

ORIGINAL ARTICLE

Adenovirus expressing interleukin-1 receptor antagonist alleviates allergic airway inflammation in a murine model of asthma

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Interleukin-1 (IL-1) is a proinflammatory cytokine and IL-1 receptor antagonist (IL-1ra) is a natural inhibitor that binds to IL-1 receptor type I without inducing signal transduction. It is suggested that IL-1 is required for allergen-specific T helper type 2 cell activation and the development of airway hyper-responsiveness (AHR), but the immunologic effect of exogenous IL-1ra in allergic asthma remains unclear. To examine the effect of IL-1ra on airway inflammation and immunoeffector cells in allergic asthma, recombinant adenovirus expressing human IL-1ra (Ad-hIL-1ra) was delivered intranasally into ovalbumin (OVA)-immunized mice. Single intranasal administration of Ad-hIL-1ra before airway antigen challenge in OVA-immunized mice

significantly decreased the severity of AHR and reduced pulmonary infiltration of eosinophils and neutrophils. Suppression of IL-5 and eotaxin with concomitant enhancement of interferon gamma in bronchoalveolar lavage fluid was also noted in OVA-immunized mice by administration of Ad-hIL-1ra. In addition, histological studies showed that Ad-hIL-1ra was able to decrease OVA-induced peribronchial inflammation. Taken together, our results indicated that administration of Ad-hIL-1ra may have therapeutic potential for the immunomodulatory treatment of allergic asthma.

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Introduction

Allergic asthma is a chronic inflammatory disease characterized by elevated production of T helper type 2 (Th2) cytokines, inflammatory cellular infiltration with muscular hypertrophy and airway hyper-responsiveness (AHR). The severity of AHR has been correlated with the degree of airways eosinophilia, neutrophilia and Th2 cytokine-producing lymphocytes.¹ Following the infiltration of inflammatory cells, a cascade of mediators are released and are involved in airway smooth muscle dysfunction. The recruitment of these inflammatory cells into the lung is linked with local immune response to inhaled antigen, inducing secretion of Th2 cytokines. Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, all contribute to the regulation of antigen-induced leukocyte recruitment within the lung.² However, a key element in the local inflammatory response is the prompt production of proinflammatory cytokines, IL-1 and, to a lesser

extent, tumor necrosis factor alpha (TNF- α), which may also alter airway responses in asthma.³

IL-1, a proinflammatory cytokine, can elicit strong proinflammatory reactions in mucosal tissues⁴ and has been associated with eosinophil migration,⁵ late asthmatic responses,⁶ altered smooth muscle responsiveness⁷ and development of AHR.⁸ It can also activate Th2 cells and enhance antibody production,^{9–15} suggesting that IL-1 functions as Th2 cytokine in the development of allergic diseases. IL-1 receptor antagonist (IL-1ra) is an endogenous receptor antagonist, which can inhibit the biological functions of IL-1 by occupying the same receptor without inducing signal transduction.^{16,17} Imbalanced production between IL-1 β and IL-1ra has been documented in serum of asthmatic patients, suggesting that dysregulation of IL-1 β /IL-1ra is critical in asthma inflammation.¹⁸

Several studies have investigated the effects of recombinant IL-1ra (rIL-1ra) in experimental models of allergic airway diseases. Pretreatment with aerosolized rIL-1ra before antigen challenge reduced antigen-induced AHR, bronchoalveolar lavage fluid (BALF) inflammation and eosinophil accumulation.^{6,19,20} However, exogenous recombinant cytokines have a short half-life *in vivo*, and systemic administration at high doses may cause substantial adverse effects. Additionally, a 100- to 1000-fold molar excess of IL-1ra over IL-1 is

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needed to ameliorate the proinflammatory effect of IL-1 in lung diseases.^{21,22} In order to achieve high local concentration of IL-1ra, we used replication-deficient adenovirus (Ad) vector system. Adenovirus-mediated gene delivery represents a potentially effective tool for gene transfer to the lung and it can induce a high level of transgene expression locally, which makes it an ideal tool for cytokine-target gene therapy in lung diseases.^{23–25}

In this study, we applied a recombinant Ad expressing human IL-1ra (Ad-hIL-1ra) to examine the effect of hIL-1ra on airway inflammation and immunoeffector cells in an ovalbumin (OVA)-sensitized murine model of asthma. Our results suggest that single intranasal delivery of Ad-hIL-1ra to OVA-sensitized mice significantly alleviates the severity of AHR, reduces pulmonary infiltration of eosinophils and neutrophils, and decreases levels of IL-5 and eotaxin with concomitant enhancement of interferon gamma (IFN- γ) in BALF.

