

The DNA Methylation Inhibitor 5-Azacytidine Increases Regulatory T Cells and Alleviates Airway Inflammation in Ovalbumin-Sensitized Mice

Cheng-Jang Wu^a Chin-Yu Yang^a Yi-Hsin Chen^a Chun-Ming Chen^a
Li-Chen Chen^c Ming-Ling Kuo^{a,b}

^aGraduate Institute of Biomedical Sciences and ^bDepartment of Microbiology and Immunology, College of Medicine, Chang Gung University, and ^cDivision of Allergy, Asthma, and Rheumatology, Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ROC

Key Words

Asthma • Airway inflammation • TH2 responses •
5-Azacytidine • T regulatory cells • CD25+ cells

Abstract

Background: Asthma is characterized as a chronic inflammatory disorder of the airways associated with an enhanced TH2 response to inhaled allergens. CD4+ T regulatory (Treg) cells are controlled by the master transcription factor FoxP3 and strictly maintain peripheral immunotolerance. Epigenetic regulation of FoxP3 by DNA methyltransferase inhibitors, such as 5-azacytidine (Aza), can generate a steady supply of functional Treg cells. Therefore, we propose that Aza can augment Treg cells in vivo to prevent the pathogenesis of asthma. **Methods:** BALB/c mice were sensitized with chicken ovalbumin (OVA) and treated with different doses of Aza. Airway hyperresponsiveness to methacholine, eosinophilia in bronchoalveolar lavage fluid, circulating titers of OVA-specific IgG1 and IgE, and stimulating levels of TH2 cytokines from splenocytes were then determined. Cellular populations were examined by flow cytometry. PC61 antibody, which depletes CD25+ cells, was used to verify the role of CD25+ cells in Aza-induced tolerance. **Results:** Administra-

tion of Aza to OVA-sensitized mice diminished airway hyperactivity, pulmonary eosinophilia, levels of OVA-specific IgG1 and IgE in serum, and secretion of TH2 cytokines from OVA-stimulated splenocytes in a dose-dependent manner. Percentages of CD25+ and FoxP3+ cells in the CD4+ cell population were notably increased in Aza-treated mice compared to sensitized control mice. Furthermore, the major symptoms of asthma were exacerbated by depleting CD25+ cells in Aza-treated mice. **Conclusions:** Aza may have applications as a novel clinical strategy to increase the production of Treg cells in order to modulate the airway inflammation associated with asthma.

Copyright © 2012 S. Karger AG, Basel

Introduction

Asthma is a chronic inflammatory disease of the airways [1]; it has emerged as a worldwide public health problem that is increasing in incidence, morbidity, and mortality [2]. Asthmatic patients are characterized by hyperactivity of the airways and infiltration of inflammatory cells. It is known that TH2 cells are responsible for the pathogenesis of asthma [3]. TH2 cytokines, such as

IL-4, IL-5, and IL-13, promote allergen-specific IgE production [4, 5] and eosinophil differentiation [6] and further enhance the severity of asthma.

During asthma attacks, medication is required to alleviate the symptoms. Medical therapies are divided into two main classes: drugs that are taken daily for long periods of time to decrease the frequency of asthma attacks, and quick-relief drugs that are taken to relieve acute airflow obstruction [7]. None of the currently available therapies can cure asthma or completely prevent asthma symptoms. Recently, several novel therapies have been proposed, including anti-IgE, anti-TH2 cytokines, and regulation of IFN- γ . Modulation of regulatory T (Treg) cells is another potential therapy for asthma [8, 9].

Treg cells, a subset of CD4⁺ T lymphocytes that have suppressive activity, play an essential role in controlling immune responses [10, 11]. Treg cells either occur naturally in the thymus or are adaptively induced in the periphery following specific tolerogenic stimulation [12]. Natural Treg cells constitute 5–10% of peripheral CD4⁺ T cells and can be characterized on the basis of high levels of CD25 expression [13]. Recently, the forkhead winged-helix transcription factor FoxP3 was shown to be specifically expressed by natural Treg cells and to be critical for their homeostasis and function [14]. It was also reported recently that the DNA methylation status of CpG islands in the *foxp3* promoter region is important for FoxP3 expression [15] and discriminates Treg cells from activated T cells [16].

In 2004, the US Food and Drug Administration (FDA) approved a chemical analogue of cytidine, 5-azacytidine (Aza), for the treatment of all subtypes of myelodysplastic syndrome (MDS) [17]. In the absence of exogenous TGF- β , Aza induces and stabilizes FoxP3 expression. In addition, adoptive transfer of TGF- β -induced T cells plus 5-aza-2'-deoxycytidine may prolong the survival of diabetic mice with pancreatic islet allografts [18]. These studies indicate that Aza may induce Treg cell development. However, the ability of this agent to expand Treg cells in vivo to prevent the development of asthma remains to be verified.

