

Original article

Effect of omalizumab treatment on peripheral eosinophil and T-lymphocyte function in patients with allergic asthma

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Background: Omalizumab is a recombinant monoclonal anti-IgE antibody with proven efficacy in allergic diseases and further anti-inflammatory potency in the treatment of asthma.

Objectives: To explore the anti-inflammatory mechanism of omalizumab, we investigated the induction of immunologic changes leading to eosinophil apoptosis and examined T-lymphocyte cytokine profiles in patients with allergic asthma. **Methods:** Nineteen patients with allergic asthma were enrolled and received omalizumab at a dose of at least 0.016 mg/kg/IgE (IU/mL) every 4 weeks. Peripheral eosinophils and T-lymphocyte cytokine profiles were evaluated by fluorescence-activated cell sorting before treatment (baseline), at 12 weeks of treatment, and 12 weeks after discontinuation of treatment with omalizumab or placebo.

Results: Markers of eosinophil apoptosis (Annexin V) were significantly increased in omalizumab recipients compared with placebo, whereas no changes in markers of necrosis (7-amino-actinomycin) or eosinophil activation CD69 or Fas receptor (CD95) were detected. GM-CSF⁺ lymphocytes were reduced in omalizumab recipients compared with placebo. Fewer IL-2⁺ and IL-13⁺ lymphocytes were evident in omalizumab recipients than in the placebo group. There were no significant differences in IL-5, IFN- γ , or TNF- α between the omalizumab and placebo groups.

Conclusion: These findings provide further evidence that omalizumab has additional anti-inflammatory activity demonstrated by induction of eosinophil apoptosis and downregulation of the inflammatory cytokines IL-2 and IL-13. Further studies are needed to determine the underlying mechanisms.

Clinical implications: These findings support the critical role of IgE in the regulation of inflammation in allergic asthma:

influencing the inflammation is the key to control the more severe type of asthma. (J Allergy Clin Immunol ■■■■;■■■: ■■■-■■■.)

Key words: Omalizumab, allergic asthma, asthma treatment, inflammation, anti-IgE, eosinophils, T-lymphocytes

Allergic asthma is an inflammatory disease of the airways, characterized by increased serum IgE levels.¹ Sensitization to an allergen results in the production of IgE by B lymphocytes, leading to an allergic inflammatory process characterized by 2 phases. The early allergic phase involves the binding of allergens to antigen-binding sites on the surface of basophils and mast cells followed by the cross-linking of IgE. The aggregation of high-affinity IgE receptors (Fc ϵ R1) then triggers the release of pre-formed and newly synthesized mediators associated with the onset of allergic symptoms. The late allergic phase is characterized by an influx of eosinophils and activated T cells in the airways. Eosinophils are one of the main effector cells in allergic inflammation and accumulate in the tissue of individuals with allergic asthma.

Preventing the allergen-induced release of inflammatory mediators from target cells provides a new strategy in the treatment of atopic diseases.² The treatment of allergic diseases, including allergic asthma, has been improved by the introduction of omalizumab, a recombinant humanized monoclonal anti-IgE antibody that binds to IgE at the same site as the high-affinity Fc ϵ RI receptor and downregulates high-affinity IgE receptors on circulating basophils and mast cells.^{3,4} The binding of omalizumab to IgE and the downregulation of high-affinity receptors result in potent inhibition of IgE-mediated release of inflammatory mediators. Omalizumab thus attenuates both the early-phase and late-phase responses to inhaled allergens in patients with asthma.⁵

In recent years, several large-scale clinical trials have evaluated the efficacy, safety, tolerability, and pharmacokinetics of single and multiple doses of omalizumab in patients with allergic asthma.^{6,7} In addition, several studies have shown improvement in allergic rhinitis, including a decrease of inflammatory mediators in patients receiving omalizumab.^{8,9} Although clinical improvements in the number of asthma exacerbation and quality of life are

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Abbreviations used

7AAD: 7-Amino-actinomycin

APC: Allophycocyanin

well documented,^{10,11} little is known about the immunologic pathways affected by the dramatic decrease of circulating free IgE that occur during treatment with omalizumab.

