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Intranasal Exposure to Protein Antigen Induces Immunological Tolerance Mediated by Functionally Disabled CD4$^+$ T Cells$^1$

Daphne C. Tsitoura,* Rosemarie H. DeKruyff,* Jonathan R. Lamb, † and Dale T. Umetsu* 

In this study we examined the immunological parameters underlying the natural immunity to inhaled nonpathogenic proteins. We addressed this question by examining the effect of intranasal exposure to OVA in both wild-type mice and mice reconstituted with OVA-TCR transgenic CD4$^+$ T cells. Intranasal administration of OVA induced an initial phase of activation during which CD4$^+$ T cells were capable of proliferating and producing cytokines. Although many of the OVA-specific CD4$^+$ T cells were subsequently depleted from the lymphoid organs, a stable population of such T cells survived but remained refractory to antigenic rechallenge. The unresponsive state was not associated with immune deviation due to selective secretion of Th1- or Th2-type cytokines, and the presence of regulatory CD8$^+$ T cells was not required. Moreover, neutralization of the immunosuppressive cytokines IL-10 and TGF-β did not abrogate the induction of tolerance. Inhibition of the interaction of T cells with CD86, but not CD80, at the time of exposure to intranasal Ag prevented the development of unresponsiveness, while selective blockade of CTLA-4 had no effect.

Our results suggest that intranasal exposure to Ags results in immunological tolerance mediated by functionally impaired CD4$^+$ T cells via a costimulatory pathway that requires CD86. The Journal of Immunology, 1999, 163: 2592–2600.

However, the immunological parameters underlying the natural immunity to inhaled proteins are poorly defined. It has been suggested that the default immune response to aeroallergens includes a strong component of Th2-like reactivity, and avoidance of allergic sensitization is achieved through early immune deviation toward the Th1 pathway (1, 2, 7). This shift in the phenotypic profile of the immune response has been mainly attributed to Ag-specific CD8$^-$ T cells that become activated following Ag inhalation and secrete enhanced amounts of IFN-γ (1, 2). Nevertheless, the experimental evidence for this process remains controversial. Normal individuals do not exhibit vigorous Th1-type reactivity against aeroallergens, but, rather, they are characterized by a lack of productive immunity. Furthermore, recent studies have concluded that CD8$^-$ T cells also play a pathogenic role in the development of airway inflammation and hyper-responsiveness (8, 9).

In contrast to CD8$^-$ T cells, the direct effect of inhaled proteins on CD4$^+$ T cell functions has not been analyzed. It is well documented that the induction of long-lasting immunity to protein Ags requires the expansion and differentiation of Ag-specific, effector CD4$^+$ T cells, while unresponsiveness has been correlated with their elimination or functional inactivation. The quality of costimulatory signaling at the time of priming has been shown to differentially regulate this process (10–12). Therefore, the principal aim of this study was to investigate in detail the effect of intranasal (i.n.)$^3$ exposure to OVA on T cell responses in vivo and define the requirements for the development of protective immunity. To address this question, we analyzed immune responses in wild-type mice as well as in mice bearing a small population of adaptively transferred OVA-TCR transgenic T cells. The latter allowed us to follow the fate of Ag-specific CD4$^+$ T cells in vivo (13). The results of our study indicate that i.n. exposure to OVA induces unresponsiveness to subsequent immunogenic challenges that is not dependent on the presence of regulatory CD8$^-$ T cells or inhibitory cytokines, but is mediated by functionally impaired Ag-specific CD4$^+$ T cells. An initial, transient phase of activation, during which the T cells produce Th1 and Th2 cytokines, precedes...
Intranasal exposure to OVA induces Ag-specific unresponsiveness to immunogenic rechallenge. BALB/c mice were exposed i.n. to 100 μg of OVA or PBS on 3 consecutive days and rechallenged i.p. with OVA and HEL in alum on day 13. Spleen cells were harvested 6 days later and cultured in vitro with increasing concentrations of OVA (A) or HEL (B) as indicated on the x-axis. The proliferation was determined by measuring [3H]Tdr incorporation. Results are expressed as the mean counts per minute (±SD) of triplicate cultures.

the development of unresponsiveness. The functional differentiation of the CD4+ T cells in response to i.n. OVA appears to be selectively dependent on CD86 (B7.2), but not CD80 (B7.1), costimulatory signals.

