

The role of CD28-B7 costimulation in allergen-induced cytokine release by bronchial mucosa from patients with moderately severe asthma

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Background: T cells play an important role in airway inflammation in asthma through the release of T_H2 cytokines. Optimal T-cell activation by antigen-presenting cells requires costimulatory signaling, such as the interaction of CD80, CD86, or both with CD28. In patients with mild allergic asthma, the fusion protein cytotoxic T-lymphocyte antigen 4Ig (CTLA-4Ig), which inhibits CD28-mediated signaling, blocks the release of IL-5 and IL-13 from bronchial explant cultures exposed to the allergen *Dermatophagoides pteronyssinus*.

Objectives: To assess costimulation in more severe forms of atopic asthma, we have compared the ability of CTLA-4Ig to block allergen-induced cytokine responses of bronchial explants and PBMCs from patients with moderately severe asthma.

Methods: Bronchial explants and PBMCs were cultured in vitro, and cytokine expression was measured by means of quantitative RT-PCR and ELISA.

Results: Constitutive mRNA transcripts for IL-5, IL-13, and GM-CSF were detected in the tissue explants, but only IL-5 mRNA increased significantly with allergen stimulation. Consistent with increased transcription, allergen-stimulated IL-5 protein release into explant supernatants, but this was not blocked by CTLA-4Ig. Allergen did not induce GM-CSF release, and IL-13 protein could not be detected in the explant supernatants under any condition. In contrast, allergen enhanced production of IL-5 and IL-13 by PBMC cultures from the same subjects, and this was inhibited effectively by CTLA-4Ig.

Conclusions: These data suggest that IL-5 production in the airways of subjects with moderately severe asthma is largely independent of CD28-mediated costimulation. The different requirements for CD28-mediated costimulation in PBMC cultures and bronchial tissue cultures emphasizes the importance of the tissue microenvironment in pulmonary inflammatory responses in severe asthma. (*J Allergy Clin Immunol* 2001;108:976-81.)

Key words: Asthma, costimulation, CTLA-4, CD28, T cell, allergen, explant, inflammation, IL

Abbreviations used

APC: Antigen-presenting cell

CTLA-4Ig: Cytotoxic T-lymphocyte antigen-4Ig

ICOS: Inducible costimulator

Atopic asthma is characterized by variable airflow obstruction, bronchial hyperresponsiveness, and airway infiltration by inflammatory cells, including activated mast cells, T_H2 -type T cells, and eosinophils.¹ T cells play an important orchestrating role in asthmatic airway inflammation by the release of T_H2 -type cytokines (IL-4, IL-5, IL-9, IL-13, and GM-CSF) encoded on chromosome 5q31-33, which promote the development, survival, activation, and recruitment of inflammatory cells to the asthmatic airways.¹ The activation of naive T cells in primary immune responses requires at least 2 signals from antigen-presenting cells (APCs): The first is provided by the cognate interaction of the T-cell receptor with MHC II complexes on APCs and the second by CD80 (B7-1) and CD86 (B7-2) on APCs interacting with CD28 receptors on T cells.²⁻⁴ Costimulation through CD28 results in T-cell activation, and ligation of its higher affinity homologue, cytotoxic T-lymphocyte antigen 4Ig (CTLA-4Ig), limits immune responses by competing with CD28 for binding with CD80 or CD86 and thus depriving the T cells of CD28-mediated activation. Lack of costimulatory signaling through CD28 has been shown to result in T-cell unresponsiveness or anergy.⁵ Recent murine studies suggest a critical role for CD28-B costimulation in the production of T_H2 cytokines, the development of airway hyperresponsiveness, and eosinophilic airway infiltration in asthma.⁶⁻⁹

CTLA-4Ig is a chimeric fusion protein of the Fc component of human IgG₁ and the extracellular component of human CTLA-4, which inhibits signaling through the CD28-B7 pathway and has been used to study the requirement for CD28-B7 costimulation in immune responses.¹⁰ CTLA-4Ig has also been shown to inhibit allergen-induced proliferation and cytokine production by PBMCs obtained from atopic donors.^{11,12}

We have previously reported that CTLA-4Ig effectively inhibits allergen-induced IL-5, IL-13, RANTES, and IL-16 release from bronchial tissue explants of subjects

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with mild atopic asthma stimulated with allergen in vitro,^{13,14} indicating a requirement for CD28-B7 costimulation for production of these proinflammatory mediators. Because responses in more severe persistent asthma have not been studied, we have performed explant cultures using bronchial biopsy specimens from patients with moderately severe asthma. Because costimulatory requirements may be specific for inflamed tissue, we compared the requirement for CD28-B7 costimulation in allergen-induced cytokine production in explants with that in PBMCs from the same patients.

