

Blockade of CTLA-4 Promotes Airway Inflammation in Naive Mice Exposed to Aerosolized Allergen but Fails to Prevent Inhalation Tolerance

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Abstract

In subjects not developing allergy, inhalation of nonpathogenic protein antigens causes no harm and is associated with tolerance induction. Repeated exposure to aerosolized ovalbumin (OVA) likewise does not evoke airway inflammation and induces inhalation tolerance in experimental animals. The present study explored the role of the inhibitory T-cell receptor CTLA-4, in preventing inflammation and in establishing inhalation tolerance in response to a protein antigen. Naive BALB/c mice were injected intraperitoneally with anti-CTLA-4 monoclonal antibody or control immunoglobulin G (IgG) and exposed daily to aerosolized saline or OVA over 10 or 20 consecutive days. OVA-specific IgE levels and the inflammatory response in airway tissues were assessed 2 days after last exposure. The OVA-specific IgE response was also evaluated in mice subjected to a subsequent immunogenic OVA challenge 18 days after last aerosol exposure. Additional mice were made tolerant by 10 days of OVA aerosol exposure and were then subjected to an immunogenic OVA challenge combined with CTLA-4 blockade or control IgG treatment. Repeated inhalation of aerosolized OVA alone did not cause a pulmonary inflammatory response. In contrast, 10 days of OVA exposure combined with blockade of CTLA-4 led to development of eosinophilic lung infiltrates, BAL fluid eosinophilia, goblet cell hyperplasia and increased OVA-specific IgE. By 20 days of OVA exposure and blockade of CTLA-4, the inflammatory response remained. Mice exposed to aerosolized OVA for 10 days exhibited greatly reduced OVA-specific IgE responses to subsequent immunogenic OVA challenge. Blockade of CTLA-4 during the period of OVA aerosol exposure did not prevent this suppression of the OVA-specific IgE response. Neither did blockade of CTLA-4 during immunogenic OVA challenge alter the allergen-specific IgE response. Our results indicate that *in vivo* blockade of CTLA-4 modulates the initial immune response to a protein antigen allowing the development of allergen-induced airway inflammation in naive mice. However, this initial exaggerated immune response is followed by the induction of inhalation tolerance, demonstrating that CTLA-4 signalling is not decisive in this process. Our findings also show that once inhalation tolerance is established it may not be disrupted by blockade of CTLA-4.

Introduction

The respiratory mucosa is continuously exposed to innocuous aeroallergens, which, in most individuals, do not induce pathology. However, an increasing number of individuals in western societies become sensitized in response to nonpathogenic antigens and develop airway

inflammatory diseases such as allergic asthma and rhinitis. The allergic inflammatory response in these diseases characteristically involves airway infiltration of eosinophils and production of allergen-specific IgE [1].

The normal immune response to innocuous antigens encountered at the respiratory mucosa is associated with

induction of tolerance. Accordingly, experimental models have shown that repeated exposure to an aerosolized protein antigen, ovalbumin (OVA), does not lead to the development of airway histopathology [2–4] but may evidently elicit a phenomenon known as inhalation tolerance [5–7]. An enigmatic issue is to explain why some individuals mount allergic immune responses to otherwise innocuous environmental antigens and why some do not. This enigma makes it of interest to characterize the immunological mechanisms that may disrupt the homeostatic balance evoking allergen-induced airway inflammation.

CD4⁺ T cells play a critical role in initiation and progression of allergic asthma [8]. The proliferation and differentiation of CD4⁺ T cells require at least two signals. The T-cell receptor interaction with antigen/MHC-II expressed on antigen-presenting cells provides the first signal, and the second is delivered by costimulatory molecules [9]. The costimulatory signal critical for initial cell-cycle progression, IL-2 production and clonal expansion of T cells, is provided by the interaction between B7-1 and B7-2 expressed on antigen-presenting cells and CD28 on T cells [9]. The crucial role of CD28/B7 signalling for initial T-cell activation and subsequent development of allergic airway inflammation have been repeatedly demonstrated in experimental models of allergic asthma [10–15].