Materials and Methods

Animals

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OVA-TCR transgenic D011.10 mice (14) were obtained from Dr. D. Y. Loh (Washington University, St. Louis, MO). D011.10 mice were backcrossed with normal BALB/c mice, and the progeny were screened by flow cytometric analysis of peripheral blood leukocytes stained with anti-CD4 and the clonotype-specific KJ1-26 mAbs (14). All mice were housed in pathogen-free conditions at the laboratory animal facilities of Stanford University, in accordance with the guidelines of National Institutes of Health. Mice used for experiments were sex and age matched.

Media, reagents, and mAbs

All cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FCS (Gemini Bioproducts, Calabasas, CA), 2 mM glutamine, 20 μg/ml gentamicin, and 5 × 10−5 M 2-ME. The hybridoma cell lines secreting the anti-CD8 (53.6.7), anti-CD80 (1G10), and anti-CD86 (GL1) mAbs were obtained from American Type Culture Collection (Manassas, VA). The cell lines secreting anti-CTLA-4 (UC10-4F10) and anti-IL-10 (JES5-2A5) mAbs were gifts from Dr. J. Bluestone (University of Chicago, Chicago, IL) and Dr. J. Abrams (DNAX, Palo Alto, CA), respectively. Hybridoma cells producing the anti-clonotypic Ab KJ1-26 were provided by Dr. P. Marrack (National Jewish Medical Center, Denver, CO). Each of the above-mentioned Abs were grown as ascites in pristane-primed BALB/c nu/nu mice (0.5 ml/mouse i.p. of 98% 2,6,10,14-tetramethylpentadecane; Sigma) and were subsequently purified by ammonium sulfate precipitation and ion-exchange chromatography. The neutralizing anti-TGF-β mAb was obtained from Genzyme (Cambridge, MA). The Ab doses we used in our experiments have been previously shown to be effective in vivo (15–19).

Induction of tolerance to i.n. Ag

Mice lightly anesthetized with methoxufurane received i.n. 30 μl of PBS containing OVA (grade V; Sigma) on 3 consecutive days. Control mice received i.n. PBS. To confirm that the i.n. administered Ag was delivered to the airways, mice were given i.n. OVA in Evans Blue dye. We found that the dye was deposited only in the respiratory tract, while there were no detectable traces in the esophagus or stomach (unpublished data) (16). Ten days later the mice were immunized i.p. with 10 μg of OVA in 2 mg of aluminum hydroxide (alum) in a volume of 0.5 ml. For the experiments with chimeric OVA-transgenic mice 100 μg of OVA in alum were used for the i.p. injection. To examine bystander suppression to an unrelated Ag, mice pretreated with i.n. OVA or PBS were coimmunized i.p. with 10 μg of OVA and 20 μg of hen egg lysozyme (HEL; Sigma) in alum.

Adoptive transfer of TRC-transgenic T cells

Lymph node and spleen cells from D011.10 mice were pooled and enriched for CD4+ T cells. In brief, erythrocytes were removed by hypotonic lysis, resting B cells were depleted by adherence to goat anti-mouse Ig-coated plates; CD8+ cells and APC were depleted by treatment with anti-CD8 (HB130), anti-HSA(J11d.2), and anti-MHC class II (MKD6) mAbs in the presence of rabbit complement (Pel-Freez, Brown Deer, WI). The percentage of CD4+, KJ1-26+ cells in the final population was determined by flow cytometric analysis, and 5–6 × 10^6 CD4+, KJ1-26+ cells were injected i.v. into unirradiated syngeneic BALB/c recipients, in a volume of 0.3 ml of normal saline.

In vitro proliferation and cytokine assays

Lymph node or spleen cells were harvested, passed through a nylon mesh, and cultured (5 × 10^5 cells/well) with or without OVA in 0.2 ml of complete DMEM. After 72 h, the cultures were pulsed with 0.25 μCi of [3H]Tdr thymidine for 12–16 h, and the incorporated radioactivity was measured in a Betaplate scintillation counter (MicroBeta Trilux, Wallac, Turku, Finland). The clonotype-specific proliferation was calculated by dividing the total OVA-specific proliferation in counts per minute by the number of KJ1-26+ CD4+ T cells in the culture (assessed by flow cytometry). To determine the secretion of cytokines in the cultures, supernatants were collected on day 4. The levels of IL-4, IL-5, IL-10, and IFN-γ in the supernatants were assayed by ELISA. The presence of TGF-β was assessed...
in a bioassay using the mink lung epithelial cell line Mv1Lu (American Type Culture Collection).

