



Interleukin-10 does not mediate inhalational tolerance in a chronic model of ovalbumin-induced allergic airway disease [☆]

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Abstract

Objective: IL-10 is a potent anti-inflammatory cytokine, and IL-10-producing regulatory T cells are effective inhibitors of murine asthmatic responses. This study determined whether IL-10-dependent mechanisms mediated the local inhalational tolerance seen with chronic inhalational exposure to antigen.

Methods: Wildtype and IL-10^{-/-} mice were sensitized with ovalbumin (OVA) and then challenged with daily OVA inhalations for 10 days or 6 weeks.

Results: The 10-day animals developed allergic airway disease, characterized by BAL eosinophilia, histologic airway inflammation and mucus secretion, methacholine hyperresponsiveness, and OVA-specific IgE production. These changes were more pronounced in IL-10^{-/-} mice. The 6-week IL-10^{-/-} and wildtype animals both developed inhalational tolerance, with resolution of airway inflammation but persistence of OVA-specific IgE production.

Conclusion: IL-10 may have anti-inflammatory effects in the acute stage of murine allergic airways disease, but the cytokine does not mediate the development of local inhalational tolerance with chronic antigen exposure.

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Keywords: Airway inflammation; Asthma; Cytokines; Regulatory T cells

1. Introduction

In the last decade, tremendous experimental efforts have been directed towards elucidating the inflammatory processes that occur in the lungs of patients with asthma. Current paradigms propose that allergic asthma occurs because of a breakdown of the normal tolerance to inhaled antigen, resulting from complex interactions between host and environmental factors. This breakdown allows for the accumulation of T-helper 2 (Th2) CD4⁺ lymphocytes in and

around the airways. Th2 cells direct the allergic inflammatory response by their production of such cytokines as IL-4, which enhances IgE synthesis; IL-5, which stimulates eosinophil proliferation; and IL-13, which increases mucus production and induces airway smooth muscle hyperreactivity [1,2]. In the normal, tolerant condition, the development of Th2 responses to inhaled antigens is suppressed by other lymphocytes that “regulate” the function of effector T-cells. Types of regulatory T cells include Th3 cells, T_R1 cells, CD4⁺CD25⁺ Foxp3⁺ cells, CD8⁺ cells, and natural killer T (NKT) cells [3,4]. It has been suggested that T_R1 cells are the key cells regulating allergic airway disease [5].

One mechanism by which T_R1 cells exert their inhibitory effects is via production of the anti-inflammatory cytokine, IL-10. IL-10 has been shown to down-regulate proliferation

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of Th2 clones and to decrease production of IL-4 and IL-5 [6]. In addition, by inhibiting the expression of CD40 on eosinophils, IL-10 accelerates eosinophil apoptosis, providing an additional mechanism to attenuate eosinophilic inflammation [7]. In allergic mice, the intranasal administration of rIL-10 inhibits airway eosinophilia in a dose-dependent manner [8]. In addition, mice expressing a transgene for human IL-10 have an impaired ability to mount specific T- or B-cell-mediated immune responses to soluble antigen [9]. It should be noted, however, that IL-10 also shows immunostimulatory effects in an ovalbumin (OVA)-induced model of allergic airway disease (AAD)¹ [9]. Conflicting findings have also been reported in human asthmatic subjects. Some studies have shown no difference between IL-10 levels in BAL fluid from asthmatics and controls [10], nor increased numbers of BAL cells positive for IL-10 mRNA in symptomatic asthmatics compared with control subjects [11]. Other studies have demonstrated decreased levels of IL-10 in BAL fluid and transcription of IL-10 in T lymphocytes from asthmatic subjects compared to healthy controls [12,13]. In addition, a genotype associated with low IL-10 production has been associated with severe asthma [14].

We have described a model in which there is a natural progression from acute AAD at 3–14 days of aerosol exposure to local inhalation tolerance (LIT) after 6–12 weeks of exposure [15,16]. Unlike other models, this model is unique in that LIT develops subsequent to an intense Th2 response in the lung. We have previously observed that IL-10 levels increase in BAL fluid with the progression from acute AAD to chronic LIT [16]. The present study used this model to determine whether the conversion from AAD to LIT is dependent upon immunomodulatory effects of IL-10.

