

Effect of GM-CSF on immune, inflammatory, and clinical responses to ragweed in a novel mouse model of mucosal sensitization

Elizabeth C. Cates, BSc,^a Beata U. Gajewska, BSc,^a Susanna Goncharova, PhD,^a David Alvarez, BSc,^a Ramzi Fattouh, BSc,^a Anthony J. Coyle, PhD,^b José-Carlos Gutierrez-Ramos, PhD,^b and Manel Jordana, MD, PhD^a *Hamilton, Ontario, Canada, and Cambridge, Mass*

Background: Conventional models of allergic airway inflammation involve intraperitoneal administration of ovalbumin in conjunction with a chemical adjuvant (generally aluminum hydroxide) to generate allergic airways inflammation. Here we have investigated the effect of respiratory mucosal exposure to a ragweed extract in the absence of chemical adjuvant on the generation of allergic responses.

Objectives: We sought to develop a mouse model of ragweed-induced allergic airway inflammation through mucosal sensitization and to investigate the role of GM-CSF in this process.

Methods: Ragweed was delivered intranasally to an airway microenvironment enriched with GM-CSF, which was achieved by means of either multiple coadministrations of recombinant GM-CSF or a single delivery of an adenoviral vector carrying the GM-CSF transgene.

Results: Administration of a purified ragweed extract leads to T_H2 sensitization (and not inhalation tolerance) accompanied by mild airway inflammation, modest clinical symptoms, and moderate production of T_H2 cytokines by splenocytes on ragweed restimulation. The administration of anti-GM-CSF antibodies in conjunction with ragweed diminished T_H2-associated cytokine production. These responses were amplified by enriching the airway microenvironment with GM-CSF. Under these conditions, all T_H2-associated immune-inflammatory responses, as well as the clinical responses, were considerably enhanced. To investigate the mechanism underlying these effects, we examined lung mononuclear cells by means of flow cytometry and detected a substantial expansion of antigen-presenting cells, particularly dendritic cells, as well as a substantially increased activation of these antigen-presenting cells, as demonstrated by the expression of B7 molecules, particularly B7.2.

Conclusion: GM-CSF plays an important role in the generation of allergic immune-inflammatory responses to ragweed. (*J Allergy Clin Immunol* 2003;111:1076-86.)

Key words: Allergic airway inflammation, asthma, experimental model, GM-CSF, murine clinical symptoms, ragweed, T_H2

Conventional models of allergic airway inflammation involve intraperitoneal administration of ovalbumin (OVA) in conjunction with a potent chemical adjuvant (generally aluminum hydroxide) to generate unequivocal allergic airways inflammation. As a consequence of this approach, these systems are intrinsically weak with respect to elucidating the subtle immunologic mechanisms that naturally elicit and modulate allergic sensitization. Moreover, it is remarkable that there is such a limited amount of animal model-derived data utilizing aeroallergens other than OVA, a truly innocuous protein.

In the present study we have investigated the effect of respiratory mucosal exposure to ragweed on the generation of allergic responses. Our data demonstrate that although repeated intranasal exposure to ragweed alone elicits T_H2-type sensitization, airway eosinophilic inflammation is minimal in the short term and absent in the long term on ragweed re-exposure. Conversely, the delivery of ragweed to an airway microenvironment enriched with GM-CSF, which is achieved by means of either multiple coadministrations of recombinant GM-CSF or a single delivery of an adenoviral vector carrying the GM-CSF transgene, results in considerable eosinophilic airway inflammation and ragweed-specific T_H2 memory responses. These findings suggest that the presence of GM-CSF in the airway microenvironment influences not only the degree but also the evolution of the adaptive immune response to ragweed.

METHODS

Animals

Female BALB/c mice (6-8 weeks old) were purchased from Charles River Laboratories (Montreal, Quebec, Canada). The mice were housed in a specific-pathogen-free environment under a 12-hour light-dark cycle. All experiments described here were approved by the Animal Research Ethics Board of McMaster University.

Protocols of respiratory mucosal sensitization

I. Exposure protocol with Ad/GM-CSF. A replication-deficient human type 5 adenoviral construct carrying mouse GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF)¹ was delivered

From ^athe Department of Pathology and Molecular Medicine and the Centre for Gene Therapeutics, Division of Respiratory Diseases and Allergy, McMaster University, Hamilton, and ^bMillennium Pharmaceuticals Inc, Cambridge.

Supported by the Canadian Institutes of Health Research. Elizabeth C. Cates is supported by an Ontario Graduate Scholarship.

Received for publication September 25, 2002; revised January 31, 2003; accepted for publication February 13, 2003.

Reprint requests: Manel Jordana, MD, PhD, HSC-4H21, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St W, Hamilton, Ontario, L8S 3Z5, Canada.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1460

Abbreviations used

Ad:	Adenoviral vector
APC:	Antigen-presenting cell
B:	Coca's buffer
BAL:	Bronchoalveolar lavage
OVA:	Ovalbumin
PE:	Phycoerythrin
rGM-CSF:	Recombinant murine GM-CSF
RW:	Ragweed

intranasally to isoflurane-anesthetized mice 24 hours before the first ragweed exposure. Ad/GM-CSF was administered at a dose of 3×10^7 pfu in 30 μ L of PBS (two 15- μ L administrations 5 minutes apart), as previously described.² Over a period of 7 consecutive days (days 0-6) after Ad/GM-CSF exposure, isoflurane-anesthetized mice were administered 200 μ g of ragweed (RW) protein extract (LPS free; Greer Laboratories, Lenoir, NC) intranasally (dropwise, alternating between nostrils) in a volume of 10 μ L. The ragweed was resuspended in Coca's buffer as per the method of Sur et al³ and passed through a 0.2- μ m filter (Pall Gelman Laboratory, Ann Arbor, Mich).

II. Exposure protocol with recombinant mouse GM-CSF. Recombinant mouse GM-CSF (rGM-CSF; Biosource International, Camarillo, Calif) was used in specific instances in place of the adenoviral construct. In these cases 1 μ g of rGM-CSF was delivered intranasally in 15 μ L of PBS daily for 5 days, followed by administration of RW (as described above, days 0-4). Only RW was administered in the last 2 days of the protocol.

III. Recall challenge (long-term) protocol. After complete resolution of the initial airways inflammation (occurring around day 30), mice were subjected to an in vivo rechallenge with RW. Mice were re-exposed to 200 μ g of RW administered intranasally daily for 2 consecutive days.

Clinical assessment

The intensity of the symptoms exhibited by RW- and GM-CSF/ragweed-exposed mice was determined to be either mild or intense on the basis of the duration of symptoms and the degree of disruption of normal activity. This classification of intensity was made in the following manner:

Sneezing. Mild sneezing was defined as lasting for less than 1 minute, disrupting the normal activity of the animal, or both (ie, it was occasional). Intense sneezing was defined as significantly disrupting the activity of the mice. In this case mice sneezed constantly or in several short bursts (lasting approximately 10 seconds), or sneezing remained occasional but lasted considerably longer (up to 2 minutes).

Nose rubbing. Mild nose rubbing was defined as often, but not always, coupled with sneezing. It did not overtly disrupt the normal behavior of the mice.

