

Antigen-specific regulatory T cells develop via the ICOS–ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity

OMID AKBARI¹, GORDON J. FREEMAN³, EVERETT H. MEYER¹, EDWARD A. GREENFIELD³,
TAMMY T. CHANG⁴, ARLENE H. SHARPE⁴, GERALD BERRY²,
ROSEMARIE H. DEKRUUFF¹ & DALE T. UMETSU¹

¹Division of Immunology and Allergy, Department of Pediatrics, and ²Department of Pathology,
School of Medicine, Stanford University, Stanford, California, USA

³Department of Adult Oncology, Dana-Farber Cancer Institute and Department of Medicine, and
⁴Immunology Research Division, Department of Pathology, Harvard Medical School, Boston, Massachusetts, USA
Correspondence should be addressed to D.T.U.; email: umetsu@stanford.edu

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Asthma is caused by T-helper cell 2 (Th2)-driven immune responses, but the immunological mechanisms that protect against asthma development are poorly understood. T-cell tolerance, induced by respiratory exposure to allergen, can inhibit the development of airway hyperreactivity (AHR), a cardinal feature of asthma, and we show here that regulatory T (T_R) cells can mediate this protective effect. Mature pulmonary dendritic cells in the bronchial lymph nodes of mice exposed to respiratory allergen induced the development of T_R cells, in a process that required T-cell costimulation via the inducible costimulator (ICOS)–ICOS-ligand pathway. The T_R cells produced IL-10, and had potent inhibitory activity; when adoptively transferred into sensitized mouse T_R cells blocked the development of AHR. Both the development and the inhibitory function of regulatory cells were dependent on the presence of IL-10 and on ICOS–ICOS-ligand interactions. These studies demonstrate that T_R cells and the ICOS–ICOS-ligand signaling pathway are critically involved in respiratory tolerance and in downregulating pulmonary inflammation in asthma.

Asthma, which has increased substantially in prevalence in the last two decades¹, is characterized by airway hyperreactivity (AHR) to a variety of specific and non-specific stimuli, by chronic airway inflammation with pulmonary eosinophilia, mucus hypersecretion and increased serum immunoglobulin E (IgE) levels². The pathology in asthma occurs as a consequence of excessive development of T-helper cell 2 (Th2) cells^{3–5}. Whereas the immunological mechanisms that induce asthma and allergy are relatively well characterized, the specific mechanisms that transpire *in vivo* to downmodulate Th2-driven allergic inflammatory responses in the lungs are poorly understood.

Because Th1 cells secreting interferon- γ (IFN- γ) cross regulate Th2 cells in some systems⁶, allergen-specific Th1 cells are thought to be critically involved in downmodulating Th2-driven airway hyperreactivity and asthma⁷. However, IFN- γ may contribute to the severity of the disease⁸, and Th1 cells exacerbate pulmonary inflammation rather than counterbalancing the effects of Th2 cells⁹. This suggests that mechanisms involving other cell types, for example, interleukin-10 (IL-10) or transforming growth factor- β (TGF- β) secreting cells¹⁰, might be more effective in relieving airway inflammation in asthma.

In support of this possibility, peripheral CD4⁺ T-cell tolerance, induced by respiratory exposure to allergen, protected against the development of Th2-biased responses and allergen induced AHR. In this tolerance process, pulmonary DCs acquired antigen inhaled into the lungs, and migrated to the bronchial lymph nodes where

they interacted with CD4⁺ T cells¹¹. These DCs induced an initial phase of allergen-specific T-cell activation, proliferation and expansion, followed by depletion of these T cells from the lymphoid organs, though a stable population of these cells survived but remained refractory to antigenic rechallenge¹². However, the precise effector mechanisms by which T-cell tolerance downmodulates Th2-driven AHR remain unclear.

Here we investigate specific effector mechanisms by which respiratory exposure to allergen protects against the development of asthma. We found that following respiratory exposure to allergen, regulatory T (T_R) cells developed, which produced high levels of IL-10. These T_R cells had potent *in vivo* and *in vitro* inhibitory activity and were highly effective in down modulating allergen-induced airway hyperreactivity in previously sensitized mice. Moreover, we found that both the development and function of these T_R cells depended on the presence of IL-10 and on a unique costimulation pathway involving inducible costimulator (ICOS), a new member of the CD28 family^{13–15}. These studies demonstrate the importance of T_R cells secreting IL-10 in the regulation of allergen-induced AHR, and of ICOS–ICOS-ligand interactions in driving the development of and in mediating respiratory tolerance and protection against asthma.

DCs producing IL-10 induce T-cell production of IL-10

After exposure to intranasally (i.n.) applied ovalbumin (OVA), pulmonary DCs transiently produced IL-10, and expressed high levels



of the costimulatory molecules B7-1 and B7-2 (ref. 11). These IL-10-producing dendritic cells (DCs) initially induced in naive CD4⁺ T cells from DO11.10 OVA-specific TCR-transgenic mice the production of IL-10, some IL-4, but not IFN- γ (Fig. 1). This CD4⁺ T-cell cytokine profile evolved over time, following several rounds of restimulation with fresh DCs isolated from tolerized mice, and resulted in the persistence of T-cell production of IL-10, but a reduction in the production of IL-4 (Fig. 1). The T cells therefore developed a cytokine profile characteristic of T_R cells^{16,17}.

T cells secreting IL-10 develop *in vivo*

Our *in vitro* findings suggested that CD4⁺ T cells producing IL-10 might also develop *in vivo* following i.n. exposure to OVA. To examine this possibility, we labeled T cells from DO11.10 T-cell receptor (TCR) transgenic mice with carboxyfluorescein diacetate succinimidyl ester (CFSE), and transferred the labeled T cells into naive BALB/c mice, which received i.n. OVA or PBS (Fig. 2) immediately following transfer. Two days after cell transfer, these T cells in mice that received i.n. OVA but not those that received i.n. PBS expressed both IL-10 and IL-4, while undergoing limited cell-cycle progression (Fig. 2). This indicated that i.n. exposure to OVA, which results in the development of IL-10-producing DCs in the bronchial lymph nodes, induced the *in vivo* development of OVA-specific T cells that initially produced IL-10 and IL-4.

