

Depletion of CD8⁺ T cells enhances airway remodelling in a rodent model of asthma

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doi:10.1111/j.1365-2567.2008.02876.x

Received 17 December 2007; revised 6 May 2008; accepted 8 May 2008.

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Summary

Airway remodelling is induced by persistent airway inflammation and may lead to severe asthma. T cells play a pivotal role in asthmatic airway inflammation but their role in remodelling is poorly understood. Although previous studies have revealed that CD8⁺ T cells inhibit the late airway response and airway inflammation in a rat model of asthma, their effects on airway remodelling have not been evaluated. The aim of this study was to examine the role of CD8⁺ T cells in airway remodelling. Brown Norway rats were sensitized with ovalbumin (OVA) on day 0. CD8⁺ T cells in rats were depleted during the repeated challenges by treating them with a CD8 α monoclonal antibody (OX-8). Control rats were treated with mouse ascites. Sensitized rats were challenged with OVA on days 14, 19 and 24 or were sham challenged with phosphate-buffered saline. On day 29, bronchoalveolar lavage and lung tissues were harvested. Repeated OVA inhalations evoked significant increases in the numbers of periodic acid–Schiff-positive epithelial cells and proliferating cell nuclear antigen-positive epithelial cells, and in airway smooth muscle mass compared to the control group. CD8-depleted rats had significant enhancement of these changes, principally affecting the large airways. These results suggest that endogenous CD8⁺ T cells have inhibitory effects on airway remodelling in this model of asthma.

Keywords: allergy; asthma; T cells

Introduction

Bronchial asthma is characterized by airflow limitation, airway hyperresponsiveness (AHR), and airway inflammation with the infiltration of eosinophils and activated lymphocytes.¹ Although these components were thought to be reversible, there is a group of asthmatic patients who manifest incomplete responses to β -agonist and persistent AHR even after prolonged corticosteroid therapy that significantly inhibits airway inflammation.² Pathological and morphometric studies of autopsy specimens or bronchial biopsies from asthmatic subjects have

demonstrated a complex series of structural alterations in the airway affecting most, if not all, airway tissues.^{3–5} Airway remodelling, a process related to airway injury and repair that leads to changes in airway structural cells and tissues, is induced by persistent airway inflammation and may contribute to the pathogenesis of chronic severe asthma. The changes of remodelling include epithelial destruction,⁶ goblet cell hyperplasia and metaplasia,⁷ subepithelial fibrosis,⁴ increased vascularity⁸ and airway smooth muscle (ASM) hyperplasia and hypertrophy.^{5,9} It has been suggested that airway remodelling provides an explanation for corticosteroid-resistant AHR and for the

Abbreviations: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; DC, dendritic cell; IFN- γ , interferon- γ ; IL, interleukin; i.p., intraperitoneal injection; LAR, late airway response; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; NK, natural killer; OVA, ovalbumin; OX-8 mAb, mouse anti-rat CD8 α monoclonal antibody; PAS, periodic acid–Schiff; P_{BM} , basement membrane perimeter; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription–polymerase chain reaction; α -SMA, α -smooth muscle actin; TBS, Tris-buffered saline; TGF- β_1 , transforming growth factor- β_1 ; Th2, T helper type 2.

accelerated decline of lung function observed in asthmatic subjects.¹⁰ Understanding the factors regulating airway remodelling in asthma may help in the identification of new therapeutic targets for this disease.

Many clinical and experimental studies have established that airway inflammation with mast cells, eosinophils and activated T lymphocytes plays a central role in asthma. CD4⁺ T cells are important in the pathogenesis of this disease and the messenger RNA (mRNA) of interleukin-4 (IL-4) and IL-5 are expressed predominantly in T cells in bronchoalveolar lavage (BAL) obtained from subjects with atopic asthma.¹¹ The activation of T cells may be related to asthmatic symptoms and to the degree of airflow limitation and AHR through the synthesis of T helper type 2 (Th2) cytokines.¹² Interleukin-4, IL-5 and IL-13 are important mediators of airway inflammation, AHR and pathological findings such as subepithelial fibrosis or mucus hypersecretion in mouse models of asthma.^{13,14} Transfer of antigen-specific CD4⁺ T cells into naïve allergen-challenged rats causes AHR and increases numbers of eosinophils and IL-5-positive cells in BAL.¹⁵

Although relatively fewer studies have been conducted compared to those on CD4⁺ T cells, CD8⁺ T cells are also involved in the regulation of airway inflammation and the late airway response (LAR) following allergen challenge in sensitized rats.^{16,17} We have previously demonstrated that resident CD8⁺ T cells down-regulate CD4⁺ T-cell-dependent LAR and airway inflammation, and that this effect is attributable to T-cell receptor- $\gamma\delta$ -bearing CD8⁺ T cells.^{18,19}

However, there have been conflicting reports showing the proinflammatory effects of CD8⁺ T cells in asthmatic airways.²⁰ In both atopic and non-atopic asthmatic patients, CD8⁺ T cells, as well as CD4⁺ T cells, express mRNA for IL-4 and IL-5.²¹ CD8-deficient mice develop less AHR and lung eosinophilia and have decreased IL-13 production in BAL; these responses are restored by the adoptive transfer of antigen-primed CD8⁺ T cells.²⁰ The mechanism by which CD8⁺ T cells contribute to the immune response in asthmatic airways merits further exploration.