The aim of the present study was to investigate whether Aza can induce Treg cells in vivo and prevent asthmatic responses. We first examined ovalbumin (OVA)-induced asthmatic responses in mice after they were treated with Aza. We also determined the proportion of CD4⁺CD25⁺ cells in Aza-treated mice and investigated the role of CD4⁺CD25⁺ cells in Aza-induced tolerance by applying an antibody to deplete CD25⁺ cells.

Materials and Methods

Animals

Female BALB/cByJNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). They were maintained and handled according to guidelines set by the Animal Care Committee of Chang Gung University (Taiwan) and the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Mouse Sensitization and Aza or Antibody Treatment

Mice aged 8 weeks were sensitized by intraperitoneal injection with 50 μ g of chicken OVA (Sigma-Aldrich, Saint Louis, Mo., USA) emulsified with 0.8 mg of aluminum hydroxide gel (Pierce Biotechnology, Rockford, Ill., USA) in a total volume of 200 μ l on days 1, 2, 3, and 14. Mice were challenged on days 14, 17, 20, 23, and 26 by inhaling 2% OVA in normal saline using an atomizer (Pulmo-Aide; DeVilbiss Health Care, Somerset, Pa., USA) for 20 min. Control mice were sensitized and challenged only with normal saline. Mice were analyzed for airway hyperresponsiveness (AHR) on day 27 and sacrificed on day 28. During the OVA sensitization periods, various concentrations of Aza (Sigma-Aldrich) in PBS were administered to the mice on days 1, 4, 7, 10, 14, 17, 20, 23, and 26 before they were challenged with OVA. In the CD25⁺ cell-depleted experiment, mice that received only 7 doses of 100 μ g Aza did not receive the last 2 doses administered to the 9-dose mouse group. Control mice received PBS alone as the control. In order to remove CD25⁺ cells, mice received 0.25 mg/ml anti-CD25 antibody on day 13 (clone PC61; Biolegend, San Diego, Calif., USA). Sensitized control and Aza-treated mice were injected with control rat IgG (Sigma-Aldrich). None of the mice exhibited any overt signs of illness during the experiment.

Airway Hyperresponsiveness

AHR was measured on day 27 with inhaled methacholine (Mch) (Sigma-Aldrich) stimulation to induce airway constriction. The mice were first exposed to no Mch, and then to cumulatively increasing concentrations of Mch (10, 20, 30, and 40 mg/ml) for 3 min per concentration. A transducer in each chamber detected changes in the respiratory airflow of each mouse. The pulmonary airway obstruction was calculated based on the formula $Penh = (Te/RT - 1) \times (PEF/PIF)$, where *Penh* is the enhanced pause (a dimensionless value), *Te* is the expiratory time, *RT* is the relaxation time, *PEF* is the peak expiratory flow (ml/s), and *PIF* is the peak inspiratory flow (ml/s). The changes were analyzed by BioSystem XA software (all instruments were from Buxco Electronics, Wilmington, N.C., USA).

Cells in Bronchoalveolar Lavage Fluid and Lung Histology

The lungs of the mice were lavaged 3 times with 1 ml of normal saline. The bronchoalveolar lavage fluid (BALF) cells were centrifuged onto poly-L-lysine-coated slides at 800 rpm for 5 min in a Shandon Cytospin 4 (Thermo Shandon, Pittsburgh, Pa., USA). The cells were then stained with LiuA dye for 30 s and LiuB dye for 90 s. Two hundred cells per slide were counted under a microscope (Olympus, Tokyo, Japan), and those with different morphology were noted. Lungs were isolated, fixed in 10% formaldehyde-PBS, and embedded in paraffin for histology. Lung sections were stained with hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) stain for assessment of pulmonary inflammation and goblet cell hyperplasia, respectively.

Determination of OVA-Specific IgG1, IgG2a, and IgE

OVA-specific IgG1 and IgG2a antibody titers in sera were determined by ELISA as described previously [19]. A previous protocol for the measurement of OVA-specific IgE levels was followed with modifications [20]. Briefly, wells in ELISA plates were coated with 3 µg/ml purified anti-IgE (BioLegend). After blocking, serum samples were diluted 1:5, and standard mouse IgE (anti-TNP, BioLegend) was added at concentrations of 3.125–100 ng/ml. Biotinylated TNP-OVA (Upstate, Lake Placid, N.Y., USA) was subsequently added to the wells, followed by avidin-conjugated horseradish peroxidase (BD Biosciences, San Jose, Calif., USA). Finally, 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (BD Biosciences) was added, and the reaction was stopped with 2 N H₂SO₄. The concentrations of antibodies were determined according to the standard curve.