Our group recently demonstrated that treatment of allergic asthma with omalizumab resulted in a significant decrease of blood eosinophils from 6.2% at baseline to 1.3% (median) after 12 weeks of treatment.¹² In addition, other recent studies have shown that treatment with omalizumab led to significant reduction of eosinophil counts in the airway tissue (8.0 at baseline to 1.5 [median] eosinophils/mm² posttreatment) and induced sputum, a lower number of cells staining for IL-4,¹³ and downregulation of dendritic cells and leukotriene release.¹⁴ The late-phase reaction and influx of eosinophils into the skin after repeated skin allergen challenge was inhibited by treatment with omalizumab.¹⁵ In our previous study, we showed that treatment with omalizumab led to a decrease in circulating IL-13.¹² However, the precise mechanism of the reduction of eosinophil counts by treatment with anti-IgE remained unclear, although a direct interaction with the IgE receptor on eosinophils might be a contributing factor.¹⁶ In a model of allergic airway inflammation, IgE was shown to contribute to tissue infiltration of eosinophils and the production of T_H2 cytokines, which could be suppressed by the application of an anti-IgE antibody.¹⁷ In addition, the regulation of mediators influencing the survival of eosinophils secreted by other cells may also have been responsible. Downregulation of eosinophils provides an important additional pathway by which omalizumab reduces allergic inflammation. The observed influence of omalizumab therapy on bronchial hyperreactivity could be partly explained by the downregulation of eosinophils and IL-13 as an underlying mechanism, whereas IgE withdrawal in animal experiments did not influence bronchial hyperreactivity.¹⁸ Holgate et al¹⁹ summarized the anti-inflammatory effects of omalizumab in their recent review.

The aims of the current study were to determine if treatment with omalizumab results in an induction of eosinophil apoptosis and to investigate the changes of intracellular cytokine profiles in T lymphocytes.

METHODS

Study design

Patients with moderate-to-severe allergic asthma participated in this study, which was conducted as part of a randomized, double-blind, placebo-controlled, parallel-group trial.²⁰ All patients had a history of allergic asthma and allergic rhinitis with a positive skin prick test to a perennial allergen (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, cat or dog) and specific IgE (CAP; Pharmacia, Uppsala, Sweden). Patients had to have been

TABLE I. Baseline characteristics

Characteristics	Omalizumab	Placebo
Patients (n)	9	10
Female/male (n)	5/4	6/4
Age, y*	38 (26-65)	30 (20-61)
FEV ₁ , % predicted*	95 (44-120)	81 (77-102)
Beclomethasone dipropionate, µg/d*	800 (400-800)	800 (400-1600)
IgE, IU/L*	209 (54-573)	189 (65-356)
β ₂ -agonist, puffs/d*	0.143 (0-3.1)	0.43 (0-6.4)
Blood eosinophils, %*	3.4 (2.2-10.9)	3 (0.9-7.8)

*Values are presented as median (range).

receiving inhaled corticosteroids at doses equivalent to budesonide 400 to 1600 µg/d for at least 3 months, with a reversibility of FEV₁ > 12 % compared with baseline values within 30 minutes of taking salbutamol (200 µg). Long-acting β-agonists were allowed during the treatment period. The study was approved by the local ethics committee and in accordance with the Declaration of Helsinki. All patients gave written informed consent to participate.

Omalizumab or placebo was administered subcutaneously at 2-week or 4-week intervals. Doses were adjusted for body weight and total IgE at screening to ensure omalizumab administration at a dose of at least 0.016 mg/kg/IgE (IU/mL) every 4 weeks.

Blood was drawn by peripheral vein puncture in heparinized syringes before the administration of omalizumab or placebo at 3 times during the study; before the start of treatment (baseline), after 12 weeks of treatment, and 12 weeks after discontinuing treatment with omalizumab or placebo.

