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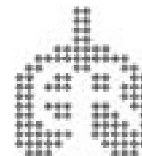

Adoptive transfer of T-helper cell type 1 clones attenuates an asthmatic phenotype in mice

K. Irifune, A. Yokoyama, K. Sakai, A. Watanabe, H. Katayama, H. Ohnishi, H. Hamada, M. Nakajima, N. Kohno and J. Higaki

ABSTRACT: **T-helper cell type 1 (Th1) cells have been postulated to have a significant role in protective immunity against allergic diseases. However, recent studies using polarised Th1 cells showed conflicting effects on both airway responsiveness and eosinophilic inflammation in a mouse asthma model. The current study explored the effects of adoptive transfer of established Th1 clones on a murine model of atopic asthma.**

Mice (BALB/c) were sensitised with ovalbumin (OVA) and challenged with aerosolised OVA (5%, 20 min) for 5 days. Just before starting the first challenge, Th1 clones ($5 \times 10^{6} \cdot body^{-1}$) or PBS alone were injected *via* the tail vein. After assessment of airway responsiveness to methacholine, bronchoalveolar lavage fluid (BALF) was obtained. Histological examination, including morphometric analysis, measurement of cytokines in the BALF and Northern blotting of lung chemokines, was also performed.

Adoptive transfer of Th1 clones showed a significantly increased total number of cells, whereas significantly decreased eosinophils were found in the BALF, when compared with mice with injection of vehicle alone or splenic mononuclear cells. Administration of Th1 clones significantly decreased the infiltration of eosinophils but increased mononuclear cells in the peribronchial area. Goblet cell hyperplasia and peribronchial fibrosis were also suppressed by Th1 clones. The transfer of Th1 cells significantly decreased airway responsiveness. Th1 injection significantly increased interferon gamma in the BALF, but significantly decreased interleukin (IL)-5 and IL-13. Eotaxin mRNA was predominantly expressed in the lungs of asthma model mice, whereas RANTES (regulated on activation, normal T-cell expressed and secreted) predominates in such mice with Th1 transfer.

In conclusion, results suggest that the adoptive transfer of T-helper cell type 1 clones can suppress both lung eosinophilia and airway responsiveness, but increase noneosinophilic inflammation in a mouse model of asthma.

KEYWORDS: **Airway responsiveness, asthma, chemokine, eosinophilic inflammation, T-helper cell type 1**

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The airway inflammation of asthma is considered to be driven by T-helper (Th) type 2 cells [1, 2]. T-cells in bronchoalveolar lavage fluids (BALF) or bronchial biopsy specimens from patients with asthma predominantly express interleukin (IL)-4 or IL-5 mRNA, suggesting these T-cells are Th2 cells. In a murine asthma model in which mice were sensitised to chicken ovalbumin (OVA) and then challenged with aerosolised OVA, the phenotype is also considered to be Th2 dominant because of Th2 pattern of cytokines in the BALF and cytokines produced by T-cells from lung lymph nodes and spleen cells. Furthermore, adoptive transfers of Th2 clones or of the polarised Th2 population into naive mice followed by transbronchial antigen challenge induced an asthma-like phenotype in the lung [3, 4].

Therefore, induction or the adoptive transfer of Th1 cells may be a possible therapeutic strategy for asthma, since Th1 cells can antagonise the Th2-driven mechanisms. Previous reports demonstrated that interferon (IFN)-γ produced by Th1 cells can inhibit proliferation of Th2 cells

For editorial comments see page 591.

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and reduce eosinophilic inflammation and airway responsiveness. Treatment of asthma model mice with Th1-inducing substances, such as IL-12, IFN-γ, or Bacille Calmette-Guellan (BCG), mycobacteria, Listeria and CpG DNA sequences can inhibit the murine asthma phenotype [5–8]. However, it is not established whether a counterbalance of the asthmatic phenotype could be induced by Th1 cells alone. The effect induced by these substances may possibly involve cell types other than Th1 cells, such as IL-10 or transforming growth factor-β-producing regulatory T cells [9–11].

The direct effect of Th1 response has been investigated by adoptive transfer of skewed Th1 cells into a mouse or rat asthma model [12–14]. The recruitment of eosinophils to the lungs was increased in a previous study [12], though suppressed in the others [13, 14]. The effect of such Th1 population on airway responsiveness also showed conflicting results [13, 14]. In these experiments, cytokine-polarised cells from rats or T cell receptor (TCR)-transgenic (Tg) mice were injected once into sensitised syngeneic rats or mice, respectively, followed by repeated exposures to an aerosolised corresponding antigen.