Results

hIL-1ra expression in BALF after Ad-IL-1ra administration

In order to elucidate the therapeutic effect of prolonged IL-1ra administration on airway inflammation and immune effector cells in OVA-sensitized mice, we applied hIL-1ra-expressing adenovirus (Figure 1a) as an effective tool for gene transfer to induce a high level of IL-1ra transgenic expression in lung tissue. Thus, we evaluated the hIL-1ra expression in BALF by various delivery routes with the same virus dose (Figure 1b). We found that hIL-1ra could not be detected in BALF if Ad-hIL-1ra was administrated by intravenous injection or intraperitoneal injection. We also found that there was no significant difference in hIL-1ra expression between intranasal delivery and intratracheal delivery. Therefore, we chose intranasal delivery to be the viral administrating route.

Then, we assayed the severity of airway inflammatory and hIL-1ra expression level in the BALF of mice receiving various doses of single intranasal administration of IL-1ra-expressing adenovirus. Mice received high dose of Ad-IL-1ra (Ad-IL-1ra-hi, 2×10^9 plaque-forming units (PFU)/mice), medium dose of Ad-IL-1ra (Ad-IL-1ra-med, 1×10^9 PFU/mice) or low dose of Ad-IL-1ra (Ad-IL-1ra-lo, 3×10^8 PFU/mice) by intranasal delivery on day 0. At 3 days after virus injection, each group of mice was challenged with OVA (100 μ g) by intranasal administration on 3 consecutive days. On day 6, all the groups of mice were killed and BALF was collected. Then, we analyzed the cellular composition and the IL-1ra protein level of the BALF. The IL-1ra expression levels in the BALF did not show significant difference between high- and medium-dose groups (Figure 1c). However, the severity of airway inflammation in the Ad-IL-1ra-hi group was greater than that of the Ad-IL-1ra-med group (even hemorrhage found in the high-dose group). So, we delivered vector at such a dose (1×10^9 PFU/mice) that would avoid serious inflammation by vectors and induce adequate hIL-1ra transgene expression. The similar dose of highly purified recombinant adenovirus was also used in a previous study.⁴⁶

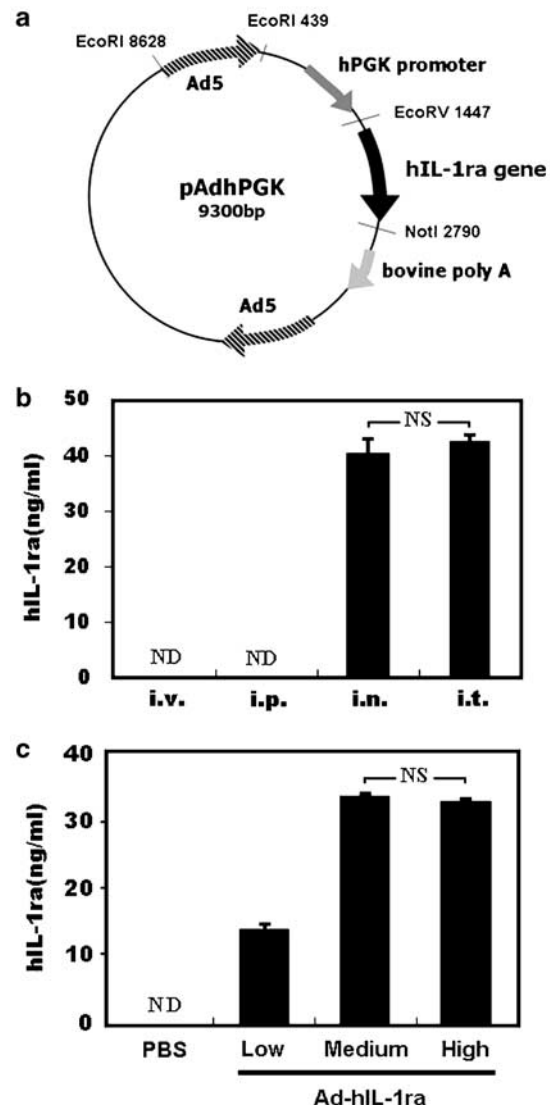


Figure 1 Schema of recombinant adenoviral vector (a) and hIL-1ra expression in BALF for different routes (b) or for different viral doses (c). (a) Recombinant adenoviral vector encoding hIL-1ra gene. The pAdhPGK vector is used for the production of adenovirus containing human IL-1ra under the control of human phosphoglycerate kinase promoter (hPGK promoter). Ad-IL-1ra is identical to Ad-mock, except for the addition of human IL-1ra gene. (b) Ad-hIL-1ra was administrated to naive 4–6-week-old female BALB/c mice via different routes with the same virus dose (virus dose = 1×10^9 PFU in 40 μ l per animal; $n = 4$ mice for each group; i.v.: injection into tail vein; i.p.: intraperitoneal; i.n.: intranasal; i.t.: intratracheal). Bronchoalveolar lavage was performed 6 days after viral administration and hIL-1ra expression in BALF was evaluated by using commercial ELISA. ND: not detectable; NS: not significant. (c) Ad-hIL-1ra was intranasally administrated to naive 4–6-week-old female BALB/c mice with different virus doses (high: 2×10^9 PFU/mice; medium: 1×10^9 PFU/mice; low: 3×10^8 PFU/mice, $n = 4$ mice for each group). Bronchoalveolar lavage was performed 6 days later after viral administration and hIL-1ra expression in BALF was evaluated by using commercial ELISA. ND: not detectable; NS: not significant.