METHODS

Subjects

Fifteen persistently symptomatic asthmatic subjects (9 men and 6 women) with a mean $\pm$ SEM age of 33 ± 2.4 years were recruited for bronchoscopy. The group had a mean FEV₁ of $81\% \pm 3.5\%$ predicted, with FEV₁ reversibility of greater than 15%, PC₂₀ histamine of 0.81 mg/mL (geometric mean), and a serum IgE level of 228 IU/mL (geometric mean). All subjects were atopic, as determined by positive skin prick test results to a panel of common aeroallergens (ALK), and were sensitized to house dust mite (*Dermatophagoides pteronyssinus*). They were all receiving regular treatment with inhaled corticosteroids (773 ± 117 mg/d [mean $\pm$ SEM] beclomethasone dipropionate) and short-acting β_2 -agonists as required for symptom relief. Three subjects received maintenance therapy with inhaled long-acting β_2 -agonists. Subjects withheld their inhaled corticosteroids for a minimum of 1 week before bronchoscopy to optimize inflammatory responses in the bronchial tissue cultures. Written informed consent was obtained before inclusion into the study, which was approved by the combined Southampton University and Hospital's Ethics committee.

Materials

AIM V (Life Technologies) medium was supplemented with 10 mmol/L HEPES, 1 mmol/L glutamine, and 2 μ mol/L 2-mercaptoethanol. The mixed *D pteronyssinus* allergen extract (Aquagen SQ, ALK) contained all the major allergen groups, and its potency was expressed in standardized quality units (SQU) per milliliter. Each batch was free of endotoxins, as determined by using an E-Toxate kit (Sigma). Lymphoprep was from NYCOMED. Cytoscreen ELISA kits were from Biosource.

Bronchoscopy

Bronchoscopy was performed with a fiberoptic bronchoscope (Olympus FB-20D) in accordance with standard published guidelines,¹⁵ as previously described.¹⁶ Six bronchial biopsy specimens were taken from the subcarinae of segmental bronchi of the right lower lobe with FB15 alligator forceps, immediately placed in AIM V serum-free culture medium, and transferred into culture wells within 30 minutes.

Bronchial biopsy culture protocol

The bronchial biopsy specimens were cultured in 500 μ L of AIM V medium alone or with *D pteronyssinus* extract (5000 SQU/mL, ALK) with or without human CTLA-4Ig (25 μ g/mL; provided by Dr P Linsley, Bristol-Myers Squibb Institute), as previously described.^{13,14} Two biopsy specimens were used in each culture condition to minimize effects caused by tissue heterogeneity. After 24 hours' culture, the biopsy specimens were then blotted to remove excess liquid before weighing and transferring to liquid nitrogen pending RNA extraction; the median weight of the biopsy tissue was 5.6 mg (interquartile range, 2.7-9.5 mg). Supernatants were clarified by means of centrifugation and stored at -80°C pending analysis.

PBMC cultures

Venous blood (30 mL) was collected into heparinized tubes, layered onto 20 mL of Lymphoprep, and centrifuged at 1000g for 20 minutes at room temperature. PBMCs were taken from the plasma/Ficoll interface and washed twice by means of centrifugation in AIM V medium. PBMCs (3×10^6 cells/mL) were cultured for 7 days, after which the medium was collected by means of centrifugation and stored at -80°C for subsequent analysis.

