

Protein/DNA vaccine-induced antigen-specific Treg confer protection against asthma

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Asthma is a chronic inflammatory disorder caused by T-cell-mediated inflammation within airways. No antigen-specific treatment has been available. Using an OVA-induced murine asthma model, we find that co-immunization of an OVA epitope peptide with a DNA vaccine encoding the same epitope is able to prevent this experimental asthma as evidenced in the marked reduction of infiltrations of eosinophils and lymphocytes into the site of the allergen challenge. We demonstrate that the prevention of experimental asthma was directly related to the induction of a population of OVA-specific T-regulatory cells (Treg) exhibiting a CD4⁺CD25⁺FoxP3⁺ phenotype and expressing IL-10, TGF- β and IFN- γ following the co-immunization. Blockade of IL-10 and TGF- β of the Treg by anti-IL-10 and TGF- β antibodies is partially able to reverse the suppression *in vitro* and *in vivo*, which caused the recurrence of the inflammation. Furthermore, adoptive transfer of the induced Treg is also able to suppress the OVA-induced asthma. To our knowledge, the combination of peptide with its cognate DNA vaccine protect experimental asthma via the induced epitope-specific Treg has not been previously reported and such strategy may lead to a novel immunotherapy against asthma in humans.

Key words: Asthma · Immune regulation · Tolerance · Treg cell · Vaccination

Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by airway obstruction, increased airway responsiveness to a variety of stimuli as well as the infiltration by many inflammatory cells, including eosinophils, macrophages, lymphocytes and mast cells into lung tissue. CD4⁺ T cells and Th2-biased cytokines (*i.e.* IL-4, IL-5 and IL-13) are believed to play a central role in the initiation and maintenance of the asthmatic response by regulating the production of IgE and the differentiation, recruitment and activation of mast cells and eosinophils [1–3].

It has been proposed that Th1 cells, which secrete interferon- γ (IFN- γ), could protect against allergic disease by inhibiting the proliferation and development of Th2 cells [4] and IgE production [5]. Approaches of redirecting immunity from a Th2 type to Th1 type have also been demonstrated to result in temporary reduction of allergic reactions [6]. However, both autoimmune disease [7] and pathogen infection [8] induced Th1-biased immune responses were found to reduce the likelihood of allergy disease. Hansen *et al.* demonstrated that antigen-specific Th1 cells were ineffective in reducing the airway hyper-reactivity induced by Th2 cells but caused serious airway inflammation [9]. Thus, immunotherapeutic approaches using Th1 cell responses against asthma are rather complicated.

Besides redirecting immunity-based approaches, several other strategies have also been considered to reduce allergic responses, including non-specific immunosuppressive drugs or monoclonal

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antibodies targeted against T or B cells [10–12]. However, this situation becomes tenuous since such long-term treatment of recipients leads to their becoming immunocompromised and results in the loss of the ability to fight infections. The recent discovery of T-regulatory cells (Treg), including the naturally occurring thymus derived CD4⁺CD25⁺ Treg has been an important advance in knowledge concerning the regulation of immune responses [13–16]. Adaptive Tr1 and mucosal-induced Th3 cells and antigen-induced CD4⁺CD25⁺ Treg have been proposed to be used as immuno-regulators of auto-reactive pathogenesis [17–19]. Various approaches have been explored to induce Treg to constrain the auto-reactive T cells. In particular, induction of antigen-specific Treg targeted to allergy, asthma and auto-immune disease antigens is considered a promising immunomodulatory strategy [20]. We recently demonstrated the induction of Treg that are able to inhibit antigen-specific T cells' function *in vivo* by co-immunization of DNA and protein vaccines encoding the same antigen [21, 22]. In this report, we further demonstrated that co-immunization of DNA and peptide vaccines against the same epitope-sequence of ovalbumin (OVA, aa 323–339)- induced antigen-specific Treg that can suppress allergic response *in vitro* and *in vivo*. We also provide evidence that such suppression is partially due to suppressive function of IL-10 and TGF- β production from the Treg and leads to the prevention of asthma in a mouse model.

Results

Co-immunization suppresses the development of asthma

We first established a murine asthma model to evaluate the effects of co-immunization of an epitope peptide and its cognate DNA vaccine on asthma allergic responses. Asthma in mice was induced by pre-sensitization and intra-tracheal challenge with OVA protein as previously described [23]. The induction of asthma-related infiltration by inflammatory cells to the lungs, mucus secretion and induction of high levels of IgE and Th2 cytokines are shown in Fig. 1.

We successfully cloned and expressed the T-cell epitopic region of OVA (aa323–339), designated as pD323 (data not

shown). We next utilized this murine asthma model to examine the ability of a co-immunization strategy to protect animals from airway hyper-responsiveness (AHR). BALB/c mice were pre-treated with the pD323 DNA vaccine and pep323 peptide (designated as pD323+pep323) or other immunogens intramuscularly twice at biweekly intervals. Subsequently, all animals except the negative control were sensitized and challenged intra-tracheally with OVA. The results demonstrated that the infiltration of inflammatory cells was significantly inhibited in the group co-immunized twice with the pD323+pep323 compared with the control groups (Fig. 1A, $p < 0.05$). In contrast, pre-treatment with the pD323 alone or pep323 alone did not suppress the infiltrations. Furthermore, no inhibition of infiltrations was observed with the control pcDNA3 vector co-immunized with pep323 (designated as vector+pep323), suggesting that the observed inhibition was not due to a non-specific factor such as a CpG motif within the plasmid backbone since the empty DNA vector contains the identical number of CpG motifs or other immunostimulatory sequences.

We next attempted to determine if the inhibition of eosinophilic inflammation induced by co-immunization is dose dependent. For this purpose, pD323 at doses of 25, 50, 100, 200 and 400 μ g were co-immunized with the pep323 at the constant dose of 100 μ g. The results, demonstrated that the infiltration of total cells and eosinophils was significantly inhibited at a pD323 dose of 100 μ g and reached maximal inhibition at 200 μ g (Fig. 1B). Doses of 25 and 50 μ g, however, exhibited a minimal or low effect on inhibitions of infiltration, respectively. In contrast, the vector + pep323 immunized animals exhibited high level of infiltrations similar to those observed in the model control at all doses tested.

