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Respiratory Tolerance Is Inhibited by the Administration of Corticosteroids

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Corticosteroids constitute the most effective current anti-inflammatory therapy for acute and chronic forms of allergic diseases and asthma. Corticosteroids are highly effective in inhibiting the effector function of Th2 cells, eosinophils, and epithelial cells. However, treatment with corticosteroids may also limit beneficial T cell responses, including respiratory tolerance and the development of regulatory T cells (TReg), which actively suppress inflammation in allergic diseases. To examine this possibility, we investigated the effects of corticosteroid administration on the development of respiratory tolerance. Respiratory exposure to Ag-induced T cell tolerance and prevented the subsequent development of allergen-induced airway hyperreactivity. However, treatment with dexamethasone during the delivery of respiratory Ag prevented tolerance, such that allergen sensitization and severe airway hyperreactivity subsequently occurred. Treatment with dexamethasone during respiratory exposure to allergen eliminated the development of IL-10-secreting dendritic cells, which was required for the induction of IL-10-producing allergen-specific TReg cells. Therefore, because allergen-specific TReg cells normally develop to prevent allergic disease and asthma, our results suggest that treatment with corticosteroids, which limit the development of TReg cells and tolerance to allergens, could enhance subsequent Th2 responses and aggravate the long-term course of allergic diseases and asthma. The Journal of Immunology, 2005, 175: 7380–7387.

S ystemic and topical corticosteroids are extremely effective as anti-inflammatory therapy for a wide variety of inflammatory disorders. For the treatment of asthma, an inflammatory disease of the lungs, the development of inhaled forms of corticosteroids has revolutionized the treatment of asthma and has emerged as a standard to which all prospective asthma therapies are compared. Indeed, by controlling local inflammation in the lung, inhaled corticosteroids have very effectively ameliorated asthma morbidity. Research in the field of corticosteroids has focused on the cellular and molecular mechanisms by which corticosteroids diminish inflammatory responses. A large number of studies have demonstrated that corticosteroids affect the immune system by modulating cytokine production in lymphocytes and altering the trafficking and function of neutrophils, eosinophils, mast cells, and endothelial cells (1, 2). In asthma, the anti-inflammatory activity of corticosteroids impairs the recruitment of eosinophils, basophils, and Th2 cells to the airways and attenuates the production of Th2 cytokines and other inflammatory mediators from both endothelial and epithelial cells (3–5). However, corticosteroids also decrease IL-12 production by APCs and dendritic cells (DCs) in both mice and humans (6–8). Because IL-12 greatly enhances IFN-γ production, corticosteroids may limit the development of Th1 responses and other responses that protect against allergic diseases and asthma in mice (9) or in humans (6, 8). Consistent with this idea, treatment of young children with corticosteroids was shown to paradoxically increase the likelihood of later developing asthma and allergy (10).

To examine other possible mechanisms by which corticosteroid therapy might exacerbate the long term course of allergic diseases and asthma, we examined the effect of corticosteroid treatment on the development of immunological tolerance. We have previously shown that respiratory tolerance is highly effective in preventing the development of airway inflammation and hyperreactivity (11) through the development of Ag-specific adaptive regulatory T cells (TReg) that express high levels of Foxp3 and the costimulatory molecule ICOS (12, 13). In this study, we show that treatment with corticosteroids prevents the protective effects of respiratory tolerance on the development of airway hyperreactivity (AHR) by inhibiting the production of IL-10 in DCs and by inhibiting the development of TReg cells. Thus, treatment with corticosteroids, while inhibiting the function of effector Th2 cells, may in the long term exacerbate Th2-polarized immune responses by blocking the development of TReg cells, thereby enhancing Th2 cell development. Therefore, because corticosteroids are so widely used in suppressing the acute symptoms of asthma and allergy, additional studies examining the effects of chronic corticosteroid use on the progression of allergic diseases and asthma are warranted.

