CTLA4-IgG Reverses Asthma Manifestations in a Mild but Not in a More "Severe" Ongoing Murine Model

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We investigated whether CTLA4-Ig can reverse established asthma manifestations in a novel murine model of ongoing disease. In BALB/c mice, sensitized to ovalbumin (OVA) without adjuvant, airway inflammation was induced by a first series of OVA aerosol challenges. Murine CTLA4-IgG was then administered, followed by a second series of OVA inhalations. In control-treated mice, two series of OVA challenges induced upregulation of OVA-specific IgE in serum, eosinophils in the bronchoalveolar lavage fluid (BALF), and IL-5 production by lung lymphocytes upon OVA restimulation *in vitro*, compared with saline-challenged mice. CTLA4-IgG significantly inhibited all of these parameters in OVA-challenged mice. Importantly, mCTLA4-IgG performed better than the gold-standard dexamethasone because this corticosteroid did not inhibit the upregulation of OVA-specific IgE in serum. In a more "severe" ongoing model, induced by sensitization to OVA emulsified in aluminum hydroxide, resulting in airway hyperresponsiveness to methacholine and stronger inflammatory responses, mCTLA4-IgG was less effective in that only the number of eosinophils in the BALF was reduced ($P = 0.053$), whereas dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes. Thus, CTLA4-Ig might be an effective alternative therapy in established allergic asthma, especially in situations of mild disease.

Allergic asthma is prevalent worldwide, but especially in developed countries its prevalence is increasing to epidemic proportions (1). Patients with asthma suffer from acute bronchoconstriction and mucus formation directly after inhalation of the allergen. Chronic symptoms include airway hyperresponsiveness to bronchospasmogenic stimuli, inflammation, and airway remodeling.

CD4+ T helper cells recognizing the allergenic peptides and differentiating into the Type 2 subset of CD4+ T helper cells (Th2 cells) are crucial for the initiation and progression of allergic asthma (2). Th2 cells are characterized by the array of cytokines they secrete, which are optimized to combat parasitic infections (3). However, Th2 cells are developed undesirably in allergic disorders such as asthma, their cytokines causing airway symptoms either directly or mediated by other immune cells and their mediators (4).

In view of the essential role of Th2 cells in allergic asthma, these cells are interesting target cells for therapy. One therapeutic strategy is the prevention of optimal activation of allergen-specific CD4+ T cells by blocking their costimulatory requirements. For complete activation, CD4+ T cells need nonspecific costimulatory signals in addition to the signal provided by the T-cell receptor (TCR) after interacting with the MHC class II/antigenic peptide complex on the same antigen-presenting cell. CD28 is the primary T-cell costimulatory molecule, constitutively present on the surface of T cells. Upon interaction with its ligands B7-1 (CD80) and/or B7-2 (CD86), CD28 transduces a signal that enhances T-cell proliferation and cytokine secretion and sustains the T-cell response. The second receptor for the B7 ligands, CTLA4 (CD152), is detectable on the cell surface 24 h after T-cell activation *in vitro* and *in vivo* and downregulates T-cell responses. Because CTLA4 has a higher affinity for the B7 ligands compared with CD28, CTLA4-Ig has been widely used to block T-cell costimulation in an array of experimental animal models of T-cell–mediated diseases (5).

Previous studies have shown that blockade of T-cell costimulation by administration of CTLA4-Ig ameliorates the asthmatic manifestations in murine models (6–10). In all of these studies, CTLA4-Ig was administered before the onset of asthma manifestations. However, asthmatic patients start therapy after the onset of T cell–mediated airway symptoms, while exposure to environmental allergen very often continues. In addition, it has been shown that memory- and recently activated effector T cells are less dependent on CD28-mediated costimulation for their activation than naive T cells (11–14). This might limit the therapeutic potential of CTLA4-Ig when administered after the development of T-cell–mediated airway symptoms in an ongoing disease.

To more closely resemble our adjuvant-free, ovalbumin (OVA)-induced animal model of allergic asthma (15, 16) to the clinical situation, we designed a new protocol in which treatment starts after the onset of airway inflammation while OVA exposures continue. In the present study, we applied murine CTLA4-IgG in this model of ongoing disease to determine if costimulatory blockade is also effective after the onset of T-cell–mediated airway inflammation. We are the first to demonstrate that, in an ongoing murine model of allergic inflammation, mCTLA4-IgG significantly inhibited the upregulation of OVA-specific IgE in serum, the number of eosinophils in bronchoalveolar lavage fluid (BALF), and IL-5 production by lung lymphocytes *in vitro*. Importantly, mCTLA4-IgG performed better than the gold standard dexamethasone because this corticosteroid did not inhibit the upregulation of OVA-specific IgE in serum. In a more severe ongoing model, induced by sensitization to OVA emulsified in aluminum hydroxide (alum) resulting in airway hyperresponsiveness and stronger inflammatory responses, mCTLA4-IgG was less effective in that only the number of eosinophils in the

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Abbreviations: aluminum hydroxide, alum; bronchoalveolar lavage fluid, BALF; digoxigenin, DIG; dose-response curve, DRC; fetal calf serum, FCS; ovalbumin, OVA; phosphate-buffered saline, PBS; standard error of the mean, SEM; T-cell receptor, TCR.

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BALF was reduced ($P = 0.053$), whereas dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes.

Materials and Methods

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific pathogen-free male BALB/c mice (6 wk old) were obtained from the Central Animal Laboratory (Utrecht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad libitum*.

Sensitization and Challenge

All mice were sensitized to OVA (chicken egg albumin, crude grade V; Sigma, St. Louis, MO). Active sensitization was performed without an adjuvant by giving seven intraperitoneal injections of 10 μg OVA in 0.5 ml pyrogen-free saline on alternate days (one injection per day). Three weeks after the last sensitization, mice were exposed to either 16 OVA challenges (2 mg/ml in pyrogen-free saline) or 16 saline aerosol challenges for 5 min on consecutive days (one aerosol per day). An additional group of mice first received eight OVA aerosols, followed by eight saline aerosols (OVA/saline, spontaneous resolution group) (*see* Table 1).

For the experiment in the more severe ongoing model, all mice were sensitized to OVA by active sensitization with two intraperitoneal injections (7 d apart) of 0.1 ml alum-precipitated antigen, comprising 10 μg OVA adsorbed onto 2.25 mg alum (AlumImject; Pierce, Rockford, IL). Two weeks after the second sensitization, mice were exposed to either six OVA challenges (10 mg/ml in pyrogen-free saline) or six saline aerosol challenges for 20 min every third day (one aerosol every third day). An additional group of mice first received three OVA aerosols, followed by three saline aerosols (OVA/saline, spontaneous resolution group) (*see* Table 2).

