
The effects of omalizumab on IgE-induced cytokine synthesis by asthmatic airway smooth muscle cells

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Background: Human airway smooth muscle cells (ASMCs) express high- and low-affinity IgE receptors and respond to IgE, thereby contributing to airway inflammation.

Objective: To determine whether anti-IgE antibodies (omalizumab) block the response of ASMCs to IgE in patients with asthma.

Methods: Airway smooth muscle cells, isolated from the biopsy specimens of patients with asthma, patients with chronic obstructive pulmonary disease, and control participants (6 in each group), were stimulated with IgE with and without omalizumab treatment, and cytokine secretion was determined by enzyme-linked immunosorbent assay, and messenger RNA (mRNA) secretion by real-time polymerase chain reaction, over 24 hours. IgE receptor expression was determined by immunoblotting.

Results: IgE-stimulated mRNA synthesis encoded for interleukin (IL) 6, IL-8, and tumor necrosis factor α in ASMCs via mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2, or p38. The secretion of the respective cytokines increased significantly: IL-6, IL-8, and tumor necrosis factor α at 6 hours and IL-4 at 24 hours. Cytokine mRNA synthesis and protein secretion were inhibited by omalizumab in a dose-dependent manner. The expression of low- and high-affinity IgE receptors was not altered by omalizumab in ASMCs.

Conclusions: Omalizumab reduced IgE-stimulated synthesis and secretion of proinflammatory cytokines by human ASMCs. These findings imply a beneficial action of omalizumab in asthma therapy. This effect is not restricted to inflammatory cells; it also includes tissue-forming cells.

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INTRODUCTION

In patients with asthma, it is assumed that the major source of proinflammatory mediators is immune-competent cells,¹ whereas the potential contribution of tissue-forming cell types (eg, fibroblasts and airway smooth muscle cells [ASMCs]) is widely ignored.² A prominent inducer of the synthesis of proinflammatory cytokines by immune cells is IgE, and IgE is enhanced in approximately 70% of patients with asthma because of an inherited condition.^{3,4} Thus, the idea of blocking IgE action as a therapy for atopic asthma was tested.^{5,6}

It has been known for some time that ASMCs express low- and high-affinity IgE receptors and respond to IgE stimulation^{7,8}; however, surprisingly, not much attention has been given to these observations regarding the role of recently available anti-IgE therapy on airway wall resident cells in

asthma.^{9,10} A summary of the possible functions of IgE receptors expressed by ASMCs and their contribution to asthma pathologies was provided by Gounni.¹¹ Therefore, the question addressed in this study was whether clinically used anti-IgE antibodies, such as the humanized anti-IgE antibody omalizumab, which is used in the treatment of allergic asthma, have a reducing effect on IgE-induced cytokine production by ASMCs.

The ASMCs not only constrict during an asthma attack, they also are a major contributor to the remodeling of the airway wall's structure and are a potential source of proinflammatory cytokines.^{2,12} Regarding IgE-mediated inflammation in patients with asthma, ASMCs have been shown to release proinflammatory cytokines in response to IgE contact, such as interleukin (IL) 8 and eotaxin, which both attract neutrophils and granulocytes to the inflamed tissue.^{7,8,12} In clinical studies, the therapy with anti-IgE antibodies reduced inflammation and other symptoms in patients with atopic asthma.^{9,10} Omalizumab, a humanized recombinant monoclonal anti-IgE antibody, has been approved for the therapy of allergic asthma and acts by reducing serum IgE levels and IgE receptor expression on inflammatory cells; however, it is unknown if the compound has any beneficial effect on other cell types.¹³

Therefore, we addressed the question of whether omalizumab (Xolair) affects IgE-induced cytokine release by primary human ASMCs obtained from patients with asthma or

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chronic obstructive pulmonary disease (COPD) or from healthy control participants.

METHODS

Primary Human ASMCs

Bronchial tissue biopsy specimens were obtained from 18 patients (6 with asthma, 6 with COPD, and 6 without asthma and without atopy) after written consent and approval by the local ethics committee. Clinical characteristics are provided in Table 1. None of the probands had used any antiasthma therapy 3 weeks before the biopsy; therefore, the drugs listed in Table 1 only indicate that the respective patient uses the drug(s) when needed at other times. The ASMCs were isolated and grown in Roswell Park Memorial Institute 1640 medium supplemented with 5% fetal calf serum, 8-mM/L L-glutamine, 20-mM/L N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonate, and 1 minimal essential medium-vitamin mix (all obtained from Gibco/BRL, Lucerne, Switzerland), as described earlier.¹⁴ Each cell line was characterized by positive immunostaining for calponin and α smooth muscle cell actin.¹⁴

Study Design

Before the experiments, ASMCs were serum starved (using 0.1% fetal calf serum for 24 hours). Then, the ASMCs were stimulated with human IgE (0, 0.01, 0.10, 1.00, and 5.00 μ g/mL) for 0, 6, 12, or 24 hours. Omalizumab (0, 0.1, 0.5, or

1.0 μ g/mL) was added, together with activated IgE, and cell culture medium was sampled at 0, 3, 6, 12, and 24 hours; 1 mL of serum-free medium was added to compensate for liquid loss.