T cells secreting IL-10 block AHR and inflammation

The *in vivo* function of the T_R cells generated *in vitro* with IL-10-producing DCs was analyzed by adoptive transfer of the T_R cells

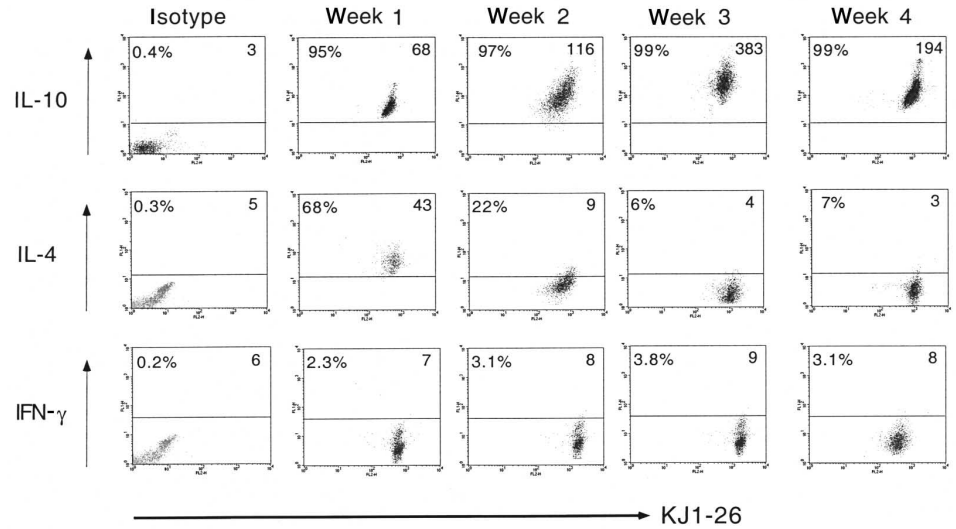


Fig. 1 T-cell lines produce IL-10 when generated with IL-10-secreting DCs. BALB/c mice were exposed on 3 consecutive days (100 μ g/d) to i.n. OVA. CD11c⁺ DCs were isolated from the bronchial lymph nodes of these mice 24 h after the last i.n. OVA administration and were cocultured with KJ1-26⁺, DO11.10, OVA-specific CD4⁺ T cells. The T cells were restimulated once a week for 4 cycles, using fresh DCs from tolerized mice, and were analyzed after each cycle for intracellular expression of cytokines. Cells were also stained with an isotype control antibody (isotype). IL-10 production in the responding CD4⁺ T cells persisted with repeated stimulation, whereas IL-4 production decreased. Mean fluorescence intensity is indicated in the upper right and percentage of positive cells is indicated in the upper left of each plot.

into OVA-sensitized BALB/c mice. The recipient mice were subsequently challenged with i.n. OVA (100 μ g on 3 consecutive days) to induce AHR. Adoptive transfer of T_R cells into OVA-sensitized mice potently inhibited the development of AHR (Fig. 3a). The inhibitory activity was dependent on the presence of IL-10, as administration of a neutralizing monoclonal antibody against IL-10 abrogated the suppressive activity of the transferred cells. Although the IL-10-producing T_R cells resembled Th2 cells, as they initially produced IL-4 (Figs. 1 and 2), the function of T_R cells clearly differed from that of classical Th2 cells; T_R cells but not Th2 cells significantly inhibited the development of AHR (Fig. 3b). Serum IgE specific for OVA as well as the number of eosinophils in the bronchoalveolar lavage (BAL) fluid of mice sensitized and challenged with OVA was greatly reduced by the IL-10-producing T_R cells (Supplementary Fig. A online).

Examination of the lungs of mice that received the IL-10-producing T cells showed a reduction in airway inflammation (Fig. 3e). Administration of anti-IL-10 abolished the inhibitory effect of the T_R cells (Fig. 3f), indicating that the anti-inflammatory effect was dependent on presence of IL-10. These studies show that the IL-10-producing OVA-specific T cells had potent *in vivo* regulatory activity against asthma and Th2-driven airway inflammation, even in OVA-sensitized recipients.

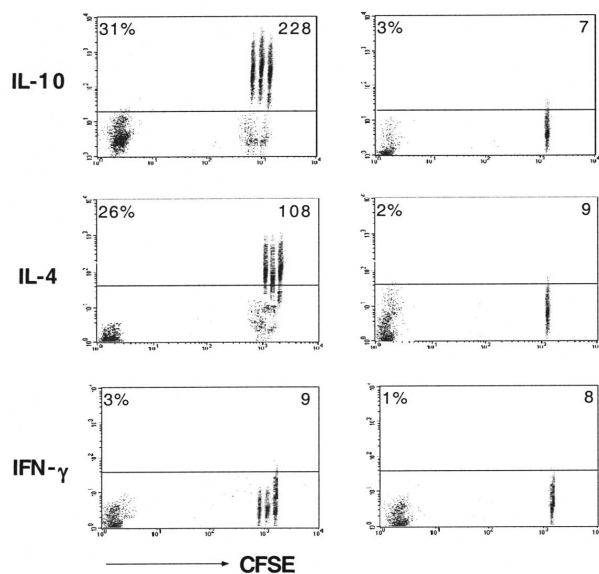


Fig. 2 Development of IL-10-producing T cells in mice exposed to i.n. OVA. 3×10^6 CFSE labeled KJ1-26⁺ (OVA-specific) T cells were transferred into naive BALB/c mice. The mice in the left panels received i.n. OVA (100 μ g) at the time of transfer and twice on the day following transfer. The mice in the right panels received PBS i.n. at the time of transfer. 2 d after cell transfer, the KJ1-26⁺ cells were isolated from the spleens of recipient mice, stained for intracellular cytokine expression and analyzed by flow cytometry, gated on KJ1-26⁺ cells. Mean fluorescence intensity is indicated in the upper right and percentage of positive cells is indicated in the upper left.

