



Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen

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Respiratory exposure to allergen induces T cell tolerance and protection against the development of airway hyperreactivity and asthma. However, the specific mechanisms by which tolerance is induced by respiratory allergen are not clear. We report here that pulmonary dendritic cells (DCs) from mice exposed to respiratory antigen transiently produced interleukin 10 (IL-10). These phenotypically mature pulmonary DCs, which were B-7^{hi} as well as producing IL-10, stimulated the development of CD4⁺ T regulatory 1-like cells that also produced high amounts of IL-10. In addition, adoptive transfer of pulmonary DCs from IL-10^{+/+}, but not IL-10^{-/-}, mice exposed to respiratory antigen induced antigen-specific unresponsiveness in recipient mice. These studies show that IL-10 production by DCs is critical for the induction of tolerance, and that phenotypically mature pulmonary DCs mediate tolerance induced by respiratory exposure to antigen.

The respiratory mucosa interfaces broadly with the environment and, therefore, a vast quantity of environmental antigens settle upon the respiratory mucosal surface and challenge the immune system. Thus, the immune system must discriminate between, and appropriately respond to, both pathogenic and innocuous antigens. In normal circumstances, soluble nonreplicating proteins delivered through intact mucosal surfaces do not provoke strong immune reactions, but instead induce a state of antigen-specific hyporesponsiveness^{1,2}. The peripheral immune system has evolved several mechanisms to maintain a state of tolerance to innocuous antigens. In particular, clonal deletion, anergy and active suppression, mediated by regulatory cells secreting transforming growth factor- β (TGF- β) and interleukin 10 (IL-10), have been implicated in the down-regulation of immune responses^{3,4}. However, the immunological events, cellular mechanisms and antigen-presenting cell (APC) types involved in inducing the T cell hyporesponsiveness to antigens inhaled into the lung are poorly defined.

The initial immunological events in the response to inhaled antigen include antigen uptake by professional APCs, processing into immunogenic peptides and presentation, in the context of major histocompatibility complex (MHC) molecules, to naïve T cells in the lymph nodes that drain the lung⁵⁻⁷. In this regard, pulmonary dendritic cells (DCs) play a critical role in sampling and presenting antigen in the lung, which leads to activation and expansion of CD4⁺ T cells and preferential production of T helper subset 2 (T_H2)-biased adaptive immune responses^{8,9}. However, respiratory exposure to allergen can induce allergen-specific CD4⁺ T cell unresponsiveness, which protects against the development of T_H2-biased responses and allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma^{10,11}. Administration of ovalbumin (OVA) intranasally resulted in the initial activation and expansion of OVA-specific CD4⁺ T cells, but this was followed by removal of most of the OVA-specific T cells and functional inactivation

of the remaining OVA-specific cells. The principal aims of this study were to define the mechanisms of antigen presentation in the development of peripheral CD4⁺ T cell tolerance to inhaled antigen and to investigate, in detail, the role of pulmonary DCs in this tolerance process.

To model the early events that occur during T cell activation in response to inhaled nonpathogenic protein, we analyzed antigen presentation by DCs in bronchial lymph nodes after intranasal (i.n.) administration of OVA. We have shown that pulmonary DCs found in bronchial lymph nodes within 24 h of i.n. exposure to OVA transiently expressed IL-10; IL-10 was detected both by reverse-transcribed polymerase chain reaction (RT-PCR) and by intracellular cytokine assay. These IL-10-producing DCs had the phenotype B7-1^{hi}B7-2^{hi}CD40⁺MHC class II^{int}CD8⁻ and induced IL-4 and IL-10 production in responding CD4⁺ T cells. In addition, adoptive transfer of IL-10-expressing DCs from IL-10^{+/+}, but not IL-10^{-/-}, donors induced OVA-specific T cell unresponsiveness in recipient mice. These results show that phenotypically mature pulmonary DCs that produce IL-10 play a critical role in initiating the induction of CD4⁺ T cell unresponsiveness after respiratory exposure to antigen.

Results

Intranasal OVA induces T cell unresponsiveness

T cells taken from mice exposed to i.n. OVA (3×100 μ g) and subsequently immunized intraperitoneally with OVA in alum proliferated poorly in response to *in vitro* stimulation with OVA (**Fig. 1a**). In contrast, T cells from control mice treated with i.n. PBS and immunized with OVA in alum proliferated vigorously when stimulated *in vitro* with OVA. The induction of CD4⁺ T cell unresponsiveness after i.n. administration of OVA was associated with the initial phase of T cell activation: proliferation and expansion^{11,12}. This was reflected by the expression, in

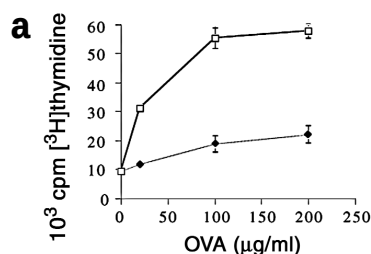
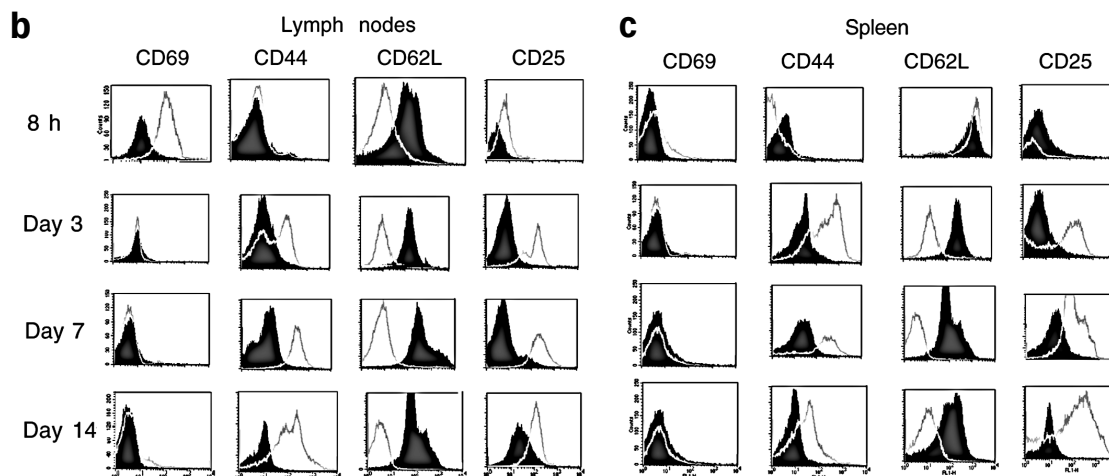