Highly purified monoclonal human IgE was obtained from Diatek, Oslo, Norway; purified human IgG (Diatek) or omalizumab alone served as the control.

Signaling of mitogen-activated protein kinases (MAPKs) was inhibited by chemical inhibitors. In MAPK inhibitory experiments, cells were pretreated for 30 minutes with either the extracellular signal-regulated kinase (ERK) 1/2 MAPK inhibitor PD98059, 10 μ M, or the p38 MAPK inhibitor SB203580, 10 μ M (both obtained from Calbiochem-Novabiochem, L  ufelfingen, Switzerland).

Cytokine Enzyme-Linked Immunosorbent Assay and Immunoblotting

Tumor necrosis factor (TNF) α , IL-4, IL-5, IL-6, IL-8, and IL-13 were detected by commercially available enzyme-linked immunosorbent assay kits (Orgenium, Helsinki, Finland). Cytokine concentrations were adjusted to cell counts, determined at the end of the 24-hour IgE stimulation, as described earlier.¹⁴

Membrane protein fractions were isolated using a MemPER kit (Pierce Biotechnology Inc, Rockford, Illinois), according to instructions. Protein samples, 10 μ g, were size fractionated by electrophoresis (100 V for 60 minutes) using 4% to 20% gradient polyacrylamide gel electrophoresis (Pierce Biotechnology Inc). Proteins were transferred onto nitrocellulose membranes by overnight diffusion in a special buffer (0.05-M sodium chloride, 2-mM/L sodium-EDTA, 0.1-mM dithiothreitol, and 10-mM Tris-hydrochloride [pH, 7.0]) at 50  C. Membranes were incubated (for 1 hour at room temperature) with blocking buffer (5% wt/vol nonfat dry milk in Tris-buffered saline and 0.1% polysorbate [Tween] 20); washed 3 times with phosphate-buffered saline; incubated overnight (at 4  C) with a primary antibody (low-affinity IgE, Santa Cruz Biotechnology, sc-70592, diluted 1:1,000; or high-affinity IgE [BD Bioscience, Basel, Switzerland], catalogue No. 551469, diluted 1:2,500) in Tris-buffered saline-polysorbate 20 (30 minutes at room temperature); and washed 3 times and incubated with horseradish peroxidase-conjugated IgG antibodies (Santa Cruz Biotechnology, sc-2005, diluted 1:5,000 to 1:10,000) for 1 hour before being visualized by electrochemiluminescence substrate (Pierce Biotechnology Inc). Densitometric analysis was performed using National Institutes of Health Image-J densitometric analysis software.

Cell Counts, Real-Time Polymerase Chain Reaction, and Statistics

After trypsin treatment and detachment, the cells were counted in an improved Neubaur chamber slide according to standard procedures.¹⁴

Total RNA was extracted from cells using a QuantiFast Multiplex polymerase chain reaction (PCR) kit (80) (Qiagen,

Table 1. Clinical Characteristics of the Study Participants^a

Diagnosis	FEV ₁ predicted, %	Age, y/sex	Treatment
Asthma	71	18/M	iGC/i β ₂
	69	25/M	iGC/i β ₂
	64	32/F	iGC/i β ₂
	74	54/F	iGC/i β ₂
	68	29/M	iGC/i β ₂
	68	42/M	iGC/i β ₂
COPD	65	36/M	NA
	61	38/M	iGC/i β ₂
	70	29/F	NA
	63	32/F	iGC/i β ₂
	64	42/F	iGC/i β ₂
	66	48/F	NA
Control	NA	24/F	None
	NA	28/F	None
	NA	35/M	None
	NA	46/M	None
	NA	26/F	None
	NA	29/M	None

Abbreviations: COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 second; i β ₂, β ₂-antagonist; iGC, glucocorticoid; NA, not applicable.

^a For the asthma group, the mean (SEM) values were as follows: FEV₁ predicted, 69.0% (3.1%); and age, 33.3 (5.7) years. For the COPD group, the mean (SEM) values were as follows: FEV₁ predicted, 64.8% (2.3%); and age, 37.5 (4.3) years. For the control group, the mean (SEM) age was 31.3 (5.7) years.

Hombrechtikon, Switzerland). Real-time PCR for all cytokines was performed using primer pairs and conditions, as described by the distributor (IL-4, QT00012565; IL-6, QT00083720; IL-8, QT00000322; and TNF- α , QT01079561; all obtained from Qiagen).

The null hypothesis was as follows: (1) "no differences between the 3 diagnostic groups" or (2) "no effect of omalizumab." Diagnostic groups were compared by analysis of

variance, and the effect of omalizumab was tested using the paired *t* test. All data are presented as mean (SEM).

