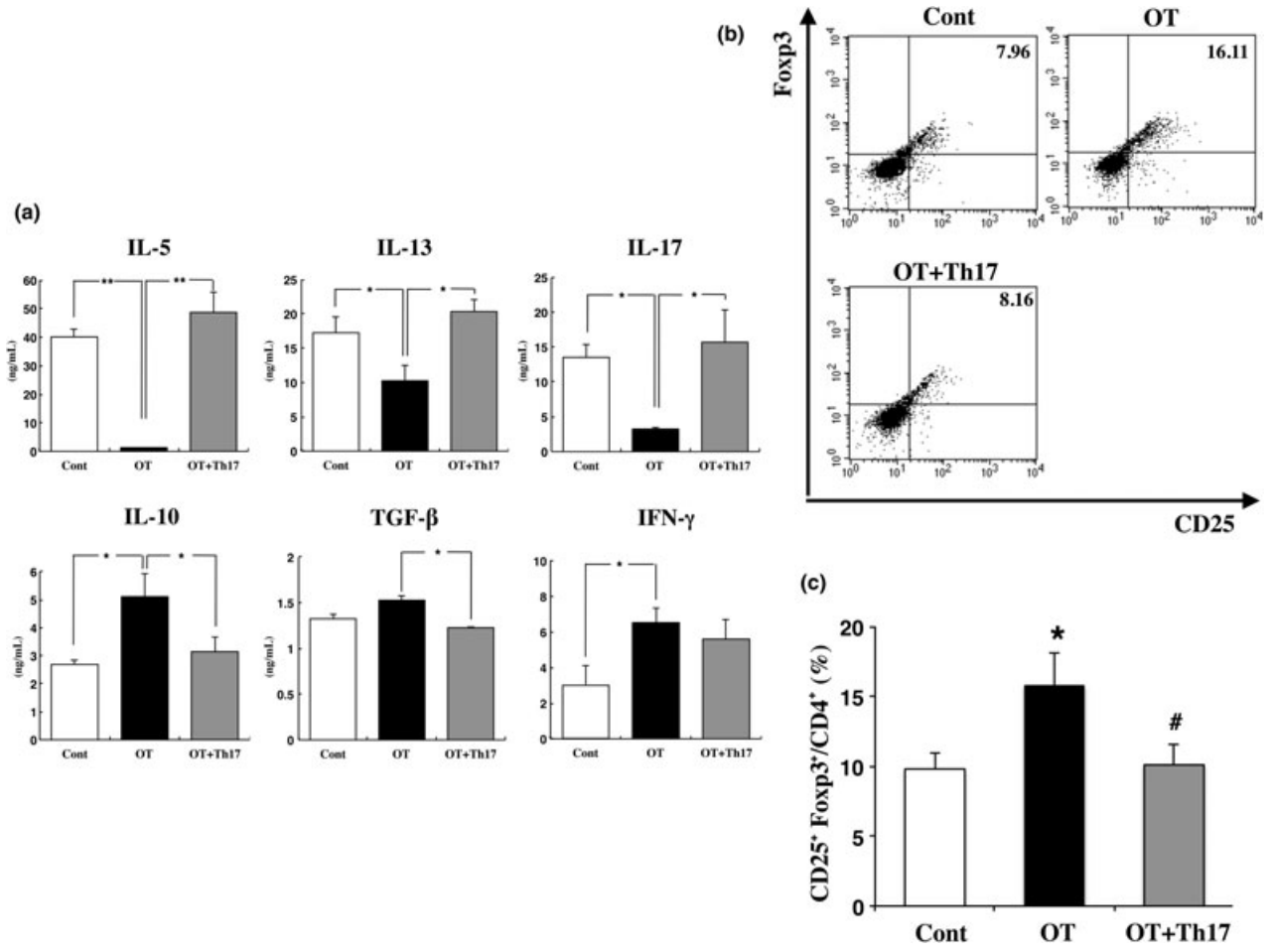

Fig. 5. *In vitro* cytokine levels (a) and flow-cytometric analysis (b, c). (a) Cytokine levels were determined in supernatants from lung CD4 T cells on BD Biocoat^{TM} T cell activation assay plates. Lung CD4 T cells from mice that received treatments were isolated as described in Materials and methods. The lung CD4 T cells (4×10^5/well) were cultured on BD Biocoat^{TM} T cell activation assay plates for 72 h. (b) Representative data of CD25 and Foxp3 expression on lung CD4 T cells by flow cytometry. (c) Percentages of $CD25^+Foxp3^+$ fraction in lung CD4 cells. Data represent means ± SEM from three independent experiments ($n = 6$). *$P < 0.05$ compared with the control group. #$P < 0.05$ compared with the OT group.

to flow cytometry and histological data, that is, the levels of IL-17 in the OT group were decreased compared with the control group, whereas levels in the OT + Th17 group were restored to similar levels as those seen in the control group (Fig. 6c). According to a previous paper, IL-6 is secreted from adherent cells such as fibroblasts, keratinocytes, epithelial and endothelial cells in response to IL-17 [15]. To determine whether the transferred Th17 cells into the mice subjected to OVA feeding were involved in the production of IL-6 in the PP, we examined the expression of IL-6 mRNA by real-time PCR and the levels of IL-6 by ELISA. We found both the IL-6 mRNA levels and levels of IL-6 in the PP of the mice subjected to OVA feeding alone were decreased as compared with that of control mice, while mRNA and protein levels of IL-6 in the PP of the mice subjected to OVA feeding + Th17 transfer were restored to similar levels to control mice (Fig. 6d, e). The protein levels of IL-6 in the PP were similar after OVA feeding and following OVA challenge (data not shown). Lending support to a previous report that IL-6 is known to inhibit the differentiation of Treg [16], the number of $CD4^+CD25^+Foxp3^+$ Treg cells in the PP obtained from the mice subjected to OVA feeding alone were significantly increased as compared with that in control mice, whereas the number of Treg cells in the PP of mice with OVA feeding + Th17 transfer was decreased to similar levels to control mice (Fig. 6f).

Role of IL-6 on the inhibitory effects of Th17 transfer against induction of oral tolerance

To determine the mechanism underlying the inhibitory effects of Th17 cell transfer on the induction of oral tolerance, we administered anti-IL-6 antibody to the mice subjected to induction of oral tolerance + transfer of Th17 cells. Anti-IL-6 antibody was administered intraperitoneally for 5 days in the same phase as the

