

Role of Regulatory T cells in Airway Inflammation in Asthma

TOMOTAKA KAWAYAMA*, TAKASHI KINOSHITA*, KAZUKO MATSUNAGA*^{***},
YOSHIKO NAITO*, JUN SASAKI*, YOSHIKAZU TOMINAGA*[†],
AND TOMOAKI HOSHINO*

**Division of Respiriology, Neurology, and Rheumatology, Department of Medicine,
Kurume University School of Medicine, Kurume 830-0011 Japan*

***Department of Respiratory Medicine, Fukuoka Sanno Hospital, Fukuoka 814-0001 Japan*

†Department of Respiratory Medicine, Asakura Medical Association Hospital, Asakura 838-0069 Japan

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Summary: Asthma is an allergic disease characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), reversibility and remodeling. Inhaled corticosteroids (ICS) are effective in many patients with asthma. However, ICS are a controlling, but not curative treatment, and there are still many patients with refractory and difficult-to-treat asthma. The evaluation of airway inflammation by induced sputum, non-specific AHR by methacholine, and asthmatic reactions by specific allergen challenge techniques are useful not only to investigate the pathogenesis of asthma but also to help develop new drugs for asthma management. Interactions between inflammation and regulation, such as between regulatory T cells (Tregs), and AHR were investigated using these techniques. The phenotypes are Tregs characterized by expression of the forkhead box P3 (Foxp3) and cytotoxic T-lymphocyte antigen 4 (CTLA4), which are potent mediators of dominant self-tolerance. Foxp3 and CTLA4 interact with each other. In patients with mild asthma, airway Tregs were decreased and airway eosinophilic inflammation was activated with accelerated AHR. Human asthmatic attack models by allergen challenge demonstrated that airway Tregs were decreased from the baseline with late asthmatic response (LAR) in patients with dual-responder asthma, and there was a significant correlation between change in airway Tregs and LAR. Airway Tregs were increased with escalation of interleukin-10 by ICS. The investigation of Tregs may lead to new strategies for management of asthma and other allergic diseases.

Key words asthma, airway inflammation, regulatory T cells, airway hyperresponsiveness, inhaled corticosteroids

INTRODUCTION

Atopic asthma is a chronic disease characterized by eosinophilic airway inflammation, airway hyperresponsiveness (AHR), asthmatic responses and bron-

chial remodeling. Elevated numbers of inflammatory cells, including activated eosinophils and T lymphocytes, are boosted further in association with allergen-induced asthmatic response and asthma attack [1–4]. Activated T lymphocytes play a central role in the

Corresponding Author: Tomotaka Kawayama, MD, PhD, Division of Respiriology, Neurology, and Rheumatology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume city, Fukuoka 830-0011, Japan. Tel: +81-942-31-7560, Fax: +81-942-31-7703. E-mail: kawayama_tomotaka@med.kurume-u.ac.jp

Abbreviations: AHR, airway hyperresponsiveness (Text); AHR, aryl hydrocarbon receptor (Fig.7); BALF, bronchial alveolar lavage fluid; BL, baseline; CTLA4, cytotoxic T-lymphocyte antigen 4; DR, dual responders; EAR, early asthmatic response; ELF, epithelial lining fluid; F_{ENO}, fraction expiratory nitrogen oxide; FEV₁, forced expiratory volume in 1 second; Foxp3, forkhead box P3; GATA, GATA ‘zinc-finger’ transcription factors; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroids; ID, DNA-binding protein inhibitor; IER, isolated early responders; IFN, interferon; IL, interleukin; ILC, immune lymphoid cells; LAR, late asthmatic response; MCh, methacholine; NK, natural killer; NS, not significant; NaCl 0.7% saline; PC₂₀-MCh, provocation concentration of methacholine causing a 20% fall in forced expiratory volume in 1 second; ROR, retinoic acid receptor-related orphan receptor; SEM, standard error of the mean; T-bet, Th1-specific T box transcription factor; TGF, Transforming growth factor; TSLP, thymic stromal lymphopoietin; Th, helper T; Tregs, regulatory T cells.

pathogenesis of asthma through cytokine production. This is supported by studies showing increased activation of T lymphocytes following allergen provocation [5, 6], and by studies showing an association between activated T lymphocytes and severity of asthma [7]. Activated CD4 positive T helper (Th) lymphocytes are subdivided into two distinct phenotypes based on the cytokines they produce [8]. Th1 lymphocytes produce cytokines such as interferon- γ (IFN- γ), interleukin (IL)-2 and IL-12, whereas Th2 lymphocytes produce cytokines such as IL-4, IL-5 and IL-13. Th1/Th2 counter-regulation has also been described, with each lymphocyte population capable of inhibiting and/or regulating the development and/or phenotype induced by the other [8]. Previous studies have demonstrated an imbalance of Th1/Th2 towards Th2 predominance, which is thought to underlie asthmatic responses [9].

Recently, a third type of T cell phenotype has been identified. The regulatory T cell (Tregs) phenotype is mainly characterized by expression of forkhead box P3 (Foxp3) protein, and these Tregs are potent mediators of dominant self-tolerance [10]. Tregs have the potential to suppress both Th1- and Th2-induced inflammation [11, 12]. Activation and expansion of Tregs may be exploited for treatment of immunologic diseases such as allergic asthma [13]. Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a homolog of CD28, which is expressed only on activated T cells, and binds to the accessory molecules B7-1 (CD80) and B7-2 (CD86) [14, 15]. Like CTLA4⁺CD4⁺CD25⁺ T cells, Tregs also have the ability to down-regulate Th1 and Th2 responses through CTLA4 signaling [16]. It is well known that CTLA4 is expressed on both surface and cytoplasm in cells. The surface CTLA4 expressed on T cells have immunosuppressive effects [17, 18]. Surface CTLA4 also play a role in sequestration of B7 molecules without cytoplasmic CTLA4 and the sequestration is regulated by the levels of surface expression of CTLA4 [19].

