

Resolution of Der p1-Induced Allergic Airway Inflammation Is Dependent on CD4⁺CD25⁺Foxp3⁺ Regulatory Cells¹

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Allergic airway inflammation (AAI) is characterized by airway hyperreactivity, eosinophilia, goblet cell hyperplasia, and elevated serum IgE, however, it is unclear what mediates natural resolution after cessation of allergen exposure. This is important because the outcome of subsequent allergen challenge may depend on the concurrent inflammatory milieu of the lung. Using a murine AAI model, we demonstrate that after exposure to a defined natural protein allergen, Der p1, the response in lungs and draining mediastinal lymph nodes (dMLN) peaks between 4 and 6 days then declines until resolution by 21 days. Der p1-specific serum IgE follows the same pattern while IgG1 continues to increase. Resolution of AAI is mediated by CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), which appear in lungs and dMLN following airway challenge. Treg depletion exacerbated lung eosinophilia, increased dMLN IL-5 and IL-13 but not IL-10 secretion, and increased allergic Ab responses. Most convincingly, transfer of CD4⁺CD25⁺Foxp3⁺ T cells from Ag naive mice (natural Tregs) abolished AAI, decreased dMLN IL-5 and IL-13 secretion, increased dMLN IL-10 secretion, abolished IgE, and decreased IgG1 Abs. Blocking IL-10 receptor function in vivo did not block the anti-inflammatory function of transferred natural Tregs but did restore dMLN IL-5 and IL-13 secretion. Thus natural Tregs can control AAI in an IL-10 independent manner. *The Journal of Immunology*, 2007, 179: 7050–7058.

Allergic asthma, which is reaching epidemic levels in the Western world (1), is triggered by inhalation of environmental allergens like cat dander, pollen, and house dust mite proteins (2). One of the major dust mites in the United Kingdom is *Dermatophagoides pteronyssinus* (2), which secretes the allergenic cysteine protease Der p1 in feces (3). Inhalation of allergens in sensitive individuals leads to a cascade of immune responses characterized by allergen-specific IgE, mastocytosis, goblet cell hyperplasia and eosinophilia, contributing to airway hyperreactivity and symptomatic disease (1–4).

There are difficulties in the study of asthma in patients with established disease, and murine models have been extensively used to examine the role of the acquired immune response in induction of allergic airway inflammation (AAI)⁵ in sensitized

animals (3, 5–11). Most of these studies examine events within the lungs 3–4 days after challenge, but there are fewer studies on the kinetics of resolution of disease markers after cessation of allergen exposure (12). The most widely used model system is OVA-induced allergy. However, although OVA is a gut allergen in humans (13), which may induce respiratory symptoms in a minority of patients when ingested (13), it is not a natural inducer of asthma. Using such models, the essential role of T cells in induction of AAI through Th2 development and the secretion of the cytokines IL-4, IL-5, IL-9, and IL-13 is well documented (14, 15). However, how T cells control the local airway immune response and their role in resolution of lung inflammation is still under characterized. It is widely accepted that allergic disease and asthma may be linked to a dysregulation in immune balance and in recent years, regulatory T cells (Tregs) have been demonstrated to play an essential role control of both innate and adaptive immune responses during Th2-mediated AAI (11, 16–18). Other studies have used murine models of OVA-induced AAI and TCR transgenic animals to determine the role of Ag-specific Tregs in induction and resolution of allergen-induced inflammation (11, 19, 20). Adoptive transfer of Ag-specific Tregs was reported to prevent induction of OVA-induced airway hyperreactivity, lung eosinophilia, and Th2 cytokine production in the lung (11). In a study using whole house dust mite extract as allergen, Lewkowich et al. (18) showed that depleting anti-CD25 Ab given to C3H mice before sensitization enhanced the allergic airway response but had no effect on the response in A/J mice and also increased the ability of lung DCs from C3H but not A/J mice to present the Ag.

However, there is still little information on the normal role of Tregs in resolution of airway inflammation or on whether transgenic Ag-specific Tregs act differently from naturally occurring or endogenously generated Tregs in sensitized wild-type animals. Naturally occurring Tregs constitutively express both CD25 and Foxp3 and constitute up to 10% of the peripheral T cell pool in naive animals (21). CD25⁺CD4⁺Foxp3⁺ T cells regulate immune

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⁵ Abbreviations used in this paper: AAI, allergic airway inflammation; dMLN, draining mediastinal lymph node; i.t., intratracheal; Treg, regulatory T cell; BAL, bronchoalveolar lavage.

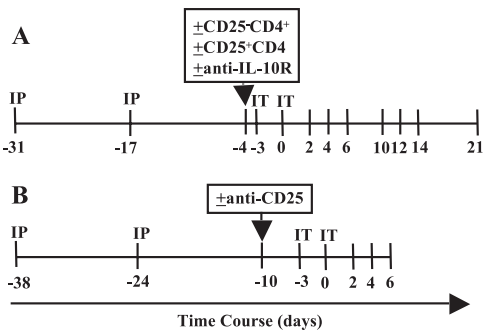


FIGURE 1. Experimental model. C57BL/6 mice were sensitized i.p. $\times 2$ with 10 μ g of Der p1 in alum and challenged i.t. $\times 2$ with either PBS (mock challenged) or 10 μ g of Der p1 in PBS (allergen challenged). To investigate resolution of inflammation, mice were culled at various time points from 2 to 21 days after challenge (protocol A). To investigate the role of IL-10 and CD25⁺ Tregs in this system, CD25⁺CD4⁺, CD25⁻CD4⁺ T cells, and anti-IL-10R (1B1.2) or isotype control Ab were administered 1 day before i.t. challenge (protocol A) or anti-CD25 (PC61), or isotype control mAb was administered 1 wk before i.t. challenge (protocol B).

responses through the secretion of immunosuppressive cytokines such as IL-10 and TGF β (21–23). Previous work showed that induction of nasal tolerance to Der p1 inhibits lung inflammation and

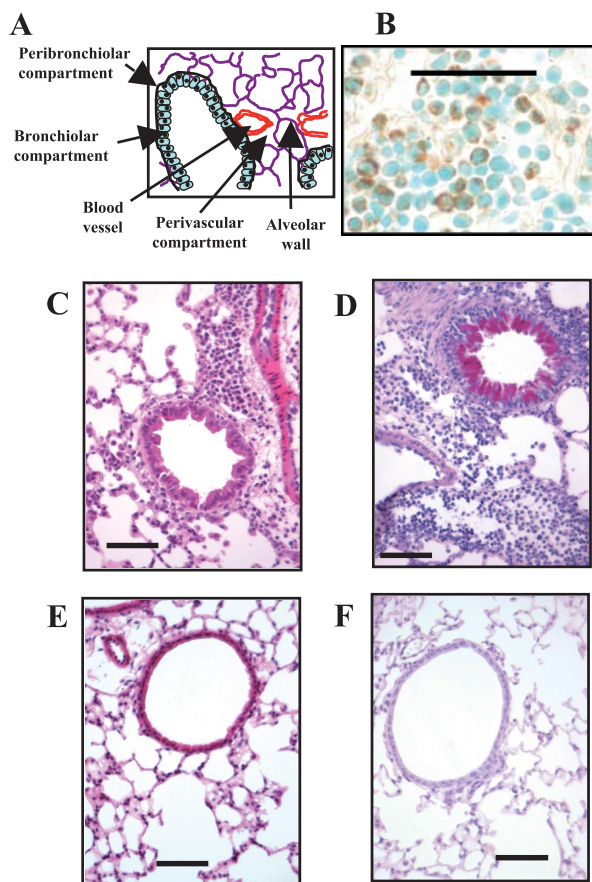


FIGURE 2. Histological analysis of lung tissue. A, Schematic representation of the lung compartments assessed for inflammation. B–D, Sections from lungs 4 days after second i.t. challenge with Der p1: peribronchiolar infiltrate stained with anti-eosinophil Ab (B); H&E stain (C); periodic acid-Schiff stain (PAS) for Goblet cells (D). E and F, Sections from lungs 4 days after second i.t. challenge with PBS: H&E stain (E); periodic acid-Schiff stain (PAS) for Goblet cells (F). Black bars, 100 μ m.

