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Wei-ping Zeng, Ph.D., Margaret M. McFarland, M.S., Baohua Zhou, Ph.D., Silva Holtfreter, Dr. rer. nat., Susan Flesher, M.D., Ambrose Cheung, M.D., Avishek Mallick, Ph.D.

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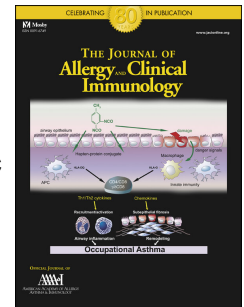
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Title: Staphylococcal Enterotoxin A-activated Regulatory T Cells Promote Allergen-specific T Helper Type 2 Response to Intratracheal Allergen Sensitization

Wei-ping Zeng, Ph.D.^{a,b,c,*}, Margaret M. McFarland, M.S.^a, Baohua Zhou, Ph.D.^d, Silva Holtfreter, Dr. rer. nat.^e, Susan Flesher, M.D.^b, Ambrose Cheung, M.D.^f, Avishek Mallick, Ph.D.^g

^aDepartment of Biochemistry and Microbiology, ^b Department of Pediatrics, Joan C. Edwards School of Medicine, Marshall University; ^c Center for Cell Development and Differentiation, Department of Biology, College of Science, Marshall University, 1 John Marshall Drive, Huntington, WV 25701

^dIndiana University School of Medicine, HB Wells Center for Pediatric Research, 1044 West Walnut, R4 272, Indianapolis, IN 46202

^e Institute of Immunology and Transfusion Medicine, University Medicine Greifswald, Greifswald, Germany

^f Department of Microbiology and Immunology, Geisel School of Medicine, Vail Building - HB 7550, Dartmouth University, Hanover, NH 03755

^g Department of Mathematics, College of Science, Marshall University, 1 John Marshall Drive, Huntington, WV 25701

* Correspondence: Wei-ping Zeng, Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine, Marshall University, 1 John Marshall Drive, Huntington, WV 25701. E-mail: zengw@marshall.edu; Tel: 304 696 3892

ABSTRACT

Background: T helper type 2 (Th2) responses are implicated in asthma pathobiology. Epidemiological studies have found a positive association between asthma and exposure to staphylococcal enterotoxins (SEs).

Objective: We used a mouse model of asthma to determine whether SEs promote Th2 differentiation of allergen-specific CD4 conventional T (Tcon) cells and asthma by activating allergen-nonspecific Treg cells to create a Th2-polarizing cytokine milieu.

Methods: Ovalbumin (OVA) specific, staphylococcal enterotoxin A (SEA)-nonreactive naïve CD4 Tcon cells were co-cultured with SEA-reactive allergen-nonspecific Treg or CD4 Tcon cells in the presence of OVA and SEA. The OVA-specific CD4 T cells were then analyzed for IL-13 and IFN- γ expression. SEA-activated Treg cells were analyzed for the expression of the Th2-polarizing cytokine IL-4 and T cell activation markers CD69 and CD62L. For asthma induction, mice were intratracheally sensitized with OVA or cat dander extract (CDE) alone or together with SEA, then challenged with OVA or CDE. Mice were also subject to transient Treg cell depletion prior to sensitization with OVA plus SEA. Asthma features and Th2 differentiation in these mice were analyzed.

Results: SEA-activated Treg cells induced IL-13 but suppressed IFN- γ expression in OVA-specific CD4 Tcon cells. SEA-activated Treg cells expressed IL-4, up regulated CD69 and down regulated CD62L. Sensitization with OVA plus SEA but not OVA alone induced asthma, and SEA exacerbated asthma induced by CDE. Depletion of Treg cells abolished these effects of SEA and IL-13 expression in OVA-specific T cells.

Conclusion: SEA promoted Th2 response of allergen-specific T cells and asthma pathogenesis by activating Treg cells.

Key Words: Asthma; Staphylococcal Enterotoxin; Th2 cells; Treg cells

Abbreviations: AHR, airway hyperresponsiveness; APC, antigen presenting cells; CD, cluster of differentiation; BALF, bronchoalveolar lavage fluid; CDE, cat dander extract; DT, diphtheria toxin; DTR, diphtheria toxin receptor; MLN, mediastinal lymph node; IFN, interferon; IL, interleukin; OVA, ovalbumin; PMA, phorbol 12-myristate 13-acetate; SE, staphylococcal enterotoxin; SEA, staphylococcal enterotoxin A; Tcon cell, conventional T cell; Th, T helper; Treg cell, regulatory T cell

Capsule Summary: We found that staphylococcal enterotoxin A activates Treg cells to promote Th2 response to allergens and asthma in mouse models, suggesting that vaccines against staphylococcal enterotoxins could prevent allergic diseases.

Clinical Implications

Our findings suggest that SEs act as natural adjuvants to promote Th2 responses to allergens. Therefore, vaccines against SEs could help prevent allergic diseases such as asthma.

INTRODUCTION

The diverse functions of CD4 T cells in immune responses are in large part mediated by different functional subsets. CD4 T cells may be categorized into the regulatory T (Treg) cells and CD4 conventional T (Tcon) cells. Treg cells protect the hosts by preventing aberrant or excessive immune responses to self as well as foreign antigens, and their development and functions are dependent on the transcription factor Foxp3¹⁻³. On the other hand, naïve CD4 Tcon cells differentiate into different T helper subsets upon antigen activation to mediate diverse immune effector functions in host defense and immunopathology⁴. The T helper type 2 (Th2) subset plays a central role in host defense against helminth infection⁵, but are also implicated in allergic diseases⁶. Upon activation by antigens in the presence of Th2 polarizing signals, naïve CD4 T cells up regulate the transcription factor GATA-3 to drive Th2 differentiation⁷. The cytokine milieu in which naïve CD4 T cells are activated is a major source of the Th2 polarizing signals. The cytokine IL-4 is a potent driver of Th2 differentiation. It activates Stat6⁸,

which together with signals generated from TCR leads to the up-regulation of GATA-3⁹,
¹⁰. Other cytokines such as IL-33, IL-25 and TSLP produced outside the lymphoid
tissues at the site of antigen exposure can indirectly, as well as directly, promote Th2
differentiation¹¹⁻¹⁴.

Asthma is a pulmonary disorder characterized by recurrent and reversible airway
obstruction¹⁵⁻¹⁷. It affects a large proportion of the world's population and its incidences
continue to rise in many countries, stressing the importance of prevention¹⁵. Although
asthma is recognized as a heterogeneous group of diseases with diverse clinical
manifestations and underlying causes¹⁸, a recent study has estimated that about half of
the clinical cases are "Th2-high" asthma where the disease pathogenesis is driven by Th2
responses to inhaled allergens¹⁹. In mouse models of asthma, the Th2 cytokine IL-13
causes airway hyperresponsiveness (AHR)^{20, 21} and stimulates mucus hyper-production
^{22, 23}, whereas IL-5 induces pulmonary eosinophilia^{24, 25}. The Th2 cytokines can also be
produced by type 2 innate lymphoid cells (ILC2)^{26, 27}. Nonetheless, at least in a mouse
model, Th2 cells remain the principal cell type responsible for asthma caused by allergen
specific recall response²⁸.