Flow cytometry
Lymph node or spleen cells were harvested at various times, and $1 \times 10^6$ cells were incubated on ice with anti-CD4-FITC (PharMingen, San Diego, CA) and the biotinylated KJ1-26 mAbs followed by streptavidin Cy3 (PharMingen). For some experiments the cells were also stained with anti-CD45RB-PE (PharMingen). After the final washing the cells were fixed with 1% paraformaldehyde and analyzed on a Becton Dickinson FACScan (Rutherford, NJ). Analysis was performed on 15,000 collected events. CD45RB expression and cell size were assessed on the gated CD4$^+$, KJ1-26$^+$ population. The total number of DO11.10 cells present in the lymphoid organs was calculated by multiplying the total number of viable lymph node or spleen cells by the percentage of CD4$^+$, KJ1-26$^+$ cells obtained by flow cytometry.

Measurement of OVA-specific Igs
Mice were bled, and OVA-specific Abs were measured using modified OVA-specific ELISAs. For the measurement of OVA-specific IgG1 and IgG2a, plates were coated overnight with 5 μg/ml OVA. After washing and blocking, serial dilutions of sera were added for 24 h. Subsequently, the plates were incubated with HRP-conjugated goat anti-IgG subclass-specific Abs (Southern Biotechnology Associates, Birmingham, AL), washed, and developed by adding o-phenylenediamine. The OD was determined at 492 nm. Anti-OVA IgG1 and IgG2a mAbs 6C1 and 3A11, respectively, were used as standards for the quantification of each IgG subclass. For the determination of OVA-specific IgE, the rat anti-mouse IgE mAb EM95 (5 μg/ml) was used as coating and developed by substrate 3,3',5,5'-tetrachloro-1,1'-phenylenediamine. The OD was determined at 492 nm. The standard for the OVA-specific IgE ELISA was sera from mice hyperimmunized with OVA that had been quantitated for IgE.

Results

Intranasal administration of protein Ag induces Ag-specific unresponsiveness
To investigate the effect of exposure to i.n. protein Ag on subsequent antigenic challenges, BALB/c mice were treated with i.n. OVA or PBS and then immunized i.p. with OVA in alum. Splenic T cells from the OVA-pretreated mice failed to expand in response to OVA in vitro in contrast to cells from mice that had received i.n. PBS, indicating that prior exposure to i.n. Ag impaired subsequent Ag priming (Fig. 1A). The inhibition of T cell responsiveness was Ag specific, because spleen cells from mice exposed to i.n. OVA and immunized i.p. with both OVA and HEL in alum proliferated normally when restimulated in vitro with HEL (Fig. 1B), but not with OVA (Fig. 1A). The T cell unresponsiveness waned with time, as mice exposed to i.n. OVA and rechallenged 2, 3, and 4 mo after the last administration of i.n. Ag slowly regained their capacity to proliferate following in vivo restimulation with OVA (Fig. 2).

To determine whether the quantity of i.n. administered Ag differentially influenced the T cell effector functions on immunogenic rechallenge, mice were exposed to 10-fold increasing doses of i.n. OVA (0.1–1000 μg). We used doses of i.n. OVA <1000 μg, because this dose greatly exceeds the level of natural exposure to aeroallergens. Doses of OVA >1 μg markedly reduced the subsequent proliferative response to OVA (Table I). Analogous reduction was observed in the levels of secreted cytokines, including IFN-γ, IL-4, and IL-5 (Table I). Production of IL-10 and TGF-β was not detected in any of the cultures. Maximal inhibition of T cell function was noticed in mice pretreated with 100 μg of i.n.

FIGURE 2. Production of anti-OVA Ab isotypes in mice pre-exposed to i.n. Ag. BALB/c mice were treated i.n. with 100 μg of OVA or PBS on 3 consecutive days and rechallenged i.p. with OVA in alum on day 13. Two weeks after the secondary immunization the mice were bled, and the levels of OVA-specific IgG1, IgG2a, and IgE in the sera were quantitated by isotype-specific ELISA. Results are expressed as the mean (± SD) of triplicate determinations.

