The Journal of Immunology

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J Immunol 2002; 169:3499-3506; ;
doi: 10.4049/jimmunol.169.7.3499
http://www.jimmunol.org/content/169/7/3499

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Chronic Exposure to Innocuous Antigen in Sensitized Mice Leads to Suppressed Airway Eosinophilia That Is Reversed by Granulocyte Macrophage Colony-Stimulating Factor[1]

Filip K. Swirski, Dusan Sajic, Clinton S. Robbins, Beata U. Gajewska, Manel Jordana, and Martin R. Stämpfli[2]

In this study we investigated the impact of chronic allergen exposure on airway inflammation and humoral responses in sensitized mice. We observed marked eosinophilia in the bronchoalveolar lavage, lung tissue, and peripheral blood after 2 wk of exposure. In contrast, eosinophilia was markedly reduced by 3 wk and completely resolved by 4 wk of exposure, despite the continued presence of Ag. Decreases in airway eosinophilia were associated with a robust humoral response. We observed that levels of OVA-specific IgE, IgG1, and IgG2a increased during the course of exposure. To assess whether continuous exposure to Ag impacts the ability of the lung to respond to subsequent Ag challenge, mice were exposed to either 2 or 4 wk of OVA in the context of GM-CSF. All groups were then rested for 28 days and exposed to OVA on three consecutive days. We observed a significant decrease in airway eosinophilia and IL-5 expression in the bronchoalveolar lavage and serum in mice initially exposed to 4 wk of OVA, compared with animals exposed to 2 wk only. However, in both groups expression of B7.2 on dendritic cells as well as CD25, CD69, and T1/ST2 on $CD4^+$ T cells was enhanced, suggesting immune activation. Delivery of rGM-CSF fully restored airway eosinophilia. This study shows that exposure to innocuous Ag alone does not lead to persistent eosinophilic airway inflammation, but rather to abrogated eosinophilia. This suppression can be reversed by GM-CSF. ***The Journal of Immunology*****, 2002, 169: 3499–3506.**

Asthma is a disorder characterized by paroxysmal or persistent symptoms, with variable airflow limitation and airway hyperresponsiveness to a variety of stimuli (1–3). It is argued that allergens contribute significantly to the initiation and persistence of airway inflammation, which is believed to be the central abnormality that leads to airway damage and dysfunction (1–3). It is now well established that expression of a distinct cytokine profile comprised particularly of IL-4, IL-5, and IL-13 leads to peribronchial and perivascular eosinophilic airway inflammation, IgE secretion, and bronchial hyperresponsiveness (4–7). All of these events are largely dependent upon interactions between allergen, APCs, T cells, and B cells, a concept that defines asthma as an Ag-induced, immune-driven process.

That allergen exposure to perennial allergens, such as house dust mite or cat dander, is rather continuous overall seems to be at variance with the "intermittent" nature of asthma. Indeed, if exacerbation of inflammation were attributed solely to allergen exposure, one would predict unabated symptoms among individuals presenting with allergic asthma. While cellular and molecular mechanisms underlying allergic sensitization and acute inflammation are subjects of intense research, comparatively little is known with regard to the impact of persistent Ag exposure on immune inflammatory processes in the airway. This understanding is further limited by the fact that research examining the effect of chronic allergen exposure on airway inflammation has produced controversial results. Studies showing that chronic allergen exposure does not lead to persistence of airway inflammation (8, 9) suggest that allergen alone is insufficient in perpetuating the inflammatory response. Others have documented persistent airway eosinophilia (10, 11); however, the focus of these studies was on airway remodeling and lung physiology rather than on immune inflammatory processes.

The objective of this study was to investigate the impact of chronic OVA exposure on immune inflammatory processes in the lung. We have recently established a model of mucosal allergic sensitization, which, like the conventional models, elicits some of the important features of asthma (12–14). In this model, mice are exposed to OVA in the context of a GM-CSF-enriched airway microenvironment for 10 consecutive days. While we argue that this is a better reflection of the route in which sensitization occurs in humans, Ag exposure is transient. Hence, to investigate immune inflammatory processes in the airway associated with chronic Ag exposure, we sensitized mice mucosally and exposed them to OVA for up to 4 wk. Expression of GM-CSF is required to allow for allergic mucosal sensitization, because exposure to OVA alone induces inhalation tolerance, as we and others have previously shown (15, 16). Importantly, GM-CSF on its own does not elicit airway eosinophilia (12); hence, its effects are likely due to its adjuvant-like properties (17–23). After 2 wk of exposure, we observed marked eosinophilia in the bronchoalveolar lavage (BAL),[3] lung tissue and peripheral blood but, despite the continuous presence of the Ag, eosinophilia was substantially diminished by 3 wk and

Department of Pathology and Molecular Medicine, Division of Respiratory Diseases and Allergy and Center for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada

Received for publication March 28, 2002. Accepted for publication July 22, 2002.

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[1] This study was supported in part by the Ontario Thoracic Society, the Hamilton Health Sciences Corporation, and St. Joseph's Hospital. F.K.S. is holder of a K.M. Hunter/Canadian Institutes for Health Research Doctoral Research Award. B.U.G. is holder of a Medical Research Council Studentship, and M.R.S. is holder of a fellowship from the Parker B. Francis Foundation.

[2] Address correspondence and reprint requests to Dr. Martin R. Stämpfli, Department of Pathology and Molecular Medicine, McMaster University, Health Sciences Center, Room 4H21A, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5. E-mail address: stampfli@mcmaster.ca

[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; DC, dendritic cell; MHCII, MHC class II; LSD, least significant difference.

0022-1767/02/$02.00

resolved after 4 wk. In contrast, similar OVA-specific IgE and increased IgG1 and IgG2a levels were observed after 4 wk, compared with 2 wk, of exposure. Next, we exposed mice to OVA for either 2 or 4 wk, rested them for 28 days, and subsequently re-exposed them to OVA only. We observed a significant decrease in airway eosinophilia as well as BAL and serum IL-5 in animals initially exposed to 4 wk, compared with 2 wk, of OVA. Nonetheless, the diminished airway eosinophilia was associated with a robust humoral response as well as activated dendritic cells (DC) and T cells. Delivery of rGM-CSF in the context of OVA recall challenge reestablished airway eosinophilia. The data demonstrate that chronic exposure to OVA does not lead to sustained airway inflammation but to a state of unresponsiveness that is overcome by GM-CSF. Therefore, we suggest that additional factors, other than Ag, are required to elicit persistent airway inflammation.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Harlan Breeders (Indianapolis, IN). Mice were maintained in a 12-h light-dark cycle with unlimited access to food and water. Cages, food, and bedding were autoclaved and all animal manipulations were conducted in a laminar flow hood by gloved, gowned, and masked personnel. All experiments were approved by the Animal Research Ethics Board of McMaster University.