Given the central role of Th2 cells in asthma, how allergen specific CD4 T cells
differentiate into Th2 cells is key to understanding asthma pathogenesis, and ultimately to
the treatment and prevention of asthma. In this regard, some clinical allergens such as
the house dust mite protein Der p2 and the cat dander protein Fel d1 and the mold
allergen *Alternaria* extract possess self adjuvant activities that promote Th2

differentiation²⁹⁻³¹. In contrast, some allergens such as the short ragweed extract and the experimental allergen ovalbumin (OVA) are considered immunologically “inert”, and Th2 response to airway exposure to these allergens requires exogenous adjuvant in the mouse model of asthma³⁰.

However, regardless of the immunological properties of the allergens, not everyone exposed to allergens, even to allergens with self-adjuvant activities, develops asthma. Therefore, other factors, both genetic and environmental, are required for the development of Th2 responses to allergens that are strong enough to cause clinical disease. For prevention, environmental factors are particularly relevant because they are amenable to manipulation. One environmental factor that may condition an individual to become susceptible to asthma is exposure to staphylococcal enterotoxins (SEs). Indeed, IgE antibodies against SEs, which are indication of prior history of SE exposure, are frequently detected in asthmatic patients³²⁻³⁴. An individual can be exposed to SEs as environmental contaminants. Colonization by *Staphylococcus aureus* could also potentially lead to SE exposure as the majority of *S. aureus* strains isolated from humans contain SE genes³⁵. In fact, meta-analysis of epidemiological studies found a statistically significant increased risk of *S. aureus* colonization in asthmatic patients³⁴, and a recent large-scale study of the U.S. population confirmed a positive association between *S. aureus* colonization and asthma between 6 – 30 years of age³⁶. However, despite ample epidemiological evidence, the immunological basis for the positive association is poorly understood. The detection of IgE antibodies to SEs implies that SEs behave as conventional antigens/allergens to which antibody responses are generated. It

has also been suggested that expansion of T cells in the lungs stimulated by the superantigen activity of SEs may contribute to asthma pathogenesis³⁷. In this study, we used staphylococcal enterotoxin A (SEA) to further investigate the mechanism for the immunological role of SEs in the mouse model of allergic asthma. There have been no previous studies that specifically investigated how SEA-activated allergen nonspecific Treg cells or CD4 Tcon cells might influence Th subset differentiation of naïve allergen specific CD4 Tcon cells. Our central hypothesis is that apart from being conventional foreign antigens, SEs can act as natural adjuvants to modulate immune responses to other allergens by activating allergen nonspecific CD4 T cells to produce Th-polarizing cytokines. We further hypothesized that SE-activated allergen nonspecific Treg cells and CD4 Tcon cells might exert different Th-polarizing effects on allergen specific CD4 Tcon cells. Indeed, we found that SEA-activated allergen nonspecific Treg cells but not CD4 Tcon cells induced Th2 differentiation of allergen specific naïve CD4 Tcon cells. Thus, our study together with previous epidemiological studies provides evidence that respiratory exposure to SEs concurrent with allergen exposure could be a common factor that confers susceptibility to asthma.

METHODS

Mice

All mice used in this study except Balb/c.Thy1.1, which was obtained from Dr. D. Fowell, were purchased from Jackson Laboratory and bred and maintained in the local animal facilities. Balb/c.Thy1.1 is a Balb/c congenic strain, which expresses Thy1.1 instead of Thy1.2 of the Balb/c mice. Animal studies were performed in compliance with

protocols approved by Institutional Animal Care and Use Committees of Marshall University and Indiana University.

In vitro cell culture

Antigen nonspecific naïve CD4 Tcon cells or Treg cells were isolated by FACS from Balb/c.Thy1.1.Foxp3.GFP (CD4⁺CD62L^{high} KJ126⁻ GFP⁻ or GFP⁺) or B6.Foxp3.GFP (CD4⁺CD62L^{high} TCR Vβ5⁻ GFP⁻ or GFP⁺) mice, both of which contain a green fluorescent protein (GFP) gene knocked in and co-expressed with Foxp3 gene³⁸. OVA specific naïve CD4 Tcon cells were isolated by FACS from DO11.10.Foxp3.GFP (CD4⁺CD62L^{high} KJ126⁺GFP⁻, Balb/c genetic background) or OTII.Foxp3.GFP (CD4⁺CD62L^{high} TCR Vβ5⁺ GFP⁻, B6 genetic background) mice, which express two different transgenic OVA-specific TCRs and a GFP gene controlled by the Foxp3 promoter³⁸. Antigen presenting cells (APC) were T and NK cell depleted spleen cells irradiated with 3000 rads of X-rays. OVA specific naïve CD4 Tcon cells were mixed with APC and naïve nonspecific Treg or CD4 Tcon cells as indicated at 1:1:1 ratios at 1 x 10⁶ cells/ml for each cell type in RPMI-1640 plus 10% FBS, 0.5 µg/ml OVA₃₂₃₋₃₃₉ peptide and 5 ng/ml IL-12 (gift of Biogen). The cells were plated in U-bottom 96-well plate, and SEA (Toxin Technology) was added to the wells as indicated at the final concentration of 5 µg/ml. On day 6 of culture, the cells were washed then stimulated with PMA (50 ng/ml) and ionomycin (1 µM) in the presence of 2 µM monensin (Sigma Aldrich) for 6 hrs. The cells were harvested and analyzed as described^{39, 40}. Briefly, the cells were first stained for surface markers, then fixed and permeabilized and stained for

intracellular IL-13 and IFN- γ . The cells were analyzed on a FACSaria II, and the data were processed with the FlowJo software (BD Bioscience).

ELISA

ELISA was carried out as previously described⁴¹ to measure IL-4 concentrations in culture supernatants. IL-4 capture and detection antibodies were purchased from BD Pharmingen.