The aerosol was performed in a plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 liters/min. Aerosol was given in groups composed of no more than eight animals.

Experimental Treatment Protocols

Both saline- and OVA-challenged mice were divided into two groups of eight mice. One group of mice received experimental treatment, and the other group received control treatment. The spontaneous resolution group received control treatment in both independent experiments.

In Experiment A, murine CTLA4-IgG fusion protein was used as the experimental treatment, consisting of murine CTLA4 fused via an ECD-immunoglobulin junction to the hinge and Fc regions of human IgG1 (9). Human total IgG was used as control treatment in this experiment. Ten minutes before the ninth aerosol, mice were intravenously injected with 110 μl saline containing either 143 μg mCTLA4-IgG or human total IgG. Ten minutes before each of the seven following aerosols, mice were subcutaneously injected with 72 μg of the antibodies in a volume of 55 μl. In Experiment B, dexamethasone (1:40 diluted in saline) was used as experimental treatment and saline as control treatment. Ten minutes before the ninth through the sixteenth aerosol, mice were injected intraperitoneally with 0.0125 mg dexamethasone in 0.25 ml pyrogen-free saline or saline alone (*see* Table 1).

In the experiment using the more severe ongoing model, the group of saline-challenged ($n = 8$) and the spontaneous resolution group ($n = 8$) received control treatment. OVA-challenged animals were divided into three groups of eight animals each, receiving either control treatment, murine CTLA4-IgG fusion protein, or dexamethasone. Ten minutes before the fourth aerosol, a single dose of control treatment (human total IgG, 280 μg in 200 μl saline) or murine CTLA4-IgG fusion protein (280 μg in 200 μl saline) was administered intravenously. This single dose resulted in very similar concentrations of the antibodies in the final sera as observed in those from the experiment in the milder ongoing model of airway inflammation. Dexamethasone treatment (0.0125 mg in 0.25 ml pyrogen-free saline) was started 10 min before the fourth aerosol and was followed by six daily intraperitoneal injections (one injection per day) during the period of the second series of OVA aerosols (*see* Table 2).

Human total IgG was purchased from ICN Pharmaceuticals (Cosa Mesa, CA) and dissolved in saline. This solution was treated with 10% (v/v) polymyxin B-agarose (Sigma, St. Louis, MO) for 1 h at 4°C to remove lipopolysaccharide. After incubation, the agarose beads were removed by centrifugation, and the supernatant was sterilized. Murine CTLA4-IgG fusion protein was kindly provided by Dr. P. Jardieu (Immunology, Genentech, South San Francisco, CA) and constructed as described previously (9). The endotoxin level was less than 1 endotoxin U/mg. Dexamethasone (Dexadreson) was purchased from Intervet Nederland BV (Boxmeer, The Netherlands).

In all experiments, airway responsiveness to methacholine, OVA-specific IgE levels in serum, cellular infiltration in the BALF, and T-cell responses in lung tissue were measured 24 h after the last challenge in each mouse.

Measurement of Airway Responsiveness *In Vivo*

Airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France) in response to inhaled methacholine (acetyl-β-methylcholine chloride; Sigma). Airway responsiveness was expressed in enhanced pause (Penh), as described in detail previously (17). Briefly, mice were placed in a whole-body chamber, and basal readings were obtained and averaged for 3 min. Aerosolized saline, followed by doubling concentrations of methacholine (ranging from 1.6–50 mg/ml saline), were nebulized for 3 min, and readings were taken and averaged for 3 min after each nebulization. Dose-response curves (DRCs) to methacholine were statistically analyzed by a general linear model of repeated measurements followed by *post-hoc* comparison between groups. Data were LOG transformed before analysis to equalize variances in all groups.

Determination of OVA-Specific IgE Levels in Serum

After measurement of *in vivo* airway responsiveness, mice were killed by intraperitoneal injection of 1 ml 10% urethane in pyrogen-free saline (Sigma). Subsequently, mice were bled by cardiac puncture, and OVA-specific IgE was measured by ELISA. Briefly, microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 2 μg/ml rat anti-mouse IgE (clone EM95) diluted in phosphate-buffered saline (PBS). The next day, the ELISA was performed at room temperature. After blocking with ELISA buffer (PBS containing 0.5% bovine serum albumin [Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ] pH 7.2) for 1 h, serum samples and a duplicate standard curve (starting 1:10), diluted in ELISA buffer, were added for 2 h. An OVA-specific IgE reference standard was obtained by intraperitoneal immunization with OVA and arbitrarily assigned a value of 10,000 experimental units/ml (EU/ml). After incubation, 1 μg/ml of OVA coupled to digoxigenin (DIG), which was prepared from a kit containing DIG-3-*o*-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide-ester (Roche Diagnostics, Basel, Switzerland) in ELISA buffer, was added for 1.5 h, followed by incubation with anti-

DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) diluted 1:500 in ELISA buffer for 1 h. Color development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/ml, Sigma) and 4 mM H_2O_2 in PBS and stopped by adding 4 M H_2SO_4. The optical density was read at 492 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). The detection limit of the ELISA was 0.5 EU/ml IgE.

Analysis of the Cellular Composition in the BALF

BAL was performed immediately after bleeding of the mice. Briefly, the airways were lavaged five times through a tracheal cannula with 1-ml aliquots of pyrogen-free saline warmed to 37°C. The recovered lavage fluid was pooled, and cells were pelleted (32 × *g*, 4°C, 5 min) and resuspended in 150 μl cold PBS. The total number of cells in the BALF was determined using a Bürker-Türk counting-chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made and stained with Diff-Quick (Dade AG, Düdingen, Switzerland). Per cytospin, 400 cells were counted and differentiated into mononuclear cells (monocytes, macrophages, and lymphocytes), eosinophils, and neutrophils by standard morphology. Statistical analysis was performed using the nonparametric Mann-Whitney *U* test.