2. Methods

2.1. Animals and sensitization protocol

Male and female C57BL/6J and B6.129P2-I110^{tm1Cgn} (IL-10^{-/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed conventionally in an animal room with ambient temperature 22–24°C and a daily light-dark cycle (0600–1800 light). Chow and water were supplied ad libitum. Animal welfare was in accordance with Institutional and Office of Laboratory Animal Welfare guidelines, and all experimental procedures were approved by the institution's Center for Laboratory Animal Care. At age 6–8 weeks, mice were sensitized to OVA as previously described [16; Fig. 1]. Briefly, they received 3 weekly intra-peritoneal injections of 25 µg of OVA (grade V; Sigma Chemical Co., St. Louis, MO) and 2 mg of aluminum hydroxide (alum) suspended in 0.5 ml of saline. One

week after the last injection the mice were exposed to 1% aerosolized OVA either 1 h/day for 7–10 days (acute), or 1 h/day for 10 days followed by 5 days/week for 4 weeks (chronic). The OVA aerosols were generated by a BANG nebulizer (CH Technologies, Westwood, NJ) into a 7.6-L, 48-port inhalation exposure chamber to which mice placed in plastic restraint tubes were attached (CH Technologies). Chamber airflow was 6 L/min. Exposure to the 1% OVA aerosol for 1 h resulted in an estimated inhaled OVA dose of 30–40 µg/mouse. Twenty-four hours after the final aerosol exposure, the mice were sacrificed by ketamine/xylazine overdose and exsanguination, and analyses of BAL fluid, lung tissue, and blood samples were performed.

2.2. BAL cellular analysis

At sacrifice, the lungs from each animal were lavaged in situ with five 1-ml aliquots of sterile saline. Total leukocytes were counted with a hemocytometer using Trypan blue dye exclusion as a measure of viability. Leukocyte differentials were determined in BAL fluid using cytocentrifuged preparations stained with May-Grünwald/Giemsa.

2.3. Histology

After sacrifice, unmanipulated lungs from separate animals ($n=4-6$ in each group) not subjected to BAL were removed, fixed with 10% buffered formalin, and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin and with periodic acid-Schiff for the specific identification of mucus. The whole lungs were fixed and sections from all five lobes were examined in their entirety in a blinded manner.

2.4. OVA-specific serum IgE levels

OVA-specific IgE levels in venous blood samples were measured by ELISA using isotype-specific capture monoclonal antibodies. IgE was captured from diluted serum using Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) coated with anti-mouse IgE (R35-72; BD PharMingen, San Diego, CA) at 2 µg/ml in 0.1 mol/L carbonate, pH 9.5. Detection was with an OVA-digoxigenin conjugate followed by horseradish peroxidase-conjugated anti-digoxigenin. Plates were developed with the TMB microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

2.5. Airway hyperreactivity

Airway responses to methacholine were assessed by whole-body barometric plethysmography [16]. Mice were studied 12 h after the third OVA aerosol in acute animals or during the final week of exposure in chronic animals. Animals were placed in the main chambers of whole-body plethysmographs (Buxco Electronics, Troy, NY) and exposed for 2 min to aerosolized saline or increasing concentrations

¹ Abbreviations used: AAD, allergic airway disease; BAL, bronchoalveolar lavage; LIT, local inhalational tolerance.

