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IMMUNOPATHOGENESIS IN A MOUSE MODEL OF HUMAN ASTHMA

by

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DISSERTATION

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Dedicated to my family.

PREFACE

Asthma is a disease that has affected the lives of millions. Trying to understand a disease process of such complexity is a challenging task. One of the biggest challenges for immunologists is to reproduce a human immunological disorder, such as asthma, in a convenient animal model. Immunologists have over the years developed a plethora of immunologic and genetic tools in mice, in order to characterize such immune disorders in detail and elucidate the pathways involved in the disease process. I am therefore very grateful to my advisor Dr. Richard M. Locksley for allowing me to pursue a complex problem and help me develop the skills required to design and execute experiments as well as challenge the hypothesis and results of other scientists. Through his continuous enthusiasm for science, he was always available for feedback and helpful comments on my projects. His broad knowledge and excitement for science has been a true inspiration for me and others who have worked with him. Another person who deserves a lot of praise, especially in teaching me the daunting task of performing mouse surgery for the assessment of airway physiology, is Dr. David Corry. David's patience, humor and knowledge had a profound impact on my graduate career. He taught me the true meaning of science - keep questioning every result.

I would also like to thank all of the lab members, both past and present for providing a fun lab environment, exchange of stories, jokes and gossips. I am particularly thankful to Ninetta Flores who helped me manage my mouse colony and Cliff McArthur for his flexibility and help in cell sorting. I would like to stress that very few labs maintain such a collegial and supportive environment, that at the same

time is very critical of science. This true spirit of collegiality and critical support was evidenced by the interactive times we had in lab meetings and beyond.

I would also like to acknowledge the help and support of the Lung Biology Center at the San Francisco General Hospital headed by Dr. Dean Sheppard. In particular Xiaozhu Huang and Xinliu Bernstein who had tremendous impact on the success of the work presented in this thesis. Their knowledge and skills have transformed the Sandler core facility that has helped our lab members and others conduct very intricate airway physiologic studies.

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Chapter 2 was presented in the following publication:

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The results and most of the text in chapter 3 was presented in:

Corry, D.B., G. Grunig, H. Hadeiba, V.P. Kurup, M.L. Warnock, D. Sheppard, D.M. Rennick, and R.M. Locksley. 1998. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol Med* 4:344-355.

Finally, without the support of my family, none of this would have been possible, I am grateful for their continuous support, especially given the fact that they are so far away. Hvala i svim Bosancima na tako dobroj zajebanciji....nebi ovo nikad zavrsio da njih nije bilo...Nek je samo zdravlja i veselja...amin!

ABSTRACT

IMMUNOPATHOGENESIS IN A MOUSE MODEL OF HUMAN ASTHMA

Husein Hadeiba

Asthma is a complex inflammatory disorder of the airways characterized by reversible episodes of airway narrowing, inflammation and airway reactivity to bronchoconstricting agents, termed airway hyperreactivity. The pathogenesis of asthma is believed to reflect allergen-induced airway inflammation initiated by Th2 cells. Our goal was to reproduce the physiologic and pathologic changes seen in human asthma in a robust mouse model in order to study immune responses to airborne allergens. The work presented in this thesis suggests that the airway response to allergens is a regulated process.

First, the potential role of modifier genes, independent of type 2 immunity to allergens, was identified by use of a spontaneous mouse model of airway hyperreactivity that requires no prior sensitization with antigen. The hyperreactive phenotype was shown to be independent of CD4+ T cells or bone-marrow derived cells. Hyperreactive mice on wild-type, immunodeficient or IL-4-deficient backgrounds displayed comparable elevated baseline airway reactivity but no inflammatory lesions in the lung.

Second, immune responses in an antigen-driven system require Th2 cells and IL-4 to mediate the disease. Adoptive transfers into immunodeficient recipients revealed that CD4+ T cells alone were sufficient to restore airway hyperreactivity and pathology associated with human asthma. Furthermore, B cell deficient mice developed airway hyperreactivity and lung inflammatory changes comparable to that in wild type mice, even in the absence of eosinophilia. The use of cytokine-deficient mice revealed an important role for IL-4, but not IL-5 in mediating antigen-driven airway pathology and bronchial reactivity.

Finally, regulatory T cells may control unwanted type 2 immune responses to innocuous allergens. Transgenic mice with lung-specific expression of antigen, crossed to antigen-specific TCR transgenic mice, revealed clonotypic CD4 T cells infiltrating the lung held in check by the accumulation of CD4+ CD25+ regulatory T cells that inhibited immune effector function in vivo. When challenged with exogenous antigens, the regulatory T cells remained highly efficient in blocking type 2 immunity but were unable to inhibit bronchial hyperreactivity. The regulatory capacity of CD4+ CD25+ T cells may be limited in chronic complex diseases allowing substantial contributions by non-T cells.

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CHAPTER 1

Introduction

Asthma remains one of the common diseases of industrialized countries, affecting approximately 6% of U.S. children (1). Despite advances in management, morbidity and mortality have not improved significantly over the past two decades. Asthma is a complex inflammatory disease of the airways, characterized by reversible episodes of airway narrowing and inflammation. The asthmatic airway is typified by chronic inflammatory lesions, eosinophilia, mucus hyperproduction, epithelial cell hyperplasia and varying degrees of subepithelial fibrosis due to collagen deposition (2-5). Airway smooth muscle contraction, excessive mucus production by goblet cells, and edema caused by the underlying inflammation lead to impaired ventilation. One of the clinical hallmarks of the disease is hyperreactivity to a variety of bronchoconstricting stimuli such as cholinergic agents, allergens or even cold air, termed airway hyperreactivity (6, 7). It is believed that the inflammation causes the airway smooth muscle to be hyperreactive to these stimuli [reviewed in(8)].

Increasing evidence has linked asthma to aberrant activation of the immune system in the airways. Activated T cells and eosinophils are present in bronchial biopsies and broncho-alveolar lavage fluid (BAL) from individuals with active disease (9-13). The degree of CD4+ T cell activation was correlated with both the severity of the symptoms and the extent of airway hyperreactivity, as well as with the number of eosinophils recovered in BAL (14). These studies suggest that asthma represents aberrant activation of CD4+ Th2 cells in the airways to inhaled allergens.

Functional subsets of CD4+ T cells have been identified based on the pattern of cytokines that they produce (15, 16). The presence of these unique cytokine repertoires has offered insights into the pathogenesis of diseases ranging from autoimmunity and

infectious diseases to allergic disorders. Naïve CD4+ T cells can develop either along the Th1 or Th2 pathway upon encounter with antigen. Th1 cells produce IL-2, LTα and IFNγ which orchestrate potent cell-mediated immune responses towards intracellular pathogens, including viruses, and when dysregulated, mediate a wide spectrum of autoimmune diseases (17). On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which stimulate IgE production from B cells, mucosal mastocytosis and eosinophilia (15, 16). Th2 cells have been implicated to play a major role in the elimination of helminth parasites and in the induction of allergic disorders including asthma. Cytokines elaborated by Th2 cells are felt to orchestrate the sequence of events leading to IgE production, mucosal mastocytosis and eosinophilia (figure 1). Several lines of evidence provide support for the hypothesis that asthma represents an aberrant activation of type 2 immune responses in the lung to inhaled allergens.

First, cytokine expression consistent with a Th2 expression pattern from the cytokine gene cluster on human chromosome 5 (mouse chromosome 11), including, IL-3, IL-4, IL-5 and GM-CSF, was significantly increased in T cells recovered from BAL fluid (9, 11, 18). Second, BAL and bronchial biopsies from persons with asthma demonstrate markedly increased numbers of eosinopils, mast cells and lymphocytes. Growth and activation factors for eosinophils (IL-5, GM-CSF) and mast cells (IL-3, IL-4, IL-9, IL-10, IL-13) can be produced by these cells themselves, thereby amplifying the disease process (19, 20). Third, IL-4-induced factors, such as elevated levels of serum IgE, an IL-4-dependent immunoglobulin isotype, are common in asthmatics and have been quantitatively related to asthma prevalence and risk (21). Using linkage analysis this IgE response to common environmental antigens was mapped close to the cytokine gene

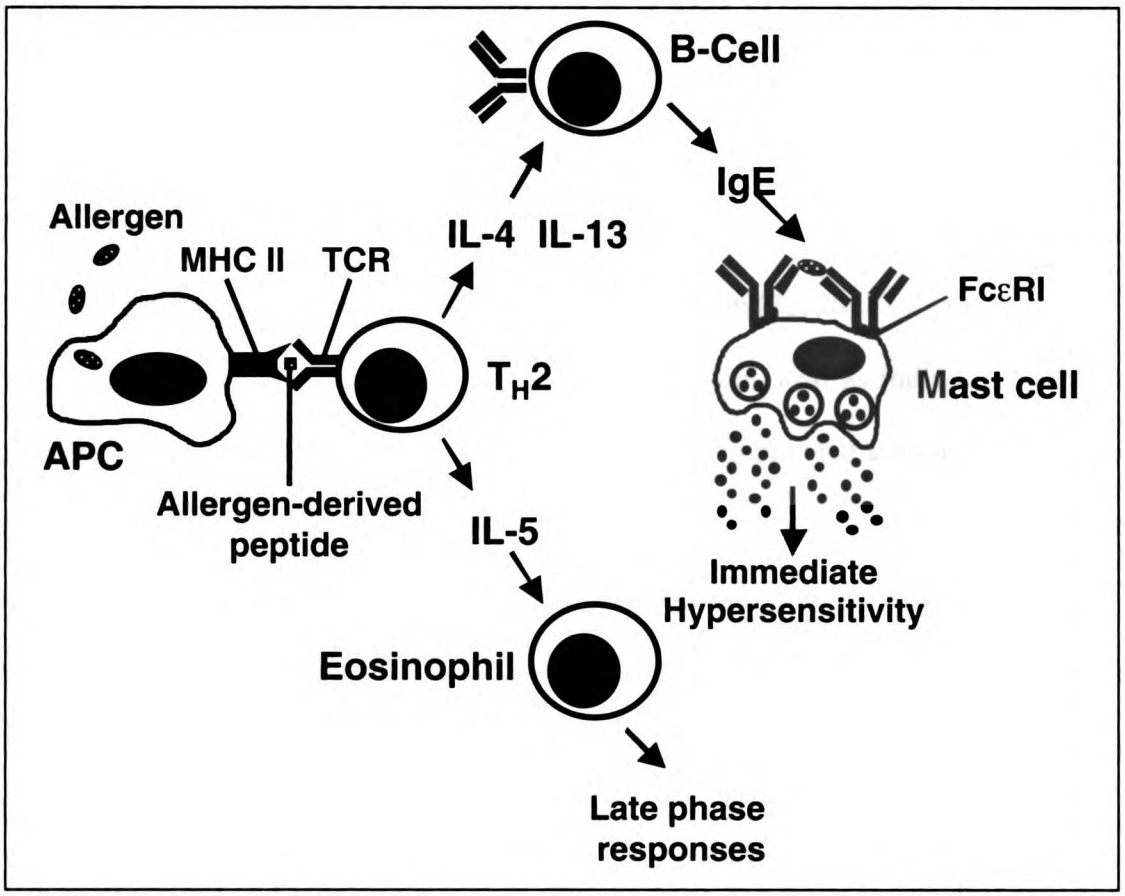

Figure 1. **Asthma is mediated by type 2 immunity to allergens**

In the sensitization phase, allergens are taken up and processed by antigen presenting cells (APCs). The allergenic peptide presented on MHC class II molecules allows for the maturation of Th2 cells that subsequently produce IL-4, IL-5 and IL-13. IL-4 and IL-13 induce isotype switching in B cells to the IgE class which binds to the high affinity Fcε-receptor on mast cells. A second encounter with the allergen, cross-links the Fcε-receptors via IgE, resulting in mast cell degranulation. Mast cell products mediate the vascular and smooth muscle response associated with immediate hypersensitivity reactions. IL-5 produced by Th2 cells, plays a role in the differentiation and survival of eosinophils in the late-phase response.

cluster on chromosome 5q that contains the IL-4 gene (22). Another Th2-dependent factor, lipoxygenase produced by mast cells (23), was also shown to be present in increased amounts in the bronchial submucosa of asthmatic patients (24). Finally, human T cell clones established from atopic individuals specific for known allergens are characterized by a Th2-type pattern of cytokine expression (25, 26).

Genetic studies utilizing genome-wide searches as well as individual candidate gene-studies among patients with allergic asthma have implicated a number of potential genes that are associated with type 2 immunity. This includes the cytokine gene cluster on human 5q (mouse chromosome 11) that contains IL-4, IL-13, and IL-5, as well as regions on human 11q and 16p that map near the high-affinity IgE receptor and the α-chain of the IL-4 receptor respectively (22, 27-29). Other recent candidates include the ADAM 33 metalloprotease on human 20p involved in shedding of cell-surface proteins such as cytokines and cytokine receptors (30), and the Tim gene family that encodes T cell membrane proteins, of which the human homolog TIM-1 is the hepatitis A virus receptor (31).

Taken together, asthma may reflect, at least in part, a genetic condition that predisposes individuals to mount exaggerated or inappropriate type 2 immune responses to common airborne allergens that leads to the recruitment of mast cells and eosinophils capable of causing chronic airway damage.

Extensive analysis suggests that the developmental pathways established in the mouse for CD4+ T helper cell differentiation are comparable in humans. Although less easily studied in humans, the critical signals for in vitro differentiation of Th1 and Th2 cells, IL-12 and IL-4, respectively, are similar in the human system (32, 33).

Furthermore, in response to a range of pathogens, human T cell clones develop into effector subsets as predicted by murine models. [reviewed in (34)]. Lacking in human studies, however, is the ability to examine the initial period of T cell activation which would allow the characterization of critical signals required for T cell effector development. Furthermore, it is almost impossible to tease apart the different effector pathways in a reductionist approach in order to delineate their role in mediating pathology.

We wanted to establish a murine model of airway hyperreactivity to environmental allergens in order to study the initiation of the immune response and delineate the checkpoints involved in the disease process. A number of studies performed by Levitt's group (35-37) demonstrated a range of airway reactivity in different untreated inbred mouse strains in response to bronchoconstricting agents (figure 2). These studies quantitated the airway pressure time index (APTI) following a single fixed dose of either intravenous acetylcholine or serotonin. These studies demonstrated a significant difference in airway reactivity among inbred mouse strains, in particular the A/J strain which showed the greatest degree of airway hyperreactivity, while the C3H strain was least hyperreactive. Other studies (38) documented similar strain distribution patterns in response to methacholine, an analogue of acetylcholine. Previous studies had indicated that the hyperreactive phenotype of the A/J strain was dependent on cyclosporin A, a pharmacologic inhibitor of T cell activation (39). Furthermore, this hyperreactive phenotype was mediated by hematopoietic cells, in particular T cells (40). Using direct measurements of murine airway resistance, we reproduced the elevated resting airway hyperreactivity of the A/J mouse strain (Chapter 2). This model of spontaneous

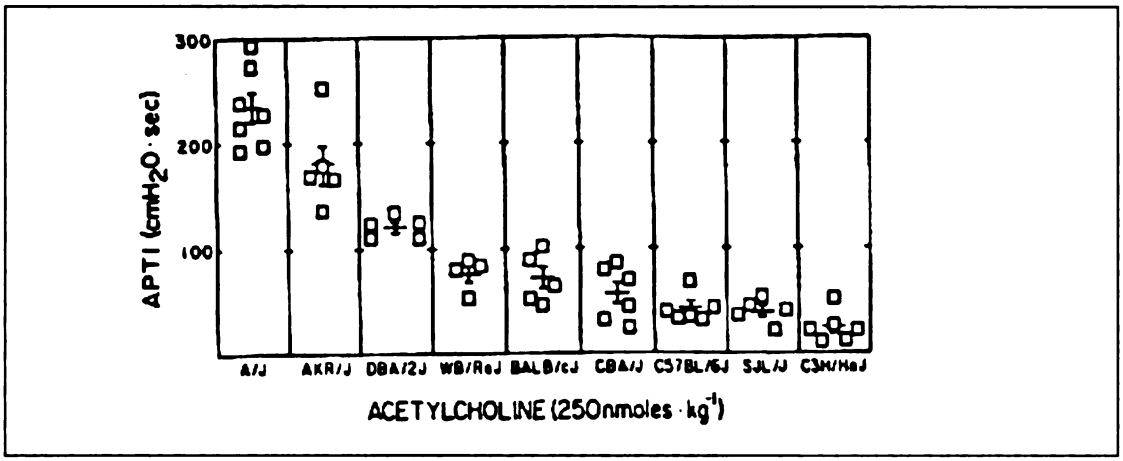

Figure 2. **Airway reactivity to Acetylcholine in different inbred mouse strains.**

Airway pressure time index (APTI) in response to intravenous Acetylcholine in different inbred mouse strains, quantitated by assessing the area under an airway pressure time curve after administration of a single intravenous bolus of acetylcholine.

(reproduced with kind permission from *levitt et al, Am. J. Physiol. 258; 157-164, 1990)*

non-atopic airway hyperreactivity allowed us to assess the importance, if any, of the immune response to either environmental antigens or, possibly, to a lung specific self antigen. The results suggest a novel mechanism of airway reactivity that is maintained independently of type 2 immunity induced by allergens.

Given a somewhat surprising result in our studies with A/J mice, we wanted to set up an antigen-dependent system of airway hyperreactivity in order to study the initiation of type 2 immune responses in the airway, and elucidate the role of various effector pathways in mediating airway hyperreactivity. Antigen-dependent mouse models offer numerous advantages towards understanding the pathogenesis of asthma. Interventions using recombinant molecules and studies in gene knock-out mice provide powerful and direct methods to examine the roles of designated effector pathways. Direct evidence suggesting that CD4+ T cells are necessary in the development of airway hyperreactivity came from a murine model of ovalbumin sensitization. In this study, depletion of CD4+ T cells with antibodies was able to curtail inflammation, eosinophilia and airway reactivity to bronchoconstricting agents (41). The role of type 2 immunity in allergen-driven airway inflammation has been elucidated by studies in IL-4- or IL-5-deficient mice (42-45) or by antibody blockade (46, 47). Furthermore, adoptive transfer of Th2 clones was found to induce airway reactivity in naïve mice (48).

Whereas no animal model perfectly reproduces human asthma, we have reproduced a murine model of airway hyperreactivity that shares both physiologic and pathologic features with the human disease. These include the antigen-specific induction of reversible increases in airway resistance and peak pressures, the influx of a similar inflammatory cell infiltrate, predominantly eosinophils, into the lung, and the presence of

activated CD4+ T cells that predominantly express Th2 cytokines. This model was used to focus on the molecular and cellular mechanisms underlying the developmental maturation of naive CD4+ T cells to pathogenic effector cells capable of mediating inflammatory and physiologic changes in the lung that are similar to human asthma. We used a pulmonary allergen, an extract of the fungus *Aspergillus fumigatus* (49), to investigate the critical components of the host response required to generate allergic airways disease in the mouse, and correlated this with the cellular and cytokine responses. Airway hyperreactivity was rigorously quantitated by measuring airway resistance in the intact animal, while maintained in a whole body plethysmograph breathing at physiological rate and tidal volume. Using adoptive transfers into immunodeficient rag-/- recipients we showed that CD4+ T cells alone were sufficient to mediate the pathology and airway reactivity that occurs in human asthma. Studies in IL-4- and IL-5-deficient mice suggested that IL-4, but not IL-5, emerged as a critical effector molecule in mediating airway hyperreactivity. Finally, depletion of eosinophils to baseline levels using neutralizing antibodies or IL-5-deficient mice, had no effect on the physiologic or immunologic changes mediated by CD4+ T cells (Chapter 3).

Taken together our findings, and those of others (41, 50-52), have established a critical role for CD4+ T cells and type 2 cytokines, in particular IL-4, in orchestrating the recruitment of inflammatory cells in acute allergic airways disease. However, chronic changes seen in human asthma, such as fibrosis and airway wall remodeling, are rarely observed in models of acute antigen-sensitized models (chapter 3). Chronic antigen deposition in the airway mucosa are difficult to carry out because of the lethality of repeated antigen administration over a long period of time. Indirect approaches have

targeted expression of CD4+ T cell products to the airways. Using a transgenic approach these studies have elucidated the role of long term type 2 cytokine deposition in the airways in mediating allergic airways disease (53-55). For example, IL-13, an important mediator of allergic airways disease (56, 57), when targeted to the airways via a lung-specific promoter, induces inflammatory changes seen in acute antigen-induced models of airway hyperreactivity, including eosinophilia, mucus production and airways obstruction. In addition, long-term changes such as Charcot-Leyden-like crystal deposition and airway fibrosis due to collagen deposition were also observed (55).

To study the effects of chronic antigen deposition in the airway mucosa on CD4+ T cell priming and to look at chronic airway changes in antigen-induced airways disease, we used a different transgenic approach. We chose chicken egg ovalbumin (OVA) as a model antigen to be placed under the transcriptional control of the surfactant protein C (SPC) promoter (58, 59), in order to study responses to an endogenous lung antigen (chapter 4). To break OVA-specific tolerance, we crossed the mice to DO11.10 TCR transgenic mice that express a TCR specific for an OVA peptide. Unexpectedly, we observed inflammatory lesions in the lung that contained a large proportion of antigen-specific CD4+ CD25+ T cells. We hypothesized that these cells were regulatory, due to their ability to suppress proliferation of effector T cells in-vitro. In an antigen-induced model of airway hyperreactivity, these regulatory cells were able to suppress type 2 immune responses in vivo, but surprisingly had no effect on the airway hyperreactive phenotype. These studies have demonstrated a role of mucosal antigen in recruiting regulatory cells to the site of antigen expression, where they are felt to inhibit Th2 effector progression in vivo and thus define a critical checkpoint. Upon exogenous

delivery of antigen, this checkpoint was breached and bronchial reactivity was induced despite inhibited type 2 immune responses in vivo. This transgenic approach defines a useful antigen-dependent system to dissect the cellular and molecular requirements for the regulation of experimental airways disease.

The following chapters presented in this thesis will discuss the regulation of the immune response in human asthma, using experimental mouse models. We begin by looking at the causes of elevated baseline airway reactivity in the absence of any sensitization to establish the role of modifier genes in mediating airway hyperreactivity. We then set up an antigen-dependent system to assess the role of cellular and molecular mediators in mediating both the pathology and the physiologic response associated with human asthma. Finally we established a chronic model of antigen-induced airways disease by examining the role of long-term exposure to a fixed model antigen using a transgenic system. This system enabled us to uncover the potential role of regulatory T cells in modulating responses to antigens encountered at the airway mucosa.

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CHAPTER 2

Baseline Airway Hyperreactivity in A/J Mice is not Mediated by Cells of the Adaptive Immune System

Summary

Human asthma is characterized by increased airway hyperreactivity to a variety of bronchoconstricting agents. Aberrant type 2 immune responses in the lung have been associated with airway hyperreactivity in both human asthma and in murine models of allergic airways disease. Despite their intrinsically elevated basal airway reactivity to smooth muscle contricting agents, A/J mice demonstrated no inherent inflammatory cell infiltration nor elevation of type 2 cytokines in the lung. Crossed bone marrow reconstitution experiments between A/J and MHC congenic B10.A mice revealed enhanced airway reactivity only in A/J recipients, irrespective of whether they had been reconstituted with A/J or B10.A hematopoietic cells. Further, A/J-derived bone marrow cells did not affect the reactivity of B10.A recipients. Although mice on RAG-deficient and IL-4-deficient backgrounds demonstrate substantial abrogation of allergen-induced airway hyperreactivity, these gene deletions had no impact on the elevated baseline reactivity when backcrossed onto A/J mice. Thus, in these mice, basal airway hyperreactivity is maintained independently of type 2 immunity induced by allergens.

INTRODUCTION

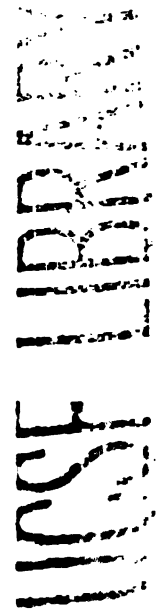
Asthma, characterized by reversible episodes of airway narrowing in the presence of chronic airway inflammation, remains an increasingly prevalent disease of industrialized nations (1, 2). Accumulating data have associated the asthma phenotype in humans with the presence of an aberrantly active type 2 immune response in the lungs (3-5). Thus, activated Th2 cells - a polarized subset of CD4+ T cells that together release the cytokines IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF - and activated eosinophils are consistently present in bronchoalveolar lavage (BAL) and bronchial biopsies obtained from patients with asthma (6-11). Together, cytokines and other mediators released by these activated cells are felt to orchestrate the recruitment of eosinophils and lymphocytes and the production of IgE that together contribute detrimentally to the chronic stimulation of mucus production and airway pathology that ultimately lead to clinical disease.

The hallmark of human asthma, airway hyperreactivity, refers to a reversible, intrinsically lower, threshold for airway narrowing in response to allergen or pharmacologic agents, such as acetylcholine and serotonin, that induce smooth muscle contractility. Genome-wide searches as well as individual candidate-gene studies among patients with allergic asthma have implicated a number of potential genes that are associated with type 2 immunity, including the cytokine gene cluster on human 5q (mouse chromosome 11) that contains IL-4, IL-13 and IL-5, as well as the high-affinity IgE receptor and the α chain of the IL-4 receptor that is shared between IL-4 and IL-13 (12-14).

Mouse models of allergen-induced airways disease share with human asthma the association of type 2 immune responses in lung tissue and the development of pathologic and physiologic changes, including airway hyperreactivity (reviewed in 15). Recent experiments using mice transgenic for the human 5q cytokine cluster through incorporation of yeast artificial chromosomes demonstrated the remarkable conservation of control in this

locus, and increased hopes that these animal models might guide the search for human susceptibility genes (16). Classic studies initiated 10 years ago identified intrinsic differences in baseline airway hyperreactivity among inbred mouse strains (17-20). Although a spectrum of reactivity was apparent, one strain, A/J, demonstrated remarkable airway hyperreactivity. Although early studies suggested a simple autosomal recessive inheritance underlying the phenotype (17, 18), subsequent studies have defined a complex polygenic trait (21, 22), one of which involves at least three major contributing loci (21).

Whereas the value of the A/J strain in identifying potential airway reactive susceptibility genes is apparent, few studies have attempted to demonstrate whether the adaptive immune response, so critically implicated in allergen-induced asthma, is involved in the baseline airway hyperreactivity that occurs spontaneously in this strain of mice. We have used baseline immunologic and T cell- depletion studies, crossed bone marrow chimeras, and genetic crosses of A/J mice to recombinase activating gene-1 (RAG) and IL-4-deficient mice to demonstrate that cells of the adaptive immune system do not contribute to the intrinsically elevated airway hyperreactivity of these animals. Despite this finding, the aberrant baseline physiology in A/J mice contributes to incrementally greater responses to allergen sensitization, thus justifying efforts to identify contributory genes as asthma susceptibility loci.



MATERIALS AND METHODS

Mice. 6-8 week old A/J and MHC-matched B10.A/SgSnJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice deficient in T- and B-cells by disruption of the RAG-1 gene and C57BL/6 mice with deletion of the IL-4 gene were purchased from Jackson Laboratories. RAG-deficient (23) and IL-4-deficient (24) mice were backcrossed six generations to A/J mice and intercrossed to generate N6 A/J RAG- and IL-4-deficient animals. Mice were typed for RAG deficiency using flow cytometric analysis to confirm the absence of peripheral blood T and B cells. Mice were typed for IL-4 deficiency by PCR analysis of tail DNA. Animals were housed in the University of California San Francisco pathogen-free animal facility.

Antigen sensitization. Mice were immunized weekly for three wk with 25 μg chicken egg ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) in 50 μl alum suspension subcutaneously at the base of the tail. Immunized mice received three aerosol exposures of 50 mg/ml OVA in PBS over 20 min, every other day, using a nose-only chamber adapted for mice (Intox Products, Albuquerque, NM) and coupled to a nebulizer (Aerotech II; CIS-US, Bedford, MA) as described (25). Airway reactivity was assessed two days after the final aerosol. Control mice were immunized with PBS in alum and exposed to a PBS aerosol.

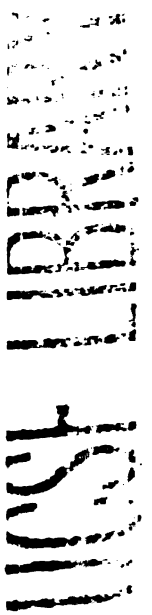
Bone marrow reconstitution. Bone marrow cells were flushed from the tibia and femur of donor mice with RPMI 1640 supplemented with 10% FBS and antibiotics, passed through a 70 μm nylon mesh, washed and resuspended at 2.5 x 10^7 cells/ml in PBS. Bone marrow cells (5 x 10^6) were injected intravenously into irradiated (900 rads) mice. Animals were maintained in pathogen-free conditions with antibiotic-supplemented water for 8 wk, at which time hematopoietic cell reconstitution was documented using flow cytometry.

T cell depletion. Where indicated, mice were injected intraperitoneally with 1 mg anti-CD4 mAb (GK1.5) 4 times at 3 day intervals. Depletion of CD4+ T cells (<1% normal) was documented using flow cytometry. Airway reactivity was assessed two days after the final antibody treatment. Control mice received matched irrelevant mAb.

Airway reactivity. Airway reactivity to acetylcholine chloride (ACh) was measured as described (25). Briefly, mice were anesthetized with etomidate (28 μg/ gm; Bedford Laboratories, CA). The trachea was cannulated and mice were ventilated with 100% oxygen at physiological rate and tidal volume using a rodent ventilator (Harvard Apparatus, South Natick, MA). Following paralysis to eliminate spontaneous respirations, mice were maintained inside a whole-body plethysmograph capable of measuring changes in air flow, as well as transthoracic pressures and resistance. After establishing a stable baseline for total lung resistance, ACh was administered intravenously over 1 s in escalating doses via the tail vein. Airway reactivity was expressed as the provocative concentration of ACh (in μg/gm body weight) required to double baseline transthoracic resistance, designated PC_{200}, as calculated by linear interpolation of appropriate dose-response curves. Significant differences were calculated using the logarithm of PC_{200} by analysis of variance using reference to the specified control groups. Baseline pulmonary resistance in the absence of ACh did not differ among the various groups of mice studied.

Analysis of bronchoalveolar lavage. BAL cells were collected after instillation and withdrawal of three sequential 1 ml PBS aliquots through the tracheal cannula. Cells were washed, counted and resuspended in RPMI 1640 with 10% FBS and antibiotics to a final concentration of 5 x 10^5 cells/ml. Aliquots (10^5 cells) were spun onto glass slides, air dried, fixed with methanol and stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL). Eosinophils, macrophages/monocytes and lymphocytes were enumerated based on morphology and staining characteristics and expressed as percentages of total BAL cells.

ELISPOT assays. Lung cell suspensions were prepared by finely mincing the lungs (devoid of any lymph node or thymic tissue) and pressing the fragments through a 70 μm nylon mesh filter. Red blood cells were lysed in hypotonic buffer and the remaining cells were washed, counted and resuspended at 10^7 cells/ml in RPMI 1640 with 10% FBS and antibiotics. The numbers of IL-4-producing cells were enumerated using enzyme-linked immunocell spot (ELISPOT) assays as described (25). Briefly, lung cell suspensions were distributed in duplicate aliquots of 10^6 cells into 96-well microtiter plates that had been precoated with anti-murine IL-4 mAb (11B11). Serial two-fold dilutions were prepared and the plates were incubated undisturbed for 18 hr at 37^0C. After washing away the cells, biotinylated secondary anti-IL-4 mAb (BVD6-24G.2) was added. Captured IL-4 was revealed using streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and developed using 5-bromo-4-chloro-indolyl-phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma) suspended in 0.6% low-melt agarose. Individual blue spots were counted after solidification of the agar using inverted microscopy.



