Resolution of airway inflammation and hyperreactivity after in vivo transfer of $CD4^+CD25^+$ regulatory T cells is interleukin 10 dependent

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Deficient suppression of T cell responses to allergen by $CD4^+CD25^+$ regulatory T cells has been observed in patients with allergic disease. Our current experiments used a mouse model of airway inflammation to examine the suppressive activity of allergen-specific $CD4^+CD25^+$ T cells in vivo. Transfer of ovalbumin (OVA) peptide–specific $CD4^+CD25^+$ T cells to OVA-sensitized mice reduced airway hyperreactivity (AHR), recruitment of eosinophils, and T helper type 2 (Th2) cytokine expression in the lung after allergen challenge. This suppression was dependent on interleukin (IL) 10 because increased lung expression of IL-10 was detected after transfer of $CD4^+CD25^+$ T cells, and regulation was reversed by anti–IL-10R antibody. However, suppression of AHR, airway inflammation, and increased expression of IL-10 were still observed when $CD4^+CD25^+$ T cells from IL-10 gene–deficient mice were transferred. Intracellular cytokine staining confirmed that transfer of $CD4^+CD25^+$ T cells induced IL-10 expression in recipient $CD4^+$ T cells, but no increase in IL-10 expression was detected in airway macrophages, dendritic cells, or B cells. These data suggest that $CD4^+CD25^+$ T cells can suppress the Th2 cell–driven response to allergen in vivo by an IL-10–dependent mechanism but that IL-10 production by the regulatory T cells themselves is not required for such suppression.

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Abbreviations used: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Cdyn, dynamic compliance; RL, lung resistance.

Asthma, which affects ~5% of the population of the Western world, is characterized by bronchial eosinophilic inflammation and airway hyperreactivity (AHR). Although it is predominantly eosinophils that are recruited to the lung, recent evidence suggests that T cells play an important effector role in this process both in human asthma and murine models because lung eosinophilia and AHR are abrogated in T cell–deficient mice (1, 2, 3). T helper type 2 (Th2) cells release IL-4, IL-5, and IL-13 and have been shown to mediate allergic reactions (4). The regulation of T cell responses is an important process in vivo in order to provide immunity to invading pathogens while preventing excessive inflammatory responses to self-antigens. There is now increasing evidence of the existence of regulatory T cell subsets capable of suppressing immune responses (5). One of the best characterized of these is the "naturally occurring" $CD4^+CD25^+$ regulatory T cells. The precise mechanisms by which $CD4^+CD25^+$ regulatory cells suppress inflammation are unknown, though there is evidence for the involvement of IL-10 and TGF-β in some settings, as administration of antibodies against these cytokines prevent the abrogation of colitis by $CD4^+CD25^+$ regulatory T cells in a mouse model and also block the suppressor activity of T regulatory cells isolated from human peripheral blood (6, 7, 8).

Although the ability of $CD4^+CD25^+$ regulatory T cells to suppress Th1 cell–mediated diseases has been extensively studied, their role in controlling Th2 cell–mediated allergic responses is the subject of more recent investigation and have thus far been limited to in vitro and complex in vivo studies. It has been shown that $CD4^+CD25^+$ regulatory T cells isolated from hay fever patients have an impaired ability

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to suppress proliferation and IL-5 production of $CD4^+CD25^-$ T cells compared with healthy controls (9). Mouse studies have shown that Th2 cell–polarized $CD4^+$ T cells depleted of the $CD4^+CD25^+$ population resulted in increased airway eosinophilia compared with unfractionated T cells after adoptive transfer of the Th2 cells (10). Moreover, double transgenic mice with T cells that express the T cell receptor for OVA (DO11.10), as well as OVA targeted to the lung epithelium, demonstrated pulmonary infiltrates containing $CD4^+CD25^+$ T cells (11). These cells were able to prevent OVA-induced proliferation of $CD4^+CD25^-$ cells in vitro. In addition, the mice were shown to be tolerant to OVA when spleen cells were removed and challenged ex vivo. However, if exogenous OVA was given to the mice, AHR still developed, although lung eosinophilia and ex vivo Th2 responses were reduced. In a different model, cotransfer of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ T cells from mice sensitized to OVA into T cell–deficient $Rag^{-/-}$ hosts inhibited both allergen-induced airway inflammation and IL-4 expression (12). Although these studies imply that $CD4^+CD25^+$ regulatory T cells may have a beneficial role in suppressing allergen-induced airway inflammation in vivo, this has not yet been clearly demonstrated.

In the present study, we have determined the effect of allergen-specific $CD4^+CD25^+$ regulatory T cells on the development of allergen-induced pulmonary inflammation using a well-characterized in vivo murine model involving systemic sensitization and airway challenge with antigen. We have demonstrated that transfer of allergen-specific $CD4^+CD25^+$ regulatory T cells from DO11.10 transgenic mice inhibited the classical pathology associated with allergic asthma, namely AHR, lung eosinophilia, and Th2 cytokine production. Blocking experiments determined that this effect is dependent on IL-10. Moreover, we have shown that $CD4^+CD25^+$ regulatory T cells exerted their suppressive effects in vivo independently of their capacity to produce IL-10 but induced IL-10 production from recipient $CD4^+$ T cells.