Intranasal delivery of adenovirus expressing hIL-1ra gene decreased the severity of AHR

To determine the effect of Ad-hIL-1ra administration on allergic asthma, both AHR and infiltrated inflammatory

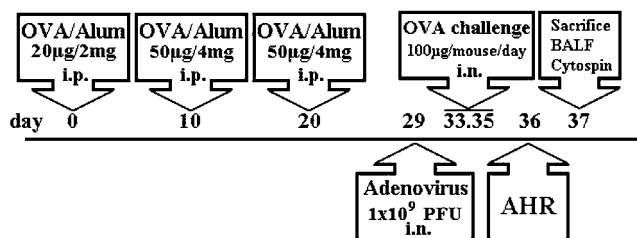


Figure 2 Treatment regimen. Time line representation of the OVA protocol used and the intranasal administration of adenovirus. i.p., intraperitoneal; i.n., intranasal.

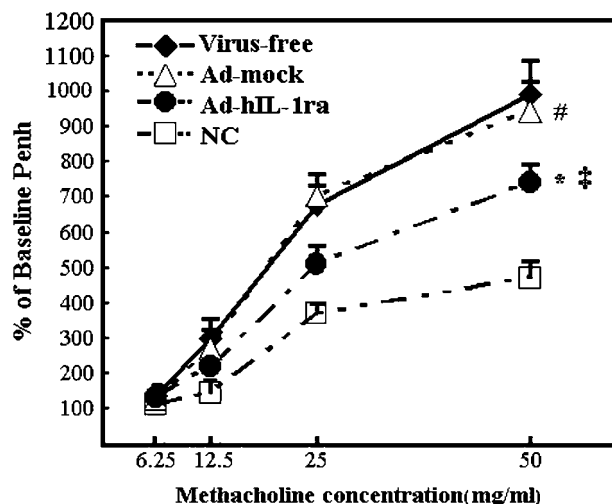
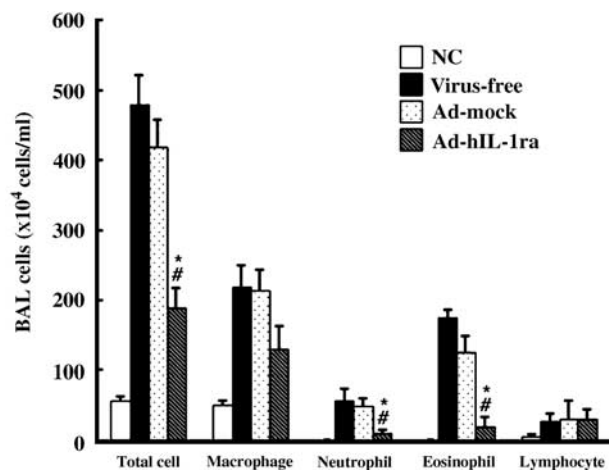


Figure 3 Effect of Ad-hIL-1ra on AHR to aerosolized methacholine in OVA-sensitized and OVA-challenged mice. One day after the last OVA challenge, AHR was measured in response to increasing concentrations of methacholine (0–50 mg/ml) in conscious mice placed in a whole-body plethysmograph. Negative control (NC) group were mice sensitized and challenged with normal saline. All virus-free, Ad-mock and Ad-hIL-1ra groups were mice sensitized and challenged with OVA. However, they were pretreated with normal saline, Ad-mock and Ad-hIL-1ra, respectively. The values are expressed as percentage of baseline Penh (aerosolized normal saline only) $\pm$ s.e.m. with five mice in each group. Data are representative of three separate experiments with similar results. $^{\#}P=0.46$ compared with the virus-free group; $^{*}P<0.05$ compared with the virus-free group; $^{\dagger}P<0.05$ compared with the Ad-mock group.

cells were measured. BALB/c mice were sensitized by three intraperitoneal injections of OVA and subsequently challenged with intranasal OVA droplet aspirations. The mice were pretreated once intranasally with 1×10^9 PFU of Ad-hIL-1ra or the control virus, Ad-mock, 3 days before the first OVA challenge (Figure 2). The time point of adenovirus administration (3 days before the first challenge) was determined by the analysis of kinetic hIL-1ra expression.²⁶ Twenty-four hours after the final challenge, airway responsiveness was assessed by barometric whole-body plethysmography. The result of AHR is shown in Figure 3. BALB/c mice that had been sensitized and challenged with OVA revealed an increase in airway responsiveness to the methacholine inhalation, as compared to normal saline-sensitized and saline-challenged mice. Similarly, the AHR was increased in