Serum IgE. Serum was prepared from whole blood collected after quantitation of airway reactivity. Total serum IgE was determined using a double mAb-based sandwich ELISA with antibody B.IE.3 as the capture antibody and biotinylated EM-95 as the detecting antibody. The plates were developed with streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch) and the substrate was 5 mM p-nitrophenyl phosphate, disodium hexahydrate (Sigma) in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma). Absorbance was quantitated at 405 nm (Bio-Tek Instruments Inc., VT) and normalized to concurrently developed standard controls.

Histopathology. Lungs were infused in situ with 1 ml of 3.7% paraformaldehyde in PBS through the tracheal cannula. The lungs were carefully removed and immersed in the same

fixative with the trachea tied closed for 24 hr. The tissues were embedded in paraffin and 2-3 μm sections were cut and stained with hematoxylin and eosin.

Flow cytometric analysis. Conjugated mAbs for flow cytometric analysis included PE-anti-B220 and FITC-anti-CD8α (Caltag Laboratories, So. San Francisco, CA), FITC-anti-Ly9.1 and PE-anti-CD4 (clone RM4-4) (PharMingen, San Diego, CA). The two anti-CD4 mAbs bind at distinct sites unaffected by the presence of the other. Stained cells were analyzed for surface expression using flow cytometry (FACScalibur; Becton Dickinson & Co., Mountain View, CA).

RESULTS

Airway hyperreactivity is increased in A/J as compared to MHC congenic B10.A mice.

A/J mice display increased airway reactivity after challenge with a number of bronchoconstricting agents (17-20). To compare airway reactivity in A/J mice with MHC congenic B10.A mice, cohorts of unimmunized animals were given escalating doses of ACh intravenously while maintained in a whole-body plethysmograph, enabling constant quantitation of transthoracic pressure and flow. A/J mice showed a marked increase in airway hyperreactivity as assessed by the significant decrease in the provocative ACh dose required to elicit a 200% increase in baseline airway resistance (PC_{200}) (Figure 1). Thus, in agreement with prior studies, unimmunized A/J mice demonstrated approximately 5-fold greater sensitivity to the airway constricting effects of ACh than did MHC-matched B10.A mice.

Immunized A/J mice develop enhanced airway hyperreactivity to aerosolized antigen.

To compare antigen-dependent airway hyperreactivity between A/J and B10.A mice, groups of mice were immunized three times with OVA and then challenged three times with OVA-containing aerosol. Two days after the third aerosol, mice were anesthetized and ventilated in a whole-body plethysmograph. As assessed by airway resistance in response to ACh, immunized A/J mice developed a further 4-fold increased reactivity, consistent with allergen-induced models of airway hyperreactivity. Similarly, immunized B10.A mice developed substantial airway hyperreactivity after sensitization and airway challenge with OVA (Figure 2A).

Figure 1. **Airway resistance in response to escalating doses of intravenous ACh.**

After establishing a stable baseline for total lung resistance, ACh was infused intravenously in escalating doses into unimmunized, age-matched, B10.A and A/J mice. ACh injections are indicated by the numbered arrowheads, 1-5, and represent incremental doses of 0.03, 0.1, 0.32, 1.0 and 3.3 μg ACh/gram bodyweight as indicated by the horizontal black triangles. The concentration of ACh required to elicit a 200% increase from baseline airway resistance, or PC_{200}, is indicated by the bold arrowhead. Results are representative of 8 experiments involving over 30 animals in each group.

Figure 1

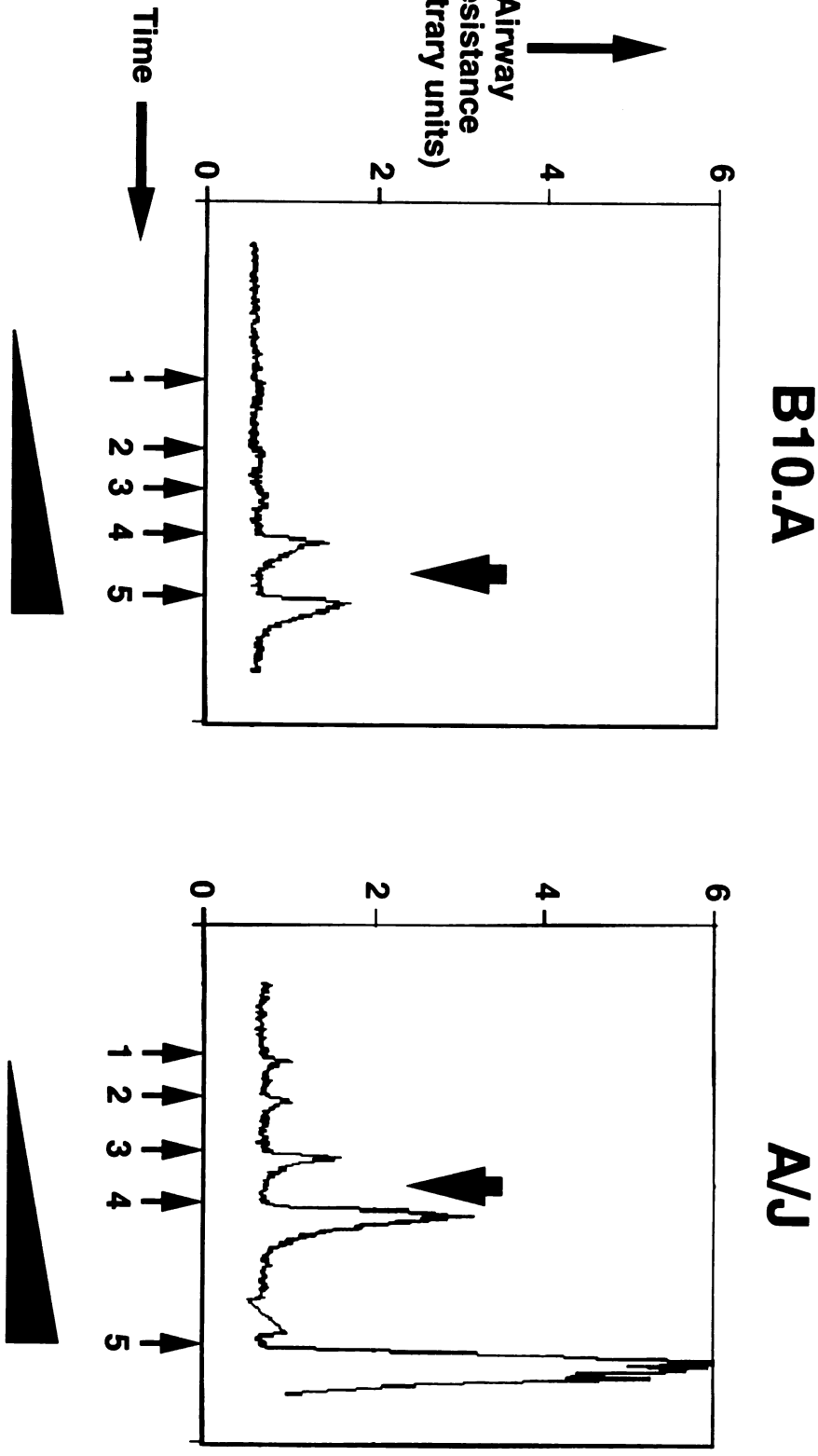


Figure 2. **Airway hyperreactivity and the immune response in control and immunized A/J and B10.A mice.**

Groups of 4-8 B10.A and A/J mice were challenged with PBS (Saline) or OVA.

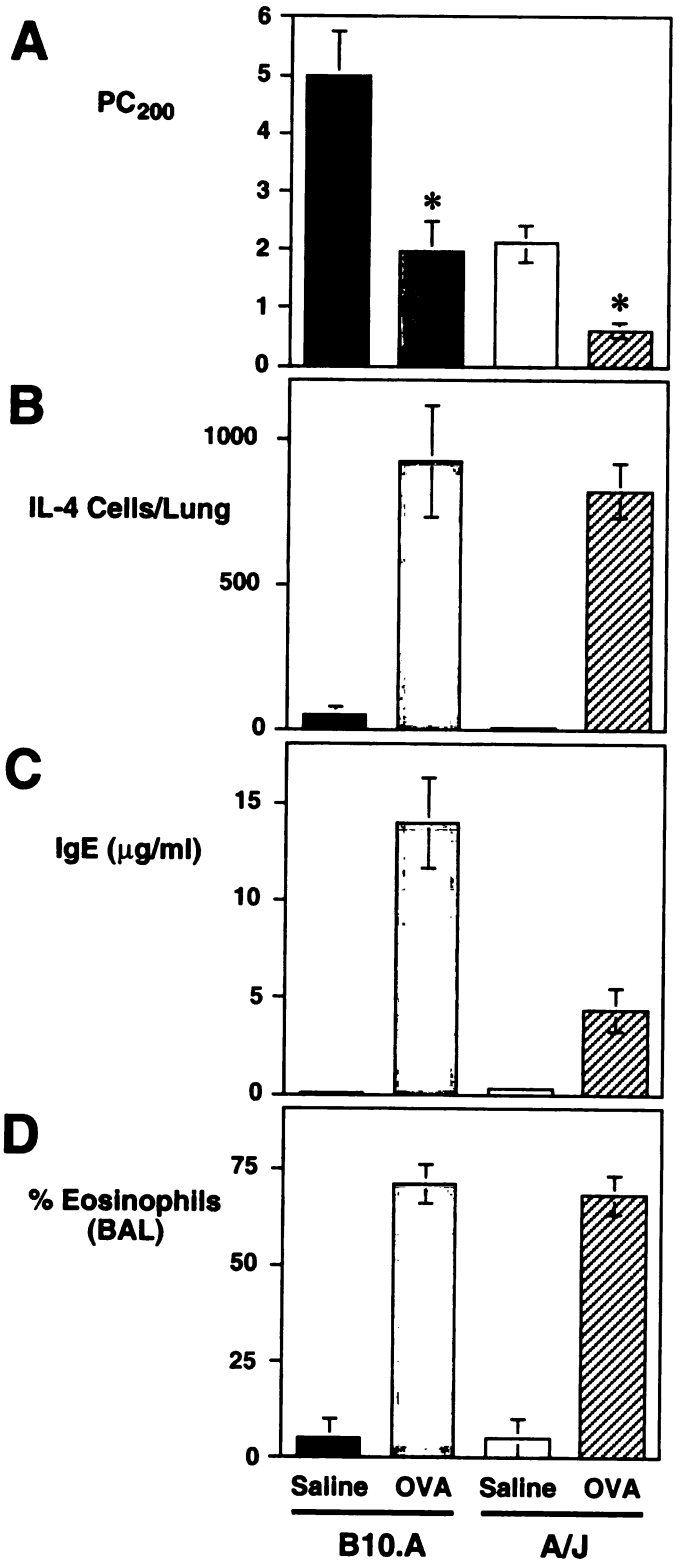
A. PC_{200} in response to escalating doses of ACh.

B. Numbers of IL-4-producing cells in the lungs of control and immunized mice as determined by ELISPOT analysis.

C. Serum IgE levels in control and immunized A/J and B10.A mice as assessed by ELISA.

D. Percentage eosinophils in total BAL cells.

In each case, results are representative of at least two experiments. Bars depict means and standard errors of the means. Significant differences ($p<0.05$) between control and OVA groups are indicated (*).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PC200
6
5
4
3
2
1
0
*
*
B
IL-4 Cells/Lung
1000
500
0
C
IgE (μg/ml)
15
10
5
0
D
% Eosinophils (BAL)
75
50
25
0
Saline
OVA
Saline
OVA
B10.A
A/J

Figure 2

Antigen-induced airway reactivity in B10.A mice was comparable to baseline airway reactivity in A/J mice (Figure 2A). This raised the possibility, considered by others (26-29), that A/J mice develop enhanced responsiveness to environmental airway allergens that results in immune-mediated elevations in baseline airway reactivity. Since airway hyperreactivity in antigen-driven systems is accompanied by type 2 immune responses, we assessed resting A/J mice for evidence of active immune responses in the lungs. First, using ELISPOT assays to determine numbers of IL-4-producing cells from whole lung preparations, no increase was observed in unimmunized A/J mice. Further, the numbers of IL-4-producing cells in the lung that appeared after OVA challenge was comparable in A/J and B10.A mice (Figure 2B). Second, serum IgE, a sensitive index of chronic IL-4- and IL-13-mediated stimulation, was low in unimmunized A/J mice and not significantly different from B10.A mice. With OVA challenge, IgE levels actually increased to greater levels in B10.A mice (Figure 2C). Finally, there was no histologic evidence of active inflammatory injury in the lungs of resting A/J mice as assessed either by enhanced numbers of eosinophils in BAL (Figure 2D) or by microscopic examination of lung tissues (Figure 3A). OVA-immunized A/J and B10.A mice demonstrated cardinal features of allergic lung disease (Figure 3B, 3C), including peribronchiolar infiltration with lymphocytes and eosinophils and the appearance of numerous eosinophils in BAL (Figure 2D). If anything, the extent of cellular inflammation was more pronounced in comparably immunized B10.A mice.

Thus, despite the marked elevation in airway reactivity in A/J mice, we could find little evidence for chronic activation of type 2 immune responses under resting conditions. In contrast, immunization and aerosol challenge in both A/J and B10.A mice resulted in highly stereotyped type 2 immune responses, including elevations in the numbers of IL-4-producing cells, in eosinophils, in tissue infiltration by lymphocytes and eosinophils, and in serum IgE.

Figure 3. **Lung histology in naive A/J and immunized A/J and B10.A mice.**

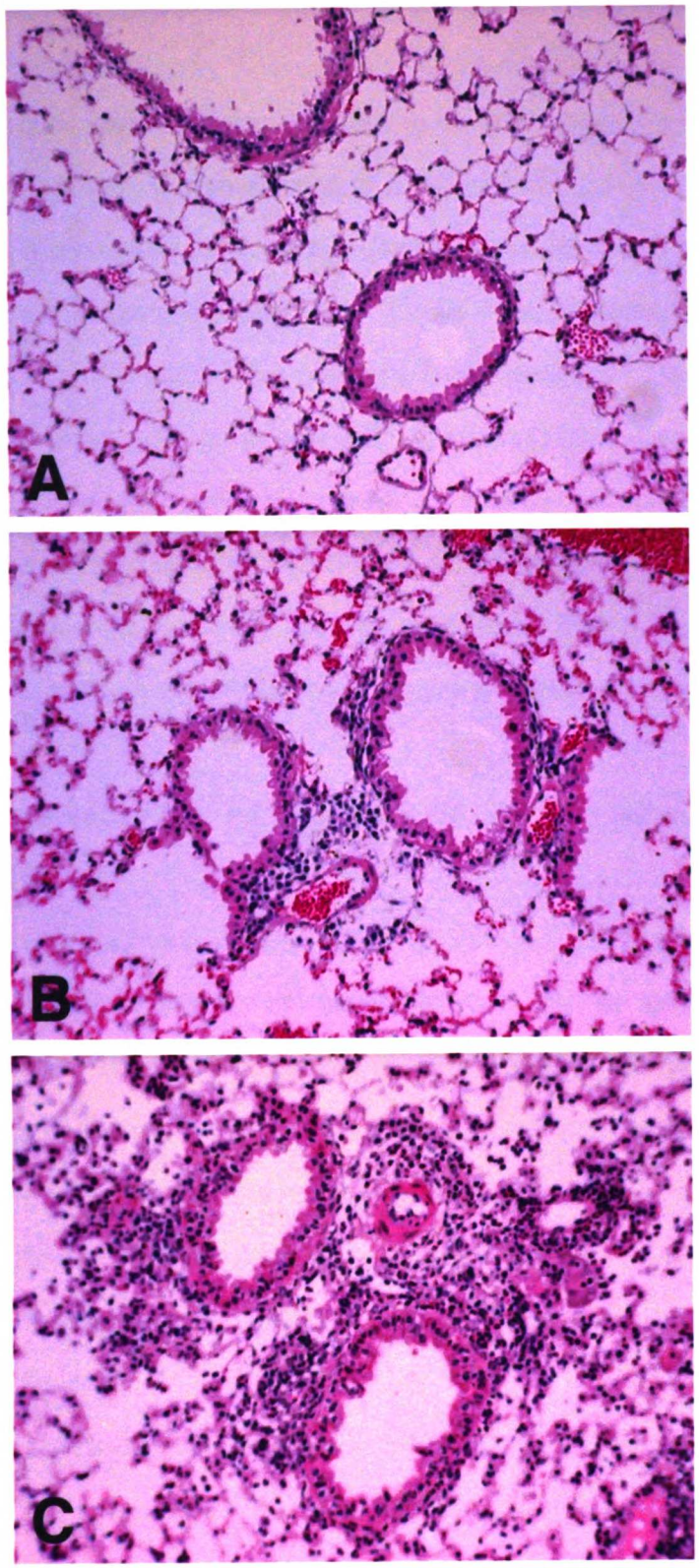
A. A/J lung after saline aerosol. No cellular infiltrate is apparent in the bronchiolar airways or alveolar spaces.

B. A/J lung after OVA aerosol. Periarterial space is filled with a lymphocyte and eosinophil rich infiltrate.

C. B10.A lung after OVA aerosol. The periarterial space is filled with an inflammatory infiltrate that spreads between two adjacent bronchiolar airways.

All sections stained with hematoxylin and eosin and viewed at 100x magnification.

Figure 3



Deletion of CD4+ T cells does not alleviate airway hyperreactivity in A/J mice.

CD4+ T cells have been demonstrated to be both necessary and sufficient to mediate airway hyperreactivity in murine models of allergic airways disease (15, 30-32). Despite our inability to document activated type 2 immune responses in unimmunized A/J mice, it remained possible that circulating CD4+ T cells were mediating airways disease by some unclear pathway. To assess this possibility, A/J mice were depleted to <1% of normal CD4+ T cells using mAb given over a 2 wk period (Figure 4A). CD4-depleted and A/J mice given matched irrelevant control antibody were compared to B10.A mice using airway reactivity to escalating doses of ACh (Figure 4B). Despite CD4+ T cell depletion, A/J mice displayed airway hyperreactivity that was no different than A/J mice given control mAb. Thus, CD4+ T cells do not mediate baseline airway hyperreactivity in A/J mice.

Airway hyperreactivity of A/J mice is not dependent on bone marrow-derived cells.

Bone marrow radiation chimeras were constructed between A/J and B10.A mice in order to evaluate the role of hematopoietic cells in mediating baseline airway hyperreactivity. The Ly9.1 surface antigen, present on A/J but not on B10.A hematopoietic cells, was used to mark donor-derived bone marrow cells. As a control, bone marrow cells were used to reconstitute each mouse strain with its own donor cells. Analysis eight wk after reconstitution confirmed that the majority of hematopoietic cells were donor-derived, although reconstitution was more complete from A/J to B10.A than vice versa (Figure 5).

Mice reconstituted with homologous or heterologous bone marrow cells were analyzed for baseline airway hyperreactivity after eight wk, at which time all mice appeared well and without apparent disease. Although there was some variability in the absolute PC_{200},

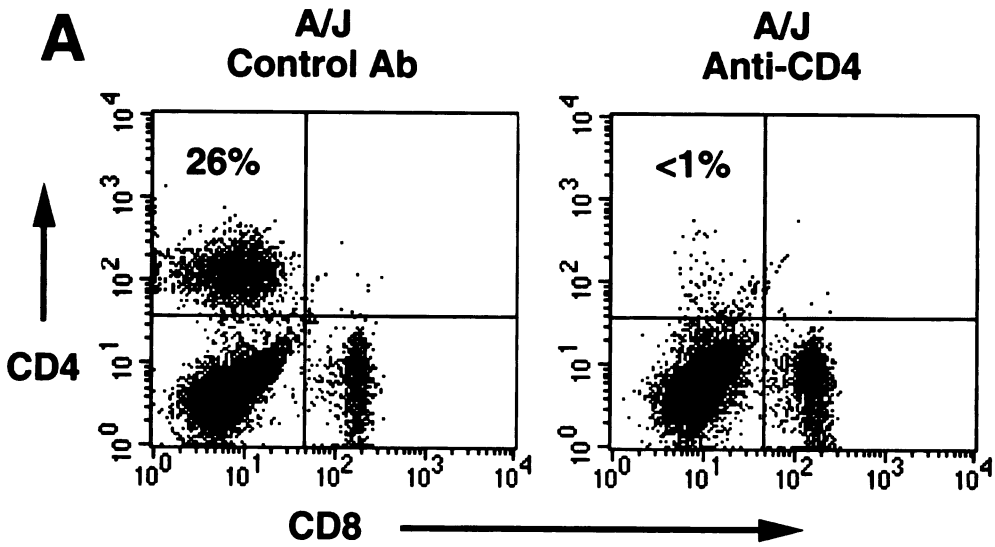
Figure 4. **Airway hyperreactivity in CD4+ T cell-depleted mice.**

A. Flow cytometric analysis of spleen cells after treatment of nonimmunized A/J mice with control and anti-CD4 mAb.

B. Groups of 4-6 nonimmunized A/J mice treated with control (ctrl Ab) or anti-CD4 mAb, or nonimmunized B10.A mice, were anesthetized and ventilated for determination of PC_{200} in response to ACh. Bars represent means and standard errors of the means. Significant differences ($p<0.05$) between the untreated B10.A and the anti-CD4-treated A/J mice are indicated (*).

Results are representative of two experiments.

Figure 4


A
A/J
Control Ab
A/J
Anti-CD4
26%
<1%
CD4
CD8

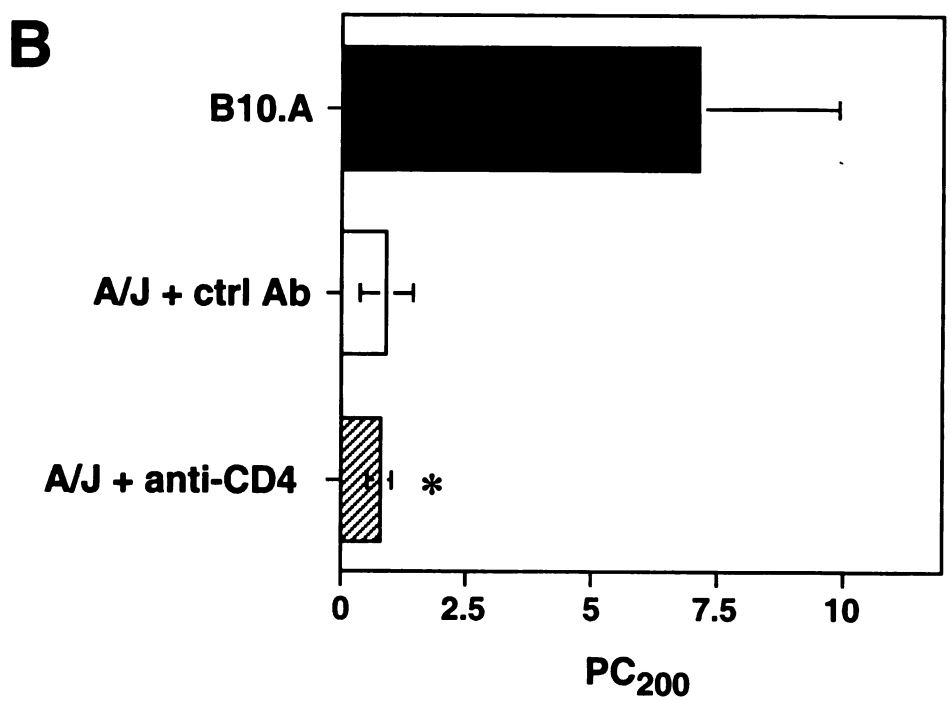
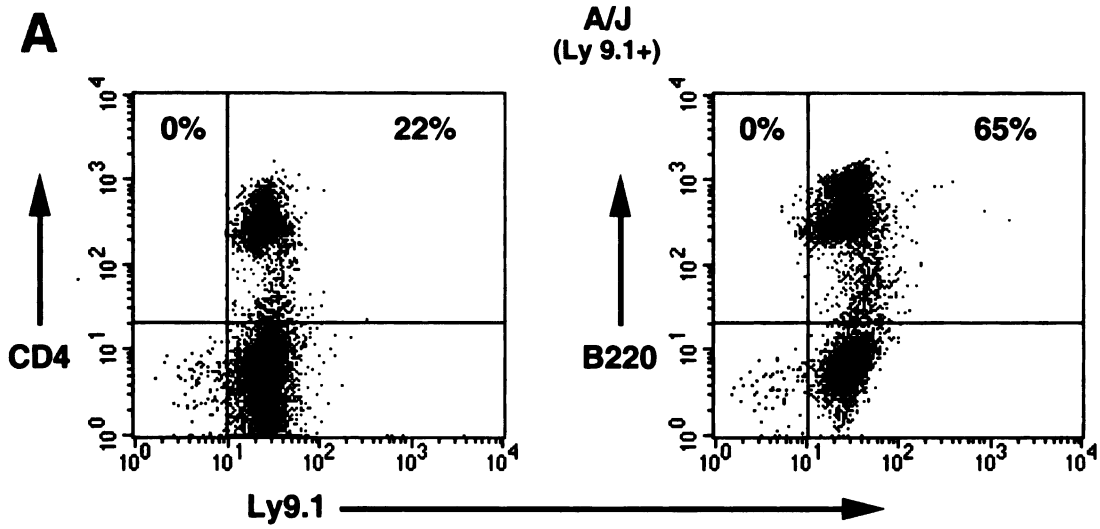
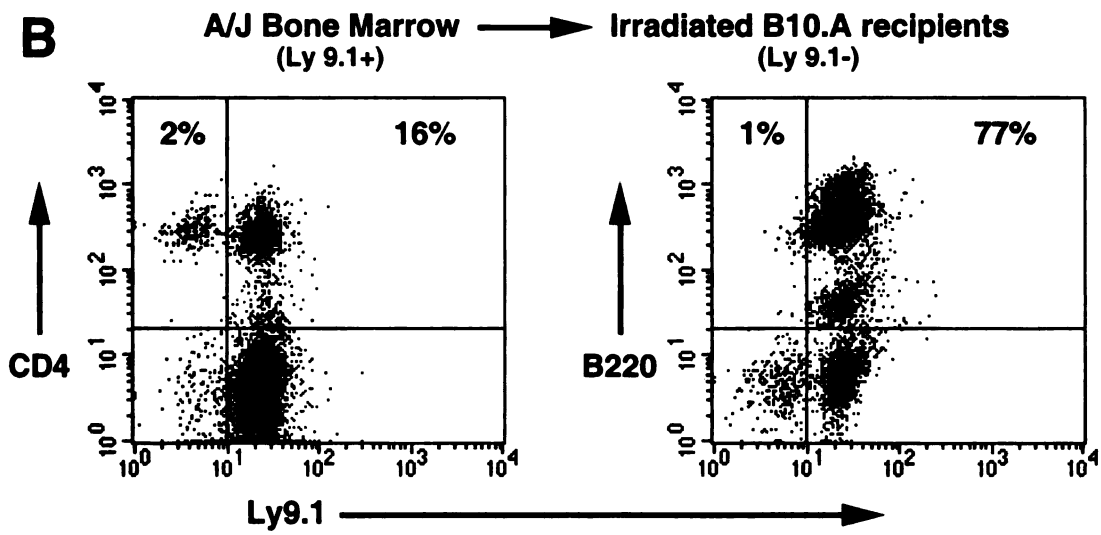

B
B10.A
A/J + ctrl Ab
A/J + anti-CD4
*
0
2.5
5
7.5
10
PC200

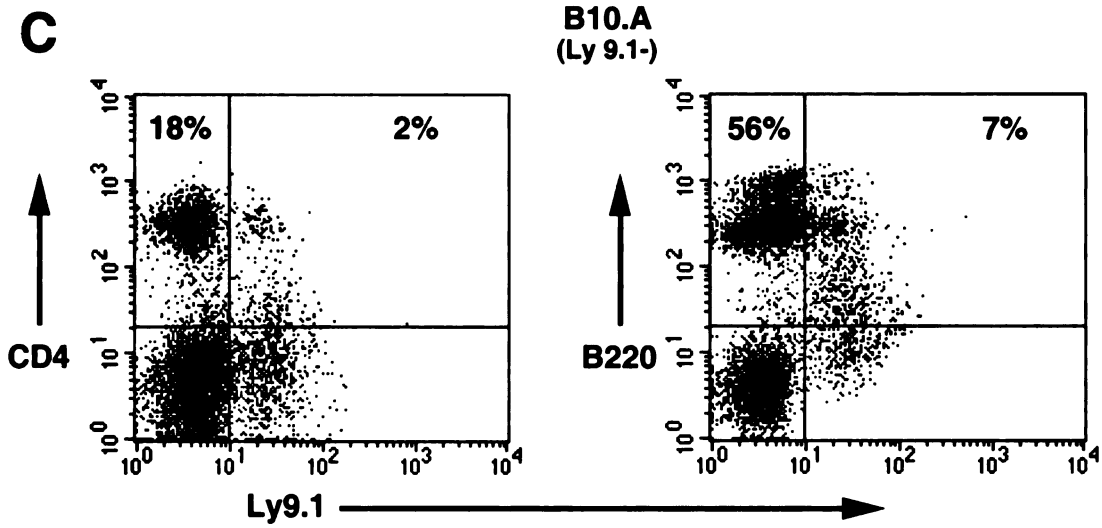
Figure 5. **Flow cytometric analysis of spleen cells from bone marrow-reconstituted radiation chimeras.**

Eight wk after reconstitution, spleen cells from untreated A/J mice (**A**), irradiated B10.A recipients reconstituted with A/J bone marrow cells (**B**), untreated B10.A mice (**C**) and irradiated A/J recipients reconstituted with B10.A bone marrow cells (**D**), were stained with mAb to Ly9.1, B220 and CD4 to mark hematopoietic cells of the A/J lineage, B cells and T helper cells, respectively. A/J hematopoietic cells are Ly9.1+ whereas B10.A cells are Ly9.1-.