RESULTS

Allergen-specific $CD4^+CD25^+$ regulatory T cells suppress T cell proliferation in vitro and migrate to the lung in vivo

Antigen-specific $CD4^+CD25^+$ T cells were isolated from spleen and lymph nodes of DO11.10 αβ TCR transgenic mice. We confirmed regulatory activity of the isolated cells using in vitro cultures stimulated with OVA and APCs. The OVA-specific $CD4^+CD25^+$ T cells did not themselves proliferate on stimulation with antigen, but suppressed proliferation of splenocytes from OVA-sensitized BALB/c mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051166/DC1).

Allergen-specific $CD4^+CD25^+$ regulatory cells were transferred into BALB/c mice 24 h before airway allergen challenge. Flow cytometric analysis of bronchoalveolar lavage (BAL), lung tissue and draining lymph nodes confirmed that transferred DO11.10 $CD4^+CD25^+$ cells were present in the airway lumen, lung, and lymph nodes of OVA-sensitized mice after allergen challenge (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20051166/DC1). Transferred cells were not detected in the sham-sensitized mice that received alum/PBS.

$CD4^+CD25^+$ regulatory T cells suppress AHR and leukocyte recruitment in vivo

AHR is characteristic of the pulmonary response to inhaled antigen in sensitized animals. Transfer of $CD4^+CD25^+$ regulatory T cells significantly decreased AHR in OVA-sensitized mice at a dose of 30 mg/ml methacholine ($P < 0.05$; Fig. 1 A).

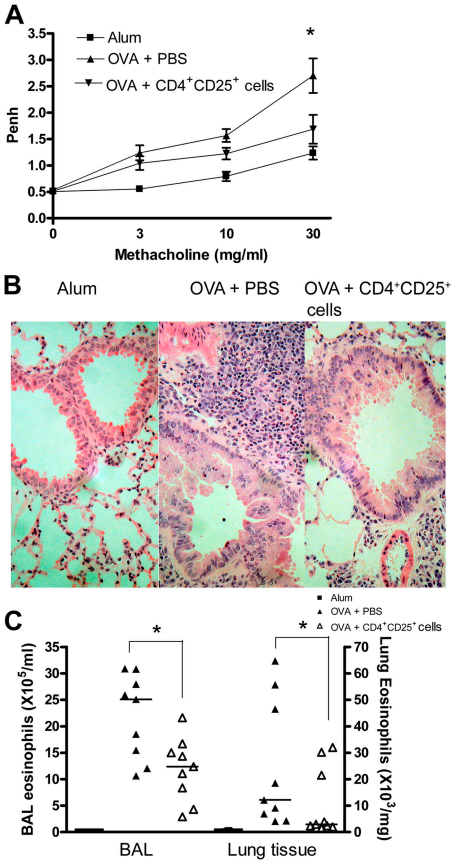

Figure 1. OVA-specific $CD4^+CD25^+$ regulatory T cells regulate allergen-induced airway inflammation in vivo. OVA-sensitized mice received either 5×10^5 $CD4^+CD25^+$ cells or an equivalent volume of PBS and were challenged through the airways with OVA. (A) AHR was measured 24 h after the final OVA challenge using a Buxco system in which mice were exposed to increasing concentrations of methacholine. Values are expressed as means ± SEM ($n = 9$–12 mice/group from two separate experiments). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS instead of $CD4^+CD25^+$ cells. (B) Lungs were fixed, sectioned, and stained with hemotoxylin and eosin. Representative sections are shown for each treatment group. BAL and lung tissue digest cells (C) were isolated as described in Materials and methods, and eosinophil numbers were determined by differential counts. Values are expressed as medians ($n = 9$–12 mice/group from two separate experiments). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS instead of $CD4^+CD25^+$ cells.

These results were confirmed by direct measurement of lung resistance (RL) and dynamic compliance (Cdyn; Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20051166/DC1). In contrast, transfer of OVA-specific $CD4^+CD25^-$ T cells had no effect on increased AHR after allergen challenge (unpublished data).

Transfer of $CD4^+CD25^+$ regulatory T cells also significantly reduced eosinophil numbers in the airway lumen (BAL cells) and lung parenchyma of OVA-sensitized and -challenged mice ($P < 0.05$; Fig. 1, B and C). In contrast, transfer of $CD4^+CD25^-$ T cells had no such effect on leukocyte recruitment to the airway lumen after allergen challenge (unpublished data).

Th2 response to allergen challenge is modulated by transfer of $CD4^+CD25^+$ regulatory T cells

Th2 cells are important effector cells in the pulmonary allergic response, and, therefore, differences in Th2 cell numbers could potentially account for the decreases in AHR and eosinophilia mediated by transfer of $CD4^+CD25^+$ regulatory T cells. Th2 cell numbers were determined in the BAL and lung tissue by costaining cells with CD4 and the Th2 cell–specific marker T1/ST2 (13). There was an increase in Th2 ($CD4^+T1/ST2^+$) cell numbers in the airway lumen and lung of OVA-sensitized and -challenged mice compared with unsensitized mice 24 h after the final OVA challenge, and this was significantly reduced by transfer of $CD4^+CD25^+$ regulatory T cells ($P < 0.05$; Fig. 2, A and B). Furthermore, antigen-induced increases in BAL IL-5 and IL-13 were significantly reduced by transfer of $CD4^+CD25^+$ regulatory T cells in OVA-sensitized mice ($P < 0.05$; Fig. 2 C). Similarly, lung homogenate IL-5 and IL-13 were also decreased by transfer of $CD4^+CD25^+$ regulatory T cells (Fig. 2 D). In contrast, production of the Th1-specific cytokine IFN-γ was unaffected by transfer of $CD4^+CD25^+$ regulatory T cells. Lung IFN-γ levels were increased in OVA-sensitized mice to 843 ± 94 pg/ml from 620 ± 53 pg/ml in alum controls. However, there was no substantial difference between OVA-sensitized mice and mice that received $CD4^+CD25^+$ cells (838 ± 106 pg/ml).

