IMMUNE REGULATORY MECHANISMS OF THE RESPIRATORY MUCOSA

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Abstract

Asthma and other allied allergic diseases represent a significant burden to health care and are recognized as endemic in the Western World. While a diverse array of effective pharmacopoeia provides reprieve from symptoms, no preventive or curative therapies are currently available. This is in part due to the paucity of understanding how allergic diseases develop. Recently, remarkable progress has been made in this regard, largely due to the discovery of regulatory mechanisms that control responsiveness to antigen in the airway. Indeed, to understand fully why people develop asthma requires an understanding of both allergic sensitization and tolerance. Work presented in this thesis contributes to our knowledge of inhalation tolerance. Using a classical mouse model of allergic airways inflammation, we show in Chapter 2 that inhalation tolerance is a persistent and active process as it prevents the generation of airway eosinophilia, antigen-specific IgE and airway hyperresponsiveness upon secondary immunogenic challenge, independently of IL-10 or IFN-γ. Building on these observations, in Chapter 3 we show in a mucosal model of allergic sensitization that inhalation tolerance cannot be broken with the expression of GM-CSF, a potent growth factor and cytokine that has been associated with asthma and allergy in both human and animal subjects. However, concomitant expression of decorin, a natural inhibitor of TGF-β, reverses inhalation tolerance, thus implicating TGF-β as putatively important in regulating responsiveness in the airway. In Chapter 4, we identify an alternative mode of tolerance. We show that chronic exposure to innocuous antigen in sensitized mice does not lead to chronic inflammation but to abrogated eosinophilia that can, nevertheless, be reversed with the expression of GM-CSF. Collectively, these findings enrich our understanding of tolerance and provide a framework for new discoveries that may, ultimately, lead to novel and powerful therapies for allergic disease.

Abbreviations

AHR – airway hyperresponsiveness
APC – antigen presenting cell
CCR – CC chemokine receptor
CD – cluster of differentiation
CTLA-4 – cytotoxic T lymphocyte-associated antigen 4
DC – dendritic cell
DNA – deoxyribonucleic acid
dsRNA – double stranded ribonucleic acid
EAE – experimental allergic encephalomyelitis
GM-CSF – granulocyte macrophage colony stimulating factor
HDM – house dust mite
IBD – inflammatory bowel disease
ICOS – inducible costimulator
IFN – interferon
Ig – immunoglobulin
IL – interleukin
LPS – lipopolysaccharide
MBP – myelin basic protein
MHC – major histocompatibility complex
MIP – macrophage inflammatory protein
MLN – mediastinal lymph nodes
OVA – ovalbumin
PAMP – pathogen associated molecular pattern
TCR – T cell receptor
TGF-β – transforming growth factor β
T_H – T helper
TNF-α – tumor necrosis factor α
T_R – T regulatory

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Chapter 1: Introduction

ALLERGY, ASTHMATIC INFLAMMATION AND TOLERANCE

Asthma is a complex and heterogeneous disease characterized by eosinophilic airway inflammation, intermittent reversible airway obstruction and airway hyperresponsiveness. The incidence of asthma, atopic dermatitis and hayfever has nearly doubled in developed countries over the last twenty years, with the present cost of care for asthmatics estimated at about six billion US dollars per year in the United States alone (1, 2). Although genetic susceptibility is a factor (3), the rate at which incidence of allergic diseases is rising implicates the environment as key in modulating, if not orchestrating, the process. Among the hypotheses put forward to explain the rise of allergic disease, the 'hygiene hypothesis' is most recent and deserving of attention (4). It proposes that decreased exposure to immune-stimulating infectious agents early in life predisposes to the development of allergic disease. Factors associated with decreased incidence of allergy, asthma or both, include infection with *Mycobacterium tuberculosis* (5), measles virus (6), and hepatitis A virus (7); increased exposure to infections through contact with older siblings (8); and attendance at a day-care facility during the first six months of life (9). The hygiene hypothesis then, "best accommodates the link between allergy and social class, the urban to rural gradient, infant diet, over-use of antibiotics and the East to West gradient of disease" (Stephen Holgate) (10).

Until the mid-1980's, investigations of allergic disease focused on allergen-specific IgE, mast cells, and immediate type 1 hypersensitivity responses. Understanding of allergic disease was, therefore, limited to this acute phase component triggered through allergen-induced cross-linking of specific IgE antibody bound to mast cells through high-affinity receptors. Mast cells release a range of granule-associated preformed mediators that are responsible for immediate symptoms of the acute allergic response. Although evidence that

inflammation is a component of asthma was initially derived from findings at autopsy in patients with fatal asthma, more recent studies have subsequently shown inflammation from bronchial biopsy specimens from patients with mild disease (4). Specifically, denudation of the airway epithelium, deposition of collagen beneath the basement membrane, mast-cell degranulation, and infiltration of the airway by lymphocytes and eosinophils have been found in patients with mild-to-moderate asthma (11-14).

The elucidation of mechanisms that lead to inflammation was finally made possible in 1986 when Mosmann and Coffman showed that (T_H1 and T_H2) cells cross-regulate each other (15). After the delineation of these subsets it became clear that CD4+ T_H2 cells are at the epicenter of the allergic process, as they control and amplify the allergic inflammatory response by, among other things, secreting key cytokines: IL-4 leads to enhanced IgE synthesis, IL-5 to eosinophil growth and differentiation, IL-9 to enhanced mast cell differentiation, and IL-13 to increased mucus production and airway hyperreactivity (16). Much research has also focused on the role of dendritic cells in the initiation and maintenance of the asthmatic phenotype. DCs are crucial in determining the outcome of antigen encounter and integrate signals that are derived from the antigen, its inflammatory context and the host environment into a signal that can be read by naïve T cells in the lymphoid tissues (17). The list of mediators of allergic disease has grown substantially over the years, and includes nearly every type of leukocyte, as well as a myriad of cytokines, chemokines, growth factors, and transcription factors (4). Currently, airway inflammation is regarded as the central component contributing to airway damage and dysfunction.