Figure 5 (A&B)


A
A/J
(Ly 9.1+)
0%
22%
0%
65%
CD4
B220
Ly9.1


B
A/J Bone Marrow
(Ly 9.1+)
Irradiated B10.A recipients
(Ly 9.1-)
2%
16%
1%
77%
CD4
B220
Ly9.1


C
B10.A
(Ly 9.1-)
18%
2%
56%
7%
CD4
B220
Ly9.1

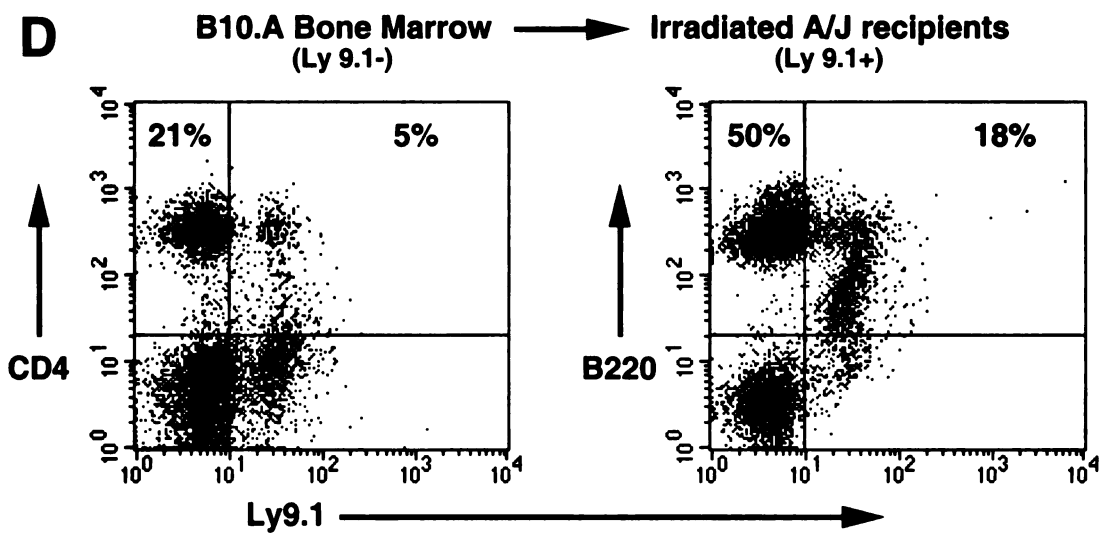
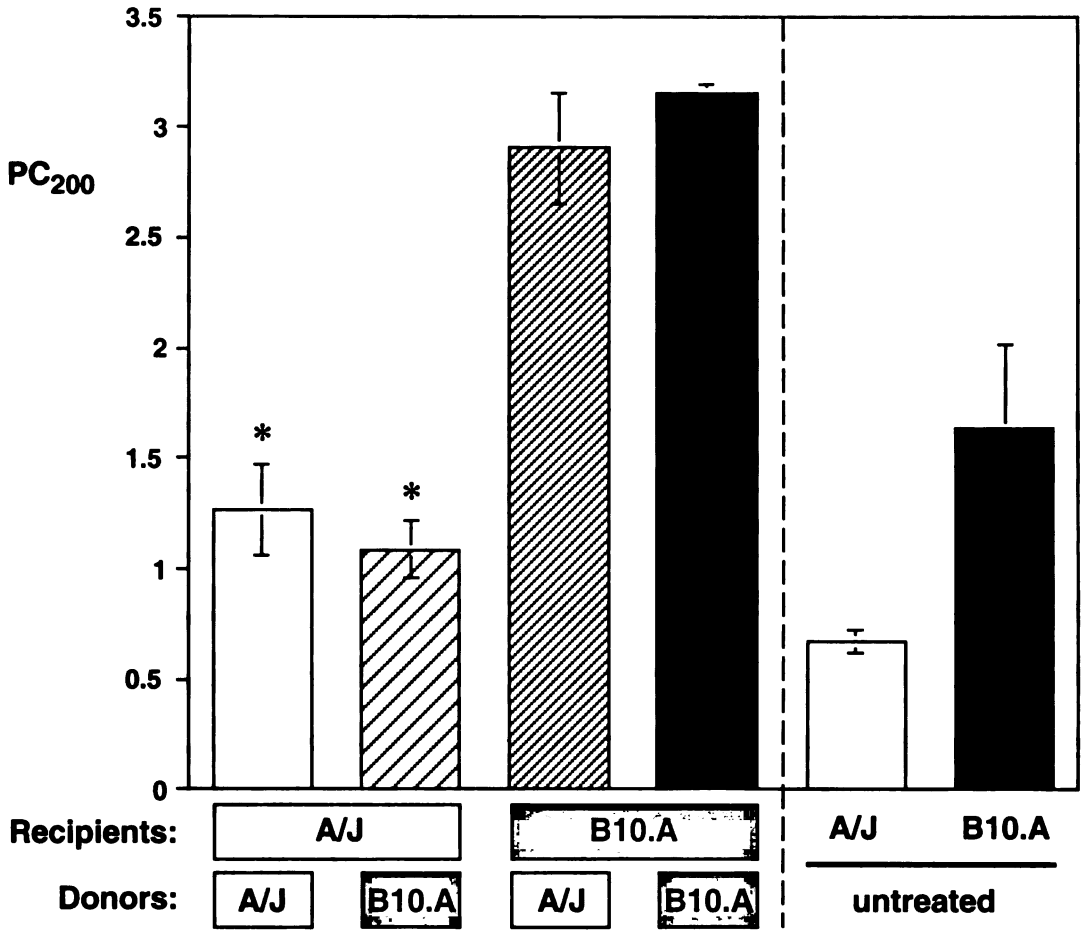

D
B10.A Bone Marrow
(Ly 9.1-)
Irradiated A/J recipients
(Ly 9.1+)
21%
5%
50%
18%
CD4
B220
Ly9.1

Figure 6. **Airway hyperreactivity in bone marrow reconstituted radiation chimeras.**

Groups of 4-8 irradiated A/J and B10.A recipients were reconstituted with either A/J or B10.A bone marrow cells and compared to untreated A/J and B10.A mice eight wk after reconstitution as indicated. Bars depict mean and standard errors of the mean PC_{200} as determined by responsiveness to escalating doses of ACh. Significant differences ($p<0.05$) between the A/J and B10.A recipient groups given either A/J or B10.A donor bone marrow, respectively, are indicated (*). Results are representative of two independent experiments.

Figure 6


3.5
3
2.5
2
1.5
1
0.5
0
PC200
*
*
Recipients:
A/J
B10.A
A/J
B10.A
Donors:
A/J
B10.A
A/J
B10.A
untreated

B10.A remained relatively unresponsive to ACh as compared to A/J mice. After irradiation and bone marrow reconstitution, all mice demonstrated enhanced PC_{200} as compared to unmanipulated mice (Figure 6). There was no evidence for graft-versus-host disease in the lungs or elsewhere, and no pulmonary infiltrations by inflammatory cells was apparent in any of the mice. Despite these consistent findings, A/J mice, whether recipients of their own bone marrow or B10.A bone marrow, demonstrated significantly increased airway hyperreactivity (decreased PC_{200}) as compared to B10.A recipients. In a comparable way, B10.A recipient mice displayed decreased responsiveness to ACh regardless of whether they had been reconstituted with A/J or B10.A cells. Thus, in this model, we could find no evidence suggesting that bone marrow-derived cells contribute to the intrinsic elevation in airway reactivity of resting A/J mice.

Airway hyperreactivity of A/J mice is independent of T and B cells or IL-4.

Despite our inability in implicating immune dysfunction in the airway hyperreactivity of resting A/J mice, it remained possible that prolonged developmental effects mediated by the immune system might produce chronic airway changes that persisted even after immune cell depletion in adult life. We took a genetic approach to this possibility by crossing A/J mice to RAG-deficient and IL-4-deficient mice. Both of these mice have been demonstrated to have abrogated airway responses following antigen challenge, with RAG-deficient mice having a more complete attenuation of the response than IL-4-deficient mice (31, 33-35). For the RAG backcross, C57BL/6 RAG-1-deficient mice were crossed six generations to A/J, and the progeny intercrossed to create N6 A/J RAG-deficient mice. Similarly, C57BL/6 IL-4-deficient mice were crossed six generations to A/J, and the progeny intercrossed to create N6 A/J IL-4-deficient mice. The success of the backcross was confirmed by PCR analysis of tail DNA to confirm the presence of the targeted, but

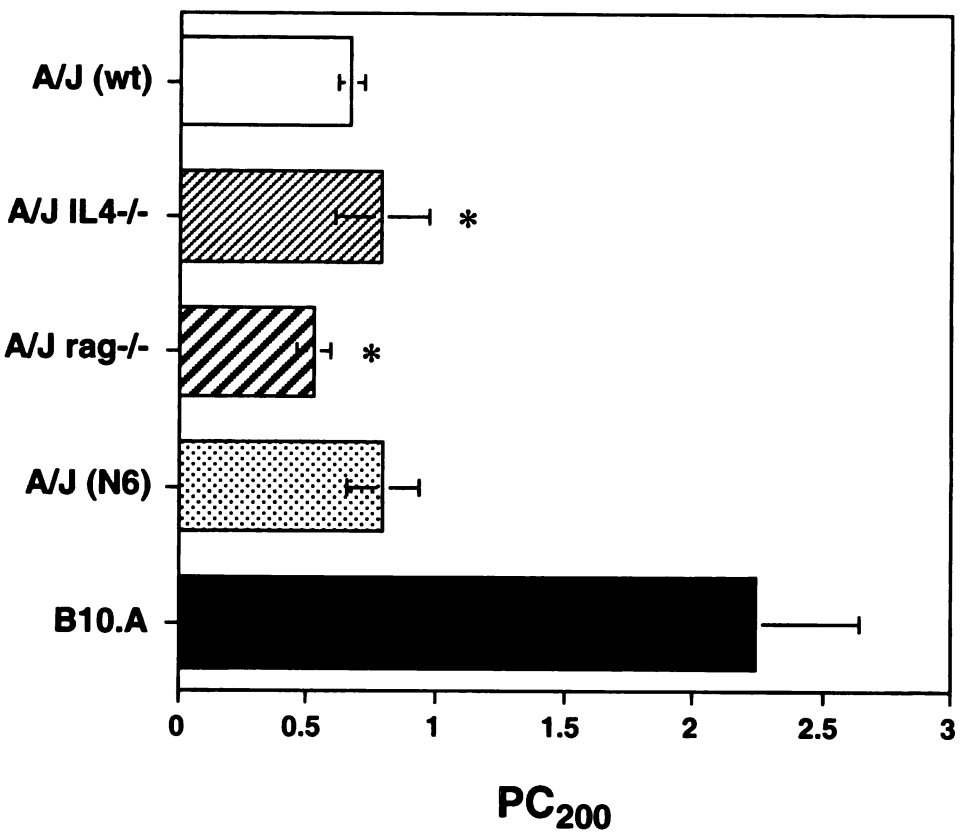
not the wild-type IL-4 allele, and by flow cytometry to confirm the absence of T and B cells in the RAG-deficient A/J mice.

A/J RAG-deficient and IL-4-deficient mice were analyzed for reactivity to ACh and compared to control A/J and B10.A mice (Figure 7). Despite the complete absence of T and B cells from birth, or the complete inability to produce the cytokine IL-4, the backcrossed A/J mice displayed airway hyperreactivity comparable to wild-type A/J mice and to control backcrossed N6 A/J mice. Although IL-13 can mediate airway hyperreactivity in the OVA model in the absence of IL-4 (33, 36), we could confirm no increase in IL-13 mRNA in A/J, A/J RAG-deficient, A/J IL-4-deficient or B10.A mice in the absence of airway sensitization with allergen (data not shown). Thus, in this model, we could define no contributions from the adaptive immune system that contribute to the underlying airway hyperreactive phenotype of A/J mice.

Figure 7. **Airway hyperreactivity in IL-4 and B- and T-cell deficient mice.**

Unimmunized groups of 4-8 IL-4 deficient and T- and B-cell deficient (rag-/-) A/J mice, wild-type A/J, B10.A, and pooled N6 A/J littermate control mice were anesthetized and ventilated for determinations of PC_{200} in response to escalating doses of ACh. Bars depict means and standard errors of the means. Significant differences ($p<0.05$) between the mutant (IL4- and rag-deficient) mice and B10.A mice are represented (*). Results are representative of three experiments.

Figure 7



DISCUSSION

The role of the adaptive immune system in mediating allergen-induced airway hyperreactivity, despite some modest controversy, seems well established (reviewed in 15). In this model, CD4+ T cells differentiate preferentially to a type 2 phenotype, leading to the activation of various effector cells and the elaboration of reactive agents that together result in the classic cell and tissue responses that characterize asthma. Many of these downstream effector functions seem to depend upon release of IL-4 and IL-13 from Th2 cells, which can mediate eosinophil recruitment, mucus hyperplasia and airway hyperreactivity by a mechanism dependent on signaling through IL-4Rα (33, 37-39) and activation of Stat6 (40, 41). Despite this knowledge, the contributions of adaptive immunity to the maintenance of baseline airway reactivity to bronchoconstricting agents remain less well studied. Various transgenic mice that express cytokines in the lung have demonstrated effects on baseline airway hyperreactivity (42-45), but whether this reflects the physiologic state is unknown.

We used A/J mice, a strain with inherently increased baseline airway hyperreactivity to pharmacologic bronchoconstricting agents, to assess the role of the adaptive immune system in mediating this physiologic response. Despite markedly increased baseline airway reactivity as compared to MHC congenic B10.A mice, we could find no evidence that cells of the adaptive immune system contribute to resting airway responsiveness. This was assessed by multiple independent strategies, including the acute depletion of CD4+ T cells, the crossed bone marrow reconstitutions between A/J and B10.A mice, and the genetic approach involving crossing A/J mice to RAG- and IL-4-deficient backgrounds. The baseline hyperreactivity of A/J mice was essentially unaltered by each of these approaches. Further, there existed no histologic evidence to support an immune-mediated mechanism for maintaining baseline reactivity in A/J mice. In contrast to immunized B10.A mice with

comparable airway hyperreactivity (Figure 2A), naive A/J mice displayed no cellular infiltrates into pulmonary tissue and no evidence for intrinsic activation of type 2 immune responses. The data suggest an intrinsic mechanism for airway hyperreactivity in A/J mice that makes this a valuable model for investigating contributions of nonimmune genes to airway physiology.

Our inability to implicate hematopoietic cells in baseline airway hyperreactivity in A/J mice was in contrast to a prior study that used a similar approach using crossed bone marrow chimeric mice. Importantly, however, the prior study used F1 A/J x C57BL/6 mice as the hyperreactive parental strain as compared to C57BL/6 control animals (26). F1 mice were used to minimize contributions by graft-versus-host disease in chimeric animals, but the discriminatory differences in airway physiology were smaller in F1 as compared to parental A/J mice. We used MHC congenic B10.A mice to both minimize the potential for graft-versus-host disease amongst recipients while maximizing the discrimination in airway physiologic parameters as compared to A/J mice. We could discern no evidence for graft-versus-host disease in reconstituted mice using histologic criteria, but observed a consistent change in the baseline airway physiology in all irradiated mice, irrespective of the source of the donor marrow cells. Despite these baseline changes, which were consistent within groups of mice, it was clear that A/J recipients demonstrated resting airway hyperreactivity that was elevated comparably whether reconstituted with A/J or B10.A-derived bone marrow cells. Conversely, B10.A recipients displayed relatively normal airway hyperreactivity even after reconstitution with A/J-derived cells.

Acute reconstitution experiments leave open the possibility that developmental processes mediated by hematopoietic cells, and specifically by immune T cells, could induce chronic airway structural alterations leading to airway hyperreactivity that might not be readily reversed in adult life. We, in agreement with a prior report (28), could demonstrate no effect on baseline airway physiology mediated by acute depletion of CD4+ T cells, but

potential chronic effects of lymphocytes have not been investigated. We used a genetic approach by crossing A/J mice to RAG-1-deficient and IL-4-deficient mice because, in each case, animals on these backgrounds have demonstrated profound deficiencies in the ability to mount airway responses to aerosolized antigen, including OVA (31, 33-35). In each case, the crossing in of A/J-derived genes resulted in increased baseline airway hyperreactivity in the N6 generation, at which point over 98% of the genome was A/J-derived. Airway hyperreactivity of control littermates was indistinguishable from parental A/J mice. In contrast to parental A/J mice, however, the backcrossed mutant mice developed similar airway hyperreactivity in the complete absence of B and T cells (RAG-1-deficient) or IL-4 (IL-4-deficient). Thus, any contributions of lymphocytes or IL-4 to baseline airway hyperreactivity in A/J mice would seem to be nonessential.

The role of non-hematopoietic cell factors in allergen-mediated airway hyperresponsiveness was illustrated here by the enhanced reactivity of A/J mice to sensitization with ovalbumin as compared to B10.A mice with the same amount of antigen challenge (Figure 2A). Similar findings were noted in comparing A/J and C3H mice after sensitization with OVA or sheep red blood cells (29). Thus, subsequent type 2 inflammation resulted in incrementally greater airway hyperreactivity at more modest levels of immune cell recruitment, as evident by microscopic examination (Figure 3). Understanding the mechanisms for such interaction with the immune response may have great implications for the role of innate factors in contributing to susceptibility to allergic airways disease.

These findings raise questions regarding the mechanism(s) that underlie intrinsic airway hyperreactivity in A/J mice. Differences in airway caliber or number were not apparent in our studies (H. Hadeiba, R. M. Locksley; data not shown). The finding that high-dose cyclosporin A could attenuate airway hyperreactivity in A/J mice is intriguing (28). The ubiquitous distribution of calcineurin, the target of cyclosporin A, in all tissues, will require

definition of the amounts and affinities of this phosphatase in discrete lung cell types. Indeed, the recent incrimination of calcineurin-dependent pathways in myocardial hypertrophy and signaling through the myocyte angiotensin receptor point out previously unappreciated roles for phosphatases of this class outside of the immune system (46). Other possibilities include the role of the autonomic nervous system control of airway smooth muscle as determined by a combination of β2-adrenergic and postganglionic muscarinic receptors (reviewed in 47). Of the various airway muscarinic receptor subtypes, the two most implicated in human asthma are designated subtypes II (M2) and III (M3). M2 receptors - present on postganglionic nerves - inhibit acetylcholine release via a negative feedback loop. Some evidence has been provided for dysfunctional M2 receptors in both human asthma (48) and in animal models of allergic airway disease (49). M3 receptors - present on airway smooth muscle, submucosal glands, epithelial and endothelial cells - mediate airway smooth muscle contraction and mucus hypersecretion (47). Some evidence also suggests that coupling of muscarinic receptors to signal-transducing G proteins might be more efficient in A/J mice, although the subtypes of muscarinic receptors involved and their distribution will require further study (50). We did note modestly enhanced expression of M3 receptor mRNA in lung tissues extracted from A/J, as compared to B10.A, mice (H. Hadeiba, R. M. Locksley; data not shown), but further study will be required to quantitate the overall stoichiometry of the various muscarinic receptor subtypes in order to discern any relationship with baseline airway hyperresponsiveness. Although recent studies have defined a role for the integrin αvβ6 in mediating lung tissue homeostasis (51), baseline histologic studies failed to provide evidence for aberrant cell infiltration in mediating the physiologic changes in A/J lung.

These studies reaffirm the importance of the A/J strain in elucidating intrinsic factors that modulate airway hyperreactivity that arise independent of the acquired immune system. The aggravated phenotype demonstrated by A/J mice, with a proportionately greater airway

response following immunization, points out the capacity of such genetic tendencies to aggravate subsequent immune-mediated airways disease. A/J mice will provide not only a valuable strain for classical genetic analysis to elucidate non-immune genes that might contribute to asthma susceptibility, but will remain a stringent test for examining the ability of immune interventions to alleviate successfully the induced allergic response.

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CHAPTER 3

Requirements for Allergen-Induced Airway Hyperreactivity in T and B Cell-Deficient Mice

Summary

The pathogenesis of asthma is believed to reflect antigen-induced airway inflammation leading to the recruitment of eosinophils and activation of mast cells through cell-associated IgE. Controversies persist however, regarding the relative importance of different pathogenic cells and effector molecules. Using direct measurements of pulmonary resistance in mice, we demonstrate that reconstitution of T and B cell-deficient recombinase-activating-gene-deficient mice (RAG-/-) with CD4+ T cells was sufficient to restore airway hyperreactivity to a potent fungal allergen, an extract of Aspergillus fumigatus. CD4+ T cell reconstitution also restored lung inflammatory changes, including eosinophilia and goblet cell hyperplasia. Sensitized B cell-deficient mice also developed airway hyperreactivity and lung inflammation comparable to wild-type animals, confirming that antibodies were dispensable. Treatment with neutralizing anti-IL-4 antibody or sensitization of IL-4-deficient mice resulted in loss of airway hyperreactivity, whereas treatment with anti-IL-5 antibody or sensitization of IL-5-deficient mice had no effect. Therefore, CD4+ T cells alone are sufficient to mediate many of the pathognomonic changes that occur in human asthma by a mechanism dependent upon IL-4, but independent of IL-5, IgE or both. Clarification of the role played by CD4+ T cells is likely to stimulate important therapeutic advances in asthma.

INTRODUCTION

Asthma is a complex clinical disorder marked by reversible episodes of airway narrowing and inflammation. Inflammation is characterized by the presence of Th2 lymphocytes that produce a spectrum of cytokines, including IL-3, IL-4, IL-5, IL-10, IL-13 and GM-CSF (1-5). Together, these cytokines are believed to mediate the recruitment and activation of eosinophils (by IL-5 and GM-CSF) and mast cells (by IL-3, IL-4, IL-9, IL-10 and IL-13) and the production of IgE antibodies (by IL-4 and IL-13), that ultimately cause pathologic changes, including damage and sloughing of the respiratory epithelium, excess mucus production, airway edema and changes in airway smooth muscle that collectively lower the threshold for airway narrowing to a variety of stimuli, termed airway hyperresponsiveness (AH) (6, 7).

Recently, mouse models of allergic airway inflammation have been used to identify host determinants involved in the inflammatory and physiologic changes associated with human asthma. As expected, T cell-deficient mice failed to develop lung inflammation and AH (8-11), and the role for Th2 cells predicted by the human studies was validated using anti-IL-4 and anti-IL-4 receptor antibodies and in IL-4-deficient mice, which failed to develop antigen-induced airway changes in most (8, 12, 13), but not all (14), studies. Activity mediated in the absence of IL-4 may reflect the capacity of IL-13, which can be coordinately expressed with IL-4 in asthma (15), to itself direct Th2 differentiation through STAT6-mediated stimulation in some strains of mice (16). Mice engineered to express excessive amounts of IL-4 in the lung developed tissue inflammation, including eosinophils, and baseline elevations of pulmonary resistance, but did not develop AH, indicating additional requirements for the full spectrum of disease (17). Roles for eosinophils, IL-5 and IgE in AH have been more variable, with some studies suggesting a

necessary role and others demonstrating either or both to be dispensable (reviewed in 18, 19, and discussed below).

The fungus Aspergillus fumigatus has been shown to be a potent pulmonary allergen (20), and is known to cause allergic airways disease in humans (21). We used an extract of Aspergillus which induced AH across a range of inbred mouse strains to investigate the critical components of the host response required to generate AH in the mouse. AH was rigorously quantitated in the intact animal while breathing at physiologic tidal volumes. As assessed in both CD4+ T cell-reconstituted, recombinase-activating-gene (RAG)-deficient mice and in B cell-deficient mice, T cells were sufficient to mediate acute AH and lung infiltration by inflammatory cells. Further, depletion of eosinophils to baseline levels had no effect on the physiologic or immunologic changes mediated by CD4+ T cells. IL-4, but not IL-5, emerged as a critical effector molecule mediating AH. This model should be useful in elucidating the minimal signals required to elicit allergen-induced lung disease.

MATERIALS AND METHODS

Mice. Female C57BL/6 (Jackson Laboratories, Bar Harbor, ME) and 129Sv/Ev (Taconic Laboratories, Germantown, NY) mice were used at 6-10 weeks of age. Mice rendered deficient in B cells by targeted disruption of the membrane exon of the immunoglobulin μ chain gene (22), and mice rendered deficient in T and B cells by disruption of the recombinase activating gene-1 (RAG-1) (23) were backcrossed 6 times to C57BL/6 mice and purchased from Jackson Laboratories. RAG-2-deficient mice (24) were on the 129SvEv background (Taconic Laboratories), IL-4-deficient mice (25) were on the 129Sv/Ev background and IL-5-deficient (26) mice were on the C57BL/6 background.

Antigen sensitization. Aspergillus fumigatus antigens represent a mixture of culture filtrate and mycelial extract prepared free of living organisms (27). Batches #5323R1 and 5325 with equivalent activities were aliquoted at 10 mg/ml and stored at -70° C prior to use. The endotoxin content of the extract was less than 0.1 EU/100 μg antigen.

For sensitization, anesthetized mice were given 100 μg Aspergillus extract in 50 μl PBS intranasally 5 times at 4-day intervals, as described (20) or, with slight modification, by first systemically priming animals with 3 subcutaneous injections of 100 μg Aspergillus extract in 50 μl PBS at 4-day intervals, followed by delivery of the same amount of antigens 3 times at 4-day intervals to anesthetized animals via a blunt-end gavage needle inserted into the upper airway by direct visualization. No physiologic or pathologic differences occurred among mice immunized by either of the two protocols (D. B. C. and G. G., unpublished data). Designated mice were given 2 mg of neutralizing anti-murine IL-5 antibody, TRFK-5 (28), or 2 mg of neutralizing anti-murine IL-4 antibody, 11B11 (29), intraperitoneally, beginning on the first day of antigen sensitization and then

concurrently with additional antigen administration every 4 days. Control mice were given an isotype-matched irrelevant monoclonal antibody, Y13-259.

Quantitation of AH. Mice were anesthetized and maintained inside a whole body plethysmograph on rodent ventilators as described (8). For convenience, animals were studied four days after the final antigen challenge, although entirely comparable results occurred when mice were investigated either immediately or one day after the final antigen challenge (D. Corry and G. Grunig; unpublished observations). Briefly, mice were ventilated using 100% oxygen under conditions that maintained physiologic pH and PCO_2. It was not possible to maintain physiologic conditions if mice were ventilated with room air, due to the severe hypoxemia incurred after administration of the higher doses of acetylcholine (D. Corry; unpublished data). After establishing a stable baseline for lung resistance (R_L), as determined by continuously quantitating $\Delta Pt/\Delta V$ (where ΔPt = change in tracheal pressure and ΔV = change in flow) at 70% tidal volume, acetylcholine chloride was administered intravenously over 1 second in escalating doses via an indwelling tail vein catheter. The provocative concentration of acetylcholine in μg/gm that caused a 200% increase in R_L, designated PC_{200}, was calculated by linear interpolation of appropriate dose-response curves. Significant differences (defined as $P < 0.05$) were calculated on the logarithm of PC_{200} by analysis of variance using reference to the specified control groups. Baseline pulmonary resistance did not differ among the various groups of mice in these studies.

Assessment of the immune response. Bronchoalveolar lavage (BAL) cells were collected by serially instilling and withdrawing 1 ml aliquots of Hanks' balanced salt solution, pH 7.2, from the tracheal cannula. Cells were washed, counted and adjusted to 10^7 cells/ml in RPMI 1640 with 5% fetal bovine serum and antibiotics. Aliquots of 10^5 cells were centrifuged onto glass slides, stained using modified Giemsa, and used to determine the absolute numbers of eosinophils.

Suspensions of lung cells were prepared by removing the whole lungs and dissecting away lymph node and thymic tissue. Lungs were finely minced and the fragments were pressed through a 0.75 μm nylon mesh filter. Red blood cells were lysed in hypotonic buffer, and the remaining cells were washed twice, counted and adjusted to 10^7 cells/ml in RPMI 1640 with 5% fetal bovine serum and antibiotics.

The numbers of IL-4-producing cells were quantitated using enzyme-linked immunocell spot (ELISPOT) assays as described (8). Briefly, duplicate cell samples were distributed to 96-well microtiter plates that had been precoated with mAb 11B11 anti-murine-IL-4 antibody, serial two-fold dilutions of the cells were carried out, and the plates were incubated undisturbed for 18 hours at 37° C. After washing away the cells, biotinylated secondary antibody against IL-4, BVD6-24G.2, was added. Captured IL-4 was revealed using streptavidin-conjugated alkaline phosphatase and developed using 5-bromo-4-chloro-indolyl-phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer suspended in 0.6% agarose. Individual blue spots were counted after solidification of the agar using inverted microscopy.

Serum was prepared from whole blood collected at the time of death for determination of total IgE and IgG levels using sandwich monoclonal antibody-based ELISA as described (8).

Tissue pathology. For histologic analysis, whole lungs were infused via the trachea with 4% formalin until distended and fixed for 24 hours. The tissues were embedded in paraffin, and 2-3 μm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff for viewing by light microscopy.

T cell reconstitutions. Spleen, inguinal and axillary lymph nodes were harvested from C57BL/6 or 129SvEv mice. The tissues were finely minced and dispersed into single-cell suspensions by passage through a 0.75 μm nylon mesh filter. Cells were washed twice, counted, and depleted of B cells, MHC class II- and CD8-bearing cells using monoclonal

antibodies Jlld, BP107, and 3.155 (all from American Type Culture Collection; Rockville, MD), respectively, and low-toxicity rabbit and guinea pig complement (Cedarlane; Ontario, Canada). The resulting cells were labelled with phycoerythrin (PE)-conjugated anti-B220, FITC-conjugated anti-Thy 1.2, and Tri-Color-conjugated anti-CD4 and sorted on a flow cytometer (FACS Star Plus, Becton and Dickinson; Mountain View, CA) to achieve final populations of 96-98% pure CD4+, Thy1+, B220- lymphocytes. CD4+ T cells were transferred to RAG-1 -/- (C57BL/6 donor cells) or RAG-2 -/- (129 SvEv donor cells) mice intraperitoneally in 0.5 ml RPMI 1640. The next day and every 4 days thereafter, mice were sensitized with 100 μg Aspergillus antigens in 50 μl PBS or PBS alone for 3 doses given subcutaneously followed by 3 doses given intranasally. At the conclusion of the experiment, aliquots of lung and spleen cells were analyzed for the presence of CD4+, CD8α+, IgM+ and CD19+ cells using the appropriate conjugated monoclonal antibodies and flow cytometry.

RESULTS

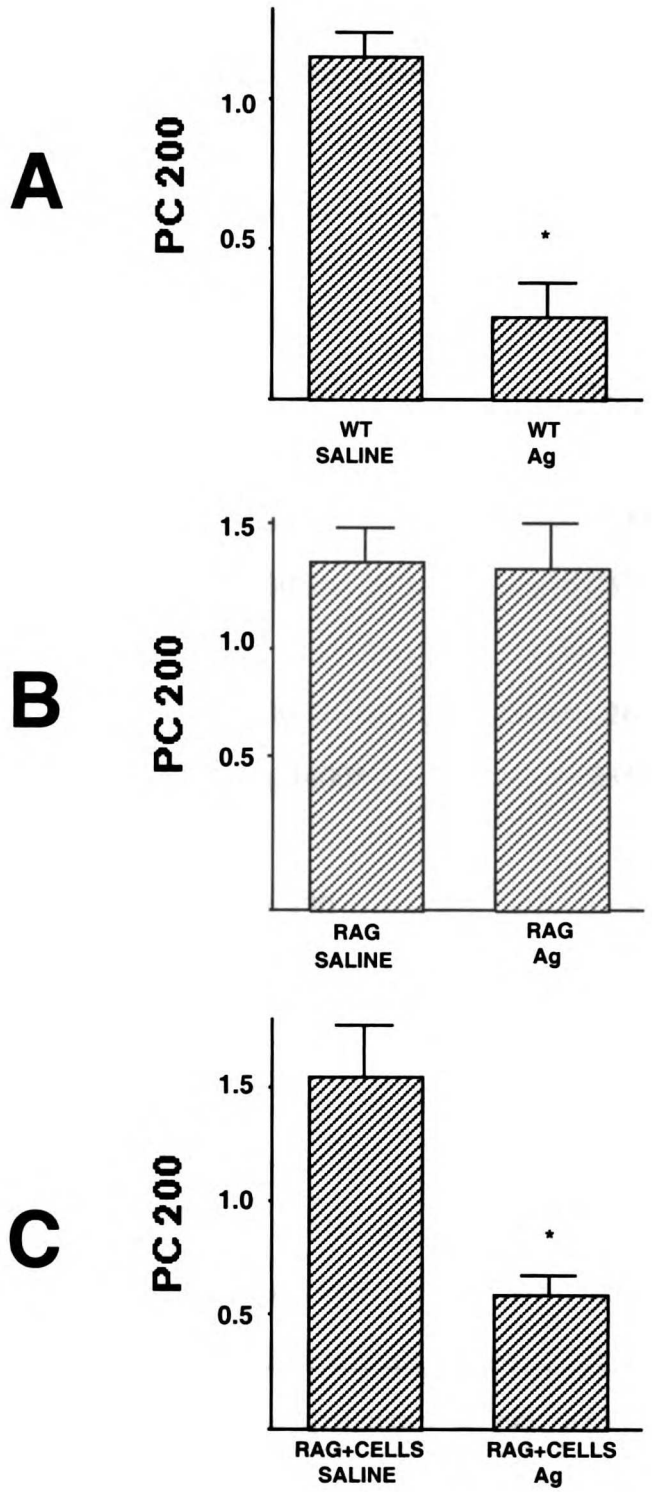
Airway responses induced by Aspergillus antigens require lymphocytes.