Materials and Methods

Mice

BALB/c mice were purchased from The Jackson Laboratory, OVA-specific TCR transgenic DO11.10 (Rag2+/–) breeder mice were provided by Dr.

1 Abbreviations used in this paper: DC, dendritic cell; TReg, regulatory T cell; AHR, airway hyperreactivity.
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For induction of tolerance, mice lightly anesthetized with methoxyflu- 
rane received intranasal OVA (100 μg) (Wortington Biochemical; 
chromatographically purified) on three consecutive days (day 0, day 1, day 2).

In some groups, mice were treated with dexamethasone i.p. (100 μg each on 
day −1 and day 1; ICN Pharmaceuticals). Dexamethasone was 
dissolved in PBS. Control mice received intranasal PBS. In some experi- 
ments, the mice were challenged 10 days later i.p. with 50 μg of OVA in 2 ml 
of aluminum hydroxide (alum), in a volume of 0.5 ml. Mice intended to 
undergo measurement of AHR were challenged with OVA intranasally (50 
μg/ml) 8 days later to induce AHR. The Stanford University Commit- 
tee on Animal Welfare (administration panel of laboratory animal care) 
approved all animal protocols used in this study.

In vitro proliferation assays

Lymph node or spleen cells were harvested and passed through a nylon 
mesh, and stimulated in round-bottom 96-well plates (5 × 10⁶ cells/well) 
with or without OVA in 0.2 ml of complete DMEM. For measurement of 
cell proliferation, the cultures were pulsed after 72 h with 0.25 μCi 
[^3H]Hydride for 16 h, and the incorporated radioactivity was measured in 
a Betaplate scintillation counter (MicroBeta TriLux; Wallac).

Isolation, purification, and adoptive transfer of cells

DCs were isolated by digestion of fragments of spleens with a mixture of 
0.1% DNase I (fraction IX; Sigma-Aldrich) and 1.6 mg/ml collagenase 
(CLS-4; Wortington Biochemical) at 37 °C for 1 h, then dissociation for 10 
min with EDTA (10 mM). Positive selection of DCs from lymph nodes was 
performed as described (11). Purification of cells was performed using 
AutoMACS (Miltenyi Biotec), according to the manufacturer’s instruc-
tions (purity >96% by flow cytometry), and cells were injected i.v. into 
BALB/c recipients (1 × 10⁶/mouse).

Culture of cells

DO11.10 cells were harvested from the spleens of DO11.10 Rag−/− mice and 
labelled with CFSE (Molecular Probes) as described (14). For assay of 
regulatory activity, mice were challenged 10 days later i.p. with 50 μg of OVA in 2 ml 
of aluminum hydroxide (alum), in a volume of 0.5 ml. Mice intended to 
undergo measurement of AHR were challenged with OVA intranasally (50 
μg/ml) 8 days later to induce AHR. The Stanford University Commit-
tee on Animal Welfare (administration panel of laboratory animal care) 
approved all animal protocols used in this study. 

Quantification of IFN-γ

Cytokine ELISAs

To distinguish the effects of corticosteroids on T cells versus DCs, 
we examined the function of purified DCs from corticosteroid-
treated mice. We showed previously that respiratory tolerance de-
creases on myeloid CD8α− DCs, and that these DCs, upon adoptive 
transfer, can transfer the Ag-specific tolerance (11). The effect of 
dexamethasone in the inhibition of intranasal tolerance was eval-
uated by adoptively transferring pulmonary DCs from mice that 
were exposed to dexamethasone during the induction of respira-
tory tolerance to OVA. As shown in Fig. 3, DCs were purified from 
unaffected mice into naive recipient mice (day 0). Recipient mice were 
immunized with OVA (100 μg) in alum (2 mg) on day 8, and spleens 
were harvested on day 15, and splenocytes were restimulated with 
OVA for 4 days. As shown in Fig. 3b, adoptive transfer of tolero-
gen DCs effectively suppressed the proliferation of T cells in vitro. 
DCs treated with dexamethasone during the induction of 
tolerance restored the proliferation of recipient T cells in vitro,
indicating that dexamethasone had suppressed the tolerogenic capacities of pulmonary DCs primed by intranasal application of OVA.