Taking advantage of the stability, the current study used long-term cultured, established Th1 clones instead of polarised cells. Adoptive transfer experiments were performed to clarify the modulating effects of Th1 cells on a phenotype of a mouse asthma model.

MATERIALS AND METHODS

Adoptive transfer of T-helper cell type 1 clones

Mice (BALB/c, 6–8 week-old female; Charles River Laboratories, Kanagawa, Japan) were sensitised with OVA (grade V; Sigma Chemical Co. St. Louis, MO, USA) plus alum (asthma model and asthma-Th1 groups) or PBS (Th1-alone and PBS groups) three times at weekly intervals (day 0, 7 and 14), as described previously [15–17]. These mice were then challenged with aerosolised OVA (5%) for five consecutive days (days 15–19). Just before starting challenges (day 15), Th1 clones (5×10^6/500 µL PBS/mouse; asthma-Th1 and Th1 alone groups), splenic mononuclear cells (SMNC; 5×10^6/500 µL PBS/mouse; asthma-SMNC group) or PBS alone (asthma model and PBS groups) were injected *via* tail vein. The characterisation of OVA-reactive Th1 clones (D2, S1, and S4) was described previously [17]. The clones were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Gibco, Grand Island, NY, USA), 100 U·mL^{-1} penicillin G, 100 µg·mL^{-1} streptomycin, 50 µM 2-mercaptoethanol (Sigma), and 10 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) (Sigma).

The challenge was performed in a 50 mL plastic tube, with OVA aerosol delivered by a nebuliser (DeVilbiss Corp., Somerset, PA, USA). Mice were killed by cervical dislocation under anesthesia 24 h after the final exposure to OVA. BALF (0.5 mL, three times) from the whole lungs was obtained by cannulating the surgically exposed trachea. Cells in the BALF were counted using a haemocytometer. The differential cell count was obtained using cytospin preparations (May-Giemsa staining).

Histology and morphometric analysis

Following bronchoalveolar lavage, the lungs were perfused in 10 mL PBS by puncturing the right ventricle. The left lung was removed and stored at -80℃ for RNA extraction. The right lung was removed and fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5-µm sections. The sections were stained with haematoxylin and eosin, elastica-Masson's trichrome, or alcian blue at pH 2.5/periodic acid-Schiff (AB-PAS).

The numbers of eosinophils, goblet cells (AB-PAS-positive cells) and total epithelial cells (total number of nuclei in the epithelium) were counted using an image analysis system as previously described [16]. Data are expressed as the number of cells per millimeter subepithelial basement membrane. The morphometric analysis of airway fibrosis was performed by the method described previously, with some modification [16]. In brief, by using recorded images of the intrapulmonary airway stained by elastica-Masson's trichrome, a basement membrane perimeter (Pi) and the external perimeter (Pe) of the area stained blue was measured. On the assumption that both Pi and Pe are circular, the area of collagen deposition under the basement membrane was given by the following:

$$(\mathrm{Pe})^2/4\pi - (\mathrm{Pi})^2/4\pi \qquad (1)$$

The mean ± SD of Pi measured were 4.64 ± 0.40, 4.71 ± 0.42, 4.60 ± 0.34 and 4.66 ± 0.39 ($\times 10^{-1}$ mm; n=7–8) for the asthma model, asthma-Th1, Th1-alone and PBS groups, respectively. There were no significant differences among the groups (p-value=0.98).