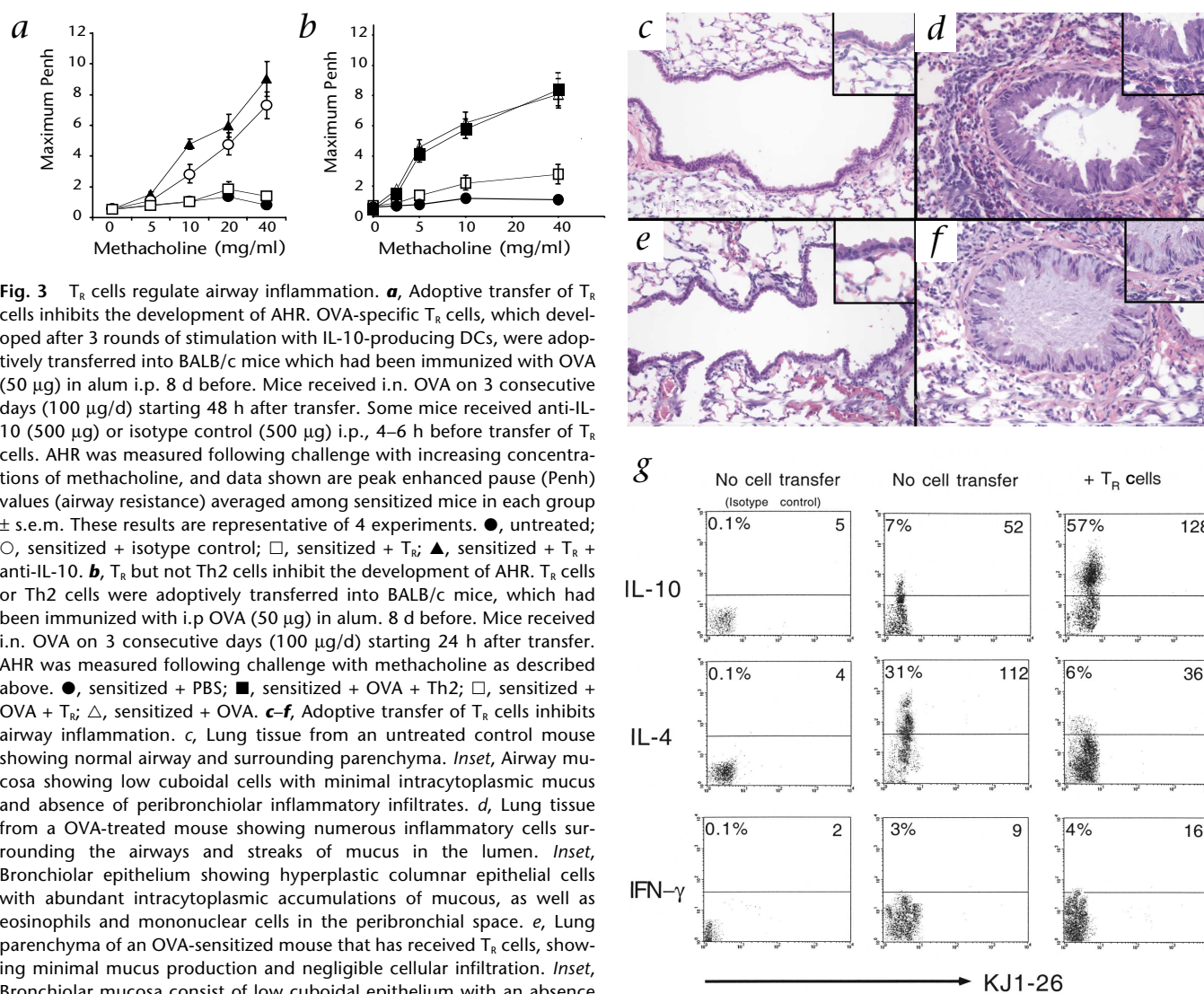


Fig. 3 T_R cells regulate airway inflammation. **a**, Adoptive transfer of T_R cells inhibits the development of AHR. OVA-specific T_R cells, which developed after 3 rounds of stimulation with IL-10-producing DCs, were adoptively transferred into BALB/c mice which had been immunized with OVA (50 μ g) in alum i.p. 8 d before. Mice received i.n. OVA on 3 consecutive days (100 μ g/d) starting 48 h after transfer. Some mice received anti-IL-10 (500 μ g) or isotype control (500 μ g) i.p., 4–6 h before transfer of T_R cells. AHR was measured following challenge with increasing concentrations of methacholine, and data shown are peak enhanced pause (Penh) values (airway resistance) averaged among sensitized mice in each group \pm s.e.m. These results are representative of 4 experiments. ●, untreated; ○, sensitized + isotype control; □, sensitized + T_R; ▲, sensitized + T_R + anti-IL-10. **b**, T_R but not Th2 cells inhibit the development of AHR. T_R cells or Th2 cells were adoptively transferred into BALB/c mice, which had been immunized with i.p. OVA (50 μ g) in alum. 8 d before. Mice received i.n. OVA on 3 consecutive days (100 μ g/d) starting 24 h after transfer. AHR was measured following challenge with methacholine as described above. ●, sensitized + PBS; ■, sensitized + OVA + Th2; □, sensitized + OVA + T_R; △, sensitized + OVA. **c–f**, Adoptive transfer of T_R cells inhibits airway inflammation. **c**, Lung tissue from an untreated control mouse showing normal airway and surrounding parenchyma. *Inset*, Airway mucosa showing low cuboidal cells with minimal intracytoplasmic mucus and absence of peribronchiolar inflammatory infiltrates. **d**, Lung tissue from a OVA-treated mouse showing numerous inflammatory cells surrounding the airways and streaks of mucus in the lumen. *Inset*, Bronchiolar epithelium showing hyperplastic columnar epithelial cells with abundant intracytoplasmic accumulations of mucous, as well as eosinophils and mononuclear cells in the peribronchial space. **e**, Lung parenchyma of an OVA-sensitized mouse that has received T_R cells, showing minimal mucus production and negligible cellular infiltration. *Inset*, Bronchiolar mucosa consist of low cuboidal epithelium with an absence of peribronchiolar inflammatory infiltrates. **f**, Increased luminal and epithelium mucous of AHR in airway lumen of an OVA-sensitized mouse that has received T_R cells and anti-IL-10. *Inset*, Inflammatory cells around the airways and abundant mucous within the lumen and lining cells. All staining, H&E. Magnifications, $\times 200$ in main panels; $\times 600$ in insets. **g**, Adoptively transferred T_R cells alter endogenous cytokine production in recipient CD4⁺ T cells. T_R cells were labeled with CFSE and transferred into recipient mice sensitized as in **a**. Bronchial lymph node cells were re-

moved 24 h after the last i.n. administration of OVA, and stained for intracellular cytokine expression. Cells were analyzed by flow cytometry, gated on the CD4⁺, CFSE-negative population. The first column represents cells from sensitized mice that received no T_R cells, and which were stained with an isotype control mAb. Mean fluorescence intensity is indicated in the upper right and percentage of positive cells is indicated in the upper left of each plot.

To determine the effect of the transferred IL-10-producing regulatory cells on cytokine production by endogenous CD4⁺ T cells in the sensitized recipient mice, the T_R cells were labeled with CFSE before transfer into the OVA-sensitized BALB/c mice. Two days after transfer, the recipient mice received i.n. OVA (100 μ g, 3 doses). 48 hours after the last OVA challenge, bronchial lymph nodes were removed, and the endogenous CD4⁺ T cells in the lymph nodes that were not CFSE-labeled were analyzed by intracellular cytokine assay. In control mice that did not receive T_R cells, the endogenous CD4⁺ T cells produced high levels of IL-4 and some IL-10 (Fig. 3g), consistent with the development of AHR in these mice. In contrast, transfer of IL-10-secreting regulatory cells resulted in the induction of IL-10 production and inhibition of IL-4 production in the endogenous CD4⁺ T cells (Fig. 3g). This suggests that the IL-10-producing T_R cells blocked the

development of AHR in part by reducing IL-4 production and enhancing IL-10 production in endogenous CD4⁺ T cells activated by antigenic challenge with OVA.