	Total cell number		Percentage (%)			
	($\times 10^3$ cells/ml)		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Negative control	563 $\pm$ 74		89.4 $\pm$ 3.5	0.6 $\pm$ 0.8	1.0 $\pm$ 1.3	9.0 $\pm$ 5.0
Virus-free	4800 $\pm$ 424		45.8 $\pm$ 5.3	11.8 $\pm$ 2.5	36.8 $\pm$ 2.4	5.6 $\pm$ 2.1
Ad-mock	4190 $\pm$ 383		50.8 $\pm$ 4.7	11.7 $\pm$ 1.5	30.2 $\pm$ 5.8	7.3 $\pm$ 6.6
Ad-hIL-1ra	1900 $\pm$ 271		68.2 $\pm$ 10.9	5.1 $\pm$ 3.0	10.8 $\pm$ 8.4	15.9 $\pm$ 8.5

Figure 4 Effect of Ad-hIL-1ra on cell infiltration to the mouse airway. Cell compositions in BALF of different groups of mice are expressed as the mean $\pm$ s.e.m. for five mice in each group. Experiments were repeated in variation two times with similar results. $^{*}P<0.05$ compared with the virus-free group; $^{\dagger}P<0.05$ compared with the Ad-mock group.

OVA-sensitized and OVA-challenged mice pretreated with Ad-mock. However, BALB/c mice that were sensitized and challenged with normal saline revealed no increase in airway responsiveness. In contrast, pretreatment with Ad-hIL-1ra decreased AHR as compared with Ad-mock- and saline-pretreated groups (Figure 3).

Ad-hIL-1ra decreased eosinophil and neutrophil infiltration in BAL cells

In order to explore whether Ad-hIL-1ra administration could alleviate the allergen-induced airway inflammation, the cellular compositions in the BALF were analyzed (Figure 4). OVA challenge significantly increased the total number of cells and eosinophil counts as compared with the negative control group. The number of eosinophils and neutrophils in the BALF of mice pretreated with Ad-hIL-1ra was significantly lower than that of other control groups. Furthermore, the administration of Ad-hIL-1ra decreased the total number of cells that infiltrated the lungs.

Ad-hIL-1ra alleviated the severity of peribronchial inflammation

Intranasal OVA challenge induced inflammatory cell infiltration in the lung interstitium around the airway and pulmonary blood vessels (Figure 5a). Pretreatment with Ad-hIL-1ra blocked cell infiltration progressively. In contrast, pretreatment with Ad-mock did not show significant inhibitory effects on peribronchial inflammation as compared with the saline pretreatment group (Figure 5a and b).