Cytokine Production by OVA-Stimulated Splenocytes

Splenocytes were stimulated *in vitro* with 100 µg/ml OVA at a density of 5×10^6 cells/ml in RPMI 1640 medium (Gibco-BRL) with 10% fetal calf serum for 6 days. Concentrations of cytokines (IL-4, IL-5, IL-10, IL-13, and IFN-γ) in culture supernatants were determined with ELISA kits specific to each cytokine (IL-5 from BD Biosciences; others from R&D Systems, Minneapolis, Minn., USA). Cytokine concentrations were calculated based on standards run in parallel with recombinant cytokines. The limits of detection were 15.6 pg/ml for IL-4, IL-5, and IL-10, 31.3 pg/ml for IFN-γ, and 39 pg/ml for IL-13.

Cytometric Analysis

Cytometric analysis was performed with a BD FACSCalibur instrument and CellQuest (BD Biosciences) software. Cells harvested from mediastinal lymph nodes (LN), spleen, or lung were stained with antibodies in PBS for 20 min at 4°C and washed with 2 ml of PBS. For intracellular FoxP3 staining, cells were stained with anti-CD4-FITC, then fixed and permeabilized with fixation and permeabilization buffers (eBioscience, San Diego, Calif., USA) according to the manufacturer's instructions, and then incubated at 4°C for 30 min with anti-FoxP3-APC or isotype control antibody. Anti-CD4-FITC (clone: GK1.5) and CD25-PE (clone: PC61) monoclonal antibodies (mAbs) were purchased from BioLegend; anti-CD8-PE (clone: 53-6.7), anti-B220-FITC (clone: RA3-6B2), anti-CD3-PE (clone: 145-2C11), anti-FoxP3-APC (clone: FJK-16s), and rat IgG2a-APC (clone: eBR2a) mAbs were purchased from eBioscience.

Statistical Analysis

Data were analyzed using the Mann-Whitney test. Results were expressed as means ± standard error of the mean (SEM). $p < 0.05$ was considered to represent significant differences between groups.

Results

Aza Ameliorates OVA-Induced Airway Inflammation

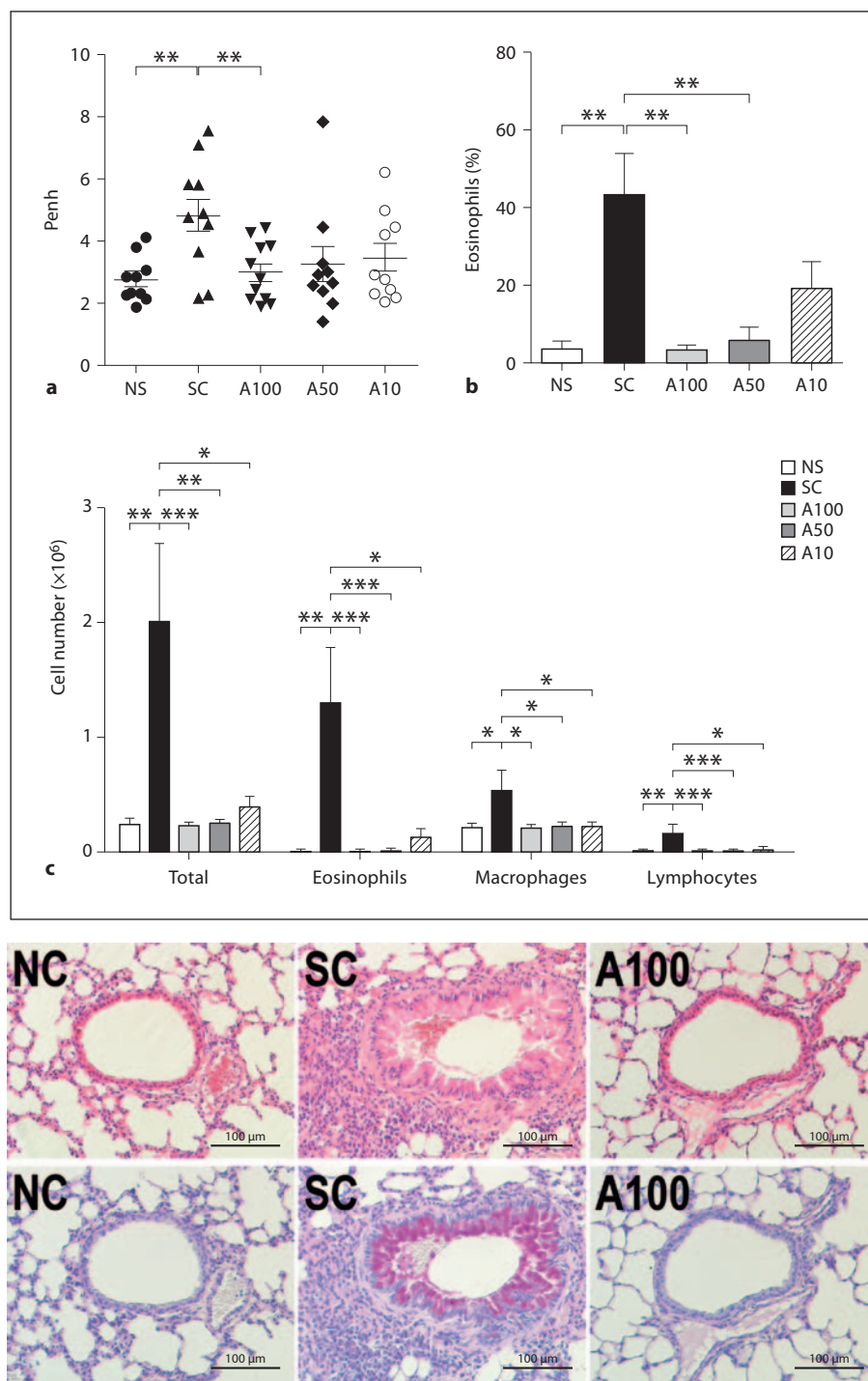
To test whether Aza is able to attenuate the development of OVA-induced allergic responses, and to determine effective concentrations of Aza, each of three groups