RESULTS

In the presence of IgE (range, 0.1–10.0 $\mu\text{g/mL}$), the release of IL-6 increased significantly in all 3 patient groups in a dose-dependent manner (Fig 1A). More important, IgE-stimulated ASMCs of patients with asthma released significantly more

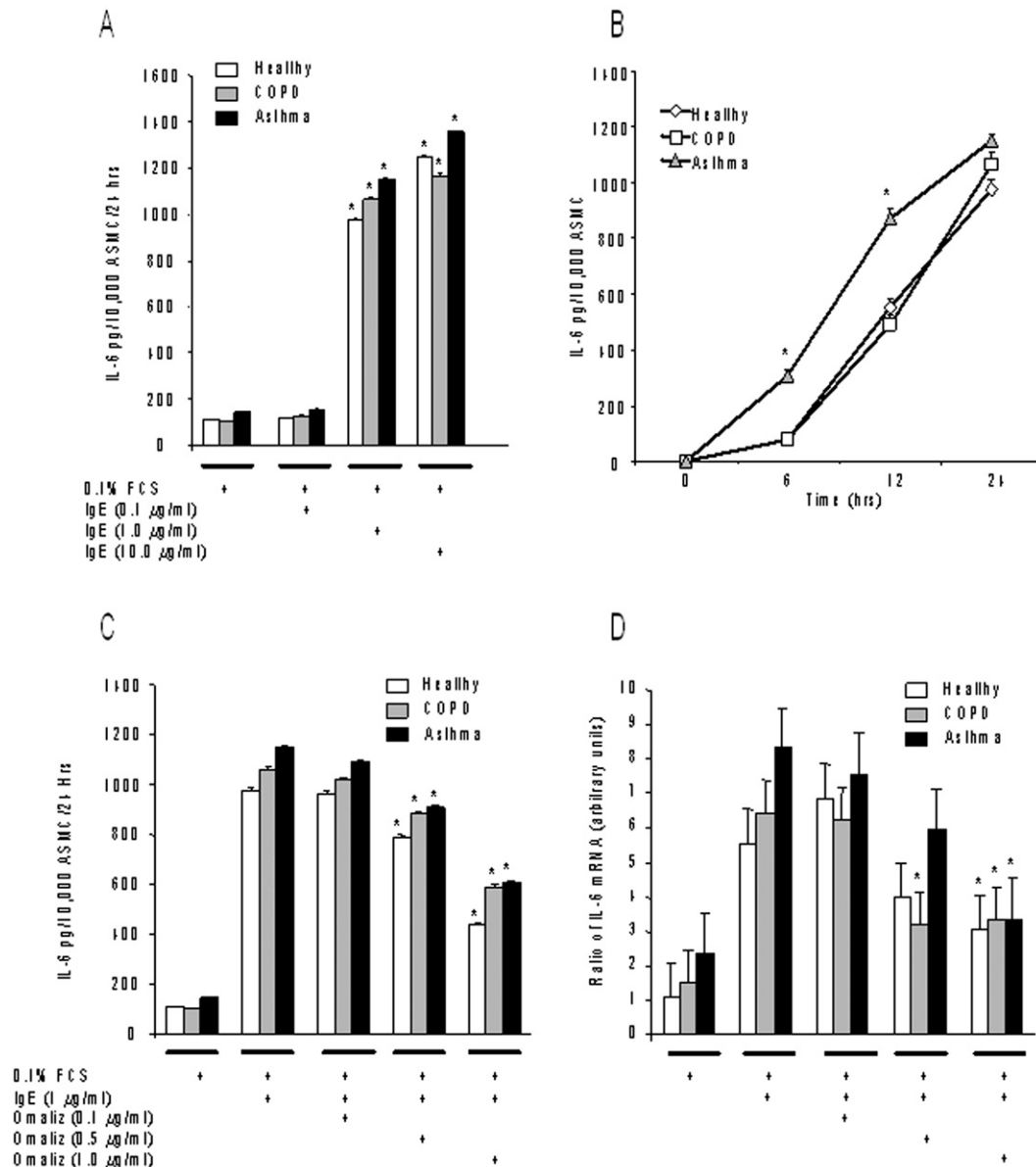


Figure 1. Disease-specific interleukin (IL) 6 secretion by airway smooth muscle cells (ASMCs) and the inhibitory effect of omalizumab (Omaliz). A, IgE-induced IL-6 secretion (12 hours). $*P < .01$. B, Kinetics of IL-6 inhibition by omalizumab. $*P < .05$. C, Omalizumab inhibits IL-6 secretion (24 hours). $*P < .05$. D, Omalizumab inhibits IgE-induced IL-6 messenger RNA (mRNA) synthesis (3 hours, real-time polymerase chain reaction). $*P < .05$. Data represent the mean (SEM) obtained in healthy controls ($n = 5$), patients with chronic obstructive pulmonary disease (COPD) ($n = 5$), and patients with asthma ($n = 6$). ACT-IgE, activated IgE; FCS, fetal calf serum.

IL-6 at early time points compared with cells of patients with COPD or of controls (Fig 1B). However, the disease-specific higher IL-6 secretion by ASMCs of patients with asthma became insignificant at 24 hours (Fig 1B). Omalizumab significantly inhibited the IgE-induced secretion of IL-6 in a dose-dependent manner by a maximum of 50% (Fig 1C). The effect of omalizumab on IL-6 messenger RNA (mRNA) synthesis by real-time PCR was determined, and the antibody maximally reduced the mRNA level by 70% (Fig 1D).