T_R cells inhibit OVA-specific T-cell proliferation

The inhibitory activity of the T_R cells was also mediated in part by a capacity to inhibit the proliferation of other OVA-specific T cells. This was demonstrated by coculture of T_R cells with naive CFSE-labeled DO11.10 T cells and bone marrow-derived DCs with OVA. In the absence of T_R cells, the OVA-specific CFSE-labeled naive T cells proliferated vigorously, with a large fraction of the T cells completing 5–6 cell divisions (Fig. 4, upper left). However, addition of the T_R cells to these cultures greatly inhibited cell-cycle progression of OVA-specific T cells, such that the largest fraction of CFSE-labeled cells completed only 2–3 cell cy-

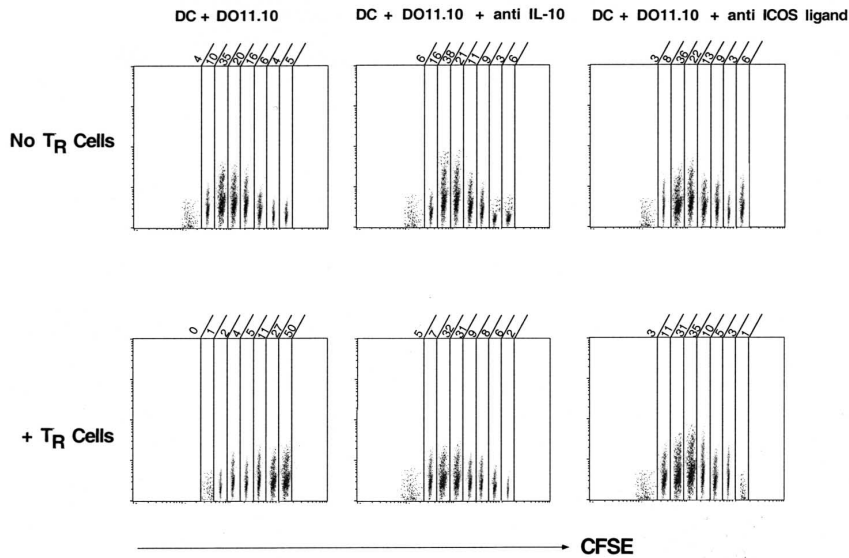


Fig. 4 Effector function of regulatory T cells is dependent on IL-10 and ICOS. KJ1-26⁺ T cells were purified from naive DO11.10 mice, labeled with CFSE and cultured with bone marrow-derived DCs and 250 $\mu\text{g/ml}$ OVA in the presence or absence of regulatory T cells from cycle 3 of restimulation described above. Where indicated, cultures contained control mAb, anti-ICOS-ligand or anti-IL-10 (100 $\mu\text{g/ml}$). After 48 h, cells were collected and analyzed by flow cytometry, gated on CFSE⁺, KJ1-26⁺ cells. Number above each column indicates the percentage of cells of the total cells gated and analyzed. The inhibitory activity of T_R cells was blocked with either anti IL-10 or anti ICOS-ligand.

cles (Fig. 4, lower left). Moreover, the inhibitory capacity of the T_R cells in these cultures was dependent on the presence of IL-10, as the addition of anti-IL-10 reversed the inhibitory effect, as shown by increased cell-cycle progression of the CFSE-labeled T cells (Fig. 4, middle). Addition of blocking anti-ICOS-ligand reversed the inhibitory effects of the T_R effector cells (Fig. 4, lower right). Identical results were obtained by blocking anti-ICOS (data not shown). These results together indicate that the inhibitory effector function of the T_R cells on T-cell proliferation was dependent on IL-10 production and the ICOS–ICOS-ligand signaling pathway.

Induction of T-cell tolerance is ICOS-dependent

Blockade of the ICOS–ICOS-ligand costimulatory pathway not only abrogated the *in vitro* function of the T_R effector cells, but also the development of T-cell tolerance induced by exposure to i.n. OVA. The CD4⁺ T cells from control mice challenged intraperitoneally (i.p.) with OVA in alum proliferated vigorously upon *in vitro* restimulation with antigen (Fig. 5a). In contrast, T cells taken from mice exposed to OVA and subsequently challenged with OVA in alum were tolerized and proliferated poorly in response to antigen. However, administration of anti-ICOS-ligand during and after OVA exposure (before challenge with OVA in alum) reversed the induction of tolerance and restored the response to OVA. Similar results were obtained with blocking anti-ICOS. These results indicated that ICOS–ICOS-ligand interactions were involved in the induction of tolerance to OVA.

Interruption of the ICOS–ICOS ligand pathway also blocked the development of tolerance to i.n. administration, and allowed the subsequent development of AHR. In control mice, exposure to i.n. OVA on days 0, 1 and 2 induced tolerance, which inhibited the development of AHR, assessed on day 21 (Fig. 5b).

However, if the mice were treated with either anti-ICOS-ligand or anti-ICOS (on days 0 and 4) when initially exposed to OVA, tolerance was prevented and severe AHR subsequently developed. Treatment with these antibodies not only prevented the development of tolerance, resulting in AHR associated with a great increase in the numbers of eosinophils in BAL fluid, and in increased production of serum OVA-specific IgE (Supplementary Fig. B online). Because inhibition of the ICOS–ICOS-ligand pathway blocks development and expression of AHR (refs. 18,19), we administered anti-ICOS or anti-ICOS-ligand only during the induction phase of tolerance (days 0 and 4) so as not to inhibit the subsequent development of the Th2-biased immune response.

To visualize the early response of T cells in mice following exposure to i.n. OVA and anti-ICOS-ligand, we labeled OVA-specific T cells with CFSE, and transferred them into BALB/c mice, which received OVA immediately following transfer. This allowed cell division to be monitored in the presence of anti-ICOS-ligand or control antibody (given 4 h before cell transfer). Treatment with the anti-ICOS-ligand greatly increased cell-cycle progression in responding OVA-specific T cells, while greatly reducing IL-10 production in these cells (Fig. 5c). This indicated that during the development of i.n. tolerance, the ICOS–ICOS-ligand costimulatory pathway limits T-cell proliferation and enhances IL-10 production in T cells.

Expression of ICOS and ICOS-ligand by T cells and DCs

At 24 hours after exposure to i.n. OVA, DCs in the bronchial lymph nodes expressed high levels of B7-1 and B7-2 (Fig. 6a)¹¹, as well as high levels of ICOS-ligand and CD40. In addition, ICOS-ligand and B7-2 expression by DC from mice exposed to i.n. OVA was greater than that expressed by control DCs, isolated from mice immunized intradermally with OVA in alum, which induces a vigorous Th2 inflammatory response (Fig. 6a). Moreover, OVA-specific T cells in the bronchial lymph nodes of the tolerized mice expressed high levels of ICOS by 72 hours following i.n. exposure to OVA, and ICOS expression remained increased for at least 14 days (Fig. 6b). CD4⁺ T cells in the lymph nodes of mice that received i.n. PBS showed no increase in ICOS (Fig. 6b) or other activation markers¹¹, whereas CD4⁺ T cells from mice that received OVA in alum or soluble OVA intradermally expressed significantly lower levels of ICOS (Supplementary Fig. C online).

