

RESEARCH ARTICLE

Adenovirus expressing Fas ligand gene decreases airway hyper-responsiveness and eosinophilia in a murine model of asthma

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Allergic asthma is characterized by airway hyper-responsiveness (AHR) and cellular infiltration of the airway with predominantly eosinophils and Th2 cells. The normal resolution of inflammation in the lung occurs through the regulated removal of unneeded cells by Fas–Fas ligand-mediated apoptosis. Fas ligand (FasL) is a member of the tumor necrosis factor family, and when bound to Fas, it induces apoptosis of the cells. To examine the effect of the FasL gene on airway inflammation and immune effector cells in allergic asthma, recombinant adenovirus expressing murine FasL (Ad-FasL) was delivered intratracheally into ovalbumin (OVA)-immunized mice. We found that a single administration of Ad-FasL in OVA-immunized mice significantly alleviated AHR and eosinophilia by inducing the

apoptosis of eosinophils and/or reducing eosinophil attractant factors, such as IL-5 and eotaxin levels. The number of infiltrated lymphocytes and Th2 cytokines, including IL-5 and IL-13, decreased in OVA-immunized mice by administration of Ad-FasL. KC and TNF- α production also decreased in Ad-FasL-treated OVA-immunized mice. These findings indicated that the administration of Ad-FasL to OVA-sensitized mice significantly suppressed pulmonary allergic responses. Although more studies are needed, these results suggested that Ad-FasL might be applied as an alternative therapy for allergic asthma.

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Introduction

Airway hyper-responsiveness (AHR) is a well-established characteristic of allergic asthma and has been attributed to chronic airway inflammation. The severity of such hyper-reactivity has been correlated with the number of Th2 cytokine-producing T cells and eosinophils, monocytes, and neutrophils in the airway.^{1,2} Several Th2 cytokines, such as IL-5 and IL-13, have the potential to modulate airway inflammation and AHR.^{3,4} IL-5 regulates eosinophil function, including development, activation, migration, and survival of eosinophils.⁵ The eosinophil is thought to be a major effector cell in the pathogenesis of allergen-mediated AHR.^{6–9} In particular, the release of cytotoxic granule proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO), which can damage the airway epithelium, contribute to this phenomenon.² IL-13 is thought to induce AHR in the allergic lung by directly affecting epithelial cells¹⁰ or by recruiting eosinophil via an IL-5- and eotaxin-dependent mechanism.^{11,12} However, the transfer of eosinophils to IL-5^{–/–} mice overcomes the intrinsic defect in T cell IL-13

production¹³ and induces the development of AHR.⁸ Thus, although the role of IL-5 in AHR is controversial,^{6–9,14–16} the relationship between eosinophils and AHR is still correlative.^{8,11–13}

Apoptosis is an important mechanism for the maintenance of homeostasis in the immune system.¹⁷ Fas (CD95), the receptor signaling component of apoptosis, is expressed either constitutively or after activation in a variety of immune cells, such as T cells, B cells, monocytes/macrophages, NK cells, neutrophils, and eosinophils.¹⁸ The other essential component involved in the Fas-mediated apoptotic pathway is the Fas ligand (FasL, CD95L). Identified as a type II membrane protein member of the tumor necrosis factor family of ligands, FasL was originally characterized in T cells as the primary effector molecule of Th1 cytotoxicity.^{19–21} It has been suggested that the normal resolution of inflammation in the lung occurs through the regulated removal by apoptosis of unneeded cells without the release of damaging histotoxins.²² Asthmatics are found to be associated with reduced apoptosis of inflammatory cells in the lung.^{23,24} We attempted to clarify whether the induction of apoptosis is beneficial in asthma by using a murine model with ovalbumin (OVA) as the allergen.

Adenovirus-mediated gene delivery is an effective tool for gene transfer to the lungs, and it can induce a high level of transgene expression, which makes it an ideal tool for gene therapy in lung diseases.^{25–27} Previous

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studies have shown that administrations of adenovirus-mediated IL-12, IL-18, or IFN- γ attenuate established allergen-induced airway inflammation and AHR.^{26,27} Furthermore, introduction of this nonreplicating adenovirus into murine lungs does not induce an inflammatory response in the lungs.^{26,27}

In this study, we generated a FasL-expressing adenovirus (Ad-FasL) to examine the effect of the FasL gene on airway inflammation and immune effector cells in an OVA-sensitized murine model. The results suggested that single administration of Ad-FasL to OVA-sensitized mice significantly alleviated development of AHR, reduced the infiltration of eosinophils and lymphocytes, decreased eosinophil chemoattractant eotaxin and proinflammatory cytokine KC and TNF- α productions, and decreased Th2 cytokines, such as IL-5 and IL-13 levels.

Results

Expression of FasL was confirmed by FACS, Reverse transcription-polymerase chain reaction (RT-PCR), and Western blot analysis

To determine the expression of FasL, bone marrow-derived dendritic cells (BMDCs) infected with Ad-FasL and the expression of FasL were analyzed. Dendritic cells (DCs) were isolated from the bone marrow of BALB/c mice and infected with a multiplicity of infection (MOI) of 250 of Ad-FasL or Ad-eGFP (control virus) on day 6.