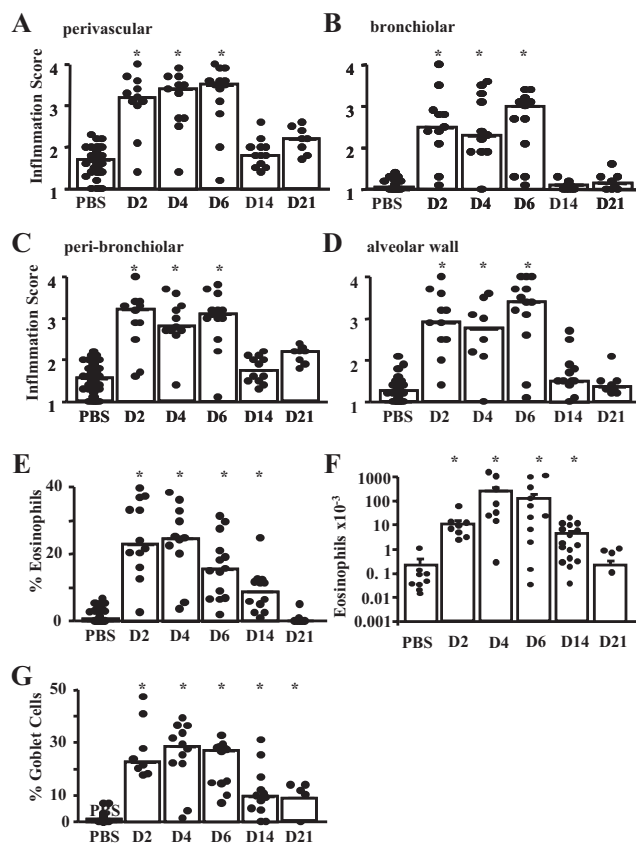


FIGURE 3. Der p1 airway challenge induces acute lung inflammation, eosinophilia, and goblet cell hyperplasia. Interstitial lung inflammation was scored in perivascular (A), bronchiolar (B), peri-bronchiolar (C), and alveolar wall compartments (D). E, Percentage of eosinophils in interstitial infiltrate; numbers of eosinophils recovered in BAL (F); percentage of goblet cells in airway epithelium (G). Data accumulated from three experiments (3–4 mice/group/experiment). (D, day post challenge). ●, Individual mice; bars, median value per group; PBS, pooled results from mock-challenged mice; *, Significant difference (Mann-Whitney) from mock-challenged mice ($p < 0.001$).

is associated with increased IL-10 secretion (10) although it was not investigated whether Tregs were involved.

This paper focuses for the first time on the role of CD25⁺CD4⁺Foxp3⁺ Tregs in control of AAI induced by the defined, natural airway allergen, house dust mite-derived native Der p1 protein, in sensitized animals with an intact nontransgenic immune system. We have used both depletion and adoptive transfer of Tregs to look at disease markers, Treg migration and function, and immune responses in both lungs and draining mediastinal lymph nodes (dMLN). Our data demonstrate a clear, novel role for naturally occurring CD25⁺CD4⁺Foxp3⁺ T cells. Upon airway challenge of Der p1-sensitized mice Tregs migrate into both lungs and dMLN and peak in number just before the start of resolution of disease markers. Depletion of CD25⁺CD4⁺Foxp3⁺ cells before airway challenge exacerbates AAI leading to elevated eosinophilia in both lung tissue and bronchoalveolar lavage, elevated titers of Ag-specific IgE, and expanded populations of Th2 cytokine-producing cells in the lymph node. Furthermore, adoptive transfer of CD25⁺CD4⁺Foxp3⁺ Treg from Ag naive syngeneic mice before airway challenge leads to their recruitment to dMLN, and both abrogates lung inflammation and the allergen-induced immune response within the dMLN. Surprisingly, blocking IL-10R function had no effect on the function of these Ag naive CD25⁺CD4⁺Foxp3⁺ Tregs in preventing lung inflammation but