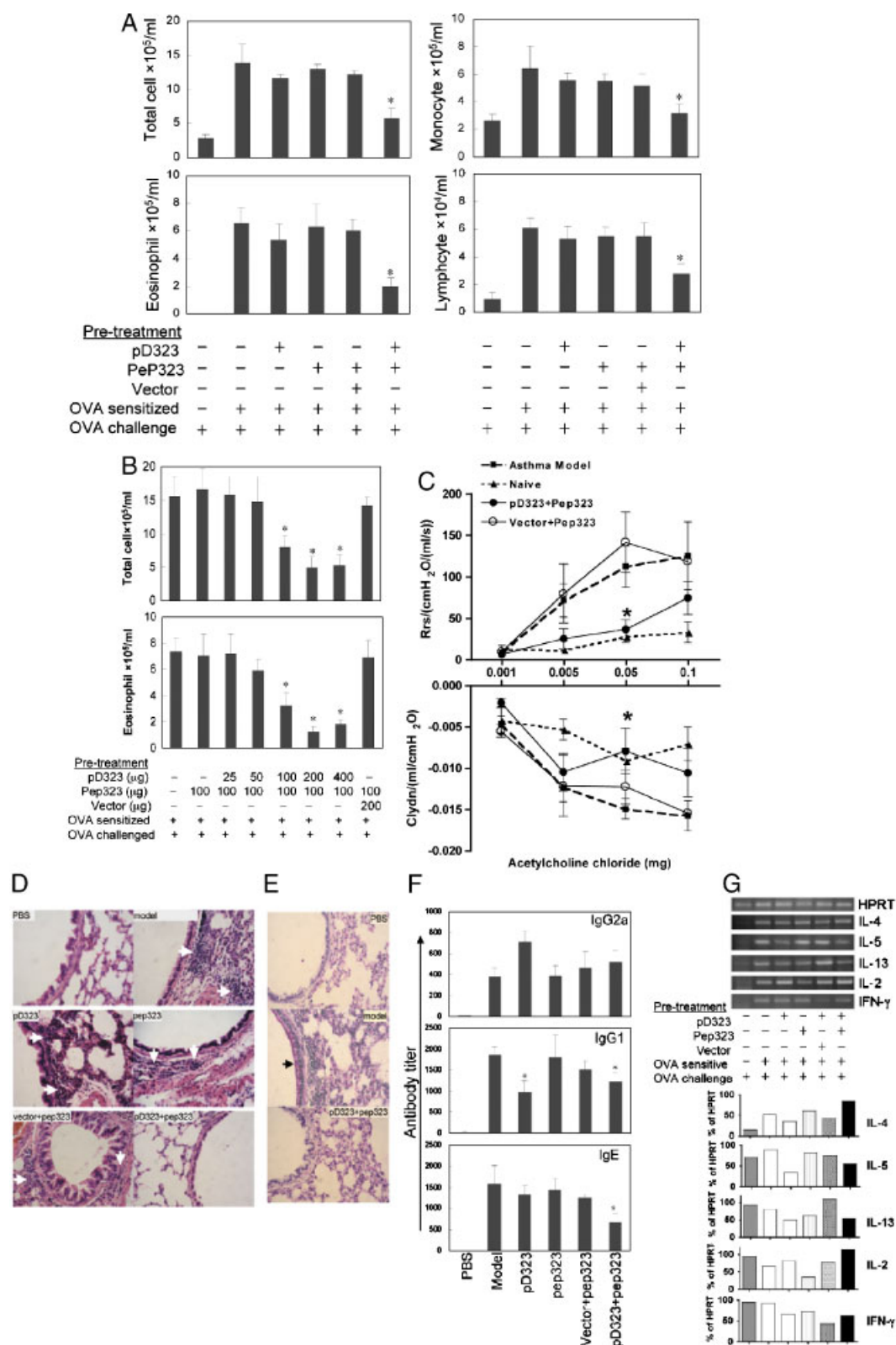
Bronchial hyper-responsiveness (BHR), a key functional indicator for asthma, was measured in mice in response to 200 μ L each of intra-jugular administration of various doses of acetylcholine chloride in the whole-body plethysmography. As shown in Fig. 1C, a less responses of Rrs and Cldyn to i.v. acetylcholine chloride at higher doses (0.05 mg) in the group co-immunized with the pD323+pep323 of OVA-sensitized mice compared with other groups as measured 72 h after the last OVA aerosol challenge.

Histological analysis of tissue sections after hematoxylin and eosin H&E staining revealed a massive infiltration in the lungs by eosinophils, monocytes and lymphocytes. In addition, weakened tissue structures were observed in the groups

Figure 1. Inhibition of the development of asthma by co-immunization of an OVA-peptide (pep323) and a DNA vaccine (pD323) encoding the same epitope. Mice were pre-treated with the indicated vaccination regimens intramuscularly on day -15 and -5, sensitized with OVA protein i.p. on day 0, and subsequently challenged with OVA intra-tracheally on days 8, 14, 16 and 18. The naïve mice without pre-treatment and sensitization, but OVA-challenged served as a negative control. Mice that were OVA-sensitized and OVA-challenged without any other treatments are defined as the model group ("asthma model"). (A) Twenty-four hours after the last challenge, BAL was analyzed. Data show the mean $\pm$ SEM of total eosinophils, monocytes and lymphocytes ($n = 5$ per group). * $p < 0.05$ in comparison with the model group. (B) Dose-dependent suppression of cellular infiltration into the BAL after co-immunization. * $p < 0.05$ in comparison with the model group. (C) Bronchial hyper-responsiveness (BHR) was measured by whole-body plethysmography in mice in response to the intra-jugular administration of methacholine at various doses (at the x-axis) and expressed as dynamic resistance (Rrs) and dynamic compliance (Cldyn) on the y-axis. The asthma model used is the induced model (i.e. OVA sensitization and challenge) without immunization. Naïve mice are as in (A). * $p < 0.05$, ($n \geq 4$) between the co-immunization group and either the asthma model or the vector+Pep323 group. Histological examination of lung tissues by H&E (D) and PAS (E) staining after challenge with OVA. The arrows show different cell infiltration (white arrow) and mucus secretion (black arrow), magnification, $\times 400$. (F) Levels of OVA-specific IgE and IgG1 were determined by ELISA on day 5 after the challenge and are presented as mean antibody levels $\pm$ SEM. * $p < 0.05$ in comparison with the model group. (G) Cytokine production in the spleens of mice on day 1 after OVA challenge was examined by RT-PCR. The density of each band determined using image software and expressed as a percentage of intensity for the HPRT band are shown in the lower panels. * $p < 0.05$.

pre-treated with the pD323, pep323, vector+pep323 and the model (Fig. 1D); whereas, mice pre-treated with co-immunization of pD323+pep323 demonstrated a normal lung structure

that was free of cell infiltration. Consistently, secreted mucus in the alveolus of lung by PAS staining was apparently normal in mice pre-treated with pD323+pep323 co-immunization



compared with the normal mice (Fig. 1E); while significant mucus secretion was observed in the model group, defined as mice that were OVA-sensitized and OVA-challenged but without any other treatments (“asthma model”). The data suggest that the histopathogenic parameters of AHR were inhibited by the pre-treatment of co-immunization with pD323+pep323.

