

# Induction of T helper type 1–like regulatory cells that express Foxp3 and protect against airway hyper-reactivity

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The range of regulatory T cell (T<sub>R</sub> cell) types that control immune responses is poorly understood. We describe here a population of T<sub>R</sub> cells that developed *in vivo* from naive CD4<sup>+</sup>CD25<sup>-</sup> T cells during a T helper type 1 (T<sub>H</sub>1)–polarized response, distinct from CD25<sup>+</sup> T<sub>R</sub> cells. These antigen-specific T<sub>R</sub> cells were induced by CD8 $\alpha$ <sup>+</sup> DCs, produced both interleukin 10 and interferon- $\gamma$ , and potently inhibited the development of airway hyper-reactivity. These T<sub>R</sub> cells expressed the transcription factors Foxp3 and T-bet, indicating that these T<sub>R</sub> cells are related to T<sub>H</sub>1 cells. Thus, adaptive T<sub>R</sub> cells are heterogeneous and comprise T<sub>H</sub>1-like T<sub>R</sub> cells as well as previously described T<sub>H</sub>2-like T<sub>R</sub> cells, which express Foxp3 and are induced during the development of respiratory tolerance by CD8 $\alpha$ <sup>-</sup> DCs.

There is now convincing evidence that regulatory CD4<sup>+</sup> T cells are essential in the control of immune responses by inhibiting the function of T helper type 1 (T<sub>H</sub>1) and T<sub>H</sub>2 effector cells. Several distinct types of CD4<sup>+</sup> T regulatory (T<sub>R</sub>) cells have been described, including CD4<sup>+</sup>CD25<sup>+</sup> T cells, that develop naturally in the thymus, constitute 5–10% of CD4<sup>+</sup> T cells from naive mice and provide potent inhibitory activity against autoreactive T cells (called ‘natural T<sub>R</sub> cells’)<sup>1</sup>. In addition, several forms of antigen-specific T<sub>R</sub> cells have been described that are induced after exposure to specific, exogenous antigen (called ‘adaptive T<sub>R</sub> cells’)<sup>2</sup>. These include T<sub>R</sub>1 cells, which develop *in vitro* in the presence of interleukin 10 (IL-10)<sup>3</sup> or in the presence of vitamin D3 and dexamethasone<sup>4</sup>, produce IL-10 and inhibit inflammatory responses in the colon and central nervous system. Adaptive T<sub>R</sub> cells also include antigen-specific T<sub>R</sub> cells that develop *in vivo* from CD25<sup>-</sup> naive T cells after epicutaneous immunization with autoantigenic peptides and inhibit experimental allergic encephalomyelitis<sup>5</sup> or that develop from CD25<sup>-</sup> naive T cells after respiratory exposure to antigen and inhibit the development of allergen-induced airway hyper-reactivity (AHR)<sup>6</sup>. Furthermore, T<sub>H</sub>3 cells have been described that develop after exposure to oral antigen and inhibit the development of experimental autoimmune encephalomyelitis<sup>7</sup>. However, because adaptive T<sub>R</sub> cells have been difficult to generate, isolate and study, the relationship between natural and adaptive T<sub>R</sub> cells, specific methods that efficiently induce the development of adaptive T<sub>R</sub> cells and the full range of adaptive T<sub>R</sub> cells that exist are not fully understood.

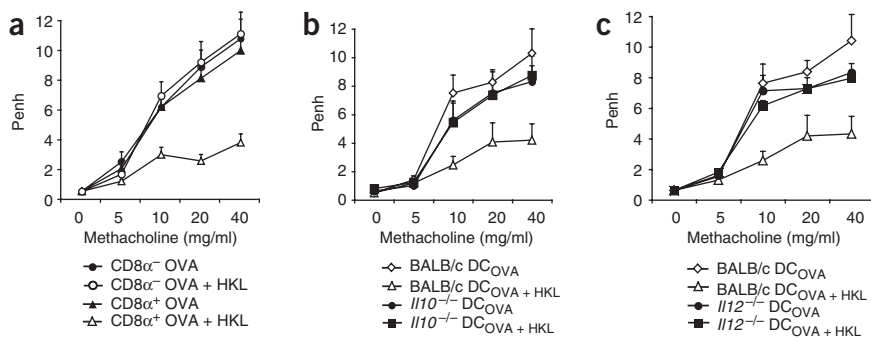
Here we describe a unique T<sub>R</sub> cell type that developed *in vivo* from naive CD4<sup>+</sup>CD25<sup>-</sup> T cells during T<sub>H</sub>1 polarized immune responses. These ovalbumin (OVA)–specific T<sub>R</sub> cells were induced by mature CD8 $\alpha$ <sup>+</sup> dendritic cells (DCs), produced both IL-10 and interferon- $\gamma$  (IFN- $\gamma$ ) and expressed the ‘master T<sub>H</sub>1 transcription regulator’ T-bet as well as large amounts of inducible costimulator (ICOS). The T<sub>H</sub>1-related T<sub>R</sub> cells also expressed Foxp3, a transcription factor previously thought to be exclusively expressed by CD4<sup>+</sup>CD25<sup>+</sup> natural T<sub>R</sub> cells. Moreover, the T<sub>R</sub> cells potently inhibited the development of allergen-induced AHR, a function that was inhibited by neutralization of IL-10 but not IFN- $\gamma$ . Because these adaptive T<sub>R</sub> cell produced IFN- $\gamma$ , were induced by CD8 $\alpha$ <sup>+</sup> DCs and expressed T-bet, these T<sub>R</sub> cells are related to but are distinct from T<sub>H</sub>1 cells. We suggest therefore that a spectrum of adaptive T<sub>R</sub> cell types exists, including T<sub>H</sub>1-like T<sub>R</sub> cells, as well as previously described T<sub>H</sub>2-like T<sub>R</sub> cells, which are induced by CD8 $\alpha$ <sup>-</sup> DCs, express the T<sub>H</sub>2 ‘master transcription factor’ GATA3 and Foxp3 and develop via an IL-4-producing intermediate stage<sup>6</sup>.