Allergic mucosal sensitization

Mice were exposed to OVA in the context of a GM-CSF-enriched airway microenvironment. Prolonged expression of GM-CSF was achieved using an adenovirus-mediated gene transfer approach, as previously described (24). Briefly, a replication-deficient human type 5 adenoviral construct carrying the transgene for GM-CSF in the E1 region of the viral genome (Ad/GM-CSF) was delivered intranasally. A dose of 3×10^7 PFU Ad/GM-CSF construct was delivered in a total volume of 30 μl of PBS vehicle (two 15-μl administrations 5 min apart) into anesthetized animals. Subsequently, mice were placed in a Plexiglas chamber ($10 \times 15 \times 25$ cm) and exposed to aerosolized OVA (1% w/v in 0.9% saline; Sigma-Aldrich, St. Louis, MO) for 20 min daily. Mice were exposed 5 days per week from Monday to Friday. The aerosol is produced by a Bennett/Twin nebulizer (Puritan-Bennett, Carlsbad, CA) at a flow rate of 10 L/min.

Delivery of rGM-CSF

In a limited number of experiments, we delivered 1 μg of rGM-CSF (BioSource International, Camarillo, CA) in 10 μl PBS intranasally over five consecutive days to achieve sustained levels of GM-CSF in the airways.

Collection and measurement from specimens

BAL was performed as previously described (25). In brief, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences, Sparks, MD). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.25 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation cell pellets were resuspended in PBS and slides were prepared by cytocentrifugation (Thermo Shandon, Pittsburgh, PA) at 300 rpm for 2 min. HEMA 3 Stain set (Biochemical Sciences, Swedesboro, NJ) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological procedures to classify the cells as mononuclear cells, neutrophils, or eosinophils. Peripheral blood was obtained using heparin-coated capillaries (Fisher Scientific, Pittsburgh, PA). Total white blood cell counts were determined after lysing RBCs and cell differentials were assessed on smears stained with the HEMA 3 Stain set. For serum, animals were bled with nonheparinized capillary tubes. Serum was prepared by incubating whole blood for 30 min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. Three-micrometer-thick sections were stained with H&E.

Cytokine and Ig measurements

IL-5 was detected using a commercially available ELISA kit (Amersham, Little Chalfont, U.K.). The threshold of detection was 5 pg/ml. Levels of OVA-specific IgE were detected with an ELISA that has been described in detail previously (25). For OVA-specific IgG1 and IgG2a, Maxi-Sorb plates (Nunc, Roskilde, Denmark) were coated with 5 μg OVA in borate buffer (pH 8.3–8.5) overnight at 4°C. Subsequently, coated wells were blocked with 1% BSA in PBS for 2 h at room temperature. After washing, serum samples were incubated overnight at 4°C, washed, and developed with biotin-labeled, anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL) overnight at 4°C. Plates were washed and incubated with alkaline-phosphatase streptavidin for 1 h at room temperature. The color reaction was developed with *p*-nitrophenyl phosphate tablets. Samples were compared with a standard serum containing OVA-specific IgG1 and IgG2a. Units correspond to maximal dilution that results in an OD that is greater than the blank + 2 SD.

Lymph node and lung cell isolation

Hilar, mediastinal, and tracheobronchial lymph nodes were dissected, ground between the frosted ends of slides, and filtered through a nylon mesh (BSH Thompson, Scarborough, Ontario, Canada). The cell suspension was centrifuged at 1200 rpm for 10 min at 4°C and resuspended in PBS. Cells were resuspended in flow cytometric analysis buffer (PBS supplemented with 0.2% BSA).

For isolation of lung cells, lungs were flushed via the right ventricle of the heart with 10 ml of warm (37°C) HBSS (calcium and magnesium free) containing 5% FBS (Sigma-Aldrich), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies, Burlington, Ontario, Canada). The lungs were then cut into small (~2 mm in diameter) pieces and shaken at 37°C for 1 h in 15 ml of 150 U/ml collagenase III (Worthington Biochemical, Freehold, NJ) in HBSS. Using a plunger from a 5-ml syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon mesh. Cells were washed twice and mononuclear cells were isolated by density centrifugation over a 30 and 60% Percoll gradient (Pharmacia Biotech, Uppsala, Sweden).

Flow cytometry

Panels of mAbs were selected to study the phenotype of cells in the lymph nodes and lung. To minimize nonspecific binding, 10^6 cells were incubated with 0.5 μg Fc Block (CD16/CD32; BD PharMingen, Mississauga, Canada) at 0–4°C for 10 min and subsequently with first-stage mAbs at 0–4°C for 30 min. Cells were then washed and treated with second-stage reagents. Data were collected using a FACScan and analyzed using WIN-MDI software (BD Biosciences, Sunnyvale, CA). The following Abs and reagents were used: anti-CD3, biotin-conjugated 145-2C11 (BD PharMingen); anti-CD4, FITC-conjugated L3T4 (BD PharMingen); anti-CD69, PE-conjugated H1 2F3 (BD PharMingen); anti-CD25, PE-conjugated PC61 (BD PharMingen); anti-T1/ST2, FITC-conjugated 3E10 (kindly provided by A. J. Coyle, Millennium Pharmaceuticals, Cambridge, MA) labeled in-house according to a standard protocol (24); anti-MHC class II (MHCII), FITC-conjugated 25-9-17 (BD PharMingen); anti-CD11c, PE-conjugated HL3 (BD PharMingen); anti B7.1, biotin-conjugated 16-10AI (BD PharMingen); anti-B7.2, biotin-conjugated GLI (BD PharMingen); and streptavidin PerCP (BD Biosciences, San Jose, CA). Titration was performed to determine the optimal concentration of each Ab.

Data analysis

Data are expressed as mean ± SEM. Statistical interpretation of results is indicated in the figures. Differences were considered statistically significant when $p < 0.05$.

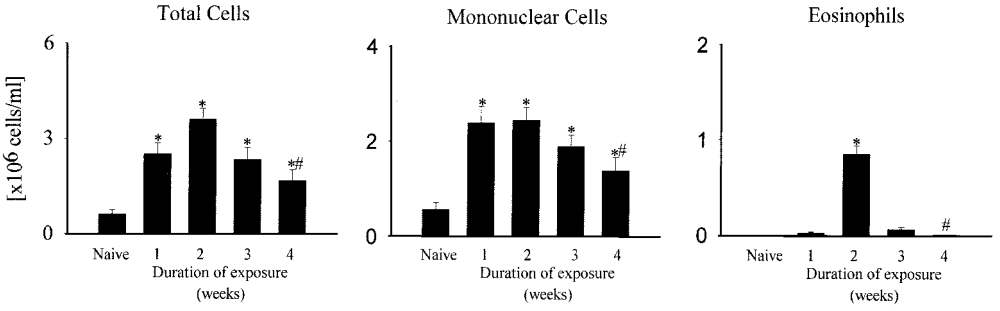
Results

Cellular profile in the BAL and peripheral blood of mice exposed to OVA for 1, 2, 3, or 4 wk

BALB/c mice were infected intranasally with 3×10^7 PFU Ad/GM-CSF. We previously reported that GM-CSF is expressed in the airways for ~10 days, with peak expression of ~80–100 pg/ml in the BAL at day 7. GM-CSF was undetectable in the serum (26). Three days later, mice were exposed to aerosolized OVA daily for 1, 2, 3, or 4 wk. Fig. 1*A* shows that we observed only few eosinophils in the BAL after the first week of OVA exposure. After 2 wk of OVA exposure, mice developed significant eosinophilia in the BAL. Despite continued exposure to OVA, airway eosinophilia was decreased by 95% after 3 wk and was completely resolved after 4 wk.