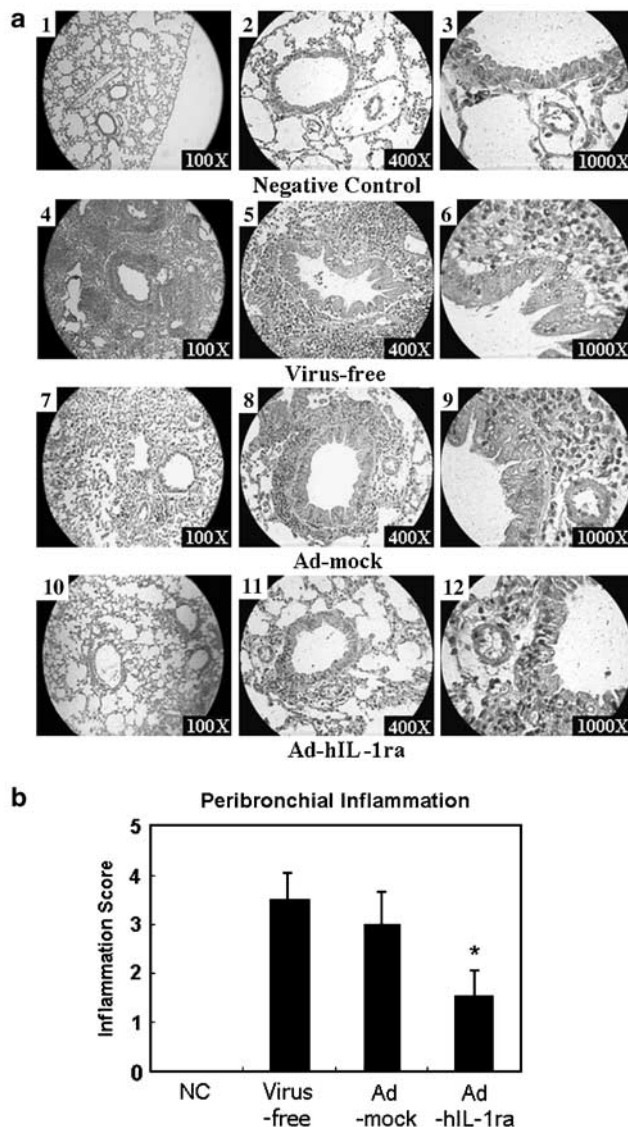


Figure 5 Histological examination of lung tissues. (a) Representative sections of lungs. Lung sections were obtained from negative control, (NC) (panels 1–3), virus-free (panels 4–6) and pretreated with Ad-mock (panels 7–9) or Ad-hIL-1ra mice (panels 10–12). Sections were stained with H&E for morphological analysis. Tissue was examined by light microscopy (original magnification: $\times 100$ (panels 1, 4, 7, 10), $\times 400$ (panels 2, 5, 8, 11) and $\times 1000$ (panels 3, 6, 9, 12)). Cell infiltration caused by OVA, virus-free (panels 4–6) was decreased by Ad-hIL-1ra (panels 10–12) compared with negative control (NC) (panels 1–3). (b) Quantitative analyses of the severity of peribronchial inflammation. Inflammation score was performed based on the method of Myou *et al.*, as described in Materials and methods. * $P < 0.05$ compared with virus-free group.

Influence of Ad-hIL-1ra on IL-5, IFN- γ , eotaxin and TNF- α levels in BALF

To test whether T-cell responses were affected in the mice receiving Ad-hIL-1ra, the levels of IL-5 and IFN- γ in the BALF of various groups of mice were measured by enzyme-linked immunosorbent assay (ELISA). IL-5 expressions decreased in mice pretreated with Ad-hIL-1ra, as compared with mice pretreated with Ad-mock or normal saline (Figure 6b). These results indicated that

Ad-hIL-1ra administration might reduce the infiltration of inflammatory cells and Th2 cytokines, and that Ad-hIL-1ra administration alleviated AHR. In addition, we observed the enhancement of BALF IFN- γ expression in mice pretreated with Ad-hIL-1ra as compared with mice pretreated with Ad-mock or saline (Figure 6c).

Further, eotaxin is the most potent chemokine for the recruitment of eosinophils.²⁷ Eotaxin levels in the BALF of different groups of mice were assayed by ELISA. Eotaxin levels in the BALF of mice pretreated with Ad-hIL-1ra were significantly lower than those in the BALF of mice pretreated with Ad-mock or saline (Figure 6d). This result indicated that Ad-hIL-1ra reduced the secretion of eotaxin and subsequently decreased the number of recruited eosinophils. We also checked the expression of TNF- α in the BALF following IL-1ra gene transfer. However, the TNF- α level in BALF was not significantly different between the four groups (Figure 6e).

Discussion

One of the key features of allergic asthma is the influx of various inflammatory cells into the airway.²⁸ A number of studies have shown that IL-1 plays a significant role in the recruitment of these cells for allergic asthma.^{5,19,29–33} IL-1 is produced primarily by monocytes and macrophages; other nucleated cells are also found to release IL-1, including endothelial cells, lymphocytes and epithelial cells.³³ IL-1 enhances human eosinophil, basophil and neutrophil adhesiveness to human umbilical vein endothelial cells through induction of the expression of adhesion molecules.³⁴ That means IL-1-IL-1RI signaling may be responsible for the induction of VCAM-1 expression, which recruits eosinophils to the lung during AHR.⁵ Baskar and Pincus³⁵ have reported that IL-1 augments the IgE-dependent activation of eosinophils.³⁵ Okada *et al.*⁶ also concluded that IL-1 might participate in the priming of eosinophils in the augmentation of late asthmatic reaction (LAR). Furthermore, IL-1 induces OX40 (CD134) expression on CD4⁺ T cells, which play an important role in Th2 cell activation.³⁶ It has been shown that antigen-specific T-cell proliferation, IL-4 and IL-5 production by T cells, and antigen-specific IgG1 and IgE secretion by B cells are reduced in IL-1 α / β ^{−/−} mice during AHR, whereas these responses increase in IL-1ra^{−/−} mice, compared with wild-type mice.⁸ The latter observations are consistent with the view that IL-1 is critical in the development of AHR through the activation of Th2 cells during the sensitization phase. Appropriately, it has been suggested that the proinflammatory disposition of IL-1 contributes to allergic asthma through upregulation of adhesion molecules on pulmonary endothelium (via changes in ICAM-1 and VCAM-1),³⁷ induction of leukocyte chemotaxis and degranulation,³⁴ B- and T-cell proliferation and activation,¹¹ and microvascular leakage.³⁸

IL-1ra inhibits the biological function of IL-1 by occupying the same receptor without exerting any agonist activity.^{16,17} The beneficial effects of the administration of recombinant IL-1ra in many animal models of allergic diseases are well documented. In a mouse model of allergic conjunctivitis, topical IL-1ra suppresses allergic eye inflammation by downregulating the recruitment of eosinophils and other inflammatory cells.²⁰ In a

