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T-cell-mediated inflammation does not contribute to the maintenance of airway dysfunction in mice

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Leigh, Richard, David S. Southam, Russ Ellis, Jennifer N. Wattie, Roma Sehmi, Yonghong Wan, and Mark D. Inman. T-cell-mediated inflammation does not contribute to the maintenance of airway dysfunction in mice. *J Appl Physiol* 97: 2258–2265, 2004. First published July 30, 2004; doi:10.1152/jappphysiol.00597.2004.—T-cell-mediated airway inflammation is considered to be critical in the pathogenesis of airway hyperresponsiveness (AHR). We have described a mouse model in which chronic allergen exposure results in sustained AHR and aspects of airway remodeling and here sought to determine whether eliminating CD4⁺ and CD8⁺ cells, at a time when airway remodeling had occurred, would attenuate this sustained AHR. Sensitized BALB/c mice were subjected to either brief or chronic periods of allergen exposure and studied 24 h after brief or 4 wk after chronic allergen exposure. In both models, mice received three treatments with anti-CD4 and -CD8 monoclonal antibodies during the 10 days before outcome measurements. Outcomes included in vivo airway responsiveness to intravenous methacholine, CD4⁺ and CD8⁺ cell counts of lung and spleen using flow cytometric analysis, and airway morphometry using a computer-based image analysis system. Compared with saline control mice, brief allergen challenge resulted in AHR, which was eliminated by antibody treatment. Chronic allergen challenge resulted in sustained AHR and indexes of airway remodeling. This sustained AHR was not reversed by antibody treatment, even though CD4⁺ and CD8⁺ cells were absent in lung and spleen. These results indicate that T-cell-mediated inflammation is critical for development of AHR associated with brief allergen exposure, but is not necessary to maintain sustained AHR.

allergy; lung; T lymphocytes; rodent; bronchial hyperreactivity

THE MECHANISMS UNDERLYING airway hyperresponsiveness (AHR) in asthma are complex and likely to be multifactorial (39). There is, however, considerable evidence to support a role for T-cell-mediated airway inflammation in the pathogenesis of AHR (6, 11, 13, 19, 42, 48, 52), and several T helper type 2 (Th2) cytokines, particularly IL-13, have the potential to modulate AHR in animal models of allergic airway inflammation and AHR (7, 12, 18, 21–23, 27, 38, 53, 55). Although these experimental models have greatly increased our understanding of the mechanisms underlying transient responses to inhaled allergen, they have all employed models of acute allergic airway inflammation that have centered on short-term exposure of sensitized rodents to allergen. Thus, despite the undoubted advances that have come from these studies, a relative limitation of these models is that the AHR is transient, disappearing 14–21 days after allergen exposure, and appears

to be related only to acute increases in inflammatory mediators. This is not equivalent to the chronic airway inflammation and sustained AHR present in individuals with asthma, and, whereas these experimental models have provided valuable information, they are unlikely to provide a complete description of the mechanisms underlying AHR.

Furthermore, the observation that profound AHR is sustained in asthma, despite prolonged treatment with anti-inflammatory corticosteroids (3, 15, 30, 44), suggests that mechanisms other than acute inflammation likely account for a major component of AHR. Evidence suggests that chronic structural changes in the airway, often termed airway remodeling, may be at least partly responsible for sustained AHR (2, 4, 5, 29, 33–35, 51, 54). These changes include thickening of the airway wall, subepithelial fibrosis, hyperplasia and hypertrophy of smooth muscle cells, and hyperplasia of fibroblasts/myofibroblasts and goblet cells (8, 10, 14, 16, 24, 25, 31, 43, 49). We have recently described a model in which AHR and aspects of airway remodeling develop in mice following chronic exposure to allergen (37). These abnormalities persist for at least 8 wk following final allergen exposure, well beyond the resolution of acute inflammatory events, and suggest that airway remodeling occurs as a consequence of allergic airway inflammation and that aspects of airway remodeling contribute independently to the ongoing, sustained airway hyperactivity.