Correspondingly, a wealth of pharmacopoeia has emerged. New therapies for allergic diseases have been developed by improving existing classes of drug or by discovering new classes of drug through research. Corticosteroids are the most effective in treating atopic

diseases and represent the first-line treatment for chronic asthma in patients of all ages and severity of disease (18, 19). Their mechanism of action involves binding to a cytosolic glucocorticoid receptor that normally translocates to the nucleus and binds as a monodimer to DNA to activate the genes. Corticosteroids may also elicit their anti-inflammatory activity irrespectively of binding to DNA (20). The principal action of corticosteroids is to suppress multiple inflammatory genes, including cytokines, inflammatory enzymes, adhesion molecules and inflammatory mediator receptors. Although a high level of anti-inflammatory action with minimal side effects has been documented, corticosteroids do not cure the disease and allergic inflammation recurs when treatment is stopped (18). Histamine H1-receptor antagonists (antihistamines) have a long history in the treatment of atopic diseases. These drugs are effective in rhinitis and reduce itch in atopic dermatitis, but have no clear benefit in asthma (21). Multiple other antagonists and modulators have been developed, or are currently in development, including antileukotrienes (22), monoclonal antibodies to IL-5 (23), or soluble IL-4 receptors (24). In addition, several strategies to inhibit inflammation are being tested in animal models, including chemokine inhibitors, anti-inflammatory cytokines, transcription factor inhibitors, and cell adhesion blockers (18). By no means an exhaustive list, it nevertheless represents a wide variety of approaches in the treatment of atopic disease. Yet, in all cases, the treatment focuses on the effector phase of an allergic response: this is likely reflective of the paucity in our understanding of mechanisms that lead to sensitization. Although it is certain that CD4+ T_H2 effector T cells have an important role in generating the inflammation that characterizes allergic disease, including airway eosinophilia and mucus hypersecretion in atopic asthmatics, less is known about how these T_H2 cell responses are first induced. The elucidation of mechanisms that lead to sensitization remains, therefore, one of the most prescient tasks facing immunologists.

The discovery in the last several years of mechanisms that limit, and in some case prevent the development of, inflammation has greatly revolutionized our understanding of how the immune system initiates the response to antigen. Until the late 1990s, immune homeostasis was regarded as relatively inert; antigen recognition was understood to lead to sensitization and subsequent inflammation only in the presence of additional signals, as provided by costimulatory molecules or certain cytokines. It was believed that the absence of such signals leads to tolerance. For the last several years we have known that tolerance is the result of an active process that involves DCs, costimulation, cytokines and regulatory T cells (25). This reconceptualization makes it possible to envision novel treatment strategies directed at preventing the development of atopic disease. Future therapies may, for example, harness the regulatory capacity of tolerogenic DCs (26-28) or T cells (29-32), thereby preventing the development of the T_H2 phenotype with greater sophistication than that afforded by the blockade of downstream mediators. While there is still very little evidence to suggest that such approaches will dominate therapies of the future, recent findings provide impetus for these studies to continue.

Work presented in this thesis enriches our knowledge of mechanisms that limit immune-inflammatory responses to inhaled innocuous antigen. The work was conducted between 1998 and 2003, a timeline that closely parallels the explosion of our understanding of mechanisms behind tolerance. My work hinges on three hypotheses, each corresponding to a chapter found in this thesis. In chapter 2, I hypothesize that *inhalation tolerance is an active process that, once induced, prevents the generation of airway inflammation upon subsequent immunogenic challenge.* Chapter 3 builds on the findings in Chapter 2 and proposes that *established inhalation tolerance can be broken in the presence of two signals: one that typically leads to sensitization (GM-CSF) and one that may interfere with established regulatory activity in the airway (decorin).* At first glance a

departure from previous studies, work described in Chapter 4 is instructive to the extent that negative regulation of immune responses is heterogeneous and largely dependent on the context of initial exposure to antigen. In the chapter, I hypothesize that *chronic exposure to innocuous antigen in sensitized animals leads to a unique and less robust state of tolerance that can be reversed with one pro-inflammatory signal, GM-CSF.* Together, my work proposes that inhalation tolerance may have both active and passive components, some of which likely involve suppression in an organ specific manner. It further argues that the relationship between tolerance and disease is fluid and interchangeable, but that the 'favoured' response is largely dictated by initial context of exposure. My work, then, not only builds on how the immune system responds to innocuous antigen, but also adds to existing paradigms that aim to delineate the conditions that lead to allergic disease.

I divided the remainder of this Introduction into two parts. In the first, I discuss the role of the dendritic cell in mucosal immune responses while in the second I focus on T cells. I have elected to take this 'cell-centric' approach for several reasons: First, I believe that an understanding of dendritic cells and T cells in mediating allergic airways inflammation and tolerance is essential background information to my thesis. While certain aspects of my work may not address directly everything that is discussed, I believe that the value of my work is greatly enhanced when viewed through this prism. Secondly, this approach allows the exploration of other aspects of allergy and tolerance, namely, the role of cytokines, chemokines, and co-stimulatory molecules. So as to avoid repetition, concepts unique to, and discussed in, subsequent chapters are left out of the Introduction. For example, the role of GM-CSF in allergic airways inflammation, the role of decorin as it pertains to TGF-β biology, and literature on chronic exposure, while discussed in their respective chapters are, to a large extent, absent in the Introduction. Chapter 5 is a 'meta-discussion' in which ideas

explored in the respective chapters are re-examined and woven together in an attempt to acquire a novel perspective on an observation.