A second receptor for B7-1 and B7-2, CTLA-4, binds to B7-1 and B7-2 with an approximately 20-fold greater affinity than CD28 [16]. CTLA-4 downregulates T-cell expansion and cytokine production by recently activated T cells [17]. CTLA-4 is expressed in low levels on resting T cells and is highly induced after T-cell activation and thus opposes the effect of the constitutively expressed CD28 [18]. The importance of CTLA-4 in downregulating T-cell activation and maintaining homeostasis is illustrated in mice that are CTLA-4 deficient. These mice develop lymphoproliferative disease, characterized by polyclonal T-cell activation, and die within 3–4 weeks after birth [19]. Furthermore, it has been demonstrated that blockade of CTLA-4 may abrogate peripheral T-cell tolerance induced by oral or i.p. administered antigens [20, 21]. Interestingly, CTLA-4 polymorphisms have been evaluated in humans, and at least some studies have shown significant associations between different CTLA-4 polymorphisms and asthma or atopy [22, 23].

Daily exposure to aerosolized OVA for 10 consecutive days does not induce airway inflammation [2–4]. In addition, this experimental protocol is known to induce a transient OVA-specific IgE production, followed by greatly reduced IgE responses to subsequent immunogenic OVA challenges, i.e. a state of T-cell and IgE unresponsiveness, also known as inhalation tolerance [5–7]. We hypothesized that B7/CTLA-4 interactions may be involved in the induction of inhalation tolerance. Using the above protocol for noninflammatory allergen exposures [2–4], the present study first examined occurrence

of airway inflammatory response in mice treated with anti-CTLA-4 or control immunoglobulin G (IgG). Then, additional experiments, involving immunogenic OVA challenges, investigated whether inhalation tolerance was induced following these treatment regimens. Also, separate experiments were carried out to elucidate if blockade of CTLA-4 may disrupt an already established inhalation tolerance.

Methods

Animals. Female BALB/c mice 6 weeks of age were purchased from M&B, Ry, Denmark. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pelleted food throughout the experimental period. All animals were used under protocols approved by the Ethics Committee of the Faculty of Medicine at the University of Lund.

Aerosolization protocol. Over a period of 10 or 20 consecutive days, mice were placed in a Plexiglas chamber and exposed for 20 min daily to aerosolized OVA (Grade III, Sigma, St. Louis, MO, USA; 1% wt/vol in 0.9% saline) or saline. The aerosols were generated into the chamber using a nebulizer (Bird 500 ml Inline Micronebulizer, Bird Co., Palm Springs, CA, USA) driven at 4 bar. Animals were sacrificed at different time-points after the final aerosol exposure to assess OVA-specific IgE levels and the inflammatory response in airway tissues (Table 1).

In vivo blockade of CTLA-4. Mice (groups 1–4 and 9–12) exposed to OVA or saline for 10 days were injected i.p. with 100 µg of anti-CTLA-4 monoclonal antibody (MoAb) or hamster IgG daily during the aerosolization period (Table 1). Mice (groups 5–8) exposed to OVA or saline for 20 days received 200 µg of anti-CTLA4 MoAb or hamster IgG on day 1, and every third day thereafter until termination of the experiment. Additional animals (groups 13–14) received 200 µg of anti-CTLA-4 MoAb or control IgG 1 day before and 2 days after immunogenic challenge (Table 1). The hamster anti-mouse CTLA-4 MoAb (clone UC10-4F10) was kindly provided by Dr J.A. Bluestone. Control hamster IgG was purchased from Jackson ImmunoResearch, West Grove, PA, USA.

Immunogenic challenge. At day 28, mice (groups 9–12) previously exposed to aerosolized OVA or saline for 10 days and treated with anti-CTLA-4 MoAb or control IgG, were given an i.p. injection with 10 µg OVA (Grade III), adsorbed to 1 mg of alum adjuvant. Animals were sacrificed at day 48 to evaluate the levels of OVA-specific IgE (Table 1). In separate experiments, mice (groups 13–14) exposed to aerosolized OVA without additional treatment were challenged at day 28 with an i.p. injection of 10 µg OVA (Grade III), adsorbed to 1 mg of alum adjuvant, combined with anti-CTLA-4 MoAb or control IgG treatment (Table 1).