IgE (range, 0.1–10.0 $\mu\text{g/mL}$) stimulated IL-8 secretion by ASMCs significantly after 12 hours in all 3 groups in a dose-dependent manner (Fig 2A). This effect was most prominent in the ASMCs of patients with asthma at 12 hours (Fig 2B). Omalizumab inhibited the IgE-induced IL-8 secretion in a dose-dependent manner, with a maximum inhibition of 100% (Fig 2C). Real-time PCR showed that the IgE-induced IL-8 secretion was preceded by transcription, which was inhibited by a maximum of 63% by omalizumab (Fig 2D).

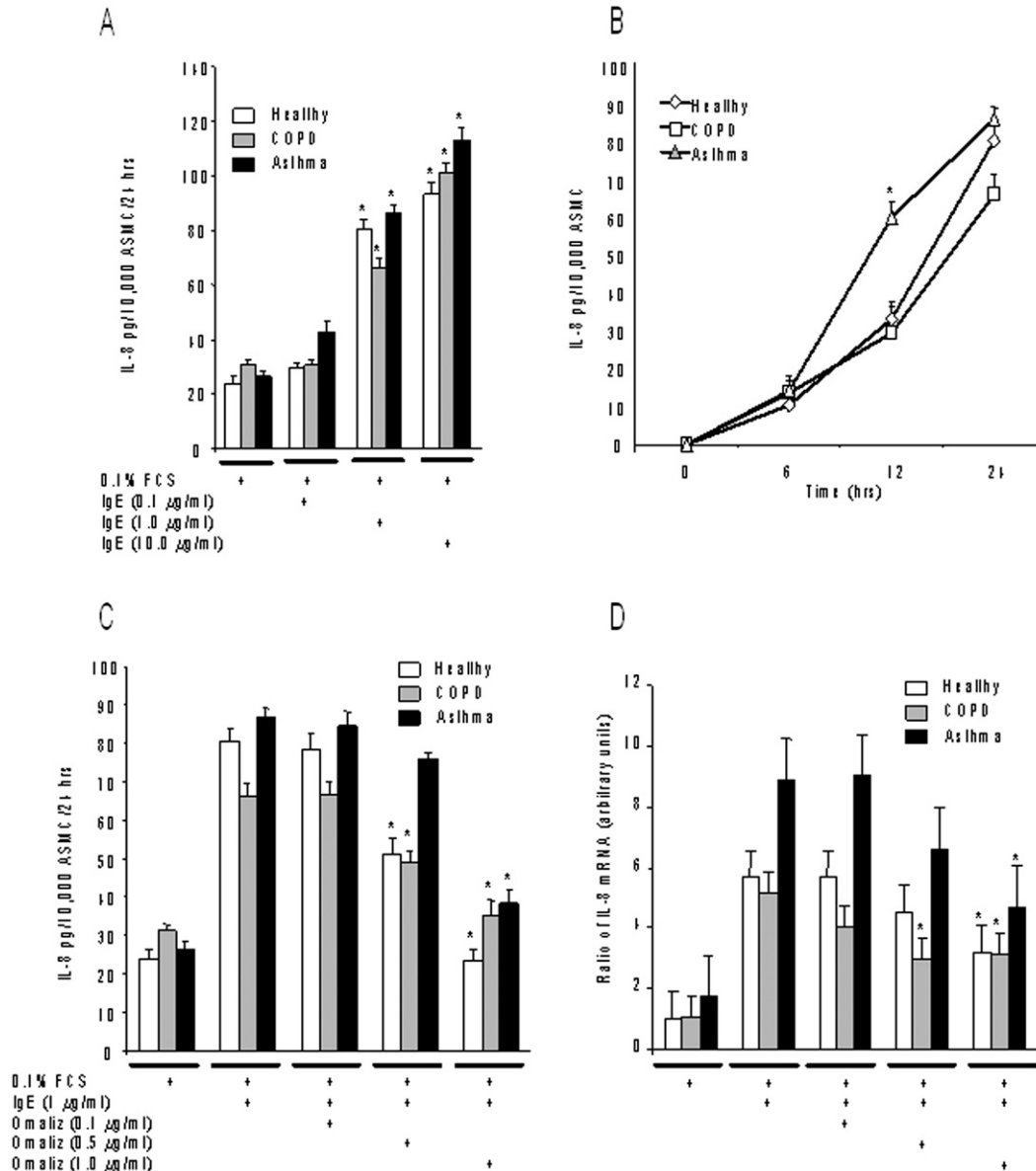


Figure 2. Disease-specific interleukin (IL) 8 secretion by airway smooth muscle cells (ASMCs) and the inhibitory effect of omalizumab (Omaliz). A, Dose-dependent IgE-induced stimulation of IL-8 secretion (12 hours). $*P < .01$. B, Interleukin 8 secretion kinetic (24 hours). $*P < .05$. C, Omalizumab blocks IgE-induced IL-8 secretion (12 hours). $*P < .05$. D, Omalizumab reduces IgE-induced IL-8 messenger RNA (mRNA) synthesis (6 hours). $*P < .05$. Data represent the mean (SEM) obtained in healthy controls ($n = 5$), patients with chronic obstructive pulmonary disease (COPD) ($n = 5$), and patients with asthma ($n = 6$). FCS indicates fetal calf serum.