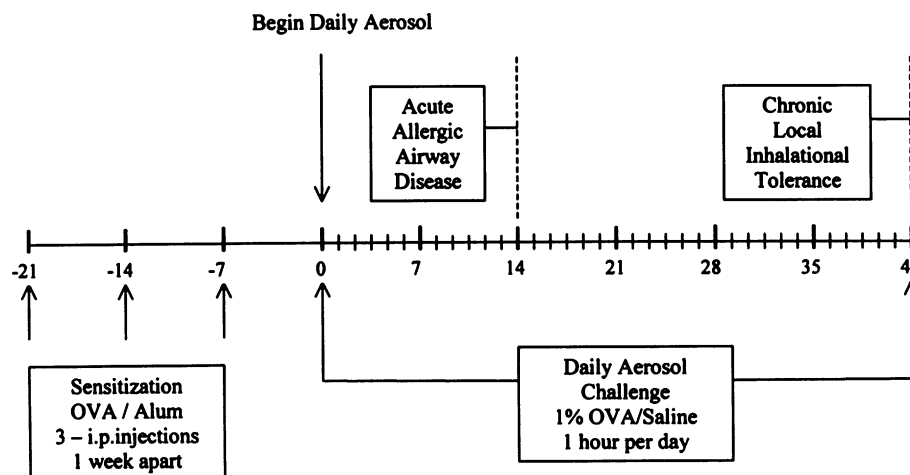


Fig. 1. Mouse sensitization and aerosol exposure protocol. Mice received 3 weekly intra-peritoneal injections of ovalbumin. One week after the last injection, the mice were exposed to 1% aerosolized OVA either 1 h/day for 10 consecutive days (acute, AAD) or 1 h/day for 10 consecutive days followed by 5 days/week for 4 weeks (chronic, LIT).

of methacholine (3–300 mg/ml). Respiratory system variables including tidal volume, respiratory frequency, inspiratory and expiratory times, and changes in box pressure were recorded before and during aerosolization and for 4 min after each exposure. The maximal enhanced pause (Penh) value response to methacholine was recorded at each dose. Sensitivity to methacholine was characterized by the interpolated concentration of methacholine needed to increase the Penh value to 2 U. Changes in Penh-2 values before and after aerosol exposures were used to characterize the development of airway hyperreactivity.

2.6. Statistical analysis

Statistical comparisons between groups were made with analysis of variance using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA). Dose–response data and serum IgE levels were compared by repeated measures analysis of variance. Changes in Penh-2 values before and after aerosol exposure were made by paired *t* tests and were compared between groups by repeated measures analysis of variance. A *p* value less than 0.05 was used to determine statistical significance.

3. Results

3.1. *IL-10*^{−/−} mice did not differ from wildtype mice in the development of AAD with acute OVA aerosol challenges and the progression to LIT with chronic OVA exposure

With exposure to OVA aerosols for 7 days, sensitized *IL-10*^{−/−} mice and wildtype mice developed AAD, characterized by marked airway eosinophilia (Fig. 2; Table 1). Relative to values in naive unchallenged animals, all cell types were increased in number; however, there was no difference in any cell number or its percentage of total BAL leukocytes between the *IL-10*^{−/−} and wildtype mice.

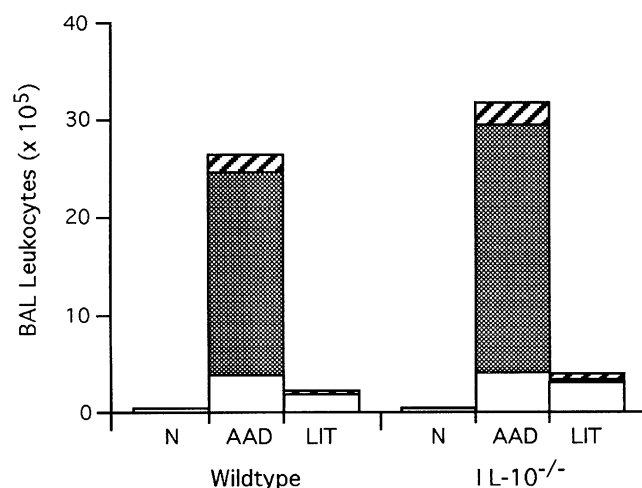


Fig. 2. BAL leukocyte counts in wildtype and *IL-10*^{−/−} mice in naive conditions and following 7 days (acute) and 6 weeks (chronic) of OVA aerosol exposures. Wildtype and *IL-10*^{−/−} mice were sensitized to OVA and then exposed to OVA aerosols as described in the text. BAL from naive animals contained almost exclusively macrophages. Acute OVA aerosol challenges resulted in robust airway eosinophilia in both wildtype and *IL-10*^{−/−} animals. This airway eosinophilia resolved with 6 weeks of continued OVA aerosol exposures. Data represent mean values for macrophages (open bars), PMNs (solid bars), eosinophils (shaded bars), and lymphocytes (striped bars); their SEM are given in Table 1.