Similarly, after 2 wk of exposure to OVA, peripheral blood eosinophilia and total cell number was significantly increased compared with naive animals (Fig. 1*B*). These levels were significantly decreased and were similar to naive levels after 4 wk of exposure.

A



B

Duration of Exposure	Total PB Leukocytes ($x10^6$ cells/ml)	Eosinophils (% of total)
Naive	3.3±0.3	1.3±1.0
2 weeks	11.0±1.4*	15.9±2.2*
4 weeks	4.3±0.6#	1.7±1.2#

FIGURE 1. Inflammatory response during prolonged exposure to OVA in the context of GM-CSF. *A*, The BAL cellular profile in naive mice and in mice exposed to 1, 2, 3, or 4 wk of OVA (mean ± SEM). *B*, The peripheral blood cellular profile from naive animals and animals exposed to either 2 or 4 wk of OVA (mean ± SEM). Statistical analysis was performed using one-way ANOVA with the Fisher least significant difference (LSD) post hoc test (∗, $p < 0.05$ compared with naive; #, $p < 0.05$ compared with 2 wk). $n = 5–8$ mice per group.

Impact of 2 and 4 wk of OVA exposure on Ig production

Next, we assessed the impact of continuous OVA exposure on Ag-specific Ig production (Table I). Mice were subjected to OVA exposure in the context of GM-CSF according to the protocol outlined above. Serum Ig levels of OVA-specific IgE increased significantly during the first 2 wk but remained similar after 4 wk. In contrast, we observed significantly increased levels of OVA-specific IgG1 and IgG2a after 2 wk, and these were further elevated after 4 wk of exposure.

Impact of 4-wk Ag exposure on subsequent in vivo Ag recall

Given that continuous exposure to OVA did not result in persistence of eosinophilia and airway inflammation, we investigated whether continuous exposure to Ag impacts the ability of the lung to respond to subsequent long-term Ag exposure. To this end, we first exposed mice to either 2 or 4 wk of OVA in the context of GM-CSF. Animals exposed to OVA for only 2 wk were subsequently subjected to saline aerosol to control for the handling. All groups were then rested for 28 days and exposed to OVA on three consecutive days. Forty-eight hours after the last exposure, mice were sacrificed and the inflammatory infiltrate in the BAL was assessed. We observed a robust mononuclear and eosinophilic response in animals initially exposed to 2 wk of OVA (Fig. 2*A*). This response was significantly greater than that observed in naive mice and similar in magnitude to the inflammation observed after 2 wk of OVA exposure, as depicted in Fig. 1. In contrast, animals initially exposed to OVA for 4 wk exhibited a significant decrease in total cell number and negligible eosinophilia following OVA recall. Indeed, the cellular profile in these animals was statistically not different from naive mice.

Table I. *Ig levels during prolonged exposure to OVA*[a]

Ig	OVA		
	Naive mice	2-wk exposed mice	4-wk exposed mice
IgE	2.33 ± 1.80	29.5 ± 9.4*	26.1 ± 7.4*
IgG1	0.246 ± 0.246	6.11 ± 0.90*	20.7 ± 1.9*†
IgG2a	0.216 ± 0.073	12.6 ± 6.5*	69.3 ± 1.5*†

[a] Mice were exposed to OVA aerosol in the context of GM-CSF for a period of 2 and 4 wk. Three days after the last aerosolization, Ig levels were measured in the serum (mean ± SEM). Data are measured as follows: IgE, $\times 10^0$ U/ml; IgG1, $\times 10^6$ U/ml; IgG2a, $\times 10^2$ U/ml. Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test.

∗, $p < 0.05$ when compared to naive.

†, $p < 0.05$ when compared to 2 wk. $n = 4$ mice per group.

Histological evaluation of lungs from animals initially exposed to either 2 or 4 wk of OVA and then rechallenged corroborated our BAL findings. While animals initially exposed to 2 wk of OVA demonstrated extensive peribronchial and perivascular inflammation, including mononuclear cells and eosinophilia (Fig. 2*B*, *i* and *ii*), animals initially exposed to 4 wk of OVA had dramatically reduced tissue inflammation, with no eosinophilia (Fig. 2*B*, *iii* and *iv*).

Next, we assessed IL-5 expression in the BAL and serum after recall challenge. Twenty-four hours after the first OVA exposure, we observed significantly reduced levels of IL-5 in both the BAL and serum in animals initially exposed to 4 wk of OVA when compared with animals initially exposed to 2 wk only (Fig. 3). A similar trend was observed 24 h after the second OVA exposure, but the levels of IL-5 expression were lower (data not shown). No IL-5 was detected in naive animals (data not shown).

Assessment of serum Igs showed similar levels of OVA-specific IgE and IgG2a between the groups and elevated levels of IgG1 in mice initially exposed to 4 wk, compared with 2 wk, of OVA (Table II). Levels of all Igs were markedly higher than those observed in naive mice (Table I).