On day 7, we determined the expression of the adenovirus-infected BMDCs by fluorescence-activated cell sorting (FACS) analysis for both enhanced green fluorescent protein (eGFP) and FasL expression. The data demonstrated that FasL expressed on the surface of Ad-FasL-infected BMDCs (Figure 1a). RT-PCR and Western blot further confirmed high levels of FasL mRNA and protein expression (Figure 1b and c). Only scarce amounts of mRNA and protein were detected in uninfected BMDCs or BMDCs infected with Ad-eGFP (Figure 1b and c).

Assay of FasL-induced apoptosis *in vitro*

To determine the biological activity of Ad-FasL, we evaluated the ability of Ad-FasL to induce apoptosis in Fas-positive target cells. COS-1 cells infected with Ad-FasL, but not infected with Ad-eGFP or noninfected, induced a marked apoptosis of Jurkat cells after coinocubation for 18 h (Figure 2).

FasL gene expression in the lungs inhibiting the development of AHR

To determine the effect of Ad-FasL administration on allergic asthma, both AHR and infiltrated inflammatory cells were measured. BALB/c mice were sensitized to OVA by three intraperitoneal injections, and later on, were challenged with intranasal OVA droplet aspirations. The mice were treated once intratracheally with 2×10^8 PFU of Ad-FasL or the control virus, Ad-eGFP, 3

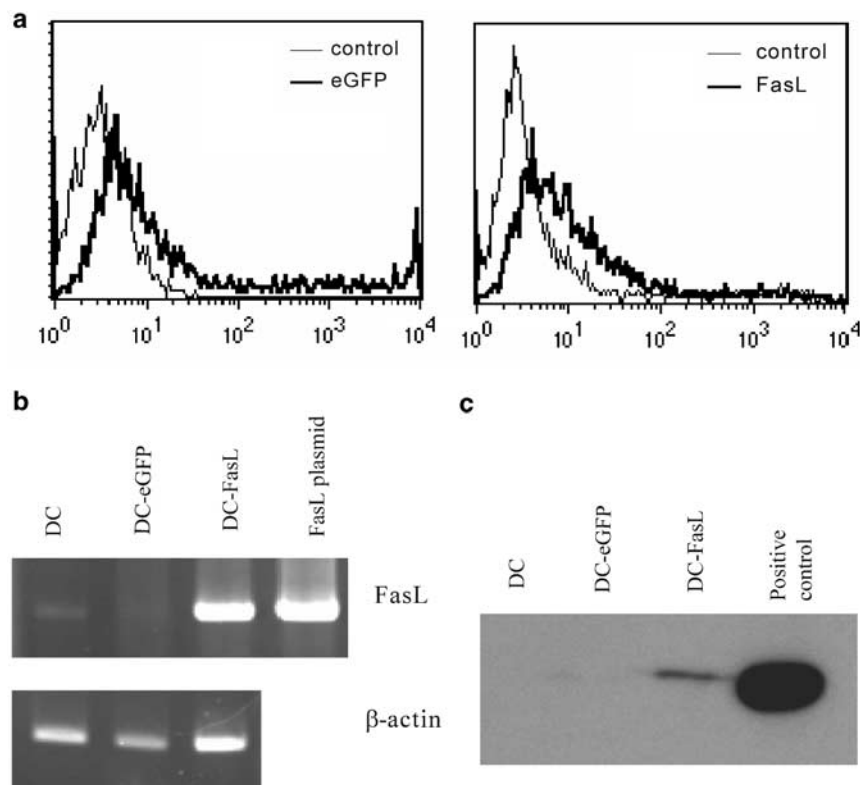


Figure 1 Expressions of FasL in Ad-FasL-infected DCs. (a) DCs were isolated from the bone marrow of BALB/c mice and infected with a MOI of 250 of Ad-FasL or Ad-eGFP (control virus) on day 6. On day 7, the expression of the adenovirus-infected BMDCs was measured by FACS analysis for both eGFP (left panel) and FasL expression (right panel). (b) Expression of FasL in the infected DCs was demonstrated by RT-PCR analysis. (c) The level of FasL in the infected DCs was demonstrated by Western blot analysis of the cell extract. DC, no virus infected DC; DC-eGFP, Ad-eGFP-infected DC; DC-FasL, Ad-FasL-infected DC.

days before the first challenge (Figure 3a, protocol 1). The time point of adenovirus administration (3 days before the first challenge) was chosen by the analysis of kinetic

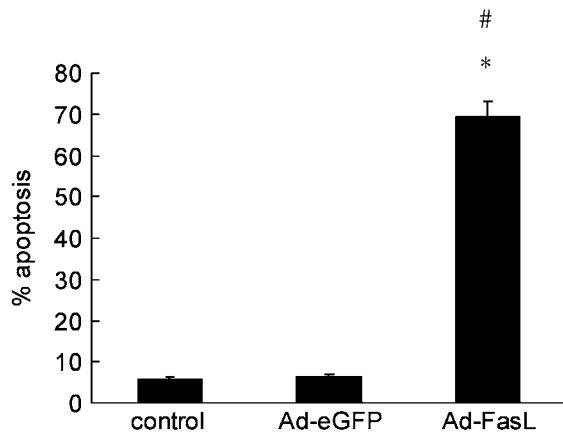


Figure 2 Detection of FasL-induced apoptosis *in vitro*. Apoptosis of Jurkat cells incubated with Ad-FasL-infected COS-1 cells for 18 h was measured by staining with PI and analyzed by flow cytometry. Data were shown as the mean $\pm$ s.d. of the percentage apoptotic cells for three experiments. * $P < 0.00005$ versus control. # $P < 0.00005$ versus Ad-eGFP.