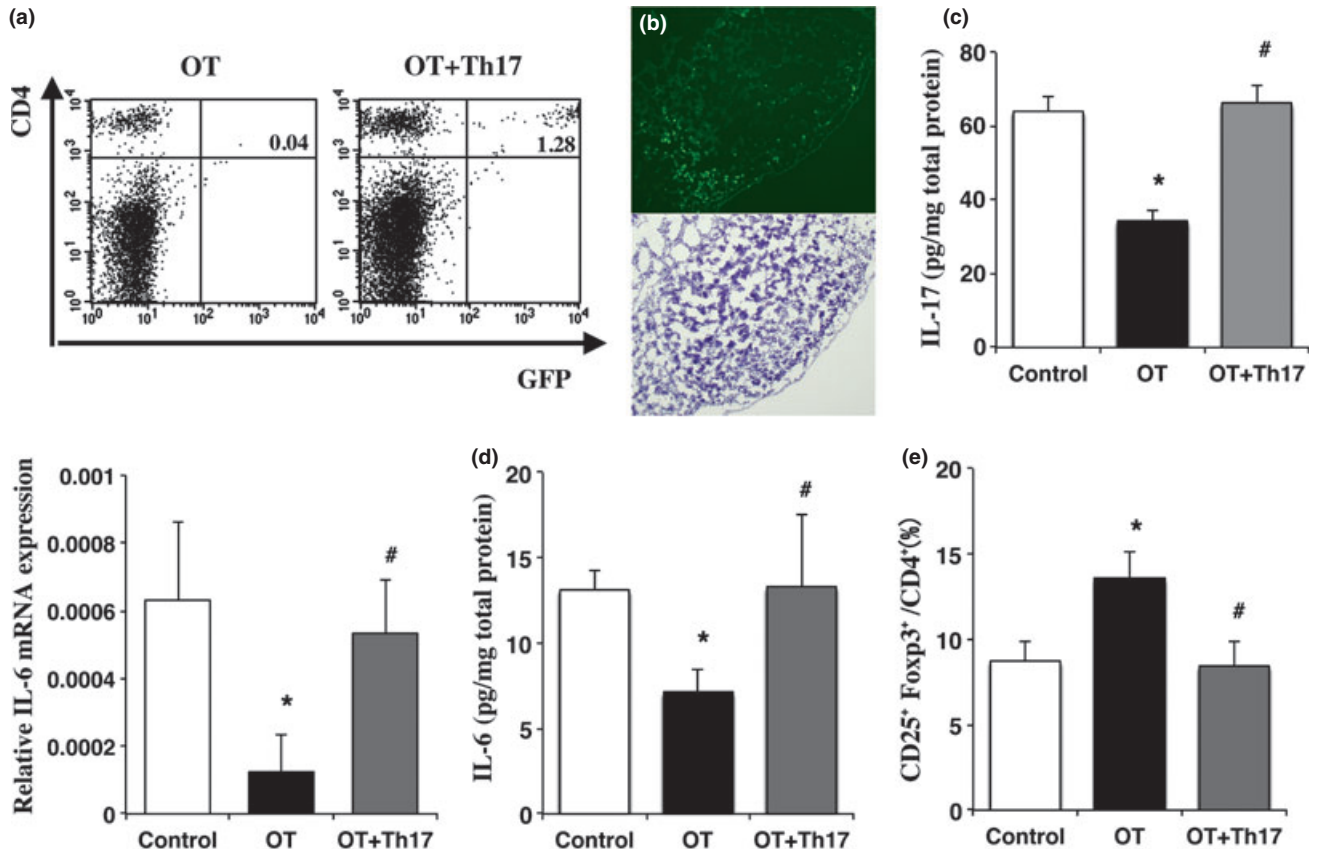

Fig. 6. The effect of transfer of Th17 cells in Peyer's patches (PP). (a) Flow-cytometric analysis for migration of the transferred Th17 cells into PP examined at 24 h after the last OVA feeding. Th17 cells generated from OT-II/GFP double-transgenic mice were detected as GFP^+CD4^+ cells. (b) Representative sections of PP tissues (magnification: ×100) obtained 24 h after the last OVA feeding by fluorescence microscopy (right photo shows a section counterstained with haematoxylin). PPs were fixed for 4 h at 4°C in periodatelysine-paraformaldehyde, washed for 4 h with PBS containing 10%, 15% and finally 20% sucrose, embedded in OCT compound (Miles, Elkhart), frozen in dry ice-acetone and cut on a cryostat (Bright) into 6-μm-thick sections. The fluorescence from the transferred Th17 cells was analysed by fluorescence microscopy. The nuclei were counterstained with haematoxylin. (c) Levels of IL-17 in the PP. Extracts from homogenized PP tissues were assessed for IL-17 by ELISA. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit. (d) IL-6 mRNA expression in the PP. Total RNA was prepared from the PP, and real-time polymerase chain reaction (PCR) analysis for IL-6 mRNA was performed. (e) Levels of IL-6 in the PP. Extracts from homogenized PP tissues were measured for IL-6 by ELISA. (f) Percentage of the $CD25^+Foxp3^+$ fraction in the PP CD4 cells. Data represent means ± SEM from three independent experiments carried out in triplicate. Control: Secondarily challenged control mice (with PBS feeding). OT: Secondarily challenged + OVA-fed mice. OT + Th17: OT mice with transfer of Th17 cells. $*P < 0.05$ compared with the control group. $\#P < 0.05$ compared with the OT group.