Cytokine mRNA quantitation with RT-PCR ELISA

RT-PCR was used to quantify changes in cytokine mRNA gene transcripts, as previously described.¹⁷ The sense and antisense primers (product size) were as follows: APRT, 5'-GCT GCG TGC TCA TCC GAA AG-3' and 3'-CCT TAA GCG AGG TCA GCT CC-5' (246 bp); IL-5, 5'-CTG AGG ATT CCT GTT CCT GT-3' and 3'-CAA CTT TCT ATT ATC CAC TC-5' (257 bp); IL-13, 5'-CGG TCA TTG CTC TCA CTT GCC TT-3' and 3'-TTA CCC CTC CCT AAC CCT CCT T-5' (500 bp); and GM-CSF, 5'-GCA TGT GAA TGC CAT CCA GG-3' and 3'-GCT TGT AGT GGC TGG CAA TC-5' (215 bp). cDNA (2.5 μ L) was amplified with 1 U of Taq DNA polymerase (Sigma) in the presence of 15 pmol of both primers, 2.5 μ L of PCR digoxigenin-labeled nucleotide triphosphate mix (0.2 mmol/L dATP, dCTP, and dGTP; 0.19 mmol/L dTTP; and 0.01 mmol/L digoxigenin-11-deoxyuridine triphosphate; Boehringer Mannheim), magnesium-free PCR buffer (50 mmol/L KCl, 10 mmol/L TRIS HCl [pH 9], 0.1 % Triton X-100 [Sigma], and 1 mmol/L MgCl₂). Target cDNA was amplified by using a 3-temperature PCR thermal cycler for 35 cycles, with optimal annealing temperature for each cytokine primer pair (56°C for APRT, 50°C for IL-5, and 60°C for GM-CSF and IL-13).

The digoxigenin-labeled PCR product (10 μ L) was denatured (PCR ELISA kit, Boehringer Mannheim) for 15 minutes. Biotin-labeled capture probes specific for each target PCR product were then hybridized to the complementary digoxigenin-labeled PCR product, and the complex was immobilized on duplicate wells of streptavidin-coated microtiter plates for 3 hours at 37°C . The primer-specific PCR capture probes were 5'-AGG GCG TCT TTC TGA ATC TC-3' for APRT, 5'-GGG AAT AGG CAC ACT GGA GA-3' for IL-5, 5'-CGA GAA GAC CCA GAG GAT GC-3' for IL-13, and 5'-AGA CCC GCC TGG AGC TGT AC-3' for GM-CSF. After washing, hybridized-bound PCR products were detected by means of incubation for 30 minutes with an anti-digoxigenin antibody conjugated to horseradish peroxidase. After washing, this was detected with H₂O₂ and 2'-azino-di-(3-ethyl benzthiazoline sulfonate). The absorbance at 405 nm was measured between 10 and 30 minutes later by using an ELISA plate reader. Mean values were calculated, normalized to APRT, and expressed as a percentage ([reading of cytokine PCR product/reading of APRT product] $\times$ 100).

Cytokine protein measurement by means of ELISA

The levels of IL-5, IL-13, and GM-CSF in the culture supernatants of biopsy explants and PBMCs were determined by using commercially available ELISA kits in accordance with the manufacturer's instructions (Cytoscreen, Biosource). The detection limits were as follows: IL-5, 4 pg/mL; GM-CSF, 3 pg/mL; and IL-13, 12 pg/mL. Cytokine levels were corrected for the weight of bronchial tissue in each well and expressed as picograms per milligram of wet weight.

Statistical analyses

Results are expressed as medians and interquartile ranges and were analyzed with the Wilcoxon signed-rank test. *P* values of .05 were accepted as statistically significant.