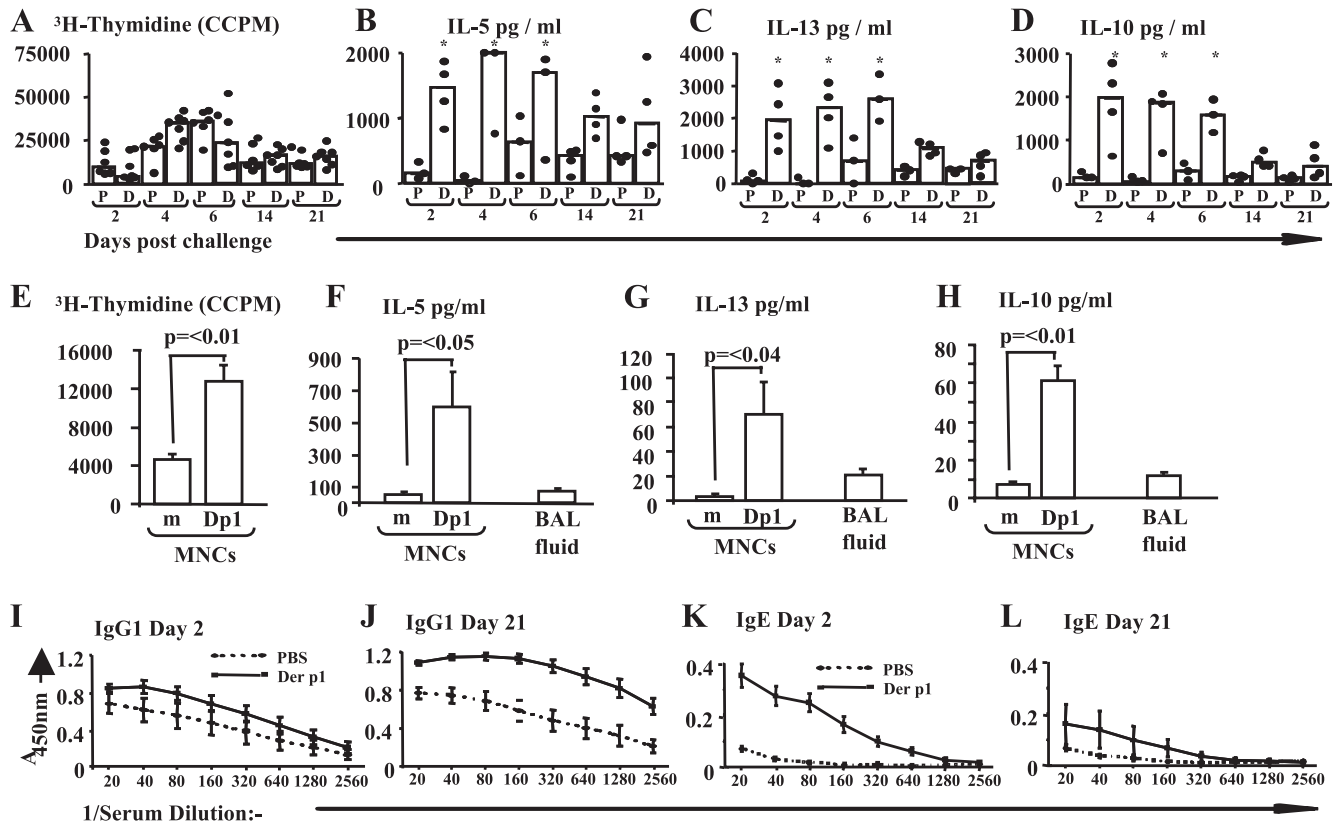


FIGURE 4. Systemic immune responses to Der p1 airway challenge. See *Results and Materials and Methods* for further details. *A–D*, In vitro responses of dMLN cells from individual mice 2, 4, 6, 14, and 21 days after Der p1 (D) or PBS challenge (P) in vivo. Representative data from four experiments ($n = 3–5$ /group/experiment); bars = median group value. *A*, [^3H]Thymidine incorporation at 72 h; *B–D*, cytokine secretion at 48 h. Cells cultured in medium alone secreted <100 pg/ml of any cytokine. *E–H*, Representative experiment ($n = 4$) showing responses of mononuclear cells isolated from Der p1-challenged lungs at 4 days post challenge. Lung mononuclear cells (MNCs) also proliferate in response to Der p1 (Dp1) but not medium only (m) and produce IL-5, IL-13, and IL-10. *F–H* also show that BAL fluid from the same animals contained detectable levels of IL-5, IL-13, and IL-10. Representative data from four experiments ($n = 3–5$ /group/experiment) showing serum Ab responses on day 2 (*I* and *K*) and day 21 (*J* and *L*) post challenge for Der p1-specific IgG1 (*I* and *J*), and IgE (*K* and *L*). A, absorbance. Differences were considered significant (Mann-Whitney) when $p < 0.05$ (*).

did restore the ability of dMLN cells to secrete Th2 cytokines suggesting that Treg control of AAI and effector T cell expansion in lymph nodes operate through different mechanisms. Overall, this study identifies a clear role for $\text{CD}25^+\text{CD}4^+\text{Foxp}3^+$ Tregs in control and in resolution of AAI.

Materials and Methods

Animals

Female C57BL/6J mice (6–8 wk old) (Harlan-Olac) were housed in conventional specific and opportunistic pathogen-free facilities. All experiments were performed in accordance with the U.K. Home Office Scientific Procedures Act (1986) and local ethical approval.

Sensitization, airway challenge, and in vivo Ab treatment

As shown in Fig. 1, mice were sensitized by two i.p. injections of $10 \mu\text{g}$ of Der p1 absorbed on 2.25 mg of aluminum hydroxide (Imject ALUM; Pierce) in $100 \mu\text{l}$. AAI was induced by two intratracheal (i.t.) instillations of $10 \mu\text{g}$ of Der p1 in $50 \mu\text{l}$ of PBS in anesthetized animals (0.1 mg/g Avertin; 2,2,2-tribromoethanol). Sensitized control mice were mock challenged with PBS. Der p1 was provided by Prof. Wayne Thomas (Institute of Child Health, Australia) or purchased from Indoor Biotechnology. Mice were treated i.p. with $250 \mu\text{g}$ of depleting anti-CD25 Ab (clone PC61), 7 days before airway challenge or with $500 \mu\text{g}$ of anti-IL-10R-blocking Ab (1B1.2) 1 day before challenge. Control mice received isotype control i.p. All Abs were purified from hybridoma supernatants using protein G-coupled Sepharose beads (Amersham Biosciences).

Cell purification

$\text{CD}4^+\text{CD}25^+$ cells were isolated from spleens of naive mice to 92% ($\pm 3\%$) purity using MACS bead mouse $\text{CD}4^+\text{CD}25^+$ Treg isolation kit

(Miltenyi Biotec). $\text{CD}4^+\text{CD}25^-$ cells were isolated from the negative fraction using MACS $\text{CD}4^+$ positive selection kit to a purity of 98% ($\pm 2\%$). All isolated $\text{CD}25^+\text{CD}4^+$ cells were also $\text{Foxp}3^+$ by flow cytometry. Preliminary experiments established that 5×10^5 Treg cells per recipient i.v. gave consistent results. To track transferred Tregs in vivo, cells were labeled with $5 \mu\text{M}$ CFSE before being transferred to Der p1-sensitized animals 1 day before i.t. challenge.

Bronchoalveolar lavage (BAL)

Mice were killed by i.p. injection of pentobarbitone. The trachea was exposed and cannulated with a 27-gauge needle encased in 0.96-mm silicon tubing (Portex). Lungs were lavaged three times with $500 \mu\text{l}$ of PBS followed by $800 \mu\text{l}$ of fresh PBS also instilled three times. Samples were centrifuged at $300 \times g$ for 5 min at 4°C , and supernatant from the first wash stored at -20°C . Cell pellets were resuspended in $500 \mu\text{l}$ of PBS and cytopins prepared by cytocentrifugation (Shandon) at 300 rpm for 3 min. Slides were air-dried, methanol fixed, and stained with Diff Quik (CellPath Store). Differential cell counts were performed blinded to experimental details (by S.E.M.H.).

Histological and immunohistochemical analysis of lung tissue

After BAL, lungs were perfused with PBS and for histological examination were inflated with and fixed in 4% neutral buffered formalin before paraffin embedding. Sections ($3 \mu\text{m}$) were stained with H&E for assessment of inflammation and periodic acid-Schiff for goblet cell hyperplasia. Inflammation was scored for each mouse at $\times 200$ magnification by averaging the score of 10 consecutive fields where the lungs were correctly inflated and the field contained a complete transsection of at least one bronchiole (less than half a field width/ $300 \mu\text{m}$ in diameter), blood vessels, and alveolar airway. Inflammation was scored on an increasing severity score of 1–4 in the perivascular compartment (1, no cells; 2, <20 cells; 3, <100 ; and 4, >100 cells); the bronchiolar epithelium (1, no cells; 2, <5 ; 3, <10 ; and 4,