OVA feeding (Fig. 1d). Interestingly, combined Th17 cell transfer + anti-IL-6 antibody administration resulted in attenuation of the degree of AHR, airway eosinophilia, Th2 cytokine elevation in BAL fluid and goblet cell metaplasia to the degree observed in the mice subjected to OVA feeding alone (Fig. 7). Representative data of the flow-cytometric analysis showed that the number of Treg cells in the lungs of animals subjected to combined Th17 cell transfer + anti-IL-6 antibody administration was increased as compared with that in the mice subjected to Th17 cell transfer alone (Fig. 7f). According to previous reports, blocking of IL-6 in the challenge phase of asthma induced amelioration of the AHR and airway allergic inflammation [31]. Anti-IL-6 antibody administered without either Th17 cell transfer or OVA feeding as control showed little effect on the degree of AHR or allergic airway inflammation (Fig. 7a, b). These data imply that while blockade of IL-6 may have effects in the tolerance induction phase, but not in the challenge phase.

Discussion

We demonstrated that both Th17 transfer and administration of IL-17 in the induction phase of oral tolerance abolished the therapeutic effects of oral tolerance by activating IL-6 in an airway allergic inflammation model. Furthermore, administration of anti-IL-17 antibody augmented the therapeutic effects of oral tolerance. These phenomena appeared to be