Following 6 weeks of OVA aerosol exposure, both the *IL-10*^{−/−} and wildtype mice developed LIT, as defined by the near resolution of the airway eosinophilia. Both groups of chronically exposed animals continued to show increased BAL leukocytes relative to naive levels. There were no differences in cell counts between the two groups of chronic animals; however, the chronic *IL-10*^{−/−} mice had a lower percentage of macrophages and a higher percentage of neutrophils in their BAL relative to chronic wildtype animals (*p* = 0.002 and *p* < 0.0001 by ANOVA, respectively).

Table 1

BAL leukocyte counts and differentials in wildtype (WT) and IL-10^{-/-} mice in naive conditions and following 7-days (acute) or 6-weeks (chronic) OVA aerosol exposures

	WT naive	WT acute	WT chronic	IL-10 ^{-/-} naive	IL-10 ^{-/-} acute	IL-10 ^{-/-} chronic
BAL WBCs ($\times 10^5$)	0.33 $\pm$ 0.05	26.3 $\pm$ 8.3	2.02 $\pm$ 0.18	0.40 $\pm$ 0.08	31.3 $\pm$ 3.0	4.29 $\pm$ 0.84
Macs (%)	0.32 $\pm$ 0.05 (>98)	3.60 $\pm$ 1.23 (14 $\pm$ 3)	1.71 $\pm$ 0.14 (84 $\pm$ 1)	0.32 $\pm$ 0.06 (80 $\pm$ 12)	3.85 $\pm$ 0.47 (13 $\pm$ 2)	2.86 $\pm$ 0.57 (67 $\pm$ 4)*
Eos (%)	0 (0)	20.9 $\pm$ 7.4 (77 $\pm$ 4)	0.09 $\pm$ 0.03 (4 $\pm$ 1)	0 (0)	25.1 $\pm$ 2.6 (80 $\pm$ 2)	0.31 $\pm$ 0.10 (7 $\pm$ 2)
PMNs (%)	<0.01 (<1)	0.09 $\pm$ 0.06 (1 $\pm$ 1)	0.10 $\pm$ 0.03 (5 $\pm$ 1)	0.08 $\pm$ 0.02 (20 $\pm$ 8)	0.30 $\pm$ 0.18 (1 $\pm$ 1)	0.59 $\pm$ 0.14 (13 $\pm$ 2)**
Lymphs (%)	<0.01 (<1)	1.76 $\pm$ 0.45 (8 $\pm$ 1)	0.12 $\pm$ 0.02 (6 $\pm$ 1)	0 (0)	2.34 $\pm$ 0.50 (7 $\pm$ 1)	0.54 $\pm$ 0.18 (13 $\pm$ 3)

Data represent means $\pm$ SE values.

* Designates $p < 0.005$.

** Designates $p < 0.0001$ between WT and IL-10^{-/-} mice exposed to the same conditions.

3.2. Histological evidence of peribronchial inflammation was present in AAD wildtype and IL-10^{-/-} mice but absent in both groups of LIT mice

Qualitative histological evaluations were made on unmanipulated, uninflated formalin-fixed lungs from separate groups of AAD and LIT wildtype and IL-10^{-/-} animals. As previously reported [15], acutely challenged wildtype mice developed pathologic changes typical of asthma, including perivascular and peribronchial inflammation characterized by infiltrates of lymphocytes, plasma cells, and eosinophils; moderate thickening of the bronchial smooth muscle layer; and widening of alveolar septa (Fig. 3A). These pathologic changes were qualitatively more pronounced in the acute IL-10^{-/-} mice relative to wildtype animals (Fig. 3B). Staining with PAS demonstrated mucus secretion and airway plugging in acutely challenged mice. Again, this finding appeared more extensive in the IL-10^{-/-} than the wildtype animals (Fig. 3C and D). In 6-week chronically exposed mice, the histologic evidence of lung inflammation had subsided. There was less cellular infiltration, less airway edema, and minimal or no mucus production. These findings were similar in both wildtype and IL-10^{-/-} animals (Fig. 3E and F) and were indistinguishable from those in naive mice.