FasL expression. The expression of FasL in the airway was measured by eGFP expression in the BAL cells of mice treated with the intratracheal injection of Ad-FasL. The eGFP expression was detectable 24 h after injection and lasted for at least 3 days. After Ad-FasL administration, the eGFP expression was detected in the bronchoepithelial cells, granulocytes, macrophages, and lymphocytes (data not shown). At 24 h after the final challenge, airway responsiveness was assessed. BALB/c mice that had been sensitized and challenged with OVA revealed an increase in airway responsiveness to the methacholine inhalation over normal saline-sensitized and -challenged mice (Figure 3b, OVA+NS group *versus* NC group). Similarly, the AHR was increased in OVA-sensitized and -challenged mice that received Ad-eGFP (Figure 3b). However, BALB/c mice that were sensitized and challenged with normal saline and were received Ad-eGFP or Ad-FasL revealed no increase in airway responsiveness (data not shown). In contrast, the administration of Ad-FasL significantly inhibited the increases in airway responsiveness after OVA challenge in the sensitized animals (Figure 3b). The administration of the Ad-FasL resulted in a slight increase in enhanced pause (Penh) in the response to normal saline inhalation, although, it was not statistically significant (Figure 3b).

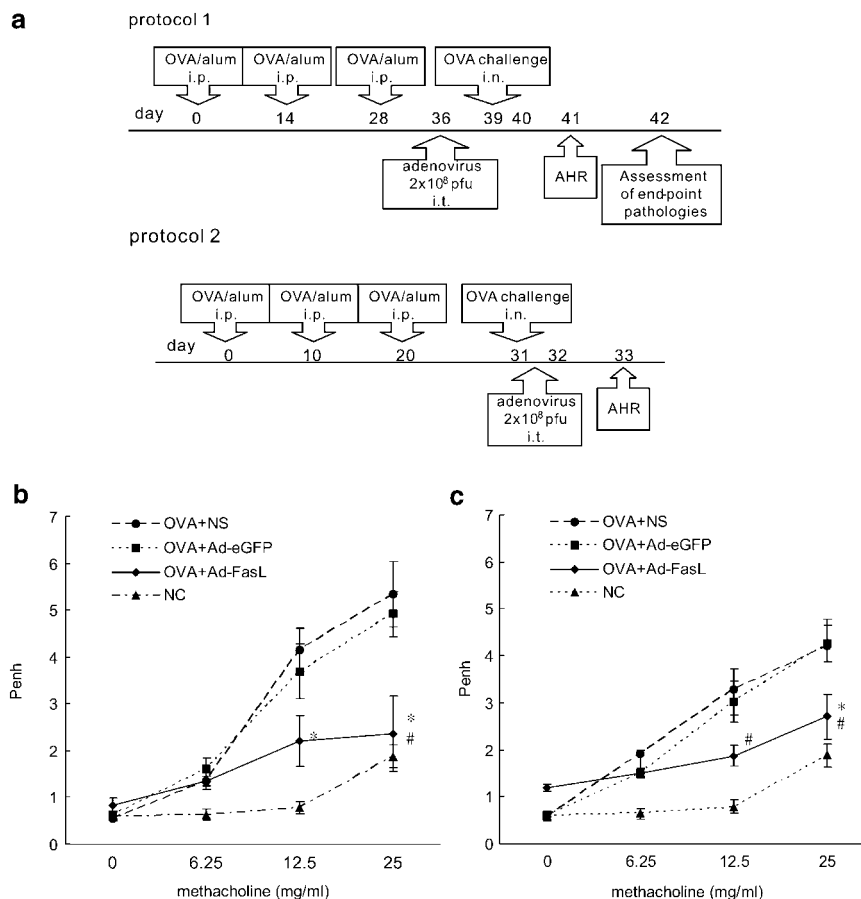


Figure 3 Effect of Ad-FasL on airway responsiveness to aerosolized methacholine in OVA-sensitized and -challenged mice. (a) Time line representation of the OVA protocol used and the intratracheal injection of adenovirus. i.p., intraperitoneal; i.n., intranasal; i.t., intratracheal. (b, c) Airway responsiveness to increasing concentrations of methacholine (0–25 mg/ml) was assessed by barometric whole-body plethysmography. Virus was given before (b) and after (c) the challenge of OVA. NC (negative control) mice were mice that were sensitized and challenged with normal saline. Penh (Enhanced pause (Penh) = pause $\times$ (peak expiratory box flow/peak inspiratory box flow)). Results were expressed as the mean $\pm$ s.e.m. with five mice in each group. Experiments were repeated in variation three times with similar results. * $P < 0.05$ as compared with the OVA+NS group. # $P < 0.05$ as compared with the OVA+Ad-eGFP group.