Eosinophils

Eosinophils were detected by a cytometric method in whole blood by identifying them as a granulocytic subpopulation with higher side and forward scatter characteristics.¹⁹ Whole blood samples (50 µL) taken from the patients were transferred into a reaction tube. Cells were washed and treated for 10 minutes at room temperature (20°C) with Becton Dickinson (BD) Lysis Buffer and washed with BD cell wash (BD Bioscience, Heidelberg, Germany).

Cells were stained by adding 5 µL antibodies for activation marker CD69 allophycocyanin (APC; BD Bioscience, San Diego, Calif) or CD95 peridinin-chlorophyll-protein. For detection of apoptosis, 5 µL Annexin V APC and 5 µL 7-amino-actinomycin (7-AAD; BD Bioscience, Heidelberg, Germany) to differentiate apoptosis and necrosis were added. Positive controls were performed by incubating cells with the apoptosis inducers camptothecin (5 µmol/L) or staurosporin (1 µmol/L; Sigma, Munich, Germany). After mixing and incubating for 25 minutes in the dark at room temperature, 400 µL Annexin Binding Buffer (BD Bioscience) was added to each tube. Negative controls were performed with isotype controls. Analysis was performed by flow cytometry (FACSCalibur; BD Bioscience, Oxnard, Calif). Results were analyzed by using Cell Quest Pro Software (Becton Dickinson, San Jose, Calif).

Intracellular cytokines

Heparinized blood 100 µL was treated with hypo-osmolar sodium chloride (NaCl) solution to lyse erythrocytes and then washed with PBS. Cells were stimulated for 6 hours with phorbol 12, 13-dibutyrate 10⁻⁷ mol/L, ionomycin 50 ng/mL, and Monesin 200 µmol/L (Sigma) in 920 µL AIM V medium (Gibco, Karlsruhe, Germany).

Cells were harvested and washed in PBS containing 10% FCS, washed, and antibodies CD3 (fluorescein isothiocyanate), CD8 (peridinin-chlorophyll-protein), CD4 (APC), or isotype control (BD

TABLE II. Eosinophils during therapy

Variable	Baseline		After 12 weeks of therapy		12 weeks after discontinuing therapy	
	Omalizumab	Placebo	Omalizumab	Placebo	Omalizumab	Placebo
CD95 ⁺	95.2 (80.1-99.8)	95.6 (85.8-99.4)	94.2 (69.8-98.5)	93.7 (71.1-98.9)	95.5 (88.0-97.5)	93.5 (78.7-98.4)
CD69 ⁺	8.3 (5.0-53.1)	8.8 (1.9-22.5)	7.1 (3.8-16.2)	9.2 (3.8-15.5)	8.1 (4.3-19.4)	7.8 (5.2-14.1)
Annexin ⁺	37.1 (11.4-89.5)	49.1 (13.5-87.5)	60.1** (26.6-95.5)	45.6 (19.4-92.8)	41.4 (27.0-70.8)	37.7 (11.2-88.8)
7AAD ⁺	13.3 (2.9-21.1)	18.9 (5.8-37.4)	10.9 (5.2-28.7)	14.5 (5.0-4.6)	16.3 (7.7-50.0)	12.5 (2.8-47.8)
Annexin ⁺ 7AAD ⁻	23.3 (3.9-86.9)	30.9 (5.6-64.2)	47.2** (18.0-90.9)	30.3 (12.2-56.4)	28.8 (20.3-57.6)	27.2 (5.2-53.3)
Annexin ⁺ 7AAD ⁺	7.5 (2.6-20.3)	15.4 (3.9-31.2)	9.0 (1.1-27.1)	13.4 (4.4-36.4)	13.1 (3.4-39.2)	8.4 (1.3-44.9)

Values are presented as median (range) of percentage of eosinophils positive for examined variables.

** $P < .01$.

Bioscience) for the identification of T-cell subtypes were added. Cells were fixed with 2% paraformaldehyde in PBS for 20 minutes at 4°C. After washing, cells were permeabilized with 0.5% saponin, washed, and 5 μ L anticytokine phycoerythrin-conjugated antibodies against IL-2, IL-5, IL-13, GM-CSF, IFN- γ , and TNF- α (BD Bioscience) were added and incubated for 30 minutes at 4°C. Cells were washed with PBS containing paraformaldehyde, resuspended, and measured in a FACS Calibur Cytometer (BD Biosciences). Analysis was performed by using Cell Quest Pro Software.