The present review discusses the correlation between chronic airway eosinophilic inflammations, asthmatic response and regulatory T cells in asthma.

Evaluation of airway inflammation and hyperresponsiveness

Airway inflammation is a major factor in the pathogenesis of asthma, as shown by the fact that inhaled corticosteroids (ICS) are the first choice of medications in patients with asthma. To understand the pathogenesis and mechanisms of asthmatic responses, the evaluation of airway inflammation is important. Many approaches are available to investigate airway inflam-

mation. Induced sputum [20], fraction expiratory nitrogen oxide (F_ENO) [21, 22], and exhaled breath condensate (EBC) [23, 24] are non-invasive techniques, whereas bronchial alveolar lavage fluid (BALF) [9, 25], bronchial epithelial lining fluid (ELF) [26] and bronchial biopsy [27, 28] are more invasive but can provide additional information. The F_ENO and EBC give us information from exhaled gases, but tell us nothing about airway cell differentiation, while BALF, ELF and biopsy use flexible endobronchoscopy. Among these examination techniques, the induced sputum technique is not only safe and easy to perform, it also enables us to obtain information about both cells and supernatants repeatedly [29]. The induced sputum technique also can assess the effectiveness of newly developed agents [30, 31]. The number of airway eosinophils in patients with mild asthma was significantly higher than non-asthmatic control subjects (control subjects) (Figure 1A and 1B) [32]. The number of airway eosinophils was also increased in patients with asthma after allergen, but not methacholine (Mch) challenge (Figure 1C) [33].

Evaluation of nonspecific AHR

The second major factor in the pathogenesis of asthma is AHR. There are two types of AHR. The first involves a nonspecific response to MCh, acetylcholine and histamine, and the other involves a specific response to antigens such as house dust mite, ragweed, and cat. [1, 34, 35]. MCh reveals the effects of bronchoconstriction via airway muscarinic receptors, and a decline of the forced expiratory volume in 1 second (FEV₁) is observed by spirometry after inhaled MCh challenge in asthma patients [34, 35].

Figure 2A shows the % changes in FEV₁ from baseline after 0.7% NaCl, and doubling doses of MCh (0.03 mg/mL to 32 mg/mL) in control subjects without history of asthma, patients with intermittent and persistent mild asthma, and patients with cough variant asthma (unpublished data). The MCh challenge tests were performed in accordance with previous studies [34, 35]. The severity of asthma was evaluated based on the Global Initiative for Asthma (GINA) guidelines [36]. The mean percentages of FEV₁ in all patients with asthma fell to under 80% of the baseline at MCh concentrations less than 8 mg/mL, although those in control subjects remained above 80% of baseline at MCh concentrations of 16 mg/mL. In patients with intermittent and persistent mild asthma, there was a significant negative correlation between airway inflammation (number of sputum eosinophils) and AHR (provocative concentration of MCh causing a 20% fall

