NOTE TO USERS

INVESTIGATION OF IMMUNOMODULATORY CONCEPTS IN A MURINE MODEL OF AIRWAY MUCOSAL SENSITIZATION TO INNOCUOUS ANTIGEN

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

IMMUNOMODULATION OF AIRWAY MUCOSAL SENSITIZATION

DOCTOR OF PHILOSOPHY (2004) McMaster University
(Medical Sciences) Hamilton, Ontario

TITLE: Investigation of Immunomodulatory Concepts in a Murine Model of Airway Mucosal Sensitization to Innocuous Antigen

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NUMBER OF PAGES: xii, 123

—ABSTRACT—

The epidemic rise in the prevalence of allergy and asthma, primarily in the "developed" world, has impelled prolific nodes of inquiry into the epidemiology, ætiology, immunology and management of these syndromes. While studies in human patients have revolutionized pharmacological treatment of asthma and allergy, and have also intimated some of the environmental agents and conditions conducive to disease expression in susceptible populations, only animal models of asthma afford the experimental flexibility upon which detailed *in vivo* analysis of immunology and pathogenesis depends. Because asthma arises through airway mucosal contact with allergens, chemical pollutants and/or infectious agents, an authentic animal model of asthma should preserve the airway as the interface of incipient contact with antigen and, by extension, as the immune microenvironment that conditions allergic sensitization. This heuristic is particularly relevant when considering questions about the immunomodulatory effects of local, anti-inflammatory intervention. The research documented in this thesis investigates several immunomodulatory concepts—including pharmacological intervention (Chapter 2), costimulatory molecule blockade (Chapter 3) and chemokinetic manipulation of cell trafficking (Chapters 4 and 5)—in a murine model of airway mucosal sensitization to an innocuous antigen. The salient message informed by these studies is that the outcome of an immune-inflammatory response is very much a reflection of the airway microenvironment in which the immune system initially processes antigen. Of substantive clinical interest, these data indicate that the efficacy of acute, therapeutic intervention must be reconciled with the status of the antigen-specific response once treatment has ceased.

—ACKNOWLEDGEMENTS—

I wish to thank the following, inevitably incomplete, list of people whose invaluable contribution to this work—and whose participation in my life—mean more to me than my words or actions can possibly convey.

My supervisor, Manel	*for being a mentor, a father, a friend*
My supervisory committee, Jack, Ken and Zhou	*for their wisdom, their gentle nudges and their support*
Our laboratory technicians, Susanna, Joanna, Tina, Meghan and Monika	*for their skill, their inscrutable standards and their extraordinary patience*
Our secretary, Mary	*for her ability to do just about everything without objection or delay*
My laboratory colleagues, Stacey, Beata, Fil, Liz, Ramzi, Mackenzie, Tree, Jill, Clint and Martin	*for their friendship, their intellectual vigour, their helpful pairs of hands and their joie de vivre*
My students, too numerous to name	*for challenging me constantly*
My parents, brother and grandmother, Deanna and Jack, Matthew and Nanny	*for loving me, unconditionally*
My partner, Brian	*for entering my life, my heart and my future*

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—List of Abbreviations—

Ag	antigen
Ab	antibody
Ad	adenovirus
AHR	airway hyperresponsiveness
APC	antigen-presenting cell
ASO	antisense oligonucleotide
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CS	corticosteroid
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	dendritic cell
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FEV1	forced expiratory volume in 1 second
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
HBSS	Hank's balanced salt solution
HDM	house dust mite
i.n.	intranasal
i.p.	intraperitoneal
ICOS	inducible costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	IFNγ-inducible protein 10
KO	knock-out
MHC	major histocompatibility complex
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PFU	plaque-forming unit
RDA	replication-deficient adenovirus
SEM	standard error of the mean
TcR	T cell receptor
Th	T helper
TNF	tumour necrosis factor

—PREFACE—

The research documented in Chapters 2 to 5 of this "sandwich" thesis is presented as four independent but thematically related articles that, as of July 2004, are in print or awaiting submission. Like most scholarly work in the health sciences, this research has required a collaborative effort, to which the multiple authors listed in each article attest. However, in each case, I, as the author of this doctoral thesis, provided the intellectual leadership (establishing research objectives, designing experiments, interpreting results) and laboratory co-ordination (mobilizing and organizing resources, executing experiments) to carry the work to a fruitful conclusion. Moreover, each of these articles is an original document that I have written in its entirety.

CHAPTER 2 Wiley RE, M Cwiartka, D Alvarez, DC Mackenzie, JR Johnson, S Goncharova, L Lundblad and M Jordana. 2004. Transient corticosteroid treatment permanently amplifies the Th2 response in a murine model of asthma. *J Immunol* 172:4995-5005.

This work was inspired by an intriguing, but very preliminary, observation (namely, heightened OVA-specific IgE production in mice treated with corticosteroids during mucosal allergic sensitization) recorded in 2000 by M. Cwiartka, at the time a technician in our laboratory, in the context of a contract with AstraZeneca Canada. From the winter of 2002 to the fall of 2003, I repeated and extended the original observation, and I designed, initiated and assembled the long-term *in vivo* studies and the extensive cellular analysis that form the crux of this article. D. Alvarez, a PhD student in our laboratory, is responsible for the assessment of airway physiology, L. Lundblad provided technical expertise with the drug

nebulizer, and S. Goncharova, a technician in our lab, assisted with cytokine detection by ELISA. D. C. Mackenzie and J. R. Johnson were undergraduate students who worked on this project under my supervision.

CHAPTER 3 Wiley RE, TM Shea, JR Johnson, S Goncharova, AJ Coyle and M Jordana. 2003. Examination of ICOS/B7RP-1 as a therapeutic target in a murine model of allergic airway inflammation. *Am J Respir Cell Mol Biol* 28:722-730.

This study was completed between the fall of 2001 and the fall of 2002, and was made possible by the availability of an anti-ICOS neutralizing antibody furnished by A. J. Coyle at Millennium Pharmaceuticals, Inc. I designed, executed and analyzed the results of all experiments. S. Goncharova provided technical support, and T. M. Shea and J. R. Johnson were undergraduate students who worked on this project under my supervision.

CHAPTER 4 Wiley RE, K Palmer, BU Gajewska, MR Stämpfli, D Alvarez, AJ Coyle, JC Gutierrez-Ramos and M Jordana. 2001. Expression of the Th1 chemokine IFNγ-inducible protein 10 in the airway alters mucosal allergic sensitization in mice. *J Immunol* 166:2750-2759.

This study, which was completed between the fall of 1998 and the spring of 2000, employs an IP-10-expressing adenoviral vector prepared by K. Palmer. B. U. Gajewska and D. Alvarez provided technical support with lung cell isolation and flow cytometry; M. R. Stämpfli, A. J. Coyle and J. C. Gutierrez-Ramos critically appraised the original manuscript and offered invaluable advice as I led this, my first independent research project.

CHAPTER 5 Wiley RE, PC Emtage, DC Mackenzie, D Oppedisano, M Hitt, J Gauldie and M Jordana. 2004. Expression of the chemokine CCL20/MIP-3α in the mouse airway expands antigen-presenting cells and facilitates mucosal sensitization to aerosolized OVA. Manuscript to be submitted to *Am J Resp Cell Mol Biol*, May 2004.

I initiated, managed and completed this study between winter 2002 and fall 2003 with the instrumental aid of the CCL20/MIP-3α adenoviral vector prepared in 1995 by P. C. Emtage, at the time a student in the laboratory of J. Gauldie. D. C. Mackenzie and D. Oppedisano were undergraduate students who worked on this project under my supervision, and M. Hitt obtained sequencing information that confirmed the identity of the transgene in the original vector.

—CHAPTER 1—

Introduction

Context

The dramatic rise in asthma and allergy prevalence, particularly in so-called developed nations in North America, Europe and southeast Asia, has impelled prolific nodes of inquiry into the ætiology, pathology and immunology of these syndromes (reviewed in [1]). In humans, this research has been predicated on two primary objectives: to identify and appraise prospective therapeutic targets that promise specific, yet robust, control of the local inflammation believed to underly the asthma diathesis; and, more recently, to understand the environmental variables that might establish biological conditions conducive to allergic sensitization. These clinical investigations, in turn, have substantially advanced our understanding of the key mediators of allergic inflammation (reviewed in [2]); have led to the development of new asthma drugs, such as leukotriene (reviewed in [3]) and IgE inhibitors (reviewed in [4]); have permitted us to draw epidemiological associations between asthma prevalence and the ultra-hygienic Western lifestyle (reviewed in [5-7]); and have begun to describe how the airway epithelium might be the key immunological interface arbitrating between protective (tolerogenic) and pathologic (allergic) responses (reviewed in [8]).

Of course, in human studies, mechanistic analysis is generally restricted to *ex vivo* manipulation of the limited variety of specimens that can reasonably be harvested from patients. This approach has proven most invaluable in the precise definition of cell/tissue phenotype and in the testing of novel biological concepts *in vitro*. Where analysis of human specimens unavoidably falls short, however, is in the integration of discrete observations about isolated tissues into the irreducible complexity of the disease process *in vivo*. Systematic evaluation of biological relevance—the dispensation of *in vivo veritas*—requires the experimental rigour and license afforded by animal models of asthma. Not only do animal

models permit a process-oriented appraisal of biological mechanism through molecular interventions that would be unthinkable in humans, but—and herein lies a source of both strength and abstraction—they also allow researchers to reproduce, reliably and precisely, features of the human condition to the exclusion of confounding factors. Unlike clinical research, where the object of study determines the scope of inquiry, animal models are created and manipulated according to the objectives of the research question.

The information an animal model of asthma can provide is therefore very much a reflection of its design, which itself is a reflection of both the research motive and the biological/immunological assumptions that inform it. Assessment of the role of a given molecule in allergic sensitization, for instance, imposes a very different set of experimental challenges and modeling parameters from those that apply to the evaluation of a novel intervention's anti-inflammatory properties; the former raises questions about the immunological conditions that best recapitulate sensitizing phenomena in humans, the latter about the kinetics and route of intervention that authentically parallel therapy in humans with established disease. Operationally, it is incumbent on an investigator to select a model apropos of the clinical paradigm under investigation—and to accept the interpretive limitations that choice entails. The research described in this document models asthma with a view to comparing interventions both during the process of sensitization and upon antigen challenge, *i.e.* in antigen-exposed animals (mice) with previous antigen experience. Although a broad range of immunomodulatory strategies is explored, each is rooted in a shared conceptualization of asthma immunology that dictates the inextricable features of the modeling instrument and the requirements of the biological analysis.

Experimental Modeling of Asthma

It seems almost tautological to say that the objective of any animal model is to reproduce key elements of the human disease. However, this objective is straightforward only insofar as researchers can agree on a standard, coherent definition of the pathology being investigated. Experimental simulation of asthma has deferred to a well-established clinical description of this syndrome as a *chronic inflammatory process* with attendant airway dysfunction, including *reversible airflow obstruction* and *airway hyperresponsiveness* (AHR). The relevance of this definition is reinforced by the therapeutic success of the two pharmaceuticals most widely prescribed for the management of asthma symptoms: corticosteroids, which arrest inflammatory processes that cause obstruction of the airway with mucous and cells; and bronchodilators, which desensitize smooth muscle fibres and relax constricted small airways. However, for the purpose of reproducing this syndrome in mice—a species that does not spontaneously develop asthma—scientists have generally focused their attention on the quintessential *immune-inflammatory phenotype* in human asthmatics. Characterized by overproduction of cardinal T helper (Th) 2 mediators (such as IL-4, IL-5 and eotaxin), accumulation of eosinophils in the lung, and circulation of IgE specific for any of a spectrum of aeroallergens (including pollens, animal danders and house dust mite), asthma is broadly understood as an *antigen-initiated allergic process*; the phenomenon that most animal models mimic, therefore, is best described as *allergic asthma* (reviewed in [9,10]).

CONVENTIONAL MODELS OF ASTHMA. Building on this conceptualization, virtually all murine models of asthma privilege allergic sensitization as the essential precursor to asthmatic airway inflammation, though these models can differ substantially in the identity of

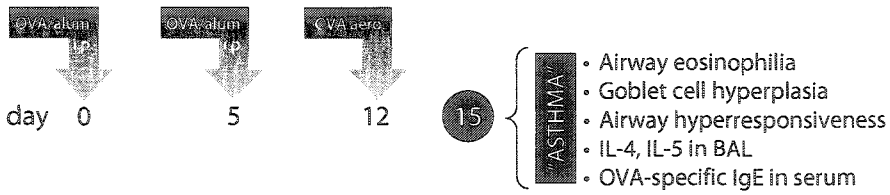

FIGURE 1. *A conventional model of antigen-induced allergic airway inflammation.* Mice are sensitized to OVA via 2 consecutive intraperitoneal (i.p.) injections of alum-adsorbed OVA on days 0 and 5. Airway responses are evoked by OVA aerosol challenge on day 12. By day 15, mice exhibit robust airway eosinophilia, airway physiological responses and elevated OVA-specific IgE in serum.

the antigen and the route of sensitization (considerations upon which sections that follow will elaborate). However, with very few exceptions, most investigators have relied on some variation of a standard model of antigen-induced allergic airway inflammation, henceforth referred to as the *conventional* model of asthma, which involves one or more intraperitoneal (i.p.) injections of aluminum hydroxide (alum)-adsorbed surrogate allergen, usually ovalbumin (OVA), followed 7 to 10 days later by an aerosol or intranasal (i.n.) antigen challenge (Figure 1) (see [11] for an example). The strength of this model lies in its faithful reproduction of some of the salient cellular, humoral and physiological outcomes of allergic airway disease. Moreover, that antigen sensitization and challenge are anatomically separate and occur at temporally discrete phases in the protocol affords the fine resolution of the cellular and molecular processes specific to allergic airway inflammation in mice with an established hypersensitivity. As a consequence, conventional models of asthma are superb instruments to evaluate the impact of a given therapeutic intervention on local inflammatory responses.

However, because conventional models have been designed to recreate fundamental elements of asthma *pathology*, they afford little insight into asthma *ætiology* or *pathogenesis*

(discussed in [9]). Indeed, injection of OVA into the peritoneum in the context of a potent chemical adjuvant bears little resemblance to the physiological route of sensitization in humans, which presumably occurs via allergen contact with the airway mucosa. From the perspective of human biology, the trafficking of immune cells in these models is awkward. During sensitization, OVA-loaded, alum-activated dendritic cells track from the peritoneum to the thoracic lymph nodes where they engage naïve T cells, while the lung—the organ of interest—remains hermetically excluded from antigen experience. According to this scheme, *bona fide* understanding of asthma is restricted to its manifestation as a local, airway inflammatory disorder: to events downstream of sensitization. The artificial, geographically distant inciting conditions preclude meaningful examination of the lung at incipient stages of pathogenesis. The particular immunology of the airway—its native response to the aeroallergen—is rendered moot by a pre-emptive, robust and remote sensitizing regimen.

INHALATION TOLERANCE. An awareness of the clinical paradigm that conventional models of asthma simulate is particularly important when one considers the outcome of passive exposure of the airway mucosa to aerosolized OVA: inhalation tolerance. A weak immunogen, OVA elicits an antigen-specific, anti-inflammatory, regulatory T cell response when delivered to the airway in the absence of an adjuvant; this response is persistent, circumventing the development of allergic inflammation upon subsequent administration of OVA in the context of appropriate sensitizing conditions (*e.g.* OVA/alum via the peritoneum) [12] (and reviewed in [13]).

Applying the logic of evolutionary adaptation, the airway's intrinsic tolerance of innocuous airborne proteins makes sense. The act of breathing, though biologically unassailable, is fraught with peril. A breath of air introduces to the lung not only life-

sustaining oxygen, but also myriad bacteria, viruses and foreign proteins, each of which disrupts the delicate immunologic homeostasis of the respiratory mucosa. This basic quandary, which complicates any biological interface, informs the inextricable challenge facing the lung: to evolve immune-inflammatory responses that effectively eliminate potentially dangerous interlopers while minimizing the attendant impairment of the lung's mechanical and physiological functions. If the immunological conundrum faced by the lung is *whether to mount an inflammatory response*, and if the biological compromise is to tolerate anything that is not accompanied by an unambiguous indication of danger, then the challenge for researchers wishing to understand allergic sensitization is to develop a model of airway antigen exposure that subverts this default pathway—to identify what factors render an innocuous aeroallergen *dangerous*.

MODEL OF MUCOSAL ALLERGIC SENSITIZATION. Our laboratory has shown that adenovirus (Ad)-mediated gene transfer of granulocyte-macrophage colony-stimulating factor (GM-CSF) to the airway at the time of OVA aerosol exposure subverts inhalation tolerance and permits the articulation of a Th2-polarized immune-inflammatory response [14]. Airway eosinophilia, the production of IL-4, IL-5 and OVA-specific IgE, and airway hyperresponsiveness are cardinal features of this allergic response (Figure 2); moreover,

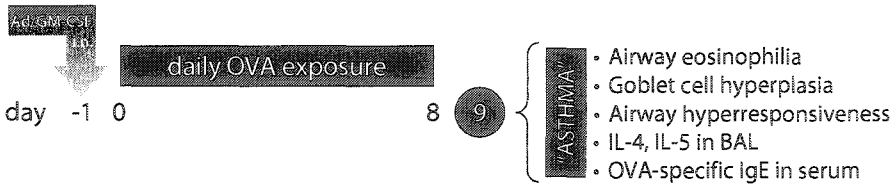

FIGURE 2. *Model of mucosal allergic sensitization to aerosolized OVA.* Mice are sensitized to OVA via daily exposure to OVA aerosol in the context of a GM-CSF-conditioned airway microenviroment. By day 9, mice exhibit robust airway eosinophilia, airway physiological responses and elevated OVA-specific IgE in serum.

despite compartmentalized OVA exposure, sensitization in this model is systemic as cultured splenocytes liberate Th2 cytokines upon stimulation with OVA. It is likely that GM-CSF mediates mucosal allergic sensitization to OVA by altering the profile of pulmonary antigen-presenting cells (APC) [15], which undergo robust expansion upon Ad/GM-CSF delivery and, perhaps most importantly, express elevated levels of the costimulatory molecules B7.1 and B7.2. It would seem, then, that it is the context in which antigen is presented—and not simply the intrinsic properties of the antigen *per se* or the peculiar predisposition of the immune system—that ultimately negotiates between tolerogenic and pathologic sensitization.

This murine asthma model involving repeated exposure to aerosolized OVA in the context of a GM-CSF-conditioned airway milieu (designated the *mucosal Th2 model*) represents a prototype system in which both sensitization and challenge are initiated locally and contiguously. Because it entails physiologically relevant trafficking of immune cells from the airway to secondary lymphoid organs (Figure 3), the mucosal Th2 model is a tractable instrument to consider questions about allergic sensitization and the temporal/spatial evolution of allergic airway disease. Moreover, the selection of GM-CSF as the agent driving

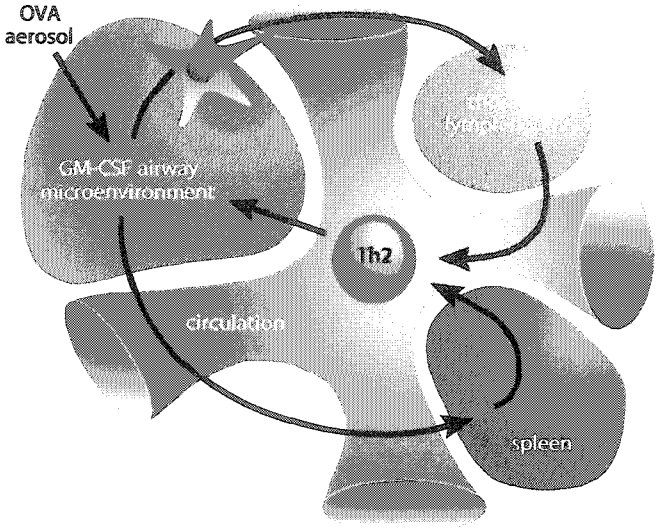

FIGURE 3. *Immune geography of the mucosal Th2 model.* GM-CSF-activated dendritic cells convey OVA from the airway to secondary lymphoid organs, where Th2 cells differentiate before returning to the lung and initiating an airway inflammatory (eosinophilic) response.

sensitization may be ætiologically apt (reviewed in [16]). GM-CSF is elevated in the lungs of asthmatics [17,18], and is secreted by airway epithelial cells [19,20] and alveolar macrophages [21] upon contact with a variety of airborne particles putatively involved allergic sensitization, including diesel exhaust [22] and proteolytic aeroallergens (*e.g.* HDM) [23,24]. From the perspective of clinical symmetry, this mucosal model of allergic sensitization and airway inflammation more closely emulates the immunology of these processes in human asthmatics, and creates opportunities to investigate questions about immunomodulation that conventional models of asthma could answer only provisionally.

THREE CLINICAL PARADIGMS. Approximately 4 weeks after the cessation of the initial period of daily OVA exposures (*i.e.* in the context of exogenous GM-CSF expression), inflammatory processes in the airway have resolved completely; subsequent rechallenge of the airway with aerosolized OVA elicits an anamnestic Th2 (eosinophilic) response in the lung and heightened IgE production systemically [14]. This protracted recall protocol ensures a decisive separation between processes peculiar to mucosal sensitization or airway inflammation, thereby differentiating between interventions whose effects are acute *vs.* long-term, local *vs.* systemic, immune-deviating *vs.* inflammation-regulating. The flexibility of the mucosal Th2 model distinguishes it from conventional models of asthma, in which aerosol challenge of mice with established disease occurs immediately following sensitization and coincides with the airway's initial contact with antigen. In particular, the mucosal Th2 model permits a comprehensive analysis of three clinically meaningful immunomodulatory paradigms: (*i*) acute effect of treatment during initial antigen exposure; (*ii*) effect of intervention during secondary antigen rechallenge of mice with established disease; and (*iii*)

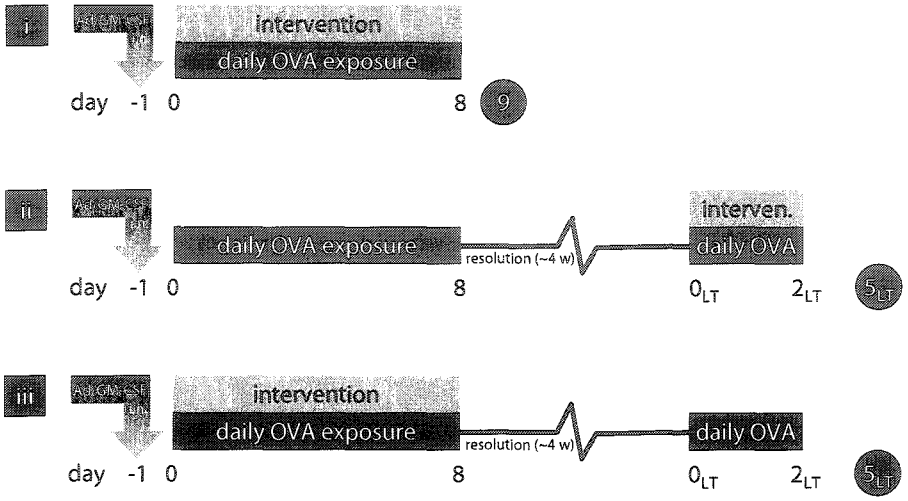

FIGURE 4. *Models of allergic airway inflammation and immunomodulation.* (*i*) Model of acute intervention: Mice are exposed daily to OVA in the context of a GM-CSF-conditioned airway microenvironment with concurrent intervention. (*ii*) Model of long-term therapeutic intervention: Mice are sensitized to OVA in the absence of treatment; inflammation is permitted to resolve (~4 weeks) before mice are rechallenged with OVA while receiving immunomodulatory treatment. (*iii*) Model of the long-term effects of acute intervention: Mice are sensitized to OVA while being treated; mice are rechallenged with OVA in the absence of further treatment. Numbers in circles indicate time-points at which outcomes are measured; LT, long-term.

effect of treatment during sensitization on long-term antigen-specific recall responses (Figure 4).

The varied immunomodulatory concepts explored in the chapters that follow illustrate how this view of asthma as a locally-initiated, systemically-maintained and evolving process—as embodied in the mucosal Th2 model—can enrich our understanding of the immune-inflammatory outcomes of a given intervention. For example, we document how a treatment such as corticosteroids, whose primary service is broadly classified as the local control of inflammation, can in fact elaborate sustained amplification of the systemic Th2 response if administered to mice early in the pathogenesis of experimental asthma. This important immunological outcome, which might otherwise have been overlooked in a conventional model driven primarily by the recreation and modulation of asthma

inflammation, was detected by giving due consideration to the kinetics of intervention and to the possible discontinuities between acute and long-term, local and systemic effects. This rigorous approach speaks to one of the key assumptions informing the mucosal Th2 model: that immunological *context* critically determines immunological *outcome*. We have previously shown that concurrent expression of Ad-derived IL-10 [25] or IL-12 [26] during GM-CSF-driven sensitization to aerosolized OVA reorients the evolving immune response toward antigen tolerance or Th1-associated pathology, respectively. With this in mind, we sought to understand not only their capacity to modulate inflammation, but also whether the immunomodulatory strategies appraised in this document could effectively modify the context in which antigen was received by the airway and interpreted by the immune apparatus.

LIMITATIONS OF THE MUCOSAL MODEL. It is important to recognize that the mucosal Th2 model, while furnishing us with a tractable instrument to investigate the immunology of allergic sensitization and asthma pathogenesis, does not in fact recreate in mice the syndrome that besets human patients. Human asthma is a chronic condition: *i.e.* it is a process of sustained airway inflammation, injury and remodeling that, though arrested or forestalled by appropriate pharmacological intervention, arises and deepens over a period of years (reviewed in [27]). The experimental model of asthma we employ in our studies, on the other hand, is decidedly acute. What we call "asthma"—eosinophilic airway inflammation and AHR—is established in mice after about 10 days of daily OVA exposure and disappears completely within weeks of exposure cessation (though airway inflammation can be recapitulated upon *in vivo* antigen recall); there is no evidence of airway remodeling, no evidence that the pathology we induce is self-perpetuating or chronic. Certainly, there are

sound experimental and logistical reasons to substantiate a compressed protocol as a satisfactory scientific compromise. However, equally important are the limitations imposed by OVA as a surrogate allergen. Even when sensitization to aerosolized OVA occurs in the context of a GM-CSF-conditioned airway microenvironment, persistent exposure to OVA over a period of weeks ultimately substitutes established Th2 immunity with active, sustained, antigen-specific tolerance [28]. In other words, continuous exposure to OVA actually precludes any effort to model chronicity.

Additionally, the use of an adenoviral construct to transfer GM-CSF to the airway introduces an irrevocable experimental artifact. Although this technique offers the advantage of sustained but transient expression of GM-CSF during the mucosal sensitization protocol—and although use of prohibitively expensive recombinant GM-CSF protein does achieve the same immune-inflammatory outcome—the presence of viral antigens and the fact that allergic sensitization depends on an *exogenous* source of GM-CSF render the system somewhat artificial. Artificiality, however, is any model's irrepressible albatross. What we have gained in terms of developing a clinically-relevant model of pure mucosal sensitization, we have sacrificed somewhat in terms of a tactical need to manipulate expediently the airway microenvironment. We take comfort in the fact that both GM-CSF and viral infection (reviewed in [29] and [30]) have been ætiologically linked to asthma pathogenesis in humans. However, it is becoming apparent that the most promising route to improve this model of mucosal Th2 sensitization is to change the inciting antigen. Indeed, recent data from our lab suggest that acute exposure to the proteolytic allergen HDM forsakes the requirement for exogenous GM-CSF, and that chronic exposure can mimic the long-term airway inflammation and remodeling that typify human asthma [31].

These caveats notwithstanding, the mucosal Th2 model has provided a supple platform to investigate diverse immunomodulatory concepts and appraise their biological effects from several inquiry trajectories: acute *vs.* long-term, local *vs.* systemic, immune *vs.* inflammatory. The work detailed in the body of this document, which covers topics in anti-inflammatory pharmacology, costimulation and chemokine biology, reinforces the notion that the airway microenvironment is a critical arbiter of the immune-inflammatory response that evolves when antigens come into contact with the lung mucosa.

Immunomodulation of Mucosal Sensitization

It is perhaps germane to begin a discussion of immunomodulation in experimental asthma by reflecting on what the term *immunomodulation* conveys. If we understand the *immuno* prefix to denote all aspects of immune-inflammatory responses, from incipient stages of T cell differentiation to the downstream expression of leukocytes' effector function, then immunomodulation can be understood broadly as the deliberate alteration of any of these processes' normal or default progression. According to this definition, a treatment that controls inflammation in the target organ while sparing the established systemic immune response is as immunomodulatory as a therapy that induces, say, a permanent shift in the phenotype of memory T cells from Th2 to Th1 or T_{reg}. However, that these two phenomena are categorically and conceptually distinct recommends a more precise definition of immunomodulation that differentiates *immunity* from *inflammation*. Immunomodulation, then, will be used to refer to interventions that substantively alter T cell memory, either by modifying the context in which naïve T cells are activated and differentiate, or by permanently altering the phenotype of established memory responses.

PHARMACOLOGICAL INTERVENTION: CORTICOSTEROIDS. Given the diverse array of potential immunomodulatory strategies that could be conceived in a mouse model of asthma, a sound experimental design and incisive appraisal critically depend on the clarity of the paradigm being investigated—on decisions about the experimental frame of reference. If, for instance, the objective of immunomodulation is therapeutic reversal of key features of the allergic response, then an understanding of the model's interaction with conventional therapy is a useful place to start. Indeed, as one of the indices of an animal model's utility is how authentically it reproduces human disease, evaluating an experimental animal's responsiveness to clinically-proven therapy can provide instructive validation of the model's practical relevance. Translating this principle to asthma, a clinically faithful model is one whose airway inflammation and attendant airway dysfunction are ameliorated by aerosolized corticosteroid (CS) delivery, which affords impressive efficacy in human patients. By inhibiting the activity of the proinflammatory transcription factor nuclear factor (NF)-κB (reviewed in [32]), inhaled CS effectively arrest the production of cytokines, chemokines and other mediators instrumental in the mobilization and effector function of airway inflammatory cells [33-35]. In essence, the pharmacological success of CS intervention resides in its *lack of specificity*—in its wholesale suspension of virtually *all* inflammatory processes, irrespective of their relationship to asthma. Novel treatments that aim to mitigate corticosteroids' indiscriminate management of inflammation by identifying robust, asthma-specific targets therefore face the daunting prospect of meeting the indomitable therapeutic standard set by corticosteroids' anti-inflammatory prowess. By extension, experimental investigation of prospective therapies in animal models logically requires the establishment of CS responsiveness as the optimal, clinically relevant control.

However, it is not simply in the provision of a meaningful therapeutic reference that CS merit study in experimental models of asthma. Although the anti-inflammatory function of CS is well documented and mechanistically understood, their effect on the immunology of an evolving inflammatory response remains an open question. For instance, the legions of asthma patients who remain dependent on inhaled CS throughout their lives have convinced researchers that CS do not cure asthma: *i.e.* that they do not modify the Th2-polarized immune response that underlies the symptoms CS so effectively suppress. While this would tend to classify CS as strictly anti-inflammatory rather than immunomodulatory drugs, other data suggest a more complex immunologic interaction. Of particular relevance to asthma, and somewhat paradoxically, CS have been shown to potentiate the polarization of Th2 cells [36-38] and to facilitate IgE isotype switching in B cells *in vitro* [39-42]; moreover, heightened serum IgE production in conjunction with selectively impaired Th1 cytokine secretion by cultured peripheral blood mononuclear cells (PBMC) has been observed in human atopics treated with steroids *in vivo* [43]. These data indicate that, under some circumstances, CS may amplify Th2-associated T and B cell responses, raising the possibility that CS exhibit immunomodulatory activity that transcends—and may in fact antagonize—their avowed anti-inflammatory efficacy in allergic airways disease.

In Chapter 1, we use the three basic immunomodulatory configurations of the mucosal Th2 model (detailed above) to investigate the short- and long-term, local and systemic effects of CS treatment. We confirm that CS treatment of mice with established disease (Model *ii* in Figure 4), consistent with clinical experience in humans, ameliorates OVA-induced lung inflammation and AHR; likewise, treatment during sensitization (Model *i*) ablates acute inflammatory processes in the airway. However, we also document a striking

discontinuity between the local and systemic effects of CS intervention during sensitization, furnishing evidence of an amplified Th2 response: OVA-specific IgE in serum and Th2 cytokine production by cultured splenocytes were significantly elevated in CS-treated mice. This effect was not transient, but reflected a permanent hyper-polarization of the OVA-specific immune response; although airway eosinophilia and AHR were not exacerbated in rechallenged mice originally introduced to OVA in the context of CS, splenocyte and IgE responses remained unusually high (Model *iii*). While the clinical relevance of this paradoxical finding is not immediately obvious—the capacity of CS to amplify Th2-polarized sensitization may not pertain to asthma/allergy patients who, by virtue of their need for CS treatment, are already sensitized—it certainly impinges on the issue of *neosensitization*, *i.e.* whether allergic sensitization is an ongoing, not an historically remote, process; it may also temper those clinical observations indicating *early* and *prolonged* use of CS to forestall the irreversible airway remodeling that attends asthmatic inflammation. Experimentally, however, the salient lesson from this study is clear: interventions designed to control local inflammatory phenomena may exhibit systemic immunomodulatory properties that are masked (or obviated) by the efficacy of treatment in the target organ.