Table 1 Study design

Groups	Aerosol exposure (day 1–10 or 1–20)	Treatment during aerosol exposure	Intraperitoneal challenge (day 28)	Treatment during intraperitoneal challenge	Termination of experiment (day)
1	OVA × 10	IgG	–	–	12
2	OVA × 10	Anti-CTLA-4	–	–	12
3	Saline × 10	IgG	–	–	12
4	Saline × 10	Anti-CTLA-4	–	–	12
5	OVA × 20	IgG	–	–	22
6	OVA × 20	Anti-CTLA-4	–	–	22
7	Saline × 20	IgG	–	–	22
8	Saline × 20	Anti-CTLA-4	–	–	22
9	OVA × 10	IgG	OVA	–	48
10	Saline × 10	IgG	OVA	–	48
11	OVA × 10	Anti-CTLA-4	OVA	–	48
12	OVA × 10	Anti-CTLA-4	Saline	–	48
13	OVA × 10	–	OVA	Anti-CTLA-4	48
14	OVA × 10	–	OVA	IgG	48

IgG, immunoglobulin; OVA, ovalbumin.

Measurement of OVA-specific IgE in plasma and analysis of cells in BAL fluid. Animals were anaesthetized with an i.p. injection of pentobarbital. Blood was drawn by cardiac puncture and placed in EDTA tubes. After centrifugation, the plasma samples were rapidly frozen. OVA-specific IgE was determined by sandwich ELISA in 96-well ELISA plates as previously described [24]. To obtain BAL fluid a tracheal cannula was inserted via a midcervical incision. The airways were lavaged twice with 0.7 ml of phosphate-buffered saline (PBS) (Life Technologies, Paisley, Scotland) as previously described [24]. The BAL fluid was immediately centrifuged (10 min, 4 °C, 160 × g). Cell pellets were resuspended in 250 µl PBS for total and differential cell counting. Differential counting was performed on May-Grünwald-Giemsa stained cytopins, 400–500 cells being counted on each cytospin. The investigator counting the cells was blinded to the treatment groups.

Histochemistry. Lung tissue specimens and extrahilar large airway segments obtained 48 h after the last OVA or saline exposure were immersed overnight in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2), rinsed repeatedly in buffer (Tyrode buffer supplemented with 10% sucrose), frozen in mounting medium (Tissue-Tek, Miles Inc., Elkhart, IN, USA) and stored at –80 °C until sectioning. Eosinophils were detected by histochemical visualization of cyanide-resistant eosinophil peroxidase activity [24, 25]. For histochemical detection of mucus-containing cells in extrahilar large airways, cryosections were stained with periodic acid-Sciff reagent (PAS) [24, 25]. Distinct purple-red granules could be observed in the mucus-containing cells. For assessment of general airway morphology, sections were stained with hematoxylin and erythrosin. For evaluation of the number of eosinophils in pulmonary tissue, 20 randomly selected areas (0.04 mm²

each) in one lung section from each animal were examined at a magnification of 400, and the mean was expressed as eosinophils/unit area. The total number of PAS-positive cells in the large airway epithelium in one section from each animal was counted. The total length of the airway epithelial basement membrane (BM) in each section was calculated using a computerized image analysis system, and the density of PAS-positive cells was expressed as cells/mm BM. All cell counts were made in a blinded fashion.

Statistics. All data are expressed as mean ± SEM. To calculate significance levels between treatment groups, Mann–Whitney rank sum test was used throughout the study. P < 0.05 were used as the generally accepted level of statistical significance for differences between groups.

Results

Repeated inhalation of aerosolized OVA alone does not cause a pulmonary inflammatory response

Mice exposed to OVA and treated with control IgG were subjected to a detailed histological analysis. No eosinophilic lung infiltrates could be detected (Fig. 1), and the number of eosinophils in lung tissue was similar in OVA and saline-exposed IgG-treated mice (Fig. 2). Likewise, the total cellular content and the percentage of eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid did not differ significantly between OVA and saline-exposed IgG-treated mice (data not shown). Furthermore, equally low numbers of mucus cells in large airways could be detected in OVA and saline-exposed IgG-treated mice (Figs 3 and 4). As previously demonstrated [2], no OVA-specific IgE was detected in OVA and saline-exposed IgG-treated mice 2 days after last aerosol exposure (Fig. 5).