The chemokines eotaxin/CCL11, MDC/CCL22, and TARC/CCL17 are thought to be involved in leukocyte recruitment during an allergic response and in particular for the recruitment of eosinophils and Th2 cells (14). By decreasing production of these chemokines, regulatory T cells could potentially suppress the Th2 response. However, BAL and lung levels of eotaxin/CCL11, MDC/CCL22, and TARC/CCL17 were unaffected by transfer of $CD4^+CD25^+$ regulatory T cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20051166/DC1, and not depicted).

Similarly, transfer of $CD4^+CD25^+$ T regulatory cells had no effect on serum IgE or OVA-specific IgE levels in OVA-sensitized mice (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20051166/DC1).

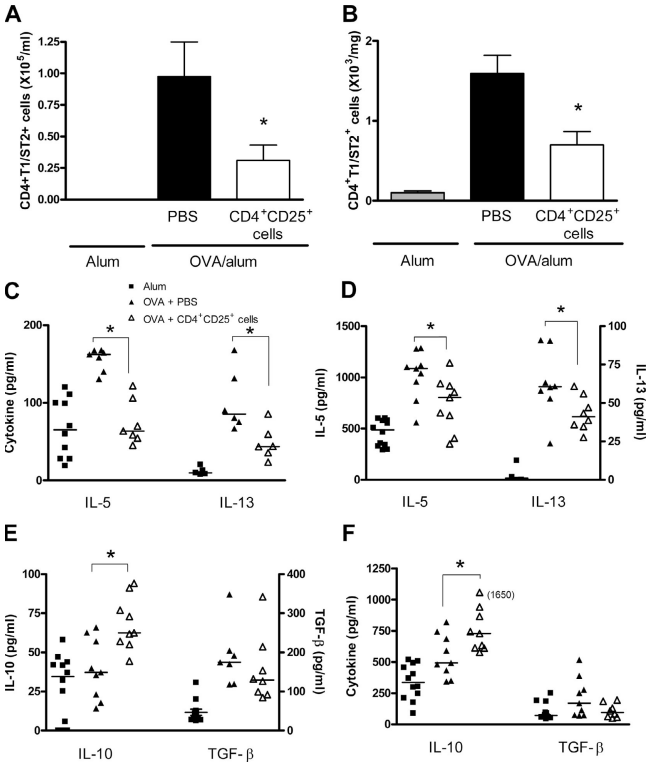

Figure 2. Transfer of $CD4^+CD25^+$ regulatory T cells reduces Th2 cell responses in the lung after allergen challenge. BAL (A) and lung tissue digest cells (B) were isolated as described in Materials and methods. Th2 cell numbers were determined 24 h after the final OVA challenge by antibody staining and flow cytometric analysis, as described in Materials and methods. Th2 cells were defined as cells that were double-stained for CD4 and the Th2 cell–specific marker T1/ST2. Data are expressed as means ± SEM. IL-5 and IL-13 levels measured in BAL fluid (C) and lung homogenate supernatant (D) by ELISA. IL-10 and active TGF-β1 levels were measured in BAL fluid (E) and lung homogenate supernatant (F) by ELISA. Data are expressed as medians ($n = 9$–12 mice/group from two separate experiments). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS instead of $CD4^+CD25^+$ cells.

$CD4^+CD25^+$ T cell transfer increases pulmonary IL-10 expression

The regulatory activity of $CD4^+CD25^+$ T cells has been attributed to IL-10 and TGF-β in some models (6, 7, 8). Therefore, these cytokines were measured in BAL fluid and lung homogenate supernatant 24 h after the final allergen challenge. The concentration of IL-10 was significantly increased in the BAL and lung of OVA-sensitized mice that received $CD4^+CD25^+$ regulatory T cells compared with OVA-sensitized mice that received PBS ($P < 0.05$; Fig. 2, E and F). In contrast, levels of TGF-β were unaffected by transfer of $CD4^+CD25^+$ regulatory T cells (Fig. 2, E and F).

Suppression of allergic inflammation and AHR by $CD4^+CD25^+$ regulatory T cells is IL-10 dependent

Because suppression of Th2 responses after transfer of OVA-specific $CD4^+CD25^+$ T cells in OVA-sensitized mice was

associated with increased expression of IL-10, we investigated the role of IL-10 in regulation. To this end, we administered a neutralizing antibody against the IL-10 receptor during the allergen challenge phase. Anti–IL-10R treatment completely abrogated the suppression of AHR and lung eosinophil infiltration by $CD4^+CD25^+$ regulatory cells (Fig. 3, A and B). Moreover, $CD4^+CD25^+$ regulatory T cells were unable to reduce Th2 cell numbers and IL-5 and IL-13 levels in the lung in the presence of anti–IL-10R (Fig. 3, C and D, and not depicted). Interestingly, anti–IL-10R treatment had no effect on the increased lung IL-10 levels associated with $CD4^+CD25^+$ regulatory T cells (Fig. 3 E).