MUCOSAL IMMUNE RESPONSES – THE ROLE OF THE DENDRITIC CELL

DCs are pivotal in controlling responses to inhaled antigen. A network of DCs is located immediately beneath the basement membrane of respiratory epithelium (33, 34). Studies using fluorescently labeled macromolecules have shown that airway DCs capture antigen and carry it to T cell rich areas of draining mediastinal lymph nodes (MLNs) (35). DCs lining the airway are specialized at capturing antigen but unable to stimulate T cells – they are immature. Under steady state conditions, these cells continually migrate to the lymphoid tissue; there, they express an intermediate array of co-stimulatory molecules and high levels of MHC class II molecules, thereby inducing the activation of naïve T cells (35). Airway DCs, then, play a sentinel role by picking up antigen in the airways and presenting it to T cells in the lymphoid tissue (36). The functional outcome of the resultant T cell activation, however, is tolerance; this may explain why continuous migration of airway DCs under baseline conditions does not lead to autoimmunity (37). Indeed, studies have shown that the T-cell priming activity of dendritic cells under steady state conditions is restricted to low-level, T_H2-skewed responses (36, 38). On the one hand, T_H2 skewing during harmless (OVA only) or self-antigen presentation protects against T_H1-associated chronic inflammatory pathology; on the other, the observed low level activation may be indicative of an airway DC network that reaches only 'partial' maturation, thus inducing an abortive proliferative response (39, 40). According to studies investigating the consequence of short or weak stimulation of the T cell receptor, this would lead to death by neglect (41). Alternatively, harmless or self-antigen may be presented by specialized airway DCs that generate regulatory T cells that inhibit subsequent

inflammatory effector responses (32, 42). The exact subtype of DC that mediates tolerance induction in the respiratory mucosa is still unknown, although naturally occurring DC subsets putatively involved in the *in vivo* generation of regulatory cells have been described in the gut mucosa, spleen and lymph nodes (43-45). Figure 1 is a conceptual representation of the response to innocuous antigen.

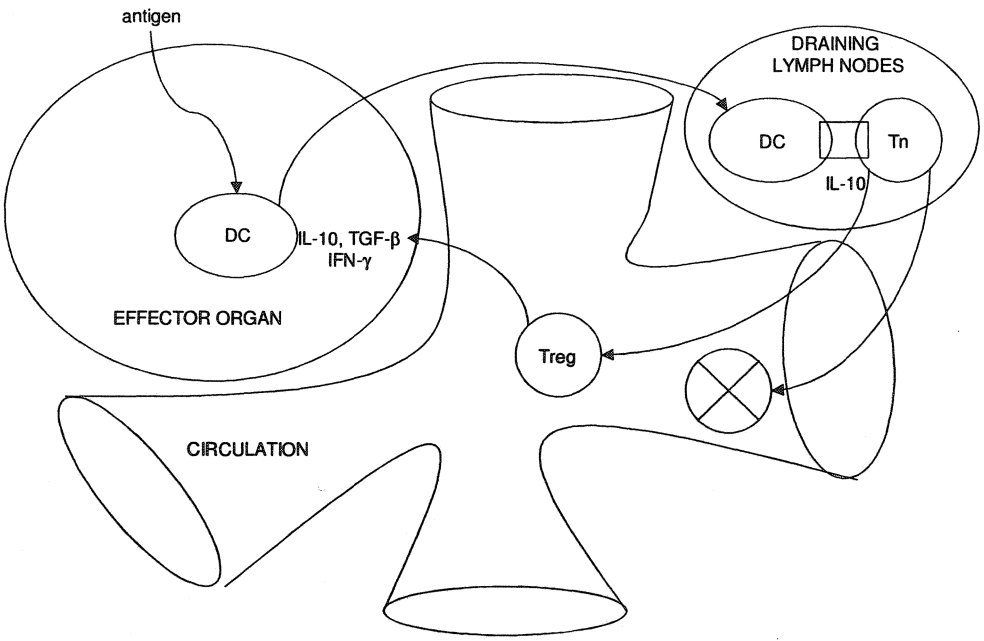

Figure 1: Schematic representation of the response to innocuous antigen during homeostatic conditions. Antigen is captured by DC in the effector organ and transported to the draining lymph nodes where it is presented to naïve T cells (Th0). This results in either anergy or death by neglect (crossed cell) and/or a regulatory phenotype (Treg) that then may migrate to the effector organ.