To be more relevant in clinical context, mice were administered with virus 2 h after the first challenge and airway responsiveness was assessed one day after the second challenge (Figure 3a, protocol 2). The result showed that administration of Ad-FasL after challenge significantly inhibited the increases in airway responsiveness after nonspecific methacholine stimulation (Figure 3c). However, the Penh in the response to normal saline inhalation was higher in mice receiving Ad-FasL after challenge than that in mice receiving Ad-FasL before challenge (Figure 3b and c).

Ad-FasL alleviated eosinophils and lymphocytes infiltration in BAL cells

In order to explore whether Ad-FasL administration could alleviate the allergen-induced airway inflammation, the cellular compositions in the bronchoalveolar lavage fluid (BALF) were analyzed. The number of total inflammatory cells in the BALF was significantly higher in mice sensitized and challenged with OVA than that in mice sensitized and challenged with normal saline. The number of eosinophils and lymphocytes in the BALF of mice administered with Ad-FasL was significantly lower than that in the BALF of mice administered with Ad-eGFP or normal saline. Further, the administration of Ad-FasL increased the number of cells that infiltrated the lungs, especially neutrophils and macrophages (Figure 4).

Eosinophils were apoptotic in the lung sections of mice receiving Ad-FasL

There are two possibilities why the number of infiltrated eosinophils in the BALF decreased in mice that received Ad-FasL. One possibility is that FasL induced the cell death of Fas-bearing eosinophils. The other possibility is that the decreased number of recruited eosinophils was due to decreased chemokine levels for the eosinophils. To understand whether apoptosis of eosinophils occurred in the OVA-sensitized and -challenged mice that received Ad-FasL, we analyzed the apoptotic cells in the lung section. There were a marked number of apoptotic cells

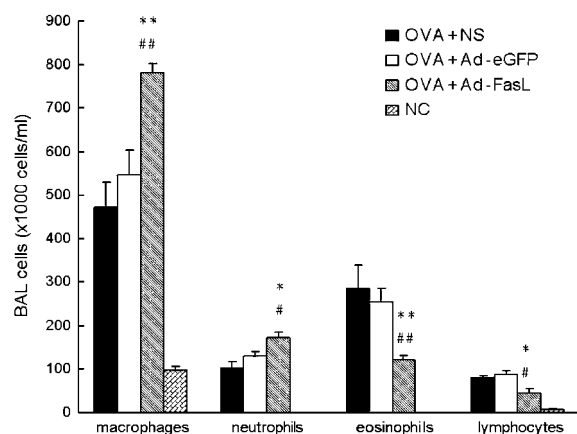


Figure 4 Effect of Ad-FasL on cell infiltration to the mouse airway. The cell compositions in BALF of different groups of mice were analyzed. Results were expressed as the mean $\pm$ s.e.m. for 5–7 mice in each group. Experiments were repeated in variation two times with similar results. * $P < 0.05$ and ** $P < 0.005$ as compared with the OVA+NS group. # $P < 0.05$ and ### $P < 0.005$ as compared with the OVA+ Ad-eGFP group.

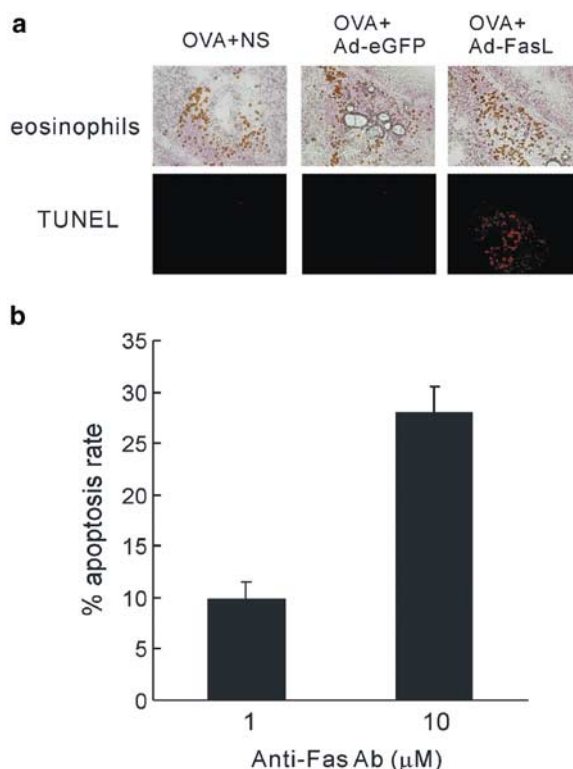


Figure 5 Eosinophils were apoptotic *in vivo* and *in vitro*. (a) Results of cyanide-resistant eosinophil peroxidase assay (upper panels) and TUNEL assay (lower panels) of lungs of different groups of mice. Representative staining of lungs of mice receiving normal saline, Ad-eGFP, or Ad-FasL were shown ($\times 200$ magnification). (b) Purified lung eosinophils were incubated with anti-Fas antibodies (1, 10 μ g/ml) for 18 h and apoptosis were measured by annexin V staining. Data were represented as (% of annexin V-positive cells of anti-Fas antibodies treatment)–(% of annexin V-positive cells of medium alone). Data were shown as the mean $\pm$ s.d. of the percentage apoptotic cells for three experiments (3–5 mice per experiment).

