

Fluticasone propionate increases CD4⁺CD25⁺ T regulatory cell suppression of allergen-stimulated CD4⁺CD25⁻ T cells by an IL-10-dependent mechanism

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Background: Corticosteroids are the most effective anti-inflammatory therapy for allergic diseases, and these drugs inhibit T_H2 T-cell activation. We previously reported that CD4⁺CD25⁺ T cells from atopic donors suppressed allergen-stimulated T cells less than those from nonatopic donors.

Objective: We sought to determine the effect of fluticasone propionate (FP) on allergen-stimulated CD4⁺CD25⁻ T cells and on the suppressive ability of CD4⁺CD25⁺ T cells.

Methods: CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were separated from peripheral blood of atopic and nonatopic volunteers and cultured alone or mixed in the presence of allergen. Effects of FP were assessed by means of addition to cultures or preincubation with CD4⁺CD25⁺ T cells.

Results: FP inhibited allergen-stimulated proliferation of CD4⁺CD25⁻ T cells in a dose-dependent manner. Preincubation of CD4⁺CD25⁺ T cells in FP increased subsequent suppressive activity of these cells in allergen-stimulated cultures with CD4⁺CD25⁻ T cells. This effect was seen when cells were obtained from both nonatopic and atopic donors but was less for cells obtained from atopic individuals. Prior exposure of CD4⁺CD25⁺ T cells to FP also increased subsequent IL-10 production by these cells when stimulated with allergen, and addition of anti-IL-10 antibody reversed the steroid-induced enhancement of suppression in mixed cultures.

Conclusion: Increased suppression by CD4⁺CD25⁺ T cells might play a role in anti-inflammatory effects of corticosteroids in asthma and allergic diseases. (*J Allergy Clin Immunol* 2004;114:296-301.)

Key words: Corticosteroids, allergy, T lymphocytes, IL-10

Abbreviations used

FP: Fluticasone propionate

SX: Salmeterol xinafoate

Activation of CD4⁺ T lymphocytes of a T_H2 cytokine profile is implicated in asthma and other allergic diseases.¹ IL-4 and IL-13, unopposed by IFN-γ, act in B-cell switching to IgE production; IL-5 and IL-9 drive eosinophil development and tissue survival; and IL-9 and IL-13 cause mucus hypersecretion.^{1,2} Increased expression of these cytokines is reported in the airway in asthma,³ rhinitis,⁴ and allergic skin⁵ and eye⁶ disease, and intervention studies in mouse models suggest they play important roles in driving pathophysiology.⁷ Suppression of T_H2 cell activation is therefore a goal of therapy for allergic disease.

Corticosteroids are the most effective and widely used anti-inflammatory agents for asthma and allergic disease, and topical corticosteroids are recommended therapy in current guidelines for asthma, rhinitis, and allergic skin disease.⁸⁻¹⁰ These drugs act by complexing to an intracytoplasmic receptor and directly increase or repress gene transcription or interact with other transcription factors.¹¹ Corticosteroids inhibit T-cell activation and production of T_H2 cytokines *in vitro*¹¹ and have been shown to reduce expression of T_H2 cytokines in the airway mucosa in asthmatic patients *in vivo*.^{12,13}

A number of types of regulatory or suppressive T cells have been described. The CD4⁺CD25⁺ T-cell subset was shown to prevent development of autoimmune pathology in mouse models, and both mouse and human CD4⁺CD25⁺ T cells can inhibit T-cell activation to a wide variety of stimuli *in vitro*.¹⁴⁻¹⁶ We and others have recently shown that human peripheral blood CD4⁺CD25⁺ T cells can inhibit allergen-stimulated proliferation and T_H2 cytokine production of CD4⁺CD25⁻ cells from both nonatopic and atopic donors.¹⁷⁻¹⁹ The suppressive activity of these regulatory T cells *in vitro* was less when cells were obtained from atopic donors than from nonatopic individuals, especially when the atopic subjects had active disease.¹⁷ This led us to suggest that increasing activity of regulatory T cells would be beneficial in control of asthma and allergic disease.