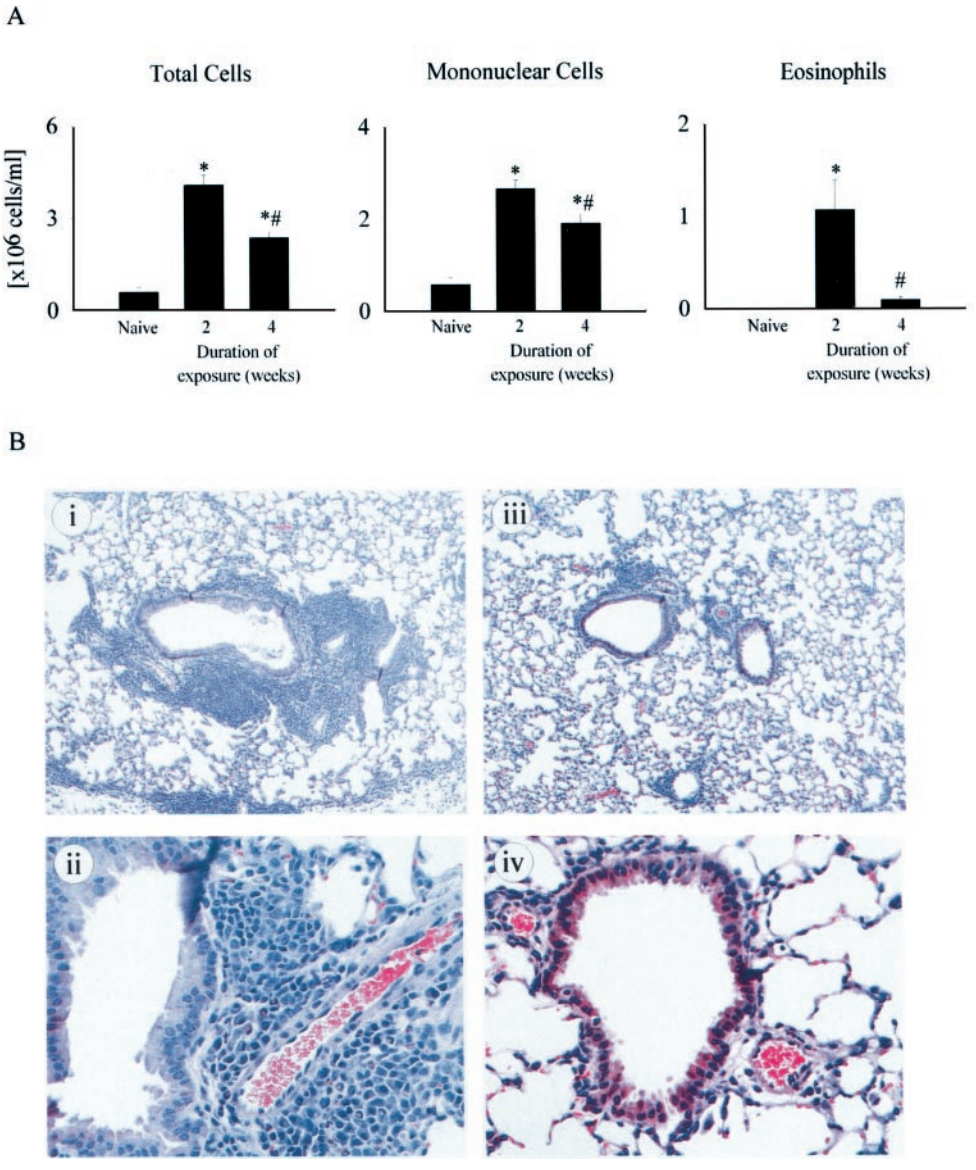


FIGURE 2. Inflammatory response during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on three consecutive days and killed 2 days later. *A*, The BAL cellular profile in naive mice and animals initially exposed to 2 and 4 wk of OVA (mean ± SEM). Statistical analysis was performed using one-way ANOVA with the Fisher LSD post hoc test (∗, $p < 0.05$ compared with naive; #, $p < 0.05$ compared with 2 wk). $n = 4–8$ mice per group. *B*, A micrograph of paraffin-embedded sections of lung tissue from animals exposed to 2 wk (*i* and *ii*) and 4 wk (*iii* and *iv*) of OVA at magnifications of ×50 (*i* and *iii*) and ×200 (*ii* and *iv*).

T cell and APC phenotype during in vivo Ag recall

Given that our histological assessment showed residual mononuclear inflammation in mice initially exposed to 4 wk of OVA, we quantified tissue mononuclear cells in the lungs and draining lymph nodes following in vivo recall to OVA. As Table III shows, lungs from animals initially exposed to 2 wk of OVA had

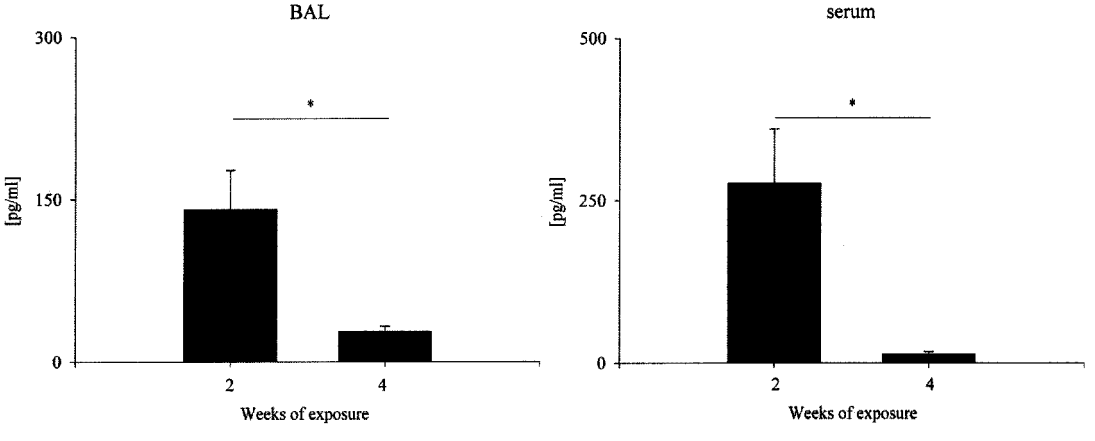


FIGURE 3. Expression of IL-5 in the BAL and serum during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest after the exposure protocol, both groups were challenged with OVA and killed 1 day later. Data show the expression of IL-5 in the BAL and serum (mean ± SEM). Statistical analysis was performed using the Student's *t* test (∗, $p < 0.05$). $n = 5$ mice per group.

Table II. *Ig levels during long-term in vivo recall*[a]

	OVA	
	2-wk exposed	4-wk exposed
IgE	46.9 ± 12.1	53.5 ± 4.5
IgG1	1.35 ± 0.38	4.11 ± 1.05*
IgG2a	41.0 ± 7.8	49.8 ± 3.3

[a] Mice were exposed to OVA aerosol in the context of GM-CSF for a period of 2 and 4 wk. Mice were then rested for 28 days. Both groups were then exposed to OVA for 3 days, and 24 h after the last exposure Ig levels were measured in the serum (mean ± SEM). Data are measured as follows: IgE, $\times 10^0$ U/ml; IgG1, $\times 10^6$ U/ml; IgG2a, $\times 10^2$ U/ml. Statistical analysis was performed using Student's *t* test.
*, $p < 0.05$. $n = 3$–8 mice per group.

approximately twice the number of mononuclear cells compared with animals initially exposed to 4 wk of OVA (11.8×10^6 vs 4.94×10^6 cells per mouse in experiment 1 and 16.2×10^6 vs 7.8×10^6 cells per mouse in experiment 2). In naive mice, we previously documented only 2.2×10^6 lung cells per mouse (27). Similarly, in the draining lymph nodes we observed substantially fewer cells at 4 wk compared with 2 wk (6.89×10^6 vs 3.64×10^6 cells per mouse in experiment 1 and 10×10^6 vs 4.5×10^6 cells per mouse in experiment 2). Naive mice had only 1.2×10^6 lymph node mononuclear cells per mouse (27).