## RESULTS

### CD8 $\alpha$ <sup>+</sup> DCs transfer suppression

Heat-killed *Listeria monocytogenes* (HKL) as an adjuvant induces an antigen-specific inhibitory response that prevents the development of and reverses established T<sub>H</sub>2 responses and AHR<sup>8</sup>. Although HKL induces the development of T<sub>H</sub>1 cells<sup>9,10</sup>, the absence of inflammation in the lungs of mice treated with HKL<sup>8</sup> suggests that

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**Figure 1** CD11c<sup>+</sup> CD8 $\alpha$ <sup>+</sup> DCs protect against AHR. (a) CD8 $\alpha$ <sup>-</sup> DCs or CD8 $\alpha$ <sup>+</sup> DCs, isolated from spleens of BALB/c mice that had been immunized previously with OVA or with OVA plus HKL were adoptively transferred into BALB/c recipients ( $1 \times 10^6$  cells/mouse), which were then immunized with OVA plus alum. Then 8 d later, mice were challenged intranasally with OVA (50  $\mu$ g, three times), and AHR was assessed 24 h later. (b,c) The inhibitory function of CD8 $\alpha$ <sup>+</sup> DC requires the production of IL-10 and IL-12. CD8 $\alpha$ <sup>+</sup> DCs were isolated from *Il10*<sup>-/-</sup> (b) or *Il12*<sup>-/-</sup> (c) mice (BALB/c background) previously immunized with OVA or with OVA and HKL and were adoptively transferred into wild-type BALB/c mice, as in a. The recipient mice were then immunized with OVA plus alum and 8 d later were challenged intranasally with OVA (50  $\mu$ g, three times) and were assessed for AHR 24 h later. Results are presented as mean peak Penh values of five mice per group  $\pm$  s.e.m.

anti-inflammatory T<sub>R</sub> cells, rather than proinflammatory T<sub>H</sub>1 cells<sup>11,12</sup>, are mainly responsible for the inhibitory effect of HKL on AHR.

Because the protective effect of HKL is abolished by treatment with mAb to CD8 $\alpha$ <sup>8</sup>, we sought to determine if cells expressing CD8 $\alpha$  might be responsible for the anti-inflammatory effect of HKL. CD8 $\alpha$ <sup>+</sup> DCs from mice immunized with OVA plus HKL had a mature phenotype (high surface expression of CD80, CD86, major histocompatibility complex class II, ICOS ligand (ICOSL), CD40, as well as CD205 (DEC-205) and OX40-L, but not B-220; **Supplementary Fig. 1** online). Adoptive transfer of these mature CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs isolated from mice immunized with OVA plus HKL inhibited the subsequent development of AHR (**Fig. 1a**), whereas transfer of CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> DCs from mice immunized with OVA plus HKL failed to inhibit the development of AHR, indicating that only the CD8 $\alpha$ <sup>+</sup> and not the CD8 $\alpha$ <sup>-</sup> DCs were responsible for the inhibitory effect. Furthermore, the inhibitory effect of the CD8 $\alpha$ <sup>+</sup> cells was not due to conventional CD8 $\alpha$  $\beta$ <sup>+</sup> T cells, as adoptive transfer of CD8 $\alpha$ <sup>+</sup> T cells purified with a mAb to CD8 $\beta$  from mice immunized with OVA plus HKL had no inhibitory effect on AHR (data not shown). Thus, CD8 $\alpha$ <sup>+</sup> DCs are effective in transferring the inhibitory effect of HKL, presumably by inducing a regulatory response that inhibited AHR.

We next evaluated the mechanisms by which CD8 $\alpha$ <sup>+</sup> DCs generated by immunization with OVA plus HKL mediated the inhibition of AHR. We examined IL-12 and IL-10 production by the DCs because CD8 $\alpha$ <sup>+</sup> DCs classically produce IL-12, and because regulatory DCs have been shown to produce IL-10<sup>13</sup>. Adoptive transfer of CD8 $\alpha$ <sup>+</sup> DCs isolated from IL-10-deficient mice immunized with OVA plus HKL failed to inhibit AHR (**Fig. 1b**). Furthermore, adoptive transfer of CD8 $\alpha$ <sup>+</sup> DCs isolated from IL-12-deficient mice immunized with OVA plus HKL also failed to inhibit AHR (**Fig. 1c**), indicating that the production of both IL-10 and IL-12 by the CD8 $\alpha$ <sup>+</sup> DCs was required for the DCs to exert their protective effects.

### Induction of T cells producing IL-10 and IFN- $\gamma$

To investigate the mechanism by which CD8 $\alpha$ <sup>+</sup> DCs exert their regulatory effects in this model, we analyzed the T cells activated by the CD8 $\alpha$ <sup>+</sup> DCs. We adoptively transferred CD8 $\alpha$ <sup>+</sup> DCs from mice immunized with OVA plus HKL without further administration of

