Interleukin 4, but Not Interleukin 5 or Eosinophils, Is Required in a Murine Model of Acute Airway Hyperreactivity

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Summary

Reversible airway hyperreactivity underlies the pathophysiology of asthma, yet the precise mediators of the response remain unclear. Human studies have correlated aberrant activation of T helper (Th) 2-like effector systems in the airways with disease. A murine model of airway hyperreactivity in response to acetylcholine was established using mice immunized with ovalbumin and challenged with aerosolized antigen. No airway hyperreactivity occurred in severe combined immunodeficient mice. Identically immunized BALB/c mice developed an influx of cells, with a predominance of eosinophils and $CD4^+$ T cells, into the lungs and bronchoalveolar lavage fluid at the time that substantial changes in airway pressure and resistance were quantitated. Challenged animals developed marked increases in Th2 cytokine production, eosinophil influx, and serum immunoglobulin E levels. Neutralization of interleukin (IL) 4 using monoclonal antibodies administered during the period of systemic immunization abrogated airway hyperreactivity but had little effect on the influx of eosinophils. Administration of anti–IL-4 only during the period of the aerosol challenge did not affect the subsequent response to acetylcholine. Finally, administration of anti–IL-5 antibodies at levels that suppressed eosinophils to $<1\%$ of recruited cells had no effect on the subsequent airway responses. BALB/c mice had significantly greater airway responses than C57BL/6 mice, consistent with enhanced IL-4 responses to antigen in BALB/c mice. Taken together, these data implicate IL-4 generated during the period of lymphocyte priming with antigen in establishing the cascade of responses required to generate airway hyperreactivity to inhaled antigen. No role for IL-5 or eosinophils could be demonstrated.

Asthma is one of the common chronic lung diseases of industrialized countries. Despite effective therapy, treatment costs, toxicity, and the need for coordinated delivery of medication limit the benefits of current regimens. Strategies designed to prevent asthma in high-risk individuals, or that reverse the pathologic processes that lead to asthma, could be far more effective than current interventions. Unfortunately, development of such strategies has been hampered by a lack of basic understanding of the pathogenesis of the disease.

Reversible airway narrowing, the physiologic hallmark of asthma, is often seen in association with evidence of immune system activation, including T cell and eosinophil infiltration of the airways. Evidence for Th2 cytokine production, including IL-4 and IL-5, and effector function, such as eosinophil infiltration and elevated levels of IgE, has been reported (1–8). A number of secondary inflammatory mediators, particularly mast cell and eosinophil products, have been shown to induce physiologic changes similar to asthma or to cause smooth muscle contraction in vitro (9, 10). However, little direct experimental evidence exists that clearly identifies the principal pathogenic cells and mediators of this complex inflammatory cascade.

Murine models of airway hyperreactivity have been used to begin to identify components of the immune response, although the immunology, cellular response, and airway physiology have infrequently been coordinately investigated (11–18). We have established a murine model and used this to elucidate critical cellular and cytokine requirements for the acute airway changes induced by exposure to antigen. Surprisingly, these data fail to support a role for two prominent components of the inflammatory response, eosinophils and IL-5, and emphasize the need for integration of immunologic and physiologic studies.

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Materials and Methods

Immunization Protocol. BALB/c, C57BL/6, and C.B-17/*scid* mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were immunized with 25 μg turkey egg OVA (Sigma Chemical Co., St. Louis, MO) administered subcutaneously with alum weekly for 4 wk and subsequently exposed to two or three aerosolizations 5 d apart of OVA or an unrelated immunodominant antigen from the protozoan *Leishmania major* (19). Aerosolization of antigens was performed using a nose-only aerosol chamber adapted for mice (Intox Products, Albuquerque, NM) coupled to a nebulizer (Aerotech II; CIS-US, Bedford, MA) driven by compressed air at a flow rate of 10 liters/min. Under these conditions, aerosol particles have a median aerodynamic diameter of 1.4 μm as measured by a cascade impactor (Intox Products). Antigen was delivered over 20 min at a concentration of 50 mg/ml in PBS. Designated groups of mice received 1 mg neutralizing anti–IL-4 mAb (11B11; rat IgG1; reference 20) intraperitoneally weekly, either throughout the experimental protocol or limited to the aerosolization period, as denoted; or a combination of 1 μg each of two neutralizing anti–IL-5 mAbs (TRFK-4 and TRFK-5; rat IgG2a and IgG1, respectively; reference 21) intraperitoneally weekly throughout the experimental period. Isotype control antibodies Y13-259 (rat IgG1) and Y13-238 (rat IgG2a) were administered to control groups.

Determination of Airway Physiology. 24 h after the final aerosol exposure, mice were anesthetized with pentobarbital (Abbott Laboratories, North Chicago, IL; 50 mg/kg intraperitoneally) and the tracheas were surgically exposed, cannulated, and connected to a rodent ventilator (Harvard Apparatus, South Natick, MA) with an in-line pressure transducer (Pd23 inside diameter; Gould, Inc., Oxnard, CA). Animals were ventilated with 100% oxygen (9 μl/g) at 120 breaths/min; under these conditions, naive mice maintain physiologic arterial blood gas parameters except for supraphysiologic arterial Pao_2 (data not shown). A paralytic agent (pancuronium bromide, 0.1 mg/kg; Gensia Laboratories, Irvine, CA) was administered to eliminate spontaneous respirations. After recording of a stable baseline airway pressure (<5% variation over 3 min), acetylcholine chloride (Ach; 1 μg/g; Sigma Chemical Co.) was infused intravenously over 1 s via the tail vein and the change in airway pressure recorded over 3 min on a standard polygraph (Grass Instrument Co., Quincy, MA). Final airway pressure changes were recorded as the maximum percentage of change in airway pressure from baseline (maximum airway pressure − baseline airway pressure/baseline airway pressure).