of mice received a different dose of Aza, i.e. 10, 50, or 100 µg/dose for the A10, A50, and A100 groups, respectively. The doses of Aza were administered by intraperitoneal injection twice a week a total of 9 times during the OVA sensitization period. After 5 challenges with OVA, the mice were stimulated by inhaling different concentrations of Mch. The Penh value was calculated to determine the extent of pulmonary airway obstruction. The mean AHR of the A100 group was significantly lower than that of the saline-treated OVA-sensitized control (SC) mouse group at 30 mg/ml Mch stimulation (fig. 1a). In addition, the percentages and numbers of eosinophils in BALF were decreased in the Aza-treated groups in a dose-dependent manner (fig. 1b, c). The total number of cells in BALF was also significantly lower in Aza-treated mice compared to SC mice (fig. 1c). The decrease in pulmonary inflammation in Aza-treated mice was also confirmed histologically with HE and PAS staining of lung tissues (fig. 1d, e). These results indicate that Aza may prevent pulmonary inflammation.

Aza Inhibits OVA-Specific Humoral and TH2 Cellular Responses

We next investigated whether Aza treatment is associated with reduced levels of OVA-specific IgG1 and IgE. The levels of OVA-specific antibody were undetectable in the nonsensitized (NS) control group (fig. 2a–c). The level of OVA-IgG1 (fig. 2a) was significantly different in the A100 and A50 groups compared to the SC group. Furthermore, the levels of OVA-specific IgE, a definitive marker for asthma, were also lower in the A100 and A50 groups compared to the SC group (fig. 2c). These results indicate that Aza inhibits OVA-specific antibody responses in a dose-dependent manner.

Because Aza was associated with lower levels of OVA-specific TH2-type antibodies, we also examined whether Aza could decrease TH2 cytokine production. The supernatants from OVA-activated splenocytes were collected, and concentrations of cytokines were determined with ELISA (fig. 2d–g). Compared to SC mice, lower levels of TH2 cytokines, including IL-4, IL-5, and IL-13, were detected in Aza-injected mice in a dose-dependent manner. It is noteworthy that suppressive cytokine IL-10, which is originally thought to be produced by TH2 cells, was also inhibited by Aza treatment (fig. 2d–g). IFN-γ, a cardinal TH1 cytokine, was not detectable in any of the groups. Cytokines in BALF were low or not detectable in our model (data not shown). The above results suggest that Aza treatment can suppress the TH2 response.



Percentage of CD25+ T Cells in the CD4+ T Cell Population Is Increased after Aza Treatment

To determine whether Aza influences the population of lymphocytes, we used flow cytometry to measure the percentage of lymphocytes, including T cells, B cells,

CD4+ cells, CD8+ cells, CD25+ cells, and CD4+FoxP3+ cells, in mediastinal LN or spleen. The proportion of cell populations in secondary lymphoid organs did not change with treatment, except for Treg cells. Compared to the SC group, the percentage of CD4+CD25+FoxP3+