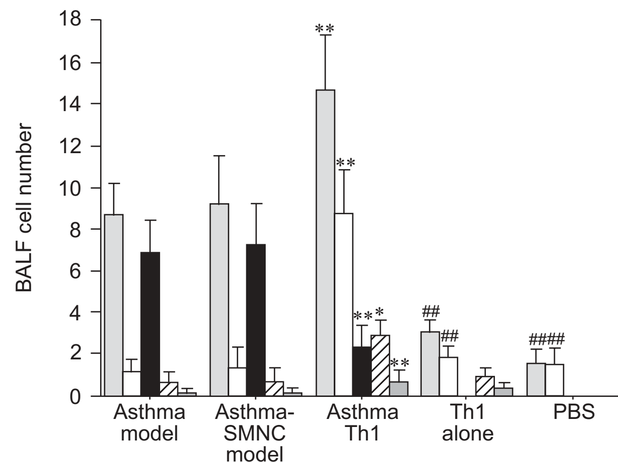

FIGURE 1. Analysis of the cells in bronchoalveolar lavage fluid (BALF) in the asthma model, asthma-splenic mononuclear cells (SMNC), asthma T-helper cell type 1 (Th1), Th1 alone, and PBS group mice. The asthma model mice showed a marked eosinophilia in the BALF. The transfer of Th1 cells but not SMNC to these model mice decreased the number of eosinophils. However, the Th1 cells increased the total number of cells in the BALF with an increased number of macrophages, lymphocytes and neutrophils. The results are expressed as mean ± SD of 6–7 mice. ▒: total cells; □: macrophages; ■ eosinophils; ▨: lymphocytes; ▒: neutrophils. *: $p<0.05$; **: $p<0.01$ *versus* the asthma-model group; ##: $p<0.01$ *versus* asthma-Th1 group.

Measurement of the airway responsiveness

Airway responsiveness was measured under conscious and unrestrained conditions by body plethysmography (Buxco, Troy, NY, USA) as described previously [15, 16, 18]. Measurements were obtained before and after exposure to aerosolised saline or an escalating dose of methacholine (MCh). Data were averaged for 3 min and expressed as the enhanced pause (Penh):

$$\text{Penh} = [(\text{Te} - \text{Tr})/\text{Tr}] \times (\text{PEP}/\text{PIP}) \qquad (2)$$

where Te is the expiratory time(s), Tr is the relaxation time (time of the pressure decay to 36% of the total box pressure during expiration), PEP is the peak expiratory pressure ($m \cdot s^{-1}$), and PIP is the peak inspiratory pressure ($m \cdot s^{-1}$). Results are expressed as the per cent increase of Penh following a challenge at each concentration of MCh, where the value of Penh obtained after saline at challenge is 100%.

Analysis of BALF and measurement of cytokines

BALF (0.5 mL × 3, total 1.5 mL) was obtained by the method described previously [15–18]. The concentrations of IFN-γ (Endogen, Boston, MA, USA), IL-5 (Endogen) and IL- 13 (R&D Systems, Minneapolis, MN, USA) were measured according to the procedures recommended by the manufacturer. The detection limits were 1.5 $pg \cdot mL^{-1}$ for IL-13, 5 $pg \cdot mL^{-1}$ for IL-5 and 15 $pg \cdot mL^{-1}$ for IFN-γ.

cDNA probes

Total RNA from the frozen lung tissues of each group was extracted by the protocol of ISOGEN (Nippon Gene, Tokyo, Japan). The total RNA for chemokine cDNA probes was extracted from the lungs of BALB/c mouse. Reverse transcription (RT) was performed at 42℃ for 60 min using a cDNA synthesis kit (Life Sciences, St. Petersburg, FL, USA), and the polymerase chain reaction (PCR) was performed at 94℃ for an initial 5 min followed by 30 cycles of