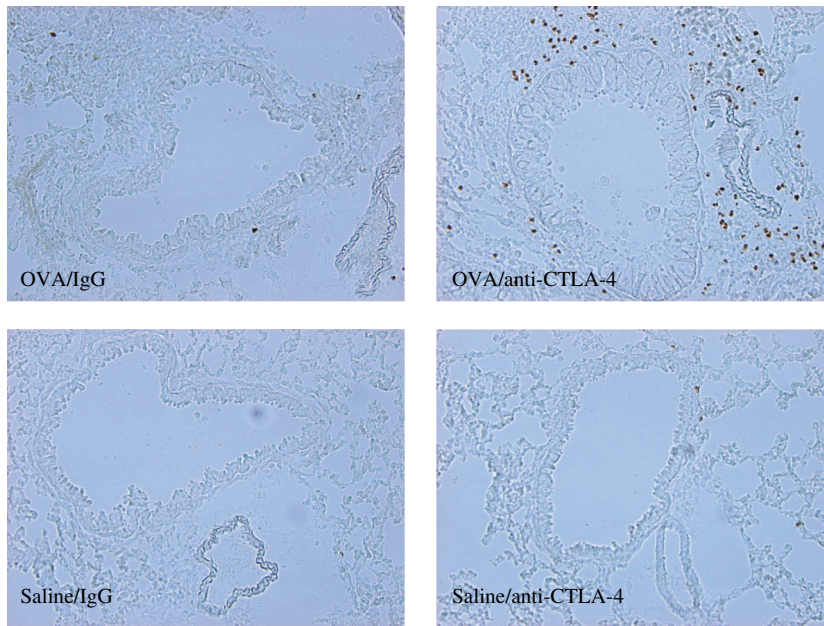


Figure 1 Lung histology. Eosinophils are visualized by histochemical demonstration of cyanide-resistant eosinophil peroxidase activity. Peribronchial and perivascular eosinophilic infiltrates are seen in anti-CTLA-4-treated ovalbumin (OVA)-exposed mice, while repeated OVA exposure alone did not result in eosinophilic infiltrates. No eosinophilic infiltrates were observed in lung tissue from saline-exposed controls treated with immunoglobulin G (IgG) or anti-CTLA-4 monoclonal antibody.

Anti-CTLA-4-treated mice develop allergic airway inflammation in response to repeated OVA inhalation

Ten days of OVA exposure combined with blockade of CTLA-4 resulted in development of a pulmonary inflammatory response. Histological evaluation revealed a moderate number of peribronchial and perivascular eosinophilic lung infiltrates in OVA-exposed anti-CTLA-4-treated mice (Figs 1 and 2). BAL fluid cell data indicated recruitment of inflammatory cells into airway lumen. Blockade of CTLA-4 during OVA exposure thus produced a significant increase in the percentage of BAL fluid eosinophils (6.6 ± 3.7 versus $0.0 \pm 0.0\%$ eosinophils in saline-exposed controls, $P < 0.01$), BAL fluid lymphocytes

(5.9 ± 1.5 versus $0.6 \pm 0.4\%$ in saline-exposed controls, $P < 0.05$) and BAL fluid neutrophils (1.2 ± 0.3 versus $0.0 \pm 0.0\%$ in saline-exposed controls, $P < 0.01$). Concomitantly, there was a decrease in the percentage of macrophages in BAL fluid (78.3 ± 4.4 versus $89.3 \pm 1.1\%$ in saline-exposed controls, $P < 0.01$). The total cellular content in BAL fluid did not differ significantly between OVA and saline-exposed anti-CTLA-4-treated mice (data not shown). In addition to the infiltration of inflammatory cells, there was a marked increase in large airway epithelial mucus cells in OVA-exposed anti-CTLA-4-treated mice (Figs 3 and 4).

Indicating that repeated exposure to OVA in anti-CTLA-4-treated mice induces a systemic antigen-specific immune response, OVA-specific IgE levels were increased in plasma in these animals (Fig. 5).

To examine the effect of CTLA-4 blockade for an extended period of OVA challenges, mice were exposed to OVA aerosol for 20 consecutive days. Histological evaluation revealed a moderate number of peribronchial and perivascular eosinophilic lung infiltrates in anti-CTLA-4-treated mice only. The number of eosinophils in lung tissue was slightly increased in anti-CTLA-4-treated mice exposed to OVA for 20 consecutive days compared with corresponding mice exposed to OVA for 10 consecutive days (7.1 ± 0.7 versus 4.6 ± 1.5 eosinophils/unit area, $P < 0.05$). However, there was no apparent evidence of an altered distribution or occurrence of eosinophilic infiltrates between the two groups. Anti-CTLA-4-treated mice exposed to OVA for 10 or 20 consecutive days exhibited similar numbers of large airway epithelial mucus cells (18.3 ± 5.0 and 21.7 ± 5.3 cells/mm BM, respectively) and levels of OVA-specific IgE (132.3 ± 36.2 and 217.8 ± 83.4 U/ml, respectively).