To compare changes in airway pressure, as measured above, with changes in resistance, separate groups of comparably treated mice were analyzed using a protocol modified from Amdur and Mead (22). Mice were anesthetized and ventilated as above, and a 27-guage needle was used to establish intravenous access in the tail vein. Animals were placed inside a plethysmograph coupled to a pressure transducer (model MP45; Validyne Engineering Corp., Northridge, CA) and constructed with ports for intravenous and ventilator access tubing. Lung resistance (R_L) was determined by continuously quantitating the quotient $\Delta Pt/\Delta V$ (where ΔPt = change in tracheal pressure and ΔV = change in flow) at points of equal lung volume (70% tidal volume) using a pulmonary mechanics analyzer (model 6; Buxco Corp., Sharon, CT). ΔPt was determined using a pressure transducer (model 267 BC; Sanborn Co., Waltham, MA) connected to the tracheal cannula. To determine ΔV, plethysmograph pressure changes were calibrated to changes in volume over the physiologic range studied. The differential of this value over time is ΔV. After establishing a stable baseline R_L (<5% variation over 3 min), Ach was administered intravenously over 1 s in increasing doses until at least a 50% increase in airway pressure and a 200% increase in R_L was obtained. The provocative concentration of Ach, in micrograms Ach per gram, that caused a 50% increase in airway pressure (PC_{50}) and a 200% increase in R_L (PC_{200}) was calculated from interpolation of appropriate dose–response curves as described (23).

Collection of Lung Cells. Immediately after collection of physiologic parameters, mice were given 0.1 ml 1:1,000 heparin intraperitoneally and killed by transecting the left renal artery for collection of blood. Bronchoalveolar lavage (BAL)[1] cells were collected by lavaging whole lung with 3 ml PBS in 0.5-ml aliquots via the tracheal cannula and withdrawing slowly while gently massaging the thorax. Whole-lung cells were prepared after removing residual blood by thorough perfusion of PBS via the main pulmonary artery until the lungs were completely blanched. The lungs were removed and minced into fine fragments that were gently dispersed into PBS using a syringe plunger and passed through a 0.75-μm nylon mesh filter. BAL and whole lung cells were washed twice, counted, and resuspended at 10^7 cells/ml in RPMI-1640 with 5% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and antibiotics.

Analysis of Cytokine Transcripts. Cells were lysed in RNAzol B (Biotecx Laboratories, Houston, TX) and RNA extracted according to the manufacturer's recommendations. RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and random hexamer primers (Promega Corp., Madison, WI). PCR was performed with a multiple cytokine competitor construct as described (24). The construct contains a sequence specific for the constitutively expressed gene encoding hypoxanthine phosphoribosyltransferase (HPRT), allowing adjustments of input cDNA to comparable amounts of HPRT expression. Adjusted cDNAs were then amplified with cytokine-specific primers, and the products were separated by electrophoresis in ethidium bromide–stained 2.5% agarose gels, thus allowing discrimination of the larger competitor construct from the wild-type cDNAs that migrate further in the gel. Photographic negatives of the gels were scanned with an imaging densitometer to quantitate band intensities and confirm visual interpretations.

FACS® Analysis. 10^5 cells were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and FITC-conjugated anti-B220 mAbs (PharMingen, San Diego, CA) and analyzed for surface expression by cytofluorimetry (FACScan®; Becton Dickinson & Co., Mountain View, CA).

Cell Differentials. 1–5 × 10^3 cells were spun onto glass slides (Cytospin 2; Shandon Instruments, Inc., Astmoor, UK), air dried, fixed with methanol, and stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL). Eosinophils and macrophages/monocytes were enumerated based on morphology and staining characteristics and expressed as percentages of total cells. Eosinophil counts were confirmed with phloxine B staining (Unopette; Beckton Dickinson & Co.).

ELISPOT Assays. Single-cell suspensions from whole lung cells were distributed in duplicate aliquots (10^6 cells in 100 μl of RPMI-1640 with 5% FBS and antibiotics) into 96-well microtiter plates (Immulon IV; Dynatech Laboratories, Inc., Chantilly, VA) that had been precoated with either mAb BVD4-1D11.2 against IL-4 or mAb R46A2 against IFN-γ. Serial threefold dilu-

[1] *Abbreviations used in this paper:* Ach, acetylcholine chloride; BAL, bronchoalveolar lavage; ELISPOT, enzyme-linked immunospot assay; FBS, fetal bovine serum; HPRT, hypoxanthine phosphoribosyl transferase.

tions were prepared, and the plates were incubated undisturbed for 8 h at 37°C. Wells were washed with PBS to remove cells and incubated with biotinylated secondary antibodies against IL-4 (BVD6-24G.2) or IFN-γ (XMG-1.2). After 1 h, wells were washed and incubated for 1 h with 100 μl of streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS with 0.05% Tween 20 and 5% FBS. Color was developed with 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma Chemical Co.) suspended in 0.6% agarose (Sea-Plaque; FMC Bioproducts Inc., Rockland, ME). After solidification of the agar, individual blue spots were counted by inverted microscopy.

Antibody Isotypes. Total serum IgE was quantitated using a double mAb-based sandwich ELISA with antibody B.IE.3 as the primary antibody and biotinylated EM-95 as the detecting antibody. Results were quantitated by comparison to a standard control (Sigma Chemical Co.).

Histopathology. Whole lungs were distended by instillation of 4% paraformaldehyde in PBS via the trachea, removed, and immersed in the same fixative with the trachea clamped for 24 h. Tissue was sliced and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin for light microscopic examination.