REGULATION OF COSTIMULATORY PATHWAYS: ICOS. Our investigation of CS in the mucosal Th2 model establishes a sound framework for the evaluation of other immunomodulatory concepts *in vivo*. Among these, regulation of costimulatory pathways has been heralded as a therapeutic touchstone broadly relevant to all immune-inflammatory processes, since the seminal discovery that naïve T cells, in addition to cognate MHC:TcR interaction, require a second, nonspecific signal to become activated and assume an effector phenotype (reviewed in [44-51]). Much of this research has focused on modulation of the APC ligands CD80

(B7.1)/CD86 (B7.2) and the T cell-expressed activatory receptor CD28, as the strength of costimulation through this pathway critically arbitrates between effector T cell activation and T cell anergy or tolerance. However, because the T lymphocytes responsible for immune-inflammatory pathologies are, by definition, *already* effector-memory cells, the CD80/CD86:CD28 axis has proven redundant as a therapeutic target for established disease [52]; moreover, ostensibly therapeutic inhibition of this pathway can simultaneously and deleteriously suppress the elaboration of protective immune-inflammatory responses to infection. For this reason, attention has shifted to the recent identification of additional costimulatory pathways, whose apparent involvement in the expression of *established* T cell effector function affords more therapeutic promise. Of particular relevance to asthma and allergy, inducible costimulator (ICOS), a receptor expressed by T cells, and its APC ligand B7-related protein 1 (B7RP-1) have been implicated in the preferential differentiation and manifestation of Th2 phenomena [53-56].

A number of studies examining experimental allergic airways disease during ICOS neutralization or in ICOS-deficient mice generally agree that ICOS ligation is instrumentally involved in Th2-polarized eosinophilic inflammation and IgE secretion during allergen challenge [57,58]. Less evident, however, is whether interruption of ICOS signaling simply retards the expression of a pre-existing Th2 effector phenotype or, depending on the kinetics of ICOS ablation, can actually alter the differentiation of the Th2 response; indeed, most studies have restricted their analysis to acute immune-inflammatory phenomena during a compressed protocol of antigen sensitization and challenge. Additionally, the apparent anti-inflammatory efficacy of ICOS ablation in models of cardinal Th1-associated pathology [59], and its proposed involvement in the development of regulatory T cells [60], cast doubt on

the strict assignment of ICOS functionality to the Th2 pole. Therefore, while the association of ICOS with Th2 cytokine secretion has been amply documented *in vitro*, analysis of ICOS *in vivo* has begun to describe a costimulatory pathway whose activity may largely depend on (a) the nature of the immunologic stimulus and (b) the maturity of the effector-memory immune response.

The research presented in Chapter 2 of this document addresses some of these questions in the mucosal Th2 model. Corroborating other groups' findings, we show that systemic neutralization of ICOS during sensitization to OVA inhibited acute eosinophilic inflammation in the airway. Unexpectedly, however, local control of inflammation was accompanied by amplified Th2 cytokine secretion by cultured splenocytes—which translated to an enhanced airway inflammatory response and heightened IgE production upon long-term antigen rechallenge. Furthermore, whereas ICOS ablation protected the lung from eosinophilic inflammation during mucosal sensitization, targeting of ICOS during antigen recall in mice with established allergic disease conferred no therapeutic benefit. Therefore, paralleling the effects of CS intervention, we observe with anti-ICOS neutralization an apparent discordance between the local and systemic effects of treatment during mucosal allergic sensitization: notwithstanding the amelioration of airway eosinophilia acutely, hyperpolarization of the OVA-specific Th2 response was irreversible. However, unlike CS—whose anti-inflammatory efficacy prevails irrespective of the maturity of allergic airways disease—ICOS exhibits a transient functionality that appears redundant in an established effector Th2 response.

THERAPY: REGULATING INFLAMMATION *VS.* IMMUNITY. Therapeutically, CS intervention and inhibition of ICOS are conceptually similar. Notwithstanding their unexpected

immunomodulatory effects in the mucosal Th2 model, both CS treatment and disabling of ICOS primarily aim to regulate Th2 inflammation by suppressing T cell effector function: CS through the wholesale arrest of proinflammatory cytokine transcription, ICOS neutralization through the specific targeting of Th2 cells. Neither strategy purports to be a cure for allergic airway inflammation—*i.e.* to reverse, suppress or deviate *permanently* the Th2-polarized systemic immune response that is engaged upon subsequent, untreated contact with antigen. To this end, more deliberate immunomodulatory concepts have been proposed. Some, such as administration of the immunoregulatory cytokine IL-10 during antigen sensitization, subvert Th2 differentiation through the dominance of antigen-specific tolerance [25]; others, by overexpressing IL-12 [26], adjust the Th1-Th2 balance by superimposing Th1 phenomena on default Th2 immune-effector programming. While the clinical relevance of these experimental manipulations is tenuous, as their immunomodulatory objectives are restricted to the kinetics and conditions of allergic sensitization, they afford important insight into the microenvironmental factors that, presumably, modify APC activity and thereby regulate the phenotypic fate of T cell activation.

CHEMOKINE IMMUNOMODULATION: *(I)* IP-10. While the ability of cytokines to condition the immunologic milieu has been well documented, it is now becoming evident that a group of chemotactic cytokines, chemokines, plays a pivotal role in establishing the cellular constituents of the immune microenvironment—and that they may therefore represent new immunomodulatory instruments. Though chemokine activity can range from bactericidal [61] to angiogenic (reviewed in [62]), chemokines are best known for the formation of chemotactic gradients that orchestrate the trafficking of immune-inflammatory cells both homeostatically (*e.g.* maintaining the architecture of lymphoid tissue) [63] and at sites of

immunologic challenge (reviewed in [64]). Of particular interest from an immunomodulatory perspective is the apparent coupling of chemokine/chemokine receptor expression and T helper phenotype. The chemokines eotaxin and thymus-activated and regulated chemokine (TARC), for example, are typically upregulated during Th2 responses, and their receptors, CC chemokine receptor (CCR) 3 and CCR4, are generally expressed exclusively by eosinophils and Th2-polarized T cells. By comparison, macrophage inflammatory protein (MIP)-1α and IFNγ-inducible protein (IP)-10 are induced by Th1 signals, and their receptors, CCR5 and CXCR3, reliably identify macrophages and Th1 lymphocytes [65-70]. Conceptualizing this relationship in a useful, if simplified, biological model, the interplay between cytokines and chemokines programs a self-perpetuating, self-selecting cascade: cytokines and other stimuli in the tissue evince the secretion of chemokines that mobilize specialized, phenotypically-relevant cells, which in turn liberate mediators—including chemokines—that facilitate even more specialized leukocyte recruitment. From the perspective of immunomodulation, manipulation of the chemokine microenvironment might be a way to transform the inflammatory phenotype in the tissue and, by implication, to alter the course of an evolving immune response.

In Chapter 3, we examine this paradigm in the mucosal Th2 model. By locally administering an adenoviral vector encoding the transgene for IP-10 during GM-CSF-driven sensitization to OVA, we were able to alter dramatically the inflammatory infiltrate in BAL. Though total airway inflammation was unchanged by IP-10 intervention, eosinophilia and Th2 lymphocyte recruitment were attenuated, dominated instead by IFNγ-secreting $CD4^+$ T cells and an elevated CD8:CD4 T cell ratio. Of particular interest, this Th1-privileged airway

microenvironment established conditions conducive to Th1-polarized antigen sensitization, as evidenced by amplified OVA-specific IgG_{2a} production and the elicitation of largely mononuclear inflammation during long-term OVA rechallenge. Mechanistically, it is unclear whether the IP-10-mediated dampening of Th2 differentiation reflected the selective recruitment of Th1-predisposed APC or the indirect conditioning of a Th1-permissive airway microenvironment through the mobilization of IFNγ-secreting lymphocytes. However, this study does illustrate that chemokinetic modulation of local inflammatory conditions can substantively and irreversibly determine the outcome of systemic immunity.

CHEMOKINE IMMUNOMODULATION: *(II)* CCL20. While the Th1/Th2 phenotypic categories have been profitably used to conceptualize chemokine function, they are by no means an exhaustive construction of chemokine biology. Another conceptual framework derives from the spatio-temporal considerations of immune responses—from innate processes in the challenged organ to immune-adaptive genesis in the lymph nodes to effector-memory expression in inflamed tissue—which depend on the timely migration of leukocytes bearing the appropriate chemokine receptor profile. Chemokines can therefore be classified loosely as homeostatic or inflammatory, innate or adaptive, local or lymphoid, immune-generating or immune-effector: a spatio-temporal paradigm whose relevance has been substantiated, for example, by the proposed distinction between lymphoid-homing, $CCR7^+$ mature DC (DC_{mat})/central-memory T cells (T_{CM}) and tissue-homing, $CCR7^-$ immature DC (DC_{imm})/effector-memory T cells (T_{EM}) (reviewed in [71]). With respect to DC in particular, the prevailing model argues that incipient inflammatory stimuli in infected tissue induce resident cells to produce the chemokine MIP-3α/CCL20, which recruits $CCR6^+$ DC_{imm} from

the circulation or other regions of the tissue to the site of antigenic challenge [72,73]. Once these DC_{imm} are activated by the harvesting and processing of antigen, they acquire a cardinal $CCR6^-$, $CCR7^+$ mature phenotype that is responsive to the constitutively-expressed, lymphoid chemokine MIP-3β/CCL19 [74-76]. Of key immunomodulatory significance, this model establishes a precedent in which the evolution of an adaptive response is orchestrated in part by the co-ordinated, sequential expression of chemokine receptors and their anatomically-restricted ligands.

That CCL20 recruits, among other cells, immature DC qualifies it as an interesting candidate to compare with GM-CSF, the immunostimulatory cytokine that drives the mucosal Th2 model. Indeed, as described above, it is likely that GM-CSF overexpression in the airway subverts inhalation tolerance to OVA through the expansion and activation of lung DC. Is it possible that substitution of GM-CSF with CCL20 can create a symmetrical, DC-rich airway microenvironment capable of disarming default tolerance mechanisms? This question is explored in Chapter 4, in which the basic architecture of the mucosal Th2 model is adapted to investigate the immunology of CCL20 in the airway. Intranasal administration of an Ad vector encoding the transgene for human CCL20 induced the marked expansion of APC of all subsets, in addition to $CD4^+$ and $CD8^+$ T cells, in the lung. That these APC bore an activated phenotype—possibly an artifact of the unavoidable presence of viral antigens—intimated that CCL20 overexpression had conditioned an immunologically primed airway microenvironment. In this immune-potentiated context, concurrent exposure to an aerosol of OVA resulted in Th1-biased sensitization, as evidenced by the production of OVA-specific IgG_{2a} and the reconstitution of mononuclear airway inflammation upon long-term OVA rechallenge *in vivo*. Using CCR6-deficient mice or CCL20 neutralization, a number of

published studies have documented the importance of CCL20 in the pathogenesis of experimental models of inflammatory bowel disease [77], multiple sclerosis [78] and asthma [79]. This study, however, is the first to demonstrate the sufficiency of CCL20 expression *per se* in the induction of immune-inflammatory responses in the lung, and implicates CCL20, which is known to be upregulated in the lung (among other organs) by inflammatory stimuli, in the ætiology of Th1-biased inflammatory disorders.

Summary

The research presented in this document employs a variety of immunomodulatory strategies to investigate the immunology of the airway mucosa in a mouse model of passive exposure to an innocuous antigen. The salient message informed by each of these studies is that the outcome of an immune-inflammatory response is very much a reflection of the airway microenvironment in which aerosolized antigen is initially interpreted by the immune system—a finding whose significance resonates with the prospective targets and kinetics of therapeutic intervention for asthma. Therefore, because they may concomitantly alter the context in which antigen is presented by airway APC, interventions ostensibly designed to modulate local lung inflammation may also condition the evolution of systemic immunity.

—CHAPTER 2—

Transient Corticosteroid Treatment Permanently Amplifies the Th2 Response in a Murine Model of Asthma

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Published in *The Journal of Immunology* 172:4995-5005 (2004).

The Journal of Immunology

Transient Corticosteroid Treatment Permanently Amplifies the Th2 Response in a Murine Model of Asthma[1]

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Corticosteroids (CS) remain the most efficacious pharmacotherapeutic option for the management of asthma. Although the acute anti-inflammatory effects of CS treatment have been amply documented both clinically and experimentally, recent human data intimate that exposure to CS may be associated with retrograde immune phenomena, including enhanced synthesis of IgE in vivo and elevated Th2 cytokine production in vitro. We have investigated the long-term immunologic effects of CS treatment in a murine model of allergic airway inflammation. CS treatment during initial exposure to OVA or upon long-term Ag rechallenge remarkably attenuated eosinophilic airway inflammation and airway hyperresponsiveness. Interestingly, however, Th2 cytokine production by cultured splenocytes from CS-treated mice was significantly elevated, while IFN-γ synthesis was depressed. Moreover, mice rechallenged with OVA several weeks after CS intervention during allergic sensitization not only developed airway inflammation, but also exhibited enhanced Th2 cytokine production in lymphoid tissues and OVA-specific IgE in serum. This amplification of the systemic immune response was associated with an intact APC compartment during CS-conditioned sensitization to OVA. These data indicate that immune processes underlying the allergic phenotype remain impervious to CS treatment and raise the possibility that treatment with CS during sensitization may amplify elements of the allergen-specific immune response. ***The Journal of Immunology,*** **2004, 172: 4995–5005.**

Corticosteroids (CS)[3] have emerged, deservedly, as the pharmaceutical of choice for patients with chronic inflammatory conditions. The development of inhaled CS, in particular, has revolutionized the treatment of asthma and is now the inimitable standard to which all prospective asthma therapies are compared. Indeed, by affording exquisite control of local inflammatory processes in the lung and therefore reversing attendant airway dysfunction, inhaled CS effectively ameliorate asthma morbidity.

In recent years, research into steroids has primarily extended our knowledge of the cellular and molecular mechanisms by which pharmacological intervention with CS might attenuate inflammation. This research has uncovered pathways, such as inhibition of the proinflammatory transcription factors AP-1 and NF-κB (transrepression), direct activation of anti-inflammatory gene transcription through glucocorticoid responsive elements on DNA (transactivation), or mobilization of apoptotic processes, that are engaged when steroids associate with endogenous glucocorticoid receptors (1). In human asthmatics, the anti-inflammatory activity of inhaled CS has been associated, for instance, with impaired recruitment of eosinophils and other leukocytes to the airway and with attenuated production of Th2 cytokines and other inflammatory mediators (2–4); in mice, inhaled CS treatment has also been shown to suppress eosinophil progenitor expansion in the bone marrow, pointing to the systemic effects of local steroid delivery (5). These studies have furnished a rich mechanistic foundation upon which the development of new generations of highly specialized steroid-based therapeutics will draw (6–9).

However, informed by the understanding that steroids are effective at managing inflammation only as long as they are taken, investigators have directed comparatively little attention to the long-term immunobiological implications of steroid delivery: whether steroids have the capacity to alter substantively the evolution and pathogenesis of allergic airway disease. Our study examines this question in a murine model of asthma in which mice are re-exposed to Ag several weeks after steroid intervention has been discontinued. Our observations derive two unequivocal conclusions: 1) that concurrent delivery of CS during mucosal allergic sensitization does not prevent the development of a local airway inflammatory response upon Ag re-exposure and 2) that CS treatment during sensitization actually amplifies cardinal systemic features of the allergic phenotype both acutely and during long-term Ag rechallenge.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed under specific pathogen-free conditions and maintained on a 12-h light-dark schedule. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University. A total of 442 mice were sacrificed during the course of these experiments.

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Received for publication July 15, 2003. Accepted for publication February 3, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded in part by the Canadian Institutes of Health Research and AstraZeneca, Inc. (Sweden). R.E.W. and D.A. hold Canadian Institutes of Health Research Doctoral Research Awards; J.R.J. is the recipient of an Ontario Graduate Scholarship.

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[3] Abbreviations used in this paper: CS, corticosteroid; Ad, adenovirus; BAL, bronchoalveolar lavage; RSR, respiratory system resistance; MCh, methacholine; MIP-1α, macrophage-inflammatory protein 1α; NoRx, no treatment; MHCII, MHC class II.

0022-1767/04/$02.00

Model of Ag-induced airway inflammation

Mice were subjected to a model of musocal allergic sensitization as previously described (10). Briefly, mice were exposed to aerosolized OVA in the context of GM-CSF overexpression in the airway microenvironment. To elicit local expression of GM-CSF, a replication-deficient human type 5 adenoviral (Ad) construct encoding murine GM-CSF cDNA in the E1 region of the viral genome was delivered intranasally to isoflurane-anesthetized animals on day −1, 24 h before the first exposure to OVA. Ad/GM-CSF was administered at a dose of 3×10^7 PFU in a total volume of 30 μl of PBS vehicle. Over a period of 10 consecutive days (days 0–9), mice were placed in a Plexiglas chamber (10 cm × 15 cm × 25 cm) and exposed for 20 min daily to aerosolized OVA (1% w/v in 0.9% saline; Sigma-Aldrich, Oakville, Ontario, Canada). The OVA aerosol was generated by a Bennett nebulizer (Pleasanton, CA) at a flow rate of 10 L/min. For the long-term in vivo rechallenge experiments, sensitized mice were allowed to recover from acute inflammation (~4 wk) and were then exposed to a 1% OVA aerosol for 20 min on 3 consecutive days (rechallenge days 0_{LT} to 2_{LT}). Models are depicted in Fig. 1.

Budesonide administration

We used a pharmaceutical grade, modified Wright Dust Feed system (AstraZeneca, Lund, Sweden) to generate a dry powder aerosol of budesonide in lactose vehicle or of lactose alone. A Battelle (Columbus, OH) two-stage inhalation chamber was used; it contains 15 inhalation ports and 1 filter sampling port, thus allowing us to expose 15 mice simultaneously. Filtered, desiccated air flowed through the Wright Dust Feed at a rate of 10 L/min or 0.63 L/min/port; the rate of evacuation from the chamber was 8 L/min. The concentration of steroid powder entering the chamber was monitored with a real-time aerosol dust monitor (Casella CEL, Kempston, Bedford, U.K.). For these studies, the generated concentration of budesonide powder in the chamber was 800 mg/m^3 or 0.8 μg/ml, and mice were exposed to budesonide for 10 min per treatment.

The inhaled dose is calculated using the following formula: $ID = ET \times RMV \times CC/BW$, where ID = inhaled dose (μg/g), ET = exposure time (min), RMV = respiratory minute volume = $4.19 \times BW^{2/3}$ (ml/min), CC = chamber concentration (μg/ml), and BW = body weight (g). Using this equation, we have calculated an inhaled budesonide dose of 12.3 μg/g for a 20-g mouse. The overall systemic bioavailability of budesonide is a factor of both lung-deposited dose and swallowed dose. In humans, lung deposition of aerosolized budesonide is on average 20% and gut deposition 50%; in mice, ~4% is deposited in the lung and 36% in the gut. Approximately 90% of the portion of drug deposited in the gut is inactivated in the liver via first-pass metabolism. The portion of drug deposited in the lung is absorbed into the circulation, but only 25% of this is inactivated by first-pass metabolism (AstraZeneca, unpublished data). Taking into account these considerations and the fact that budesonide has a M_r of 430.53 g, the nominal maximum bioavailable dose of budesonide mice received was 0.86 nmol/g via the lung and 1.0 nmol/g via the gastrointestinal tract, for a total systemic bioavailability of 1.86 nmol/g; however, because of inherent inefficiency of the apparatus and concomitant overestimation of budesonide concentration by the dust monitor, the actual bioavailable dose was typically one-third the nominal dose (for a final bioavailability of 0.62 nmol/g). By comparison, the bioavailable dose achieved in humans taking 3.2 g of budesonide daily is approximately 0.018 nmol/g.

Collection and measurement of specimens

Twenty-four hours after the ninth OVA exposure (day 9) or 72 h after the third OVA exposure during long-term in vivo recall (day 5_{LT} of the rechallenge protocol), mice were sacrificed and bronchoalveolar lavage (BAL) was obtained as previously described (11). In brief, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences, Oakville, Ontario, Canada). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation, supernatants were stored at −20°C for measurement of cytokines by ELISA; cell pellets were resuspended in PBS and smears were prepared by cytocentrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. Diff-Quik (Baxter, McGraw Park, IL) was used to stain all smears. Differentiation of leukocyte subsets in BAL was determined by counting at least 500 white blood cells using standard hemocytological procedures to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Blood smears were prepared from peripheral blood collected in heparinized capillary tubes; leukocytes were differentiated by counting at least 300 white blood cells. Finally, lung tissue was fixed in 10% Formalin and embedded in paraffin. Three-μm-thick sections were stained with H&E for visualization of leukocytes and histopathological features.

Cytokine and Ig measurement

ELISA kits for GM-CSF, IFN-γ, IL-4, IL-5, and IL-13 were purchased from R&D Systems (Minneapolis, MN); each of these systems has a threshold of detection of 1.5–7 pg/ml. Levels of OVA-specific IgE were detected using an Ag-capture (biotinylated OVA) ELISA method as described previously (11); anti-mouse IgE Abs were obtained from Southern Biotechnology Associates (Birmingham, AL). This ELISA was standardized with serum obtained from mice sensitized to OVA according to a conventional i.p. sensitization model and bled 7 days following the second sensitization (11); Ig levels, therefore, are expressed in units per milliliter relative to this standard serum.

Splenocyte and lymph node mononuclear cell culture

Using the plunger from a 5-ml syringe, spleens or thoracic lymph nodes were triturated through a 40-μm nylon cell strainer (BD Falcon, Bedford, MA) into HBSS. RBC in splenocyte preparations were lysed by resuspending dispersed cells from each spleen in 1 ml of ACK lysis buffer for 1 min. Splenocytes were then washed twice in supplemented RPMI 1640 (containing 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 0.1% mercaptoethanol); lymph node mononuclear cells were washed once. Splenocytes and lymph node mononuclear cells were cultured in 96-well plates at a density of 8×10^5 or 6×10^5 cells/well, respectively, in a total volume of 200 μl of RPMI 1640. Cells were stimulated with OVA at a concentration of 40 μg/well for 5 days, at which point supernatants were harvested and stored at −20°C for detection of cytokines.

ELISPOT assay

OVA-specific, IL-4-, IL-5-, or IFN-γ-producing T cell clones in the spleen were detected with an ELISPOT assay (BD Biosciences). Dispersed splenocytes were plated at a density of 8×10^5 cells/well in a 96-well ELISPOT plate that had been precoated with the appropriate anti-mouse cytokine Ab; cells were cultured in medium alone or stimulated with 40 μg/well OVA for 72 h, after which immobilized, secreted IL-4, IL-5, or IFN-γ was detected with the corresponding biotinylated anti-mouse cytokine Ab according to the protocol included with the ELISPOT kit. Each plate was developed via streptavidin-HRP reaction for 5 min, desiccated, and imaged using AxioVision software (Zeiss, Thornwood, NY); QWin software (Leica Microsystems, Richmond Hill, Ontario, Canada) was adapted for automated, color-dependent enumeration of spots.

Assessment of airway hyperresponsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (RSR) to increasing doses of methacholine (MCh) injected into the internal jugular vein of Avertin-anesthetized mice as previously described (12). Exposed tracheas were cannulated and a constant inspiratory flow was delivered by mechanical ventilation (RV5; Voltek Enterprises, Toronto, Ontario, Canada). Paralysis was achieved using pancuronium (0.03 mg/kg i.v.) to prevent respiratory effort during measurement. RSR was measured following consecutive i.v. injections of saline, followed by 10, 33, 100, 330, and 1000 μg/kg MCh (ACIC, Brantford, Ontario, Canada), each delivered in a 0.2-ml bolus. Evaluation of airway responsiveness was based on the peak RSR measured in the 30 s following the saline and MCh challenges.

Lung cell isolation and flow cytometric analysis of lung cell subsets

Lungs were perfused with 10 ml of HBSS through the right ventricle, cut into small (~2 mm in diameter) pieces, and agitated at 37°C for 1 h in 15 ml of collagenase III (Life Technologies, Rockville, MD) at a concentration of 150 U/ml in HBSS. Using the plunger from a 5-ml syringe, the lung pieces were triturated through a 40-μm nylon cell strainer (BD Falcon) into HBSS. Mononuclear cells were isolated at the interphase between layers of 30 and 60% Percoll following density gradient centrifugation. Cells were washed twice and stained for flow cytometric analysis. For each Ab combination, 1.0×10^6 cells were incubated with mAbs at 0 - 4°C for 30 min; the cells were then washed and treated with second-stage reagents. Intracellular cytokine staining was executed according to the protocol detailed in the Cytofix/Cytoperm GolgiPlug kit (BD Biosciences). Briefly, dispersed lung mononuclear cells were cultured in six-well plates at a density of 2×10^6 cells/ml in the context of GolgiPlug reagent (brefeldin A) alone (1 μl/ml supplemented RPMI 1640) or concurrent LPS stimulation (1.0

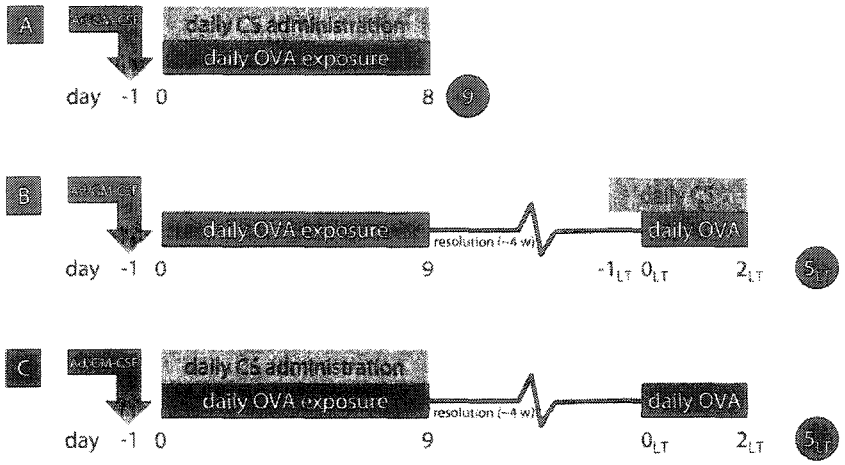

FIGURE 1. Models of Ag-induced allergic airway inflammation and CS administration. *a*, Model of acute CS intervention: Mice were exposed daily to OVA in the context of a GM-CSF-conditioned airway microenvironment with concurrent CS treatment. *b*, Model of long-term therapeutic CS intervention: Mice were sensitized to OVA in the absence of CS treatment; inflammation was permitted to resolve (~4 wk) before mice were rechallenged with OVA while being treated with CS. *c*, Model of the long-term effects of acute CS intervention: Mice were sensitized to OVA while being treated with CS; mice were rechallenged with OVA in the absence of further CS treatment. Numbers in circles indicate time points at which outcomes were measured.

μg/ml); cells were harvested after an 8-h incubation and, after surface staining, cells were fixed in Cytofix/Cytoperm solution (4% paraformaldehyde in PBS) and permeabilized in Perm/Wash buffer (saponin/sodium azide concentrate, diluted 1/10 in distilled water) for intracellular staining.

All data were collected using a FACScan (BD Biosciences) for three-color flow cytometry and were analyzed using WinMDI software (The Scripps Research Institute, La Jolla, CA). The following Abs and reagents were used: mouse IgG_3 anti-mouse MHC class II (MHCII; I-A^d), FITC-conjugated and biotinylated (39-10-8); rat IgG_{2b} anti-mouse CD11b, FITC- and PE-conjugated (M1/70); hamster IgG anti-mouse CD11c, FITC- and PE-conjugated (HL3); hamster IgG anti-mouse B7.1, biotinylated (16-10A1); rat IgG_{2a} anti-mouse B7.2, biotinylated (GL1); rat IgG_1 anti-mouse IL-12, PE-conjugated (C15.6); hamster IgG anti-mouse CD3ϵ, PE-conjugated and CyChrome-conjugated (145-2C11); rat IgG_{2a} anti-mouse CD4, FITC-conjugated and CyChrome-conjugated (RM4-5); hamster IgG anti-mouse CD69, PE-conjugated (H1.2F3) (all purchased from BD PharMingen, Mississauga, Ontario, Canada); rat IgG_1 anti-mouse TI/ST2, FITC-conjugated (produced in-house by Millennium Pharmaceuticals, Cambridge, MA); all appropriate isotype control Abs and streptavidin-PerCP (BD PharMingen). The Abs were titrated to determine optimal concentration.

Data analysis

Data are expressed as mean ± SEM, unless otherwise indicated. Results were interpreted using Student's *t* test or ANOVA with Tukey's post hoc test, where appropriate. Differences were considered statistically significant when $p < 0.05$.

Results

CS treatment during sensitization acutely depresses local inflammatory processes in the airway

To assess the acute anti-inflammatory effects of CS intervention, mice were exposed to aerosolized OVA for 20 min on 9 consecutive days (0–8) beginning 24 h after intranasal administration of Ad/GM-CSF (day −1); a subset of mice was treated with nebulized budesonide at a concentration of 0.8 μg/ml for 10 min immediately before each OVA exposure (model A in Fig. 1). Fig. 2*A*

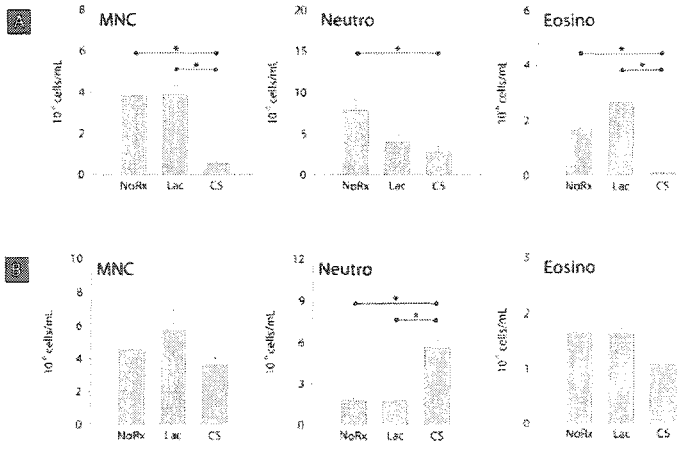

FIGURE 2. CS intervention during allergic mucosal sensitization to OVA arrests inflammation in the airways of mice. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS treatment and sacrificed at day 9 of the aerosolization protocol. Graphs depict mononuclear cells (MNC), neutrophils (Neutro), and eosinophils (Eosino) in the BAL (*A*) and peripheral blood (*B*) of OVA-sensitized, untreated control mice (NoRx) or mice exposed to OVA in the context of either lactose vehicle (Lac) or powdered budesonide (CS). n = 5–12/group (pooled) from two experiments; *, $p < 0.05$.

Table I. *Cytokine and Ig content in BAL and serum at day 9 of the aerosolization protocol*[a]

	NoRx	CS
Cytokines and Chemokines[b] (pg/ml)		
IL-4	16 ± 6.4	10 ± 3.5
IL-5	83 ± 21	20 ± 8.3*
IL-13	432 ± 62	31 ± 9.4*
IFN-γ	17 ± 3.4	8.3 ± 1.3*
MIP-1α	39 ± 3.6	23 ± 2.0*
RANTES	14 ± 2.2	3.1 ± 1.8*
Igs[c] (μ/ml)		
OVA-specific IgE	123 ± 39	240 ± 32*

[a] Groups of mice were mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment and sacrificed 24 h after the ninth OVA exposure (day 9). Cytokines, chemokines, and Igs were measured by ELISA in BAL[b] or serum.[c] Data represent mean + SEM; $n = 7$ 8/group.
*, $p < 0.05$.

illustrates inflammation in BAL at day 9 of the aerosolization protocol. CS treatment dramatically inhibited inflammation in the airways compared with both untreated sensitized mice (NoRx) and mice receiving nebulized lactose vehicle; eosinophilia, in particular, was completed abrogated by CS intervention. The attenuated cytokine and chemokine content in BAL of CS-treated mice mirrors these cellular data (Table I); indeed, levels of the Th2-affiliated cytokines IL-5 and IL-13, of the chemokines macrophage-inflammatory protein 1α (MIP-1α) and RANTES, and of IFN-γ were all reduced in the context of CS intervention, asserting the unequivocal anti-inflammatory properties of CS in this model at the molecular level. In contrast, data in Fig. 2*B* demonstrate that CS therapy elicited no statistically significant qualitative or quantitative changes in the peripheral blood leukocyte profile, except the expected elevation in neutrophils due to the well-documented, CS-induced demargination of these cells, compared with untreated and lactose-treated controls. Moreover, and paradoxically, OVA-specific IgE in serum was elevated 2-fold following CS treatment (Table I).