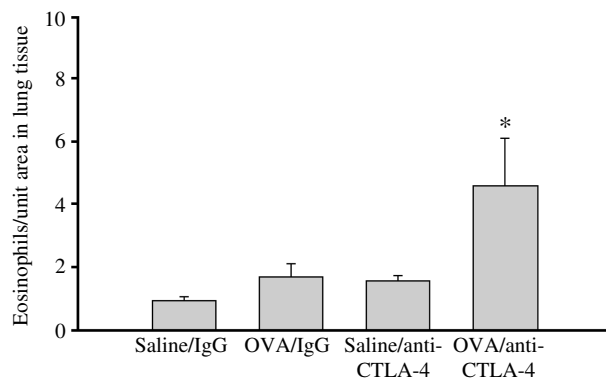
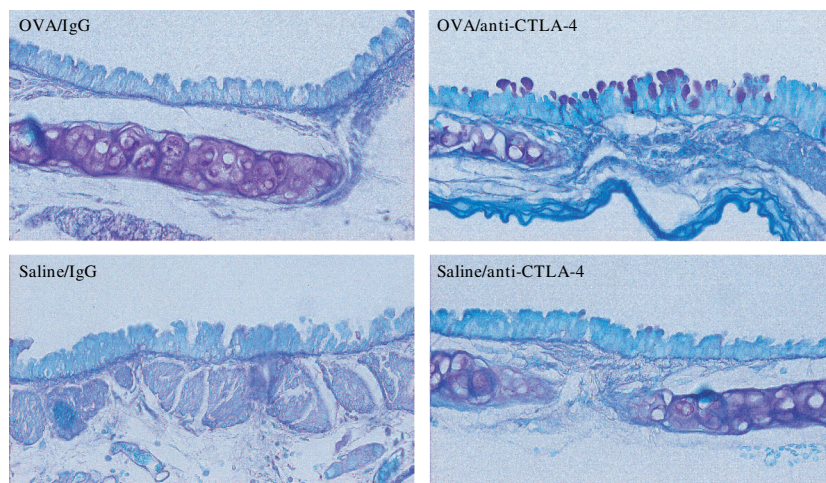


Figure 2 Number of eosinophils in lung tissue from anti-CTLA-4 or control immunoglobulin G (IgG)-treated mice 48 h after last exposure to aerosolized saline or ovalbumin (OVA). Data are presented as mean $\pm$ SEM ($n = 4$ –10 in each group). An increased number of eosinophils in lung tissue was observed in OVA-exposed anti-CTLA-4-treated mice compared with corresponding saline-exposed animals (* $P < 0.05$). No significant difference was observed between OVA and saline-exposed IgG-treated animals.

Figure 3 Light micrographs of periodic acid-Schiff reagent (PAS)-stained large airway cryosections obtained from anti-CTLA-4 or control immunoglobulin G (IgG)-treated mice 48 h after last exposure to aerosolized saline or ovalbumin (OVA). After saline exposure or exposure to OVA alone, there were very few PAS-positive cells in the airway epithelium. In contrast, after OVA exposure combined with anti-CTLA-4 treatment, there was a marked increase of PAS-positive cells in the airway epithelium.



CTLA-4 blockade fails to prevent induction of inhalation tolerance

To investigate if CTLA-4 signalling is decisive in the induction of inhalation tolerance, mice received an immunogenic challenge with an i.p. injection of OVA 18 days after last aerosol exposure. OVA-specific IgE responses were much lower in animals exposed to aerosolized OVA as compared with the control group exposed to aerosolized saline (Fig. 6). Thus, as previously demonstrated [6, 7], repeated exposure of mice to aerosolized OVA markedly reduce their capacity to mount IgE responses to an immunogenic challenge with OVA. Blockade of CTLA-4 during the period of OVA aerosol exposure did not abrogate this suppression of the OVA-specific IgE response. A moderate increase, although statistically insignificant, in OVA-specific IgE levels was detected after immunogenic challenge of OVA-exposed anti-CTLA-4 treated mice compared with corresponding IgG-treated mice (Fig. 6). However, similar

levels of OVA-specific IgE were detected in OVA-exposed anti-CTLA-4 treated mice i.p. challenged with OVA or saline. Hence, these slightly increased levels of OVA-specific IgE reflect production during the exaggerated initial immune response to aerosolized OVA rather than a response to the i.p. OVA challenge.