Induction of T-cell tolerance by DCs is ICOS-dependent

To demonstrate the importance of DCs and the ICOS–ICOS-ligand costimulatory pathway in the development of T-cell tolerance, we isolated pulmonary DCs from draining lymph nodes of mice exposed to i.n. OVA 24 hours after exposure to OVA. The DCs were incubated with anti-ICOS-ligand mAb, and then adoptively transferred into naive mice, which were also treated with anti-ICOS-ligand mAb. Seven days after transfer, the mice were challenged with OVA in alum, i.p. Adoptive transfer of DCs from

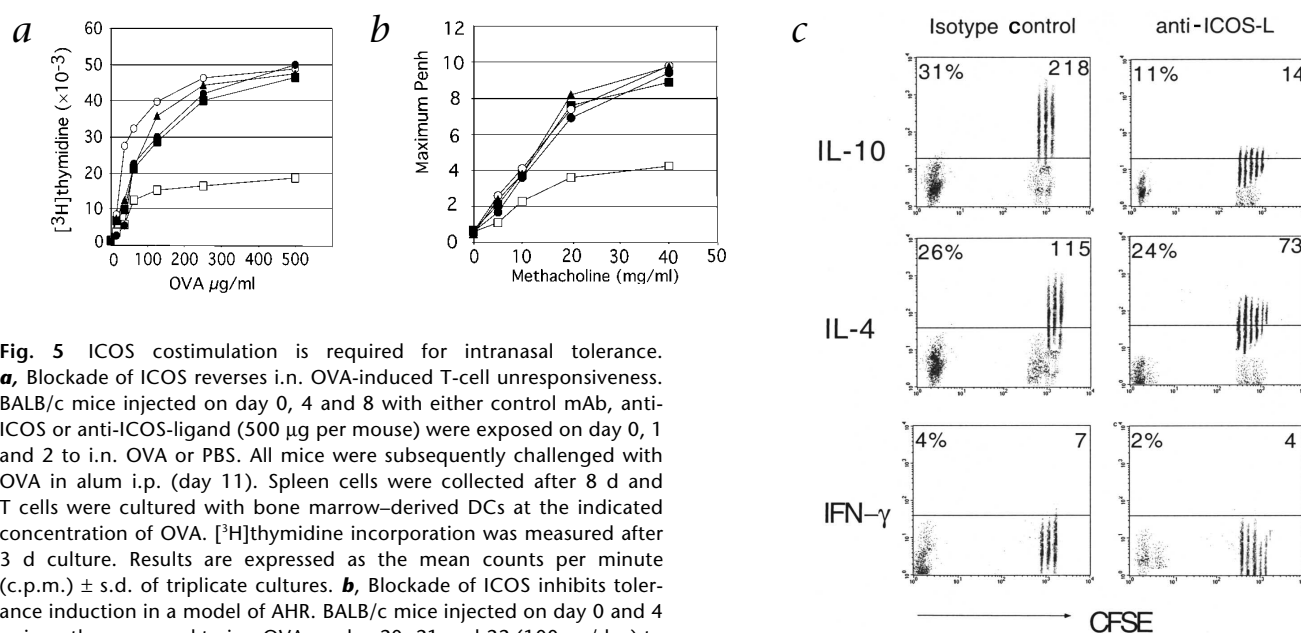


Fig. 5 ICOS costimulation is required for intranasal tolerance. **a**, Blockade of ICOS reverses i.n. OVA-induced T-cell unresponsiveness. BALB/c mice injected on day 0, 4 and 8 with either control mAb, anti-ICOS or anti-ICOS-ligand (500 µg per mouse) were exposed on day 0, 1 and 2 to i.n. OVA or PBS. All mice were subsequently challenged with OVA in alum i.p. (day 11). Spleen cells were collected after 8 d and T cells were cultured with bone marrow-derived DCs at the indicated concentration of OVA. [³H]thymidine incorporation was measured after 3 d culture. Results are expressed as the mean counts per minute (c.p.m.) ± s.d. of triplicate cultures. **b**, Blockade of ICOS inhibits tolerance induction in a model of AHR. BALB/c mice injected on day 0 and 4 as in **a**, then exposed to i.n. OVA on day 20, 21 and 22 (100 µg/day) to induce AHR. AHR was measured after challenge with increasing concentrations of methacholine, and data represent peak Penh values averaged among sensitized mice in each group ± s.e.m. In **a** and **b**, □, OVA + isotype control; ■, OVA + anti-ICOS ligand; ○, PBS + isotype control; ●, PBS + anti-ICOS ligand; ▲, OVA + anti-ICOS. **c**, ICOS costimulation of T cells is required for IL-10 production following i.n. OVA. CFSE-labeled KJ1-26⁺ (OVA-specific) T cells (3×10^6 cells per mouse) were transferred into naive BALB/c mice treated 4 h previously with anti-ICOS-ligand (500

µg per mouse) or an isotype control mAb. The mice received i.n. OVA at the time of transfer and twice the day after. 2 d after cell transfer, the KJ1-26⁺ cells were isolated from the spleens of recipient mice, stained for intracellular cytokine expression and analyzed by flow cytometry, gated on KJ1-26⁺ cells. Mean fluorescence intensity is indicated in upper right and percentage of positive cells is indicated in the upper left of each plot. For IL-10, isotype control versus anti-ICOS-ligand, $P = 0.0005$; for IL-4 and IFN-γ, $P > 0.5$ (χ^2 analysis).

mice exposed to i.n. OVA, transferred T-cell tolerance as demonstrated by reduced *in vitro* proliferation of T cells of recipient mice upon restimulation with OVA *in vitro* (Fig. 6c). In contrast, treatment of the DCs from tolerized mice with anti-ICOS-ligand abolished the capacity of these DCs to transfer T-cell tolerance. Comparable results were obtained from measurement of IL-2 production (data not shown). Moreover, whereas anti-ICOS-ligand inhibited the induction of tolerance, it did not inhibit T-cell priming, since treatment with anti-ICOS-ligand did not affect T-cell proliferation in control mice receiving DCs from mice immunized with OVA-alum intradermally, or control mice receiving DCs from mice exposed to i.n. PBS.

Finally we demonstrated that the requirement for the ICOS and ICOS-ligand pathway in the development of i.n. tolerance had implications for the development of AHR. Thus, we showed that in control mice, transfer of DCs from tolerized mice transferred OVA-specific tolerance, such that recipient mice did not develop AHR, airway eosinophilia or elevated serum IgE when exposed to i.n. OVA (Fig. 6d and Supplementary Fig. C online). However, if the DCs were treated before transfer with blocking anti-ICOS-ligand, the transfer of tolerance by the DCs was abolished, and the recipient mice subsequently developed severe AHR, airway eosinophilia and elevated serum IgE when exposed to i.n. OVA. These results together demonstrated that ICOS-ligand expression by DCs has a critical role in the induction of T-cell tolerance, which protects against the development of AHR and airway inflammation.