**Table I. Intranasal tolerized T cells fail to produce cytokines upon antigenic restimulation**

<table>
<thead>
<tr>
<th>OVA (μg/dose)</th>
<th>cpm</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
<th>TGF-β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (PBS)</td>
<td>105,505 ± 2,304</td>
<td>98 ± 2.3</td>
<td>5.3 ± 1.1</td>
<td>30 ± 0.8</td>
<td>8.3 ± 0.8</td>
<td>&lt;40</td>
</tr>
<tr>
<td>0.1</td>
<td>97,148 ± 2,102</td>
<td>106 ± 1.9</td>
<td>4.1 ± 0.5</td>
<td>27 ± 1.6</td>
<td>6.2 ± 0.5</td>
<td>&lt;40</td>
</tr>
<tr>
<td>1</td>
<td>68,247 ± 3,767</td>
<td>80 ± 1.7</td>
<td>1.9 ± 0.4</td>
<td>16 ± 1.9</td>
<td>2.1 ± 0.9</td>
<td>&lt;40</td>
</tr>
<tr>
<td>10</td>
<td>33,218 ± 4,638</td>
<td>&lt;5</td>
<td>&lt;0.156</td>
<td>7.5 ± 0.6</td>
<td>&lt;0.195</td>
<td>&lt;40</td>
</tr>
<tr>
<td>100</td>
<td>22,616 ± 131</td>
<td>&lt;5</td>
<td>&lt;0.156</td>
<td>&lt;0.185</td>
<td>&lt;0.195</td>
<td>&lt;40</td>
</tr>
<tr>
<td>1000</td>
<td>20,916 ± 2,242</td>
<td>&lt;5</td>
<td>&lt;0.156</td>
<td>&lt;0.185</td>
<td>&lt;0.195</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

* BALB/c mice exposed to i.n. PBS or 10-fold increasing doses of OVA three times were rechallenged i.p. 10 days later with OVA in alum. Spleen cells were harvested after 6 days and restimulated in vitro with OVA. The proliferation and cytokine production in response to 100 μg/ml OVA is shown from one representative experiment out of four using four mice/group. Spleen cells cultured in vitro without OVA failed to proliferate and produce cytokines. Results are expressed as the mean (± SD) for duplicate or triplicate samples.
The induction of OVA-specific unresponsiveness is not mediated by CD8 T cell compartment may be responsible for the down-regulation of Th2 immunity that is associated with i.n. exposure to a protein Ag.

Immunosuppressive cytokines, such as TGF-β and IL-10, have been implicated in the development of peripheral tolerance (5, 6). To examine whether the presence of these cytokines was essential for the induction of unresponsiveness by i.n. Ag, mice treated with neutralizing anti-IL-10 or anti-TGF-β mAbs were exposed to i.n. OVA. The anti-cytokine Abs were administered by i.p. injection starting 24 h before the first dose of i.n. OVA and thereafter every 2 days until day 15. However, after systemic priming splenic T cells from such mice proliferated in response to OVA in vitro as poorly as cells from mice that were not treated with anti-cytokine Abs (Fig. 4, b and c), indicating that the absence of IL-10 or TGF-β did not influence the development of T cell unresponsiveness to secondary antigenic challenge.

**Exposure to i.n. Ag induces a persistent activated population of CD4+ T cells**

To examine the involvement of CD4+ T cells in the down-regulation of T cell responses after i.n. exposure to OVA, we used a chimeric model in which a small population of OVA-TCR transgenic CD4+ T cells from D011.10 mice was adoptively transferred into syngeneic BALB/c mice. The recipient mice were subsequently treated with i.n. OVA or PBS, and the fate of D011.10 cells was monitored by flow cytometry using the clonotype Ab KJ1-26. CD4+, KJ1-26+ cells were undetectable in normal BALB/c mice even after immunization with OVA in adjuvant (data not shown). Exposure of the chimeric mice to i.n. OVA three times resulted in considerable expansion of the D011.10 T cells in vivo. In the peribronchial lymph nodes, the main site of accumulation of inhaled Ags (16), a considerable increase in cellularity occurred 2 days after the last administration of OVA, with a 15-fold increase in the percentage of CD4+, KJ1-26+ T cells (Fig. 5a). This change represents a 49-fold expansion of the absolute number of OVA-transgenic T cells per mouse. On day 5 the total number of CD4+, KJ1-26+ T cells bronchial lymph nodes was 3.5 × 10^6 in the PBS-pretreated mice compared with 171 × 10^6 in the OVA-treated group (Fig. 5a). The CD4+, KJ1-26+ T cells in the OVA-exposed mice presented an activated phenotype, as demonstrated by an increase in cell size (Fig. 5b) and down-regulation of CD45RB expression, which remained reduced 1 wk later (Fig. 5c). Similar expansion of D011.10 cells was also observed in the spleen (Fig. 6b) and other non-draining lymph nodes (data not shown), providing evidence for systemic activation of the OVA-specific CD4+ T cells. To examine whether the in vivo activated Ag-specific T cells from mice exposed to i.n. OVA were initially functionally responsive, bronchial lymph node cells were restimulated in vitro with OVA. The cells showed strong proliferation accompanied by the production of both Th1-type (IFN-γ) and Th2-type (IL-4 and IL-5) cytokines (Table II). The initial phase of T cell reactivity following exposure to i.n. OVA was also confirmed in normal BALB/c mice (data not shown).