in the lung of mice that received Ad-FasL but not in those that received control Ad-eGFP or normal saline as demonstrated by *in situ* TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling) staining (Figure 5a). By identifying eosinophils, we demonstrated that eosinophils were apoptotic in the lung sections of mice receiving Ad-FasL (Figure 5a). To understand whether eosinophils were susceptible to Fas-mediated apoptosis, the susceptibility of eosinophils to Fas-induced apoptosis was measured. Purified eosinophils were treated with different concentrations of anti-Fas antibodies, and apoptosis was measured by annexin V staining. After 18 h of culture, eosinophils treated with anti-Fas antibodies induced cell death in a dose-dependent manner (Figure 5b).

Eotaxin, IL-5, IL-13, KC, and TNF- α levels reduced in the BALF of mice receiving Ad-FasL

Eosinophil-active chemokines include RANTES, macrophage chemotactic protein (MCP)-2, MCP-3, MCP-4, macrophage inflammatory protein (MIP)-1 α , and eotaxin. Eotaxin is the most potent chemokine for the recruitment of eosinophils.^{28,29} Eotaxin levels in the BALF of different groups of mice were assayed by

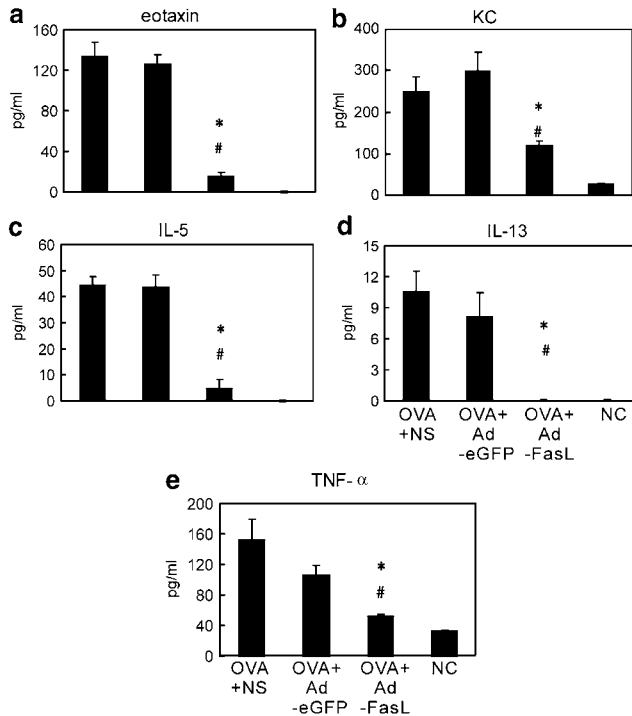


Figure 6 The eotaxin (a), KC (b), IL-5 (c), IL-13 (d), and TNF- α (e) levels in the BALF of different groups of mice were measured by ELISA. Results were expressed as the mean $\pm$ s.e.m. for 5–7 mice in each group. Experiments were repeated in variation two times with similar results. * $P < 0.005$ as compared with the OVA+NS group. # $P < 0.005$ as compared with the OVA+ Ad-eGFP group.

enzyme-linked immunosorbent assay (ELISA). Eotaxin levels in the BALF of mice administered with Ad-FasL were significantly lower than those in the BALF of mice administered with Ad-eGFP or normal saline (Figure 6a). This result indicated that Ad-FasL reduced the secretion of eotaxin, and subsequently decreased the number of recruited eosinophils. To test whether T-cell responses were affected in the mice receiving Ad-FasL, the levels of IL-5 and IL-13 in the BALF of various groups of mice were measured by ELISA. IL-5 and IL-13 expressions decreased in mice receiving Ad-FasL, when compared with mice receiving Ad-eGFP or normal saline (Figure 6c and d). This result was correlated with the reduced number of lymphocytes in the BALF of Ad-FasL-treated mice (Figure 4). Moreover, by double staining with anti-CD4 and TUNEL, CD4+ T cells were found apoptotic in the lung sections of mice receiving Ad-FasL (data not shown). These results indicated that Ad-FasL administration might reduce the infiltration of T cells and Th2 cytokines, and subsequently, alleviated AHR. TNF- α , a proinflammatory cytokine, is produced by activated mast cells and mediates the chemoattractance of neutrophils and eosinophils in the lungs.³⁰ Mouse KC, a member of the alpha (CXC) chemokine family of inflammatory cytokines, is a functional homology of human IL-8 protein and may function as the major proinflammatory chemokine in mice.³¹ KC and TNF- α levels in the BALF were also significantly decreased in mice that received Ad-FasL (Figure 6b and e).