When exposure to inhaled OVA is accompanied by inflammatory stimuli, such as GM-CSF, TNF-α, IL-1, or bacterial and viral products such as LPS or dsRNA, migration of DCs to lymph nodes is accelerated and is associated with full maturation, characterized by a rapid and dramatic increase in the ability of these cells to induce T-cell immunity (36, 40, 46, 47). The distribution of chemokines is vital in regulating DC migration from peripheral tissues to the draining lymph nodes. In the early phase of inflammation, local production of

chemokines such as macrophage inflammatory protein (MIP)-3α will attract immature DC to non-lymphoid tissue; immature DC express CCR6, a receptor for MIP-3α (48). Upon antigen uptake DC lose their responsiveness to MIP-3α by downregulating CCR6. Concurrently, they upregulate the expression of CCR7, a chemokine receptor specific for chemokines constitutively expressed in the paracortical areas of lymphoid tissue, most important of which are MIP-3β (ELC) and 6-C-kine (SLC). Consequently, maturing CCR7-bearing DC leave the inflamed tissue, enter the lymph stream and migrate to peripheral lymphoid tissue (49-51). The arriving DC may themselves become a source of MIP-3β and 6-C-kine, thereby amplifying and maintaining the chemotactic signal (49). It is likely that these chemokines facilitate the interaction between mature DC and naïve T cells, which are also responsive to 6-C-kine and MIP-3β (52, 53).

In the presence of strong but non-polarizing danger signals, the functional outcome of OVA exposure are fully mature DCs that lead to stable T_H2 immunity, as assessed by T_H2 recall responses in the airway mucosa (54-57). Moreover, exposure to commonly encountered environmental allergens such as HDM or ragweed leads to T_H2 immunity, characterized by airway eosinophilic inflammation, and likely resulting from the inherent protease activity or other danger signals associated with allergens (58-60). In this regard, exogenous proteases have been shown to degrade proteins forming tight junctions in airway epithelium, thereby facilitating antigen access to the subepithelial DC network (61). It is likely that allergens interact with epithelial cells to induce the release of signals that favour the maturation and activation of DC (56). The discrepancy between the inability of OVA in generating strong T_H2 responses on its own, and the tendency of environmental allergens such as HDM to overcome tolerance and lead to T_H2-type inflammation, leads to at least two important

conceptualizations: one, that the extent to which OVA is a surrogate allergen is limited to its biochemical but not its immunological properties – it is a protein, the peptides of which are presented on MHC class II molecules by DC, but it does not have the built-in danger signal that real-life allergens possess. Secondly, the observation that OVA acquires allergen-like properties when concomitantly exposed to naturally occurring pro-inflammatory signals such as GM-CSF (55), suggests that the nature of a real-life allergen depends on built-in or external signals that are supplementary to the secondary structure of the molecule. Consequently, OVA will continue to be used as a surrogate allergen because it may, among other things, clarify the precise nature of those signals.

Concurrent exposure to OVA and strong T_H1 polarizing signals such as high doses of LPS, leads to stable T_H1 responses (62). The tendency of the respiratory mucosa, and mucosal tissues in general, to induce T_H2 polarized responses is consistent with the low-level T_H2 activation as observed under steady state conditions, and may depend on the particular profile of resident cells in the tissue (38, 56, 63).

It is becoming increasingly clear that initiation of both effector and regulatory T cell immunity requires antigen presentation in the lymphoid tissue in the context of a specific costimulatory molecule profile (64-66). Although all the major costimulatory pathways such as CD28/B7, ICOS/ICOSL, and OX40/OX40L have been implicated in generating polarized responses, it is unclear to what extent a particular costimulatory molecule profile dictates the polarization. Indeed, a growing number of studies have implicated these pathways not only in T_H2 (67-69) but also in T_H1 responses (70-73). Costimulatory molecules are also important to tolerance. In this regard, Umetsu and colleagues have shown that the ICOS/ICOSL pathway is critical to the generation of regulatory T_R cells that control hyperresponsiveness and airway inflammation during antigen recall (32). Other molecules of different pathways

may also be involved (74, 75). Notable among these is cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a CD28 homologue expressed on T cells. It binds to B7.1 and B7.2 and provides a negative signal for T cell activation (76). CTLA-4 is known to inhibit the development of colitis (77) although these findings are still controversial (30). Importantly, it had been reported that the induction of T cell anergy *in vivo* is due to an abortive T cell response that requires recognition of B7 molecules by CTLA-4, since blocking B7 maintained T cells in an inactivated but functionally competent state (78). While this study did not address the suppressive potential of anergic T cells (see below), it provided additional evidence that costimulation may not be as important to polarization as it is to the generation of effective T cell responses.

Clearly, other DC-generated signals are necessary to educate T cells to perform the necessary function. Studies have shown that DC-generated IL-12 influences T_H1 polarization, while its relative absence leads to T_H2 responses. For example, IL-12 deficient mice fail to prime for T_H1 responses but develop T_H2-type cells (79). The T_H1 polarization that occurs during high doses to LPS, peptidoglycan, or CpG motifs, involves the induction of IL-12 by DCs (80), while exposure to nematodes leads to T_H2 polarization, likely because nematodes do not stimulate DC IL-12 production (81). Notable other cytokines shown to influence IL-12 production, and consequently T cell polarization, are IL-10 and IL-6 (82). Indeed, freshly isolated, lung DCs produce IL-10 and IL-6, suggesting that the primary physiological role of these cytokines is in controlling the generation of T_H1 responses (57). IL-10 may also be important in tolerance. While it inhibits MHC class II and costimulatory molecule expression on DCs (83, 84), IL-10 also controls the development of eosinophilic airway inflammation (85), and may be involved in generating tolerogenic DCs (42, 44).