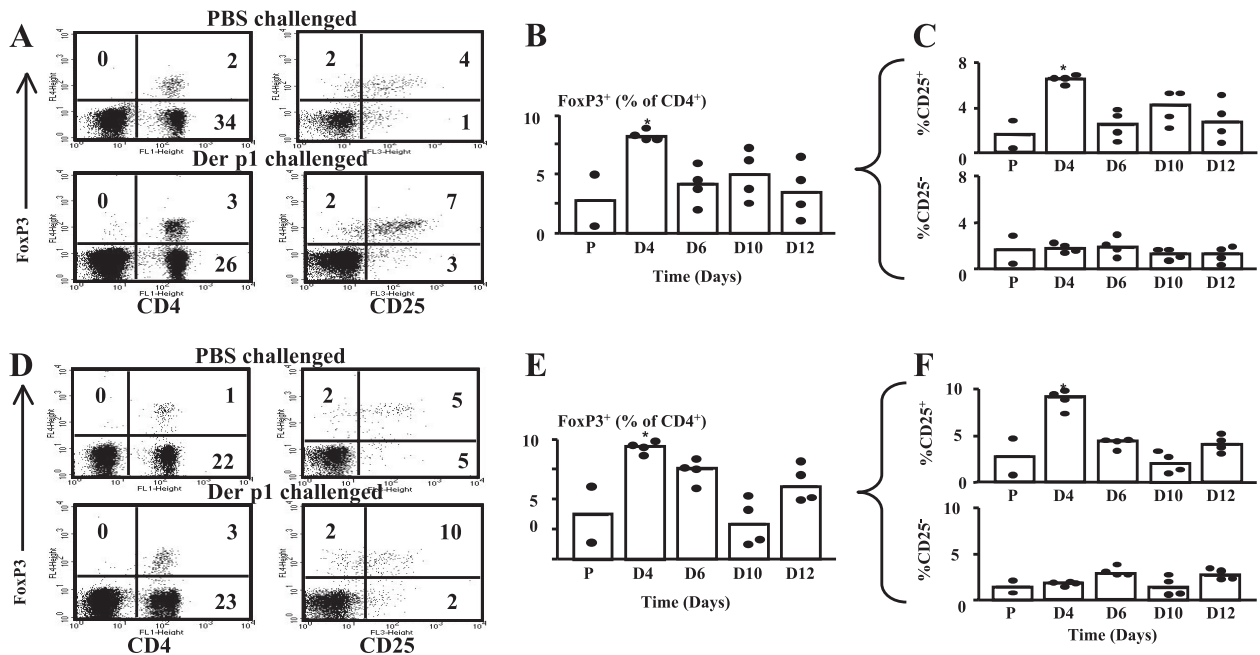


FIGURE 5. Detection of $CD4^{+}Foxp3^{+}CD25^{+}$ and $CD25^{-}$ cells in draining lymph node and lung during AAI. Representative data of two experiments, $n = 4-8$ mice/group/experiment. dMLN (A–C) and lung (D–F) mononuclear cells from Der p1- or PBS-challenged mice were stained for CD4, CD25, and Foxp3. To obtain sufficient cells for analysis after Der p1 challenge, dMLN and lungs were pooled from sets of two animals (4 pools, 8 mice, per experimental group) 2, 4, 6, 10, and 12 days after challenge; PBS-challenged mice (P) had very small lymph nodes and no lung inflammation so cells were pooled from sets of 4 animals (2 pools, 8 animals) on day 4 only. Representative dot blots are shown (A and D), followed by graphical representation of the percentage of Foxp3⁺ cells in the CD4 gate (B and E) and then the percentage of $CD4^{+}Foxp3^{+}CD25^{+}$ and $CD4^{+}Foxp3^{+}CD25^{-}$ cells in dMLN (C) and lungs (F). Bars, median group value. *, Significant difference from PBS-treated controls (Mann-Whitney, $p < 0.05$).

>10 cells); the peribronchiolar alveolar tissue (1, no cells; 2, <20; 3, <100; and 4, >100 cells); and the alveolar walls (1, normal; 2, focal cellular expansion of the alveolar walls by 2–3 cells; 3, by 4–5 cells; 4, >5 cells). Eosinophilia was determined as the percentage of infiltrating cells in lung tissue (not airspaces) and was additionally confirmed in some experiments with a monoclonal eosinophil-specific Ab supplied by Dr. J. Lee (Mayo Clinic, Rochester, MN) (24). Goblet cell hyperplasia was scored on 10 airways at a magnification of $\times 400$ and is expressed as the average percentage of goblet cells/airway. Histological scores were performed blinded to experimental details (S.E.M.H.).

Lymph node and lung lymphocyte restimulation

Single cell suspensions of dMLN and PBS perfused whole lungs were made by passing tissues through $40\text{-}\mu\text{m}$ sterile sieves. Lung cells were then spun over Lympholyte-M (V_{H} -Bio) to isolate mononuclear leukocytes. Cells were washed, resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, and 50 μM 2-ME; Sigma-Aldrich) plated in 96-well microplates at 5×10^5 cells/well and cultured with medium alone or 50 $\mu\text{g}/\text{ml}$ Der p1. Because numbers of cells were always limiting, preliminary experiments indicated that removing 48 h supernatants for cytokines, labeling the same wells with 0.5 μCi [^3H]thymidine and counting incorporation at 72 h in a beta plate scintillation counter (Wallac U.K.) gave consistent results. Supernatants were stored at -80°C before analysis using a mouse inflammatory cytokine bead array (BD Biosciences) and commercially available ELISA kits (IL-5, BD Biosciences; IL-13, R&D Systems). In some experiments, proliferation was measured by flow cytometry using a live lymphocyte gate following incubation of cells with 5 μM CFSE for 15 min at 37°C , followed by washing, before culturing for 72 h as above.

Analysis of Tregs

Single cell suspensions from dMLN or lungs were resuspended at 5×10^6 cells/ml and stained with extracellular CD4 and CD25 (BD Pharmingen) and intracellular Foxp3 as recommended by the manufacturer (eBioscience). CD25 depletion was confirmed by analysis with αCD25 Abs (clones 7D4 and 3C7) and flow cytometry on a BD FACSCalibur using CELLQuest software.

Der p1-specific IgG1, IgG2a, and IgE

Microtiter wells were coated with 0.25 μg of Der p1 in 50 μl of 0.05 M carbonate bicarbonate buffer (pH 9.6). Nonspecific binding was blocked with 3% BSA in PBS. For IgG1 and IgG2a^b analysis, sera were double diluted from a 1/20 dilution in PBS-T (PBS containing 0.05% Tween 20). Bound Ab was detected using biotinylated rat anti-mouse IgG1 (clone LO-MG1-2; Serotec) or biotinylated mouse anti-mouse IgG2a^b (clone 5.7; BD Pharmingen). For IgE measurement, sera were depleted of IgG using protein G-coupled beads (25) and double diluted from a 1/10 dilution in PBS-T. Bound Ab was detected using biotinylated rat anti-mouse IgE (clone R35-118; BD Pharmingen). Binding was visualized using streptavidin-HRP (R&D Systems) and tetramethylbenzidine (R&D Systems).

Statistical analysis

Mann-Whitney tests were used to determine statistical differences between groups, where a value of $p < 0.05$ was considered to be significant.