Blockade of CTLA-4 fails to disrupt established inhalation tolerance

Blockade of CTLA-4 during the subsequent immunogenic OVA challenge did not affect the following OVA-specific IgE response. Thus, equally low levels of OVA-specific IgE were detected in mice made tolerant by 10 days of OVA aerosol exposure and then subjected to either anti-CTLA-4 or control IgG treatment during immunogenic challenge (Fig. 6).

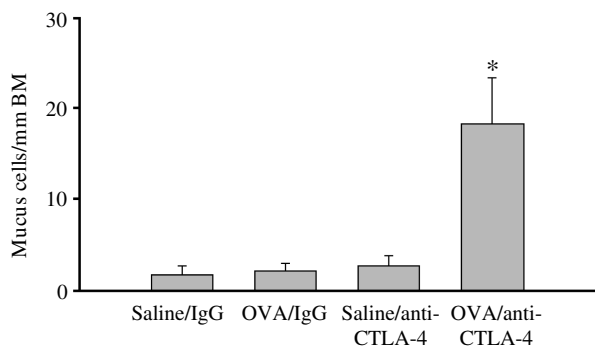


Figure 4 Quantification of mucus cells in periodic acid-Schiff reagent (PAS)-stained large airway cryosections obtained from anti-CTLA-4 or control immunoglobulin G (IgG)-treated mice 48 h after last exposure to aerosolized saline or ovalbumin (OVA). Data are presented as mean ± SEM ($n=4-10$ in each group). Increased numbers of PAS-positive cells are seen in OVA-exposed anti-CTLA-4 treated mice. * $P<0.05$ compared with corresponding saline-exposed mice. BM, basement membrane.

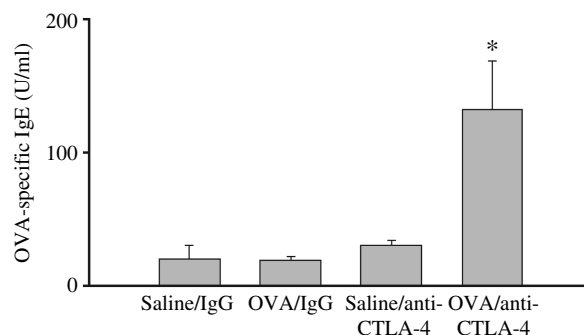


Figure 5 Ovalbumin (OVA)-specific IgE in plasma was measured using ELISA. Plasma was obtained after centrifugation of blood drawn by cardiac puncture from mice 48 h after the last aerosol exposure. Data are presented as mean ± SEM ($n=4-10$ in each group). Enhanced levels were detected in anti-CTLA-4 treated OVA-exposed mice only. Values below actual detection limit were seen in controls. * $P<0.01$ compared with corresponding saline-exposed mice.

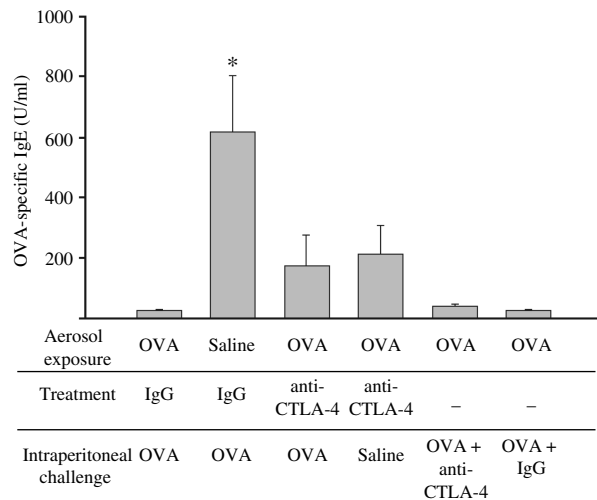


Figure 6 Ovalbumin (OVA)-specific IgE in plasma was measured using ELISA. Plasma was obtained after centrifugation of blood drawn by cardiac puncture from mice 20 days after immunogenic challenge. Data are presented as mean $\pm$ SEM ($n = 4-5$ in each group). * $P < 0.01$ compared with OVA aerosol-exposed and control immunoglobulin G (IgG)-treated mice.