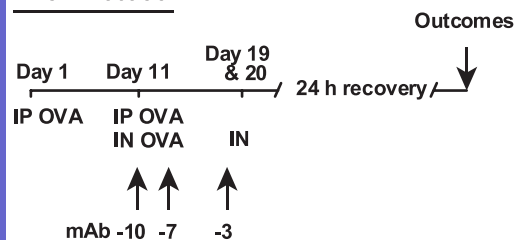
We now hypothesized that the sustained AHR present in our model is independent of T-cell-orchestrated acute inflammatory events but is instead the result of chronic structural airway remodeling. Our underlying hypothesis does not question that the initiating events that ultimately result in this remodeling include antigen-induced, T-cell-dependent acute airway inflammation but focused instead on whether the ongoing sustained dysfunction was due to the chronic structural changes rather than T-cell-dependent acute inflammatory events. The purpose of this study was, therefore, to explore whether the sustained AHR that occurs following chronic exposure to allergen is independent of ongoing T-lymphocyte responses. This observation would provide evidence that airway dysfunction in allergic airway diseases, such as asthma, may be dependent on factors other than ongoing immune responses.

To do this, we performed a series of experiments in which we initially sought to confirm what other investigators have already demonstrated, namely, that depletion of CD4⁺ and CD8⁺ T cells prevented the development of acute, T-cell-mediated airway inflammation and associated transient AHR in

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Brief Protocol



Chronic Protocol

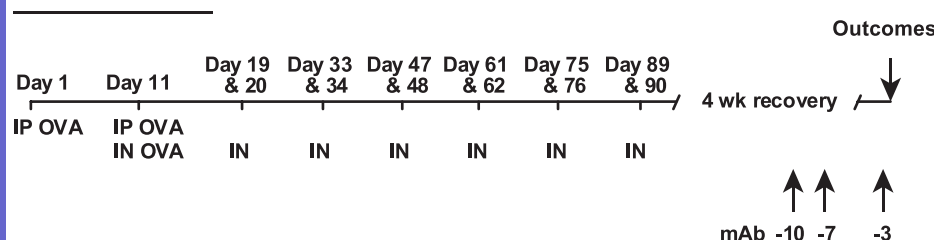


Fig. 1. Study protocols. Sensitization and challenge protocols were used in brief and chronic challenge models. Intraperitoneal (IP) sensitization with ovalbumin (OVA) was followed by intranasal (IN) challenge with OVA, while control mice received saline (SAL). Note that MAb/diluent (DIL) treatment was given 10, 7, and 3 days before outcome measurements being made in each protocol.

mice, following a period of brief allergen exposure (20). In the second series of experiments, mice were exposed to our chronic allergen-challenge protocol and then, at a later point, treated with anti-CD4⁺ and anti-CD8⁺ MAb. Consistent with our hypothesis, we expected that T-cell depletion, on a background of established airway remodeling, was unlikely to attenuate the sustained AHR observed in the model.

MATERIALS AND METHODS

Animals. Female BALB/c mice, aged 10–12 wk, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in environmentally controlled specific pathogen-free conditions for 1 wk before study and for the duration of the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University and conformed to National Institutes of Health guidelines for the experimental use of animals.

Sensitization. Mice were sensitized with intraperitoneal ovalbumin conjugated to aluminum potassium sulfate, as described by our laboratory previously (28).

Challenge and treatment intervention. Sensitized mice were subjected to either brief or chronic periods of allergen exposure, as described by our laboratory previously (37). Control mice were subjected to the same sensitization protocol but received saline challenges. Allergen-challenged and control mice were studied at 24 h after the final exposure (day 21) in the brief allergen-challenge protocol and at 4 wk after the final exposure in the chronic allergen-challenge protocol (Fig. 1). Immunodepletion studies were completed by using MAb GK1.5 (anti-CD4; ATCC, Manassas, VA) and 2.43 (anti-CD8; ATCC). Hybridoma ascites fluid (100 μ l) for each MAb was diluted in phosphate-buffered saline (total volume, 500 μ l) and administered intraperitoneally on days -10 , -7 , and -3 before the outcome day in each protocol (Fig. 1). Flow cytometry confirmed that the depletion of CD4⁺ and CD8⁺ T cells was consistently $>98\%$. The following outcome measurements were made: 1) *in vivo* airway responsiveness to intravenous methacholine (MCh); 2) total and differential cell counts in bronchoalveolar lavage (BAL) fluid; 3) CD4⁺ and CD8⁺ cell counts using fluorescence-activated cell sorter (FACS) analysis; and 4) airway morphometry using a computer-based image analysis system. Separate groups of 10 mice were studied in each treatment arm of each protocol.