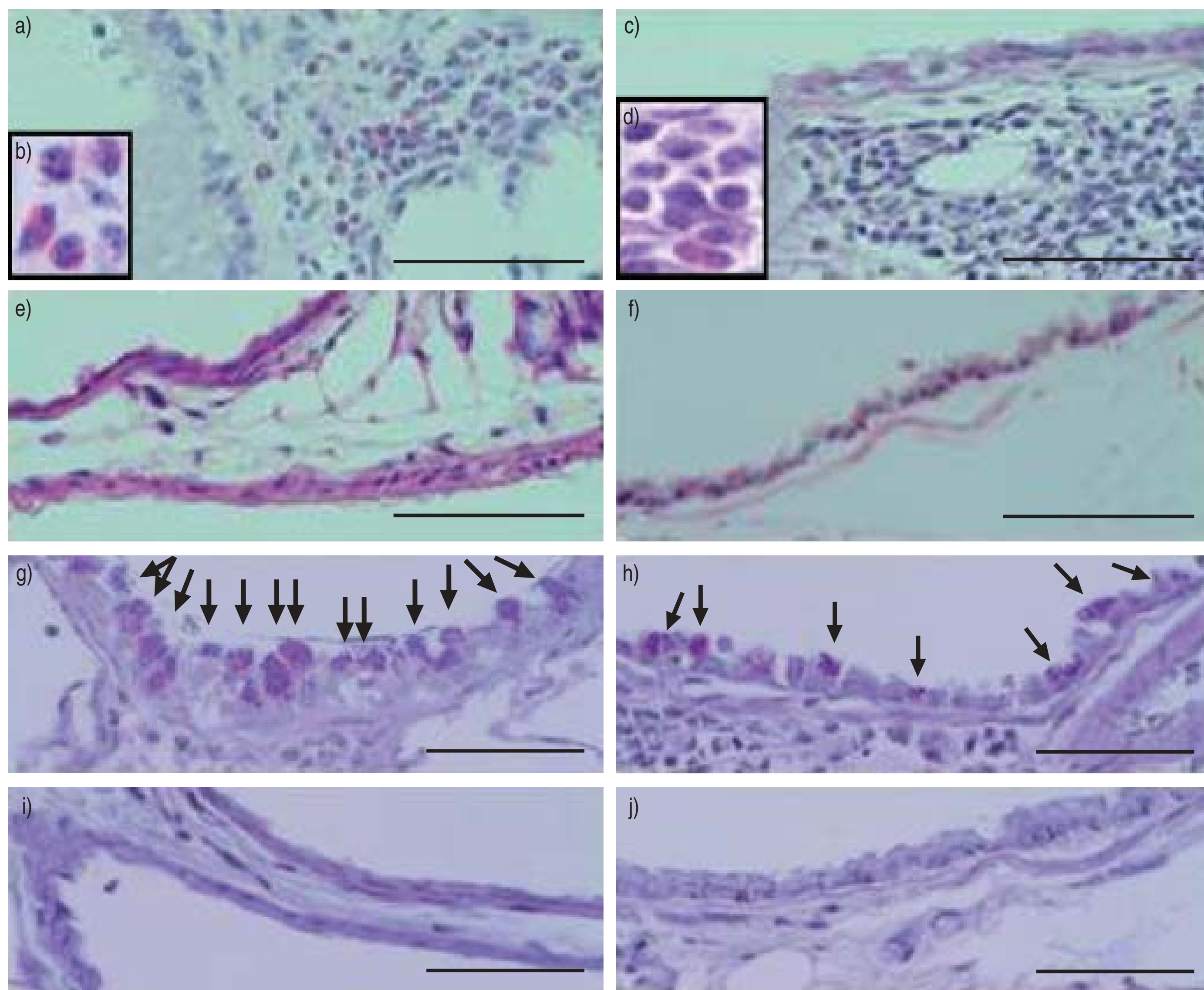

FIGURE 2. Effects on histological changes in the lung. Representative photos of the lungs from the asthma model group (a, g), the asthma-T-helper cell type 1 (Th1) group (c, h), the Th1-alone group (e, i), and the PBS group mice (f, j). Lung sections were stained with haematoxylin and eosin (a–f), or alcian blue/periodic acid-Schiff (AB-PAS; g–j). Arrows show AB-PAS-positive airway epithelial cells. Scale bar = 100 μm.

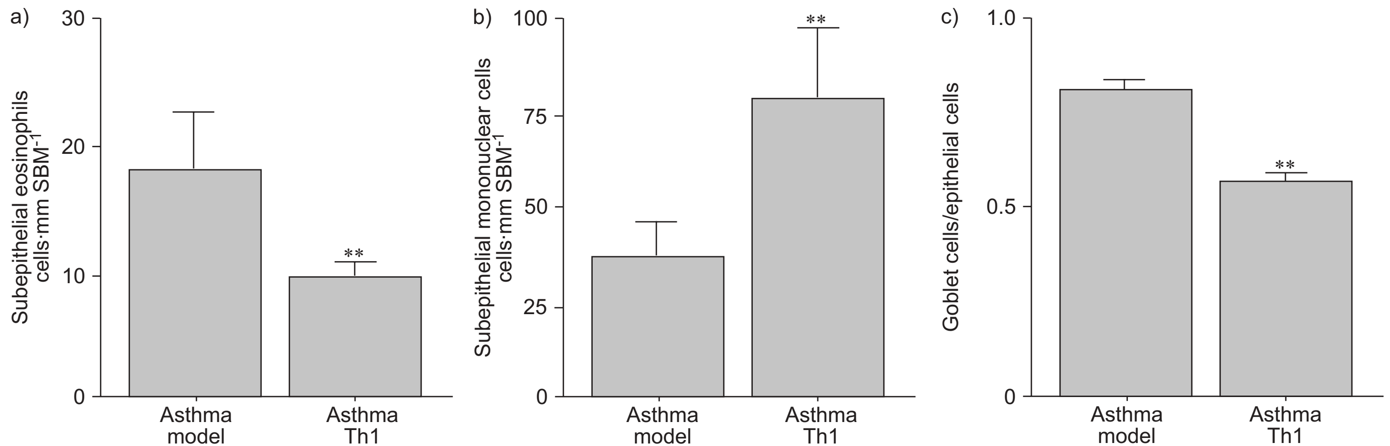

FIGURE 3. Morphometric analysis of airway inflammation of mice from the asthma-model group and the asthma T-helper cell type 1 (Th1) group. The subepithelial eosinophils (a), subepithelial mononuclear cells (b), and the goblet cell hyperplasia (c) are shown. Data are expressed as the number of cells per mm subepithelial basement membrane (SBM), or the goblet cells/epithelial cells ratio (n=7–8, mean±SD). **: $p<0.01$ *versus* asthma-model group.