Allergen sensitization and challenge

Mice (3 – 4 mice/experimental group) were i.t. instilled with 100 μ g OVA protein (Sigma-Aldrich) or 8 μ g (protein content) of cat dander extract (CDE) (Greer Laboratory) with or without 0.5 μ g SEA in 60 μ l PBS. In experiments for OVA tetramer staining of endogenous T cells, 2.5 μ g OVA₃₂₃₋₃₃₉ was included in OVA sensitization to increase the tetramer specific T cell population because the tetramer is designed to detect TCR specific for this peptide. Mice (3 – 4 mice/experimental group) also received i.p. injection of diphtheria toxin (DT) (EMC Millipore) at 50 ng/g body weight on 2 consecutive days prior to allergen sensitization to deplete Treg cells in B6.Foxp3^{DTR} mice that express the humanized DT receptor under the control of the Foxp3 promoter⁴². To reconstitute the Treg cell-depleted B6.Foxp3^{DTR} mice with DT resistant Treg cells, 5 x 10⁵ Treg cells isolated from B6. Foxp3.GFP mice were i.v. injected into B6.Foxp3^{DTR} mice one day prior to sensitization with OVA plus SEA. On day 7 after sensitization, mice were subject to i.t. challenge with the same doses of OVA or CDE; the challenge was repeated 2 times every other day. For Treg cell depletion prior to allergen challenge,

mice (3 mice/experimental group) received 2 consecutive daily injections of DT prior to the first challenge. Three days after the last challenge, BALF was collected by flushing the lungs with 0.75 ml PBS plus 1% FBS 3 times from mice under anesthesia. Afterwards, blood was drained from the lungs by intracardiac extraction. Lungs and mediastinal lymph nodes (MLN) were collected, and lungs were fixed in neutral formalin. BALF cell differentials, 2×10^5 BALF cells were used to prepare BALF cell smear using a Cytospin Cytofuge. The slides will be fixed and stained with Diff-Quick solutions (Fisher Scientific). Eosinophils, macrophages, lymphocytes and neutrophils were counted under a microscope. At least 200 cells were counted for each slide. Lung tissues were analyzed for inflammatory infiltration by hematoxylin and eosin (H&E) staining, and mucus production was determined by Periodic acid–Schiff (PAS) staining.

Airway hyperresponsiveness (AHR) assay

AHR was measured by airway resistance and compliance⁴³, which produces less variation than the whole body plethysmography method. Three days after the last allergen challenge, an individual mouse was anesthetized, and a small cannula was inserted into the trachea. Total of 4 mice/ experimental group were analyzed simultaneously. The mice were mechanically ventilated by the Buxco Elan Series RC instrument (200 breath per min; 0.2 ml tidal volume), and challenged with included inhalation of increasing concentrations of aerosolized solutions (10 μ l) of methacholine (6.25, 12.5, 25, 50 mg/ml, 3 minutes for each challenge). Pulmonary resistance was measured after each dose. Statistical significance of difference was determined by Student's t-test.

Tetramer analyses of OVA specific T cell responses

Mice sensitized with OVA protein and peptide with or without SEA were challenged with OVA protein and peptide for about 10 hrs. Tetramer staining was carried out as previously described⁴⁴. Briefly, BALF and MLN cells from the mice were incubated with a mixture of PE labeled OVA-2C:IA^b and OVA-3C:IA^b tetramers (gifts of Dr. M. Jenkins) at the final concentration of 10 nM of each tetramer in complete cell culture medium supplemented with 2 μ M monensin (Sigma-Aldrich) at room temperature for 1 hr. After washing, the cells were surface stained with fluorochrome-conjugated antibodies against CD4, CD8 and TCR β at 4°C. The cells were then fixed, permeabilized and stained for IL-13 and IFN- γ and analyzed by flow cytometry as described⁴⁴.

RESULTS

Regulation of OVA specific Th differentiation by SEA-activated CD4 T cells

Staphylococcal enterotoxins (SEs) are superantigens that can acutely activate polyclonal CD4 T cells to release cytokines⁴⁵, which in turn could instruct Th differentiation of allergen specific CD4 Tcon cells. To test this, we took advantage of the OVA specific TCR transgenic mice DO11.10, which express a V β 2 TCR that is unresponsive to staphylococcal enterotoxin A (SEA)⁴⁶. OVA specific naïve CD4 Tcon cells were isolated from DO11.10.Foxp3.GFP mice (Balb/c background), and antigen nonspecific naïve Treg cells and CD4 Tcon were isolated from Balb/c.Thy1.1.Foxp3.GFP mice. In mix culture experiments, the OVA specific naïve CD4 Tcon cells and antigen nonspecific

naïve Treg cells were stimulated with OVA₃₂₃₋₃₃₉ peptide and SEA, respectively, in the presence of APC and IL-12, a major cytokine produced by APC in response to *S. aureus* infection⁴⁷. After the differentiation culture, we analyzed the OVA specific CD4 Tcon cells by intracellular staining of the Th1 cytokine IFN- γ and the Th2 cytokine IL-13. As shown in Fig 1A (right panel), in the presence of SEA-activated Treg cells, IFN- γ expression was almost completely abolished whereas IL-13 expression was induced, demonstrating a polarization to Th2 differentiation of the OVA specific CD4 Tcon cells. As expected, without Treg cells the OVA specific CD4 Tcon cells showed robust Th1 differentiation (Fig 1A, left panel). Treg cells must be stimulated by SEA to modulate the Th differentiation of the OVA specific CD4 Tcon cells because Treg cells did not affect the Th differentiation without SEA stimulation (Fig S1). When antigen nonspecific CD4 Tcon instead of Treg cells were used in the mix culture experiments, no IL-13 expression was induced in the OVA specific CD4 Tcon cells and IFN- γ expression was modestly enhanced (Fig 1B).

Expression of IL-4 by Treg cells activated by SEA

IL-4 is the most potent polarizing cytokine for Th2 differentiation. To investigate whether SEA can stimulate IL-4 expression by Treg cells, naïve Treg cells derived from Balb/c.Foxp3.GFP mice were stimulated with APC plus SEA and IL-12. After 4 days, IL-4 was detected in the culture supernatant by ELISA (Fig 2A). The IL-4 was produced by Treg cells because SEA-stimulated non-Treg cells did not produce IL-4 (Fig 2A). We further analyzed acute expression of IL-4 at single cell level by intracellular cytokine staining. Ex vivo spleen cells of Balb/c.Foxp3.GFP mice were stimulated with SEA.

Significant numbers of IL-4 producing cells (~ 4.5%) were detected in the Treg cell population (Fig 2B). To further demonstrate the activation of Treg cells by SEA, we analyze the expression of the T cell activation markers CD69 and CD62L, which are up and down regulated following T cell activation, respectively. Splenic Treg cells of Balb/c.Foxp3.GFP mice i.v. injected with SEA showed up-regulation of CD69 and down-regulation of CD62L as compared with splenic Treg cells from mice i.v. injected with PBS (Fig 2C).

Induction of asthma by sensitization with OVA plus SEA

The experimental allergen OVA is immunologically “inert” in the sense that it does not have intrinsic adjuvant activity³⁰, and therefore is ideal for studying the immune modulatory or “adjuvant” activity of SEA in allergen sensitization. For this purpose, Balb/c mice were i.t. sensitized with OVA alone or OVA plus SEA. One week later, the mice were i.t. challenged with OVA or PBS 3 times and analyzed for asthma features. As shown in Fig 3A and Fig S2, very few ($< 2 \times 10^5$) total BALF cells were detected in mice sensitized and challenged with OVA alone, and those few BALF cells were predominantly macrophages ($> 80\%$). In contrast, in mice sensitized with OVA plus SEA and challenged with OVA alone, total BALF cells dramatically increased ($> 8 \times 10^5$), and eosinophils became the dominant cell type ($> 60\%$) whereas the percentage of macrophages declined ($< 40\%$). The increases of BALF cellularity and eosinophils were OVA specific because they were not observed in mice sensitized with OVA plus SEA and challenged with PBS. H&E staining of lung tissue sections also showed inflammatory infiltration only in the mice sensitized with OVA plus SEA and challenged

with OVA (Fig 3B). PAS staining of the lung tissue sections showed massive mucus production in mice sensitized with OVA plus SEA and challenged with OVA, whereas only modest mucus staining was observed in mice sensitized and challenged with OVA alone (Fig 3C). Airway hyperresponsiveness to bronchial methacholine challenge is a key pathophysiological feature of asthma. Like mucus production, heightened airway resistances to increasing doses of inhaled methacholine were detected in mice sensitized with OVA plus SEA and challenged with OVA, compared with mice sensitized and challenged with OVA alone (Fig 3D).