To translate our findings describing the impact of local CS administration during concomitant mucosal allergic sensitization to a different clinical paradigm, we treated previously sensitized animals with CS during long-term Ag rechallenge. To this end, mice were sensitized to aerosolized OVA according to our 10-day mucosal sensitization regimen and permitted to recover from acute inflammatory phenomena, which are completely resolved by day 28. Groups of mice were then treated with nebulized budesonide on day -1_{LT} of the rechallenge protocol, followed by three daily 20-min exposures to aerosolized OVA preceded by CS therapy (days 0_{LT} to 2_{LT}); mice were sacrificed on day 5_{LT}, the peak of airway inflammation following this Ag recall procedure (R. E. Wiley, B. U. Gajewska, J. R. Johnson, and M. Jordana, unpublished data; model B in Fig. 1). Data in Fig. 3*A* illustrate that the accumulation of all inflammatory cells, especially eosinophils, in BAL was abolished in mice concurrently receiving CS compared with untreated mice and mice receiving lactose (data not shown; it should be noted that, for simplicity, the lactose control has been excluded from this and subsequent figures since lactose intervention had no effect on any immune-inflammatory parameter examined). In agreement with findings in BAL, histological examination of lung tissue from CS-treated mice revealed attenuated peribronchial and perivascular inflammation and reduced goblet cell hyperplasia and mucous secretion compared with untreated mice (representative sections are displayed in Fig. 3, *C* and *D*). In contrast and consistent with observations in mice treated during sensitization, levels of OVA-specific IgE, which are substantially elevated at recall, were unaffected by CS intervention (Fig. 3*B*),

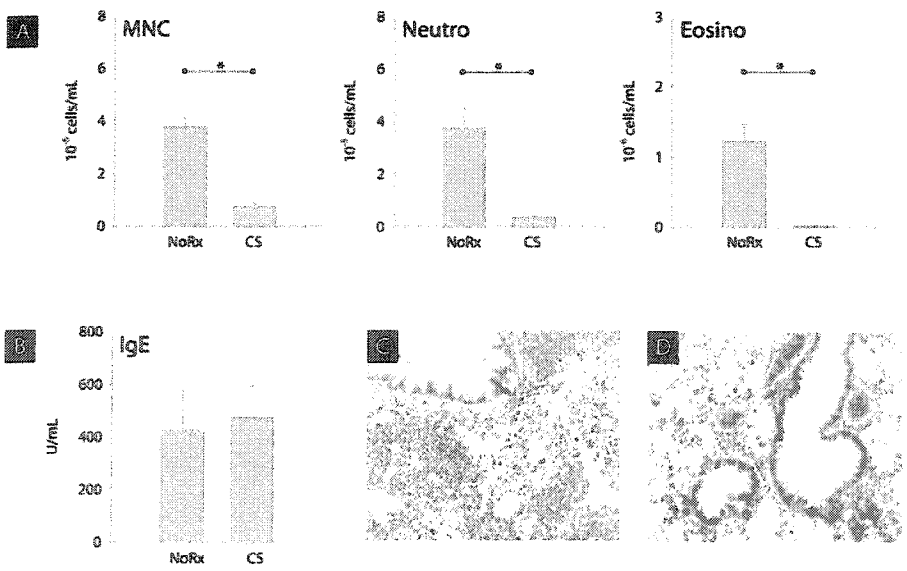

FIGURE 3. CS treatment during long-term OVA rechallenge of mice with established allergy ablates airway inflammation but has no impact on circulating OVA-specific IgE. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu, and acute inflammatory processes were subsequently permitted to resolve (~4 wk); mice were then sacrificed 72 h following three daily re-exposures to aerosolized OVA in the presence or absence of concurrent CS delivery. Graphs depict mononuclear cells (MNC), neutrophils (Neutro), and eosinophils (Eosino), in the BAL (*A*) and OVA-specific IgE in serum (*B*) of OVA-sensitized, untreated control mice (NoRx) or mice rechallenged with OVA in the context of powdered budesonide (CS). *C* and *D*, Representative H&E-stained cross-sections from the lungs of untreated and steroid-treated mice, respectively. Original magnification, ×100. $n = 7$ 10/ group (pooled) from two experiments; *, $p < 0.05$.

Table II. *Peripheral blood leukocytes in mice treated with CS during OVA rechallenge*[a]

	NoRx	CS
Total cells	11.5 ± 1.91	12.2 ± 3.99
Mononuclear cells	8.30 ± 1.62	3.52 ± 1.24
Neutrophils	2.37 ± 0.23	8.36 ± 3.06
Eosinophils	0.79 ± 0.15	0.28 ± 0.06*

[a] Groups of mice that had been mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment were sacrificed 72 h after long-term OVA rechallenge. Data indicate 10^{-6} cells/ml of blood; $n = 5$–12/group.
*, $p < 0.05$.

Table III. *ELISPOT analysis of the frequency of IL-5-producing cells in the spleens of mice at day 9 of the aerosolization protocol*[a]

	Medium	OVA
NoRx	0.0009 ± 0.0003	0.0883 ± 0.0064
CS	0.0002 ± 0.0001	0.0636 ± 0.0184

[a] Groups of mice were mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment and sacrificed 24 h after the ninth OVA exposure (day 9). Spleens were harvested, and 8.0×10^6 dispersed cells/ml were cultured in medium alone or stimulated with OVA in an IL-5-detecting ELISPOT plate. Data display the mean ± SEM fraction (percent) of seeded cells positively expressing IL-5 following a 72-h incubation: $n = 6$–9/group. Results are representative of two independent experiments.

although there was a modest reduction in eosinophil content in peripheral blood (Table II).

CS treatment during mucosal allergic sensitization hyperpolarizes the systemic Th2 response and amplifies immune-inflammatory phenomena upon long-term in vivo recall

Impelled by the observation that acute CS administration, while ablating the local inflammatory response, does not appreciably affect peripheral blood leukocytosis and actually results in elevated levels of serum IgE, we examined OVA-specific immune activity in the spleen. Fig. 4 illustrates Th2 cytokine production by cultured splenocytes harvested from untreated and CS-treated mice at day 9 of the aerosolization protocol. Interestingly, splenocytes from CS-treated mice liberated 2- to 3-fold more IL-4, IL-5, and IL-13 upon stimulation with OVA than cells from untreated controls, while IFN-γ production was significantly depressed. This elevation in Th2 cytokine production, moreover, cannot be attributed to the disproportionate retention of OVA-specific clones in the spleens of CS-treated mice, as ELISPOT analysis of cultured splenocytes identified no difference in the frequency of IL-5-producing cells upon OVA stimulation (Table III); parallel enumeration of IL-4- and IFN-γ-secreting splenocytes also showed similar clone frequencies in CS-treated and untreated mice (data not shown).

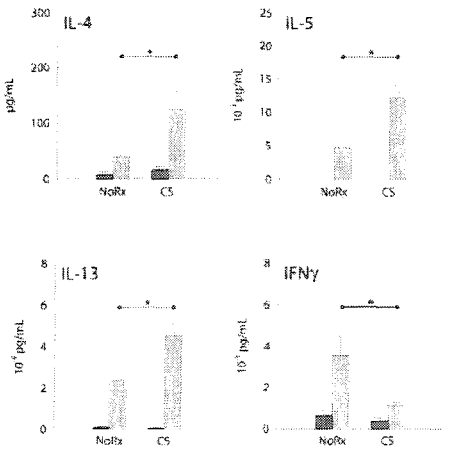

FIGURE 4. CS intervention during allergic mucosal sensitization to OVA enhances Th2 and attenuates Th1 cytokine production by cultured splenocytes. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS treatment and sacrificed at day 9 of the aerosolization protocol; spleens were harvested and dispersed splenocytes were cultured for 5 days in medium alone (■) or in the context of OVA stimulation (▨). Graphs depict IL-4, IL-5, IL-13, and IFN-γ in culture supernatants of splenocytes from OVA-sensitized, untreated control mice (NoRx), or mice exposed to OVA during concurrent delivery of powdered budesonide (CS). Data are representative of two independent experiments: $n = 7$–8/group; *, $p < 0.05$.

Informed by the findings in the spleen, we investigated the implications of acute CS therapy for the long-term immune-inflammatory response to OVA. Mice were mucosally sensitized to OVA according to the 10-day aerosolization regimen in the presence or absence of concomitant CS treatment. Following the resolution of inflammation in the lung (by day 28), mice were re-exposed to aerosolized OVA on 3 consecutive days without additional CS intervention and sacrificed 72 h after the last OVA challenge (model C in Fig. 1). Fig. 5 indicates elevated OVA-specific Th2 cytokine production by splenocytes in vitro, verifying observations in the spleen at day 9. Data from cultured mononuclear cells harvested from thoracic lymph nodes following in vivo OVA recall (Table IV) complement the findings in the spleen and underscore the long-term systemic effects of steroid therapy, i.e., OVA-induced production of IL-4, IL-5, and IL-13 was consistently higher in lymph node cells from rechallenged mice sensitized to OVA in the context of CS therapy; in contrast, we were unable to detect OVA-specific IFN-γ production above baseline levels in unstimulated lymph node cells from CS-treated mice (data not displayed).

To elaborate these findings, we evaluated the consequences of this alleged sustained hyperpolarization of the Th2 phenotype on the allergic response in vivo. In particular, we examined inflammation in the BAL (Fig. 6*A*) and histopathologically (Fig. 6, *C* and *D*) following long-term rechallenge of mice initially sensitized to OVA in the absence or presence of concurrent CS treatment; we documented no overt changes in cellular inflammation 72 h after the last OVA exposure, with the exception of a small reduction in

FIGURE 5. Enhanced Th2 cytokine production by cultured splenocytes from mice mucosally sensitized to OVA in the context of corticosteroid treatment persists during long-term OVA rechallenge. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS intervention and acute inflammatory processes were subsequently permitted to resolve (~4 wk); mice were then sacrificed 72 h following three daily re-exposures to aerosolized OVA in the absence of further CS treatment. Spleens were harvested, and dispersed splenocytes were cultured for 5 days in medium alone (■) or in the context of OVA stimulation (▨). Graphs depict IL-4, IL-5, and IL-13 in culture supernatants of splenocytes from OVA-sensitized, untreated control mice (NoRx) or rechallenged mice originally sensitized to OVA in the context of powdered budesonide delivery (CS). $n = 4$/group from a single experiment; *, $p < 0.05$.

Table IV. *Cytokine production by cultured mononuclear cells from thoracic lymph nodes following long-term OVA rechallenge of mice treated with CS during sensitization*[a]

	NoRx		CS	
	Medium	OVA	Medium	OVA
IL-4	0	180	23.4	>2,000
IL-5	5.20	4,385	1,385	28,238
IL-13	62.4	15,464	12,878	>100,000

[a] Groups of mice that had been mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment were sacrificed 72 h after long-term OVA rechallenge. Dispersed mononuclear cells from draining thoracic lymph nodes were pooled and cultured at a density of 6.0×10^6 cells/ml in medium alone or in the context of OVA stimulation. Cytokines were measured by ELISA in supernatants following a 5-day incubation. Data are expressed in picograms per milliliter; $n = 5$ (pooled)/group. Results are representative of two independent experiments.

BAL neutrophilia, a finding of nominal significance given the minor contribution of neutrophils to the inflammatory profile at this time point. The indistinguishable nature of the airway inflammatory profile between untreated and CS-treated mice was verified by flow cytometric analysis of enriched lung mononuclear cells. Gated $CD3^+CD4^-$ cells expressed similar levels of the Th2 marker T1/ST2 (10 15%) (13,14) and of the activation marker CD69 (24–28%) in the two treatment groups (data not shown graphically), intimating similar effector capacity of lung T cells. Importantly, however, levels of OVA-specific IgE in serum were considerably elevated, on the order of 3-fold, suggesting CS-mediated amplification of one of the cardinal immunological features of allergic airway disease (Fig. 6*B*). Peripheral blood eosinophilia was also elevated by ~80% upon rechallenge of mice sensitized to OVA in the context of CS treatment (Table V).

To ascertain the long-term physiological/functional impact of acute CS therapy and the associated potentiation of OVA-specific IgE, airway responsiveness to i.v. administered MCh was measured upon long-term OVA rechallenge of mice with established allergy. As indicated in Fig. 7, budesonide treatment during OVA recall resulted in a dramatic reduction in airway resistance (to naive levels) compared with sensitized controls challenged without concurrent pharmacological intervention. In contrast, mice initially treated with CS during sensitization were afforded no protection against the airway physiological response during long-term recall, exhibiting a responsiveness curve similar to that observed in untreated controls.

CS treatment during sensitization does not inhibit the differentiation and activation of APC in the lung

That CS intervention during sensitization did not diminish, and even amplified, the long-term systemic immune response to OVA intimates that GM-CSF-induced differentiation and activation of the APC compartment was impervious to CS administration. We therefore used flow cytometry to analyze the phenotype of APC subsets in mononuclear cell-enriched lung homogenates between days 7 and 9 of the aerosolization protocol, the peak of pulmonary APC activity in this model (15). Although total numbers of mononuclear cells were predictably reduced in CS-treated mice (data not shown), the fraction of cells coexpressing MHCII and high levels

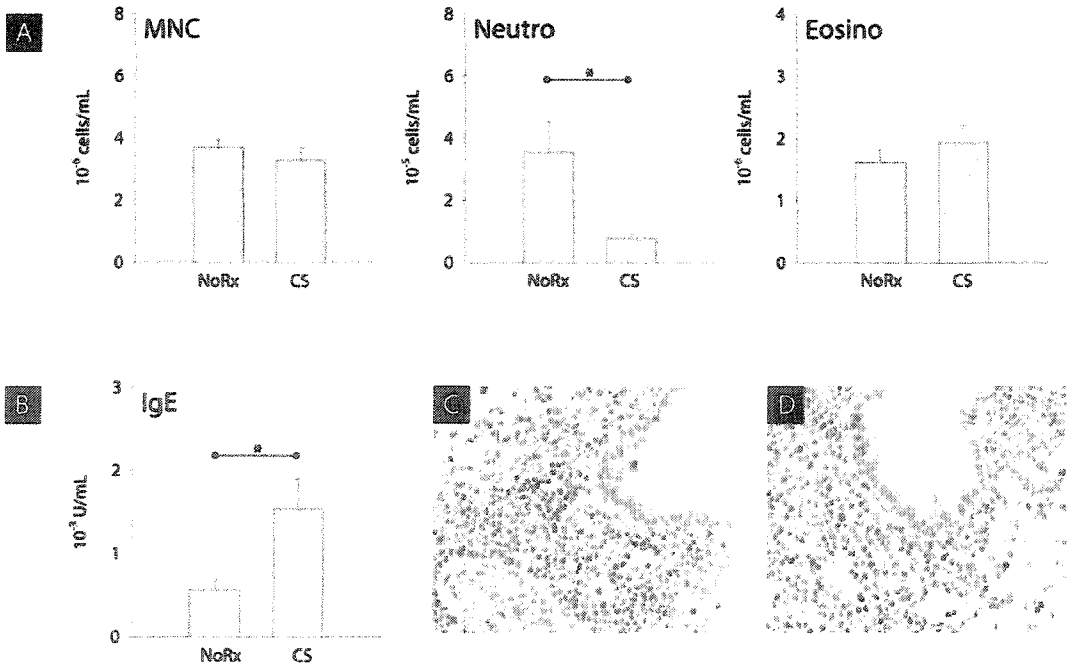

FIGURE 6. CS treatment during allergic mucosal sensitization has no impact on inflammation in the airways of mice during long-term OVA rechallenge, but results in dramatically elevated levels of circulating OVA-specific IgE. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS intervention and acute inflammatory processes were subsequently permitted to resolve (~4 wk); mice were then sacrificed 72 h following three daily re-exposures to aerosolized OVA in the absence of further CS treatment. Graphs depict mononuclear cells (MNC), neutrophils (Neutro), and eosinophils (Eosino) in the BAL (*A*), and OVA-specific IgE in serum (*B*) of OVA-sensitized, untreated control mice (NoRx) or rechallenged mice originally sensitized to OVA in the context of powdered budesonide delivery (CS). *C* and *D*, Representative cross-sections from the lungs of untreated and steroid-treated mice, respectively. Original magnification, ×200. n = 7–10/group (pooled) from two experiments; *, $p < 0.05$.

Table V. *Peripheral blood leukocytes following OVA rechallenge in mice treated with CS during sensitization*[a]

	NoRx	CS
Total cells	12.4 ± 1.19	10.9 ± 1.52
Mononuclear cells	8.65 ± 0.95	7.22 ± 1.25
Neutrophils	3.04 ± 0.46	2.35 ± 0.22
Eosinophils	0.75 ± 0.12	1.34 ± 0.22*

[a] Groups of mice that had been mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment were sacrificed 72 h after long-term OVA rechallenge. Data indicate 10^{-6} cells/ml blood; $n = 7$/group.
*, $p < 0.05$.

of CD11c (dendritic cells) or CD11b (macrophages) was essentially identical to untreated controls and, importantly, dramatically higher than levels observed in naive mice (Fig. 8*A*); with respect to dendritic cells in particular, there was a characteristic and visually unambiguous elevation in the intensity of MHCII staining on $CD11c^{high}$ cells from CS-treated and untreated mice compared with naive controls. Moreover, analysis of costimulatory molecule expression on gated $MHCII^+CD11c^{high}$ and $MHCII^+CD11b^{high}$ cells furnished evidence that the activated phenotype displayed by APC populations during sensitization was not depressed by concomitant steroid treatment (Fig. 8*B*). Indeed, the definitive elevation in B7.1 and B7.2 expression on dendritic cells and macrophages observed in untreated mice was preserved and in the case of B7.1 enhanced by CS intervention; APC from naive mice (data not shown) express comparatively low levels of these costimulatory molecules (15). We supplemented this phenotypic analysis of the APC compartment by examining expression of IL-12, whose attenuation in the context of in vitro steroid treatment has been advanced as an explanation for the observed potentiation of Th2 cytokine production (16–23). However, intracytoplasmic staining of lung mononuclear cells harvested from control mice or mice treated with steroids in vivo showed similar levels of IL-12 expression in gated $MHCII^+CD11c^+$ and $MHCII^+CD11b^+$ populations both spontaneously and following stimulation with LPS (Table VI).

Discussion

Research into synthetic CS has generally focused on the pharmacological properties and immunobiological targets that account for the unimpeachable efficacy of these drugs in the treatment of inflammatory diseases. However, notwithstanding their broad clinical applications, CS do not cure immune-inflammatory conditions and are therefore only provisionally therapeutic, their anti-inflammatory effects extinguished once treatment has been terminated. Building on this apparently intrinsic limitation of CS pharmacology, we have investigated the long-term effects of transient CS delivery in a model of asthma in which mice were mucosally sensitized to OVA in the context of CS treatment and, following resolution of acute inflammatory events in the airway, rechallenged with this Ag in the absence of further treatment.

Administration of CS to mice during Ag exposure, either at sensitization or long-term rechallenge, remarkably attenuated local inflammatory processes in the lung. Indeed, the acute, Th2-polarized response elicited upon repeated daily exposure of mice to aerosolized OVA in the context of a GM-CSF-enriched airway milieu was averted by concurrent delivery of nebulized budesonide. Airway eosinophilia, the hallmark of this inflammatory response, was completely ablated, and other leukocyte subsets were similarly reduced in BAL (Fig. 2*A*). Consistent with these cellular data, the Th2 cytokines IL-5 and IL-13 were appreciably lower in BAL of budesonide-treated mice, as were the chemokines MIP-1α and RANTES; IL-4 content in BAL, which is detected at very low levels in this model, was not significantly different from that of controls (Table I). Paralleling these observations, budesonide exhibited a potent anti-inflammatory effect when administered to mice during long-term OVA rechallenge: airway eosinophilia, as assessed quantitatively in BAL, was dramatically reduced, and there was diminished evidence of peribronchial/perivascular inflammation and goblet cell hyperplasia histopathologically (Fig. 3, *A*, *C*, and *D*). Although the therapeutic effect of CS in mice with established allergic disease extended to peripheral blood eosinophilia (Table II), likely reflecting impaired recruitment and activation of IL-5-producing T cells in the lung, the content of OVA-specific IgE in serum was identical in treated and control mice (Fig. 3*B*), intimating that the systemic immune response to OVA was unaffected by steroid treatment.

Given the well-documented efficacy of steroids in the clinical management of inflammation associated with asthma, the anti-inflammatory effect of CS delivery to mice undergoing Ag challenge served to validate the clinical relevance of steroid intervention in our model of allergic airway inflammation. We were surprised, however, by an apparent discordance between the local and systemic effects of CS when administered during mucosal allergic sensitization to OVA. In contrast to unequivocal therapeutic effects in the lung, CS treatment elicited a 2-fold increase in the titer of OVA-specific IgE in serum (Table I). Moreover, splenocytes harvested from mice sensitized to OVA in the context of CS delivery liberated substantially higher levels of Th2 cytokines, but far less IFN-γ, when stimulated with OVA in vitro (Fig. 4), a finding that cannot be explained by the disproportionate retention of OVA-specific, Th2-polarized clones in steroid-treated mice (Table III). Paradoxically, then, CS treatment, while at once affording outstanding therapeutic efficacy in the target organ, appears to

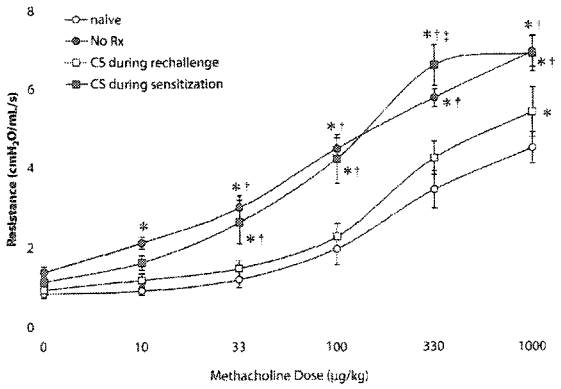

FIGURE 7. Airway hyperresponsiveness during long-term OVA recall of allergic mice is attenuated when CS are delivered during rechallenge, but not if treatment occurs exclusively during sensitization. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS intervention and acute inflammatory processes were subsequently permitted to resolve (~4 wk); airway responsiveness to i.v. administered MCh was assessed 72 h following three daily re-exposures to aerosolized OVA in the presence or absence of additional CS treatment. Graph depicts airway resistance to increasing doses of MCh in naive mice, OVA-sensitized, untreated control mice (NoRx), in allergic mice treated with powdered budesonide during OVA rechallenge (CS during rechallenge), or in mice originally sensitized to OVA in the context of budesonide and rechallenged without further pharmacological intervention (CS during sensitization). $n = 6$–8/group from a single experiment; *, $p <$ 0.05 compared with naive; †, $p < 0.05$ compared with CS during rechallenge; ‡, $p < 0.05$ compared with NoRx.

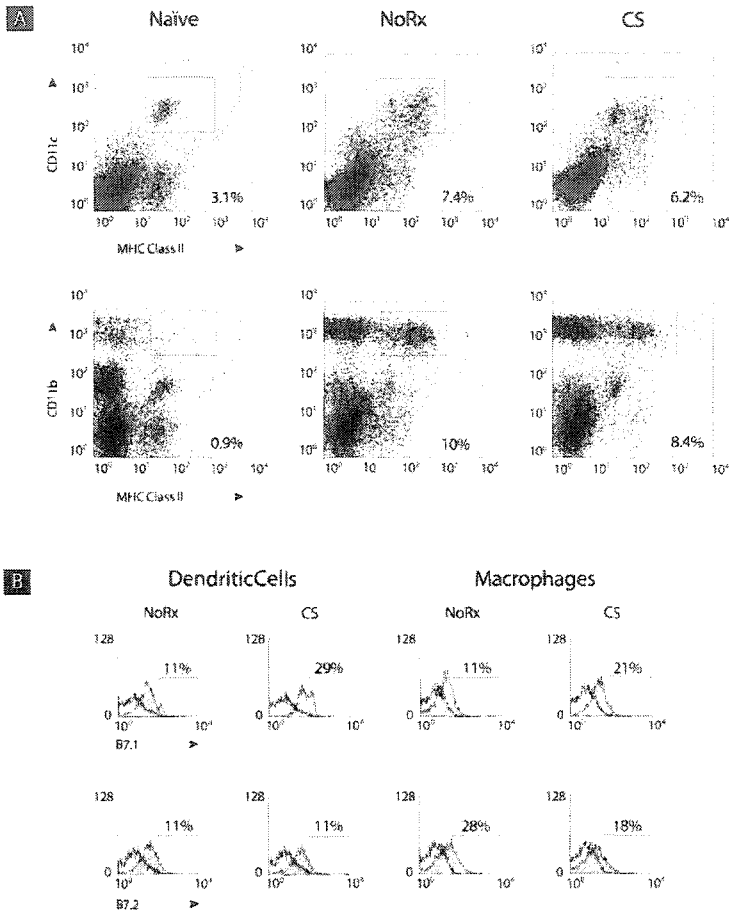

FIGURE 8. CS intervention during mucosal allergic sensitization has no impact on pulmonary APC maturation. Mice were sensitized to OVA in the context of a GM-CSF-conditioned airway milieu with or without concurrent CS treatment and sacrificed at day 7 of the aerosolization protocol; lungs were harvested and enriched mononuclear cells from each group were pooled and stained for flow cytometric analysis. Dot plots in *A* display the fraction of cells in the mononuclear cell gate expressing a dendritic cell ($MHCII^+CD11c^{high}$) or macrophage ($MHCII^+CD11b^{high}$) phenotype in naive, untreated control (NoRx) or budesonide-treated (CS) mice. *B*, Gated dendritic cells and macrophages were assessed for expression of B7.1 and B7.2. The analysis is based on the collection of 100,000 events in the mononuclear cell gate and is representative of two independent experiments; $n = 8–10$ (pooled)/group.

have conditioned the hyperpolarization of the systemic Th2 immune response to OVA. We have considered and dismissed the possibility that this potentiation of OVA-specific Th2 immunity is an epiphenomenon of extended adenovirus-mediated GM-CSF expression, and therefore enhanced adjuvant activity, due to the suppression of antiviral immunity. We detect no difference in the level of GM-CSF protein in BAL of CS-treated compared with control mice at day 9 (data not shown), when previous kinetics studies in our model have shown that transgene expression has waned; moreover, the low dose of adenovirus we administered (3×10^7 PFU) results in negligible inflammation per se and therefore should not be especially susceptible to immunomodulation by CS (10). This interpretation is entirely consistent with the results of Kolb et al. (24), who have demonstrated that the kinetics and magnitude of IL-6 expression following a single, high-dose intranasal administration of Ad/IL-6 are not affected by concurrent delivery of CS to the airway.

These results lend compelling insight into the immunological effects of CS treatment and identify an immunomodulatory function that transcends straightforward regulation of inflammation. Indeed, that Th2-affiliated, OVA-specific T cell responses in vitro are enhanced, while Th1 phenomena are depressed, when sensitization occurs in the context of CS intervention suggests a reprogramming of the immune response that one would expect to persist in the memory T cell pool. To test this hypothesis and to translate our findings at day 9 to a more clinically relevant experimental paradigm, we sensitized mice to OVA in the presence or absence of concurrent CS treatment and then, following the resolution of acute inflammation and in the absence of further pharmacological intervention, we rechallenged the mice with aerosolized OVA. The first salient finding emerging from this study is that treatment with CS during sensitization did not attenuate the long-term inflammatory response to allergen challenge in the lung: i.e., allergic airway disease has been neither cured nor exacerbated (Fig. 6, *A*, *C*, and *D*). We have also demonstrated the physiological implications of this unaltered inflammatory response; whereas CS delivery during long-term OVA rechallenge significantly improved airway hyperresponsiveness in mice with established allergic disease, a history of CS intervention during primary OVA exposure did not ameliorate the physiological response when mice were rechallenged in the absence of further CS treatment (Fig. 7). These results confirm that steroids do not prevent allergic sensitization and underscore the clinical importance of administering efficient doses of CS during asthma symptoms and exacerbations.

Interestingly, this clear and (given clinical experience) somewhat predictable dissociation between the acute and long-term effects of CS on inflammation in the target organ was not reproduced systemically. The capacity of both splenocytes and lymph node mononuclear cells to elaborate Th2 cytokines in vitro remained substantially greater in rechallenged mice originally sensitized to

Table VI. *Intracytoplasmic flow cytometric analysis of spontaneous and LPS-induced IL-12 expression by APC isolated from the lungs of mice at day 9 of the aerosolization protocol*[a]

	NoRx		CS	
	Medium	LPS	Medium	LPS
$MHCII^+$ $CD11c^+$ gate	7.8	25	7.0	19
$MHCII^+$ $CD11b^+$ gate	6.9	15	7.0	13

[a] Groups of mice were mucosally sensitized to OVA in the presence (CS) or absence (NoRx) of concurrent steroid treatment and sacrificed 24 h after the ninth OVA exposure (day 9). Dispersed mononuclear cells from lungs were pooled and cultured at a density of 2.0×10^6 cells/ml in medium alone or in the context of LPS stimulation. Cells were harvested after an 8-h incubation, and IL-12 was detected by intracytoplasmic flow cytometry in gated $MHCII^+$ $CD11c^+$ and $MHCII^+$ $CD11b^+$ populations. Data are expressed as the fraction (percent) of gated cells coexpressing IL-12. The analysis is based on the collection of 100,000 events in the mononuclear cell gate and is representative of two independent experiments: $n = 6$–9 (pooled)/group.

OVA in the context of CS treatment (Fig. 5 and Table IV). Likewise, titers of OVA-specific IgE in serum, which had been twice as high as controls at day 9, were now 3-fold higher in rechallenged mice whose initial exposure to OVA was conditioned by CS (Fig. 6*B*); peripheral blood eosinophilia was also elevated in these mice (Table V). Thus, we observed an uncoupling of the long-term local and systemic effects of acute CS intervention: although treatment with CS during sensitization, despite unequivocal efficacy acutely, did not affect the inflammatory or physiological response in the airway upon OVA rechallenge, the potentiated systemic allergic response associated with CS delivery persisted and was even further polarized upon subsequent Ag exposure. This discordance may reflect the nature of our rechallenge protocol, which involves only three consecutive OVA exposures; perhaps a protracted protocol or one involving a tertiary challenge (i.e., OVA exposure following the resolution of inflammation during the initial recall) would have detected subtle changes in airway inflammation, although detailed kinetic studies are beyond the scope of this article.

These results are particularly intriguing in the light of experimental evidence and published clinical precedent. Several groups have concluded that CS strongly potentiate IL-4-induced IgE synthesis by PBMC in vitro (25–28), such that supplementation of culture medium with CS actually supplants the need for concomitant T cell help in IgE isotype switching (29). Using B cells from allergic patients, Bohle et al. (30) have shown that this CS-mediated IgE production is polyclonal and cannot be attributed to the selective enhancement of allergen-specific IgE. Clinically, a number of studies, some dating back several decades, have noted that therapeutically efficacious CS intervention is not associated with the reversal of seasonal, allergen-provoked increases in serum IgE (31, 32). Others have even reported an association between treatment with CS and a subsequent, if transient, elevation in serum IgE titer in asthma/allergy patients (33, 34) but not healthy volunteers (35). Although these studies do not impute an association between CS-induced changes in IgE and an exacerbation of the particular atopic condition responsible for a patient's allergic manifestations, it is significant that the salient finding in these clinical reports, namely, that IgE is elevated in allergy patients following steroid treatment, mirrors the experimental findings we have documented systematically in mice.

Of considerable interest is the immunological mechanism that might account for steroid-mediated potentiation of systemic Th2-polarized immunity. That we observed an irreversible hyperpolarization of T cell responses and sustained elevation of OVA-specific IgE intimates CS-mediated modulation of the APC compartment, details of which have been examined in a number of primarily in vitro studies. Although local steroid treatment predictably impairs the recruitment of APC to, for instance, the nasal mucosa in patients with allergic rhinitis (36, 37), there is less consensus on the implications of CS for APC function. Depending on the developmental status of APC and the culture conditions in which they are propagated, CS can retard differentiation of dendritic cells from monocytic (38, 39) and $CD34^+$ precursors (40), and can also impair the terminal maturation and costimulatory capacity of monocytes in the presence of IFN-γ (41) and of a dendritic cell line in the presence of T cells or LPS (42). In contrast, Vanderheyde et al. (43) have shown that CD40 ligand-dependent maturation of in vitro-derived dendritic cells is unaffected by concurrent CS treatment and that GM-CSF-dependent up-regulation of MHCII on airway dendritic cells (44) or alveolar macrophages (45) is not attenuated in the presence of therapeutic doses of CS. In general, then, CS may be understood to arrest APC differentiation and function at stages upstream of Ag presentation, effects that are therefore obviated when APC are matured in the presence of potent activatory signals (18, 44, 46).