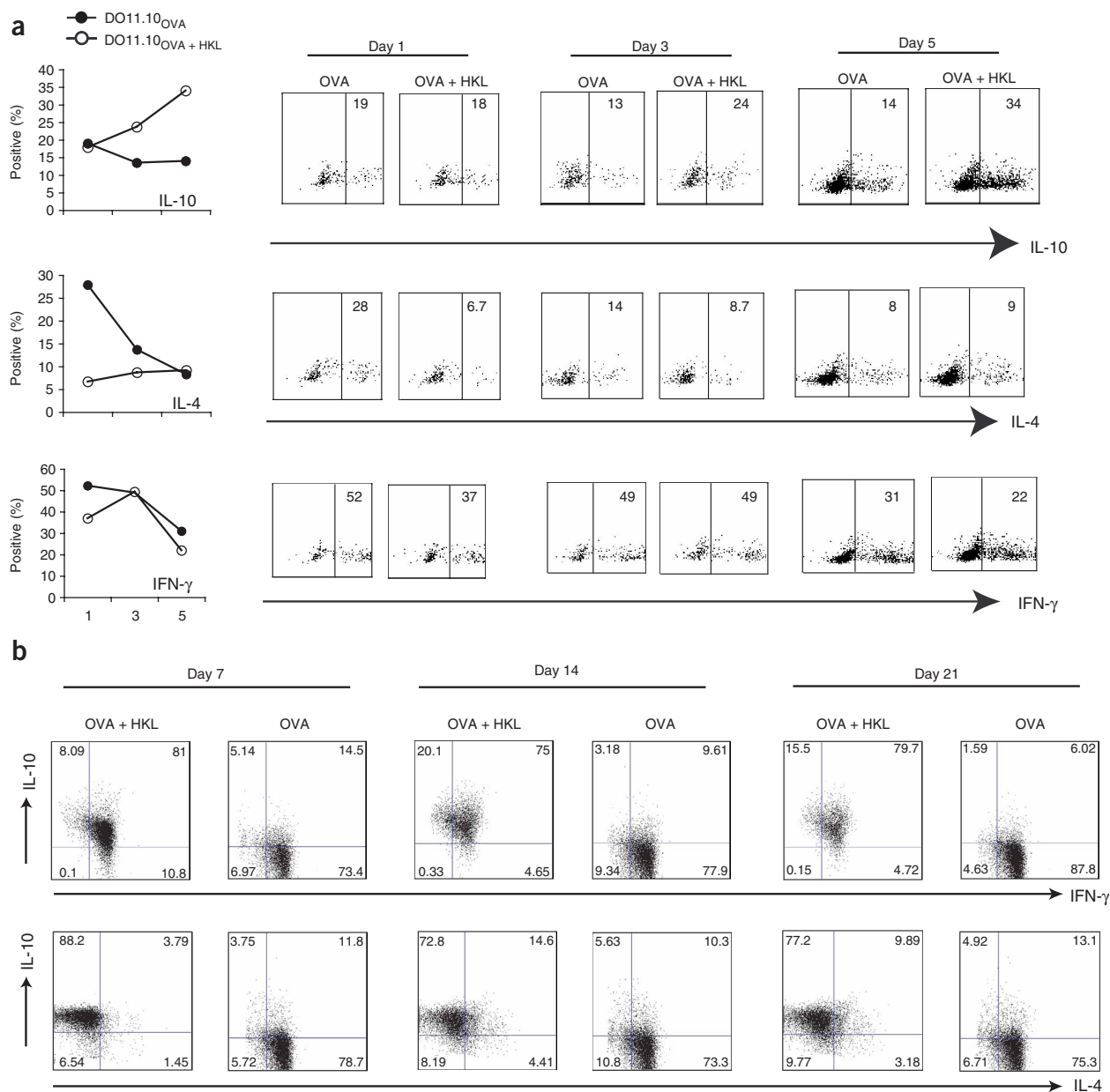
antigen and examined the differentiation in mice that received adoptively transferred naive OVA-specific CD4<sup>+</sup> DO11.10 T cell receptor (TCR)-transgenic T cells (from DO11.10 recombination activating gene 2-deficient (*Rag2*<sup>-/-</sup>) mice, which lack CD25<sup>+</sup> T<sub>R</sub> cells<sup>14</sup>). Over the course of 5 d, the DO11.10 cells isolated from mice receiving CD8 $\alpha$ <sup>+</sup> DCs exposed to HKL produced large amounts of IL-10 (**Fig. 2a** and **Supplementary Fig. 2** online). In contrast, we noted production of IL-4 only in DO11.10 cells at early time points after adoptive transfer of CD8 $\alpha$ <sup>+</sup> DCs exposed to OVA alone. The production of IL-10 in the DO11.10 cells was dependent on the exposure of the DCs to HKL, because the DO11.10 cells that developed in mice receiving CD8 $\alpha$ <sup>+</sup> DC generated in the absence of HKL did not produce IL-10 (**Fig. 2a** and **Supplementary Fig. 2** online). In addition, approximately half of the DO11.10 cells generated in the presence of HKL-stimulated DCs produced IFN- $\gamma$  3 d after transfer of DCs.

The IFN- $\gamma$ -producing T cells induced with CD8 $\alpha$ <sup>+</sup> DCs were distinct from T<sub>H</sub>1 cells, because most of the cytokine-producing DO11.10 examined 7 d after adoptive transfer were positive for both IL-10 and IFN- $\gamma$ , as shown by intracellular staining of cells positive for KJ1-26, a clonotypic mAb for DO11.10 T cells. These cells producing both IL-10 and IFN- $\gamma$  did not produce IL-4, as determined by double staining for IL-10 and IL-4. In contrast, the KJ1-26<sup>+</sup> cells generated in the absence of HKL produced IL-4 and some IFN- $\gamma$  but not IL-10. The phenotype of T<sub>R</sub> cells producing both IL-10 and IFN- $\gamma$  seemed to be stable, as production of both cytokines persisted when examined on days 14 and 21, after the mice received additional CD8 $\alpha$ <sup>+</sup> DCs on days 7 and 14 (**Fig. 2b** and **Supplementary Table 1** online).

### T<sub>R</sub> cells express ICOS, Foxp3 and T-bet

To better characterize the T cells producing both IL-10 and IFN- $\gamma$ , we examined them for expression of other markers of T<sub>H</sub> cells and T<sub>R</sub> cells. The T cells producing both IL-10 and IFN- $\gamma$  expressed CD25, CD44, CD69 and ICOS, a costimulatory molecule associated with IL-10 expression in T cells<sup>6,15–20</sup>, but small amounts of CD62L (**Fig. 3a**). In contrast, the DO11.10 T cells generated in the absence of HKL expressed CD25, CD69, CD62L, some CD44 and small amounts of ICOS. The T cells producing both IL-10 and IFN- $\gamma$  generated with HKL-stimulated DCs, but not T cells generated by DCs stimulated only with OVA (*T*<sub>OVA</sub>), also expressed mRNA for the transcription factor Foxp3, previously shown to be expressed only by natural CD25<sup>+</sup> T<sub>R</sub> cells, as determined by conventional RT-PCR (data not shown) or by quantitative RT-PCR (**Fig. 3b**). We also noted expression of Foxp3 in another IL-10-producing T<sub>R</sub> cell type previously demonstrated to develop after respiratory exposure to allergen with CD8 $\alpha$ <sup>-</sup> DCs<sup>6</sup> (T<sub>R</sub> pulmonary cells)<sup>6</sup>. Thus, two different types of antigen-specific adaptive T<sub>R</sub> cells induced *in vivo* expressed Foxp3. In contrast, Foxp3 was not expressed by CD25<sup>-</sup> spleen cells, a CD25<sup>+</sup> T cell line (IL-2-dependent CTLL) or naive DO11.10 T cells.

We also examined the T cells that were generated with HKL and produced both IL-10 and IFN- $\gamma$  for expression of the T<sub>H</sub>1 ‘master transcription regulator’ T-bet and the T<sub>H</sub>2 ‘master transcription factor’ GATA3. T cells producing both IL-10 and IFN- $\gamma$  that were generated with HKL, but not those generated in the absence of HKL,