Effects of transient Treg cell depletion prior to allergen sensitization

To further investigate whether Treg cells activated by SEA at the time of allergen exposure promote asthma pathogenesis in the mouse model of asthma, we wished to use the B6.Foxp3^{DTR} mice in which Treg cells can be transiently depleted by i.p. injection of diphtheria toxin (DT)⁴². The cell depletion in these mice is specific for Treg cells but not other cells⁴⁸. Since the B6.Foxp3^{DTR} mice are on the B6 background, we first confirmed that like the mice of the Balb/c background Treg cells of B6 background also induced Th2 differentiation of allergen specific CD4 Tcon cells. The OTII mice express a transgenic V β 5 TCR specific for OVA that like the DO11.10 TCR is nonreactive to SEA^{46, 49}. In vitro mix cultures were performed like in Fig 1A. In the presence of SEA-activated Treg cells of B6.Foxp3.GFP mice, the OTII CD4 Tcon cells acquired a Th2 phenotype as demonstrated by the expression of IL-13 but not IFN- γ (Fig S3).

We then proceeded to deplete Treg cells in B6.Foxp3^{DTR} by 2 consecutive daily i.p. injections of diphtheria toxin (DT) prior to the day of OVA plus SEA inoculation. Two DT injections deplete > 95% of Treg cells in the B6.Foxp3^{DTR} but not wild type B6 mice on day 3, and the Treg cell population recovers thereafter⁴². For comparison, a group of B6 mice also received the DT injections. B6 mice without DT injections were also included in the study. As shown in Fig 4A, like Balb/c mice, B6 mice sensitized and challenged with OVA alone had very few inflammatory cells in the BALF, and most of these cells were macrophages. In contrast, B6 mice sensitized with OVA plus SEA and challenged with OVA showed dramatic increase of BALF cellularity, and eosinophils became the dominant cell type in the BALF. As expected, DT treatment of B6 mice prior to sensitization with OVA plus SEA resulted in no remarkable changes in the BALF cellularity and cell differentials. In stark contrast, DT treatment of B6.Foxp3^{DTR} mice sensitized with OVA plus SEA and challenged with OVA abolished inflammatory infiltration in the BALF so that the BALF cellularity and cell differentials were similar to those of the B6 mice sensitized and challenged with OVA alone. We further compared the histological features of the DT treated B6 and B6.Foxp3^{DTR} mice (Fig 4B). Lung sections of the DT treated B6 mice showed substantial inflammatory infiltration as revealed by H&E staining. Substantial mucus production was also detected in the lungs of these mice by PAS staining. However, it must be pointed out that both mucus staining and H&E staining of inflammatory cells were weaker than those of the Balb/c mice in Figure 3 perhaps due to strain variation. Unlike in the DT treated B6 mice, H&E and PAS staining showed little inflammatory infiltration and mucus production in the lungs of DT treated B6.Foxp3^{DTR} mice (Fig 4B). Similarly, DT treated B6 but not B6.Foxp3^{DTR}

mice showed elevated airway resistance in response to bronchial challenge with inhaled methacholine (Fig 4C). In contrast to Treg cell transient depletion prior to sensitization, Treg cell transient depletion prior to challenge greatly increased BALF cellularity, and the BALF cells were co-dominated by both eosinophils and lymphocytes (Fig S4). Thus, SEA-activated Treg cells acted specifically in the allergen sensitization phase to promote asthma pathogenesis.

Increase of severity of CDE-induced asthma by SEA

OVA is an experimental and immunologically inert allergen. To further investigate whether SEA can also impact asthma in the mouse model induced by a different allergen that is clinically related and with self-adjuvant activity, we used cat dander extract²⁹ to induce asthma in B6.Foxp3^{DTR} mice. When used alone to sensitize the mice without Treg cell deletion, CDE was sufficient to induce inflammatory infiltration after challenges with CDE as judged by high BALF cellularity ($\sim 1 \times 10^6$). The BALF cells in these mice consisted of a substantial number of eosinophils (> 30%), but macrophages were the more abundant cell population (> 65%) (Fig 5A). When the mice were sensitized with CDE plus SEA and challenged with CDE alone, BALF cellularity increased almost 5-fold and eosinophils became the dominant cell population (> 72%) over macrophages (~16%) (Fig 5A). However, when Treg cells were transiently depleted prior to sensitization, the enhancement of BALF cellularity and eosinophilia was largely abolished, and they were only slightly above those of the mice sensitized with CDE alone (Fig 5A). In airway resistance assays, B6 mice sensitized with CED alone or CDE plus SEA both showed strong airway resistance in response to bronchial challenge with

inhaled methacholine. Nonetheless, a significantly greater response at a high dose of 50 mg/ml of methacholine was observed in mice sensitized with CDE plus SEA (Fig 5B).

Characteristics of early allergen specific T cell responses

Although Th2 responses to allergen are believed to be critical for triggering the clinical symptoms of asthma, T cells of the in vivo immune responses expressing endogenously generated allergen specific TCR (as opposed to transgenic TCR) following allergen challenge have not been well characterized. To address this issue, we used a mixture of OVA-2C:I-A^b and OVA-3C:I-A^b tetramers, which bind to TCRs recognizing 2 distinct epitopes within the OVA₂₂₃₋₃₃₉ peptide⁵⁰, to detect endogenous OVA specific T cells in the OVA/SEA sensitization model. To increase the number of tetramer positive T cells, we supplemented OVA protein with OVA₃₂₃₋₃₃₉ peptide in both sensitization and challenge. Within 8 – 9 hrs following the first OVA challenge, we found that outside the MLN, the T cells were most abundant in the BALF. Within the TCRβ⁺ cells in the BALF, the OVA tetramer⁺ cells were predominantly CD4⁻ although CD4⁺ tetramer⁺ cells were also present, and this was the case for mice either with or without Treg cell transient depletion prior to sensitization (Fig 6A middle panels). As expected, almost all the CD4⁻ tetramer⁺ were also CD8⁻. For detecting OVA-induced cytokine expression, we fixed and permeabilized the BALF cells immediately following tetramer and surface marker staining, then stained intracellular IFN-γ and IL-13. More than a quarter of the CD4⁻ tetramer⁺ BALF T cells were stained positive for IL-13 in mice without Treg cell transient depletion prior to sensitization. IL-13⁺ cells were also detected in the CD4⁺ tetramer⁺ BALF T cells in the same mice but to a lesser extent (~5%). In both cell

populations, no IFN- γ was detected (Fig 6A, upper left and right panels). The negative staining of IFN- γ was not due to technical failure because IFN- γ was robustly stained positive controls. Unlike in the Treg cell sufficient mice, in mice with Treg cell transient depletion prior to sensitization neither IL-13 nor IFN- γ was detected in either the CD4⁻ or the CD4⁺ tetramer⁺ BALF T cells (Fig 6A, lower left and right panels).