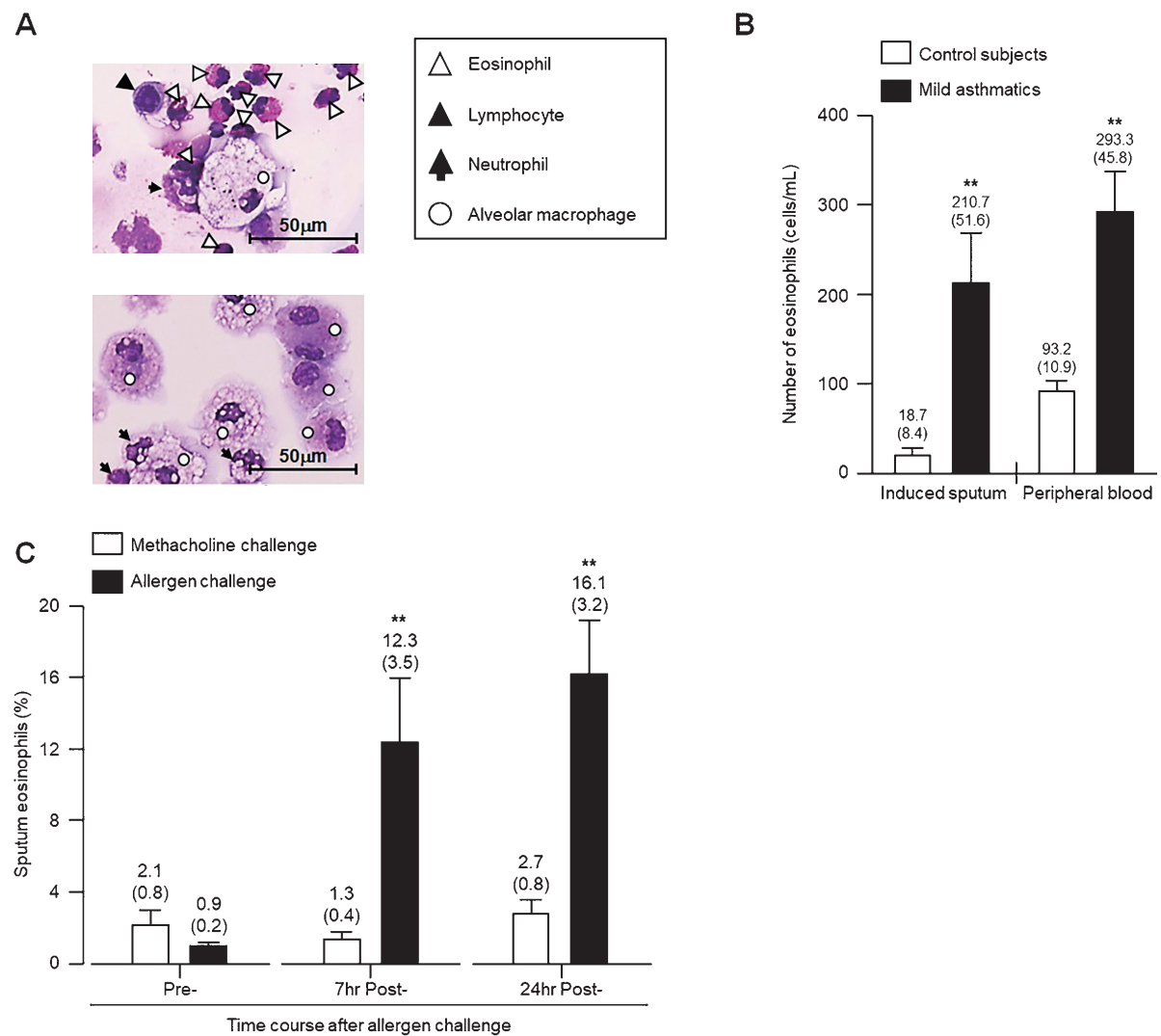


Fig. 1. Induced sputum between asthmatics and control subjects, and sputum eosinophils after methacholine and allergen challenge tests

Notes: A) The findings of induced sputum with May-Giemsa stain ($\times 200$, PZ-9000®, Keyence co., Tokyo) after cytopsin preparation. The sputa were obtained from a stable patient with asthma (32 years old, male, GINA step I) (top) and a control subject (34 years old, male, non-smoker). B) Comparison in the number of sputum eosinophils between stable mild asthma ($n=16$) and control subjects ($n=12$). The tests of inductions of sputum were performed after written consents in each subject at the Respiratory Disease Center, Kurume University School Hospital (A and B) (unpublished data). Data were expressed as mean (Standard error from mean, SEM). ** $p<0.01$ versus healthy subjects. C) Kinetics of number of sputum eosinophils after MCh and allergen challenge [34]. Data were expressed as mean (SEM). ** $p<0.01$ versus pre-challenge.

in FEV_1 (PC_{20}) (Figure 2B) (unpublished data)). However, the airway inflammation as measured by proportion (%) of eosinophils in induced sputum was unchanged after MCh provocation in patients with asthma (Figure 1C) [33].

Allergen challenge tests as human asthma attack models

Patients with atopic, but not non-atopic, mild asthma are divided into 2 phenotypes, namely isolated early responders (IER) and dual responders (DR) [1]. Figure 3 shows the changes in $\%FEV_1$ from baseline

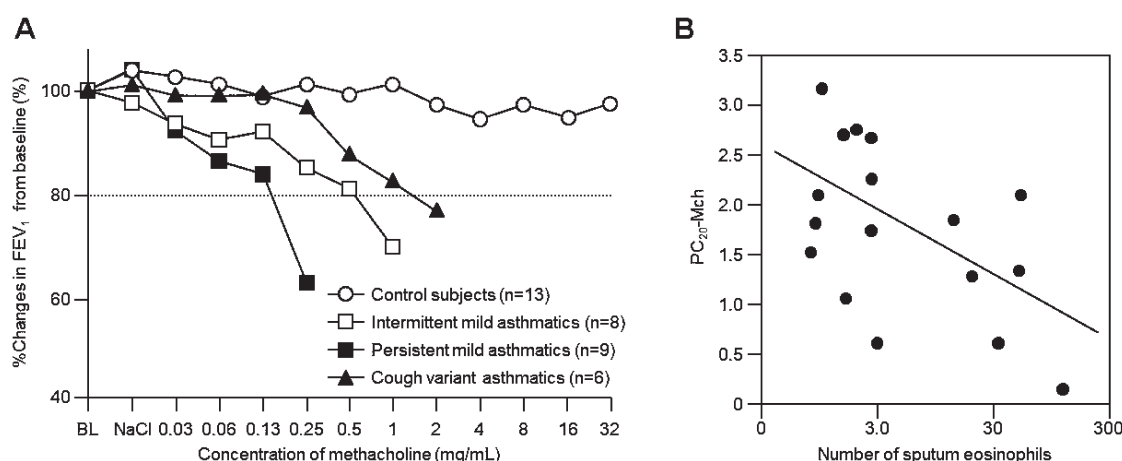


Fig. 2. Airway hyperresponsiveness and sputum eosinophils in patients with asthma
 Notes: A) The mean %changes in FEV₁ were plotted after MCh challenge from baseline (unpublished data). B) The correlation between geometric PC₂₀-MCh values (mg/mL) and number of sputum eosinophils ($\times 10^4$ cells/mL, Log value) at baseline by Spearman tests ($r = -0.53$, $p = 0.029$) (unpublished data). The tests of MCh challenge and inductions of sputum were performed after written consents in each subject at the Respiratory Disease Center, Kurume University School Hospital. Abbreviations: BL, baseline; FEV₁, forced expiratory volume in 1 second; NaCl, 0.7% saline; MCh, methacholine; PC₂₀-MCh, provocative concentration of MCh causing a 20% fall in FEV₁.