In the experimental models of asthma, it has been shown that repeated allergen exposure causes structural alterations including epithelial thickening,²² increased ASM mass,^{23,24} goblet cell hyperplasia,^{22,25} increased collagen deposition^{25,26} and increased vascularity.²⁶ Recently it was reported that antigen-specific CD4⁺ T cells regulate both proliferation and apoptosis of ASM cells and induce an increase of ASM mass in the Brown Norway rat model of asthma.²⁷ Treatment with an anti-CD4 monoclonal antibody (mAb) prevents the development of AHR and the pathological findings of airway remodelling that are observed in sensitized, chronically exposed mice.²⁸ Currently there is little information concerning the possible role of CD8⁺ T cells in airway remodelling, which contrasts with the information on acute experimental

asthma. In the present study, we address the hypothesis that CD8⁺ T cells have an inhibitory effect on airway remodelling in the Brown Norway rat model of asthma.

Materials and methods

Animals

Highly inbred male Brown Norway rats, 6–7 weeks of age and weighing 95–150 g, were purchased from Sankyo Labo Service Corp. (Tokyo, Japan) and were maintained in an animal facility at Tokyo Medical and Dental University (Tokyo, Japan). Experimental procedures were approved by an institutional animal care committee.

Sensitization and challenges

All rats were actively sensitized with 1 mg ovalbumin (OVA) (grade V; Sigma-Aldrich, St Louis, MO) in 0.5 ml phosphate-buffered saline (PBS) containing 4.28 mg aluminium hydroxide by a subcutaneous injection on day 0. Simultaneously, 6×10^9 heat-killed *Bordetella pertussis* bacilli were administered intraperitoneally (i.p.) as an adjuvant. On days 14, 19 and 24, rats were sedated with xylazine (7 mg/kg) i.p. and anaesthetized 15 min later with pentobarbital sodium (30 mg/kg) i.p. Orotracheal intubation was then performed using a 6-cm length of polyethylene tubing (PE240; Becton Dickinson, Sparks, MD). Rats received inhalational challenge with PBS or OVA (5% wt/v) for 5 min from an ultrasonic nebulizer (Model NE-U17; Omron, Kyoto, Japan).

Study protocol

The protocol used to induce ASM remodelling in the rat was that previously published by Sapienza *et al.*²³ Animals were challenged with PBS or OVA on days 14, 19 and 24 and additionally were administered an i.p. injection of either 250 μ g mouse anti-rat CD8 α mAb (OX-8; a gift from Dr P. M. Renzi, University of Montreal) or the same dose of BALB/c control ascites fluid (CL8100; CEDARLANE, Hornby, ON, Canada) as a control on days 13, 19 and 25. The CD8⁺ T cells of animals treated with 250 μ g OX-8 on days 13, 19 and 25 were depleted by over 90% during the period from day 14 to day 29. Rats were divided into three groups: (1) rats sensitized with OVA, treated with control ascites and challenged repeatedly with PBS (PBS/Cont group; $n = 7$), (2) rats sensitized with OVA, treated with control ascites and challenged repeatedly with OVA (OVA/Cont group; $n = 7$), (3) rats sensitized with OVA, treated with OX-8 and challenged repeatedly with OVA (OVA/OX-8 group; $n = 8$). On day 29 all animals were given an overdose of pentobarbital sodium and exsanguinated by cardiac puncture; BAL and lung tissue samples were obtained.

Fluorescence-activated cell sorting analysis

Immunofluorescent staining of lymphocytes from rat cervical lymph nodes was performed to quantify the effectiveness of OX-8 in depleting CD8⁺ T cells.

Lymphocytes were obtained by disrupting the isolated lymph node tissues between slide glasses and were placed in PBS. Cells in PBS were filtered through a 100- μ m nylon mesh (Cell Strainer; BD Falcon, Billerica, MA) and centrifuged. Subsequently, cell pellets were incubated for 10 min in 3 ml NH₄Cl to remove red blood cells. The lymphocytes were incubated with phycoerythrin (PE)-Cy5 mouse anti-rat CD8 α mAb (CL004TC; CEDARLANE) in 5% naïve rat serum in PBS. The PE-Cy5 mouse immunoglobulin G1 isotype control (CLCMG106; CEDARLANE) was used as the control antibody. The cells were fixed with 1% paraformaldehyde. Each sample was counted by fluorescence-activated cell sorting using a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA).

Bronchoalveolar lavage and cell counting

On day 29, lavage of the right lung was performed with three volumes of 4 ml PBS via the endotracheal tube. Cytospin slides of BAL were prepared with 6×10^4 cells/slide (Cytospin2; Shandon Inc., Pittsburgh, PA) and stained with Diff-Quick (International Reagents Corp., Kobe, Japan). Total BAL cell numbers were counted and cells were identified as neutrophils, eosinophils, lymphocytes and macrophages from a total count of 300 cells. The percentage and absolute numbers of each cell type were calculated.