The MLN cells were subjected to the same analyses of tetramer staining and cytokine expression (Fig 6C). Modest numbers of tetramer⁺ T cells were detected in mice without Treg cell transient depletion. However, the number of tetramer⁺ T cells increased dramatically in the MLN of mice with Treg cell transient depletion prior to sensitization such that all the CD4⁺ T cells were tetramer⁺. Unlike in the BALF, tetramer⁺ T cells in the MLN were predominantly CD4⁺ although CD4⁻ populations were also detected in both types of mice (Fig 6C, middle panels). Unlike in BALF, IL-13 was not detected in the tetramer⁺ T cells in the MLN (Fig 6C, left and right panels).

We further determined whether reconstituting the Treg cell-depleted mice with Treg cells could restore Th2 cytokine expression. Treg cells isolated from B6.Foxp3.GFP mice were adoptively transferred to the B6.Foxp3^{DTR} mice on the same day of the second DT injection. The mice were sensitized with OVA plus SEA and challenged with OVA as described above. BALF cells derived from the Treg cell constituted and non-constituted mice were analyzed by OVA tetramer and cytokine staining. Similar to mice untreated with DT, IL-13 expression was detected in CD4 negative OVA tetramer positive T cells

from the Treg cell-constituted and DT treated mice but not the non-constituted mice (Fig 7).

DISCUSSION

Although ILC2 cells may also contribute to Th2 cytokine production, Th2 cells are the principal cells responsible for asthma caused by antigen specific recall response to allergen challenge²⁸. However, the fundamental question of why certain individuals but not others develop strong Th2 responses sufficient to drive asthma pathogenesis remains unanswered. Here we showed that SEA promoted asthma caused by sensitization with the inert experimental allergen OVA, which when administered alone, does not induce asthma but instead induces tolerance^{51, 52}. SEA also exacerbated asthma caused by the clinical allergen CDE, which by itself was able to induce asthma with mild eosinophilia. In both cases, the asthma-promoting activity of SEA was dependent on the activation of Treg cells by SEA at the time of allergen sensitization. Like SEA, other SEs are also superantigens, therefore should be able to activate polyclonal Treg cells and promote asthma pathogenesis. A recent large-scale epidemiological study found that 40.7% of asthmatics (vs 28.0% of non-asthmatics) were serum positive for anti-SE IgE indicative of prior exposure to SEs, and there is a strong positive association between anti-SE IgE and asthma (odds ratio of 2.10)⁵³. However, this study measured IgE antibodies against only 3 SEs. So far, a total of 24 SEs (including the classical SEs, SE-like toxins and TSST-1) have been identified⁵⁴. If antibodies against all 24 SEs were measured, the percentage of asthmatics positive for anti-SE antibodies could be much higher. Thus,

while exposure to SEs may not always lead to asthma, SE exposure concurrent with allergen exposure could be a common factor for susceptibility to asthma.

To dissect the immunological mechanisms for the association between SEs and asthma, we have shown that SEA, by activating antigen nonspecific Treg cells, promotes Th2 and concomitantly suppresses Th1 differentiation of OVA specific CD4 T cells. We further demonstrated that SEA-activated Treg cells produced IL-4. Although IL-4 is by far the most potent Th2-polarizing factor, the cellular sources of IL-4 for promoting Th2 responses in various in vivo conditions remain elusive⁵⁵. Basophils have been shown to produce IL-4 in response to the food allergen papain^{56, 57}, which may also cause occupational asthma in workers in the food processing industry⁵⁸. While we could not exclude the contribution of IL-4 derived from other cell types, our studies showed that SE-activated Treg cells could be another source of IL-4 for inducing Th2 responses to inhaled allergens that are more commonly associated with asthma than papain. It should also been pointed out that in addition to promoting Th2 differentiation, our in vitro culture experiments also showed that SEA-activated Treg cells attenuated Th1 differentiation of OVA-specific CD4 Tcon cells. Similar function of Treg cells in modulating Th1/Th2 balance may explain the excessive production of Th1 cytokines in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome caused by the loss-of-function mutation of the Foxp3 gene hence the lack of Treg cells⁵⁹. In fact, the ratio of GATA3 (Th2 master regulator⁷) to T-bet (Th1 master regulator⁶⁰) was lower in Foxp3 deficient mice than in wild type mice⁶¹. Furthermore, Treg cells with

defects in the micro RNA miR-146a and the Itch E3 ubiquitin ligase cause excessive Th1 and Th2 responses, respectively^{62, 63}.

The frequency and ability of Treg cells to produce IL-4 may explain some discrepancies of epidemiological findings in humans. The positive association between asthma and *S. aureus* colonization and/or SE exposure is most evident in adolescents and young adults^{36, 53}, whereas one study found that neonatal colonization with *S. aureus* is not associated with asthma later in life⁶⁴. A potential explanation for the discrepancy could be the degrees of the maturity of the immune system. In mice, it is known that there is a lack or paucity of Treg cells in neonates (35, 36). In humans, a general lack of Treg cells may not be a feature of the neonatal immune system, but there are clear variations of Treg cell frequencies among individuals. In fact, higher frequencies of Treg cells in neonates are positively associated with asthma in later life⁶⁵, which is consistent with our finding that SE-activated Treg cells promote allergen specific Th2 differentiation in the sensitization phase. Studies of the ability of human Treg cells to produce IL-4 upon stimulation by SEs will further clarify the role of Treg cells in asthma.