Figure 1. Administration of i.n. OVA induces T cell unresponsiveness and expression of T cell activation antigens. (a) BALB/c mice were exposed to i.n. OVA (open symbol) or PBS (closed symbol) on 3 consecutive days and subsequently challenged with OVA in alum. Spleen cells were collected 8 days later and T cells were cultured with bone marrow-derived DCs at the indicated concentrations of OVA. [³H]thymidine incorporation was measured after 3 days of culture. Data are mean ± s.d. of triplicate cultures. (b,c) BALB/c mice that had received DO11.10 T cells were treated with i.n. OVA (open histograms) or PBS (filled histograms) on days 1–3 after cell transfer. Bronchial lymph nodes (b) and spleen cells (c) were collected at the indicated times after OVA exposure. Cells were stained with anti-clonotypic TCR (mAb KJ1-26) and mAbs to the indicated T cell activation markers, then analyzed by flow cytometry. Expression of CD69, CD44, CD62L and CD25 on gated KJ1-26⁺ T cells are shown.

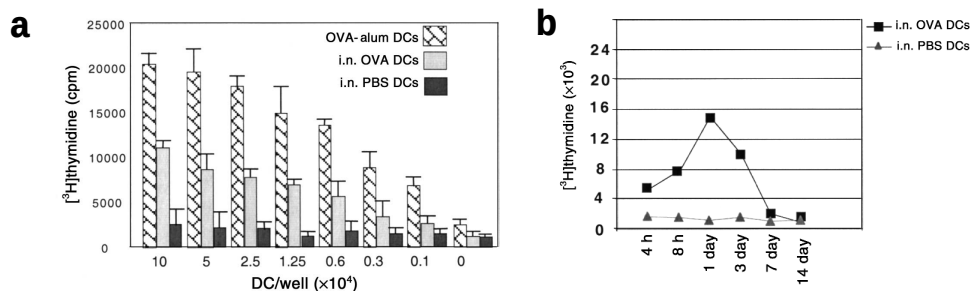


OVA-specific CD4⁺ T cells from the bronchial lymph nodes, of cell surface markers characteristic of activated and/or mature T cells. Within 8 h of exposure to i.n. OVA, bronchial lymph node, but not splenic, CD4⁺ T cells in mice given OVA-specific T cell receptor (TCR)–transgenic T cells from DO11.10 RAG-2^{−/−} mice expressed increased amounts of CD69, an early activation marker, and reduced amounts of CD62L (also known as L-selectin), a marker of naïve cells (Fig. 1b,c). This indicated that i.n. exposure to OVA resulted in rapid activation of T cells within the pulmonary compartment but not in the spleen. However, OVA-specific CD4⁺ T cells in both the bronchial lymph nodes and spleen developed 3 days after i.n. exposure to OVA: an activated phenotype, which was characterized by down-regulation of CD62L and up-regulation of CD44. In contrast, T cells from control mice exposed to i.n. PBS showed no signs of maturation and retained their naïve phenotype (CD69^{lo}CD62L^{hi}CD44^{lo}) throughout the course of the experiment.

Characteristics of OVA-exposed pulmonary DCs

We next examined the role of pulmonary DCs in mediating T cell tolerance induced by i.n. exposure to antigen. Twenty-four hours after antigen exposure, DCs were purified from the pulmonary draining lymph nodes of mice exposed to i.n. OVA (or PBS) or from mice immunized intradermally with OVA in alum and their capacity to activate OVA-specific TCR–transgenic T cells *in vitro* compared. DCs from mice immunized intradermally with OVA in alum induced the production of high concentrations of IL-2 in OVA-specific T cells, whereas DCs from mice exposed to i.n. OVA had a reduced capacity to induce IL-2 production by T cells (Fig. 2a). As expected, DCs from mice that received i.n. PBS did not stimulate T cells; this was because the mice were not exposed to OVA and OVA was not added to the cell cultures. Kinetic analysis of DC antigen presentation indicated that DCs from mice exposed to i.n. OVA showed the highest capacity for antigen presentation when the DCs were

Figure 2. Capacity and kinetics of antigen presentation by pulmonary DCs from mice exposed to i.n. OVA. (a) BALB/c mice were treated with i.n. 3 × 100 μg of OVA (i.n. OVA DCs) or PBS (i.n. PBS DCs) or intradermally with OVA in alum (OVA-alum DCs). Twenty-four hours after OVA exposure, CD11c⁺ cells were positively selected from bronchial lymph nodes and cultured with naïve DO11.10 T cells for 72 h. Culture supernatants were collected and IL-2 expression analyzed with the IL-2-dependent cell line CTLL. Data are mean ± s.d. of triplicate cultures. (b) DCs were isolated from bronchial lymph nodes of BALB/c mice at various time-points after administration of i.n. OVA, then cultured with naïve DO11.10 T cells for 72 h. Culture supernatants were collected and IL-2 expression analyzed with CTLL cells. Data are mean ± s.d. of triplicate cultures.