Discussion

Here we demonstrate that respiratory tolerance, which protects against the development of pulmonary inflammation and

asthma, was mediated by antigen-specific CD4⁺ T_R cells producing IL-10. The OVA-specific IL-10-producing CD4⁺ T cells had potent *in vivo* and *in vitro* inhibitory activity: when adoptively transferred into OVA-sensitized mice in a mouse model of asthma, the CD4⁺ T cells inhibited the development of allergen-induced AHR, and when cultured *in vitro*, they inhibited the proliferation of OVA-specific T cells. The inhibitory capacity of the T_R effector cells was abrogated by anti-IL-10 or anti-ICOS-ligand. Interference with the ICOS–ICOS-ligand signaling pathway not only abrogated the inhibitory capacity of T_R cells, but also blocked the development of IL-10 production and the induction of T-cell tolerance. These studies thus demonstrate the importance of T_R cells in mediating respiratory tolerance and protection against asthma, and demonstrate the critical role of ICOS–ICOS-ligand pathways in initiating and mediating CD4⁺ T-cell unresponsiveness following respiratory exposure to antigen.

We show that T_R cells develop during respiratory tolerance and inhibit the development of AHR. By contrast, it has been thought that Th1 cells, by inhibiting the function of Th2 cells, are the primary cell type that provides protective immunity in asthma. The idea that Th1 cells are beneficial in allergy and asthma, is supported by indirect evidence that is generally based on data developed in infectious disease models, indicating that Th1 cells mediate resistance, whereas Th2 cells mediate susceptibility to infection^{20–28}. However, direct examination of the role of antigen-specific Th1 effector cells in asthma demonstrated that although Th1 cells were able to reduce mucus production and airway eosinophilia, they failed to inhibit Th2 cell-induced airway hyperreactivity^{9,29}. These studies suggest that other forms of immunity might be effective in downmodulating inflammation in asthma. We demonstrate that IL-10-secreting T_R cells, induced

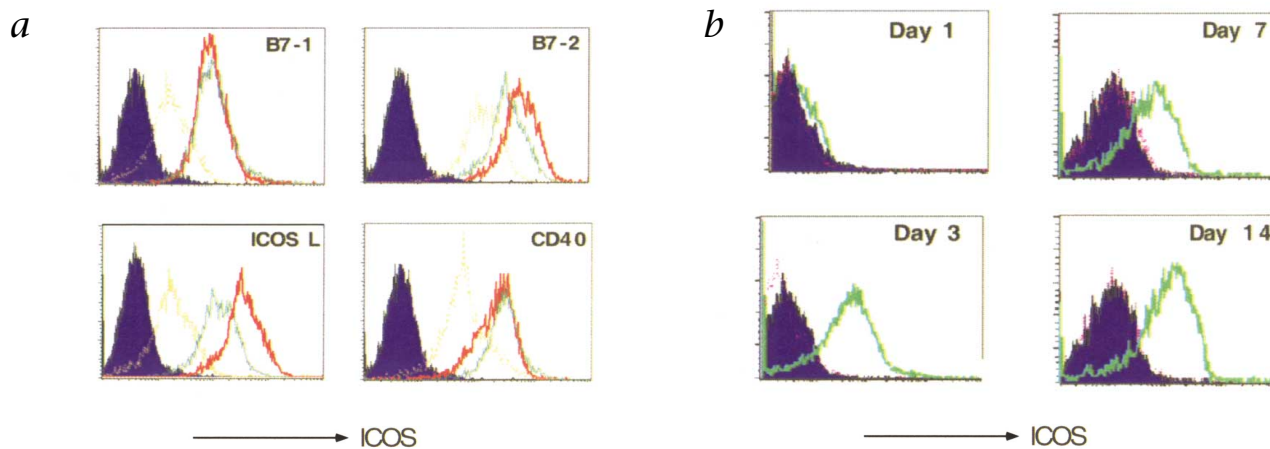
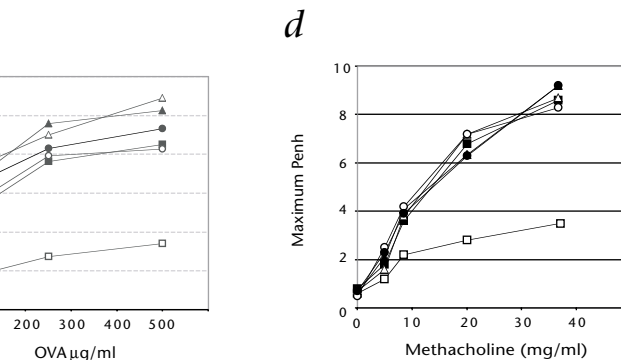


Fig. 6 ICOS-L expression on DCs is required for i.n. tolerance. **a**, Expression of costimulatory molecules by pulmonary DCs from mice exposed to i.n. OVA. DCs were isolated by magnetic cell sorting from bronchial lymph nodes of BALB/c mice 24 h after administration of i.n. OVA (red line), intradermal OVA in alum (green line) or PBS i.n. control (yellow line) and stained for expression of surface markers. Shaded histogram depicts staining with isotype control antibody. **b**, T cells from mice exposed to intranasal OVA express ICOS. BALB/c mice that had received DO11.10 T cells were treated with i.n. OVA (thick lines) or PBS (thin line) on days 1–3 after cell transfer. Spleen cells were collected at the indicated times after OVA exposure. Cells were stained with biotin conjugated, anti-clonotypic TCR mAb KJ1-26 followed by streptavidin-PE and FITC conjugated, anti-ICOS mAb 7E.17G9, and analyzed by flow cytometry. ICOS expression was analyzed by gating on KJ1-26⁺ cells. **c**, Blocking ICOS-ligand on DCs inhibits their capacity to transfer tolerance. CD11c⁺ DCs were isolated from bronchial lymph nodes of BALB/c mice 24 h after exposure to i.n. OVA or PBS i.n. In parallel, as a positive control, DCs were isolated from lymph nodes of mice challenged intradermally with OVA in alum. DCs were treated *in vitro* with 100 $\mu\text{g}/\text{ml}$ anti-ICOS-ligand or isotype control for 6 h and 1×10^6 DCs were adoptively transferred i.v. into naive BALB/c mice which also received 500 μg anti-ICOS-ligand mAb or isotype control i.p. All mice were subsequently challenged with OVA in alum i.p. after 7 d. Spleen cells were isolated 8 d later and T cells were cultured with bone marrow-derived DCs at the indicated concentration of OVA. [³H]thymidine incorporation was measured after 3 d of culture. Results are expressed as the mean c.p.m. (\pm s.d.) of triplicate cultures. **d**, Blocking