As depicted in Figure 3A, the secretion of TNF- α was significantly increased when stimulated with IgE at 12 and 24 hours and was significantly higher in the ASMCs of patients with asthma compared with those of other groups (Fig 3A). However, the data imply that TNF- α secretion had not reached its maximum at 24 hours (Fig 3B). Omalizumab inhibited TNF- α protein secretion, with a significantly lower

effect on asthma ASMCs compared with COPD and control cells (Fig 3C). Tumor necrosis factor α mRNA synthesis was down-regulated by omalizumab, with no difference in the effect when comparing the 3 groups (Fig 3D).

Of the remaining cytokines tested, only IL-4 was secreted at detectable amounts by ASMCs at 24 hours of IgE stimulation and was similar in the 3 groups at this time (Fig 4A).

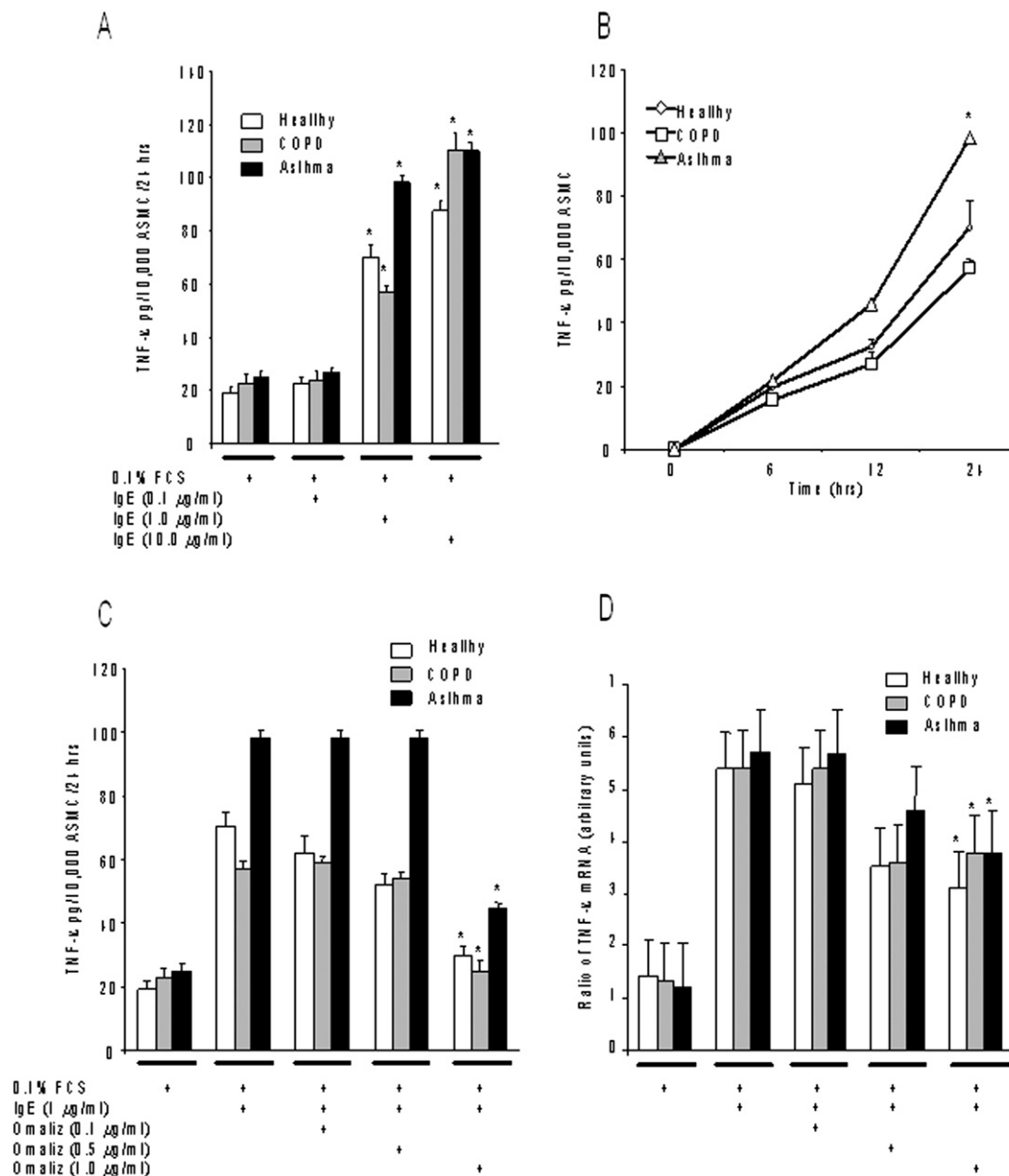


Figure 3. Disease-specific IgE-induced tumor necrosis factor (TNF) α synthesis by airway smooth muscle cells (ASMCs) and the inhibitory effect of omalizumab (Omaliz). A, IgE-induced TNF- α secretion. $*P < .01$. B, Disease-specific TNF- α kinetic. $*P < .05$. C, Inhibition of TNF- α secretion (24 hours) by omalizumab. $*P < .05$. D, Inhibition of TNF- α messenger RNA (mRNA) synthesis (12 hours) by omalizumab. $*P < .05$ compared with IgE-stimulated cells. Data represent the mean (SEM) in controls (n = 5), patients with chronic obstructive pulmonary disease (COPD) (n = 5), and patients with asthma (n = 6). FCS indicates fetal calf serum.