The accumulation of D011.10 cells in the bronchial lymph nodes and spleens of mice exposed to i.n. OVA declined after day 5 (Fig. 6). Nevertheless, not all CD4+, KJ1-26+ cells were eliminated, and on day 13, the time of the i.p. immunogenic challenge, a substantially larger population of D011.10 cells remained in the OVA-pretreated mice compared with their control counterparts (Fig. 6). Particularly in the peribronchial area, these mice had 16-fold higher numbers of D011.10 cells with an activated phenotype (Fig. 5c). Thus, exposure to i.n. OVA results in an initial phase of T cell activation followed by some degree of deletion, possibly due...
to apoptosis. However, a small activated population of OVA-specific CD4+ T cells persists, implying that deletion is not the primary cause underlying the unresponsiveness to further in vivo immunogenic challenges with OVA.

The development of i.n. tolerance is due to the presence of functionally impaired Ag-specific CD4+ T cells

The immunological basis underlying the failure of T cells to respond to antigenic restimulation after i.n. exposure to OVA was investigated. Mice injected with D011.10 cells and then exposed to i.n. OVA or PBS were immunized i.p. with OVA in alum 10 days later. Spleen or mesenteric lymph node cells from these mice were retrieved after 4 days and stained with anti-CD4 and KJ1-26 Abs. The results are representative of five experiments using five or six mice per group.

FIGURE 5. Intranasal exposure to OVA induces initial activation and expansion of Ag-specific CD4+ T cells in vivo. BALB/c recipients of DO11.10 T cells were administered i.n. PBS (top) or 100 μg of OVA (bottom) on days 1–3. On day 5 bronchial lymph nodes were pooled and stained with anti-CD4 and KJ1-26 Abs for FACS analysis (a). The cell size was examined by gating on the KJ1-26+ and CD4+ cell population (b). Bronchial lymph nodes cells harvested on day 13 were also stained with an anti-CD45RB (c). The results are representative of five experiments using five or six mice per group.

FIGURE 6. Kinetics of the in vivo response of DO11.10 T cells to i.n. OVA. BALB/c recipients of DO11.10 T cells were exposed i.n. to OVA (100 μg) or PBS (days 1–3). Bronchial lymph node (a) and spleen (b) cells were harvested on days 5, 9, and 13; stained with anti-CD4 and KJ1-26 Abs; and analyzed by flow cytometry. The total number of KJ1-26+ T cells is shown as the mean (±SD) of five or six mice per group.
Representative results from one of four experiments are shown. Alternatively, this may be subject to the influence of inhibitory mechanisms mediated by regulatory cells derived from the host. To investigate whether i.n. protein Ag leads to active suppression, D011.10 cells were transferred into mice preexposed to i.n. OVA or PBS, which were then challenged i.p. with OVA in alum. In both PBS- and OVA-pretreated mice the KJ1-26+, CD4+ T cells expanded equally well, because the numbers of OVA-transgenic cells detected in the lymph nodes and spleen were similar (Fig. 8), suggesting that a suppressive environment was not created. Moreover, adoptive transfer of bronchial lymph node and spleen cells or purified CD4+ T cells (up to 20 × 10^6 cells) from i.n. tolerized mice (wild type, as well as chimeric) into naive BALB/c recipients failed to confer a state of OVA-specific hyporesponsiveness to subsequent immunization analogous to that of the original donors (data not shown).

The induction of i.n. tolerance is dependant on CD86-mediated, but not CTLA-4-mediated, costimulation

Our observation that activation of Ag-specific CD4+ T cells occurs initially following i.n. exposure to OVA prompted us to examine the contribution of costimulation in the induction of tolerance. BALB/c mice exposed to i.n. OVA or PBS also received injections of anti-CD80 (B7.1) or anti-CD86 (B7.2) Abs and were rechallenged i.p. with OVA in alum. The administration of anti-B7 Abs did not prevent T cell responses to i.p. immunization with OVA 10 days after the completion of the Ab treatment (Fig. 9). Mice receiving i.n. OVA and anti-CD80 Ab were still unresponsive to secondary challenge (Fig. 9A). In contrast, treatment with anti-CD86 Ab resulted in abrogation of tolerance induction (Fig. 9B). The possibility that selective interaction of CD86 with CTLA-4 was required for the development of i.n. tolerance was also examined by treating the mice with anti-CTLA-4 Ab at the time of administration of i.n. OVA. Blocking the CTLA-4-mediated costimulation, however, failed to abolish the induction of i.n. hyporesponsiveness, and no marked difference in the proliferation was