Results

Acute Der p1-induced AAI resolves within 21 days

To establish a time course of AAI resolution, a highly reproducible, minimal sensitization and challenge model was established in C57BL/6J mice. Mice were sensitized i.p. twice with Der p1 in alum adjuvant and challenged i.t. twice with Der p1 in PBS. Control sensitized mice were mock challenged with PBS (Fig. 1). Animals that received no sensitizing injection, only one sensitizing injection, or only one i.t. challenge did not develop AAI (data not shown). The time between last sensitization and first challenge could vary between 14 and 28 days without effect on AAI (data not shown). After the second challenge, mononuclear cells and eosinophils infiltrated into perivascular, peribronchiolar and bronchiolar lung tissue and into alveolar walls (Fig. 2). By day 2 post challenge, mice given Der p1 had moderate to severe lung inflammation in all compartments (Fig. 3, A–D), which resolved by

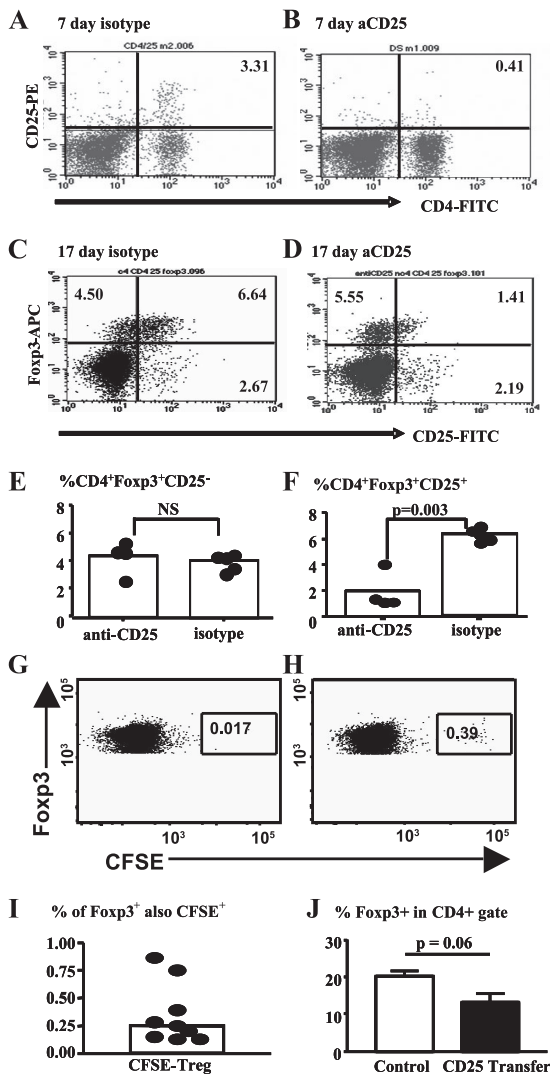


FIGURE 6. Analysis of depletion and transfer of CD4⁺Foxp3⁺CD25⁺ Tregs. *A–F*, Mice were given α CD25 (clone PC61) or isotype control Ab i.p. *A* and *B*, Representative dot plots stained with α CD4 and α CD25 (clone 3C7) Abs 7 days after treatment; *C* and *D*, representative dot plots of cells gated on CD4 and stained with α Foxp3 and α CD25 (clone 7D4) Ab 17 days after treatment; *E* and *F*, CD4⁺Foxp3⁺CD25⁺ but not CD4⁺Foxp3⁺CD25⁻ are still depleted from mice 17 days after treatment with α CD25 ($n = 4$) but not isotype control ($n = 5$). *G* and *H*, CFSE-labeled Tregs from naive mice were transferred i.v. into sensitized recipients before i.t. challenge. Four days after challenge (9 days after transfer), labeled cells were detected in the dMLN Foxp3⁺ population: *G* and *H*, representative dot plots showing CFSE-labeled cells in transferred (*H*) but not control (*G*) dMLN; *I*, the percentage of CD4⁺Foxp3⁺ cells in dMLN was not significantly different from those of control mice (*J*); ($n = 8$).

day 21. The maximal eosinophil influx in lung tissue and BAL coincided with the peak in inflammation and gradually declined over time reaching control levels by day 21 (Figs. 2*B*; and 3, *E* and *F*). Goblet cell hyperplasia (Figs. 2*D* and 3*G*) peaked on day 4 post challenge but was not completely resolved by day 21. PBS-challenged mice were included at each time point but never developed AAI (Fig. 2, *E* and *F*) and are represented as a pooled PBS group (Fig. 3).

Local and systemic immune response are initiated upon airway challenge

Local (dMLN) and systemic (serum antibody) adaptive immune responses to airway challenge were determined. Although mock-

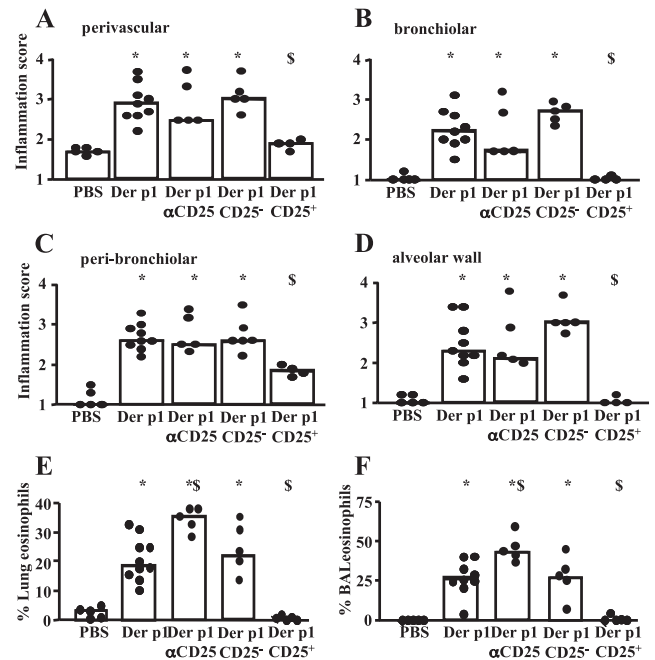


FIGURE 7. Depletion of CD25⁺Foxp3⁺ Tregs exacerbates eosinophilia whereas adoptive transfer abrogates inflammation and eosinophilia. Mice were sensitized and then either depleted of CD25⁺ cells or given isotype control Ab; or had i.v. transfer of CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells from naive syngeneic mice as in Fig. 1. Responses of individual mice challenged i.t. with Der p1 or PBS at 6 days post challenge. *A–D*, Lung inflammation. The percentage of eosinophils in the tissue infiltrate (*E*) and in BAL (*F*) was determined. Cumulated data from two experiments ($n = 4–5$ /group/experiment). Bars, median group value. Significant difference from mock challenged (*) or relevant control group (\$) ($p < 0.05$, Mann-Whitney).

challenged (PBS) mice had very small lymph nodes compared with Der p1-challenged animals, dMLN cells from both groups proliferated when incubated with Der p1 because they had all been sensitized. This indicated that Ag-reactive memory cells had spread through the lymphoid system after sensitization (Fig. 4*A*). The proliferative response of dMLN cells in medium alone were always <1000 cpm (not shown). Cells from Der p1-challenged mice produced high levels of IL-5, IL-10, and IL-13 in response to Ag recall. IL-4 was measured in preliminary experiments but levels were low and variable at the 48 h time point, and it was decided to concentrate on IL-5 and IL-13 as indicators of allergic Th2 activation. dMLN cells produced <100 pg/ml of any cytokine when grown in medium alone (data not shown). Cytokine release and proliferation peaked in cells cultured from day 4 post challenge and declined thereafter (Fig. 4, *A–D*). This indicated that as the lung response resolved, the numbers of effector cells in dMLN decreased. Despite showing equivalent proliferative responses to Der p1, dMLN cells from mock-challenged mice secreted much less IL-5 and IL-13 and essentially no IL-10 when stimulated with Der p1 (Fig. 4, *B–D*). This indicated that although Der p1-reactive memory was similar in lymphoid tissue of both groups, the challenged mice had expanded allergic effector cell populations. IFN- γ was always undetectable in cells from mock or Der p1-challenged mice.