denaturation at 94℃ for 30 s, annealing at 58℃ for 45 s, and extension at 72℃ for 60 s. The primers for eotaxin were sense, 5′-CGCGGATCCACCATGCAGAGCTCCACA-3′; antisense, 5′-CCGCTCGAGGTTGTTTATGGTTTTGGA-3′ and for RANTES were sense, 5′-CGCGGGATCCATGAAGATCTCTGCA-3′; antisense, 5′-GAATCTAGAAACCCTCTATCCTAGC-3′. The reduced glyceraldehyde phosphate dehydrogenase (GAPDH)-specific PCR primers (R&D Systems, Minneapolis, MN, USA) for RT-PCR of internal control were used. The cDNA probes were labelled with (α-^{32}P) deoxycytosine triphosphate by the random oligonucleotide primer technique using a Prime It II kit (Stratagene, La Jolla, CA, USA).

Nothern blotting of lung chemokines

Denatured RNAs (10 µg) were size fractionated by gel electrophoresis on 1% agarose/5% formaldehyde gels and transferred to a nylon membrane (Hybond-N$^+$; Amersham, Buckinghamshire, UK). The membrane was pre-hybridised for 15 min at 68℃ in QuickHyb Hybridisation Solution (Stratagene) with 20 µg·mL^{-1} salmon sperm DNA (Sigma) and hybridised in the same buffer containing 50 ng·mL^{-1} heat-denatured α-^{32}P-labelled probes at 68℃ for 1 h. After hybridisation, the membrane was washed twice in 2×saline sodium citrate (SSC) (1×SSC=150 mM sodium chloride and 15 mM sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min and in 0.1×SSC with 0.1% SDS at 60℃ for 30 min. The blots were exposed to X-ray film at -80℃ for 48 h.

Statistical analysis

Data are expressed as the mean±SD, unless otherwise stated. Airway responsiveness was analysed by Dunnett *post hoc* test following ANOVA. Variables were analysed using the Mann-Whitney U-test for the comparison of two variables.

RESULTS

As shown in figure 1, mice from the asthma model group had a markedly increased number of total cells and eosinophils in BALF. Th1 administration to these mice (asthma-Th1 group) decreased the number of eosinophils. However, the number of total cells, macrophages, neutrophils and lymphocytes were significantly increased. These effects were not observed in mice of the asthma-SMNC group, suggesting these are not non-specific effects of transferred cells. Mice of the Th1-alone group showed significantly more total cells, macrophages, neutrophils, or lymphocytes in BALF than mice of PBS group. Mice of the PBS group showed few inflammatory cells and no eosinophils in BALF. Although the data of a clone D2 are shown, essentially the same results were obtained in the other Th1 clones (S1 and S4; data not shown).

Mice from the asthma model group mounted a dramatic eosinophilic inflammatory response in the peribronchial area and a marked increase of AB-PAS-positive goblet cells in the airway epithelium (fig. 2a, g, fig. 3a, c). These findings were essentially the same in the lungs from asthma-SMNC group (data not shown). Mice from the asthma-Th1- group showed further inflammation, but the inflammation consisted primarily of mononuclear cells with a small number of eosinophils observed (fig. 2c, fig. 3a, b). The number of goblet cells was significantly less in mice from the asthma-Th1 group than those from the asthma model group (fig. 2h, fig. 3c). Mice from the Th1-alone group did not show significant inflammation over background in the airway (fig. 2e). These results indicate that the Th1 cells transferred into the asthma model mice can generate noneosinophilic inflammation in the airway. Collagen deposition around the airways was observed in the asthma model mice and asthma-Th1 group mice, but not the other group of mice (fig. 4a, b). However, the area of collagen deposition in mice from the asthma-Th1 group was significantly less than that of the asthma model group (fig. 4b).