Determination of Cytokine Production by OVA-Restimulated Lung Cells *In Vitro*

Cytokine production by antigen-restimulated T cells in lung tissue was determined as described previously (16). Briefly, the lungs were lavaged as described above and perfused via the right ventricle with 4 ml saline containing 100 U/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands) to remove any blood and intravascular leukocytes. Complete lung tissue was removed and transferred to cold sterile PBS. Lungs were then minced and digested in 3 ml RPMI 1640 containing 2.4 mg/ml collagenase A and DNase I (grade II) (both from RocheDiagnostics) for 30 min at 37°C. Collagenase activity was stopped by adding fetal calf serum (FCS). The lung tissue digest was filtered through a 70-μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) with 10 ml RPMI 1640 to obtain a single-cell suspension. The lung-cell suspension was washed, resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% glutamax I, and gentamicin [all from Life Technologies, Gaithersburg, MD]) and 50 mM β-mercaptoethanol (Sigma), and the total number of lung cells was determined using a Bürker-Türk counting-chamber. Lung cells (8×10^5 lung cells/well) were cultured in round-bottom 96-well plates (Greiner Bio-One GmbH, Kremsmuenster, Austria) in the presence of OVA (10 μg/ml) or medium only. As a positive control, cells were cultured in the presence of plate-bound rat anti-mouse CD3 (clone 17A2, 50 μg/ml, coated overnight at 4°C). Each *in vitro* stimulation was performed in triplicate. After 5 d of culture at 37°C, the supernatant was harvested, pooled per stimulation, and stored at −20°C until cytokine levels were determined by ELISA.

The IFN-γ, IL-4, IL-5, IL-10, and IL-13 ELISAs were performed according to the manufacturer's instructions (PharMingen, San Diego, CA). The detection limits of the ELISAs were 160 pg/ml for IFN-γ, 16 pg/ml for IL-4, 32 pg/ml for IL-5, and 100 pg/ml for IL-10 and IL-13.

Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM). Unless stated otherwise, the differences between groups were statistically analyzed using a Student's *t* test (two-tailed, homosedastic). Results were considered statistically different at the $P < 0.05$ level. Statistical analyses were performed using SPSS for Windows version 10.0.05 (SPSS, Chicago, IL).

Results

Table 1 shows the design of two independent experiments using a novel murine model of ongoing disease. Experimental treatments were mCTLA4-IgG in Experiment A and the gold standard dexamethasone in Experiment B. Group 5 was used to compare the effect of experimental treatment on established asthma manifestations (Group 4) to stopping with OVA aerosols while continuing with saline aerosols (hereafter referred to as the spontaneous resolution group).

Airway Hyperresponsiveness *In Vivo* Is Induced by the First Series of OVA Aerosols but Disappears After the Second Series of Aerosols

As shown previously, one of the advantages of whole-body plethysmography is that the airway responsiveness *in vivo* of experimental animals can be measured several times during an experiment because it is performed in conscious, unrestrained animals (18). To verify that hyperresponsiveness was induced in OVA-sensitized mice by the first series of OVA aerosol challenges, airway responsiveness to aerosolized methacholine was measured after 24 h in each of the two independent experiments after the first series of aerosols, just before treatment and the second series of aerosols. A DRC of doubling concentrations methacholine was performed in each mouse. The DRC to methacholine of OVA-challenged animals was significantly different ($P < 0.01$, Experiment A and $P < 0.01$, Experiment B) from those of saline-challenged mice. Figure 1A shows the responses to the methacholine DRC of saline- and OVA-challenged animals from Experiment A.

To determine if mCTLA4-IgG or dexamethasone could reverse established hyperresponsiveness, airway responsiveness was measured again 24 h after the second series of

TABLE 1

Design of the two experiments using a novel murine model of ongoing disease

Group	Sensitization	Challenge: Aerosol (1–8)	Challenge: Aerosol (9–16)	Treatment: Experiment A	Treatment: Experiment B
1	OVA	Saline	Saline	Human total IgG	Saline
2	OVA	Saline	Saline	Murine CTLA4-IgG	Dexamethasone
3	OVA	OVA	OVA	Human total IgG	Saline
4	OVA	OVA	OVA	Murine CTLA4-IgG	Dexamethasone
5	OVA	OVA	Saline	Human total IgG	Saline

Definition of abbreviation: OVA, ovalbumin.
Mice were sensitized, challenged, and treated as described in MATERIALS AND METHODS. Each experimental group consisted of at least six animals.

aerosols in Experiments A and B. However, in control-treated groups, OVA challenge did not result in hyperresponsiveness to methacholine compared with saline challenge; Penh values of OVA-challenged mice were just as low as those from saline-challenged animals at all concentrations of the methacholine DRC in both experiments. In Figure 1B, the maximal responses to 50 mg/ml methacholine of Experiment A are shown. Hence, an effect of mCTLA4-IgG or dexamethasone on established, OVA-induced airway hyperresponsiveness could not be determined in this model of ongoing disease.

Thus, OVA-challenged mice show airway hyperresponsiveness *in vivo* compared with saline-challenged mice after the first series of aerosols, but this nonspecific hyperresponsiveness disappears after the second series of aerosols.

CTLA4-IgG Inhibits the Upregulation of Serum OVA-Specific IgE in the Ongoing Murine Model of Airway Inflammation

In Experiments A and B, which evaluated mCTLA4-IgG and dexamethasone as experimental treatment, respectively, serum levels of OVA-specific IgE were determined in all mice 24 h after the second series of aerosols.

In control-treated mice, OVA challenge induces a significant upregulation of serum OVA-specific IgE compared with saline challenge (373% increase, $P < 0.01$ and 250% increase, $P < 0.01$; shown in Figures 2A and 2B, respectively). CTLA4-IgG-treated, OVA-challenged mice show significantly reduced levels of OVA-specific IgE compared with control-treated, OVA-challenged animals (43% inhibition, $P < 0.05$). This level of allergen-specific IgE in serum was very similar to that observed in the sera of mice from the spontaneous resolution group (Group 5, Table 1) (56% inhibition compared with control-treated, OVA-challenged mice, $P < 0.01$) (Figure 2A). In contrast, dexamethasone treatment has no effect on the OVA-induced upregulation of serum antigen-specific IgE (Figure 2B).

Thus, mCTLA4-IgG is able to partially inhibit the upregulation of serum OVA-specific IgE levels in the ongoing model of airway inflammation.