Since allergic antigens trigger IgE and IgG1 that can mediate AHR [24–26], we next investigated if the co-immunization of pD323+pep323 could inhibit the induction of anti-OVA IgE and IgG1 antibodies. The levels of anti-OVA IgE and IgG1 antisera were therefore measured on day 5 after the last intra-tracheal challenge and were observed to reduce significantly in mice co-immunized with pD323+pep323 compared with the model group (Fig. 1F). Moreover, although pD323 alone immunization induced higher level of IgG1, the production of IgE was not modulated.

High levels of Th2-related cytokine productions, including the IL-4, IL-5 and IL-13 have been associated with the severity of allergic responses [27–30]. We examined if different profiles of these cytokines were affected by the co-immunization regimens. Mice from the model group, pre-treatment with vector+pep323 or pep323 alone were induced to produce higher levels of expressed mRNA for IL-5 and IL-13 (Fig. 1G); whereas, mice co-immunized with pD323+pep323 were induced to produce relatively low levels of these cytokines, suggesting that an anti-inflammatory immune regulatory function was associated with co-immunization with pD323+pep323. Pre-treatment with pD323 alone could also suppress the levels of IL-5 and IL-13, suggesting that the DNA vaccination elicited a polarized immunity toward the Th1 immunity.

These results demonstrated that the co-immunization could suppress the development of asthma outcomes, and further indicated that the suppression was not likely due to the redirecting the T-helper cells from Th2 to Th1.

CD4⁺CD25⁺ Treg cells are not responsible for the suppression

To test whether the co-immunization initiated the suppression by the induction of Treg, we first investigated whether frequency of the natural CD4⁺CD25⁺ Treg was influenced by the co-immunization. On day 7 after the second co-immunization, the frequency of CD4⁺CD25⁺ T cells was analyzed by FACS in the splenocytes isolated from the co-immunized or other groups. The results demonstrated that there was no significant difference in the frequency of CD4⁺CD25⁺ T cells among the co-immunized group, the model group and other immunized groups (Fig. 2A).

To directly test whether natural CD4⁺CD25⁺ Treg participated in the suppression of eosinophilic inflammation elicited by co-immunization, mice were pre-treated by the co-immunization or control immunizations, and followed by OVA sensitizations. Neutralizing antibody against CD25 was intravenously injected into the mice during the first challenge with OVA. As shown in Fig. 2B, the blocking of CD4⁺CD25⁺ T cells did not affect the suppression of infiltration by inflammatory cells in the group pre-treated with the pD323 and pep323 co-immunization, suggesting that the natural CD4⁺CD25⁺ T cells did not play a role in the suppression of eosinophilic inflammation induced by the co-immunization.

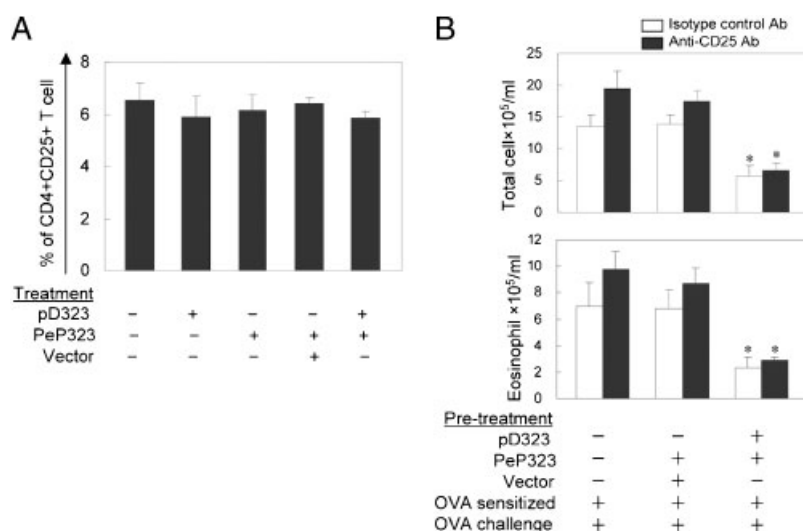


Figure 2. CD4⁺CD25⁺ T cells did not contribute the observed inhibition of experimental asthma. (A) The co-immunization regimen does not modulate the number of CD4⁺CD25⁺ T cells. Twenty-four hours after the third challenge, a single-cell suspension was prepared from the spleen of mice, and the number of CD4⁺CD25⁺ T-cells was analyzed by flow cytometry. Data are presented as the mean percentage of total CD4⁺ T cell numbers in the spleen ($n = 5$ per group). (B) Ab-blocking of CD4⁺CD25⁺ T cells *in vivo* does not reverse the suppression of inflammatory infiltrated cells induced by co-immunization. After co-immunization and OVA sensitization, mice were treated with a neutralizing Ab against CD25 at the time of both the first and second intra-tracheal challenge. Twenty-four hours after the last challenge, BAL was collected and the number of infiltrating cells (total) and eosinophils assessed by Diff-Quik staining. Results are representative of three experiments. * $p < 0.05$, compared with the model group (i.e. OVA-sensitized and OVA-challenged mice without any other treatments), $n = 5$ per group.