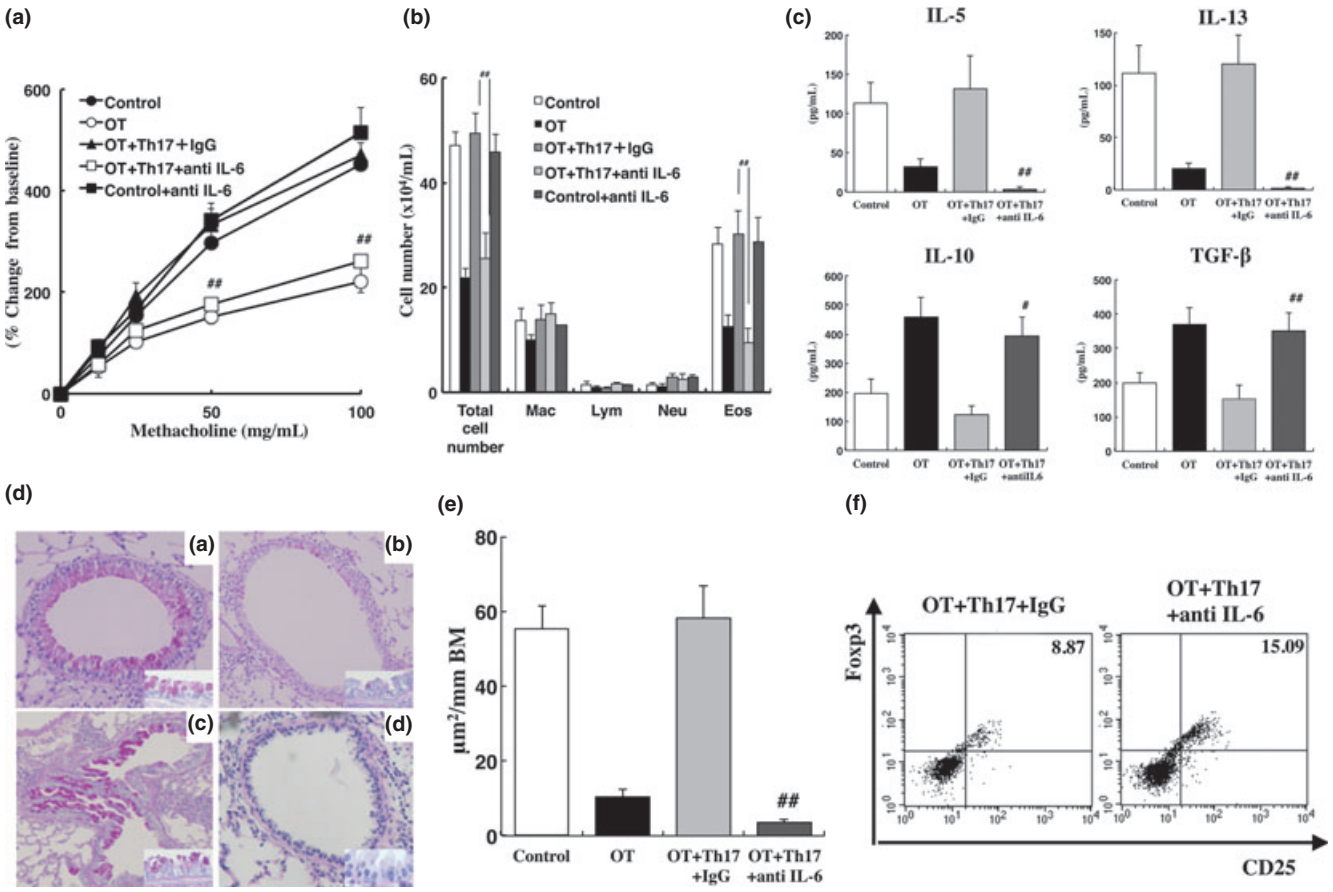

Fig. 7. Effects of administration of anti-IL-6 antibody after transfer of Th17 cells on lung allergic responses. (a) Changes in respiratory resistance following OVA challenge. (b) Bronchoalveolar lavage (BAL) cellular composition. (c) Cytokine levels in BAL fluid. (d) Representative periodic acid-Schiff (PAS)-stained histological sections of lung tissues (original magnification: ×100, insets: ×400) obtained 24 h after the last challenge in a) 2nd control, b) 2nd OT, c) OT mice + Th17, d) OT mice + Th17 + anti-IL-6. (e) Quantitative analysis of PAS-positive cells. (f) Representative data of CD25 and Foxp3 expression on lung CD4 T cells by flow cytometry. Data represent means ± SEM from three independent experiments ($n = 9$). $\#P < 0.05$ or $\#\#P < 0.01$ compared with OT mice + Th17 + IgG mice or as indicated. Control: Secondarily challenged control mice (with PBS feeding), OT: Secondarily challenged + OVA-fed mice. OT + Th17 + IgG: OT mice with simultaneous transfer of Th17 cells and administration of control rat IgG. OT + Th17 + anti-IL-6: OT mice with simultaneous transfer of Th17 cells and administration of anti-IL-6 antibody. Control + anti-IL-6: Secondarily challenged control mice and administration of anti-IL-6 antibody (with PBS feeding). Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils; BM, basement membrane.

specific to Th17/IL-17, because we confirmed that transfer of Th2 cells, which are a critical T cell subset for airway allergic inflammation, did not similarly attenuate the effects of oral tolerance (Fig. 2). When we attempted to identify the transferred cells in the lung parenchyma after the challenges by using Th17 or Th2 cells originating from the OT-II/GFP double-transgenic mice, few cells were found to reside in the lung (data not shown). Therefore, we speculate that the transferred cells attenuated the induction of oral tolerance.