Pulmonary DCs from mice immunized with intranasal OVA expressed high levels of CD80, CD86, CD40, and ICOS-L compared with naive DC (Fig. 4a), indicating that the regulatory DCs expressed a mature phenotype. Although the levels of these markers were similar in pulmonary DCs immunized with intranasal OVA in the presence of dexamethasone, Fig. 4b shows that the tolerogenic DCs primed by intranasal application of OVA expressed high levels of IL-10 mRNA. Production of IL-10 was blocked by dexamethasone, because DCs primed in the presence of dexamethasone

**FIGURE 1.** Dexamethasone blocks the development of T cell tolerance. a, Proliferation of T cells. BALB/c mice were treated intranasally with OVA (100 μg × 3) on days 0, 1, and 2 in the absence or presence of dexamethasone (100 μg i.p. each on days −1 and 1). Positive controls received neither intranasal OVA nor dexamethasone. On day 10, all mice were immunized i.p. with OVA (50 μg) in alum (2 mg), and spleens were harvested 8 days later (day 18). Splenocytes were restimulated in vitro (5 × 10^5 per well) with indicated doses of OVA for 48 h, then pulsed with [3H]thymidine for 18 h. Incorporation of thymidine was assessed. Data are mean ± SD of triplicate cultures and are representative of four experiments, four to five mice per group. b, Development of AHR. Treatment of mice is as shown in a. On day 18, mice were challenged with OVA (50 μg × 3) intranasally, and AHR was assessed 24 h later (day 19). Values are means of five to seven mice per group ± SD, and are representative of three experiments.

**FIGURE 2.** Secretion of cytokines in peribronchial lymph nodes. Mice were treated as described in Fig. 1b. On day 20, lymphocytes were isolated from peribronchial lymph nodes and restimulated in vitro for 4 days with indicated amounts of OVA. Cumulative levels of cytokines in the culture supernatants were determined by ELISA. The data were generated from pooled lymph node cell cultures of five to seven mice. One representative experiment of three is shown.
produced much lower levels of this cytokines. Production of IL-12, TNF-α, or IFN-α remained largely unaltered by addition of corticosteroids.

Dexamethasone inhibits the induction of TReg cells

We previously showed that exposure of mice to intranasal OVA induced T cell tolerance, mediated by pulmonary DCs, which transiently produced IL-10, and expressed high levels of the costimulatory molecules B7-1 and B7-2 (11). As demonstrated previously (12), culture of these IL-10-producing DCs with naive CD4+ T cells from DO11.10 OVA-specific TCR transgenic mice induced T cell production of IL-10, some IL-4, but not IFN-γ (Fig. 5a, thick lines). These data indicate that dexamethasone treatment of mice during the generation of regulatory DCs in vivo inhibited the development of T cells with a cytokine profile of TRegs (high amounts of IL-10, but low levels of IL-4 and IFN-γ (22, 23)).

To analyze the suppressive capacity of pulmonary DCs, we examined their effects on naive DO11.10 T cells. As shown in Fig. 5b, naive DO11.10 cells cultured in vitro with DCs primed by OVA/alum and labeled with CFSE proliferated vigorously in response to DCs plus OVA, completing several rounds of cell division over 48 h. In contrast, use of DCs primed by OVA intranasally (Fig. 5b) inhibited the proliferation of the CFSE-labeled cells,
such that the majority of the input T cells remained in a nondividing state. As shown previously, this reflects the tolerogenic potential of pulmonary DCs primed by OVA intranasally (12). Such DCs were isolated by MACS from bronchial lymph nodes of BALB/c mice 24 h after administration of intranasal OVA in the presence or absence of dexamethasone or after intradermal OVA in alum (open histograms). The filled histograms show staining of isotype control Ab. B7-1, B7-2, CD40, and ICOS-L expression by DCs were increased to comparable amounts in all groups (one representative experiment of three is shown). b. Expression of IL-10 in pulmonary DCs. DCs were isolated as shown in a, lysed, and mRNA was isolated, and real-time PCR was performed. The bars show relative amounts of cytokine message compared with naive, unstimulated DCs (relative differences in CT values, ΔΔCT) (one representative experiment of two is shown). *, p < 0.005 vs OVA intranasal.