Discussion

This study demonstrates that *in vivo* blockade of CTLA-4 may modulate the initial immune response to an inhaled protein, allowing development of allergen-induced airway inflammation in animals that otherwise do not mount an inflammatory response to repeat allergen exposures. This initially enhanced immune response was not sustained, and it was followed by the induction of inhalation tolerance, demonstrating that CTLA-4 signalling is not decisive in this process. Our findings also show that once inhalation tolerance has been established it may not be disrupted by introducing blockade of CTLA-4 in association with subsequent immunogenic challenge. These data are of interest for our understanding of development of allergic inflammatory conditions.

The present protocol involving 20 min exposures to aerosolized OVA over a period of 10 consecutive days has been used previously to study inhalation tolerance [5–7]. Our data confirm previous findings that repeated exposure of mice to aerosolized OVA markedly reduce their capacity to mount IgE responses to an immunogenic challenge with OVA. This study also confirms previous reports [2–4] that mice treated according to this protocol do not show any evidence of an ongoing inflammatory response supporting the utility of this model in studies of inhalation tolerance. Using this model, we demonstrated that repeated OVA exposure combined with blockade of CTLA-4 result in development of a pulmonary inflammatory response involving eosinophilic lung infiltrates, recruitment of inflammatory cells into airway lumen,

marked increase in large airway epithelial mucus cells and increased OVA-specific IgE levels in plasma. These findings indicate that CTLA-4 may regulate the intensity of the initial immune response to inhaled OVA. Indeed, it has been shown using a protocol involving repeated OVA exposure that the establishment of tolerance and the characteristic T-cell unresponsiveness to aeroallergens is an active process initiated by a transient phase of CD4+ T-cell activation with increased proliferation, expression of activation markers and cytokine production [26]. The activated-CD4+ T cells are subsequently depleted to leave a stable population of functionally impaired T cells, which are refractory to antigen [26]. Hence, we speculate that the transient phase of CD4+ T-cell activation and cytokine production are reinforced by deficient CTLA-4 signalling, thus promoting the present inflammatory reaction in response to OVA. In agreement, a number of studies involving active immunization, in contrast to our approach using a model of inhalation tolerance, have demonstrated that blockade of CTLA-4 enhances the immune response and the following inflammatory reaction to a number of pathogenic and nonpathogenic antigens [27–30]. For example, Hellings *et al.* [27] demonstrated enhanced airway inflammation upon inhalatory allergen challenge in BALB/c mice that had been injected with anti-CTLA-4 MoAb during i.p. sensitization with OVA. Furthermore, it was recently shown that blockade of CTLA-4 during oral sensitization to peanut protein exaggerates the following allergic reaction with enhanced IgE responses and elevated mast cell degranulation [28]. Blockade of CTLA-4 in these prior studies led to increased production of Th2-associated cytokines which may enhance the immune response and the subsequent inflammatory reaction. For example, an increased IL-4 and IL-5 production was demonstrated in lymph nodes obtained a few days after infection with a nematode in mice treated with anti-CTLA-4 MoAb [29]. Moreover, *in vitro* studies have shown that the relative contribution of CD28 and CTLA-4 costimulatory signals influences the differentiation of naive T cells. CD28 ligation has been demonstrated to promote the production of Th2-type cytokines by naive murine T cells via an IL-4-dependent mechanism [31]. On the contrary, CTLA-4 has been reported to be a potent inhibitor of Th2 differentiation and polarize naive T cells towards the Th1 phenotype [32, 33]. Thus, it may be speculated that the allergen-induced inflammatory response described in the present study depends on Th2-promoting effects of CTLA-4 blockade. CTLA-4 has also been shown to play an important role in the function of CD25+CD4+ regulatory T cells, which produce IL-10 and TGF- β and downregulate Th1 and Th2 responses [34–36]. Thus, blockade of CTLA-4 may disturb the suppressive action of regulatory T cells and thereby allow excessive Th-cell activation, leading to an inflammatory response. However, further studies on these possibilities

are required, involving characterization of T-cell subsets and cytokine profiles.