ICOS-ligand on DCs inhibits tolerance in AHR model. CD11c⁺ DCs were isolated from bronchial lymph nodes of BALB/c mice 24 h after exposure to i.n. OVA or PBS i.n. In parallel, as a positive control, DCs were isolated from lymph nodes of mice challenged intradermally with OVA in alum. DCs were treated *in vitro* with 100 $\mu\text{g}/\text{ml}$ anti-ICOS-ligand or isotype control for 6 h and 1×10^6 DCs were adoptively transferred i.v. into naive BALB/c mice which also received 500 μg anti-ICOS-ligand or isotype control i.p. All mice were subsequently challenged with OVA in alum i.p. after 9 d. Mice then received i.n. OVA on 3 consecutive days (100 $\mu\text{g}/\text{d}$) starting 9 d after receiving OVA in alum to induce AHR. AHR was then measured and data shown represent peak Penh values averaged among sensitized mice in each group \pm s.e.m. For **c** and **d**, \square , OVA DC + isotype; \blacksquare , OVA DC + anti-ICOS ligand; Δ , DC OVA Alum + isotype; \blacktriangle , DC OVA Alum + anti-ICOS ligand; \circ , PBS + isotype; \bullet , PBS + anti-ICOS ligand.

by respiratory exposure to allergen by pulmonary DCs transiently producing IL-10 and expressing high level of ICOS-ligand, potentially inhibited the development of airway inflammation and airway hyperreactivity in a mouse model of asthma.

The role of IL-10 in limiting airway inflammation and AHR has been controversial. IL-10 has been considered to be an essential Th2 cytokine⁶, particularly as IL-10 inhibits Th1 cytokine production by inhibiting IL-12 synthesis³⁰ and B7 expression³¹, and because IL-10^{-/-} mice develop poor Th2 responses and resist the development of AHR (ref. 32). However, IL-10 may have several roles in asthma, not only by having a critical function in initiating the development of Th2 polarized responses, but also by playing an important regulatory role late during immune responses, by downmodulating Th2-driven inflammation^{32–34}. Although previous studies show that administration of IL-10 inhibits the expression of AHR, we show for the first time that T_R cells producing IL-10 prevent the development of AHR even in

allergen-sensitized mice, strongly suggesting that T_R cells normally develop during respiratory exposure to allergen and protect against allergic asthma.

IL-10-secreting T_R cells have been previously generated by culturing T cells in the presence of large quantities of exogenous IL-10 (ref. 16), culturing T cells with immature DCs (ref. 35) or using immunosuppressive drugs³⁶. Although those results suggested that T_R cells might be related to anergic cells^{37,38}, we show that antigen-specific T_R cells in the respiratory tract develop following receipt of a specific set of costimulatory signals from DCs including ICOS-ligand and IL-10, as well as B7-2, which preferentially costimulates IL-10 production^{13,14,39}. Although a variety of other mechanisms may be important for the development of other previously described regulatory cells, such as Th3 (ref. 40), CD4⁺CD25⁺ (ref. 41,42), antigen-specific T_R1 cells^{43,16} or with CTLA-4 mediated suppression⁴⁴, ICOS–ICOS-ligand signaling in the presence of IL-10 seem to

be critically important in the development of T_R cells in the lung.

Recent studies indicate that the ICOS–ICOS-ligand is important in the activation and function of effector T cells, and that it induces CD28-independent T-cell proliferation and cytokine production^{13,14}, promoting preferential IL-10 production, along with production of IL-4, IL-5 and GM-CSF (refs. 45–47) but little IL-2 (ref. 14). Mice deficient in ICOS have major reductions in IgE production¹⁸, Th2 cytokine production and in the development of AHR, indicating an important role for ICOS in the development of allergic respiratory inflammatory responses¹⁹. In addition to its role in amplifying the development of Th2 responses, we now demonstrate that ICOS costimulation pathways are critically important in immune regulation and tolerance, and that ICOS costimulates the induction of T_R cells that inhibit the function of antigen-specific T cells and inhibit the development of AHR. Whereas this role for ICOS was unexpected, in retrospect the role of ICOS in immune regulation is not surprising given that ICOS costimulation induces large quantities of T-cell IL-10 production⁴⁸, which is important in the development of T-cell tolerance^{16,34}. Moreover, ICOS^{-/-} mice seem to be prone to the development of experimental allergic encephalomyelitis⁴⁹.

The idea that ICOS–ICOS-ligand interactions costimulate the development of both Th2-driven inflammation and T_R cell-mediated tolerance suggests that these distinct processes are in fact related. Both Th2 cells and T_R cells are associated with respiratory mucosal responses, require costimulation through CD28 and ICOS for induction and produce both IL-4 and IL-10, though in relatively different quantities. Th2 cells produce primarily IL-4, IL-13 and IL-10, whereas T_R cells produce primarily IL-10 and low levels of IL-4. Moreover, we have recently observed that T_R cells do not produce detectable levels of IL-13, whereas Th2 cells produce large quantities of IL-13 (T_R cells, <15 ng/ml; Th2 cells, 390 ng/ml). These differences may explain the distinct function of T_R and Th2 cells.

Our findings also demonstrate that pulmonary DCs from mice exposed to intranasal OVA induced T-cell production of both IL-4 and IL-10 initially, but with subsequent stimulation, IL-4 production waned, whereas production of IL-10 was maintained. The specific signals that preferentially induce development of T_R cells rather than Th2 cells are not entirely clear, but may involve IL-10 production by DCs. We propose that, in the presence of IL-10-producing DCs, T_R cells develop, whereas in the absence of IL-10, for example, when antigen is presented by DCs from IL-10-deficient mice or from mice with allergic asthma¹¹, T_R cells fail to develop. Furthermore, the development of Th2 cells and of allergic diseases might represent an aberration of T_R -cell development, due to inadequate production of IL-10 (refs. 3,11). In other words, allergic asthma may develop as a consequence of limited IL-10 and enhanced IL-4 and IL-13 production and from the failure to develop allergen-specific T_R cells. Whether Th2 cells and T_R cells arise as a single population or in parallel is not clear, and further study of the signals that differentially affect Th2 cell and T_R cell development are needed to fully understand the development of tolerance and its role in the pathogenesis of allergic asthma.

In summary, we showed that during the induction of T-cell tolerance by respiratory exposure to allergen, CD4⁺ T cells secreting high levels of IL-10 develop. The IL-10-secreting T_R cells expressed significant *in vivo* and *in vitro* inhibitory activity and blocked the development of allergen-induced AHR. The growth

of the allergen-specific T_R cells required mature DCs expressing high levels of ICOS-ligand, as well as B7-1 and B7-2, and was prevented by neutralization of IL-10 or by blockade of ICOS–ICOS-ligand signaling. These studies strongly suggest that respiratory exposure to allergen normally induces the development of T-cell tolerance, mediated in part by T_R cells, which potentially impede the development of allergic inflammation in asthma. Future therapies therefore, using DCs expressing IL-10 and ICOS-ligand to induce allergen-specific T_R cells, might be extremely effective in downmodulating pulmonary inflammation in allergic asthma.