Discussion

Allergic asthma is a chronic inflammatory lung disease characterized by elevated Th2 cytokine, IgE antibodies, lung eosinophilia, and AHR. AHR plays an important role in the pathogenesis of bronchial asthma.^{1–4} The severity of this hyper-reactivity has been correlated with the number of Th2 cytokine-producing T cells and various inflammatory cells, predominantly eosinophils, in the airway.^{1–4} Fas/FasL-mediated apoptosis is an important mechanism in the downregulation of T-cell-mediated immune responses and resolution of inflammatory responses.^{19–22,32} The *gld* mutant mice, which express a dysfunctional FasL protein, develop prominent infiltration of inflammatory cells in the airway.³³ Asthmatic individuals show an increase in the number of eosinophils, a decrease in the expression of FasL, and a decrease in the number of apoptotic cells in the airway but not that of healthy persons.^{23,34} Moreover, the number of apoptotic eosinophils is reduced within the airway of asthmatic individuals compared with patients with other eosinophilic disorders of the lung, such as chronic bronchitis.²⁴ During allergic airway inflammation induced by OVA in mice, FasL mRNA and protein is markedly reduced in the airway epithelium.³³

These studies suggest that inflammatory cell apoptosis, especially eosinophils apoptosis is clinically relevant in asthma, and apoptosis may represent a mechanism that promotes the resolution of inflammation in asthma. It will be interesting to study whether the induction of apoptosis is beneficial in asthma. In the present study, administration of Ad-FasL in a murine model with OVA immunization significantly abolished AHR and induced apoptosis of eosinophils and lymphocytes (Figures 3–5). These results were similar to previous studies in which administration of anti-Fas antibody was able to induce apoptosis in the infiltrated eosinophils of the lungs and reduced AHR in a murine model of asthma.^{32,35} In our experiments, a single administration of adenoviral vector expressing FasL significantly alleviated airway responsiveness. In contrast, multiple administrations and/or high doses of anti-Fas antibodies were needed in the previous studies to achieve a similar effect.^{32,35} Taken together, these data suggest that the induction of Fas-mediated apoptosis of the infiltrated eosinophils in the airway could be a novel therapeutic strategy for asthma.

We and others demonstrated that stimulation of purified eosinophils *in vitro* with a monoclonal anti-Fas antibody induced apoptosis in a dose-dependent manner,³² and eosinophils were apoptotic in the lung sections of mice receiving Ad-FasL (Figure 5). In addition, eotaxin and IL-5 levels in the BALF were dramatically decreased in mice that received Ad-FasL (Figure 6a and b). These findings indicate that FasL administration reduces the number of eosinophils by inducing apoptosis of Fas-bearing eosinophils and/or decreasing eosinophil attractant factor (such as IL-5 and eotaxin) production. IL-5, one product of Th2 cells, has been shown to provide an essential signal for the expansion and mobilization of eosinophils from the bone marrow into the lung after allergen exposure.^{1–4} In our experiments, IL-5 and IL-13 levels and the number of lymphocytes in the BALF were lower in mice receiving Ad-FasL than in control mice (Figure 4, 6c and d). Eotaxin has been found to be the most potent chemokine for the recruitment of

eosinophils.^{28,29} It has been reported that eotaxin can be produced by various cells, including lung epithelial cells, endothelial cells, and lung fibroblast. Further, eotaxin production by these cells can be induced by certain cytokines, such as IL-4 and IL-13.^{11,36,37} It is likely that reduced eotaxin was due to the reduced IL-13 (Figure 6c and d). These results indicate that FasL administration prevents AHR and eosinophilia, either directly inducing apoptosis of eosinophils or indirectly affecting Th2 cells or eotaxin-producing cells to reduce eosinophilia.

As in our study, administration of Ad-FasL or anti-Fas antibody in a murine model with OVA immunization significantly abolishes AHR and eosinophilia.^{32,35} However, administration of anti-Fas antibody or recombinant soluble FasL in the lungs of normal animals results in acute pulmonary inflammation and pulmonary fibrosis.^{38–40} Although apoptosis has been implicated as a homeostatic mechanism, extensive apoptosis may result in disease. Systemic administration of excess anti-Fas antibody or Ad-FasL to normal animals would cause hepatic failure and death,^{41,42} suggesting that excess apoptosis causes disease. It is possible that administration of Ad-FasL or anti-Fas antibody to OVA-immunized mice causes apoptosis of Fas-expressed activated inflammatory cells, while administration of Ad-FasL or anti-Fas antibody to naïve normal mice induces apoptosis of alveolar epithelial cells and inflammation. We cannot rule out the possibility that apoptosis of alveolar epithelial cells and inflammation occurred in our study, although the levels of some inflammatory response-related cytokine or chemokine, such as TNF- α , KC, and MIP2, decreased (Figure 6b and e and data not shown).