A total of <10⁵ mononuclear cells could be isolated from lungs of mock-challenged mice so it was not possible to determine their responses at each time point. It was only possible to isolate sufficient numbers of cells for in vitro restimulation from Der p1-challenged lungs at the peak of inflammation on day 4 after challenge. Results from a representative experiment are shown in Fig.

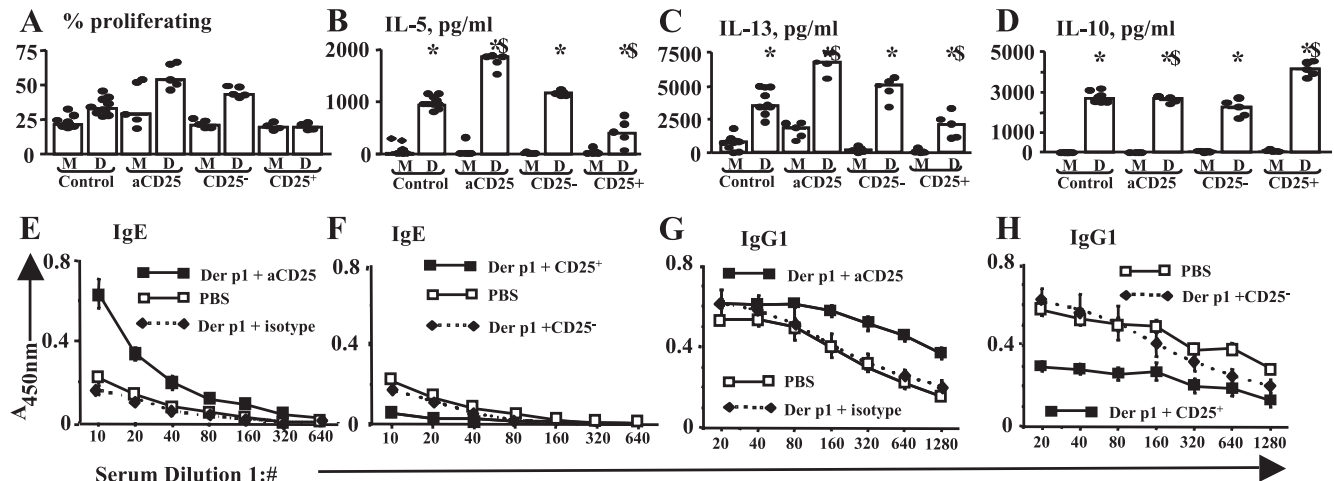


FIGURE 8. Depletion of CD25⁺Foxp3⁺ Tregs enhances, whereas adoptive transfer reduces systemic immune responses to airway challenge. Mice were sensitized, challenged, and treated as in Fig. 1. On day 6 post Der p1 challenge, dMLN cells were cultured in medium alone (M) or with Der p1 (D). A, Percentage of proliferating cells at 72 h (CFSE incorporation); B–D, 48 h cytokine secretion; shown for individual mice. Bars, median group value. Representative results from three experiments ($n = 4$ –5/group/experiment). Significant differences to medium only (*) or relevant control group (\$) ($p < 0.05$, Mann-Whitney). E–H, Sera from Der p1 sensitized mice, treated with anti-CD25 or isotype control (E and G) or CD4⁺CD25⁺ or CD4⁺CD25⁻ cells (F and H) before challenge, analyzed for Der p1-specific IgG1 (G and H) and IgE (E and F) 6 days post challenge.

4, E and F, and show that responses of Der p1-reactive effector cells in lung tissue were similar to those in dMLN and that IL-5, IL-13, and IL-10 were secreted. Levels of IL-5, IL-13, and IL-10 in BAL at the same time point were low but detectable, and IFN- γ and IL-4 were undetectable. BAL collected at all other time points or from mock-challenged mice at any time point contained no detectable cytokines (not shown).

Sera from sensitized mice 2 days after challenge with PBS or Der p1 had similar levels of specific IgG1 (Fig. 4I). However, by day 21, Der p1-challenged mice had enhanced serum IgG1 (Fig. 4J). In contrast, Der p1-specific IgE titers were enhanced at day 2 post challenge with Derp1 (Fig. 4K), but by day 21 this declined almost to the level of mock-challenged mice (Fig. 4L). Der p1-specific IgG2a^b was undetectable at all time points (not shown).

CD4⁺CD25⁺Foxp3⁺ Tregs increase in the lung and dMLN upon Ag challenge

Endogenous CD4⁺CD25⁺Foxp3⁺ Tregs have been implicated in suppression of responses to foreign Ag, thus we aimed to investigate whether Tregs were involved in resolution of Der p1-induced AAI. Fig. 5 shows that low numbers of Treg cells were found in both dMLN and lungs of mock-challenged mice. After allergen challenge the numbers of CD4⁺Foxp3⁺CD25⁺ Tregs significantly increased at day 4 and declined over time. In both dMLN and lung there was a consistent, detectable population of CD4⁺Foxp3⁺CD25⁻ cells, which was not changed either by allergen challenge or over time.

CD4⁺CD25⁺Foxp3⁺ Tregs can modulate allergic pulmonary eosinophilia and lung inflammation

Having shown that Tregs accumulated in lungs and dMLN after allergen challenge we wished to know whether or not they were involved in resolution of AAI. Mice were given a single dose of 250 μ g of anti-CD25 Ab (PC61) i.p. to deplete endogenous CD25⁺ Tregs or received additional (5×10^5) purified Tregs i.v. from allergen naive syngeneic donors. Preliminary experiments established that CD4⁺Foxp3⁺CD25⁺ cells were undetectable in spleens of PC61-treated animals 7 days after Ab administration and were still 75% depleted after 17 days,

whereas treatment had no effect on the CD4⁺Foxp3⁺CD25⁻ population (Fig. 6). To test Treg function, depleting Ab was given 14 days after second sensitization (Fig. 1) when effector T cells were not detectable in dMLN, and mice were then rested for 7 days before first i.t. challenge to allow clearance of the anti-CD25 Ab.