Our own in vivo data generally concur with these in vitro observations. Flow cytometric analysis of dispersed lung cells from CS-treated mice revealed no diminution of the proportional expansion of both dendritic cells and macrophages during allergic sensitization (Fig. 8*A*). Likewise, expression of the costimulatory molecules B7.1 and B7.2 by these APC were comparable to levels detected on cells from untreated controls (Fig. 8*B*). The differentiation of a phenotypically mature APC compartment in the context of CS intervention likely reflects the continued presence of exogenous GM-CSF, the expression of whose receptor is enhanced by CS (47); CS, therefore, may establish a highly permissive environment for sensitization in our model (48–50). This interpretation explains why transient CS delivery to mice during sensitization neither impaired allergic sensitization nor mitigated the memory response in the absence of further CS treatment; it does not, however, decisively account for the potentiation of systemic features of Th2 immunity in the presence of CS. To this end, a number of investigators have argued that the ability of glucocorticoids to extinguish IL-12 secretion by APC (20, 21, 23) accounts for impaired Th1, and therefore enhanced Th2, cytokine secretion by T cells activated in vitro by CS-treated APC (16–19, 22). However, we observed no appreciable difference in the fraction of IL-12-producing APC in the lungs of mice treated with CS during sensitization compared with untreated controls (Table VI), suggesting that CS-mediated inhibition of APC-derived IL-12 may not account for the selective potentiation of Th2 immunity in vivo. Perhaps the observed hyperpolarization of Th2 cells and concomitant enhancement of IgE production in CS-treated mice reflects direct interaction of CS with glucocorticoid receptors in T cells (51, 52), which may directly suppress Th1 polarization (53).

Our study is, to our knowledge, the first to demonstrate experimentally in vivo that CS, while affording an outstanding therapeutic index for local allergic airway inflammatory responses during treatment, do not ameliorate the underlying immune pathology responsible for allergic disease and actually amplify significant features of the allergic response when administered to mice during sensitization. Although it is difficult to infer a direct comparison of local vs systemic bioavailability of budesonide in humans and mice or to ascertain what constitutes a high vs a low dose of CS in mice, the in vivo biological effects of CS documented in this study are unequivocal and are consistent with published findings in humans. Of course, extrapolation of our data in mice to human asthma must be tempered by an awareness of the limitations of the model; the mucosal model is just one of many experimental systems that simulate human asthma in mice and, like most of these

other models, it does not adequately mimic the chronicity or airway remodeling characteristic of human disease. These caveats notwithstanding, the clinical implications of this research are manyfold, but may have particular resonance in debates about when CS treatment should begin as an immune-inflammatory condition evolves. For instance, evidence that early and sustained intervention with steroids may forestall some of the irreversible airway dysfunction in asthma patients (54–56) would seem to recommend early initiation of steroid treatment (1). In contrast, our own data would caution against wholesale endorsement of such a therapeutic strategy, as CS treatment, particularly during sensitization, might accentuate features of the underlying immune pathology. It is certainly germane to distinguish our model, in which treatment occurs during sensitization, from clinical praxis, in which steroid treatment begins in already sensitized patients presenting asthmatic symptoms. However, it is by no means clear that allergic sensitization is a remote, immunologically hermetic, temporally discrete event impervious to subsequent alteration. If sensitization is an ongoing process, interventions that have the potential to modify sensitization should be evaluated even in patients with established disease.

Steroids should, and undoubtedly will, continue to be the treatment of choice for chronic inflammatory processes such as asthma; the kinetics of optimal pharmaceutical intervention, however, remain an open question. We hope that this study will encourage clinical practitioners to titrate CS treatment to minimal effective doses, to avoid overtreatment of asthma with steroids, and to include additional immunological metrics, such as serum IgE measurements or PBMC responses in vitro, as they investigate the advantages of early or prolonged steroid treatment in the management of allergic airway inflammatory responses.

Acknowledgments

The secretarial assistance of Mary Kiriakopoulos and the technical support of Joanna deJong are gratefully acknowledged.

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—CHAPTER 3—

Evaluation of ICOS/B7RP-1 as a Therapeutic Target in a Murine Model of Allergic Airway Inflammation

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Published in *American Journal of Respiratory Cell and Molecular Biology* 28:722-730 (2003)

Evaluation of Inducible Costimulator/B7-Related Protein-1 as a Therapeutic Target in a Murine Model of Allergic Airway Inflammation

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Given its primary role in the execution of T cell, and especially Th2, effector activity, the inducible costimulator (ICOS)/B7-related protein (RP)-1 costimulatory pathway is currently being heralded as a promising therapeutic target for immune-inflammatory disorders such as asthma. This study investigates the merits of ICOS blockade in a murine model of experimental asthma in which mice are sensitized to ovalbumin (OVA) through the respiratory mucosa. Intraperitoneal treatment of mice with anti-ICOS neutralizing antibody during sensitization resulted in a marked reduction in airway eosinophilia and IL-5 in bronchoalveolar lavage, but had no effect on interleukin (IL)-4, IL-13, and eotaxin content in bronchoalveolar lavage or the production of OVA-specific immunoglobulin E in serum. Cultured splenocytes from mice sensitized to OVA in the context of ICOS ablation produced enhanced levels of IL-4 and IL-5 upon stimulation with OVA, and this correlated with elevated inflammation and immunoglobulin E secretion upon long-term *in vivo* OVA recall; the deleterious effects ICOS blockade, however, were not associated with reduced IL-10 production by splenocytes. Peculiarly, anti-ICOS intervention during OVA rechallenge had no effect on airway inflammation or immunoglobulin production, despite high levels of ICOS expression on infiltrating CD4+ T cells. This study provides *in vivo* evidence of an exacerbated long-term immune-inflammatory response following acute ICOS blockade, and suggests that ICOS costimulation is functionally redundant in established allergic disease.

The premise that activation and differentiation of naive T cells critically require the delivery of two signals—cognate interaction between the T cell receptor and the peptide:MHC complex on antigen-presenting cells (APC), and engagement of CD28 by B7 molcules—has been instrumental to our understanding of the initiation and regulation of immune responses. The therapeutic applications of this paradigm have been vigorously investigated, with intervention strategies proposed for transplantation (1–3), cancer (4, 5), and some immune-inflammatory disorders (3, 6, 7). However, CD28:CD80/86-mediated costimulation appears to be essentially redundant for T cell effector function, suggesting that CD28 blockade may have limited value in established disease (8). It is for this reason that a number of recently-described costimulatory pathways, including inducible costimulator (ICOS)/B7-related protein (RP)-1 (9–15) and PD-1/B7H-1 (16, 17), have received considerable attention as a potential avenue to ameliorate established, T cell–mediated inflammatory disorders.

In this regard, ICOS, the third member of the CD28/CTLA-4 superfamily and the receptor for B7RP-1, has emerged as a costimulatory pathway with compelling therapeutic promise (18–20). ICOS/B7RP-1 has been characterized primarily as a costimulatory pathway that orchestrates events downstream of T cell activation (11, 21–23), including humoral immunity (24–27). ICOS is distinguished in particular by its association with Th2 effector activity (11, 21, 27–29) and, as such, ICOS has received attention in models of asthma and allergic airway inflammation (30). Indeed, inhibition of ICOS function during antigen challenge has been shown to attenuate eosinophil accumulation in the airway, and immunoglobulin (Ig)E production and cytokine content in bronchoalveolar lavage (BAL) in models of antigen-induced allergic airway inflammation (31, 32). Importantly, ICOS blockade does not alter the course of allergic sensitization in these models, indicative of the putative role of ICOS in the elaboration of effector activity in previously differentiated T cells.

Sperling and colleagues (19) have speculated that ICOS/B7RP-1 may represent a costimulatory pathway with unique therapeutic advantages. Unlike CD28/B7 costimulation, whose inhibition may lead to generalized immunosuppression, ICOS/B7RP-1 activity is apparently restricted to activitated, differentiated T cells; by extension, its suppression may preferentially modulate the established responses characteristic of immune-inflammatory disorders. However, although intriguing, this therapeutic postulate has not been investigated comprehensively, as most studies to date have examined the effects of ICOS inhibition during incipient stages of experimental disease. We therefore executed this study of ICOS function in two models of antigen-induced airway inflammation in which mice were mucosally sensitized to aerosolized ovalbumin (OVA) in the context of a granulocyte macrophage–colony-inducing factor (GM-CSF)– or GM-CSF and interleukin (IL)-12–enriched airway microenvironment, which elicit prototypic Th2- (33) and Th1-polarized (34) immune-inflammatory responses, respectively. We report three key findings: (*i*) ICOS blockade

(*Received in original form October 18, 2002 and in revised form December 11, 2002*)

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Abbreviations: adenovirus, Ad; B7-related protein-1, B7RP-1; bronchoalveolar lavage, BAL; enzyme-linked immunosorbent assay, ELISA; granulocyte-macrophage colony-stimulating factor, GM-CSF; Hanks' balanced salt solution, HBSS; inducible costimulator, ICOS; immunoglobulin E, IgE; interleukin, IL; monoclonal antibody, mAb; ovalbumin, OVA; phosphate-buffered saline, PBS.

Am. J. Respir. Cell Mol. Biol. Vol. 28, pp. 722–730, 2003
DOI: 10.1165/rcmb.2002-0220OC
Internet address: www.atsjournals.org

during acute OVA exposure inhibits Th2- but not Th1-associated inflammation in the airway; however, (*ii*) inhibition of ICOS does not suppress Th2-polarized sensitization to OVA and ultimately exacerbates the response to OVA recall *in vivo*; and (*iii*) therapeutic neutralization of ICOS during OVA recall in mice with established allergic disease does not ameliorate allergic airway inflammation.

Materials and Methods

Animals

Female Balb/c mice (6–8 wk old) were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed under specific pathogen–free conditions and maintained on a 12 h light-dark schedule. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada). A total of 322 mice were killed during the course of these experiments.

Models of Antigen-Induced Airway Inflammation

Mice were subjected to our Th1- or Th2-polarizing protocols as previously described (33, 34). Briefly, mice were exposed to aerosolized ovalbumin in the context of an airway microenvironment conditioned by GM-CSF (Th2 model) or GM-CSF and IL-12 (Th1 model). To elicit local expression of GM-CSF or IL-12, a replication-deficient human type 5 adenoviral (Ad) construct encoding murine GM-CSF or IL-12 cDNA in the E1 region of the viral genome was delivered intranasally to isoflurane-anesthetized animals on Day −1, 24 h before the first exposure to OVA. Ad/GM-CSF and Ad/IL-12 were administered at doses of 3×10^7 and 1×10^7 pfu, respectively, in a total volume of 30 μl of phosphate-buffered saline (PBS) vehicle. Over a period of seven consecutive days (Days 2–8), mice were placed in a Plexiglas chamber (10 cm × 15 cm × 25 cm) and exposed for 20 min daily to aerosolized OVA (1% wt/vol in 0.9% saline; Sigma-Aldrich, Oakville, ON). The OVA aerosol was generated by a Bennett nebulizer at a flow rate of 10 liters/min. For the long-term *in vivo* rechallenge experiments, sensitized mice were allowed to recover from acute inflammation (~ 4 wk) and were then exposed to a 1% OVA aerosol for 20 min on three consecutive days (Days 0–2).

In Vivo Neutralization of ICOS Activity

ICOS fusion protein (ICOS-Ig) (11) or anti-ICOS neutralizing monoclonal antibody (mAb) 12A8 (31, 36) were administered intravenously or intraperitoneally at a dose of 100 μg in 100 μl or 500 μl PBS, respectively, on Days 2, 4, 6, and 8 of the aerosolization protocol. To assess the therapeutic potential of ICOS ablation in sensitized mice, 100 μg anti-ICOS in 500 μl PBS was administered intraperitoneally on each of Days 0, 2, and 4 of the long-term *in vivo* rechallenge protocol. An equivalent dose of human IgG (control Ig) (Sigma-Aldrich) was used as a control for all experiments.

Collection and Measurement of Specimens

Twenty-four hours after the seventh OVA exposure (Day 9), or 72 h after the third OVA exposure during long-term *in vivo* recall (Day 5 of the rechallenge protocol), mice were killed and BAL was obtained as previously described (35). In brief, the lungs were dissected and the trachea cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation, supernatants were stored at −20°C for measurement of cytokines by enzyme-linked immunosorbent assay (ELISA); cell pellets were resuspended in PBS and smears were prepared by cytocentrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. Diff-Quik (Baxter, McGraw Park, IL) was used to stain all smears. Differentiation of leukoctye subsets in BAL was determined by counting at least 500 white blood cells using standard hemocytologic procedures to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Blood smears were prepared from peripheral blood collected in heparinized capillary tubes; leukocytes were differentiated by counting at least 300 white blood cells.

Cytokine and Immunoglobulin Measurement

ELISA kits for IL-4, IL-10, IL-13, and eotaxin were purchased from R&D Systems (Minneapolis, MN), and the kit for IL-5 was obtained from Amersham (Buckinghamshire, UK); each of these systems has a threshold of detection of 1.5 to 5 pg/ml. Levels of OVA-specific IgE were detected using an antigen-capture (biotinylated OVA) ELISA method as described (35); anti-mouse IgE antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). This ELISA was standardized with serum obtained from mice sensitized to OVA according to a conventional intraperitoneal sensitization model and bled 7 d following the second sensitization (35); immunglobulin levels, therefore, are expressed in units (U)/ml relative to this standard serum.

Splenocyte Culture

Spleens were triturated between the frosted ends of glass slides to disperse mononuclear cells; the resulting cell suspension was filtered through nylon mesh. Red blood cells (RBC) were lysed by resuspending dispersed cells from each spleen in 1 ml ACK lysis buffer for 1 min. Splenocytes were then washed twice in supplemented RPMI (containing 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 0.1% mercaptoethanol) and cultured in 96-well plates at a density of 8×10^5 cells/well in a total volume of 200 μl RPMI. Cells were stimulated with OVA at a concentration of 40 μg/well for 5 d, at which point supernatants were harvested and stored at −20°C for detection of cytokines.

Lung Cell Isolation and Flow Cytometric Analysis of Lung Cell Subsets

Lungs were perfused with 10 ml Hanks' balanced salt solution (HBSS) through the right ventricle, cut into small (~ 2 mm diameter) pieces and agitated at 37°C for 1 h in 15 ml collagenase III (Life Technologies, Rockville, MD) at a concentration of 150 U/ml in HBSS. Using the plunger from a 5-ml syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon membrane. Mononuclear cells were isolated at the interphase between layers of 30% and 60% Percoll following density gradient centifugation. Cells were washed twice and stained for flow cytometric analysis. For each antibody combination, 1.0×10^6 cells were incubated with monoclonal antibodies at 0–4°C for 30 min; the cells were then washed and treated with second stage reagents. Data were collected using a FACScan (Becton Dickinson, Sunnyvale, CA) for three-color flow cytometry or a FACSCalibur (Becton Dickinson,

Sunnyvale, CA) for four-color flow cytometry, and were analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA). The following antibodies and reagents were used: hamster IgG anti-mouse CD3ε, PE-conjugated and Cy-Chrome–conjugated (145–2C11); rat IgG_{2a} anti-mouse CD4, FITC-conjugated, biotinylated, and APC-conjugated (RM4–5); rat IgG_{2b} anti-mouse CD44, FITC-conjugated (IM7); rat IgG_{2a} anti-mouse CD62L, PE-conjugated (MEL-14) (all purchased from BD PharMingen, San Diego, CA); rat IgG_1 anti-mouse T1/ST2, PE-conjugated; rat IgG_{2b} anti-mouse ICOS, biotinylated (both produced in-house by Millennium Pharmaceuticals, Cambridge, MA); all appropriate isotype control antibodies, Streptavidin PerCP, and Streptavidin Cy-Chrome (BD PharMingen). The antibodies were titrated to determine optimal concentration.

Data Analysis

Data are expressed as mean ± SEM, unless otherwise indicated. Results were interpreted using Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

Results

ICOS Ablation during Sensitization Inhibits Th2- but Not Th1-Polarized Inflammation in BAL, but Has No Effect on Production of OVA-Specific IgE

To investigate the function of ICOS in antigen-induced airway inflammation, and to ascertain the T helper specificity of ICOS activity, we administered ICOS fusion protein (ICOS-Ig), anti-ICOS neutralizing antibody or control Ig systemically to mice undergoing Th1- or Th2-polarized sensitization to aerosolized OVA; as previously reported, the anti-ICOS (12A8) mAb antagonizes the association of ICOS with its ligand and does not deplete $ICOS^+$ cells (36). On Day −1, mice were injected intranasally with 3×10^7 pfu Ad/GM-CSF with (Th1 model) or without (Th2 model) concurrent instillation of 1×10^7 pfu Ad/IL-12; from Days 2–8 inclusive, mice were exposed daily to aerosolized OVA, and then killed on Day 9. In untreated mice (i.e., sensitized in the absence of concurrent control Ig or ICOS intervention; data not shown), and as previously reported (33, 34), this regimen resulted in robust accumulation of mononuclear cells and eosinophils (Th2 model) or neutrophils (Th1 model) in BAL—an inflammatory profile that was unaltered in mice treated intravenously (Figures 1A and 1B) or intraperitoneally (Figure 1C) with control Ig. In the Th2 model, neutralization of ICOS—either through intravenous administration of ICOS-Ig (Figure 1B) or intraperitoneal injection of anti-ICOS (Figure 1C)—attenuated the accumulation of eosinophils in BAL by 50–75% compared with control Ig–treated mice, a result consistent with the documented participation of ICOS in Th2 effector function and allergic airway inflammation. In contrast, ablation of ICOS activity in mice undergoing Th1-privileged sensitization to OVA had no effect on mononuclear cell or neutrophil accumulation in BAL (Figure 1A); this confirmed the putative association of ICOS activity with Th2-dominant processes, and justified the exclusion of the Th1 model from further investigation. No consistent changes were observed following ICOS intervention with respect to neutrophils in the Th2 model, in which neutrophil accumulation is comparably small

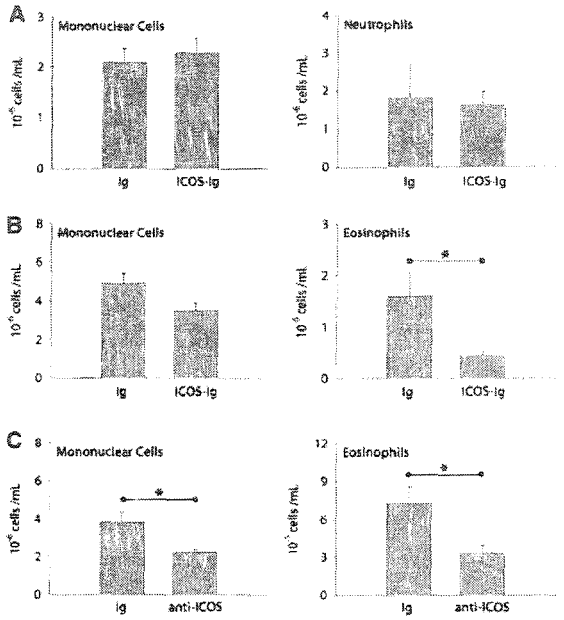

Figure 1. Effect of ICOS neutralization on Th1- or Th2-polarized inflammation in BAL at Day 9 of the aerosolization protocol. Mice were exposed repeatedly to OVA in the context of a GM-CSF (*B* and *C*) or GM-CSF/IL-12 (*A*) airway milieu to elicit Th2- or Th1-polarized immune-inflammatory responses, respectively. One hundred micrograms of ICOS-Ig, anti-ICOS, or Ig control were administered intravenously (ICOS-Ig) or intraperitoneally (anti-ICOS) on Days 2, 4, 6, and 8 of the aerosolization protocol; mice were killed at Day 9. Graphs describe mononuclear cell, neutrophil (*A*), and eosinophil (*B* and *C*) accumulation in BAL. Data are expressed as mean ± SEM; $n = 3$–4 (*A* and *B*) or 7–10 (*C*) per group; $^*P < 0.05$ by Student's *t* test compared with Ig control.

(33), or eosinophils in the Th1 model, in which eosinophils are virtually absent (34). It should be noted that, to be exhaustive, we have presented data in Figure 1 from both ICOS-Ig– and anti-ICOS–treated mice, thereby validating the similar effects of these two well-documented reagents in our model; wishing to avoid redundancy in our experimental design, we included only anti-ICOS intervention in subsequent studies.

The effects of anti-ICOS intervention on local inflammatory phenomena can, in part, be understood in terms of local cytokine production and systemic changes in peripheral blood. Table 1 displays the leukocyte profile in peripheral blood, OVA-specific IgE in serum, and levels of key cytokines and chemokines in BAL at Day 9 of the aerosolization protocol following intraperitoneal treatment of mice with anti-ICOS neutralizing antibody. Whereas concentrations of IL-4, IL-13, and the IL-4/IL-13–inducible chemokine eotaxin remain unaltered in mice treated with anti-ICOS, levels of IL-5 have been reduced by ∽ 50%—a degree of inhibition comparable to that observed for eosinophil accumulation in BAL, and indicative of impaired activation

TABLE 1

Peripheral blood leukocytes, OVA-specific IgE in serum, and cytokines/chemokines in BAL following inhibition of ICOS during sensitization (Day 9)

	Control Ig	Anti-ICOS
Peripheral blood leukocytes		
mononuclear cells	255 ± 36	214 ± 55
eosinophils	7.9 ± 2.5	15 ± 3.3
Cytokines*, chemokines*, and immunoglobulins†		
OVA-specific IgE	29 ± 2.2	27 ± 2.2
IL-4	47 ± 9.0	46.8 ± 15.1
IL-5	212 ± 48	121 ± 13‡
IL-13	948 ± 323	980 ± 82
Eotaxin	76 ± 18	105 ± 5.7

Mice were treated during sensitization with control Ig or anti-ICOS and killed on Day 9 of the aerosolization protocol. Leukocytes in peripheral blood were differentiated by standard hemocytometric analysis of blood smears and are displayed as 10^{-3} cells/ml. Cytokines, chemokines, and immunoglobulins were detected by ELISA in *BAL or †serum and are expressed in *pg/ml or †U/ml. Data represent mean ± SEM; $n = 4$–10 per group, pooled from two independent experiments; ‡$P < 0.05$ by Student's t test compared to Ig control.

or mobilization of eosinophils from bone marrow. It should be noted, however, that ICOS blockade had no statistically significant effect on eosinophil or mononuclear cell content in peripheral blood, suggesting a deficiency in leukocyte migration across the vascular endothelium into the lung parenchyma rather than attenuated bone marrow eosinopoeisis.

ICOS Ablation Inhibits the Recruitment of Th2 Cells to the Lung but Does Not Impair the Maturation of a Th2 Phenotype *In Vivo*

To examine the effects of anti-ICOS intervention on $CD4^+$ T cells, flow cytometric analysis was performed on dispersed cells from the lungs of mice killed on Day 9 of the aerosolization protocol. That ICOS is upregulated in our model of allergic airway inflammation, and that intraperitoneal administration of anti-ICOS extinguishes endogenous ICOS expression, are documented in the *upper panel* of Figure 2; indeed, although ICOS was detected on ∽ 19% of $CD3^+CD4^+$ cells from the lungs of Ig-treated and untreated (data not shown) mice, ICOS was expressed by only ∽ 6% of $CD4^+$ T cells following anti-ICOS intervention—a level comparable to that observed in naive mice. Consistent with the putative role of ICOS in Th2 effector function, it is noteworthy that the fraction of $CD4^+$ T cells expressing ICOS in Ig-treated mice is similar to that of T1/ST2 (16%), a prototypic marker of Th2 effector cells (37–39). In this regard, a modest reduction in expression of T1/ST2 by lung-derived T helper cells in anti-ICOS–treated mice suggests impaired influx, rather than differentiation, of effector Th2 cells, as four-color flow cytometric analysis of $CD4^+$ T cells indicates a preferential amplification of the $CD44^{lo}CD62L^{hi}$ (naive) phenotype, and a concomitant depression of $CD44^{hi}CD62L^{lo}$ (memory/effector) T cell infiltration (Table 2). Moreover, analysis of ICOS expression on naive and memory/effector $CD4^+$ T cells is instructive: although ∽ 46% of $CD4^+CD44^{hi}CD62L^{lo}$ cells coexpress ICOS, it is virtually undetectable on $CD4^+CD44^{lo}CD62L^{hi}$ cells, entirely consistent with the proposed role of ICOS in the elaboration of T cell effector activity (Table 2).

That Th2 differentiation is intact following anti-ICOS intervention is illustrated in Figure 3, which documents spontaneous and OVA-stimulated IL-4 and IL-5 production by cultured splenocytes from mice killed on Day 9 of the aerosolization protocol. Indeed, splenocytes from mice treated with anti-ICOS *in vivo* liberate significantly *higher* levels of IL-4 and IL-5 than cells from Ig-treated mice, both in the context of OVA and, in the case of IL-5, constitutively (medium alone). Similar trends were observed for IL-13 in culture supernatants (data not shown). These results corroborate previous reports of preserved Th2 differentiation in the absence of ICOS activity, and suggest that ablation of ICOS may in fact exaggerate Th2 polarization *in vivo*.

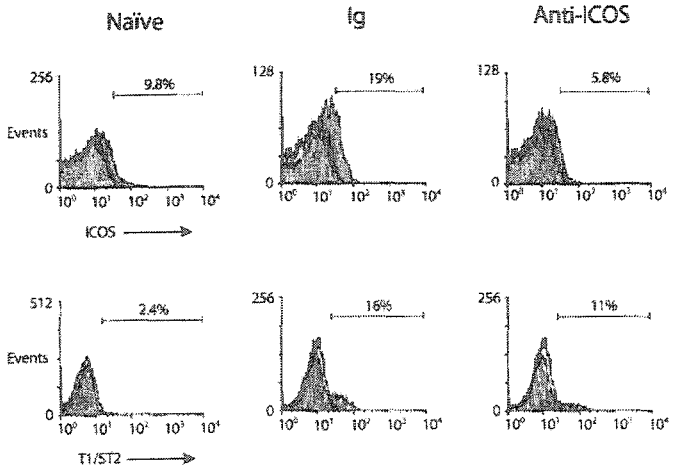

Figure 2. Flow cytometric analysis of T cells from the lungs of anti-ICOS–treated mice acutely exposed to OVA. Mice were sensitized to OVA according to the Th2-polarizing regimen, treated with anti-ICOS or control Ig intraperitoneally between Days 2 and 8, and killed at Day 9. Enriched mononuclear cells from lung tissue were pooled from each group and stained for flow cytometry. Data depict ICOS (*upper panels*) or T1/ST2 (*lower panels*) expression on gated $CD3^+CD4^+$ cells from naive (untreated, unsensitized) mice and mice treated with control Ig or anti-ICOS concurrent with OVA exposure. 50,000 events were collected in the mononuclear cell gate; $n = 5$ per group.

TABLE 2

Four-color flow cytometric analysis of ICOS expression on naive and memory/effector T cell populations in lung tissue at Day 9 of the aerosolization protocol

	Naive T cells		Memory/Effector T cells	
	$CD4^+$ gate $CD44^{lo}CD62L^{hi}$	$CD44^{lo}CD62L^{hi}$ gate $ICOS^+$	$CD4^+$ gate $CD44^{hi}CD62L^{lo}$	$CD44^{hi}CD62L^{lo}$ gate $ICOS^+$
Control Ig	32	3.4	54	46
Anti-ICOS	53	3.9	37	16

Mice were treated with anti-ICOS or control Ig during sensitization and killed on Day 9 of the aerosolization protocol. Mononuclear cells were isolated and pooled from the lungs of five mice per group and stained with anti-CD4, anti-CD44, anti-CD62L, and anti-ICOS antibodies. Data in column 1 represent the fraction of $CD4^+$ cells expressing a $CD44^{lo}CD62L^{hi}$ (naive) or $CD44^{hi}CD62L^{lo}$ (memory/effector) phenotype; data in column 2 indicate the proportion of $CD4^+CD44^{lo}CD62L^{hi}$ or $CD4^+CD44^{hi}CD62L^{lo}$ cells expressing ICOS. A total of 50,000 events were collected in the mononuclear cell gate.

ICOS Impairment during Sensitization Exacerbates the Long-Term Immune-Inflammatory Response to OVA *In Vivo*

To date, reports of ICOS function have generally examined events during antigen sensitization and acute antigen exposure, and have therefore not addressed the long-term consequences of ICOS/B7RP-1 intervention *in vivo*. To elucidate the long-term impact of anti-ICOS delivery during sensitization to OVA, groups of mice were permitted to recover from acute inflammation in the lungs (~ 4 wk) and were then rechallenged with aerosolized OVA. In untreated (data not shown) and Ig-treated mice, *in vivo* recall elicited robust mononuclear cell and eosinophil infiltration into the airway at Day 5 of the rechallenge protocol (Figure 4); there was also evidence of a matured humoral response, with elevated levels of OVA-specific IgE detected in serum (~ 10-fold higher than levels at Day 9) (Figure 4). Not unexpectedly, these phenomena were not impaired in mice sensitized to OVA in the context of ICOS ablation—unequivocal evidence that ICOS is not required for Th2 differentiation (Figure 4). On the contrary, and consistent with the trend toward higher spontaneous and OVA-stimulated cytokine production by splenocytes from anti-ICOS–treated mice both at Day 9 (Figure 3) and following *in vivo* rechallenge (Table 3), intervention with anti-ICOS during sensitization actually *exacerbated* mononuclear and eosinophilic inflammation and IgE production upon *in vivo* recall, resulting in levels that were 50–100% higher than those observed in Ig-treated mice (Figure 4).

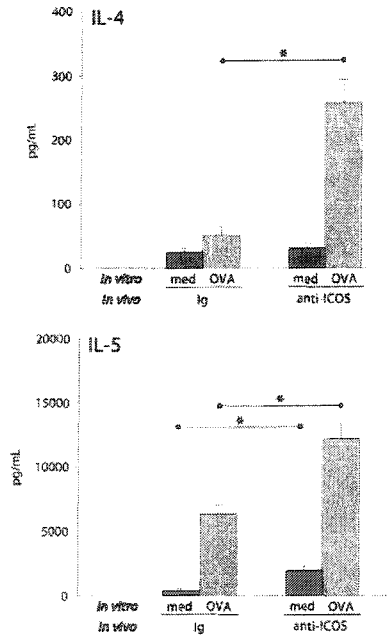

Figure 3. IL-4 and IL-5 production by cultured splenocytes from mice acutely exposed to aerosolized OVA in the context of ICOS ablation. Mice were sensitized to OVA under Th2-polarizing conditions, treated with anti-ICOS or control Ig intraperitoneally between Days 2 and 8, and killed at Day 9 of the aerosolization protocol. Splenocytes were cultured for 5 d in medium alone (med) or with OVA stimulation (OVA); IL-4 and IL-5 content in supernatants was detected by ELISA. Cells were plated at a density of 8×10^5 cells/well. Data are expressed as mean ± SEM; $n = 7$–12 per group; $^*P < 0.05$ by Student's t test compared with Ig control.

To investigate whether the deleterious long-term effects of ICOS blockade were related to impaired T cell immunoregulatory function, IL-10 was measured in supernatants from cultured splenocytes harvested either at Day 9 of the aerosolization protocol or following *in vivo* rechallenge (Table 4). Interestingly, spontaneous and OVA-specific IL-10 production was generally enhanced in splenocytes from mice treated with anti-ICOS during sensitization, irrespective of the time of harvesting.

Therapeutic Delivery of Anti-ICOS to Mice with Established Allergic Airway Disease Has No Effect on Airway Inflammation or IgE Production During OVA Rechallenge

We next evaluated the therapeutic potential of ICOS attenuation in mice with an established allergic phenotype. We therefore sensitized mice to OVA in the absence of anti-ICOS intervention and, following the resolution of acute inflammation, rechallenged mice in the context of ICOS blockade. Whether anti-ICOS was administered intraperitoneally on two occasions (the first and last challenges on Days 0 and 2, respectively) or on three occasions (Days 0, 2 and 4), mice mounted an equivalent inflammatory re-

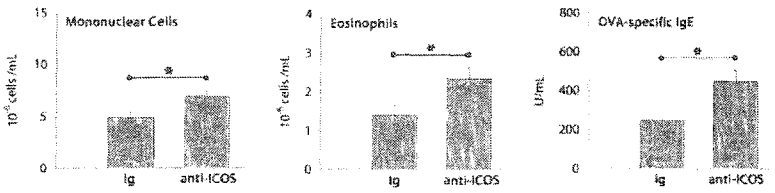

Figure 4. Effect of ICOS neutralization during sensitization on inflammation in BAL and IgE in serum upon long-term antigen rechallenge. Mice were exposed repeatedly to OVA in the context of a Th2-polarizing airway microenvironment and were concurrently treated with 100 μg anti-ICOS or control Ig intraperitoneally on each of Days 2, 4, 6, and 8 of the aerosolization protocol. Acute inflammatory phenomena were allowed to resolve (∽ 4 wk) before mice were rechallenged with OVA. Graphs depict mononuclear cell and eosinophil accumulation in BAL, and OVA-specific IgE content in serum 72 h after the final OVA recall challenge (Day 5). Data are expressed as mean ± SEM; n = 7–9 per group; $^*P < 0.05$ by Student's t test compared with Ig control.

sponse in BAL and produced similar levels of OVA-specific IgE in serum compared with untreated (data not shown) and Ig-treated mice (Figure 5). This observation is particularly striking given the robust degree (∽ 39%) of ICOS expression on $CD4^+$ T cells from the lungs of control mice, and its virtual nondetection in anti-ICOS–treated mice, according to flow cytometric analysis (Figure 6). This observation, coupled with the unaltered distribution of T1/ST2 on T helper cells from mice rechallenged in the context of anti-ICOS intervention, would suggest that ICOS expression is redundant for both the recruitment and function of T cells in mice with established allergic airway disease.