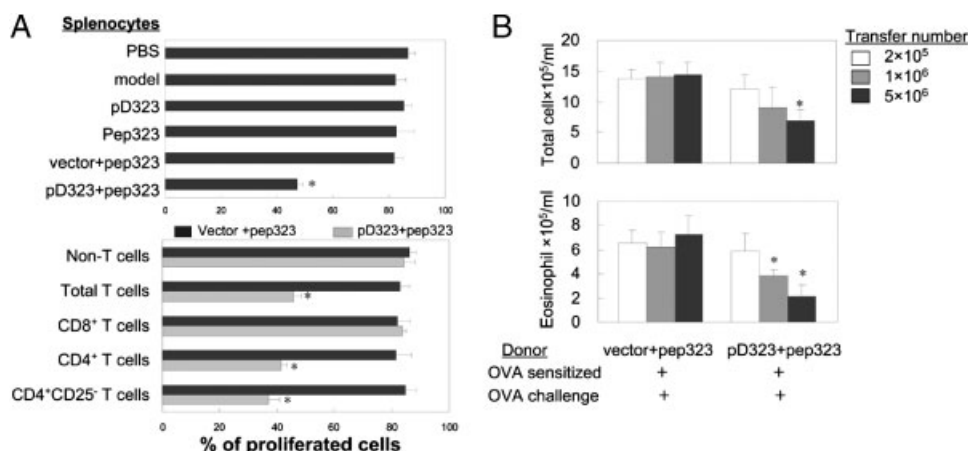


Figure 3. CD4⁺CD25⁻ T cells were responsible for the observed inhibition of experimental asthma. (A) CD4⁺ T cells from DO11.10 mice as responder cells were labeled with CFSE and added to 24-well-plates in the presence of BMDC for 48 h *in vitro*. At the same time, splenocytes as regulatory cells from the different experimental groups indicated were added to the same plate. Similarly, non-T cells, total T cells, purified CD8⁺, CD4⁺ or CD4⁺CD25⁻ T cells were isolated from the spleens of mice immunized with vector+pep323 or pD323+pep323, and added as regulatory cells. After 48 h of culture, proliferation was examined by flow cytometry. Data depict the mean and standard error of the percentage of proliferated cells ($n = 3$, * $p < 0.05$ in comparison with vector+pep323). (B) To test the regulatory function of the co-immunization *in vivo*, CD4⁺CD25⁻ T cells were isolated from splenocytes of mice immunized with vector+pep323 or pD323+pep323, and adoptively transferred, at the time of the first two OVA challenges, into syngeneic OVA pre-sensitized mice. After the third intra-tracheal challenge with OVA, the number of total infiltrating cells and eosinophils from BAL/lung was analyzed. Data are the mean cell numbers+SEM ($n = 5$, * $p < 0.05$).

CD4⁺CD25⁻ T cells are responsible for the suppression

To identify whether lymphocytes induced by the co-immunization can mediate the suppression, a proliferation assay was performed in the co-cultivation of OVA-specific T cells with the T cells obtained from the co-immunized mice. The CD4⁺ T cells from DO11.10 mice were used as the responder cells with bone marrow-derived dendritic cells (BMDC) from syngeneic BALB/c mice as the APC cells. In addition, the OVA protein was utilized as the antigen-specific stimulant, and splenocytes from the co-immunized group or other control groups were added into the co-cultures at ratio 1:5 with the responder cells in order to examine their regulatory function to inhibit the recalled proliferative responses *in vitro*. As demonstrated in Fig. 3A, addition of splenocytes from mice co-immunized with pD323+pep323 significantly inhibited the recalled OVA-specific T-cell responses. By contrast, splenocytes from mice of the model group, immunized by vector+pep323, or those from naive mice failed to inhibit the OVA-specific T cells' proliferative responses (Fig. 3A, upper panel). This indicates that certain cells within the splenocyte population generated during the co-immunization can suppress the antigen-specific T-cell proliferative response.

To further delineate what type of lymphocytes was induced by the co-immunization, we purified total T cells, non-T cells, or subsets of T cells from the spleen of mice co-immunized with pD323+pep323 or controls and tested them individually for suppression of the recalled proliferation as described above. Significant inhibitions were observed with either purified T cells, CD4⁺ cells or CD4⁺CD25⁻ cells from the mice co-immunized with pD323+pep323, but not from cells from other immunized groups (Fig. 3A, lower panels).

We utilized an adoptive transfer procedure to further examine this CD4⁺CD25⁻ T regulatory function *in vivo*. Syngeneic recipient mice previously sensitized by OVA protein were injected with CD4⁺CD25⁻ T cells (*i.e.* by adoptive transfer) isolated from animals co-immunized with pD323+pep323 or vector+pep323. The infiltration by inflammatory cells was assessed 24 h after the last challenge on all recipient mice. The results showed that CD4⁺CD25⁻ T cells from mice immunized with the pD323+pep323, but not from the vector+pep323 immunization, were able to suppress infiltration (Fig. 3B). Thus, *in vitro* and *in vivo* results were consistent and indicated that CD4⁺CD25⁻ T cells mediated the suppressive function of AHR.

CD4⁺CD25⁻ T cells from the co-immunization mediate antigen-specific suppression

To investigate the potential role CD4⁺CD25⁻ T cells play in an antigen-specific inhibition, we adoptively transferred CD4⁺CD25⁻ T cells taken from Balb/c mice previously co-immunized with pD323+pep323 into syngeneic recipient mice that were subsequently immunized twice at a biweekly interval with specific-OVA or a non-specific-BSA antigen emulsified in complete Freund's adjuvant. On day 7 after the last immunization, T cells were isolated and tested for their ability to proliferate to either OVA or BSA in an *in vitro* stimulation assay. As shown in Fig. 4, T cells from the recipient mice that were subsequently immunized with the OVA did not respond to the OVA-induced stimulation *in vitro*; whereas, T cells from recipient mice subsequently immunized to BSA responded well with BSA stimulation, but not to the OVA stimulation *in vitro*. This

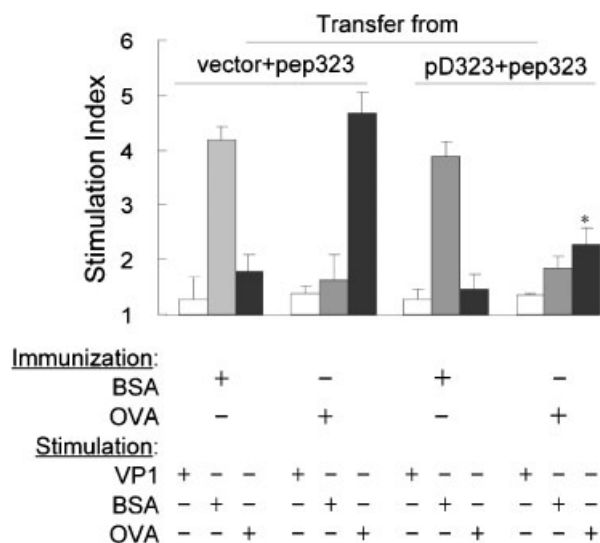


Figure 4. Co-immunization of DNA and peptide vaccines induces antigen-specific suppression of experimental asthma. A total of 1×10^6 CD4⁺CD25⁻ T cells were purified from the splenocytes of pD323+pep323 or vector+pep323 immunized mice and adoptively transferred into syngeneic naïve mice. The mice were then immunized with either OVA or BSA on the day of adoptive transfer. After 14 days, CD4⁺ T cells were isolated and their proliferation after the indicated *in vitro* stimulation assessed. VP1, derived from VP1 capsid protein of foot and mouth disease virus served as an irrelevant antigen control [20]. Results shown are mean stimulation index+SD and are representative of two independent experiments. * $p < 0.05$ compared with vector+pep323 transfers, $n = 5$ per group.

result indicates that adoptively transferred CD4⁺CD25⁻ T cells only inhibits the OVA antigen-specific T-cell priming and proliferation *in vivo*; while the responses from the irrelevant antigen-specific T cells were not affected.