Labored breathing. Mild labored breathing was defined as short lived (<2 minutes) and intermittent in nature. In the case of labored breathing, mice assume a hunched posture, and the effort required to breathe is observable (ie, there is a pronounced abdominal component). Mice assume this posture intermittently after short periods of normal activity and cannot be moved with gentle prodding. Intense labored breathing was defined as distressed posture of a mouse maintained for a longer period of time (≥ 30 seconds), with several minutes passing before normal activity was resumed.

Collection and measurement of specimens

At various time points, mice were killed, bronchoalveolar lavage (BAL) was performed, and differential cell counts were determined according to a previously reported protocol.⁴ In addition, blood was collected by means of retro-orbital bleeding. Serum was obtained

by means of centrifugation after incubating whole blood for 30 minutes at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin, Congo red, and Alcian blue (pH 2.5).

Splenocyte culture

Spleens were harvested into sterile tubes containing sterile HBSS (Life Technologies Inc, Burlington, Ontario, Canada). Splenocytes were isolated as previously described.⁴ Cells (8×10^5 cells/well) were cultured in medium alone, medium with Coca's buffer, or medium with 1.6 μ g of RW per well (as determined on the basis of dose response, data not shown) in flat-bottom 96-well plates (Becton Dickinson, Franklin Lakes, NJ). After 5 days of culture, supernatants were harvested for cytokine measurements.

Cytokine and immunoglobulin measurement

ELISA kits for mouse IL-4, IL-5, and IL-13 were purchased from R&D Systems (Minneapolis, Minn). These assays have thresholds of detection of less than 2, 1.5, and 7 pg/mL, respectively.

RW-specific IgG1 and total IgE levels were measured in the following manner: 96-well plates were coated overnight at 4°C with 100 μ L of a 2 μ g/mL solution of RW in Coca's buffer. Coated wells were blocked with 1% BSA in PBS for 2.5 hours at room temperature. The ELISA was performed by using 4 serial dilutions of each serum sample prepared with the reagent diluent (Tris-Tween solution with 0.05% BSA). These dilutions were 1:20, 1:200, 1:2000, and 1:20,000 and 1:2, 1:20, 1:100, and 1:500 for RW-specific IgG1 and total IgE, respectively. Antibody titers were calculated by using the following formula:

$$1/(X \cdot \text{ODX}) \cdot 0.05,$$

where X equals the dilution factor closest to but greater than twice the background optical density reading and ODX is the optical density reading of X.

Anti-GM-CSF-neutralizing antibody treatment

In specific instances mice were treated with polyclonal goat anti-mouse GM-CSF-neutralizing antibody (μ GM-CSF, R&D Systems) to block the effects of endogenous GM-CSF. In these cases mice received 50 μ g of μ GM-CSF in a 10- μ L volume, concurrent with their RW exposure, for the first 3 days of the exposure protocol. The dose of μ GM-CSF was reduced to 25 μ g in a 10- μ L volume for the last 4 days of the exposure protocol. Polyclonal goat IgG (R&D Systems) was used as a control in these experiments.

Isolation of mRNA from tissues and generation of cDNA

Thoracic lymph nodes and lungs were collected, placed in RNeasy lysis buffer (Qiagen, Crawfordsville, Ind), and stored at -20°C for future RNA extraction. Total RNA was extracted by using RNeasy spin columns (Qiagen). Genomic DNA was eliminated by using the DNA-free kit from Ambion. Finally, cDNA was generated by using the RETROscript kit from Ambion and either random decamers or oligo-dT as primers.

Real-time quantitative PCR (TaqMan)

For real-time quantitative PCR, primers and FAM-labeled probes were designed for GATA-3 and T1/ST2 with PrimerExpress version 1.5 software (Applied Biosystems, Foster City, Calif). GATA-3 primers and probes were used as described in the study by Ritz et al.⁵ In the case of T1/ST2, the forward primer was 5'-ACGCCAGGCAAGTGTCTTACA-3' the reverse primer was 5'-CACCGACTGAGCAACTCAGCTA-3' and the probe was 5'-

TTGAATTTTGCCAATAGATATGAGCCCTGCC-3'. For IL-4, IL-5, and IL-13, primers and FAM-labeled probes and GAPDH primers and VIC-labeled probes were obtained from Applied Biosystems. PCR was done by using the TaqMan Universal PCR Master Mix in the ABI Prism 6700 Sequence Detection System operated by Sequence Detector version 1.7 software (Applied Biosystems). Gene expression is quantified relative to the expression of GAPDH, with naive samples defined as 1, and all other values are plotted relative to them.

Flow cytometric analysis

Flow cytometric analysis was performed on lung cells isolated as described previously.⁴ The cells were then stained with a panel of fluorescently conjugated antibodies (PharMingen, Mississauga, Ontario, Canada). The following antibodies were used: anti-CD11c-FITC, anti-MHC II-FITC, anti-CD4-FITC, anti-B220-FITC, anti-CD69-phycoerythrin (PE), anti-CD11b-PE, anti-B7-1-PE, anti-CD3-PE, anti-CD3-CY, anti-MHC II-biotin, anti-B7-2-biotin, and anti-CD4-CY. Appropriate isotype controls were also purchased from PharMingen. The anti-T1/ST2-FITC antibody was provided by Millennium Pharmaceuticals Inc (Cambridge, Mass). To minimize nonspecific binding, 10^6 cells were preincubated with Fc block (CD16/CD32, PharMingen). For each antibody combination, 10^6 cells were incubated with mAbs at 0°C to 4°C for 30 minutes; the cells were then washed and treated with second-stage reagents. Streptavidin PerCP (Becton Dickinson Immunocytometry Systems, San José, Calif) was used as a second-step reagent for detection of biotin-labeled antibodies. Titration was used to determine the optimal concentration for each antibody. Cells were fixed in 1% paraformaldehyde and counted on a FACScan (Becton Dickinson and Co). Analysis was performed with WinMDI software (The Scripps Research Institute, La Jolla, Calif). A total of 100,000 events were acquired. The percentages of the various cell types, obtained by means of flow cytometry, were subsequently used to approximate the number of each cell type on the basis of the total number of cells isolated per mouse, and data are expressed as fold increases over data from naive mice, with the values obtained from naive mice being represented as 1.

Data analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed by using SigmaStat version 2.03 software. Differences were considered significant at a *P* value of less than .05 by means of ANOVA with the Fisher post hoc test, where applicable.

RESULTS

Clinical assessment

Mice receiving RW alone experienced clinical symptoms that consisted of mild sneezing and face rubbing. Clinical manifestations were observed when the mice had recovered from anesthesia. Symptoms began consistently as early as the third RW administration in all groups. In contrast to mice that received RW alone, mice that received RW in conjunction with Ad/GM-CSF (GM/RW) displayed symptoms that increased in severity with subsequent RW administrations. These animals typically showed, toward the end of the exposure protocol, severe sneezing and, during the last 2 days of the exposure protocol, labored breathing. Labored breathing was often mild but was found to be severe in older animals. No symptoms were observed in mice that received Coca's buffer in the context of Ad/GM-CSF (GM/B) or rGM-CSF alone.