Airway responsiveness. The underlying contributors to airway responsiveness, namely airway reactivity [slope of increase in total

respiratory system resistance (Rrs) for a given increase in MCh dose], airway sensitivity (lowest dose of MCh to produce bronchoconstriction), and the maximum inducible bronchoconstriction (maximum Rrs) were measured following brief or chronic allergen challenge. These components of airway responsiveness were measured based on the response of Rrs to saline and increasing (10, 33, 100, and 330 μ g/kg) intravenous doses of MCh (Fig. 2). Rrs was measured by using the flow interrupter technique, modified for use in mice, and described in detail elsewhere (28, 37, 50).

BAL. Following airway physiology measurements, BAL was performed as described by our laboratory previously (28). Differential cell counts were performed on 400 cells, counted by one investigator blinded to the experimental conditions. Cells were classified, based on morphological criteria, as macrophages, mononuclear cells, neutrophils, or eosinophils.

FACS analysis. FACS analysis was performed on cells extracted from lung tissue by enzymatic digestion as described by our laboratory previously (56). The viability of the extracted lung cells was $>95\%$. A total of 1×10^6 cells underlaid with purified anti-mouse CD16/CD32 MAb were incubated for 30 min with saturating amounts of fluorescein isothiocyanate-anti-mouse CD4 MAb, CyChrome-anti-mouse CD8a MAb, and phycoerythrin-anti-mouse CD90.2 MAb. Analysis was performed on a Becton Dickinson (Franklin Lakes, NJ) FACScan flow cytometer, by using Cellquest and WinMDi software

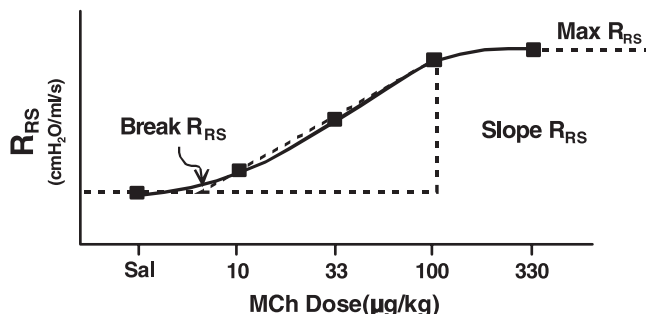


Fig. 2. Airway responsiveness methods. Total respiratory system resistance (Rrs) was measured in response to increasing doses of intravenous methacholine (MCh). Using the resulting Rrs-MCh dose-response curve, indexes of airway reactivity (Slope Rrs), airway sensitivity, or the lowest dose to produce bronchoconstriction (Break Rrs) and maximal degree of bronchoconstriction (Max Rrs) were measured.

Table 1. Total and eosinophil cell counts in BAL fluid following brief or chronic exposure to saline or ovalbumin

	Brief Challenge			Chronic Challenge		
	SAL/DIL	OVA/DIL	OVA/MAb	SAL/DIL	OVA/DIL	OVA/MAb
Total cell counts ($\times 10^4/\text{ml}$)	17.0	106.8*	54.1†	21.9	38.7	30.7
Eosinophils ($\times 10^4/\text{ml}$)	0.1	70.2*	19.8†	0.0	0.1	0.0

Mice were either treated with diluent (DIL) or anti-CD4/anti-CD8 MAb. SAL, saline; OVA, ovalbumin. * $P < 0.001$ compared with OVA-challenged/DIL-treated mice. † $P < 0.001$ compared with SAL-challenged/DIL-treated mice.

packages (BD Biosciences, Oakville, ON). CD90.2⁺CD4⁺ (CD4⁺) and CD90.2⁺CD8⁺ (CD8⁺) stained cells were identified by detection of FL-1 (fluorescein isothiocyanate), FL-2 (phycoerythrin), and FL-3 (CyChrome) and expressed as percentages of T cells (all antibodies: Pharmingen, Mississauga, ON). The numbers of positive cells were calculated by multiplying total cell count obtained after lung digestion.

Lung histology and morphometry. The lungs were dissected and processed as described by our laboratory in detail previously (37). Three-micrometer-thick transverse sections were cut and assessed with the following stains: hematoxylin and eosin for the presence of eosinophils; picrosirius red to demonstrate the presence of collagen, and periodic acid Schiff to demonstrate the presence of mucin within goblet cells. Additional sections were prepared for immunohistochemistry by using a MAb (clone $\alpha\text{sm-1}$, Novacastra Laboratories, Newcastle upon Tyne, UK) against α -smooth muscle actin to identify contractile elements. Morphometric quantification of the stained lung sections was performed by using a customized digital image analysis system (Northern Eclipse, Empix Imaging, Mississauga, ON), as described by our laboratory previously (17, 37).