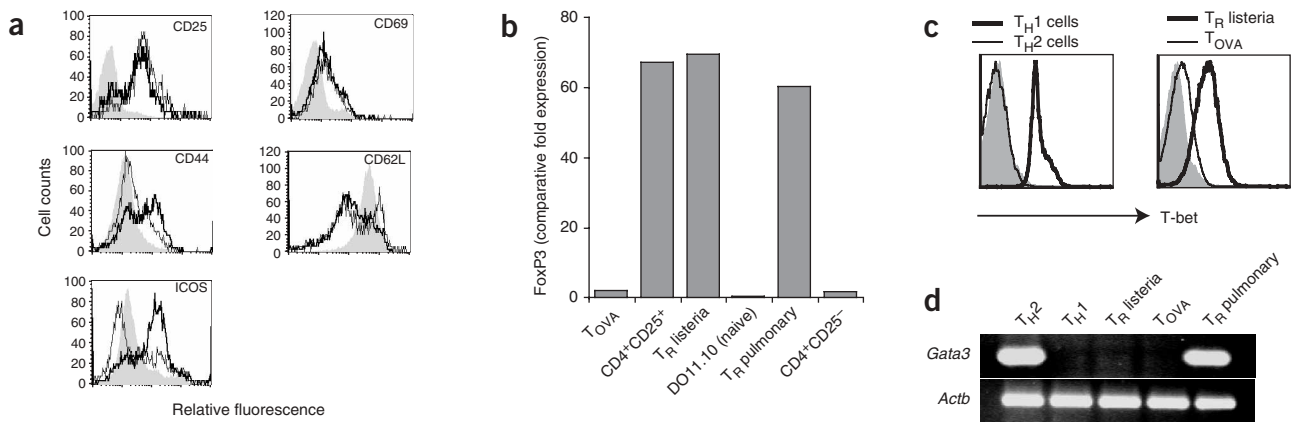
**Figure 2** T cells induced by the regulatory CD8 $\alpha^+$  DC express IL-10 and IFN- $\gamma$ . **(a)** CD8 $\alpha^+$  DCs isolated from spleens of BALB/c mice that had been immunized previously with OVA or with OVA plus HKL were adoptively transferred along with naive DO11.10 cells into BALB/c recipient mice. On days 1, 3 and 5, KJ1-26 $^+$  cells were purified from the spleens of these mice and intracellular cytokine production was assessed by flow cytometry, gating on KJ1-26 $^+$  cells. Numbers in dot plots represent the percentage of cytokine-producing cells, summarized as graphs (left). Vertical axes on dot plots indicate forward scatter. Results are from one experiment representative of five. **(b)** As described in **a**, CD8 $\alpha^+$  DCs from mice previously immunized with OVA plus HKL were adoptively transferred (on day 0) into recipient mice that also received naive DO11.10 cells. Some mice received additional CD8 $\alpha^+$  DCs from mice previously immunized with OVA with or without HKL on days 7 and 14. DO11.10 cells were isolated from the spleens of recipient mice on days 7, 14 and 21, were double-stained for intracellular cytokines and were assessed by flow cytometry, gating on cytokine producing DO11.10 cells. Numbers in quadrants indicate the percentage of cells in that quadrant. Results are from one experiment representative of three.

expressed T-bet (**Fig. 3c**). However, these T cells producing both IL-10 and IFN- $\gamma$  did not express GATA3 (**Fig. 3d**). In contrast, the IL-10-producing T<sub>H</sub>2 cells induced by respiratory exposure to allergen<sup>6</sup> (T<sub>R</sub> pulmonary cells) expressed GATA3 but not T-bet. Thus, the T cells producing both IL-10 and IFN- $\gamma$  have characteristics of T<sub>H</sub>1 cells (expressing T-bet and IFN- $\gamma$  and generated by CD8 $\alpha^+$  DCs), but are distinct from T<sub>H</sub>1 cells by having characteristics of T<sub>R</sub> cells (expressing ICOS, Foxp3 and IL-10). In contrast, the T<sub>R</sub> pulmonary cells have

characteristics of T<sub>H</sub>2 cells (expressing GATA3 and generated by CD8 $\alpha^-$  DCs via an IL-4-producing intermediate stage)<sup>6</sup>.

#### *In vivo* function of T<sub>H</sub>1-like T<sub>R</sub> cells

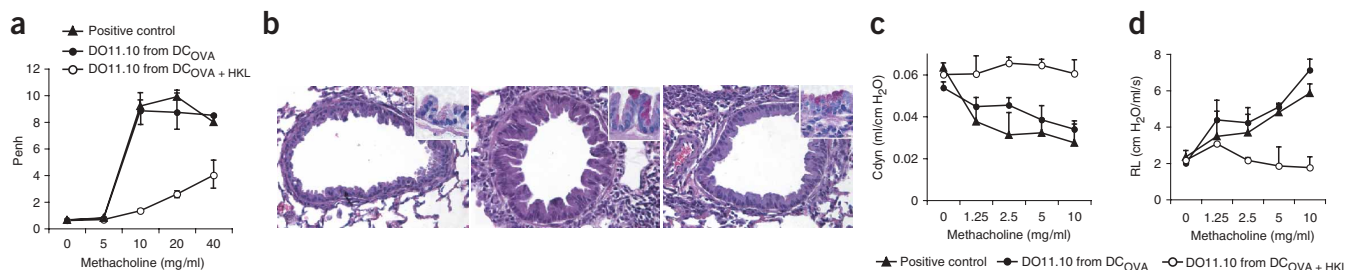
We examined the capacity of the T<sub>H</sub>1-like T<sub>R</sub> cells producing both IL-10 and IFN- $\gamma$  to inhibit the development of AHR. We isolated DO11.10 cells from BALB/c mice immunized with CD8 $\alpha^+$  DCs exposed to HKL. We adoptively transferred these OVA-specific



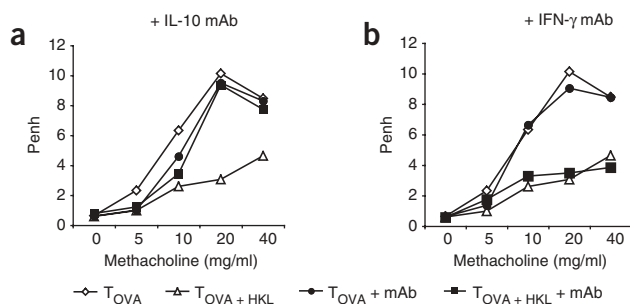
KJ1-26 $^+$  cells into recipients that had been sensitized with OVA in Al(OH)<sub>3</sub> (alum) 8 d before. At 24 h after transfer, we challenged the recipient mice intranasally with OVA to induce AHR. Adoptive transfer of T<sub>H</sub>1-like T<sub>R</sub> cells notably reduced the development of AHR, whereas transfer of control T cells generated with DCs in the absence of HKL did not (**Fig. 4a**). The reduction in AHR by the T<sub>H</sub>1-like T<sub>R</sub> cells was accompanied by a notable reduction in airway inflammation (**Fig. 4b**). Thus, transfer of the T<sub>H</sub>1-like T<sub>R</sub> cells but not naive DO11.10 cells greatly reduced the peribronchiolar infiltrate and mucus production in the airways (**Fig. 4b**). Transfer of T cells generated with DCs in the absence of HKL also did not inhibit airway

inflammation, such that large numbers of inflammatory cells and abundant mucus in pulmonary epithelial cells were present in the airways (**Fig. 4b**). We confirmed the inhibitory effect of the T<sub>H</sub>1-like T<sub>R</sub> cells on AHR by assessing AHR using direct invasive assays for dynamic compliance (**Fig. 4c**) and lung resistance (**Fig. 4d**) in mice that were anesthetized, tracheostomized and mechanically ventilated.