Methods

Mice and antigen administration. BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). OVA-TCR transgenic D011.10 mice were obtained from D.Y. Loh and backcrossed to RAG-2-deficient mice (from A. Abbas). For induction of tolerance, mice received i.n. OVA (100 µg) (Worthington Biochemical Corp, chromatographically purified) on three consecutive days and challenged as previously described^{11,12}. The Stanford University Committee on Animal Welfare (A-PLAC, administration panel of laboratory animal care) approved all animal protocols used in this study.

Monoclonal antibodies. Hybridoma cells producing the anti-clonotypic mAb KJ1-26 were provided by P. Marrack⁵⁰. Monoclonal anti-IL-10 JES-2A5 (from M. Howard) was purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. Anti-ICOS mAb 7E.17G9 was generated as described¹³. Anti-38C13 idiotype mAb 4G10 (rat IgG2a)⁵¹ was provided by S. Levy and was used as isotype control. The anti ICOS-ligand mAbs were prepared according to standard immunization protocols. Hybridomas 16F.8D11 and 16F.7E5 (both rat IgG2b, κ light-chain) were chosen for analysis on the basis of blocking the interaction of ICOS and ICOS-ligand and robust staining.

CFSE labeling and adoptive transfer of TCR-transgenic T cells. KJ1-26⁺ T cells were purified to >95% by magnetic cell sorting. Lymph node and spleen cells of D011.10 mice were pooled and incubated with biotin coupled KJ1-26, followed by streptavidin conjugated microbeads, and positively selected using the Vario-MACS (Miltenyi Biotec Inc., Auburn, California). In some experiments, labeling of KJ1-26⁺ cells with 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon) was performed as described⁵².

Generation and functional analysis of T_R and Th2 cells. T_R cells were generated by culture of purified DO11.10 T cells with pulmonary DCs purified as described¹¹. T cells were restimulated every 10 d with similarly isolated DCs.

For assay of regulatory activity, 1×10^4 regulatory cells were cocultured with 4×10^4 purified naive CD4⁺ DO11.10 T cells labeled with CFSE, in the presence of OVA (250 µg/ml) and 1×10^4 bone marrow-derived DCs. For some cultures, T_R cells were incubated in 100 µg of anti-IL-10, anti-ICOS-ligand or isotype control for 4 h, washed before coculture, and maintained in culture in the presence of mAb at the same concentration. After 48 h, cells were collected and analyzed by FACS. OVA-specific Th2 lines were generated from spleen of DO11.10 mice as described⁹. For OVA-specific IgE assay, mice were bled, and serum levels of OVA-specific IgE antibody was measured using a modified OVA-specific ELISA as described⁵³.

Adoptive transfer of T_R cells. T_R cells (3×10^6) generated following 3 rounds of stimulation, were adoptively transferred into BALB/c mice which had been immunized with OVA (100 µg) in alum i.p., 8 d before. Mice received i.n. OVA on 3 consecutive days (50 µg/d) starting 48 h after transfer.

Induction of airway hyperreactivity and measurement of airway responsiveness. For measurement of AHR, mice were immunized and challenged as described^{9,10}. Airway responses were assessed by methacholine-induced airflow obstruction from awake mice placed in a whole-body plethysmograph (Buxco Electronics Inc., Troy, New York) as described^{9,10}.

Collection of BAL fluid and lung histology. Animals were injected i.p. with a lethal dose of phenobarbital (450 mg/kg). The trachea was cannulated, the lung was then lavaged with 1 ml of PBS 3 times, and the fluid pooled. Cells in the lavage fluid were counted and analyzed as described⁹. In some animals, no BAL was performed but lungs were removed, washed with PBS, fixed in 10% formalin and stained with H&E.

In vivo antibody treatments. Mice were injected i.p. with anti-ICOS-ligand 16F.7E5 or anti-ICOS on days 0, 4 and 8 (500 µg) or isotype control 4G10 (500 µg). Mice were exposed on 3 consecutive days (day 0–2) to i.n. OVA, and mice were challenged on day 11 i.p. with 10 µg of OVA in 2 mg of aluminum hydroxide (alum), in a volume of 0.5 ml.

Preparation of bone marrow-derived DCs. Bone marrow–derived DCs were generated as described^{54,55} with some modifications⁵⁶. Briefly, 5×10^6 bone-marrow cells were cultured in petri dishes (9-cm diameter; Nunc, Inc.) in 10 ml culture medium containing 20–25 U/ml GM-CSF. Loosely adherent cells were transferred onto a second dish on day 6 of culture. From day 6 to 10, these transferred cells were used as a source of APC.

Adoptive transfer of DCs. CD11c⁺ DCs were isolated from bronchial lymph nodes of BALB/c mice as described¹¹, 24 h after exposure to OVA or PBS (3 × 100 µg) i.n. treatment. DCs were treated *in vitro* with anti-ICOS ligand (100 µg/ml) or isotype control for 6 h. 1×10^6 DCs were adoptively transferred i.v. into naive BALB/c mice, which also received 500 µg anti-ICOS ligand or isotype control i.p. In some experiments, as a positive control, CD11c⁺ DCs were isolated from draining lymph nodes of mice 24 h after immunization with OVA in alum intradermally.

Flow cytometry and FACS analysis. Analytical flow cytometry was carried out using a FACScan instrument (Becton Dickinson, Franklin Lakes, New Jersey). Lymph node or spleen cells were collected at various times, and 1×10^6 cells were incubated on ice with biotin-conjugated anti-KJ1-26 mAb followed by streptavidin PE (PharMingen, San Diego, California) using standard procedures. FITC-conjugated anti-ICOS was used on cells already stained with the biotin-conjugated, clonotype-specific KJ1-26 monoclonal antibodies⁵⁷. Flow-cytometric measurement of cytokine production in T cells was performed according to a standard protocol with some modifications^{58,59}. Briefly, T cells were stimulated with 20 ng/ml PMA plus 500 ng/ml ionomycin. After 2.5 h, 1 µl/ml of GolgiStop (BD PharMingen) was added and the cells were cultured for an additional 2.5 h. Fixation and permeabilization was performed on collected cells using Cytofix/Cytoperm and Perm/Wash (BD PharMingen) according to manufacturer's instructions. Staining for cytoplasmic IL-10, IL-4 and IFN-γ was performed by staining with fluorochrome-labeled mAbs for intracellular staining (BD PharMingen).

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

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Competing interests statement

The authors declare that they have no competing financial interests.

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