

Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects

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Background: IL-10 is an anti-inflammatory cytokine made by lymphocytes, monocytes-macrophages, and eosinophils, and it may have an important role in regulating the asthmatic inflammatory response. IL-10 levels have been reported to be reduced in asthmatic airways, potentially contributing to more intense inflammation.

Objective: We sought to determine whether IL-10 levels were deficient in patients with mild asthma compared with controls and to determine whether IL-10 levels were associated with the resolution of eosinophilic inflammation.

Methods: We quantified IL-10 levels in the bronchoalveolar lavage (BAL) fluid (ELISA), BAL cells (quantitative immunocytochemistry), purified alveolar macrophages-monocytes studied ex vivo (ELISA), before (day 1) and after (24 hours [day 2], 1 week [day 9], and 2 weeks [day 16]) segmental antigen challenge (SAC), and investigated the effect of glucocorticoid treatment on ex vivo macrophage-monocyte IL-10 production.

Results: IL-10 levels were significantly higher in the BAL fluid of mild asthmatic subjects who demonstrated a dual reaction (both early and late) after whole lung ragweed inhalation challenge compared with nonallergic, nonasthmatic control subjects before and 24 hours and 1 week after SAC. Macrophages-monocytes obtained before and after SAC from asthmatic patients also secreted increased amounts of IL-10 ex vivo than those from controls. Dexamethasone did not significantly change spontaneous IL-10 secretion from macrophages-monocytes in vitro. Quantitative immunocytochemical analysis of BAL cells demonstrated increased IL-10 in macrophages 24 hours after SAC and a similar trend in eosinophils.

Conclusion: IL-10 is not deficient in mild asthma. Furthermore, BAL IL-10 levels are significantly higher in asthmatic subjects with a dual response than in control subjects before

and after SAC. The increase in IL-10 was coincident with the initial increase in BAL eosinophils, although BAL eosinophilia persisted after IL-10 levels had returned to baseline, suggesting that the increased IL-10 levels could not promptly terminate this localized eosinophilic response. (*J Allergy Clin Immunol* 2000;106:880-6.)

Key words: Interleukin-10, allergic asthma, cytokines, macrophages, eosinophils, glucocorticoids, segmental antigen challenge, bronchoalveolar lavage, immunocytochemistry

IL-10, initially described as a product of murine T_H2 lymphocytes that inhibits cytokine synthesis by T_H1 cells,¹⁻³ is now known to be produced by numerous other cells besides T_H2 cells, including T_H0 cells, T_H1 cells, B cells, monocytes-macrophages, and eosinophils.⁴⁻⁶ It may play a critical role in regulating the asthmatic inflammatory response through its suppressive effects on T_H1 and T_H2 cells⁷⁻⁹ and cytokines including TNF- α , IL-1 β , IL-6, IL-8, and GM-CSF,^{10,11} as well as through effects on expression of class II molecules on antigen-presenting cells¹² and by inhibiting eosinophil survival.¹³

Given its potent anti-inflammatory effects, it has been suggested that a deficiency in this cytokine could lead to a more intense inflammatory response in asthmatic subjects. However, available data are conflicting. Although Borish et al¹⁴ reported reduced levels of IL-10 in the bronchoalveolar lavage fluid (BAL) of asthmatics at baseline and after antigen challenge compared with control subjects, Robinson et al¹⁵ reported that mild asthmatic subjects had higher IL-10 levels compared with control subjects at baseline, and IL-10 mRNA in the BAL cells of asthmatics increased after allergen inhalation challenge compared with diluent challenge of the same subjects.

Glucocorticoids, a mainstay of asthma therapy, have been found to have varying effects on IL-10 production. In vitro data suggest that glucocorticoids suppress LPS-stimulated IL-10 production in macrophages-monocytes in healthy subjects,^{14,16} whereas in vivo data have shown that IL-10 production is increased in alveolar macrophages of asthmatic subjects who are being treated with an inhaled glucocorticoid.^{16,17} It is unclear what mechanisms permit this difference in the modulation of IL-10 production.