Discussion

The recent identification of new costimulatory pathways with specialized effector or inhibitory activities has afforded novel, prospective therapeutics for a variety of immunologic disorders. The ICOS/B7RP-1 axis, in particular, has received attention based on its putative role in T cell effector function (11, 21–23), which may predispose it to modulation that selectively blocks the *expression of disease* rather than indiscriminately impairing the *development of an adaptive response* generally. In this vein, ICOS/B7RP-1 represents a costimulatory target of therapeutic relevance to *established* disease; unlike the CD28/CD80/CD86 system, which is critically engaged during the initial stages of antigen presentaton and T cell activation, ICOS/B7RP-1 may be indispensable to the execution of an immunologic program during periods of antigen challenge (19). Here we have appraised this hypothesis in a murine model of allergic airway inflammation in which ICOS/B7RP-1 costimulation has been discretely impaired during mucosal allergic sensitization or during *in vivo* antigen recall in mice with established allergic disease.

Consistent with the observations of others (31, 32), we have shown that blockade of the ICOS/B7RP-1 pathway in mice during initial encounter with antigen (OVA) attenuates Th2-polarized allergic airway inflammation, but does not alter systemic features of sensitization. In this model of mucosal allergic sensitization, mice are exposed to an OVA aerosol in the context of airway GM-CSF expression; this results in a marked eosinophilic infiltrate in the airway after ∽ 10 d, accompanied by Th2 cytokines in the BAL, OVA-specific IgE in serum, and the production of prototypic cytokines by splenocytes stimulated with OVA (33). Compared to untreated or Ig-injected controls, concurrent intraperitoneal or intravenous treatment of mice with either anti-ICOS antibody or ICOS fusion protein during sensitization significantly reduced airway eosinophilia, and, in a complementary manner, IL-5 content in BAL at Day 9 of the protocol, but did not impair OVA-specific IgE or the capacity of splenocytes to yield Th2 cytokines. These findings argue persuasively for the preferential involvement of ICOS/B7RP-1 costimulation in the expression, rather than the genesis, of T helper phenotype, and generally concur with published findings in conventional murine models of antigen-induced allergic airway inflammation (which typically involve intraperitoneal delivery of OVA in the context of an adjuvant (31, 32)). Moreover, flow cytometric analysis of T cells at Day 9 demonstrates that ICOS is expressed in our model of antigen-induced airway inflammation, and that the neutralizing antibody used in our studies clearly affected its expression/detection. Whereas 15–20% of $CD4^+$

TABLE 3

IL-5 and IL-13 production by cultured splenocytes from mice treated with anti-ICOS during sensitization and rechallenged with OVA following the resolution of acute inflammatory events

	IL-5		IL-13	
	Medium	OVA	Medium	OVA
Control Ig	138 ± 138	6,742 ± 2,269	2,023 ± 693	17,223 ± 3,870
Anti-ICOS	1,757 ± 417*	10,765 ± 1,432	15,351 ± 2,337*	28,185 ± 2,960

Mice were treated with anti-ICOS or control Ig during sensitization and killed on Day 5 of the *in vivo* recall protocol. Splenocytes were harvested and cultured for 5 d at a density of 8×10^5 cells/well in medium alone or in the context of OVA stimulation. IL-5 and IL-13 were detected by ELISA in culture supernatants. Data represent mean ± SEM; n = 4–5 per group; $^*P < 0.05$ by Student's t test compared to Ig control.

TABLE 4
IL-10 production by cultured splenocytes from mice treated with anti-ICOS during sensitization

	Control Ig		Anti-ICOS	
	Medium	OVA	Medium	OVA
Acute				
Experiment 1	143 ± 71	766 ± 105	525 ± 82*	1,995 ± 263*
Experiment 2	76 ± 19	485 ± 108	514 ± 158*	1,361 ± 348
Experiment 3	145 ± 50	1,285 ± 170	323 ± 58*	2,072 ± 374
Rechallenge	92 ± 24	928 ± 48	418 ± 59*	1,536 ± 124*

Mice were treated with anti-ICOS or control Ig during sensitization and killed at Day 9 (Acute) in three separate but identical studies (Experiments 1–3) or on Day 5 of the *in vivo* recall protocol (Rechallenge). Splenocytes were harvested and cultured for 5 d at a density of 8×10^5 cells/well in medium alone or in the context of OVA stimulation. IL-10 was detected by ELISA in culture supernatants and is expressed in pg/ml. Data represent mean ± SEM; $n = 3$–6 per group; *$P < 0.05$ by Student's t test compared to Ig control.

T cells expressed ICOS in OVA-exposed control mice—an expression profile comparable to the distribution of the Th2 marker T1/ST2 (37–39)—ICOS was virtually undetectable on an equivalent population of cells in anti-ICOS–treated mice; as shown by Özkaynak and coworkers (36), who used the same antibody in a murine model of allograft rejection, this is indicative of receptor neutralization *in vivo* rather than depletion of $ICOS^+$ cells. Interestingly, that ICOS was detected on ~ 50% of $CD44^{hi}$ $CD62L^{lo}CD4^+$ cells of the memory/effector T cell compartment, but only on < 5% of $CD44^{lo}CD62L^{hi}CD4^+$ naive cells, in OVA-exposed mice at Day 9 is commensurate with its putative role in the effector activity of differentiated T cells.

Mechanistically, the effects of ICOS neutralization on acute eosinophilic inflammation in BAL reflect a diminution in the capacity of activated, polarized Th2 cells to execute their effector program upon cognate recognition of OVA in the airway. Indeed, that ICOS blockade failed to modify the magnitude or phenotype of airway inflammation in our model of Th1-polarized sensitization to OVA illustrates the Th2 selectivity of the ICOS pathway (34). Moreover, the ~ 50% reduction in IL-5 in the BAL of anti-ICOS–treated mice, coupled with unaltered eosinophil content in peripheral blood, suggests that the downstream effects of ICOS ablation may include reduced expression of cell-adhesion molecules or chemokine receptors by eosinophils, rather than impaired mobilization of eosinophils from the bone marrow. Of note, however—and in contrast to evidence of modest IL-5 inhibition—anti-ICOS–treated mice exhibited unaltered levels of IL-4, IL-13, and eotaxin, the prototypic eosinophil-recruiting chemokine, in BAL. It would seem, then, that the activity of ICOS in this model does not extend to all Th2 processes, but is restricted to the potentiation of particular proinflammatory phenomena.

That neutralization of ICOS attenuates allergic airway inflammation during sensitization and initial respiratory exposure to antigen, however, does not necessarily bear on the long-term consequences of ICOS ablation *in vivo*, nor on the potential of the ICOS/B7RP-1 pathway as a therapeutic target in established disease. To address this question, we examined the effects of anti-ICOS delivery on the long-term response (i.e., after the resolution of acute inflammation) to *in vivo* rechallenge with aerosolized OVA. As expected, intraperitoneal treatment with anti-ICOS during mucosal sensitization to OVA (acute exposure) did not inhibit airway eosinophilia upon OVA rechallenge (chronic exposure). This observation extends our *in vitro* findings, which demonstrate that ICOS blockade *in vivo* did not compromise T cell sensitization, and provides novel *in vivo* verification that the anti-inflammatory effects of ICOS/B7RP-1 neutralization are both transient and secondary to the development and maturation of an adaptive Th2-polarized response. In fact, and peculiarly, mice treated with anti-ICOS during sensitization presented evidence of an *exacerbated* allergic phenotype upon long-term rechallenge: airway eosinophilia and OVA-specific IgE in serum were both elevated (on the order of 50–100%) compared with Ig-treated controls. The mechanism by which impairment of ICOS during sensitization might magnify immune-inflammatory phenomena upon *in vivo* recall merits further

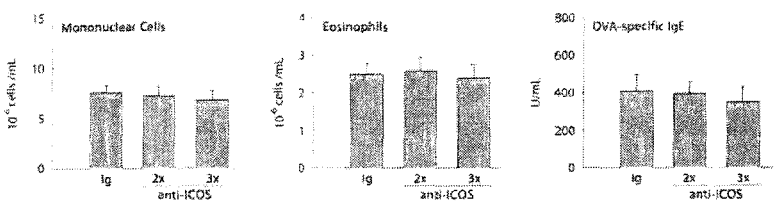

Figure 5. Effect of therapeutic anti-ICOS intervention on inflammation in BAL and IgE in serum during long-term antigen rechallenge of mice with established allergic airway disease. Mice were sensitized to aerosolized OVA in the context of a Th2-polarizing airway microenvironment. Acute inflammatory phenomena were allowed to resolve (~ 4 wk) before mice were rechallenged with OVA on Days 0–2 and concurrently treated with anti-ICOS or control Ig on Days 0 and 2 (2×) or Days 0, 2, and 4 (3×) of the *in vivo* recall protocol. Graphs display mononuclear cell and eosinophil accumulation in BAL, and OVA-specific IgE content in serum 72 h after the final OVA recall challenge (Day 5). Data are expressed as mean ± SEM; $n = 7$–10 per group.

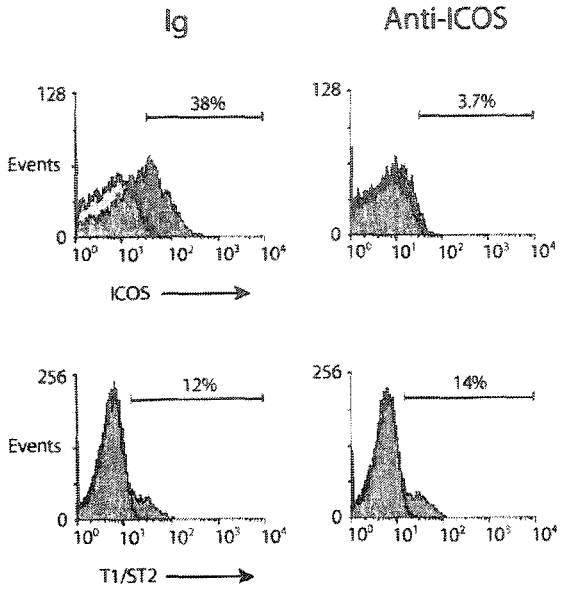

Figure 6. Flow cytometric analysis of T cells from the lungs of sensitized mice following long-term OVA rechallenge in the context of ICOS ablation. Mice were sensitized to OVA according to the Th2-polarizing regimen and rechallenged with OVA while receiving treatment with anti-ICOS or control Ig. Mice were killed at Day 5 of the *in vivo* recall protocol; lungs were homogenized and enriched mononuclear cells were pooled from each group and stained for flow cytometry. Data depict ICOS (*upper panels*) or T1/ST2 (*lower panels*) expression on gated $CD3^+CD4^+$ cells from mice treated with control Ig or anti-ICOS at the time of OVA rechallenge. 50,000 events were collected in the mononuclear cell gate; $n = 5$ per group.

study. However, that splenocytes from mice treated with anti-ICOS *in vivo* produce higher levels of IL-4 and IL-5 when stimulated with OVA is consistent with an hypothesis of T cell *hyperpolarization* or *dysregulation* in the absence of ICOS signaling. Likewise, reports of exacerbated clinical manifestations and accelerated mortality in mice treated with anti-ICOS during the sensitization phase of EAE (29) lend credence to the notion that ICOS function may not be restricted to the expression of an effector program in differentiated T cells, and may extend more broadly to T cell activation, education, and regulation. Indeed, as Akbari and colleagues (40) have recently demonstrated, ICOS–ICOS ligand interactions may be critically involved in the development of the IL-10–producing regulatory T cells that mediate inhalation tolerance and attenuate the expression of an asthma phenotype in mice. Although we found that the capacity of splenocytes to produce IL-10 was not impaired—and was generally enhanced—in mice treated with anti-ICOS during sensitization, suggesting that IL-10 deficiency is not responsible for the long-term effects of ICOS neutralization in our model, the possibility that the immunoregulatory activities of particular T cell subsets fail to mature in the absence of ICOS signaling is an hypothesis under active investigation in our laboratory.

Of particular therapeutic interest, and in striking contrast to observations in acutely-exposed mice, our studies unexpectedly show that intraperitoneal administration of anti-ICOS *during* long-term rechallenge of sensitized mice does not alter the magnitude or phenotype of lung inflammation, or the content of OVA-specific IgE in serum. The inefficacy of this treatment, moreover, cannot be attributed to kinetics of ICOS expression: ~ 40% of $CD4^+$ T cells isolated from the lungs of control mice co-expressed ICOS during *in vivo* OVA recall—a marked amplification of levels observed at Day 9 of the protocol and consonant with the presumed expansion of memory lymphocytes at rechallenge. Paradoxically, then, ICOS expression, although robustly identifying lymphocytes mobilized during a memory response, may be functionally irrelevant to the elaboration of the memory response. It is not clear why ICOS should exhibit this biological redundancy, nor why its activity during antigen sensitization and acute exposure should differ from its role in established disease. However, it may be that ICOS operates in a narrow window between CD28/B7-mediated T cell activation and irrevocable commitment of cells to a memory/effector phenotype. That is, ICOS ligation may modulate (positively or negatively depending, for example, on the strength of the activation signal) the differentiation of activated T cells into Th subsets either directly or by regulating the secretion of Th-polarizing cytokines. Once the memory/effector T cell pool has expanded and matured—a protracted process that may take several rounds of stimulation, and which may explain the apparently discordant conclusions in more compressed models of antigen sensitization and challenge (31, 32)—cells become hyporesponsive to further modulation by ICOS. This explanation is somewhat reminiscent of Sperling and Bluestone's "strength of signal" hypothesis of ICOS function (41): as the strength of the TCR/B7 activation signal increases (or, in the case of a memory response, the requirements for activation decrease), the need for ICOS costimulation diminishes. Additionally, there may also exist a subset of Th2-polarized memory cells that do not express ICOS and whose activity compensates for the functional deficiency of ICOS-dependent lymphocytes.

Our study, therefore, challenges the notion that the ICOS/B7RP-1 costimulatory axis represents a promising therapeutic target for immune-inflammatory disorders such as asthma. Although neutralization of ICOS during sensitization and acute antigen exposure attenuated airway inflammation, this intervention ultimately exacerbated immune-inflammatory phenomena upon long-term *in vivo* recall. Moreover, blockade of ICOS signaling during rechallenge of mice with established allergic disease elicited no therapeutic effect, intimating that ICOS may be functionally redundant in mature memory responses.

Acknowledgments: The secretarial assistance of Mary Kiriakopoulos is gratefully acknowledged. This research has been funded by a CIHR operating grant. R.E.W. is supported by a CIHR Doctoral Research Award. J.R.J. is supported by an Ontario Graduate Scholarship, and M.J. holds a Canada Research Chair.

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—CHAPTER 4—

Expression of the Th1 Chemokine IFNγ-Inducible Protein 10 in the Airway Alters Mucosal Allergic Sensitization in Mice

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Published in *The Journal of Immunology* 166:2750-2759 (2001)

Expression of the Th1 Chemokine IFN-γ-Inducible Protein 10 in the Airway Alters Mucosal Allergic Sensitization in Mice[1]

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Although the preliminary characterization of chemokines and their receptors has been prolific, comparatively little is known about the role of chemokines in the evolution of immune responses. We speculate that the preferential recruitment of a particular immune cell population has implications for the short- and long-term features of an adaptive response. To test this hypothesis, we employed adenovirus-mediated gene transfer to express the Th1-affiliated, CXC chemokine IFN-γ-inducible protein (IP) 10 in the airways of mice undergoing a mucosal sensitization regimen known to result in a Th2-polarized allergic response. This resulted in a ~60–75% inhibition of eosinophils in the bronchoalveolar lavage (BAL); these inflammatory changes were accompanied by enhanced IFN-γ, ablated IL-4, and, peculiarly, unaltered IL-5 and eotaxin levels in the BAL. The effect of IP-10 expression was shown to be dependent on IFN-γ, as there was no statistically significant reduction in BAL eosinophilia in IFN-γ knockout mice subjected to the IP-10 intervention. Flow cytometric analysis of mononuclear cells in the lung revealed a ~60% reduction in the fraction of $CD4^+$ cells expressing T1/ST2, a putative Th2 marker, and a parallel increase in the proportion expressing intracellular IFN-γ following IP-10 treatment. The effect of IP-10 expression at the time of initial Ag encounter is persistent, as mice rechallenged with OVA following the resolution of acute inflammation exhibited reduced eosinophilia and IL-4 in the BAL. Collectively, these data illustrate that local expression of the chemokine IP-10 can introduce Th1 phenomena to a Th2-predisposed context and subvert the development of a Th2 response. ***The Journal of Immunology,*** **2001, 166: 2750–2759.**

Chemokines are a diverse superfamily of small secreted proteins with a well-established function as chemoattractant cytokines for leukocytes. However, that some of these molecules affect T cell costimulation (1–3), myelopoiesis (4), and oral tolerance (5) suggests that the function of chemokines transcends chemotaxis; in particular, these observations have engendered theories about the role of chemokines in the orchestration of immune-inflammatory responses. Indeed, recent research suggests that the regulated expression of chemokines and chemokine receptors is an important component of an integrated immune response. It has been shown in an in vivo model of *Leishmania donovani* infection that expression of IFN-γ-inducible protein (IP)[3] 10, and therefore the accumulation of granuloma-promoting mononuclear cells, is initiated by T cell-independent mechanisms but is ultimately amplified and sustained by activated T cells at the site of inflammation (6). Likewise, Lloyd et al. (7) have documented temporal regulation of chemokines in a murine model of allergic airways disease; they have shown that as the immune response evolves in a repeatedly challenged airway, eotaxin, which is initially dominant in the recruitment of $CCR3^+$ Th2 cells, is ultimately supplanted by the CCR4 ligand macrophage-derived chemokine. Collectively, these observations strongly suggest that the elaboration of an immune response may in part hinge on the coordinated expression of chemokines.

It has also been proposed that the acquisition of a chemokine and chemokine receptor repertoire is an integral part of Th differentiation; indeed, although Th2 cells are associated with the chemokines eotaxin, thymus and activation-regulated chemokine, macrophage-derived chemokine, and the chemokine receptors CCR3, CCR4, and CCR8, Th1 cells correlate with the chemokines IP-10, monokine induced by IFN-γ, and macrophage-inflammatory protein (MIP) 1α and the chemokine receptors CXC chemokine receptor (CXCR) 3 and CCR5 (8–13). The association between chemokines, chemokine receptors, and Th phenotype is convincingly illustrated by IP-10. IP-10 is chemoattractant for T cells, monocytes (14–16), and NK cells (17) but not for neutrophils (18, 19), and has been shown to facilitate selective recruitment of Th1 cells that preferentially express the receptor CXCR3 both in vitro (9) and in vivo (20). Indeed, abundant CXCR3 expression has been reported on T cells infiltrating Th1-associated multiple sclerosis lesions (21) and rheumatoid arthritis synovial fluid (22), and sustained, protective expression of IP-10 has been described in murine models of Th1-polarized leishmaniasis (12, 23). IP-10 has also been implicated in the recruitment of lymphocytes to sites of atheroma formation (24) and has been detected in the bronchoalveolar lavage (BAL) fluid of patients with pulmonary sarcoidosis (25). The relationship between IP-10 and IFN-γ is unequivocal: stimulation with IFN-γ elicits IP-10 expression by activated human bronchial epithelial cells (26) and neutrophils (27), whereas levels of IP-10 mRNA are markedly reduced in lung interstitial macrophages in

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Received for publication September 5, 2000. Accepted for publication November 30, 2000.

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[1] This research was supported in part by the Medical Research Council of Canada/Canadian Institutes of Health Research. R.E.W. holds a Doctoral Research Award from the Medical Research Council/Canadian Institutes of Health Research and has been supported by an Ontario Graduate Scholarship; M.R.S. holds a Parker B. Francis Fellowship. We also recognize the support of the Hamilton Health Sciences Corporation and St. Joseph's Hospital (Hamilton).

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[3] Abbreviations used in this paper: IP, IFNγ-inducible protein; MIP, macrophage-inflammatory protein; CXCR, CXC chemokine receptor; BAL, bronchoalveolar lavage; KO, knockout; Ad, adenovirus; i.n., intranasal; RDA, replication-deficient Ad; PAS, periodic acid-Schiff; MCP, monocyte chemoattractant protein; Rx, treatment.

0022-1767/01/$02.00

IFN-γ receptor knockout (KO) mice (28). On the other hand, IP-10 has been documented to induce IFN-γ expression in cultured human PBMC (29). IP-10, therefore, may serve not only to mobilize differentiated Th1 cells but also to reinforce the evolution of a Th1 response.

We speculate that the preferential recruitment of a particular immune cell population by chemokines has implications for the short- and long-term features of an adaptive immune response. To test this hypothesis, we used an adenovirus (Ad)-mediated gene transfer approach to express IP-10, a prototype Th1 chemokine, in the airways of mice subjected to a mucosal sensitization regimen that results in a Th2-polarized allergic response. We have previously shown that intranasal administration of Ad/GM-CSF to mice followed by 10 daily exposures to aerosolized OVA results in cardinal Th2 events (30). In this study, we demonstrate that concurrent expression of IP-10 in the airway microenvironment significantly attenuates eosinophilia and elaborates Th1 phenomena in an IFN-γ-dependent fashion. Moreover, OVA rechallenge long after the clearance of IP-10 and resolution of acute inflammation elicits an inflammatory response that is primarily mononuclear and not eosinophilic, indicating that airway expression of IP-10 affects sensitization and the maturation of T cell memory. Collectively, these data suggest that IP-10, through preferential recruitment of Th1-privileging immune-inflammatory cells, can subvert a Th2-polarized response in vivo and attribute to this chemokine an immunoregulatory function that transcends chemotaxis.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Female IFN-γ KO mice on a BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions following a 12-h light-dark cycle. A total of 623 mice was sacrificed during the course of these experiments. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Aerosolization protocol

Over a period of 10 consecutive days (days 0–9), mice were placed in a Plexiglas chamber (10 cm × 15 cm × 25 cm) and exposed for 20 min daily to aerosolized OVA (1% w/v in 0.9% saline; Sigma-Aldrich, Oakville, Ontario, Canada). The OVA aerosol was generated by a Bennett (Kansas City, MO) nebulizer at a flow rate of 10 L/min. For the rechallenge experiment, mice were exposed to a 1% OVA aerosol for two 1-h periods separated by 4 h on day 50 of the protocol.

Administration of adenoviral constructs

To elicit local expression of GM-CSF, IP-10, or IFN-γ, a replication-deficient human type 5 Ad construct carrying murine GM-CSF, human IP-10, or human IFN-γ cDNA in the E1 region of the viral genome was delivered intranasally (i.n.) to anesthetized animals on day −1, 24 h before the first exposure to OVA; the human IP-10 sequence was selected since it has been characterized extensively and is the standard genetic instrument in murine systems (31). Ad/GM-CSF, Ad/IP-10, and Ad/IFN-γ were administered at doses of 3×10^7, 1×10^8, and 1×10^8 PFU, respectively, in 30 μl of PBS vehicle; an appropriate dose of an E1-deleted replication-deficient adenovirus (RDA) was used to control for the higher viral burden in the Ad/IP-10 and Ad/IFN-γ groups. The Ad/IFN-γ vector was a kind gift from J. Kolls (Louisiana State University Medical Center, New Orleans, LA), and the Ad/IP-10 (31) and Ad/GM-CSF (32) vectors were engineered and characterized previously by our laboratory.

Collection and measurement of specimens

Two days after the last OVA exposure (day 11) and at various time points during the aerosolization regimen, mice were sacrificed and BAL was obtained as previously described (33). In brief, the lungs were dissected and the trachea cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation, supernatants were stored at −20°C for cytokine measurements by ELISA; cell pellets were resuspended in PBS and smears were prepared by cytocentrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. Diff-Quik (Baxter, McGraw Park, IL) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Finally, lung tissue was fixed in 10% Formalin and embedded in paraffin. Sections (3-μm thick) were stained with hematoxylin and eosin or with periodic acid-Schiff (PAS) to distinguish mucous production in goblet cells.

Cytokine and Ig measurement

ELISA kits for IL-4, IFN-γ, eotaxin, monocyte chemoattractant protein (MCP) 1, MIP-1α, and RANTES were purchased from R&D Systems (Minneapolis, MN), while the kit for IL-5 was obtained from Amersham (Buckinghamshire, U.K.). IP-10 was measured by ELISA according to a standard alkaline phosphatase/streptavidin assay protocol (R&D Systems). Briefly, IP-10 was captured with an anti-human IP-10 Ab in the solid phase and developed with biotinylated anti-human IP-10 (R&D Systems); recombinant human IP-10 (PeproTech, Rocky Hill, NJ) was used as a standard. Levels of OVA-specific IgE were detected using an Ag-capture (biotinylated OVA) ELISA method that has been described previously (33): the ELISA was standardized with serum obtained from mice sensitized to OVA according to a conventional i.p. sensitization model (34) and bled 24 h after the second sensitization. OVA-specific IgG2a was measured by sandwich ELISA with OVA in the solid phase. Ninety-six-well plates were coated with 5 μg/ml OVA in borate buffer (100 μl/well) for 1 h at 37°C, 3 h at room temperature, and then overnight at 4°C. Plates were blocked for 2 h at room temperature with 150 μl/well 1% BSA in PBS before loading samples (50 μl/well). Plates were then incubated overnight at 4°C and washed before adding 50 μl of 0.25 μg/ml biotinylated anti-mouse IgG2a Ab (Southern Biotechnology Associates, Birmingham, AL) to each well. Following a 2-h incubation at room temperature, plates were washed, incubated with alkaline phosphatase/streptavidin for 1 h at room temperature (50 μl/well at a concentration of 1:1000), and developed with *p*-nitrophenyl phosphate substrate dissolved in diethanolamine buffer. This ELISA was standardized with serum obtained from mice sensitized to OVA according to our Th1-polarized mucosal sensitization model (33) and bled at day 11 of the protocol. Ig levels are expressed in units per milliliter relative to standard sera.

Lung cell isolation and flow cytometric analysis of lung cell subsets

Lungs were perfused with 10 ml of prepared buffer (10% FBS, 1% penicillin/streptomycin in HBSS) through the right ventricle, cut into small (~2-mm diameter) pieces, and agitated at 37°C for 1 h in 15 ml of collagenase III (Life Technologies, Rockville, MD) at a concentration of 150 U/ml in HBSS. Using the plunger from a 5-ml syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon mesh. Mononuclear cells were isolated at the interphase between layers of 30 and 60% Percoll following density gradient centrifugation. Cells were washed twice and stained for flow cytometric analysis. For each Ab combination, 0.5×10^6 cells were incubated with mAbs at 0–4°C for 30 min; the cells were then washed and treated with second-stage reagents. For detection of intracellular IFN-γ, cells were first polyclonally stimulated with PMA/ionomycin in 4-h cultures and then permeabilized with saponin according to a standard protocol (BD PharMingen catalogue, PharMingen, San Diego, CA). Data were collected using a FACScan (Becton Dickinson, Sunnyvale, CA) and analyzed using WinMDI software (The Scripps Research Institute, La Jolla, CA). The following Abs and reagents were used: anti-CD3, PE conjugated (145-2C11); anti-CD3, CyChrome conjugated (145-2C11); anti-CD4, FITC conjugated (RM4-5); anti-CD4, CyChrome conjugated (RM4-5); anti-CD8α, FITC conjugated (53-6.7); anti-CD69, PE conjugated (H1.2F3); anti-IFN-γ, PE conjugated (XMG1.2) (all purchased from BD PharMingen); anti-T1/ST2, PE conjugated (Millennium Pharmaceuticals, Cambridge, MA); and streptavidin PerCP (BD PharMingen). The Abs were titrated to determine optimal concentration.

Data analysis

Data are expressed as mean ± SEM, unless otherwise indicated. Results were interpreted using ANOVA followed by the Student-Newman-Keuls

post hoc test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Effect of Ad/IP-10 intervention on the cellular profile in the BAL

To investigate the impact of expressing a Th1-affiliated chemokine on a system that is otherwise predisposed to the development of a Th2-polarized response, we used a mucosal model of allergic airways inflammation in which mice are exposed daily to aerosolized OVA over a period of 10 consecutive days in the context of GM-CSF expression in the airway. In this study, 1×10^8 PFU Ad/IP-10 or control virus (RDA) were coadministered i.n. with Ad/GM-CSF to BALB/c mice, which were then subjected to the OVA exposure regimen. Administration of Ad/IP-10 to naive mice resulted in sustained expression of IP-10 in the airways for ~7–10 days, with peak expression of ~2100 pg/ml in the BAL 1 day after vector administration, followed by resolution at day 4 and a secondary peak of IP-10 (~600 pg/ml) in the BAL at day 7. Two days after the last OVA exposure, mice were sacrificed and the cellular profile in the BAL was assessed (Fig. 1). Mice exposed to OVA in the presence of IP-10 but in the absence of GM-CSF had modestly elevated mononuclear cells in the BAL. As expected, exposure to OVA in the context of GM-CSF expression resulted in marked mononuclear and eosinophilic inflammation in the BAL; concurrent administration of RDA did not significantly alter this inflammatory response. Although concurrent IP-10 expression did not appreciably affect the quantity of inflammation in mice treated with GM-CSF and OVA, it did change the phenotype of the infiltrate; in particular, IP-10 reduced the number of eosinophils in the BAL by ~60–75%. To verify that this reduction in airways eosinophilia was not the consequence of eosinophil retention in the circulation, peripheral blood leukocytes were assessed; IP-10 expression in the airway resulted in a >50% decrease in eosinophil counts in the circulation (Table I).

Histological evaluation of lung tissue

We conducted a detailed histological analysis that demonstrates a correlation between processes occurring in lung tissue and the findings in BAL at day 11 of the aerosolization protocol. Exposure to OVA in the context of GM-CSF led to marked peribronchial and perivascular inflammation that was distinctly eosinophilic in nature (Fig. 2*A*). Evidence of goblet cell hyperplasia was confirmed in PAS-stained tissue (Fig. 2*E*), which documents pervasive expansion of mucous-producing cells. Analysis of lung tissue from RDA-treated mice revealed similar histopathological phenomena (data not shown). In contrast, concurrent expression of IP-10 reduced the accumulation of eosinophils in lung tissue, although robust mononuclear inflammation persisted (Fig. 2, *B* and *D*); moreover, there was significant, if somewhat attenuated, goblet cell hyperplasia along the airway epithelium of these mice (Fig. 2*F*). Exposure to OVA in the context of IP-10 expression alone

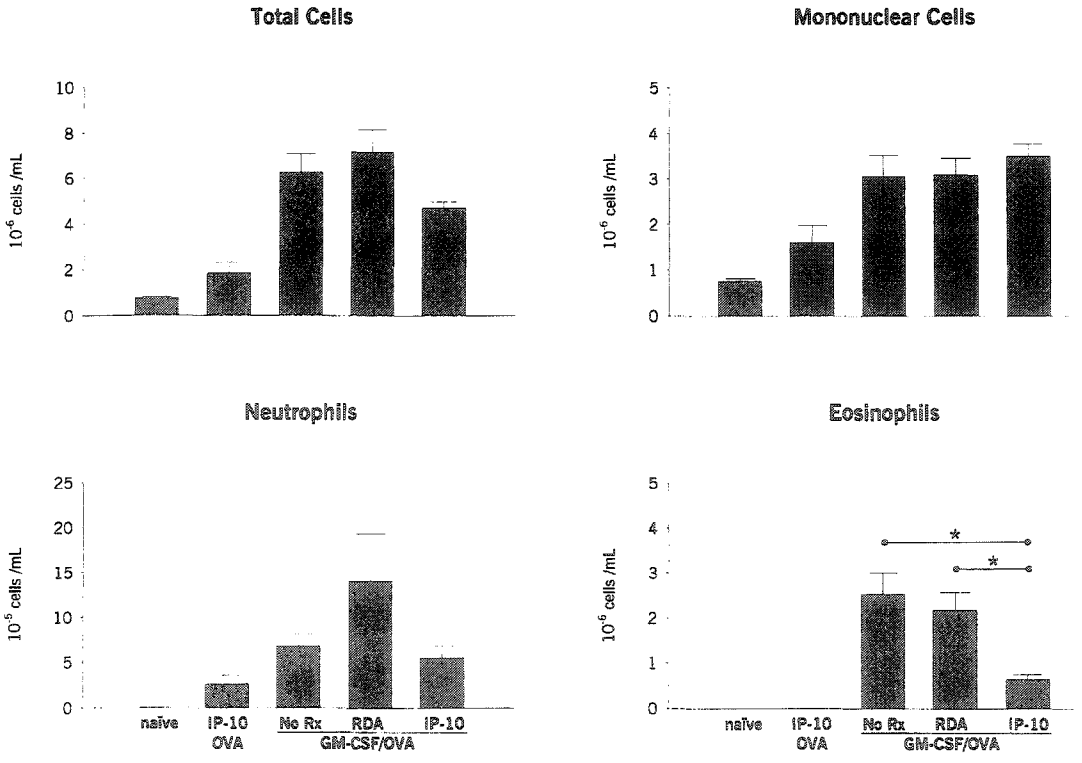

FIGURE 1. Effect of IP-10 expression on inflammation in the BAL of mice sensitized to OVA in the context of a GM-CSF airway microenvironment. Groups of mice were treated with Ad/GM-CSF with or without concurrent administration of Ad/IP-10 or RDA before proceeding through the OVA aerosolization protocol. Bars represent untreated mice (naive), mice exposed to OVA in the context of IP-10 expression (IP-10 OVA), and mice sensitized to OVA in the context of GM-CSF with no additional treatment (no Rx), with concurrent Ad/IP-10 intervention (IP-10) or with coincident infection with control virus (RDA). Data show total cells, mononuclear cells, neutrophils, and eosinophils in BAL obtained 48 h after the last OVA exposure (mean ± SEM; n = 4–10/treatment group; ANOVA was used to assess statistical significance; *, $p < 0.05$).