3.3. IL-10^{-/-} mice developed more airway hyperreactivity than wildtype mice with acute OVA aerosol exposures, but both groups lost their hyperreactivity with chronic exposure

Increasing doses of methacholine elicited dose-dependent increases in Penh compared to basal and saline values (Fig. 4). Relative to the pre-OVA baseline responses, Penh responses increased after 3 days of OVA aerosols in wildtype mice. The Penh responses returned to baseline after 6 weeks of OVA aerosols, consistent with the development of LIT. Characterization of respiratory responsiveness by p2 values demonstrated that naive wildtype and IL-10^{-/-} mice had similar baseline reactivity (Fig. 4). Wildtype animals became 2.5-fold more reactive to methacholine (95% Confidence Interval: 1.8–3.5-fold) with acute OVA exposure, while the IL-10^{-/-} mice became 7.0-fold more reactive (95% C.I.: 3.8–12-fold; $p = 0.017$ vs. wildtype response). With

chronic OVA exposure, methacholine sensitivity returned to baseline values in both groups of mice.

3.4. Serum OVA-specific IgE levels increased with OVA aerosol exposure and were higher in IL-10^{-/-} mice than in wildtype animals

OVA-specific IgE was not detected in naive wildtype or IL-10^{-/-} mice but was present in acute and chronic OVA-exposed animals. IgE levels were log transformed to normalize variance prior to analysis. The geometric mean OVA-specific IgE was 3.6-fold higher in acute IL-10^{-/-} mice than in acute wildtype mice ($7.34 \pm 0.22 \mu\text{g/ml}$ vs. $2.02 \pm 0.09 \mu\text{g/ml}$; $p = 0.01$; Fig. 5). The OVA-specific IgE levels increased further in both groups of mice ($p = 0.026$ vs. acute levels by analysis of variance). The continued systemic production of OVA-specific IgE despite the attenuation of lung inflammatory responses with chronic OVA aerosol exposure is a cardinal feature of the LIT that develops in this model [16].

4. Discussion

As previously observed in wildtype C57BL/6 mice [16], OVA-sensitized IL-10^{-/-} mice underwent a biphasic (AAD to LIT) response to aerosolized OVA challenge. With acute OVA exposure, the mice developed AAD, characterized by airway eosinophilia, peribronchial inflammation, and bronchial mucus production, increased responsiveness to inhaled methacholine, and increased OVA-specific IgE production. In contrast, during chronic OVA exposure, the mice established LIT, with ablation of the lung and airway changes but continuation of OVA-specific IgE production. We have previously reported that LIT is dependent on continuous exposure to antigen and demonstrates nonspecific bystander suppression of airway eosinophilia induced by an unrelated antigen [16].

The maintenance of homeostasis at mucosal surfaces constantly exposed to antigens is a complex process, involving many different regulatory and effector lymphocytes, antigen-presenting cells, cytokines, and other biochemical mediators. One important regulatory cytokine is IL-10. In the airway, IL-10 exerts direct inhibitory effects on CD4⁺ T lymphocytes [17]. In addition, the cytokine suppresses the