Histological examination

Tissue samples removed from the right lungs were fixed with an intrabronchial perfusion of 10% neutral buffered formalin at a pressure of 25 cm H₂O at room temperature for 24 hr and embedded in paraffin. Sections (4 μ m) were stained with haematoxylin & eosin for histological assessment. Additionally, periodic acid–Schiff (PAS) staining was performed to identify mucus production in epithelial cells and the number of positive cells per unit length of basement membrane perimeter (P_{BM}) was determined. Airways were classified into two categories based on the length of P_{BM} , small ($P_{BM} < 2.0$ mm) and large ($P_{BM} \geq 2.0$ mm).

Immunohistochemistry for α -smooth muscle actin

ASM in the airway was detected by α -smooth muscle actin (α -SMA) immunohistochemistry. Sections were deparaffinized with xylene and rehydrated in a series of ethanol solutions. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Non-specific binding was blocked with 20% normal horse serum (S-2000; Vector Laboratories, Burlingame, CA) in 2% bovine serum albumin

in Tris-buffered saline (TBS). Sections were then incubated at 37° for 60 min with primary antibody: monoclonal anti- α -SMA antibody (1 : 2000, Clone 1A4; Sigma-Aldrich), followed by two washes with TBS. The secondary antibody was biotinylated horse anti-mouse immunoglobulin G (1 : 300, BA-2000; Vector Laboratories) and incubated at room temperature for 45 min. After washing in TBS, incubation with a peroxidase-linked avidin–biotin complex solution (Vectastain Elite ABC Standard Kit, PK-6100; Vector Laboratories) was performed according to the manufacturer's protocol. Diluent for antibodies was 2% bovine serum albumin in TBS. Visualization was achieved by applying 3,3'-diaminobenzidine tetrahydrochloride (DAB-4HCl) (Histofine Simple Stain DAB Solution; Nichirei Corp., Tokyo, Japan). Sections were counterstained with Mayer's haematoxylin, dehydrated in graded ethanols and xylene, and mounted with coverslips. The amount of ASM was measured as α -SMA-positive area using image analysis software (MICROANALYZER; Nihon Poladigital Corp., Tokyo, Japan). The ASM mass was evaluated as the area divided by the square of P_{BM} .

Cell proliferation analysis in airway epithelium and ASM

The proliferating cell nuclear antigen (PCNA) immunohistochemistry of paraffin sections was performed to detect cell proliferation. To detect PCNA-positive epithelial cells, autoclave unmasking in antigen retrieval solution (H3300; Vector Laboratories) was performed and sections were blocked with horse serum, followed by the incubation with anti-PCNA mouse mAb (Ab-1) (Cat.NA03; Oncogene, Cambridge, MA) at 37° for 60 min. The procedures after that were the same as in the α -SMA staining. Furthermore, the double immunostaining of α -SMA and PCNA was performed to detect cell proliferation in ASM. The procedure was based on that described by Ramos-Barbon *et al.*²⁷ with a slight modification. First, PCNA immunohistochemistry was performed using an anti-PCNA mouse mAb, secondary antibody (biotinylated horse anti-mouse immunoglobulin G) and an alkaline phosphatase linked avidin–biotin complex solution (Vectastain ABC-AP Standard Kit, AK-5000; Vector Laboratories) as well as a visualizing agent, BCIP/NBT chromogen substrate (SK5400; Vector Laboratories). The sections were then rinsed in tap water and subsequently α -SMA immunohistochemistry was performed as described above. The number of PCNA-positive cells in the epithelium was counted and expressed per unit length of P_{BM} , and that in ASM was expressed per the square of P_{BM} .

Semiquantitative reverse transcription–polymerase chain reaction of cytokine mRNA expression in lung tissues

Tissue samples removed from the left lung were sliced and frozen immediately in liquid nitrogen, and stored at –80°.

Frozen tissue samples were ground in a liquid nitrogen-cooled mortar and total RNA was extracted from the lung tissue using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Concentration of RNA was measured using a spectrophotometer (DU530; Beckman, Fullerton, CA). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). For the RT-PCR 0.5 µg of total RNA was used with a TITANIUM One-Step RT-PCR Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The PCR primers were designed using online utilities from the National Center for Biotechnology Information database and the PRIMER3 program. Cytokine mRNA expression was determined by the size of the PCR product in 2.0% agarose gels, stained with ethidium bromide under ultraviolet light. The band density of IL-4, IL-5, IL-13, interferon- γ (IFN- γ) and transforming growth factor- β_1 (TGF- β_1) was measured by densitometry using the SCION IMAGE software (version beta 4.0.2; Scion Corporation, Frederick, MD, USA) and expressed as a ratio with respect to the expression of the housekeeping gene, cyclophilin A. Optimal PCR conditions, including the annealing temperature and the number of cycles, were determined for each cytokine. Cycles of amplification were adjusted to stay within the linear range of amplification for quantification.

Statistical analysis

Data are expressed as the mean $\pm$ SE. Comparisons among groups were analysed using the Kruskal–Wallis test. When appropriate, a non-parametric *post hoc* multiple comparison test (Steel–Dwass test) was used to evaluate differences between the groups. *P*-values less than 0.05 were considered to be significant.