After sensitization with antigens from Aspergillus, control mice (C57BL/6 or 129), but not RAG-1- or RAG-2-deficient mice that lacked T and B cells, developed substantial increases in airway hyperreactivity, as demonstrated by the significant decrease in the dose of acetylcholine, a potent airway constrictor, required to elicit a 200% increase in pulmonary resistance (provocative dose 200%, or PC_{200}) (Figure 1). RAG-deficient mice also failed to develop the degree of airway inflammation - airway eosinophils and increases in the numbers of IL-4-producing cells in the lung - that characterized the response in wild-type mice, although some increases in BAL eosinophils could be consistently shown (Table 1). The inflammatory changes were markedly suppressed as compared to wild-type mice, however, as revealed in multiple histologic sections from lungs, and this did not differ between RAG-1 -/- and RAG-2 -/- mice (Figure 2). Thus, lymphocytes were required to mediate both the physiologic and pathologic changes that occured in the airways of mice challenged with the fungal antigens.

Figure 1. **Provocative concentrations of acetylcholine (μg/gm) that increased baseline airway resistance 200% (PC_{200}).**

Groups of 4-18 C57BL/6 (wild-type, WT) **(A)**, RAG-1-deficient (RAG) **(B)** and RAG-1 or RAG-2-deficient mice reconstituted with purified CD4+ T cells (RAG + Cells) **(C)** were sensitized with either saline (Saline) or Aspergillus antigens (Ag). Decreases in PC_{200} indicate enhanced airway hyperreactivity, with bars representing means and standard errors of the means. Significant differences ($P < 0.05$) between control and Ag groups are indicated (*).

Figure 1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RAG+CELLS
SALINE
RAG+CELLS
Ag

Table I. **Immune responses to inhaled *Aspergillus* antigens.**

Mice	Total IL-4-producing cells/lung	No. eosinophils in BAL fluid (%)	IgE (μg/ml)
C57BL/6			
saline	130	0.8 ± 0.5 (1)	< 0.1
Aspergillus	1692 ± 356	295 ± 27* (69)	4.5 ± 1.4*
RAG -/-			
saline	< 30	0.5 ± 0.4 (1)	< 0.04
Aspergillus	< 30	53 ± 13* (21)	< 0.04
RAG -/- + CD4 T cells			
saline	< 30	26 ± 6 (41)	< 0.04
Aspergillus	2400 ± 1400*	335 ± 50* (77)	< 0.04
μMT			
saline	208 ± 130	3 ± 2 (1)	< 0.04
Aspergillus	614 ± 137*	259 ± 31* (65)	< 0.04
Aspergillus + anti-IL-5	N.D.	6 ± 2 (2)	< 0.04
Aspergillus + anti-IL-4	N.D.	128 ± 81* (30)	< 0.04
IL-5 -/-			
saline	N.D.	0 (0)	<0.04
Aspergillus	N.D.	2.7 ± 0.8 (6)	5.6 ± 0.5*

Groups of 4-18 mice from the designated groups were used to prepare single-cell suspensions of lung tissue for quantitation of numbers of IL-4-secreting cells by ELISPOT assay. Bronchoalveolar lavage (BAL) cells were stained to assess the absolute numbers ($x10^4$) and percentages (in parenthesis) of eosinophils in total BAL cells. Serum IgE was quantitated by ELISA. Numbers represent mean ± SEM, with significant differences between saline and Aspergillus challenge indicated (*, $P < 0.05$). N.D., not done.

RAG-deficient mice reconstituted with CD4+ T cells manifest allergen-induced physiologic and pathologic findings in the lungs.

To confirm that T cells alone, and particularly CD4+ T cells, could mediate the pathologic changes associated with asthma, spleen and lymph node CD4+ T cells from the appropriate matched donor animals were purified by flow cytometry and were passively transferred into RAG-1- or RAG-2-deficient mice. The reconstituted mice were sensitized with antigen or saline, and the pathologic and physiologic changes were assessed. In contrast to nonreconstituted RAG-deficient mice, mice reconstituted with CD4+ T cells developed AH to acetylcholine (Figure 1), as well as airway eosinophilia, tissue IL-4-producing cells and peribronchiolar inflammatory changes in the lung (Table 1 and Figure 2). Flow cytometric analysis confirmed the presence of CD4+ and the absence of CD8+ T cells and CD19+ B cells in the reconstituted, but not the nonreconstituted RAG-deficient mice (Figure 3). Serum immunoglobulins (IgG, IgE) were below the limits of detection of the ELISA in these reconstituted mice, suggesting that T cells mediated these changes in the absence of antibodies, including IgE.

Allergen-sensitized B cell-deficient mice develop AH and lung inflammation.

To confirm that antibodies were not required to mediate AH, mice with targeted disruption of the immunoglobulin μ heavy chain gene, which are deficient in B cells and antibody (21), were immunized with Aspergillus antigens. These sensitized B cell-deficient mice developed AH, as quantitated by decreases in PC_{200}, that was comparable to that seen in B cell-replete mice (Figure 4). Moreover, eosinophil recruitment to the airways and tissues, the appearance of lung Th2 cells, the degree of peribronchial/peribronchiolar inflammation, and the presence of goblet cell hyperplasia, were all preserved in the sensitized B cell-deficient mice (Table 1 and Figure 2). Flow cytometric analysis of spleen

Figure 2. **Photomicrographs of representative tissue sections prepared from lungs of designated mice 4 days after the final antigen challenge.**

A. μMT lung sensitized with saline. Minimal lymphoid cell infiltrate is apparent in the periarterial space adjacent to a normal bronchiole. Alveolar spaces are not involved. This was not different than that seen in C57BL/6 mice sensitized with saline. Inset: PAS staining reveals absence of goblet cells in epithelial cell layer.

B. C57BL/6 lung sensitized with Aspergillus extract. Periarteral space is filled with an eosinophil-rich infiltrate. Airway contains many pale-staining, vacuolated goblet cells (arrow). Inset: PAS stain highlights hyperplasia of purple-staining goblet cells in epithelial cell layer.

C. μMT lung sensitized with Aspergillus extract. The periarterial space is filled with an eosinophilic inflammatory infiltrate that spreads to the adjacent bronchiole.

D. μMT lung sensitized with Aspergillus extract and treated with anti-IL-5 antibody. The perivascular and peribronchiolar spaces are filled with a lymphoid infiltrate containing only rare eosinophils.

E. RAG-deficient lung reconstituted with CD4+ T cells and sensitized with Aspergillus extract. A heavy perivascular infiltrate with eosinophils is present surrounding the peribronchiolar space.

F. High-power field of section D demonstrating marked reduction in numbers of eosinophils after treatment with anti-IL-5 antibody.

G. High-power field of section E demonstrating homogeneous eosinophil-rich infiltrate.

All sections stained with hematoxylin and eosin. A-E x 250 magnification; F, G x 1500 magnification.

Figure 2

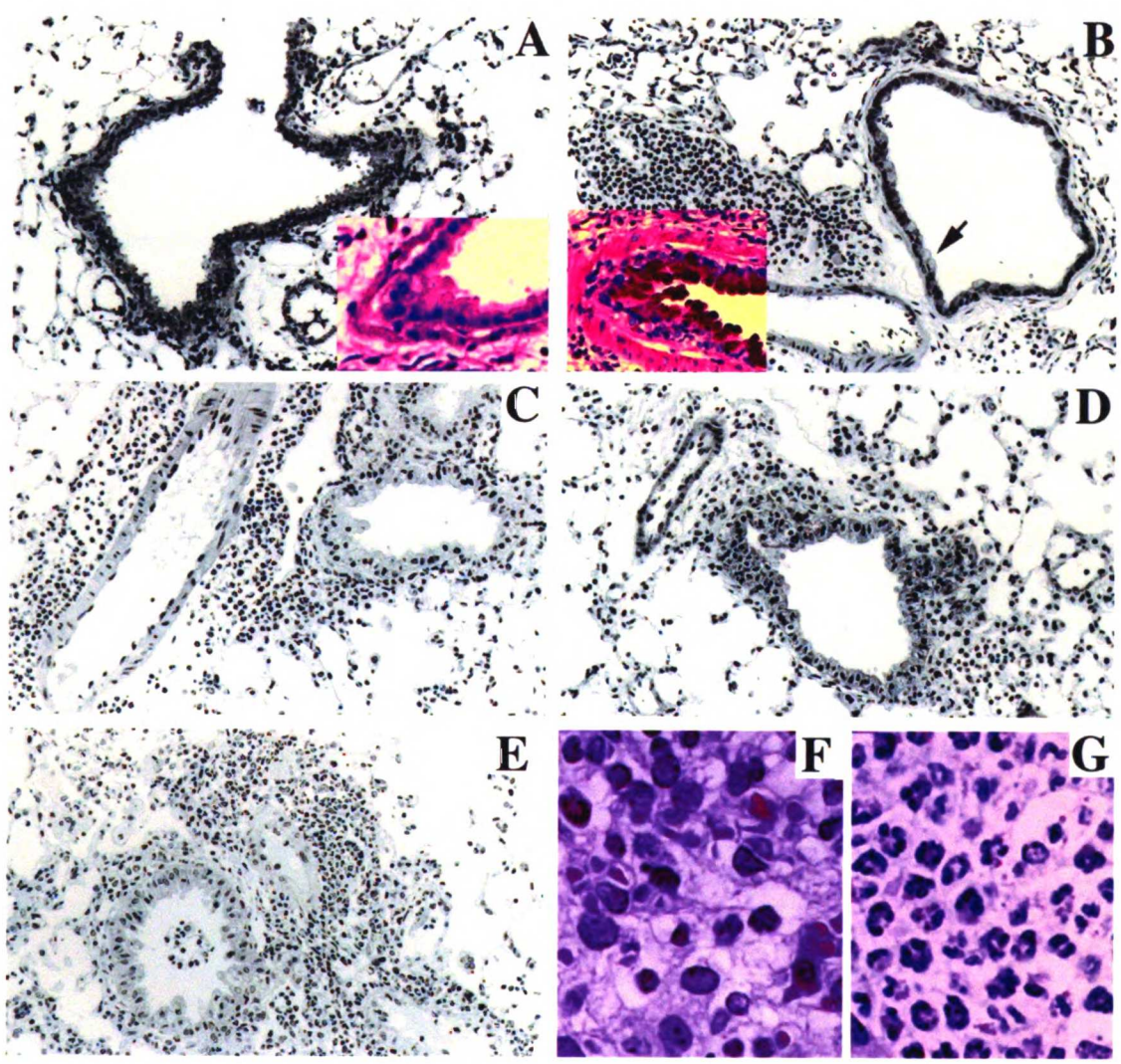


Figure 3. **Flow cytometric analysis of spleen cells from CD4+ T cell-reconstituted RAG-deficient mice.**

Spleen cells from RAG-1 or RAG-2-deficient that had been either untreated (Nonreconstituted) or repleted with purified CD4+ T cells (Reconstituted with CD4+ T cells) were stained with fluorescence-conjugated monoclonal antibodies to CD4, CD8α or CD19 (black shaded areas) to mark T helper, T cytotoxic and B lymphocytes, respectively. Gates were adjusted such that background staining with isotype control-matched fluorescence-conjugated monoclonal antibody (gray lines) was < 3%.

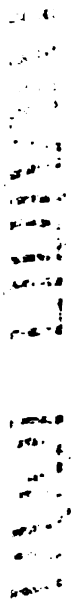


Figure 3

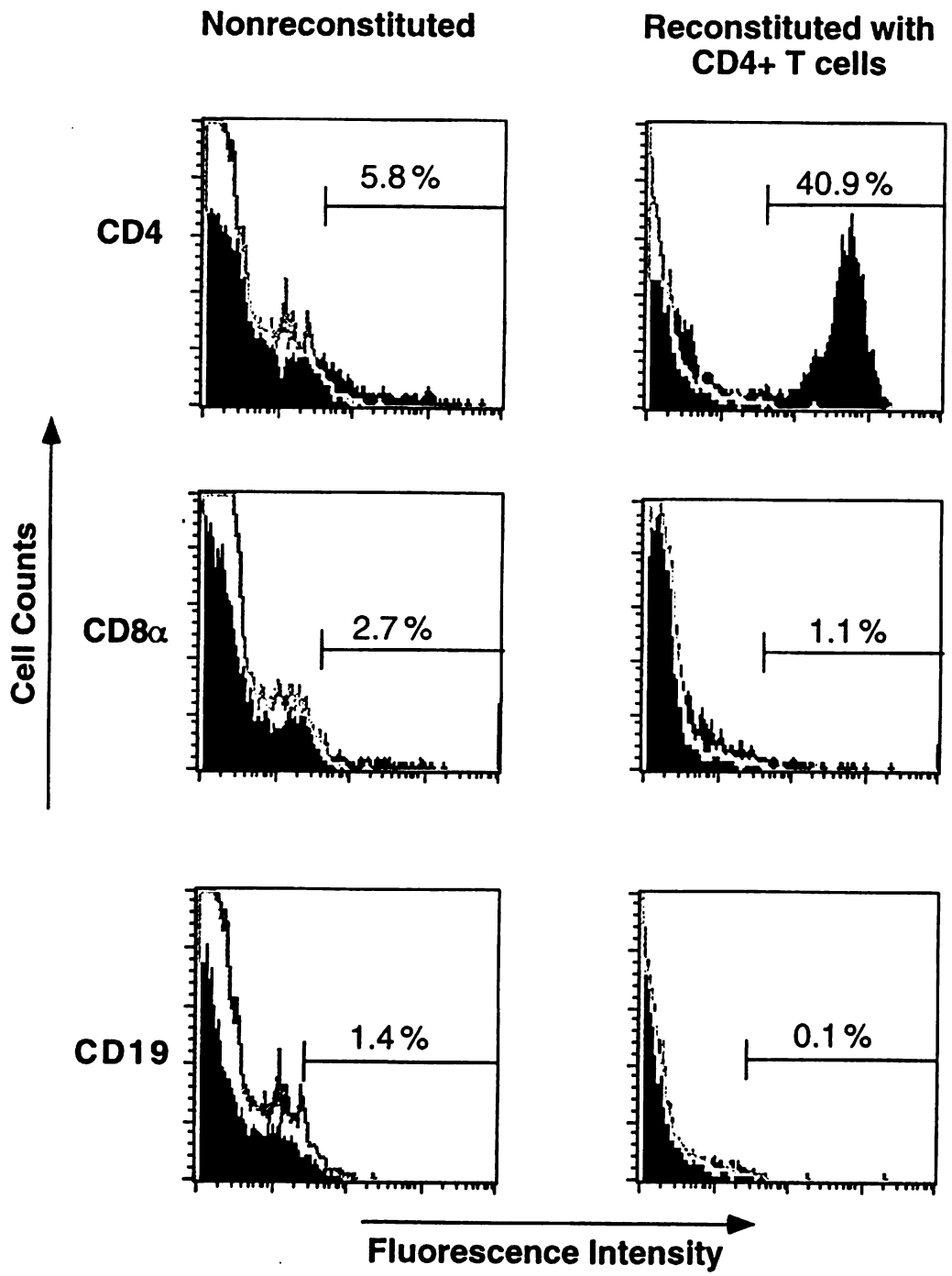
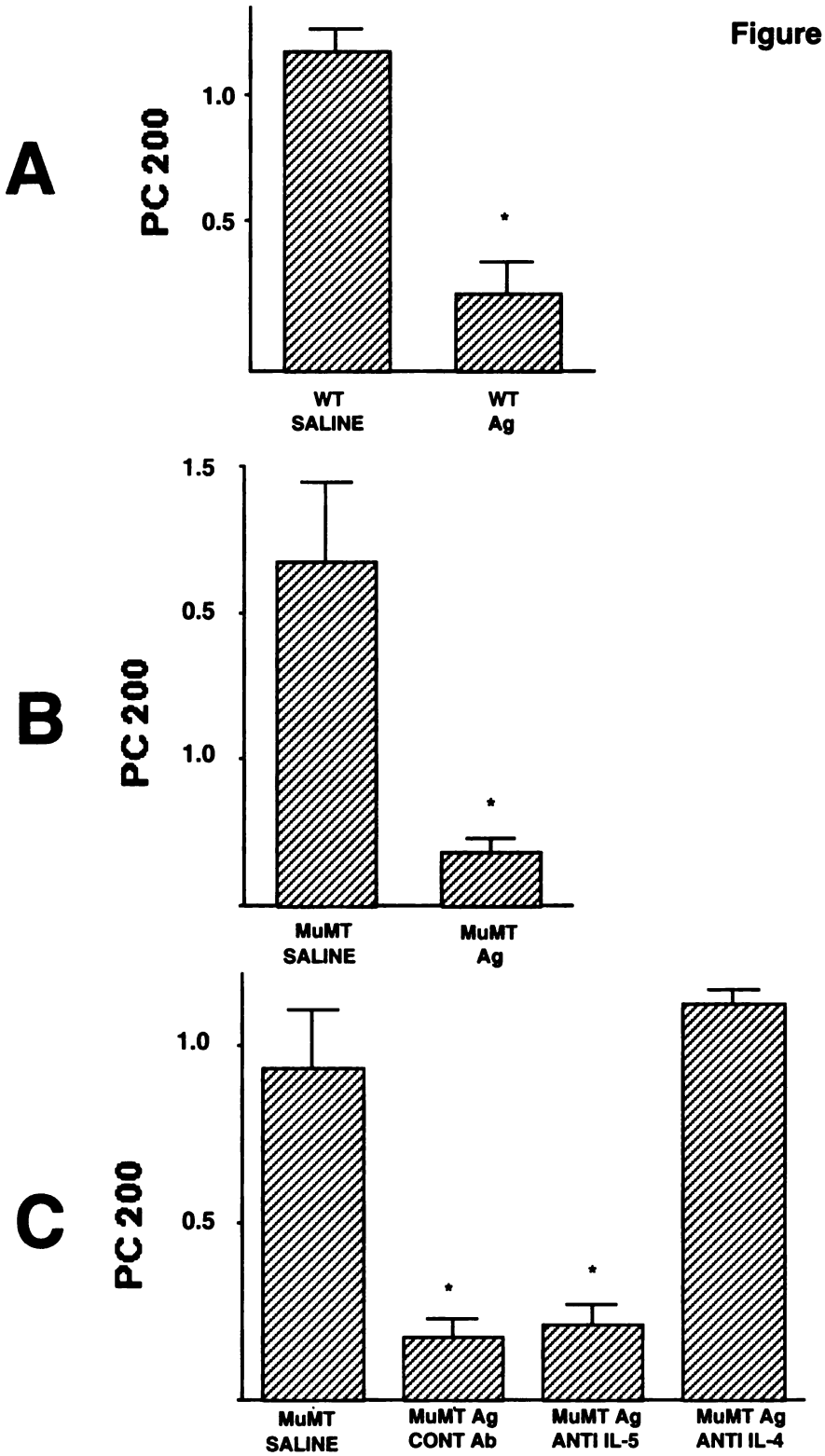

Nonreconstituted
Reconstituted with CD4+ T cells
CD4
5.8%
40.9%
CD8α
2.7%
1.1%
CD19
1.4%
0.1%
Cell Counts
Fluorescence Intensity

Figure 4. **Airway hyperreactivity in B cell-deficient mice.**

Groups of 4-18 C57BL/6 (WT) **(A)** or B cell-deficient (MuMT) mice **(B)** were sensitized with either saline (Saline) or Aspergillus antigens (Ag). Designated groups of MuMT mice were treated with anti-IL-5, anti-IL-4 or control (Cont Ab) monoclonal antibodies throughout the sensitization period **(C)**. The provocative concentrations of acetylcholine (μg/gm) that increased the baseline airway resistance by 200% (PC_{200}) are represented by bars and standard errors of the means. Significant differences ($P < 0.05$) between the antigen and control groups are indicated (*).

Figure 4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MuMT Ag
ANTI IL-4

cells recovered at the end of the experiments, as well as serum immunoglobulin determinations, confirmed that these animals lacked B cells and antibody.

IL-4, but not eosinophil recruitment mediated by IL-5, is required to mediate AH.

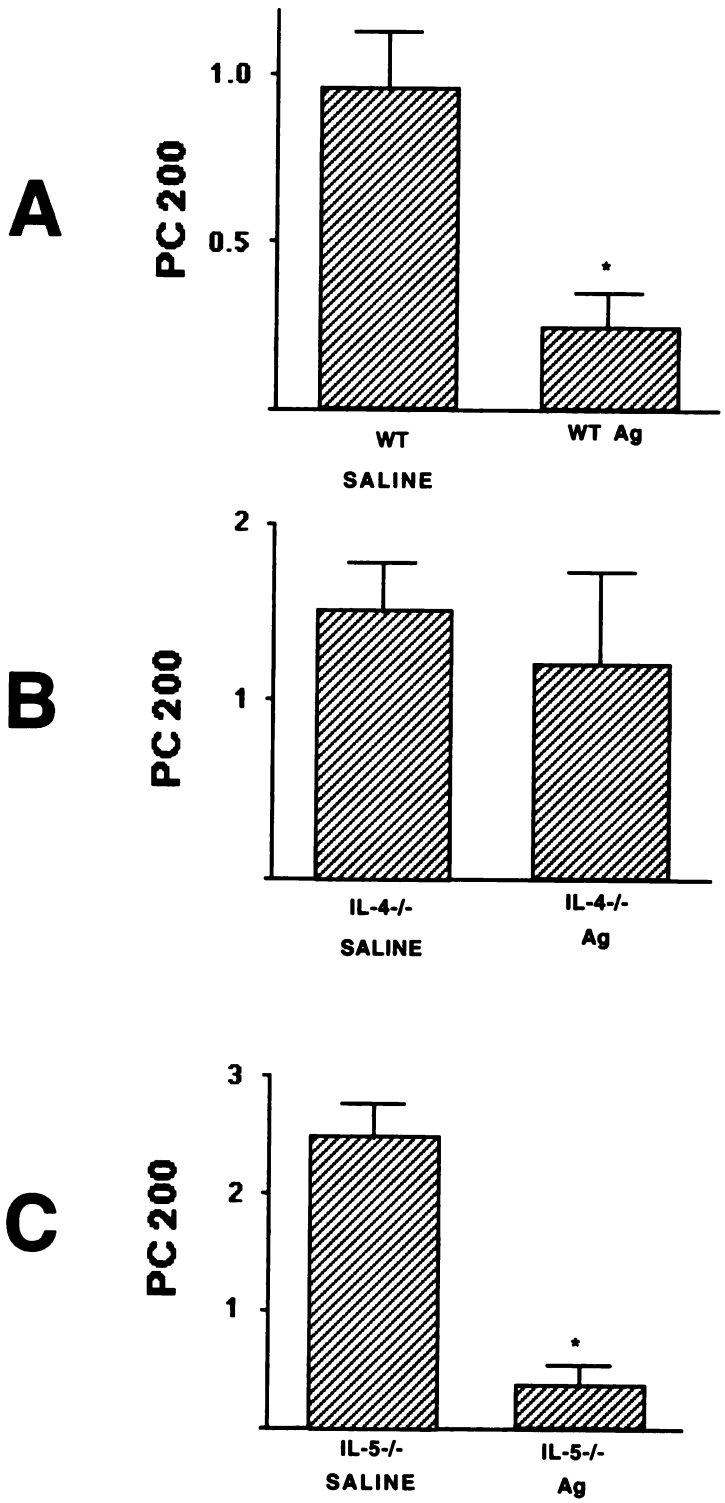
In prior experiments using mice sensitized with ovalbumin, we demonstrated requirements for IL-4, but not IL-5, using neutralizing antibodies administered during the period of antigen priming (8). Similarly, neutralization of IL-4 during the period of antigen sensitization in B cell-deficient mice completely abolished the development of AH. Eosinophil recruitment to the lung was diminished by approximately 50% in these mice (Figure 4 and Table 1). In contrast, administration of anti-IL-5 antibody such that eosinophils were reduced to levels seen in nonimmunized control animals had no effect on the induction of AH (Table 1, Figures 2 and 4).

To further confirm the IL-4-dependence and IL-5-independence of the airway phenotype mediated by sensitization with Aspergillus antigens, IL-4-deficient and IL-5-deficient mice were sensitized and analyzed for AH. Cohorts of wild-type mice were analyzed concurrently. Although the wild-type and IL-5-deficient mice had comparable induction of AH by the stringent physiologic criteria used, the IL-4-deficient mice had no airway reactivity (Fig. 5). As anticipated, sensitized IL-5-deficient mice did not substantially increase the numbers of eosinophils recovered in BAL as compared to wild-type mice, but generated levels of IgE that were comparable to control animals (Table 1).

Figure 5. **Allergen-induced airways disease in cytokine-deficient mice.**

Cohorts of 4-6 wild-type (WT), IL-4-deficient (IL-4 -/-) or IL-5-deficient (IL-5 -/-) mice with sensitized 5 times intranasally with either saline (Saline) or Aspergillus antigens (Ag). Four days after the last intranasal challenge, mice were anesthetized and the airway resistance quantitated as the PC_{200}, which is represented by bars and standard errors of the means. Significant differences ($P < 0.05$) between the saline and Ag groups are indicated (*).

Figure 5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IL-5-/-
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DISCUSSION

These studies demonstrate that T cells, in the absence of B cells, IgE or elevations in the numbers of eosinophils, were capable of inducing acute AH in mice sensitized to a potent airway allergen. Further, the recruitment of eosinophils and the establishment of tissue inflammation were unimpeded by the absence of B cells and antibody. These studies are in accord with prior findings regarding inflammation in B cell-deficient mice by investigators using different antigenic challenges (30, 31), but extend these observations by the concomitant evaluation of pulmonary resistance in vivo. We also noted marked hyperplasia of goblet cells, an additional histologic hallmark of asthmatic airways (32), in antigen-sensitized, B cell-deficient mice. At least in this model, inflammation induced by the cross-linking of immunoglobulin Fc receptors on mast cells or eosinophils was not required to establish these key pathologic and physiologic components that occur in human asthma. Although eosinophils and mast cell-associated IgE undoubtedly contribute to the chronicity of human disease through their own elaboration of type 2 cytokines and other toxic factors (6, 7, 33-39), these data establish that CD4+ T cells, in an IL-4-dependent manner, were alone capable of initiating the process, an important observation that may be relevant to therapeutic strategies applicable to human disease. This extends earlier reports indicating the capacity to transfer AH in rodents using sensitized Thy-1+ or CD4+ lymphocytes adoptively given into animals with otherwise intact immune systems (40, 41), in rigorously defining the capacity of helper T cells to alone mediate the key pathologic and histologic markers of airway disease.

Despite intensive study, the role of IgE and eosinophils, both in murine models of allergen-induced lung disease and in human asthma, remains controversial. Studies in B cell-deficient and IgE-deficient mice have demonstrated that IgE is not required to mediate

pulmonary inflammation, including the recruitment of eosinophils, in allergen-induced airways disease (30, 42, 43). In contrast, other investigators have reported a critical role for IgE in eosinophil recruitment as studied using anti-IgE antibodies (44) or in analysis of hyper-IgE-producing lines of mice (45), or in inducing tracheal smooth muscle hyperresponsiveness (31, 46, 47). Similarly, studies in mice rendered eosinophil-deficient by targeted disruption of the IL-5 gene (48) or in mice administered anti-IL-5 antibodies (14, 45) demonstrated a role for IL-5-dependent eosinophilia in mediating airway hyperreactivity, whereas other studies using neutralizing antibodies could demonstrate no role for IL-5 or elevated tissue eosinophil numbers (8, 49). These investigations have used different techniques for quantitating airway physiology, and a variety of antigens for sensitization, some of which display genetic differences among various inbred strains of mice (18). It remains possible that multiple pathways exist for the creation of allergic airways disease - one IL-4/CD4+ T cell-dependent (8), one IL-5/eosinophil-dependent (14, 48) - although, as shown here and elsewhere (8), we have been unable to define a necessary role for IL-5/eosinophils in mediating increases in lung resistance over a range of inbred mouse strains and allergens. In addition, by simultaneously assessing the importance of different immune cells and molecules in an optimized system, we have clearly demonstrated the greater importance of CD4+ T cells, and IL-4, over other effector moieties.

The demonstration that CD4+ T cells could mediate acute allergen-induced airways disease raises questions regarding the mechanism(s) involved. The mechanism is ultimately quite different than that mediated by immune complexes or antigen in Arthus-like reactions. Pulmonary inflammation was markedly reduced in mice deficient in either the substance P receptor, NK-1R, or the C5a anaphylatoxin receptor when challenged with antigen-antibody complexes (50), and cutaneous and systemic Arthus reactions were completely abrogated in mice with disruption of high- and low-affinity Fc receptors (51).

The absence of immunoglobulin in the B cell-deficient mice or T cell-reconstituted RAG-deficient mice precluded activation of these types of inflammatory cascades. Potential interactions between Th2 cells and resident pulmonary cells that might serve as sources of distinct inflammatory pathways mediated by chemokines (11, 52-56), interleukins (57), or the induction of homing and adhesion molecules (11, 58), merit further study. Studies in B cell-deficient and RAG-reconstituted mice should be informative in defining the necessary and sufficient roles for such effector molecules. Although the requirement for IL-4 suggests that Th2 cells were required and sufficient to mediate disease, further studies will be needed to establish whether IL-4 production is necessary only from CD4+ T cells, or also from endogenous cells of the innate immune system, or if IL-4 contributes directly or indirectly to the development of AH. Of note, these experiments could not define a necessary role for either CD8+ T cells or γδ T cells, although their possible regulatory contributions remain open.

An acknowledged caveat of these findings is their relevance to human asthma, a complex disease of unclear etiology that may encompass several different phenotypes. It is noteworthy that allergic diseases, including asthma, have been reported among rare individuals who completely lack eosinophils (59, 60). Further, an inverse relationship was noted between the predilection for asthma and the degree of CD4+ T cell depletion among patients infected with HIV, despite the common association of elevated IgE levels with progressive disease (61). Thus, the association of elevated IgE and eosinophil levels with asthma may not indicate a causal role in the pathogenesis. In murine intestinal helminth infections, systems in which protective immunity is mediated by Th2 cells, no requisite role has been demonstrated for either IgE or eosinophils in immunity, despite their marked elevations in vivo (26, 62).

The prevalence of asthma has risen steadily, such that 6% - 14% of children in the United States and Great Britain are affected (63, 64). As noted above, a number of the

pathologic and physiologic aspects of asthma are similar to those induced in this murine model. Human asthma is felt to be induced by a number of relatively common environmental antigens. Genetic studies in human populations have suggested linkage to areas near the cytokine locus that encodes IL-4, IL-5, IL-13, IL-9 and GM-CSF on chromosome 5q (65, 66), raising the possibility that aberrant type 2 responses may occur in the lung due to genetic factors that concordantly link these cytokine responses. The inverse relationship demonstrated in population studies between the intensity of delayed type hypersensitivity, a type 1-mediated response, and the incidence of atopy and asthma, provides additional support for this hypothesis (67). The data reported here suggest that early interventions that target the activation and/or effector development of T cells specific for candidate environmental allergens might abrogate the subsequent development of lung disease by preventing the chronic recruitment of additional cells and mediators that amplify the tissue destructive process. The model described should be useful for revealing the minimal interactions between CD4+ T cells and endogenous cells of the lung that mediate airway pathology.