In cell transfer experiments, mice were given 5×10^5 naive CD4⁺CD25⁺ or 5×10^5 CD25⁻CD4⁺ T cells i.v. 24 h before first airway challenge (Fig. 1). All CD25⁺CD4⁺ T cells isolated were also Foxp3⁺ by flow cytometry. To track migration of transferred cells, 5×10^5 naive CFSE-labeled Tregs were transferred as above. Four days after Der p1 challenge (9 days after transfer), when endogenous Treg numbers peaked in lungs and dMLN (Fig. 5), lungs, dMLN and spleens were removed and labeled cells detected by flow cytometry. Although numbers were very low, transferred cells were only found in the dMLN suggesting that they were recruited to and retained in the activated lymphoid tissue (Fig. 6G).

To assess Treg function in AAI, tissues were analyzed at day 6 post challenge when inflammation was well established, but starting to resolve (Figs. 2–4). In depletion experiments, isotype Ab i.p. and PBS i.p. were both used as controls for PC61 administration. Because these control treatments gave equivalent results in three separate experiments ($p > 0.05$), they are expressed as a total control group. Anti-CD25 Ab had no effect on overall lung inflammation (Fig. 7, A–D) indicating that mice were still capable of mounting a response to allergen challenge, i.e., depletion had not removed memory cells. However, there was increased eosinophilia in the pulmonary tissue (Fig. 7E) and BAL (Fig. 7F). In contrast, transfer of CD4⁺CD25⁺Foxp3⁺ T cells from naive mice before airway challenge reduced disease as lung inflammation and eosinophilia was almost completely absent (Fig. 7). Transfer of CD4⁺CD25⁻ cells had no effect.

CD4⁺CD25⁺Foxp3⁺ Tregs control both local and systemic immune responses to airway allergen exposure

Transfer and depletion of CD25⁺Foxp3⁺ T cells before airway challenge had profound effects on lung pathology. Whether transfer or depletion also reduced or exacerbated the adaptive

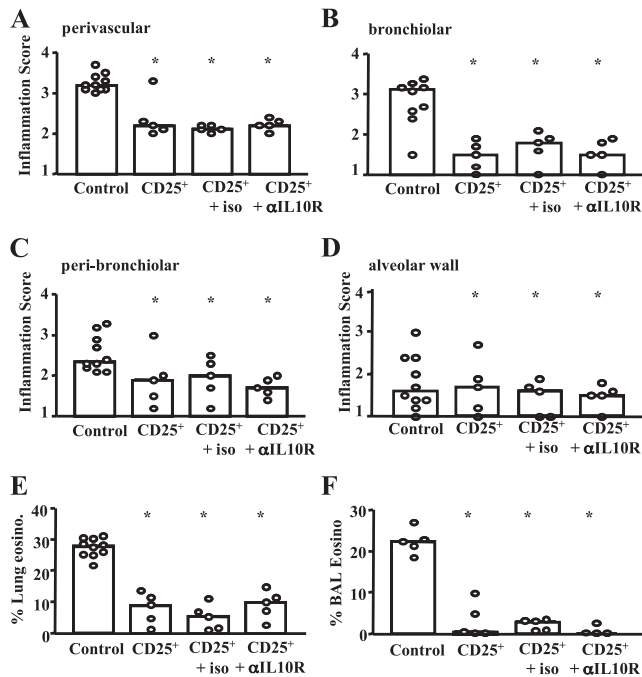


FIGURE 9. Blockade of IL-10R and transfer of CD25⁺Foxp3⁺ Tregs prevents lung inflammation and eosinophilia. Mice sensitized, treated (PBS or isotype control Ab; or CD25⁺Foxp3⁺ cells only; or isotype control and CD25⁺Foxp3⁺ cells; or anti-IL-10R Ab and CD25⁺Foxp3⁺ cells) and challenged with Der p1. On day 6 post challenge, lung inflammation was assessed in perivascular (A), bronchiolar (B), peribronchiolar (C), and alveolar wall (D) compartments. Pulmonary (E) and BAL eosinophils (F) were also assessed. Data representative of three comparable experiments. Bars, median group value. Significant difference (*) to control ($p < 0.05$, Mann-Whitney).

allergic responses was investigated by determining the dMLN T cell response and serum Ab levels. Depletion of CD25⁺Foxp3⁺ T cells enhanced proliferation of dMLN cells to recall Ag (Fig. 8A). This coincided with elevated levels of the Th2 cytokines, IL-5 (Fig. 8B), and IL-13 (Fig. 8C) but there was no effect on IL-10 secretion (Fig. 8D). In contrast, adoptive transfer of

CD25⁺Foxp3⁺ T cells abrogated proliferation and decreased secretion of IL-5 and IL-13 (Fig. 8, A–C), but enhanced levels of IL-10 compared with CD25[−] cell recipients or control mice (Fig. 8D). Depletion of CD25⁺ T cells increased IgG1 and IgE whereas transfer inhibited Ab titers compared with control mice (Fig. 8, E–H).

Suppression of lung inflammation by CD25⁺Foxp3⁺ Tregs is independent of IL-10R function

CD25⁺Foxp3⁺ transfer increased production of IL-10 by dMLN cells from Der p1-challenged mice. To resolve whether IL-10 was important in prevention or early resolution of pulmonary inflammation, anti-IL-10R-blocking Ab was administered at the time of cell transfer. Blockade of IL-10R at the time of cell transfer had no effect on the inhibition of lung inflammation (Fig. 9, A–D) or on eosinophilia (Fig. 9, E and F).

IL-10R function is essential for control of allergen-induced draining lymph node cell proliferation and cytokine secretion by transferred CD25⁺Foxp3⁺CD4⁺ T cells

Fig. 10, A–D, shows that, as above, adoptive transfer of CD25⁺Foxp3⁺CD4⁺T cells decreased dMLN cell proliferation and this coincided with decreased IL-5 and IL-13 and increased IL-10 secretion. Administration of anti-IL-10R Ab at the time of transfer prevented the decreased proliferation and cytokine secretion.

Treg influence on systemic Ab response is independent of IL-10R function

As above, transfer of CD25⁺Foxp3⁺ cells before airway challenge prevented secretion of Der p1-specific IgG1 and IgE Abs. Blockade of IL-10R binding at the time of transfer did not reverse this response in cell recipients (Fig. 10, E and G). Levels of IgG2a^b were low in all groups of mice (Fig. 10F).

Discussion

This paper reports that endogenous Tregs are important in resolution of Th2-mediated AAI induced by the native form of a natural human airway allergen, Der p1 in mice with an intact immune system. We show that endogenous Tregs from Ag naive syngeneic