Statistical analysis

Between-group comparisons were made by using the Mann-Whitney U test. A 2-tailed P value of less than .05 was considered statistically significant. All tests compared the treatment groups in terms of percentage changes from baseline to week 12 unless otherwise noted. The in-group (before and after treatment) comparisons were made by using the Wilcoxon signed-rank test. P values (2-tailed) of less than .05 were considered statistically significant.

RESULTS

Patient characteristics

Nineteen patients were enrolled in the study (Table I). Baseline characteristics were well balanced between the treatment groups.

Eosinophils

Binding to Annexin V as marker of eosinophil apoptosis was significantly ($P = .004$) increased in the eosinophils of the omalizumab-treated group compared with placebo, whereas 7AAD was unchanged, indicating that cell death caused by necrosis was not important for this effect. Also, a significant ($P = .0039$) increase of Annexin-positive eosinophils was detectable comparing baseline to after 12 weeks of treatment in the omalizumab-treated group. No difference at the time of treatment was observed in the placebo group (Table II; Fig 1). There were no significant differences in the eosinophil activation marker CD69. The Fas receptor CD95 did not differ between the treatment groups (Table II; Fig 1).

Intracellular cytokines

Significant ($P = .018$) downregulation of GM-CSF⁺ lymphocytes in the omalizumab-treated group was detected compared with placebo, but also comparing baseline levels with those after 12 weeks of treatment

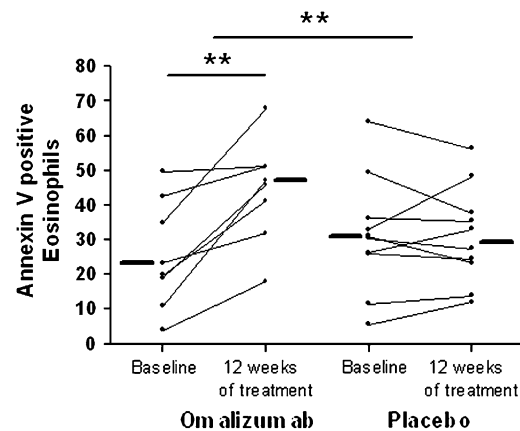


FIG 1. Eosinophil apoptosis. Individual values at baseline and week 12 of omalizumab therapy. The omalizumab group ($n = 9$) demonstrated a significant decrease in Annexin-positive eosinophils compared with placebo ($n = 10$). In the omalizumab-treated group, a significant decrease of Annexin-positive eosinophils comparing baseline with 12 weeks of treatment was found. Horizontal bars represent medians. ** $P < .01$.

($P = .0039$) in the omalizumab-treated group (Table III). This effect was predominantly a result of CD4 lymphocytes, as shown by a significant decrease in GM-CSF⁺ cells in this lymphocyte subset in the omalizumab-treated group comparing treatment groups, but also individual changes over the treatment period; no change was detected in the number of GM-CSF⁺ CD8 lymphocytes (Fig 2).

The number of IL-2⁺ lymphocytes was significantly lower ($P = .027$) in the omalizumab-treated group than in the placebo group. In the omalizumab-treated group a significant reduction of IL-2/CD3⁺ lymphocytes ($P = .0078$) was also detected comparing baseline values with those after 12 weeks of treatment. The number of CD4⁺ cells was significantly decreased ($P = .02$), whereas the number of IL-2⁺ CD8 lymphocytes was also significantly ($P = .034$) reduced (Fig 3).