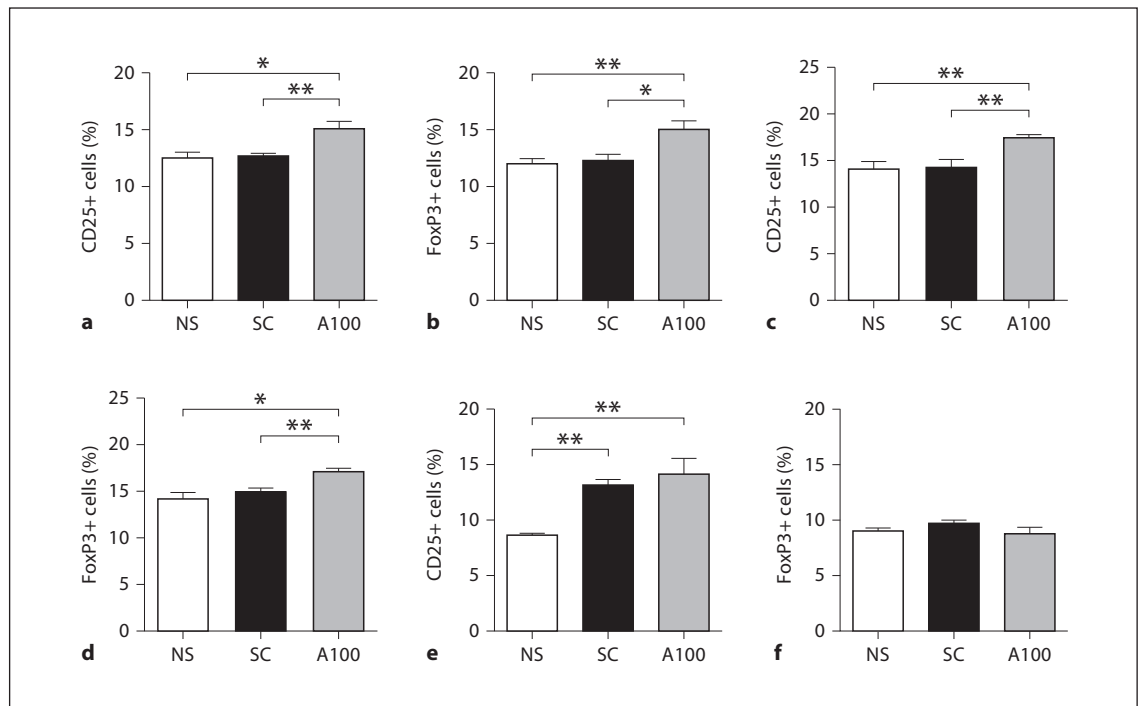


Fig. 3. Proportions of CD25+ or FoxP3+ cells in the CD4+ cell population were increased in the periphery in Aza-treated mice. Cells from mice in figure 1 were stained with anti-CD4-FITC/anti-CD25-PE or anti-CD4-FITC/anti-FoxP3-APC and then analyzed by flow cytometry. Percentages of CD4+CD25+ and CD4+FoxP3+ within the CD4+ cell population in mediastinal LN (**a, b**), spleen (**c, d**), and lung (**e, f**) are shown. Data are presented as means $\pm$ SEM (n = 6–15) from 3 experiments. * $p < 0.05$; ** $p < 0.01$.

in the levels of OVA-specific IgG1 and IgG2a between the 7A and 7AP groups (data not shown). Compared to the 7A group, TH2 cytokines were higher in the 7AP group (fig. 4g–i). Collectively, our findings point to a critical role for CD4+CD25+ T cells in Aza-induced tolerance.