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CHAPTER 4

Lung CD25 CD4 Regulatory T Cells Suppress Type 2 Immune Responses but Not Bronchial Hyperreactivity

Summary

To study the effects of chronic antigen deposition in the airway mucosa on CD4+ T cell priming and subsequent airways disease, ovalbumin (OVA) was expressed under the transcriptional control of the surfactant protein C promoter. CD4 T cells from these mice were tolerant to OVA but this was overcome among spleen CD4 T cells by crossing to OVA-specific DO11.10 TCR transgenic mice. Lungs from the double transgenic mice developed lymphocytic infiltrates and modest mucus cell hyperplasia. Infiltrating cells were unaffected by the absence of either Rag-1 or Stat6, although the latter deficiency led to the disappearance of mucus. In the lung of double transgenic mice, a large number of antigen-specific CD4 T cells expressed CD25 and functioned as regulatory T cells. The CD25+ CD4 T cells suppressed proliferation of CD25- CD4 T cells in vitro and inhibited type 2 immune responses induced by aerosolized antigens in vivo. Despite their ability to suppress allergic type 2 immunity in the airways, however, CD25+ CD4 regulatory T cells had no effect on the development of bronchial hyperreactivity.

INTRODUCTION

Asthma is a widely prevalent airways disease believed to reflect the cumulative effects of dysregulated type 2 immune responses to inhaled antigens (1-5). Bronchial hyperreactivity is thought to result from years of chronic inflammation by infiltrating T cells and eosinophils, leading to mucus cell hyperplasia and destructive airway tissue remodeling (6). Animal models of allergic airways disease have contributed many insights towards understanding the pathogenesis of asthma, including the central role of various chemokines and cytokines associated with type 2 immunity that serve to orchestrate complex downstream effects (7). In general, animal models mirror more closely acute allergic airways disease, whereas chronic changes such as fibrosis and airway wall remodeling seen in human asthma, are rarely observed (8, 9). Indirect attempts, by targeting the expression of CD4 T cell products to the airways, have elucidated the role of long term type 2 cytokine production in mediating chronic allergic airway changes (10-12). Models that more closely mimic the chronic exposure to respiratory allergens as occurs in the human disease would be of much value.

In an effort to create a model for chronic allergen exposure at the respiratory mucosa, we generated transgenic mice that expressed ovalbumin (OVA) under control of the surfactant protein C (SPC) promoter (13, 14). Despite expression in a number of tissues among numerous founders, the tissue consequences in these transgenic animals were essentially restricted to the lung. Although intrinsically tolerant to OVA, mice crossed to OVA-specific TCR transgenic animals allowed us to examine the consequences of

chronic antigen exposure at the mucosa in the setting of a reactive T cell repertoire. Although CD4 T cells that infiltrated the lungs of these double transgenic mice were primed to produce IL-4 in vitro, they were held in check in vivo by the accumulation of CD25 CD4 regulatory T cells that inhibited immune effector function. When challenged with exogenous antigens, the regulatory T cells remained highly efficient in blocking type 2 effector function by CD4 T cells, but were unable to affect the induction of airway hyperreactivity. The regulatory capacity of CD25 T cells may be limited in chronic complex diseases with substantial contributions by non-T cells.

MATERIALS AND METHODS

Transgenic mice. Mice expressing ovalbumin (OVA) under control of the surfactant protein C (SPC) promoter were produced to target antigen expression to lung type II pneumocytes in the alveoli (13, 14). A chicken egg OVA cDNA derived from pETOV (kindly provided by Cor Turnnir, Stressgen Biotechnologies Corp., Victoria, Canada) was inserted into the SPC.TRK plasmid (15). After confirmation by sequencing, the construct was linearized and injected into C57BL.6 X 129 F1 oocytes to create transgenic founders. One line, SPC.OVA, was selected for further study after confirming OVA mRNA and protein in lung together with low serum OVA levels. SPC.OVA was backcrossed 10 generations onto BALB/c. Transgenic integration was confirmed from tail DNA using OVA-specific primers: 5'-GCGCAGCAAGCATGGAAT-3' and 5'-GGAAACACATCTGCCAAA-3'

Mice. BALB/c and BALB/c DO11.10 OVA-specific T cell receptor (TCR) transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). BALB/c TCR-Cα-deficient (16), Rag-1-deficient (17), Stat6-deficient (18), and 4get mice containing a bicistronic knockin enhanced green fluorescent protein linked via an IRES element with the IL-4 gene (19) were bred and maintained in the UCSF specific pathogen-free animal care facility. Intercrossing was used to generate SPC.OVA x DO11.10, SPC.OVA x DO11.10 x Rag-1-deficient, SPC.OVA x DO11.10 x Stat6-deficient, and SPC.OVA x DO11.10 x 4get mice. Crosses were confirmed using flow cytometry with monoclonal antibody KJ1-26 specific for the DO11.10 TCR (20), and DNA typing.

OVA quantitation. Designated organs were frozen in liquid nitrogen, pulverized, and lysed in RNAzol B (Biotecx Laboratories, Houston, TX) for preparation of RNA. After reverse transcription (murine Moloney leukemia virus reverse transcriptase; GIBCO BRL, Gaithersburg, MD) with random hexamer primers (Promega Corporation, Madison, WI), polymerase chain reaction was used to amplify OVA cDNA using the above primers. Expression of OVA message was compared to the constitutively expressed hypoxanthine phosphoribosyltransferase (HPRT) using primers as described (8).

OVA protein in serum and BAL was determined using ELISA. Briefly, rabbit anti-chicken OVA (Sigma Chemical Company, St. Louis, MO) was purified by ammonium sulfate precipitation and used at 5 μg/ml, to coat flat-bottom microtiter wells (Immulon 4HBX; Dynex Technologies Inc., Chantilly, VA). After blocking with 3% bovine serum albumin in PBS and extensive washing, samples were titrated and incubated for 2 hrs at room temperature. After washing, purified biotinylated rabbit anti-chicken OVA was added at 1 μg/ml for 1 hr. Wells were developed using streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Chicken OVA (Sigma) was used as a standard.

Cell preparation and analysis. Where designated, CD4 T cells from indicated mice were enriched from either dispersed lung or spleen cell preparations by antibody- and complement-mediated lysis of CD8, MHC class II and heat stable antigen-bearing cells as described (21). Otherwise, populations of CD4 T cells were fractioned, based on CD25 cell surface expression, using flow cytometry. Lungs were minced and dispersed into single-cell suspensions. Red blood cells were lysed in hypotonic buffer and the

remaining cells were washed and maintained in tissue culture medium (RPMI 1640 with 10% heat-inactivated fetal calf serum, 50 μM β2-mercaptoethanol, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin). Dispersed spleen cells were purified over Ficoll prior to suspension in tissue culture medium. Cells were purified (>98%) by flow cytometry (Mo-Flo Multi-Laser Flow Cytometer, Cytomation, Ft. Collins, CO) after staining with FITC-anti-CD4 (Caltag laboratories, So. San Francisco, CA), PE-anti-CD25 (Pharmingen, San Diego, CA), TriColor-anti-B220 (Caltag) and TriColor-anti-CD8 (Caltag).

Proliferation assays were performed after distribution of indicated numbers of CD4 T cells with 10^6 irradiated antigen presenting cells (APC) prepared from TCR-Cα-deficient spleen cells and titrated concentrations of OVA peptide. After incubation for 48 hrs, 1 μCi [^{3}H]-thymidine was added for 18 hrs and cultures were harvested for determination of radioactive uptake.

Polarization assays were performed by incubating indicated numbers of CD4 T cells with 10^7 irradiated APC and 1 μM OVA peptide under neutral (100 U/ml recombinant human IL-2) or Th2 conditions (100 U/ml recombinant human IL-2, 50 ng/ml recombinant murine IL-4, 50 μg/ml neutralizing anti-IFNγ mAb [XMG1.2]), as described (22).

Intracellular cytokines were assessed after stimulation of CD4 T cells with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) and ionomycin (2 μg/ml; Sigma). After 2 hrs, brefeldin A (Sigma) was added to a final concentration of 10 μg/ml, for an additional 2 hrs to promote intracellular cytokine accumulation. Cells were fixed in 4% formaldehyde

in PBS, permeabilized in 0.5% saponin in 1% FBS/PBS, and analyzed using TriColor-anti-CD4 (Caltag), FITC-anti-IFNγ (Pharmingen), and PE-anti-IL-4 (Pharmingen) or isotype control as described (23).

Adoptive transfers. Rag-1-deficient mice were reconstituted with CD4 T cell populations, purified by flow cytometry, at the indicated numbers. Cells were transferred in 0.3 ml PBS by intravenous injection into the tail vein, 24 hrs prior to antigen sensitization.

Immunizations and airways sensitization. Mice were immunized at the base of the tail with 25 μg OVA in Alum. After 1 week, mice were reimmunized i.p. with 25 μg OVA in Alum. After 5 days, CD4 T cells were enriched from the draining lymph nodes and used in proliferation and cytokine assays as described above. For airways sensitization, mice were treated 5 times intranasally at 2-day intervals with 2.5 mg OVA in 50 μl PBS or with PBS alone.

Airways reactivity. Anesthetized mice were analyzed 2 days after the final OVA sensitization. The trachea was cannulated and mice were ventilated with 100% oxygen at physiologic rate and tidal volume using a rodent ventilator (Harvard Apparatus, S. Natick, MA). After paralysis to eliminate spontaneous respirations, mice were maintained inside a whole-body plethysomgraph for measurements of air flow, transthoracic pressure and resistance. After establishing a stable baseline, acetylcholine (ACh) was injected intravenously over 1 s in escalating doses via the tail vein. Airway reactivity was expressed as the provocative concentration of ACh in μg/gm body weight required to double the baseline transthoracic resistance, designated PC_{200}, as calculated by

linear interpolation of appropriate dose-response curves. Significant differences were calculated using the logarithm of PC_{200} by analysis of variance using reference to the specified control groups. Baseline pulmonary resistance in the absence of ACh did not differ among the various groups of mice studied.

Histopathology. Lungs were infused *in situ* with 1 ml of 3.7% formaldehyde in PBS through the tracheal cannula. The lungs were removed and immersed in the same fixative with the trachea tied closed for 24 hr. The tissues were embedded in paraffin and 2-3 μm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff for light microscopy.

Serum IgE. Serum IgE was determined by a double mAb-based sandwich ELISA as described (8).

RESULTS

Generation and analysis of transgenic mice.

To target high levels of antigen expression to the airway alveoli, chicken ovalbumin (OVA) was expressed as a transgene under the control of the human surfactant protein C (SPC) promoter (Figure 1A). The construct was linearized and injected into C57BL/6 x 129 F1 oocytes to create transgenic founder lines. After screening six founder lines, we selected for further study one line, designated SPC.OVA, based on robust lung tissue mRNA expression and high levels of OVA protein in bronchial lavage fluid (Figure 1B, 1C). Despite ubiquitous OVA tissue expression as based on PCR analysis of mRNA, functionally significant protein was found in the lung, as assessed by histologic examination of multiple tissues (see below). SPC.OVA was backcrossed 10 generations to BALB/c without change in the distribution of OVA mRNA or protein expression (data not shown).

CD4 T cell tolerance to OVA in SPC.OVA mice was overcome after crossing in the OVA-specific DO11.10 TCR transgene.

After immunization with OVA, spleen T cells from SPC.OVA failed to proliferate to a broad range of OVA peptide that induced strong proliferative responses in similarly immunized control BALB/c mice (Figure 2A, left panel). The response to OVA by SPC.OVA T cells did not differ from the response by cells from unimmunized mice, confirming the deletion and/or tolerance of OVA-specific T cells, as expected by the

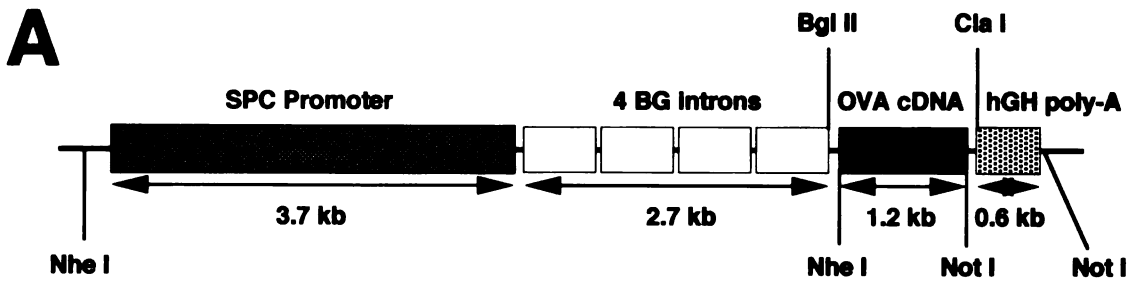
Figure 1. **Generation and characterization of SPC.OVA transgenic mice.**

(A) Schematic of the SPC.OVA construct used for creating transgenic mice. The OVA cDNA was liberated by digestion with Nhe I and Not I and the plasmid containing the SPC promoter was digested with Cla I and Bgl II. Sticky ends were filled in with T4 polymerase. The OVA cDNA was ligated into the SPC-containing plasmid between the bovine growth hormone (BG) introns and the human growth hormone (hGH) poly A tail. The proper orientation of the OVA cDNA insert was confirmed by DNA sequencing. The ligated plasmid was linearized by digestion with Nde I and Not I.

(B) RT-PCR analysis of OVA mRNA transcripts from tissues of SPC.OVA transgenic mice. Concentrations of input cDNA were standardized to comparable amounts of the constitutively expressed hypoxanthine phosphoribosyltransferase (HPRT) gene. Results are representative of 5 mice of the SPC.OVA founder line.

(C) Serum and bronchoalveolar lavage (BAL) OVA protein levels assessed by OVA-specific ELISA. Bars represent means ± SEM from 5 mice of the SPC.OVA founder line. The transgene littermate controls (not shown) express values below the limit of detection of <0.5ng/ml.

Figure 1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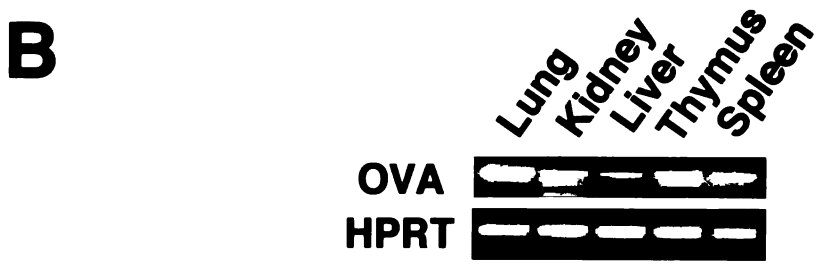
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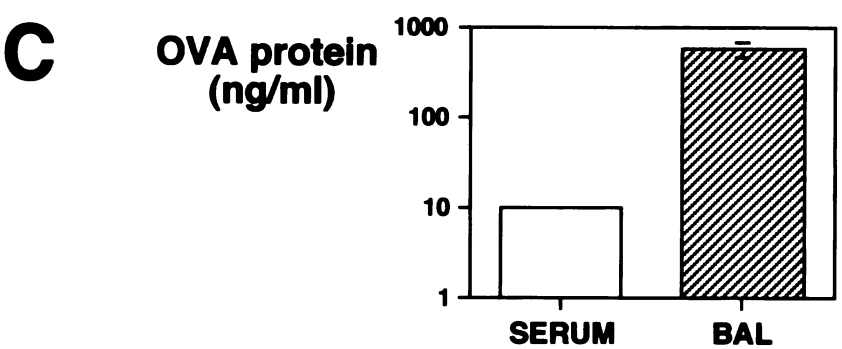

C
OVA protein
(ng/ml)
1000
100
10
1
SERUM
BAL

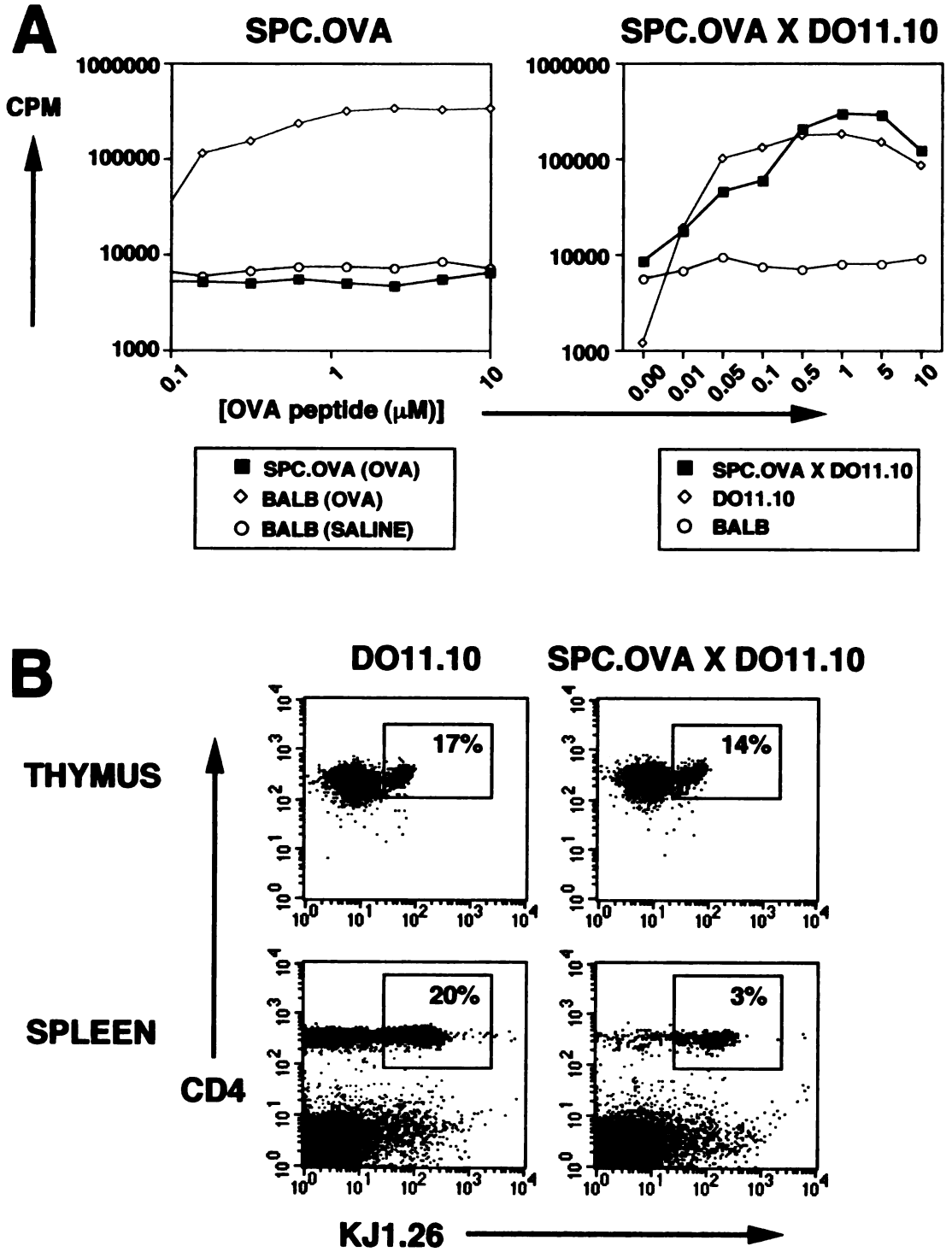
Figure 2. **Functional analysis of OVA-specific CD4 T cells in SPC.OVA and SPC.OVA X DO11.10 mice.**

(A) Left Panel. Spleen CD4 T cells were purified from SPC.OVA mice immunized with OVA (solid squares) or control BALB/c mice immunized with OVA (open diamonds) or saline control (open circles) and incubated at 10^5 cells/well with irradiated APC and the designated concentrations of OVA peptide for 48 hrs. Proliferation was analyzed by [^{3}H]-thymidine incorporation over the final 18 hrs. Data represent results from one of three comparable experiments.

Right Panel. Spleen CD4 T cells were purified from nonimmunized SPC.OVA x DO11.10 (solid squares), DO11.10 (open diamonds) or control BALB/c mice (open circles) and analyzed as previously described. Comparable numbers of KJ1-26+ cells from transgenic mice were present at the initiation of the assay. Data represent results from one of three comparable experiments.

(B) Thymi and spleens from DO11.10 and SPC.OVA x DO11.10 mice were dispersed and analyzed for the presence of clonotypic cells using mAb KJ1-26 and anti-CD4 mAb. Percentages in boxes represent fraction of total lymphoid cells. Flow cytometric analysis is representative of studies in >10 single and double transgenic mice.

Figure 2


A
SPC.OVA
SPC.OVA X DO11.10
CPM
1000000
100000
10000
1000
0.1
1
10
[OVA peptide (μM)]
0.00
0.01
0.05
0.1
0.5
1
5
10
SPC.OVA (OVA)
BALB (OVA)
BALB (SALINE)
SPC.OVA X DO11.10
DO11.10
BALB
B
DO11.10
SPC.OVA X DO11.10
THYMUS
SPLEEN
CD4
17%
14%
20%
3%
KJ1.26

presence of OVA mRNA in thymus and OVA protein in serum (Figure 1B, 1C). In comparable transgenic antigen models in other organs, co-expression of an antigen-specific transgenic TCR has promoted escape from negative selection, presumably driven by the high thymic precursor frequency established by the TCR transgene (24-26). To assess whether similar outcomes might occur in the lung, we crossed the SPC.OVA mice to OVA-specific DO11.10 TCR transgenic mice which contain CD4 T cells that recognize OVA peptide in the context of I-A^d MHC class II molecules (27). The clonotypic, OVA-specific CD4 T cells from the spleen of double transgenic mice, designated SPC.OVA x D011.10, proliferated in a dose-dependent response that was comparable to clonotypic cells from DO11.10 TCR transgenic mice (Figure 2A, right panel). Thus, despite high-level antigen expression, clonotypic T cells from the double transgenic mice maintained function. Analysis of numbers of clonotypic cells in thymus and spleen, however, revealed comparable numbers and percentages in the thymus but a 6-fold reduction in numbers of cells in the spleen as compared to control D011.10 mice (Figure 2B, and data not shown).

Lymphocytic accumulation in the lungs of SPC.OVA X DO11.10 mice independent of Rag-1 or Stat6.

The reduction in numbers of clonotypic D011.10 T cells in the spleen of the double transgenic mice raised the possibility of either activation induced cell death (28) or recruitment into tissues where OVA was expressed at high levels. Although lungs of D011.10 TCR transgenic mice did not differ from normal tissues (Figure 3A, and data not

shown), the lungs of SPC.OVA x DO11.10 mice revealed peribronchiolar and perivascular lymphocytic infiltrates (Figure 3B). Organized bronchus-associated lymphoid structures, or BALT, were scattered throughout the lungs (Figure 3C). Examination of the larger airways using PAS stains revealed areas of mucin-containing goblet cells adjacent to the infiltrates that were not apparent in control D011.10 mice (Figure 3D, and data not shown). Despite detectable OVA mRNA in the kidney and liver, the histologic appearance of these organs were not different than wild-type mice (data not shown).

Analysis of the cellular infiltrate revealed the presence of clonotypic OVA-specific T cells (see below), although the possibility remained that some of the T cell specificity was driven through use of non-clonotypic TCRs from endogenous TCR-α genes. To restrict the T cell specificity to the single clonotype, transgenic D011.10 TCR and SPC.OVA x D011.10 mice were further crossed to create SPC.OVA x D011.10 x Rag-1-deficient mice. Despite this reduction in TCR specificity, the accumulation of pulmonary infiltrates into organized, BALT-like structures, as well as the presence of scattered regions of mucin-producing goblet cells, was unchanged (Figure 3E, 3F).

In various models of airway hyperreactivity, mucin production by airway epithelial cells is largely dependent on IL-4Rα-mediated signaling via Stat6 (29, 30). To assess whether the modest amounts of goblet cell hyperplasia that occurred in SPC.OVA x D011.10 mice was similarly Stat6-dependent, we additionally intercrossed mice to generate SPC.OVA x D011.10 x Stat6-deficient mice. Although the accumulation of

Figure 3. **Lung pathology in SPC.OVA X DO11.10 mice.**

(A) DO11.10 lung (H&E).

(B) SPC.OVA x DO11.10 lung with peribronchial and perivascular infiltrates (H&E).

(C) SPC.OVA x DO11.10 lung with bronchus-associated lymphoid tissue in interstitium (H&E).

(D) SPC.OVA x DO11.10 lung with mucin-producing cells indicated by arrow (PAS).

(E) SPC.OVA x DO11.10 x Rag-1-/- lung with persistent infiltrates and BALT (H&E).

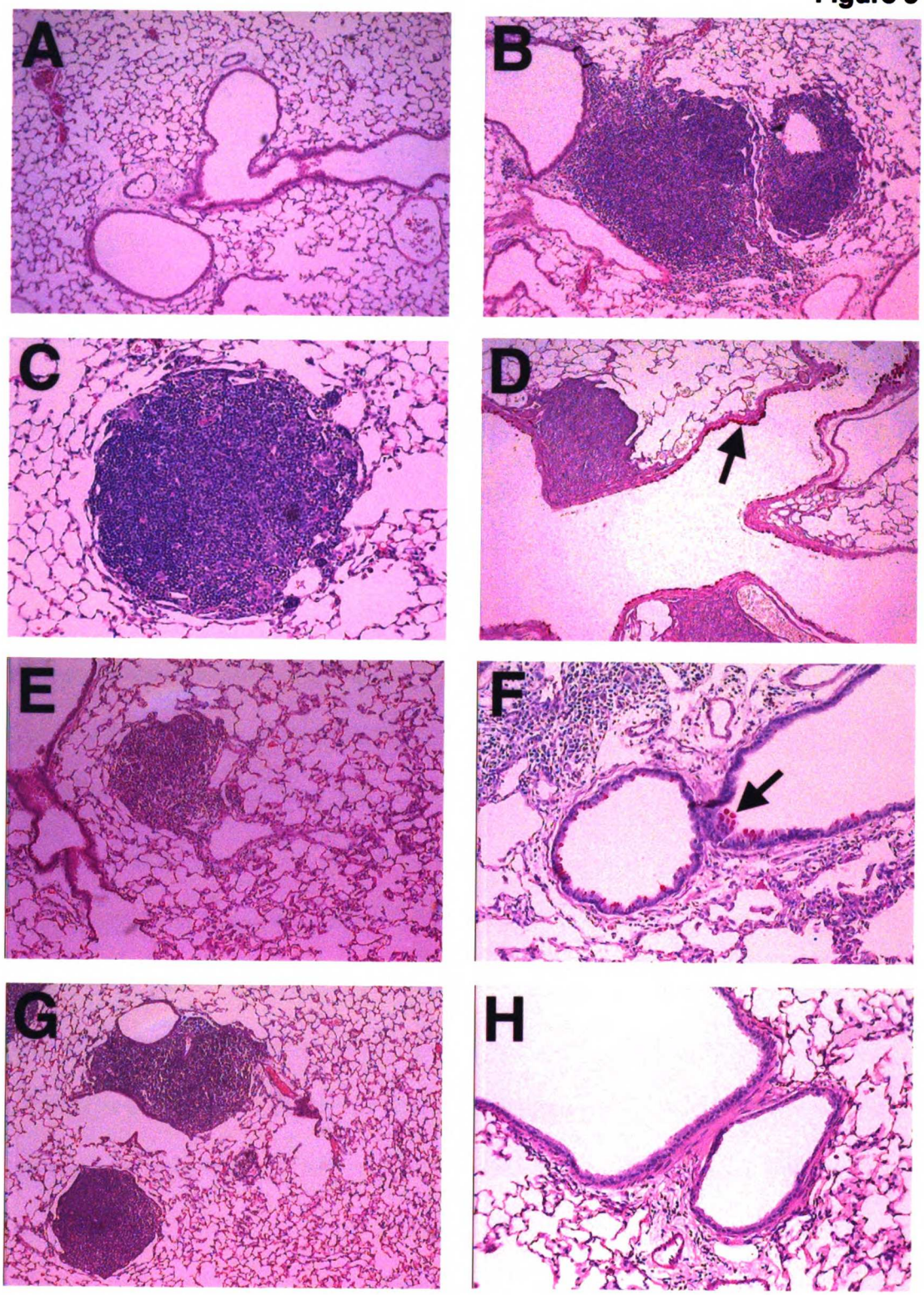
(F) SPC.OVA x DO11.10 x Rag-1-/- lung with mucin-producing cells indicated by arrow (PAS).

(G) SPC.OVA x DO11.10 x Stat6-/- lung with persistent infiltrates and BALT (H&E).

(H) SPC.OVA x DO11.10 x Rag-1-/- lung with absence of mucin-producing cells (PAS).

Each photograph represents 100X magnification, except **(C)** which was 250X magnification. At least 4 independent mice were examined to confirm representative nature of pulmonary histology.

Figure 3



spontaneous lung infiltrates remained unchanged, mucin production was entirely ablated on the Stat6-deficient background (Figure 3G, 3H).

Accumulation of CD25+ clonotypic T cells in lungs of SPC.OVA x D011.10 mice.

Dispersal of lung cells and analysis using surface markers revealed increased percentages of CD4 T cells and B cells in the SPC.OVA x D011.10 mice compared to DO11.10 controls (Figure 4A). Despite the presence of some mucin-containing goblet cell hyperplasia consistent with aspects of type 2 immunity, no eosinophils were detected. As compared to cells from control D011.10 animals, substantial numbers of clonotypic CD4 T cells from the double transgenic mice displayed an activated phenotype consisting of marked upregulation of CD25 and CD69, and downregulation of CD62L (Figure 4B). The appearance of CD25+ CD4 T cells did not require the expression of endogenous TCRs, since SPC.OVA x D011.10 x Rag-1-deficient mice also accumulated activated OVA-specific T cells in lung and spleen (Figure 4C). However transgenic antigen expression was a prerequisite on the Rag-1-deficient background for the generation of these cells, because they were not present in single transgenic D011.10 x Rag-1-deficient mice (Figure 4C). Crossing the double transgenic mice onto the Stat6-deficient background, also did not affect the accumulation of CD25+ clonotypic T cells relative to the wildtype background (Figure 4C and data not shown). Thus, introduction of the TCR transgene into mice concomitant with antigen expression in the airway mucosa, resulted in the appearance of antigen-specific, CD25+ CD4 T cells in spleen and their recruitment into pulmonary tissue.