Statistical analysis. Reported values are expressed as means $\pm$ SE. Comparisons with respect to airway reactivity (slope of the Rrs-log-transformed MCh dose-response curve), maximal bronchoconstriction (maximal MCh-induced Rrs), cell counts, and indexes of airway remodeling between saline control mice and mice receiving either brief or chronic allergen exposure, treated with either MAb or diluent, were made by using ANOVA. Post hoc multiple-comparison testing was performed by using Duncan's test to assess for significant effects. All comparisons were two-tailed, and P values < 0.05 were considered to be significant.

RESULTS

Treatment with anti-CD4 and anti-CD8 MAb during brief allergen exposure abrogates allergen-induced airway inflammation and prevents the development of AHR. Following a period of brief allergen challenge, mice treated with diluent (sham) exhibited a significant increase in total cell counts and eosinophils in BAL compared with mice exposed to saline and treated with diluent ($P < 0.001$) (Table 1). Concurrent treatment with anti-CD4/anti-CD8 MAb during the period of brief allergen challenge (Fig. 1) resulted in significant attenuation of the allergen-induced increase in total cell counts and eosinophils in lavage fluid ($P < 0.001$) (Table 1). The magnitude of BAL eosinophilia in response to brief allergen challenge and the subsequent attenuation by MAb treatment were similar to those seen in the airway tissue (data not shown).

FACS analysis of lung tissue from these allergen-challenged/diluent-treated animals demonstrated increased numbers of CD4⁺ and CD8⁺ cells compared with saline-challenged/diluent-treated control mice (Fig. 3). Treatment with anti-CD4/anti-CD8 MAb resulted in the complete elimination of CD4⁺ and CD8⁺ T cells from both lung (Fig. 3) and spleen tissue (data not shown) in these mice.

Following brief exposure to allergen, mice treated with diluent exhibited significant increases in airway reactivity ($P = 0.03$) and maximal bronchoconstriction ($P < 0.001$), compared with the saline control groups (Fig. 4). We were also able to confirm, as others have shown before (20), that anti-CD4/anti-CD8 MAb treatment during the period of brief allergen challenge completely abrogated the development of airway hyper-reactivity and the increased maximal bronchoconstriction present in allergen-challenged/diluent-treated mice (Fig. 4).

Chronic allergen exposure results in aspects of airway wall remodeling and sustained AHR, which are not attenuated by anti-CD4 and anti-CD8 MAb treatment. As expected, minimal numbers of eosinophils were observed in the BAL fluid of diluent-treated (sham) and anti-CD4/anti-CD8 MAb-treated mice 4 wk after chronic allergen exposure, and these values were not significantly different from baseline values observed in saline control mice (Table 1). CD4⁺ and CD8⁺ cells were absent from the lungs (Fig. 5) and spleens (data not shown) of mice treated with anti-CD4/anti-CD8 MAb.

There was a significant increase in the amount of mucin containing periodic acid Schiff-positive goblet cells (Figs. 6

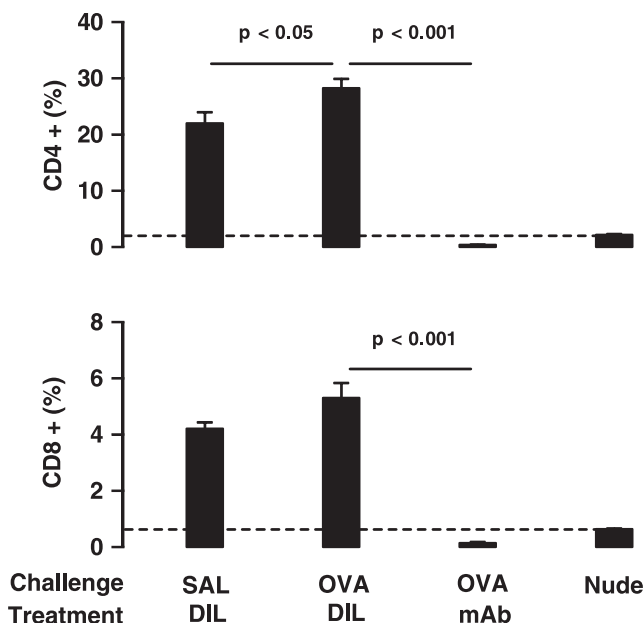


Fig. 3. Lung CD4⁺ and CD8⁺ T cells following brief exposure to SAL or allergen. Percentages of lung CD4⁺ and CD8⁺ T cells, determined by fluorescence-activated cell sorter (FACS) analysis are shown, following brief challenge with either SAL or OVA and treatment with either DIL or anti-CD4/anti-CD8 MAb. FACS analysis on lung tissues from 2 nonsensitized, unchallenged, untreated nude mice (Jackson Laboratory, Bar Harbor, ME) was performed to establish baseline levels for both CD4⁺ and CD8⁺ cells. Values are means $\pm$ SE.