Characterization of the induced CD4⁺CD25⁻ T cells

To determine if CD4⁺CD25⁻ T cells induced by co-immunization express well-documented Tr markers, CD4⁺CD25⁻ cells were isolated from the spleen of mice immunized with vector+pep323 or pD323+pep323 on days 1, 3, 7 and 14 post immunization. Cytokines in T cells were analyzed by FACS using intracellular staining with specific fluorescent labeled antibodies. Over the course of 14 days, the CD4⁺CD25⁻ T cells from mice co-immunized with pD323+pep323 expressed high levels of IL-10 (Fig. 5A), IFN- γ (Fig. 5B), FoxP3 (Fig. 5C), and a minimal amount of IL-4 (Fig. 5D). In contrast for the mice immunized with vector+pep323, CD4⁺CD25⁻ T cells induced a higher level of IL-4 and little expression of FoxP3, IL-10 or IFN- γ . Since the transcriptional factor Foxp3 has been demonstrated to be a hallmark of the Treg [31, 32], the co-immunization-induced CD4⁺CD25⁻ Treg can be thus categorized into the regulatory class of T cells with a unique phenotype.

Besides IL-10, TGF- β has been also demonstrated to be an important cytokine related to Tr's function [17, 33, 34]. We tested

TGF- β expression using RT-PCR in the CD4⁺CD25⁻ T cells from the co-immunized mice (Fig. 5E). The results demonstrated that a higher level of expression of TGF- β was induced in the CD4⁺CD25⁻ T cells from the mice co-immunized with pD323+pep323, but not from the vector+pep323 immunized or naïve mice, indicating that TGF- β might be involved in the Tr-mediated suppression.

IL-10 and TGF- β mediate the Treg's function *in vitro*

Having demonstrated the expression of IL-10 and TGF- β in Treg, we next examined whether they contributed to regulatory function. The CD4⁺ T cells from DO11.10 mice were selected as the responder cells, BMDC from BALB/c mice as the APC cells, OVA protein as the antigen and CD4⁺CD25⁻ T cells from the co-immunized group were added into the co-cultures system as the regulatory cells. At the same time, neutralizing antibody against IL-10, IFN- γ or TGF- β was added into the co-cultures to block each cytokine's function. As shown in Fig. 6, blocking IL-10 or TGF- β , but not to the IFN- γ significantly reversed the inhibition of antigen-specific T-cell recalled responses *in vitro*. Moreover, blocking of both IL-10 and TGF- β strengthened synergistically the reversion (Fig. 6), indicating that both IL-10 and TGF- β contributed to the regulatory function of Treg obtained from mice after co-immunization.

Blockade of IL-10 and TGF- β functions *in vivo*

To further examine the role of IL-10 and TGF- β *in vivo*, the endogenous IL-10 or TGF- β were blocked in mice pre-treated by pD323+pep323 or vector+pep323, followed by OVA sensitization and rechallenge. As shown in Fig. 7, blocking of IL-10, TGF- β or both could reverse suppression of infiltrated inflammatory cells by pre-treatment with pD323+pep323. Therefore, the results confirmed that IL-10 and TGF- β contributed to the regulatory function of CD4⁺CD25⁻ Treg obtained from the co-immunization *in vivo*.

Discussion

Our studies demonstrated that co-immunization with an OVA epitopic peptide and DNA vaccine encoding the same epitope-induced adaptive Treg that inhibited the development of experimental asthma induced by OVA challenge *in vivo*. These Treg exhibited a phenotype of CD4⁺CD25⁻Foxp3⁺ and expressed IL-10, TGF- β and IFN- γ with associated suppression of allergic infiltration of inflammatory cells *in vivo* and T-cell recalled proliferation, in an antigen-specific manner *in vitro*. The suppressive function of Treg was apparently in part mediated by both IL-10 and TGF- β *in vitro* and *in vivo*. Although our previous study shown the induction of IL-10, but no TGF- β , by the co-immunization of DNA and protein vaccines derived from the flea saliva antigen [22], the level of TGF- β is clearly induced by