after inhaled allergen challenge test and the mean geometric PC₂₀-MCh values pre and post inhaled allergen challenge tests (unpublished data) [38]. Early (EAR) and late asthmatic responses (LAR) occur after inhaled provocation of specific allergens in patients with atopic mild asthma [1, 37]. EAR is shown by the decline of FEV₁ with bronchoconstriction within 30 min after provocation of allergen, and the LAR is defined as bronchoconstriction with recruited airway inflammation 3 hrs after EAR (Figure 3A). The IER have only EAR, whereas the DR have both EAR and LAR [1]. The population of patients with atopic mild asthma is evenly divided between IER and DR [1]. The responses are reproduced just like human asthma attacks after inhaled provocation of allergen in asthmatics with DR (Figure 1C and Figure 3A). Interestingly, the AHR is exacerbated after allergen provocation, as the mean geometric PC₂₀-MCh values are decreased after inhaled allergen challenge (Figure 3A). In accordance with these results, the first line of treatment should focus on prevention of provocation by allergens, rather than medicines, in patients with atopic asthma, when specific allergens associated with exacerbations of asthma are found [36]. For example, the efficacy of inhaled corticosteroids (ICS) [37-39], the main management strategy for asthma, have been investigated using human allergen challenge models in DR (Figure 3C and 3D) [37]. Pre-treatments with ICS, ciclesonide

40 and 80 μ g/day, attenuate LAR (Figure 3C), and also attenuate allergen-induced airway eosinophilic inflammation when compared with placebo (Figure 3D) [37]. However, the effects of ciclesonide are not dose dependent.

Airway regulatory T cells (Tregs) in patients with asthma

The populations of cytotoxic T-lymphocyte-associated protein 4 (CTLA4)⁺, forkhead box P3 (Foxp3)⁺ and CTLA4⁺Foxp3⁺ Tregs on CD25^{high}CD4⁺ T cells in sputum and blood were measured by flow cytometry in patients with adult stable mild asthma [32]. Tregs cell profiles differed between sputum and blood in both patients with mild asthma and control subjects (Figure 4A). The populations of Foxp3⁺ and CTLA4⁺Foxp3⁺, but not CTLA4⁺, Tregs in sputum are significantly lower than those in blood in patients with asthma, whereas all types of Tregs in sputum are significantly higher than those in blood in control subjects (Figure 4A). The populations of sputum Foxp3⁺ and CTLA4⁺Foxp3⁺, but not CTLA4⁺, Tregs in patients with asthma are significantly lower than those in control subjects (Figure 4A). Asthma may be a local airway, but not systemic, disease associated with lower populations of Tregs. In patients with asthma, the population of CTLA4⁺, but not Foxp3⁺, Tregs is associated negatively with sputum eosinophils and AHR