CTLA4-IgG and Dexamethasone Inhibit the Number of Eosinophils in the BALF in the Ongoing Murine Model of Airway Inflammation

The number of cells in the BALF was used as a measure for the infiltration of cells in the airways and determined in all mice 24 h after the second series of aerosols. In the lavage fluid of saline-challenged mice no (or sometimes only a few) eosinophils can be observed. However, OVA challenge results in a significant ($P < 0.01$) increase in the number of eosinophils compared with saline challenge of the same treatment group in each of the two independent experiments (Figure 3). Both mCTLA4-IgG- or dexamethasone-treated, OVA-challenged mice show significantly reduced numbers of eosinophils in the BALF com-

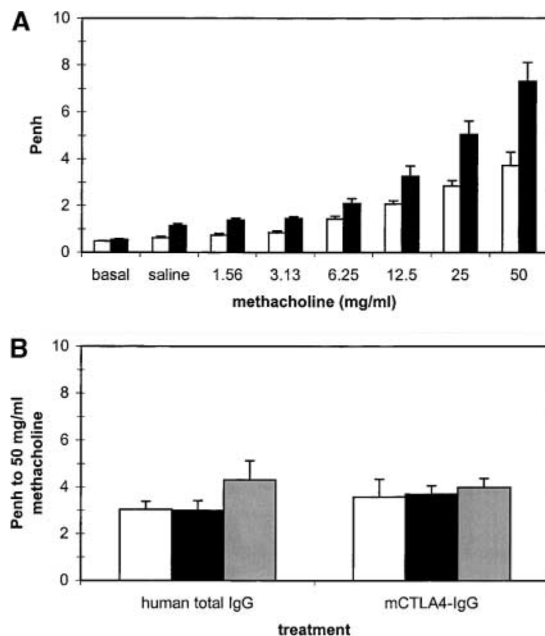

Figure 1. Airway hyperresponsiveness is induced by the first series of OVA aerosols but disappears after the second series of aerosols. Airway responsiveness to aerosolized methacholine was measured in conscious, unrestrained mice 24 h after the first (*A*) and second (*B*) series of aerosols. (*A*) BALB/c mice were OVA-sensitized and challenged with either eight saline aerosols (n = 16; *open bars*) or eight OVA aerosols (n = 32; *closed bars*). (*B*) Subsequently, mice were challenged with eight saline aerosols (*open bars* and *shaded bars*) or eight additional OVA aerosols (*closed bars*). During this second series of aerosols, mice received control treatment (human total IgG) or mCTLA4-IgG. Values are expressed as mean ± SEM (n = 8 per group). Results of Experiment A, representative for both experiments, are shown.

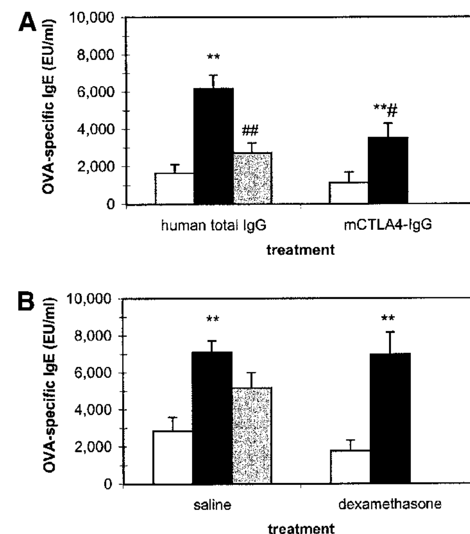

Figure 2. CTLA4-IgG inhibits the upregulation of serum OVA-specific IgE in the ongoing murine model of airway inflammation. Serum OVA-specific IgE was determined in all mice 24 h after the second series of aerosols. BALB/c mice were OVA-sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*), or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (*A*), or dexamethasone (*B*). Values are expressed as mean ± SEM (n = 6–8 per group). **$P < 0.01$, as compared with saline-challenged animals of the same treatment group. #$P < 0.05$ and ##$P < 0.01$, as compared with control-treated, OVA-challenged animals.

pared with control-treated, OVA-challenged mice (83% inhibition, $P < 0.01$ and 97% inhibition, $P < 0.01$; shown in Figures 3A and 3B, respectively). In mice from the spontaneous resolution group, a similar reduction compared with control-treated, OVA-challenged mice was observed (82% inhibition, $P < 0.01$ and 90% inhibition, $P = 0.052$; shown in Figures 3A and 3B, respectively).

Thus, both mCTLA4-IgG and dexamethasone are able to inhibit the number of eosinophils in the BALF in the ongoing model of airway inflammation.

In each of the two independent experiments, no significant differences in the absolute number of neutrophils were observed in the BALF between the experimental groups (data not shown). In Experiment A, the absolute numbers of mononuclear cells (monocytes, macrophages, and lymphocytes) were not significantly different between the experimental groups. In Experiment B, dexamethasone treatment of OVA-challenged animals resulted in a reduction in the number of mononuclear cells compared with control-treated, OVA-challenged animals (32% inhibition, $P < 0.05$).

CTLA4-IgG Inhibits Lung Cell IL-5 Production After OVA Restimulation *In Vitro* in the Ongoing Murine Model of Airway Inflammation

A single-cell suspension of complete lung tissue of each mouse was prepared 24 h after the second series of aerosols. To determine T-cell responses upon antigenic restimulation, lung cells were cultured for 5 d in the presence of OVA. In agreement with previous studies from our laboratory (16), no IFN-γ or IL-4 could be detected in the supernatant of these total lung-cell cultures after 5 d. In addition, we could not detect IL-10 and IL-13 in the supernatant of these OVA-restimulated lung-cell cultures in this study. Lung-cell cultures derived from control-treated, OVA-challenged mice show significantly increased levels of IL-5 after restimulation with OVA *in vitro* compared with cultures from control-treated, saline-challenged animals (369% increase, $P < 0.01$ and 221% increase, $P < 0.05$; shown in Figures 4A and 4B, respectively). Allergen-stimulated lung-cell cultures derived from mCTLA4-IgG-treated, OVA-challenged mice show significantly reduced levels of IL-5 in the supernatant compared with cultures from control-treated, OVA-challenged animals (48% inhibition, $P < 0.05$). This degree of inhibition was similar to that observed in the cultures from mice of the spontaneous resolution group (48% inhibition, $P < 0.05$) (Figure 4A).

Dexamethasone treatment of OVA-challenged mice slightly decreased the IL-5 production by lung tissue cells after restimulation with OVA *in vitro*, compared with lung cells from control-treated, OVA-challenged animals (Figure 4B).

Polyclonal stimulation of T cells with anti-CD3 resulted in high levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 in the supernatant of lung-cell cultures derived from all experimental groups, indicating that there was no intrinsic T-cell defect to produce any of these cytokines (data not shown).