Results

Efficacy of CD8 cell depletion by OX-8

We have previously shown that a single administration of 250 µg OX-8 almost completely depletes both

CD8 $\alpha\alpha^+$ and CD8 $\alpha\beta^+$ T cells in rat cervical lymph nodes after 24 hr.¹⁹ In this study we examined the duration of effective depletion and we verified that CD8 $^+$ T cells from rat cervical lymph nodes given 250 µg OX-8 were depleted by over 90% during the period from 24 hr to 7 days after antibody treatment. We therefore concluded that an administration of OX-8 every 6 days was enough to effectively deplete CD8 $^+$ T cells continuously.

Effects of OX-8 on BAL cells

The number of total cells in the OVA/OX-8 group was significantly greater than that in the PBS/Cont group ($P < 0.05$) and the number of macrophages in the OVA/OX-8 was significantly increased over that in the PBS/Cont group ($P < 0.05$). The number of eosinophils was significantly increased in the OVA/Cont and OVA/OX-8 groups compared with that in the PBS/Cont group ($P < 0.05$ and $P < 0.05$, respectively). There were no significant differences in the total or differential cell counts between the OVA/Cont and OVA/OX-8 groups (Table 1).

The effects of OX-8 on goblet cell numbers

For measurement of the number of PAS-positive epithelial cells, a mean of 26 airways per rat was studied. There was no significant difference in the number of airways analysed among the three experimental groups (21.7 ± 1.2 versus 28.1 ± 2.3 versus 26.9 ± 1.9). Compared to the PBS/Cont group, the numbers of PAS-positive epithelial cells in OVA/Cont and OVA/OX-8 groups were significantly greater in both small airways ($1.57 \pm 0.51/\text{mm } P_{\text{BM}}$ versus 2.17 ± 0.43 versus 3.04 ± 0.78 , $P < 0.01$, $P < 0.01$, respectively) and in large airways (4.41 ± 1.60 versus 9.05 ± 1.31 versus 15.4 ± 1.52 , $P < 0.01$, $P < 0.01$, respectively). In addition, the difference between the OVA/Cont and the OVA/OX-8 groups was also significant only in large airways (9.05 ± 1.31 versus 15.4 ± 1.52 , $P < 0.05$) (Figs 1 and 4a–c).

Group	Total cells	Macrophages	Neutrophils	Eosinophils	Lymphocytes
PBS/Cont ($n = 7$)	4.71 ± 0.96	4.42 ± 0.91	2.18 ± 0.76	16.9 ± 15.0	10.1 ± 4.05
OVA/Cont ($n = 7$)	8.02 ± 1.20	6.87 ± 1.01	5.31 ± 1.65	91.6 ± 36.8^1	17.7 ± 6.19
OVA/OX-8 ($n = 8$)	10.30 ± 1.73^1	9.46 ± 1.50^1	5.61 ± 1.44	59.3 ± 21.8^1	14.5 ± 5.41

Units: $\times 10^5$ cells in total cells and macrophages, $\times 10^3$ cells in neutrophils, eosinophils and lymphocytes.

PBS/Cont, phosphate-buffered saline-challenged sham-depleted group; OVA/Cont, ovalbumin-challenged sham-depleted group; OVA/OX-8, OVA-challenged CD8 $^+$ T-cell-depleted group. Data are expressed as average $\pm$ SE.

¹ $P < 0.05$ versus PBS/Cont.

Table 1. Total cell count and cell profile in bronchoalveolar lavage cells

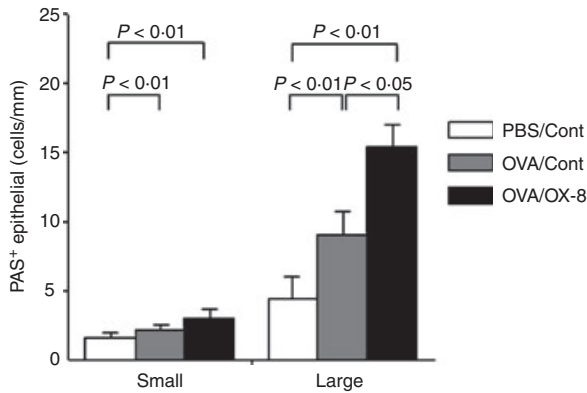


Figure 1. Morphometric quantification of mucus-containing cells in epithelium by periodic acid–Schiff staining. The number of positive cells per unit length of the basement membrane perimeter (P_{BM}) in small ($P_{BM} < 2.0$ mm) and large ($P_{BM} \geq 2.0$ mm) airways in each of three experimental groups is shown. Data are expressed as the number of positive cells per millimetre and error bars indicate SE.

Effects of OX-8 on allergen-induced DNA synthesis in airway epithelial cells

To measure the number of PCNA-positive epithelial cells, a mean of 16 airways per rat was studied. Compared to the PBS/Cont group, the numbers of PCNA-positive epithelial cells in the OVA/Cont and the OVA/OX-8 groups were significantly greater in both small airways ($4.05 \pm 0.38/\text{mm}$ P_{BM} versus 6.04 ± 0.44 versus 7.63 ± 0.74 , $P < 0.05$, $P < 0.01$, respectively) and large airways (3.44 ± 0.41 versus 6.09 ± 0.74 versus 9.13 ± 0.74 , $P < 0.05$, $P < 0.01$, respectively). Moreover, the depletion of CD8⁺ T cells resulted in a significant increase compared to the OVA/Cont group in large airways (6.09 ± 0.74 versus 9.13 ± 0.74 , $P < 0.05$) (Figs 2 and 4d–f).