Discussion
In the present study, we showed that treatment with corticosteroids prevents the protective effects of respiratory tolerance on the development of AHR. Respiratory tolerance is highly effective in preventing airway inflammation, and is thought to limit immune responses against the large quantities of innocuous Ags that enter the lungs suspended in inspired air (11, 24–27). We found that treatment with dexamethasone prevented the development of respiratory tolerance, thereby allowing Ag-specific T cell proliferation and cytokine secretion as well as AHR to develop. Treatment with dexamethasone also abolished IL-10 production in DCs from the lungs of tolerized mice, and eliminated the capacity of these DCs to transfer tolerance and to induce the development of Ag-specific TReg cells. Thus, although corticosteroids can reduce acute inflammation in allergy (4), they may also hinder the development of respiratory tolerance, an immune response that down-regulates Th2-driven allergic pulmonary inflammation.
Corticosteroids are known to be very effective in the treatment of inflammatory diseases, because corticosteroids potently reduce cytokine production and the function of critical effector cells in many diseases. In asthma, corticosteroids inhibit acute allergic inflammation and improve airway hyperresponsiveness in both mice and humans (5), by limiting cytokine production in T cells and epithelial cells and impairing the recruitment and growth of eosinophils and other inflammatory cells (4, 28). By all measures, corticosteroids, delivered either systemically or locally in the respiratory tract by the inhaled route, is the pharmaceutical of choice for both acute and chronic asthma (29, 30).

However, although the anti-inflammatory effects of corticosteroids have been amply documented both clinically and experimentally, corticosteroid therapy has limitations. For example, despite widespread use of corticosteroids, the prevalence of asthma and allergic diseases has increased dramatically over the past two decades. Corticosteroids do not alter the underlying disease processes, and when treatment is discontinued, symptoms rapidly return (31). In addition, investigators recently showed that administration of corticosteroids during allergen exposure did not prevent allergen sensitization, but in fact enhanced sensitization (9). In these experiments, which used an unusual sensitization method with adenovirus expressing GM-CSF as adjuvant, corticosteroid treatment increased OVA-specific IgE and Th2 cytokine production by cultured splenocytes while reducing IFN-γ synthesis.

Corticosteroids might enhance Th2 allergic sensitization by several mechanisms. First, corticosteroids have been shown to potentiate IL-4-induced polyclonal IgE synthesis by peripheral blood B cells in vitro (32–36). In vivo, corticosteroid treatment has been shown to increase serum IgE levels transiently (37, 38). In addition, corticosteroid therapy has been shown to potenti ate Th2 differentiation, by either reducing IL-12 secretion from APC (6–8, 39–43) or by directly suppressing Th1 cell polarization (44). Corticosteroids do not appear to diminish the expression of costimulatory molecules on DCs (8), allowing corticosteroid-treated DCs to present Ag to T cells and enhance immune deviation toward Th2 differentiation. Moreover, our results demonstrate an additional mechanism by which corticosteroid therapy might enhance allergen sensitization, and that is by inhibiting the development of respiratory tolerance and TReg cell development (11), was abolished by corticosteroid treatment. Furthermore, DCs from corticosteroid-treated mice were unable to transfer respiratory tolerance and were unable to induce the development of TReg cells. We found that DC cell production of IL-10, which has been shown to be critical in induction of respiratory tolerance and TReg cell development (11), was abolished by corticosteroid treatment. The effects might be mediated through several mechanisms by which corticosteroids have been shown to function, for example by increasing IgE synthesis (45), inhibited histone acetyltransferases activity or by recruiting histone deacetylases to the activated inflammatory gene complex (46). Our results suggest that TReg cell development is more sensitive to corticosteroid therapy than Th2 cell development (47), resulting in enhanced Th2 cell but reduced TReg Cell development following corticosteroid treatment.