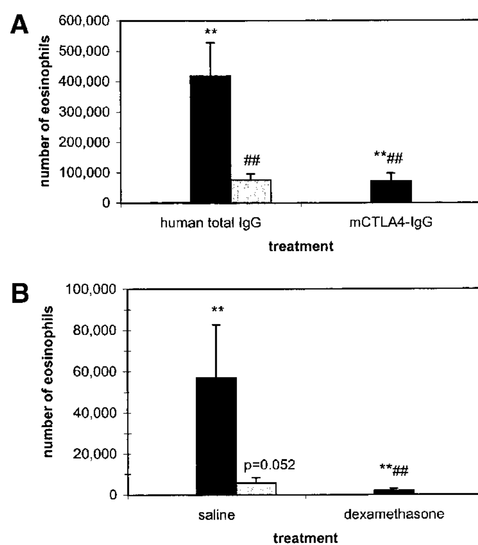

Figure 3. CTLA4-IgG and dexamethasone inhibit the number of eosinophils in the BALF in the ongoing murine model of airway inflammation. The number of eosinophils in the BALF in all mice was determined 24 h after the second series of aerosols. BALB/c mice were OVA sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*), or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (*A*), or dexamethasone (*B*). Values are expressed as mean ± SEM ($n = 6$–8 per group). $^{**}P < 0.01$, as compared with saline-challenged animals of the same treatment group. $^{\#\#}P < 0.01$, as compared with control-treated, OVA-challenged animals.

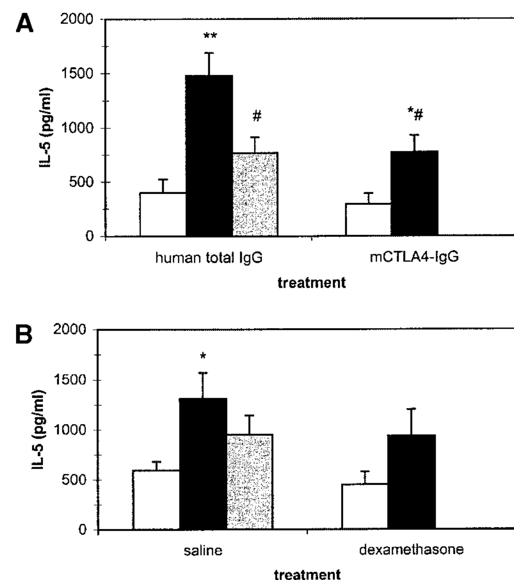

Figure 4. CTLA4-IgG inhibits lung-cell IL-5 production after OVA restimulation *in vitro* in the ongoing murine model of airway inflammation. Total lung-cell cultures prepared from each mouse 24 h after the second series of aerosols were cultured for 5 d in the presence of OVA. BALB/c mice were OVA sensitized and challenged with 16 saline aerosols (*open bars*), 16 OVA aerosols (*closed bars*), or 8 OVA/8 saline aerosols (*shaded bars*). During the second series of aerosols, mice received control treatment, mCTLA4-IgG (*A*), or dexamethasone (*B*). Values are expressed as mean ± SEM ($n = 11$–14 per group) averaged from two independent experiments (*A*) or as mean ± SEM (n = 6–8 per group) (*B*). $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared with saline-challenged animals of the same treatment group. $^{\#}P < 0.05$, as compared with control-treated, OVA-challenged animals.

Thus, mCTLA4-IgG is able to inhibit the IL-5 production by lung-cell cultures after *in vitro* restimulation with allergen in the ongoing model of airway inflammation.

In a More Severe Ongoing Murine Model, the Effects of mCTLA4-IgG Are Less Pronounced

The experiments described above are performed in a relatively mild model of airway inflammation; that is, animals are sensitized to OVA without adjuvant and challenged with low doses of OVA. Most research groups, however, use a protocol in which animals are allergen sensitized in the presence of the Th2-dominant response-inducing alum as adjuvant and challenged with high doses of allergen. This protocol results in a model with stronger responses and appears to represent a more severe type of an inflammatory response. Whereas the extent of airway hyperresponsiveness is similar, ~ 10 times as much serum OVA-specific IgE and at least twice as many eosinophils in BALF can be observed after a first series of OVA aerosols when using this protocol, compared with the mild protocol (D.T. Deurloo, unpublished observations). Interestingly, it has been shown that the various models respond differently to modulation (19, 20). Therefore, we wanted to evaluate the effects of mCTLA4-IgG and dexamethasone in a more severe ongoing model as well (adapted from Refs. 17 and 21). Table 2 shows the design of this experiment.

TABLE 2
Experimental design to determine the effects of mCTLA4-IgG and dexamethasone in a more severe ongoing model

Group	Sensitization	Challenge: Aerosol (1–3)	Challenge: Aerosol (4–6)	Treatment
1	OVA/alum	Saline	Saline	Human total IgG
2	OVA/alum	OVA	OVA	Human total IgG
3	OVA/alum	OVA	OVA	Murine CTLA4-IgG
4	OVA/alum	OVA	OVA	Dexamethasone
5	OVA/alum	OVA	Saline	Human total IgG

Definition of abbreviations: alum, aluminum hydroxide; OVA, ovalbumin.
Mice were sensitized, challenged, and treated as described in MATERIALS AND METHODS. Each experimental group consisted of at least six animals.

In the more severe ongoing model, airway responsiveness to methacholine was measured in all mice 24 h after the second series of aerosols. In contrast to the milder ongoing model of airway inflammation, OVA challenge induces airway hyperresponsiveness to methacholine compared with saline challenge not only after a first series of aerosols (D.T. Deurloo, unpublished observations) but also after two series of aerosols in control-treated mice ($P < 0.05$) (Figure 5A). CTLA4-IgG- or dexamethasone-treated, OVA-challenged mice show reduced nonspecific responsiveness at the higher concentrations of methacholine, but the complete DRCs to methacholine are not significantly different compared with the DRC of control-treated, OVA-challenged mice. Also, the mice from the spontaneous resolution group (Group 5, Table 2) showed no significantly reduced airway responsiveness compared with control-treated, OVA-challenged mice (Figure 5A).

Figure 5B shows that, in comparison with the milder model of ongoing disease, much higher serum levels of OVA-specific IgE can be observed in all experimental groups of the more severe ongoing model 24 h after the second series of aerosols (compare Figure 5B with Figure 2). In control-treated mice, OVA challenge induces a significant upregulation of serum OVA-specific IgE compared with saline challenge (380% increase, $P < 0.01$). Both mCTLA4-IgG and dexamethasone have no effect on the OVA-induced upregulation of specific IgE levels. Notably, the mice from the spontaneous resolution group show no significantly different levels of serum IgE compared with control-treated, OVA-challenged mice (Figure 5B).

