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

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Allergic asthma, which is reaching epidemic levels in the Western world, is triggered by inhalation of environmental allergens like cat dander, pollen, and house dust mite proteins. 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) 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, which may induce respiratory symptoms in a minority of patients when ingested, 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...
The production of nasal tolerance to Der p1 inhibits lung inflammation and IL-10 and CD25 from 2 to 21 days after challenge (protocol A). To investigate the role of gate resolution of inflammation, mice were culled at various time points (protocol B). Black bars, 100 μm of Der p1 in alum and challenged i.t. with PBS (mock challenged) or 10 μg of Der p1 in PBS (allergen challenged). To investigate the role of IL-10 and CD25 Tregs in this system, 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 TGFβ (21–23). Previous work showed that induction of nasal tolerance to Der p1 inhibits lung inflammation and 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 Tregs in preventing lung inflammation but...
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 CD25+ cells 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 μg of Der p1 absorbed on 2.25 mg of aluminium hydroxide (Imject ALUM; Pierce) in 100 μl. AAI was induced by two intratracheal (i.t.) instillations of 10 μg of Der p1 in 50 μl of PBS in anesthetized animals (0.1 mg/g Avetin; 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 injected with 250 μg of depleting anti-CD25 Ab (clone PC61), 7 days before airway challenge or with 500 μg of anti-IL-10R-blocking Ab (1B1.2) 1 day before challenge. Control mice received isotype control i.p. of 500 μg of anti-IL-10R-blocking Ab (clone 1B1.2) or PBS. 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 (l, m, and L) 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 (*).

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 μl of PBS followed by 800 μl of fresh PBS also instilled three times. Samples were centrifuged at 300 × g for 5 min at 4°C, and supernatant from the first wash stored at −20°C. Cell pellets were resuspended in 500 μl of PBS and cytospins 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 μm) were stained with H&E for assessment of inflammation and periodic acid-Schiff for goblet cell hyperplasia. Inflammation was scored for each mouse at ×200 magnification by averaging the score of 10 consecutive fields where the lungs were correctly inflated and the field contained a complete transection of at least one bronchiole (less than half a field width/300 μm 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,
spun over Lympholyte-M (VH-Bio) to isolate mononuclear leukocytes. Using CELLQuest software.

Belonging the same wells with 0.5 ml of 0.05 M carbonate bicarbonate buffer (pH 9.6). Nonspecific binding was blocked with 3% BSA in PBS. For IgG1 and IgG2a 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 (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, peribronchial and bronchial 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

>10 cells): the peribronchial 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-μm sterile sieves. Lung cells were then spun over Lympholyte-M (VH-Bio) to isolate mononuclear leukocytes. Cells were washed, resuspended in complete medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 μM 2-ME; Sigma-Aldrich) plated in 96-well microplates at 5 x 10⁵ cells/well and cultured with medium alone or 50 μg/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 x 10⁶ 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 aCD25 Abs (clones 7D4 and 3C7) and flow cytometry on a BD FACS Calibur using CELLQuest software.
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-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. 4A). 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 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 <10^5 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. 4C. Results from a representative experiment are shown in Fig. 4D.

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in BAL at the same time point were low but detectable, and IFN-γ cells in lung tissue were similar to those in dMLN and that IL-5, and lung there was a consistent, detectable population of significantly increased at day 4 and declined over time. In both dMLN allergen challenge the numbers of CD4+ 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 CD4+CD25− 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 deletion 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

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**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.
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) 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
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 × 10⁶ 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 donos 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.

References


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