IL-10 was initially described as a T_H2 cell product that inhibited T_H1 cytokine synthesis but is now shown to be

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produced by both T_H1 and T_H2 cells in addition to antigen-presenting cells and a variety of other cell types.²⁰ Activation of human or mouse CD4 or CD8 T cells in the presence of corticosteroids led to a cell type that produced high concentrations of IL-10 and reduced T_H1 or T_H2 cytokines on restimulation.²¹⁻²³ Addition of vitamin D3 with corticosteroids generated IL-10-producing regulatory T cells.²⁴

Thus corticosteroids can promote a regulatory T-cell subtype, at least *in vitro*. However, there is no information on the effects of corticosteroids on the suppressive activity of CD4⁺CD25⁺ T cells. If future therapy aimed at boosting regulation by CD4⁺CD25⁺ T cells is to be developed for allergic disease, it will likely be used in conjunction with corticosteroids. We hypothesized that corticosteroids would increase the suppressive activity of human peripheral blood CD4⁺CD25⁺ T cells. To test this hypothesis, we therefore examined the effects of the corticosteroid fluticasone propionate (FP) on CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in allergen-stimulated *in vitro* cultures.

METHODS

Volunteers were recruited from among the staff of Imperial College London and by advertisement. The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals NHS Trust. Volunteers were assessed by questionnaire on allergic symptoms and skin prick tests (ALK, Horsholm, Denmark) and assigned to atopic (positive history and at least one positive skin prick test result of 3 mm or larger in diameter than that elicited by the negative control) or nonatopic (no history of allergic symptoms and negative skin prick test responses) groups.

Two hundred milliliters of blood was drawn, and PBMCs were separated by means of centrifugation in Histopaque (Sigma Chemical, Poole, United Kingdom). CD4⁺ and CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were then separated by means of negative and positive immunomagnetic selection, respectively by using MACS (Miltenyi Biotech, Bisley, United Kingdom), as previously described.¹⁷ CD4⁺ isolation provided 95% to 97% purity, and CD4⁺CD25⁺ T cells provided 75% to 90% purity. Cells were cultured at a density of 2×10^6 /mL in triplicates in 96-well flat-bottomed plates in RPMI 1640 supplemented with penicillin and streptomycin and 5% human AB serum (Sigma) with 100 μ g/mL cat or grass pollen allergen extracts (depending on which elicited a positive skin test response in the donors, ALK) for 6 days. Cultures were of PBMCs or CD4⁺CD25⁻ cells with added CD4⁺CD25⁺ or CD4⁺CD25⁻ cells in a ratio of 2:1, as previously described.¹⁷ In all cultures of isolated T cells, irradiated PBMCs were added as antigen-presenting cells (2×10^5 cells/well). In certain cultures FP (GlaxoSmithKline, Greenford, United Kingdom) was added at various concentrations, either alone or with 10^{-8} mol/L salmeterol xinafoate (SX; GlaxoSmithKline). The FP was dissolved in DMSO (to 10^{-1} mol/L, Sigma) and then diluted in medium, whereas SX dissolved in methanol (to 10^{-1} mol/L, Sigma) was then diluted in medium, and these were added to the medium control as appropriate. For preincubation experiments, separated CD4⁺CD25⁺ cells were placed in wells with medium alone or medium containing 10^{-7} mol/L FP and left unstimulated in a 37°C, 5% CO₂ incubator overnight. CD4⁺CD25⁻ T cells were similarly kept overnight in medium alone. After overnight incubation, cells were washed to minimize any carryover of drug to the cocultures, and then cells were set up in mixed cultures stimulated with allergen extracts as above but at a ratio

of 4 CD4⁺CD25⁻ T cells to 1 CD4⁺CD25⁺ T cell. This 4:1 ratio was used here for 2 reasons: to provide conditions of submaximal suppression to allow demonstration of an increase after preincubation in FP and for the practical reason that insufficient CD4⁺CD25⁺ T cells could be isolated to allow a 2:1 ratio with and without drug. In all cultures, proliferation was assessed by means of addition of 18.5 kBq of tritiated thymidine for the last 8 hours of culture, followed by cell harvest and liquid scintillation counting. Before addition of tritiated thymidine, 100 μ L of supernatant was taken and frozen for subsequent analysis of IL-10 by means of cytometric bead array (Becton Dickinson, Oxford, United Kingdom). Anti-IL-10 antibody (10 μ g/mL) or isotype control (R&D systems, Abingdon, United Kingdom) was added to block IL-10 in some cultures.