The anti-inflammatory effects of the transferred cells were not due to conventional antigen-specific T<sub>H</sub>1 cells, because adoptively transferred T<sub>H</sub>1 cells cannot inhibit, but instead greatly exacerbate, airway inflammation and AHR in sensitized mice<sup>11,12</sup>. Moreover, the inhibitory effects of the T<sub>H</sub>1-like T<sub>R</sub> cells were blocked by a mAb to IL-10



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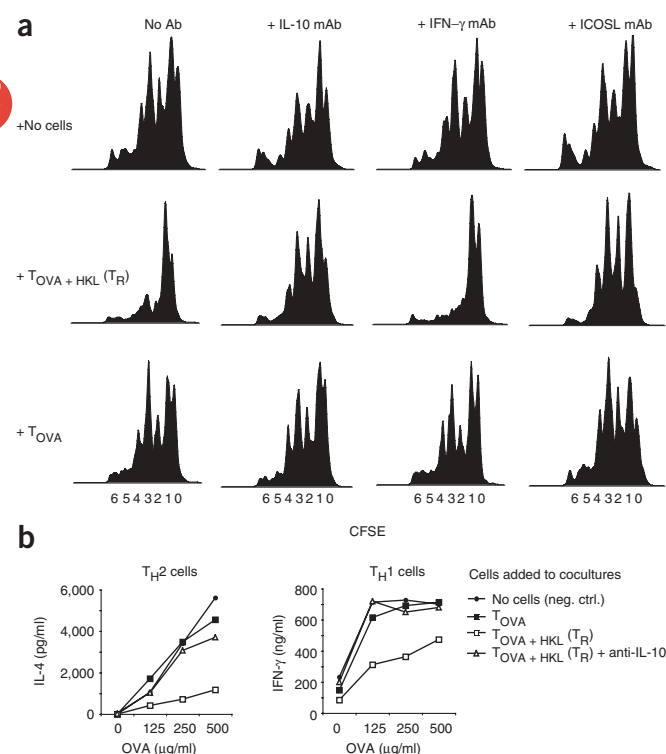


**Figure 5** The regulatory effects of the  $T_R$  cells depend on IL-10 but not IFN- $\gamma$ .  $T_R$  cells were generated and adoptively transferred into mice sensitized to OVA+alum as described in **Figure 4a**. Cells were incubated for 4 h at 37 °C with mAb to IL-10 (**a**) or mAb to IFN- $\gamma$  (**b**) and were adoptively transferred together with 500  $\mu$ g of the same mAb as used for the incubation. AHR was measured after challenge with methacholine; data represent Penh values averaged among sensitized mice in each group.

(**Fig. 5a**) but not by a mAb to IFN- $\gamma$  (**Fig. 5b**), indicating that the production of IL-10 and not IFN- $\gamma$  by the  $T_H1$ -like  $T_R$  cells was required for their regulatory effects. We confirmed the requirement for IL-10 but not IFN- $\gamma$  production by the  $T_H1$ -like  $T_R$  cells to reduce AHR by invasive measurements of compliance and resistance of the lungs (data not shown). Thus,  $T_H1$ -like  $T_R$  cells are distinct from  $T_H1$  cells and have a potent anti-inflammatory function that reverses established  $T_H2$  responses.

#### Analysis of the *in vitro* function of $T_H1$ -like $T_R$ cells

To further analyze the suppressive capacity of  $T_H1$ -like  $T_R$  cells, we examined their effects on naive DO11.10 T cells and on OVA-specific  $T_H1$  and  $T_H2$  effector cells. In the absence of the  $T_H1$ -like  $T_R$  cells, naive DO11.10 cells labeled with 5- (and 6-) carboxyfluorescein diacetate succinimidyl diester (CFSE) proliferated vigorously in



response to DCs plus OVA, completing three to four rounds of cell division over 48 h (**Fig. 6a**). The addition of the  $T_H1$ -like  $T_R$  cells notably inhibited the proliferation of the CFSE-labeled cells. The inhibitory effect of  $T_H1$ -like  $T_R$  cells was dependent on IL-10 and the ICOS-ICOSL pathway, because the addition of neutralizing mAb to IL-10 or mAb to ICOSL to the cultures restored the proliferation of naive DO11.10 T cells. In contrast, the addition of mAb to IFN- $\gamma$  produced little or no effect on the function of  $T_H1$ -like  $T_R$  cells. Control T cells generated in the absence of HKL ( $T_{OVA}$  cells) did not inhibit the proliferation of the naive DO11.10 T cells. Thus, the  $T_H1$ -like  $T_R$  cells inhibit antigen-specific T cell proliferation in an IL-10- and ICOS-dependent but IFN- $\gamma$ -independent way.

To investigate whether  $T_H1$ -like  $T_R$  cells could inhibit the function of polarized effector T cells, we cultured established OVA-specific  $T_H1$  or  $T_H2$  cells in the presence or absence of  $T_H1$ -like  $T_R$  cells. The addition of  $T_H1$ -like  $T_R$  cells reduced the production of IL-4 by  $T_H2$  cells and IFN- $\gamma$  by  $T_H1$  cells (**Fig. 6b**). In contrast, the addition of control T cells generated in the absence of HKL to the cultures did not alter the release of IL-4 by  $T_H2$  cells or of IFN- $\gamma$  by  $T_H1$  cells. The inhibitory effects of the  $T_H1$ -like  $T_R$  cells on effector  $T_H2$  and  $T_H1$  cells were dependent on IL-10, because neutralization of IL-10 inhibited their suppressive effects and restored the production of IL-4 and IFN- $\gamma$ , respectively. These results demonstrate that  $T_H1$ -like  $T_R$  cells have functions distinct from those of  $T_H1$  cells, in that they inhibit the proliferation of naive cells and suppress IL-4 and IFN- $\gamma$  production in polarized effector T cells *in vitro* in an ICOS- and IL-10-dependent way.

#### DISCUSSION

In this study we have described a unique antigen-specific adaptive  $T_R$  cell producing both IL-10 and IFN- $\gamma$  that was induced by mature CD8 $\alpha^+$  DCs producing IL-10 and IL-12. The cytokine profile of these  $T_R$  cells, their expression of T-bet and the requirement for CD8 $\alpha^+$  DCs producing IL-12 suggest that these  $T_R$  cells are related to  $T_H1$  cells. However, these  $T_H1$ -like  $T_R$  cells are distinct from  $T_H1$  cells because they potently inhibited established  $T_H2$  responses and allergen-induced AHR, a function that cannot be accomplished by conventional  $T_H1$  cells<sup>11</sup>. Moreover, these *in vivo*-generated  $T_H1$ -like  $T_R$  cells expressed IL-10 and ICOS, which were required for their function, and Foxp3, a transcription factor that was previously thought to be restricted to CD25<sup>+</sup>  $T_R$  cells<sup>21</sup> but that we suggest may be common to *in vivo*-generated T cells with potent regulatory capacities.