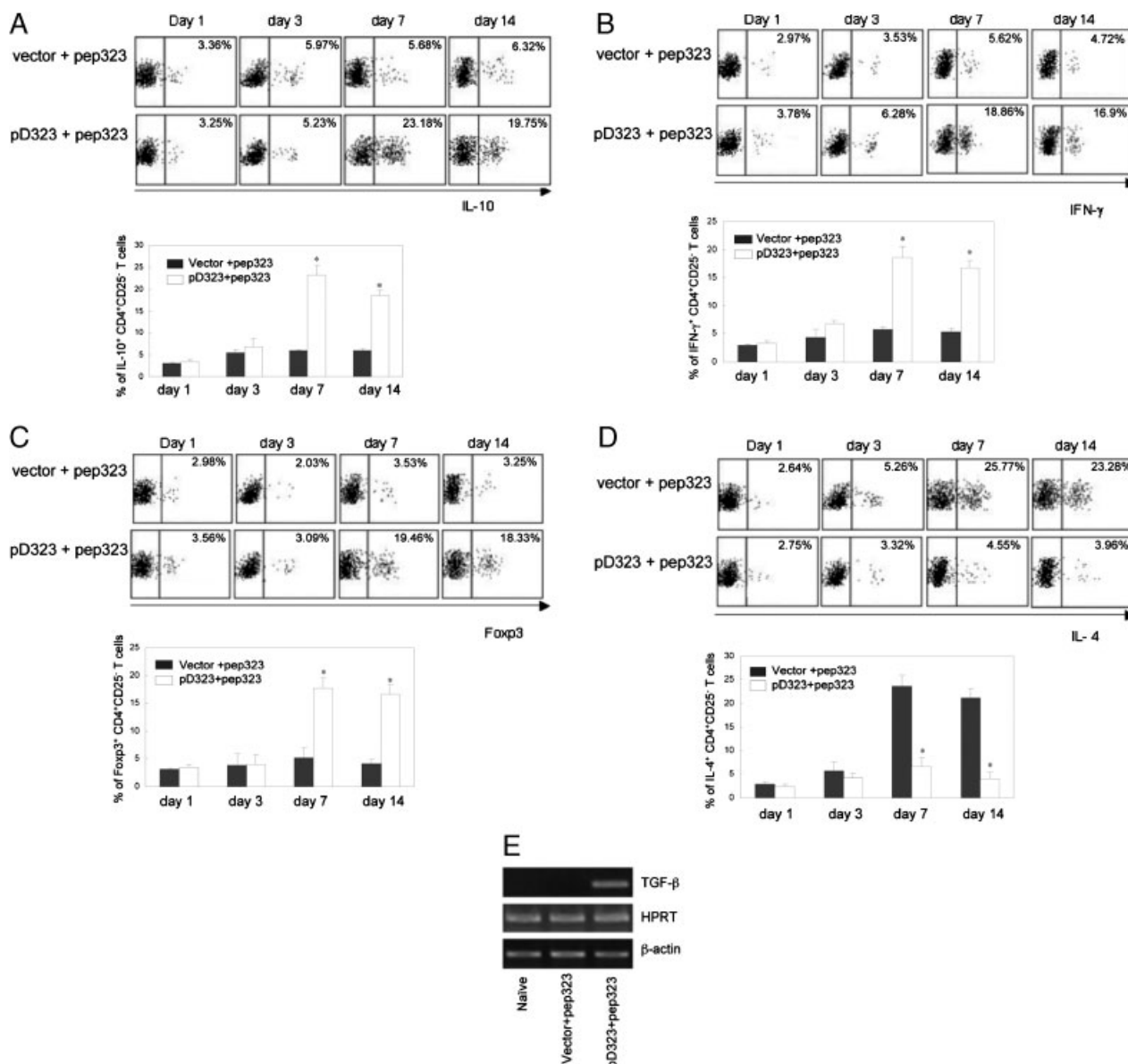


Figure 5. Analysis of cytokine production by CD4⁺CD25⁻ Treg. Following co-immunization, CD4⁺CD25⁻ T cells were purified from the spleens of vector+pep323 and pD323+pep323 immunized mice on days 1, 3, 7 and 14 and intracellular cytokine production analyzed by flow cytometry. Representative plot of three independent experiments represents the percentage of positive cells within CD4⁺CD25⁻ Treg in each plot (upper panel). The combined data showing the mean percentage plus SEM ($n = 5$, lower panel) are shown for IL-10 (A), IFN- γ (B), Foxp3 (C) and IL-4 (D). * $p < 0.05$ compared with vector+pep323 group. Analysis of TGF- β (E) production by RT-PCR. Lanes are the RNA from naïve, vector+pep323 and pD323+pep323 groups, respectively.

the co-immunization as demonstrated in this study and involved in the suppression as evidenced by the anti-TGF- β antibody blocking *in vitro* and *in vivo*. The difference on the induction of TGF- β may be due to the different antigenic epitope or sequence; however, it remains to be determined further.

Asthma is characterized by the overproduction of the Th2 cytokines IL-4, IL-5 and IL-13, which initiates and maintains the allergic inflammatory response by enhancing the production of IgE and the recruitment and activation for the inflammatory cells [1–3]. Recent studies demonstrated that, in some cases, the development of inflammation and autoimmune diseases were

directly related to a new type of Th17 subpopulation [35, 36], but to neither Th2 nor Th1 cells. These findings suggested that under certain circumstances the Th2 hypothesis should be reconsidered. No matter what the situation, these previous studies suggest that there are some other forms of responses that regulate the development of inflammation. For instance, IL-10 expressing Th1 cell is recently identified in parasite-infected animals as characterized as CD4⁺/CD25⁻/Foxp3⁺/IL-10⁺/IFN- γ phenotype [37]. However in this study, we identify the T cell with a phenotypic resemblance to Tr1 of CD4⁺/CD25⁻/Foxp3⁺/IL-10⁺/IFN- γ /TGF- β ⁺ after the induction of co-immunization with DNA and protein

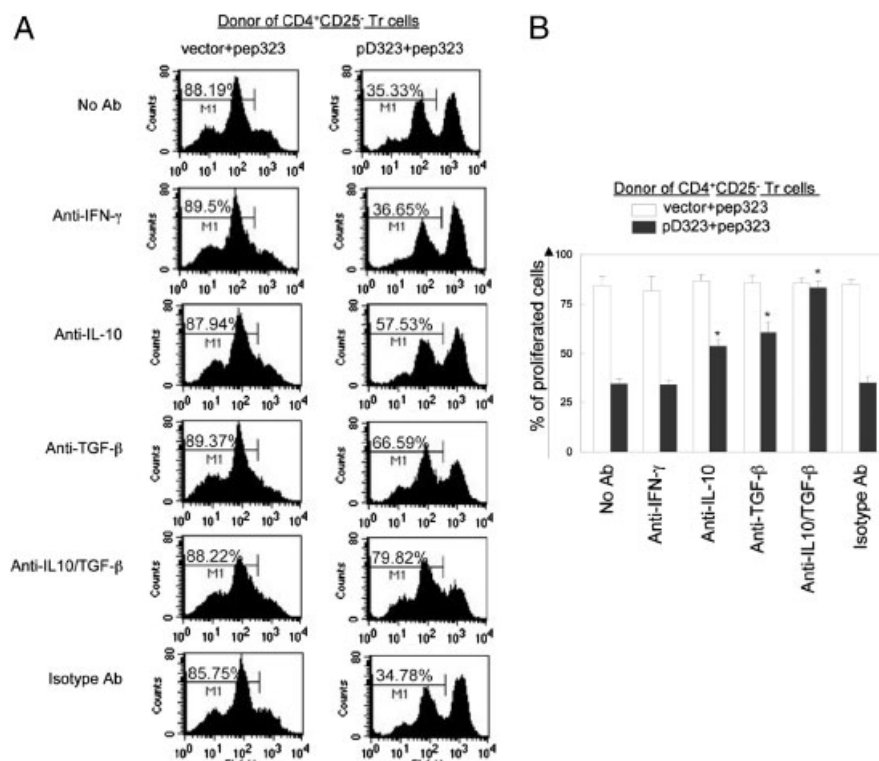


Figure 6. Both IL-10 and TGF- β are responsible for the regulatory function of Treg induced by co-immunization *in vitro*. A total of 5×10^5 CD4⁺ T cells from DO11.10 mice (responder cells) were labeled with CFSE and added to 24-well plates. These responder cells were stimulated with OVA protein (20 μ g/mL) in the presence of 1×10^5 bone marrow derived DC for 48 h *in vitro*. A total of 1×10^5 CD4⁺CD25⁻ T cells were isolated from the spleens of mice immunized with vector+pep323 or pD323+pep323, and were added before the stimulation as the regulatory cells. At the same time during the addition of regulatory cells, anti-IL-10, anti-IFN- γ , anti-TGF- β , anti-IL-10+anti-TGF- β or an isotype control Ab were added to the co-cultures. After 48 h, proliferation was examined by flow cytometry as detailed in the representative flow cytometry plots (A). Percentage of M1 in each plot represents the level of proliferation. The results are representative of three independent experiments. The bar graph (B) depicts mean percentage of the proliferated cells $\pm$ SEM, $n = 5$, * $p < 0.05$, compared with the isotype control group for pD323+pep323.

protocol, this Tr1 like cells can successfully suppress the development of asthma-like inflammations in an antigen-specific manner in mice.