The throat is the primary site of *S. aureus* colonization in the respiratory tracks^{66, 67}. Therefore, trachea and lower airways are natural sites of exposure to SEs excreted by the throat-colonizing bacteria or inhaled as environmental contaminants. Our studies demonstrated that a single i.t. inoculation of allergen plus SEA was an effective model of allergen sensitization for asthma. The 70% lethal dose of SEA in mice is 0.3 µg/g body weight⁶⁸, so the SEA dose used in this study for asthma induction (~ 0.025 µg/g body

weight) is a safe dose. The lethal dose of SEA in humans or primates has not been determined, there is also a lack of information on the precise levels of SEA resulted from *S. aureus* colonization or septic shock. In addition, human T cells are much more sensitive to SEs than mouse T cells. Therefore, it is difficult to draw comparison between our experimental dose of SEA and the exposure levels in humans. Nonetheless, using our mouse model and OVA:IA^b tetramers, we unveiled several unique features of early allergen specific T cell responses in the airways and MLN. Within less than 10 hrs of the first allergen challenge, OVA specific T cells in BALF were dominated by CD4⁻ population while significant numbers of CD4⁺ T cells were also detected. Unlike in the BALF, in the MLN, CD4⁺ T cells were the dominant OVA specific T cells although OVA specific CD4⁻ T cells were also found. Whether the CD4⁻ T cells in the BALF, as well as in the MLN, were derived from CD4⁺ T cells that had lost CD4 expression upon allergen stimulation or they were originally CD4⁻ remains a question. Nonetheless, this finding suggests that targeting CD4 may not be an effective strategy for mitigating allergen specific T cell responses in asthmatic patients.

OVA-stimulated IL-13 expression was detected mostly in the OVA specific CD4⁻ T cell population and to a lesser extent in the CD4⁺ T cell population in the BALF of Treg cell sufficient mice but not of mice with Treg cell transient depletion prior to allergen sensitization. This demonstrated a key role of Treg cells for in vivo Th2 responses. In contrast, OVA-stimulated IL-13 expression was not detected in the T cells of the MLN of mice regardless of Treg cell transient depletion. Here we propose two alternative explanations for the difference in Th2 cytokine expression between BALF and MLM T

cells. In the first alternative, we assume that the T cells in the BALF are originated from MLN, and that priming of allergen specific T cells in the MLN occurs over a period of time. Since release of cytokines such as IL-4 by SEA-activated Treg cells is a transient event, only the early wave(s) of allergen specific T cells are subject to Th2 polarization by the SEA-activated Treg cells. Such early-activated T cells are more likely to exit the MLN and migrate to the airways. In contrast, the T cells remaining in the MLN are likely those primed at later time points when the Th2 polarizing effect of SEA-activated Treg cells has dissipated so that these T cells remain undifferentiated therefore do not produce IL-13. In the second alternative, we suspect that the T cells in the BALF are primed in situ and preferentially differentiate into Th2 cells due to the direct exposure of antigen nonspecific Treg cells to SEA. In support of this scenario, Constant and colleagues have shown that antigen-loaded dendritic cells do indeed stay in the lung and prime T cells in situ without the involvement of lymph nodes and spleen ⁶⁹.

In conclusion, we have presented experimental evidence that SEs act as natural adjuvants by activating allergen nonspecific Treg cells to promote Th2 responses to allergens, thereby promote asthma pathogenesis in the mouse model of asthma. These findings suggest that attenuating the activation of Treg cells by SEs in the allergen sensitization phase could help prevent asthma and possibly other allergic diseases.

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Figure Legends

Fig. 1. Regulation of Th differentiation of OVA specific CD4 T cells by SEA. (A) OVA specific naïve CD4 Tcon cells ($CD4^+CD62L^{high}KJ126^+GFP^-$) from DO11.10.Foxp3.GFP mice were stimulated with APC plus OVA₃₂₃₋₃₃₉ peptide, IL-12 and SEA in the presence or absence of naïve Treg cells ($CD4^+CD62L^{high}KJ126^-GFP^+$) from Balb/c.Thy1.1.Foxp3.GFP mice. Cells were analyzed for cytokine expression on day 6 of culture. (B) Experiments were set up as in (A) except that naïve antigen nonspecific CD4 Tcon cells of Balb/c.Thy1.1.Foxp.GFP mice were used in the co-cultures. After the

differentiation culture, the cells were stimulated with PMA and ionomycin and analyzed for intracellular cytokine expression by flow cytometry. Dot plots of IL-13 and IFN- γ expression in CD4⁺KJ126⁺GFP⁻ cells of representatives of 3 experiments are shown. Numbers in the dot plots are the percentages of cells.

Fig. 2. IL-4 expression by SEA-activated Treg cells. (A) Naïve Treg cells from Balb/c.Foxp3.GFP mice mixed with APC or APC alone were stimulated SEA in the presence of IL-12. On day 4, IL-4 in the culture supernatants was measured by ELISA. (B) Ex vivo spleen cells of Balb/c.Foxp3.GFP mice were either un-stimulated or stimulated with SEA in the presence of monensin for 6 hrs. IL-4 expression was detected by intracellular cytokine staining. Dot plots of Treg cells (CD4⁺GFP⁺) are shown. (C) Balb/c.Foxp3.GFP mice were i.v. injected with either PBS (-SEA) or 2 μ g SEA (+SEA), 6 – 7 hrs later spleen cells were stained for cell surface marker. Dot plots of CD69 and CD62L staining on gated Treg cells (CD4⁺GFP⁺) are shown. Numbers in the dot plots indicate the percentages of cells. Representatives of one of two experiments are shown.

Fig. 3. Analyses of asthma features of OVA sensitized mice. (A) Balb/c mice were i.t. sensitized with OVA alone or together with SEA, and i.t. challenged with OVA or PBS as indicated. On day 3 after the last challenge, BALF were collected from the mice. Total BALF cells and percentages of eosinophils and macrophages were determined. Averages and standard deviations of 3 mice per group of a representative of 3 experiments are shown. (B) Histology of H&E stained lung sections of mice in (A). (C) PAS staining of lung sections of mice sensitized with OVA or OVA plus SEA and

challenged with OVA in (A). (D) Mice sensitized with OVA or OVA plus SEA were challenged with OVA as in above. On day 3 after the last challenge, the mice were subject to bronchial challenge with increasing doses of methacholine. Pulmonary resistance at each dose of methacholine challenge was measured. Averages and standard deviations of 4 mice per group in 1 of 2 experiments are shown.

Fig. 4. Effects of Treg cell transient depletion prior to sensitization with OVA plus SEA on asthma. (A) Wild type B6 mice or B6.Foxp3^{DTR} mice (FDR) received 2 consecutive daily i.p. injections of DT prior to the day of sensitization with OVA plus SEA. Mice were challenged with OVA as in Figure 3 A. Total cell numbers and percentages of eosinophils and macrophages in the BALF were determined. Averages and standard deviations of 3 mice per group of a representative of 3 experiments are shown. (B) Sections of lungs of the same mice as in (A) were subject to H&E or PAS staining. (C) Mice treated as in (A) were subject to bronchial methacholine challenges, and pulmonary resistances were measured. Averages and standard deviations of 4 mice per group in 1 of 2 experiments are shown.