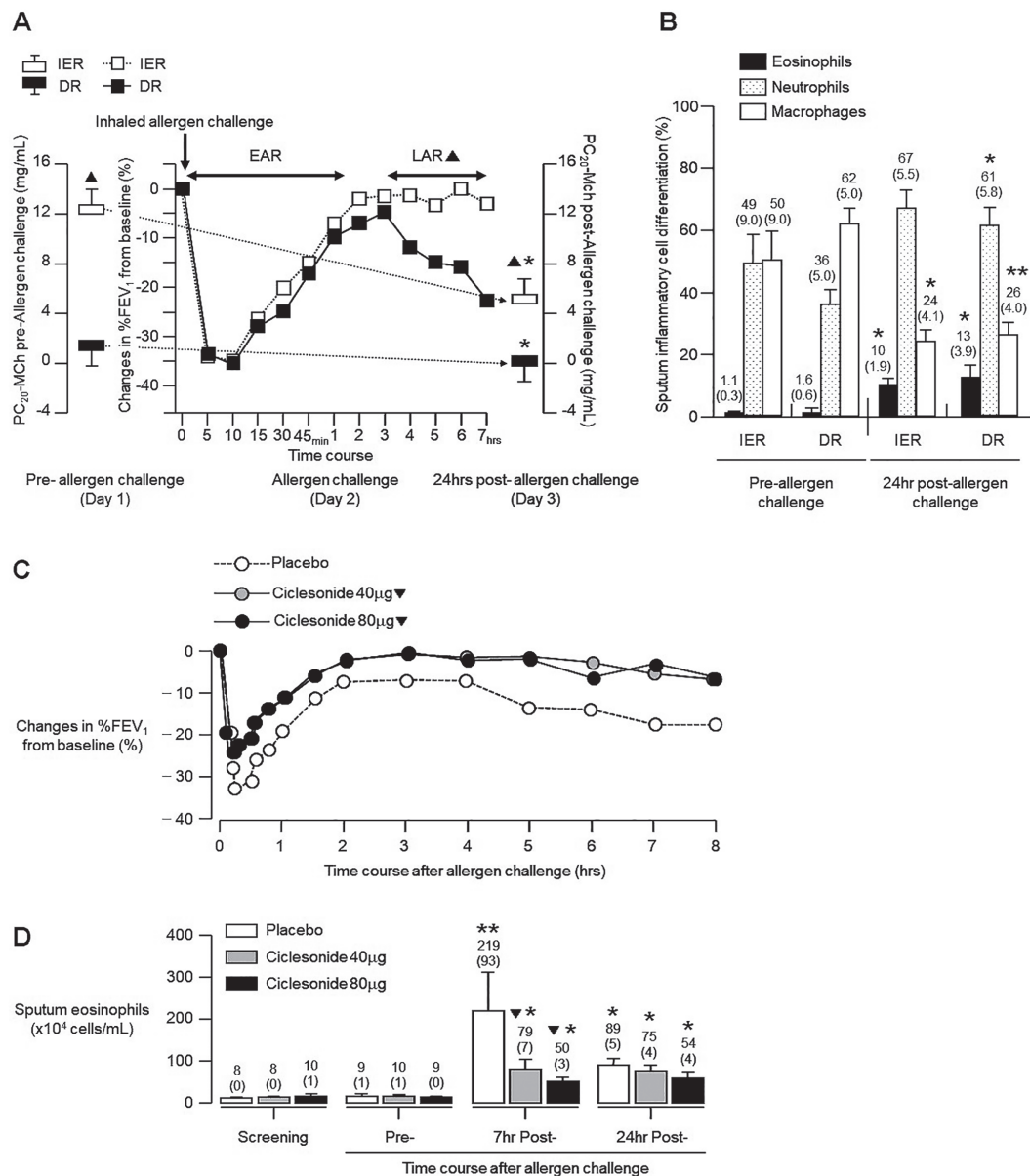


Fig. 3. Allergen induced human asthma attack models and evaluations of inhaled corticosteroids by using allergen challenge tests

Notes: A) Comparison of changes in FEV₁ and mean geometric PC₂₀-MCh values between IER and DR after allergen challenge (Unpublished data were obtained at McMaster University after written consents in each subject) [37]. The geometric PC₂₀-MCh values with both IER and DR were significant decreased after allergen challenge. The areas under the curve (AUC) of LAR with DR was significantly greater than those with IER. $\blacktriangle$ $p < 0.05$ between IER and DR. * $p < 0.05$ between pre- and post-allergen challenge. B) Comparison of sputum inflammatory cell differentiations between IER and DR after allergen challenge [40]. Data were expressed as mean (SEM). Sputum total cell counts (TCC, $\times 10^6$ cells/mL) with IER and DR were increased, but not significantly from pre- to 24hrs post-allergen challenge (both, $p > 0.05$), and there was no significant difference in sputum TCC between IER and DR pre- and post-allergen challenge ($p > 0.05$) (data not shown) [40]. * $p < 0.05$ and ** $p < 0.01$ versus pre-allergen challenge. C) Comparison of AUC of %changes in FEV₁ after allergen challenge among pre-treatments (7days) with ciclesonide 80 μ g/day, ciclesonide 40 μ g/day, and placebo [37]. There was no significant difference in the AUC between ciclesonide 80 μ g/day and ciclesonide 40 μ g/day. $\blacktriangledown$ $p < 0.05$ versus placebo. D) Comparison of AUC of changes in number of sputum eosinophils after allergen challenge among pre-treatments (7days) with ciclesonide 80 μ g/day, ciclesonide 40 μ g/day, and placebo [37]. There was no significant difference in the number of sputum eosinophils between ciclesonide 80 μ g/day and ciclesonide 40 μ g/day. * $p < 0.05$ and ** $p < 0.01$ versus pre-allergen challenge.

Abbreviations: DR, dual responders; EAR, early asthmatic response; FEV₁, forced expiratory volume in 1 second; IER, isolated early responders; LAR, late asthmatic response; MCh, methacholine; PC₂₀-MCh, provocation concentration of MCh causing a 20% fall in FEV₁

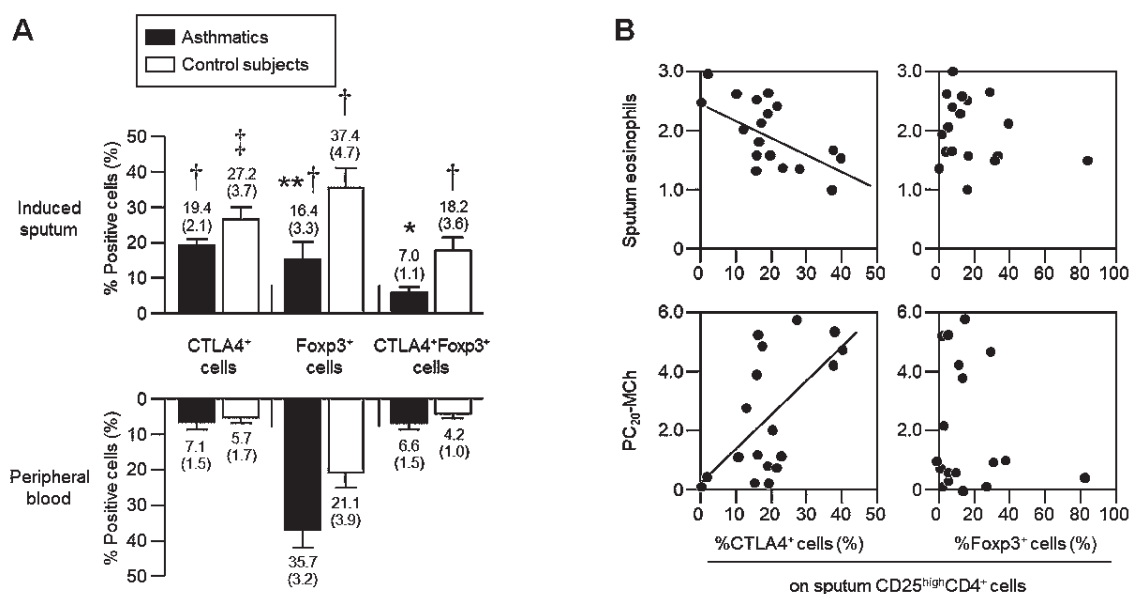


Fig. 4. Airway and peripheral regulatory T cells between asthmatics and control subjects

Notes: A) Comparison of %CTLA4⁺, Foxp3⁺, and CTLA4⁺Foxp3⁺ Tregs in induced sputum and peripheral blood between patients with stable mild asthma and non-asthmatic subjects as controls [42]. Data were expressed as mean (SEM). * $p < 0.05$ and ** $p < 0.01$ versus controls. † $p < 0.05$ and ‡ $p < 0.01$ versus peripheral blood. B) The correlation between sputum %CTLA4⁺ and Foxp3⁺ Tregs, and sputum eosinophils (×10³ cells/mL, Log values) and geometric PC₂₀-MCh values (mg/mL) [42]. Sputum %CTLA4⁺, but not Foxp3⁺, Tregs are significantly associated with sputum eosinophils ($r = -0.60, p = 0.008$) and geometric PC₂₀-MCh values ($r = 0.60, p = 0.009$).