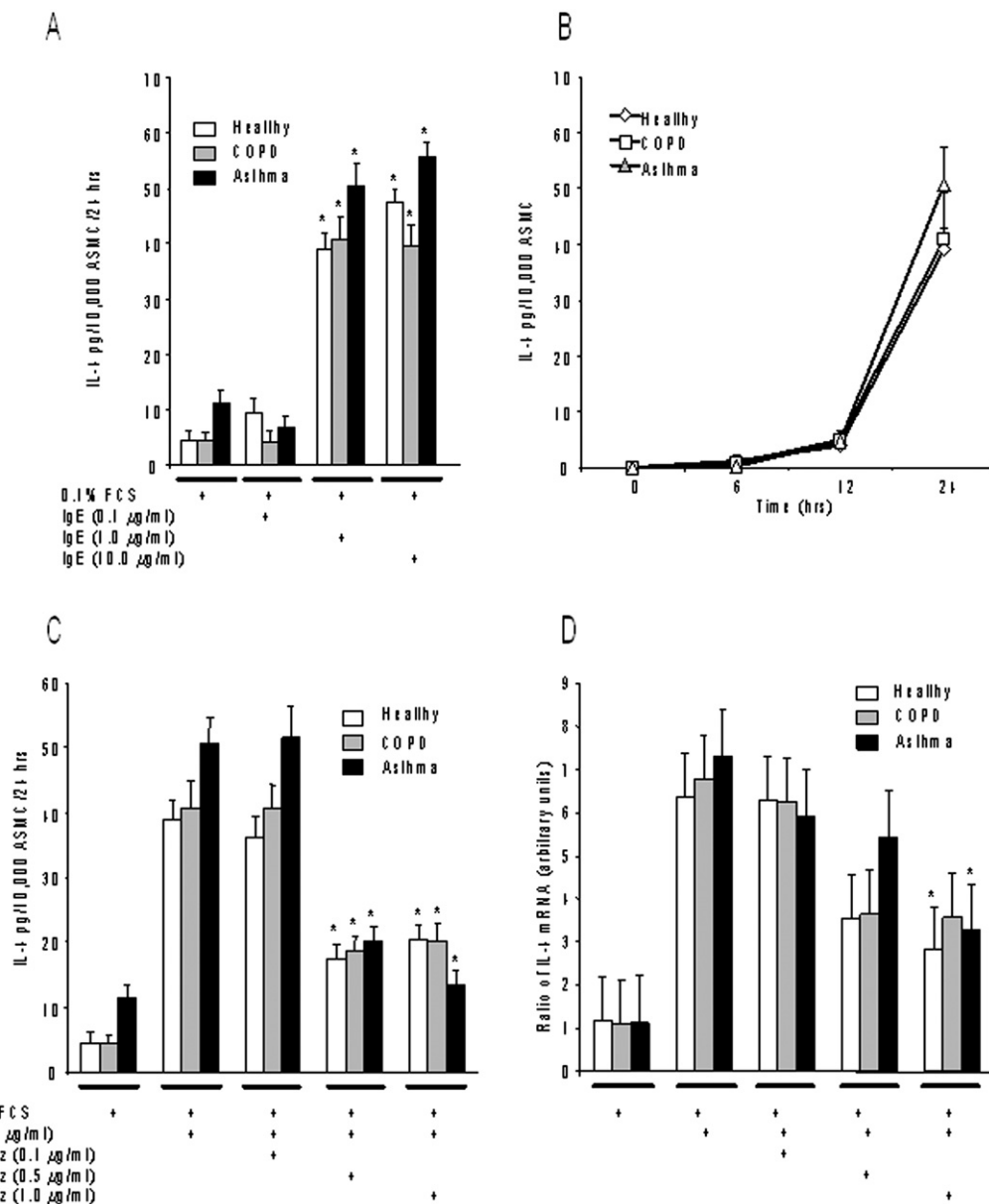


Figure 4. IgE-induced interleukin (IL) 4 synthesis by airway smooth muscle cells (ASMCs) and the inhibitory effect of omalizumab (Omaliz). A, IgE-induced IL-4 secretion (24 hours). $*P < .01$. B, Kinetics of IL-4 secretion. $*P < .05$. C, Omalizumab inhibits IL-4 secretion (24 hours). $*P < .05$. D, Omalizumab inhibits IL-4 messenger RNA (mRNA) synthesis (12 hours). $*P < .05$. Data represent the mean (SEM) of controls (n = 5), patients with chronic obstructive pulmonary disease (COPD) (n = 5), and patients with asthma (n = 6). FCS indicates fetal calf serum.