In accordance with previous reports [15, 18] asthma model mice developed a marked airway responsiveness to MCh (fig. 5). Th1 administration into the asthma model mice (asthma-Th1 group) caused significantly less airway responsiveness than the asthma model mice. Airway responsiveness in asthma model mice was not affected by administration of SMNC (data not shown). Airway responsiveness in mice with

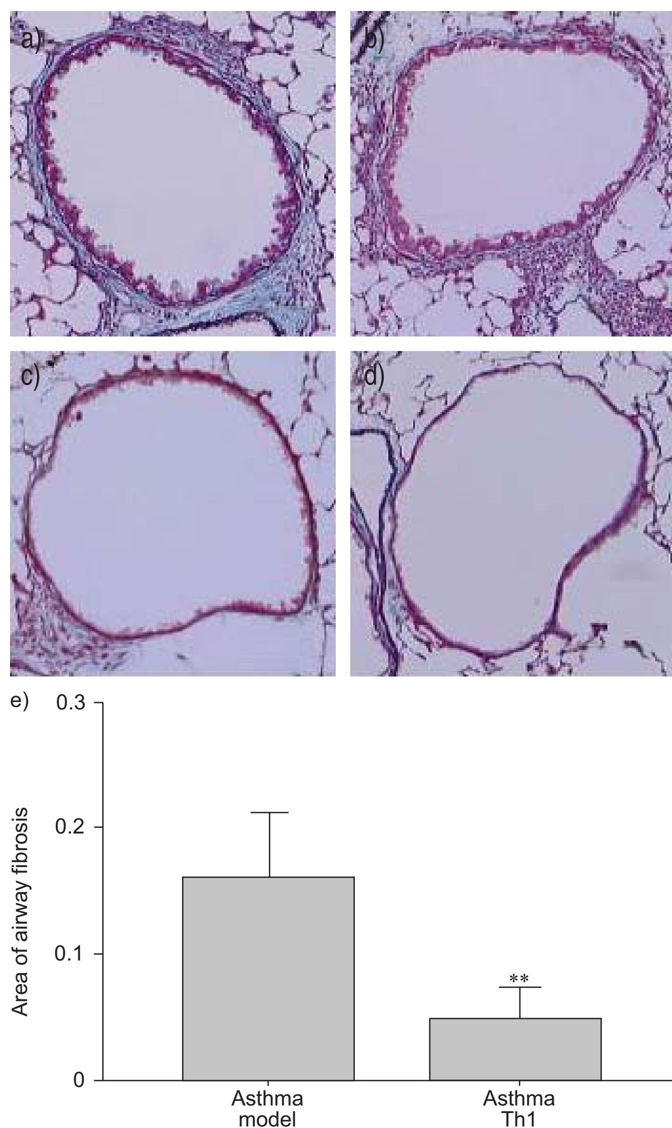

FIGURE 4. Effects on peribronchial fibrosis. Representative photos of the lungs from the asthma-model group (a), the asthma T-helper cell type 1 (Th1) group (b), the Th1-alone group (c), and the PBS group mice (d). The specimens were stained with elastica-Masson's trichrome. e) Morphometric analysis of the peribronchial fibrosis of mice from the asthma model group and the asthma-Th1 group. **: $p<0.01$ *versus* the asthma-model group. Scale bar=100 μm.

Th1 transfer alone was the same as the negative control group. Penh before exposure to aerosolised MCh (baseline Penh) did not differ significantly between the asthma model group and the asthma model with Th1 transfer group (0.41 ± 0.02 *versus* 0.46 ± 0.02, respectively). Although data of a clone D2 are shown, essentially same results were obtained in the other Th1 clones (S1 and S4; data not shown).

The BALF cytokine levels are shown in figure 6. The IFN-γ level in BALF was markedly increased in the mice of asthma-Th1 group, but it was detected only by a small amount in the mice from the asthma model group. The levels of Th2 cytokines, IL-5 and IL-13 were significantly lower in the mice from the asthma-Th1 group than that of asthma model group.

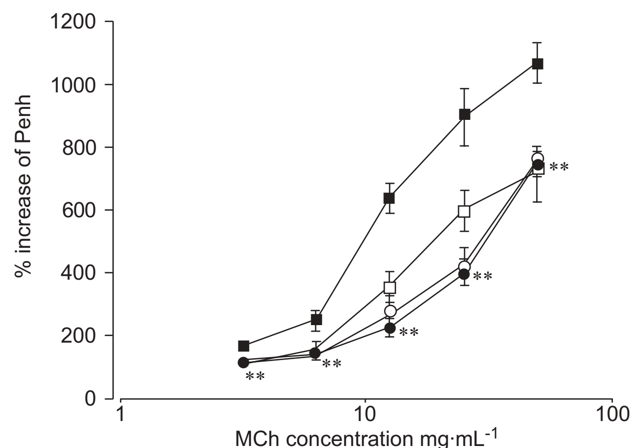

FIGURE 5. Effect on airway responsiveness. Data are expressed as mean % enhanced pause (Penh) ± SEM of 6–8 mice per group. Baseline Penh after saline challenge in each mouse was considered 100%. MCh: methacholine. ■: asthma model; ●: asthma T-helper cell type 1 (Th1); ○: Th1 alone; □: PBS. **: $p<0.01$ *versus* the asthma-model group.