Results

Establishing a Murine Model of Airway Hyperreactivity. Groups of BALB/c mice that had been immunized with OVA were challenged with aerosolized OVA, an irrelevant antigen, or saline; an additional nonimmunized group was challenged with aerosolized OVA. 1 d after the final aerosolization, mice were anesthetized and paralyzed, and, after stabilization on a rodent ventilator, airway pressure and resistance were measured after intravenous administration of 1 μg/g Ach (Fig. 1). Mice immunized with OVA and aerosolized with OVA demonstrated a marked bronchomotor response to Ach and a marked delay in the return to baseline airway pressure that was not present in any of the other groups. To assess airway responsiveness by an alternative method, airway resistance was measured and compared with simultaneously calculated airway pressure responses (Fig. 2). High doses of Ach could elicit exaggerated bronchomotor reactivity as assessed by increases in both airway pressure and resistance in control groups of mice. However, the dose–response curves were significantly shifted in the immunized group challenged with OVA (PC_{50}: 1.7 ± 0.2 and PC_{200}: 1.8 ± 0.3 μg/g) compared with saline (PC_{50}: 6.5 ± 1.7 and PC_{200}: 6.2 ± 0.8 μg/g; $P = 0.003$ and 0.03, respectively). Baseline values of airway pressure and resistance did not differ significantly between groups of mice before injection of Ach. These physiologic findings were reproducible in multiple experiments and were used to define antigen-specific airway hyperreactivity. Results in the subsequent experiments are displayed as airway pressure changes in response to 1 μg/g Ach.

The requirement for antigen specificity suggested by these experiments was consistent with a role for cells of the acquired immune system. Immunization and aerosolization of SCID mice, which are deficient in T and B cells, resulted in no induction of airway reactivity to Ach under the same conditions (Fig. 1).

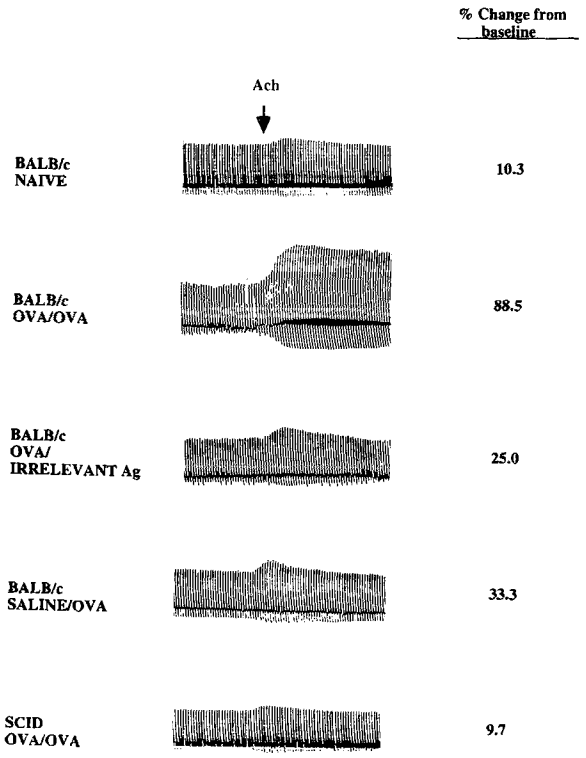

Figure 1. Airway pressure tracings in response to Ach. BALB/c or SCID mice were untreated (*naive*), immunized with OVA (*OVA*) and aerosolized with OVA (*OVA/OVA*), immunized with OVA and aerosolized with an irrelevant antigen (*OVA/irrelevant Ag*), or immunized with saline and aerosolized with OVA (*saline/OVA*). After stabilization of airway pressures on a rodent ventilator, Ach (1 μg/g) was infused intravenously and the airway pressure changes continuously monitored. The time of Ach administration is indicated by the arrowhead. The maximum percent change in airway pressure relative to baseline is indicated on the right of the tracings. Results are representative of five experiments with three to five animals per group.

Characterization of the Cellular Response Accompanying Airway Hyperreactivity. Having established a model of airway hyperreactivity, we next characterized the cells recruited to the lung and airways after antigen challenge (Table 1). Analysis of the cells in BAL and whole lung confirmed the marked increase in total cells, particularly eosinophils and

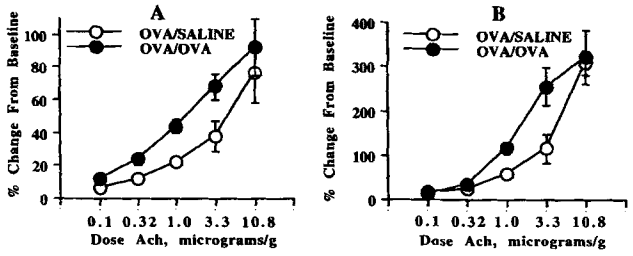

Figure 2. Dose–response curves to intravenous Ach. Groups of five BALB/c mice were immunized with OVA and challenged with either OVA (*solid circles*) or saline (*open circles*) aerosol. Airway pressure (*A*) and resistance (*B*) were quantitated as described in the Materials and Methods. Results represent mean ± SEM and are representative of two separate experiments.