The effect of IgE on IL-4 secretion was dose dependent (Fig 4B), and omalizumab inhibited IL-4 secretion (Fig 4C) and IL-4 mRNA synthesis (Fig 4D) significantly.

IgE did not stimulate the release of IL-5 or IL-13 by ASMCs within the observation period.

In addition, we evaluated the effect of omalizumab (0.1 and 1.0 μ g/mL) on the expression of low- and high-affinity IgE receptors by ASMCs after 24 hours of stimulation of IgE (0.1 and 1.0 μ g/mL). The ASMCs of all 3 groups expressed

the high- and the low-affinity IgE receptor without any significant difference comparing the groups by semiquantitative immunoblot analysis. Omalizumab did not affect the expression of IgE receptors by ASMCs within 24 hours (Fig 5A). The data were confirmed by immunostaining (not shown).

By using chemical inhibitors for MAPKs, we found that most of the IgE-stimulated cytokine synthesis and secretion was controlled by either ERK 1/2 or p38 MAPKs, which were phosphorylated by IgE stimulation; this was blocked by

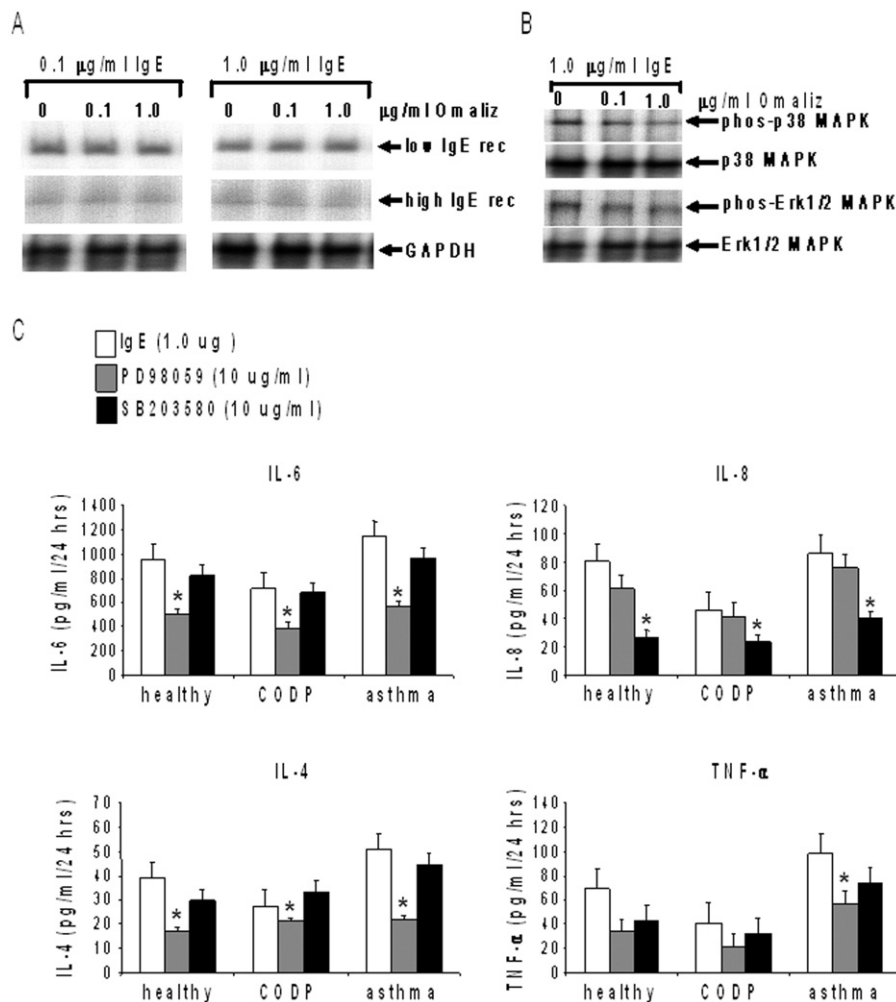


Figure 5. The effect of omalizumab (Omaliz) on IgE receptor expression and signaling in airway smooth muscle cells (ASMCs). A, Representative immunoblot of IgE receptor expression at 24 hours: similar results were obtained in 2 additional cell lines and glyceraldehyde phosphate dehydrogenase as equal protein loading control. B, Representative immunoblot of extracellular signal-regulated kinase (ERK) 1/2 and p38 MAP kinases (MAPKs) phosphorylation (phos) at 15 minutes: similar results were obtained in 2 additional cell lines. C, Role of ERK 1/2 and p38 MAPKs in IgE signaling (PD, PD98059, 10 μ M; SB, SB203580, 10 μ M). Data represent the mean (SEM) of triplicate experiments in nonasthmatic cells. * $P < .05$. COPD indicates chronic obstructive pulmonary disease; IL, interleukin; and TNF, tumor necrosis factor.

omalizumab (Fig 5B). Blocking ERK 1/2 MAPK by PD98059, 10 μ M, resulted in a significant down-regulation of the secretion of IL-6, TNF- α , and IL-4, whereas the secretion of IL-8 was reduced by the inhibition of p38 MAPK by SB203580, 10 μ M (Fig 5C).