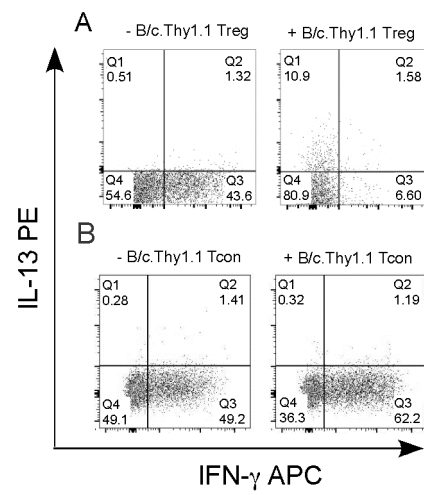
Fig. 5. Effects of SEA and Treg cell transient depletion on CDE-induced asthma. (A) B6.Foxp3^{DTR} mice were treated with or without 2 consecutive i.p. injection of DT prior to i.t. sensitization with CDE alone or plus SEA, and challenged with CDE. Total cell numbers and percentages of eosinophils and macrophages in the BALF were determined. Averages and standard deviations of 3 mice per group are shown. (B) B6.Foxp3^{DTR} mice sensitized with CDE alone or plus SEA and challenged with CDE as in (A) were subject

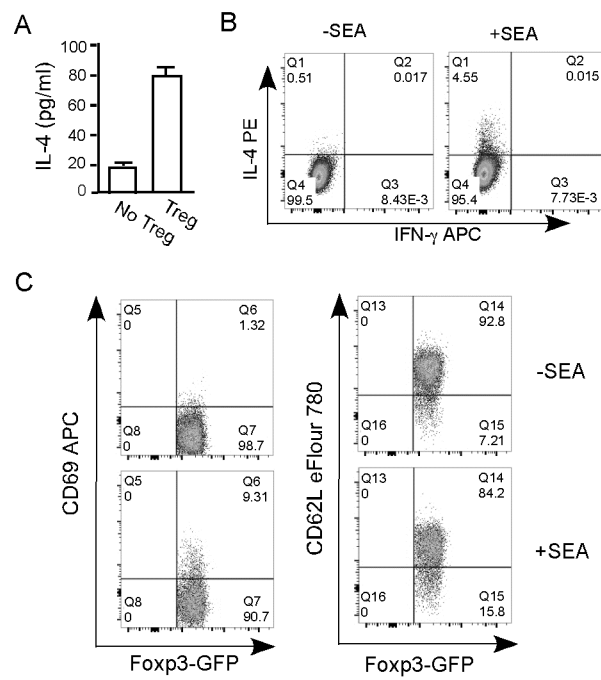
to bronchial challenge with increasing doses of methacholine. Pulmonary resistances were measured. Averages and standard deviations of 4 mice per group are shown. Statistical significance of difference was determined by Student's t-test. Representatives of 2 experiments are shown.

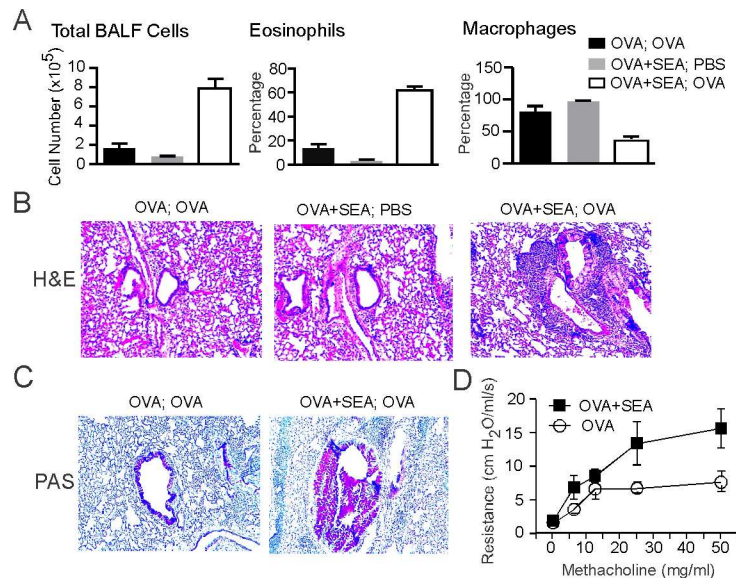
Fig. 6. Characterization of early T cell response to allergen challenge. (A) B6.Foxp3^{DTR} mice (FDR) were treated with or without 2 consecutive daily i.p. injections of DT prior to i.t. sensitization with OVA and OVA₃₂₃₋₃₃₉ peptide plus SEA, then i.t. challenged once with OVA and OVA₃₂₃₋₃₃₉ peptide for 8 – 9 hrs. The BALF cells were sequentially stained with PE-labeled OVA-2C:IA^b and OVA-3C:IA^b tetramers and a cocktail of antibodies against surface markers. The cells were then fixed, permeabilized and stained for IL-13 and IFN- γ . Middle panels are dot plots of CD4 and OVA tetramer staining gated on the TCR β ⁺ single T cells. Dot plots of IL-13 and IFN- γ staining of the CD4⁺ (left) and CD4⁺ tetramer⁺ (right) cells are shown. (B) BALF cells of B6 mice sensitized and challenged with keyhole limpet hemocyanin (KLH) were stained with the OVA tetramers and CD4, as negative control of tetramer staining. (C) MLN cells of the same mice as in (A) were treated and analyzed as in (A). (D) MLN cells of the same mice as in (B), as negative control of tetramer staining of MLN cells. Numbers in all plots are percentages of the cell populations. Representatives of 3 experiments are shown.

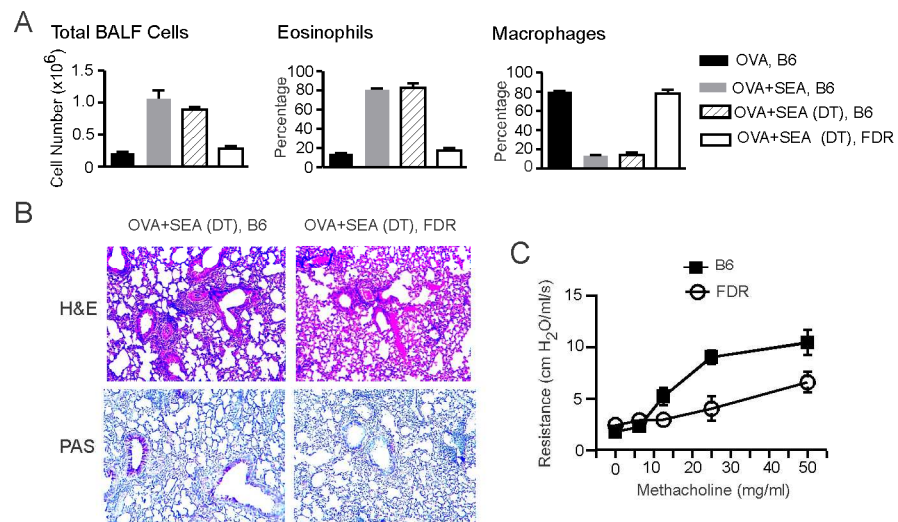
Fig. 7. Restoration of IL-13 expression by Treg cell reconstitution. B6.Foxp3^{DTR} mice (FDR) were treated with 2 consecutive daily i.p. injections of DT prior to i.t. sensitization with OVA and OVA₃₂₃₋₃₃₉ peptide plus SEA. On the day of the second DT injection,

801 mice received either adoptive transfer of Treg cells derived from B6.Foxp3.GFP mice
802 (FDR + DT + Treg) or PBS (FDR + DT). The mice were then sensitized and challenged,
803 and BALF cells were analyzed as in Fig 5. Middle panels are dot plots of CD4 and OVA
804 tetramer staining gated on the TCR β^+ T cells. Dot plots of IL-13 and IFN- γ staining of
805 the CD4 $^-$ (left) and CD4 $^+$ tetramer $^+$ (right) cells are shown. Numbers in all plots are
806 percentages of the cell populations. Representatives of 2 experiments are shown.