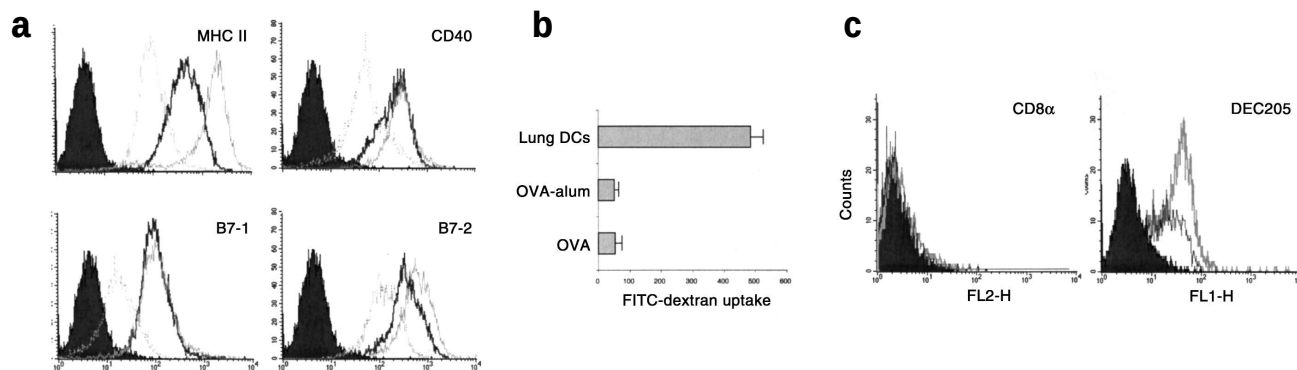


Figure 3. Phenotypic analysis of DCs from mice exposed to i.n. OVA. (a) Expression of costimulatory molecules by lymph node DCs exposed to i.n. OVA. DCs were isolated by MACS from bronchial lymph nodes of BALB/c mice 24 h after administration of i.n. OVA (open thick-lined histograms), intradermal OVA in alum (open thin-lined histograms) or PBS control (open dotted-lined histograms) and stained for expression of surface markers. The filled histograms show staining with isotype control antibody. MHC class II, CD40, B7-1 and B7-2 expression by CD11c⁺ DCs were increased by exposure to i.n. OVA. (b) Reduced endocytic uptake of FITC-dextran by DCs from mice exposed to i.n. OVA. DCs were isolated by MACS from lungs of untreated BALB/c mice (lung DCs) or from bronchial lymph nodes of BALB/c mice 24 h after administration of intradermal OVA in alum (OVA-alum) or i.n. OVA (OVA). DCs were incubated with 1 mg/ml of FITC-dextran for 30 min at 4 °C or 37 °C; their capacities to take up FITC-dextran were compared. Data are mean $\pm$ s.d. change in MFI between 37 °C and 4 °C. (c) Bronchial lymph node DCs exposed to i.n. OVA expressed DEC205 but not CD8 α . DCs were isolated, by MACS, from bronchial lymph nodes of BALB/c mice 24 h after administration of i.n. OVA (open thick-lined histograms) or PBS control (open thin-lined histograms) and stained for expression of CD8 α and DEC205 markers on gated CD11c⁺ DCs. The filled histograms show staining with isotype control antibody.

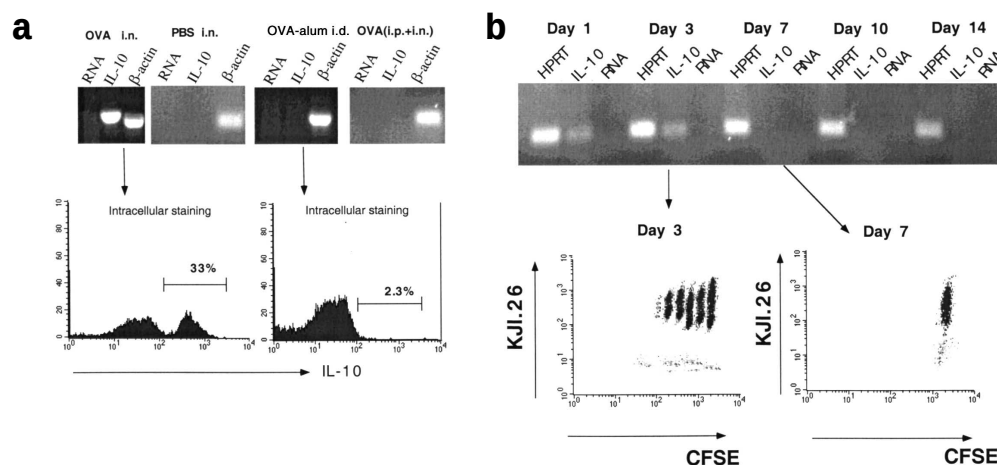
taken from the lymph nodes 24 h after the last i.n. exposure; this activity declined by day 7 and was absent by day 14 (Fig. 2b). Thus, DCs from mice exposed to i.n. OVA transiently developed the capacity to process and present the administered OVA, which was consistent with the initial expansion of OVA-specific T cells in mice exposed to i.n. OVA¹¹.

The limited capacity of DCs from mice exposed to i.n. OVA to activate CD4⁺ T cells was not due to reduced expression of costimulatory molecules. DCs isolated from bronchial lymph nodes 24 h after exposure to i.n. OVA expressed increased amounts of B7-1, B7-2 and CD40, although they expressed intermediate amounts of MHC class II (Fig. 3a). The MHC class II⁺ cells represented DCs and not B cells, as 98% of the cells in the DC population expressed the DC marker, CD11c, but

not the B cell marker, CD19 (data not shown). In addition, DCs from mice exposed to i.n. OVA expressed an additional characteristic of a mature phenotype: a reduced capacity to endocytose fluorescein isothiocyanate (FITC)-dextran (Fig. 3b). Similarly, DCs from mice immunized intradermally with OVA in alum expressed B7-1, B7-2 and CD40, expressed higher amounts of MHC class II and had a reduced capacity for endocytosis. In contrast, DCs from control mice that had received i.n. PBS expressed lower amounts of MHC class II, CD40, B7-1 and B7-2 and had a high capacity for endocytosis. None of the DCs expressed CD8, and they were DEC205^{lo} (Fig. 3c), which suggested a myeloid origin for these cells^{13–15}. These results indicated that the administration of i.n. OVA drove the migration of pulmonary DCs from the

Figure 4. Expression and kinetics of IL-10 in pulmonary DCs.