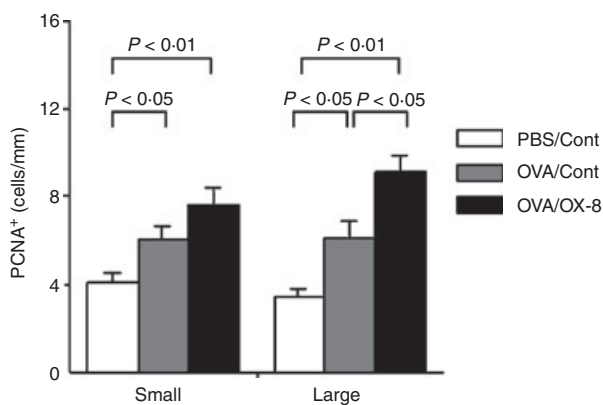


Figure 2. Morphometric quantification of proliferating cell nuclear antigen (PCNA)-positive proliferating epithelial cells by PCNA immunohistochemistry. The number of positive cells per unit length of the basement membrane perimeter (P_{BM}) in small ($P_{BM} < 2.0$ mm) and large ($P_{BM} \geq 2.0$ mm) airways in each of three experimental groups is shown. Data are expressed as the number of positive cells per millimetre and error bars indicate SE.

The effects of OX-8 on allergen-induced increase in ASM

For measurement of ASM mass, a mean of 10 airways per rat was studied. In small airways, the amount of ASM was significantly increased, not in the OVA/Cont group but in the OVA/OX-8 group compared to that in the PBS/Cont group (PBS/Cont; 1.52 ± 0.11 versus OVA/OX-8; $3.50 \pm 0.35 \times 10^{-3}$, $P < 0.01$). In large airways, on the other hand, there were significant differences in the amount of ASM between the PBS/Cont group and the OVA/Cont group (1.18 ± 0.12 versus $1.61 \pm 0.12 \times 10^{-3}$, $P < 0.05$) and the OVA/OX-8 group showed a significant increase over that in the OVA/Cont group (2.21 ± 0.16 versus $1.61 \pm 0.12 \times 10^{-3}$, $P < 0.05$) (Figs 3a and 4g–i).

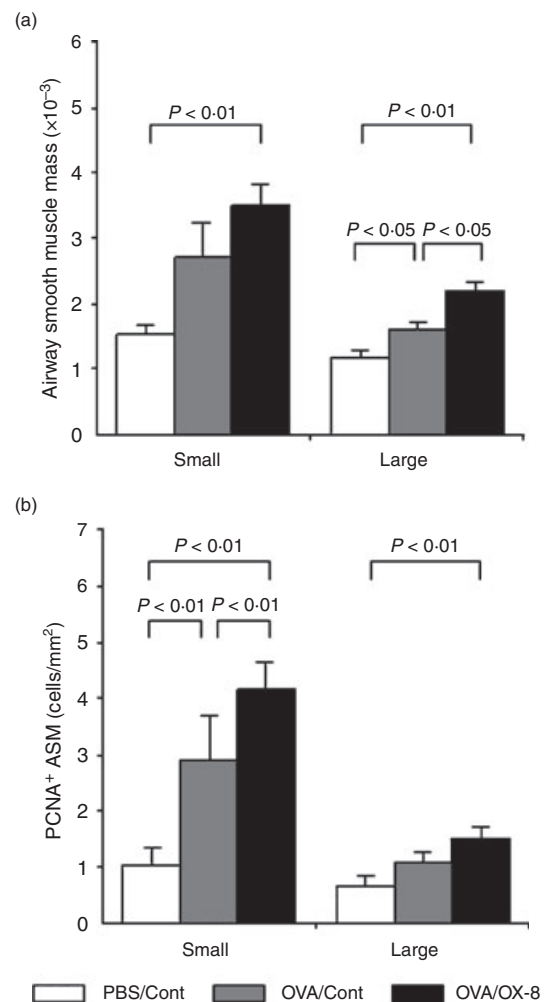


Figure 3. (a) Morphometric quantification of airway smooth muscle (ASM) mass. Data are represented as values corrected for airway size and are dimensionless units ($\times 10^{-3}$) and error bars indicate SE. Airways are classified into two categories, small [basement membrane perimeter (P_{BM}) < 2.0 mm] and large ($P_{BM} \geq 2.0$ mm). (b) Morphometric quantification of proliferating cell nuclear antigen (PCNA)-positive ASM cells. Data are expressed as the number of positive cells per P_{BM} (mm)² and error bars indicate SE.