Table I. *Effect of IP-10 intervention on circulating leukocytes*[a]

	GM-CSF/OVA		
	No Rx	RDA	IP-10
Total cells	114 ± 14	103 ± 13	82 ± 15
Mononuclear cells	79 ± 9.3	78 ± 11	59 ± 12
Neutrophils	26 ± 4.3	17 ± 1.2*	19 ± 2.9
Eosinophils	9.5 ± 1.4	8.5 ± 1.4	3.7 ± 1.1*

[a] Mice were bled retro-orbitally on day 11 of the mucosal sensitization protocol and blood smears were prepared. Data are expressed as 10^{-5} cells/ml of peripheral blood and represent the mean ± SEM; $n = 6$–7/treatment group. ANOVA was used to assess statistical significance.

*, $p < 0.05$ vs both no Rx and RDA controls.

resulted in a comparably modest mononuclear cell infiltrate (Fig. 2*C*), with no evidence of mucous-secreting cells (data not shown).

Endogenous production of cytokines, chemokines, and Igs

To evaluate IP-10-mediated changes in the Th1/Th2 balance of the immune response to OVA, we assayed a number of definitive mediators (Table II); we report only peak levels of expression, which occurred on day 7 of the protocol for cytokines and chemokines in the BAL and on day 11 for Igs in serum. As shown in Table II, delivery of RDA did not significantly alter the levels of IFN-γ, IL-4, and IL-5 detected in mice exposed to OVA in the context of GM-CSF. In contrast, IP-10 intervention resulted in a 4- to 10-fold increase in the level of IFN-γ in the BAL, while IL-4 was virtually undetectable and IL-13 was significantly attenuated; IL-5 content in the BAL was also reduced by about 40%, although this change did not reach statistical significance. Moreover, mice treated with IP-10 presented 2- to 3-fold higher levels of the proinflammatory chemokines MCP-1, MIP-1α, and RANTES in the BAL and produced three to four times more OVA-specific IgG2a, a Th1-affiliated Ig, than mice treated with GM-CSF alone or concurrently infected with GM-CSF and RDA control. It is noteworthy that IP-10 treatment did not result in a statistically significant reduction in levels of OVA-specific IgE, nor did it alter expression of eotaxin, hallmarks of the eosinophilic response, an intriguing observation given the dramatic reduction in BAL, tissue, and peripheral blood eosinophilia in IP-10-treated animals.

Role of IFN-γ in IP-10-mediated suppression of eosinophilia

Given the demonstrated association between IP-10 and IFN-γ (26–29), and the dramatic up-regulation of IFN-γ in the BAL elicited by IP-10 expression in the airway, we proceeded to explore mechanistically the role of this prototype Th1 mediator in IP-10-mediated inhibition of airways eosinophilia. Therefore, we executed the exposure regimen in IFN-γ KO mice. In this setting, airway expression of IP-10 did not attenuate BAL eosinophilia, indicating that the modulatory effect of IP-10 is dependent on IFN-γ (Fig. 3*A*). To expand this observation and understand more comprehensively the role of IFN-γ in this system, we substituted Ad/IP-10 with an adenoviral construct expressing the transgene for IFN-γ; this construct results in sustained expression of IFN-γ in the BAL for ~10 days and reaches levels of 2000 pg/ml in the BAL 7 days after i.n. delivery. In mice exposed to OVA in the context of GM-CSF, concurrent expression of IFN-γ completely abrogated BAL eosinophilia, indicating that IFN-γ per se, when expressed at levels comparable to those elicited by Ad/IP-10, is sufficient to prevent the airways eosinophilia to which this protocol is predisposed (Fig. 3*B*).

Flow cytometric analysis of T cell populations in the lung

We used flow cytometric analysis of dispersed lung mononuclear cells to evaluate the effect of IP-10 expression on the pattern of T lymphocyte recruitment to the airway. In particular, we examined $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells at day 7 of the aerosolization protocol for expression of the early activation marker CD69. Table III indicates that approximately two to three times as many $CD8^+$ T cells were isolated from the lungs of mice treated with IP-10 and exposed to OVA in a GM-CSF milieu compared with RDA-treated control mice; compared with GM-CSF alone (data not shown), delivery of RDA had no distinguishable effect on any of the T cell parameters we examined. These differences are particularly pronounced when expression of the early activation marker CD69 is examined (Fig. 4), as IP-10 treatment resulted in an increase in the fraction of both $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells in the airway compared with RDA-treated control mice. Moreover, these T cells expressed a phenotype consistent with the alleged Th1 bias of IP-10; as shown in Table IV, while the proportion of $CD3^+CD4^+$ cells expressing the putative Th2 marker T1/ST2 (35–37) declined by 40–50% in IP-10-treated mice, there was a concomitant 50% increase in the frequency of $CD4^+$ cells expressing intracellular IFN-γ. These data indicate that the Th1-biased immune phenomena observed in both the inflammatory and cytokine responses of IP-10-treated animals may have practical implications for T cell phenotype.

In vivo rechallenge of mucosally sensitized mice

To determine whether expression of IP-10 in the airway at the time of the initial mucosal sensitization affected the development of T cell memory, we investigated the long-term in vivo response of treated mice to aerosolized OVA. On day 50, several weeks following the resolution of airways inflammation, mice were re-exposed to aerosolized OVA for 1 h on two occasions 4 h apart. Seventy-two hours later, mice were sacrificed and the cellular profile of the BAL was assessed (Fig. 5). Although there was a reconstitution of airways eosinophilia in mice treated with GM-CSF alone or with GM-CSF and RDA, IP-10 expression at the time of initial Ag exposure resulted in markedly attenuated eosinophilia upon OVA rechallenge, indicating that the OVA-specific immune response was substantively altered by IP-10. Flow cytometric analysis of T cell subsets isolated from homogenized lungs of mice sacrificed 72 h after in vivo OVA recall indicates that while IP-10 intervention did not appreciably alter the recruitment of $CD4^+$ and $CD8^+$ T cells to the airways of mice exposed to OVA in the context of GM-CSF and RDA, the ratio of activated (i.e., $CD69^+$) CD4 to CD8 T cells changed considerably, from ~3 in control mice to 0.9 in mice exposed to OVA in the presence of both GM-CSF and IP-10. This change was the consequence of a ~50% increase in the number of activated $CD8^+$ T cells and a parallel ~50% reduction in activated $CD4^+$ T cells in the airways of IP-10-treated mice compared with mice initially exposed to OVA in the context of GM-CSF and RDA (Fig. 6*A*); RDA intervention had no distinguishable effect on the lymphocyte profile following OVA rechallenge compared with mice receiving GM-CSF alone (data not shown). Cytokine content in the BAL was assessed 24 h following OVA rechallenge (Fig. 6*B*); interestingly, although similar levels of IFN-γ and IL-5 were detected in all groups, mice initially treated with IP-10 produced considerably less IL-4 than mice sensitized to OVA in the context of GM-CSF alone (data not shown) or GM-CSF and RDA.

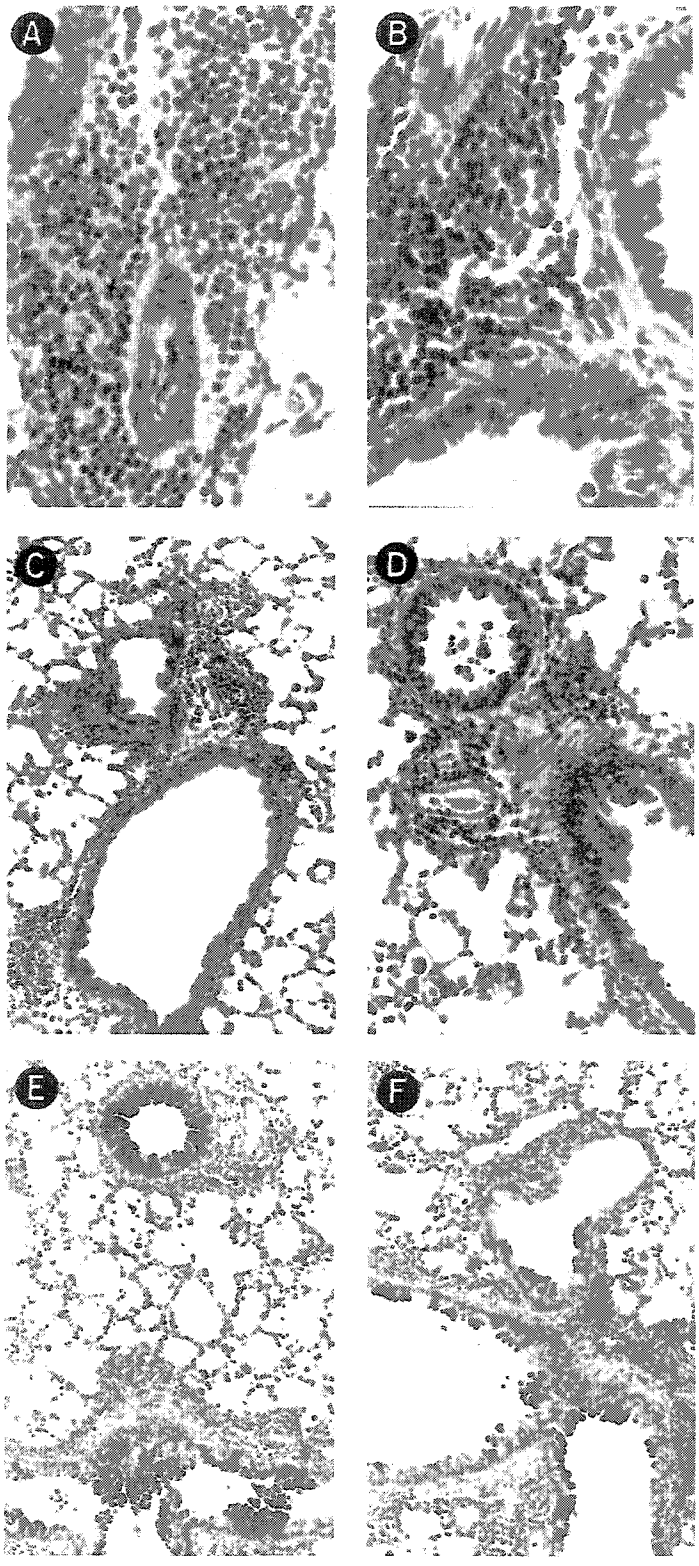

FIGURE 2. Light photomicrograph of paraffin-embedded sections of lung tissue. Mice were exposed to OVA in the context of a GM-CSF or IP-10 microenvironment and sacrificed on day 11. *A–D* were stained with hematoxylin and eosin; *E* and *F* were stained with PAS to visualize goblet cells (magenta). Panels represent exposure to OVA in the context of GM-CSF alone (*A* and *E*), in the context of IP-10 alone (*C*), or in the context of concurrent expression of IP-10 and GM-CSF (*B*, *D*, and *F*). Original magnification of panels: *A*, ×400; *B*, ×640; *C*, ×160; *D*, ×200; *E*, ×160×; and *F*, ×160.

Table II. *Cytokine, chemokine, and Ig levels in IP-10-treated mice*[a]

	GM-CSF/OVA		
	No Rx	RDA	IP-10
Cytokines			
IFN-γ	85 ± 18	274 ± 70	994 ± 164*
IL-4	22 ± 5.3	24 ± 7.3	0.92 ± 2.1*
IL-13	235 ± 53	255 ± 53	93 ± 24*
IL-5	105 ± 28	97 ± 22	63 ± 15
Chemokines			
MCP-1	47 ± 15	57 ± 7.7	167 ± 20*
MIP-1α	43 ± 1.7	54 ± 7.5	92 ± 12*
RANTES	32 ± 13	82 ± 15	175 ± 20*
Eotaxin	37 ± 6.9	59 ± 0.89	69 ± 20
Igs			
OVA-specific IgG2a	93 ± 41	78 ± 24	328 ± 44*
OVA-specific IgE	139 ± 39	127 ± 27	66 ± 23

[a] Mice were sacrificed at day 7 (cytokines and chemokines) or day 11 (Igs) of the aerosolization protocol; cytokine and chemokine content in BAL and Ig content in serum were measured by ELISA. Data are expressed in pg/ml (cytokines and chemokines) or U/ml (Igs) and represent the mean ± SEM; n = 3–12/treatment group. ANOVA was used to assess statistical significance.

*, $p < 0.05$ vs both no Rx and RDA controls.

Discussion

We hypothesize that the function of chemokines can transcend chemotaxis depending on whether a chemokine recruits immune or inflammatory cells. For example, a chemokine specific for a receptor expressed predominantly by neutrophils fulfills a direct role in recruiting these granulocytes to the site of inflammation. However, a chemokine specific for a subset of immune cells (i.e., T lymphocytes) has implications for the nature and long-term outcome of the immune-inflammatory response that evolves in the target tissue. To test this latter concept, we employed an adenovirus-mediated gene transfer approach to express IP-10 (Ad/IP-10), a chemokine that preferentially recruits Th1 cells, in the airway microenvironment under conditions otherwise predisposed to the development of a Th2-polarized response. We elected to use adenoviral gene transfer technology because it offers the advantage of sustained, though transient, expression of a desired transgene product; while the attendant antiviral immune response admittedly introduces complexity to data interpretation, our ability to control for this variable systematically (through the use of transgene-deficient RDA) equips us with a robust and incisive in vivo experimental system. Indeed, our previously described murine model of

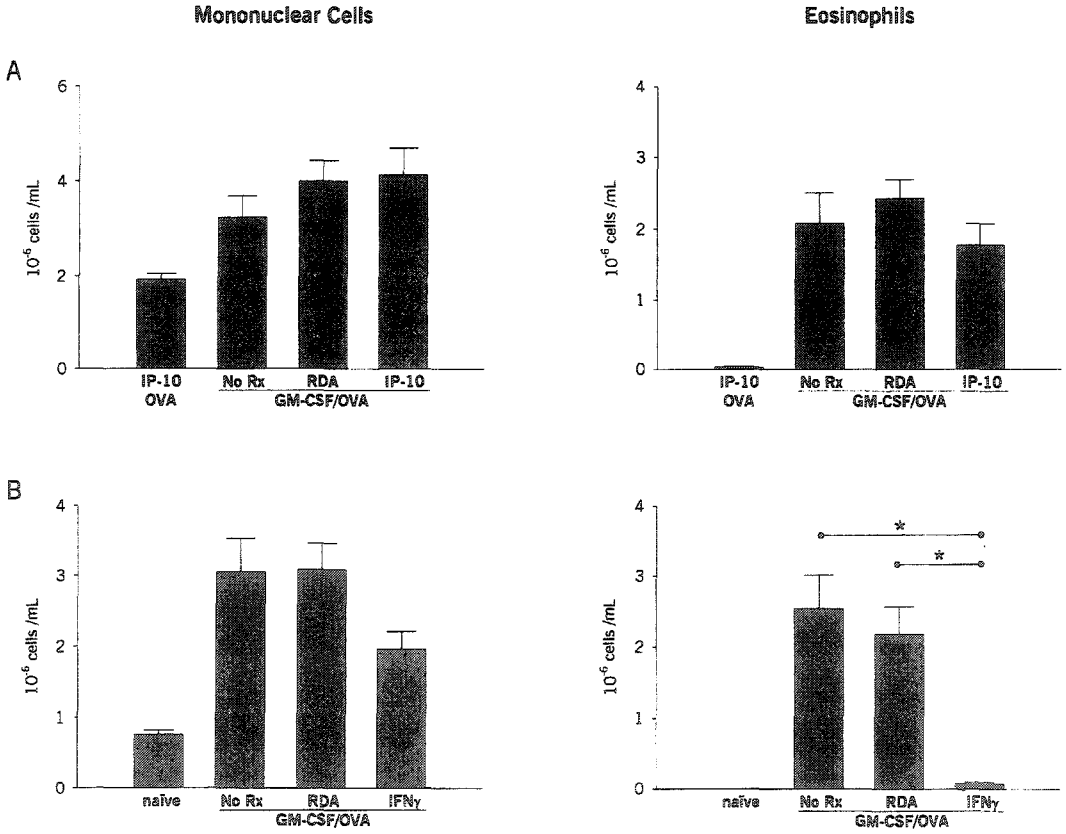

FIGURE 3. Role of IFN-γ in IP-10-mediated inhibition of eosinophilia. *A*, IFN-γ KO mice were treated with GM-CSF with or without concurrent administration of IP-10 or RDA before proceeding through the OVA aerosolization regimen. Bars represent mice exposed to OVA in the context of IP-10 expression (IP-10 OVA) and mice sensitized to OVA in the context of GM-CSF with no additional treatment (no Rx), with simultaneous IP-10 expression (IP-10) or with coincident infection with control virus (RDA). *B*, Effect of IFN-γ per se on inflammation in the BAL of mice sensitized to OVA in the context of a GM-CSF airway microenvironment. On day −1, BALB/c mice received i.n. instillations of Ad/GM-CSF; additional groups received concurrent administration of 1×10^8 PFU Ad/IFN-γ or RDA. Bars represent untreated mice (naive) and mice sensitized to OVA in the context of GM-CSF expression with no additional treatment (no Rx), with concurrent IFN-γ expression (IFN-γ) or with coincident infection with control virus (RDA). Data show mononuclear cells and eosinophils in BAL obtained 48 h after the last OVA exposure (mean ± SEM; n = 3–8/treatment group; ANOVA was used to assess statistical significance; *, $p < 0.05$).

Table III. *Flow cytometric analysis of T cell populations in the lung at day 7 of the aerosolization protocol*[a]

	Naive	GM-CSF/OVA	
		RDA	IP-10
Expt. 1			
$CD3^+CD4^+$	3.8	10.9	13.8
$CD3^+CD8^+$	1.2	3.1	7.3
Expt. 2			
$CD3^+CD4^+$	ND	6.3	9.0
$CD3^+CD8^+$		1.7	4.9

[a] Mice were sacrificed at day 7 of the aerosolization protocol; mononuclear cells were isolated from whole-lung homogenates pooled from three to four mice per treatment group. Cells were stained for flow cytometric analysis of CD3, CD4, and CD8 expression; 20,000 events within the mononuclear cell gate were collected for analysis. Data indicate the number ($\times 10^{-5}$) of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells per lung.

GM-CSF-driven mucosal sensitization to aerosolized OVA results in a prototypic Th2-polarized allergic response characterized by airways eosinophilia, expression of IL-4 and IL-5, production of OVA-specific IgE, and long-term Ag-specific memory (30). Expression of IP-10 at the time of mucosal allergic sensitization alters the Th effector composition in the lung (as assessed by expression of T1/ST2 and intracellular IFN-γ), which results in changes in the molecular and cellular profile both locally and systemically. In particular, airway expression of IP-10 attenuates IL-4 and IL-13 production, induces vigorous IFN-γ and OVA-specific IgG2a responses, and elicits a cascade of proinflammatory chemokines, including MCP-1, RANTES, and MIP-1α. These molecular changes correlate with a 60–75% inhibition of BAL eosinophilia and the recruitment of mononuclear cells and neutrophils, inflammatory changes that were confirmed by histopathological analysis of lung sections.

Several observations, however, are not satisfactorily explained by the conclusion that IP-10 expression in the context of a GM-CSF milieu merely elicits a Th1-polarized response. Our data at early time points show that significant levels of OVA-specific IgE, the cardinal humoral feature of the Th2 phenotype, are produced in animals exposed to OVA in the context of IP-10. In the effector organ, i.e., the airway, the two prototypic Th2 cytokines IL-5 and IL-13 are readily detected, whereas histopathological features associated with a Th2 response, such as goblet cell hyperplasia and mucous production, persist. On the other hand, that IP-10 simultaneously induces a robust IFN-γ response and substantially elevated levels of OVA-specific IgG2a in serum suggests that IP-10 expression, rather than deviate the system away from Th2 polarization, superimposes a competing Th1 response upon an assiduous Th2 background.

However, although IL-5 and IL-13 are detected in the BAL of IP-10-treated mice, the levels of these cytokines are diminished—significantly in the case of IL-13—compared with control mice exposed to OVA following concurrent administration of RDA and GM-CSF. We argue that these attenuated Th2 phenomena are the consequence of the diminished influx of Th2 cells into the tissue; our data, which demonstrate a ~40% reduction in the proportion of $CD4^+$ T cells expressing the bonafide Th2 marker T1/ST2, support this hypothesis. Moreover, that both the level of IL-13 and the pervasiveness of goblet cell hyperplasia in the airway are reduced, but persistent, in IP-10-treated mice agrees with the demonstrated role of IL-13 in promoting epithelial remodeling and mucous production (38); the presence of IL-13 may also account

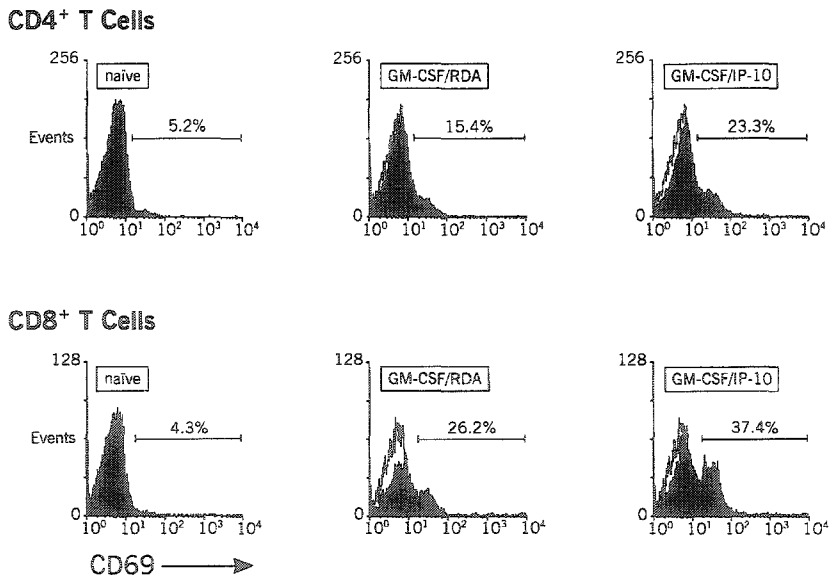

FIGURE 4. Flow cytometric analysis of T cell populations in the lung. On day 7 of the aerosolization protocol, mononuclear cells were isolated from dispersed lung cells of mice treated with GM-CSF with or without concurrent administration of IP-10 or RDA. Cells from each group of four mice were pooled and stained for flow cytometric analysis of CD3, CD4, CD8, and CD69 expression; for each Ab mixture, 20,000 events within the mononuclear cell gate were collected for analysis. Data show the flow cytometry histograms for CD69 expression on gated $CD3^+CD4^+$ or $CD3^+CD8^+$ populations in untreated mice (naive), mice exposed to OVA in the context of IP-10 (GM-CSF/IP-10), or with coincident infection with control virus (RDA). The shaded region depicts the distribution of events from samples stained with PE-conjugated anti-CD69 Ab; the unshaded region represents the corresponding isotype control. Results are representative of two independent experiments.

Table IV. *Flow cytometric analysis of T1/ST2 and intracellular IFN-γ expression on CD4⁺ cells*[a]

	T1/ST2⁺ (%)	IFN-γ⁺ (%)
Naive	5.04	ND
IP-10/OVA	8.90	8.37
GM-CSF/OVA		
No RxRDA	20.0	8.64
RDA	19.3	9.24
IP-10	13.0	14.2

[a] Mice were sacrificed at day 7 of the aerosolization protocol; mononuclear cells were isolated from whole-lung homogenates pooled from four mice per treatment group. Cells were stained for flow cytometric analysis of CD3, CD4, and T1/ST2 expression or for CD4 and IFN-γ expression following short-term stimulation with PMA/ionomycin; 20,000 events within the mononuclear cell gate were collected for analysis. Data indicate the fraction of $CD3^+CD4^+$ cells expressing T1/ST2 or the proportion of all $CD4^+$ cells expressing intracellular IFN-γ. Results are representative of two independent experiments.

for the significant levels of OVA-specific IgE detected in IP-10-treated animals (39). Finally, the comparative reduction in IL-5 in mice exposed to OVA in the context of IP-10 and GM-CSF is consistent with reduced, but not absent, peripheral blood eosinophilia in these animals. Indeed, it may be that dramatically reduced levels of IL-4 in BAL prevent the up-regulation of VCAM-1 on pulmonary endothelium, resulting in the compartmentalization of eosinophils in the circulation (40). As a future consideration, the competing Th1 phenomena elicited by IP-10 may provide a prolific experimental setting to explore the role of eosinophils, IgE, and Th2 mediators in the development of bronchial hyperreactivity.

The dramatically elevated levels of IFN-γ in the BAL of IP-10-treated animals prompted us to explore the role of this cytokine in the subversion of the Th2-polarized, GM-CSF-driven response to aerosolized OVA. We therefore executed the same treatment regimen in IFN-γ KO mice and found that IP-10 expression did not significantly inhibit BAL eosinophilia, demonstrating that the effect of IP-10 on this outcome is dependent on IFN-γ. To evaluate more comprehensively the role of IFN-γ in immune deviation in this experimental setting, we directly investigated the effect of IFN-γ airway expression in wild-type (i.e., IFN-γ sufficient) mice. We observed complete abrogation of eosinophilia, and a reduction in mononuclear cell inflammation generally, in the BAL of Ad/IFN-γ-treated animals, indicating that overexpression of IFN-γ is sufficient to subvert the eosinophilic character of the GM-CSF-driven inflammatory response to OVA. These findings inform a comparison with the apparently parallel effect induced by airway expression of IL-12 in this model of mucosal allergic sensitization (33). Expression of IL-12 in this setting results in an Ag-specific, Th1-polarized immune response that is IFN-γ independent, whereas the ultimately similar effect elicited by IP-10 is dependent on IFN-γ. This suggests different pathways capable of Th2 subversion: although IL-12 can directly mediate Th1 polarization at the level of the APC (33), we hypothesize that IP-10 initiates a cascade of events, such as preferential recruitment and activation of IFN-γ-producing cells, that introduce Th1 phenomena to an existing Th2 context.

The levels of IP-10 protein in BAL exhibit a curious time course: there is an initial burst of IP-10 in BAL 1 day after i.n. administration of the vector, followed by a secondary, less robust peak on the seventh day. We suggest that this second peak is mediated by IFN-γ. In this regard, Gangur et al. (29, 41) have shown that rIP-10 selectively enhances polyclonally driven and Ag-specific IFN-γ production by cultured PBMC, and a variety of leukocyte subsets and structural cells have been reported to express IP-10 upon stimulation with IFN-γ (26–28). Additionally, Xie et al. (20) have shown that IP-10 injected into the peritoneum of mice preferentially recruits adoptively transferred, Th1-polarized $CD4^+$ T cells, which in turn may serve as a cellular source of IFN-γ. Collectively, these observations support the hypothesis that the secondary peak of IP-10 expression 7 days after vector administration (and 3 days after resolution of the initial burst of vector-derived IP-10) may in fact reflect IFN-γ production by resident cells or by IFN-γ-producing cells preferentially recruited by exogenous IP-10.

To explore the viability of this hypothesis, we used flow cytometry to examine T cell subsets in the lung at day 7 of the aerosolization protocol, the point at which we observe peak expression of IFN-γ in the BAL of IP-10-treated mice. We found that mice exposed to OVA in the context of GM-CSF and IP-10 had elevated $CD8^+$ T cell populations in the lung compared with control mice.

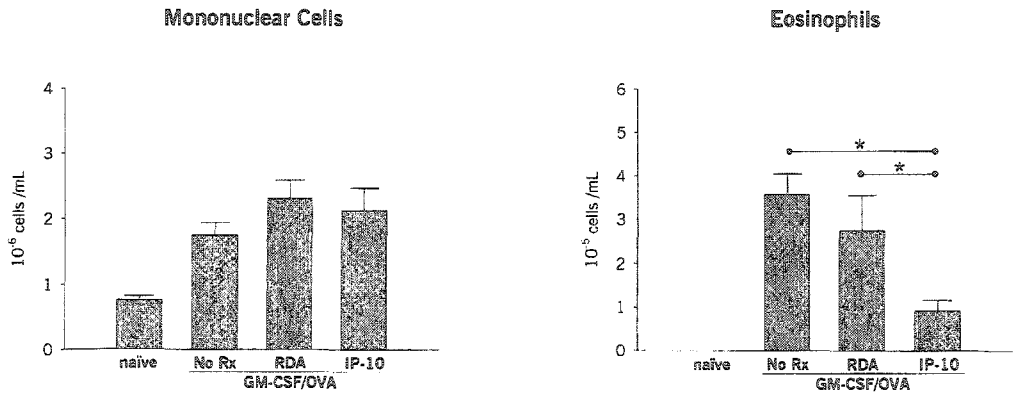

FIGURE 5. Effect of Ad/IP-10 intervention on inflammation in the BAL of mice rechallenged with aerosolized OVA. Groups of mice were mucosally sensitized to OVA in the context of GM-CSF with or without concurrent expression of IP-10; after completion of the daily OVA exposure regimen, airways inflammation was allowed to resolve (~30 days) before mice were rechallenged with aerosolized OVA. Bars represent untreated mice (naive) and mice sensitized to OVA in the context of GM-CSF with no additional treatment (no Rx), with concurrent IP-10 expression (IP-10) or with coincident infection with control virus (RDA). Data show mononuclear cells and eosinophils in BAL obtained 72 h after OVA aerosol rechallenge at day >50 (mean ± SEM, n = 5–8/treatment group; ANOVA was used to assess statistical significance; *, $p < 0.05$).

A. Flow Cytometric Analysis of Lymphocyte Subsets in the Lung

	GM-CSF/OVA	
	RDA	IP-10
CD4 T Cells		
$CD3^+CD4^+$	3.4 (29)	2.8 (24)
$CD3^+CD4^+CD69^+$	0.38 (11)	0.18 (6.7)
CD8 T Cells		
$CD3^+CD8^+$	1.2 (10)	1.1 (9.7)
$CD3^+CD8^+CD69^+$	0.14 (12)	0.21 (18)

B. Cytokine Content in BAL

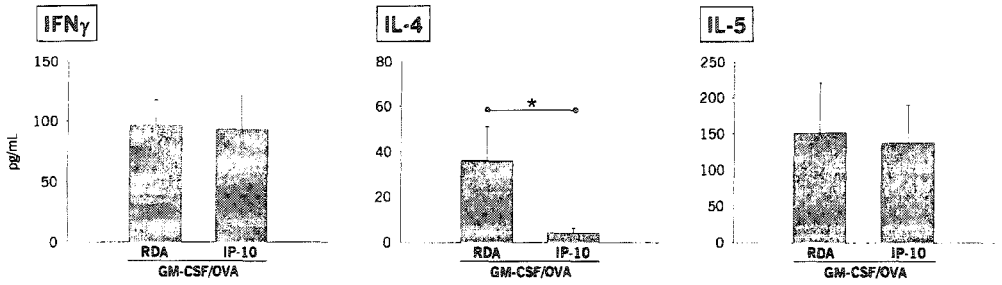

FIGURE 6. Effect of IP-10 expression on lymphocytes and cytokines following in vivo OVA recall. *A,* Groups of mice were mucosally sensitized to OVA in the context of GM-CSF with or without concurrent expression of IP-10; after completion of the daily OVA exposure regimen, airways inflammation was allowed to resolve (~30 day) before mice were rechallenged with aerosolized OVA. Seventy-two hours after secondary exposure, mononuclear cells were isolated from the dispersed lung cells of four mice per treatment group and stained for flow cytometric analysis of CD3, CD4, CD8, and CD69 expression: for each Ab mixture, 20,000 events within the mononuclear cell gate were collected for analysis. Data show the number ($\times 10^{-4}$) of $CD3^-CD4^+$ and $CD3^+CD8^+$ cells per lung as well as the number of these cells coexpressing CD69; numbers in parentheses represent the percentage of $CD4^+$ and $CD8^+$ T cells in the mononuclear cell gate or the percentage of $CD69^+$ cells within the $CD3^+CD4^+$ or $CD3^+CD8^+$ gate. *B,* Mice were sacrificed 24 h after long-term Ag rechallenge, and cytokines were measured in BAL by ELISA. Bars represent mice initially exposed to OVA in the context of GM-CSF and IP-10 expression (IP-10) or following concurrent administration of GM-CSF and RDA. Data represent mean ± SEM; $n = 3$/treatment group; Student's t test was used to assess statistical significance; *, $p < 0.05$.