(a) (Upper panels) Pulmonary DCs from mice exposed to i.n. OVA produce IL-10. RT-PCR analysis was done to examine IL-10 expression by CD11c⁺ cells isolated from bronchial lymph nodes of BALB/c mice immunized i.n. with OVA (OVA i.n.) or PBS (PBS i.n.) or from draining lymph nodes of mice injected intradermally with OVA in alum (OVA-alum i.d.) or from draining lymph nodes of mice immunized with OVA in alum intraperitoneally and challenged with OVA intranasally (OVA i.p.+i.n.). RNA, a negative control that demonstrated the absence of DNA contamination in the RNA samples (omission of RT before the PCR reaction). β -actin, a positive, loading, control. (Lower panels) Pulmonary DCs from mice exposed to i.n. OVA produced IL-10, as shown by intracellular cytokine staining. DCs were isolated from bronchial lymph nodes of BALB/c mice 24 h after i.n. administration of OVA or intradermal administration of OVA in alum. DCs were examined by flow cytometric analysis: expression of IL-10 in gated CD11c⁺ DCs (filled histograms). (b) (Upper panels) Kinetics of IL-10 expression in pulmonary DCs. RT-PCR analysis was done to examine IL-10 expression by CD11c⁺ cells isolated from bronchial lymph nodes of BALB/c mice at various time-points after treatment with 100 μ g of i.n. OVA. HRPT, a positive, loading, control. RNA, a negative control, as in a. (Lower panels) CFSE-labeled OVA-specific T cells proliferated in mice exposed to i.n. OVA. BALB/c mice were treated with i.n. OVA. After 3 and 7 days, 10⁶ CFSE-labeled KJ1.26⁺ (OVA-specific) T cells were injected intravenously; spleen cells from recipient mice were analyzed 3 days later by flow cytometry.



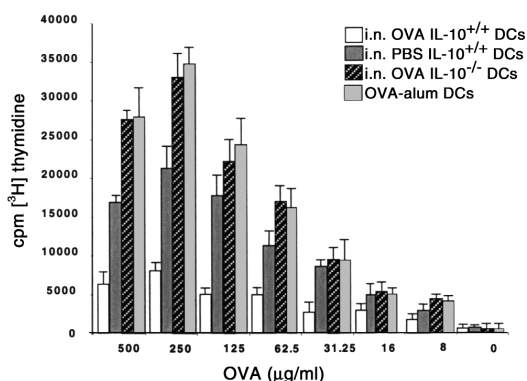


Figure 5. DCs from IL-10^{+/+}, but not IL-10^{-/-}, mice exposed to i.n. OVA transfer T cell tolerance. CD11c⁺ DCs were isolated from bronchial lymph nodes of IL-10^{+/+} or IL-10^{-/-} C57BL/6 mice 24 h after exposure OVA or PBS i.n.; 10⁶ DCs were adoptively transferred into naive C57BL/6 mice (intravenously). As a positive control, DCs isolated from lymph nodes of mice immunized intradermally with OVA in alum, were adoptively transferred into naive C57BL/6 mice. After 1 week, all four groups were challenged intraperitoneally with 100 µg of OVA in alum. Spleen cells were taken from the mice 7 days after the secondary challenge and cultured with increasing concentrations of OVA in vitro. Supernatants were collected after 48 h and IL-2 production analyzed by assessing [³H]thymidine incorporation in CTLL cells, an IL-2-dependent cell line. Data are mean±s.d. of triplicate cultures.

lung to the bronchial lymph nodes and stimulated differentiation from an immature B7^{lo} DC phenotype¹⁶ toward a mature B7^{hi} DC phenotype with a reduced capacity for endocytosis.

IL-10 production by OVA-exposed DCs

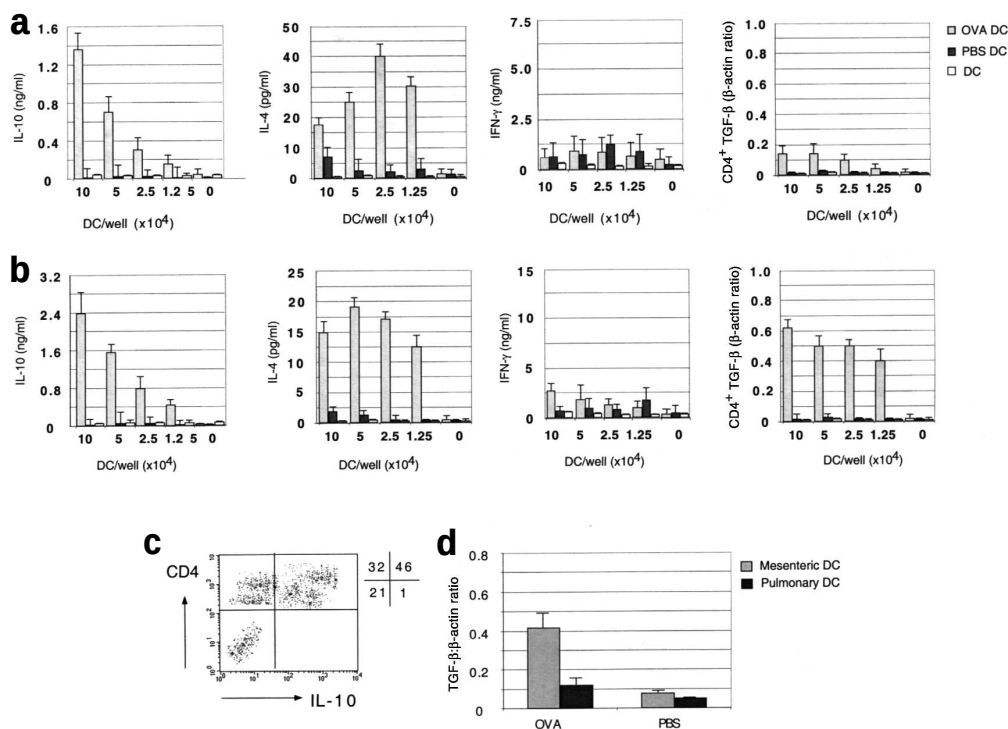
We next determined whether the tolerance-inducing pulmonary DCs from mice exposed to i.n. OVA expressed IL-10. Bronchial lymph node DCs isolated 1 day after i.n. administration of OVA expressed IL-10 mRNA, as detected by RT-PCR (**Fig. 4a**, upper panels). In contrast, neither DCs taken from control mice exposed to i.n. PBS nor DCs from mice vaccinated intradermally with OVA in alum, which induces a vigorous T_H2 inflammatory response, expressed mRNA for IL-10. Pulmonary DCs taken from mice that developed AHR after i.n. exposure