To examine whether the inflammatory response observed in OVA aerosol-exposed mice treated with anti-CTLA-4 reflected a sustained inhibition of tolerance induction, experiments were performed evaluating the OVA-specific IgE response to immunogenic challenge with OVA. As previously demonstrated [6, 7], the present mice exposed to aerosolized OVA for 10 days produced greatly reduced OVA-specific IgE responses to subsequent immunogenic OVA challenge. Interestingly, blockade of CTLA-4 during the period of OVA aerosol exposure did not prevent this suppression of the OVA-specific IgE response, demonstrating that CTLA-4 signalling does not have a crucial role in the establishment of inhalation tolerance. Previous studies have indicated that blockade of CTLA-4 may abrogate peripheral T-cell tolerance induced by oral or i.p. administered antigens [20, 21]. However, this issue remains controversial as it has also been reported that CTLA-4 blockade fails to inhibit the induction of tolerance in similar models. For example, it was recently shown that blockade of CTLA-4 could prevent neither antigen-unresponsiveness following oral exposure to antigen, nor unresponsiveness induced by 3 days of intranasal antigen exposure [26, 28].

The present study also demonstrated that the inflammatory response evoked by CTLA-4 blockade during OVA exposure was maintained for a period of 20 days. Notably, there was no general enhancement of the response despite the prolonged antigen exposure concomitant with CTLA-4 blockade. These results suggest that other mechanisms than CTLA-4 engagement may reduce T-cell expansion preventing further progress of allergen-induced inflammation in this model. In fact, two inhibitory B7-recognizing receptors in addition to CTLA-4 have recently been identified, PD-1 and BTLA. All three inhibitory B7-recognizing receptors are expressed on and regulate previously activated-T cells in ongoing immune responses [37]. PD-1 and BTLA may have a major role to suppress cytokine production and inhibit T-cell expansion leading to tolerance induction. Interestingly, the interaction between these receptors and B7 molecules may induce bidirectional signalling, delivering signals into both T cells and B-7 expressing cells, representing a fine-tuned network of regulatory signals [37]. Hence, it will be of interest to learn about the relative contribution of the three known inhibitory B7-recognizing receptors and their ligands for the induction of inhalation tolerance in this model.

Importantly, the present study also showed that once inhalation tolerance is established it may not be disrupted by blockade of CTLA-4. To date, only one study has shown that established inhalation tolerance can be broken *in vivo*. To accomplish this effect, a complex interaction was required involving concomitant expression of

granulocyte-macrophage colony-stimulating factor (GM-CSF) and decorin, the latter a natural inhibitor of TGF- β [38]. This singular report emphasizes that the immune system has evolved powerful regulatory mechanisms to control responsiveness to innocuous allergen. Disrupting tolerance may then require at least two kinds of interventions, one that allows for allergic sensitization and one that inhibits regulatory activity [38]. In contrast to the paucity of reports on disruption of established tolerance, several studies have demonstrated inhibition of tolerance induction when animals were exposed to inhalatory antigen concomitant with acute respiratory virus infection, airborne endotoxin, glucan or GM-CSF [2, 39–42]. This implies that the context of initial exposure to antigen where a regulatory role of CTLA-4 is indicated (this study) has significant influence on the subsequent immune response, and that this critical stage is more easily disturbed than established tolerance. It may further be assumed that a profound influence on the immunological state is achieved in the studies involving viral infections, etc. Hence, it is not surprising that the present blockade of one single receptor, CTLA-4, was not sufficient to prevent induction of tolerance.

Conclusions

In conclusion, this study demonstrates that *in vivo* blockade of CTLA-4 may modulate the initial immune response to an inhaled protein antigen allowing the development of allergic airway inflammation. However, this initially exaggerated immune response is followed by the induction of inhalation tolerance, demonstrating that CTLA-4 signalling is not decisive in this important process. Our findings also show that once inhalation tolerance is established, it may not be broken by blockade of CTLA-4. Our study thus describes a mechanism by which the homeostatic balance in the lung is modified to permit development of allergen-induced inflammation. Nevertheless, tolerance induction is not prevented, suggesting participation of other important regulating mechanisms in addition to CTLA-4 signalling.

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