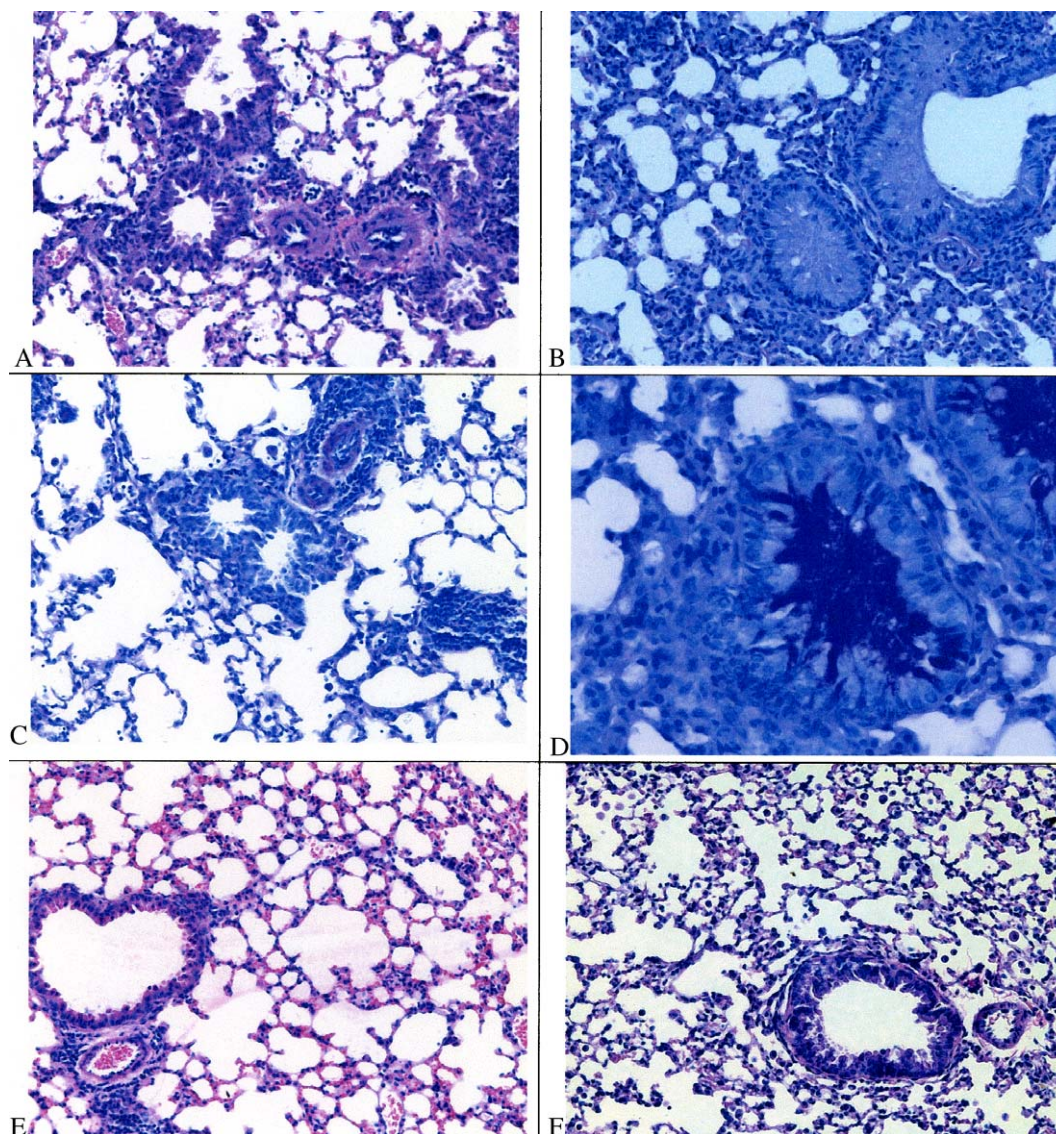


Fig. 3. Lung histology from 10-day and 6-week OVA aerosol challenged wildtype and IL-10^{-/-} animals. Top panels: The 10-day acute stage AAD was characterized by dense peribronchial mixed inflammation mainly comprising eosinophils and lymphocytes. These findings were qualitatively more pronounced in IL-10^{-/-} (B) than wildtype mice (A). Middle panels: PAS staining of AAD wildtype and IL-10^{-/-} animals showed minimal mucus plugging of airways in wildtype animals (C) but dense mucus plugging in IL-10^{-/-} animals (D). Lower panels: the chronic stage LIT animals had minimal peribronchial eosinophil and lymphocyte infiltration, and mild bronchial lining cell hyperplasia in both wildtype (E) and IL-10^{-/-} animals (F) indicating reversal of the inflammatory changes seen in AAD animals.

antigen presenting capacity of antigen presenting cells, thereby limiting their ability to induce CD4⁺ effector T cell stimulation [17]. IL-10 has been implicated as a mediator of nasal tolerance, a process whereby exposure of the nasal mucosa to an antigen prevents the development of subsequent airway and systemic allergic responses to the antigen [5]. Exposure to inhaled antigen induces the production of IL-10-secreting CD4⁺ T cells in bronchial lymph nodes [18]. These regulatory T cells, in turn, inhibit the murine asthmatic response [5,18]. Thus, IL-10 is generally considered to exert anti-inflammatory effects in the lung [5,7,13,18–22]; however, in certain circumstances IL-10 may also demonstrate pro-inflammatory capabilities [5,8,14,19,23,24]. Indeed, Lee *et al.* [19] have shown that transgenic mice

overexpressing IL-10 in the lung exhibit baseline pro-inflammatory responses (i.e., mucus metaplasia, lymphocytic inflammation, and airway remodeling) but also inhibition of endotoxin-induced inflammation.