Table 1. *Cellular Response and Maximal Airway Pressure to Ach in Designated Experimental Groups*

Group	Total Cells		Eosinophil		PMN		Lymphocyte		Mono/Macro		Airway Pressure
	BAL	Lung	BAL	Lung	BAL	Lung	BAL	Lung	BAL	Lung	
	$\times 10^6$				%						*cm H_2O*
Experiment 1											
Sham/OVA	ND	0.74	0	0.7	0	0.4	1.0	20.0	99.0	79.0	6 ± 2
OVA/sham	0.25	1.3	0	1.9	0	1.5	0	24.0	99.0	73.0	10 ± 4
OVA/OVA	2.7	5.6	63.0	20.0	2.8	0.8	9.2	30.0	25.0	49.0	36 ± 3
Experiment 2											
OVA/OVA + anti-IL-5	1.0	3.0	1.9	0.85	1.4	18.0	11.0	30.0	86.0	43.0	22 ± 8.8
OVA/OVA	5.0	7.0	44.0	23.0	1.1	8.8	18.0	39.0	37.0	29.0	22 ± 7.7

Mice in the designated groups were killed 1 d after the final aerosol challenge, and BAL and total lung cells were purified as described in Materials and Methods. The percentages of the designated cell types were highly comparable in two separate experiments and were derived from pooled cells representing five mice in each group. Maximal airway pressure change in response to 1 μg Ach/g mouse was quantitated as described in Materials and Methods and is expressed as mean ± SEM.

lymphocytes, in animals that were both immunized and aerosolized with OVA. Lymphocyte recruitment in BAL was biased toward $CD4^+$ T cells, as demonstrated by elevation of the $CD4^+/CD8^+$ cell ratio as assessed by cytofluorimetric analysis ($CD4^+/CD8^+$ = 2.7 for sham aerosol, 6.1 for OVA). Light microscopy of fixed lung specimens revealed a lymphocytic infiltrate with numerous eosinophils around veins and bronchoarterial bundles in immunized and aerosolized mice that was not present in control animals (Fig. 3).

Cytokine Expression in Mice with Airway Hyperreactivity. Cytokine expression by immune cells recruited to the lungs

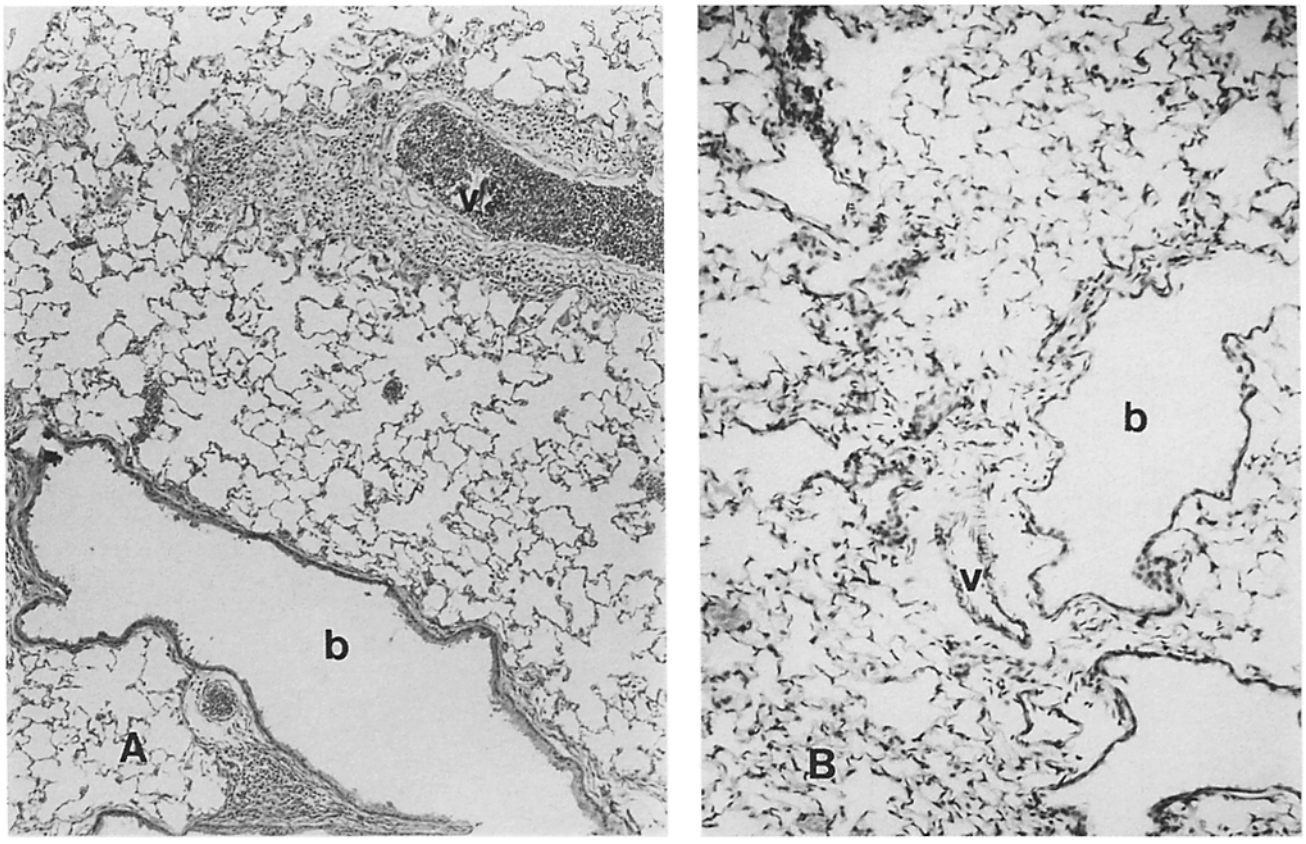

Figure 3. Histology of the lung in immunized mice. (*A*) BALB/c mouse immunized with OVA subcutaneously and aerosolized with OVA. Note marked peribronchovascular infiltrates. (*B*) BALB/c mouse immunized with OVA subcutaneously and aerosolized with saline. *v*, vessel; *b*, bronchiole. Hemotoxylin and eosin stained sections. ×100 (*A*); ×160 (*B*).