Lung eotaxin mRNA was predominantly expressed in the asthma model mice, but not the other group of mice (fig. 7). The transfer of Th1 decreased the expression of eotaxin mRNA, but markedly increased RANTES mRNA.

DISCUSSION

The transfer of Th1 clones to the asthma model mice induced attenuation of the eosinophilic airway inflammation, airway responsiveness, goblet cell hyperplasia and peribronchial fibrosis. These effects are accompanied by an inhibition of Th2 cytokines and an increase of IFN-γ in the BALF. However, such treatment augmented noneosinophilic airway inflammation. This may be at least partly caused by the different expression of chemokines in the lung. Th1 reduced the expression of eotaxin mRNA and augmented RANTES. These results indicate that the adoptive transfer of Th1 clones in asthma model mice diminishes the pathophysiology of asthma but augments noneosinophilic airway inflammation.

The inhibition of eosinophilic airway inflammation, goblet cell hyperplasia and peribronchial fibrosis may be due to the inhibition of Th2 cytokines and an increase of IFN-γ in the BALF. Th2 cytokines IL-4 and IL-13 contribute to goblet cell hyperplasia and peribronchial fibrosis [19, 20] and IL-5 to eosinophilic inflammation [21]. Therefore, inhibition of these cytokines contributes to the attenuation of the eosinophilic airway inflammation, goblet cell hyperplasia and fibrosis. IFN-γ inhibits proliferation of Th2 cells, and suppresses eosinophilia [22]. In addition, since IFN-γ is a potent inhibitor for the synthesis of collagen, the active suppression by increased IFN-γ also contributes to the reduced fibrosis [23]. Inhibition of airway responsiveness may also be explained by the suppression of both IL-4 and IL-13, and increased IFN-γ. IL-13 is known as the inducer of airway responsiveness and IFN-γ is known to have the ability to inhibit airway responsiveness [24].

While the transfer of Th1 cells suppressed Th2-mediated pathology, such treatment augmented the noneosinophilic

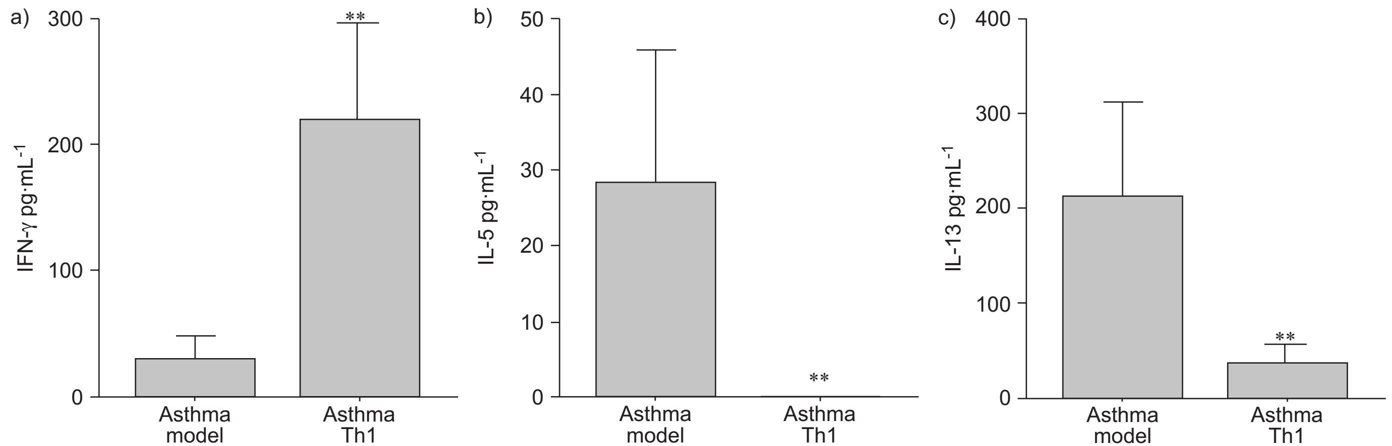

FIGURE 6. Profiles of cytokine levels in the bronchoalveolar lavage fluid from the asthma-model group and the asthma T-helper cell type 1 (Th1) group. The level of interferon (IFN)-γ was significantly increased (a), whereas interleukin (IL)-5 (b) and IL-13 (c) levels were significantly decreased in the asthma-Th1 group model mice, as compared with the asthma-model mice. Data are expressed as mean±SD. **: $p<0.01$ *versus* the asthma-model group.