Previous studies using IL-10^{-/-} mice to analyze the role of this cytokine in acute allergic responses have also reported contradictory results. In one model, challenge of sensitized IL-10^{-/-} mice with OVA resulted in decreased production of IL-5, infiltration of eosinophils, and production of mucus in the airways, suggesting that endogenous IL-10 might promote pulmonary eosinophilic inflammation via induction of IL-5 production [24]. In other models, IL-10^{-/-} mice develop enhanced pulmonary eosinophilic inflammation [20,21], suggesting an inhibitory effect for

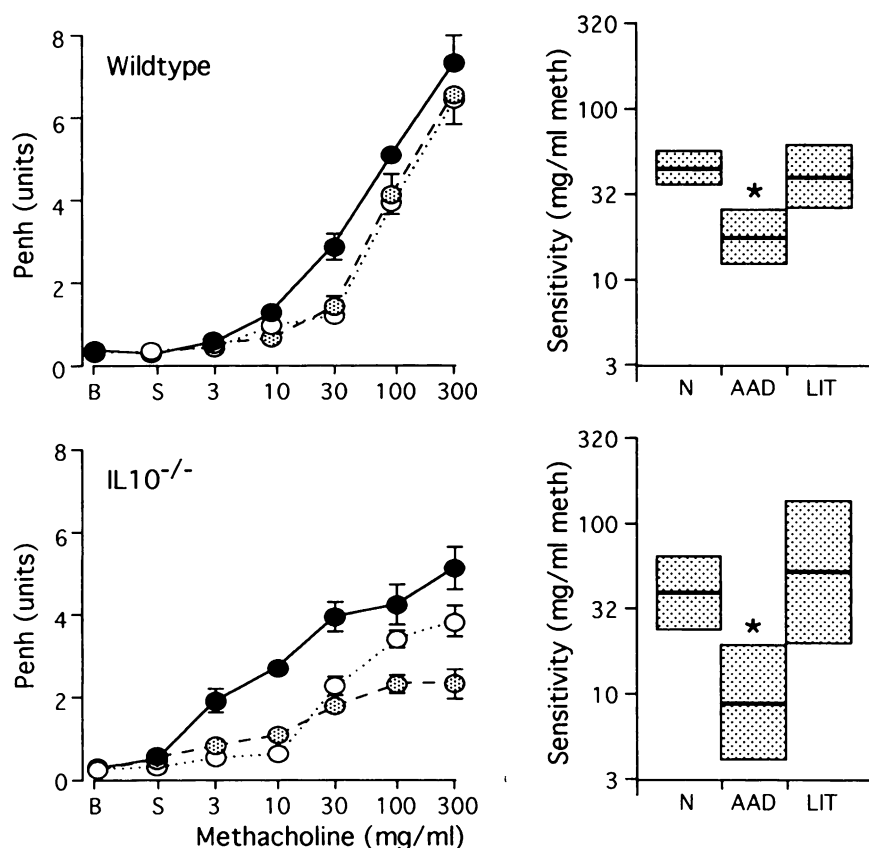


Fig. 4. Comparison of methacholine Penh responses at baseline and after acute and chronic OVA aerosol exposure. Left panels: aerosolized methacholine elicited dose-dependent increases in Penh relative to baseline (B) and saline (S) Penh levels in both wildtype (upper, $n = 11$) and IL-10^{-/-} mice (lower, $n = 6$). As compared to control responses in naive animals (open circles), methacholine responses increased during AAD (solid circles) and returned to baseline during LIT (shaded circles). Data represent means $\pm$ SEM responses. Right panels: methacholine sensitivity was characterized by the dose eliciting a Penh value of 2 U. Relative to naive animal sensitivity (N), both groups of animals became hyperreactive to methacholine after 3 days of OVA aerosols (AAD). There was a significant enhancement in responsiveness to methacholine challenge in the IL-10^{-/-} mice as compared to wildtype. Following 6 weeks of continuous OVA aerosol exposure, methacholine reactivity had returned to baseline in both groups (LIT). Data represent geometric mean values $\pm$ 95% confidence intervals. * Designate $p < 0.05$ from naive response.