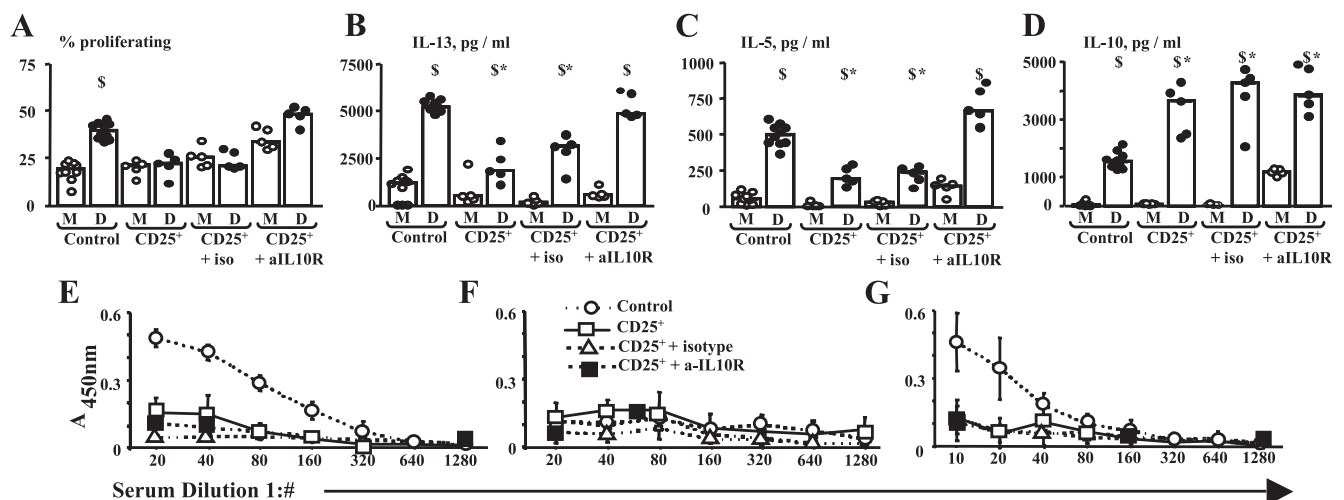


FIGURE 10. IL-10R blockade dysregulates cytokine production and isotype class switching. Mice sensitized with Der p1, treated (CD25⁺Foxp3⁺ cells only, isotype control and CD25⁺Foxp3⁺ cells or anti-IL-10R Ab and CD25⁺Foxp3⁺ cells), and challenged with Der p1. On day 6 post challenge, dMLN cells were cultured with medium alone (M) or with 50 μg ml^{−1} Der p1 (D). A, Percentage of cells proliferating after 72 h was assessed by CFSE incorporation. B–D, Concentration of cytokine secreted at 48 h. Bars, median. Serum was analyzed for Der p1-specific IgG1 (E), IgG2a (F), and IgE (G). Representative results ($n = 4–5$ /group/experiment). Significant differences to control group (*) or medium alone (\$) ($p < 0.05$, Mann-Whitney).

mice down-regulate eosinophil recruitment into lungs and Th2 effector cell function in draining lymph nodes. We also demonstrate that Treg function is IL-10R independent in the lung, but IL-10R dependent in the dMLN.

Previous studies reported on induction and modulation of AAI in murine models (15, 26) and TCR transgenic OVA-specific Tregs have been shown to down-regulate AAI in an IL-10-dependent fashion (11). However, this study did not determine whether endogenous Tregs were involved in induction and resolution of AAI, nor whether Tregs must be allergen-specific to control AAI. Using whole house dust mite extract as allergen, Lewkowich et al. (18) showed that depletion of natural CD25⁺CD4⁺ T cells before sensitization increased disease in C3H but not A/J mice and that this was due to alterations in lung dendritic cells. This study demonstrated that natural Tregs are important in determining whether allergy results from allergen encounter but did not determine whether disease can be modulated by endogenous Tregs in already sensitized animals.

To determine whether Tregs were involved in AAI resolution and associated immune responses, we first established the time course of lung inflammation and systemic immunity in Der p1-sensitized and -challenged mice. Lung inflammation and eosinophilia peaked between 2 and 6 days after challenge and had largely returned to baseline levels by 21 days (Figs. 2 and 3). Serum Der p1-specific IgE levels were transiently boosted by i.t. challenge but decreased by 21 days whereas serum IgG1 levels were boosted by challenge and continued to increase 21 days later (Fig. 4). Recall responses in dMLN cells showed that effector cells capable of secreting IL-5, IL-13, and IL-10 were present in draining lymph nodes from 2 days after challenge and decreased by 21 days (Fig. 4).

We further demonstrated that CD25⁺CD4⁺Foxp3⁺ cells are recruited into both lungs and draining lymph nodes of sensitized mice, peaking at 4 days after challenge when resolution starts (Fig. 5). To study whether these cells were functionally involved in resolution and/or disease induction we took a "two sided" approach of in vivo endogenous Treg depletion and adoptive transfer. When using depleting anti-CD25 Ab in sensitized animals it was a concern that we may simply remove effector T cells so sufficient time had to be left after sensitization to allow activated allergen-specific CD25⁺ effector cells to revert to "memory" i.e., CD25⁻ status. That depletion before lung challenge did not appear to alter the overall lung inflammation (Fig. 7) and enhanced serum Ab responses (Fig. 8) indicated that treatment did not prevent generation of effector cells from pre-existing memory cells. However, Treg depletion resulted in elevated eosinophilia (Fig. 7) and enhanced Der p1-specific serum IgG1 and IgE responses (Fig. 8). This indicates that Tregs normally "dampen down" AAI but cannot prevent eosinophilia and allergic Ab production induced by lung challenge. The results are consistent with previous reports that have suggested that Tregs are important in control of Th2 immune responses and lung eosinophilia (11, 20).

Other studies reported that Ag-specific Treg from DO11.10 transgenic mice can suppress lung eosinophilia. To establish whether or not endogenous Treg from allergen naive, wild-type mice would also be functional in AAI modulation, we transferred 5×10^5 CD4⁺CD25⁺Foxp3⁺ T cells from naive mice into sensitized animals 1 day before allergen challenge. These cells were recruited to and retained in dMLN after Ag challenge (Fig. 6). Treg transfer had the opposite effect of depletion and greatly reduced lung eosinophilia (Fig. 7) and goblet cell differentiation (data not shown) as well as inhibiting production of Der p1-specific serum IgE and IgG1 (Fig. 8).

Treg depletion enhanced levels of the Th2 cytokines, IL-5 and IL-13 secreted by dMLN cells while transfer of additional Tregs from allergen naive mice had opposite effects (Fig. 8). In contrast, IL-10 secreted by dMLN cells was unaffected by depletion but enhanced by transfer. The question arose from this whether IL-10 was necessary for Treg function in control of Der p1-induced AAI. To answer this, we treated mice with IL-10R blocking Ab at the time of Treg transfer. Unlike results reported for TCR transgenic OVA-specific Tregs (11), control of AAI in our model is independent of IL-10R signaling as administration of anti-IL-10R Ab at the time of Treg transfer had no effect (Fig. 10). In contrast, in the dMLN response, blockade of IL-10 signaling at the time of transfer prevented the inhibition of IL-5 and IL-13 seen when Tregs alone were given (Fig. 10). This indicates that IL-10 is necessary for Treg control of dMLN IL-5 and IL-13 secretion. Levels of IL-10 secretion by dMLN cells were unaffected by receptor blockade (Fig. 10).

Overall these results show that endogenous Tregs control resolution of airway inflammation and eosinophilia in the lung in an IL-10 independent manner while draining lymph node allergic Th2 cytokine responses are controlled by the same cell population in an IL-10-dependent manner. We believe that our results showing that natural/endogenous Tregs are important for resolution of AAI and that their function can be boosted by transferring cells from Ag naive donors add important information on the biology of AAI and credence to the idea that endogenous Tregs may be a therapeutic target.

Disclosures

The authors have no financial conflict of interest.

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