Modulation of allergic inflammation and AHR is not dependent on $CD4^+CD25^+$ T cell–derived IL-10

Because we had demonstrated that the inhibition of allergen-induced airway inflammation by $CD4^+CD25^+$ regulatory T cells was IL-10 dependent, we investigated whether production of IL-10 from the regulatory T cells themselves was necessary for their suppressive activity. We therefore derived $CD4^+CD25^+$ T cells from IL-$10^{-/-}$ DO11.10 animals and transferred them during allergic airway disease. AHR, eosinophil recruitment, and Th2 cytokine expression were suppressed after transfer of $CD4^+CD25^+$ regulatory T cells derived from either wild-type or IL-10–deficient mice (Fig. 4, A–D). Interestingly, transfer of $CD4^+CD25^+$ regulatory T cells from IL-10 knockout mice still resulted in increased expression of IL-10 in the lung tissue of allergen-challenged mice (Fig. 4 E).

IL-10 production from $CD4^+$ T cells is increased by transfer of $CD4^+CD25^+$ regulatory T cells

Because we had established that IL-10 production from the $CD4^+CD25^+$ regulatory T cells was not necessary for their suppressive activity, we sought to determine the source of

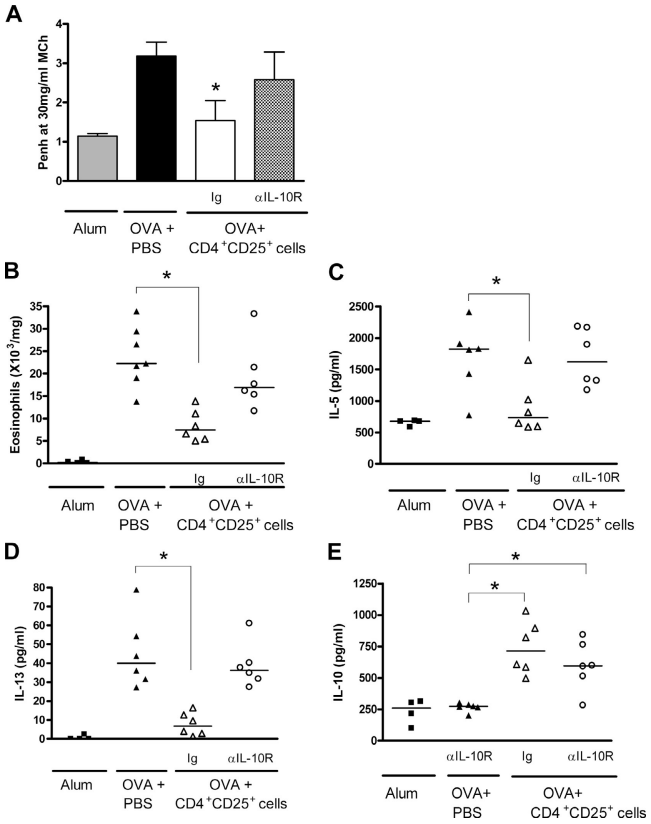

Figure 3. Suppression of allergen-induced airway inflammation is IL-10 dependent. Mice were treated with anti–IL-10R antibody or control Ig during the allergen challenge phase of allergic inflammation. AHR (A) and lung eosinophilia (B) were quantified as described in Materials and methods. IL-5 (C), IL-13 (D), and IL-10 (E) levels were determined in lung homogenate supernatant by ELISA. Data are expressed as means ± SEM (A) or medians (B–E; $n = 4$–6 mice/group). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS and control Ig.

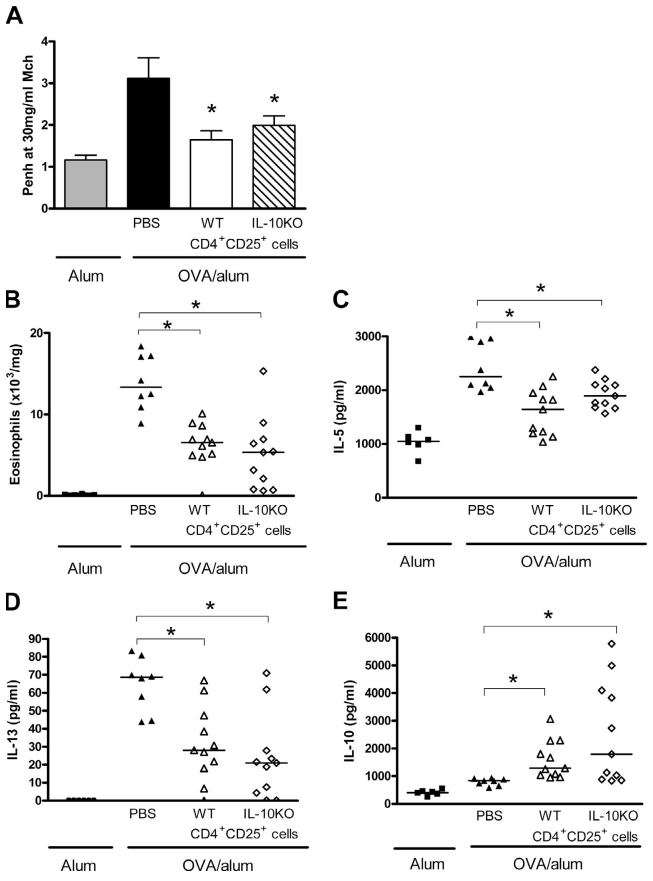

Figure 4. Transfer of IL-10–deficient $CD4^+CD25^+$ regulatory T cells suppresses allergen-induced airway inflammation. Mice received either wild-type $CD4^+CD25^+$ regulatory T cells, IL-10–deficient $CD4^+CD25^+$ regulatory T cells, or an equivalent volume of PBS as a control. AHR (A) and lung eosinophilia (B) were determined as described in Materials and methods. IL-5 (C), IL-13 (D), and IL-10 (E) levels were measured in lung homogenate supernatant by ELISA. Data are expressed as means ± SEM (A) or medians (B-E; $n = 6$–11 mice/group from two separate experiments). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS instead of $CD4^+CD25^+$ cells.