Abbreviations: CTLA4, cytotoxic T-lymphocyte antigen 4; Foxp3, forkhead box P3; PC₂₀-MCh, provocation concentration of methacholine causing a 20% fall in forced expiratory volume in 1 second; SEM, standard error of the mean

(Figure 4B). Taken together, lower Tregs may affect airway inflammation and AHR in asthma.

Kinetics of Tregs after allergen challenge in adult atopic mild asthma

To investigate the kinetics of Tregs, allergen challenge tests were performed in adult atopic mild asthma with IER and DR [40]. The % Tregs (Foxp3⁺/CD4⁺ cells) were significantly decreased in DR, but not IER, after allergen challenge (Figure 5A). The change in Tregs (delta Tregs) from pre- (0hr) to 24 post-allergen challenge (24hrs) was significantly associated with the maximum %fall in FEV₁ during LAR (from 3hrs to 7hrs after allergen challenge) in DR (Figure 5B). The magnitude of the decrease in Tregs induced by the allergen may affect the severity of LAR.

Our results [32, 40] suggest that airway Tregs may be decreased, and that the decreased Tregs may be associated with increased airway inflammation in asthma. This inflammation may contribute to bronchoconstriction. However, the mechanisms of the decrease in Tregs

are still unclear.

Efficacy of ICS on airway Tregs in patients with asthma

A randomized double-blind, cross-over, placebo control trial investigated whether treatment with ICS (Fluticasone propionate, GSK, Japan) could increase the population of sputum CTLA4⁺ Tregs, decrease sputum eosinophils, and increase interleukin (IL)-10 (Figure 6) [41]. The effect of ICS on Foxp3⁺ Tregs has not yet been investigated in patients with asthma.

Natural immunity and acquired immunity in asthma

Immune lymphoid cells 2 (ILC2) are well known as one of the inflammatory cell types in natural immunity [42, 43]. ILC2 may affect the molecular mechanisms of corticosteroid insensitivity in asthma [44]. ILC2, similar to Th2 cells, may play a role in allergic response, ILC1, like NK and Th1 cells, may play a role in autoimmune disorders, and ILC3, like Th17, may be associated with neutrophilic response in ac-