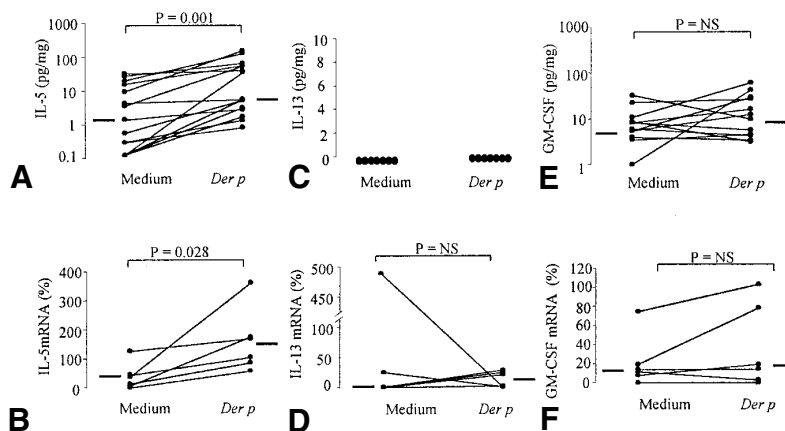


FIG 1. Cytokine protein concentration and mRNA gene expression in bronchial explant cultures of subjects with moderately severe asthma after culture in medium alone or after ex vivo stimulation with *D pteronyssinus* (*Der p*) allergen extract (5000 SQU/mL). **A**, IL-5 protein concentration (n = 15); **B**, IL-5 mRNA (n = 6); **C**, IL-13 protein concentration (n = 9); **D**, IL-13 mRNA (n = 6); **E**, GM-CSF protein concentration (n = 12); **F**, GM-CSF mRNA (n = 6). NS, Not significant.

RESULTS

Allergen-induced cytokine production in bronchial explant cultures of subjects with moderately severe asthma

Consistent with previous explant studies in mild asthma, the spontaneous release of IL-5 from bronchial explants of subjects with moderately severe asthma was extremely low (median, 1.09 pg/mg; interquartile range, 0-12.5 pg/mg). On exposure to *D pteronyssinus* extracts, IL-5 production was significantly increased (median, 4.43 pg/mg; interquartile range, 2.2-46.5 pg/mg; $P = .01$), although there was considerable variability between individual subjects (Fig 1, A). Where samples were available, parallel measurements of mRNA levels confirmed expression of the gene encoding IL-5 on exposure to *D pteronyssinus* (Fig 1, B).

In contrast to our previous observations in mild asthma,^{13,18} we were unable to detect IL-13 protein in the explant supernatants of subjects with moderately severe asthma (Fig 1, C), although low levels of IL-13 mRNA were observed by means of RT-PCR both at baseline and on exposure to *D pteronyssinus* (Fig 1, D). GM-CSF was constitutively expressed and released from the explant cultures (median, 7.1 pg/mg; interquartile range, 4.4-10.3 pg/mg; Fig 1, E) and was not significantly increased by allergen stimulation (median, 11.1 pg/mg; interquartile range, 4.3-28.7 pg/mg; $P \geq .05$; n = 12). GM-CSF mRNA transcripts were not increased by allergen stimulation in the bronchial explants (Fig 1, F).

In selected experiments CTLA-4Ig (25 μ g/mL) was added to the bronchial explant cultures simultaneously with the allergen extract. In previous experiments we have shown that CTLA-4Ig alone or an isotype control antibody had no effect on IL-5 RNA expression or cytokine protein secretions.^{13,14} Although the CTLA-4Ig fusion protein appeared to partially inhibit IL-5 protein production in 5 of 12 explant cultures and IL-5 and IL-13

mRNA expression in 3 of 6 cases studied, when the group was analyzed as a whole, this failed to reach statistical significance (Fig 2, A, B, and D). Levels of GM-CSF, which were not increased by allergen stimulation, were not significantly affected by CTLA-4Ig either at the protein or mRNA level (Fig 2, E and F).

Allergen-induced cytokine release in PBMC cultures of subjects with moderately severe asthma