Also in the more severe ongoing model, no eosinophils are found in the BALF of saline-challenged animals 24 h after the second series of aerosols. In control-treated mice, OVA challenge induces a significant ($P < 0.01$) increase in the number of eosinophils in the BALF compared with saline challenge (Figure 5C). The absolute number of eosinophils observed in the BALF of OVA-challenged mice in the more severe ongoing model is enormous compared with the number of eosinophils in the BALF recovered from OVA-challenged mice in the milder ongoing model of allergic inflammation (compare Figure 5C with Figure 3). CTLA4-IgG-treated, OVA-challenged mice have reduced numbers of eosinophils compared with control-treated, OVA-challenged animals, but this suppression does not reach the level of significance (40% inhibition, $P = 0.053$). In contrast, dexamethasone-treated, OVA-challenged mice demonstrate significantly reduced numbers of eosinophils compared with control-treated, OVA-challenged animals (73% inhibition, $P < 0.01$). In mice from the spontaneous resolution group, a similar reduction compared with control-treated, OVA-challenged mice was observed (82% inhibition, $P < 0.01$) (Figure 5C).

No significant differences in the absolute number of neutrophils were observed in the BALF between the experimental groups (data not shown). In control-treated mice, OVA challenge induces a significant increase in the number of BALF mononuclear cells (301% increase, $P < 0.01$) compared with saline challenge. Both in the spontaneous resolution group and in the group of dexamethasone-treated, OVA-challenged animals, a significant reduction in the absolute number of mononuclear cells was observed compared with control-treated, OVA-challenged animals (36% inhibition, $P < 0.05$ and 49% inhibition, $P < 0.01$).

Also in this experiment, we prepared a single-cell suspension of complete lung tissue of each mouse 24 h after the second series of aerosols to restimulate lung cells *in vitro*.

Similar to the lung-cell cultures from the milder ongoing model of airway inflammation, these lung-cell cultures produced no IFN-γ or IL-4 after 5 d of culture in the presence of OVA. However, lung cells obtained from control-treated, OVA-challenged mice produced significantly more IL-5—and in this more severe ongoing model also IL-10 and IL-13—compared with control-treated, saline-challenged animals upon antigenic restimulation (277% increase, $P < 0.05$; 677% increase, $P < 0.01$; and 886% increase, $P < 0.05$, respectively) (Figure 5D). The lung-cell cultures derived from mice treated with mCTLA4-IgG and challenged with OVA produced similar levels of these cytokines after OVA restimulation *in vitro* as those of control-treated, OVA-challenged animals. Also in the super-

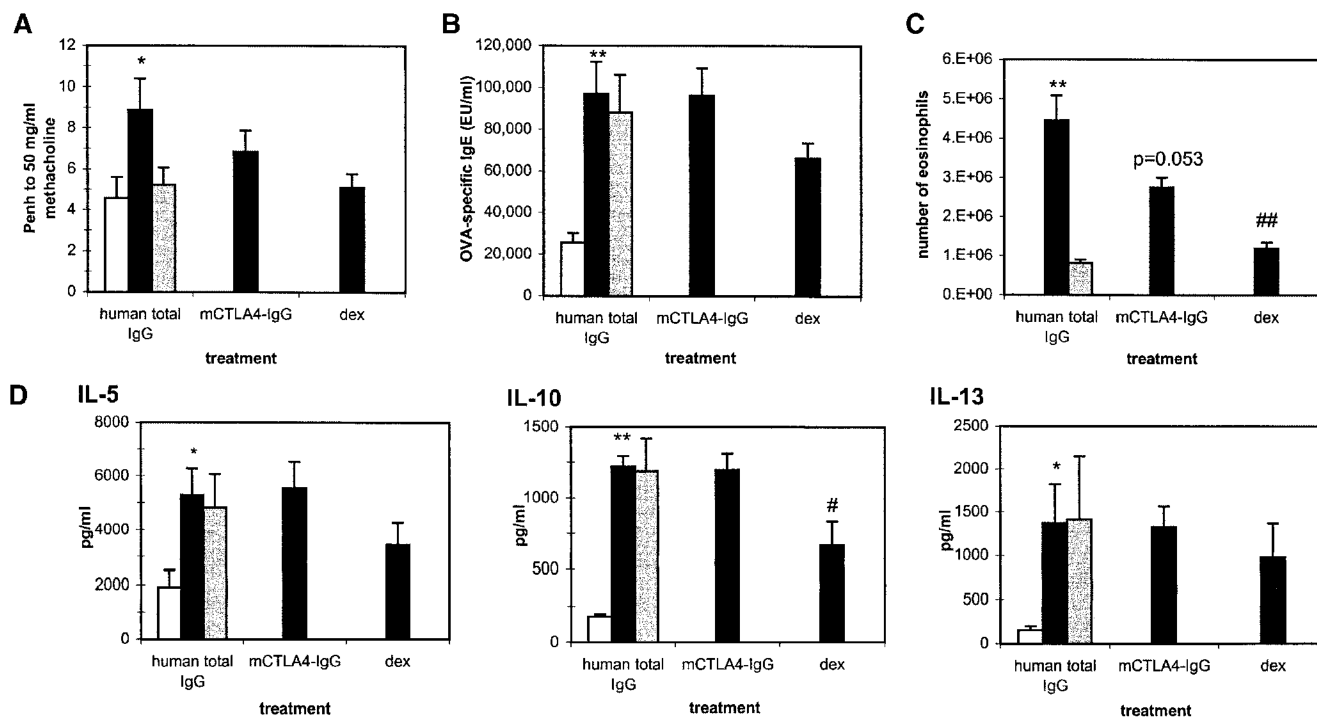

Figure 5. The effects of mCTLA4-IgG and dexamethasone in a more severe ongoing murine model. BALB/c mice were OVA sensitized and challenged with six saline aerosols (*open bars*), six OVA aerosols (*closed bars*), or three OVA/three saline aerosols (*shaded bars*). Mice received control treatment (human total IgG), mCTLA4-IgG, or dexamethasone (dex). Treatment started just before the second series of aerosols. Airway responsiveness to aerosolized methacholine was measured in conscious, unrestrained mice 24 h after the second series of aerosols. The response to the highest concentration (50 mg/ml) of the methacholine dose response curve is depicted (*A*). Serum OVA-specific IgE (*B*), the number of eosinophils in the BALF (*C*), and cytokine production by OVA-restimulated lung cells *in vitro* (*D*) was determined in all mice 24 h after the second series of aerosols. Values are expressed as mean ± SEM (n = 6–8 per group). $*P <$ 0.05 and $**P < 0.01$, as compared with saline-challenged animals. $\#P < 0.05$, as compared with control-treated, OVA-challenged animals.

natant of lung-cell cultures obtained from mice from the spontaneous resolution group, no difference in the cytokine levels was observed when compared with control-treated, OVA-challenged mice. In contrast, dexamethasone significantly inhibited IL-10 production by the lung-cell cultures (45% inhibition, $P < 0.05$), whereas IL-5 and IL-13 production was slightly reduced (Figure 5D).