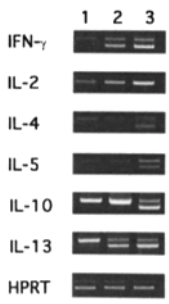

Figure 4. Cytokine transcripts from BAL cells of BALB/c mice immunized with OVA and challenged with aerosol saline (lane *1*), immunized with saline and challenged with aerosol OVA (lane *2*), or immunized with OVA and challenged with aerosol OVA (lane *3*). Concentrations of input cDNA were standardized by equalizing amplification of the constitutively expressed HPRT transcript by use of a polycompetitor construct containing tandemly arrayed amplification products that contain an irrelevant insert enlarging their size such that they migrate more slowly in ethidium bromide–stained agarose gels. After standardizing HPRT products, cytokine transcripts were amplified and compared by quantitating amplification of the wild-type (*lower band* for each amplification product) and the competitor (*upper band* for each product) products using imaging densitometry. Results are representative of three independent experiments.

of mice with airway hyperreactivity was quantitated using both RNA- and protein-based assays. Using a competitive reverse transcription–PCR approach, transcripts for IL-4, IL-5, IL-10, and IL-13, and to a lesser extent, IFN-γ, were substantially induced in mice that had been both immunized and aerosolized with OVA (Fig. 4). IFN-γ, but not the Th2 cytokines, was induced by aerosol alone, even in nonimmunized mice. ELISPOT assays were performed immediately after removing cells from the lung in the absence of added antigen in vitro to assess spontaneous cytokine production. Mice immunized and aerosolized with OVA had 2- to 3-fold increases in numbers of IFN-γ–secreting cells and 200-fold increases in the numbers of IL-4–secreting cells per lung compared with nonimmunized mice that received aerosolized OVA (Table 2). The marked elevation in IL-4 production was consistent with the significantly raised levels of serum IgE in mice immunized with OVA (nonimmunized BALB/c mice: <1 μg/ml; immunized BALB/c mice: 10 ± 0.8 μg/ml; $P < 0.05$). Treatment with GK1.5, a mAb that depletes $CD4^+$ T cells in vivo, during the period of aerosolization of OVA completely abrogated the induction of Th2 cytokines (data not shown).

Effects of Cytokine Neutralization on Airway Hyperreactivity, Cell Recruitment, and Cytokine Expression. To elucidate the role of IL-5 and eosinophils, mice were given two neutralizing anti-IL-5 mAbs weekly beginning with the first subcutaneous immunization with OVA; isotype-matched control antibodies were given to controls. Despite substantial reduction in eosinophil numbers in BAL and whole lung, in some animals to <1%, airway pressures of mice treated with anti–IL-5 were not substantially different than animals that received isotype-matched antibodies (Fig. 5). Abrogation of eosinophil infiltration into the lung in mice treated with anti–IL-5 antibodies was corroborated by enumeration of BAL and isolated lung cells (Table 1) and by light microscopic examination of fixed tissue sections (data not shown).

In marked contrast to mice treated with anti–IL-5, mice treated with neutralizing anti-IL-4 mAb throughout the period of immunization had substantial blockade of airway hyperreactivity (Fig. 6 *A*). Treatment of mice with anti–IL-4 had little effect on levels of eosinophils in the lung, suggesting that the effects of IL-4 are independent of effects on eosinophils, in agreement with earlier studies using different models (25). Importantly, when anti–IL-4 was given only during the period of aerosolization of OVA, and not during the subcutaneous priming period, there was no effect on airway hyperreactivity (Fig. 6 *B*).

Comparison of Airway Hyperreactivity between BALB/c and C57BL/6 Mice. The results using anti–IL-4 suggested that production of IL-4 early, during the period of T cell priming, was critically associated with the later manifestation of airway hyperreactivity. BALB/c mice, compared with C57BL/6 animals, produce enhanced amounts of IL-4 during priming of $CD4^+$ T cells (26), a characteristic that may underlie the peculiar susceptibility of BALB/c mice to *L. major* (27). If production of IL-4 during initial antigen exposure is associated with airway hyperreactivity in this model, BALB/c mice might be expected to generate increased responses compared with other strains of mice. In side-by-side comparisons of similarly treated animals, BALB/c

Table 2. *Cytokine Protein Production of Isolated Whole-Lung Cells from OVA-immunized and -aerosolized Mice*

	Cytokines			
	IFN-γ		IL-4	
Group	Cell frequency	Total cells/lung	Cell frequency	Total cells/lung
	per 10^6 cells		*per 10^6 cells*	
OVA/OVA	61 ± 31	376 ± 221	265 ± 49	1929 ± 514
OVA/irrelevant Ag	25 ± 5.8	35 ± 13	77 ± 13	96 ± 10
Sham/OVA	320	128	24	9.6

Mice in the designated groups were killed 1 d after the final aerosol challenge. Purified lung cells were assessed for spontaneous secretion of IFN-γ and IL-4 using an ELISPOT assay as described in Materials and Methods. Data represent mean ± SEM from three animals and are representative of three separate experiments.

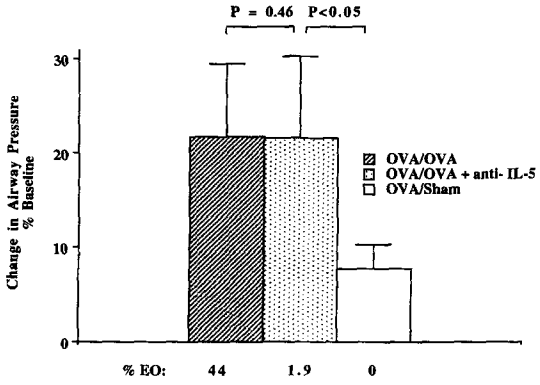

Figure 5. Effect of IL-5 neutralization on bronchial hyperreactivity. Groups of BALB/c mice were immunized with OVA and challenged with OVA or saline aerosol. Designated groups were treated weekly with neutralizing anti–IL-5 or isotype control antibodies throughout the experimental protocol. Results are expressed as mean maximum percentage of change in airway pressure ± SEM relative to baseline after challenge with intravenous Ach and are representative of two comparable experiments. Percentages of eosinophils in recovered BAL cells are depicted beneath the bar graphs (% *Eo*).

mice demonstrated consistently greater airway hyperreactivity than did C57BL/6 (percent change in baseline airway pressure 30 ± 6 versus 7 ± 3 for BALB/c versus C57BL/6, respectively; $P < 0.05$).