Replication-deficient adenovirus vectors have become a popular tool for gene transfer into mammalian cells and have gained widespread application in a variety of preclinical disease models and clinical gene therapy trials.^{25–27,43} It has been demonstrated that replication-defective adenovirus without transgene could inhibit eosinophilic inflammation in the airway of OVA-sensitized mice.^{25,44} Furthermore, administration of adenovirus expressing Th1-related cytokines (such as IL-12, IL-18 and IFN- γ) reduced AHR and lung inflammation.^{26,27} As another strategy in addition to Th1/Th2 modulation, we demonstrated that administration of Ad-FasL to OVA-sensitized mice reduced AHR and lung eosinophilia. However, the recruitment of macrophages and neutrophils was increased in OVA-sensitized and challenged mice receiving Ad-FasL. Although more studies are needed, the recruitment of neutrophils might be due to the chemotactic effect of FasL.^{45,46}

In summary, we constructed a bioactive adenoviral vector expressed murine FasL *in vitro*. In a murine model of OVA-induced asthma, the pulmonary expression of adenoviral vector expressing FasL before the airway challenge could significantly alleviated the development of AHR, reduced the infiltration of eosinophils and lymphocytes, and decreased eosinophil chemoattractant eotaxin, proinflammatory cytokines KC, and TNF- α production, and Th2 cytokines IL-5 and IL-13 production. Taken together, these results indicate that FasL administration prevents AHR and eosinophilia either directly inducing apoptosis of eosinophils or affecting Th2 cells or eotaxin-producing cells to reduce the number of infiltrated eosinophils, indirectly. With more

studies, Ad-FasL might be applied as an alternative therapy for allergic asthma.

Materials and methods

Mice

Female BALB/c mice aged 6–8 weeks were obtained from the Animal Center of the College of Medicine, National Taiwan University. Animal care and handling protocols were approved by the Animal Committee of National Taiwan University.

Construction and purification of Ad-FasL

Recombinant adenovirus was constructed using the AdEasy system that has been described previously.⁴³ Briefly, the 950-bp cDNA containing murine FasL.2 (from Dr SL Hsieh, National Yang-Ming University, Taiwan) was inserted into a recombinant adenoviral shuttle vector (pAdTrack-CMV) and cotransformed into *Escherichia coli* BJ5183 cells with an adenoviral backbone plasmid (pAdEasy-1). The linearized recombinant plasmid was transfected into the adenovirus packaging cell line (293CrmA cell line). Production of Ad-FasL was markedly reduced in 293 packaging cell lines because of apoptosis in 293 cell lines. We substituted 293CrmA cell line (from Dr Joseph T. Bruder, GenVec, Inc., MD, USA) as the packaging cell line.⁴⁷ Viruses were purified from infected cells 42–48 h after infection by three freeze-thaw cycles followed by successive banding on CsCl gradients. The purified virus was dialyzed and stored at -70°C until needed. Viral titers were measured by standard end point dilution assay using 293CrmA cells. Ad-eGFP was identical, but contained no transgene in the expression cassette.

Preparation and infection of bone marrow dendritic cells (BMDCs)

BMDCs were prepared as described previously.⁴⁸ Briefly, bone marrow cells were cultured in RPMI 1640/5% FCS in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (750 U/ml) and interleukin-4 (IL-4) (1000 U/ml) (Pepro Tech Inc., Rocky Hill, NJ, USA). OVA (500 $\mu\text{g}/\text{ml}$) were added to the BMDCs cultures on day 5. On day 6 of the culture, nonadherent cells (BMDCs) were collected and washed extensively to remove free antigens. They were subsequently infected with Ad-FasL or Ad-eGFP ($\text{moi} = 250$) and incubated for one more day. The purity of the BMDCs ($>65\%$) was analyzed by flow cytometry, examining the expression of MHC class II, B7-1, B7-2 and CD11c.

FACS analysis

Surface FasL expression was assessed by immunofluorescent staining with 1 $\mu\text{g}/\text{ml}$ of monoclonal antibodies against FasL (MLF3) or control IgG (hamster IgG) (both from PharMingen, San Diego, CA, USA). The apoptosis of purified eosinophils was stained with annexin V (PharMingen, San Diego, CA, USA). Samples were analyzed using a FACScan (Becton Dickinson, San Jose, CA, USA).

RT-PCR

Total RNA from adenovirus-infected BMDCs was isolated using TRIzol reagent (Life Technology Inc., Grand Island, NY, USA) and mixed with an oligo-dT primer, dNTPs and reverse transcriptase (Superscript II, Life Technology Inc.) for synthesis of single-stranded cDNA. cDNA was then amplified in 30 cycles of the PCR with dNTPs, FasL primers and Tag polymerase. The sequences of murine FasL primers were as follows: sense, 5'-ATGCAGCAGCCCATGAAT-3'; antisense, 5'-AAGCTTATACAAGCCGAA-3'. The cycling condition chosen was denaturing for 1 min at 95°C, annealing for 30 s at 50°C, then extending 1 min at 72°C. An additional 7 min at 72°C were extended after the last cycle. The amplified products (837 bp) were separated on 1% agarose gel and stained with ethidium bromide, and then were visualized by ultraviolet illumination. β -actin was used as an internal control.