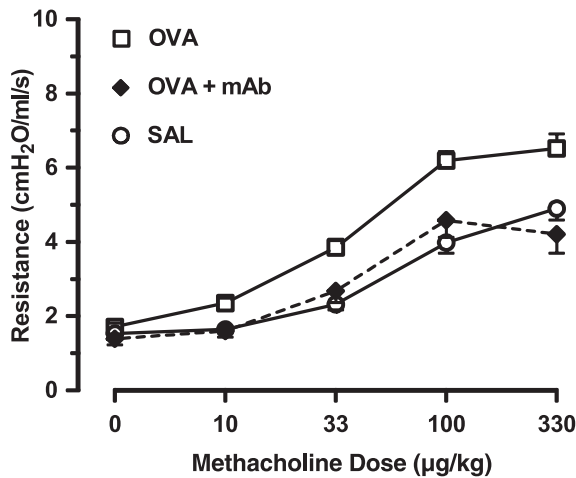


Fig. 4. Airway responses following brief exposure to SAL or allergen. Maximum airway bronchoconstriction and airway reactivity, calculated as the dose-response slope to intravenous MCh, measured 24 h following brief exposure to SAL or allergen, are shown. Values are means $\pm$ SE. Reactivity (slope of the response) of the OVA mice was greater than that of the SAL and OVA + MAb mice ($P < 0.05$), which were not different from each other ($P > 0.05$).

and 7, A and B), subepithelial collagen deposition (Figs. 6 and 7, D and E), and α -smooth muscle actin staining (Figs. 6 and 7, G and H) in the airways of the diluent-treated mice 4 wk after chronic allergen exposure, compared with saline control mice ($P < 0.01$). As in the brief challenge protocol, MAb treatment was given during the 10 days before outcome measurements were being made, but the timing of this intervention in the chronic protocol meant that treatment with MAb was only given some 3 wk after the final allergen challenge (Fig. 1), at a time when remodeling was likely to have become estab-

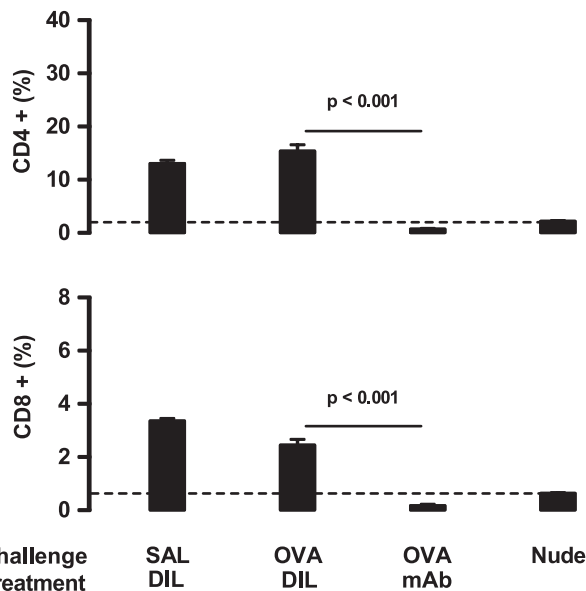


Fig. 5. Lung CD4⁺ and CD8⁺ T cells following chronic exposure to SAL or allergen. Percentages of lung CD4⁺ and CD8⁺ T cells, determined by FACS analysis are shown, following chronic challenge with either SAL or OVA and treatment with either DIL or anti-CD4/anti-CD8 MAb. FACS analysis on lung tissues from 2 nonsensitized, unchallenged, untreated nude mice (Jackson Laboratory) was performed to establish baseline levels for both CD4 and CD8 cells. Values are means $\pm$ SE.