to OVA did not express IL-10. These results were confirmed by an intracellular cytokine-staining assay for IL-10, which showed that 33% of the CD11c⁺MHC class II⁺ DCs from mice exposed to i.n. OVA expressed intracellular IL-10, whereas only 2% of DCs from control mice immunized with OVA in alum expressed intracellular IL-10 (**Fig. 4a**, lower panels). Thus, a large fraction of DCs from the bronchial lymph nodes of mice exposed to i.n. OVA, but not to an immunizing dose of OVA in alum, expressed IL-10.

IL-10 expression by DCs was transient: it was present for only 24–72 h after the last i.n. exposure to OVA and absent by day 7 after i.n. exposure to OVA (**Fig. 4b**, upper panels). Although production of IL-10 often limits the capacity of APCs to present antigen, IL-10 production by DCs correlated with the induction of OVA-specific T cell proliferation *in*

Figure 6. Stimulation of T cells with pulmonary versus mesenteric DCs. (a) DCs from mice exposed to i.n. OVA induced production of IL-4 and IL-10 in OVA-specific CD4⁺ T cells.

Bronchial lymph node cells were isolated from BALB/c mice 24 h after the last i.n. administration of OVA. CD11c⁺ cells were positively selected and cocultured at the indicated concentrations with 5×10⁵ KJ1-26⁺CD4⁺ DO11.10 T cells, which were OVA-specific. After 72 h, supernatants and cells were collected and cytokine production analyzed by ELISA or RT-PCR. ELISA data are representative of four separate experiments (n=four mice for each group) and are mean±s.d. for triplicate samples. TGF-β analysis was done by RT-PCR, with cDNA from the CD4⁺ cells purified by MACS sorting after culture with DCs. Data are the ratios of TGF-β band intensity:β-actin band intensity, measured by densitometry, and are mean±s.d. from three separate experiments. (b) Mesenteric lymph node DCs, isolated after oral administration of OVA, induced production of IL-4, IL-10 and TGF-β in OVA-specific CD4⁺ T cells. Mesenteric lymph node cells were isolated from BALB/c mice 24 h after the last oral administration of OVA (2×5 mg). CD11c⁺ cells were positively selected from draining lymph nodes and cocultured at the indicated concentrations with 5×10⁵ KJ1-26⁺CD4⁺ DO11.10 T cells, which were OVA-specific. ELISA data are representative of four separate experiments (n=four mice for each group) and are mean±s.d. for triplicate samples. TGF-β analysis was done as in a. (c) The majority of OVA-specific CD4⁺ T cells cultured with IL-10-producing DCs expressed intracellular IL-10. CD4⁺ T cells were removed from culture on day 4 and stained for intracellular IL-10. Numbers denote percentage of cells in each quadrant. For details of fixation and permeabilization see Methods. (d) Expression of TGF-β in mesenteric and pulmonary DCs after OVA treatment. TGF-β analysis was done, by RT-PCR, on CD11c⁺ cells isolated from either bronchial lymph nodes or pulmonary lymph nodes after OVA or PBS treatment.





vitro (Fig. 2b) and *in vivo*. Thus, naïve transgenic DO11.10 T cells labeled with the fluorescent dye 5,6-carboxyfluorescein diacetate-succinimidylester (CFSE), to visualize cell division¹⁷, proliferated *in vivo* when adoptively transferred into BALB/c mice 3 days, but not 7 days, after the mice had received i.n. OVA (Fig. 4b, lower panels). This indicated that the OVA-specific T cells were activated during the early phase of tolerance induction, after i.n. exposure to antigen when IL-10 was being produced by the DCs, but not late after i.n. OVA exposure, after dissipation of the i.n.-administered OVA.

Adoptive transfer of IL-10-producing DCs

We asked whether pulmonary DCs from mice exposed to i.n. OVA had the capacity to transfer T cell unresponsiveness. Pulmonary DCs from draining lymph node of IL-10^{+/+} and IL-10^{-/-} mice exposed to i.n. OVA were isolated 24 h after i.n. exposure to OVA and adoptively transferred into naïve mice, which were subsequently immunized with OVA in alum. Adoptive transfer of DCs from mice exposed to i.n. OVA blocked subsequent OVA sensitization by immunization with OVA in alum; spleen cells from recipient mice immunized with OVA proliferated poorly when stimulated *in vitro* with OVA, as indicated by reduced proliferation (Fig. 5). IL-2 production correlated directly with spleen cell proliferation (data not shown). In contrast, spleen cells from mice that had received IL-10^{-/-} DCs or DCs from mice that were intradermally immunized with OVA in alum proliferated vigorously. This showed that T cell unresponsiveness induced by exposure of mice to i.n. OVA could be transferred with IL-10-producing pulmonary DCs.