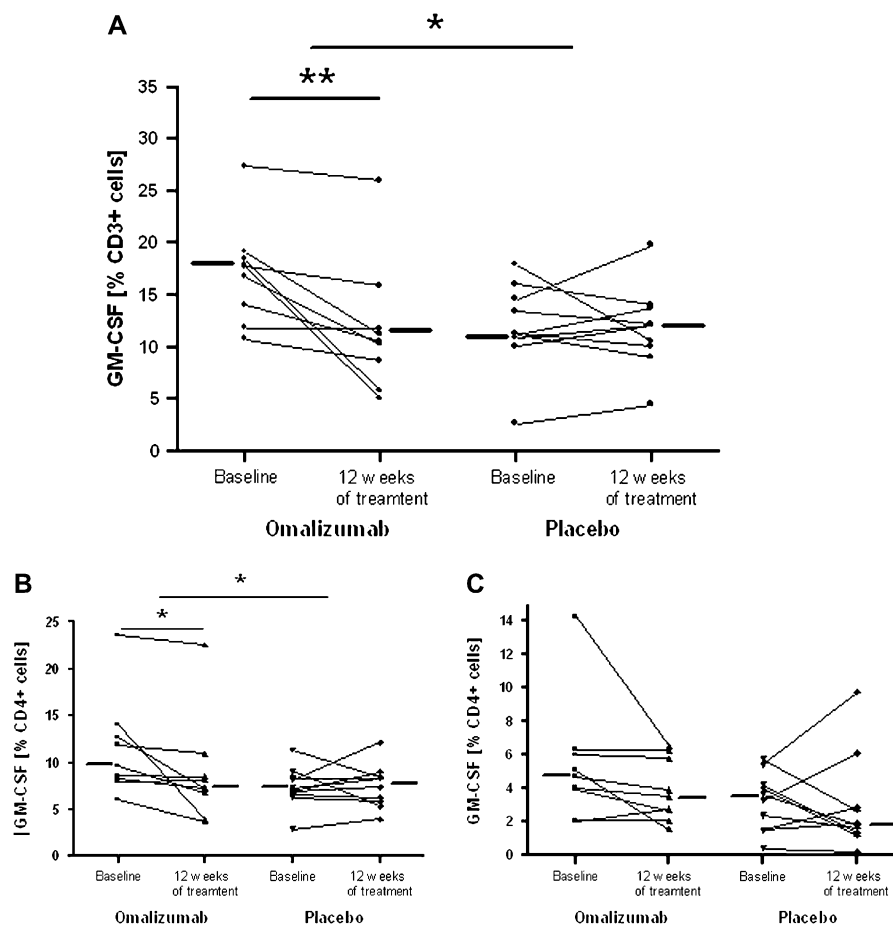
Lymphocytes with a positive signal for IL-13 were significantly decreased ($P = .027$) in the omalizumab-treated group compared with placebo. Comparing the values at baseline with 12 weeks of treatment in the omalizumab group, a significant reduction ($P = .0039$)

TABLE III. Cytokine-positive cells during therapy†

Variable	Baseline		After 12 weeks of therapy		12 weeks after discontinuing therapy	
	Omalizumab	Placebo	Omalizumab	Placebo	Omalizumab	Placebo
IL-2 CD3 ⁺	30.0 (17.6-37.0)	23.2 (13.4-33.5)	19.0** (6.9-29.4)	22.3 (11.5-35.1)	23.8 (17.5-41.3)	19.8 (12.9-37.0)
IL-2 CD4 ⁺ /CD3 ⁺	21.5 (16.4-34.5)	17.6 (12.6-28.3)	17.6* (6.4-27.1)	18.5 (9.8-29.7)	21.7 (13.8-34.6)	19.1 (13.8-31.8)
IL-2 CD8 ⁺ /CD3 ⁺	3.00 (1.2-7.6)	1.00 (0.32-4.2)	1.62** (0.13-6.5)	1.65 (0.52-6.1)	2.07 (0.34-7.0)	1.29 (0.48-4.2)
IL-13 CD3 ⁺	1.70 (1.07-6.0)	0.77 (0.17-1.5)	1.14** (0.17-3.1)	0.81 (0.19-2.7)	1.24 (0.76-3.1)	0.98 (0.26-3.8)
IL-13 CD4 ⁺ /CD3 ⁺	1.75 (0.92-4.8)	0.83 (0.18-1.7)	1.30 (0.21-2.4)	0.71 (0.28-1.5)	1.21 (0.77-3.4)	1.00 (0.24-3.0)
IL-13 CD8 ⁺ /CD3 ⁺	0.52 (0.18-2.07)	0.16 (0.06-0.4)	0.29* (0.2-1.07)	0.22 (0.07-0.83)	0.29* (0.07-0.79)	0.27 (0.06-0.8)
GM-CSF CD3 ⁺	17.7 (10.8-27.4)	11.2 (2.6-17.9)	10.5** (5.0-26.0)	12.1 (4.5-19.8)	13.4 (9.4-37.5)	9.4 (6.7-14.5)
GM-CSF CD4 ⁺ /CD3 ⁺	9.5 (6.0-23.0)	7.1 (2.8-11.3)	7.3* (3.7-22.5)	7.8 (3.9-12.1)	8.3 (6.2-30.5)	6.9 (4.8-15.9)
GM-CSF CD8 ⁺ /CD3 ⁺	4.7 (2.0-14.3)	3.5 (0.46-5.8)	3.5 (1.6-6.6)	1.9 (0.2-9.8)	3.0 (1.7-7.0)	2.9 (0.7-13.6)