These differences were particularly pronounced when the activation status of these lymphocytes was considered: IP-10 intervention resulted in markedly enhanced expression of CD69 on both $CD4^+$ and $CD8^+$ T cells, an observation consistent with the finding that IP-10 preferentially recruits activated T cells (15, 19). To characterize more comprehensively the phenotype of T lymphocytes in the lung, we studied T cells for surface expression of T1/ST2, a putative marker of Th2 differentiation (35–37), and for intracellular IFN-γ, the prototypic Th1 effector cytokine. Although $CD8^+$ T cells, irrespective of treatment regimen, expressed only basal levels of T1/ST2 and exhibited no change in intracellular IFN-γ (data not shown), there was a reduction in the proportion of $CD4^+$ T cells displaying T1/ST2 and a corresponding increase in the fraction of $CD4^+$ cells expressing intracellular IFN-γ. Thus, it is tempting to envision a model in which IP-10, through the preferential recruitment of activated $CD8^+$ and Th1 cells, establishes an airway microenvironment conducive to the differentiation and amplification of a Th1-polarized response to OVA.

To elucidate these Th1-biased phenomena and to investigate whether IP-10 intervention altered the memory response to OVA, we subjected mice to an OVA aerosol challenge well after resolution of acute inflammation in the airway and clearance of the vector-encoded IP-10 and GM-CSF transgenes. We observed that OVA-rechallenged mice whose initial Ag encounter occurred in the context of an IP-10/GM-CSF milieu exhibited significantly less eosinophilia, though comparable total inflammation, in the BAL compared with mice initially treated with GM-CSF alone. This observation argues strongly for the persistence of a distinct, Ag-specific memory lymphocyte population and, concomitantly, suggests that the airway microenvironment established by IP-10 during primary OVA exposure influenced the evolution of the adaptive response to OVA. Examination of cytokine production in the BAL and of lymphocyte populations in the lung following OVA rechallenge complement findings during primary Ag exposure. Indeed, concurrent IP-10 expression in the airway at the time of initial OVA delivery privileges the recruitment of activated

$CD8^+$ T cells to the lung upon in vivo OVA recall. Moreover, although IFN-γ and IL-5 content in the BAL of IP-10-treated mice is unaltered—observations that speak to the heterogeneity of the Th1/Th2 balance and the persistence of Th2 phenomena following IP-10 intervention—IL-4 production is ablated, indicating that the underlying Th2 character of GM-CSF-mediated sensitization to OVA has been permanently impoverished by concurrent expression of IP-10.

In summary, we have shown that expression of the chemokine IP-10 can introduce a competing Th1 phenotype to an immunological setting otherwise predisposed to the development of allergic airways inflammation and can thereby subvert Th2 features of the ensuing response. Extrapolated to a broader immunological and clinical context, these data illustrate the plasticity of T helper responses and reinforce the importance of the microenvironment in elaborating and maintaining such responses. That an IP-10 immunological milieu has the capacity to alter the nature of an Ag-specific, Th2-polarized immune-inflammatory response attributes to this chemokine an immunomodulatory function that transcends chemotaxis.

Acknowledgments

The expert technical support of Susanna Goncharova, Meghan Cundall, Monika Cwiartka, Joanna deJong, and Doriana Oppedisano and the secretarial assistance of Mary Kiriakopoulos are gratefully acknowledged. We also extend thanks to Filip Swirski, Stacey Ritz, and Clinton Robbins for provocative discussion and critique.

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—CHAPTER 5—

Overexpression of the Chemokine CCL20/MIP-3α in the Mouse Airway Expands Antigen-presenting Cells and Facilitates Mucosal Sensitization to Aerosolized OVA

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To be submitted to *American Journal of Respiratory Cell and Molecular Biology*, July 2004.

1. Abstract

Several recent studies have implicated CCL20 (MIP-3α) in the recruitment of dendritic cells to sites of immunologic challenge. To investigate the functional implications of this chemotactic activity in the lung, we administered a replication-deficient adenovirus (Ad) encoding the transgene for human CCL20 to the airways of mice. 6 days following intranasal delivery, Ad/CCL20 resulted in a 20- to 50-fold enrichment in the number of lung mononuclear cells coexpressing MHC class II and CD11c (dendritic cells) or CD11b (macrophages) compared to administration of an equivalent dose of replication-deficient adenovirus (RDA) control; there was also an expansion of B lymphocytes, $CD4^+$ and $CD8^+$ T cells in the lungs of Ad/CCL20-treated mice. To investigate the immunological implications of this inflammatory milieu, Ad/CCL20- or RDA-treated mice received 10 consecutive daily exposures to aerosolized OVA; while no inflammatory response was observed in RDA controls, OVA exposure in the context of CCL20 expression resulted in a mononuclear/neutrophilic inflammatory infiltrate in the BAL of mice that was indistinguishable from that elicited by Ad/CCL20 alone. However, in sharp constrast to treatment with RDA control or Ad/CCL20 alone, long-term *in vivo* rechallenge with aerosolized OVA elicited a mononuclear/neutrophilic recall inflammatory response in BAL of mice initially exposed to OVA in a CCL20-conditioned airway microenvironment. This local response in the airway was associated with amplified levels of OVA-specific IgG_{2a}, a Th1-associated immunoglobulin, in serum. These data underscore the potential importance of the CCL20/CCR6 axis in the immunology of the lung, and suggest that CCL20, through the recruitment/expansion of APC subsets in the airway, facilitated Th1-biased sensitization to OVA.

2. Introduction

The CC chemokine ligand (CCL) 20, often designated by its familiar name, macrophage inflammatory protein (MIP) 3α, has been described as one of the most potent chemokines involved in the recruitment of dendritic cells (DC) to sites of immunologic challenge. Its receptor, CC chemokine receptor (CCR) 6, was originally identified on B lymphocytes, $CD4^+$ and $CD8^+$ T cells (1), and immature DC (2, 3). Although initial data suggested that CCL20 preferentially recruited DC that had differentiated from $CD34^+$ progenitors, more recent data indicate that this putatively restricted pattern of CCR6 expression on particular DC subsets may be doctrinaire (4-8) and that CCL20 may be understood as a chemokine broadly relevant to DC biology.

Seminal research in the skin intimated that CCL20 functioned primarily in the constitutive recruitment of immature DC, while a separate panel of chemokines, such as CCL2 (MCP-1), mobilized myeloid, "inflammatory" DC during periods of immunologic challenge (9-11). Far from simply a regulator of homeostatic DC migration, however, CCL20 is also preferentially upregulated at sites of inflammation and may be part of a series of complementary chemokine gradients that sequentially mobilize DC, guide them to the pathogen and shunt them to the lymphatic system (12, 13). CCL20 has been detected in tonsils and lesional psoriatic skin (14, 15), in the central nervous system of mice undergoing experimental autoimmune encephalomyelitis (EAE) (16), in lesional skin tissues of patients with atopic dermatitis (17), in colonic specimens from Crohn's sufferers (18), and in the inflamed synovium of rheumatoid arthritis patients (19-22). Moreover, its critical role in the regulation of experimental inflammatory bowel disease (23), intestinal immunity (4) and allergic airway inflammation (24) has been documented in CCR6-deficient mice. Thus, the

prevailing paradigm argues that $CCR6^+$ DC, once they have been recruited to the site of immunologic challenge, lose responsiveness to CCL20 and acquire a phenotype that prepares them for migration to the draining lymph nodes. Indeed, stimulation of *in vitro*-derived DC with inflammatory signals, such as TNF-α or lipopolysaccharide (LPS), reduces CCR6 expression, aborts responsiveness to CCL20 and upregulates CCR7, whose ligands CCL19 (MIP-3β) and CCL21 (secondary lymphoid tissue chemokine [SLC]) are constitutively expressed in lymphoid tissue (25-30).

Notwithstanding significant progress in our understanding of CCL20 as a chemokinetic regulator of DC migration, investigators have yet to reach consensus *vis-à-vis* the role of this chemokine in the elaboration of immune responses. Moreover, with the exception of a handful of studies showing CCL20 upregulation in appropriately stimulated airway epithelia (31-33), little is known about the immunoregulatory activity of CCL20 in the lung *in vivo*. In this study, we sought to advance this knowledge by exploring the inflammatory effects of adenovirus-mediated local gene transfer of CCL20 to the lungs of mice. We report that CCL20 expression in the airway elicited robust accumulation of APC and T lymphocyte populations in the lung, preferentially expanding dendritic cells bearing a potent costimulatory phenotype. We further demonstrate that this inflammatory context subverted inhalation tolerance to an otherwise innocuous protein, facilitating Th1-biased sensitization and the establishment of long-term immunologic memory to OVA. Overall, this study furnishes novel evidence that CCL20 expression at the site of immunologic challenge can orchestrate an inflammatory response that has important implications for the evolution of adaptive immunity.

3. Materials and Methods

Animals. Female Balb/c mice (6-8 weeks old) were purchased from Charles River Laboratories (Montreal, PQ, Canada). The mice were housed under specific pathogen-free conditions following a 12 h light-dark cycle. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Administration of Adenoviral Constructs. To elicit local expression of CCL20, a replication-deficient human type 5 adenoviral (Ad) construct encoding murine CCL20 cDNA in the E1 region of the viral genome was delivered intranasally (i.n.) to anæsthetized animals on day –1 (original CCL20 sequence furnished by Dr. John White, GlaxoSmithKline, King of Prussia, PA). The vector was constructed by homologous recombination in 293 cells using the Ad genomic plasmid pJM17 (34). Ad/CCL20 was administered at doses of 3×10^7 or 9×10^7 pfu in 30 μL of PBS vehicle; an appropriate dose of an E1-deleted replication-deficient adenovirus (RDA) was used to control for the immunologic effects of viral infection *per se.*

Aerosolization Protocol. Over a period of ten consecutive days (days 0 to 9), mice were placed in a Plexiglass chamber (10 cm × 15 cm × 25 cm) and exposed for 20 min daily to aerosolized OVA (1% wt/vol in 0.9% saline) (Sigma-Aldrich, Oakville, ON). The OVA aerosol was generated by a Bennett nebulizer at a flow rate of 10 L/min. For the rechallenge experiment, mice were exposed to a 1% OVA aerosol for 20 minutes on three consecutive days beginning ~4 w after the initial ten OVA exposures had been completed.

Collection and Measurement of Specimens. At various time points after adenovirus administration or during the aerosolization regimen, mice were sacrificed and bronchoalveolar lavage (BAL) was obtained as previously described (30). In brief, the lungs

were dissected and the trachea cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged twice with PBS (0.25 mL followed by 0.2 mL). Approximately 0.3 mL of the instilled fluid was consistently recovered. Total cell counts were determined using a hæmocytometer. After centrifugation, supernatants were stored at -20°C for cytokine measurements by ELISA; cell pellets were resuspended in PBS and smears were prepared by cytocentrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. Diff-Quik (Baxter, McGraw Park, IL) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hæmocytological criteria to classify the cells as neutrophils, eosinophils, lymphocytes or macrophages/monocytes. Additionally, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Lung tissue was fixed in 10% formalin and embedded in paraffin. 3-μ-thick sections were stained with hematoxylin and eosin for visualization of leukocytes and histopathological features.

Cytokine and Immunoglobulin Measurement. ELISAs for the measurement of IFNγ, IL-4, IL-5, IL-10 and IL-13 were purchased from R&D Systems; each of these ELISAs has a threshold of detection between 1.5 and 7.0 pg/mL. OVA-specific IgG_{2a} was measured by sandwich ELISA with OVA in the solid phase. 96-well plates were coated with 5 μg/mL OVA in borate buffer (100 μL/well) for 1 h at 37°C, 3 h at room temperature and then overnight at 4°C. Plates were blocked for 2 h at room temperature with 150 μL/well 1% BSA in PBS before loading samples (50 μL/well). Plates were then incubated overnight at 4°C and washed before adding 50 μL of 0.25 μg/mL biotinylated anti-mouse IgG_{2a} antibody (Southern Biotechnology Associates, Birmingham, AB) to each well. Following a 2-h

incubation at room temperature, plates were washed, incubated with alkaline phosphatase/streptavidin for 1 h at room temperature (50 μL/well at a concentration of 1:1000) and developed with p-nitrophenyl phosphate substrate dissolved in diethanolamine buffer. This ELISA was standardized with serum obtained from mice sensitized to OVA according to our Th1-polarized mucosal sensitization model (35) and bled at day 11 of the protocol. Levels of OVA-specific IgE were detected using an antigen-capture (biotinylated OVA) ELISA method as described (36); anti-mouse IgE antibodies were obtained from Southern Biotechnology Associates (Birmingham, AB). This ELISA was standardized with serum obtained from mice sensitized to OVA according to a conventional intrapertioneal sensitization model and bled 7 d following the second sensitization (36). Immunglobulin levels are expressed in units (U)/mL relative to standard sera.

Lymph Node/Lung Cell Isolation and Flow Cytometric Analysis of Lymph Node/Lung Cell Subsets. Lungs were perfused with 10 mL prepared buffer (10% FBS, 1% penicillin/streptomycin in HBSS) through the right ventricle, cut into small (~2 mm diameter) pieces and agitated at 37°C for 1 h in 15 mL collagenase III (Life Technologies, Rockville, MD) at a concentration of 150 U/mL in HBSS. Using the plunger from a 5 cc syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon mesh. Mononuclear cells were isolated at the interphase between layers of 30% and 60% Percoll following density gradient centifugation. Cells were washed twice in PBS and stained for flow cytometric analysis. Thoracic lymph nodes were triturated between the frosted ends of glass slides to disperse mononuclear cells; the cells were filtered through nylon mesh and washed once in PBS before staining. For each

antibody combination, 1×10^6 cells were incubated with monoclonal antibodies at 0-4°C for 30 min; the cells were then washed and treated with second stage reagents. Data were collected using a FACScan® (Becton Dickinson, Sunnyvale, CA) and analyzed using WinMDI software (Scripps Research Institute, La Jola, CA). The following antibodies and reagents were used: hamster IgG anti-CD3ε, PE-conjugated and Cy-Chrome™-conjugated (145-2C11); rat IgG_{2a} anti-CD4, FITC-conjugated and Cy-Chrome™-conjugated (RM4-5); rat IgG_{2a} anti-CD8α, FITC-conjugated (53-6.7); mouse IgG_3 anti-MHC class II, FITC-conjugated (39-10-8); hamster IgG anti-CD11c, PE-conjugated (HL3); rat IgG_{2b} anti-CD11b, PE-conjugated (M1/70); rat IgG_{2a} anti-B220, PE-conjugated (RA3-6B2); hamster IgG anti-B7.1, biotinylated (16-10A1); rat IgG_{2a} anti-B7.2, biotinylated (GL1); all relevant control isotypes; and Streptavidin PerCP (all purchased from BD PharMingen, San Diego, CA). The antibodies were titrated to determine optimal concentration.

Data Analysis. Data are expressed as mean±SEM, unless otherwise indicated. Results were interpreted using Student's t-test or ANOVA with Tukey *post hoc* test, where appropriate. Differences were considered statistically significant when $p<0.05$.

4. Results

4.1 *Adenoviral gene transfer of CCL20 elicits mononuclear and neutrophilic inflammation in BAL.*

To characterize the biological impact of localized expression of CCL20 in the airway, 9×10^7 pfu Ad/CCL20 or 9×10^7 pfu control RDA were administered intranasally to mice on day -1, groups of which were sacrificed at days 3 or 6. The accumulation of macrophages, neutrophils and lymphocytes corresponded to peak expression of exogenous (human) CCL20 in BAL (Figure 1), though eosinophils (not illustrated) were not detected; no changes in cell content were observed in RDA-treated mice, whose BAL profile resembled that of naïve mice (historical control not shown). Exogenous CCL20 expression was extinguished by day 6, though inflammation in BAL persisted. There was no statistically significant change in endogenous CCL20 expression in BAL of Ad/CCL20-treated mice compared to RDA controls (data not shown).

4.2 *CCL20 expression enriches antigen-presenting cell and T lymphocyte populations in the lung.*

To investigate the impact of local expression of CCL20 on the antigen-presenting cell (APC) and T lymphocyte profile in the lung, we administered 3×10^7 or 9×10^7 pfu Ad/CCL20, or 9×10^7 pfu control RDA intranasally to Balb/c mice on day -1; mice were sacrificed at days 3 or 6 (coincident with robust inflammation in BAL), and mononuclear cells were isolated from whole lung homogenates and stained for flow cytometric analysis of APC and T cells. Exogenous CCL20 activity yielded virtually undetectable changes in mononuclear cell content in whole lung at day 3. By day 6, however, CCL20 expression had induced substantial inflammation in the lung. Intervention with 9×10^7 pfu Ad/CCL20, in

particular, resulted in a 20- to 50-fold expansion of MHC Class II (MHCII)$^+$CD11c$^+$ dendritic cells, MHCII$^+$CD11b^{high} macrophages and MHCII$^+$B220$^+$ B lymphocytes in the lung at day 6 compared to administration of an equivalent dose of RDA (Figure 2). Moreover, this dramatic alteration in the overall level of inflammation translated to the preferential enrichment of CD11c$^+$ and CD11b^{high} APC populations, each representing approximately 2 to 3% of pulmonary mononuclear cells at day 6 following RDA administration and rising to ~8% in mice treated with 9×10^7 pfu Ad/CCL20 (proportional data presented in numbers above bars in Figure 2); 3×10^7 pfu Ad/CCL20 resulted in a comparable enrichment of macrophages at day 6. No substantial changes in the relative magnitude of these APC subsets were observed for either dose at day 3 (data not presented). In addition to augmenting APC subsets in the lung, instillation of 9×10^7 pfu Ad/CCL20 elicited a complementary 10-fold expansion of both CD4$^+$ and CD8$^+$ T cells at day 6 compared to RDA control (Figure 3), indicating that CCL20 expression *in vivo* exhibits proinflammatory activity (either directly or indirectly) for a broad spectrum of mononuclear cells.

4.3 *Flow cytometric analysis of costimulatory molecules on the APC compartment in the lung.*

We used flow cytometry to characterize the costimulatory phenotype of APC infiltrating the lung following Ad/CCL20 administration. In particular, we examined expression of the costimulatory molecules B7.1 and B7.2 on MHCII$^+$CD11c$^+$ dendritic cells, MHCII$^+$CD11b$^+$ macrophages and MHCII$^+$B220$^+$ B cells at days 6 and 11 following Ad/CCL20 or RDA administration at a dose of 9×10^7 pfu (adenovirus was administered at

this dose in all experiments that follow). Exogenous CCL20 expression resulted in a dramatic increase in the numbers of $B7.1^+$, and especially $B7.2^+$, APC of all subsets at both time points compared to naïve animals (numbers in parentheses in Table I); by contrast, RDA intervention elicited a relatively modest amplification of these APC populations. Moreover, exogenous CCL20, to a greater extent than RDA alone, preferentially enhanced the fraction of APC expressing B7.2 at days 6 and 11 (Table I); that is, Ad/CCL20 established an airway microenvironment that privileged the enrichment of APC bearing a costimulatory phenotype.

4.4 A *CCL20-conditioned airway microenvironment permits phenotypically mixed, Th1-biased sensitization to OVA.*

Given the impressive expansion of mononuclear cells in the lung following airway gene transfer of CCL20, and the privileged enhancement of professional APC in particular, we investigated the effect of this inflammatory environment on the response to aerosolized OVA, to which passive exposure typically elicits immunologic tolerance. Mice were treated with Ad/CCL20 or RDA and were exposed concurrently to an aerosol of OVA on ten consecutive days. In BAL and whole lung homogenates, it was impossible to distinguish the OVA-specific inflammatory response from that elicited by Ad/CCL20 alone; indeed, CCL20 expression *per se* resulted in a robust accumulation of mononuclear cells that obviated the effect of OVA (data not shown). However, in the thoracic lymph nodes, the putative site of differentiation and articulation of the immune response, CCL20 intervention in the context of OVA exposure induced a 3-fold greater expansion of APC, including dendritic cells, than CCL20 expression alone (*i.e.* in the presence of viral antigens only) at day 6 of the

aerosolization protocol (Table II); the magnitude of the CCL20/OVA-induced lymphoid response was approximately 20-fold greater than that observed in RDA controls.

To ascertain whether CCL20 established conditions conducive to OVA-specific sensitization, mice were rechallenged with aerosolized OVA on three consecutive days once exogenous CCL20 expression had waned and acute inflammatory processes had resolved (recall exposures began ~ 4 weeks after the initial 10-day OVA exposure regimen had been completed). OVA-naïve control mice originally treated with Ad/CCL20 in the absence of concurrent OVA exposure established a baseline against which OVA-specific inflammatory responses during rechallenge could be appraised (Figure 4). By comparison, mice sensitized to OVA in the context of CCL20 mounted a robust, primarily mononuclear inflammatory response in the lung as assessed in BAL (Figure 4); this contrasts with the negligible airway inflammation evoked in mice initially treated with RDA. In particular, the inflammatory infiltrate evoked in mice sensitized to OVA in a CCL20 milieu was characterized by dramatically elevated levels of lymphocytes; RDA intervention did not yield this lymphocytic response upon rechallenge with OVA. Consistent with observations at earlier time points, eosinophils were not detected in BAL of either group (not illustrated). Cellular findings in BAL were confirmed histopathologically (Figure 5). OVA-rechallenged mice sensitized to OVA in the context of CCL20 overexpression exhibited pervasive peribronchial and perivascular mononuclear inflammation, with clear evidence of goblet cell hyperplasia. In contrast, relatively modest baseline inflammation persisted in the lung tissue of OVA-naïve, Ad/CCL20-treated control mice, whose airway epithelium was punctuated by only occasional foci of goblet cell hyperplasia; the level of inflammation observed in rechallenged mice originally treated with RDA was similarly low.

To characterize further the phenotype of the CCL20-conditioned, OVA-specific immune response, we quantified immunoglobulin levels in serum both acutely (*i.e.* after the tenth sensitizing exposure to OVA) and following long-term *in vivo* recall. Though immunoglobulin titres were low in all groups at the early time-point, post-rechallenge levels of OVA-specific IgG_{2a}, a Th1-associated immunoglobulin, were several fold higher in mice sensitized to OVA in the context of CCL20 than in RDA control mice (Figure 4); levels of OVA-specific IgE were uniformly low in all groups (data not displayed), while there was a trend toward elevated OVA-specific IgG_1, an isotype of arguably neutral/mixed phenotype, in the CCL20 group (Figure 4). Notably, this humoral response is consistent with the cytokine profile in BAL of mice during OVA sensitization. Compared to concurrent RDA/OVA treatment, OVA exposure in the context of CCL20 expression resulted in a trend toward elevated IFNγ content in BAL immediately following the tenth OVA exposure (data not shown); irrespective of experimental intervention, only negligible levels of the Th2-affiliated cytokines IL-4, IL-5, IL-10 and IL-13 were detected in BAL. Combined, these humoral and inflammatory *in vivo* recall data indicate both the presence and persistence of Th1-biased, OVA-specific immunologic memory in mice intially exposed to OVA in an airway microenvironment modified by CCL20.

5. Discussion

As the most potent subset of antigen-presenting cells capable of activating naïve T cells, dendritic cells (DC) are generally instrumental in the initiation of immune-inflammatory responses in lymphatic organs. That they migrate to draining lymph nodes upon maturation means that resident DC need to be replenished in immune-challenged tissue. Given the documented ability of CCL20 to recruit primarily immature DC, it is not surprising that a handful of recent studies have intimated the critical role of the CCL20/CCR6 axis in the elaboration of immune-inflammatory responses. The availability of anti-CCL20 neutralizing antibodies and the CCR6-deficient mouse, in particular, have afforded researchers indispensable instruments to appraise CCL20 activity in homeostasis and in models of disease—to which our emerging understanding of the complex role of CCL20 in cutaneous and intestinal immunity (4, 23, 37-39), in the development of experimental allergic encephalomyelitis (EAE) (40), and in the expression of the allergic phenotype (24) attest. While this research has begun to elucidate the processes and pathways in which CCL20 activity is *necessary*, very little is known about the *sufficiency* of CCL20: about the immune-inflammatory cascade that CCL20 expression *per se* initiates (41). Moreover, though CCL20 is expressed constitutively in the lung (42, 43) and its receptor has been detected on lung dendritic cells (3), the function of CCL20 in the immunology of the airway mucosa remains relatively uncharted. The research presented in this study begins to address these questions, furnishing evidence that amplified expression of CCL20 in the lung establishes an immune-potentiating microevironment that can subvert tolerance to an otherwise innocuous inhaled antigen.

We have found that overexpression of human CCL20 in the lungs of mice resulted

in a marked accumulation of APC and T lymphocytes in both BAL and dispersed lung tissue. Compared to intranasal administration of control RDA, which elicited no detectable inflammation, Ad/CCL20 administration resulted in the infiltration of macrophages, neutrophils and lymphocytes, but no eosinophils, into BAL following peak expression of exogenous CCL20; this cellular response, moreover, mirrored the kinetics of exogenous CCL20 expression in the lung. Although activated human neutrophils are known to express CCR6 (44), which may account for their accumulation in the BAL of Ad/CCL20-treated mice, it also possible, perhaps even likely, that this neutrophilia was an indirect consequence of the potentiation of anti-viral immunity by CCL20-responsive APC.

Flow cytometric characterization of $MHCII^+$ APC susbets in lung tissue showed a dramatic expansion of $CD11c^-$ (dendritic cell), $CD11b^{high}$ (macrophage) and $B220^+$ (B cell) populations in Ad/CCL20-treated mice compared to RDA controls, though the 4-fold proportional expansion of dendritic cells and macrophages was especially pronounced. From published data, one can reasonably assume that CCL20 expression primarily recruited cells of a myeloid (*i.e.* $CD11b^+$) rather than a lymphoid (*i.e.* $CD8^+$) pedigree (6). However, it is also important to consider the intrinsic biological complexity of sustained, Ad-mediated CCL20 expression *in vivo* and the resulting inability to distinguish direct recruitment of $CCR6^+$ cells from indirect, downstream inflammatory pathways. While it is likely that exogenous CCL20 in the lung directly mobilized $CCR6^+$ dendritic cells from the circulation, in addition to B lymphocytes, $CD4^+$ and $CD8^+$ effector/memory T cells—all of which have been shown to express CCR6 and respond to CCL20 (45-51)—it is also possible that incipient recruitment of these cells engaged a self-perpetuating inflammatory cascade potentiated, in part, by the persistence of viral antigens. This intrinsic artifact notwithstanding, it is clear that airway

gene transfer of CCL20 established a proinflammatory microenvironment that could not be achieved with RDA alone.

We also quantified the expression of B7.1 and B7.2 on gated APC subsets from dispersed lung tissue and found that exogenous CCL20 expression elicited an early and sustained enrichment of $B7.2^+$ APC subsets compared to naïve controls. Interestingly, the presence of viral antigens was apparently instrumental to this amplification of the APC costimulatory profile: RDA-treated mice exhibited a similar, if delayed and slightly attenuated, enhancement in B7.2 expression on lung dendritic cells. However, in terms of cell number, CCL20 expression resulted in an expansion of both $B7.1^+$ and $B7.2^+$ APC that far exceeded that achieved by RDA alone, pointing to a significantly heightened immune potential in Ad/CCL20-treated mice.

Sustained, Ad-driven expression of CCL20 in the lung provides an interesting demonstration of the chemotactic potential of the CCL20/CCR6 axis at the airway mucosa, an anatomical and immunological site that has received relatively little attention in the CCL20 literature. We wished to parlay these observations to a more rigorous functional analysis by investigating the impact of CCL20-mediated APC accumulation on immune activity, particularly given the expansion of APC subsets bearing a costimulatory phenotype. To this end, we passively exposed Ad/CCL20- or RDA-treated mice to an aerosol of OVA, an antigen that, in the absence of additional activatory signals, typically induces inhalation tolerance (52, 53). Although there was some suggestion that the level of IFNγ in the BAL of mice exposed to OVA during concurrent CCL20 expression was higher than that detected in RDA-treated controls sacrificed immediately following termination of the OVA challenge protocol, it was impossible to disentangle OVA-specific phenomena from the robust

inflammatory response elicited by CCL20 *per se*. We therefore waited several weeks to allow acute inflammatory processes to resolve and Ad-mediated CCL20 expression to expire before subjecting mice to a secondary recall challenge with aerosolized OVA. In this setting, Ad/CCL20-treated mice not previously exposed to OVA had a negligible inflammatory profile in BAL that was virtually indistinguishable from that of OVA-rechallenged mice initially introduced to OVA in the context of RDA. By comparison, mice whose first encounter with OVA was conditioned by CCL20 expression mounted an impressive inflammatory response upon antigen recall. Macrophages, neutrophils and especially lymphocytes were substantially elevated in BAL of these mice; eosinophils could not be detected. The magnitude and phenotype of this recall response suggested unequivocally that CCL20 expression during incipient OVA exposure had conditioned an inflammatory milieu in the airway conducive to sensitization to OVA.

These data indirectly confirm the importance of APC *expansion*, in addition to *activation*, in the initiation and elaboration of a productive immune-inflammatory response. Although RDA infection *per se* matured resident APC in the airway, only CCL20 expression provided a potent stimulus to increase these APC populations; it was likely Ad-dependent activation, in addition to the non-specific amplification of endogenous signals in the inflamed airway of Ad/CCL20-treated mice, that matured the accumulated APC and established a critical mass of activated dendritic cells. This concept was verified in the thoracic lymph nodes, where airway CCL20 expression facilitated a 20-fold expansion of dendritic cells in OVA-exposed mice compared to that observed in RDA controls. It would seem, then, that CCL20 expression in the airway mobilized the migration of professional APC to the lung; the presence of exogenous antigen (OVA), in turn, harnessed this APC

response as the recruited cells, having sequestered antigen and acquired a mature phenotype, migrated to the lymph nodes to initiate an antigen-specific T cell response.

The profusion of research into the role of CCL20 in various inflammatory processes has tended to favour a model that prioritizes CCL20 activity in Th1-related phenomena, such as inflammatory bowel disease (4, 18, 23, 37-39), various skin pathologies (14, 15), rheumatoid arthritis (19-22), neurogenic inflammation (16, 40) and, underscored by CCL20's recently-described antimicrobial properties, bacterial infection (32, 54-58). However, that CCR6 deficiency attenuated inflammation in a murine model of allergic asthma (24), and that human airway epithelia secrete CCL20 when stimulated with IL-4, IL-13 and ambient particulate matter (31)—a putative agent in the ætiology of asthma (59, 60)—would challenge the strict assignment of CCL20 to a particular pole on the Th1/Th2 spectrum. While the local inflammatory response we documented in Ad/CCL20-treated mice was certainly consistent with an interpretation of a default Th1 bias, assessment of systemic humoral immunity confirmed the Th1 phenotype of the CCL20-conditioned immune response to OVA. Unlike RDA treatment, which facilitated the production of persistently low levels of OVA-specific immunoglobulins, CCL20 expression resulted in dramatically amplified production of Th1-polarized IgG_{2a} and essentially no change in IgE. It can be inferred from these data that CCL20 expression conditioned a Th1-biased immune-inflammatory response to concurrent OVA exposure.

This paper is one of the first to describe the immune-inflammatory processes elaborated by CCL20 overexpression, and furnishes some insight into the functional role CCL20 might play in the immunology of the airway mucosa. We have also shown that the APC-enriched airway microenvironment conditioned by Ad-mediated CCL20 expression

facilitates Th1-biased sensitization to an innocuous antigen, fortifying the concept that CCL20/CCR6 could be targeted for both the management and therapeutic induction of immune-inflammatory responses.

6. References

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7. Figure Legends

FIGURE 1 *Kinetics of CCL20 expression and concomitant inflammation in BAL.* Mice were injected intranaslly with 9×10^7 pfu Ad/CCL20 or RDA on day -1, and groups were serially sacrificed at days 3 and 6. The upper panel displays total inflammation in BAL (bar graph) of Ad/CCL20-treated (gray) and RDA control-treated (black) mice with respect to changes in expression of exogenous (human) CCL20 protein in BAL (line graph). The lower panels describe the phenotype of cells accumulating in BAL. Data indicate mean±SEM; n=3-7 per treatment group; * $p<0.05$ (*vs.* RDA).

FIGURE 2 *Flow cytometric analysis of antigen-presenting cell populations in the lung following compartmentalized expression of CCL20.* Mice were treated intranasally with 3×10^7 or 9×10^7 pfu Ad/CCL20 or 9×10^7 pfu RDA on day –1 and sacrificed at days 3 and 6. Mononuclear cells were purified from dispersed lung cells, pooled from 3-4 mice per group and stained for flow cytometric analysis of dendritic cells ($MHCII^+CD11c^+$), macrophages ($MHCII^+CD11b^{high}$) and B cells ($MHCII^+B220^+$); for each antibody cocktail, 20000 events within the mononuclear cell gate were collected for analysis. Data show the number of $MHCII^+CD11c^+$, $MHCII^+CD11b^{high}$ and $MHCII^+B220^+$ cells isolated per lung at days 3 (black bars) and 6 (gray bars) from naïve mice, mice infected with 9×10^7 pfu RDA, and mice treated with 3×10^7 or 9×10^7 pfu Ad/CCL20. Numbers above bars indicate the fraction (%) of $MHCII^+CD11c^+$, $MHCII^+CD11b^{high}$ or $MHCII^+B220^+$ cells per lung in the mononuclear cell

gate at day 6.