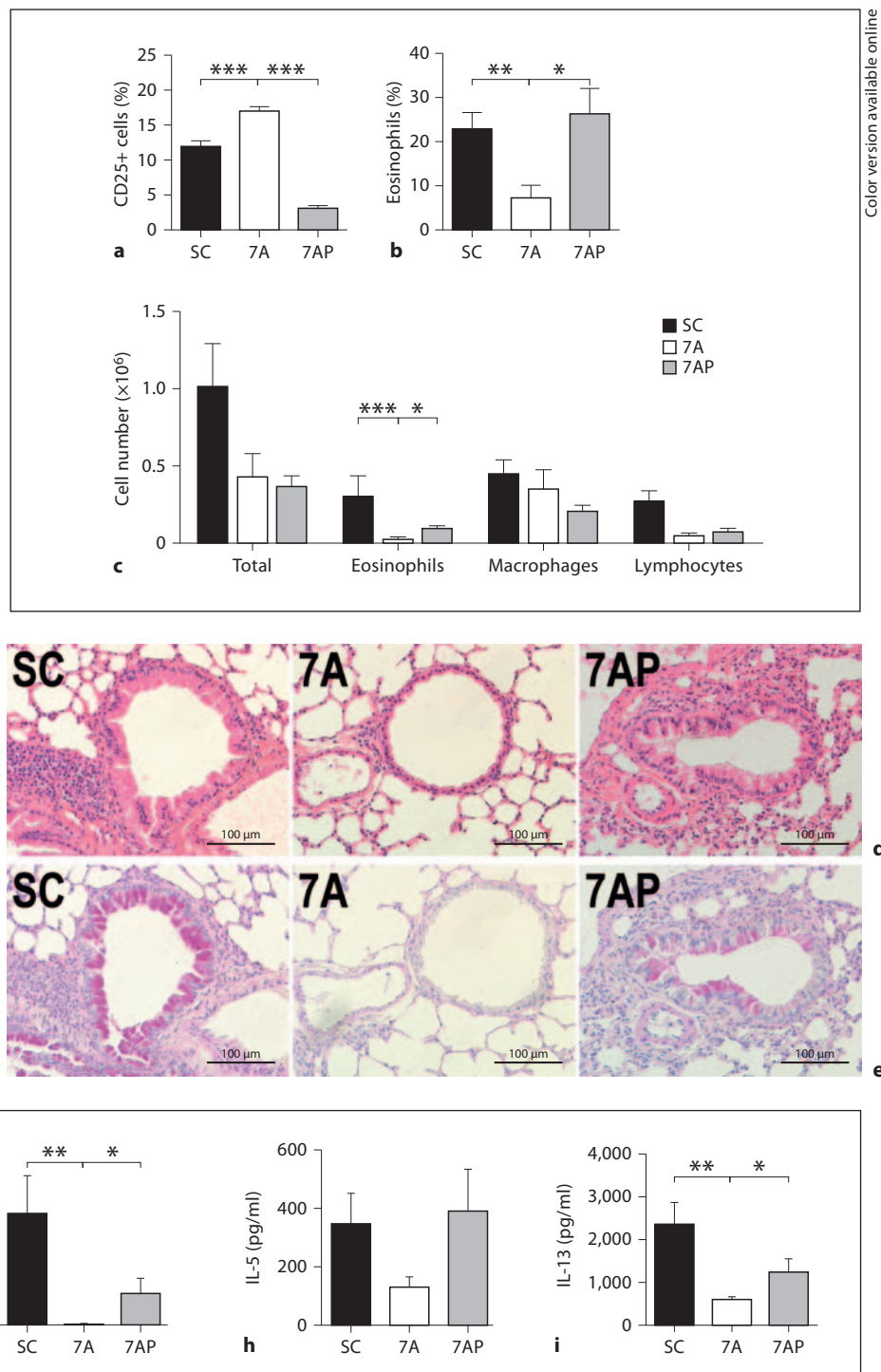
Discussion

In this study, we investigated whether different doses of Aza, administered at the beginning of OVA sensitization in a mouse asthma model, could prevent the development of asthma. We found that, compared to OVA-sensitized mice that were not treated with Aza, Aza treatment was associated with a lower AHR Penh value, less eosinophil infiltration in the lung, lower OVA-specific IgG1 and IgE levels in serum, and lower levels of TH2 cytokines (i.e. IL-4, IL-5, and IL-13). All of these changes were dose dependent. Changes in the percentage of types of leukocytes were also examined with flow cytometry. The data showed that the percentage of CD25+ and FoxP3+ cells

in the CD4+ cell population was significantly increased in Aza-treated animals. However, Aza has been reported to cause an autoimmune disease with lupus-like symptoms [23]. To improve the modulation of asthma symptoms without inducing autoimmunity, we treated the asthmatic mice with 100- μ g doses of Aza as previous described. After 6 months, we observed that there were no pathological changes in liver and kidney histological sections (data not shown).

We next addressed whether Aza can induce CD4+CD25+ Treg in vivo and modulate the symptoms of asthma. Not all of the symptoms of asthma were restored in 9AP mice (9 doses of Aza), which were treated with Aza and injected with anti-CD25 antibody to deplete CD25+ cells. Only the levels of serum OVA-specific IgE, the secretion of IL-4, and the proliferation of splenocytes were significantly reversed. We also injected anti-CD25 antibody into OVA-sensitized mice that had received Aza doses a total of 7 times during the OVA sensitization period, missing the last 2 doses administered to the 9AP mice. The symptoms of asthma were significantly re-

Fig. 4. Asthmatic responses were ameliorated in CD25+ cell-depleted mice. **a** The percentage of CD25+ cells in the CD4+ cell population was reduced after PC61 injection. OVA-sensitized and OVA-challenged mice were treated with 7 doses of 100 μ g Aza (7A) or normal saline as an SC. To remove CD25+ cells, mice received 0.25 mg/ml PC61 antibody on day 13 (7AP). Splenocytes were stained with anti-CD4-FITC/anti-CD25-PE and analyzed by flow cytometry. Percentages (**b**) and numbers (**c**) of eosinophils in BALF were determined as previously described. Depletion of CD25+ cells restored asthmatic response in Aza-treated mice. Histological sections of lung tissue were stained with HE (**d**) and PAS (**e**) staining. Scale bars = 100 μ m. Aza-treated and CD25+ cell-depleted mice had restored inflammatory infiltrate and goblet cell hyperplasia. The levels of OVA-specific IgE in sera (**f**) and TH2 cytokines from OVA-stimulated splenocytes (**g-i**) were significantly higher in the 7AP group compared to the 7A group. Results were determined as in previous experiments and are representative of 3 independent experiments ($n = 4-5$ mice per group). Data are presented as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



versed, especially the percentage of eosinophil infiltration (fig. 4). The percentage of CD4+CD25+ cells was 7% in the 9AP group, but in the 7AP group only 3% of CD4+ cells were CD25+. In the 9AP group, Aza doses were administered up until day 26, two days before sacrifice, in-

ducing new CD4+CD25+ cell formation. It is possible that the most recently formed CD4+CD25+ cells comprised the additional 4% of CD25+ cells seen in the 9AP group, and there was not enough time for the anti-CD25 antibody to completely deplete these CD25+ cells prior to

sacrifice. This might inhibit the expression of IL-5 and IL-13 and also influence the maturation and recruitment of eosinophils. In addition, asthma symptoms were still inhibited in the 7A group, similar to what was observed in the 9A group. This indicates that the effect of Aza can be sustained when OVA-sensitized mice have been subjected to continuing challenge with OVA.