DISCUSSION

IgE stimulated the de novo synthesis and secretion of IL-4, IL-6, IL-8, and TNF- α by ASMCs. The stimulatory IgE effect was stronger for IL-6, IL-8, and TNF- α in cells obtained from patients with asthma compared with cells obtained from patients with COPD or from controls. Omalizumab significantly reduced cytokine mRNA and protein expression in all 3 groups in a dose- and time-dependent

manner. The IgE-induced cytokine synthesis was mediated by ERK 1/2 and/or p38 MAPKs in a cytokine-specific manner, and both signaling pathways were inhibited by omalizumab. The expression of low- and high-affinity IgE receptors was not altered in the ASMCs by omalizumab.

Omalizumab is an example of a novel type of IgE-reducing and mast cell-stabilizing drug, which was approved and used for the treatment of atopic asthma.^{10,13} Omalizumab therapy significantly improved asthma symptoms,¹⁵ reduced the frequency of asthma exacerbations, and lowered the use of inhaled corticosteroids¹⁶; thus, the anti-IgE antibody improves the quality of life of patients with allergic asthma.

In isolated human ASMCs, we observed a disease-specific significantly higher secretion of IL-6, IL-8, and TNF- α by

cells of patients with asthma in response to IgE stimulation. This may be linked to either an increase of disease-specific IgE receptor responsiveness or subsequent intracellular signaling pathways,^{11,17,18} or it may be the result of modified expression of IgE receptors in the ASMCs by patients with asthma.^{7,8,11} These findings suggest that ASMCs, diseased or healthy, respond to IgE; however, cells from patients with asthma respond more strongly. Such an asthma-specific increase of IL-6 and TNF- α secretion was described when cells were infected with rhinovirus or lipopolysaccharide.¹⁹ The asthma-specific increase of certain cytokines is supported by a study showing that IL-6 secretion by asthma ASMCs was significantly elevated when stimulated by Fc-activated OX40 via protein kinase C β_2 .²⁰ The inhibition of TNF- α in asthmatic lungs reduced a wide range of inflammatory processes²¹; similar to our observation, omalizumab inhibited the IgE-induced IL-6 and TNF- α secretion by human skin mast cells.²² In the ASMCs, the inhibitory effect of omalizumab on cytokine expression was based on a down-regulation of the de novo mRNA synthesis that most probably was the result of the inhibition of MAPKs. The role of IL-6 in asthma is unclear, but it may contribute to asthma pathologies, because ASMCs respond to it when a soluble receptor, gp80, is present.²³ Therefore, its down-regulation by omalizumab may reduce remodeling.

Increased IL-8 levels and subsequently increased infiltration of neutrophil into the lung have been described in house dust mite-provoked asthma²⁴ or after suspension of steroid treatment.²⁵ The ASMCs respond to IL-8 and, therefore, a reduction of IL-8 secretion by omalizumab may reduce its contribution to immune cell activity²⁶ and may explain a reduction of neutrophil infiltration in vivo.²⁷

The IL-4 was linked to asthma severity and TNF- α levels.²⁰ This may explain the observed late increase of IL-4 secretion by IgE-stimulated ASMCs, indicating an indirect activation via TNF- α .²⁰ Therefore, it is possible that the observed reduction of IL-4 secretion in omalizumab-treated ASMCs is the result of the compound's inhibitory effect on TNF- α secretion.

In contrast to others, we did not observe an increase of IgE receptor expression in omalizumab-treated ASMCs within 24 hours. This difference may be the result of different species, different cell culture conditions, or different lengths of the observation period.¹⁸ Furthermore, our data indicate that the high-affinity IgE receptor plays a prevalent role in IgE-dependent cytokine secretion by ASMCs via 2 MAPKs. This finding is in line with data obtained in human mast cells, in which both MAPKs ERK 1/2 and p38 were activated by IgE and mediated the secretion of IL-4 and IL-8, but not of TNF- α .²⁸ Furthermore, in human B cells, T cells, and natural killer cells, both MAPKs mediated allergen-induced secretion of IL-8 and TNF- α .²⁹ In human intestinal epithelial cells of allergic patients, ERK 1/2 MAPK alone was sufficient to mediate allergen-IgE complex-stimulated IL-8 secretion.³⁰ Thus, the involvement of MAPKs in IgE signaling seems to be cell type specific and needs further investigation.

One repeated concern when studying the response of cells isolated from patients who use asthma therapies is that there might be a long-lasting "spillover" effect. However, such an effect is unlikely because none of the probands included in this study had used any antiasthma drug within 3 weeks before the biopsy specimen was obtained. Furthermore, the period between the isolation of the cells from bronchus material and the experiments is at least 8 weeks and includes 4 to 6 passages of cells. Finally, the effect of glucocorticoids and β_2 -agonists is short lasting and has no known long-lasting inhibitory effect on gene activity.¹² Thus, we can exclude that any of the observed effects is the result of a long-term effect of other antiasthma therapies in this study.

In summary, our findings suggest that the anti-inflammatory action of omalizumab is not restricted to immune-reactive cells, but that the drug also reduces the contribution of tissue-forming cells, such as ASMCs, to the pathogenesis of asthma.

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