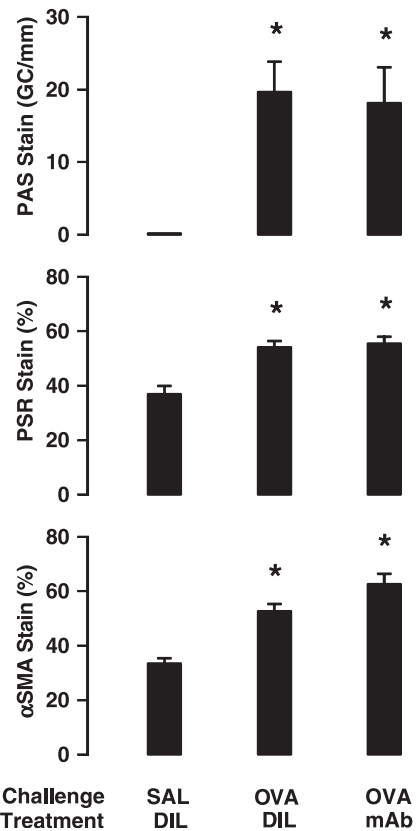


Fig. 6. Morphometric changes in airways of mice following chronic exposure to SAL or allergen. Morphometric quantification of mucin containing goblet cells (GC) [periodic acid Schiff (PAS)], collagen [picosirius red (PSR)], and contractile elements [α -smooth muscle action (α -SMA)] in the airways of mice following chronic challenge with SAL or OVA and treatment with DIL or anti-CD4/anti-CD8 MAb are shown. Data are expressed as the no. of GC staining for mucin (PAS positive) expressed per length of airway wall (GC/mm) and as the percentage of positively stained tissue in the region of interest in the PSR- and α -SMA-stained sections. Values are means $\pm$ SE. * $P < 0.05$ compared with SAL-challenged mice.

lished (37). Anti-CD4/anti-CD8 MAb treatment had no effect on the amount of mucin present in the airway epithelium (Figs. 6 and 7C), the amount of airway subepithelial collagen deposition (Figs. 6 and 7F), or in the amount of airway wall contractile tissue staining (Figs. 6 and 7I).

After chronic allergen exposure, diluent-treated mice exhibited a significant and sustained increase in airway reactivity ($P < 0.01$) and maximal bronchoconstriction ($P < 0.01$) (Fig. 8), compared with saline control mice. However, in contrast to our observations in the brief exposure model, treatment with anti-CD4/anti-CD8 MAb after chronic allergen exposure, at a time when airway remodeling was likely to have become established, had no attenuating effect on either this sustained airway hyperreactivity or the sustained increase in maximal bronchoconstriction (Fig. 8).

DISCUSSION

In this study, we have shown that anti-CD4 and anti-CD8 MAb treatment of mice during a period of brief allergen exposure resulted in attenuation of T-cell-mediated airway inflammation and prevented the associated transient AHR. Thus these observations confirm the critical role of CD4⁺ and CD8⁺ T cells in