Our results indicated that IL-17 secreted from the transferred Th17 cells induced IL-6 production, resulting in the suppression of expansion of Treg cells. Conversely, blocking of endogenous IL-17 by anti-IL-17 antibody during OVA feeding with suboptimal dose (20 mg/day) also induced reduction of AHR and airway eosinophilia (Fig. 3). Schnyder-Candrian et al. reported that reduction in OVA-induced Th2 inflammation was observed following IL-17 administration, and exacerbation of Th2 inflammation following anti-IL-17 administration in wild-type mice [6]. However, the systems used in our study differed from that of Schnyder-Candrian et al. First, a different study using a similar protocol reported that airway inflammation was exacerbated, including AHR [32]. This finding indicates that the negative regulator function of IL-17 in allergic model may be controversial. Second, Schnyder-Candrian et al. administered IL-17 or anti-IL-17 antibodies locally (intratreacheally) at doses differing from ours. Third, our data involve a tolerance model. In the gut, Th17 is thought to be a negative regulator for the

induction of Treg cells [13]. Peron et al. also reported that oral tolerance in EAE induced a reduction of Th17 cells and inflammation in the periphery and CNS [33]. From these data, it may be concluded that IL-17 plays a role in regulating oral tolerance. With regard to Treg cells, Hauet-Broere et al. reported that the critical mechanism of induction of oral tolerance was expansion of Tregs in the GALT, such as PP and MLN [29]. Exogenous antigens are processed by the GALT system and utilized for an active suppression mechanism to render the host immunologically unresponsive to them [13]. Since our data indicated that the number of Tregs in the lung increased after the induction of oral tolerance and presumably decreased by the influence of IL-17/IL-6, it can be speculated that the Tregs generated in the PP or MLNs may immigrate to the lung and ameliorate the airway allergic inflammation. The results of flow cytometry and histological examination for the PP demonstrated that the transferred Th17 cells had immigrated into the PP and induced upregulation of IL-6 production, resulting in suppression of the expansion of Treg cells (Fig. 5).

Neutralization of IL-6 in accordance with Th17 transfer was quite effective for restoration of oral tolerance in this study. Reciprocal development of Foxp3-Tregs and Th17 cells has recently been discovered, because TGF-β triggers the expression of Foxp3 in naïve T cells, whereas IL-6 inhibits the TGF-β-driven expression of Foxp3, and TGF-β plus IL-6 together induce retinoid-related orphan receptor (ROR)-gt, triggering the developmental programme of Th17 cells [16]. Korn et al. reported that the blockade of IL-6 signalling using genetic deficiency of the signalling subunit glycoprotein 130 (gp130) likely changed the immunogenic vaccination protocols into a tolerogenic regimen with the induction of antigen-specific Foxp3 Tregs in experimental allergic encephalomyelitis [34]. In our preliminary data, *in vitro* blocking of endogenous IL-6 from Th17 cells by anti-IL-6 antibody altered production of IL-17 from Th17 cells (data not shown). These results support our data with respect to IL-6 blockade as a powerful candidate to boost the tolerogenic effect in immunotherapy.

In the study by Doganci et al. [31], nasal administration of anti-IL-6R or gp130-fraction contrast (gp130-Fc), which causes blockade of trans-signalling of IL-6, in the antigen challenge phase of asthma in a murine model showed suppression of expansion of the Th2-type cells in the lung and induction of $CD4^+CD25^+Foxp3^+$ Tregs in the lung. To determine whether anti-IL-6 antibody was more effective in the tolerance induction phase or the challenge phase, we examined the effects in mice subjected to anti-IL-6 antibody administration without transfer of Th17 cells + OVA feeding as controls, and found that anti-IL-6 had little effects on the degree of AHR and allergic airway inflammation (Fig. 7a, b). These data indicate that blockade of IL-6 in our study was effective in the tolerance induction phase, but not in the challenge phase. Doganci et al. described the different roles of membrane-bound IL-6R (mIL-6R) and soluble IL-6R (sIL-6R) in lung immune responses [31, 35]. In other words, local blockade of sIL-6R in a murine model of asthma after OVA sensitization by gp130Fc led to suppression of the Th2 cells in the lung. In contrast, local treatment of the lung with anti-IL-6R antibody for blockade of mIL-6R and sIL-6R induced Th1-CD4 cells as well as $CD4^+CD25^+Foxp3^+$ Treg cells, with immunosuppressant effects in the lung. In our study, anti-IL-6 antibody was used for neutralization of IL-6, thereby our system was expected to show dual effects theoretically. However, the effect of blockade of IL-6R on the induction of oral tolerance in an airway allergic inflammation model must be elucidated in a future study.