In this series of experiments we sought to further explore these disparate results and to test two major hypotheses: (1) mild asthmatics show a deficiency of

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TABLE I. Subject demographics

	Asthmatics (single)	Asthmatics (dual)	Nonallergic controls
No.	4	9	9
Sex (male/female)	4/0	6/3	6/3
Mean age (y) (range)	31 (25-37)	29 (24-38)	27 (22-36)
Methacholine PC ₂₀ (mg/mL) (geometric mean)	0.64	2.61	>32
FEV ₁ (L) (% predicted)	4.14 (95)	3.63 (105)	3.88 (103)
FEV ₁ /forced vital capacity ratio (%)	77	80	80

Methacholine PC₂₀, Concentration of methacholine producing a 20% fall in FEV₁.

Abbreviations used

- AAD (or AD): Allergic asthmatics having a dual (early and late) airway response after whole-lung ragweed challenge
- AAS (or AAS): Allergic asthmatics having a single (early) airway response after whole-lung ragweed challenge
- BAL: Bronchoalveolar lavage
- RWAU: Ragweed antigen unit
- SAC: Segmental antigen challenge

BAL levels of IL-10 before and after segmental allergen challenge (SAC) and (2) increased IL-10 levels are associated with the resolution of eosinophilic inflammation after SAC. To address these questions, IL-10 levels in BAL fluid and IL-10 expression by mixed BAL cells and purified alveolar macrophages-monocytes were quantitated from subjects before and after SAC over a 2-week period. Additionally, we examined the effects of in vitro dexamethasone treatment on the ex vivo production of IL-10 by airway macrophages-monocytes. These series of observations permitted us to address the following specific questions concerning the biology of IL-10: (1) Is IL-10 production reduced in mild allergic asthmatics after segmental antigen challenge? (2) What are the kinetics of the production of IL-10 after SAC of human asthmatics? (3) Are BAL cells an important source of IL-10 after SAC? (4) Is the ex vivo production of IL-10 by alveolar macrophages-monocytes modulated by corticosteroid treatment in vitro?

MATERIAL AND METHODS

Subject characterization

Nine non-ragweed allergic healthy volunteers and 13 ragweed-allergic asthmatic volunteers who met standard criteria for the diagnosis of asthma by the National Institutes of Health/National Heart, Lung, and Blood Institute expert panel¹⁸ were enrolled in this study, which was approved by Jefferson Medical College Institutional Review Board. Asthmatics took no long-term medication; rather, they were treated only with as-needed β -agonist. More detailed information concerning subject characterization and methods for intradermal ragweed testing (short ragweed antigen, 0.001-10 units/mL in normal saline solution with 0.03% human serum albumin [1 ragweed antigen unit (RWAU) = 0.005 μ g ragweed antigen E (Amb aI), Greer Laboratories, Lenoir, NC]) and for performing methacholine and whole-lung ragweed challenge has been published.^{19,20} Asthmatic subjects were divided into single reactors (those having only an immediate airway response [$>20\%$ fall in

FEV₁ during the first hour after whole-lung ragweed challenge]) (AAS) and dual reactors (those having both an early and a late response [$>15\%$ fall in FEV₁ during hours 2 through 8 after ragweed challenge]) (AAD). Subject demographics and physiologic parameters are listed in Table I.