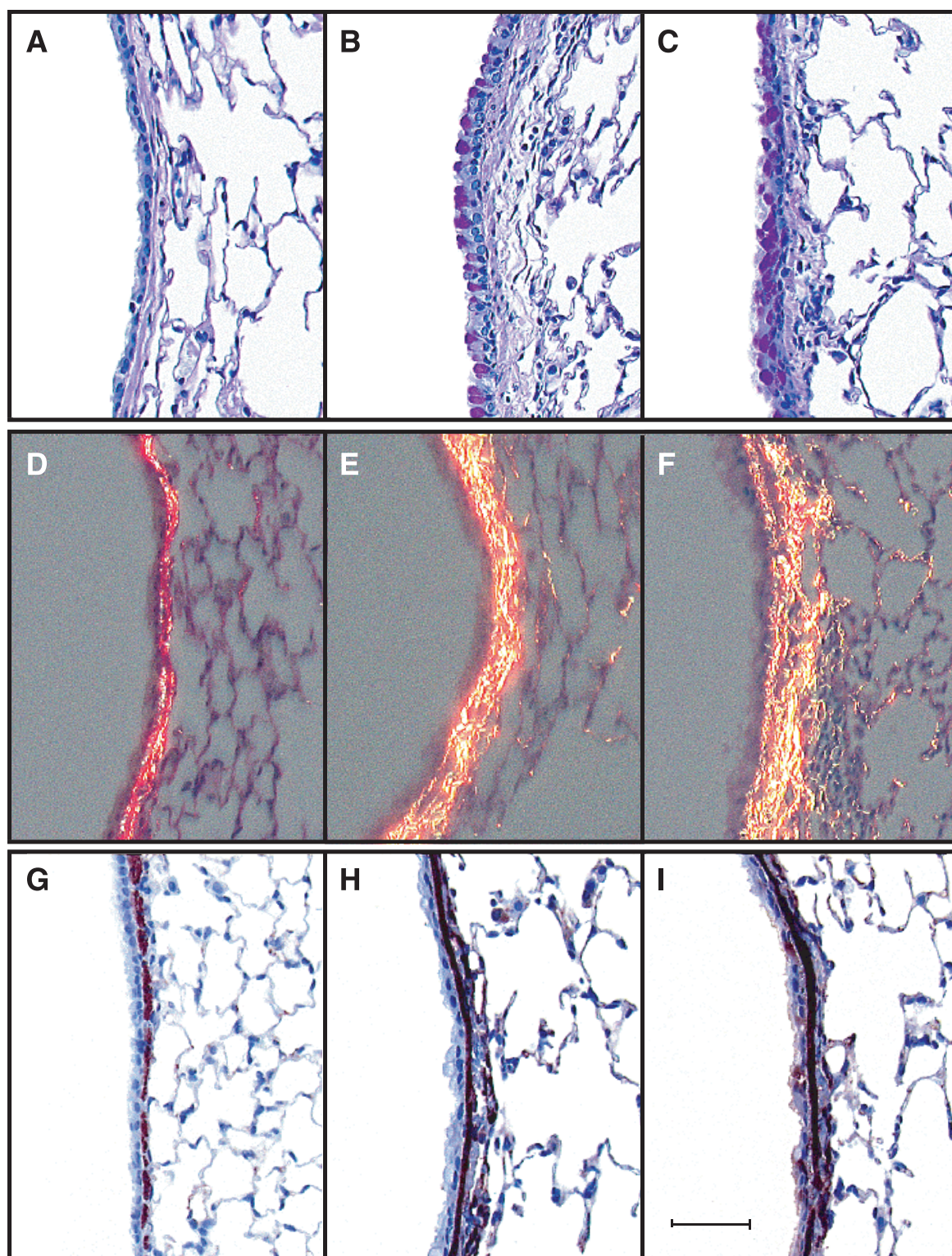


Fig. 7. Histological sections of airway wall from chronically challenged mice. Staining for PAS-positive GC (A–C), collagen deposition (PSR, viewed using polarized light microscopy) (D–F), and contractile elements (α -SMA) (G–I) in the airways of mice following chronic exposure to SAL with DIL treatment (A, D, and G), allergen with DIL treatment (B, E, and H), or allergen with MAb treatment (C, F, and I) are shown. Bars = 50 μ m.

initiating the acute inflammatory events that lead to transient AHR in this model and are consistent with other published reports, in which T-cell depletion has resulted in a reduction of the cellular inflammatory infiltrate and AHR (20, 40).

However, this is the first study to directly examine the role of CD4⁺ and CD8⁺ cells in established, sustained AHR. We have now shown that depleting CD4⁺ and CD8⁺ cells (Fig. 5), at a time when chronic allergen exposure has already resulted

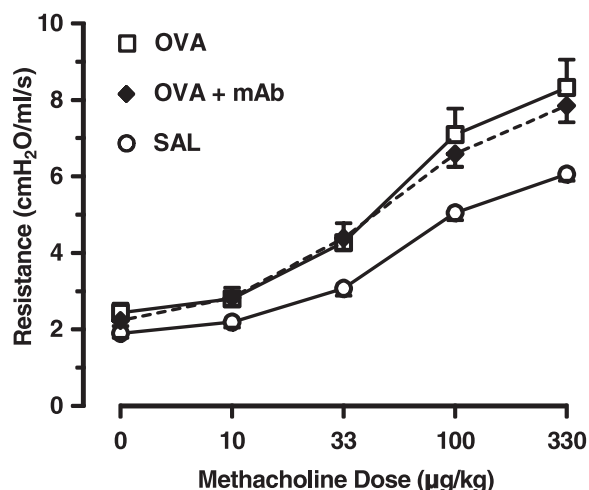


Fig. 8. Airway responses following chronic exposure to SAL or allergen. Maximum airway bronchoconstriction and airway reactivity, calculated as the dose-response slope to intravenous MCh, measured 4 wk following chronic exposure to SAL or allergen is shown. Values are means $\pm$ SE. Airway reactivity of the OVA and OVA + MAb mice was greater than that of the SAL mice ($P < 0.05$) but were not different from each other ($P > 0.05$).

in the establishment of airway remodeling (Figs. 6), does not attenuate the sustained AHR observed in the model (Fig. 8). These observations are consistent with our underlying hypothesis, namely that T-cell-mediated inflammation is not required to maintain sustained AHR, and further strengthen the concept that the sustained AHR is a consequence of airway remodeling, rather than ongoing cellular inflammation.