* $P < .05$; ** $P < .01$.

†Values are presented as median (range) of percentage cells positive for examined variables.

**FIG 2.** Intracellular GM-CSF in T lymphocytes. Individual values at baseline and week 12 of omalizumab therapy in (A) GM-CSF⁺/CD3⁺ lymphocytes, (B) GM-CSF⁺/CD4⁺ lymphocytes, and (C) GM-CSF⁺/CD8⁺ lymphocytes. Horizontal bars represent medians. ** $P < .01$; * $P < .05$.

of IL-13/CD3⁺ lymphocytes was detected. CD8 lymphocytes were responsible for the reduction in the number of positive cells in the CD3⁺ fraction, because a significant decrease was observed in the CD8⁺ subpopulation, but not in the CD4⁺ population (Fig 4). After another

statistical examination of the results, excluding the outlying data pair in Fig 2, C, Fig 3, A, and Fig 4, A and B, most results still turned out to be statistically significant. There were no significant differences in IL-5, IFN- γ , or TNF- α between the omalizumab and placebo-treated groups.

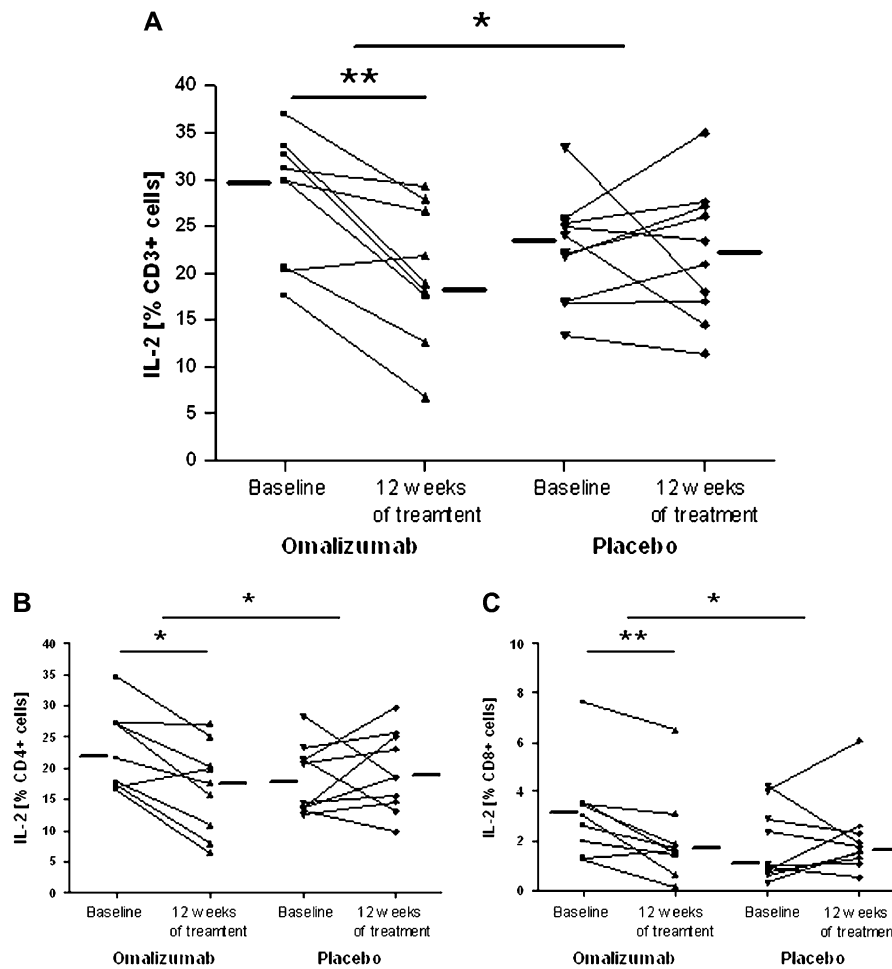


FIG 3. Intracellular IL-2 in T lymphocytes. Individual values at baseline and week 12 of omalizumab therapy in (A) IL-2⁺CD3⁺ lymphocytes, (B) IL-2⁺CD3/CD4⁺ lymphocytes, and (C) IL-2⁺CD3/CD8⁺ lymphocytes. Horizontal bars represent medians. ***P* < .01; **P* < .05.

DISCUSSION

This study shows that treatment with omalizumab leads to increased eosinophil apoptosis. Furthermore, we found a reduction in cellular production of GM-CSF, an important factor in eosinophil growth and survival.²¹ The fact that CD4 lymphocytes are the predominant subpopulation involved in this effect is important, because this population of lymphocytes orchestrates the late-phase allergic response that is accompanied by an influx of eosinophils. Furthermore, IL-2 and IL-13 producing lymphocytes were also reduced, which is compatible with our recent observation of decreased serum IL-13 in patients receiving omalizumab.