Inflammatory responses in the airway

There were no significant differences between naive mice and mice treated in vivo with GM/B. Exposure to RW alone resulted in an increase in mononuclear cells of approximately 2-fold and a level of eosinophilia of 6.6% compared with 0% in naive mice (Fig 1, *A* and *B*). These increases, however, did not reach statistical significance. In contrast, when mice received GM/RW, we observed dramatic changes in the inflammatory infiltrate. Under these conditions, the total number of cells increased to 3 million from approximately 500,000 in naive mice (data not shown), and the cell distribution consisted of 69% mononuclear cells and 20% eosinophils (Fig 1, *A* and *B*).

Groups of mice were sensitized with rGM-CSF in place of Ad/GM-CSF to eliminate the potentially confounding effect of the adenoviral construct (Fig 1, *C* and *D*). In mice that received rGM-CSF alone (rGM), we observed a significant increase in the total cell number (to approximately 3.5 million), which was entirely mononuclear in nature (data not shown). Mice that received rGM-CSF and RW (rGM/RW) exhibited a similar increase in the total number of cells (to approximately 5 million); however, eosinophils comprised approximately 36% of the cells in this infiltrate.

Histology

As can be seen in Fig 2, *A* and *B*, there was no evidence of inflammation in naive mice or mice exposed to GM/B. Interestingly, and in agreement with our BAL findings, lungs from mice exposed to RW alone displayed a very mild inflammation in some peribronchial and perivascular regions, including the presence of a few eosinophils (Fig 2, *C*). In contrast, mice exposed to GM/RW presented with severe peribronchial and perivascular inflammation, as well as inflammation of the surrounding parenchyma (Fig 2, *D*). This was accompanied by goblet cell hyperplasia, as well as mucous production (Fig 2, *E*).

Evidence of T_H2 sensitization in RW- and GM/RW-treated animals

Because the draining lymph nodes are the preeminent site for the generation of a primary immune response, we examined the thoracic lymph nodes for evidence of T_H2 differentiation. Specifically, we assessed the relative expression of the T_H2 effector molecules IL-4, IL-5, and IL-13 by means of TaqMan. As shown in Fig 3, *A*, *C*, and *E*, considerable upregulation of each of these genes was observed in mice that had been exposed to GM/RW in vivo. Specifically, we observed a 90-fold increase in IL-4, a 15-fold increase in IL-5, and a 40-fold increase in IL-13 mRNA expression. Importantly, an upregulation of these cytokine genes was also seen in mice that were exposed to RW alone in vivo. In this case we observed an 80-fold increase in IL-4, a 17-fold increase in IL-5, and a 15-fold increase in IL-13.

Splenocytes were isolated from mice exposed to GM/B, RW, or GM/RW and stimulated with RW in vitro to determine whether ragweed-specific T_H2 cytokine

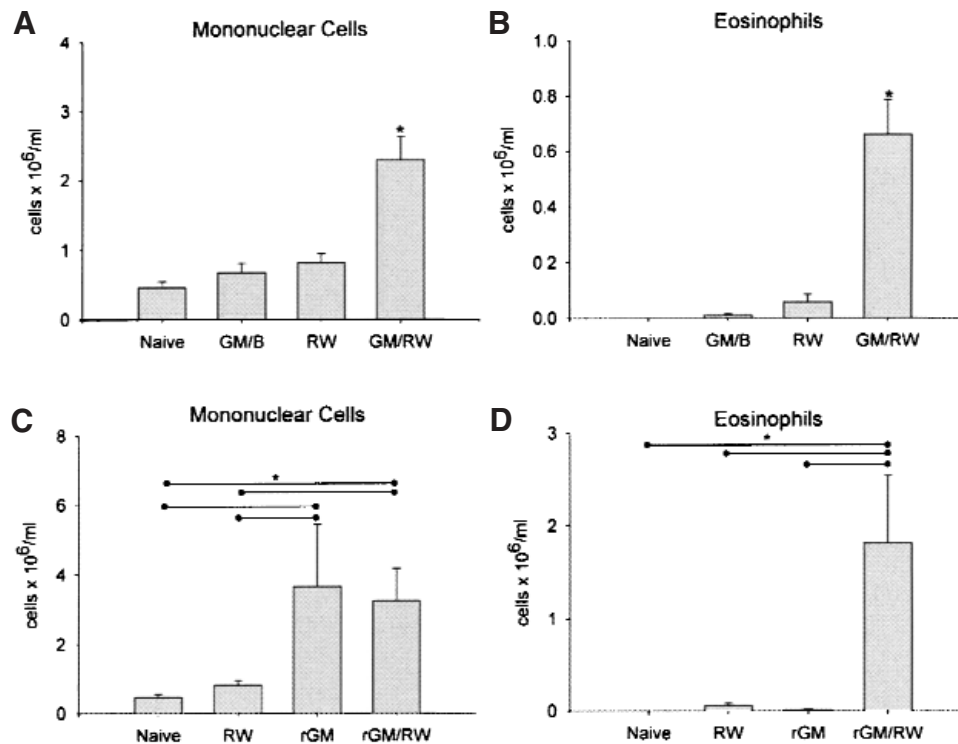


FIG 1. Airway inflammation in the BAL fluid of BALB/c mice exposed to ragweed extract (RW) or Coca's buffer (B) intranasally. Mice were subjected to exposure protocol I (A and B) and exposure protocol II (C and D) and killed 72 hours after the last exposure (n = 5-10, *P < .05).

production occurs in animals exposed to ragweed in vivo. We detected significant T_H2 cytokine production by splenocytes from GM/RW-treated animals (Fig 3, B, D, and F). RW stimulation did not elicit production of T_H2 cytokines by splenocytes obtained from mice exposed to GM/B in vivo. Interestingly, RW stimulation did induce significant production of IL-13 by splenocytes from mice exposed to RW alone in vivo (Fig 3, F). A similar trend was observed for IL-4 and IL-5 (Fig 3, B and D), although the difference, compared with the levels seen in naive mice, did not reach statistical significance. Splenocytes that were unstimulated or exposed to Coca's buffer did not produce any of these cytokines.

RW-specific IgG1 and total IgE levels were measured in the sera of mice exposed to all of the in vivo treatment conditions. As shown in Table I, mice exposed to GM/RW produced significantly greater amounts of these immunoglobulins compared with mice in the other treatment groups.

Evidence of T_H2 effector function in the lungs of sensitized mice

To seek evidence of T_H2 effector activity in the target organ (ie, the lung), we examined the levels of activated T_H cells (CD3⁺/CD4⁺/CD69⁺) and T_H2 effector cells (CD3⁺/CD4⁺/T1ST2⁺) by means of flow cytometry at day 9 of the exposure protocol. RW alone led to a minimal expansion of both activated and effector T cells, 2.3- and 2.1-fold increases over values seen in naive mice, respec-

tively (Fig 4, A and B). rGM-CSF led to a greater but still modest expansion of these cells, a 3.5-fold increase over that seen in naive mice of CD4⁺/CD69⁺ cells and a 3.1-fold increase in CD4⁺/T1ST2⁺ cells. Mice exposed to rGM/RW showed a 5.5-fold increase in the number of activated CD4⁺ cells and a 10.6-fold increase over naive mice in terms of T_H2 effector cells.