Because there was little effect of CTLA-4Ig on allergen-induced cytokine release from the explants, control experiments were performed with PBMCs from the same volunteers. Using these cultures, spontaneous IL-5 production was low (median, 21.7 pg/mL; interquartile range, 6.0-44.7 pg/mL) but increased significantly with allergen stimulation (median, 106.6 pg/mL; interquartile range, 52.2-217.4 pg/mL; $P = .005$; Fig 3, A). Similarly, the baseline production of IL-13 was low (median, 0 pg/mL; interquartile range, 0-22.6 pg/mL) but increased significantly with allergen stimulation (median, 345 pg/mL; interquartile range, 51.1-727.0 pg/mL; $P = .05$; Fig 3, C). GM-CSF demonstrated a trend for increased production with allergen stimulation (median, 18.5 pg/mL; interquartile range, 5.3-74.3 pg/mL vs median, 25.7 pg/mL; interquartile range, 11.8-168.8 pg/mL); however, this did not reach statistical significance ($P = .092$; n = 12; Fig 3, E). In contrast to the bronchial explant cultures, the allergen-induced release of IL-5 (median, 150 pg/mL; interquartile range, 91-770 pg/mL) was effectively suppressed by CTLA-4Ig (median, 46.1 pg/mL; interquartile range, 26.7-95.0 pg/mL; Fig 3, B). In similar fashion to IL-5 production, the allergen-induced release of IL-13 in the PBMC cultures of subjects with moderate asthma was significantly inhibited by the addition of CTLA-4Ig (median, 11.4 pg/mL; interquartile range, 0-100.1 pg/mL; $P = .028$; Fig 3, D).

had been receiving prolonged inhaled corticosteroid therapy until 1 week before the biopsy specimens were obtained.¹³ However, in some cases the production of IL-5 was low, even on exposure to allergen.

IL-13 is produced predominantly by T lymphocytes, but mast cells and basophils are also capable of releasing IL-13. Although we demonstrated IL-13 mRNA expression in the explants from subjects with moderately severe asthma, we did not detect IL-13 protein production, which is in contrast to our findings in mild disease.¹⁸ It is possible that failure to detect IL-13 and the low levels of IL-5 in some explant cultures was related to the persistent action of inhaled corticosteroids in these particular patients. However, studies have shown that withdrawal of inhaled corticosteroids, even for 1 week, results in the return of asthma symptoms^{21,22} and an associated increase in airway inflammation.²²⁻²⁴ Whether withholding corticosteroids for 1 week is sufficient to completely reverse any effect on IL-5 and IL-13 production is at present unknown. Given the risk of unacceptable exacerbation of asthma symptoms, it was believed ethically unacceptable to withhold corticosteroids for more than 1 week.

GM-CSF plays an important role in promoting airway inflammation by promoting the maturation of dendritic cells and prolonging the survival of neutrophils and eosinophils in the airways.²³ Constitutive release of GM-CSF was observed in the explant cultures of the subjects with moderately severe asthma, although production was not significantly augmented by allergen. This differs from the explant studies in mild asthma, where, under identical culture conditions, we were unable to detect GM-CSF either spontaneously or with stimulation. This difference suggests a potential role for GM-CSF in perpetuating the airway inflammation at the severe end of the disease spectrum. Because bronchial epithelial cells, mast cells, myofibroblasts, and macrophages generate plentiful amounts of GM-CSF,^{25,26} these cells are likely sources of GM-CSF that would not be expected to require CD28-mediated costimulation.

In contrast to the suppression of T_H2 cytokine responses by CTLA-4Ig in mild asthma,¹⁴ we found that it did not significantly inhibit IL-5 or GM-CSF release in the explants of subjects with moderately severe asthma. However, its ability to suppress IL-5 and IL-13 production in the PBMC cultures suggests that activation of circulating T cells of these subjects still requires CD28-mediated costimulation, as found in mild-to-moderate disease.¹⁸ These data suggest that allergic responses in the bronchial mucosa in moderately severe asthma are less dependent on CD28-mediated costimulation when compared with circulating mononuclear cells from the same subjects. This proposal is consistent with a recent report that T-lymphocyte chemotactic activity of allergen-stimulated bronchial explants is suppressed by antagonists of CD28-mediated costimulation in subjects with mild, but not moderately severe, asthma.²⁷ Although it is possible that relatively higher doses of the fusion protein may be required to inhibit costimulation in the bronchial tissue in more severe asthma, this seems

unlikely in view of its efficacy in the PBMC cultures. Reduced penetration of CTLA-4Ig into the biopsy tissue also seems an unlikely explanation for its lack of effect because *D pteronyssinus* allergen itself has protein components with molecular weights at least as great as those of CTLA-4Ig and yet evoked a clear T_H2 cytokine response from the tissue. Furthermore, the protocol adopted for this study was identical to that in mild asthma, where a substantial inhibitory effect was observed,¹³ and the efficacy of the fusion protein has been demonstrated in a variety of in vivo models using either systemic⁶ or topical administration,²⁸ further suggesting that tissue penetration is not a problem.