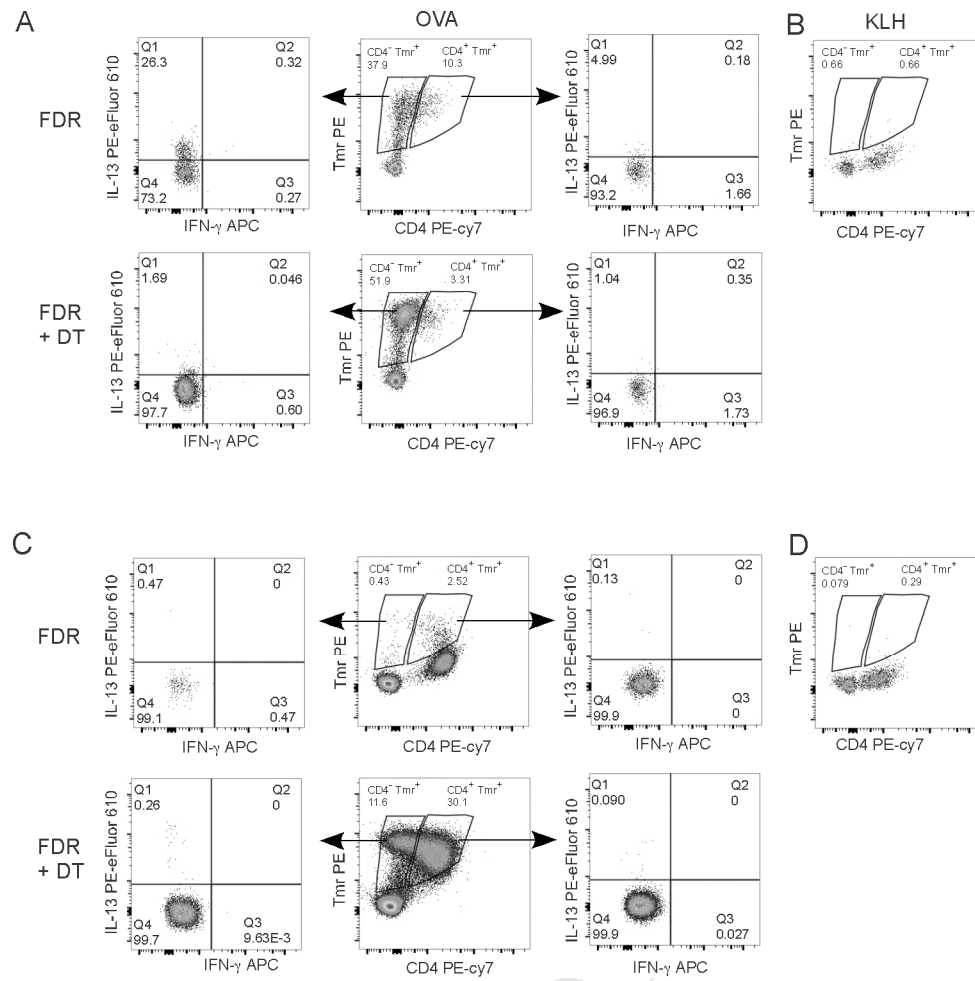


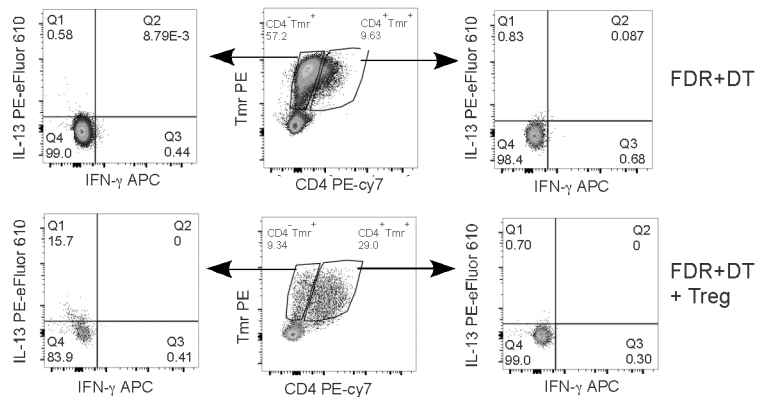












Supplemental Figure Legends

Figure S1. In vitro Th differentiation of OVA specific CD4 Tcon cells in the absence of SEA. OVA specific naïve CD4 Tcon derived from DO11.10.Foxp3.GFP mice were mixed with or without antigen nonspecific naïve Treg cells derived from Balb/c.Thy1.1.Foxp3.GFP mice. The cells were stimulated with APC plus OVA₃₂₃₋₃₃₉ peptide and IL-12. On day 6 of culture, cells were stimulated with PMA and ionomycin. IFN- γ and IL-13 were measured by intracellular cytokine staining. Dot plots of CD4⁺KJ126⁺GFP⁻ populations are shown. Numbers are percentages of cells in each quadrant.

Figure S2. BALF cell differential staining. Balb/c mice were i.t. sensitized with OVA or OVA plus SEA, and challenged with either OVA or PBS as indicated. BALF smear slides were stained with Diff-Quick solutions. Microscopic pictures of the stained slides are shown.

Figure S3. In vitro Th differentiation of OVA specific CD4 Tcon cells of OTII.Foxp3.GFP mice. OVA specific naïve CD4 Tcon cells derived from OTII.Foxp3.GFP mice were mixed with or without antigen nonspecific naïve Treg cells derived from B6.Foxp3.GFP mice. The cells were stimulated with APC plus OVA₃₂₃₋₃₃₉ peptide, SEA and IL-12. On day 6 of culture, cells were stimulated with PMA and ionomycin. IFN- γ and IL-13 were measured by intracellular cytokine staining. Dot plots

of the OTII CD4 Tcon cells ($CD4^+TCR V\beta5^+$) are shown. Numbers are percentages of cells in each quadrant.

Figure S4. Effects of Treg cell deletion after sensitization and prior to allergen challenge on asthma. B6.Foxp^{DTR} mice were i.t. sensitized with OVA or OVA plus SEA for 1 week. Prior to i.t. challenge with OVA, mice were i.p. injected with or without DT to deplete Treg cells. The mice were then i.t. challenged 3 times with OVA. BALF from the mice were collected. Total numbers of cells, percentages of eosinophils, macrophages and lymphocytes in the BALF are shown.