Although many aspects of the mechanisms by which the regulatory cells function remain to be elucidated, it has been well established that Treg use both cell-to-cell interactions and/or the production of IL-10 and TGF- β [33, 34, 38, 39]. Treg, particularly for the thymic-derived CD4⁺CD25⁺ Treg, express the Foxp3 gene and mediate the suppressive function in an antigen-non-specific manner [40, 41]. The expression of Foxp3 gene has reported to be induced *via* IL-10 or TGF- β in the population of CD4⁺CD25⁻ T cells and leads to develop the Tr1 cells *in vitro* [42]. This population is relatively small and less characterized, but importantly induced by antigen compared with the CD4⁺CD25⁺ Foxp3⁺ Treg [32, 43]. Such induction of adaptive Tr1 cells has been observed *via* sub-activation of DC *in vitro* [44, 45]. Although the induction of regulatory Th3 cell *via* oral delivery of antigen(s) has been demonstrated, it is not clear how Tr1 or other antigen-specific Treg is induced systematically. Thus, the co-immunization strategy by using the antigenic peptide and DNA vaccine encode the same sequence induces an antigen-specific expansion of CD4⁺/CD25⁻/Foxp3⁺/IL-10⁺/TGF- β ⁺/IFN- γ ⁺ Treg, which then inhibits asthma response in an

antigen-specific manner, presents a novel means to induce such Treg systematically. Whether the co-immunization of pD323+pep323 presents an alternative signal to the antigen-presenting cells as “sub-optimal” remain to be determined. Nevertheless, the exploitation of this novel strategy may lead to develop an effective therapeutic/prophylactic treatment against allergic and other related type immune disorders in both humans and animals.

Materials and methods

Animals

Adult female BALB/c mice (8–10 wk of age) were from the Animal Institute of Chinese Medical Academy (Beijing, China). DO11.10 mice (8–10 wk of age) were obtained from SLAC Lab Animal Co. (Shanghai, China). All animals received pathogen-free food and water, were maintained in the permitted facility by the local authority and following proper animal protocols.

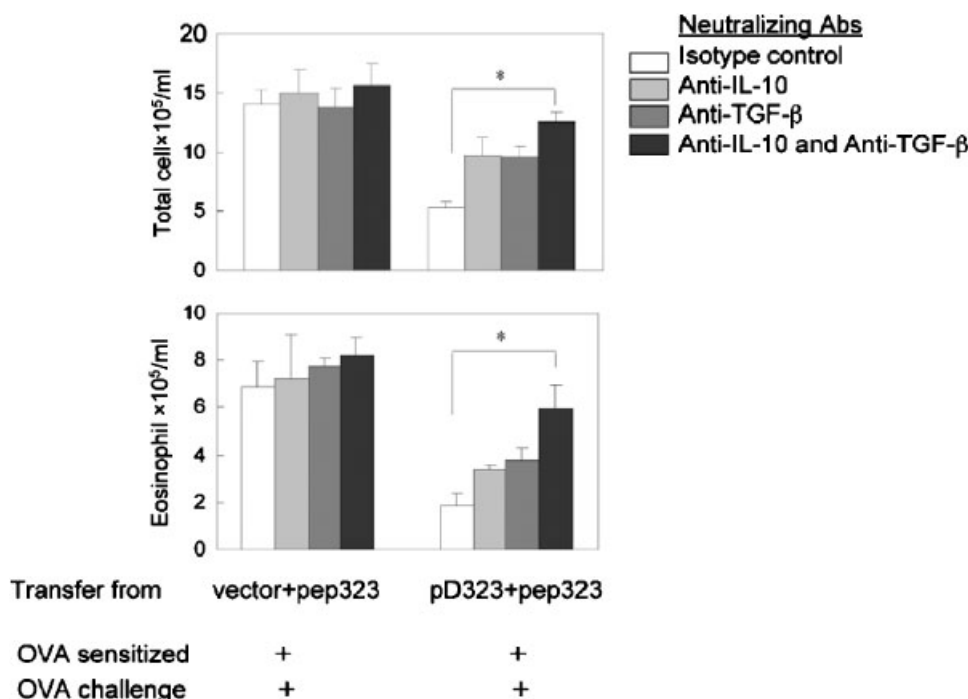


Figure 7. Both IL-10 and TGF- β are responsible for the regulatory function of Treg induced by co-immunization *in vivo*. A total of 5×10^5 CD4⁺CD25⁺ T cells were isolated from the splenocytes of mice immunized with vector+pep323 or pD323+pep323, and adoptively transferred into syngeneic sensitized mice, which were pre-sensitized with OVA protein and challenged intra-tracheally three times with OVA. The mice were treated with neutralizing Abs against IL-10, TGF- β , or matched isotype control Ab at the time of the first and the second challenge. Twenty-four hours after the third and final challenge, BAL was collected and the number of infiltrating total cells or eosinophils assessed by Diff-Quik staining. The mean number of infiltrating cells plus SEM ($n = 5$ per group) is shown and the results are representative of two independent experiments. * $p < 0.05$ between the groups as indicated.

Neutralizing antibodies

The anti-CD25 mAb PC61.5.3 [46], anti-IL-10 mAb JES5-2A5 [47], anti-IFN- γ mAb R4-6A2 [48] and anti-TGF- β mAb HB 9849 [49] were isolated from hybridoma culture supernatants using cells purchased from the ATCC (Manassas, VA, USA).

Allergen sensitization and challenge protocol

OVA (1 mg/mL; Sigma, USA) in PBS was mixed with an equal volume of 10% aluminum potassium sulfate (alum; Sigma) in distilled water with shaking for 2 h at room temperature. After centrifugation at $750 \times g$ for 5 min, the OVA/alum complexes were resuspended into the distilled water with the original volume. Mice were sensitized with 100 μg OVA (0.1 mL of 1 mg/mL OVA/alum complexes) on day 0. Intra-tracheal challenges were performed as previously described and with some modifications [23]. In brief, mice were anesthetized by i.p. injection of 0.2 mL mixture of ketamine and xylazine (0.44 and 6.3 mg/mL) in saline. This was followed by delivery of 100 μg (100 μL of 1 mg/mL) OVA to the back of the tongue of each animal on days 8, 14, 16 and 18. Control mice received with saline delivered intra-tracheally on days 8, 14, 16 and 18.

Vaccines and vaccinations

Synthesized OVA peptides (aa323–339, named as pep323) were emulsified in incomplete Freund's adjuvant (IFA).

A eukaryotic expressing construct encoding the epitope of OVA at aa323–339 region, designated as pD323, was dissolved in saline at 1 mg/mL. The pcDNA3 vector was dissolved in saline at a concentration of 1 mg/mL.