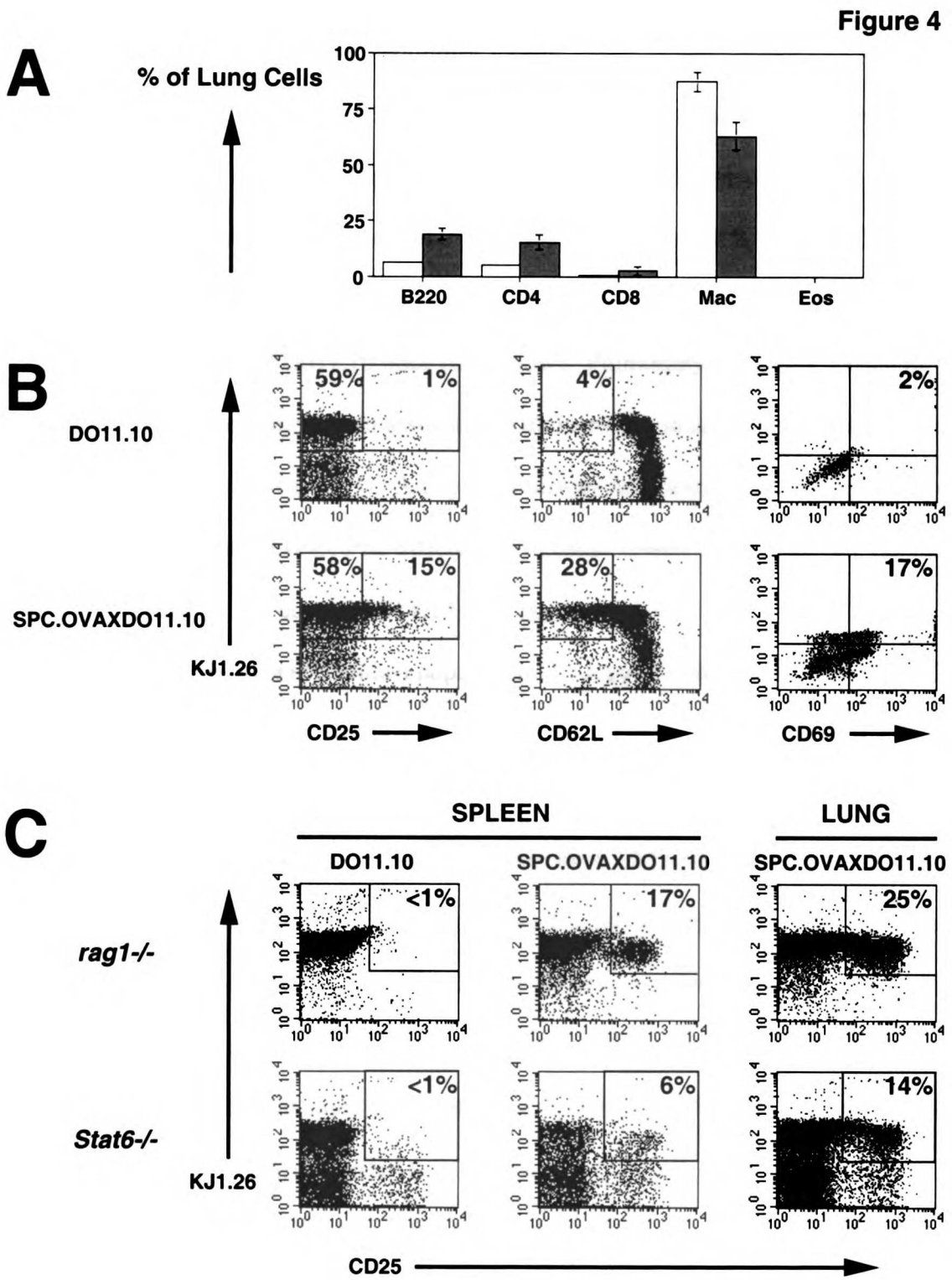
Figure 4. **Characterization of lung infiltrating cells in SPC.OVA X DO11.10 mice.**

(A) Dispersed lung cells from SPC.OVA x DO11.10 (filled bars) and DO11.10 (open bars) mice were analyzed for percentages of CD4 and CD8 T cells and B220+ B cells using flow cytometry after staining with appropriate mAb. Macrophages and eosinophil percentages were evaluated after staining cytospin preparations. Bars represent means and SEM.

(B) Dispersed lung cells from DO11.10 and SPC.OVA x DO11.10 mice were analyzed after gating on CD4 cells for surface expression of the clonotypic TCR, using mAb KJ1-26, and CD25, CD62L and CD69 using appropriate mAb. Depicted percentages represent boxed gates as a proportion of total CD4 lymphocytes.

(C) CD4 gated cells from spleen and lung cells of DO11.10 or SPC.OVA x DO11.10 mice that were crossed onto Rag-1-deficient (Rag-1-/-) or Stat6-deficient (Stat6-/-) backgrounds were analyzed for surface expression of the clonotypic TCR (KJ1-26) and CD25. Depicted percentages represent boxed gates as a proportion of total CD4 lymphocytes.

Figure 4


A
% of Lung Cells
100
75
50
25
0
B220
CD4
CD8
Mac
Eos
B
DO11.10
SPC.OVAXDO11.10
KJ1.26
59%
1%
4%
2%
58%
15%
28%
17%
CD25
CD62L
CD69
C
SPLEEN
LUNG
DO11.10
SPC.OVAXDO11.10
SPC.OVAXDO11.10
rag1-/-
Stat6-/-
KJ1.26
<1%
17%
25%
<1%
6%
14%
CD25

Clonotypic CD25+ CD4 T cells recruited to the lungs of SPC.OVA x D011.10 mice have the functional phenotype of regulatory T cells.

The presence of infiltrating CD25+ CD4 T cells raised the possibility that these cells were effector cells activated by exposure to cognate antigen expressed in large amounts in lung tissues. Analysis of intracellular cytokine production of IL-4 and IFN-γ after PMA/ionomycin stimulation, however, revealed no difference in cells collected from SPC.OVA x D011.10 and D011.10 mice (see below). Pulmonary infiltrates consisting of Th2 or Th1 effector cells have been associated with increases in airway hyperreactivity to bronchoconstricting agents (31-33). Analysis of airway resistance to increasing doses of acetylcholine, however, revealed no differences in basal airway hyperreactivity between SPC.OVA x D011.10 and control D011.10 mice (see below).

Regulatory T cells are a specialized population of CD4 T cells that express the IL-2 receptor α chain (CD25) and strongly suppress antigen-induced activation of T cells in vitro and in vivo (34). They have been invoked in a variety of autoimmune, infectious and inflammatory diseases (35). To assess whether the CD25+ CD4 T cells in SPC.OVA x D011.10 mice functionally resembled regulatory T cells, we separated CD4 T cells into CD25+ and CD25- subsets and examined their capacity to proliferate either alone or together in response to escalating doses of the cognate OVA peptide. Since SPC.OVA x D011.10 x Rag-1-deficient mice accumulated comparable CD25+ clonotypic T cells (Figure 4C), we performed similar experiments using fractionated cells from these animals. The CD25- CD4 T cells from both double transgenic and double transgenic/Rag-1-deficient mice proliferated to OVA in a dose-dependent fashion, in

contrast to the CD25+ CD4 T cells from both groups of mice which remained unresponsive (Figure 5A). When cultured with the CD25- T cells at the 1:4 ratio of CD25+:CD25- T cells that was present in the lungs of the mice, the CD25+ CD4 T cells suppressed proliferation to OVA peptide. Thus, whether on the wild-type or Rag-1-deficient background, the CD25+ CD4 T cells had the phenotype of regulatory T cells.

The appearance of peribronchial lymphocytic infiltrates together with induction of Stat6-dependent goblet cell hyperplasia suggested that aspects of type 2 immunity were induced in the double transgenic SPC.OVA x D011.10 mice. To assess the status of IL-4 expression, we further crossed the SPC.OVA x D011.10 mice to 4get mice, which contain a bicistronic knockin IL-4 gene modified with an IRES and eGFP, thus enabling sensitive tracking of IL-4-producing cells (19). When analyzed directly from mice, neither lung nor spleen CD4 T cells expressed eGFP, indicating that the IL-4 gene was not actively transcribed in the SPC.OVA x D011.10 x 4get T cells (Figure 5B). When activated under Th2 conditions with IL-2 to overcome potential regulatory T cell function (36, 37), CD4 T cells from lung and spleen were induced to express eGFP at levels not different from cells taken from D011.10 x 4get mice (Figure 5B, and data not shown). Activation under neutral conditions, however, using IL-2 without IL-4 and anti-IFNγ, suggested that T cells in the lung, in contrast to the spleen, were poised to express IL-4 more readily. Indeed, IL-4 expression, as quantitated using eGFP fluorescence, was comparable under neutral or polarizing Th2 conditions in lung T cells (Figure 5B).

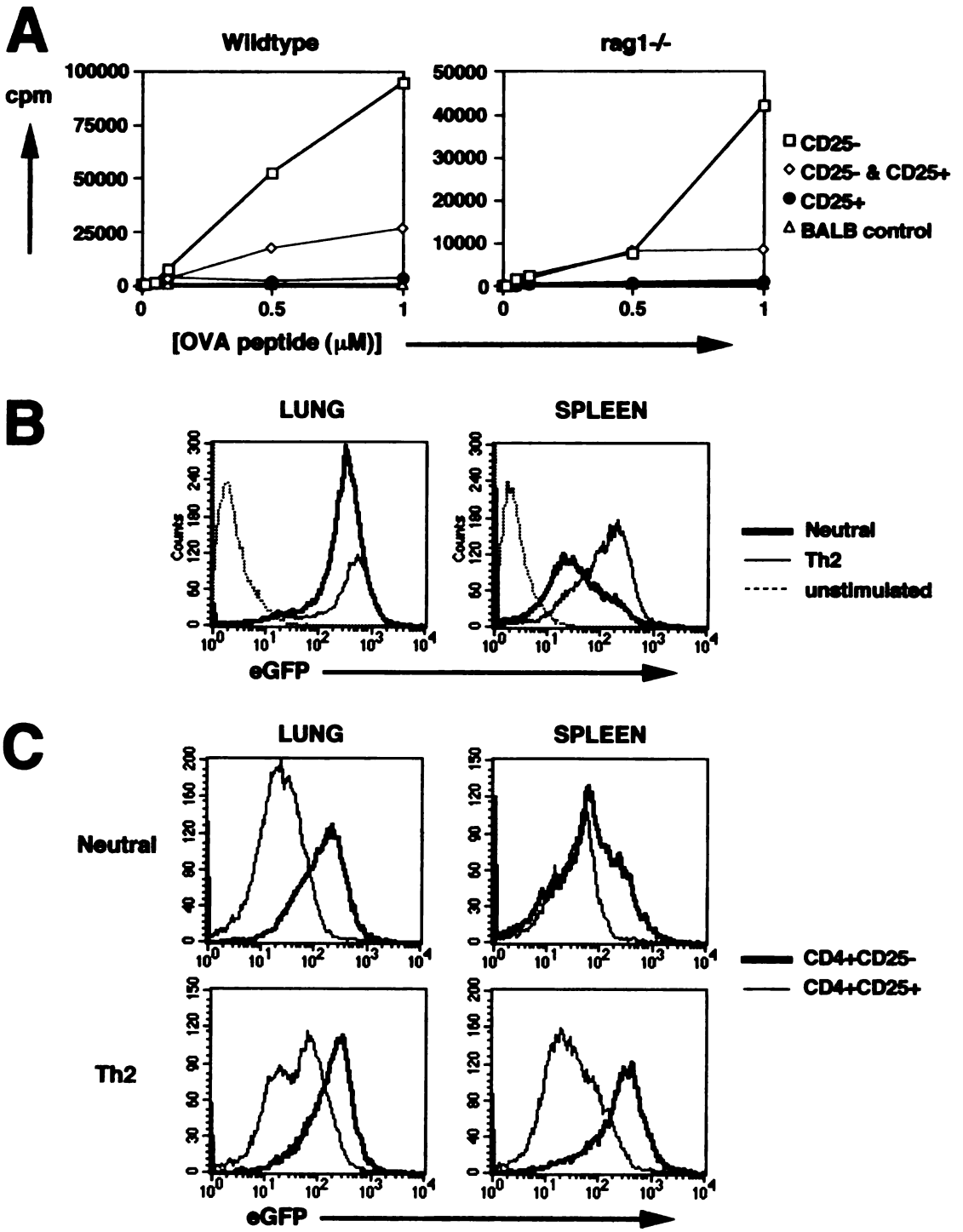
Figure 5. **Lung CD25+ CD4 T cells from SPC X DO11.10 mice inhibit CD25- CD4 T cells and maintain resistance to IL-4 induction.**

(A) CD4 T cells from the lungs of SPC.OVA x DO11.10 and SPC.OVA x DO11.10 x Rag-1-/- mice were fractionated by flow cytometry into CD25+ and CD25- populations and examined either alone or together (ratio of 4:1 for CD25- to CD25+ T cells) for proliferation to OVA peptide at varying concentrations with irradiated APC as assessed by radioactive thymidine incorporation. Data represent results from one of two comparable experiments.

(B) Purified CD4 T cells from lung and spleen of SPC.OVA x DO11.10 x 4get mice were examined for eGFP fluoresence either directly ex vivo (thin broken line), or 1 wk after activation with OVA peptide and APCs in the presence of IL-2 (thick solid line; Neutral) or IL-2 plus IL-4 and anti-IFNγ mAb (thin solid line; Th2). Data represent results from the live CD4+ gate from one of two comparable experiments.

(C) CD4 T cells from the lung and spleen of SPC.OVA x DO11.10 x 4get mice were separated by flow cytometry into CD25+ (thin solid line) and CD25- (thick solid line) populations and independently examined 1 wk after activation with OVA peptide and APCs under neutral or Th2 conditions noted in **(B)**. Data represent results from the live CD4+ gate from one of two comparable experiments

Figure 5


A
Wildtype
rag1-/-
cpm
100000
75000
50000
25000
0
50000
40000
30000
20000
10000
0
0.5
1
[OVA peptide (μM)]
CD25-
CD25- & CD25+
CD25+
BALB control
B
LUNG
SPLEEN
Counts
eGFP
Neutral
Th2
unstimulated
C
LUNG
SPLEEN
Neutral
Th2
eGFP
CD4+CD25-
CD4+CD25+

To assess the individual contributions of the CD25+ and CD25- CD4 T cell subsets, we isolated the respective cell populations from the lung and spleen and examined their capacity to express eGFP after priming in the presence of IL-2 alone or in Th2-polarizing conditions (Figure 5C). Under both conditions, the CD25- CD4 T cells expressed a more robust IL-4 response, and this was particularly evident using lung T cells. The CD25+ CD4 T cells were consistently more refractory to the induction of IL-4.

Taken together, the CD25+ CD4 T cells that accumulate in the lungs of double transgenic mice do not proliferate in response to their cognate antigen, suppress the proliferation of CD25- CD4 T cells, and maintain resistance to IL-4 induction even under Th2 conditions in vitro.

CD25+ CD4 regulatory T cells from lung suppress Th2 effector function in vivo but do not inhibit antigen-induced airway hyperreactivity.

Despite the accumulation of pulmonary infiltrates, saline treated SPC.OVA x D011.10 mice have basal airway reactivity to acetylcholine that is not different from similarly treated single transgenic D011.10 mice (Figure 6A). Repeated intranasal administration of OVA induces airway hyperreactivity and activated T cell responses in D011.10 mice (38). Despite the expression of OVA in the lungs of the SPC.OVA x D011.10 mice, we tested the capacity of additional exogenous OVA to induce airway hyperreactivity, perhaps due to activation of innate inflammatory systems. Indeed, after sensitization 5 times with OVA, airway hyperreactivity was induced to a comparable degree in both D011.10 and in SPC.OVA x D011.10 mice (Figure 6A). Since the CD25+ CD4

Figure 6. **Antigen-treated SPC.OVA X DO11.10 mice exhibit elevated airway hyperreactivity and suppressed type 2 immune reponses.**

Groups of 4-6 DO11.10 and SPC.OVA X DO11.10 mice were challenged intranasally with PBS (Saline) or OVA.

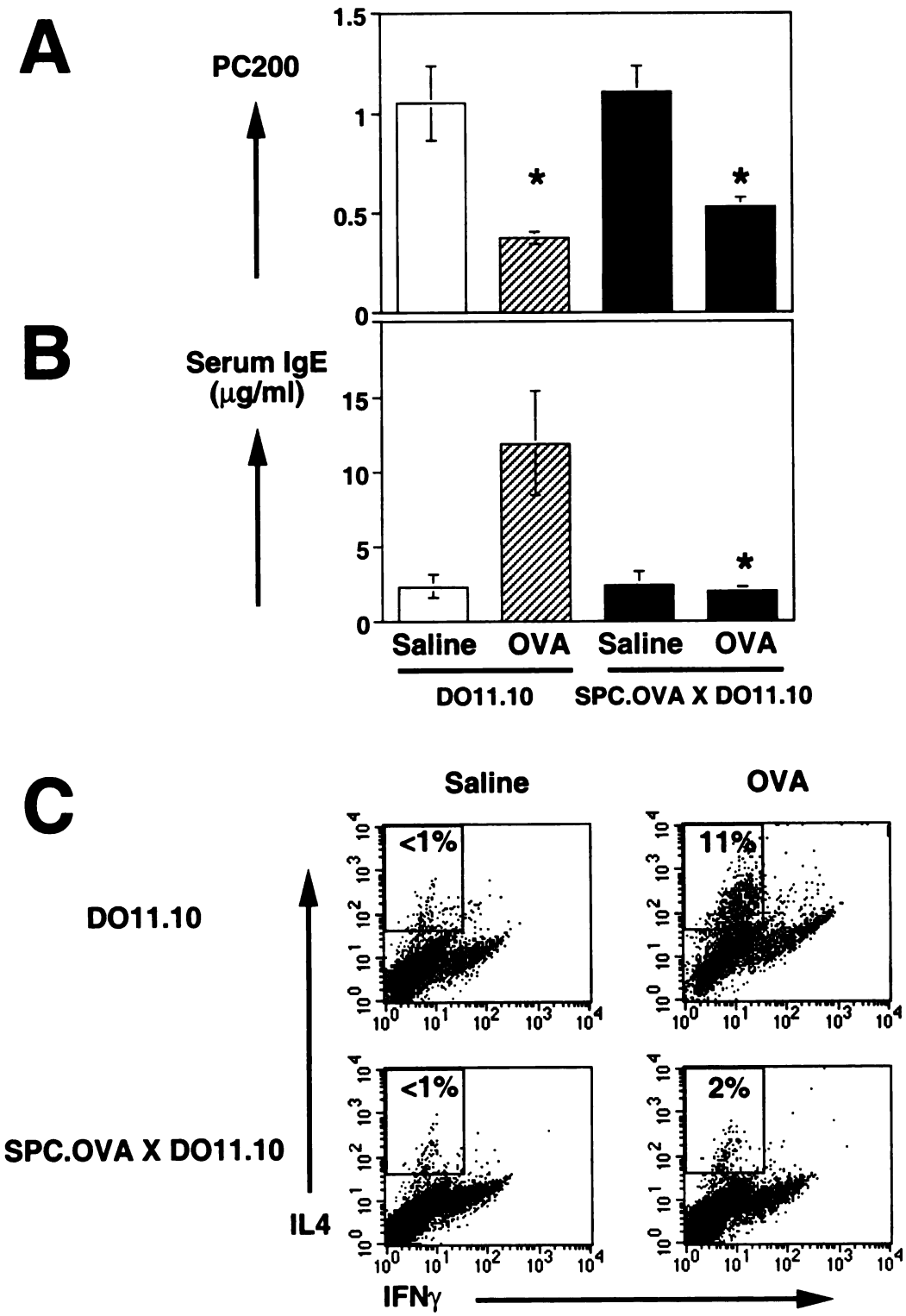
(A) The concentration of ACh, quantitated as μg of ACh per gram body weight, required to elicit a 200% change increase from baseline airway resistance or PC_{200}. Significant differences ($p<0.05$) between control and OVA groups are indicated by an asterisk.

(B) Serum IgE levels in control and sensitized mice as assessed by ELISA. Significant differences ($p<0.05$) between single and double transgenic groups given OVA are indicated by an asterisk.

(C) Lung cell suspensions were stimulated with PMA/Ionomycin for 4hrs and analyzed for the intracellular accumulation of IL-4 and IFNγ. Numbers indicate percentages of IL4+ cells in the lymphocyte gate. Lines delimit isotype antibody controls.

In each case, results are representative of at least two experiments. Bars depict means±SEM.

Figure 6


A
PC200
1.5
1
0.5
0
*
*
B
Serum IgE
(μg/ml)
15
10
5
0
*
Saline
OVA
Saline
OVA
DO11.10
SPC.OVA X DO11.10
C
Saline
OVA
DO11.10
SPC.OVA X DO11.10
<1%
11%
<1%
2%
IL4
IFNγ

regulatory T cells were not ablated by OVA sensitization (data not shown), we assessed whether antigen sensitization had completely overcome their regulatory function in vivo. Whether assayed using serum IgE or intracellular IL-4 production, the SPC.OVA x D011.10 mice however, revealed a marked suppression of type 2 effector function (Figure 6B, 6C). These data indicated that antigen-induced airway hyperreactivity continued to be manifest even in the presence of antigen-specific regulatory T cells that functionally suppressed type 2 immunity in vivo.

To investigate the dissociation between airway hyperreactivity and inhibition of type 2 immunity more fully, we reconstituted T and B cell-deficient Rag-1 knock-out mice with CD25- CD4 T cells from D011.10 mice in the presence or absence of CD25+ CD4 T regulatory cells isolated from the lungs of SPC.OVA x D011.10 mice. The recipients were reconstituted at the ratio of CD25-:CD25+ cells present in the lungs of double transgenic mice (4:1). After repeated intranasal sensitization with OVA, cohorts of mice were examined for airway hyperreactivity, IL-4 production by CD4 T cells and histologic criteria of type 2 immunity, including lung infiltration and goblet cell hyperplasia. In two independent experiments, airway hyperreactivity after OVA sensitization did not differ in the presence or absence of CD25+ regulatory T cells (Figure 7A). Despite the lack of effect on airway hyperreactivity, however, IL-4 expression by lung-infiltrating CD4 T cells was inhibited over 5-fold in mice that received CD25+ CD4 regulatory T cells (Figure 7B). Additionally, lung infiltrates and mucus production by goblet cells were markedly attenuated (Figure 7C). Thus, by this independent analysis, regulatory T cells

capable of inhibiting antigen-specific type 2 immune responses in the lung had no effect on the development of airway hyperreactivity.

Figure 7. **Lung CD25+CD4 T cells from SPC.OVA X DO11.10 mice suppress type 2 immune responses but not antigen-induced airway hyperreactivity.**

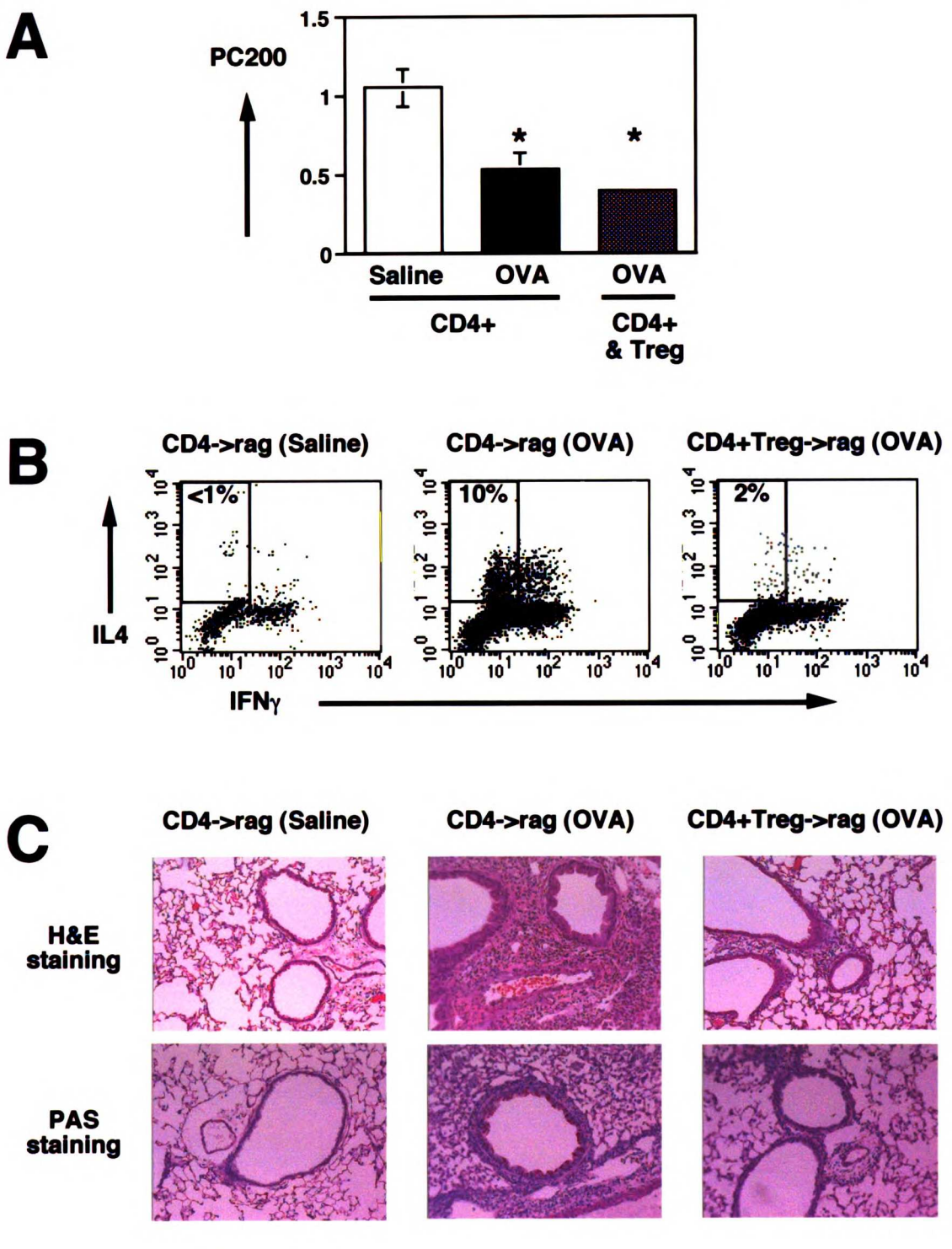
Splenic CD25- CD4 T cells from DO11.10 mice (CD4+) and lung CD25+ CD4 T cells from SPC.OVA X DO11.10 mice (T_{reg}) were purified by flow cytometry. $2x10^6$ CD4+ cells were adoptively transferred into rag-1-/- recipients (rag). Cohorts of 4-6 reconstituted mice were sensitized intranasally with PBS (Saline) or OVA. An additional cohort of 4-6 rag-1-/- recipients were reconstituted with $2x10^6$ CD4+ T cells and 0.5 x 10^6 T_{reg} cells and sensitized intranasally with OVA.

(A) PC_{200} in response to escalating doses of ACh. Bars depict means±SEM. Significant differences ($p<0.05$) between control and OVA groups are indicated by an asterisk.

(B) Lung cell suspensions were stimulated with PMA/Ionomycin for 4hrs and analyzed for the intracellular accumulation of IL-4 and IFNγ. Numbers indicate percentages of IL4+ cells in the lymphocyte gate. Lines delimit isotype antibody controls.

(C) Paraffin embedded lung sections from mice of the designated reconstituted and challenged groups, examined using H&E and PAS stains. The purple-staining cells in the lower middle panel depict mucus-producing goblet cells. In each case, results are representative of two independent experiments. Magnification 100X

Figure 7


A
PC200
1.5
1
0.5
0
*
*
Saline
OVA
OVA
CD4+
CD4+ & Treg
B
CD4->rag (Saline)
CD4->rag (OVA)
CD4+Treg->rag (OVA)
<1%
10%
2%
IL4
IFNγ
C
CD4->rag (Saline)
CD4->rag (OVA)
CD4+Treg->rag (OVA)
H&E staining
PAS staining

DISCUSSION

The role for type 2 immunity in mediating antigen-induced airway responses has been established in multiple animal models (7). Associations with asthma have further implicated these effector pathways in human disease (39). In an effort to mimic more closely the chronic exposure to airway antigens that characterizes human asthma, we generated transgenic mice that express a model antigen, OVA, in the airways, and crossed these onto an OVA-specific TCR transgenic background to overcome tolerance. A number of novel findings were made using these mice. First, despite driving the accumulation of lung-infiltrating lymphocytes, evidence for spontaneous effector type 2 immunity in the airways was minimal; although Stat6-dependent goblet cell mucin production was evident, airway hyperreactivity, eosinophils and IL-4-producing CD4 T cells were absent. Second, the co-expression of self-antigen and high-avidity TCR led to the accumulation of CD25+ CD4 T cells in the lung that expressed regulatory function in vitro and in vivo capable of suppressing antigen-induced type 2 immunity. Indeed, these cells likely played a role in mitigating the spontaneous expression of type 2 effector function in the double transgenic mice. Finally, despite the clear ability of the CD25+ CD4 regulatory T cells to attenuate type 2 responses, these cells had little effect on acute antigen-induced airway hyperreactivity.

It is intriguing to compare the outcome of forced expression of self-antigen at the mucosa, as done here, with forced expression at systemic non-mucosal tissue sites, such as the liver or pancreas. In most systemic models, various checkpoints appear to interrupt

a default type 1 immune differentiation pathway that, if unchecked, leads to tissue injury (24, 25, 40, 41). Indeed, suppression of type 1 responses to Bordetella infection of the respiratory tract by regulatory T cells has been reported (42). In contrast, our forced expression of self-antigen at the mucosa suggested the presence of a similar checkpoint appearing to interrupt default type 2 allergic tissue injury. Deposition of exogenous antigen on respiratory epithelia typically engenders type 2 immunity (43), perhaps reflecting release of specific epithelial chemokines and cytokines involved in allergic responses, such as eotaxin (44, 45). Alternatively, respiratory tract dendritic cells may intrinsically activate a type 2 differentiation pathway after migration and maturation to regional mediastinal lymph nodes (46). Although no IL-4 was produced in these double transgenic mice as assessed directly ex vivo or by inference with respect to normal IgE levels, IL-4 was readily revealed when T cells were activated in the presence of IL-2. Activation with IL-2 overcomes inhibition by regulatory T cells (36, 37), leaving the effector T cells to reveal evidence for priming as Th2 cells by their high levels of IL-4 gene expression. Indeed, IL-4 expression was comparable to that achieved by Th2 polarized cells from control TCR transgenic mice. Further, the capacity of these effector T cells to express a type 2 cytokine program was blocked by co-transfer of regulatory T cells, demonstrating directly the capacity of these cells to inhibit type 2 immunity in vivo. Together, these data provide evidence for an endogenous type 2 bias in response to high antigen load at the respiratory mucosa that is kept in check through recruitment of regulatory T cells.

Additional evidence supporting a poised type 2 immune state in the double transgenic mice was the consistent appearance of mucus production at levels above those seen in wild-type or single transgenic mice. Although not as widespread as the goblet cell hyperplasia that occurred in antigen-sensitized mice (9), mucus production remained Stat6-dependent, suggesting an underlying IL-13-mediated activation pathway (47, 48). We were unable to demonstrate IL-13 protein levels above background using a sensitive ELISA assay from lung T cell supernatants from double transgenic mice (data not shown), although this does not exclude a role for IL-13 produced by non-T cells. If so, the capacity of regulatory T cells to suppress cytokine effector function may be incomplete with respect to non-T cells.

The lung CD25+ CD4 regulatory T cells we describe have the phenotype of regulatory T cells generated in numerous autoimmune and inflammatory models (49). Their capacity to suppress type 2 immune responses has been infrequently examined, however. As we demonstrated, the lung regulatory T cells suppressed type 2 immunity in vivo and in vitro. The mechanisms by which these cells inhibit effector function remain inconclusive. Most prior in-vitro experimental systems have suggested a contact-mediated, T cell-to-T cell mechanism as opposed to cytokines such as TGFβ or IL-10 (36, 37). Although regulatory T cells express high levels of CTLA-4 (50, 51), we were unable to relieve suppression by treating mice with either CTLA-4/Ig fusion protein or with anti-CTLA-4 Fab (data not shown).