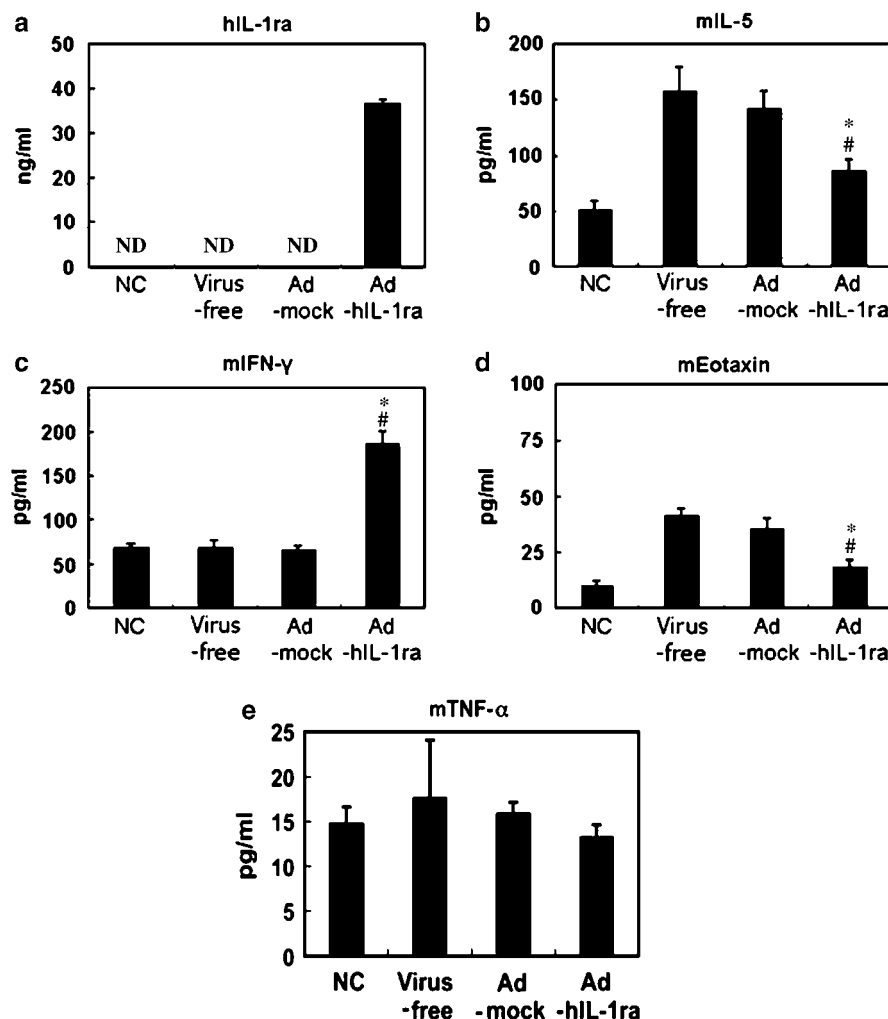


Figure 6 ELISA determinations of hIL-1ra (a), mIL-5 (b), mIFN- γ (c) and m-eotaxin (d) mTNF- α (e) levels in BALF of different groups of mice. Results are expressed as the mean $\pm$ s.e.m. for five mice in each group. Experiments were repeated in variation two times with similar results * P < 0.05 compared with the virus-free group; # P < 0.05 compared with the Ad-mock group. ND: not detectable.

guinea-pig model of asthma, intravenous administration of IL-1ra before antigen exposure reduces the generation of LAR by modulating the activation of eosinophils.⁶ In a similar model, aerosolization of IL-1ra immediately before antigen challenge confers marked protection against bronchial hyper-reactivity and pulmonary eosinophil accumulation.³⁹ Taube *et al.*⁴⁰ have demonstrated that inhibition of IL-1, using IL-1ra, effectively decreases levels of neutrophil chemokines as well as neutrophil numbers in BALF following allergen exposure in an OVA-sensitized murine model of asthma. Moreover, pretreatment of isolated rabbit tracheal rings with IL-1ra abrogates both IL-5 and IgE immune complex-induced changes in airway smooth muscle responsiveness.⁴¹ Mao *et al.*¹⁸ found that decreased production of IL-1ra might significantly predispose to nonatopic asthma. They also found a similar phenomenon in atopic asthma, and concluded that imbalanced production between IL-1 β and IL-1ra in bronchial asthma might be an important determinant of asthma.