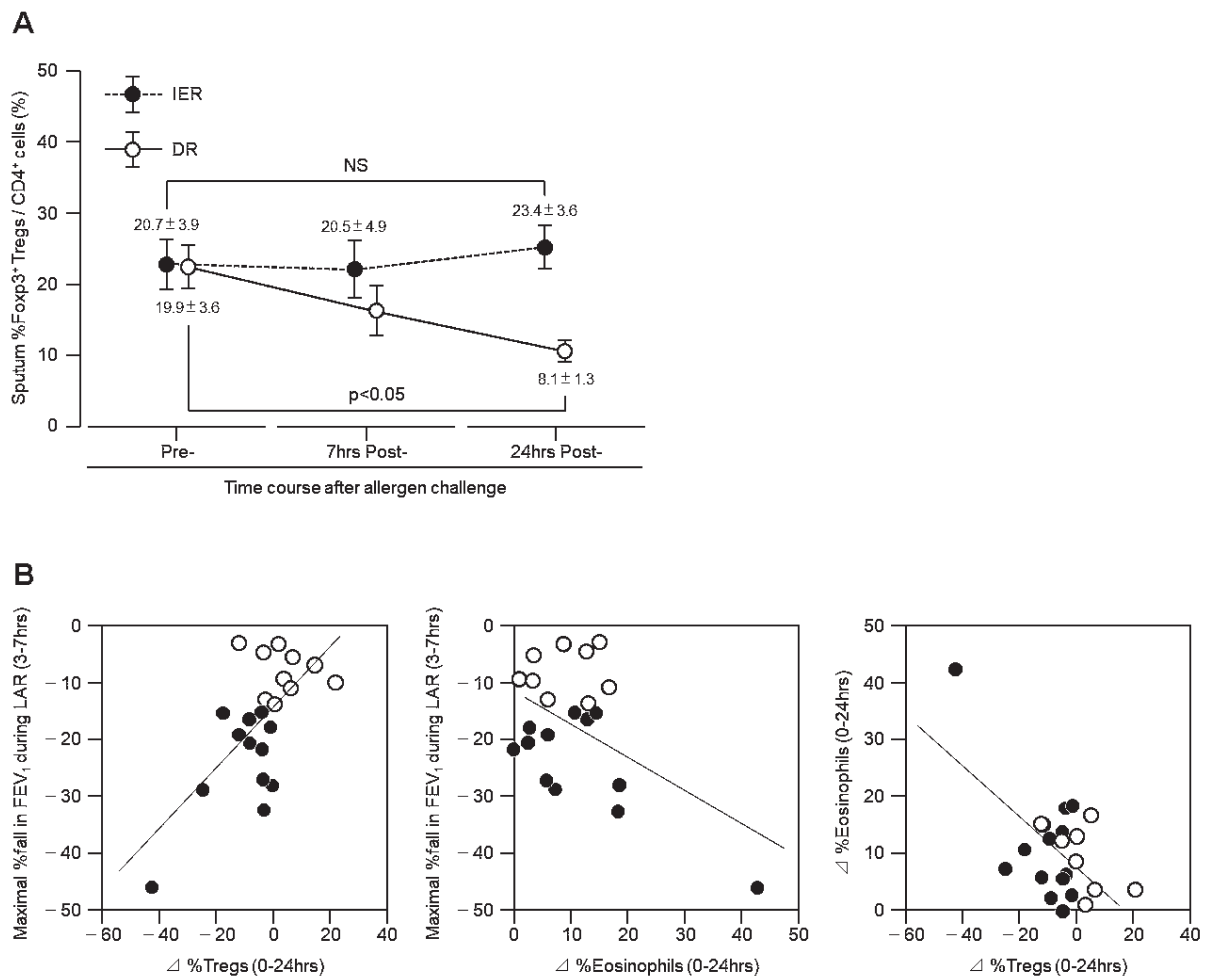


Fig. 5. Kinetics of sputum regulatory T cells in atopic mild asthmatics after allergen challenge tests

Notes: A) Kinetics of sputum %Foxp3⁺ Tregs after allergen challenge in stable atopic mild asthmatic with IER and DR [40]. The %Foxp3⁺ Tregs with DR were significantly lower than those with IER 24hrs post-allergen challenge ($p < 0.05$). Data were expressed as mean $\pm$ SEM. B) The correlation between sputum %Tregs, sputum %eosinophils, and LAR (including unpublished data) [40]. The correlation (r) between delta %sputum Tregs (0-24hrs) and %maximal %fall in FEV₁ during LAR (3-7hrs) was 0.65 ($p = 0.0011$). The correlation between delta %sputum eosinophils (0-24hrs) and %maximal %fall in FEV₁ during LAR (3-7hrs) was -0.52 ($p = 0.0150$) (unpublished data). The correlation between delta % sputum Tregs (0-24hrs) and delta %sputum eosinophils (0-24hrs) was -0.59 ($p = 0.0047$) (unpublished data). Data of DR and IER were expressed as closed and opened circles, respectively.

Abbreviations: DR, dual responders; IER, isolated early responder; FEV₁, forced expiratory volume in 1 second; Foxp3, forkhead box P3; LAR, late asthmatic response; NS, not significant; Tregs, regulatory T cells; SEM, standard error of the mean

quired immunity (Figure 7) [45, 46]. However, Tregs-like cells working in the innate immune system have not been found, whereas Tregs expressing Foxp3⁺ and CTLA4⁺ CD4 are able to modulate inflammation as an acquired immune response.

Conclusion

The interactions between airway inflammation and AHR in asthma were examined. ICS as anti-in-

flammatory agents are the first choice for management of asthma, but they are just controllers. Curing asthma is still difficult, although anti-IgE and anti-IL-5 therapies have shown results in severe asthma that are as good as other anti-allergic inflammatory agents [47-49]. Development of new treatment strategies utilizing Tregs could be useful in attenuating allergic reactions and achieving a cure for asthma.