Differences in the requirement for CD80 and CD86 costimulation have been shown in the circulation compared with in the bronchial mucosa of subjects with mild asthma. Larche et al²⁹ reported that the allergen-induced proliferation of T cells isolated from bronchoalveolar lavage fluid was inhibited by anti-CD86, but not by anti-CD80, antibodies, whereas in explant studies in mild asthma, we found that allergen-induced IL-5 and IL-13 production was inhibited by anti-CD80 and, to a lesser extent, by anti-CD86 antibodies. However, we also found that cytokine production by PBMCs of subjects with mild asthma was not affected by anti-CD80 but was reduced by anti-CD86 and was strongly attenuated with a combination of both antibodies.¹⁸ Furthermore, although allergen-induced cytokine production and proliferation of PBMC cultures from subjects with mild asthma are inhibited by either CTLA-4Ig or anti-CD86,²⁹ when the responses of allergen-specific peripheral blood CD4⁺ T-cell lines are assessed, responses are independent of CD80 or CD86 costimulation. This supports in vitro studies showing that ongoing, memory-type, T cell-dependent immune responses are less dependent on CD28-B7 costimulation than are primary immune responses involving naive T cells.³⁰

Our present results indicate that CD28-B7 costimulation may not be critical for IL-5 production in the bronchial mucosa of subjects with moderately severe asthma. One explanation for this observation is that T cells resident in the airways of subjects with severe asthma may behave more like T-cell lines, being independent of CD28-B7 costimulatory signals. Alternatively, other costimulatory pathways, including ligation of Ox40-Ox40L,³¹ inducible costimulator (ICOS) signaling,³² or T1/ST2 member of the IL-1 receptor family,³³ may play a more important role in T_H2-mediated immune responses in the lung mucosa. In ovalbumin-sensitized mice, ICOS mRNA is markedly reduced in the lungs after antigen challenge, and systemic administration of an ICOS-Ig fusion protein markedly inhibited antigen-induced IL-5 production in parallel with an 80% reduction in eosinophil infiltration.³⁴ It will now be important to test the recently available human ICOS-Ig in the severe bronchial asthmatic explant system. A third explanation for our observations is that proinflammatory cytokine production in the airway tissue of subjects with more severe asthma is derived from cells distinct from T cells,

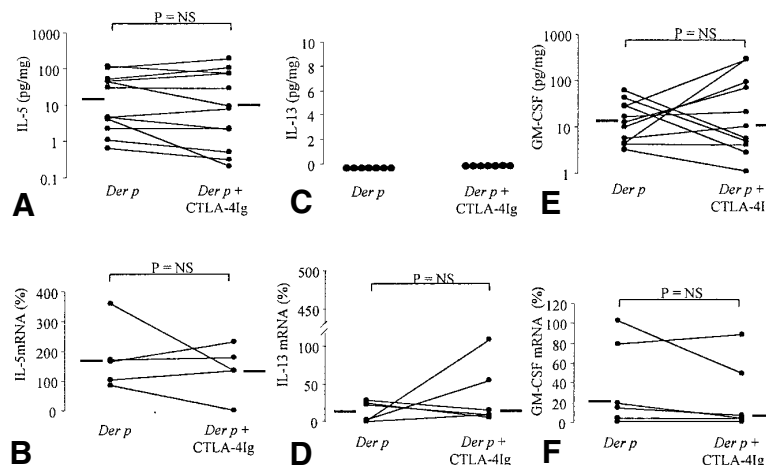


FIG 2. Cytokine protein concentration and mRNA levels in bronchial explants of subjects with moderately severe asthma after culture in the presence of *D pteronyssinus* (*Der p*) allergen extract (5000 SQU/mL) in the absence or presence of CTLA-4Ig (25 μ g/mL). **A**, IL-5 protein concentration (n = 12); **B**, IL-5 mRNA (n = 5); **C**, IL-13 protein concentration (n = 9); **D**, IL-13 mRNA (n = 6); **E**, GM-CSF protein concentration (n = 11); **F**, GM-CSF mRNA (n = 6). NS, Not significant.