¹² Although IL-5 is known to be an important growth and survival factor of eosinophils, it was not affected significantly by omalizumab treatment in this study. The efficacy of omalizumab in the improvement of asthma symptoms has been described in numerous studies in the last decade.^{2,5-7,10,11} The described effects include the regulatory potency of omalizumab on the number of circulating and tissue eosinophils.^{12,13} Our study offers a possible

explanation for this finding, because we could demonstrate increased eosinophil apoptosis. Although the precise mechanism remains to be elucidated, we provide a possible mechanism for the induction of eosinophil apoptosis by the reduced production of GM-CSF in lymphocytes. It has to be considered that the reduction of mediators in T cells was measured because of the experimental design in stimulated lymphocytes to reach mediator levels measurable in the flow cytometry. Therefore, this study could not answer whether these effects are also present under physiological conditions *in vivo* without stimulation. However, our findings provide a further clue to explain noted clinical effects. Numerous studies have shown that eosinophil viability is strongly dependent on cytokines such as IL-5 and GM-CSF.²²⁻²⁵ From our data, it is impossible to determine definitely whether increased apoptosis was a result of the direct effects of interaction with eosinophils or secondary effects of secreted cytokines. As a consequence of omalizumab therapy, a downregulation of IgE receptors was reported on dendritic cells, basophils, and mast cells.^{3,4,26} This effect might also