Recent studies show that DCs may be important not only in the generation of an immune response but also in its maintenance. There is, for example, an 80-fold increase in the number of myeloid DCs in the airway mucosa and BAL fluid of mice and rats with experimentally induced asthma (86-88). These DCs have a mature phenotype, suggesting an interaction with primed T cells in the airway. Interaction between airway DC and T memory cells in the airway mucosa may also lead to local maturation of DC function (89). The observation that airway DC produce chemokines that selectively attract CCR4-expressing memory T_H2 cells makes it more plausible that DCs interact locally in the lung (90). There is, moreover, increased migration of airway DCs to the lymphoid tissue during antigen challenge of primed mice, implying that re-stimulation of resting central memory T cells takes place, thereby inducing their proliferation and differentiation into effector T cells (17, 39, 89, 91). Finally, while some studies have shown that the administration of DCs induces and exacerbates T_H2 eosinophilic inflammation (92, 93), potentially through the OX40/OX40L pathway (94), removal of DC from sensitized mice eliminates asthmatic features induced by antigen aerosol (17, 34). It is still unclear whether, and to what extent, DCs maintain tolerance in the lung. Figure 2 is a simple conceptualization of an inflammatory response to antigen.

Although DCs are the most potent APCs and are believed to be indispensable to the initiation of T cell immunity (95) other APCs may also be involved in the pathogenesis of asthma and airway inflammation. Alveolar macrophages are scavenger cells that ingest particulate antigen and suppress adaptive immunity (96). When depleted, both primary and secondary immune responses are greatly enhanced (97). Macrophages are, therefore, predominantly protective against T_H2 responses. Although B cells can function as APCs during cognate interaction with T_H2 cells, sensitized B-cell-deficient mice show no signs of

reduced levels of T_H2 type effector cytokines, airway eosinophilia or goblet cell hyperplasia (98). Epithelial cells and eosinophils have also been shown to present antigen, but little is known as to their role *in vivo* (17).

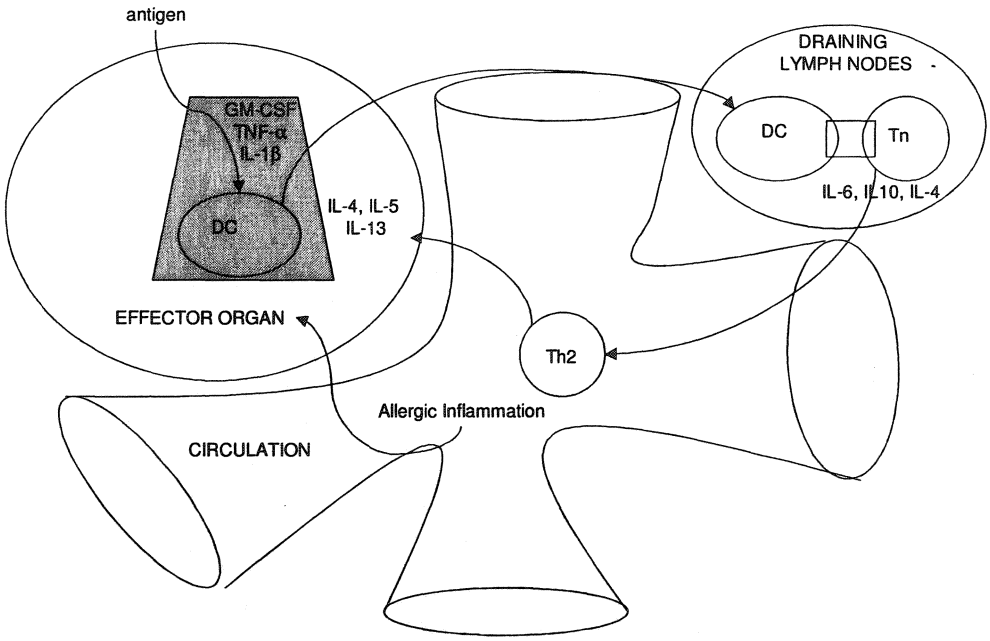

Figure 2: Schematic representation of the inflammatory response to antigen. In the presence of a danger signal such as GM-CSF, antigen is captured by DC in the effector organ and transported to the draining lymph nodes where it is presented to naïve T cells (Tn). This results in generation of Th2 cells that then may migrate to the effector organ and orchestrate allergic airway inflammation.

MUCOSAL IMMUNE RESPONSES – T CELLS AND BEYOND

It has been known for a long time that atopic individuals express high levels of allergen specific IgE, but the reason for these responses were not well understood until the discovery of T_H1 and T_H2 subsets of CD4 T cells (15, 99). T_H1 cells are defined by their ability to produce IL-2, IFN-γ and TNF-α, whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. T_H1 cytokines mediate cytotoxic and inflammatory functions as well as delayed-type hypersensitivity reactions (100, 101); in contrast, T_H2 cytokines mediate inflammatory

responses that involve mast cells and eosinophils as well as IgE (102). A number of immune responses *in vivo* appear to be predominantly mediated by T_H1 or T_H2 polarized T cells, and it is now well established that asthma and other allied allergic inflammatory diseases are mediated by T_H2 cytokines. The presence of T_H2 cytokines at sites of allergic inflammation is consistent with a T_H2 etiology of the disease (103-106). A more definite proof that CD4+ T_H2 cells are responsible for the generation of allergic inflammation was shown when T-cell receptor transgenic T_H2, but not T_H1, cells were adoptively transferred to mice (107-110). Although other cell types such as CD8+ T cells have been shown to participate in asthma pathophysiology, particularly in mediating airway hyperresponsiveness (111, 112), CD4+ T_H2 cells are the main orchestrators of allergic-type inflammation.