Discussion

The presence of activated T cells in bronchial biopsies and BAL fluid from patients with asthma (3–6, 28, 29) and of lymphocytes in the airways and submucosa of patients dying of the disease (30, 31) have focused attention on the role of cells of the acquired immune system in the pathogenesis of asthma. Activated $CD4^+$ T cells have been recovered that express a range of cytokines, particularly IL-4, IL-5, and GM-CSF, consistent with a Th2-like profile (3–6). Allergen-specific T cell clones established from atopic individuals consistently produce Th2 cytokines (32), and allergen-induced provocation has caused expression of these cytokines at sites of challenge (33, 34). Although activated T cells constitute a significant source of these cytokines, both activated mast cells (35, 36) and eosinophils (37) are capable of contributing Th2-like cytokines that might be anticipated to compound pathology mediated by these cytokines in an autocrine fashion. Patients have been identified with expanded numbers of Th2-like cells or clones who present with atopic symptoms, including sinopulmonary pathology (38, 39). These various data have suggested a model of asthma in which aberrant activation of immune cells to produce Th2-type cytokines in response to unknown respiratory antigens leads to the recruitment of mast cells, T cells, and eosinophils capable of causing chronic damage to the airway.

The murine model used in these studies constitutes a reproducible system for inducing airway hyperreactivity in response to antigen that permits the identification of factors that are required for this physiologic response. As demonstrated using SCID mice, the phenotype of airway hyperreactivity required cells of the specific immune system. Studies elsewhere have demonstrated that depletion of T cells, or $CD4^+$ T cells, up to 1 wk before challenge successfully abrogated the airway response (11, 18). As shown here, IL-4 was required during the period of T cell priming to OVA, since neutralization of IL-4 abrogated the physiologic response when administered during subcutaneous sensitization with antigen, but not during the period of aerosol challenge. A number of in vitro and in vivo systems have demonstrated that IL-4 is required for the priming of Th2 cells (40), suggesting that $CD4^+$ T cell priming with IL-4 is a prerequisite for the development of airway inflammation and hyperreactivity to Ach. Similar results were observed in studies using IL-4 knock-out mice; airway inflammation and bronchial responses to carbachol and serotonin were substantially less than in wild-type mice (41). The results here would extend those findings to a greater requirement for IL-4 in the inductive, rather than effector, stages of airway pathology, despite the fact that we were able to quantitate marked production of IL-4 during the time that bronchial hyperreactivity was determined.

A large body of literature has documented the association of pulmonary eosinophil infiltration and airway hyperreactivity in both asthmatic humans and animal models of airway inflammation (1, 9, 42). Studies from humans with asthma have demonstrated large quantities of eosinophil cationic protein and major basic protein in BAL fluid and deposited in the submucosa of airway biopsies, indicating active eosinophil degranulation (9, 42). Eosinophil products are postulated to contribute to airway hyperreactivity through damage to the airway epithelium, leaving underlying smooth muscle more susceptible to nonspecific contractile mediators, or perhaps by contributing to the airway remodeling and narrowing observed in patients with

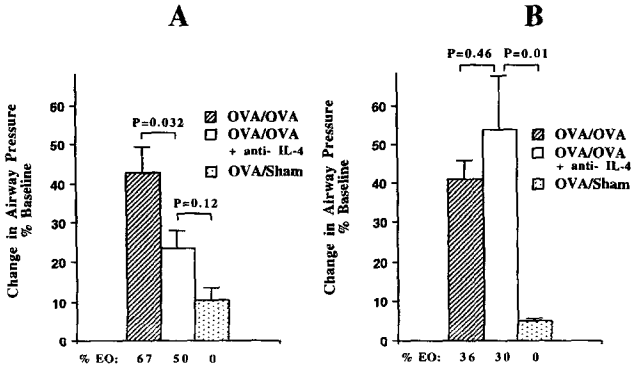

Figure 6. Effect of IL-4 neutralization on bronchial hyperreactivity. Groups of BALB/c mice were immunized with OVA and challenged with OVA or saline aerosol. Designated groups were treated weekly throughout the experiment with neutralizing anti–IL-4 antibody or isotype control (*A*) or only during the period of aerosol challenge (*B*). Results are expressed as mean maximum percentage of airway pressure ± SEM relative to baseline after challenge with intravenous Ach and are representative of two comparable experiments. Percentages of eosinophils in recovered BAL cells are depicted beneath the bar graphs (% *Eo*).

chronic disease (43, 44). Although a role for eosinophils in contributing to chronic airway changes or late-phase asthmatic responses is not excluded, the data here do not support a role for either IL-5 or eosinophils in mediating the acute airway hyperreactivity seen in this model. Studies of intestinal helminth infection, another system in which eosinophils are prevalent, have also been unable to define a role for eosinophils in mediating either host defense or pathologic responses (45).

The demonstration that IL-4 was required at the time of parenteral sensitization but not at the time of challenge leaves unanswered the source of the early IL-4 and the mechanism by which airway effects are mediated. Transfer of wild-type $CD4^+$ T cells into IL-4 knock-out mice suggested that $CD4^+$ T cells themselves were capable of generating the IL-4 required for Th2-like responses in the absence of mast cells or basophils (46). Recent studies have focused attention on a subset of $CD4^+$ T cells restricted by nonpolymorphic class I molecules, including CD1 (47), that generated essentially all of the IL-4 produced after administration of anti-CD3 or superantigens (48). These T cells show a marked bias toward expression of Vβ8 T cell receptors (49). Interestingly, $V\beta8^+$ T cells were demonstrated both to expand after immunization of BALB/c mice with OVA and to be capable of transferring airway hyperreactivity to naive recipients (50), raising the possibility that this unusual $CD4^+$ T cell population might contribute to the development of airway hyperreactivity.