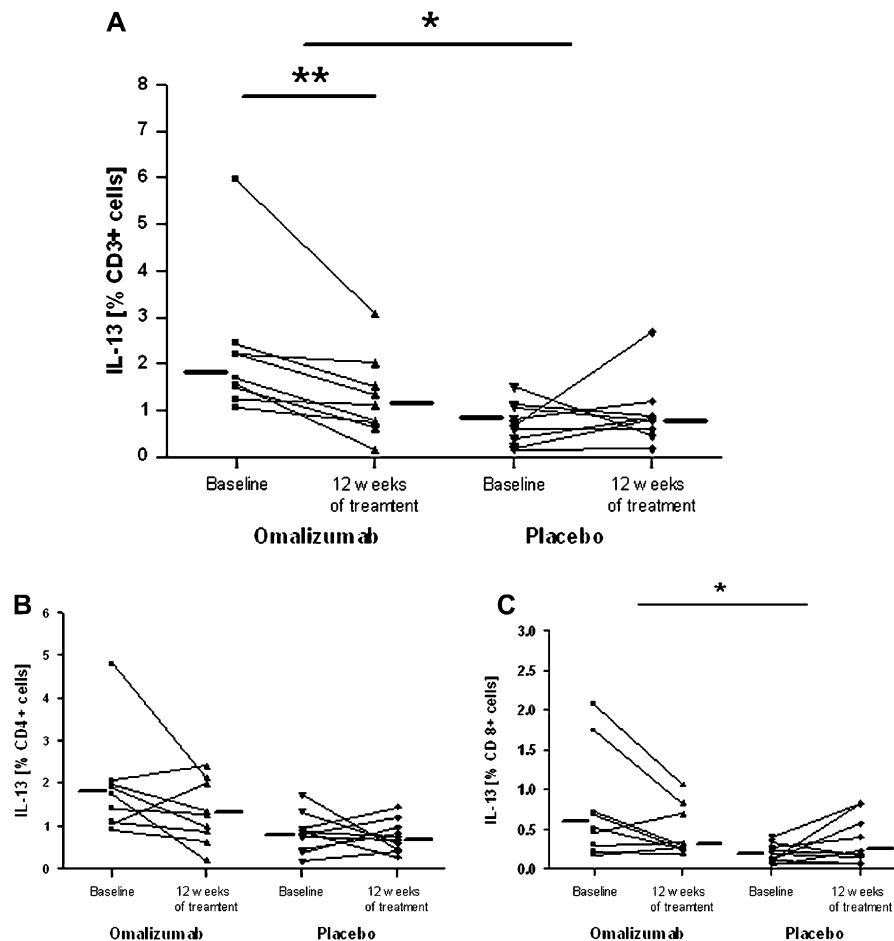


FIG 4. Intracellular IL-13 in T lymphocytes. Individual values at baseline and week 12 of omalizumab in (A) IL-13⁺CD3⁺ lymphocytes, (B) IL-13⁺CD3/CD4⁺ lymphocytes, and (C) IL-13⁺CD3/CD8⁺ lymphocytes. Horizontal bars represent medians. ** $P < .01$; * $P < .05$.

account for changes in lymphocytes. IgE possibly plays a role in directly maintaining eosinophils. The elimination of circulating IgE might result in increased apoptosis of eosinophils.

Omalizumab is an effective therapy for patients with allergic diseases, influencing both the early and late allergic response. The decrease in IL-2⁺ cells might be an additional effect of omalizumab, because as well as having proliferative effects, IL-2 influences eosinophil activity.^{27,28}

In view of the outstanding regulatory potential of IL-13 in the pathophysiology of asthma, it might be of importance that we describe a decrease of IL-13⁺ T cells. Recently we were able to demonstrate a decrease of serum IL-13. The reduced number of IL-13⁺ T cells might offer an explanation for the finding in circulating levels and provides a possible source of IL-13. This finding is particularly important for the asthma pathogenesis because IL-13 is related to bronchial hyperreactivity, IgE production, and further variables characterizing asthma.²⁹

In conclusion, our results give insight into the anti-allergic and anti-inflammatory properties of anti-IgE,

demonstrated by an increase in eosinophil apoptosis and decrease in GM-CSF⁺, IL-2⁺, and IL-13⁺ T lymphocytes in omalizumab-treated patients. Our findings underline additional anti-inflammatory activity related to eosinophil and T-lymphocyte function, which is consistent with the findings of other recent studies. Further investigation is needed to determine whether a direct interaction via IgE receptors or secondary mechanisms involving decreased liberation of mediators is responsible for these anti-inflammatory effects. In addition to its proven clinical efficacy, omalizumab provides a valuable tool for examining allergic inflammation in the absence of circulating free IgE.

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