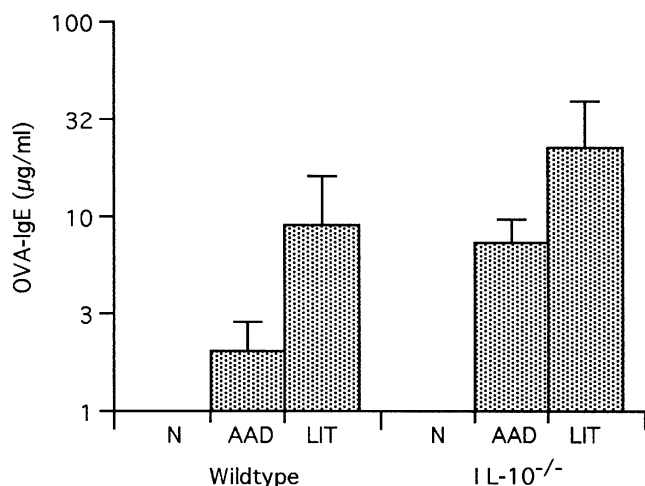


Fig. 5. Serum OVA-specific IgE levels in wildtype and IL-10^{-/-} mice after acute and chronic OVA aerosol exposures. OVA-specific IgE was not detected in naive wildtype or IL-10^{-/-} mice but was present in both wildtype and IL-10^{-/-} acute and chronic OVA-exposed animals. Data represent geometric means $\pm$ SEM values ($n = 6-10$ in each group).

endogenous IL-10 in these conditions. Moreover, the injection of OVA-specific CD4⁺ T cells genetically engineered to express IL-10 suppressed the development of airway hyperreactivity and eosinophilia in OVA-sensitized and challenged mice [22]. In the present study, OVA-sensitized IL-10^{-/-} mice showed the same progression from acute AAD to chronic LIT as wildtype animals. Acutely, the IL-10^{-/-} mice tended to have more severe disease than the wildtype animals, as manifested by increased histologic inflammation and mucus production, greater reactivity to inhaled methacholine, and higher systemic OVA-specific IgE levels. These data support the concept that IL-10 has a role in down regulating acute airway inflammatory responses to antigen.

The previous studies only investigated the role of IL-10 in the acute development of AAD. The present study is unique in that it investigates the effects of IL-10 in the development of LIT in the chronic stage of this asthma model. Although IL-10 may have exerted an anti-inflammatory effect in the acute stage of this model, the progression to LIT was not dependent on the presence of IL-10. The IL-10^{-/-} mice showed resolution of airways and

histologic inflammation and of methacholine hyperresponsiveness, similar to that observed in wildtype mice. Systemic IgE levels remained higher in the chronically exposed IL-10^{-/-} animals relative to chronic wildtype mice, consistent with the known inhibitory effect of IL-10 on IgE production [25]. The persistent elevation in serum IgE levels implied that our LIT is a different homeostatic process than nasal tolerance. Nasal tolerance is characterized by the suppression of subsequent IgE production in response to aerosol challenge with antigen. In our model, OVA-specific IgE levels remain elevated, and in fact increase further with chronic OVA exposure. Accordingly, while nasal tolerance has been shown to involve local IL-10 production [5,18], the tolerance that developed in our mice was independent of IL-10.

The pathophysiology of the asthmatic response is extremely complex and multifactorial, manifested by several very different altered physiologic responses. The apparent contradictions in the literature regarding the issue of whether IL-10 is anti- or pro-inflammatory may be reconciled with the notion that the cytokine most likely can be both or either depending on the type of injury induced and the ensuing physiologic consequences. In addition, other cytokines (e.g., IL-19, IL-20, IL-22, IL-24, and IL-26) have some homology with IL-10 and can reciprocate IL-10 function when needed [26]. In our acute model, IL-10 appeared to have an anti-inflammatory role, such that allergic inflammatory responses were increased in the absence of IL-10. In contrast, chronic exposure to inhaled antigen resulted in the development of LIT, with resolution of the acute local responses but persistence of systemic IgE production. This tolerance was independent of IL-10, in that it developed to a similar degree in wildtype and IL-10^{-/-} animals. Collectively, these findings suggest that IL-10 may have an anti-inflammatory role in acute AAD and asthma; however, other mechanisms must be involved in the development of chronic LIT. These other, as yet unidentified regulatory mechanisms may be important in the spontaneous resolution of asthma that occurs in some children and adults with the disorder.

Acknowledgments

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