The effects of neutralization of IL-4 during the aerosol phase of the model suggests that IL-4 is not required during the effector phase of airway hyperreactivity. IL-4 is required for IgE isotype switching, and cross-linking of Fcε receptors on mast cells by IgE would be consistent with the activated mast cell phenotype seen in asthma. Indeed, the mast cell–deficient W/W^v mouse had diminished eosinophil infiltration when used in a comparable airway model (13). However, IgE-deficient mice were capable of developing anaphylaxis and pulmonary function changes comparable to wild-type mice (51), suggesting additional pathways capable of mediating pathologic responses. The critical role for IL-4 during antigen priming in establishing the downstream cascade required for airway hyperreactivity suggests that interdictions capable of reprogramming $CD4^+$ subset development to common respiratory allergens might constitute effective vaccine approaches to the control of asthma. Such interventions are amenable to testing in the animal model used for these studies.

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References

1. Bousquet, J., P. Chanez, J.Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, P. Godard, and F.-B. Michel. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323:1033–1039.
2. Hamid, Q., M. Azzawi, S. Ying, R. Moqbel, A.J. Wardlaw, C.J. Corrigan, B. Bradley, S.R. Durham, J.V. Collins, P.K. Jeffery, et al. 1991. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* 87: 1541–1546.
3. Azzawi, M., P.W. Johnston, S. Majumdar, A.B. Kay, and P.K. Jeffery. 1992. T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. *Am. Rev. Respir. Dis.* 145:1477–1482.
4. Walker, C., M.K. Kaegi, P. Braun, and K. Blaser. 1991. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allergy Clin. Immunol.* 88:935–942.
5. Walker, C., E. Bode, L. Boer, T.T. Hansel, K. Blaser, and J.-C. Virchow, Jr. 1992. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146:109–115.
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: 298–304.
7. Del Prete, G., M. De Carli, M.M. D'Elios, P. Maestrelli, M. Ricci, L. Fabbri, and S. Romagnani. 1993. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur. J. Immunol.* 23:1445–1449.
8. Kerstjens, H.A., J.P. Schouten, P.L. Brand, D.F. Schoonbrood, P.J. Sterk, and D.S. Postma. 1994. Importance of total serum IgE for improvement in airways hyperresponsiveness with inhaled corticosteroids in asthma and chronic obstruc-

tive pulmonary disease. The Dutch CNSLD Study Group. *Am. J. Respir. Crit. Care Med.* 151:360–368.
9. Gleich, G.J. 1990. The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.* 85:422–436.
10. Wasserman, S.I. 1994. Mast cells and airway inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 150:S39–S41.
11. Gavett, S.H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine $CD4^+$ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10:587–593.
12. Lack, G., H. Renz, J. Saloga, K.L. Bradley, J. Loader, D.Y. Leung, G. Larsen, and E.W. Gelfand. 1994. Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J. Immunol.* 152:2546–2554.
13. Lukacs, N.W., R.M. Strieter, S.W. Chensue, and S.L. Kunkel. 1994. Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 10:526–532.
14. Nakajima, H., A. Nakao, Y. Watanabe, S. Yoshida, and I. Iwamoto. 1994. IFN-α inhibits antigen-induced eosinophil and $CD4^+$ T cell recruitment into tissue. *J. Immunol.* 153: 1264–1270.
15. Nakao, A., H. Nakajima, H. Tomioka, T. Nishimura, and I. Iwamoto. 1994. Induction of T cell tolerance by pretreatment with anti-ICAM-1 and anti-lymphocyte function-associated antigen-1 antibodies prevents antigen-induced eosinophil recruitment into the mouse airways. *J. Immunol.* 153: 5819–5825.
16. Brusselle, G., J. Kips, G. Joos, H. Bluethmann, and R. Pauwels. 1995. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 12:254–259.
17. Kung, T.T., D. Stelts, J.A. Zurcher, H. Jones, S.P. Umland, W. Kreutner, R.W. Egan, and R.W. Chapman. 1995. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am. J. Respir. Cell Mol. Biol.* 12:404–409.
18. Garlisi, C.G., A. Falcone, T.T. Kung, D. Stelts, K.J. Pennline, A.J. Beavis, S.R. Smith, R.W. Egan, and S.P. Umland. 1995. T cells are necessary for Th2 cytokine production and eosinophil accumulation in airways of antigen-challenged allergic mice. *Clin. Immunol. Immunopathol.* 75:75–83.
19. Mougneau, E., F. Altare, A.E. Wakil, S. Zheng, T. Coppola, Z.-E. Wang, R. Waldermann, R.M. Locksley, and N. Glaichenhaus. 1995. Expression cloning of a protective *Leishmania* antigen. *Science (Wash. DC).* 268:563–566.
20. Ohara, J., and W.E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature (Lond.).* 315:333–336.
21. Schumacher, J.H., A. O'Garra, B. Shrader, A. van Kimmenade, M.W. Bond, T.R. Mossman, and R.L. Coffman. 1988. The characterization of four monoclonal antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent. *J. Immunol.* 141:1576–1581.
22. Amdur, M.O., and J. Mead. 1958. Mechanics of respiration in unanesthetized guinea pigs. *Am. J. Physiol.* 192:364–368.
23. Peat, J.K., W.R. Unger, and D. Combe. 1994. Measuring changes in logarithmic data, with special reference to bronchial responsiveness. *J. Clin. Epidemiol.* 47:1099–1108.
24. Reiner, S.L., S. Zheng, D.B. Corry, and R.M. Locksley. 1993. Constructing polycompetitor cDNAs for quantitative PCR. *J. Immunol. Methods.* 165:37–46.
25. Coffman, R.L., B.W.P. Seymour, S. Hudak, J. Jackson, and D. Rennick. 1989. Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science (Wash. DC).* 245:308–310.
26. Hsieh, C.-S., S.E. Macatonia, A. O'Garra, and K.M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med.* 181: 713–721.
27. Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol.* 13:151–177.
28. Azzawi, M., B. Bradley, P.K. Jeffery, A.J. Frew, B. Assoufi, J.V. Collins, S. Durham, and A.B. Kay. 1990. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthmatics. *Am. Rev. Respir. Dis.* 142: 1407–1413.
29. Gratziou, C., M. Carroll, A. Walls, P.H. Howarth, and S.T. Holgate. 1992. Early changes in T lymphocytes recovered by bronchoalveolar lavage after local allergen challenge of asthmatic airways. *Am. Rev. Respir. Dis.* 145:1259–1264.
30. Poston, R.N., P. Chanez, J.Y. Lacoste, T. Litchfield, T.H. Lee, and J. Bousquet. 1992. Immunohistochemical characterization of the cellular infiltration in asthmatic bronchi. *Am. Rev. Respir. Dis.* 145:918–921.
31. Kleinerman, J., and L. Adelson. 1987. A study of asthma deaths in a coroner's population. *J. Allergy Clin. Immunol.* 80: 406–409.
32. Parronchi, P., D. Macchia, M.-P. Piccinni, P. Biswas, C. Simonelli, E. Maggi, M. Ricci, A.A. Ansari, and S. Romagnani. 1991. Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA.* 88:4538–4542.
33. Kay, A.B., S. Ying, V. Varney, M. Gaga, S.R. Durham, R. Moqbel, A.J. Wardlaw, and Q. Hamid. 1991. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J. Exp. Med.* 173:775–778.
34. Durham, S.R., S. Ying, V.A. Varney, M.R. Jacobson, R.M. Sudderick, I.S. Mackay, A.B. Kay, and Q.A. Hamid. 1992. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J. Immunol.* 148:2390–2394.
35. Plaut, M., J.H. Pierce, C.J. Watson, J. Hanley-Hyde, R.P. Nordan, and W.E. Paul. 1989. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature (Lond.).* 339:64–67.
36. Bradding, P., J.A. Roberts, K.M. Britten, S. Montefort, R. Djukanovic, R. Mueller, C.H. Heusser, P.H. Howarth, and S.T. Holgate. 1994. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am. J. Respir. Cell Mol. Biol.* 10:471–480.
37. Broide, D.H., M.M. Paine, and G.S. Firestein. 1992. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest.* 90:1414–1424.
38. Cogan, E., L. Schandene, A. Crusiaux, P. Cochaux, T. Velu, and M. Goldman. 1994. Clonal proliferation of type 2 helper T cells in a man with the hypereosinophilic syndrome. *N. Engl. J. Med.* 330:535–538.
39. Field, E.H., R.J. Noelle, T. Rouse, J. Goeken, and T. Wald-