FIGURE 3 *Effect of compartmentalized CCL20 expression on T lymphocyte populations in the lung.* Groups of mice were treated intranasally with 3×10^7 or 9×10^7 pfu Ad/CCL20 or 9×10^7 pfu RDA on day –1 and sacrificed at days 3 and 6. Mononuclear cells were purified from dispersed lung cells, pooled from 3-4 mice per group and stained for flow cytometric analysis of $CD4^+$ T cells ($CD3^+CD4^+$) and $CD8^+$ T cells ($CD3^+CD8^+$); for each antibody cocktail, 20000 events within the mononuclear cell gate were collected for analysis. Data show the number of $CD4^+$ and $CD8^+$ T cells isolated per lung at days 3 (black bars) and 6 (gray bars) from naïve mice, mice infected with 9×10^7 pfu RDA, and mice treated with 3×10^7 or 9×10^7 pfu Ad/CCL20. Numbers above bars indicate the fraction (%) of CD3+CD4+ or CD3+CD8+ cells per lung in the lymphocyte gate (T cell subsets) at day 6.

FIGURE 4 *Effect of initial exposure to OVA in the context of CCL20 expression on the long-term immune-inflammatory response during in vivo OVA recall.* Groups of mice were treated with 9×10^7 pfu RDA or Ad/CCL20 and were concurrently exposed to aerosolized OVA on ten consecutive days; airway inflammation was allowed to resolve (>4 weeks) before mice were rechallenged with aerosolized OVA on three consecutive days. Bars represent Ad/CCL20-treated mice that were not exposed to OVA during exogenous CCL20 expression (CCL20), and rechallenged mice initially exposed to OVA in the context of coincident RDA intervention (RDA/OVA) or CCL20 expression

(CCL20/OVA). Upper panels show macrophages, lymphocytes and neutrophils in BAL obtained 72 h after the last OVA recall exposure; lower panel displays OVA-specific IgG_{2a} in serum of mice 48 h following the tenth sensitizing OVA exposure (black bars) or 72 h after OVA rechallenge (gray bars). Data indicate mean±SEM; n=4-12 per treatment group; * $p<0.05$.

FIGURE 5 *Histopathological analysis of lung tissue from OVA-rechallenged mice.* Groups of mice were treated with 9×10^7 pfu RDA or Ad/CCL20 and were concurrently exposed to aerosolized OVA on ten consecutive days; airway inflammation was allowed to resolve (>4 weeks) before mice were rechallenged with aerosolized OVA on three consecutive days. Lungs were harvested 72 h after the last recall exposure, fixed in formalin, sectioned and stained with hematoxylin and eosin. Panels display representative sections from Ad/CCL20-treated mice that were not exposed to OVA during exogenous CCL20 expression (CCL20 only), and rechallenged mice initially exposed to OVA in the context of coincident RDA intervention (RDA/OVA) or CCL20 expression (CCL20/OVA). Original magnification: 100×.

Figure 1

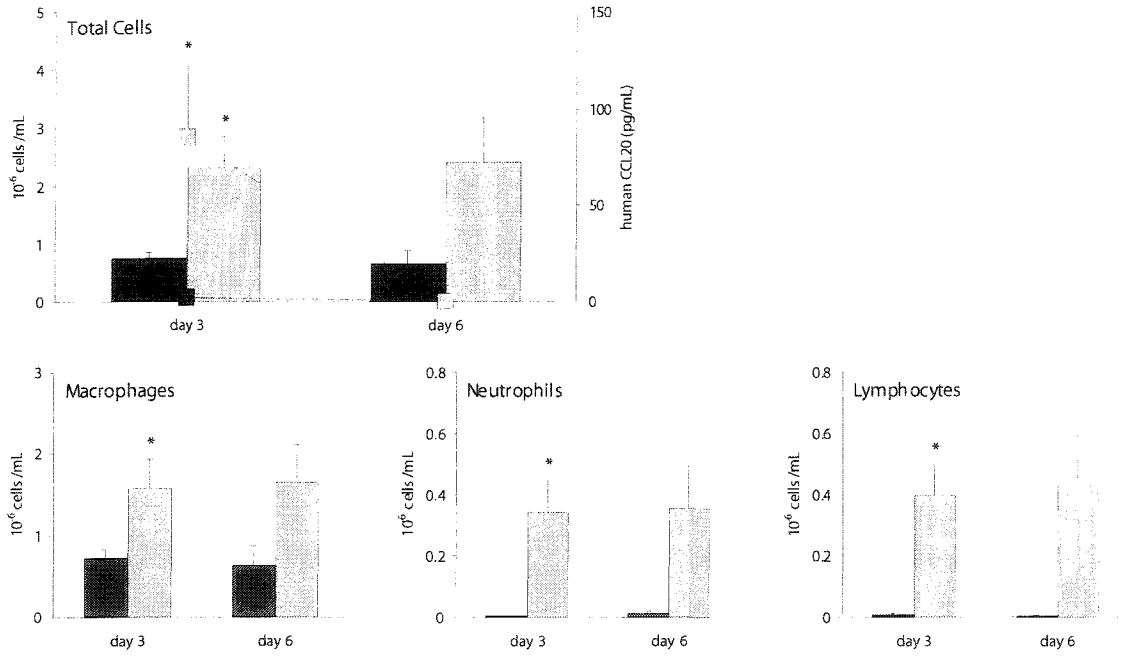
Total Cells
10^6 cells/mL
human CCL20 (pg/mL)
day 3
day 6
Macrophages
Neutrophils
Lymphocytes

Figure 2

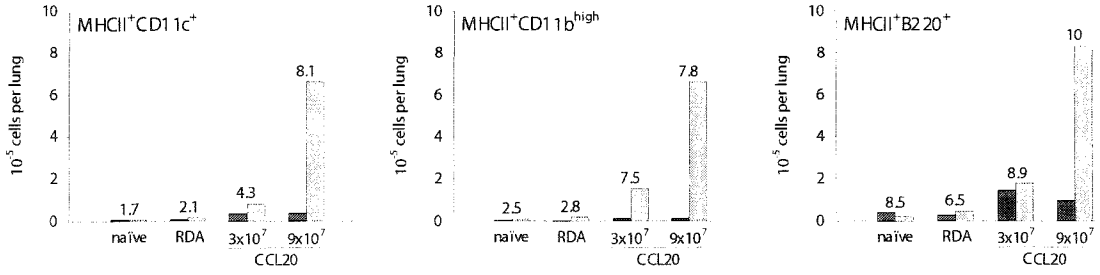
MHCII+CD11c+
MHCII+CD11bhigh
MHCII+B220+
10-5 cells per lung
0
2
4
6
8
10
1.7
2.1
4.3
8.1
2.5
2.8
7.5
7.8
8.5
6.5
8.9
10
naïve
RDA
3x10^7
9x10^7
CCL20

Figure 3

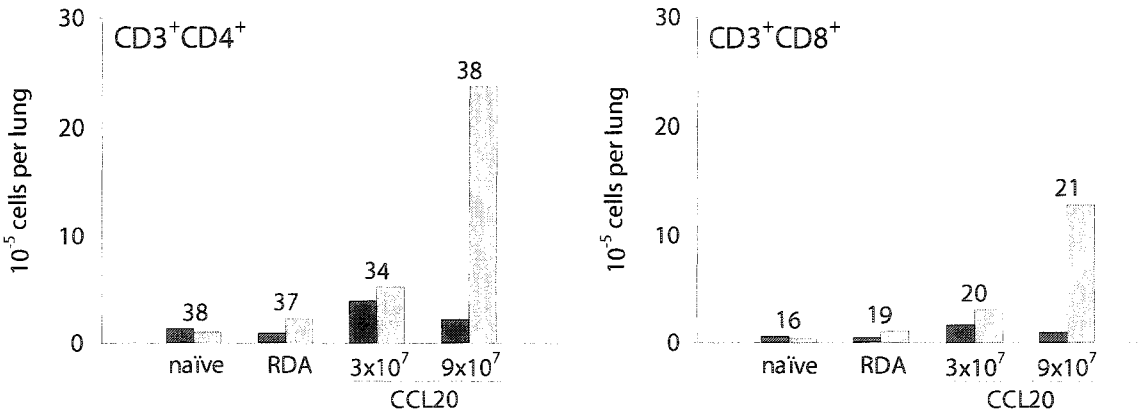
CD3+CD4+
10-5 cells per lung
30
20
10
0
38
37
34
38
naïve
RDA
3x10^7
9x10^7
CCL20
CD3+CD8+
10-5 cells per lung
30
20
10
0
16
19
20
21
naïve
RDA
3x10^7
9x10^7
CCL20

Figure 4

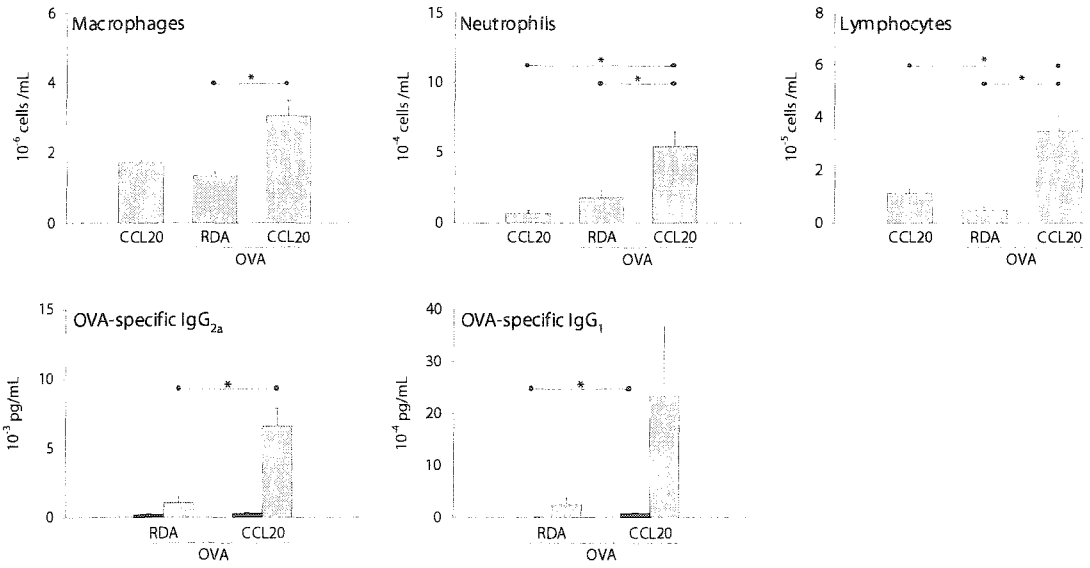
Macrophages
10^{-6} cells/mL
Neutrophils
10^{-4} cells/mL
Lymphocytes
10^{-5} cells/mL
OVA-specific IgG_{2a}
10^{-3} pg/mL
OVA-specific IgG_1
10^{-4} pg/mL
CCL20
RDA
OVA

Figure 5

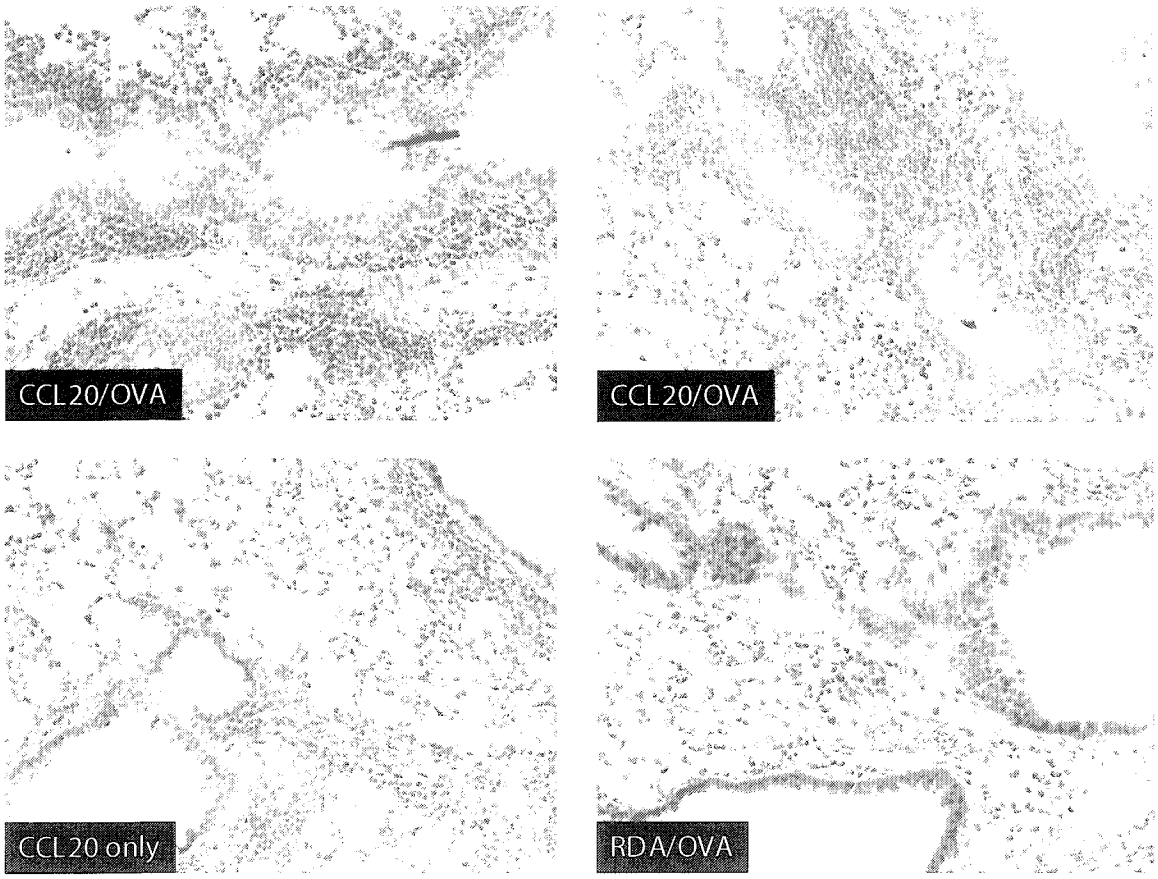

Table I Expression of costimulatory molecules by $MHCII^+CD11c^+$ APC.

	Day 6		Day 11	
	B7.1	B7.2	B7.1	B7.2
naïve	12 (0.04)	9.9 (0.03)	13 (0.11)	10 (0.08)
RDA	6.4 (0.07)	11 (0.14)	11 (0.50)	30 (1.3)
CCL20	6.3 (1.3)	24 (5.1)	17 (2.1)	44 (5.3)

Mice were sacrificed at days 6 and 11 after intranasal instillation of Ad/CCL20 or RDA on day -1. Mononuclear cells were isolated from whole lung homogenates pooled from 3 to 4 mice per treatment group. Cells were stained for flow cytometric analysis of MHC class II (MHCII), CD11c (dendritic cells), CD11b (macrophages), B220 (B cells), B7.1 and B7.2 expression; 20000 events within the mononuclear cell gate were collected for analysis. Data indicate the fraction (number $\times 10^{-4}$ per lung) of $B7.1^+$ and $B7.2^+$ cells in the $MHCII^+CD11c^+$ gate; analysis of $CD11b^+$ and $B220^+$ subsets yielded comparable results (data not displayed).

Table II Expansion of $MHCII^+CD11c^+$ APC in thoracic lymph nodes.

naïve		0.15
no OVA	RDA	0.38
	CCL20	5.6
OVA	RDA	0.65
	CCL20	15

Mice were sacrificed at day 6 of the OVA aerosolization protocol. Mononuclear cells were isolated from thoracic lymph nodes pooled from 3 to 4 mice per treatment group. Cells were stained for flow cytometric analysis of MHC class II (MHCII), CD11c (dendritic cells), CD11b (macrophages) and B220 (B cells) expression; 20000 events within the mononuclear cell gate were collected for analysis. Data indicate 10^{-5} cells in the $MHCII^+CD11c^+$ gate; analysis of $CD11b^+$ and $B220^+$ subsets yielded comparable results (data not displayed).

—CHAPTER 6—

Conclusion

Immunomodulation of Mucosal Sensitization: Interpretation

The initiation, pathogenesis and exacerbation of asthma are perpetrated (in suitably susceptible individuals) by airway mucosal contact with allergens, chemical pollutants or infectious agents. To model authentically the immunology of asthma in mice one should therefore preserve the airway as the interface of incipient contact with antigen and, by extension, as the immune microenvironment that conditions allergic sensitization. This heuristic becomes particularly salient when considering questions about the immunomodulatory effects of local, anti-inflammatory intervention. While conventional models of asthma lend important insight into the regulation of inflammation in established disease, that these models entail spatial and temporal discontinuities between antigen sensitization and challenge obscures consideration of airway immunology as the adaptive response evolves. Employing a model of airway mucosal sensitization, the research presented in this document underscores the importance of the airway microenvironment in the establishment and modulation of systemic immunity. Of substantive clinical interest, these data indicate that the efficacy of acute, therapeutic intervention in experimental asthma must be reconciled with the status of the antigen-specific response once treatment has ceased.

ANTI-INFLAMMATORY IMMUNOMODULATION. In no instance did this caveat prove more illuminating than in our investigation of corticosteroid (CS) treatment in the mucosal Th2 model (Chapter 2). As one of the most widely prescribed pharmaceuticals for the management of asthma symptoms, CS represented an unimpeachable therapeutic standard to validate the clinical relevance of the mucosal Th2 model. To this end, it was not surprising to observe that CS intervention, either during initial OVA exposure or in rechallenged mice

with established allergic airways disease, attenuated eosinophilic airway inflammation and ameliorated methacholine-induced AHR. However, it proved equally true that therapeutic success locally belied immune phenomena systemically. Indeed, even as surrogate asthma "symptoms" improved in the lungs of CS-treated mice during sensitization, a retrograde, OVA-specific Th2 response evolved in the lymphoid organs (Models *ii* and *iii* in Figure 4, Introduction). Elevated levels of circulating OVA-specific IgE and heightened Th2 cytokine secretion by cultured splenocytes and lymph node mononuclear cells persisted for several weeks in mice initially exposed to OVA in the context of CS intervention, pointing to the establishment of an amplified Th2 response. Though the mechanism by which CS treatment inculcated this therapeutically counterintuitive Th2 phenotype remains unclear—and may reflect Th2-biased conditioning of the APC compartment—these data do help to unify a number of largely *in vitro* studies that have intimated the Th2-amplifying disposition of CS [36-42].

Given the utility of the mucosal Th2 model in elucidating the immunomodulatory complexity of CS, whose therapeutic purview was ostensibly restricted to inflammation, it was of comparative interest to investigate the immune-inflammatory nuances of the costimulatory receptor ICOS (Chapter 3). That ICOS is putatively associated with the priming and execution of Th2 effector function has qualified it as a prospective therapeutic target for asthma [53-56]. In fact, it is the specialized therapeutic implication of those two words—*Th2 effector*—that, if substantiated, renders ICOS such a conceptually attractive target. They imply that, like CS, an ICOS inhibitor could regulate an established inflammatory response—while offering a tangible advantage over CS in the treatment of asthma: specificity for Th2 cells, thereby sparing concurrent, potentially protective, Th1

responses. Consistent with the findings of other groups [57,58], ICOS neutralization during initial OVA exposure in the mucosal Th2 model (but not in the complementary mucosal Th1 model) partially inhibited airway inflammation. However, mimicking the paradoxical uncoupling of local and systemic effects observed with CS treatment, anti-ICOS intervention also resulted in the sustained hyper-polarization of the evolving, OVA-specific Th2 response—ultimately resulting in an amplified airway inflammatory response upon long-term antigen rechallenge in the absence of further treatment. In essence, the anti-inflammatory efficacy of ICOS neutralization in the lung masked deleterious immunomodulatory effects whose significance was only realized once the allergic response had fully matured. This anti-inflammatory efficacy, moreover, enjoyed only a narrow window of opportunity. Notwithstanding significantly elevated levels of ICOS expression on infiltrating T cells, ablation of ICOS during long-term rechallenge of mice with established allergic disease had no effect on the magnitude or phenotype of the airway inflammatory recall response.

Since the December 2002 approval of the manuscript "Evaluation of inducible costimulator/B7-related protein-1 as a therapeutic target in a murine model of allergic airway inflammation" for publication by *Am J Resp Cell Mol Biol*, a number of new studies have produced data that may advance the interpretation of our original observations. First, there is an emerging consensus that the specialization of ICOS for Th2 responses is overstated [80,81]. While it may be true that, in idealized, highly abstracted polarizing conditions, ICOS signaling is most efficient at elaborating Th2 cytokine production [82], *in vivo* ICOS is as instrumental to cardinal cell-mediated processes, such as myocarditis [83], cardiac transplant rejection [84,85] and rheumatoid arthritis [86], as it is to asthma and allergy. The therapeutic promise of Th2 specificity may therefore be illusory. Moreover, depending on the organ, the

antigen-presenting cell and the magnitude of ICOS expression, ICOS signaling may also be required for *negative* regulation of T cell effector function. High levels of the ICOS ligand B7-related protein (RP)-1 are constitutively expressed by lung DC in non-inflamed conditions [87], consistent with the previously documented role of ICOS in the establishment of inhalation tolerance [60] (reviewed in [88]); it is also apparent that the strength of the T cell response to antigen presentation by B cells is downregulated through ICOS/B7RP-1 [89]. The unexpected immunomodulatory activity of anti-ICOS intervention in the mucosal Th2 model may therefore reflect interference with the lung's intrinsic immune-regulatory apparatus. On the other hand, the dichotomous roles of ICOS in T cell activation and suppression may be related to differences in levels of ICOS expression by T cells. Indeed, that $ICOS^{hgh}$ T cells from naïve mice are selectively enriched for IL-10-secreting clones [90] may help to explain why ICOS ablation was therapeutically redundant during long-term OVA recall, despite amplified levels of ICOS on T cells recruited to the airway. Speculatively, ICOS may potentiate (Th2-polarized) immunity during incipient stages of the immune-inflammatory response, when ICOS expression is comparatively low; as the response matures and ICOS expression peaks, the ICOS/B7RP-1 pathway may become a self-limiting regulator of the very effector activity it engendered.

EXPERIMENTAL SYSTEMS AS CLINICAL PARADIGMS. Although the immunoregulatory properties of both CS and ICOS are certainly of scientific interest, that they were observed in the context of allergic sensitization begs the question of clinical relevance. It is tautological to say that asthma patients cannot be treated *before* the onset of symptoms (or before the diagnosis of disease), but this is effectively the clinical paradigm that was replicated in the mucosal Th2 model. How tractable is a mature adaptive response? Can CS treatment, by

extension, amplify the systemic Th2 response in patients with *established* asthma? This has become a question of urgent clinical moment given the recent focus on data recommending *early* and *prolonged* use of inhaled CS to forestall the irreversible airway remodeling associated with asthma progression [32]. It certainly is not clear whether any intervention, let alone CS, can modify the adaptive response in a patient who is, by definition, already sensitized. Even if CS were able to amplify established systemic Th2 immunity, it is unlikely that this would ever normally be detected in asthma patients, whose indefinite reliance on CS for the routine management of symptoms would likely mask the deleterious immunological consequences of sustained treatment. However, it is clear that the phenotype of an allergic response is not necessarily static. In mice, repeated inhalation of OVA can subvert a pre-existing OVA-specific allergic response and elaborate antigen tolerance [28]; in humans, the avowed success of allergen immunotherapy proves that patients can, after a protracted regimen of allergen injections, be desensitized to these allergens (reviewed in [91]). It is possible that allergen desensitization depends more on the particular biochemical properties of certain innocuous proteins than on the immune-inflammatory microenvironment [31]. However, the phenomenon of allergen desensitization illustrates that permanent modification of an established adaptive response is conceivable, making the immunomodulatory activity of CS and ICOS both biologically interesting and clinically germane.

IMMUNOMODULATION THROUGH CELL TRAFFICKING. If Chapters 2 and 3 investigated immunomodulation by *anti-inflammatory* interventions, the research detailed in Chapters 4 and 5 was conceptually reversed, impelled by questions about immunomodulation through the *selective recruitment of inflammatory cells.* To this end, the specialized ability of chemokines to mobilize and direct the migration of particular leukocyte populations was of outstanding

biological interest. The basic hypothesis driving these studies went something like this: overexpression of a given chemokine in the airways of mice should influence the evolving immune response to concurrent OVA exposure in a manner consistent with the cell sub-population or phenotype for which that chemokine has a predilection. For instance, in Chapter 4 we found that Ad-mediated gene transfer of IP-10, a chemokine associated with the recruitment of Th1-associated cells, to mice subjected to the mucosal Th2 model supplanted the default OVA-specific Th2- by superimposing a Th1-dominant immune-inflammatory response. Presumably, the *de facto* cytokine microenvironment that IP-10 established through the preferential mobilization of IFNγ- or IL-12-producing cells altered the context in which antigen was harvested by local APC and transmitted to naïve T cells in the draining lymph nodes. In Chapter 5, we substituted GM-CSF, the APC-activating cytokine that drives Th2 sensitization in the mucosal Th2 model, with the chemokine MIP-3α/CCL20, whose role in the recruitment of immature DC positioned it as an immunostimulatory signal that might subvert inhalation tolerance to OVA. Indeed, OVA exposure in the context of a CCL20-conditioned airway microenvironment bypassed tolerance but, in contrast to GM-CSF, introduced instead a Th1-polarized immune-inflammatory response. Though it can only be inferred from the ensuing immune phenotype that CCL20 overexpression preferentially mediated the infiltration of Th1-biased APC, it is unequivocally true that CCL20 marshaled a critical mass of DC that heightened the immunogenicity of an otherwise innocuous protein.

The manuscript "Expression of the Th1 chemokine IFNγ-inducible protein 10 in the airway alters mucosal allergic sensitization in mice", published in *J Immunol* in February 2001,

has recently been the subject of a spate of ostensibly conflicting data concerning the pleiotropic role of IP-10 in allergic airways disease. On the one hand, Lambert *et al.* [92] have demonstrated that exposure of mice to ultrafine carbon particles—an environmental stimulus putatively involved in asthma exacerbation and ætiology—diminishes RSV-induced expression of IP-10 mRNA in BAL. Symmetrically, allergen immunotherapy in mice with established experimental asthma has been shown to be more efficacious if Th1-polarizing CpG oligodeoxynucleotides and low-dose allergen are injected concurrently; apparently, the amelioration of airway eosinophilia upon subsequent antigen challenge is associated in part with the induction of IP-10 [93]. While these and other [94] studies reinforce our contention that the IP-10/CXCR3 axis antagonizes, or is at least incompatible with, the manifestation of an allergic phenotype, data from Medoff *et al.* [95] are less sanguine about this conceptualization of IP-10 biology. Using a conventional model of intraperitoneal sensitization to OVA, these authors have shown that constitutive overexpression of IP-10 in transgenic mice amplifies cardinal features of the allergic response, while genetic IP-10 deficiency dampens it. It is difficult—if tempting—to speculate on whether the dissonance between these and our own data are simply an artifact of the very different experimental systems the two studies employed. However, a recent paper from Thomas *et al.* [96] helps to reconcile these discordant results by postulating a nuanced regulatory role for IP-10 in allergic airway inflammation. On the one hand, neutralization of IP-10 during allergen challenge of cockroach antigen-sensitized mice exacerbated eosinophil accumulation in the lung and concomitant AHR; on the other, instillation of exogenous IP-10 into the airway during challenge also amplified eosinophil infiltration and, following an initial depression, the magnitude of the physiological response. One interpretation of these data is that the

capacity of IP-10 to modulate allergic responses through the preferential introduction of Th1 phenomena is offset by its eosinophil-recruiting properties. By comparison, that IP-10 overexpression in the mucosal Th2 model was induced during sensitization—and therefore before a Th2-biased, eosinophil-yielding immunological predisposition had even been programmed—obviated this complication.

Immunomodulation in the Context of Asthma Therapy

This discussion of the immunomodulatory possibilities in asthma begs more strategic questions about what constitutes a *bona fide*, meritorious treatment for allergic airways diseases. Should the object of intervention be to interrupt a single molecular pathway or to modulate an immunologic programme? What molecular targets hold the most promise? The extensive knowledge compiled over the last decade on the relevance of cytokine, chemokine, costimulatory and intracellular signalling networks in the pathogenesis of allergic asthma has impelled researchers in academia and, particularly, the pharmaceutical industry to investigate a variety of strategies to interfere with the biological actions of these immune mediators and, ultimately, to develop novel asthma therapeutics. The ponderous number of studies that have been reported to date impart, in our opinion, two main conclusions: first, the actual testing of intervention strategies has, irrespective of outcome, clearly advanced our understanding of the role of these molecules in the pathogenesis of asthma; and second, none of these interventions appears to promise to become an important addition to, or replacement for, the existing therapeutic armamentarium. It may be informative to speculate and elaborate on the reasons underlying this, admittedly argumentative, second conclusion.

- If a strategy is developed as a therapeutic, which implies an ability to ameliorate clinical features of an already established allergic phenotype, then the candidate therapy must face, and survive, the challenge of comparison with currently available medicines. That short- and long-acting bronchodilators and, especially, inhaled corticosteroids introduce a rather outstanding "therapeutic index" makes this challenge particularly steep. It is conceivable, however, that an intervention, such as gene transfer, could be employed to enhance the activity of corticosteroids. In corticosteroid-resistant asthma, for instance, the pharmacologically deleterious accumulation of c-fos, the inducible component of activating peptide (AP)-1, diminishes the binding of glucocorticoid receptor (GR):corticosteroid complexes to transcription regulatory elements on DNA [97]; Lane *et al.* [98], however, have demonstrated that pretreatment of PBMCs from steroid-resistant patients with antisense oligonucleotides (ASO) targeted to c-fos mRNA enhanced GR binding DNA.
- In light of our appreciation of molecular networks and, notably, of molecular redundancy, the notion that interference with a single effector molecule will confer a profound and sustained effect on the expression of the asthmatic phenotype is tenuous. Indeed, the overlapping functions of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, or of the Th2-affliated cytokines IL-4, IL-9 and IL-13, in the development and presentation of allergy weave a complex, highly resilient immunologic fabric. Intuitively, a more compelling approach would be to intervene simultaneously with several targets or to interrupt shared intracellular signalling

molecules, as proposed by ASO-mediated blockade of the Th2-affiliated transcription factor GATA-3 [99].

- We further suggest that a fundamental element in the appraisal of novel therapeutics is to consider the intrinsic heterogeneity of asthma. For example, there is an increasing consensus that bronchial hyperreactivity can be elicited by several mechanisms, some IgE-dependent, others IgG_1-dependent (reviewed in [100]), and yet others altogether independent of immunoglobulins [101]. This suggests that a given intervention engineered for the selective inhibition of IgE (through, for instance, the provision of antagonistic cytokines or the immobilization of costimulatory pathways) will likely have a sectarian impact on the correction of bronchial hyperreactivity and its associated symptoms.
- Asthma, moreover, is not a static disease. There is a history of progression that can be affected by numerous ætiological factors including smoking, environmental exposure, treatment, etc. We suggest that underlying this diversity of clinical progressions is an equally diverse cellular and molecular network. In other words, the incipient trigger of the allergic phenotype is not necessarily what maintains or perpetuates this phenotype at later stages of disease. The clinical ramifications of this proposition are clear: the therapeutic impact of a given cytokine-targeted strategy in an asthmatic whose near-normal pulmonary function necessitates only occasional use of bronchodilators will likely be vastly different in an asthmatic whose FEV1 is 65% and who requires regular, low-dose, inhaled steroids to control symptoms.

Where does this rather complicated perspective leave us? It forces us to consider strategically the kinds of modalities that afford the best chance of supplanting existing pharmacopoeia. In

particular, inhaled steroids and bronchodilators, while therapeutically unimpeachable, do not cure asthma: that is, they do not permanently reverse the immunologic and pathophysiologic phenomena that account for the persistence of disease. It is in this light that treatments combining allergens and immunomodulatory signals, rather than treatments to neutralize effector cytokines or their molecular pathways, introduce a unique therapeutic tangent: to modify the very nature of allergen-initiated and, even, allergen-perpetuated events, in all likelihood by conditioning the context in which the allergen is presented by APC to T cells. Admittedly, this prospect is tricky, especially considering that such therapy would be implemented in asthmatics who, by definition, have already been sensitized; nonetheless, this re-education of the immune system is conceptually plausible given that allergen immunotherapy, though cumbersome, protracted and poorly standardized, is a remarkably effective treatment in some patients. However, to contemplate such therapies renders oneself vulnerable to the same limitations to which other modalities have succumbed: the reductionist tendency to simplify the molecular underpinnings of allergic sensitization. In other words, if the goal is therapeutic re-education of the immune response to allergens, the design of the therapeutic instrument must be informed by an integrated conceptualization of immune function—both molecularly and topographically. With these caveats in mind, however, we argue that it is in the manipulation of allergen-specific immunity—the conversion of an inflammatory response to a persistently inert response upon recognition of allergen—where the greatest promise of novel therapies for asthma rests.

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