Function of lung versus gastrointestinal tract DCs

We examined the capacity of pulmonary DCs to induce cytokine production in OVA-specific CD4⁺ T cells. Pulmonary DCs from the draining lymph nodes of mice exposed to i.n. OVA induced T cell production of IL-10 and IL-4, but not interferon- γ (IFN- γ) or TGF- β (Fig. 6a). In contrast, DCs from the mesenteric lymph nodes of mice tolerized with oral OVA induced T cell production of IL-10, IL-4 as well as TGF- β (Fig. 6b). The IL-10 detected in the culture supernatants was produced by the T cells rather than the DCs because the majority of the CD4⁺ T cells stained positively for intracellular IL-10 after 4 days of culture (Fig. 6c). On the other hand, the CD4⁺ cell population, which included DCs, did not express intracellular IL-10 after 4 days of culture. Although DCs from pulmonary lymph nodes did not induce increased amounts of TGF- β production in responding T cells, DCs from mesenteric lymph nodes, which expressed elevated amounts of TGF- β (Fig. 6d), enhanced TGF- β production in CD4⁺ T cells (Fig. 6b). These results indicated that DCs in bronchial lymph nodes were distinct from those in mesenteric lymph nodes with regard to the capacity to induce TGF- β production and distinct CD4⁺ T cell responses.

Discussion

We examined the cellular and molecular events involved in the development of T cell tolerance to inhaled antigen and the role that DCs play in this process. We showed that, within 24 h of respiratory exposure to OVA, pulmonary DCs found within the bronchial lymph nodes expressed IL-10. In addition, *in vitro* culture of these IL-10-producing DCs with OVA-specific CD4⁺ T cells induced the development of CD4⁺ T cells producing IL-4 and high concentrations of IL-10, a combination associated with regulatory T cells¹⁸. Adoptive transfer of the IL-10-producing DCs from IL-10^{+/+}, but not IL-10^{-/-}, mice induced OVA-specific T cell unresponsiveness in recipient mice, which indicated that IL-10 production by DCs was critical for the induction of T cell tolerance in the respiratory tract.

Pulmonary DCs play a key role in activating and expanding CD4⁺ T cells in the pulmonary compartment^{8,16,19–21}. Within the lung, DCs exist primarily as immature cells, with low expression of costimulatory molecules B7-1 and B7-2, and function as highly endocytic sentinel cells, continuously sampling and acquiring antigen in the lung. When these DCs are transferred to naïve mice, they induce primarily T_H2 responses and appear to be essential for the development and maintenance of T_H2-biased immune responses in allergic asthma. Published data therefore suggest that DCs in the lung function primarily to amplify and enhance inflammatory responses, rather than to down-modulate immune responses or to induce T cell hyporesponsiveness.

Our results indicate that the role of pulmonary DCs in the lung is complex: the function of DCs depends on the context in which they become activated while acquiring antigen in the lung. Although pulmonary DCs from mice exposed to OVA under conditions that resulted in airway hyperreactivity induced vigorous inflammatory responses and did not produce detectable amounts of IL-10, pulmonary DCs from mice exposed to i.n. OVA in the absence of inflammation produced high concentrations of IL-10. The production of IL-10 and the associated capacity to induce T cell tolerance was maximal 24 h after the administration of i.n. OVA, but waned over the subsequent 48 h. Production of IL-10 has been observed in mouse DCs from Peyer's patches in the intestine¹², in DCs from the lung (detected by RT-PCR)⁵, in human monocyte-derived CD1 α ⁺ DCs²² and in human CD14⁺ monocyte-derived DCs²³. In all these instances, the DCs expressed low amounts of CD86 (immature phenotype) and preferentially induced T_H2 cytokines. IL-10 production by DCs has not previously been associated with the induction of T cell tolerance. This is possibly because the induction of T cell unresponsiveness by DCs requires higher concentrations of IL-10 production than was observed and requires the expression of a particular set of costimulatory molecules, as we have shown.

The IL-10-producing pulmonary DCs that developed after respiratory exposure to OVA expressed features associated with mature DCs. These DCs had high expression of B7-1 and B7-2 and a low capacity for endocytosis, which are hallmarks of mature DCs that normally induce vigorous T inflammatory responses. The progression, after exposure to i.n. OVA, from the entirely immature DC phenotype within the pulmonary tissues to the more mature phenotype in the bronchial lymph nodes was consistent with published data, which indicates that DCs mature as they migrate from peripheral tissues into the regional lymph nodes after acquiring antigen. The "tolerogenic DCs" in our studies contrast with other studies of tolerance, which show that immature DCs—expressing low amounts of B7 molecules—induced tolerance, presumably by inducing T cell activation in the absence of costimulation^{24,25}. In most of these models, the tolerance-inducing immature DCs required extensive propagation *in vitro* in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and could not be shown in freshly isolated tissues. In contrast, the tolerance-inducing phenotypically mature DCs we studied were generated *in vivo* under more physiological conditions and were freshly isolated from bronchial tissues.

One unusual property of the IL-10-producing DCs was that these cells induced the production, in naïve CD4⁺ T cells, of IL-4 and high concentrations of IL-10 but not IFN- γ . Whereas IL-4 and IL-10 are T_H2 cell-associated cytokines, production of high concentrations of IL-10 is associated with regulatory cells such as T regulatory subset 1 (T_R1) cells or T_H3 cells, which mediate a form of "tolerance" or a type of immune deviation^{18,26}. T_R1 cells, which have a low proliferative capacity, produce high IL-10, low IL-2 and TGF- β and actively down-regulate pathological antigen-specific immune response *in vivo*¹⁸. T_H3 cells similarly suppress antigen-specific inflammatory responses (for example, experimental



autoimmune encephalomyelitis), produce various amounts of IL-4, IL-10 and TGF- β and have been derived by oral administration of antigen²⁶.