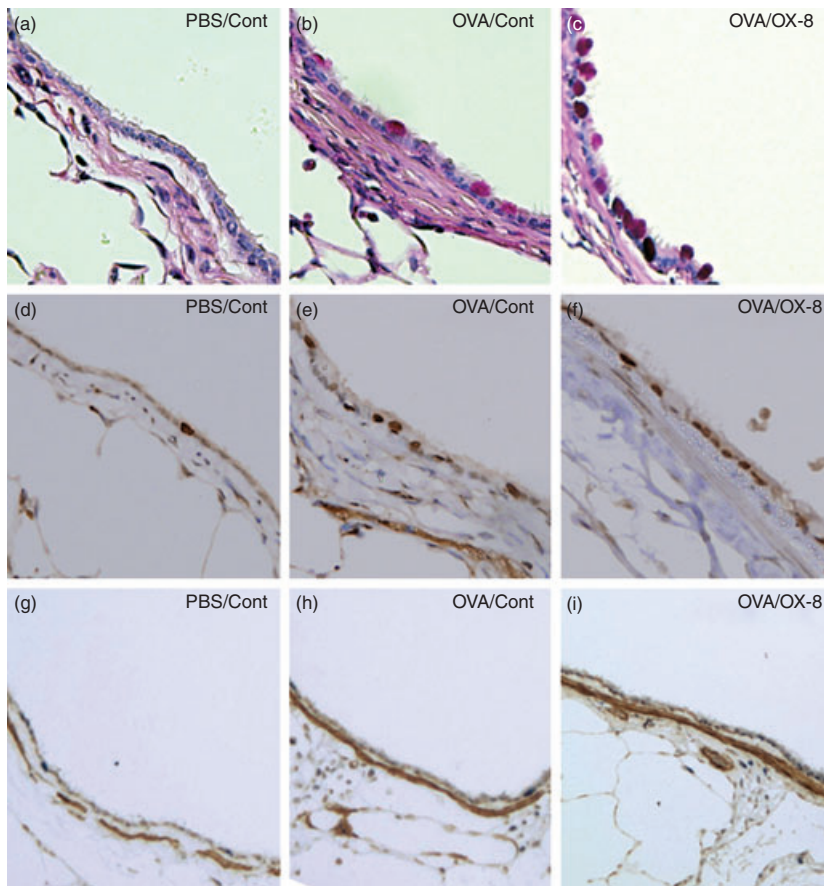


Figure 4. Histological sections of airways from rats chronically challenged with phosphate-buffered saline (PBS), treated with control ascites (PBS/Cont group), rats chronically challenged with ovalbumin (OVA), treated with control ascites (OVA/Cont group) and rats chronically challenged with OVA, treated with OX-8 monoclonal antibody (OVA/OX-8 group). Periodic acid-Schiff staining for the detection of mucus-containing cells (a–c), proliferating cell nuclear antigen immunohistochemistry for the detection of proliferating epithelial cells (d–f), and α -smooth muscle actin immunohistochemistry for the detection of airway smooth muscle (g–i) in rat airways.

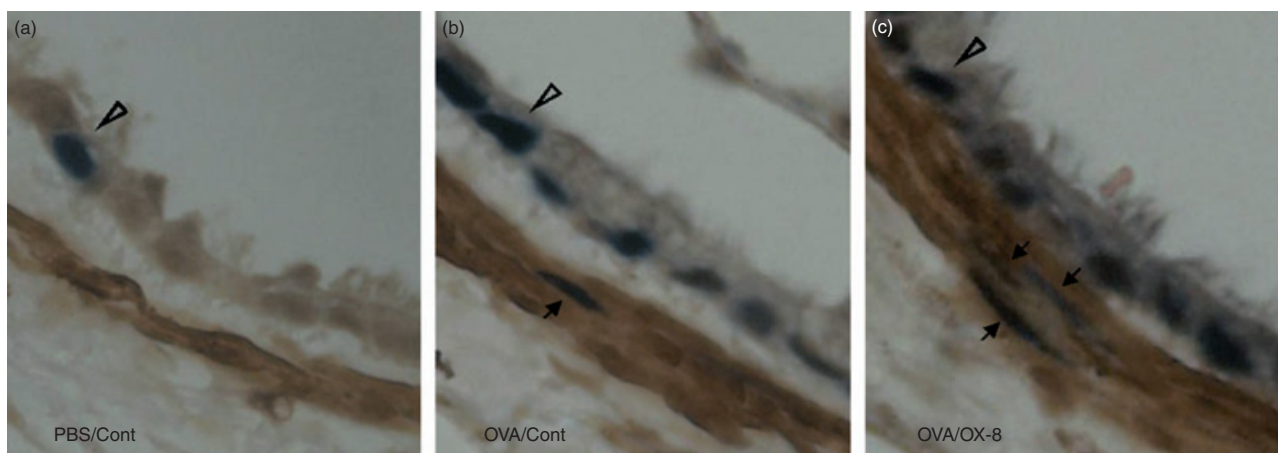


Figure 5. Detection of proliferating airway smooth muscle (ASM) cells by double immunostaining of α -smooth muscle actin (α -SMA) and proliferating cell nuclear antigen (PCNA). ASM area was detected as α -SMA-positive brown coloured signal and PCNA-positive ASM cells were detected as dark-blue-coloured signal (arrows). PCNA-positive cells were also identified in the airway epithelium (open arrowheads).