In this study, we adopted the therapeutic protocol used in the allergic airway inflammation model, because of application of a similar protocol in the clinical setting. In general, three patterns have been used to develop the oral tolerance model: feeding before sensitization and feeding between sensitization and challenges, the so-called prophylactic and more popular protocols, and the third is feeding after the first challenge, as used in our study, the so-called therapeutic protocol [36]. With regard to the effects, the first two are associated with a greater susceptibility to oral tolerance than the third [36–38]. Actually, we observed little effect on the degree of AHR and airway inflammation with fewer doses (20 mg/day/mouse) of OVA (Fig. 3). However, in the clinical setting, BA patients never receive immunotherapy before the occurrence of the disease; therefore, we adopted the therapeutic protocol of feeding in this study.

In summary, we investigated the inhibitory effects of IL-17 on the induction of oral tolerance in an allergic airway inflammation model. Administration of IL-17 as well as the transfer of Th17 cells was found to have the potential to abolish the therapeutic effects of oral tolerance such as amelioration of the degree of AHR, airway allergic inflammation, by presumably augmenting endogenous IL-6 production in PP. On the other hand, administration of anti-IL-17 antibody on the induction phase of oral tolerance induced the augmentation of therapeutic effects of oral tolerance. Based on the finding that transfer of Th2 did not induce similar effects, we concluded that this phenomenon was specific to IL-17. In the flow-cytometric analysis, increase in the number of Foxp3-positive Treg cells in the lungs was observed in the OVA-fed mice, whereas a decrease in the number of these cells was noted in the animals

subjected to OVA feeding + Th17 cell transfer. Neutralization of IL-6 by anti-IL-6 antibody at the same time as the transfer of Th17 was able to restore the effects of oral tolerance. These data suggest that IL-17 may inhibit the induction of tolerance to antigen in asthma, and it is possible that modulation of IL-17/Th17 cells may offer great therapeutic benefits in the immunotherapy for BA.

Acknowledgements

The authors express their gratitude for the expert help of Dr Naofumi Imai and Ms Keiko Yamagiwa in performing the histological studies and for the expert care of the animals by Ms Akiko Hayafuji.

Conflict of interest: The authors declare that they have no competing interests.