Bronchoscopy, BAL, and SAC

Most of these procedures have been described previously.²⁰ All subjects were premedicated with albuterol (4 puffs by metered-dose inhaler); lidocaine was used locally in the airways. BAL was performed (150 mL of 37°C saline solution in 3 aliquots of 50 mL each) after the bronchoscope was wedged in one of the left lower lobe segments, followed by brush biopsies (2 to 4 samples/session) of the lavaged subsegment (to obtain epithelial samples) and then endobronchial forceps biopsies (3 to 5 samples/session, proximal to site of brush biopsies). After these day 1 samples were obtained, an SAC was performed in the right middle lobe with 5 mL of ragweed solution in a concentration 100-fold higher than that producing a positive intradermal skin reaction. In the 13 asthmatics, 4 were challenged with a concentration of 0.1 RWAU/mL, 1 with 1 RWAU/mL, and 8 with 10 RWAU/mL. In the 9 controls, 2 were challenged with 1 RWAU/mL, 3 with 10 RWAU/mL, and 4 with 100 RWAU/mL. The solution was instilled in a "semiwedged" position with efforts made to accomplish instillation across two subsegmental bronchi to optimize future bronchoscopic sampling. The antigen-challenged segment was sampled (BAL, bronchial brush biopsies, endobronchial forceps biopsies) 24 hours later (day 2), at which time a second antigen challenge procedure was performed in the lingula. This second challenged segment was sampled 7 days later (day 9). Finally, the initial antigen-challenged segment was resampled 7 days later (day 16). The entire protocol included a total of four bronchoscopies, two SACs, and four BALs, endobronchial brush biopsies, and forceps biopsies per subject.

Sample handling and IL-10 assay

Procedures for handling BAL fluid, epithelial cells, biopsy specimens, cell staining, and cell counting have been previously described.^{19,21,22} For macrophage-monocyte experiments, 500,000 macrophages per well in 1 mL of RPMI medium were plated in a series of 15.6-mm diameter (24 wells/plate) tissue culture plates and cells were allowed to adhere for 2 hours. Nonadherent cells were removed by washing twice with fresh RPMI medium, and the resulting adherent cells were then incubated with and without dexamethasone 1 μ mol/L for 24 hours at 37°C. After 24 hours culture supernatants were harvested and stored frozen until analyzed for IL-10 (ELISA). Remaining BAL cells were prepared as cytopins (40,000 cells/slide), treated with paraformaldehyde, washed in PBS, and stored frozen until used for immunostaining. BAL fluid was concentrated 14-fold (Centriprep YM10, Amicon) and all samples were stored at -80°C until assayed by ELISA (Biosource International, Camarillo, Calif, and R&D Systems, Minneapolis, Minn) with a minimum detectable level for IL-10 of ~1 pg/mL.

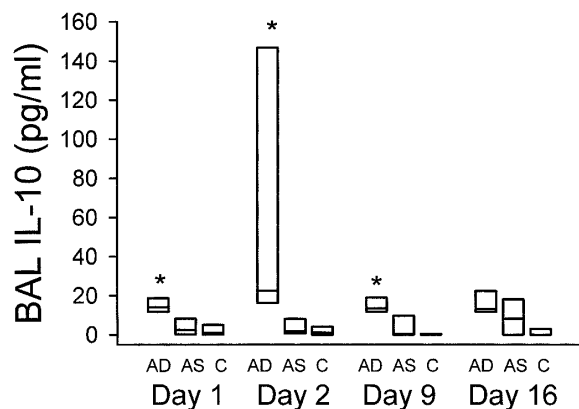


FIG 1. IL-10 levels in the BAL fluid of allergic asthmatic and control subjects. Asthmatic subjects were divided into two groups, those with both early and late (dual) reactions (AD, $n = 8$) and those with only a single early reaction after whole-lung antigen challenge (AS, $n = 4$). The control group (C) consisted of eight subjects. Data are presented as modified box plots with extremes of the boxes representing the 25th and 75th percentiles, respectively, and the horizontal line in the box representing the median. When all days were considered together, a Kruskal-Wallis ANOVA on ranks revealed a significant difference between groups ($P < .001$) with a pairwise multiple comparison procedure by Dunn's methods revealing significant differences between the AD and AS ($P < .05$) and AD and control ($P < .05$) groups. When individual days were analyzed with these same procedures, the AD group was significantly different from the control group on days 1, 2, and 9 (shown by asterisk).

Immunostaining

Immunostaining of IL-10 was performed on cytospins with slides blocked in pure goat serum/0.1% saponin for 1 hour and subsequently incubated in IL-10 mAb (2 $\mu\text{g/mL}$ in 10% goat serum in PBS) at 4°C overnight in humidifying chambers. After being washed, slides were incubated in a 1:300 dilution of secondary antibody, biotinylated anti-mouse antibody (Zymed), for 30 minutes. Streptavidin-alkaline phosphatase at a 1:100 dilution was added after washes and incubated for 30 minutes. Slides were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium with 1 mmol/L levamisole. Additional cytospins were treated with irrelevant isotype-matched mouse antibody substituted for the primary IL-10 antibody as a negative control.