IL-10 during allergen-induced airway inflammation. Intracellular cytokine analysis of lung tissue digest cells showed that IL-10 is produced primarily from $CD4^+$ T cells (Fig. 5, A and B). We could not find appreciable IL-10 production from other cells such as $CD8^+$ T cells, macrophages, dendritic cells, B cells, or granulocytes during allergic lung inflammation. Moreover, transfer of $CD4^+CD25^+$ regulatory T cells increased the percentage of $CD4^+$ cells producing IL-10 compared with PBS alone (Fig. 5, A and B). This increase in $CD4^+IL\text{-}10^+$ cells was not dependent on the ability of the transferred $CD4^+CD25^+$ regulatory T cells to produce IL-10 because a similar increase in $CD4^+IL\text{-}10^+$ cells was seen after transfer of IL-10–deficient $CD4^+CD25^+$ regulatory T cells. Thus, $CD4^+CD25^+$ regulatory T cells suppressed allergen-induced airway inflammation by inducing IL-10 production from $CD4^+$ cells in the lungs of OVA-sensitized mice.

A

Population	% of population producing IL-10 in the lung (Median (range))		
	OVA + PBS	OVA + WT $CD4^+CD25^+$ cells	OVA + IL-10KO $CD4^+CD25^+$ cells
$CD4^+$ T cells	0.9 (0.3-1.6)	4.0 (2.8-6.6) *	4.2 (3.3-5.2)*
$CD8^+$ T cells	0.0	0.0	0.0
Macrophages	0.0	0.0	0.0
Dendritic cells	0.0	0.0	0.0
B cells	0.3 (0.1-0.4)	0.3 (0.2-0.5)	0.3 (0.2-0.5)
Granulocytes	0.0	0.0	0.0

B

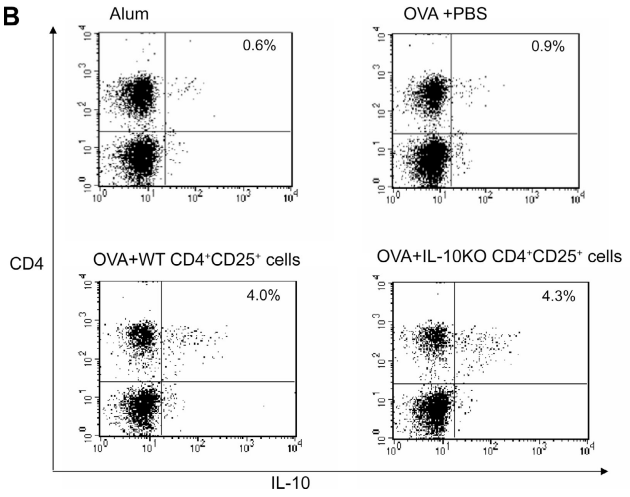

Figure 5. IL-10 is produced by $CD4^+$ T cells during allergen-induced airway inflammation and is increased by transfer of $CD4^+CD25^+$ regulatory T cells. Lungs were digested with collagenase and DNase as described in Materials and methods. Digest cells were stimulated by PMA/Ionomycin in the presence of Brefeldin A for 6 h. Cells were phenotyped by staining for CD4, CD8, CD11b (macrophages), CD11c (dendritic cells), and B220 (B cells). Granulocytes were defined by forward and side scatter. (A) Data are expressed as median cell types producing IL-10 with interquartile range ($n = 6$–14 mice/group in three separate experiments). *, $P < 0.05$ compared with OVA-sensitized mice that received PBS instead of $CD4^+CD25^+$ cells. (B) Data are shown as representative FACS plots showing costaining of lung tissue digest cells with CD4 and IL-10. Percentages in the top right quadrants refer to median percentages of CD4 cells expressing IL-10 ($n = 6$–14 mice/group).

DISCUSSION

In this paper we show for the first time that allergen-specific $CD4^+CD25^+$ T regulatory cells can suppress multiple pathophysiological features of allergic airway disease in vivo. Transfer of these allergen-specific $CD4^+CD25^+$ T regulatory cells reduced AHR, Th2 cell and eosinophil recruitment, and Th2 cytokine secretion. Importantly, we show that this effect is dependent on IL-10 because a neutralizing anti–IL-10R antibody abrogated suppression by $CD4^+CD25^+$ regulatory T cells. Moreover these effects were found to be independent of production of IL-10 by the $CD4^+CD25^+$ regulatory cells themselves.

Previous studies have determined that $CD4^+CD25^+$ regulatory T cells are able to regulate autoimmune immunopathologies, which are generally of a Th1 cell type (7, 15, 16), although a recent study using a *Leishmania major* infection model in SCID mice confirmed that both Th1 and Th2 cell function can be suppressed by naturally occurring $CD4^+CD25^+$ T regulatory cells (17). Other studies have implied that $CD4^+CD25^+$ T cells may be able to suppress some aspects of allergic pathology, but these have all involved complex transgenic models or cell transfer into T cell–deficient recipient mice (10–12). We have shown that transfer of $CD4^+CD25^+$ T regulatory cells into a classical model of systemic allergen sensitization in immunocompetent mice reduces multiple pathophysiological parameters, including both allergen-induced airway inflammation and AHR. These findings support our conclusions from human studies in vitro, which argue that an imbalance between suppression and activation exists in patients with allergic disease (9).