Despite the capacity to inhibit type 2 immunity in the presence of large amounts of antigen and effector T cells, the antigen-specific regulatory T cells did not affect acute

airway hyperreactivity after antigen challenge. Even in the presence of endogenous antigen, exogenous administration of OVA was required to induce airway hyperreactivity. We hypothesize that chronic antigen expression at high levels in the airways induces strong immunosuppressive pathways in alveolar macrophages for example (52), which have been shown to interrupt the antigen presenting capacity of resident dendritic cells (53). Exogenous OVA may overcome the inherent macrophage homeostatic function, and achieve antigen delivery by pulmonary dendritic cells which subsequently favors type 2 immunity (46). In the presence of CD25 regulatory T cells, Th2 effector function was markedly attenuated, but expression of type 2 immunity by non-T cells may be unaffected. These latter pathways may underlie the development of mucus cell hyperplasia and airway hyperreactivity that escapes the regulatory capacity of the CD25 CD4 T cells. As such, the mechanisms that drive innate type 2 immune responses to mucosal antigen challenge in the airway remain an important focus for further study.

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CHAPTER 5

Requirements for Airway Hyperreactivity

Summary

The work presented in this thesis suggests that airway responses to inhaled allergens is a well regulated process. We have first identified the potential role of modifier genes, independent of type 2 immunity to allergens, in a spontaneous murine model of airway hyperreactivity. To study the immune response in an antigen-dependent system, we used a potent extract from a fungal allergen to characterize T-cell responses in the airway. We were able to assess the importance of CD4+ T cells and IL-4, in particular, in mediating the pathologic and physiologic changes that underlie the human disease. Finally, we established the role of tolerance to a model antigen expressed chronically in the airway mucosa. This form of tolerance was mediated by regulatory T cells that inhibit spontaneous CD4+ T cell effector responses to a mucosal antigen. We were therefore able to establish a novel role for regulatory T cells in suppressing type 2 immune responses in vivo.

The aim of this discussion is to address three main aspects involved in the induction of airway hyperreactivity:

(I) The role of modifier genes, independent of type 2 immunity to allergens?

(II) The role of type 2 immune responses to airborne allergens?

(III) The role of tolerance to airborne allergens?

I. ROLE OF MODIFIER GENES

Initial genetic studies have suggested a simple autosomal recessive inheritance underlying the hyperreactive phenotype of the A/J strain (1, 2). However, subsequent studies have defined a complex polygenic trait (3, 4), one of which involves at least three major contributing loci (3). Some of these loci were found to lie close to immune relevant genes such as IL-1β (on chromosome 2), IL-2 receptor β chain and IL-3 receptor β chain (on chromosome 15), TNFα (on chromosome 17) (3) and the IL-5 receptor (on chromosome 6) (4). However other loci were found to map close to immune independent genes such as G_s protein alpha (Gnas) and the acetylcholine receptor (Acra) (3) which are clearly involved in airway smooth muscle physiology to cholinergic stimuli. Interestingly no data to date have suggested any linkage of the A/J hyperreactive trait to the Th2 cytokine gene cluster on mouse chromosome 11, whereas linkage studies in human populations have implicated the syntenic cytokine gene cluster on chromosome 5q (5).

Taken together, our findings (chapter 2) and those of others suggest an important role of modifier genes affecting airway responsiveness that is independent of type 2 immunity to inhaled allergens. A number of alternative mechanisms could account for the genetic differences in the airway hyperreactive phenotype between low and high responder strains of mice that are independent of type 2 immunity.

First, the autonomic nervous control of airway smooth muscle may differ between strains of mice. Control of airway smooth muscle tone is mediated via stimulation of postganglionic muscarinic cholinergic receptors and β2-adrenergic receptors on airway

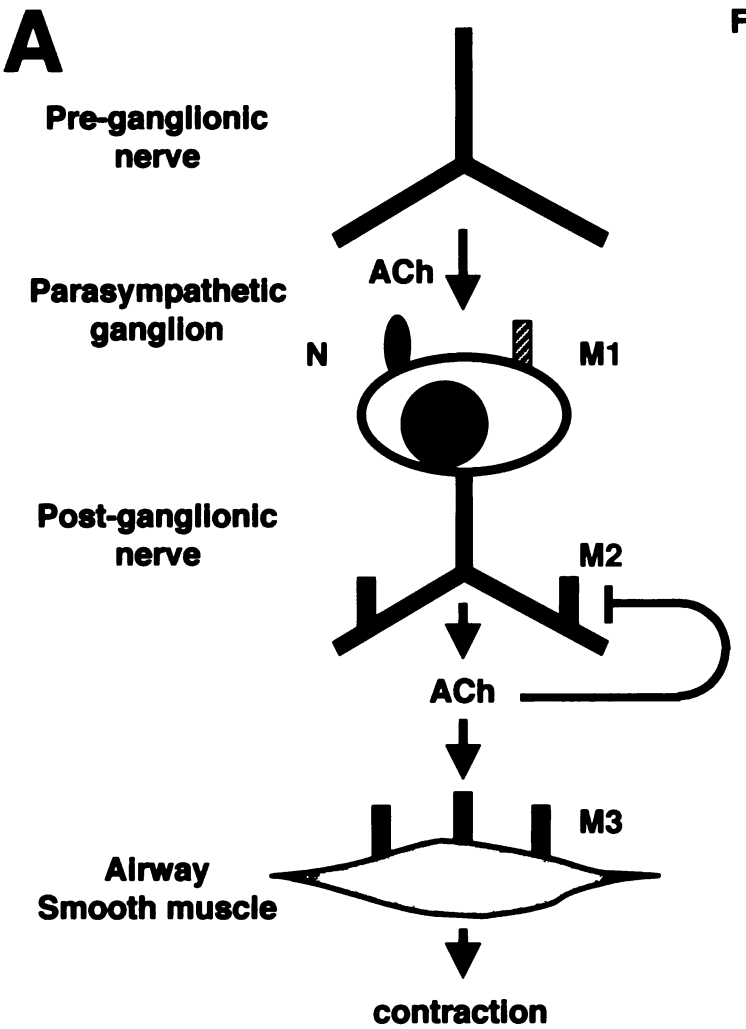
smooth muscle [reviewed in (6)]. There are up to five different muscarinic receptor subtypes in the airways. The two most important subtypes implicated in asthma are subtype II (M2) and III (M3) (6, 7). M2-receptors act as autoreceptors on post-ganglionic cholinergic nerves and inhibit acetylcholine release via an inhibitory feedback loop (figure 1A). There is some evidence to suggest that they are dysfunctional in human asthmatics (8) and in animal models of asthma (9). M3 receptors are present on airway smooth muscle, submucosal glands, epithelial and endothelial cells, and their stimulation leads to smooth muscle contraction and increased mucus secretion (6). Recent studies have compared β2-adrenergic and muscarinic cholinergic receptor properties and their coupling to guanine nucleotide binding proteins (G proteins) in peripheral lung membrane fractions from responsive A/J and hyporesponsive C3H strains (10). No significant differences were found in β2 adrenergic or muscarinic receptor densities or antagonist affinities between the strains, however muscarinic receptors were shown to be more effectively coupled to G proteins in A/J lungs. Since the Scatchard binding data using a cholinergic antagonist cannot distinguish between the different muscarinic receptor subtypes, we used a quantitative RT-PCR approach to examine the expression of the predominant M3 receptor at the message level. We found only a slight elevation in the expression of M3 message in A/J whole lung mRNA while B10.A message levels were lower (figure 1B). However the overall stoichiometry of muscarinic receptor subtypes in the lung needs to be evaluated to account for the difference in airway responsiveness between these mouse strains. For example, the enhanced contractile responsiveness of A/J smooth muscle could be due to elevated levels of the M3 receptor at the protein level and/or suppressed levels of the inhibitory M2 receptor.

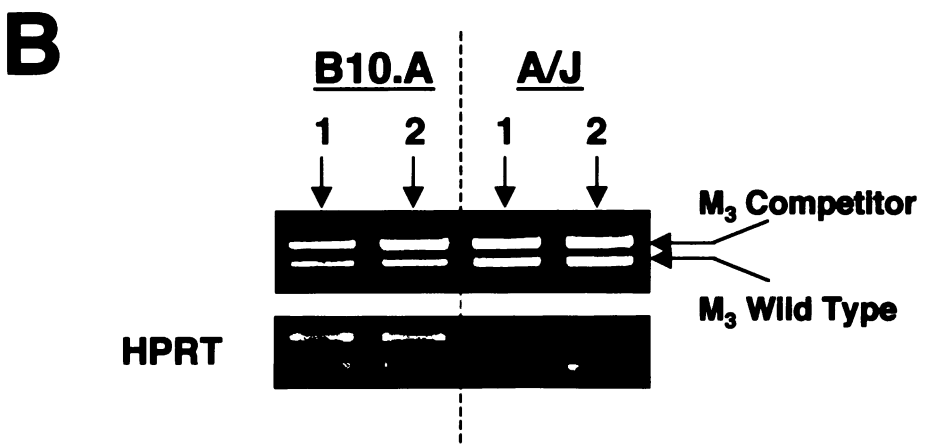
Figure 1. **Muscarinic receptor subtypes involved in bronchial hyperreactivity.**

A. Ganglionic transmission is mediated by nicotinic (N) and muscarinic receptors (M1). M2 receptors at the postganglionic nerve inhibit the release of acetylcholine in a negative feedback loop. M3 receptors are expressed on airway smooth muscle, submucosal glands, epithelial cells and endothelial cells causing airway smooth muscle contraction, increased mucus secretion, increased ciliary beating and vasodilation respectively. (Adapted from Barnes, P.J. 1993. *Life Sci* 52:521-527).

B. Competitive RT-PCR analysis of M3 mRNA transcripts from A/J or MHC-matched B10.A whole lung preparations. Concentrations of input cDNA were standardized to comparable amounts of the constitutively expressed hypoxanthine phosphoribosyltransferase (HPRT) gene. Results indicate 2 mice from each group.

Figure 1


A
Pre-ganglionic nerve
ACh
Parasympathetic ganglion
N
M1
Post-ganglionic nerve
M2
ACh
M3
Airway Smooth muscle
contraction


B
B10.A
A/J
1
2
1
2
M3 Competitor
M3 Wild Type
HPRT

Second, a more trivial explanation is a possible anatomical difference in airway wall geometry that could affect airway responsiveness. Thus an overall smaller airway radius might lead to increased airway resistance for a set degree of smooth muscle contraction induced by the bronchoconstricting agent. However, the lack of any difference in baseline airway resistance between the mouse strains, makes differences in baseline airway caliber an unlikely explanation. Furthermore, under plain histologic examination of age and size matched animals it was difficult to compare the right types of airways.

Finally, selective expression of integrins, cadherins, or other cell-adhesion molecules, could account for differences in airway hyperreactivity. Integrins for example, are important in mediating cell-cell contact between epithelial cells and extracellular matrix in normal airways [reviewed in (11)]. Therefore any defects in integrin expression might affect the repair process of the airway epithelium which could ultimately affect airway hyperreactivity. Although recent studies have defined a role for the integrin $\alpha_v\beta_6$ in mediating lung tissue homeostasis (12), baseline histologic studies failed to provide evidence for aberrant cell infiltration in mediating the physiologic changes in the lung.

II. ROLE OF TYPE 2 IMMUNITY

We have shown that CD4+ T cells alone are sufficient to mediate many of the pathologic changes that occur in a murine model of human asthma by a mechanism dependent upon IL-4, but independent on IL-5, IgE or both (chapter 3). Those include pathways independent on IL-5-mediated eosinophilia and IgE-mediated mast cell degranulation (figure 2). Previous studies consistently reveal a critical role for Th2 cells and type 2 cytokines in animal models of airway hyperreactivity [reviewed in (13)]. In this section we will look at the effects of different components of type 2 immunity in the induction of allergic airways disease.

CD4+ Th2 cells and type 2 cytokines.

Th2 differentiation from naïve CD4+ T cells is largely driven by IL-4 [reviewed in(14)]. Several studies have suggested that genes regulating IL-4 expression may be altered in allergic airways disease. Firstly, a polymorphism in the regulatory sequences of the IL-4 gene has been shown to correlate with high serum IgE levels and enhanced IL-4 expression (15). Furthermore, mutations in the IL-4 receptor α chain associated with increased IL-4 signaling were linked with atopy (16). Finally, alterations in a number of transcription factors, such as the NFAT protein family members that control the expression of a number of genes involved in asthma, including IL-4, may lead to the development of the allergic phenotype (17-19). A loss of function mutant of the transcription factor T-bet, critical in Th1 development, for example, has been shown to develop a spontaneous type 2 immune response in the airways (20).

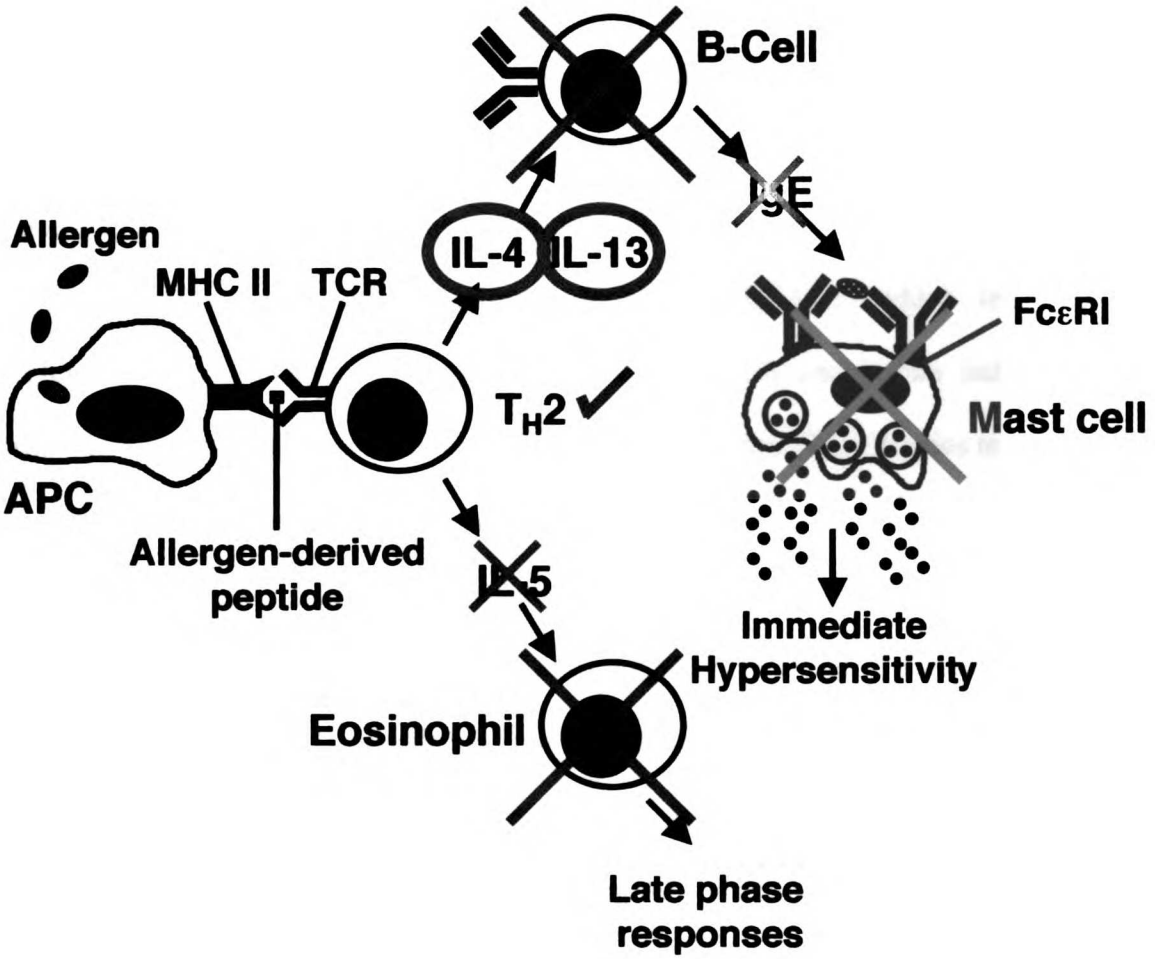


Figure 2. **CD4+ T cells alone can mediate allergic airways disease by an IL-4-dependent mechanism.**

CD4+ T cells alone are sufficient to mediate type 2 immune responses in allergic asthma by a mechanism dependent upon IL-4, but independent of IL-5, IgE or both, implicating IL-5-mediated eosinophilia, and/or IgE-mediated mast-cell degranulation to be dispensable.

Using IL-4 deficient mice, we have shown a key role for IL-4 in mediating airway hyperreactivity. IL-4 induces a wide variety of cellular responses important in type 2 immunity. It acts as an important immunoglobulin isotype (IgE) switch factor in B cells (21), it is a growth factor in conjunction with IL-3 for mast cells (22), it induces the expression of vascular cell adhesion molecule-1 (VCAM-1) which leads to the preferential recruitment of eosinophils into tissues (23) and it can induce mucus production by upregulating the expression of the MUC5 gene (24). Studies in which IL-4 has been depleted using either IL-4-deficient mice (25, 26), antibody-blockade (27-29) or soluble IL-4 receptor treatment (30), have shown an inhibition of both pathology and airway reactivity associated with type 2 immunity in the airways. However, IL-4 may play two important roles in mediating type 2 immunity to airway antigens. It can either prime naïve CD4+ T cells to mature into Th2 cells and/or the subsequent IL-4 released from these cells can mediate an important role in the effector phase of the disease. Using IL-4 deficient mice we were unable to dissect these individual roles for IL-4, because the immune response to airway antigen switched to a predominantly IFNγ-producing type 1 response which inhibited airway hyperreactivity. A murine model involving repetitive subcutaneous immunization with ovalbumin (OVA), followed by aerosol challenge with the antigen, showed that IL-4 depletion during the early subcutaneous priming stage prevented the induction of disease (29). However, depletion during the aerosolization phase had no effect on airway hyperreactivity. In other studies, adoptive transfer of IL-4-deficient Th2 cells into the airways was able to induce mucus production (31). Together, these studies demonstrate a dominant role for IL-4 in priming CD4+ T cells to develop into Th2 cells, whereas type 2 cytokines other than IL-4 may mediate many of the

pathognomonic changes seen in asthma. To distinguish whether IL-4 plays a role in the priming or effector phase of the response, we transferred in vitro activated Th2 cells from DO11.10 transgenic mice into syngeneic recipients that received an OVA aerosol and anti-IL-4 antibody treatment. These studies showed that airway hyperreactivity was unimpaired in antigen-challenged mice that received either the anti-IL-4 or control antibody (figure 3A). Further, transfer of in-vitro activated Th2 cells from DO11.10 mice into IL-4 receptor α deficient mice abolished some of the bronchial reactivity relative to wildtype recipients, suggesting an important role for the IL-4 signaling pathway in the effector phase of the response. Some studies were able to show IL-4 receptor independent effects on airway hyperreactivity (32) which explains the partial airway reactive phenotype we observed in antigen challenged IL-4 receptor α deficient recipients (figure 3B). Other studies have also confirmed the importance of IL-4-mediated signaling in allergic airways disease using antibody blockade of the IL-4 receptor (33). In addition, Stat6, a critical cellular transducer of IL-4 signals, was shown to be required for the induction of airway eosinophilia, mucus production, elaboration of type 2 cytokines and airway hyperreactivity (34, 35). Similar studies to our own have shown that transfer of IL-4-deficient Th2 cells into Stat6 deficient recipients abrogated allergic airways disease that was normally seen in wildtype recipients (36). Taken together, these studies suggest that Th2 cytokines other than IL-4 can mediate effector functions associated with human asthma through the IL-4 receptor and Stat6 signaling pathway, both of which are implicated in IL-4 as well as IL-13 signaling (figure 4).

Subsequent landmark studies have shown that IL-13 plays the key role in the effector phase of asthma. Depletion studies using a soluble IL-13 receptor-F_c fusion

Figure 3. **Type 2 cytokines, other than IL-4, can mediate airway hyperreactivity, in an IL-4 receptor dependent fashion.**

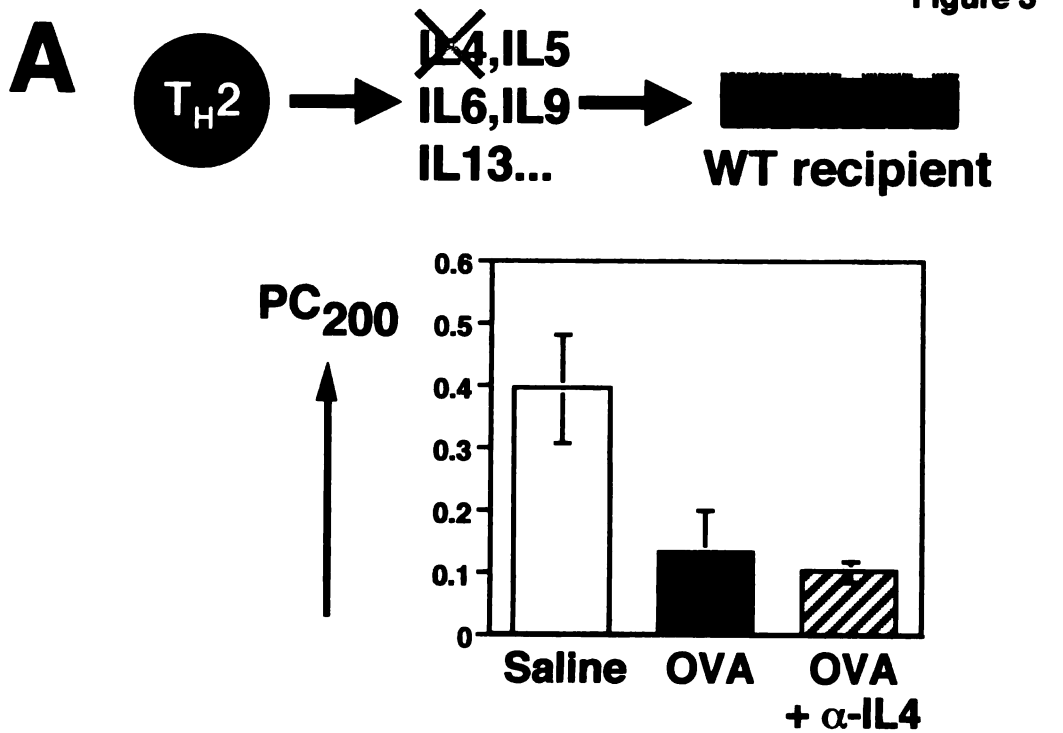
Purified CD4+ T cells from DO11.10 mice were cultured in-vitro with T-depleted splenic APCs, OVA peptide and recombinant IL-2 and IL-4 as well as anti-IFNγ (XMG1.2) for 1 week (as described in chapter 4, materials and methods). The cells were ficolled and restimulated for an additional week under the same polarizing conditions. Purified cells were transferred intravenously into designated recipients.

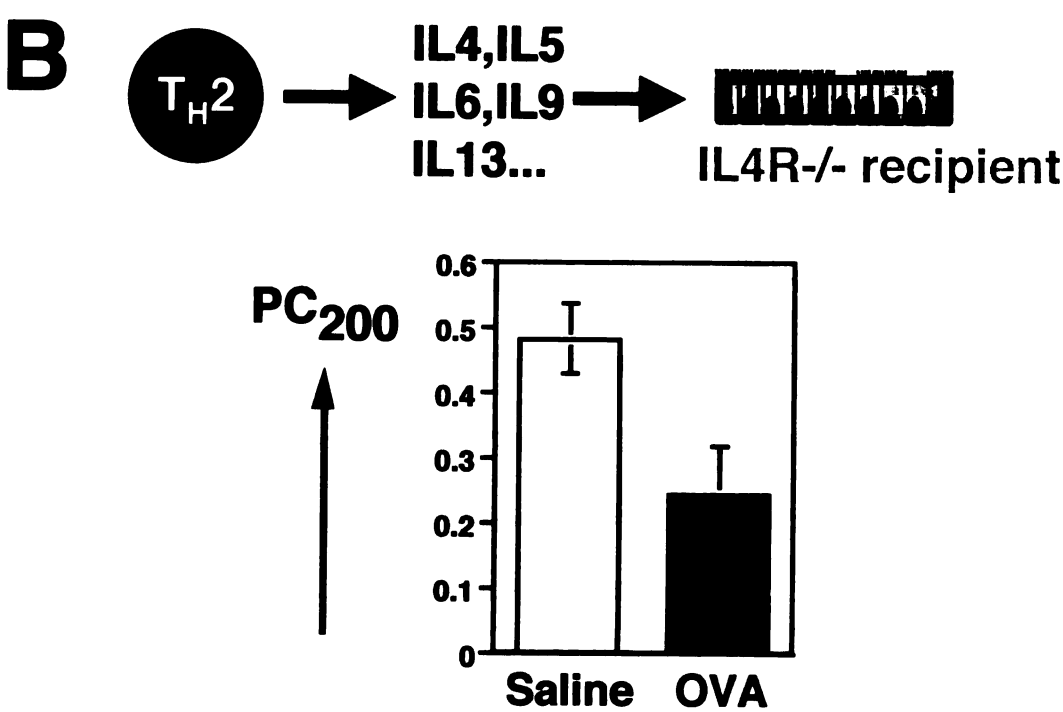
A. The concentration of acetylcholine required to elicit a 200% increase from baseline airway resistance (PC_{200}) in wildtype Balb/C recipients aerosolized with OVA or PBS (as described in chapter 2, materials and methods). A designated cohort of sensitized mice were given anti-IL-4, 11B11 (5 mg) prior to aerosolization at a dose required to inhibit the induction of type 2 immune responses in-vivo

B. PC_{200} in response to escalating doses of acetylcholine in IL-4 receptor α-deficient recipients aerosolized with OVA or PBS.

Bars depict means ± SEM.

Figure 3


A
T_H2
IL4,IL5
IL6,IL9
IL13...
WT recipient
PC_{200}
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
OVA
OVA
+ α-IL4


B
T_H2
IL4,IL5
IL6,IL9
IL13...
IL4R-/- recipient
PC_{200}
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
OVA

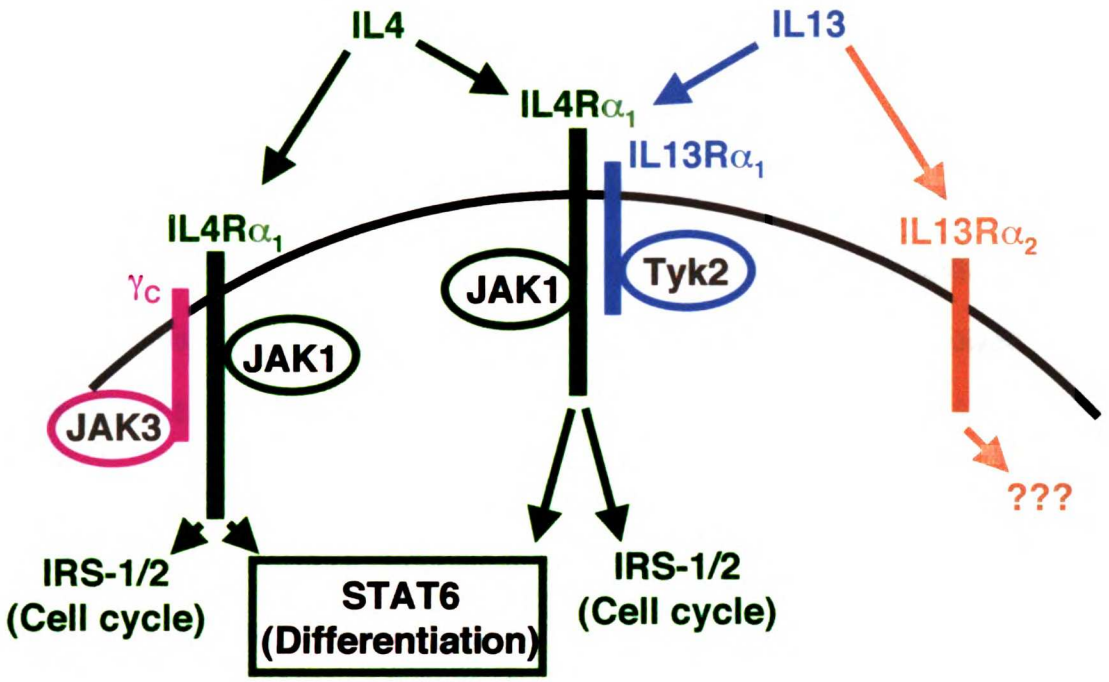


Figure 4. **Similarities between IL-4 and IL-13 signaling**

Two different forms exist for the IL-4 receptor (IL-4R), classical and alternative. The Classical IL-4R consists of IL4-Rα_1 and the IL-2 receptor gamma c (γ_c) chains. The alternative form consists of IL-4Rα_1 and IL-13Rα_1 chains that is also used for IL-13 mediated signaling. Phosphorylation of various Janus Kinases (JAKs) leads to the phosphorylation, dimerization and subsequent translocation of Signal Transducer and Activator of Transcription-6 proteins (STAT6) into the nucleus. IL-4Rα_1-mediated signaling is required for STAT6 activation. A decoy receptor for IL-13, IL-13Rα_2 has been proposed, but its function remains unknown.

protein, in an antigen-dependent model, showed IL-13 to be necessary in mediating the airway response (37, 38). Furthermore, intranasal administration of recombinant IL-13 to unimmunized wildtype or immunodeficient recipients revealed that IL-13 was sufficient to mediate pathology and airway reactivity (37, 38). These studies were confirmed using a transgenic approach which targeted IL-13 expression to the airways (39). Reports of IL-4-independent pathways of asthma (40) can therefore be rationalized by the importance of IL-13 in the disease process. IL-13 was also shown to be important for priming Th2 responses both in vivo and in vitro (41). Similar studies in helminth parasite models have also revealed an important requirement for IL-13 and stat6 in the expulsion of *Nippostrongylus Braziliensis* (42, 43).

Future studies will address the direct effects of IL-13 on resident airway tissue such as epithelium, goblet cells or smooth muscle in mediating the disease. It is known that the IL-13 receptor, composed of the IL-4 receptor α and IL-13 receptor α_1 chain (figure 4) is predominantly expressed on non-hematopoietic cells [reviewed in (44)]. A recent study has already revealed direct effects of IL-13 on epithelial cells to be the cause of airway hyperreactivity and mucus production in asthma (45).

Eosinophils.