Initially, a dose-response analysis was performed for the effect of FP on CD4⁺CD25⁻ T cells cultured with allergen extract. Data from 6 separate donors were expressed as means and SEs because these data were normally distributed.

The hypothesis that corticosteroids increase suppressive activity of CD4⁺CD25⁺ regulatory T cells was examined by comparing proliferative responses (as counts per minute) from triplicate wells in allergen-stimulated *in vitro* assays. Data were compared between cultures of cells with either medium (with vehicle) or FP alone or the combination of FP with SX. Because data were not normally distributed, nonparametric statistical analysis was used: either the Wilcoxon paired test for comparison of cell cultures from the same donors with and without drug or the Mann-Whitney *U* test for comparison of treatment-induced differences between groups (Δ values for atopic vs nonatopic donors). Data were expressed as percentage suppression by expressing the reduction in counts per minute for cocultures of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells relative to counts per minute for cultures of CD4⁺CD25⁻ T cells alone to allow representation of the suppressive ability of CD4⁺CD25⁺ T cells.

RESULTS

Effect of FP on allergen-stimulated proliferation of CD4⁺CD25⁻ T cells

Inhibitory effects of corticosteroids on T-cell proliferation are well described.¹¹ However, there are no reports of inhibition of isolated CD4⁺CD25⁻ T cells. We previously showed that these CD4⁺CD25⁻ T cells, in the absence of regulatory CD4⁺CD25⁺ T cells, show increased proliferation to allergen stimulation when compared with unseparated PBMCs.¹⁷ First, we examined the effects of different concentrations of FP on allergen-stimulated proliferation of CD4⁺CD25⁻ T cells from 6 atopic subjects. Proliferation was inhibited in a dose-dependent manner with an inhibitory concentration of 50% of 8×10^{-11} mol/L (95% CI, 6×10^{-12} to 1.0×10^{-9} mol/L; Fig 1).

Effect of low-dose FP with or without SX on suppression of allergen-stimulated CD4⁺CD25⁻ T-cell proliferation by CD4⁺CD25⁺ T cells

To examine the effect of FP on suppression by CD4⁺CD25⁺ T cells, we added a concentration that did not inhibit proliferation of CD4⁺CD25⁻ T cells (because that would obscure any change in suppression). Because SX has been reported to potentiate the effects of FP in some culture systems,²⁵ we also tested the effects of

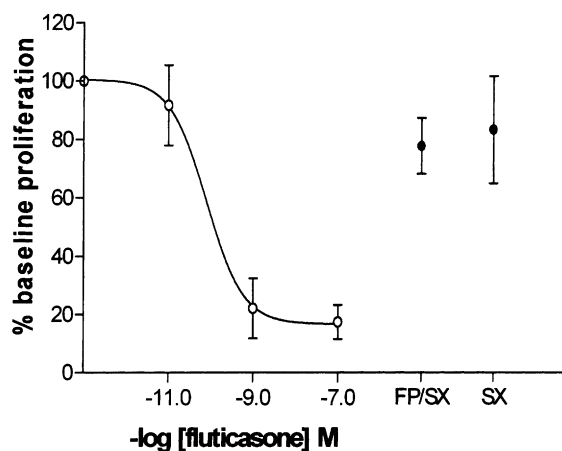


FIG 1. Dose-response effect of added FP to allergen-stimulated 6-day culture of CD4⁺CD25⁻ T cells. Data are means and SEs from 6 separate atopic donors. Counts per minute varied from 8000 to 40,000 between donors.

adding combined FP (10^{-11} mol/L) with SX (10^{-8} mol/L) in allergen-stimulated cultures of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. Neither low-dose FP nor FP plus SX had an effect on suppression of proliferation by CD4⁺CD25⁺ T cells (Fig 2). The suppression of allergen-stimulated proliferation by CD4⁺CD25⁺ T cells from atopic donors was significantly less than that seen by regulatory cells from nonatopic donors ($P = .03$), confirming our previous findings.¹⁷

Effect of preincubation of CD4⁺CD25⁺ T cells in FP on their suppressive activity: Comparison of nonatopic and atopic donors

We then asked whether higher doses of FP would inhibit or potentiate suppression by CD4⁺CD25⁺ T cells. We could not add the drug throughout the culture because inhibition of CD4⁺CD25⁻ T-cell proliferation by the steroid would obscure any suppression by CD4⁺CD25⁺ T cells. We therefore preincubated the CD4⁺CD25⁺ T cells in FP at a concentration of 10^{-7} mol/L overnight before mixing the cells with CD4⁺CD25⁻ T cells and stimulating with allergen. Preincubation with FP increased suppression by CD25⁺ T cells. This effect was seen with cells from both atopic and nonatopic donors but was significantly greater for nonatopic donors (between-group comparison, $P = .03$, Fig 3).