DNA synthesis in ASM cells

To measure the number of PCNA-positive ASM cells, a mean of 10 airways per rat was studied. In small airways, there were significant differences in the number of PCNA-positive ASM cells between the PBS/Cont group and the

OVA/Cont group (1.04 ± 0.35 versus 2.92 ± 0.74 , $P < 0.01$) and furthermore the OVA/OX-8 group showed a significant increase over that in the OVA/Cont group (4.17 ± 0.50 versus 2.92 ± 0.74 , $P < 0.01$). In large airways, however, the number of PCNA-positive ASM cells was significantly increased not in the OVA/Cont group but

in the OVA/OX-8 group as compared to that in the PBS/Cont group (PBS/Cont; 0.67 ± 0.14 versus OVA/OX-8; 1.49 ± 0.21 , $P < 0.01$) (Figs 3b and 5).

Cytokine expression in lung tissues

Using RT-PCR, we examined mRNA expression for IL-4, IL-5, IL-13, IFN- γ and TGF- β_1 in rat lung tissues from the three groups. Compared to the PBS/Cont group, the expression of IFN- γ was significantly higher in the OVA/Cont group and in the OVA/OX-8 group (0.012 ± 0.005 versus 0.074 ± 0.023 versus 0.222 ± 0.053 , $P < 0.05$, $P < 0.01$, respectively). CD8 depletion had no significant effect on IFN- γ expression between the OVA/Cont group and the OVA/OX-8 group. There were no significant differences in mRNA expression for IL-4, IL-5, IL-13 and TGF- β_1 among the three groups (Table 2).

Discussion

In the present study, depletion of CD8⁺ T cells enhanced DNA synthesis and mucus production in epithelial cells and increased the ASM mass evoked by repeated OVA inhalations, especially in large airways, in the Brown Norway rat model of asthma. This depletion had no obvious effects on the cellular composition of BAL. These results indicate that resident CD8⁺ T cells have an inhibitory effect on these measures of airway remodelling in this rat model of asthma.

Increased DNA synthesis in epithelial cells has been found in animal models of chronic allergen exposure.^{24,25} There are studies demonstrating increased epithelial DNA synthesis predominantly in large airways²⁵ and also diffusely throughout the airways.²⁴ Endothelin-1, IL-1 β , tachykinin or tryptase have been thought to have effects on epithelial cell proliferation in airways. Our results support a previous report in which the number of epithelial cells increased and the epithelium showed high levels of the antiapoptotic marker Bcl-2 and of the marker of proliferation PCNA²⁹ in the airways of patients with asthma.

It has been proposed that there are two phenotypes of increase in ASM mass in asthmatic patients. The first phenotype demonstrates hyperplasia predominantly in the larger airways unaccompanied by hypertrophy. The second shows mild hyperplasia in the larger airways and mainly shows hypertrophy distributed throughout the airways, which is particularly noteworthy in the small peripheral airways.⁹ Likewise, in experimental models, several investigators showed that the increase of ASM amount following sensitization and repeated allergen exposures was observed predominantly in larger airways^{24,30} and others reported that this finding was seen throughout the airway tree from small to large airways.^{23,27} Our findings are consistent with the former observation, showing the predominant localization of the structural changes to the larger airways. The reason for this phenomenon may be ascribed to a preferential deposition of aerosolized antigen in the central airways or may be the result of the difference of ASM phenotype between large and small airways.

There are differences in the reported patterns of ASM cell proliferation in relationship to airway size. A dominance of DNA proliferation of ASM cells in larger airways²⁵ and a more diffuse pattern of response involving large and small airways have been described.²⁴ In the current study, we detected increased DNA synthesis in ASM cells mainly in the small airways, although increased ASM mass was demonstrated predominantly in the large airways. The explanation for the greater sensitivity of the proliferation marker of the ASM in small airways compared to large airways in our experimental model is not clear but it seems probable that differences in rates of apoptosis account for the observed discrepancies between PCNA-positive cells and ASM mass. Proliferation of ASM has been attributed to various stimulatory or inhibitory mediators of ASM cell proliferation, including tumour necrosis factor- α , TGF- β_1 , IL-1, IL-6, platelet-derived growth factor; epidermal growth factor, insulin-like growth factor, histamine, endothelin-1, tryptase, activin A and cysteinyl leukotrienes.^{31,32} In addition, the direct interaction of T cells with ASM cells is also proposed as

Table 2. Messenger RNA expression of cytokines from rat lung tissues

Group	IL-4	IL-5	IL-13	IFN- γ	TGF- β_1
PBS/Cont ($n = 7$)	0.15 ± 0.04	0.28 ± 0.09	0.25 ± 0.10	0.012 ± 0.005	0.60 ± 0.12
OVA/Cont ($n = 7$)	0.27 ± 0.05	0.41 ± 0.09	0.30 ± 0.10	0.074 ± 0.023^1	1.15 ± 0.43
OVA/OX-8 ($n = 8$)	0.22 ± 0.04	0.30 ± 0.06	0.41 ± 0.14	0.222 ± 0.053^2	1.14 ± 0.28

Results are evaluated as the ratio to cyclophilin A expression and data are expressed as average $\pm$ SE.

PBS/Cont, phosphate-buffered saline-challenged sham-depleted group; OVA/Cont, ovalbumin-challenged sham-depleted group; OVA/OX-8, OVA-challenged CD8⁺ T-cell-depleted group; IL-4, interleukin-4; IFN- γ , interferon- γ ; TGF- β_1 , transforming growth factor- β_1 .