To investigate the phenotype of the residual inflammatory infiltrate, we assessed with flow cytometry the expression of B7.1 and B7.2 on DCs and the activation markers CD69 and CD25 on $CD4^+$ T cells. Furthermore, we characterized the expression of T1/ST2, a surface marker found on Th2 $CD4^+$ T cells. DCs were identified on the basis of MHCII/CD11c expression (28). Fig. 4 shows increased expression of B7.2 on lung DCs in both groups. We observed 19.5 and 28.7% of B7.2 in animals initially exposed to 2 and 4 wk, respectively, while historical data shows that only 5.4% of lung DCs express this molecule in naive animals (27). Similarly, we observed increased expression of B7.2 in the lymph nodes. Compared with naive mice (3.4%) (27), 27.5 and 33% of lymph node DCs expressed B7.2 in animals exposed to 2 and 4 wk of OVA, respectively. No differences in the expression of B7.1 were observed between the groups in either the lung or the lymph nodes (Fig. 4 and Ref. 27). Fig. 5 shows that the levels of CD69 and CD25 in animals exposed to OVA for 2 or 4 wk were similar, and were substantially higher than the levels we observed and previously documented in naive mice (15, 27). Specifically, in the lungs 20.2 and 27.8% of $CD4^+$ T cells expressed CD69 in animals exposed to 2 and 4 wk of OVA, respectively. Likewise, CD25 was expressed at 17.8 and 20% in 2- and 4-wk exposed animals, respectively, with <2% expression in naive animals. A similar pattern of expression was observed in the lymph nodes (data not shown). Finally, T1/ST2 was expressed on 12% of lung $CD4^+$ T cells in animals exposed to OVA for 2 wk, and on 12.7% of lung $CD4^+$ T cells in mice exposed for 4 wk, while typically 3.6% of $CD4^+$ T cells of naive animals express this molecule (27).

Table III. *Lung and lymph node mononuclear cells during long-term in vivo Ag recall*[a]

Cells	Exposure	Expt. 1 ($\times 10^6$ cells/mouse)	Expt. 2 ($\times 10^6$ cells/mouse)
Lungs	2 wk OVA	11.8	16.2
	4 wk OVA	4.94	7.80
Lymph nodes	2 wk OVA	6.89	10.0
	4 wk OVA	3.64	4.50

[a] Mice were exposed to OVA in the context of GM-CSF for 2 or 4 wk. After 28 days of rest, mice were exposed to OVA on three consecutive days. Forty-eight hours after the last exposure mice were sacrificed, their lungs and thoracic draining lymph nodes removed and pooled (four mice), and the mononuclear cells were isolated. Two representative experiments are shown.

Impact of rGM-CSF administration at time of long-term recall

Next, we investigated whether the expression of GM-CSF at the time of long-term recall could reconstitute airway eosinophilia. Mice were exposed to OVA for 4 wk and then rested for 28 days. Subsequently, we delivered rGM-CSF intranasally on five consecutive days and exposed the animals concurrently to OVA daily for nine consecutive days, with the first challenge coinciding with the day of the first rGM-CSF delivery. We observed that animals exposed to OVA in the context of rGM-CSF, but not PBS, had significantly higher levels of eosinophilia in the airway (Fig. 6). The total cell number and level of airway inflammation was similar to that observed in animals initially exposed to OVA for 2 wk, as depicted in Fig. 2*A*. In contrast, inflammation could not be recapitulated in control animals receiving OVA and PBS.

Discussion

An understanding of processes underlying allergic inflammatory diseases has benefited from experimental models that have aimed to recapitulate, in animals, the human pathology. Conventional mouse models of Ag-induced airway inflammation generally involve two distinct phases: a sensitization procedure conducted i.p. followed by aerosol challenge (25, 29, 30). While these models are, and will continue to be, of great value, their route of sensitization and the acute nature of the airway challenge sharply contrast with allergen exposure in humans. Therefore, in the present studies mice were sensitized using a protocol of mucosal allergic sensitization (12). To investigate immune inflammatory processes in the airway associated with chronic Ag exposure, mice were exposed to OVA for up to 4 wk.

First, we investigated the cellular changes in the airway during chronic exposure to OVA. We found that 2 wk of exposure resulted in peak inflammation, both eosinophilic and mononuclear (Fig. 1). That we observed eosinophilic airway inflammation at this time point corroborates our previous findings (12). Note that GM-CSF is expressed in the airway for ~10 days (12) and precedes peak inflammation by ~1 wk. After 3 and 4 wk of OVA exposure airway eosinophilia was dramatically reduced. Similarly, peripheral blood eosinophilia peaked after 2 wk and was resolved after 4 wk. That both airway eosinophilia and peripheral blood eosinophilia were decreased after 4 wk of exposure argues against an impairment in eosinophil recruitment.

While we observed decreased cellular responses in the lung during prolonged exposure, the increased levels of Igs in the serum (Table I), particularly IgG1 and IgG2a, indicate that immune responsiveness was not fully silenced. This finding suggests that processes involved in isotype switching and Ig production were not affected. That we did not observe preferential up-regulation of the Th1-associated IgG2a, over IgG1, which is Th2 associated (31), argues against a Th2→Th1 skew during prolonged exposure to Ag.

Our observation in the airway is in agreement with studies by Haczku et al. (8) and Cui et al. (9), who have shown that chronic allergen exposure in rats does not lead to persistence of inflammation. In sharp contrast, other studies have documented persistent airway eosinophilia (10, 11). With respect to Igs, it has been shown in one study that prolonged exposure leads to persistent IgE production (11), while in another, prolonged exposure leads to transient IgG and IgE expression (9). While these discrepancies may reflect differences in the experimental models, our observations demonstrate that, in the mouse, chronic exposure to Ag does not