The adjuvant used in our studies to induce  $T_H1$ -like  $T_R$  cells, HKL, potently induces  $T_H1$  responses that might counter allergic responses mediated by  $T_H2$  cells<sup>9,10,22</sup>. However, the potency of HKL as an adjuvant to inhibit established  $T_H2$ -driven inflammatory responses

**Figure 6**  $T_R$  cells suppress naive and effector T cells. (**a**) Naive DO11.10 cells ( $4 \times 10^4$  cells/well) were labeled with CFSE and cultured with bone marrow-derived DCs ( $1 \times 10^4$ ) and OVA (250  $\mu$ g/ml) in the presence of  $T_R$  cells ( $1 \times 10^4$ ) generated with HKL ( $T_{OVA} + HKL(T_R)$ ) or control T cells generated without HKL ( $T_{OVA}$ ). Top, cultures contained no mAb or mAb to IL-10, IFN- $\gamma$  or ICOSL (100  $\mu$ g/ml). After 48 h, cells were analyzed by flow cytometry, gated on KJ1-26<sup>+</sup> cells. Results are one experiment representative of three. (**b**)  $T_H2$  cells ( $4 \times 10^4$  cells/well) or  $T_H1$  cells were cultured with bone marrow-derived DCs ( $1 \times 10^4$ ) and OVA (250  $\mu$ g/ml) and either no other cells (pos. ctrl) or in the presence of  $T_R$  cells ( $1 \times 10^4$  cells/well) generated with HKL ( $T_{OVA} + HKL(T_R)$ ) or T cells generated without HKL ( $T_{OVA}$ ) (as in **a**). + anti-IL-10, addition of neutralizing antibody to IL-10. Supernatants were collected after 96 h and cumulative amounts of cytokines were determined by ELISA. Results are one experiment representative of three.

may not be solely due to the development of  $T_{H1}$  responses but may also be due to the development of a modified  $T_{H1}$   $T_R$  response. The inhibitory effect of HKL on AHR and airway inflammation was blocked not only by neutralization of IL-12 (ref. 8) but also by neutralization of IL-10 (ref. 23), suggesting that  $T_R$  cells were involved. In addition, although mAb to CD8 $\alpha$  abolishes the inhibitory effect of HKL on AHR and airway inflammation<sup>8</sup>, we have shown here that the CD8<sup>+</sup> cells that mediated the HKL effect were CD8 $\alpha^+$  DCs producing IL-10 as well as IL-12 and not CD8<sup>+</sup> T cells, which have the capacity in some systems to protect against airway hyperreactivity<sup>24,25</sup>. Also, conventional  $T_{H1}$  cells by themselves are ineffective in dampening established  $T_{H2}$  responses, because  $T_{H1}$  cells in the respiratory mucosa are proinflammatory rather than anti-inflammatory<sup>11,12</sup>. Instead, T cells producing IL-10 or transforming growth factor- $\beta$  have much greater anti-inflammatory activity and are much more effective in limiting airway inflammation and hyperreactivity<sup>6,26,27</sup>. We suggest therefore that HKL is a complex adjuvant that potently induces not only conventional  $T_{H1}$  responses but also modified  $T_{H1}$  responses characterized by  $T_R$  cells producing IFN- $\gamma$  and IL-10. These inhibitory responses may develop normally *in vivo* during stimulation with listeria to downmodulate the robust  $T_{H1}$  responses that are induced by listeria.

The combined production of IL-10 and IFN- $\gamma$  in the  $T_{H1}$ -like  $T_R$  cells may be a synergistic combination that inhibits effector T cell responses. Production of IFN- $\gamma$  in combination with IL-10 has been shown to be induced in T cells by IL-12 (ref. 28) and by certain intracellular pathogens such as leishmania, borrelia or mycobacteria<sup>29–32</sup>. The combined production of IL-10 with IFN- $\gamma$  has been postulated to occur in immunoregulatory T cells that protect against severe inflammatory pathology and that help to maintain pathogen-specific immunological memory<sup>29,30</sup>. We have demonstrated that involvement of CD8 $\alpha^+$  DCs producing both IL-10 and IL-12 is essential in the induction of such  $T_R$  cells. The precise signals that induce the development of the  $T_{H1}$ -like  $T_R$  cells but not conventional  $T_{H1}$  cells are not yet apparent, however, but may include CD8 $\alpha^+$  DC production of IL-10 and IL-12 together in the absence of IL-4 and/or expression of a specific set of costimulatory molecules, including ICOSL, induced by intracellular pathogens such as listeria or leishmania.

The expression of different costimulatory molecules on distinct types of DCs may greatly influence the development of the type of  $T_R$  cell that develops. For example, immature CD8 $\alpha^-$  (refs. 33–35) or CD8 $\alpha^+$  (refs. 36–40) DCs expressing limited quantities of costimulatory molecules have been linked to the induction of tolerance and to the silencing of pathogenic self-reactive CD4<sup>+</sup> or CD8<sup>+</sup> T cells that have escaped negative selection in the thymus, by inducing anergy or deletion, or the development of regulatory cells. In contrast, plasmacytoid (B220<sup>+</sup>) DCs, characterized by their potential to secrete large amounts of type I interferons in response to viral infection<sup>41–44</sup>, as well as mature CD8 $\alpha^-$  DCs in the respiratory tract<sup>13</sup>, maintain tolerance by inducing adaptive  $T_R$  cells. In addition, DC exposed to *Bordetella pertussis* produce IL-10 (ref. 45) and induce bordetella-specific  $T_R$  cells. Our studies have demonstrated that mature CD8 $\alpha^+$  DCs producing IL-12, which had been thought to induce mainly  $T_{H1}$  cell differentiation rather than tolerance<sup>46,47</sup>, can in fact induce  $T_{H1}$ -like  $T_R$  cells.

Although the  $T_{H1}$ -like  $T_R$  cells described here have similarities to  $T_{H1}$  cells, they also have similarities to previously described  $T_R$  cells that developed in the respiratory tract from naive CD4<sup>+</sup>CD25<sup>-</sup> T cells after respiratory exposure to antigen<sup>6</sup>. Both respiratory-induced  $T_R$  cells ( $T_R$  pulmonary) and  $T_{H1}$ -like  $T_R$  cells derive from naive CD4<sup>+</sup>CD25<sup>-</sup> T cells, express the transcription factor Foxp3 and potentially inhibit the development of AHR by pathways involving

IL-10 and the regulatory ICOS-ICOSL signaling pathway<sup>6</sup>. The two adaptive  $T_R$  cell types are distinct, however, in that the respiratory-induced  $T_R$  cells were induced with CD8 $\alpha^-$  rather than CD8 $\alpha^+$  DCs, expressed GATA3 and developed through a stage in which they transiently produced IL-4, suggesting a modified  $T_{H2}$ -like lineage. Thus, a spectrum of antigen-specific adaptive  $T_R$  cells exist, developing in a variety of conditions, and including  $T_R$  cells related to  $T_{H1}$  cells ( $T_{H1}$   $T_R$  cells) or to  $T_{H2}$  cells ( $T_{H2}$   $T_R$  cells).