Effector activity in the lung was assessed by using TaqMan to examine the levels of expression of IL-4 and IL-13 mRNA. As shown in Fig 4, C and D, we detected a significant upregulation of each of these genes in mice that had been exposed to GM/RW in vivo; specifically, we observed 35- and 130-fold increases in IL-4 and IL-13, respectively. In mice treated with RW alone, a much lower but still significant level of upregulation was observed that was approximately 13-fold for IL-4 and 40-fold for IL-13.

Flow cytometric analysis of T cells and antigen-presenting cells in the lungs of mice sensitized using rGM-CSF

Lung cells were isolated for flow cytometric analysis at day 9 of the exposure protocol to further define the types of cells present at the effector site. Cells were labeled to allow for an evaluation of changes in the subsets of antigen-presenting cells (APCs). As shown in Fig 5, exposure to RW alone had a very small effect on these cells. We observed a 2.9-fold increase over numbers in

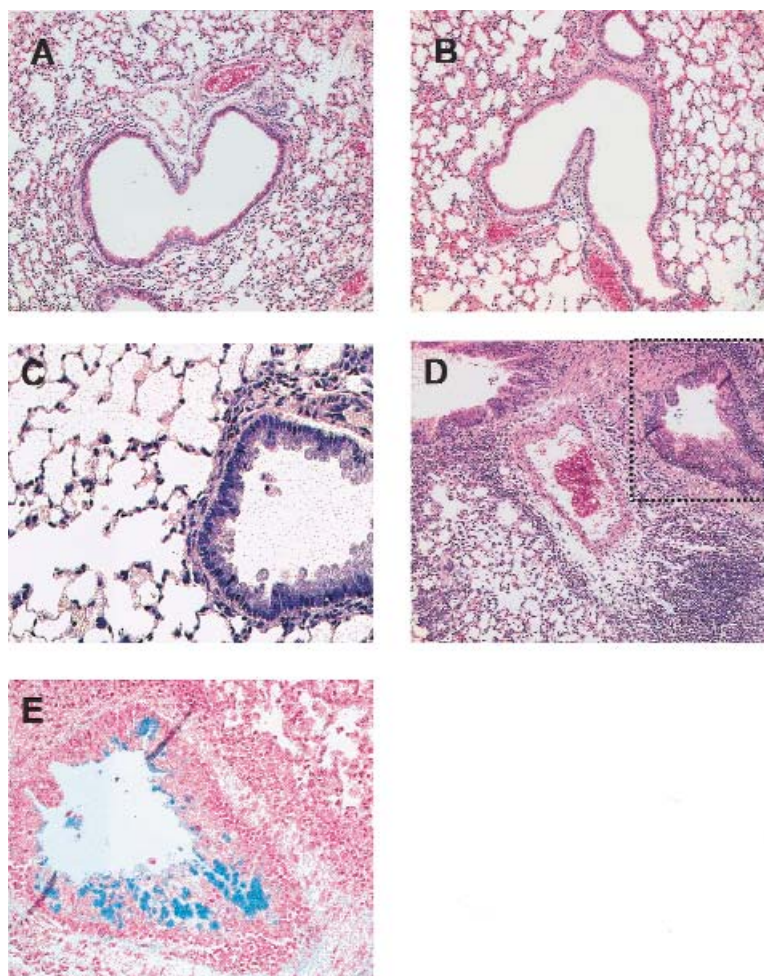


FIG 2. Light photomicrograph of paraffin-embedded sections of lung tissues obtained 72 hours after completion of exposure protocol I. Sections were stained with hematoxylin and eosin (**A**, **B**, and **D**), Congo Red (**C**), or Alcian Blue (pH 2.5; **E**). Panels represent naive mice (Fig 2, **A**), mice exposed to GM/B (Fig 2, **B**), mice exposed to RW alone (Fig 2, **C**), and mice exposed to GM/RW (Fig 2, **D** and **F**). Original magnification was 63 $\times$ for Fig 2, **A**, **B**, and **D**; 160 $\times$ for Fig 2, **C**; and 126 $\times$ for Fig 2, **E**.

TABLE I. Serum immunoglobulin levels in BALB/c mice exposed to ragweed in the context of GM-CSF expression

Immunoglobulins	In vivo treatment conditions			
	Naive	GM/B	RW	GM/RW
RW-specific IgG1	0 $\pm$ 0	0 $\pm$ 0	41.2 $\pm$ 26.6	1233.6 $\pm$ 368.2*
Total IgE	103.1 $\pm$ 25.5	55.0 $\pm$ 16.0	97.6 $\pm$ 2.1	899.5 $\pm$ 395.3*

Mice were killed at day 9 of exposure protocol I, and the immunoglobulin content in the sera was measured by means of ELISA. Data are expressed in units per milliliter (mean $\pm$ SEM; n = 4). Statistical analysis was performed by using 1-way ANOVA with the Fisher post hoc test: *significant from all groups, $P < .05$.

naive mice of all APCs (on the basis of MHC II expression), which corresponded to a 1.4-fold increase in B cells (MHC II⁺/B220⁺), a 3.1-fold increase in macrophages (MHC II⁺/CD11b⁺), and a 2.1-fold increase in dendritic cells (MHC II⁺/CD11c⁺). Interestingly, rGM-CSF led to a greater expansion of APCs, notably dendritic cells. Compared with naive mice, we observed a 2.2-fold increase in B cells, a 4.9-fold increase in macrophages, and an 8.6-fold increase in dendritic cells.

When rGM-CSF and RW were delivered together, the effects appeared to be synergistic in nature. There was a 10.9-fold increase over naive mice in all APCs, which was primarily due to an expansion of macrophages and dendritic cells. These cell types increased by 11.4-fold and 7.5-fold, respectively, whereas B cells only increased by 2.5-fold. APC activation was assessed on the basis of the expression of the costimulatory molecules B7-1 and B7-2. RW alone had a very modest effect on APC activa-