Because T_H1 cells develop in the presence of IL-10, and T_H3 cells are associated with the regulation of mucosal responses, we suggest that IL-10-producing DCs from the lungs or from the gastrointestinal tract may be particularly effective in inducing the development of T_H1 and T_H3 cells. Although T_H1 and T_H3 regulatory cells, as well as classical T_H2 cells, preferentially develop at mucosal sites and are associated with IL-10 production, our data indicate that the signals required for the induction of T_H1, T_H3 and T_H2 cells are distinct. Thus, we showed that pulmonary DCs induced T_H1-like cells that produced minimal amounts of TGF- β , whereas mesenteric lymph node DCs induced T_H3-like cells that produced much more TGF- β . The induction of TGF- β mRNA in responding CD4⁺ T cells correlated with the quantity of TGF- β mRNA in the stimulating DCs. However, other factors—for example, costimulatory signals delivered *via* ICOS^{27,29}, B7-H1 molecules³⁰ or CD40L—may also play a major role in determining cytokine production in CD4⁺ T cells and the type of T cell that develops.

Under normal circumstances, soluble proteins that are encountered in the lungs are processed by DCs and result in the induction of antigen-specific tolerance. However, in individuals with allergic asthma, processing of these proteins result in the induction of antigen-specific T_H2-biased inflammatory responses that cause AHR and asthma³¹. The specific events that alter antigen processing and presentation in the lungs of these individuals who have allergic asthma and that abrogate tolerance are not yet clear, but we suggest that DC function in these individuals is altered. For example, DC production of IL-10, which has been thought to be reduced in patients with asthma^{32,33}, or expression of important costimulatory molecules that critically regulate T_H1 cell development may be affected, for example, by viral infection¹⁰. If the events and alterations that affect the induction of respiratory tolerance were identified, new therapeutic interventions for allergic asthma could be developed, for example, by utilizing vaccination strategies that induce allergen-specific T_H1 cell development and tolerance, but hinder the survival of allergen-specific T_H2 cells.

Methods

Mice and antigen administration. BALB/c and C57BL/6 IL-10^{-/-} mice were from Jackson Laboratory (Bar Harbor, ME). OVA TCR-transgenic DO11.10 mice were from D. Y. Loh (Washington University, St. Louis, MO); they were backcrossed to RAG-2^{-/-} mice (from A. Abbas, UCSF, CA) to generate DO11.10 RAG-2^{-/-} mice. All mice were housed in pathogen-free conditions at the laboratory animal facilities of Stanford University and were treated in accordance with National Institutes of Health guidelines. Mice used for experiments were sex- and age-matched. Mice lightly anesthetized with methoxyflurane administered *via* i.n. OVA (Worthington Biochemical Corp., Lakewood, NJ, chromatographically purified) on 3 consecutive days. Control mice received i.n. PBS. Ten days later, mice were immunized intraperitoneally with 10 μ g of OVA in 2 mg of alum, in a volume of 0.5 ml. In oral tolerance experiments, mice were given 5 mg of OVA in PBS, or PBS alone, by intragastric gavage with the use of animal feeding needles, as described³⁴.

Reagents and monoclonal antibodies (mAbs). Hybridoma cells producing the anti-clonotypic mAb KJ1-26 were from P. Marrack (National Jewish Medical Center, Denver, CO)³⁵. The hybridoma was grown as ascites in pristane-primed BALB/cByJ^{mu} mice (0.5 ml of 98% 2,6,10,14-tetramethylpentadecane per mouse, administered intraperitoneally, Sigma, St. Louis, MO); mAb was subsequently purified from ascites by ammonium sulfate precipitation and ion-exchange chromatography. Anti-38C13 Id mAb 4G10 (rat IgG2a)³⁶ was from S. Levy (Stanford University, Stanford, CA) and was used as isotype control.

Preparation of bone marrow-derived DCs. Bone marrow-derived DCs were generated as described^{37,38}, with some modifications³⁹, and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, Gemini Bioproducts, Calabasas, CA), 2 mM glutamine, 20 μ g/ml of gentamycin and 5 $\times$ 10⁻⁵ M 2-mercaptoethanol. Briefly, 5 $\times$ 10⁶ bone marrow cells were cultured in petri dishes (9-cm diameter; Nunc, Roskilde, Denmark) in 10 ml of culture medium containing 20–25 U/ml of GM-CSF. Loosely adherent cells were transferred onto a second dish on day 6 of culture. From days 6–10, these transferred cells were used as a source of DCs.

Magnetic cell sorting of DCs. Lymph nodes were digested with a cocktail of 0.1% DNase I (fraction IX; Sigma) and 1.6 mg/ml of collagenase (CLS4; Worthington Biochemical Corp.) at 37 °C for 1 h. Positive selection of DCs from lymph nodes was done by magnetic cell sorting (MACS) of DCs after incubation with N418 (anti-CD11c) magnetic beads, with the Vario-MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Positively selected cells and the nonselected fractions were collected for further analysis.