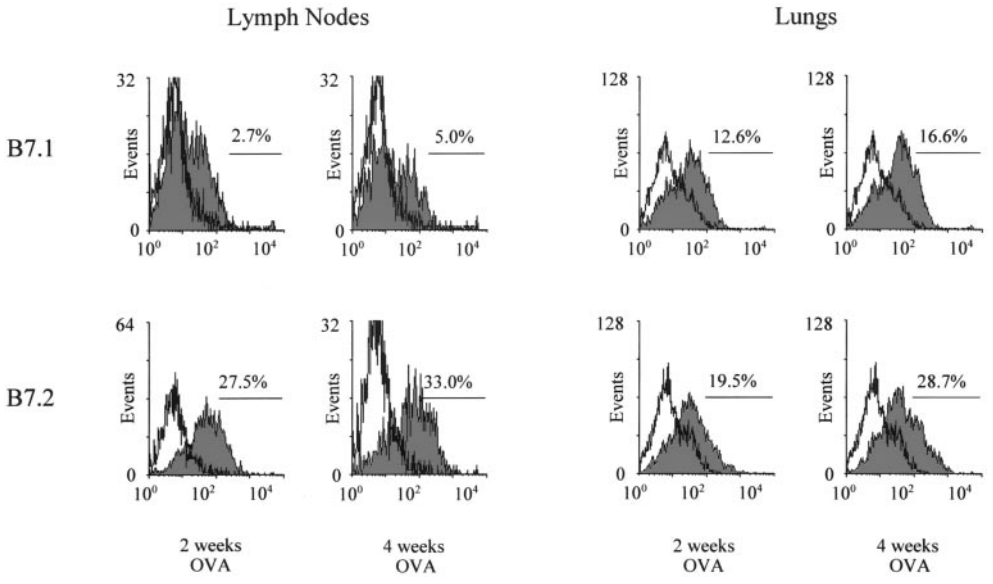


FIGURE 4. Costimulatory molecule expression on DCs during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on two consecutive days and killed 1 day later. Data show the expression of B7.1 and B7.2 on $MHCII^+CD11c^+$ cells in the draining lymph nodes and lungs of 2- and 4-wk exposed mice. $n = 5$ mice per group. One of two representative experiments is shown.

result in persistence of airway and peripheral blood eosinophilia but does result in increased or sustained Ig expression.

The diminished airway eosinophilia but elevated Ig levels during prolonged Ag challenge led us to investigate whether such exposure influenced in vivo memory recall responses. To this end, following either 2 or 4 wk of OVA challenge, mice were rested for 28 days and rechallenged with OVA. Animals initially exposed to 4 wk of OVA and subsequently rechallenged had significantly reduced airway eosinophilia (Fig. 2) and reduced BAL and serum IL-5 levels (Fig. 3) compared with 2-wk exposed animals. In contrast, Ig levels were similar (IgE and IgG2a) or increased (IgG1) in the 4-wk, compared with the 2-wk, exposed mice (Table II). These findings may suggest that levels of IgE may be of limited predictive value for inflammatory responses in the lung. We are currently pursuing studies assessing the impact of chronic Ag exposure on airway hyperresponsiveness.

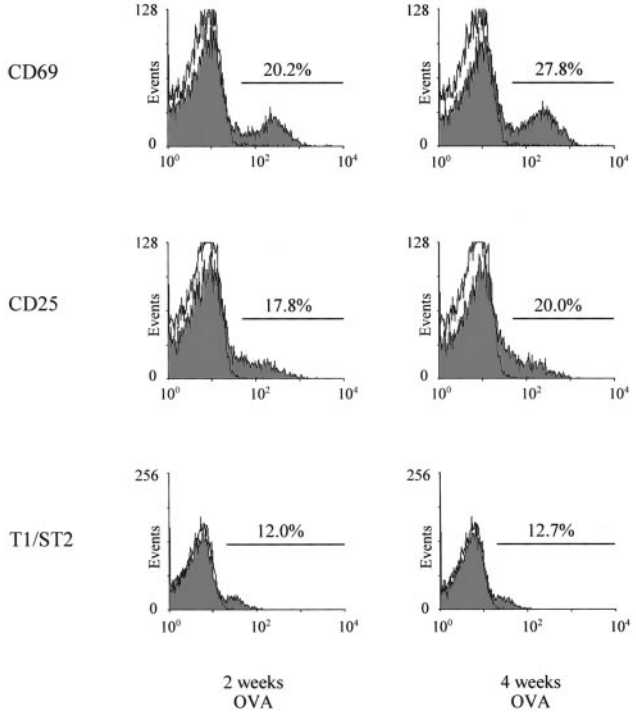


FIGURE 5. Activation of T cells in the lungs after long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on three consecutive days and killed 2 days later. Data show the expression profile of CD69, CD25, and T1/ST2 on $CD3^+CD4^+$ cells in the lungs of 2- and 4-wk exposed mice. $n = 5$ mice per group. One of two representative experiments is shown.

Airway eosinophilia is a terminal event that relies on DC presenting Ag to T cells (32) in the context of the appropriate costimulatory signals (33). To investigate whether chronic exposure altered the phenotype of DCs in 4-wk exposed animals at the time of in vivo recall challenge, we assessed the number and activation status of these cells. We observed elevated levels of B7.2 on DCs in the lymph nodes and lungs of animals initially exposed to 4 wk of OVA (Fig. 4), suggesting DC activation.

To investigate whether the changes observed in the APC compartment were associated with T cell activation, we evaluated the phenotype of $CD4^+$ T cells in the lungs at the time of in vivo recall challenge (Fig. 5). $CD4^+$ T cells from animals initially exposed to 2 and 4 wk of OVA expressed not only similar levels of the early activation marker CD69 (34, 35) and the IL-2R CD25 (36, 37), but also similar levels of T1/ST2, a marker of Th2 differentiation and a necessary factor in the development of eosinophilic airway inflammation (38–40). The level of expression of these molecules was substantially higher than previously documented in naive animals. Interestingly, these phenotypic observations did not translate into expression of the Th2-associated cytokine, IL-5. Our data suggest that chronic exposure to OVA does not alter T cell activation while preventing the generation of airway eosinophilia upon in vivo recall. Importantly, that a seemingly differentiated Th2 $CD4^+$ T cell is incapable of eliciting airway eosinophilia may argue for the presence of regulatory mechanisms in the airway microenvironment, as has previously been suggested (41, 42).

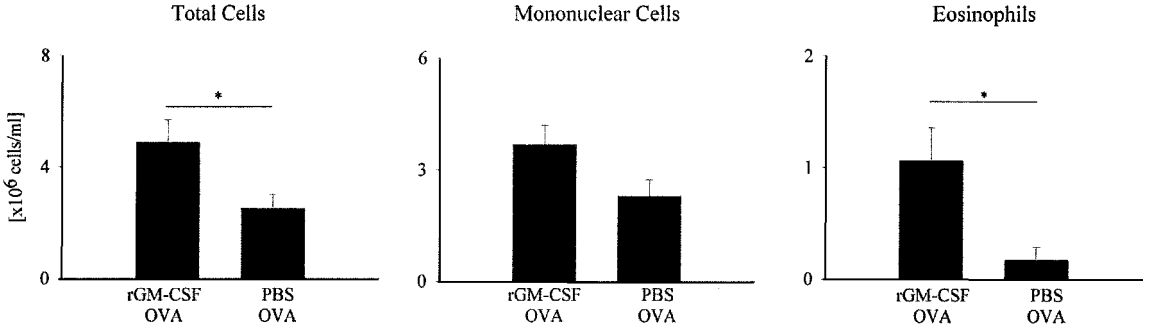


FIGURE 6. Exposure to OVA in the context of rGM-CSF during long-term in vivo recall. Mice were exposed to 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, mice were challenged with OVA on nine consecutive days and killed 1 day later. One group of mice received intranasal delivery of rGM-CSF on the first five consecutive days of the rechallenge protocol, while another group received intranasal delivery of vehicle control (PBS). Data represent the BAL cellular profile from animals receiving either rGM-CSF or PBS in the context of OVA recall challenge (mean ± SEM). Statistical analysis was performed using the Student's *t* test (*, $p < 0.05$). $n = 8$ mice per group.