The individual roles of T_H2 cytokines in mediating allergic airway inflammation have now been described. Cytokines involved in generating airway eosinophilia include IL-5 and IL-13 (113-120), while IL-4 and IL-9 are important in enhancing the response (121-124). Mucus hypersecretion has been attributed to IL-13 and IL-9 (117-119, 122, 124); and airway hyperresponsiveness to IL-13 (117, 118, 120). General effects on T_H2 type responses, including T_H2 differentiation and IgE isotype class switching have been associated with IL-4 and IL-13 (125-130).

Central to the T_H1/T_H2 paradigm is the idea that T_H1 and T_H2 responses cross regulate one another (15, 101, 131). This gave rise to the notion that T_H1 responses can prevent asthma. Initial findings have shown that allergen specific T cells generated from non-allergic individuals express a T_H1 cytokine profile (132). Additional studies have shown that allergen immunotherapy for allergic disease converts T_H2 cytokine profiles into T_H1 profiles, correlating with symptomatic improvements in both human subjects (133-135) and in animal models (136-138). These findings are consistent with the hygiene hypothesis, which, as

discussed earlier, suggests that decreased exposure to T_H1-type infections leads to the development of T_H2 responses and increased incidence of allergic disease (5, 6). The findings also correlate with the concept of immune deviation as a mechanism for tolerance (139). To this end, it has been shown that the suppression of IgE responses in an animal model of allergic inflammation depends on the expression of IFN-γ (140), although these findings have been contested (141, 142). Several other lines of evidence argue against T_H1 responses as protective for the generation of asthmatic airway inflammation. Not only have T_H2 allergic diseases been on the rise in recent years, but the prevalence of T_H1-associated diseases such as Inflammatory Bowel Disease (IBD) and Type I diabetes has also risen (2). Given that T_H1 cells are pro-inflammatory, the development of a T_H1-associated inflammatory response is unlikely to downmodulate asthma and allergic disease (143, 144). In human subjects, inhalation of recombinant IFN-γ resulted in increased number of lymphocytes in the airways (145, 146). Together, these findings argue against T_H1-based therapy as a likely treatment option for allergic disease.

The characterization in recent years of T cell subsets that suppress the function of other cells has greatly revolutionized our understanding of immune processes, challenging investigators to expand, if not abandon, the T_H1/T_H2 paradigm. To date, at least four populations of regulatory cells have been described: CD4+CD25+ cells, T_R1 cells, T_H3 cells, and T_R cells. First identified by Sakaguchi and colleagues (147, 148), CD4+CD25+ cells are the most widely studied (149, 150). These naturally occurring, poorly-proliferating, thymus derived CD4+ T cells constitutively express CD25, express low levels of CD45RB and high levels of CTLA-4 (30, 77, 151, 152), and constitute ~10% of the peripheral murine and human CD4+ T cell population (153-156). They possess potent regulatory activity both *in vitro* (30, 157) and *in vivo* (77, 147, 148, 158); they inhibit autoimmune diabetes in mice and rats (151, 154),

induce tolerance to alloantigens (159, 160), impede anti-tumor immunity (161), and prevent the development of gastritis (155) and colitis (77). CD4+CD25+ T_R cells likely use multiple mechanisms by which they suppress immune responses, although the ability to inhibit proliferation of other T cell populations by specifically inhibiting the production of IL-2 is a defining feature of their function (30, 157, 162). Suppression *in vitro* requires activation of CD4+CD25+ T_R cells via their TCR (163), does not involve killing of the responder cells and is mediated through cell contact (30, 157). Strikingly, although CD25+ T_R cell activity depends on antigen-specific activation, once activated, these cells inhibit both CD4+ and CD8+ T cell responses in an antigen-nonspecific manner (163).

The role of suppressive cytokines, particularly IL-10 and TGF-β, is still controversial. Although addition of neutralizing antibodies specific to IL-10 or TGF-β does not reverse suppression (30, 157, 162, 164-167), and CD25+ T cells from IL-4 or IL-10 KO mice are competent suppressors *in vitro* (30), the plasticity of the immune system, as well as possible involvement of a cell-bound cytokine, or a cytokine acting over short distances, keeps this issue from being resolved (150). Studies by Nakamura and colleagues suggest, for example, that suppression should be reversed with high concentrations of antibody; otherwise, the interface between the CD4+CD25+ and CD4+CD25- T cells is difficult to penetrate (168). The controversy is further underscored by different *in vivo* studies that implicate either IL-10 or TGF-β in controlling autoimmune disease (169, 170). These studies caution against relying on *in vitro* studies for the analysis of regulatory cell function. Collectively, the phenotypic and functional characterization of CD4+CD25+ T cells has not only enriched our knowledge of central and peripheral tolerance, but also bridged the gap between anergy and active suppression, mechanisms of tolerance formerly believed to be unconnected (139, 155). Compelling evidence currently links anergy with suppression and

APC function (171-173). Identification of markers that will reliably distinguish suppressor T cells from other T cell populations, as well as the characterization of molecular pathways responsible for mediating suppression, are only two of many challenges facing investigators in this field (174-176).

When human or murine CD4+ T cells are stimulated *in vitro* to allogeneic antigen in the presence of IL-10, anergic clones are generated (177) that, upon repetitive stimulation secrete high amounts of IL-10, moderate amounts of TGF-β, and inhibit immune responses (29). Termed T_R1, these T cell clones suppress the immune response of other T cells *in vitro* and *in vivo*, and inhibit the development of colitis (29), and other inflammatory processes (178), including, possibly, T_H2-associated diseases like asthma (179). Whether T_R1-like cells occur naturally, and if so, whether they are ontologically or functionally related to CD4+CD25+ T_R is still to be determined, although recent studies argue against such association (180). Interestingly, *in vitro* culture of bone marrow cells in the presence of IL-10 induces the differentiation of a distinct subset of dendritic cells with the capability to generate T_R1 cells (44).