***In vitro* proliferation and cytokine assays.** Lymph node or spleen cells were collected and passed through a nylon mesh. They were stimulated in round-bottomed 96-well plates (2 $\times$ 10⁵ cells/well) with or without OVA in the presence of 2 $\times$ 10⁴ bone marrow-derived DCs/well in 0.2 ml of complete DMEM. For measurement of cell proliferation, the cultures were pulsed after 72 h with 0.25 μ Ci of [³H]thymidine for 16 h, and the incorporated radioactivity measured in a Betaplate scintillation counter (MicroBeta Trilux, Wallac, Turku, Finland). For measurement of IL-2 production, aliquots of supernatants were removed after 48 h of culture and tested for IL-2 content with the IL-2-dependent cell line, CTLL. In some experiments, CD11c⁺ cells purified from bronchial or mesenteric lymph nodes of immunized mice were incubated with OVA-specific naïve CD4 T cells purified from lymph nodes and spleens of DO11.10 mice, according to established methods, but with some modifications⁴⁰. Cytokine production was assessed by analysis of culture supernatants collected on day 2 for measurement of IL-2 by bioassay, and on day 4 for measurement of IL-4, IL-10 and IFN- γ by ELISA. ELISAs were done as described⁴¹. The mAb pairs for capture and biotinylated detection were as follows: for IL-4, BVD4-1D11 and BVD6-24G2; for IFN- γ , R4-6A2 and XMG1.2; for IL-10, 2A5 and Sxc.1. Recombinant cytokines were used as standards, with curves generated in 1:2 dilutions from 39–500 pg/ml for IL-4, from 0.078–10 ng/ml for IL-10 and from 0.156–20 ng/ml for IFN- γ .

Adoptive transfer of TCR-transgenic T cells. Lymph node and spleen cells from DO11.10 mice were pooled and positively selected with biotin-coupled KJ1-26 mAb. This was followed by incubation with streptavidin-conjugated microbeads. Isolated cells in the final population were examined by flow cytometry and 5.0 $\times$ 10⁶ CD4⁺KJ1-26⁺ cells were injected intravenously into nonirradiated syngeneic BALB/c recipients, in a volume of 0.3 ml of normal saline.

CFSE labeling and T cell transfer. KJ1-26⁺ T cells were purified to >95% with MACS. Labeling of KJ1-26⁺ cells with CFSE (Molecular Probes, Inc, Eugene, OR) was done as described⁴². In brief, cells were washed and resuspended at a concentration of 10⁷/ml in PBS. CFSE was added at a final concentration of 5 μ M and incubated for 15 min at 37 °C at a concentration of 10⁷ cells/ml, washed once and transferred intravenously.

Flow cytometry and FACS[®] Analysis. Analytical flow cytometry was carried out with a FACScan[®] (Becton Dickinson, San Jose, CA) and the data were processed with Cellquest software (Becton Dickinson). Lymph node or spleen cells were collected at various times and 1 $\times$ 10⁶ cells were incubated on ice with biotin-conjugated KJ1-26 mAbs followed by phycoerythrin (PE)-streptavidin (PharMingen, San Jose, CA), using standard procedures. FITC-conjugated antibodies to CD44, CD69, CD62L and CD 25 (PharMingen) were used on cells already stained with the clonotype-specific KJ1-26 mAbs⁹. Flow cytometric measurement of cytokine production in DCs was done according to a standard protocol, with some modifications^{17,43}. Briefly, 1 $\times$ 10⁶ DCs were isolated from lymph nodes 24 h after i.n. administration of OVA. After blocking of Fc receptors with excess anti-Fc (HB197) and cell-surface staining with PE-CD11c and biotin-anti I-A^d (MKD6) followed by cytochrome-streptavidin (PharMingen), cells were washed twice with cold PBS. Fixation and permeabilization was done according to cytofix-cytoperm plus with the GolgiPlug protocol (PharMingen). Staining for cytoplasmic IL-10 was done by adding FITC-anti-IL-10 (PharMingen) to permeabilized cells (30 min on ice) and washing twice with cold PBS.

FITC-dextran uptake. Cells were suspended in RPMI 1640, 10% FCS and 25 mM HEPES (pH 7.4) and incubated with 1 mg/ml of FITC-dextran (*M_w* = 40,500, Sigma) for 30 min at 4 °C or 37 °C. Cells were washed three times with ice-cold PBS, 0.1% bovine serum albumin and 0.01% NaN₃, and labeled on ice with PE-conjugated appropriate mAbs. The uptake was calculated as the change in mean fluorescence intensity (MFI) between cell samples incubated at 37 °C and 4 °C.

RNA isolation and RT-PCR assay. DCs isolated 24 h after i.n. OVA administration were analyzed for mRNA expression. Cells were processed using Qiagen RNA isolation kits (Qiagen, Valencia, CA). Reverse transcription was done with 200 ng of RNA, 2 μ g of oligo(dT) (Life Technologies, Grand Island, NY) and 1 U of Superscript II Reverse transcriptase at 60 °C for 60 min. Samples were stored at -20 °C until further use. Primers specific for β -actin (forward 5'-TGACCGGCTTGATGCTATC-3'; reverse 5'-CAGTGTAGCCAGGATATAG-3'), IL-10 (forward 5'-ATGCAGGACTTTAAGGGTTACTTG-3'; reverse 5'-ATTCCAGGTTCTGTGCCACAGAT-3') and TGF- β (forward 5'-ACCGCAACAACGCCATCTAT-3'; reverse 5'-GTAACGCCAGGAATTGTTGC-3') were designed and synthesized at the Stanford PAN (Protein and Nucleic Acid) facility (Stanford, CA). cDNA (10 ng) was mixed with 10 $\times$ buffer, dNTPs (0.2 mM final), MgCl₂ (2.5 mM final), primers and Taq DNA polymerase (1 U/reaction, Life Technologies) in a final volume of 25 μ l. PCR was done in a DNA thermal cycler (MJ Research, Cambridge, MA) for 35 cycles, and products were visualized by electrophoresis. In TGF- β expression studies, RT-PCR was done on CD4⁺ cells after *in vitro* stimulation



with DCs and the β -actin ratio was calculated as described⁴⁴. The semiquantitative PCR was done, with some modifications, with the use of primers for β -actin. In brief, aliquots of cDNA were analyzed for β -actin by placing serial dilutions of the experimental cDNA against a fixed concentration of the competitor construct and examining the ratio of competitor:wild-type band intensity after amplification. The PCR products were separated by electrophoresis in 2% agarose gels that contained ethidium bromide.

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