Transfer of allergen-specific $CD4^+CD25^+$ T cells into OVA-sensitized mice reduced eosinophilia and AHR, together with Th2 cell numbers and Th2 cytokine production. This implies that there is an effect on either Th2 cell trafficking or in local expansion of allergen-specific Th2 cells. However, the lack of effect on Th2-attracting chemokines such as CCL22, CCL17, and CCL11 suggests that Th2 cell recruitment can still occur. Although we saw decreases in Th2 cytokine secretion after transfer of $CD4^+CD25^+$ T cells, there was no accompanying reduction in serum IgE production. This might seem surprising because $CD4^+CD25^+$ T cells have previously been reported to suppress IgE in a transgenic model of hyper IgE (18). In addition, patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome who lack $CD4^+CD25^+$ regulatory T cells because of a mutation in Foxp3 exhibit elevated serum IgE levels (19). However, IL-10 has been shown to actually increase IgE production from B cells that have already switched but inhibits germline class switch (20). The lack of change in IgE in our model probably reflects the comparably short time course of the model and the timing of $CD4^+CD25^+$ T cell transfer.

The mechanism of suppression by allergen-specific $CD4^+CD25^+$ T regulatory cells is controversial. In vitro, the phenomenon is contact dependent but can be observed in the

absence of either IL-10 or TGF-β (9, 21, 22). In contrast, in vivo experiments in a colitis model suggest that IL-10 and TGF-β are involved in the suppression mediated by these cells (15). However, the effect may be model dependent because it was not observed in a gastritis model (23). We show that IL-10 but not TGF-β is up-regulated in the lung after transfer of allergen-specific $CD4^+CD25^+$ T regulatory cells and that this is observed together with an abrogation of AHR and allergic inflammation. Moreover, we highlight the importance of this IL-10 by showing that neutralizing anti–IL-10R antibody treatment abolished the suppressive effect of the $CD4^+CD25^+$ regulatory T cells so that AHR, eosinophilia, and Th2 cytokine levels were comparable to those seen in the absence of regulatory cells. IL-10 has previously been reported to be a potential regulatory factor in allergen-induced airway inflammation because transfer of engineered IL-10–producing T cells reduced AHR and inflammation in a murine model (24). Although this study showed that neutralization of IL-10 in the lung worsened allergic inflammation, we did not find that anti–IL-10R treatment had any effect on OVA-sensitized mice in the absence of transferred $CD4^+CD25^+$ regulatory T cells. This may be because of differences in the time of IL-10 neutralization. Oh et al. administered anti–IL-10 during both sensitization and challenge phases of allergen-induced airway inflammation, whereas we have blocked during the challenge phase only (24).

We have shown that the suppression by $CD4^+CD25^+$ regulatory T cells was not dependent on IL-10 production from allergen-specific T regulatory cells themselves, because the suppressive effect was preserved in $CD4^+CD25^+$ cells isolated from OVA-transgenic IL-10 $^{-/-}$ mice. Interestingly, Asseman et al. previously reported that IL-$10^{-/-}$ $CD4^+$ $CD25^+$ T cells could inhibit colitis induced by transfer of naive but not antigen-experienced T cells (25). In our model, suppression was seen after transfer of IL-$10^{-/-}$ $CD4^+$ $CD25^+$ T cells into mice that had been sensitized to OVA and, thus, presumably OVA-experienced Th2 cells were present. The differential dependence on IL-10 may reflect differences in the degree of inflammation and T cell activation in the gut in the colitis model compared with our lung model but is in keeping with data from a gastritis model (23). IL-10 expression in the airway was still elevated after transfer of IL-$10^{-/-}$ $CD4^+CD25^+$ T cells, suggesting that the regulatory T cells may induce IL-10 production in another cell population within the lung. These could be $CD4^+CD25^-$ T cells, which, in humans, have been reported to acquire a regulatory phenotype dependent on IL-10 production after in vitro co-culture with $CD4^+CD25^+$ T cells (26). In addition, a recent report also suggests that $CD4^+CD25^+$ T cells might not be the only source of IL-10 in a model of infectious tolerance with $CD4^+CD25^+$ T cells during experimental allergic encephalomyelitis (27). Here, we show for the first time that the percentage of pulmonary $CD4^+$IL-10–producing cells is increased after transfer of $CD4^+CD25^+$ regulatory T cells in vivo. Although we cannot exclude the possibility that other cells contribute to IL-10 production, we did not find expression in macrophages, dendritic cells, or granulocytes. Interestingly, we found IL-10 production by $CD4^+$ cells was similarly increased after transfer of IL-10–deficient $CD4^+CD25^+$ regulatory T cells. This implies that $CD4^+CD25^+$ regulatory T cells suppress inflammation via induction of IL-10 from $CD4^+$ T cells and that production of IL-10 from the regulatory cells themselves is not required. Thus, our data may reconcile the apparent contradiction in previous in vitro and in vivo reports because suppression in vivo may rely on the induction of IL-10 from recipient $CD4^+$ T cells.