Cumulatively, these studies have spurred the clinical evaluation of IL-1ra in allergic lung diseases where IL-1

plays an important pathophysiological role. Taube *et al.*⁴⁰ demonstrated that early neutrophil response following allergen challenge of sensitized animal is regulated by IL-1. However, they found that IL-1ra-mediated inhibition of early airway neutrophilia cannot affect development of AHR. They suggested the reason for their inconsistencies is because the single dose of IL-1ra blocks IL-1RI only transiently because of its short half-life.^{26,35} In addition, despite near-equal affinities of IL-1 β and IL-1ra for IL-1RI, a 100- to 1000-fold molar excess of IL-1ra is required to inhibit IL-1 activity *in vivo*.^{21,22} Thus, to induce locally substantial levels of IL-1ra for down-regulating IL-1, we used an Ad vector system for gene transfer. Replication-deficient Ad are considered as gene delivery vectors for the genetic treatment of a variety of diseases, especially in the transfer of exogenous genes to airway epithelial cells *in vivo*.^{23–25} In our study, single intranasal administration of Ad-hIL-1ra could induce significantly high levels of hIL-1ra expression within the murine lung, even 8 days after virus administration (Figure 6a). This observation is consistent with a previous report showing that adenovirus-based vectors

can be used to induce high levels of hIL-1ra expression within the lung.⁴² The latter authors suggested that human IL-1ra binds to murine IL-1RI and inhibits IL-1-mediated response. Therefore, human IL-1ra can inhibit the biological effect of IL-1 in mice that are believed to involve IL-1 as an endogenously generated mediator.

Consistent with the findings of previous studies, our results clearly demonstrate that single intranasal administration of Ad-hIL-1ra in OVA-sensitized murine asthma model is capable of downregulating allergic inflammation such as AHR, eosinophilia and neutrophilia in BALF (Figures 3 and 4). Histological studies also revealed that Ad-hIL-1ra is able to reduce OVA-induced peribronchial inflammation (Figure 5a and b). In addition, Ad-hIL-1ra-treated mice showed significantly decreased levels of IL-5 and eotaxin with concomitant enhancement of IFN- γ in BALF compared with control mice (Figure 6b–d). Suzuki *et al.*²³ reported that replication-deficient Ad can lead to increased expression of IFN- γ in BALF and subsequent inhibition of allergic responses, but suggested that there could be potentially enhanced efficacy of recombinant Ad with transgene-expressing antagonizing molecule for host Th2-mediated reactions. However, other reports have not observed such an immune response to Ad-mock.^{24,25} Our results are consistent with the latter studies. We found no evidence of an IFN- γ response in Ad-mock-treated control mice (Figure 6c). Irikura *et al.*⁴³ found that overproduction of IL-1ra during primary listeriosis decreases macrophage major histocompatibility complex class II expression, yet increases IFN- γ levels in the serum. They concluded that IL-1ra overproduction from its endogenous promoter causes a dysregulation of cytokine network that ultimately results in increased production of IFN- γ , supporting a model wherein overproduction of the anti-inflammatory cytokine IL-1ra leads to an increase in proinflammatory cytokine production *in vivo*. Additionally, Lin *et al.*¹⁴ showed that IL-1ra downregulates antigen-specific IL-4 and IgE responses, with enhancement of IFN- γ and IgG2a responses *in vivo*. They suggested that such a Th1-promoting effect of IL-1ra is not mediated by enhancing IL-12 synthesis by macrophages. Furthermore, it has been shown that enhancement of IFN- γ expression in antigen-challenged lung is associated with a significant decrease of both IL-4 and IL-5, eosinophilia and AHR.^{24,44} IFN- γ can also prevent antigen-induced CD4⁺ T-cell infiltration into the tissue and thereby decrease the subsequent CD4⁺ T cell and IL-5-dependent eosinophil recruitment into the lung.⁴⁵ Although the exact molecular mechanisms of IL-1ra in regulating Th1/Th2 responses *in vivo* remain to be further investigated, our findings are consistent with other studies indicating that IL-1ra can downregulate not only IL-1-mediated non-specific inflammatory responses but also the development of antigen-specific allergic inflammation.

Although recombinant adenovirus-mediated gene therapy has been demonstrated great promise for delivery of therapeutic genes to pulmonary epithelium, dose-dependent inflammation and host local immune response induced by vectors could abbreviate transgene expression. High virus dose may result in marked lung injury and accelerate the adenovirus clearance with lower transgene expression. However, low virus dose cannot achieve the therapeutic levels of transgene expression in the lung. Furthermore, McCoy *et al.*⁴²

concluded that adenovirus-based vectors can induce high levels of hIL-1ra expression in murine lung. Nevertheless, such an expression was unable to prevent adenoviral vector-induced inflammation.⁴² To weigh the transgene expression and inflammation caused by Ad vectors, we delivered vectors at a dose that can achieve therapeutic levels and only induce a very modest inflammatory response.⁴⁶ However, dependent effects of hIL-1ra in antigen-specific allergic inflammation remain to be further investigated.