Although there have been other reports of animal models in which chronic allergen or fungal exposure has resulted in structural airway changes and AHR (26, 41, 45–47), our model is fundamentally different in that the sustained AHR that we observe is present for at least 8 wk after the final allergen challenge, at a time when acute, immune-mediated inflammatory responses have resolved (37). This is in contrast to the AHR described in the other models of chronic allergen or fungal exposure, where AHR is observed at times when cellular airway inflammation is still marked (26, 41, 45, 46).

We recognize that earlier Th2 immune-mediated inflammatory events are likely to be critical in the initial pathogenesis of functionally important airway remodeling processes. Foster and coworkers (19) have already reported that CD4⁺ T-cell depletion at the time of chronic allergen exposure results in attenuation of AHR and aspects of airway remodeling. Their observations indicate that T cells contribute to the remodeling process during a period of chronic allergen exposure. Our study was not designed to determine whether T-cell depletion had any effect on indexes of airway remodeling, but instead examined the effects of depleting CD4⁺ and CD8⁺ T cells, at a time when they were still present in lung tissue (Fig. 5) and when aspects of airway remodeling were already established and sustained AHR was present. Our principal hypothesis related to whether ongoing T-cell-mediated inflammation was required to maintain sustained AHR; our *a priori* expectation was that the brief period of T-cell depletion used in this study was unlikely to have any attenuating effect on indexes of airway remodeling.

T-cell depletion in these experiments was $\sim 98\%$, raising the question of how many T cells would have been required to

maintain AHR under chronic allergen conditions. Obviously, the precise answer to this question is not known. However, we observed that the same degree of T-cell depletion in the brief allergen-challenge protocol was completely effective at abrogating AHR. We, therefore, assumed that any ongoing T-cell-dependent AHR in the chronic model would also have been abrogated by the same antibody treatment protocol.

We elected to treat mice with anti-CD4/anti-CD8 MAb for a relatively brief 10-day period in this study. Whereas this was sufficient to deplete both CD4⁺ and CD8⁺ cells in lung and spleen tissue and to address our study hypothesis, it also provides an opportunity to speculate on the potential effects of extended anti-CD4/anti-CD8 MAb treatment. We assume that concomitant MAb treatment throughout the period of chronic allergen exposure might have resulted in abrogation of Th2 immune-mediated airway inflammation, with the subsequent attenuation of aspects of airway remodeling and AHR, as has been observed by Foster and colleagues (19). It is perhaps more intriguing to speculate whether prolonged MAb treatment, given after a period of chronic allergen exposure, might have facilitated some resolution of the AHR and associated airway remodeling. Formal testing of this hypothesis has important implications for targeted anti-CD4 or more specific immune-based treatments as a potential therapy for asthma and is clinically relevant, in that the majority of patients with asthma already have aspects of airway remodeling present at the time of clinical presentation (9). If prolonged abrogation of T-cell-mediated airway inflammation is unable to attenuate aspects of established airway remodeling and AHR, then it is clear that other approaches will be needed to fully address airway dysfunction in asthma. These may take the form of earlier immune-based interventions, at a time before significant airway remodeling has occurred, or intervention with other agents that specifically address the functionally important aspects of airway remodeling. Addressing these issues may also provide a better understanding as to why anti-CD4 MAb treatment (32), as well as anti-IL-5 (36) and anti-IL-4 treatment (1), have provided disappointing results in clinical studies of asthma to date.

In summary, we have demonstrated that depletion of CD4⁺ and CD8⁺ cells during brief allergen challenge, at a time when acute, T-cell-mediated airway inflammation is associated with transient AHR, results in significant attenuation of the acute inflammation response and the prevention of the transient AHR. In contrast, despite the depletion of CD4⁺ and CD8⁺ cells after chronic, repeated allergen exposure at a time when airway remodeling has already become established has no effect on the sustained AHR present in our model at that time. Our results strongly support the paradigm that the transient airway hyperreactivity, occurring after brief exposure to allergen, is dependent on T-cell-mediated airway inflammation. However, our observations extend that understanding by providing substantial novel evidence that T cells are not necessary to maintain established, sustained AHR, resulting from chronic allergen exposure.

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GRANTS

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