References

1 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999; **17**:255–81.
2 Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001; **344**:350–62.
3 Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002; **3**:715–20.
4 Chakir J, Shannon J, Molet S *et al*. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003; **111**:1293–8.
5 Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010; **104**:1131–7.
6 Schnyder-Candrian S, Togbe D, Couillin I *et al*. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 2006; **203**: 2715–25.
7 Nakae S, Komiyama Y, Nambu A *et al*. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002; **17**:375–87.
8 Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008; **8**:218–30.
9 Weiner HL, Friedman A, Miller A *et al*. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* 1994; **12**:809–37.
10 Smith KM, Eaton AD, Finlayson LM, Garside P. Oral tolerance. *Am J Respir Crit Care Med* 2000; **162**:S175–8.
11 Faria AM, Weiner HL. Oral tolerance: therapeutic implications for autoimmune diseases. *Clin Dev Immunol* 2006; **13**:143–57.
12 Kraal G, Samsom JN, Mebius RE. The importance of regional lymph nodes for mucosal tolerance. *Immunol Rev* 2006; **213**:119–30.
13 Tsuji NM, Kosaka A. Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol* 2008; **29**:532–40.
14 Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol* 2009; **9**:883–9.
15 Fossiez F, Banchereau J, Murray R, Van KC, Garrone P, Lebecque S. Interleukin-17. *Int Rev Immunol* 1998; **16**:541–51.
16 Bettelli E, Carrier Y, Gao W *et al*. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006; **441**:235–8.
17 Wang GY, Sun B, Kong QF *et al*. IL-17 eliminates the therapeutic effects of myelin basic protein-induced nasal tolerance in experimental autoimmune encephalomyelitis by activating IL-6. *Scand J Immunol* 2008; **68**:589 –97.
18 Bousquet J, Hejjaoui A, Dhivert H, Clauzel AM, Michel FB. Immunotherapy with a standardized dermatophagoides pteronyssinus extract. Systemic reactions during the rush protocol in patients suffering from asthma. *J Allergy Clin Immunol* 1989; **83**:797–802.
19 US Department of Health and Human Services. National Institutes of Health. National Heart L, and Blood Institute. *Expert panel report 3. Guidelines for the diagnosis and management of asthma*, 2007. Available at http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm (accessed 1 August 2011).
20 Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003; **97**:726–33.
21 Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010; **104**:1131–7.
22 Xystrakis E, Kusumakar S, Boswell S *et al*. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Invest* 2006; **116**:146–55.
23 Hartl D, Koller B, Mehlhorn AT *et al*. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007; **119**: 1258–66.
24 Wakashin H, Hirose K, Maezawa Y *et al*. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am J Respir Crit Care Med* 2008; **178**:1023–32.
25 Hayashi M, Koya T, Kawakami H *et al*. A prostacyclin agonist with thromboxane inhibitory activity for airway allergic inflammation in mice. *Clin Exp Allergy* 2010; **40**:317–26.
26 Koya T, Takeda K, Kodama T *et al*. RANTES (CCL5) regulates airway responsiveness after repeated allergen challenge. *Am J Respir Cell Mol Biol* 2006; **35**:147–54.
27 Koya T, Kodama T, Takeda K *et al*. Importance of myeloid dendritic cells in persistent airway disease after repeated allergen exposure. *Am J Respir Crit Care Med* 2006; **173**:42–55.
28 Koya T, Matsuda H, Takeda K *et al*. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J Allergy Clin Immunol* 2007; **119**:1241–50.
29 Hauet-Broere F, Unger WW, Garssen J, Hoijer MA, Kraal G, Samsom JN. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *Eur J Immunol* 2003; **33**:2801–10.

30 Zinselmeyer BH, Dempster J, Gurney AM *et al*. In situ characterization of CD4+ T cell behavior in mucosal and systemic lymphoid tissues during the induction of oral priming and tolerance. *J Exp Med* 2005; **201**:1815–23.
31 Doganci A, Eigenbrod T, Krug N *et al*. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J Clin Invest* 2005; **115**:313–25.
32 Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 2009; **180**: 720–30.
33 Peron JP, Yang K, Chen ML *et al*. Oral tolerance reduces Th17 cells as well as the overall inflammation in the central nervous system of EAE mice. *J Neuroimmunol* 2010; **227**:10–7.
34 Korn T, Mitsdoerffer M, Croxford AL *et al*. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2008; **105**:18460–5.
35 Doganci A, Sauer K, Karwot R, Finotto S. Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. *Clin Rev Allergy Immunol* 2005; **28**:257–70.
36 Russo M, Nahori MA, Lefort J *et al*. Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* 2001; **24**:518–26.
37 Chung YH, Means RE, Choi JK, Lee BS, Jung JU. Kaposi's sarcoma-associated herpesvirus OX2 glycoprotein activates myeloid-lineage cells to induce inflammatory cytokine production. *J Virol* 2002; **76**:4688–98.
38 Razafindratsita A, Saint-Lu N, Mascarell L *et al*. Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol* 2007; **120**: 278–85.