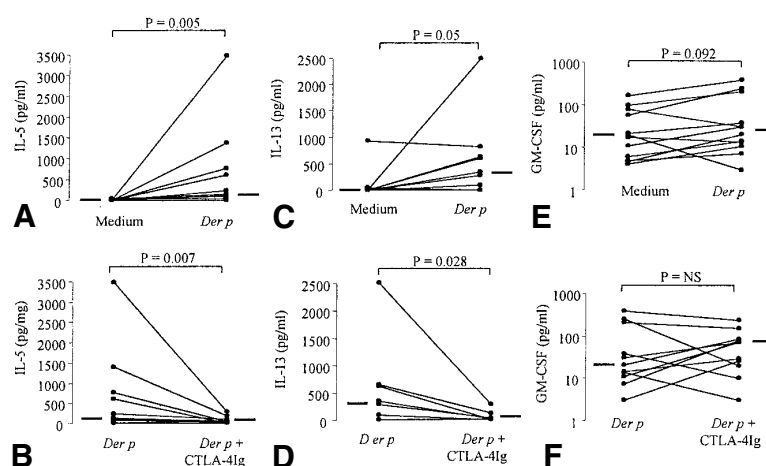


FIG 3. Cytokine protein concentration in PBMC cultures from subjects with moderately severe asthma after culture in either medium alone or house dust mite allergen extract *D pteronyssinus* (*Der p*; 5000 SQU/mL) in the absence or presence of the fusion protein CTLA-4Ig. **A** and **B**, IL-5 protein concentration (n = 11); **C** and **D**, IL-13 protein concentration (n = 9); **E** and **F**, GM-CSF protein concentration (n = 12).

Allergen stimulation augmented the release of GM-CSF from 17 pg/mL (interquartile range, 5-57 pg/mL) to 20.4 pg/mL (interquartile range, 11.1-212 pg/mL; $P = .092$; n = 11), but the addition of CTLA-4Ig had no effect.

DISCUSSION

The bronchial explant culture model is a useful integrated cell system for assessing allergic responses in vitro.¹³ Unlike isolated cell-culture systems, the explants include structural elements, such as the airway epithelium and also the inflammatory cells present in the airways. Immunohistochemistry applied to a subset of biopsy specimens that were fixed after culture confirmed the

presence of CD3⁺, CD4⁺, and CD8⁺ T-lymphocyte subsets, EG2⁺ eosinophils, AA1⁺ mast cells, NE⁺ neutrophils, CD20⁺ B lymphocytes, and occasional CD1a⁺ dendritic cells (data not shown).

A number of studies support the role of IL-5 in promoting the development, recruitment, and activation of eosinophils in inflamed asthmatic airways.^{19,20} In the present study we have demonstrated IL-5 production by explant and PBMC cultures of subjects with moderately severe asthma, with a significant increase in IL-5 on allergen stimulation. Overall, the levels of IL-5 in the explant cultures from these subjects with moderately severe asthma were comparable with those found in subjects with mild asthma, despite the fact that these patients

which may not require costimulation for cytokine production. Although T cells are considered to be the principal source of IL-5 in asthma, other cells have the capacity to generate and release this cytokine, including mast cells, eosinophils, and the bronchial epithelium.^{17,35}

The clinical utility of CTLA-4Ig has been demonstrated in patients with severe psoriasis, where it caused a reduction both in T cell-mediated inflammatory skin lesions and clinical parameters of disease severity.³⁶ Because we found that allergen responsiveness and proinflammatory cytokine production by cells in airway tissue of subjects with moderately severe asthma was insensitive to suppression by CTLA-4Ig, this suggests that airway cytokine production in more severe asthma may be less amenable to modulation by antagonists of CD28-mediated costimulation than mild asthma. Further studies are required to clarify the precise role of CD28-B7 costimulation in asthmatic airway inflammation and to assess the importance of the tissue microenvironment in pulmonary inflammatory responses in severe asthma.

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