The fact that animals were first exposed to OVA in a GM-CSF-enriched environment led us to investigate whether re-exposure to OVA with GM-CSF at the time of in vivo recall could recapitulate the eosinophilic airway inflammation. To this end, animals initially exposed to OVA for either 2 or 4 wk were rechallenged with the Ag in the context of GM-CSF. To avoid an immune response to the adenovirus that may confound the interpretation of our data, we opted to use the recombinant protein rather than the adenovirally encoded GM-CSF. We found that animals initially exposed to OVA for 4 wk and subsequently re-exposed to the Ag in the context of GM-CSF developed robust airway eosinophilia. Our data suggest that persistent airway inflammation is dependent on not only Ag, but also additional factors such as GM-CSF (Fig. 6). Therefore, GM-CSF not only is required for sensitization and development of airway eosinophilia in a protocol that otherwise leads to inhalation tolerance (12), but it may also be required for the persistence of airway inflammation in the context of continued Ag exposure.

The ability to reconstitute eosinophilic airway inflammation with the help of GM-CSF in seemingly unresponsive animals is likely of clinical relevance. It has been shown that exposure to environmental pollutants, as well as viral and bacterial agents, upregulates GM-CSF production (43–46). Indeed, these agents have been associated with exacerbation of asthma. Therefore, we hypothesize that exacerbation of symptoms among asthmatics may require not only Ag, but also additional agents that, along with Ag, generate sustained Th2-mediated eosinophilic airway inflammation. This may serve to explain, at least in part, why exacerbations are intermittent even if the Ag is continuously present. Therefore, we suggest that our experimental protocol provides a good model system to study mechanisms that regulate inflammation in the context of chronic exposure to innocuous Ag.

In summary, we show that prolonged exposure to OVA in the context of GM-CSF leads to abrogated eosinophilic airway inflammation, which is nevertheless associated with a robust humoral response and an activated $CD4^+$ T cell and DC phenotype. This unresponsiveness is reversible with GM-CSF. Understanding the principles that lead to or prevent chronic inflammation elicited by innocuous Ag is key to our understanding of allergic diseases. We propose that the elucidation of these principles and mechanisms may help us to reveal intrinsic protective mechanisms and design new ways of controlling allergic diseases.

Acknowledgments

We gratefully acknowledge the technical help of Susanna Goncharova and the secretarial assistance of Mary Kiriakopoulos. We also thank Dr. Gerard Cox, David Alvarez, Stacey A. Ritz, and Ryan E. Wiley for helpful discussion.

References

1. Boulet, L. P., A. Becker, D. Berube, R. Beveridge, and P. Ernst. 1999. Summary of the recommendations of the Canadian Consensus Conference on Asthma 1999: Canadian Asthma Consensus Group. *CMAJ 161:SF1.*
2. O'Byrne, P. M., and D. S. Postma. 1999. The many faces of airway inflammation: asthma and chronic obstructive pulmonary disease. Asthma Research Group. *Am. J. Respir. Crit. Care Med. 159:S41.*
3. Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. Beasley, O. P. Twentyman, R. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis. 142:434.*
4. Bentley, A. M., Q. Meng, D. S. Robinson, Q. Hamid, A. B. Kay, and S. R. Durham. 1993. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am. J. Respir. Cell Mol. Biol. 8:35.*
5. Bradley, B. L., M. Azzawi, M. Jacobson, B. Assoufi, J. V. Collins, A. M. Irani, L. B. Schwartz, S. R. Durham, P. K. Jeffery, and A. B. Kay. 1991. Eosinophils, T-lymphocytes, mast cells, neutrophils and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol. 88:661.*
6. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. R. Durham, and A. B. Kay. 1992. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med. 326:298.*
7. Jaffar, Z., K. Roberts, A. Pandit, P. Linsley, R. Djukanovic, and S. Holgate. 1999. B7 co-stimulation is required for IL-5 and IL-13 secretion by bronchial biopsy tissue of atopic asthmatic subjects in response to allergen stimulation. *Am. J. Respir. Cell Mol. Biol. 20:153.*
8. Haczku, A., R. Moqbel, W. Elwood, J. Sun, A. B. Kay, P. J. Barnes, and K. F. Chung. 1994. Effects of prolonged repeated exposure to ovalbumin in sensitized brown Norway rats. *Am. J. Respir. Crit. Care Med. 150:23.*
9. Cui, Z. H., B. E. Skoogh, T. Pullerits, and J. Lotvall. 1999. Bronchial hyperresponsiveness and airway wall remodelling induced by exposure to allergen for 9 weeks. *Allergy 54:1074.*
10. Kips, J. C., C. A. Cuvelier, and R. A. Pauwels. 1992. Effect of acute and chronic antigen inhalation on airway morphology and responsiveness in actively sensitized rats. *Am. Rev. Respir. Dis. 145:1306.*
11. Palmans, E., J. C. Kips, and R. A. Pauwels. 2000. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am. J. Respir. Crit. Care Med. 161:627.*
12. Stämpfli, M. R., R. E. Wiley, G. S. Neigh, B. U. Gajewska, X. F. Lei, D. P. Snider, Z. Xing, and M. Jordana. 1998. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest. 102:1704.*
13. Stämpfli, M. R., G. S. Neigh, R. E. Wiley, M. Cwiartka, S. A. Ritz, M. M. Hitt, Z. Xing, and M. Jordana. 1999. Regulation of allergic mucosal sensitization by interleukin-12 gene transfer to the airway. *Am. J. Respir. Cell Mol. Biol. 21:317.*
14. Stämpfli, M. R., M. Cwiartka, B. U. Gajewska, D. Alvarez, S. A. Ritz, M. D. Inman, Z. Xing, and M. Jordana. 1999. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am. J. Respir. Cell Mol. Biol. 21:586.*
15. Swirski, F. K., B. U. Gajewska, D. Alvarez, S. A. Ritz, M. J. Cundall, A. J. Coyle, J.-C. Guttierrez-Ramos, M. Jordana, and M. R. Stämpfli. 2002. Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin. Exp. Allergy 32:411.*
16. Holt, P. G., J. E. Batty, and K. J. Turner. 1981. Inhibition of specific IgE responses in mice by pre-exposure to antigen. *Immunology 42:409.*