Effect of FP preincubation of CD4⁺CD25⁺ T cells on IL-10 production

Glucocorticoids have been reported to increase T-cell IL-10 production in a number of settings. One possible mechanism for the observed increase in suppressive activity of CD4⁺CD25⁺ on exposure to FP was therefore induction of IL-10 production. When IL-10 production was measured in allergen-stimulated cultures of CD4⁺CD25⁺ T cells pre-exposed to FP (cultures of CD4⁺CD25⁺ T cells alone), there was an increase in

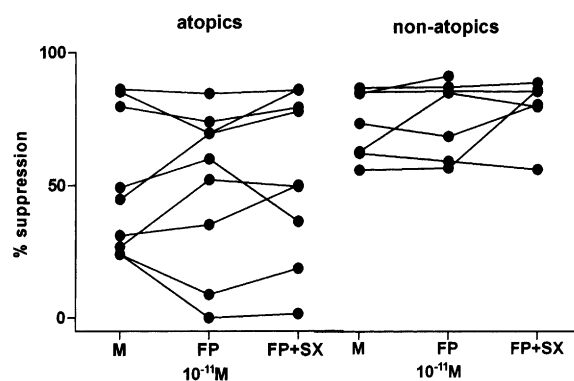


FIG 2. Effect of low-dose FP (10^{-11} mol/L) with or without SX (10^{-8} mol/L) on suppression of allergen-stimulated CD4⁺CD25⁻ T cells from atopic or nonatopic donors by autologous CD4⁺CD25⁺ T cells. M, Medium.

IL-10 in the supernatant at day 6 in cultures of cells from both atopic and nonatopic donors (Fig 4, A). There was a nonsignificant trend for an increase in IL-10 in supernatants from mixed cultures of CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells when the latter had been preincubated in FP (median increase for atopic donors was 74 pg/mL [range, 25.0-118.0 pg/mL; $n = 6$], and median increase for nonatopic donors was 17 pg/mL [range, 4.0-218.0 pg/mL; $n = 5$]). This might reflect consumption of IL-10 by CD4⁺CD25⁻ T cells in these mixed cultures. When anti-IL-10 antibody was added to cultures of cells when CD4⁺CD25⁺ T cells had been preincubated in FP, the enhancement of suppression was largely reversed (Fig 4, B).

DISCUSSION

We have recently reported that the suppressive activity of CD4⁺CD25⁺ regulatory T cells in allergen-stimulated *in vitro* cultures is diminished when cells from atopic allergic donors are compared with those from nonatopic control subjects.¹⁷ Corticosteroids are the mainstay of anti-inflammatory treatment for asthma. We now report that the corticosteroid FP inhibited allergen-stimulated proliferation of CD4⁺CD25⁻ T cells. Furthermore, preincubation with FP increased the suppressive activity of CD4⁺CD25⁺ T cells. This enhancement of suppression was seen in both atopic and nonatopic donors but was less in cells from atopic donors. The increased suppressive activity of CD4⁺CD25⁺ T cells after exposure to FP was associated with induction of IL-10 in cultures and was blocked by anti-IL-10 antibodies.