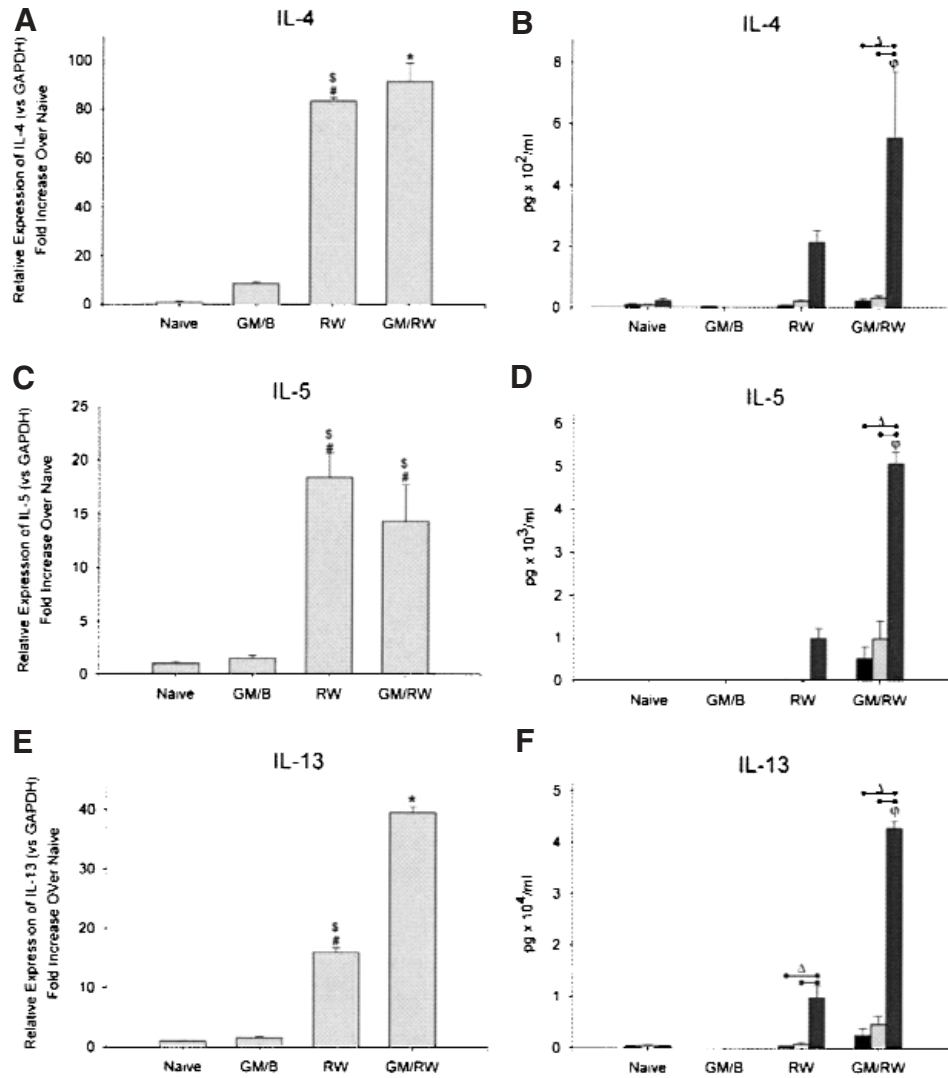


FIG 3. A, C, and E, Evidence of T_H2 effector activity in the thoracic lymph nodes of mice exposed to RW in the context of GM-CSF expression. RNA was isolated from thoracic lymph nodes on day 9 of exposure protocol I, and the relative expression of IL-4, IL-5, and IL-13 was assessed by means of TaqMan (mean $\pm$ SEM of triplicate measurements; *significantly different from all groups, $P < .05$; #significant from naive, $P < .05$; \$significant from GM/B, $P < .05$). B, D, and F, In vitro production of RW-specific IL-4, IL-5, and IL-13 by splenocytes. Cells were cultured in RPMI alone (black bars), RPMI plus Coca Buffer (light gray bars), or RPMI plus 1.6 μ g of RW per well (dark gray bars; $n = 1-4$; Δ significant differences within groups, $P < .05$; □ significantly different from all groups after RW stimulation, $P < .05$).

tion. B7-1 and B7-2 were upregulated 3-fold and 4-fold, respectively, over numbers in naive mice on MHC II⁺ cells. As with the expansion of the various cell types, rGM-CSF alone had a greater effect on the activation of these cells than did RW alone, a 4.6-fold increase in B7-1 and a 9.5-fold increase in B7-2. When mice received rGM/RW, the activation of APCs was increased dramatically, notably with respect to B7-2. Under this condition, the expression of B7-1 and B7-2 was increased to 12.9-fold and 33.5-fold over the expression seen in naive mice, respectively.

In vivo rechallenge of mucosally sensitized mice

In some groups of mice, the inflammation caused by the initial exposure to GM/RW was allowed to resolve, and the mice were rechallenged with RW approximately 30 days later. Resolution of inflammation was determined by killing mice at various time points over the exposure protocol and assessing the extent of eosinophilia both in the BAL fluid (Fig 6, A) and in peripheral blood (data not shown). By day 30, the BAL fluid was

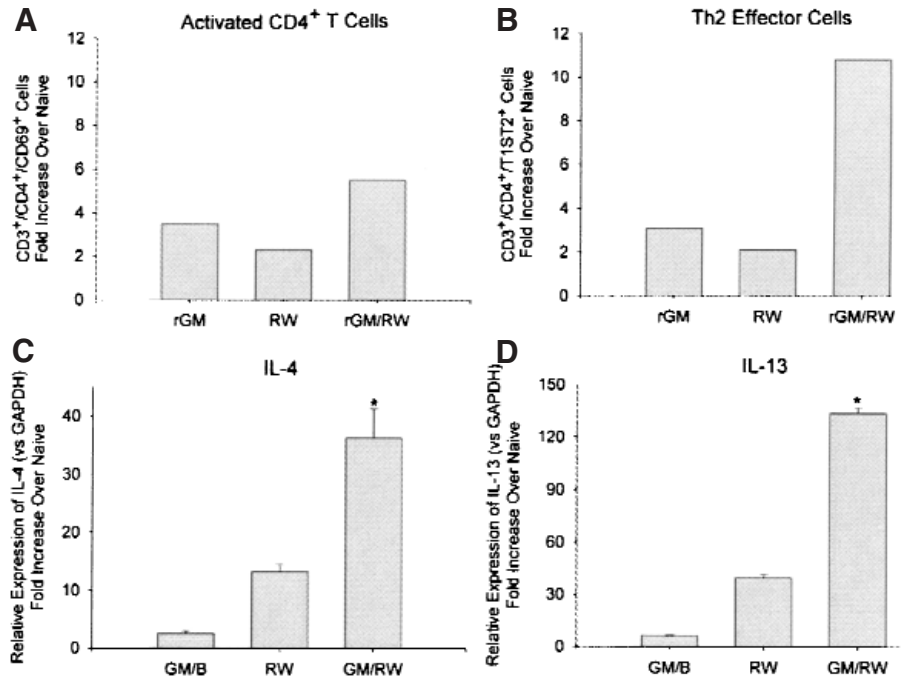


FIG 4. Evidence of T_H2 effector activity in the lungs of mice exposed to RW in the context of GM-CSF expression. **A** and **B**, Fold increase over values in naive mice of CD4⁺/CD69⁺ T cells and CD4⁺/T1ST2⁺ T cells, respectively, as assessed by means of flow cytometry. Cells were obtained on day 9 of exposure protocol I. **C** and **D**, Relative expression of IL-4 and IL-13 mRNA from lung tissue. RNA was isolated from lung tissue on day 9 of exposure protocol I, and IL-4 and IL-13 relative expression was assessed by means of Taq-Man (mean $\pm$ SEM of triplicate measurements; * $P < .05$).

found to be devoid of eosinophils, and total cell counts were back to naive levels (data not shown).

RW re-exposure resulted in the recapitulation of the initial inflammatory response. It consisted of approximately 3 million cells in the BAL fluid and a peak in BAL eosinophilia of 13% occurring 120 hours after rechallenge (Fig 6, *B* and *D*).

In vitro cytokine production by splenocytes from mice treated in vivo with an anti-GM-CSF-neutralizing antibody

A group of mice was treated with a goat anti-mouse GM-CSF-neutralizing antibody concurrent with the ragweed exposures to examine the role of GM-CSF in the responses elicited by ragweed alone more closely. Splenocytes from these mice showed a significant decrease in their production of IL-4 and IL-13 when restimulated with RW in vitro compared with splenocytes from mice that received RW alone or RW plus control antibody in vivo. Compared with mice treated with control antibody, $\square$ GM-CSF treatment resulted in a 74.5% decrease in IL-4 production (241.8 vs 61.7 pg/mL) and an 89% reduction in IL-13 production (46.5 vs 5.1 ng/mL). IL-5 production was decreased by 35% (1925.7 vs 1252.3 pg/mL), but this did not reach statistical significance.