Expression of both IL-10 and Foxp3 is a characteristic not only of 'our' adaptive  $T_{H1}$   $T_R$  cells, but also of CD25<sup>+</sup> natural  $T_R$  cells<sup>21</sup>. Therefore, the expression of IL-10 and Foxp3 is likely to be an important, perhaps defining, characteristic of both antigen-induced adaptive  $T_R$  cells and CD25<sup>+</sup> natural  $T_R$  cells, although  $T_R$  cells induced with myelin basic protein peptide do not produce detectable IL-10, transforming growth factor- $\beta$ , IFN- $\gamma$  or IL-4 or express Foxp3 (ref. 5), and IL-10-secreting  $T_R$  cells induced with IL-10 also do not express Foxp3 (ref. 48). However, prolonged subcutaneous infusion of a low dose of peptide with an osmotic pump implanted in mice transforms mature T cells into CD4<sup>+</sup>25<sup>+</sup>  $T_R$  cells that do express Foxp3 (ref. 49).

The precise function of Foxp3 is not known, but an absence of Foxp3 in humans results in IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked)<sup>50–52</sup>. Patients with IPEX syndrome share many phenotypic features with scurfy mice, which have a natural mutation in *Foxp3*, lack natural CD25<sup>+</sup>  $T_R$  cells and develop an extensive autoimmune-mediated wasting syndrome<sup>53</sup>. However, it is not apparent whether these patients and scurfy mice also lack adaptive  $T_R$  cells that differentiate in the periphery from CD25<sup>-</sup> T cells. As these patients develop eczema, food allergy, increased immunoglobulin E and peripheral eosinophilia, associated with increased production of  $T_{H2}$  cytokines<sup>52</sup>, we suggest that they may indeed lack all forms of  $T_R$  cells, including natural CD25<sup>+</sup>  $T_R$  cells as well as antigen-specific adaptive  $T_R$  cells, which express Foxp3 and regulate  $T_{H2}$  responses to allergens.

In summary, we have described a previously unknown adaptive  $T_R$  cell type that expresses IFN- $\gamma$ , T-bet, IL-10 and Foxp3 and has a potent inhibitory function. Our results suggest that a spectrum of distinct adaptive  $T_R$  cells expressing Foxp3 with distinct cytokine profiles develop *in vivo* after antigen exposure to downregulate immune responses. These  $T_R$  cells include the  $T_{H1}$   $T_R$  cells that express T-bet and develop in (modified)  $T_{H1}$ -biased conditions, using CD8 $\alpha^+$  DCs. In addition, the spectrum includes  $T_{H2}$   $T_R$  cells, which express IL-10, GATA3 and Foxp3, are induced by plasmacytoid DCs or CD8 $\alpha^-$  DCs and develop in (modified)  $T_{H2}$ -biased conditions through an IL-4-producing intermediate state. The expression of IL-10 and Foxp3 may characterize all of these adaptive  $T_R$  cells, suggesting that adaptive  $T_R$  cells are indeed close 'cousins' of CD25<sup>+</sup>  $T_R$  cells.

## METHODS

**Mice.** BALB/c and IL-12-deficient mice were purchased from The Jackson Laboratory. IL-10-deficient mice, purchased from The Jackson Laboratory, had a C57BL/6 background and were backcrossed for ten generations to BALB/c in our laboratory. *Rag2*<sup>-/-</sup> breeder mice transgenic for an OVA-specific TCR (DO11.10) were provided by A.K. Abbas (Department of Pathology, University of California, San Francisco, San Francisco, California). These mice lack CD25<sup>+</sup> natural  $T_R$  cells<sup>13</sup> and were used as donors of OVA-specific CD4<sup>+</sup>CD25<sup>-</sup> T cells.

Mice were injected intraperitoneally with 200  $\mu$ g OVA (ICN Biomedical) in incomplete Freund's adjuvant (IFA) or with 200  $\mu$ g OVA plus 4  $\times$  10<sup>8</sup> HKL in IFA on day 0. On day 5, the mice were killed and spleens were collected for further studies. Mice intended to undergo measurement of airway hyperreactivity (AHR) were injected intraperitoneally with OVA (100  $\mu$ g/mouse) adsorbed to 2 mg of alum. Then, 8 d later, mice were challenged on

3 consecutive days with OVA (three times, each 50 µg/mouse) and AHR was assessed 24 h after the last challenge. The Stanford University Committee on Animal Welfare (Administration Panel of Laboratory Animal Care) approved all animal protocols used in this study.

**Isolation, purification and adoptive transfer of cells.** DCs were isolated by digestion of fragments of spleens at 37 °C for 1 h with a 'cocktail' of 0.1% DNase I (fraction IX; Sigma) and 1.6 mg/ml of collagenase (CLS4; Worthington Biochemical) followed by dissociation for 10 min with 10 mM EDTA. CD8 $\alpha$ <sup>+</sup> DCs were purified from spleens with the CD8 $\alpha$ <sup>+</sup> Dendritic Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were purified with AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions (purity, >96% by flow cytometry) and cells were injected intravenously into BALB/c recipients (1 × 10<sup>6</sup> cells/mouse). Donors of spleen cells were BALB/c, IL-10-deficient or IL-12-deficient mice.

**Generation of regulatory cells *in vivo*.** Mice were injected intraperitoneally with OVA in IFA or with OVA plus HKL in IFA on day 0 (described above)<sup>21</sup>. On day 5, the mice were killed and spleens were collected for the purification of CD8 $\alpha$ <sup>+</sup> DCs stimulated with OVA or OVA plus HKL (CD8 $\alpha$ <sup>+</sup> DC<sub>OVA</sub> or CD8 $\alpha$ <sup>+</sup> DC<sub>OVA + HKL</sub>, respectively). For the generation of regulatory cells, DO11.10 OVA TCR-transgenic CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from spleens of naive DO11.10 *Rag2*<sup>-/-</sup> mice, which do not contain CD25<sup>+</sup> T<sub>R</sub> cells<sup>14</sup>, and were injected intravenously into recipients (5 × 10<sup>6</sup> cells/mouse). Simultaneously, CD8 $\alpha$ <sup>+</sup> DC<sub>OVA</sub> or CD8 $\alpha$ <sup>+</sup> DC<sub>OVA + HKL</sub> were injected intravenously into the same recipient mice (1 × 10<sup>6</sup> cells/mouse) without further immunization with antigen. Then, 5 d later, DO11.10 cells were isolated by magnetic-activated cell sorting from the spleens of the recipients with mAb KJ1-26 (clonotype specific). In some experiments, those KJ1-26<sup>+</sup> cells were injected intravenously into mice (3 × 10<sup>6</sup> cells/mouse) that had been immunized with OVA and alum (described above) 7 d earlier. These mice were then challenged intranasally with OVA (described above) on three consecutive days, starting 1 d after the transfer of T<sub>R</sub> cells.