It is well established that corticosteroids inhibit T-cell activation *in vitro*,¹¹ and we and others have previously shown that corticosteroids reduce expression of T_H2 cytokines in the airway mucosa in asthmatic patients *in vivo*.^{12,13} Our hypothesis here was that at least part of this activity might be through increased suppressive activity of CD4⁺CD25⁺ regulatory T cells. However, we also considered it important to establish whether FP directly inhibited activation of allergen-stimulated CD4⁺CD25⁻

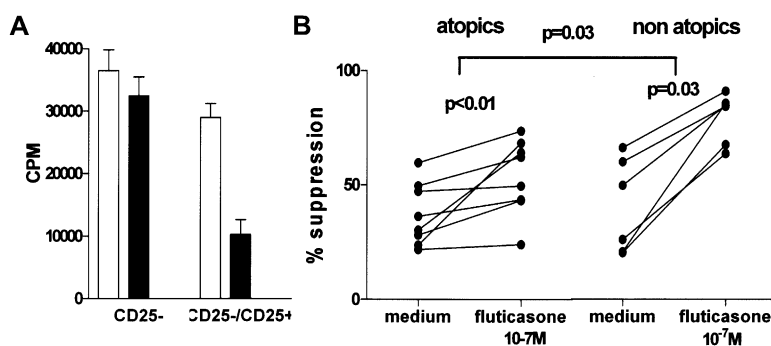


FIG 3. FP increases suppression by CD4⁺CD25⁺ T cells. **A**, An example of counts per minute with CD4⁺CD25⁺ T cells alone and CD4⁺CD25⁺ T cells mixed at a ratio of 4:1 with CD4⁺CD25⁺ T cells either preincubated in medium (open bars) or 10⁻⁷ mol/L FP (filled bars) overnight. **B**, Percentage suppression of allergen-induced CD4⁺CD25⁺ T-cell proliferation by CD4⁺CD25⁺ T cells preincubated in either medium or FP for both atopic and nonatopic donors.

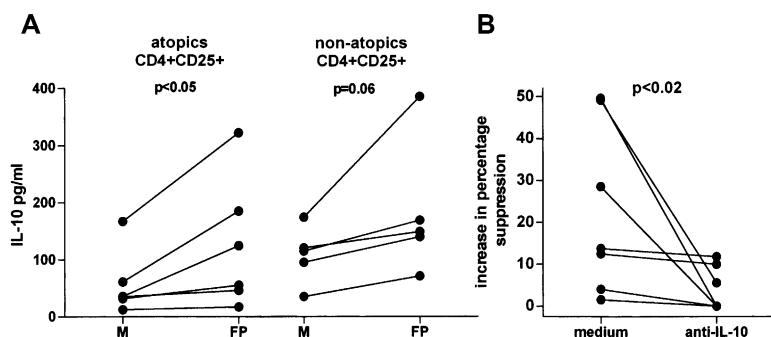


FIG 4. Preincubation of CD4⁺CD25⁺ T cells in FP increases their IL-10 production. **A**, IL-10 production by CD4⁺CD25⁺ T cells stimulated with allergen for 6 days after overnight incubation in medium (M) or FP at 10⁻⁷ mol/L. **B**, Anti-IL-10 antibody abrogates the enhanced suppression by CD4⁺CD25⁺ T cells that have been incubated in FP overnight.

T cells from atopic donors. We show here that FP inhibited activation of CD4⁺CD25⁺ T cells by means of allergen stimulation. We assume that this was the result of a direct effect on gene expression by CD4⁺CD25⁺ T cells, although we cannot exclude an indirect effect through antigen-presenting cells because these were present in the cultures. The activity of FP in inhibiting CD4⁺CD25⁺ T-cell responses to allergen meant that we could not add FP directly to mixed cocultures to test our hypothesis, except at suboptimal doses (10⁻¹¹ mol/L), which did not inhibit proliferation of the responder CD25⁺ T cells. We saw no effect of such low doses of corticosteroids and therefore went on to use preincubation experiments to determine the effect of FP on suppression by CD4⁺CD25⁺ T cells.

SX has been reported to potentiate the anti-inflammatory effects of FP both *in vivo* and in a number of *in vitro* culture systems, possibly through an effect on the glucocorticoid receptor.²⁵ Addition of long-acting β -agonists with inhaled steroids is the recommended treatment for persistent asthma symptoms, despite low-dose inhaled corticosteroid treatment.⁸ We saw no evidence of a synergistic effect of SX combined with low-dose FP in culture of mixed CD4⁺CD25⁺ and

CD4⁺CD25⁺ T cells stimulated with allergen. We were unable to test the combination of FP and SX in preincubation experiments because of limited cell numbers for culture, and therefore we cannot exclude an effect of SX and FP on CD4⁺CD25⁺ T-cell regulatory function.