In summary, our study demonstrates the immunomodulatory effect of single intranasal administration of Ad-hIL-1ra in a murine model of OVA-induced asthma. The substantial pulmonary expression of hIL-1ra via Ad vectors before airway antigen challenge may significantly alleviate the severity of AHR and reduce the infiltration of both eosinophils and neutrophils. Histological studies revealed that Ad-hIL-1ra is able to decrease OVA-induced peribronchial inflammation. Our results indicate that administration of Ad-hIL-1ra prevents AHR, eosinophilia and neutrophilia either by directly inhibiting IL-1 proinflammatory activity or by indirectly suppressing IL-5 and eotaxin, with concomitant enhancement of IFN- γ in BALF. These data suggest that the inhibition of IL-1 activity *in vivo* is of potential benefit in allergic airway inflammation, and that the intranasal administration of Ad-hIL-1ra has therapeutic potential for the immunomodulatory treatment of allergic asthma.

Materials and methods

Mice

Female 6–8-week-old BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University. Animals were age matched within each experiment. Animal care and handling protocols were approved by the Animal Committee of National Taiwan University.

Adenoviral vectors

Recombinant adenoviral vector was constructed using the Ad-Easy system that has been described previously.⁴⁷ Ad-hIL-1ra (Figure 1a) was kindly provided by Dr D-M Chang, and Ad-mock was identical with Ad-hIL-1ra structurally, but contained no transgene in the expression cassette. After propagation in 293 cells, the recombinant viruses were purified from infected cells 42–48 h after infection by three freeze–thaw cycles followed by successive banding on cesium chloride density gradient centrifugation. The purified virus was dialyzed and stored at -70°C until needed. Viral titers were measured by standard end-point dilution assay using 293 cells.

Sensitization and challenge of mice

Mice were sensitized intraperitoneally by injection of 20 μg of OVA (Grade V; Sigma Chemical Co., St Louis, MO, USA) emulsified in 2 mg of aluminum hydroxide (AlumImuject; Pierce Chemical, Rockford, IL, USA) in a total volume of 200 μl at day 0, boosted twice with 50 μg of OVA emulsified in 4 mg of aluminum hydroxide in a total volume of 200 μl at days 10 and 20, and challenged with intranasal administration of OVA (100 μg in a total volume 40 μl) on 3 consecutive days (days 33–35).

Negative group were mice that were sensitized and challenged with normal saline.

Administration of Ad-hIL-1ra

Ad-hIL-1ra (1×10^9 PFU) was delivered intranasally into slightly anesthetized mice 3 days before the first challenge with OVA. A mock virus (Ad-mock) or normal saline was used as a control. The 1×10^9 PFU of adenovirus was chosen by preliminary titration data (data not shown).

Measurement of AHR

Airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography (Buxco Electronics Inc., Sharon, CT, USA) by recording respiratory pressure curves in response to inhaled methacholine (acetyl- β -methylcholine chloride; Sigma), as detailed previously.⁴⁸ Briefly, mice were placed in the main chamber of a whole-body plethysmograph and challenged, first with aerosolized normal saline and subsequently with increasing doses of methacholine (6.25–50 mg/ml). Each nebulization lasted for 3 min. After each nebulization, records were taken and averaged over 3 min. Results are expressed as mean enhanced pause (Penh) $\pm$ s.e.m. as percentage of baseline Penh (aerosolized normal saline only; Penh = pause $\times$ (peak expiratory box flow/peak inspiratory box flow)).

Analysis of cellular composition in the BALF

One day after the measurement of the pulmonary function parameters, the mice were killed and the tracheas were immediately lavaged three times via a tracheal cannula with 1 ml of Hank's balanced salt solution (HBSS, free of ionized calcium and magnesium). Supernatants of BALF were kept frozen at -20°C until needed. The BALF cells were washed and resuspended in 1 ml of HBSS. The total number of cells in the BALF was counted with a standard hemocytometer. Differential cell counts were performed by counting at least 200 cells on cytocentrifuged preparation (cytospin 2; cytospin, Shandon Ltd, Runcorn, Cheshire, UK), stained with Liu's stain and differentiated by standard morphological criteria.

Histological examination of lung tissue

Lungs removed from the chest cavity were fixed by injection of 10% natural formalin into the tracheal cannula and immersed in the same fixative for at least 24 h. Tissues were paraffinized, cut into 5- μm sections and stained with hematoxylin and eosin (H&E) for histological analysis. To determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5-point scoring system described by Myou *et al.*⁴⁹ Briefly, the scoring system was as follows: (0) normal; (1) few cells; (2) a ring of inflammatory cells one cell layer deep; (3) a ring of cells 2–4 cells deep; (4) one ring of cells >4 cells deep. Scoring of inflammatory cells was performed in at least three different fields for each lung section. Mean scores were obtained from three to four animals.

Measurement of BALF cytokine

Quantifications of human IL-1ra, murine IL-5, murine eotaxin and murine IFN- γ in the BALF supernatants

were evaluated using commercially available ELISA (DuoSet R&D, Minneapolis, MN, USA).

Statistical analysis

Results were expressed as the mean $\pm$ s.d. or mean $\pm$ s.e.m. and evaluated using a one-way analysis of variance, following by an unpaired two-tailed Student's *t*-test for comparison between two groups. Significance levels were set at a *P*-value of 0.05.

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