T_H3 regulatory cells were first isolated from mesenteric lymph nodes of animals rendered orally tolerant to low dose feeding of MBP in a TCR-transgenic Experimental Allergic Encephalomyelitis (EAE) model. These cells produce high levels of TGF-β and moderate levels of IL-4 and IL-10 (181, 182). T_H3 cells suppress both T_H1 and T_H2 cells, are triggered in an antigen-specific fashion requiring B7.2 engagement, but suppress non-specifically (31). It is likely that anergic T cells, T_R1 cells and T_H3 cells are derived from the same population: they have a similar phenotype and usually mediate their suppressive activities via the release of the cytokines TGF-β and IL-10 (149).

At least two regulatory mechanisms specific to the respiratory mucosa have been described. We, and others, have shown that respiratory administration of OVA induces inhalation tolerance, as characterized by inhibition of AHR and airway inflammation upon subsequent immunogenic challenge (141, 183). These studies were consistent with earlier findings that depicted IgE hyporesponsiveness and reduction of peripheral blood eosinophilia in tolerant animals (142, 184). Because production of OVA-specific IgG1 and IgG2a was increased in tolerant animals suggested that immune deviation or divergent tolerance was induced. Importantly, exposure to OVA involved activation of OVA-specific T cells, as assessed by expression of activation markers CD69, CD25, CD44, and maturation of DCs as assessed by high levels of B7.1, B7.2, CD40 and ICOSL (42). Mature DCs from tolerant animals produced IL-10 as depicted by RT-PCR or intracellular cytokine staining, and induced OVA-specific T cells to proliferate and produce IL-4 and IL-10 but not IFN-γ or TGF-β (42, 131). When these T_R cells were adoptively transferred into mice previously primed with OVA they inhibited the development of AHR and airway inflammation via an IL-10 dependent mechanism, since neutralization of IL-10 with anti-IL-10 mAb reversed the inhibitory effect. The inhibitory function was also dependent on the ICOS-ICOSL costimulatory pathway (32). Umetsu and colleagues have argued that, since both T_R and T_H2 cells require ICOS-ICOSL costimulation to develop, and both produce IL-4 and IL-10 (albeit at different concentrations), suggests that these two forms of immunity are related (131). They propose that normal exposure to respiratory allergen should result in the development of T_R cells and tolerance; T_H2 cells develop as an aberration of T_R cell development, possibly as a result of inadequate production of IL-10 or enhanced IL-4 and IL-13 (131). The observation that tolerance develops in the absence of IL-10, however, suggests other mechanisms may be involved (141).

Another possible candidate in mediating tolerance is TGF-β. Studies have shown that T cells engineered to secrete TGF-β reduce airway inflammation and AHR (185). The effect is dependent on TGF-β since neutralization with anti-TGF-β abolishes the effect. Moreover, blockade of TGF-β signaling enhances airway inflammation and AHR in animals sensitized to OVA, suggesting a role for TGF-β dependent regulation (186). The first evidence that TGF-β may play a role in regulating inhalation tolerance *in vivo* came from our recent studies (Chapter 3). In this study, we demonstrate that over-expression of the pro-inflammatory cytokine GM-CSF is insufficient to break established tolerance, and that additional immune modulation in the form of TGF-β sequestrations is concurrently needed. We argue that two signals are needed to break established tolerance *in vivo*: a signal that leads to sensitization (GM-CSF) and a signal that interferes with regulation (decorin). Most likely both IL-10 and TGF-β-dependent mechanisms are important in regulating responsiveness in the airway and future studies will determine their relative contribution. The role of IFN-γ in regulating tolerance has been hotly disputed in the past, although interest in the molecule has dwindled in recent years (141, 142, 187).

Although it is well established that T cells are the central orchestrators of allergic airways inflammation and asthma, many questions remain as to the precise mechanisms involved. It is still unclear, for example, to what extent T cells influence airway remodeling. Nonetheless, since the recognition that immunologically mediated responses are integrally linked to the development of airway inflammation and, therefore, the inception, persistence, and severity of disease, treatment of asthma is now directed at those factors. Importantly, in order to understand fully why people develop asthma, mechanisms governing both

sensitization and tolerance must be elucidated. Our increased understanding of these mechanisms will yield novel approaches to the treatment of disease.

Chapter 2: Inhalation of a harmless antigen (Ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice.

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Exposure to aerosolized harmless antigen such as ovalbumin (OVA) has previously been shown to induce inhalation tolerance, a state characterized by inhibition of IgE synthesis and airway inflammation, upon secondary immunogenic antigen encounter. In this study, we investigated cellular and molecular mechanisms underlying this state of unresponsiveness. We show that initial exposure to OVA establishes a programme that prevents the generation of fully functional immune-inflammatory processes, including airway eosinophilia and airway hyperresponsiveness. These data describe inhalation tolerance in the airway and provide a foundation for subsequent studies. I generated the data and wrote the article. B. Gajewska helped with flow cytometry; D. Alvarez and M.D. Inman helped with AHR; S.A. Ritz and M.J. Cundall developed the RT-PCR technology; A.J. Coyle and J.-C. Gutierrez-Ramos provided the probes for RT-PCR; M. Jordana was instrumental in developing the project; and M.R. Stampfli supervised the project.