DISCUSSION

Ragweed is an aeroallergen of considerable clinical relevance, particularly in North America. Despite its importance, there is a limited amount of data available with respect to airway immune-inflammatory responses to ragweed in murine systems. Although a number of groups have established models of ragweed-induced allergic conjunctivitis⁶⁻¹² and eosinophilic peritonitis,¹³⁻¹⁵ data on airway inflammatory responses to ragweed, as compared with OVA, are surprisingly scarce. A limitation of using OVA in models of allergic airway disease is that it is now well known that repeated passive exposure to aerosolized OVA in mice results in inhalation tolerance.¹⁶⁻¹⁹ This has forced researchers to devise strategies to overcome the intrinsic innocuousness of OVA, such as introducing antigens into the peritoneal cavity in conjunction with powerful chemical adjuvants (eg, aluminum hydroxide). Although these models have been useful in many respects, they further alienate the system from the natural way in which human subjects are exposed and sensitized to allergens. The current body of work surrounding airway inflammatory responses to ragweed makes use predominantly of these conventional models.^{3,20-24} In 1998, our laboratory reported an experimental model of allergic airway inflammation² in which OVA is delivered to the respiratory mucosa in the

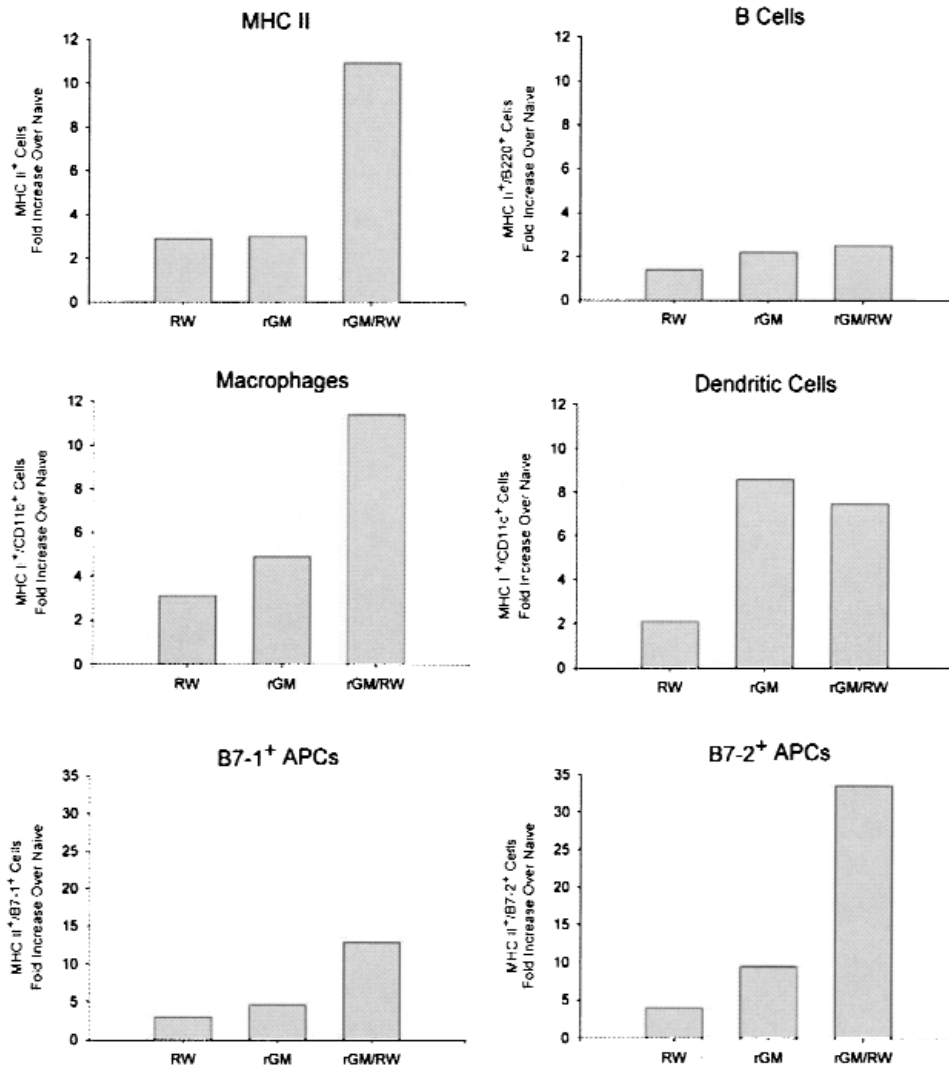


FIG 5. Expansion of APCs in the lungs of mice on day 9 of exposure protocol II. The percentages of the various APCs were assessed by means of flow cytometry on the basis of the expression of the molecules indicated. Activation of the APCs was assessed on the basis of their expression of the costimulatory molecules B7-1 and B7-2.

absence of chemical agents but in conjunction with GM-CSF (a natural adjuvant that has been demonstrated to be relevant in asthma²⁵). We believe that this is a significant advance in modeling both allergen exposure in human subjects and the processes of mucosal sensitization. The present study was performed to investigate immune-inflammatory responses locally and systemically to a purified ragweed extract.

Our data show that, in contrast to OVA, intranasal exposure to ragweed alone elicits T_H2 sensitization. This is supported by evidence of *in vivo* upregulation of T_H2 cytokine expression in the lymph nodes, production of T_H2 cytokines by splenocytes upon RW restimulation *in vitro*, and modest production of RW-specific IgG1. However, this immune response translates into only marginal airway eosinophilic inflammation and mild clinical symptoms that appeared to be limited to the upper air-

way. These low-grade responses were unlikely to have been due to an insufficient exposure protocol because increasing the exposure from 7 to 10 days yielded similar results (data not shown). Importantly, no long-range ragweed-specific memory response was observed in mice exposed to RW alone.

We found it notable that despite clear evidence of a RW-specific immune response, mice exposed to RW alone had only minimal airway inflammation and clinical symptoms. We hypothesized that this apparent discrepancy was the consequence of a relatively weak immune response and reasoned that intervening to expand and further activate the APC compartment would influence the outcome. To achieve this, we enriched the airway microenvironment by using a natural adjuvant with precisely this capability, GM-CSF.²⁶ Our data show that the addition of exogenous GM-CSF to the airway