Image analysis procedure

IL-10 stained slides from each time point for four asthmatic subjects and four healthy control subjects were assessed for stain density and cell area (Bioquant System 4 software, R & M Biometrics). Although IL-10-stained cells could be identified morphologically in many cases, this could not be done for every cell in all cases. Therefore cell area, used in conjunction with the differential cell count from Wright (Accustain, Sigma)-stained slides, was used to infer cell type. Eosinophils, neutrophils, lymphocytes, and macrophages, identified according to standard morphologic criteria in Accustain-stained slides (prepared for differential cell counts), were measured for area (50 cells in each category) and a mean $\pm$ SD was obtained. The range of cell area for eosinophils and neutrophils overlapped; therefore these were grouped as granulocytes. However, eosinophils were present in much greater numbers than neutrophils in asthmatic subjects

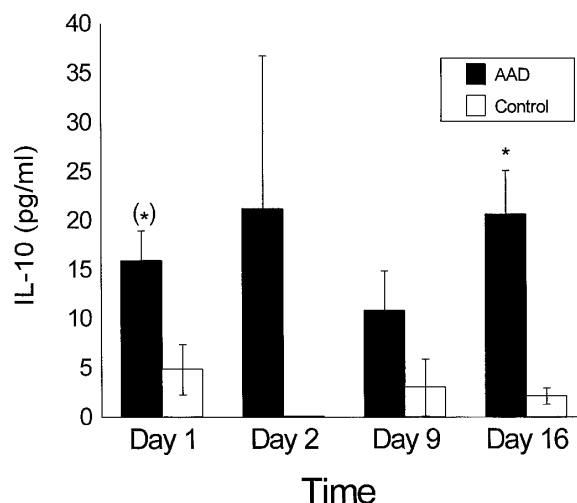


FIG 2. IL-10 levels in macrophages of allergic and control subjects. Adherent cells from both asthmatics (AAD group, $n = 8$) and controls ($n = 3$) were studied for the ex vivo production of IL-10. When all days were considered, a Mann-Whitney rank sum test revealed a significant difference between groups ($P = .036$). A significant difference between AAD and controls was noted for day 16 ($P = .038$, t test) and a trend for day 1 ($P = .07$, t test).

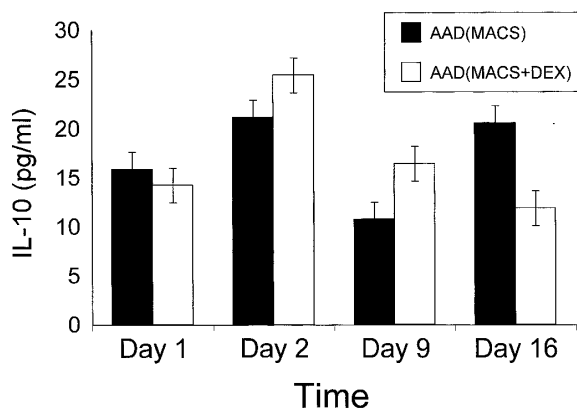


FIG 3. Effect of dexamethasone on IL-10 production by asthmatic macrophages. The effect of in vitro dexamethasone treatment (1 $\mu\text{mol/L}$) on the spontaneous secretion of IL-10 from asthmatic adherent BAL cells (AAD, $n = 8$) was studied. Wilcoxon signed rank test revealed no difference between treatments when all data were considered ($P = .922$), and pairwise comparisons (paired t tests) for each day also revealed no significant differences.

after SAC; therefore the contribution provided by neutrophils in this case was negligible. (See cell differentials and cell counts in Fig 5, A.) Based on the mean $\pm$ 1 SD for cell area, lymphocytes were found to group within 0 to 125 μm^2 and granulocytes within 126 to 250 μm^2 , and macrophages were $>250 \mu\text{m}^2$. All cells in randomly selected frames were measured until 100 cells were assessed for area and stain density on each IL-10-stained slide. Microscope light intensity and System 4 contrast settings were held constant for all slides. Background density within each frame was subtracted from cell density. Measurements for cells were sorted according to cell area on the basis