Table II. OVA-specific CD4+ T cells activated following exposure to i.n. OVA are initially functionally responsive

<table>
<thead>
<tr>
<th>Intranasal Treatment</th>
<th>cpm</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
<th>TGF-β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>2,552 ± 703</td>
<td>&lt;5</td>
<td>&lt;0.15</td>
<td>&lt;0.18</td>
<td>&lt;0.19</td>
<td>&lt;40</td>
</tr>
<tr>
<td>OVA</td>
<td>63,978 ± 8,090</td>
<td>40 ± 1.9</td>
<td>4.1 ± 0.9</td>
<td>10.3 ± 0.8</td>
<td>0.8 ± 0.2</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

* BALB/c recipients of D011.10 T cells were administered 100 µg OVA or PBS i.n. on 3 consecutive days and 2 days later the bronchial lymph node cells were harvested and cultured in vitro with OVA. Proliferation and cytokine production were measured at day 4 of culture. Representative results of the mean response (± SD) to 100 µg/ml OVA are shown.

Differences were found in the numbers of OVA-transgenic cells in mesenteric lymph nodes (data not shown). The lack of efficient accumulation of D011.10 cells in the tolerized mice may reflect a defect in their capacity to proliferate. To examine this possibility, spleen cells from these mice were cultured in vitro with OVA, and the proliferative response per transgenic T cell was estimated. Our results revealed a substantial decrease in the proliferative potential of D011.10 cells recovered from mice preexposed to i.n. OVA (Fig. 7B). The ability of the tolerized T cells to secrete cytokines was also reduced (data not shown). Similar results were obtained when these cultures were performed using purified CD4+ T cells from the spleens of tolerized mice (data not shown).

The restricted clonal expansion observed upon restimulation in the i.n. tolerized mice may be due to dysregulated TCR-mediated signaling. Alternatively, this may be subject to the influence of inhibitory mechanisms mediated by regulatory cells derived from the host. To investigate whether i.n. protein Ag leads to active suppression, D011.10 cells were transferred into mice preexposed to i.n. OVA or PBS, which were then challenged i.p. with OVA in alum. In both PBS- and OVA-pretreated mice the KJ1-26+, CD4+ T cells expanded equally well, because the numbers of OVA-transgenic cells detected in the lymph nodes and spleen were similar (Fig. 8), suggesting that a suppressive environment was not created. Moreover, adoptive transfer of bronchial lymph node and spleen cells or purified CD4+ T cells (up to 20 × 10^6 cells) from i.n. tolerized mice (wild type, as well as chimeric) into naive BALB/c recipients failed to confer a state of OVA-specific hyporesponsiveness to subsequent immunization analogous to that of the original donors (data not shown).

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are expressed as the mean counts per minute (±SD) of triplicate cultures. Observations were made for splenic T cells from mice treated before rechallenge with anti-CTLA-4 and i.n. OVA or i.n. OVA alone (Fig. 9C).

Discussion
The inhalation of nonpathogenic environmental Ags has opposing immunological outcomes in individuals who are susceptible to respiratory allergy and those who are not. In the latter, inhaled Ag does not prime productive immunity, but, on the contrary, induces a state of specific nonresponsiveness. In this study we report that intranasal exposure to protein Ag, in this instance OVA, disables the Ag-specific CD4+ T cells and renders them refractory to subsequent systemic immunization. The development of unresponsiveness is an active process that is dependent upon costimulation through the CD86 molecule.