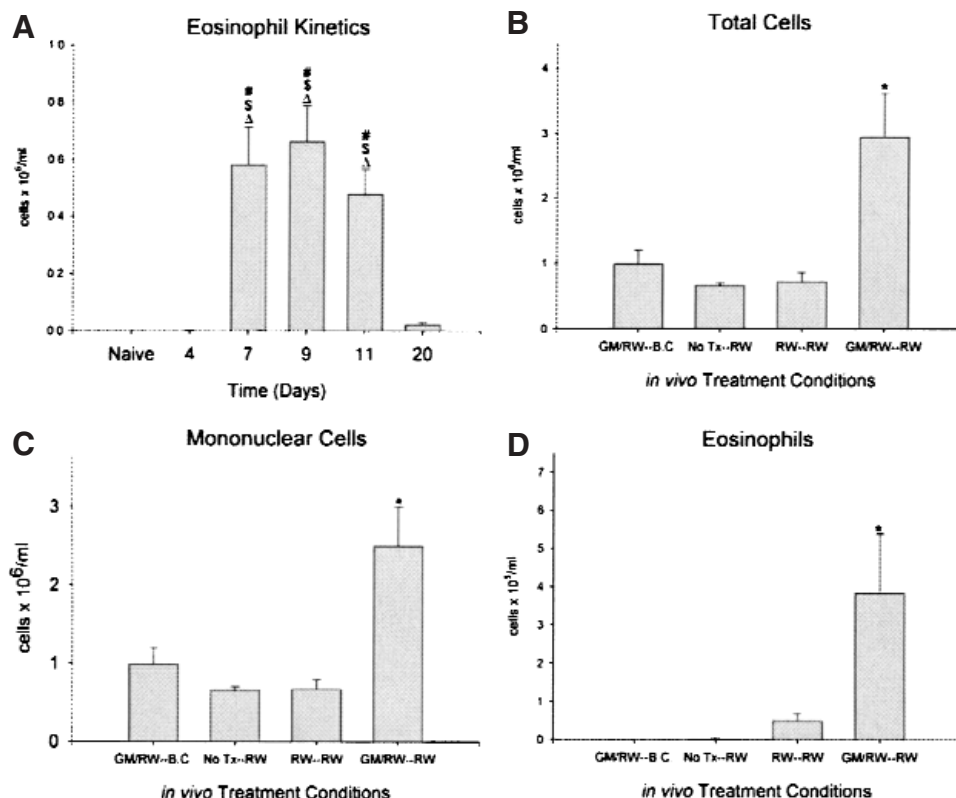


FIG 6. A, Eosinophil kinetics in the BAL fluid of BALB/c mice exposed to GM/RW. Mice were killed at various time points during exposure protocol I, as shown. B-D, In vivo rechallenge of BALB/c mice with RW. Mice were exposed daily for 7 consecutive days to either RW or GM/RW or were left untreated (No Tx). Mice were then subjected to exposure protocol III and killed 5 days after the last challenge ($n = 2-9$; *significant from all, $P < .05$; #significant from naive mice, $P < .05$; \$significant from day 4, $P < .05$; □ significant from day 20, $P < .05$). BC, Before challenge.

through the introduction of a replication-deficient adenoviral vector carrying the GM-CSF transgene markedly enhanced the response to ragweed, as indicated by increased expression of T_H2 cytokines in the thoracic lymph nodes in vivo, increased production of T_H2 cytokines by splenocytes on in vitro recall, and significantly increased serum levels of RW-specific IgG1 and total IgE. Notably, these exposure conditions also led to a robust airway eosinophilic response. To eliminate the potentially confounding effects of the adenoviral vector, we carried out a number of experiments using recombinant GM-CSF, which yielded similar results. The enhanced inflammatory response was associated with a distinct clinical effect. Not only was the severity of clinical symptoms increased, but their nature also changed in that we observed symptoms consistent with reversible lower airway obstruction. In fact, the severity of clinical symptoms in mice exposed to RW in the context of GM-CSF was such that we were compelled to decrease exposure from our customary 10 days (when using OVA) to 7 days.

To evaluate the immune response in the lung more comprehensively and, in particular, to ascertain the effect of the introduction of exogenous GM-CSF to the

airway on the APC compartment, we carried out a detailed flow cytometric analysis. For clarity of interpretation, we performed these experiments with rGM-CSF. Delivery of RW alone resulted in a minimal expansion of all APC subsets, whereas administration of rGM-CSF led to a marked expansion of dendritic cells. Concurrent exposure to GM-CSF and ragweed synergistically expanded the number of macrophages in the lung but did not lead to any further increase in dendritic cells. The rather modest expansion of B cells that we detected is consistent with the finding that these cells play a minimal role in antigen processing and presentation in the lung.²⁷ To evaluate the activation of APCs, we assessed the expression of the costimulatory molecules B7.1 and B7.2. Exposure to rGM/RW led to a substantial increase in the number of APCs expressing these molecules. This increase was remarkable in the case of B7.2, which is in agreement with evidence that B7.2 privileges T_H2 responses.²⁸ The preferential expansion and activation of dendritic cells observed in mice exposed to rGM/RW had an effect on the T-cell compartment, as demonstrated by increases in the number of activated $CD4^+$ T cells and $CD4^+$ T cells expressing T1/ST2 (T_H2 effector cells).²⁹⁻³¹

A comparative appraisal of the responses elicited by exposure to RW alone and ragweed in conjunction with exogenous GM-CSF would initially suggest that GM-CSF does not determine the evolution of the adaptive immune response; rather, it influences the degree of this response and, most importantly, its pathologic effect on the airway. However, that exposure to RW alone produced evidence of T_H2 sensitization led us to consider whether this phenomenon could be mediated by endogenously produced GM-CSF. To address this question, we treated mice with anti-GM-CSF antibodies and assessed the most robust outcome measurement: production of T_H2-associated cytokines upon splenocytes upon RW restimulation. Our data show that IL-4 and IL-13 production by splenocytes from mice treated with anti-GM-CSF were significantly reduced. IL-5 production was also decreased by anti-GM-CSF treatment, but this did not reach statistical significance. These findings support the concept that the default pathway promoted by GM-CSF might indeed be a T_H2 response. Whether GM-CSF can facilitate a T_H1 response might depend on the presence of additional signals in the airway microenvironment; for example, we have previously shown that concurrent gene transfer of GM-CSF and IL-12 to the airway shifts an OVA-specific immune response from T_H2 to T_H1.³²

Our study raises a number of questions concerning the effect of GM-CSF on the development of allergic airway diseases. First, many lung/airway cells are able to produce GM-CSF, including epithelial cells, fibroblasts, and macrophages.³³⁻³⁶ Although, to our knowledge, there is no precedent for the ability of RW to induce the production of GM-CSF by lung/airway cells, there is evidence that other allergens, notably house dust mite, do so.³⁷ Clearly, delineation of the potential cellular sources of GM-CSF upon RW exposure and of the molecular mechanisms underlying this interaction is an important venue of future investigation. Moreover, our data compel us to consider the effect of allergen-independent production of GM-CSF on the development of allergic airway disease. For example, there is abundant evidence that a number of pollutants can induce GM-CSF production by human airway cells.^{38,39} Thus clinically overt airway disease to allergens, such as RW, might be influenced by concurrent exposures that establish an airway microenvironment that amplifies these responses. We believe that the model we have developed provides a useful foundation to explore these questions.

We thank Joanna deJong, Stacey A. Ritz, Clinton Robbins, Theresa Shea, Martin R. Stämpfli, and Ryan E. Wiley for both their assistance with experiments and their critical appraisal of the manuscript. We further acknowledge the generous administrative assistance of Mary Kiriakopoulos.