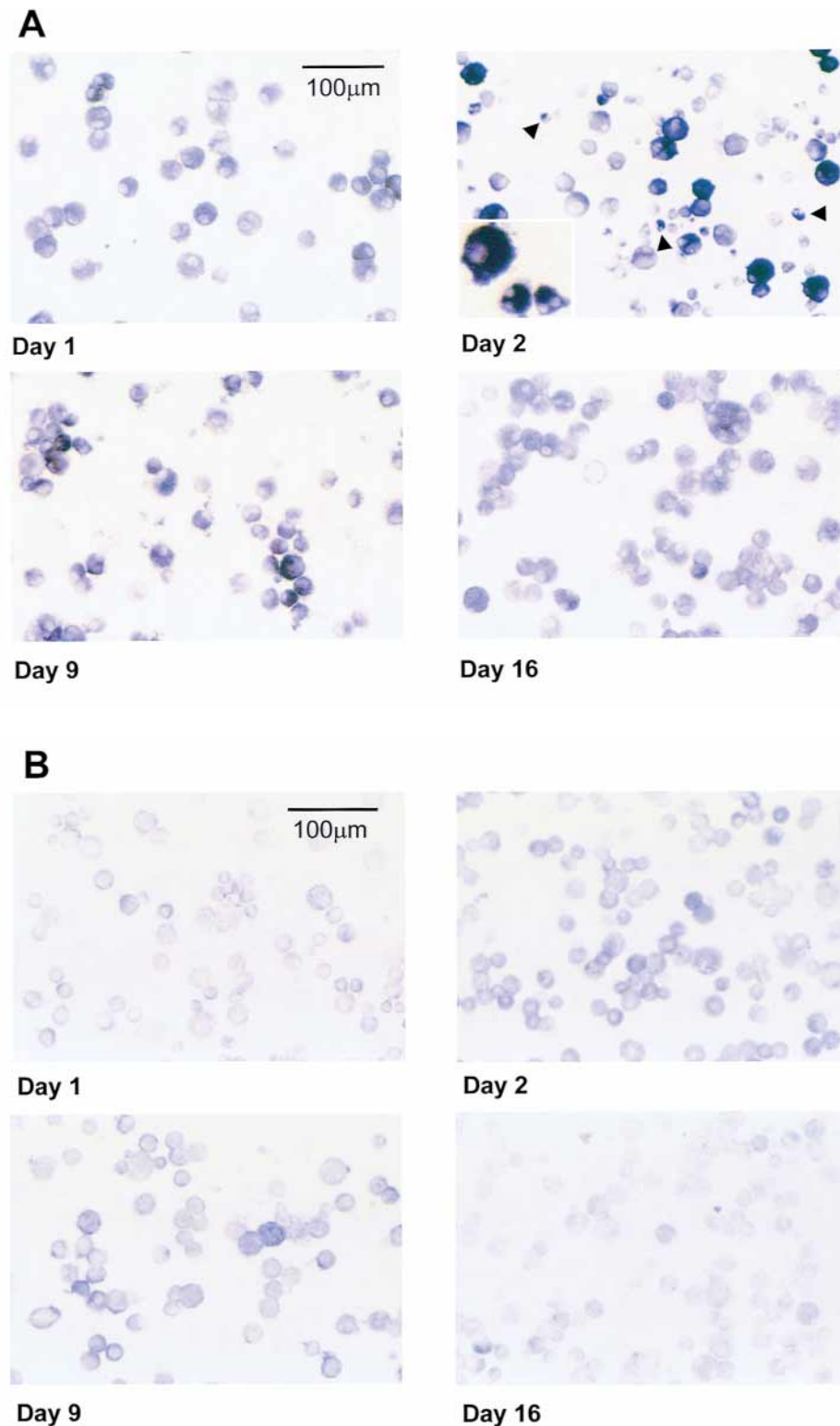


FIG 4. Cytospins immunostained with anti-human IL-10. BAL leukocyte cytospin preparations from an allergic asthmatic subject (**A**) and a control subject (**B**) at baseline (day 1) and 24 hours (day 2), 1 week (day 9), and 2 weeks (day 16) after SAC were stained with monoclonal anti-human IL-10. Note that some small cells having bilobar nuclei (eosinophils) stain intensely for IL-10 (*arrowheads*). All micrographs were printed at the same magnification (marker = 100 µm), except for the magnified insert in **A** (day 2), which suggests that stain is cytoplasmic rather than plasma membrane bound.

of the above categories, and the mean IL-10 stain density for each cell group for each subject was calculated.

Statistical analysis

Data were analyzed with use of Microsoft Excel '97 and SigmaStat for Windows (version 2.03, SPSS Science Software) statistical packages. SigmaStat tests data for normality and equal variance. If these tests failed, data were analyzed by nonparametric means (Kruskal-Wallis ANOVA with pairwise multiple comparisons by Dunn's method, Mann-Whitney rank sum, or Wilcoxon signed rank tests); otherwise, data were analyzed by parametric tests (multivariate ANOVA, unpaired or paired *t* tests). Eosinophil-IL-10 correlations were done with the Spearman rank order correlation. Data in Fig 1 are presented as a modified box plot and data in Figs 2, 3, and 5 as mean $\pm$ SEM because all the pairwise comparisons in these figures (except two comparisons in Fig 2) met normality and equal variance requirements. Statistical details are present in the figure legends. A *P* value of $<.05$ was considered significant, and $.05 < P < .010$ was considered a trend, except for data in Fig 1, where three pairwise comparisons were performed and a *P* $< .017$ was required.

RESULTS