IL-10 inhibits T-cell proliferation and cytokine production largely through effects on antigen-presenting cells but also through direct T-cell effects.²⁰ Previous reports have shown that corticosteroids enhance IL-10 production by both CD4⁺ and CD8⁺ T cells at low doses,²¹⁻²³ although high doses of corticosteroids inhibited LPS-stimulated IL-10 production by PBMCs and whole blood cultures.²⁶ Increased IL-10 production by alveolar macrophages has previously been reported after steroid therapy for asthma.²⁷ Corticosteroids combined with vitamin D3 could induce development of an IL-10-producing regulatory T-cell subset that is distinct from CD4⁺CD25⁺ T cells.²⁴ We found increased IL-10 production in allergen-stimulated cultures of CD4⁺CD25⁺ T cells after the cells had been preincubated with FP. Furthermore, addition of a blocking anti-IL-10 antibody reversed the FP-induced increase in suppressive activity of CD4⁺CD25⁺ T cells in allergen-stimulated cultures. Our

data suggest that IL-10 might be responsible for the increased suppression seen in mixed cultures after preincubation of CD4⁺CD25⁺ T cells with FP.

However, anti-IL-10 antibodies did not reduce suppression to less than the baseline seen with CD4⁺CD25⁺ T cells that had not been exposed to FP, suggesting that this basal suppressive activity might be IL-10 independent. This would be in agreement with previous reports showing that anti-IL-10 did not block *in vitro* suppression by CD4⁺CD25⁺ T cells from either mice or human subjects, and regulatory activity persisted in *IL10* gene-disrupted mice.¹⁴⁻¹⁷ In contrast, in a number of *in vivo* disease models, suppression by CD4⁺CD25⁺ T cells is IL-10 dependent.¹⁶

In contrast to previous descriptions of steroid-induced increase in T-cell IL-10 production²¹⁻²³ and induction of IL-10–producing regulatory T cells by means of stimulation in the presence of dexamethasone and vitamin D3,²⁴ we did not activate CD4⁺CD25⁺ T cells during preincubation with FP. The subsequent allergen-stimulated mixed cultures were performed in the absence of drugs at a ratio of 4 CD4⁺CD25[−] T cells to 1 CD4⁺CD25⁺ T cell. This ratio was used because of limited cell numbers and to give submaximal suppression and thus allow detection of enhanced regulation. It is possible that different mechanisms of suppression operate at differing ratios of suppressor/responder T cells. Certainly our blocking antibody experiments suggest a role for IL-10 in the enhanced suppression seen in the mixed culture conditions used here after preincubation of CD4⁺CD25⁺ T cells with FP.

It will be of interest to determine whether corticosteroids affect regulatory function of CD4⁺CD25⁺ regulatory T cells *in vivo*. Such studies will be difficult in human airway tissues because CD25 is expressed by activated and memory effector T cells in addition to regulatory T cells, and it is difficult to obtain sufficient cells for functional analysis. Nonetheless, CD4⁺CD25⁺ regulatory T cells have been obtained from lung tissue²⁸ and rheumatoid synovial fluid,²⁹ and therefore investigation of the effects of treatment might be possible.

We found less enhancement of suppression by FP treatment of CD4⁺CD25⁺ T cells from atopic donors than those from nonatopic donors. This is in accord with our previous finding of diminished regulation of allergen-stimulated cultures by cells from atopic subjects, which we have confirmed here.¹⁷ Whether this is because the CD4⁺CD25⁺ population from atopic donors contains less regulatory cells than those from nonatopic donors or whether this represents a fundamental defect in suppression is uncertain. It will be important to determine the steroid sensitivity of these cells from atopic and nonatopic subjects in terms of steroid binding and gene expression studies. It has previously been reported that cells from asthmatic volunteers made less IL-10 on stimulation than did those from nonatopic control subjects,²⁶ and it is possible that differences in *IL10* gene activation underlie the differential effects of corticosteroids we found on CD4⁺CD25⁺ T-cell suppression.

In conclusion, our data suggest that corticosteroids can enhance the suppressive effect of CD4⁺CD25⁺ T cells for allergen-stimulated CD4⁺CD25[−] T-cell cultures and that this effect is at least in part IL-10 dependent. Whether this is important for the anti-inflammatory effects of steroids in asthma and allergic disease and whether it can be manipulated to improve therapy will be important areas for future research.

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