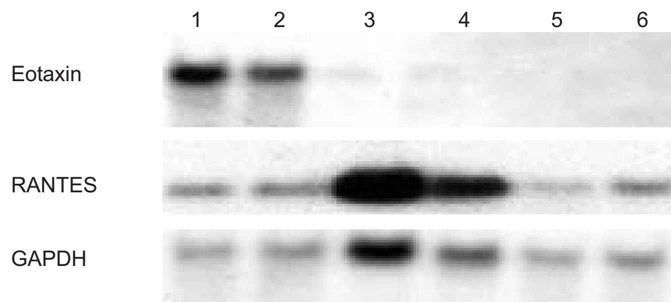

FIGURE 7. Northern blot analysis of eotaxin and RANTES (regulated on activation, normal T-cell expressed and secreted) mRNA in the lungs from the asthma model and the asthma-T helper cell type-1 (Th1) groups. The mouse glyceraldehyde phosphate dehydrogenase (GAPDH) probe was used as a control for the same blots. Representative results are shown among three independent experiments with the same results. Lanes 1 and 2: asthma model mice; lanes 3 and 4: asthma-Th1 group of mice; lanes 5 and 6: PBS group of mice.

airway inflammation. The results from the current study clearly demonstrated that the transferred Th1 cells had a definite effect on the expression of the chemokines *in vivo*. Th1 cells inhibit the expression of eotaxin, which was strongly expressed in the asthma model. The mRNA for RANTES was induced by the transfer of Th1 cells, which was not induced in the asthma model (fig. 7). It is known that both IL-4 and IL-13 are strong inducers and IFN-γ is a strong inhibitor of eotaxin [24, 25]. However, IFN-γ is a strong inducer for RANTES. Eotaxin is known as a potent specific chemoattractant for eosinophils. RANTES is known as a chemokine for not only eosinophils but also monocytes. The transient over-expression of RANTES to the bronchial epithelium induced a dramatic increase in the number of mononuclear cells but not eosinophils [26]. Such a differential expression of chemokines may explain the changes of the inflammatory cells appearing in the lungs and BALF.

The reports showing direct effects of Th1 cells on the asthma model showed conflicting results, as described previously [12–14]. The reasons for the differences observed in the previous studies is obscure at present. The differences in experimental animals used, *i.e.* mouse or rat and clonality of the Th1 population, *i.e.* monoclonal from TCR-Tg mice or polyclonal from wild-type animals, may be factors for the differences. Another possible explanation is that the differences may be due to the purity or maturity of the polarised Th1 population. The polarised population in skewing culture condition for 3 weeks had been used in the previous studies. Although the stability of these cells had been demonstrated, the population still contains Th0 cells, which will further differentiate into Th1 or Th2 cells under the influences of the inducing cytokines [27, 28]. Furthermore, careful treatment for 3 weeks would be essential for commitment of the polarised cells, since it is known that the premature cells would be phenotypically reversible when cultured in the opposite conditions [29]. Therefore, the characteristics of at least some of the polarised Th1 population may possibly be able to change *in vivo* under a particularly polarised Th2 environment, such as asthmatic airways. However, the phenotype of established T-cell clones had been demonstrated to be irreversible and stable even under stimulation with IL-4. For this reason, long-term, established T cell clones were used in the present study.

In conclusion, the concomitant presence of the T-helper cell type 1 response in the asthma model mice diminishes both airway responsiveness and eosinophils in the airway, but augments noneosinophilic inflammation *via* the suppression of T-helper cell type 2 cytokines and causes changes in the chemokines expression in the lung. These results suggest that the administration of T-helper cell type 1 cells may be therapeutically useful. However, further studies need to be carried out in order to determine if there are undesirable functional consequences of the treatment. They also indicate that successful treatment of T-helper cell type 1-inducing agents, such as Bacille Calmette-Guellan and CpG, which is not accompanied by increased inflammation, may not be caused by the induction of the T-helper cell type 1 response alone.

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