REFERENCES

- Xing Z, Ohkawara Y, Jordana M, Graham FL, Gaudie J. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* 1996;97:1102-10.
- Stämpfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei X-F, Snider DP, et al. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J Clin Invest* 1998;102:1704-14.
- Sur S, Lam J, Bouchard P, Sigounas A, Holbert D, Metzger WJ. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *J Immunol* 1996;157:4173-80.
- Gajewska BU, Alvarez D, Vidric M, Goncharova S, Stämpfli MR, Coyle AJ, et al. Generation of experimental allergic airways inflammation in the absence of draining lymph nodes. *J Clin Invest* 2001;108:577-83.
- Ritz SA, Cundall MJ, Gajewska BU, Alvarez D, Gutierrez-Ramos J-C, Coyle AJ, et al. Granulocyte macrophage colony-stimulating factor-driven respiratory mucosa sensitization induces Th2 differentiation and function independently of interleukin-4. *Am J Respir Cell Mol Biol* 2002;27:428-35.
- Merayo-Llones J, Zhao TZ, Dutt JE, Foster CS. A new murine model of allergic conjunctivitis and effectiveness of nedocromil sodium. *J Allergy Clin Immunol* 1996;97:1129-40.
- Magone MT, Chan C-C, Rizzo LV, Kozhich AT, Whitcup SM. A novel model of allergic conjunctivitis. *Clin Immunol Immunopathol* 1998;87:75-84.
- Whitcup SM, Chan C-C, Kozhich AT, Magone MT. Blocking ICAM-1 (CD54) and LFA-1 (CD11a) inhibits experimental allergic conjunctivitis. *Clin Immunol* 1999;93:107-13.
- Strauss EC, Larson KA, Brenneise I, Foster CS, Larsen GR, Lee NA, et al. Soluble P-selectin glycoprotein ligand 1 inhibits ocular inflammation in a murine model of allergy. *Invest Ophthalmol Vis Sci* 1999;40:1336-42.
- Magone MT, Whitcup SM, Fukushima A, Chan C-C, Silver PB, Rizzo LV. The role of IL-12 in the induction of late-phase cellular infiltration in a murine model of allergic conjunctivitis. *J Allergy Clin Immunol* 2000;105:299-308.
- Magone MT, Chan C-C, Beck L, Whitcup SM, Raz E. Systemic or mucosa administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. *Eur J Immunol* 2000;30:1841-50.
- Miyazaki D, Liu G, Clark L, Ono SJ. Prevention of acute allergic conjunctivitis with immunostimulatory DNA sequences. *Invest Ophthalmol Vis Sci* 2000;41:3850-5.
- Walker C, Checkel J, Cammisuli S, Leibson PJ, Gleich GJ. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J Immunol* 1998;161:1962-9.
- Robinson SD, Frenette PS, Rayburn H, Cumiskey M, Ullman-Cullere M, Wagner DD, et al. Multiple, targeted deficiencies in selectins reveal a predominant role for P-selectin in leukocyte recruitment. *Proc Natl Acad Sci U S A* 1999;96:11452-7.
- Kaneko M, Schimming A, Gleich GJ, Kita H. Ligation of IgE receptors causes an anaphylactic response and neutrophil infiltration but does not induce eosinophilic inflammation in mice. *J Allergy Clin Immunol* 2000;105:1202-10.
- Holt PG, Batty JE, Turner KJ. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 1981;42:409-17.
- McMenamin C, Pimm C, McKersy M, Holt PG. Regulation of IgE responses to inhaled antigen in mice by antigen-specific T cells. *Science* 1994;265:1869-71.
- Seymour BWP, Gershwin LJ, Coffman RL. Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require CD8+ or T cell receptor (TCR)- $\alpha\beta$ T cells or interferon (IFN)- γ in a murine model of allergen sensitization. *J Exp Med* 1998;187:721-31.
- Swirski FK, Gajewska BU, Alvarez D, Ritz SA, Cundall MJ, Cates EC, et al. Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin Exp Allergy* 2002;32:411-21.
- Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284-93.
- Wild JS, Sigounas A, Sur N, Siddiqui MS, Alam R, Kurimoto M, et al. IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. *J Immunol* 2000;164:2701-10.
- Shibata Y, Foster LA, Bradfield JF, Myrvik QN. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. *J Immunol* 2000;164:1314-21.
- Van Scott MR, Justice P, Bradfield JF, Enright E, Sigounas A, Sur S. IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L667-74.

24. Justice PJ, Shibata Y, Sur S, Mustafa MF, Van Scott MR. IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L363-8.
25. Ritz SA, Stämpfli MR, Davies DE, Holgate ST, Jordana M. On the generation of allergic airway diseases: from GM-CSF to Kyoto. *Trends Immunol* 2002;23:396-402.
26. Daro E, Butz E, Smith J, Teepe M, Maliszewski C, McKenna H. Comparison of the functional properties of murine dendritic cells generated in vivo with flt3 ligand, gm-csf and flt3 ligand plus gm-csf. *Cytokine* 2002;17:119-30.
27. Masten BJ, Lipscomb MF. Comparison of lung dendritic cells and B cells in stimulating naive antigen-specific T cells. *J Immunol* 1999;162:1310-7.
28. Delgado M, Leceta J, Sun W, Gomariz R, Ganea D. VIP and PACAP induce shift to a Th2 response by upregulating B7.2 expression. *Ann NY Acad Sci* 2000;921:68-78.
29. Xu BD, Chan WL, Leung BP, Huang F-P, Wheeler R, Piedrafita D, et al. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med* 1998;187:787-94.
30. Lohning M, Stroehmann A, Coyle AJ, Grogan JL, Lin S, Gutierrez-Ramos J-C, et al. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Immunology* 1998;95:6930-5.
31. Coyle AJ, Lloyd C, Tian J, Nguyen T, Eriksson C, Wang L, et al. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosa immune responses. *J Exp Med* 1999;190:895-902.
32. Stämpfli MR, Neigh GS, Wiley RE, Cwiartka M, Ritz SA, Hitt M, et al. Regulation of allergic mucosa sensitization by interleukin-12 gene transfer to the airway. *Am J Respir Cell Mol Biol* 1999;21:317-26.
33. Broudy V, Kaushansky K, Segal G, Harlan J, Adamson J. Tumor necrosis factor 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A* 1987;83:7467-71.
34. Lee M, Kaushansky K, Ralph P, Ladner M. Differential expression of M-CSF, G-CSF and GM-CSF by human monocytes. *J Leukoc Biol* 1990;47:275-82.
35. Marini M, Soloperto M, Mezetti M, Fasoli A, Mattoli S. Interleukin 1 binds to specific receptors on human bronchial epithelial cells and upregulates granulocyte-macrophage colony-stimulating factor synthesis and release. *Am J Respir Cell Mol Biol* 1991;4:519-24.
36. Zucali J, Dinarello C, Oblon D, Gross M, Anderson L, Weiner R. Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E2. *J Clin Invest* 1986;77:1857-63.
37. King C, Brennan S, Thompson P, Stewart G. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998;161:3645-51.
38. Devalia J, Bayram H, Abdelaziz M, Sapsford R, Davies R. 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* 1999;118:437-9.
39. Bayram H, Sapsford R, Abdelaziz M, Khair O. Effect of ozone and nitrogen dioxide on the release of proinflammatory mediators from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients in vitro. *J Allergy Clin Immunol* 2001;107:287-94.