Several independent groups have reported that IgE Ab induction to an immunogenic challenge is inhibited by prior exposure to aerosolized OVA, whereas specific IgG1 and IgG2a responses remain intact (3, 20). Much attention has focused on the role of CD8+ T cells in both the regulation of Th2 responses and peripheral tolerance to inhaled Ags. However, the experimental evidence has been controversial. Initial studies in rodents led to the hypothesis that immunologic homeostasis to inhaled proteins is mediated by a population of Ag-specific CD8+ T cells secreting IFN-γ (1, 2, 21). In contrast, in other models of Aeroallergen sensitization the activated CD8+ T cells participated in the development of airway inflammation and hyper-responsiveness (8, 9). In our study the failure to prevent the induction of i.n. tolerance when CD8+ T cells were deleted in vivo, by Ab treatment, suggests that they do not contribute to the development of the unresponsive state. Similarly, it was recently shown that administration of nebulized OVA to CD8+ and γδ-deficient mice reduced IgE responses and blood eosinophilia to subsequent challenges to the same degree as in normal wild-type mice (3). Holt and co-workers have suggested that the down-regulation of Th2 immunity following exposure to inhaled Ags is due to immune deviation toward the Th1 pathway as a result of enhanced production of IFN-γ (1, 7, 20). However, this was not confirmed in our model, because the dose-dependent inhibition of T cell expansion in the i.n. tolerized mice was associated with the reduction of both Th1- and Th2-type cytokine secretion. Moreover, we did not detect TGF-β or IL-10 secretion by T cells from the spleens or lymph nodes of OVA-unresponsive mice. The lack of a substantial role for these immunosuppressive cytokines was further confirmed by the finding that i.n. tolerance induction was not abrogated by the in vivo administration of neutralizing Abs specific for either TGF-β or IL-10. Inhibitory cytokines are involved in the regulation of immune responses in gut mucosa. It has been reported that oral administration of low doses of protein Ag leads to tolerance mediated by T cell-derived TGF-β and Th2 cytokines, while feeding high doses of Ag promotes deletion or anergy (4–6). Nevertheless, oral tolerance could be generated in TGF-β knockout mice regardless of the amount of the ingested Ag, indicating redundancy and that multiple mechanisms of tolerance may coexist (22). In general, much lower levels of Ag exposure are sufficient to initiate a tolerogenic response in the lungs compared with the gut. This may be due to differences in the local microenvironment in which the immune system first encounters the Ag and may differentially influence the mechanisms employed for the maintenance of nonresponsiveness to innocuous Ags.

To establish whether the CD4+ T cell functions were affected by prior exposure to inhaled protein Ags, we followed the fate of a small population of OVA-specific TCR transgenic CD4+ T cells in mice treated with i.n. OVA. We observed that exposure to i.n. Ag resulted in activation and initial expansion of the specific CD4+ T cells both in the draining lymph nodes and systemically. Systemic activation takes place as early as 6 h after ingestion, indicating that oral Ag, intact or degraded, enters the bloodstream and is rapidly distributed to secondary lymphoid organs (23). It is possible that ingested proteins also enter the blood. OVA-specific CD4+ T cells isolated from the peripheral lymph nodes 2 days after the i.n. treatment were capable of secreting IFN-γ and IL-4 when restimulated. Many recent reports support the idea that activation before the loss of responsiveness is a necessary step for T cell tolerance induction (13, 24, 25). In our model the number of OVA-specific CD4+ T cells initially expanded in the tolerant mice, declined rapidly with time, presumably due to apoptosis. Nevertheless, a population of cells with an activated phenotype persisted, but was refractory to further antigenic stimulation both in vitro and in vivo. It has been proposed that the presence of inflammatory stimuli at the time of priming is the deterministic factor for the functional outcome of Ag-T cell interaction. Inflammatory cytokines, such as...
TNF-α and IL-1, can rescue CD4+ T cells from deletion after exposure to superantigens or soluble peptide and promote the acquisition of an effector phenotype (17, 26). Costimulation alone is insufficient to abrogate tolerance in the absence of inflammation, and this is further illustrated by the observation that tolerance can be induced in transgenic mice overexpressing CD80 or CD86 on their APCs (17, 27). We also have found that coadministration of OVA with cytokines i.n. prevents the induction of nonresponsiveness to further antigenic challenges (D. C. Tsitoura, unpublished observations). It is possible that in atopic individuals a genetic predisposition for enhanced secretion of IL-4 contributes to the breaking of mucosal tolerance and the development of Th2 immunity. Proinflammatory molecules, such as IL-6, IL-8, and GM-CSF, present in the airways of atopic subjects (28, 29) may facilitate this process.

It is not clear why a population of unresponsive CD4+ T cells survives deletion. We noted, however, that these cells could not maintain the state of peripheral tolerance for periods longer than 3 mo. This may simply reflect that in the absence of Ag the frequency of tolerant T cells decreases with time, while the number of naïve, potentially reactive T cells increases. Alternatively, it is possible that tolerance is reversible. Similar to our findings Pape and colleagues recently reported that an unresponsive population of Ag-specific CD4+ T cells with an intrinsic defect in cytokine production is induced by i.v. injection of a high dose OVA peptide (25). The cells could retain their unresponsive status only as long as some peptide persisted in vivo, implying that some TCR-transduced signals are required to maintain tolerance. It has been suggested that in the absence of inflammation TCR signaling is perturbed, leading eventually to abortive cell differentiation with defective transcription of growth factor and other lymphokine genes (30, 31). Production of repressor proteins, within the tolerized cells, that are responsible for the unresponsive state has also been postulated (32).