Fig 1 shows IL-10 levels in the BAL fluid of allergic asthmatic (divided into dual [AD] and single [AS] reactors after whole-lung ragweed challenge) and control subjects. When all days were analyzed by Kruskal-Wallis ANOVA on ranks, a significant difference was observed among groups (*P* $< .001$) with the AD group being significantly different from both the AS and the control groups (*P* $< .05$, Dunn's method). The IL-10 level was significantly higher in the AD compared with the control groups on day 1 (before SAC), day 2 (24 hours after SAC), and day 9 (1 week after SAC).

Because significant elevations in IL-10 levels were only seen in the dual reacting (AAD) group of asthmatics, experiments characterizing the cellular production of IL-10 were only performed in this subgroup in comparison with controls. IL-10 levels in the macrophages of AAD were significantly higher than in a subgroup of controls (Fig 2) when all days were analyzed by the Mann-Whitney rank sum test (*P* = .036), with the difference between groups significantly different on day 16 (*P* = .038) and a similar trend observed on day 1 (*P* = .07). The administration of dexamethasone to the macrophages of the AAD group had no effect on the spontaneous secretion of IL-10 levels (Fig 3).

Fig 4 shows cytopspins stained for IL-10 from one asthmatic subject (A) and one control subject (B) at baseline (day 1) and 24 hours (day 2), 1 week (day 9), and 2 weeks (day 16) after SAC. The insert (Fig 4, A, day 2) shows stain concentrated in the cytoplasmic areas of the cells without an enhancement of stain intensity at the cell edges. This suggests that the IL-10 is intracellular, not just absorbed to the cell surface. The increase in stain intensity for cells from asthmatics in the 126 to 250 μm^2 size 24 hours after SAC (day 2) (90% eosinophils) tended toward significance (Fig 5, A, *P* = .088), whereas that for cells $> 250 \mu\text{m}^2$ (macrophages) was significant (Fig 5, B, *P* = .021). Note that an increase in both eosinophil concentration (asthmatics $90 \times 10^4/\text{mL}$, controls $0.12 \times$

$10^4/\text{mL}$) and macrophage concentration (asthmatics $35 \times 10^4/\text{mL}$, controls $13 \times 10^4/\text{mL}$) was observed at this 24-hour time point in asthmatics versus controls, so that both increased cell numbers and increased stain intensity contributed to the IL-10 response in asthmatics.