¹ $P < 0.05$.

² $P < 0.01$ versus PBS/Cont.

one of the important mechanisms for ASM cell proliferation.²⁷ Whether some or any of these mechanisms explains the differences observed in different-sized airways requires further exploration.

In the Brown Norway rat model of acute asthma, adoptive transfer of antigen-primed CD4⁺ T cells induced AHR and BAL eosinophilia after antigen challenge¹⁵ and CD8⁺ T cells inhibited LAR apparently through inhibitory effects on CD4⁺ T cells. We have also reported a suppressive effect of CD8⁺ $\gamma\delta$ T cells on the LAR and airway eosinophilia through the secretion of IFN- γ .³³ If we hypothesize that CD8⁺ T cells also inhibit the function of CD4⁺ T cells in rats that are repeatedly challenged with OVA, we might expect that CD8 depletion would lead to a greater degree of activation of CD4⁺ T cells and then these CD4⁺ T cells could elicit the enhancement of airway remodelling.²⁷ In this study, however, a significant increase of IFN- γ expression in both the OVA/Cont and OVA/OX-8 groups compared to the PBS/Cont group was observed and there was a trend towards higher IFN- γ levels in the OVA/OX-8 group compared to the OVA/Cont group, although this was not significant. These results were the opposite of previous reports which described IFN- γ inhibition by CD8 depletion in an acute asthma model.^{19,34} The inhibitory effect of CD8⁺ T cells on IFN- γ in this study is unknown. In our protocol, BAL and lung tissue samples were obtained 5 days after the last OVA challenge because the findings of remodelling were apparent at this time-point. Therefore the number of total cells and eosinophils in BAL and the expression of the Th2 cytokines in lung tissues were not very high in the OVA/Cont group, which might have made the effect of OX-8 unclear.

Other mechanisms for the inhibitory effects on CD4⁺ T cells by CD8⁺ T cells have been demonstrated in previous studies. CD8⁺ T cells could induce T-cell receptor V β -specific suppression of CD4⁺ T cells expressing Qa-1-peptide ligands³⁵ or Fas-mediated apoptosis of CD4⁺ T cells.³⁶

However, the role of CD8⁺ T cells is controversial because other studies have demonstrated that CD8⁺ T cells have proinflammatory effects in a murine asthma model.²⁰ Indeed, bronchial CD8⁺ T-cell infiltration was correlated with the annual decline in post-bronchodilator measurements of forced expiratory volume in 1 second in patients with asthma.³⁷ Leigh *et al.* evaluated the effect of the simultaneous depletion of CD4⁺ and CD8⁺ T cells after the formation of airway remodelling by chronic allergen exposures in BALB/c mice.³⁸ They showed that the depletion of both CD4⁺ and CD8⁺ T cells had no effect on the degree of airway remodelling once it was established.

There is a hypothetical possibility that OX-8 treatment might have affected macrophages or dendritic cells (DCs) in our model because these cells in rats have been

reported to express the CD8 α molecule.^{39,40} This study showed no significant differences in the number of macrophages in BAL between the OVA/Cont and the OVA/OX-8 groups, suggesting that a depletion of CD8 α ⁺ macrophages was not a significant factor and did not affect the macrophage population. We have shown in a previous report that the CD8⁺ T-cell depletion was induced through both complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity, but macrophages were not influenced by OX-8.¹⁹ Furthermore, the possibility that the function of DCs could be influenced by OX-8 seems to be unlikely because CD8 α ⁻ DCs mainly induce Th2 responses to OVA challenge compared to CD8 α ⁺ DCs.⁴¹ However, we cannot exclude the possibility that macrophage function was altered in some as yet undefined way or that the capacity of CD8 α ⁺ DCs to induce Th1 polarization⁴¹ was restrained by OX-8. As natural killer (NK) cells and natural killer T (NKT) cells also express CD8 α ,^{42,43} it is possible that OX-8 could inhibit the function of these cells. Most of the NK cells are CD8⁺ cells and it has been reported that depletion of NK1.1⁺ cells inhibits pulmonary eosinophil and T-cell infiltration in a murine model of asthma.⁴⁴ A small subset of invariant NKT cells expresses CD8 α in humans and Th2 cytokines from invariant NKT cells are required for the development of allergen-induced AHR in mice.⁴⁵ Based on these findings it seems unlikely that the potential suppression of NK and NKT cells by OX-8 could contribute to the enhancement of airway remodelling.

In conclusion, we have demonstrated for the first time that CD8⁺ T cells have inhibitory effects on Brown Norway rat airway remodelling as assessed by ASM mass, epithelial cell proliferation and mucus production. CD8 depletion had no significant effect on mRNA expression of IFN- γ in rat lung tissues. The inhibitory mechanism of resident CD8⁺ T cells in this model is unknown. The effects were most marked in the large airways corresponding to the pattern of remodelling seen in severe human chronic asthma. The mRNA expression of cytokines in lung tissues of this model showed no particular pattern of alteration. Further investigation will be needed to elucidate the precise mechanism of CD8⁺ T-cell participation in airway remodelling.

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