For the depletion of cytokines, T<sub>R</sub> cells were incubated for 4 h *in vitro* with 100 µg antibody to IL-10 (anti-IL-10; 2A5), anti-IFN- $\gamma$  (XMG1.2), anti-ICOSL (16E7E5) or isotype control antibody as described<sup>6</sup>. T<sub>R</sub> cells (3 × 10<sup>6</sup>) were adoptively transferred intravenously into recipients, which also received 500 µg of the corresponding antibody or isotype control intraperitoneally.

**CFSE labeling and coculture of cells.** DO11.10 cells were collected from the spleens of DO11.10 *Rag2*<sup>-/-</sup> mice and were labeled with CFSE (Molecular Probes) as described<sup>54</sup>. For assay of regulatory activity, 1 × 10<sup>4</sup> regulatory or control cells were cocultured with 4 × 10<sup>4</sup> purified and CFSE-labeled DO11.10 cells, T<sub>H1</sub> or T<sub>H2</sub> cells, in the presence of OVA (250 µg/ml) and 1 × 10<sup>4</sup> bone marrow-derived DCs, as described<sup>6</sup>. For some cultures, T<sub>R</sub> cells were incubated for 4 h in 100 µg of anti-IL-10, anti-IFN- $\gamma$ , anti-ICOSL or isotype control and were washed before coculture (mAbs were maintained in the cultures at 100 µg/well). After 48 h (CFSE), cells were collected and analyzed by flow cytometry (CFSE). For analysis of cumulative cytokines, cell culture supernatants were collected after 96 h and analyzed by enzyme-linked immunosorbent assay (ELISA). OVA-specific T<sub>H1</sub> and T<sub>H2</sub> lines were generated from spleens of DO11.10 mice as described<sup>11</sup>.

**Flow cytometry.** A FACScan (Becton Dickinson) was used for analytical flow cytometry and data were processed with CellQuest Pro (Becton Dickinson) or FlowJo (TreeStar) software as described<sup>13</sup>. T cells were stained with antibodies to CD44, CD69, CD62L and CD25 (PharMingen) and ICOS<sup>19</sup> and DCs were stained with antibodies to CD80, CD86, CD40, CD8 $\alpha$ , B220 (PharMingen), ICOSL<sup>6</sup>, OX-40L (eBioscience), major histocompatibility complex class II (purified from clone MKD6; American Type Culture Collection) and DEC-205 (Cedarlane Laboratories). Flow cytometry of cytokine production in T cells was done according to a standard protocol, with some modifications<sup>55,56</sup>. Cells were isolated from spleens and Fc receptors were blocked with excess anti-Fc (HB197). Cell surfaces were stained with fluorescent (fluorescein isothiocyanate or phycoerythrin) or biotin-coupled antibodies, followed by CyChrome-streptavidin (PharMingen) where appropriate. Cells were washed twice with cold PBS. For intracellular cytokine assays, T cells were stimulated for 6 h with phorbol 12-myristate 13-acetate (20 ng/ml) plus ionomycin (500 ng/ml).

Collected cells were fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash (BD PharMingen) according to the manufacturer's instructions. For staining for cytoplasmic IL-10, IL-4 or IFN- $\gamma$  (PharMingen) or for T-bet<sup>57</sup> (4B10; Santa Cruz Biotechnology), the appropriate phycoerythrin-labeled antibodies were added to permeabilized cells (30 min on ice) followed by washing twice with cold PBS.

**Cytokine ELISA.** ELISAs were done as described<sup>11</sup>. The mAb pairs used were as follows, listed as capture-biotinylated detection mAb: IFN- $\gamma$ , HB170-XMG1.2; IL-4, BVD4-BVD6-24G2; IL-10, SXC.2-SXC.1.

**RT-PCR.** For *Foxp3* and *Gata3* analysis, total RNA was prepared from purified T cells by TRIzol. RT-PCR was done for 30 cycles as described<sup>13</sup>. The annealing PCR temperature was 57 °C (*Foxp3*) or 55 °C (*Gata3*) and the primer sequences were as follows<sup>20</sup>: for *Foxp3*, 5'-CAGCTGCCTACAGTGCCCTA G-3' (forward) and 5'-CATTGCCCAGCAGTGGGTAG-3' (reverse); for *Gata3*, 5'-AGGCAAGATGAGAAAGAGTGCCTC-3' (forward) and 5'-CTCGACTTA-CATCCGAACCCGGTA-3' (reverse).

**Real-time RT-PCR for *Foxp3*.** For *Foxp3* analysis, total RNA was prepared from purified T cells by TRIzol. The DNA was generated as described<sup>13</sup>. The expression of *Foxp3* and 18S ribosomal RNA was quantified by real-time PCR with a sequence detection system (ABI Prism 7900; Applied Biosystems) using the TaqMan 1000 RXN Gold with Buffer A Pack (Applied Biosystems) as well as the following primers and internal fluorescent probes<sup>49</sup>: *Foxp3*, 5'-GGCCCTTCTCCAGGACAGA-3', 5' GCTGATCATGGCTGGGTTGT-3' and 5'-5-carboxyfluorescein-ACTTCATGCATCAGCTCTCCACTGTGGAT-N,N,N', N'-tetramethyl-6-carboxyrhodamine-3' ; For both *Foxp3* and 18S mRNA quantification, each sample was run in duplicate. *Foxp3* mRNA was normalized to 18S mRNA for each sample.

**Measurement of airway responsiveness.** AHR responses were assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics), as described<sup>11,58</sup>. Peak enhanced pause (Penh) results were confirmed by analysis of AHR in anesthetized and tracheostomized mice, which were mechanically ventilated, with a modified version of published methods<sup>59</sup>. Aerosolized methacholine was administered for 20 breaths in increasing concentrations (1.25, 2.5, 5 and 10 mg/ml of methacholine). Lung resistance and dynamic compliance were continuously computed by fitting of flow, volume and pressure to an equation of motion.

Note: Supplementary information is available on the Nature Immunology website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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