Finally, in the 12 asthmatics in whom BAL eosinophil concentrations and IL-10 concentrations were available, correlations were performed. For reference, eosinophil concentrations were 0.04 (median) (0.01, 0.32) (25th percentile, 75th percentile) $\times 10^4/\text{mL}$ on day 1, 47.85 (4.21, 73.13) $\times 10^4/\text{mL}$ on day 2, 10.8 (1.02, 29.02) $\times 10^4/\text{mL}$ on day 9, and 4.30 (0.24, 19.74) $\times 10^4/\text{mL}$ on day 16. The correlations between eosinophil concentration and IL-10 concentration were -0.116 (*P* = .699, day 1), 0.776 (*P* = .002, day 2), 0.454 (*P* = .130, day 9), and -0.068 (*P* = 0.817, day 16). Therefore a significant correlation was observed only 24 hours after SAC. Eosinophils persisted in the airways long after IL-10 levels had waned.

DISCUSSION

IL-10 has been characterized as having a wide variety of anti-inflammatory properties.^{4,7-13,23} In this series of experiments, we demonstrated that mild allergic asthmatics produce comparable or increased quantities of IL-10 in comparison with control subjects in terms of quantities in BAL fluid before and after SAC, in terms of that produced by alveolar macrophages-monocytes studied *ex vivo*, and in terms of the immunocytochemical signal produced by alveolar macrophages and eosinophils. Our data are consistent with the results obtained by Robinson et al¹⁵ as well as those of Magnan et al,¹⁷ who found IL-10 mRNA to be significantly higher in asthmatic compared with control subjects. Although other investigators examined BAL cells for IL-10 gene transcription, our study evaluated IL-10 protein production, which has not been reported by other investigators, because either of insufficient BAL fluid sampling¹⁵ or the suboptimal timing of BAL fluid analysis.¹⁴ These results are in contrast to those reported by some other investigators.^{14,24} Differences in patient populations could account for this difference because we have studied very mild asthmatics whereas others have studied patients with severe persistent asthma.²⁴ Together, these findings suggest that any defect in IL-10 production in asthmatics is likely to be an acquired, rather than a more fundamental, phenomenon.

Our observations also suggest that both basal IL-10 production by airway macrophages-monocytes and that triggered by *in vivo* antigen challenge are unresponsive to glucocorticoids. However, they do not directly address previous work concerning the disparate effects of glucocorticoids on IL-10 production when given *in vitro* (an inhibitory effect on LPS-stimulated IL-10 production) versus *in vivo* (where corticosteroids stimulated IL-10 production). Borish et al¹⁴ and John et al,¹⁶ who reported an inhibitory effect of glucocorticoids on LPS-stimulated IL-10 production, did not report the effect of glucocorticoids on basal IL-10 production and their data reflect only the response seen in control subjects.