Before allergen sensitization and challenge, animals were immunized with different vaccines. Mice (six per group) were immunized with 0.1 mL of 100 μg pep323 in IFA, or 100 μg of pD323, or combinations of 100 μg of synthesized peptides with different doses of pD323, or a mixture of pcDNA3 vector and pep323 in IFA. All groups were immunized on day –15 and boosted on day –5 into tibialis anterior muscle before OVA sensitization. In the *in vivo* blocking experiments, the mice were given 50 μg of neutralizing antibodies against either IL-10 or TGF- β , or both, at both times during the intra-tracheal challenges with the OVA. An isotype-matched irrelevant Ig was used as a negative control.

Plethysmography

Measurements of respiratory system dynamic resistance (Rrs) and compliance (Cldyn) were measured by placing mice in a

whole-body plethysmograph (model AniRes2005; Beijing Bestlab Technology Co. Beijing, China). Briefly, 72 h after final challenge, mice were anaesthetized, tracheotomized and connected to the ventilator. Mechanical ventilation was carried out, dynamic airway pressure (ΔP) and volume of chamber (ΔV) were recorded, and peak resistance and compliance were automatically measured as $Rrs = \Delta P / (\Delta V / \Delta T)$ and $Clydn = \Delta V / \Delta P$ after each 200 μ L of intra-jugular administration of various doses of acetylcholine chloride (mg) [50].

Bronchoalveolar lavage (BAL) collection

Twenty-four hours after the last challenge, the mice were euthanized with a lethal dose of pentobarbital. BAL was collected using 0.8 mL saline twice from the main stem bronchus. The BAL collected from one mouse was pooled, centrifuged, and the cells were resuspended in 100 μ L saline containing 0.1% BSA. Total BAL cells were counted by FACS. Cells were stained with Diff-Quik (Merck, Darmstadt, Germany), and cell types were identified by morphological criteria. Two hundred cells were examined per slide for a differential count.

Histology analysis

Twenty-four hours after the last intra-tracheal challenge, lung samples from mice were collected from every group and fixed in 4% paraformaldehyde and embedded in paraffin blocks. Sections were then cut and fixed. Antigen retrieval was accomplished by boiling the slides in 0.01 M citrate buffer (pH 6.0) followed by staining with H&E or with periodic acid-Schiff (PAS)/hematoxylin for evaluation of mucus production, and analyzed under a light microscope for determining histology changes.

Dendritic cell (DC) culture

DC were cultured from mouse bone marrow, as previously described [51]. Briefly, bone marrow cell suspensions were cultured with RPMI-1640 (Gibco, Eggenstein, Germany) supplemented with 10% FBS, 20 ng/mL murine recombinant GM-CSF. At day 8, the CD11c⁺ DC were purified by magnetic beads (e-Biosciences, USA).

T-cell recalled responses

The CD4⁺ T cells isolated from the spleen of mice were cultured at 5×10^4 cells/well in triplicate in 96-well plates containing RPMI-1640 (Gibco)/10% FBS, and the APC cells were bone marrow derived DC (1×10^4 cells/well) from the syngeneic mice, and then stimulated with 20 μ g/mL of OVA for 48 h. Following stimulation, cell proliferation was assessed by a colorimetric reaction after the addition of 20 μ L of an MTS-

PMS (Pormaga, USA) solution for 2–4 h; color density was determined at 490 nm by a plate reader (Magellan, Tecan Austria GmbH).

The assay for the inhibition function of Treg

The capacity of Treg from co-immunized animals to inhibit proliferation of OVA-specific CD4⁺ T cells from DO11.10 mice was determined using an *in vitro* assay. A total of 5×10^5 CD4⁺ T cells were isolated from DO11.10 mice and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in 48-well plates as the responder cell. The bone marrow derived DC (1×10^5 cells/well) from the syngeneic mice served as the APC cells. OVA at 20 μ g/mL was used as the stimulation antigen. A total of 1×10^5 CD4⁺CD25[−] T cells isolated from the co-immunized or indicated groups were added into the same plate as the regulatory cells. In some cases, cytokine functions were blocked by addition of neutralizing Abs to co-cultures against the IL-10, IL-4, IFN- γ , TGF- β or rat IgG isotype control. Cells were harvested after 48 h of co-culturing, and the proliferation was determined based on CFSE dilution by FACS.

Measurement of antigen-specific antibodies

Serum concentration of anti-OVA IgG1 and IgE isotypes were measured using OVA antigen-coated plates by ELISA, and detection with specific horseradish peroxidase-conjugated rat anti-IgG1 and IgE antibodies (SouthernBiotech, Birmingham, USA); absorbance (450 nm) was measured using an ELISA plate reader (Magellan, Tecan Austria GmbH).

RT-PCR

Total RNA was isolated from BAL 24 h after the last challenge using a TRIzol reagent (Promega). cDNA was synthesized and PCR was performed with each of the following primers: HPRT, IL-2, IFN- γ , IL-4, IL-5, IL-13 and TGF- β [52, 53]. PCR products were visualized using 2% agarose gels and EtBr staining, and quantitations were performed by using computer software (AlphaImager 2200, Alpha Innotech Corp.). The mRNA level represented by each of the PCR samples was determined indirectly by comparing the intensity of the sample band to that of the HPRT band.

Isolation of CD4⁺CD25[−] T cells and adoptive transfer

Single splenocyte suspensions were prepared from mouse spleen and CD4⁺CD25[−] T cells were negative isolated and purified by

using the MagCelect Mouse CD4⁺CD25⁺ T-Cell Isolation Kit according to the manufacturer's protocol (R&D Systems, USA). The purity of the selected cell populations was 96–98%. The purified cells (1×10^6 per mouse) were adoptively transferred intravenously into mice.

Flow cytometry

Flow cytometry of cell surface or intracellular cytokine production in cells was performed according to a standard protocol, with some modification [54]. Single cell suspensions were prepared from the animals spleens and Fc receptors were blocked with excess anti-Fc (BD PharMingen, USA). Cells were washed with ice-cold PBS. Cell surface CD4 and CD25 were stained with PE-conjugated antibodies to CD25 on ice (eBioscience, CA, USA). For intracellular cytokine staining, T cells were stimulated overnight with Con A (Sigma-Aldrich) in the presence of anti-CD28 mAb (BD PharMingen). Collected cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin (Sigma-Aldrich). For staining of cytoplasmic IL-10, IL-4, IFN- γ or FoxP3, the appropriate concentrations of phycoerythrin-labeled antibodies (eBioscience) were added to permeabilized cells for 30 min on ice followed by washing twice with cold PBS. Samples were processed and screened using FACSCalibur and data were analyzed with Cell Questpro software (BD).

Statistic analysis

Two group comparisons were statistically analyzed using the unpaired Student's *t*-test. Differences were considered statistically significant with **p* < 0.05 and ***p* < 0.01.

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