Polyclonal stimulation of T cells with anti-CD3 resulted in high levels of IFN-γ, IL-4, IL5, IL-10, and IL-13 in the supernatant of lung-cell cultures derived from all experimental groups (data not shown).

Thus, in a more severe ongoing model, mCTLA4-IgG was less effective compared with the milder ongoing model of airway inflammation in that only airway hyperresponsiveness was attenuated at the higher concentrations of the methacholine DRC, and the number of eosinophils in the BALF was reduced. In comparison, dexamethasone not only attenuated hyperresponsiveness at the higher concentrations of the methacholine DRC, but it also significantly inhibited BALF eosinophilia and lung IL-10 production *in vitro* together with a reduction in IL-5 production.

Discussion

In the present study, we sought to determine if costimulatory blockade by murine CTLA4-IgG can reverse established asthma manifestations in an OVA-induced murine model of ongoing disease. CTLA4-IgG inhibited the upregulation of OVA-specific IgE in serum, BALF eosinophilia, and lung-cell IL-5 production *in vitro* in a mild ongoing model of airway inflammation. Whereas airway hyperresponsiveness to methacholine was present in this ongoing model in OVA-sensitized mice after the first series of OVA aerosol challenges that induced airway inflammation, this nonspecific hyperresponsiveness had disappeared after the second series of OVA aerosols. Hence, the effect of mCTLA4-IgG on this parameter could not be determined. Importantly, mCTLA4-IgG performed better than the gold standard dexamethasone because this corticosteroid did not inhibit serum levels of OVA-specific IgE. In a more severe ongoing model (adapted from Refs. 17 and 21), mCTLA4-IgG was less effective in that only the number of eosinophils in the BALF was reduced ($P =$ 0.053). However, in this more severe ongoing model, airway hyperresponsiveness was present after the second series of OVA aerosols and attenuated by mCTLA4-IgG administration. Dexamethasone inhibited both BALF eosinophilia and cytokine production by lung lymphocytes in this more severe ongoing murine model.

Thus, we are the first to demonstrate that in a murine model of ongoing disease, costimulatory blockade by mCTL A4-IgG can also be effective after the onset of asthma

manifestations. During the time course of this ongoing model of the disease, the types and numbers of the various allergen-specific CD4+ T-cell subsets, based on their activation status, constantly changes. After sensitization, OVA-specific memory T cells have developed. In the two models of ongoing disease, recently activated effector T lymphocytes are present after the first series of OVA aerosol challenges at the time of treatment. In addition, naive T cells might be recruited and activated during the two series of OVA aerosols. *In vitro* studies have demonstrated that memory- and previously activated effector T cells are less dependent on CD28-mediated costimulation than naive T cells (11, 13, 14), whereas *in vivo* studies on the effectiveness of giving CTLA4-Ig after priming of immune responses have yielded conflicting results (12). An example of a successful study is the recent clinical report on the administration of CTLA4-Ig to patients with psoriasis vulgaris in a phase I trial that produced a dose-dependent improvement in skin lesions (22). It is possible that the effectiveness of CTLA4-Ig on established disease depends on its ability to block the development and contribution to disease of newly recruited naive CD4+ T cells. If this is true, especially in our mild ongoing model, the contribution of newly recruited naive CD4+ T cells may be considerable. Alternatively, CTLA4-Ig may be effective in an ongoing or a memory response under some conditions, determined by the type of antigen inducing the immune response, the lymphoid microenvironment, or the T-cell cytokine pattern elicited during the immune response (12, 13).

We investigated the role of the CD28/B7 T-cell costimulatory pathway in two murine models of ongoing disease. The crucial role of CD28-mediated costimulation in the generation of effective T-cell responses has been well documented (5). However, two new pairs of the CD28/B7 families have been identified recently (reviewed in 23). Inducible costimulatory molecule (ICOS), as indicated by its name, is expressed on the surface of T cells upon activation and binds to B7RP-1 (also known as B7h, B7-H2, GL-50, ICOSL, or LICOS) present on a variety of cells. Interestingly, on the basis of many recent studies, it appears that ICOS functions primarily to induce T-cell effector function. For example, engagement of ICOS appeared to be important for the cytokine responses of recently activated effector T cells. Although ICOS engagement might support activation of naive T cells, it could be more important for enhancing ongoing and/or memory responses. The other new member of the CD28 family, programmed death 1 (PD-1), like CTLA4, appears to mediate an inhibitory signal to T cells upon binding to its ligands PD-L1 (or B7H-1) and PD-L2. In contrast to CTLA4 deficient mice, which all die at 3–4 wk of age, severe autoimmune symptoms are observed only in approximately half of the PD-1 deficient mice, even at 14 mo of age, suggesting that PD-1 is not the primary inhibitory signal for T cells (23). Still more B7 family members are being identified at the time of writing this report (24, 25). A detailed understanding of the roles and interplay of these T-cell costimulatory pathways might result in new therapeutic possibilities for the manipulation of T cell–mediated diseases (e.g., a treatment consisting of CTLA4-Ig in combination with an agent blocking other costimulatory pathway[s]).

Blocking costimulation might not only result in the suppression of the immune response but in some cases might induce antigen-specific tolerance. When T cells receive an antigen-specific signal through the TCR in the absence of costimulatory signals, this results in nonresponsiveness upon subsequent optimal T-cell activation, according to the classic model of T-cell anergy (26, 27). Alternatively, some research groups have suggested that *in vivo* administration of CTLA4-Ig might leave small amounts of B7 uncovered that bind to CTLA4 due to its higher affinity. This would imply that CTLA4-Ig administration results in the absence of CD28 ligation, whereas part of the CTLA4 molecules are ligated. CTLA4 ligation has been implicated in the induction of peripheral tolerance (28, 29). Of course, tolerance induction is very attractive in the therapy of asthmatic patients because this would mean that after a single period of treatment, patients have improved clinical status for a considerable time after cessation of therapy. Therefore, we are currently investigating whether the effects of mCTLA4-IgG are transient or long lasting in the (ongoing) murine models of allergic asthma.