The data presented in this paper suggest that induction of IL-10 in vivo by T regulatory cells may represent a novel treatment for allergic asthma. Indeed, it has been previously demonstrated that nonallergic individuals have a higher percentage of allergen-specific IL-10–producing cells, whereas allergic individuals are characterized by a higher percentage of allergen-specific IL-4–producing cells (8). Strategies for inducing IL-10 may include expansion of the naturally occurring $CD4^+CD25^+$ population or enhancement of regulatory T cell function by drugs such as corticosteroids, as shown previously in vitro (28). Alternatively it may be possible to induce populations of IL-10–producing or TGF-β surface-positive T regulatory cells in vivo, as has been described in several different murine systems using mycobacterial exposure, airway allergen delivery before sensitization and challenge, or in vitro derivation by stimulation in the presence of immunosuppressive drugs (29, 30, 31, 16). Allergen immunotherapy has been used for many years to control symptoms in a variety of allergic diseases, and current data suggest that this may induce IL-10–producing regulatory T cell populations (32, 33). Peptide therapy induced IL-10–producing regulatory cells in mice (34), and recent data suggest that allergen-derived T cell peptides may also reduce features of asthma in humans through induction of IL-10 (35).

In conclusion, we provide for the first time direct evidence to show that transfer of allergen-specific $CD4^+$ $CD25^+$ T regulatory cells to sensitized mice abrogates the features of allergic airway disease in vivo. These include reduced eosinophil and Th2 cell recruitment, AHR, and Th2 cytokine production. Down-regulation of inflammation was observed with a concomitant increase in pulmonary IL-10 production from $CD4^+$ T cells. Blocking studies showed that the suppressive effect was dependent on IL-10, but, importantly, the effect was not dependent on IL-10 production from the $CD4^+CD25^+$ regulatory T cells themselves. Thus, our data suggest that strategies designed to maximize the function of T regulatory cells in vivo could be of benefit to allergic asthmatic patients in the future.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Harlan. As a source of allergen-specific $CD4^+CD25^+$ regulatory T cells, DO11.10 mice expressing the transgene for the DO11.10 αβ TCR, which recognizes residues 323–339

of chicken OVA in association with I-A^d, were used. These mice were originally generated by D. Loh (Washington University, St. Louis, MO) (36) and were provided by J. Skok (Imperial College, London, UK). Animals were housed at the Imperial College London animal facility and were used at 6–8 wk of age. Food and water were supplied ad libitum. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 were strictly observed. DO11.10 mice crossed to IL-10–deficient mice on a BALB/c background were a gift of K. Rajewsky (University of Cologne, Cologne, Germany) (37).

Isolation of CD4$^+$CD25$^+$ regulatory T cells

DO11.10 and DO11.10 IL-10–deficient mice were killed by cervical dislocation, and OVA-specific CD4$^+$CD25$^+$ cells were isolated from the spleen and peripheral lymph nodes using a CD4$^+$CD25$^+$ regulatory T cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec). Purity was assessed by flow cytometric analysis (as described in Staining of BAL...) and was typically >90%.

In vitro assessment of regulatory capacity

BALB/c mice were sensitized using OVA (Sigma-Aldrich) at a concentration of 0.1 mg/mouse in 0.2 ml alum (Au-Gel-S; SERVA Electrophoresis) i.p. 1 wk later, mice were killed by cervical dislocation. Spleens were recovered from mice, and single-cell suspensions were prepared. Splenocytes were cultured in triplicate at 5×10^5 splenocytes/well in flat-bottomed 96-well plates in the presence of complete media alone or with 100 μg/ml OVA for 48 h. Complete media consisted of RPMI 1640 (Sigma-Aldrich) containing 10% FCS (Invitrogen), 5mM L-glutamine (Invitrogen), and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). The cells were incubated for a further 18 h in the presence of 1 μCi/well [^{3}H]thymidine (MP Biochemicals) to quantify cell proliferation. To assess the suppressive capacity of freshly isolated CD4$^+$CD25$^+$ regulatory T cells, these cells were isolated from DO11.10 spleen and peripheral lymph nodes and added at 1.25×10^5 cells/well. To assess the proliferative capacity of isolated CD4$^+$CD25$^+$ cells alone, splenocytes were irradiated at 3,000 rad and cultured with CD4$^+$CD25$^+$ cells. [^{3}H]thymidine incorporation data in cpm was converted to stimulation index (fold increase = cpm with OVA / cpm of medium only condition).

Induction of AHR and eosinophilia

BALB/c mice were sensitized using OVA at a concentration of 0.1 mg/mouse in 0.2 ml alum on day 0. Control mice received the same volume of PBS in alum. On day 6, mice received either 5×10^5 CD4$^+$CD25$^+$ cells intravenously in PBS or the same volume of PBS as a control. In some experiments, OVA-specific CD4$^+$CD25$^-$ T cells were injected as a further control. All groups of mice were challenged daily with 5% OVA (aerosolized for 20 min) via the airways between days 7 and 12. In some experiments, mice were dosed with 250 μg anti–IL-10R (1B1.3) 30 min before the first, third, and fifth OVA challenge. Mice were killed by exsanguination under terminal anesthesia at 24 h after the final OVA challenge.