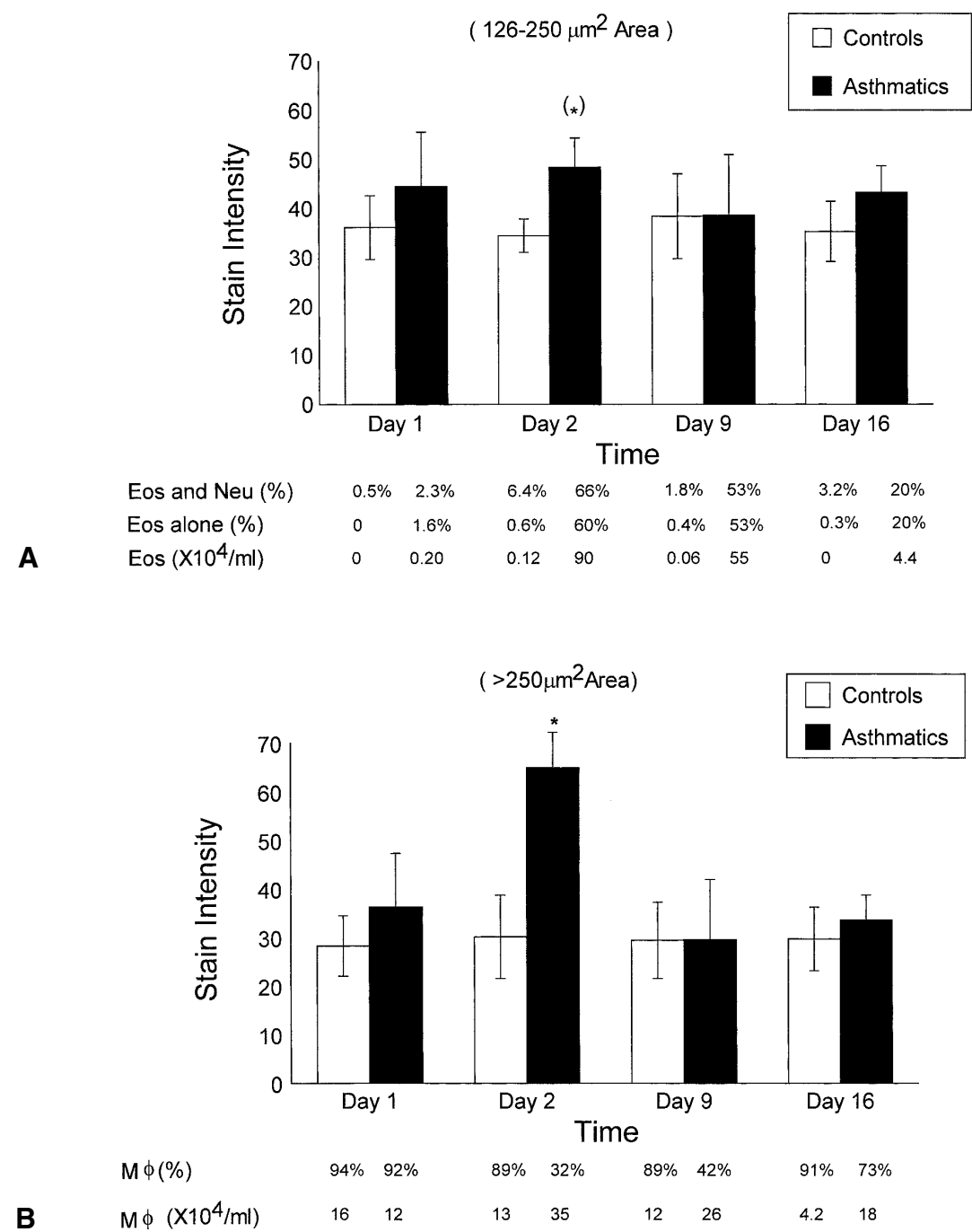


FIG 5. Quantitation of IL-10 staining intensity of BAL leukocytes according to leukocyte size. Samples from 4 asthmatics and 4 controls were analyzed. **A**, A trend for greater IL-10 stain intensity was observed in the asthmatic subjects 24 hours after SAC (day 2) compared with the control group ($P = .088$, t test) for cells in the 126 to 250 μm^2 size range. This size category contains both eosinophils (*Eos*) and neutrophils (*Neu*), but the majority of the cells in this size category were eosinophils in asthmatics at the 24-hour (day 2) and 1-week (day 9) time points. This is shown in the tabulated data from the differential cell counts for controls and for asthmatics beneath their respective bars. Absolute cell numbers (cells $\times 10^4/\text{mL}$) for eosinophils are also given. **B**, IL-10 stain intensity was found to be greater in the asthmatic group compared with the control group ($P = .021$, t test) on day 2 for cells in the size range $>250 \mu\text{m}^2$. This size category contains predominantly macrophages (*M ϕ*), as determined by the differential cell counts and absolute numbers for the asthmatic and control subjects given beneath their respective bars.

Finally, we observed a spike in IL-10 production 24 hours after SAC with a return of IL-10 levels to baseline thereafter. BAL eosinophilia persisted after IL-10 levels had returned to baseline, suggesting that the increased IL-10 levels could not rapidly terminate this eosinophilic inflammatory response.

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