IL-4 may induce allergic airways disease by recruiting eosinophils into the airways, since IL-4 blockade has been shown to prevent eosinophilia (25-27, 33). IL-4 may mediate pulmonary eosinophilia through its role in priming Th2 cells, that subsequently produce IL-5. Alternatively IL-4 can upregulate VCAM-1 expression on endothelium (23) or stimulate the release of eotaxin from resident airway cells (46). IL-5

has been shown to be a critical factor for eosinophil differentiation, activation and survival and therefore seems to mediate an important role in eosinophilia and subsequent airway hyperreactivity (47). We have shown, on the other hand, that IL-5-deficient mice still present with substantial airway hyperreactivity, despite absent airway eosinophilia. Furthermore disabling both the IgE and eosinophil mediated pathways in anti-IL-5 treated B cell deficient mice had no effect on airway reactivity. Studies in B cell and IgE deficient mice have also shown that IgE is not required to mediate eosinophilia in allergic airways disease (48-50). The importance of IL-5 in airway eosinophilia has been established by other groups as well, by either antibody blockade (29, 40, 51, 52) or gene targeting (53). However, a conflict exists as to the requirement of eosinophilia in mediating airway hyperreactivity. Studies in IL-5-deficient mice (53) or in mice treated with anti-IL-5 antibodies (40, 52) have demonstrated a role for IL-5-dependent eosinophilia in mediating airway hyperreactivity, as have results from transgenic mice that overexpress IL-5 in the airways (54). However, other studies using anti-IL-5 antibody treatment in Balb/C mice, that are genetically predisposed to developing type 2 immunity revealed no role for IL-5 dependent eosinophilia in the airway response (29). Furthermore, in our studies on the C57BL/6 genetic background, we were unable to define a role for IL-5-mediated eosinophilia in causing elevated airway reactivity. These conflicting results may be explained by the recent finding that airway eosinophils from certain strains of mice do not degranulate after antigen challenge. In addition these studies have utilized different techniques for assessing airway physiology and they vary in the nature and mode of antigen administration, some of which display genetic differences among various inbred strains of mice. Given that the role of eosinophilia in

mediating bronchial reactivity has been established through IL-5 depletion, it is highly likely that IL-5 may mediate other pathways, independent of eosinophils, that culminate in airway hyperreactivity [reviewed in (55)].

In other studies IL-5 overexpression in mice have shown a marked elevation in circulating eosinophils whereas tissue eosinophilia remained unaltered (56), suggesting the role of additional factors such as chemokines that contribute to the recruitment of eosinophils to inflamed tissue. These include the C-C chemokines RANTES, MCP-3, MCP-1 and in particular eotaxin (57, 58). Upon recruitment to inflammed tissue, eosinophils release a myriad of toxic mediators and cytokines. These include: (a) protein mediators such as eosinophilic cationic protein (ECP), major basic protein (MBP) and eosinophil derived neurotoxin (EDN); (b) lipid mediators such as LTs, PGE2 and PAF and (c) cytokines including IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF, IL-12, IFNγ, TGFα and TGFβ (59). The release of the cytotoxic MBP and ECP proteins from eosinophil granules may damage the airway epithelium. MBP may induce enhanced airway reactivity by inhibiting M2 receptor function on parasympathetic nerves, resulting in increased release of acetylcholine (60). M2 receptors are involved in a negative feedback loop regulating acetylcholine release from postganglionic nerves that inervate the airway smooth muscle (figure 1).

Mast cells.

Numerous studies have shown evidence of mucosal mastocytosis and activation in the lungs of asthmatics (61). Mast cells are thought to be activated via an IgE-dependent pathway, thus implicating the role of a preformed factor from the adaptive immune

response in activating an innate effector cell. IgE produced by allergen reactive B cells binds to the high affinity $F_c\varepsilon$ receptors on mast cells and basophils. Upon re-encounter with allergen these receptors become crosslinked via IgE and induce mast cells to degranulate releasing a plethora of vasoactive mediators, chemotactic factors and cytokines that promote leukocyte infiltration, edema, mucus production and smooth muscle contraction. Mast cell mediators fall into 4 groups: (a) Vasoactive amines such as histamine and (b) Lipid mediators such as PGD2, PAF and LTC4 that induce smooth muscle contraction, vascular leakage and edema; (c) cytokines such as TNFα, IL-1, IL-3, GM-CSF, IFNγ, IL-4, IL-5, IL-6 and IL-8 as well as chemokines such as MCP-1, MIP-1B, MIP1α and RANTES that contribute to the inflammatory process in the late phase response; (d) enzymes such as tryptase that contribute to airway wall remodeling by affecting fibroblast growth, collagen synthesis and subsequent connective tissue turnover (62).

Some studies however have reported that mast cell-deficient mice were able to exhibit allergic airways disease in an antigen-dependent system, suggesting that alternative pathways, independent of mast cells, can contribute to the inflammatory process and subsequent airway physiology (26, 63).

In summary, the exact mechanism by which Th2 cells mediate inflammation and subsequent airway hyperreactivity is still unclear. Th2 cytokines are believed to mediate the recruitment and activation of eosinophils (by IL-5 and GM-CSF) and mast cells (by IL-3, IL-4, IL-9, IL-10 and IL-13) and the production of IgE antibodies (by IL-4 and IL-13), that ultimately cause pathologic changes, including hypertrophy of the respiratory

epithelium, excess mucus production, airway edema, eosinophilia and changes in smooth muscle that collectively lower the threshhold for airway narrowing to a variety of stimuli, termed airway hyperreactivity (figure 5). Such pathology may reflect the effects of toxic eosinophil and mast cell products that are chronically deposited following IgE-mediated stimulation. The underlying inflammation, therefore, induces alterations in airway wall geometry, resulting in a net decrease in airway radius. Bronchoconstricting stimuli to an already thickened airway wall result in even further narrowing of the airway lumen. Furthermore, the subsequent inflammation has been shown to affect neural regulation of airway smooth muscle, in particular the M2 autoreceptor on the nerve ending that innervates airway smooth muscle (figure 1). There is evidence to suggest that they are dysfunctional in human asthmatics (8) and in animal models of allergic airways disease (9). Other studies suggest that cytokines such as TNFα and IL-1 alter β-adrenoreceptor signaling mechanisms that lead to impaired smooth muscle relaxation (64). It is clear from our studies and those of others that the induction of airway hyperreactivity is the integrated end result of a number of pathways, initiated by Th2 cells and the cytokines they produce. Therefore, blocking one pathway can still allow bronchial reactivity via alternative pathways.

Figure 5. **Central role of Th2 cells in initiating multiple effector pathways in allergic airways disease**

Th2 cytokines are believed to mediate inflammation by the recruitment and activation of eosinophils and mast cells and the production of IgE antibodies, that ultimately cause pathologic changes, including hypertrophy of the respiratory epithelium, excess mucus production, airway edema, eosinophilia and changes in airway smooth muscle that collectively induce airway hyperreactivity to a variety of stimuli. Such pathology may reflect the effects of toxic eosinophil and mast cell products that are chronically deposited following IgE-mediated stimulation.

Figure 5

T_H2
IL4
B cell
IgE
IL4,IL13
Epithelial cells
Chemokines
IL3,IL9
Mucosal mast cells
Cytokines
Histamine
lipid mediators
Vascular/
smooth muscle
response
Inflammation
Enzymes
(e.g. Tryptase)
IL13
Goblet cells
Mucus
IL5
Eosinophils
Enzymes
(e.g. eosinophil peroxidase)
Tissue remodeling

III. ROLE OF PERIPHERAL TOLERANCE TO MUCOSAL ANTIGENS

The role of tolerance to tissue-specific antigens has been extensively studied in transgenic mouse models employing the rat insulin promoter to target antigen expression to the islet cells of the pancreas (65). Mechanisms of this form of peripheral tolerance may include anergy, deletion, ignorance or suppression of T cells and each of these mechanisms has been defined for CD4+ T cells in several experimental models [reviewed in (66)]. It is well established that tolerance to a wide plethora of antigens at mucosal sites has to be maintained in order to prevent detrimental immune responses to commensal gut flora, food antigens or even innocuous airborne allergens. Breakdown in tolerance towards these antigens can potentially lead to conditions such as inflammatory bowel disease (IBD) and atopic disorders, such as allergy and asthma.

Regulatory T cells.

It was known since the early 1970's that a population of T cells existed that could suppress immune responses in vivo (67). However, the cellular and molecular mechanisms that mediated this suppression remained unknown. In the mid 1990's, Sakaguchi and associates rekindled this area of research by discovering that a minor population of CD4+ T cells (approximately 10% of peripheral CD4+ T cells) that co-express the IL-2 receptor α chain (CD25) can suppress T cell responses both in vitro and in vivo (68, 69). One major form of tolerance that has been implicated at mucosal surfaces is the active suppression of CD4+ T cells by such regulatory T cells (70).

Antigen deposition in the airway mucosa generated IL-4- and IL-10-producing CD4+ T cells, a phenotype consistent with T_R1 regulatory cells (71), whereas deposition in the gut mucosa generated IL-4-, IL-10- and TGFβ-producing CD4+ T cells, consistent with Th3 regulatory cells (72). It is believed that these suppressive cytokines can inhibit APC or T cell function, or even effector functions of T cells.

In an effort to study the effects of chronic allergen exposure in the airways on CD4+ T cell development and subsequent airways disease, we generated transgenic mice that expressed OVA in the lung (chapter 4). By crossing to OVA-specific TCR transgenic mice (DO11.10), we found cellular infiltrates in the lungs that consisted of a surprisingly large population of CD4+ CD25+ T cells. We were able to show that these cells suppressed the proliferation of CD4+ CD25- effector cells in vitro, thus providing formal proof of their regulatory ability. Since no hallmarks of airway T cell effector function, such as pulmonary cytokines, eosinophilia or airway hyperreactivity was evident, we hypothesized that effector progression was kept in check by this population of regulatory T cells. We were also able to show in antigen-induced models of airway hyperreactivity, that lung regulatory T cells were able to suppress type 2 immune responses in vivo, but had no effect on the airway hyperreactive phenotype. Thus, similar to other models of mucosal tolerance (71, 73), we suggest that long-term mucosal exposure to antigens, recruits regulatory T cells that prevents active type 2 immune responses to innocuous airway allergens. Previous studies employing the same promoter and the viral hemagglutinin protein have shown tolerance in the CD8+ T cell compartment, but have failed to show tolerance in the CD4+ compartment (74). However in our studies, crossing in the antigen-specific TCR transgene has allowed for

the thymic selection of a high number of antigen-specific regulatory cells which are felt to suppress CD4+ T cell effector function in the lung. The generation of these regulatory cells can be explained by a recent study which shows that the presence of self antigen can allow for the selection of regulatory T cells, provided that the TCR on the developing T cells has a high affinity for the self peptide (75). Our findings, however are in sharp contrast to a recent study which suggests that CD4+ CD25+ regulatory T cells do not suppress type 2 immune responses in the airways, rather they enhance Th2-mediated airway inflammation (76). This system, however, depletes CD25+ T cells from whole-spleen preparations, which could deplete newly activated CD4+ T cells, rendering the remaining preparation enriched in naïve T cells.

Mechanism of suppression by regulatory T cells.

The exact mechanisms by which regulatory T cells mediate their suppressive effects remain unknown. Although T:T contact is required in vitro (77, 78), it is not yet clear if they target the APCs or the responder T cell population. It has also been shown that CD4+ CD25+ murine T cells constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4), associated with inhibiton of T cell activation (79, 80). Blocking CTLA-4 interactions with their ligands CD80/CD86 revealed conflicting results. For example, in vivo blockade of CTLA-4 was able to inhibit suppression of autoimmune IBD or gastritis (79, 80), however, in vitro suppression was not abrogated by CTLA-4 blockade (78, 81, 82). Most studies have also failed to identify the role of soluble suppressive cytokines in vitro, such as IL-4, IL-10 or TGFβ (77, 78), however, in vivo studies have shown that they play crucial roles in suppression of autoimmune diseases in adoptive transfer models

(83-86). Some studies have even implicated cross-linking of CTLA-4 to be associated with the production of TGFβ (87).

We were unable to establish a role for CTLA-4 in suppression of effector T cell function in the lungs of our double transgenic mice, using either anti-CTLA-4 F_{ab} fragments or the CTLA4-Ig fusion protein. Furthermore, we were unable to detect the presence of either IL-4 or IL-10 in the lungs of these mice. In addition, inflammatory lesions and regulatory T cell recruitment were still intact on the stat6-deficient background, which is defective in IL-4-mediated signaling.

Mucosal tissue milieu in type 2 immune responses.

Our findings have also revealed clues about the role of the tissue milieu in priming CD4+ T cells. In our studies the type 2 immune potential of lung effector T cells was revealed after the addition of IL-2 and TCR signals in-vitro. This finding was further augmented by the stat6-dependency on airway mucus production. Thus, the presence of antigen in airway mucosa clearly has the potential to recruit and prime CD4+ T cells into IL-4 producing cells, but effector progression appears to be kept in check by the population of CD4+ CD25+ regulatory T cells recruited to the inflammatory lesions. In other studies, exposure to foreign antigens via mucosal surfaces has also been shown to favor type 2 immune responses in the airways (88) or the gut (89). For instance, under conditions of an antigen/mouse strain combination that strongly favors type 1 immunity in non-mucosal tissue, deposition of antigen in the airway mucosa was found to favor a type 2 immune response (90). The airway mucosa might, therefore, represent a unique environment that promotes Th2 development through the elaboration of cytokines and

chemokines by the inflammed tissue. It has been shown, for example, that inflamed airway epithelium is a source of chemokines such as eotaxin (91, 92) that promote eosinophilia and subsequent type 2 immunity. Resident APCs in the tissue may also play a role in affecting the outcome of adaptive immune responses. Distinct populations of dendritic cells (DCs) with the capacity to induce preferential priming for either Th1 or Th2 subsets have been identified [reviewed in (93)]. For example, respiratory tract dendritic cells were found to preferentially stimulate Th2 responses in an adoptive transfer model (94). The most prevalent transgenic animal models that have targeted antigens to non-mucosal tissue sites employed the rat insulin promoter that targets expression to the endocrine islets of the pancreas. These studies have almost always confirmed strong cell-mediated type 1 immune responses in the islet tissue upon breakdown of tolerance [reviewed in (65)]. Other studies targeting antigen to non-mucosal tissues have shown similar results. For example, targeted OVA deposition in the liver was shown to mediate liver injury upon transfer of OVA-specific Th1 but not Th2 cells (95). These studies clearly suggest that the tissue plays a strong role in priming CD4+ T cell responses to their cognate antigens.

Breakdown of tolerance.

In an attempt to break unresponsiveness to OVA in our model system, we sensitized the double transgenic mice intranasally with either OVA or a complex mixture of potent antigens with strong adjuvant properties that favor type 2 immunity (figure 6). One such antigen mixture is *Aspergillus fumigatus* which represents a mixture of culture filtrate and mycelial extract prepared free of living organisms (96). Sensitization with

Figure 6. **Elevated antigen-dependent bronchial hyperreactivity in SPC.OVA X DO11.10 mice with limited type 2 immune responses depending on the antigen type.**

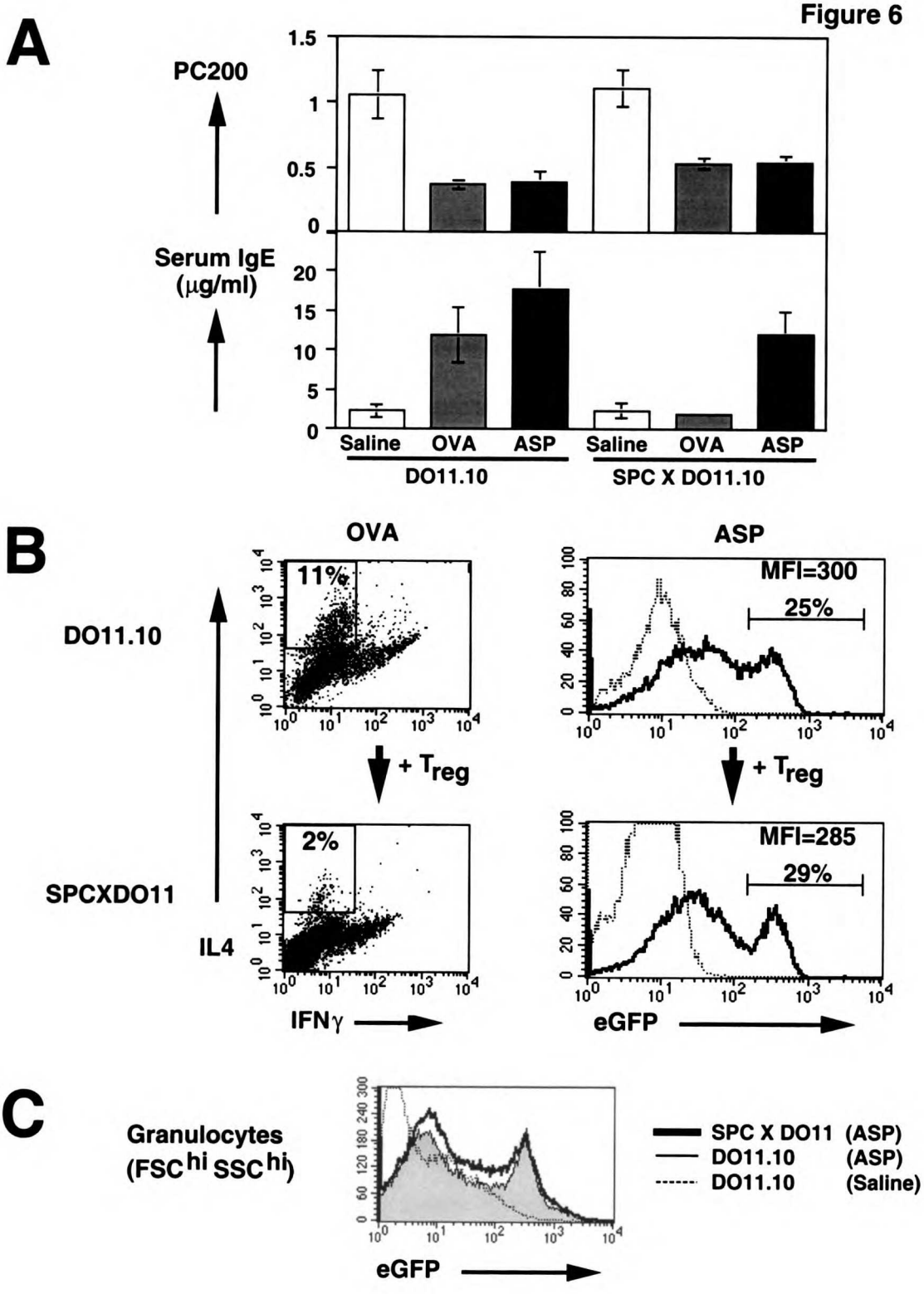
Groups of four to six DO11.10 and SPC.OVA X DO11.10 mice were challenged intranasally with PBS (saline), OVA or Aspergillus antigens (ASP).

A. PC_{200} in response to escalating doses of acetylcholine (top) and serum IgE levels as assessed by ELISA (bottom).

B. Lung cell suspensions were either stimulated with PMA/Ionomycin for 4 hrs and analyzed for intracellular IL-4 and IFNγ, or analyzed directly ex-vivo for GFP fluorescence from mice on the 4-get background. Percentages of IL-4+/GFP+ cells in the lymphocyte gate are shown. MFI of GFP denotes the mean fluorescence intensity of the GFP signal.

C. eGFP fluorescence from lung cells in the granulocyte gate (FSC^{hi}, SSC^{lo}).

Figure 6


A
PC200
Serum IgE (μg/ml)
1.5
1
0.5
0
20
15
10
5
0
Saline
OVA
ASP
Saline
OVA
ASP
DO11.10
SPC X DO11.10
B
OVA
ASP
DO11.10
SPCXDO11
11%
MFI=300
25%
+ Treg
2%
MFI=285
29%
IL4
IFNγ
eGFP
C
Granulocytes (FSChi SSChi)
eGFP
SPC X DO11 (ASP)
DO11.10 (ASP)
DO11.10 (Saline)

antigen was able to induce airway hyperreactivity. However only in the OVA sensitized mice were type 2 immune responses such as IL-4- and serum IgE-production suppressed; mice that received the more potent Aspergillus extract demonstrated enhanced lung GFP and serum IgE-levels (figure 6). This difference in the type of antigens used reflects the fact that complex antigen mixtures such as Aspergillus, might recruit and activate more innate immune cells into the lungs than OVA. In fact, Aspergillus-treated mice present with profound inflammatory lesions predominantly composed of eosinophils (chapter 3). It has been proposed that recognition of unique molecular signatures on type 2 pathogens or allergens by pattern recognition receptors (PRR) on dendritic cells (DCs), could induce type 2 cytokines independent of the Toll-like receptor (TLR) pathway, and ultimately influence adaptive immunity [reviewed in (97)]. It is not clear whether these innate signals can modulate regulatory T cell activity. It seems unlikely, however, that the presence of regulatory T cells in the double transgenic mice modulates IL-4 production from innate cells due to comparable GFP levels in the granulocyte gate of single transgenic DO11.10 versus double transgenic SPC X DO11 mice (figure 6). Complex antigen mixtures such as Aspergillus may induce higher levels of type 2 cytokine production by DCs that could subsequently modulate regulatory T cell function by inhibiting their suppressive activity. This would then enhance an effector immune response to the antigen (figure 7). In the OVA system however, most of the type 2 cytokine contribution might indeed come from the adaptive immune response which was held in check by regulatory T cells in the double transgenic mice.

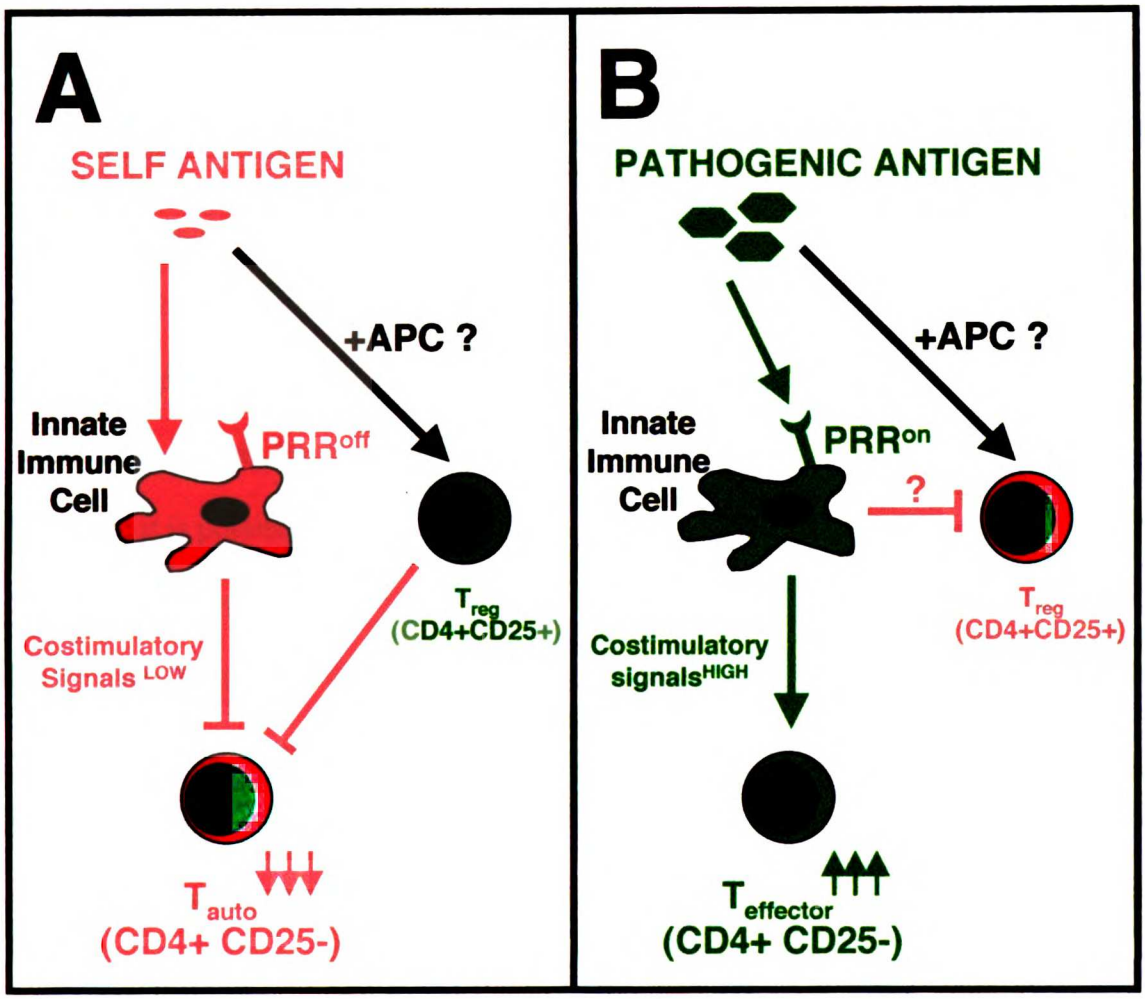
Figure 7. **Role of regulatory T cells in the mucosal response to antigens**

The airway mucosa has to distinguish between innocuous allergens/self proteins and pathogenic microbes.

A. Self antigens or innocuous airborne allergens, might be presented by immature DCs or alveolar macrophages in the absence of costimulatory ligands. Furthermore recruitment of regulatory T cells to the site of antigen exposure would keep any T cell activation in check.

B. Pathogenic microbes potently activate DCs, through their recognition by pattern recognition receptors (PRRs) expressed on the surface of the cells. The subsequent release of proinflammatory cytokines and expression of costimulatory ligands could inhibit regulatory T cell function and induce a potent T cell effector response.

Figure 7


A
SELF ANTIGEN
+APC ?
Innate
Immune
Cell
PRR^off
T_reg
(CD4+CD25+)
Costimulatory
Signals LOW
T_auto
(CD4+ CD25-)
B
PATHOGENIC ANTIGEN
+APC ?
Innate
Immune
Cell
PRR^on
?
T_reg
(CD4+CD25+)
Costimulatory
signals^HIGH
T_effector
(CD4+ CD25-)

Two key questions remain however:

Firstly, why is intranasal OVA administration necessary to induce AHR and low levels of IL4 production in double transgenic mice that already express OVA in the airways as a self-antigen?

Secondly how is AHR possible in the absence of significant type 2 immunity and airway pathology?

Firstly, the dose and route of antigen administration can play a key role in determining the outcome of an immune response. Resident airway APCs for example, display different properties. It has been shown that resident alveolar macrophages are immunosuppressive rather than stimulatory for T cell responses (98). They also have the potential to inhibit the antigen presenting capacity of pulmonary dendritic cells (99). Therefore it is possible to envision that antigen-presentation in the transgenic mice, might be favored by resident alveolar macrophages due to the high OVA protein levels in BAL. On the other hand, if the OVA is supplied as an immunogen intranasally, pulmonary DCs might be more important in presenting OVA to T cells. Pulmonary DCs have already been shown to favor type 2 immune responses (94). The subsequent Th2 immune response might then be suppressed by CD4+ CD25+ regulatory cells that have migrated into the lung where the cognate antigen is expressed. Indeed, inhaled exposure to antigen alone without prior priming induced T cell unresponsiveness (73) or T-cell tolerance due to the recruitment of regulatory T cells (71). This could be a potential mechanism by which the airway mucosa might distinguish between innocuous allergens and microbial pathogens (figure 7). Mucosal allergens for example, might be presented by immature DCs in the absence of costimulatory signals. Indeed, interstitial DCs from the lung have

been shown to have an immature phenotype (100). Alveolar macrophages and B cells isolated from the lung have also been shown to have poor antigen presenting capabilities (98, 101). Furthermore, recruitment of regulatory T cells to the site of antigen exposure would keep the possibility of T cell activation in check. In asthmatics this tolerance network might be impaired due to environmental and/or genetic factors. A pathogenic microbe, on the other hand, can potently activate DCs through their recognition by pattern recognition receptors (PRRs) expressed on DCs. The subsequent release of proinflammatory cytokines and expression of costimulatory ligands might inhibit regulatory T cell function and induce a potent T cell effector response.

Finally bronchial reactivity is maintained in mice with suppressed immune responses possibly due to low level inflammation, mucus production and tissue eosinophilia that is above a threshold required to induce airway hyperreactivity. Alternatively, there could have been a transient increase in type 2 cytokines produced by non-T cells, such as pulmonary DCs , eosinophils or airway epithelia that conditioned the airway smooth muscle and rendered it more hyperreactive to bronchoconstricting agents. Therefore, the regulatory capacity of CD4+ CD25+ T cells may be limited in chronic complex disorders, allowing substantial contributions by non-T cells.

FUTURE DIRECTIONS

An unanswered question in Th2 development remains the source of the initial IL-4 burst that is required for Th2 differentiation in vivo. Although mast cells and basophils can generate IL-4, this generally requires cross-linking of FcεIg receptors by IgE (102), and the latter would not be present during the initial encounter with a foreign antigen. CD4+ T cells stimulated in vitro do not generate sufficient amounts of IL-4 for Th2 differentiation (103). However, transfer of highly purified CD4+ T cells into IL-4-deficient mice that were challenged with a Th2-inducing nematode, *Nippostrongylus braziliensis*, demonstrated that IL-4 from CD4+ T cells themselves was sufficient to drive Th2-like differentiation in vivo (104). Recent data have excluded the possibility that a novel lineage of CD4+ T cells that express a highly restricted αβ TCR and NK-like markers, including NKRP-1 and Ly-49, can provide the initial burst of IL-4 during Th2 development (105, 106). Using adoptive transfers of 4-get CD4+ T cells into immunodeficient rag-/- recipients rendered deficient in IL-4 and IL-13 (107), the requirements of different wild type populations of innate cells can be addressed by adoptive co-transfer. Sensitization with antigen would then permit the readout of the transferred CD4+ T cell population.

The advantages of experiments in TCR transgenic mice include the ability to access relatively large numbers of antigen-specific cells, to isolate responses to discrete peptides, to generate larger quantitative airway responses due to artificial expansion of antigen-reactive T cells, to critically implicate discrete cytokines due to the availability of congenic knock-out strains, and to evaluate potential interventional/vaccine strategies that

might redirect T cell development and hence abrogate the pathologic airway response. While the OVA system described can accomplish some of these aims, these experiments will lay the groundwork for establishing a more relevant TCR transgenic mouse model important for the understanding of human airway disease. This can be achieved by generating TCR transgenic mice specific for known human allergens associated with asthma. TCR transgenic mice expressing T cell receptors specific for epitopes from the dust mite, Dermatophagoides pteronyssius (Der p I) implicated in human asthma for example, can be prepared from Der p I-specific Th2 clones. These can be established from the draining lymph node cells of mice immunized with the allergen. Clones can be tested in transfer experiments, to identify clones that confer airway reactivity to the inhaled cognate antigen. Evidence for possible immunodominance can be demonstrated through the use of common TCR variable region segments. A representative immunodominant T cell clone capable of transferring the airway response to naive mice can then be used as the donor for genomic transgenes using standard TCR cassettes developed for generating TCR transgenic mice. Such mice would be invaluable in testing vaccine approaches based on our earlier experiments in a system that would be specific for known human airway-provoking antigens.

CONCLUSION

In conclusion, the work presented in this thesis suggests that airway responses to inhaled allergens is a regulated process. First, tolerance must be maintained in the airway mucosa to innocuous airborne allergens. This is felt to be under the suppressive control of regulatory T cells and/or unprimed naïve APCs such as DCs or even alveolar macrophages that lack costimulatory ligands. Upon breakdown of tolerance, due to genetic or environmental factors, effector T cells are released from this checkpoint and develop, under the influence of the mucosal milieu, into Th2 cells. Th2 cells, through the elaboration of type 2 cytokines are able to orchestrate the cellular and molecular mediators required to induce the disease state, i.e., the pathology and the physiologic response characterized by elevated airway hyperreactivity to bronchoconstricting agents. These include experimental agents such as cholinergic neurotransmitters; however in the physiologic context they could include environmental agents such as cold air, a typical symptom of full-blown asthma. Modifier genes, independent of type 2 immunity to allergens, might be an additional genetic requirement to bypass a certain threshold required to enter the symptomatic stage. Out of all the effector pathways, it is most evident that the IL-4/IL-13 pathway is necessary and sufficient to mediate disease. Murine models of airway sensitization to antigen have enabled us to understand the pathogenesis of asthma. Interventions using recombinant molecules and studies in gene knock-out mice have provided us with powerful and direct methods to examine the roles of designated effector pathways. Finally, a controlled antigen-specific model offers the

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