Measurement of AHR

Airway responsiveness was measured in mice 24 h after the final OVA challenge by recording respiratory pressure curves by whole-body plethysmography (Buxco Electronics) in response to inhaled methacholine (Sigma-Aldrich) at concentrations of 3–30 mg/ml for 1 min, as described previously (38). Results are shown for Penh after allergen challenge. Penh results were confirmed by direct measurements of RL and Cdyn in anaesthetized and tracheostomized mice in response to inhaled methacholine at concentrations of 3–100 mg/ml in a Buxco system (Buxco Electronics) in a modified version of previously described methods (39).

Cell recovery

Airway lumen. BAL was performed as previously described (3). In brief, the airways of the mice were lavaged three times with 0.4 ml PBS via a tracheal cannula. BAL fluid was centrifuged at 200 *g* for 5 min at 4°C. Cells were counted and pelleted onto glass slides by cytocentrifugation (5×10^4 cells/slide). Differential cell counts were performed on Wright-Giemsa–stained (Thermo) cytospins. Percentages of eosinophils, lymphocyte/mononuclear cells, neutrophils, and macrophages were determined by counting their number in eight randomly selected high power fields (40×; total area = 0.5 mm^2/area) and dividing this number by the total number of cells counted. To obtain absolute numbers of each leukocyte subtype, these percentages were multiplied by the total number of cells obtained in the lavage fluid. All differential counts were performed blind and in a randomized order at the end of the study by the same observer (J. Kearley).

Lung parenchyma. To disaggregate the cells from the lung tissue, one lobe (~100 mg) of lung was incubated at 37°C for 1 h in digest reagent (0.15 mg/ml collagenase [type D; Roche], 25 μg/ml DNase [type 1; Roche]) in complete media. The recovered cells were filtered through a 70-μm nylon sieve (Falcon), washed twice, resuspended in complete media, and counted in a hemocytometer (VWR). Cytocentrifuge preparations were prepared and Wright-Giemsa stained, and differential counts were performed as for BAL.

Draining lymph nodes. Draining lymph nodes were removed, weighed, and filtered through a 70-μm nylon sieve. The resulting single cell suspension was centrifuged at 200 *g* for 5 min at 4°C, resuspended in complete media, and counted in a hemocytometer.

Lung tissue histopathology

Lungs were fixed in 10% normal buffed formalin. 4-μm paraffin-embedded sections were stained with hemotoxylin and eosin.

Staining of BAL, lung, and lymph node cells for flow cytometric analysis

Suspensions of BAL and lung tissue and lymph node cells were stained in PBS containing 1% FCS and 0.01% sodium azide. To reduce nonspecific binding, cells were incubated with rabbit serum (Sigma-Aldrich) for 15 min before staining. The antibodies used were anti–mouse CD4, anti–mouse CD11b, anti–mouse CD11c, anti–mouse B220, anti–mouse CD8, anti–mouse IL-10 and its isotype control (all obtained from BD Biosciences), anti–mouse T1/ST2 (Morwell Diagnostics), and anti–mouse KJ1-26 and its isotype control (Caltag). Cells were stained for 20 min on ice, washed twice, and fixed in cellfix (Becton Dickinson). For intracellular cytokine staining, cells were stimulated with PMA/ionomycin (Merck) in the presence of Brefeldin A (Sigma-Aldrich) for 6 h before extracellular staining. After staining and fixing, cells were permeabilized using 0.5% saponin (Sigma-Aldrich) and stained with anti–IL-10 antibody or the appropriate isotype control. Flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson).

Cytokine analysis

Cytokines were analyzed in BAL samples and lung-tissue homogenate supernatants. Lung tissue was homogenized at 50 mg/ml in HBSS (Invitrogen) and centrifuged at 800 *g* for 10 min and the supernatant wascollected. Paired antibodies for murine IFN-γ and TGF-β (BD Biosciences), IL-5 (Endogen), eotaxin/CCL11, and TARC/CCL17 (R&D Systems) were used in standardized sandwich ELISAs according to the manufacturer's protocol. MDC/CCL22 levels were measured using a sandwich ELISA generated by coating ELISA plates with anti–mouse MDC (a gift from ICOS Corp., Bothell, WA) and detecting bound antibody with biotinylated anti–mouse MDC (R&D Systems) against a standard curve generated using recombinant MDC (R&D Systems) as previously described (40). ELISA kits to measure IL-13 and IL-10 were purchased from R&D Systems and eBioscience, respectively, and were used according to the manufacturer's protocol.

IgE

Levels of total IgE were measured in serum by ELISA using paired antibodies according to the manufacturer's instructions (BD Biosciences). Levels of

anti-OVA IgE were measured in serum and lung homogenate supernatant by ELISA as described previously (41).

Data analysis

Data are expressed as medians unless otherwise stated. Statistical significance between groups was tested using a Mann-Whitney U Test. A p-value of <0.05 was considered significant. Graph generation and statistical analysis were performed by using Prism software (version 4.00; GraphPad).

Online supplemental material

Fig. S1 shows that allergen-specific $CD4^+CD25^+$ regulatory T cells suppress allergen-induced proliferation in vitro. Fig. S2 shows that allergen-specific $CD4^+CD25^+$ regulatory T cells are able to migrate to the airway lumen, lung tissue, and draining lymph nodes in vivo. Fig. S3 shows that transfer of $CD4^+CD25^+$ regulatory T cells suppresses changes in RL and Cdyn. Fig. S4 shows that proinflammatory chemokine levels in the lung are unaffected by transfer of $CD4^+CD25^+$ regulatory T cells. Fig. S5 shows that transfer of $CD4^+CD25^+$ regulatory T cells has no effect on serum IgE levels.

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