schmidt. 1993. Evidence for excessive Th2 $CD4^+$ subset activity in vivo. *J. Immunol.* 151:48–59.
40. Seder, R.A., and W.E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* 12:635–673.
41. Brusselle, G.G., J.C. Kips, J.H. Tavernier, J.G. van der Heyden, C.A. Cuvelier, R.A. Pauwels, and H. Bluethmann. 1994. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin. Exp. Allergy.* 24:73–80.
42. Djukanovic, R., J.W. Wilson, K.M. Britten, S.J. Wilson, A.F. Walls, W.R. Roche, P.H. Howarth, and S.T. Holgate. 1990. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am. Rev. Respir. Dis.* 142:863–871.
43. Kamm, R.D., and J.M. Drazen. 1992. Airway hyperresponsiveness and airway wall thickening in asthma. A quantitative approach. *Am. Rev. Respir. Dis.* 145:1249–1250.
44. Kuwano, K., C.H. Bosken, P.D. Pare, T.R. Bai, B.R. Wiggs, and J.C. Hogg. 1993. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* 148:1220–1225.
45. Urban, J.F., K.B. Madden, A. Svetic, A. Cheever, P.P. Trotta, W.C. Gausse, I.M. Katona, and F.D. Finkelman. 1992. The importance of Th2 cytokines in protective immunity to nematodes. *Immunol. Rev.* 127:205–220.
46. Schmitz, J., A. Thiel, R. Kuhn, K. Rajewsky, W. Muller, M. Assenmacher, and A. Radbruch. 1994. Induction of interleukin 4 (IL-4) expression in T helper (Th) cells is not dependent on IL-4 from non-Th cells. *J. Exp. Med.* 179:1349–1353.
47. Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse $NK1.1^+$ T lymphocytes. *Science (Wash. DC).* 268: 863–865.
48. Yoshimoto, T., and W.E. Paul. 1994. $CD4^{pos}$, $NK1.1^{pos}$ T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179:1285–1295.
49. Bix, M., and R.M. Locksley. 1995. Natural T cells: cells that co-express NKRP-1 and TCR. *J. Immunol.* 155:1020–1022.
50. Renz, H., K. Bradley, J. Saloga, J. Loader, G.L. Larsen, and E.W. Gelfand. 1993. T cells expressing specific Vβ elements regulate immunoglobulin E production and airways responsiveness in vivo. *J. Exp. Med.* 177:1175–1180.
51. Oettgen, H.C., T.R. Martin, A. Wynshaw-Boris, C. Deng, J.M. Drazen, and P. Leder. 1994. Active anaphylaxis in IgE-deficient mice. *Nature (Lond.).* 370:367–370.