Allergen-specific IgE in serum, BALF eosinophilia, and IL-5 production by lung cells are very characteristic features seen in patients with asthma. These manifestations were significantly inhibited by mCTLA4-IgG in the mild ongoing murine model of airway inflammation. Because IL-5 is involved in the growth, migration, and activation of eosinophils, the decreased IL-5 production by lung cells isolated from mCTLA4-IgG-treated OVA-challenged mice correlates with the inhibition of BALF eosinophilia observed in this group. Unfortunately, we cannot draw any conclusions about the effect of mCTLA4-IgG on OVA-induced airway hyperresponsiveness because hyperresponsiveness to methacholine had disappeared in control-treated, OVA-challenged animals after the second series of aerosols. We can only speculate on the mechanism of this disappearance of airway hyperresponsiveness. One possibility is that two series of eight OVA aerosols might induce tolerance. This would mean that in our milder ongoing murine model, partial tolerance is induced, namely for nonspecific hyperresponsiveness and not for the other parameters, suggesting that the loss of airway hyperresponsiveness is probably not due to immunologic tolerance. Unless hyperresponsiveness is regulated by a different subset of (allergen-specific) CD4+ T cell than the allergen-specific CD4+ T cell responsible for both isotype switching of B cells to IgE and BALF eosinophilia. This T cell most likely is also CD4+ because airway hyperresponsiveness is abolished by *in vivo* depletion of CD4+ T cells (30).

Another explanation comes from a study by Palmans and colleagues evaluating the effect of prolonged allergen exposure on airway function and structure in rats (31). Although airway hyperresponsiveness to aerosolized carbachol and increased total- and inner airway wall area were present after 2 wk of OVA exposure in sensitized rats, these features were absent after 12 wk of OVA exposure, whereas the influx of eosinophils into and around the airways persisted, as did IgE synthesis. The authors suggest that the loss of hyperresponsiveness is related to the development of compensatory mechanisms, the loss of which could contribute to the persistent airway hyperresponsiveness in hu-

man asthma. However, we wonder how likely these compensatory mechanisms apply to our mild ongoing model because both the time span of antigen exposures and the dose of antigen are much smaller. Future studies might clarify this issue.

In the more severe ongoing model, mCTLA4-IgG is less effective compared with the milder ongoing model. The levels of circulating OVA-specific IgE and lung IL-5 production *in vitro* are not inhibited, and the number of eosinophils in the BALF is reduced to a smaller extent compared with the milder model of airway inflammation. However, nonspecific airway hyperresponsiveness is present after the second series of OVA aerosols in this more severe ongoing model and mCTLA4-IgG attenuated OVA-induced airway hyperresponsiveness (Figure 5A). The stronger responses of this ongoing model compared with the mild ongoing model of airway inflammation (compare Figure 5 with Figures 2–4)—hence the designation more "severe"—might be more difficult to inhibit. After induction of asthma manifestations by a first series of OVA aerosols, saline challenge of mice during the second series of aerosols (spontaneous resolution group) also resulted in no or a small diminution of allergic asthma manifestations compared with control-treated, OVA-challenged animals.

Interestingly, it has been shown that the various murine models of allergic asthma induced by different sensitization and challenge protocols respond differently to modulation. For example, no airway hyperresponsiveness was developed in mast-cell–deficient mice following sensitization and challenge with OVA, whereas OVA sensitization and challenge in congenic littermates did induce nonspecific hyperresponsiveness. However, OVA-sensitized, mast-cell–deficient mice developed nonspecific hyperresponsiveness by increasing the frequency and antigen dose of challenge (19). These and other findings suggest that the relative contribution of allergen-specific IgE-dependent mast-cell activation and BALF eosinophilia to the development of nonspecific airway hyperresponsiveness not only depends on the strain of mice used but also on the sensitization and challenge protocol (32). Thus, apart from the quantitative differences between the two murine models of ongoing disease, there might be qualitative differences as well, perhaps indicating differential activation of T-cell signaling pathways under the various conditions of allergen exposure.

From the results obtained from this and previous studies (18, 33), we conclude that in our mild and more severe (ongoing) models, neither allergen-specific IgE nor BALF eosinophilia are solely responsible for the development or maintenance of airway hyperresponsiveness. Additional evidence for the dissociation of BALF eosinophilia and nonspecific hyperresponsiveness in the more severe ongoing model comes from an experiment in which two different routes of administration for mCTLA4-IgG were compared. Whereas local (intranasal) administration of mCTLA4-IgG results in a diminution of hyperresponsiveness similar to systemic (intravenous) administration of mCTLA4-IgG compared with control-treated OVA-challenged animals, only systemic administration of mCTLA4-IgG resulted in reduced BALF eosinophilia (D.T. Deurloo, unpublished observation).

For optimal treatment of asthma patients, it remains to be determined which (ongoing) model best represents their clinical situation. The use of various mouse strains combined with various sensitization and challenge protocols might be meaningful because the group of patients with asthma is very heterogeneous, both genetically and in the way they are exposed to environmental allergens. As an advantageous feature of the mild (ongoing) model, it can be said, however, that the percentage of eosinophils found in the BALF is very similar to that observed in the lavage fluid of mild to severe asthmatics (10–20%, 24 h after allergen provocation [34]).

The gold standard dexamethasone performed equally well as mCTLA4-IgG in the mild ongoing model of airway inflammation, except that circulating OVA-specific IgE levels were not inhibited. This observation on allergen-specific IgE levels is in agreement with previous studies both in humans and mice (35, 36). In the more severe ongoing model, however, dexamethasone treatment of OVA-challenged mice was more effective in comparison with mCTLA4-IgG administration. If we extrapolate the results from this study to the human situation, these data imply that patients with mild disease can benefit from specific treatment with CTLA4-Ig, whereas the more generally suppressive drug dexamethasone, perhaps in combination with CTLA4-Ig, is required for patients suffering from more severe disease.

In summary, we described the effects of mCTLA4-IgG after induction of asthma manifestations in two murine models of ongoing disease, which differed in severity of the disease. In a mild ongoing model of allergic airway inflammation, mCTLA4-IgG was very effective, whereas in a more severe ongoing model, its effects were less pronounced. We conclude from this study that CTLA4-Ig might be an effective alternative therapy for established allergic asthma, especially in situations of mild disease. Future experimental studies investigating the long-term effects of mCTLA4-IgG in (ongoing) murine models of allergic asthma will further clarify its therapeutic potential.

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