Protein electrophoresis and Western blot analysis

After 24 h of culture, BMDCs were collected, centrifuged into a pellet, and lysed with 100 μ l 8 M urea, 4% Triton $\times$ 100, 2 mM DTT, 40 mM Tris base, and 10% glycerol for 30 min on ice, and then were centrifuged at 12000 r.p.m. for 30 min at 4°C. The protein levels were measured by a BCA protein assay kit (PIERCE, Rockford, IL, USA). The protein (1 μ g/lane) was separated by a vertical dodcyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), and subsequently transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked in TBS/5% skim milk overnight and then incubated with anti-hFasL (BD Transduction laboratories) for 1 h. After washing with TBS/Tween, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (PIERCE, Rockford, IL, USA). After another washing, proteins were detected using the ECL system (Amersham, Little Chalfort, Buckinghamshire, UK).

Detection of apoptosis in vitro

Jurkat cells were cocultured with Ad-FasL-infected or Ad-eGFP-infected COS-1 cells for 18 h. DNA fragmentation was analyzed by flow cytometry following staining with propidium iodide (PI) using the method of Nicoletti and colleagues.⁴⁹

Sensitization and challenge of mice

The mice were sensitized intraperitoneally by injection of 20 μ g of OVA (Grade V; Sigma Chemical Co., St Louis, MO, USA) emulsified in 2 mg of aluminum hydroxide (AlumImject; Pierce Chemical, Rockford, IL, USA) in a total volume of 200 μ l, boosted with 50 μ g of OVA emulsified in 2 mg of aluminum hydroxide and challenged with OVA (100 μ g in a total volume 40 μ l) by intranasal administration on consecutive days (Figure 3a).

Administration of Ad-FasL

Ad-FasL (2×10^8 PFU) was delivered intratracheally (i.t.) into anaesthetized animals 3 days before (protocol 1) or 2 h after (protocol 2) the first challenge with OVA. A mock virus (Ad-eGFP) or normal saline was used as a control (Figure 3a). The 2×10^8 PFU of adenovirus was chosen by preliminary titration data (data not shown).

For intratracheal injection, viruses in 30 μ l of normal saline solution were injected with a catheter fitted with 0.61 mm (OD) of polyethylene tubing.

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 described in detail previously.^{50–52} Briefly, mice were placed in the main chamber of a whole-body plethysmography and challenged with aerosolized normal saline, later challenged with increasing doses of methacholine (6.25–25 mg/ml). Each nebulization lasted for 3 min. After each nebulization, records were taken and averaged over 3 min. Airway responsiveness was expressed as enhanced pause ($Penh = \text{pause} \times (\text{peak expiratory box flow} / \text{peak inspiratory box flow})$).

Analysis of the cellular composition in the BALF

A day after the measurement of the pulmonary function parameters, the mice were killed and the tracheas were immediately lavaged three times via a trachea 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 BAL cells were washed and resuspended in 1 ml of HBSS. The total number of cells in the BAL was counted with a standard hemocytometer. Differential cell counts were performed by counting at least 200 cells on cytocentrifuged preparation (cytospin 2; cytopsin, Shandon Ltd., Runcorn, Cheshire, UK), stained with Liu's stain, and differentiated by standard morphological criteria.

Measurement of BALF cytokine

Quantifications of IL-5, IL-13, KC, eotaxin, and TNF- α in the BALF supernatants were evaluated using commercially available ELISA (Duoset; R&D, Minneapolis, MN, USA).

Induction and purification of lung eosinophils

To induce a lung eosinophilia, BALB/c mice were immunized and challenged with OVA as described above. At 48 h after the final challenge, the mice were anesthetized with pentobarbital, and the BALF were collected as described above. Red cells were deleted with a RBC lysis buffer. Macrophages were removed from the cell suspension by adherence to a plastic dish for 2 h. Eosinophil purity was determined by staining cytopsin preparations and analyzing for standard morphological criteria. Eosinophil purity was between 70 and 80% in all experiments.

Induction of eosinophil apoptosis in vitro by anti-Fas mAb

Eosinophils (5×10^5 /well) were stimulated with anti-Fas mAb (clone: Jo2, PharMingen, San Diego, CA, USA) (1 and 10 μ g/ml) and cultured in 48-well plates for 18 h. To determine whether anti-Fas induced cell death by apoptosis, annexin V (PharMingen, San Diego, CA, USA) were stained and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

In situ TUNEL apoptosis staining

Lungs embedded in OCT (Sakura Finetek, Torrance, CA, USA) and cryostat sections 4 µm thick were prepared and mounted on poly-L-lysine-coated slide (Dako, Japan) at -20°C. The TUNEL apoptosis staining kit was purchased from Roche (Roche Molecular Biochemicals, Mannheim, Germany), and samples were stained according to the manufacturer's instructions.

Cyanide-resistant eosinophil peroxidase assay

Cryostat section was fixed with formal-acetone buffer for 10 min and incubated with substrate (diamino-benzidine; DAB) and potassium cyanide (KCN) for 15 min. After counterstaining with hematoxyline, sections were examined using a microscope.

Statistical analysis

Results were expressed as the mean ± standard deviations (s.d.) or mean ± standard error of the mean (s.e.m.) and evaluated using a one-way analysis of variance (ANOVA), 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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