A recent publication shows that pretreatment of 5-Aza-2'-deoxycytidine 2 weeks before sensitization prevents the alleviation of airway inflammation in a similar experimental asthma model [24]. Inhibition of DNA methylation at the IFNG promoter locus by Aza changes the TH1/TH2 cytokine production and then inhibits the TH2 response. However, it has been demonstrated that activated T cells exposed to the 5-Aza-2'-deoxycytidine downregulate *IFN* gene expression [25]. In our model, Aza treatment is conducted prior to sensitization on the same day and throughout the sensitization. IFN- γ was barely detected in any of the treatments, and the titer of OVA-specific IgG2a, a TH1 cytokine-regulated isotype, was greatly decreased in mice treated with Aza. Our findings provide evidence that Treg cells play a role in Aza-induced tolerance.

In recent years, Treg cells were suggested to be key modulators that could prevent allergic sensitization and progression to established allergic diseases [26, 27]. Treg cells are required to maintain immune homeostasis and self-immunological tolerance. Depleting Treg cells leads to the development of autoimmune diseases such as gastritis and thyroiditis [28] and promotes antigen-induced TH2 cell differentiation [29]. Thus, increasing the number of Treg cells in vivo could be a means of preventing or modulating the symptoms of asthma [26, 30]. Endogenous [31] and adoptive transfer [30] of Treg cells can resolve allergic airway inflammation; however, the role of IL-10 is still controversial. IL-10 was elicited in our asthmatic model, and its secretion was suppressed by Aza treatment (fig. 2f). In addition, TGF- β was barely detected in Aza-treated animals in our experiments. Taken together, these findings suggest that Aza treatment might augment the effectiveness of natural Treg cells.

FoxP3 is the most important transcription factor for the development and function of natural Treg cells [32]. Recent studies have focused on epigenetic regulation of the *foxp3* gene. The first intron and the upstream enhancer of the mouse *foxp3* locus contain CpG islands [15, 18]. Methylation at these sites counteracts the binding of CREB/ATF and recruits MeCP2, MBD2, DNMT1, and DNMT3b to inhibit FoxP3 expression [18]. The DNA methyltransferase inhibitor Aza has been shown to in-

duce and stabilize FoxP3 expression, and it was approved by the FDA for the treatment of all subtypes of MDS [17]. Based on these reasons, we hypothesized that Aza might prevent the development of asthma by increasing the percentage of Treg cells in vivo.

Zheng et al. [33] found considerable alterations in the cellularity and composition of thymocytes in mice treated with 5-aza-2'-deoxycytidine. We found similar changes 1 day after a 100- μ g dose of Aza [unpubl. data]. The size of the thymus, as well as the number of cells in the thymus, were substantially smaller than in untreated mice. In addition, the frequency of CD4+CD8+ T cells in the thymus was lower, and the percentages of single-positive and double-negative T cells were conversely increased. However, the proportion of thymocytes that underwent apoptosis was similar among the 4 populations and between Aza-treated and control mice. Most importantly, higher frequencies of CD25+ and FoxP3+ cells in the CD4+ population were also observed in the thymi of Aza-treated mice. The significance of CCR5 in Treg egress from the thymus was addressed previously [34]; its expression may be modulated by epigenetic regulation [35]. The role of CCR5 in the changes observed in the thymi of Aza-treated mice should be the subject of further investigation.

In summary, our data demonstrate that Aza treatment efficiently suppresses the symptoms of asthma and increases the number of CD4+CD25+ Treg cells in vivo. Furthermore, when CD25+ cells were depleted in vivo in Aza-treated mice, the majority of asthma symptoms were present. Therefore, Aza, which is already used to treat MDS, should be investigated as a novel strategy to increase the production of Treg cells and modulate allergic diseases.

Acknowledgements

We would like to thank Wen-Lin Chen for technical assistance. This study was supported by grants from National Science Council (NSC95-2320-B-182-043-MY3 and NSC98-2320-B-182-030-MY3), and Chang Gung Memorial Hospital (CMRPD190512 and CMRPD1A0171), Taiwan.