17. Morrissey, P. J., L. Bressler, L. S. Park, A. Alpert, and S. Gillis. 1987. Granulocyte-macrophage colony-stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cell. *J. Immunol. 139: 1113.*
18. Fischer, H.-G., S. Frosch, K. Reske, and A. B. Reske-Kunz. 1988. Granulocyte-macrophage colony-stimulating factor actives macrophages derived from bone marrow cultures to synthesis of MHC class II molecules and to augmented antigen presentation function. *J. Immunol. 141:3882.*
19. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor. *J. Exp. Med. 179:1109.*
20. Chang, C.-H., M. Furue, and K. Tamaki. 1995. B7-1 expression of Langerhans cells is up-regulated by proinflammatory cytokines, and is downregulated by interferon-γ or by interfeukin-10. *Eur. J. Immunol. 25:394.*
21. Tao, M.-H., and R. Levy. 1993. Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature 362:755.*
22. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*
23. Disis, M. L., H. Bernhard, F. M. Shiota, S. L. Hand, J. R. Gralow, E. S. Huseby, S. Gillis, and M. A. Cheever. 1996. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood 88: 202.*
24. Xing, Z., Y. Ohkawara, M. Jordana, F. L. Graham, and J. Gauldie. 1996. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest. 97:1102.*
25. Ohkawara, Y., X.-F. Lei, M. R. Stämpfli, J. S. Marshall, Z. Xing, and M. Jordana. 1997. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *Am. J. Respir. Cell Mol. Biol. 16:510.*
26. Lei, X.-F., Y. Ohkawara, M. R. Stämpfli, J. Gauldie, K. Croitoru, M. Jordana, and Z. Xing. 1998. Compartmentalized transgene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse lung enhances allergic airways inflammation. *Clin. Exp. Immunol. 113:157.*
27. Gajewska, B. U., F. K. Swirski, D. Alvarez, S. A. Ritz, S. Goncharova, M. Cundall, D. P. Snider, A. J. Coyle, J.-C. Gutierrez-Ramos, M. R. Stämpfli, and M. Jordana. 2001. Temporal-spatial analysis of the immune response in a murine model of ovalbumin-induced airways inflammation. *Am. J. Respir. Cell Mol. Biol. 25:326.*
28. Vremec, D., and K. Shortman. 1997. Dendritic cell subsets in mouse lymphoid organs. *J. Immunol. 159:565.*
29. Riffo-Vasquez, Y., D. Spina, M. Thomas, T. Gilbey, D. M. Kemeny, and C. P. Page. 2000. The role of CD23 on allergen-induced IgE levels, pulmonary eosinophilia and bronchial hyperresponsiveness in mice. *Clin. Exp. Allergy 30: 728.*
30. Suzuki, M., S. Suzuki, N. Yamamoto, S. Komatsu, S. Inoue, T. Hashiba, M. Nishikawa, and Y. Ishigatsubo. 2000. Immune responses against replication-deficient adenovirus inhibit ovalbumin-specific allergic reactions in mice. *Hum. Gene Ther. 11:827.*
31. Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature 334:255.*
32. Lambrecht, B. N., B. Salomon, D. Klatzmann, and R. A. Pauwels. 1998. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *J. Immunol. 160: 4090.*
33. Tsuyuki, S., J. Tsuyuki, K. Einsle, M. Kopf, and A. J. Coyle. 1997. Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (TH2) immune response and altered airway responsiveness. *J. Exp. Med. 185: 1671.*
34. Marzio, R., J. Mauel, and S. Betz-Corradin. 1999. CD69 and regulation of the immune function. *Immunopharmacol. Immunotoxicol. 21:565.*
35. Kennedy, J. D., C. A. Hatfield, S. F. Fidler, G. E. Winterrowd, J. V. Haas, J. E. Chin, and I. M Richards. 1995. Phenotypic characterization of T lymphocytes emigrating into lung tissue and the airway lumen after antigen inhalation in sensitized mice. *Am. J. Respir. Cell Mol. Biol. 12:613.*
36. Haczku, A., P. Macary, T. J. Huang, H. Tsukagoshi, P. J. Barnes, A. B. Kay, D. M Kemeny, K. F. Chung, and R. Moqbel. 1997. Adoptive transfer of allergen-specific $CD4^+$ T cells induces airway inflammation and hyperresponsiveness in brown-Norway rats. *Immunology 91:176.*
37. Kaminuma, O., H. Fujimura, K. Fushimi, A. Nakata, A. Sakai, S. Chishima, K. Ogawa, M. Kikuchi, H. Kikkawa, K. Akiyama, and A. Mori. 2001. Dynamics of antigen-specific helper T cells at the initiation of airway eosinophilic inflammation. *Eur. J. Immunol. 31:2669.*
38. Coyle, A. J., C. Lloyd, J. Tian, T. Nguyen, C. Erikkson, L. Wang, P. Ottoson, P. Persson, T. Delaney, S. Lehar, et al. 1999. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med. 190:895.*
39. Townsend, M. J., P. G. Fallon, D. J. Matthews, H. E. Jolin, and A. N. McKenzie. 2000. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J. Exp. Med. 191:1069.*
40. Lambrecht, B. N., M. De Veerman, A. J. Coyle, J.-C. Gutierrez-Ramos, K. Thielemans, and R. A. Pauwels. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest. 106:551.*
41. Holt, P. G., and C. McMenamin. 1989. Defence against allergic sensitization in the healthy lung: the role of inhalation tolerance. *Clin. Exp. Allergy 19:255.*
42. Hoyne, G. F., K. Tan, M. Corsin-Jimenez, K. Wahl, M. Stewart, S. E. Howie, and J. R. Lamb. 2000. Immunological tolerance to inhaled antigen. *Am. J. Respir. Crit. Care Med. 162:S169.*
43. Subauste, M. C., D. B. Jacoby, S. M. Richards, and D. Proud. 1995. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J. Clin. Invest. 96:549.*
44. Saitou, M., S. Kida, S. Kaise, S. Suzuki, M. Ohara, and R. Kasukawa. 1997. Enhancement of eosinophil survival by lipopolysaccharide through releasing granulocyte-macrophage colony stimulating factor from mononuclear cells from patients with bronchial asthma. *Fukushima J. Med. Sci. 43:75.*
45. Devalia, J. L., H. Bayram, M. M. Abdelaziz, R. J. Sapsford, and R. J. Davies. 1999. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int. Arch. Allergy Immunol. 118:437.*
46. Sanders, S. P., J. Kim, K. R. Connolly, J. D. Porter, E. S. Siekierski, and D. Proud. 2001. Nitric oxide inhibits rhinovirus-induced granulocyte macrophage colony-stimulating factor production in bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol. 24:317.*