The fact we did not observe bystander suppression, immunosuppressive cytokines, or transfer tolerance into naïve mice suggests that regulatory cells are not induced in our model. Indeed, not all forms of peripheral tolerance occur due to suppression (33). Regulatory T cells with a suppressor phenotype have been shown to be involved in protection from autoimmunity (34). How these cells develop in the periphery and what their exact phenotype is remain to be determined. They may appear early during thymic development and constitute specialized subsets of the T cell repertoire that mainly function to maintain homeostasis against self Ags (35, 36). Alternatively, the localization, amount, and duration of availability of the Ag may regulate their selection and expansion in the periphery (37). Brief exposure of T cells to exogenous, innocuous Ags may lead to transient immune responses, while Ag persistence in the lymphoid tissues may be necessary for the generation of protective regulatory cells. Furthermore, there is evidence that repeated exposure of CD4+ T cells to inhibitory cytokines, such as IL-10, can generate a suppressor phenotype associated with reduced capacity of the cells to expand (38).

In vitro studies with cell lines led to the conclusion that T cell tolerance arises as a result of TCR activation in the absence of costimulation (10, 11). However, recent experimental evidence suggests that this concept may not be applicable in vivo. B7 antagonists, such as CTLA-4-Ig, do not promote peptide-induced energy, but keep the T cells in a naïve, functionally competent state (12). It was proposed that tolerance may arise from engagement of B7 molecules by the CTLA-4 receptor, instead of CD80, and not from lack of costimulation (12). The initial activation of CD4+ T cells following i.n. delivery of OVA implies that costimulatory signals derived from APCs are at least partially intact during the primary encounter with Ag. Potentially altered kinetics or dysregulation of the expression of CTLA-4 and the ligands, CD80 and CD86, may contribute to the development of i.n. tolerance. However, the in vivo administration of anti-CTLA-4 or anti-CD80 Abs neither enhanced nor diminished the inhibitory effects of the OVA protein in our system, whereas an anti-CD86 Ab abrogated tolerance completely. Distinct inhibitory effects of anti-CD80 and -CD86 Abs have been described for T cell responses to both nominal and allo-Ags (39–41). Signaling through CD86 is associated with Th2 differentiation (39, 40), and inhibition of this costimulatory pathway has been shown to result in decreased expression of IL-4, IL-5, and IL-13 genes (42, 43). In the respiratory tract, allergen-induced Th2-type inflammation and airway hyper-responsiveness have been found to be susceptible to selective blockade of CD86-mediated, but not CD80-mediated, costimulation in animal models (16) as well as in patients with atopic asthma (44). CD86, in contrast to CD80, is constitutively expressed on mature dendritic cells, macrophages, and resting B cells and can, therefore, mediate some costimulatory signals to T cells even in the absence of an inflammatory environment (45, 46). Thus, it is possible that the selective involvement of CD86 costimulation in the induction of i.n. tolerance is due to its prominent role in the initiation of immune responses. Furthermore, CD86 might be implicated in the early, albeit transient in the case of i.n. tolerance, synthesis of Th2-type cytokines that characterizes mucosal immunity. Blocking the interaction of B7 with CTLA-4 did not prevent the induction of unresponsiveness in our system. However, CTLA-4-mediated signaling appeared to negatively control the initial expansion of CD4+ T cells following i.n. exposure to OVA, because we found that the number of OVA-specific CD4+ T cells in the bronchial lymph nodes was markedly enhanced when anti-CTLA-4 Ab was also administered (D. C. Tsitoura, unpublished observations). In contrast, it has been reported that CTLA-4 signaling may be involved in the induction of high dose oral tolerance (47).

In conclusion, our results suggest that i.n. administration of protein Ags prevents the induction of Th2 immunity to subsequent immunogenic challenges. The lack of responsiveness is associated with an early CD86-dependent activation of specific CD4+ T cells, a proportion of which survive deletion. There is no evidence for CD8+ T cells, immunosuppressive cytokines, or immune deviance being the mechanisms leading to tolerance in this system. It is likely that unresponsiveness arises from the failure of CD4+ T cells to develop normal effector function in the tolerant mice. The initial phase of activation has many similarities with allergic respiratory responses. However, in contrast to the situation in health, in allergic individuals the inflammatory response persists, and tolerance is not generated. The functional outcome of immunity or tolerance in response to inhaled Ags can be influenced by both genetic and environmental factors that remain to be defined. The information reported in this study is relevant to understanding the regulation of Th2 immunity to aeroallergens and may be of practical value in the advancement of immunotherapy.

References