References

- Akdis CA: Allergy and hypersensitivity: mechanisms of allergic disease. *Curr Opin Immunol* 2006;18:718–726.
- Beasley R, Crane J, Lai CK, Pearce N: Prevalence and etiology of asthma. *J Allergy Clin Immunol* 2000;105:S466–S472.
- Busse WW, Lemanske RF Jr: Asthma. *N Engl J Med* 2001;344:350–362.
- Romagnani S: The Th1/Th2 paradigm. *Immunol Today* 1997;18:263–266.
- McKenzie AN: Regulation of T helper type 2 cell immunity by interleukin-4 and interleukin-13. *Pharmacol Ther* 2000;88:143–151.
- Nakajima H, Takatsu K: Role of cytokines in allergic airway inflammation. *Int Arch Allergy Immunol* 2007;142:265–273.
- Chu EK, Drazen JM: Asthma: One hundred years of treatment and onward. *Am J Respir Crit Care Med* 2005;171:1202–1208.
- Holgate ST, Polosa R: Treatment strategies for allergy and asthma. *Nat Rev* 2008;8:218–230.
- Ryanna K, Stratigou V, Safinia N, Hawrylowicz C: Regulatory T cells in bronchial asthma. *Allergy* 2009;64:335–347.
- Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T: Immunologic tolerance maintained by CD25+CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001;182:18–32.
- Xystrakis E, Boswell SE, Hawrylowicz CM: T regulatory cells and the control of allergic disease. *Expert Opin Biol Ther* 2006;6:121–133.
- O'Garra A, Vieira P: Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004;10:801–805.
- Sakaguchi S: Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Ann Rev Immunol* 2004;22:531–562.
- Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057–1061.
- Kim HP, Leonard WJ: CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med* 2007;204:1543–1551.
- Baron U, Floess S, Wiczorek G, Baumann K, Grutzkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, Turbachova I, Hamann A, Olek S, Huehn J: DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol* 2007;37:2378–2389.
- Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R: FDA drug approval summary: azacitidine (5-azacytidine, vidaza) for injectable suspension. *Oncologist* 2005;10:176–182.
- Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS: Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol* 2009;182:259–273.
- Wu CJ, Chen LC, Kuo ML: Attenuated *Salmonella typhimurium* reduces ovalbumin-induced airway inflammation and T-helper type 2 responses in mice. *Clin Exp Immunol* 2006;145:116–122.
- Brewer JP, Kisselgof AB, Martin TR: Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. *Am J Respir Crit Care Med* 1999;160:1150–1156.
- Burchell JT, Wikstrom ME, Stumbles PA, Sly PD, Turner DJ: Attenuation of allergen-induced airway hyperresponsiveness is mediated by airway regulatory T cells. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L307–L319.
- Sumi T, Fukushima A, Fukuda K, Kumagai N, Nishida T, Takeuchi M, Taguchi O, Ueno H: Thymus-derived CD4+CD25+ T cells suppress the development of murine allergic conjunctivitis. *Int Arch Allergy Immunol* 2007;143:276–281.
- Richardson B: DNA methylation and autoimmune disease. *Clin Immunol* 2003;109:72–79.
- Brand S, Kesper DA, Teich R, Kilic-Niebergall E, Pinkenburg O, Bothur E, Lohoff M, Garn H, Pfefferle PI, Renz H: DNA methylation of TH1/TH2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol* 2012;129:1602–1610.
- Sanchez-Abarca LI, Gutierrez-Cosio S, Santamaria C, Caballero-Velazquez T, Blanco B, Herrero-Sanchez C, Garcia JL, Carrancio S, Hernandez-Campo P, Gonzalez FJ, Flores T, Ciudad L, Ballestar E, Del Canizo C, San Miguel JF, Perez-Simon JA: Immunomodulatory effect of 5-azacytidine (5-azac): potential role in the transplantation setting. *Blood* 2010;115:107–121.
- Shi HZ, Qin XJ: CD4CD25 regulatory T lymphocytes in allergy and asthma. *Allergy* 2005;60:986–995.
- Taylor A, Verhagen J, Akdis CA, Akdis M: T regulatory cells in allergy and health: a question of allergen specificity and balance. *Int Arch Allergy Immunol* 2004;135:73–82.
- Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S: Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;162:5317–5326.
- Jaffar Z, Sivakuru T, Roberts K: CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J Immunol* 2004;172:3842–3849.
- Kearley J, Barker JE, Robinson DS, Lloyd CM: Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;202:1539–1547.
- Leech MD, Benson RA, De Vries A, Fitch PM, Howie SE: Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J Immunol* 2007;179:7050–7058.
- Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003;4:330–336.
- Zheng Q, Xu Y, Liu Y, Zhang B, Li X, Guo F, Zhao Y: Induction of Foxp3 demethylation increases regulatory CD4+CD25+ T cells and prevents the occurrence of diabetes in mice. *J Mol Med* 2009;87:1191–1205.
- Kroetz DN, Deepe GS Jr: An aberrant thymus in CCR5–/– mice is coupled with an enhanced adaptive immune response in fungal infection. *J Immunol* 2011;186:5949–5955.
- Tausendschon M, Dehne N, Brune B: Hypoxia causes epigenetic gene regulation in macrophages by attenuating jumoni histone demethylase activity. *Cytokine* 2011;53:256–262.