In our studies, we analyzed the immunological effects of corticosteroids by examining DCs purified from corticosteroid-treated mice, isolated away from the T cells from the treated mice. This allowed us to separate the effects of corticosteroids on distinct cell types, in particular because corticosteroids have direct effects on APCs, effector T cells, and on the development of TReg cells. For
example, the presence of corticosteroids and vitamin D3 has been shown to directly induce the development of T<sub>Reg</sub> cells that produce IL-10 (48). The T<sub>Reg</sub> cells induced with corticosteroids do not express Foxp3, but have potent inhibitory activity in models of colitis (49). In human T cells, the presence of corticosteroids enhanced Foxp3 expression, but only by 2-fold (50), and enhanced IL-10 production, but also only by 2-fold (51). The increases in Foxp3 and IL-10 expression by corticosteroids were accompanied by only a modest increase in the suppressor activity of the human T<sub>Reg</sub> cells. In contrast, in our model corticosteroid therapy completely prevented the development of respiratory tolerance, which allowed the subsequent development of allergen-induced AHR to occur. Moreover, our data examining the effects of corticosteroids on purified DCs indicate that corticosteroids have major effects on the function of DCs involved in the induction of respiratory tolerance and Ag-specific T<sub>Reg</sub> cells. Corticosteroid treatment abolished the capacity of DCs to transfer tolerance, and greatly reduced IL-10 production and the ability of DCs to induce T<sub>Reg</sub> cells that inhibit allergen-specific T cell proliferation. These results together suggest that corticosteroids indeed have potent effects in enhancing Th2 responses by abolishing the function of DCs producing IL-10, thereby preventing the development of respiratory tolerance.

Finally, our studies have very important implications regarding the use of corticosteroids in the treatment of allergic inflammatory diseases in humans. Although corticosteroids have clear anti-inflammatory effects and are valuable in limiting the function of Th2 cells in asthma, the inhibitory effects of corticosteroids on T<sub>Reg</sub> cell development may impede insidious side effects that may inexcorably enhance the severity of subsequent immune responses that occur on re-exposure to allergen. Allergen-specific T<sub>Reg</sub> cells are thought to be present in higher frequency in non-allergic than in allergic individuals, to be responsible for down-modulating allergic responses and asthma (27), and appear to be induced with allergen immunotherapy (52, 53). Therefore, inhibition of T<sub>Reg</sub> cell development by corticosteroids would block these normal regulatory mechanisms that control allergy and asthma. Although in humans, corticosteroids are administered both systemically (orally and i.v.) and topically (generally by inhalation), whereas in our study, we administered dexamethasone i.p., we suggest that both systemic and topical (inhaled) corticosteroids have significant effects on respiratory DCs and on respiratory tolerance. Because corticosteroids may both enhance Th2 sensitization (6, 7, 9, 35) and limit T<sub>Reg</sub> cell development, subsequent exposure to allergens that occur in the absence of T<sub>Reg</sub> cells would result in more robust Th2 responses, as might occur when young children are treated with corticosteroids (10). We speculate therefore that as the use of corticosteroids has increased greatly over the past 20 years in industrialized countries, chronic corticosteroid therapy may have contributed to the great increase in prevalence of atopic diseases (asthma, allergic rhinitis, atopic dermatitis, and food allergy) that has occurred over the past two decades (54). However, because corticosteroids are the most effective current therapy that inhibits acute allergic inflammation and prevents late phase allergic reactions, corticosteroids should continue to be used as treatment for acute allergic reactions, asthma, and anaphylaxis, but at the lowest possible doses and for the shortest periods of time.

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Disclosures
The authors have no financial conflict of interest.
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