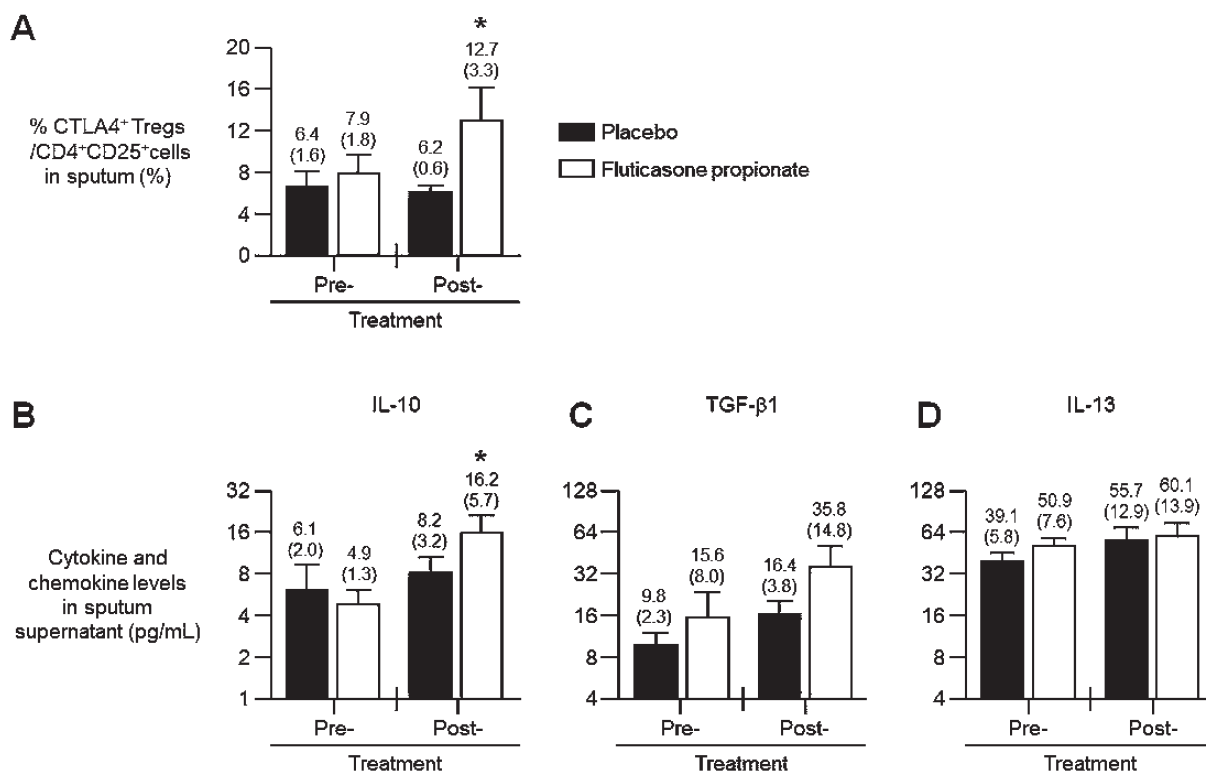


Fig. 6. Sputum regulatory T cells in mild asthmatics after treatment with inhaled corticosteroids

Notes: A) Comparison of changes in sputum %CTLA4⁺ Tregs between ICS and placebo [41]. Data were expressed as mean (SEM). * $p < 0.05$ versus pre-treatment. B) Comparison of changes in IL-10, TGF- β 1 and IL-13 levels of sputum supernatants between ICS and placebo [41]. Data were expressed as mean (SEM). * $p < 0.05$ versus pre-treatment

Abbreviations: CTLA4, cytotoxic T-lymphocyte antigen 4; ICS, inhaled corticosteroids; IL, interleukin; SEM, standard error of the mean; TGF, Transforming growth factor; Tregs, regulatory T cells

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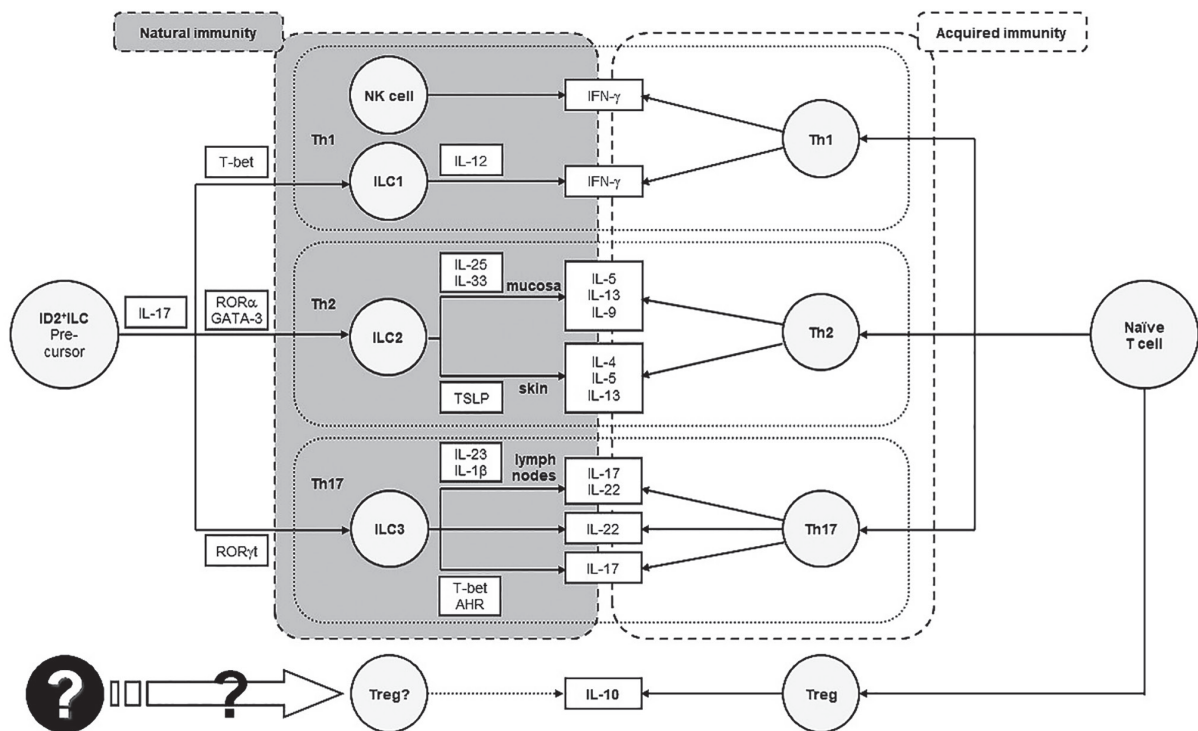


Fig. 7. Inflammatory and regulatory cells in natural and acquired immunity

Notes: In natural immunity, ILC1, ILC2, and ILC3 are differentiated from pre-cursor cells (ID2⁺ILC) via IL-17 by T-bet, ROR α and GATA-3, and ROR γ t, respectively. The ILC2 and ILC3 have 2types in each. The profiles of cytokine productions are difference between mucosa and skin ILC2, and lymph nodes and other ILC3. In acquired immunity, Th1, Th2, Th17 and Tregs are differentiated from naïve CD4⁺ T cells. The pre-cursors of Tregs-like cells are still unknown.

Abbreviations: AHR, aryl hydrocarbon receptor; GATA, GATA 'zinc-finger' transcription factors; ID, DNA-binding protein inhibitor; IFN, interferon; IL, interleukin; ILC, immune lymphoid cells; NK, natural killer cells; ROR, retinoic acid receptor-related orphan receptor; T-bet, Th1-specific T box transcription factor; TSLP, thymic stromal lymphopoietin; Th, helper T; Tregs, regulatory T cells

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