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Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice

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Summary

Background Exposure to aerosolized harmless antigen such as ovalbumin (OVA) has previously been shown to induce inhalation tolerance, a state characterized by inhibition of IgE synthesis and airway inflammation, upon secondary immunogenic antigen encounter. Immune events associated with this phenomenon are still poorly understood.

Objective The aim of this study was to investigate cellular and molecular mechanisms underlying this state of 'unresponsiveness'.

Methods; After initial repeated OVA exposure, mice were subjected to a protocol of antigen-induced airway inflammation, encompassing two intraperitoneal injections of OVA adsorbed to aluminium hydroxide followed by airway challenge. We assessed immune events in the draining lymph nodes after sensitization, and in the lungs after challenge.

Results In animals initially exposed to OVA, we observed, at the time of sensitization, considerable expansion of T cells, many of which expressed the activation markers CD69 and CD25, as well as increased numbers of antigen-presenting cells, particularly B cells. While these animals produced low levels of IgE, the observed elevated levels of IgG1 signified isotype switching. Splenocytes and lymph node cells from OVA-exposed mice produced low levels of IL-4, IL-5, IL-13 and IFN-γ, indicating aborted effector function of both T helper (Th)2- and Th1-associated cytokines. Real time quantitative polymerase chain reaction (PCR) (TaqMan) analysis of costimulatory molecules in the lungs after *in vivo* challenge showed that B7.1, B7.2, CD28 and CTLA-4 mRNA expression was low in animals initially exposed to OVA. Ultimately, these events were associated with abrogated airway inflammation and attenuated airway hyper-responsiveness. The decreased inflammation was antigen-specific and independent of IL-10 or IFN-γ.

Conclusion Initial exposure to OVA establishes a programme that prevents the generation of intact, fully functional inflammatory responses upon secondary antigen encounter. The absence of inflammation, however, is not associated with categorical immune unresponsiveness.

Keywords airway hyper-responsiveness, allergy, costimulation, cytokine, immunoglobulin, inflammation, inhalation tolerance, ovalbumin

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Introduction

The respiratory tract is, by necessity, constantly exposed to a multitude of environmental agents, both pathogenic and non-pathogenic. While the ability to mount inflammatory responses against harmful viral, bacterial and parasite antigens is critical for survival, such responses are unwarranted for innocuous agents such as aeroallergens. That the prevalence of allergy in the industrialized world is *c.* 15%, implies that *c.* 85% of the population tolerates aeroallergens, in spite of likely universal exposure [1,2]. This attests to the fine, and extremely effective, regulatory networks that maintain homeostasis in the airway/lung compartment [3,4].

Peripheral tolerance, a process that may involve immune deviation, suppression, anergy or clonal deletion, has been described in the gastrointestinal, urogenital and respiratory mucosae [5–8]. With respect to the respiratory tract, it has been shown that initial exposure to aerosolized antigens such as ovalbumin (OVA) induces inhalation tolerance in rodents [9–11], characterized by inhibition of both OVA-specific IgE synthesis and peripheral blood eosinophilia. More recent data show that OVA delivery into the nasal cavity inhibits subsequent airways inflammation and bronchial hyper-reactivity [12]. The cellular and molecular bases underlying this phenomenon

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remain to be elucidated, and evidence supporting specific mechanisms is, in some instances, controversial [10,11,13–15].

In this study, we investigated immune events following initial repeated OVA inhalation in mice subjected to an established protocol of allergic airways inflammation. To this end, mice received 10 consecutive exposures of OVA aerosol, or saline control, followed by two intraperitoneal OVA/aluminium hydroxide injections [16]. We show that, at the primary site of sensitization, the thoracic lymph nodes, initial OVA exposure correlated with clear evidence of immune activity, as assessed by expansion of T and B cells and up-regulation of the activation markers CD69 and CD25 on T cells. Systemically, this was associated with an altered immunoglobulin response, from IgE to IgG1. However, this immune response was, from an effector, pro-inflammatory standpoint, aborted. Indeed, lymph node cells and splenocytes from mice initially exposed to OVA were unable to produce either Th2 or Th1 cytokines, when recalled with OVA *in vitro*. Moreover, lung mononuclear cells from mice initially exposed to OVA, compared with those exposed to saline, expressed low levels of mRNA for the costimulatory molecules B7.1, B7.2, CD28 and CTLA-4. The inability to trigger an effector programme translated into remarkably blunted inflammatory and physiological responses in the lung. This phenomenon was antigen-specific and independent of IL-10 and IFN-γ.

Materials and methods

Animals

Female Balb/c mice (6–8 weeks old) were purchased from Harlan (Indianapolis, IN, USA). C57BL/6, IL-10 knock-out (KO) (C57BL/6-Il10^{tm1Cgn}) and IFN-γ KO (Balb/c-Ifng^{tm1Ts}) mice (6–8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in level B housing conditions in a 12-h light–dark cycle. Level B is an access-restricted area; cages, food and bedding are autoclaved, and all mice manipulations are carried out in a laminar flow hood by gloved, gowned and masked personnel. All experiments described here were approved by the Animal Research Ethics Board of McMaster University.

Initial exposure to aerosolized antigen

Mice were placed in a plexiglass chamber (10cm × 15cm × 25 cm) and exposed for 20min daily over a period of 10 consecutive days to aerosolized OVA (1% wt/vol. in 0.9% saline). Control mice were exposed to saline only. The aerosol was produced by a Bennet nebulizer at a flow rate of 10L/min. The mice were then left for a period of 2 days before undergoing the sensitization and antigen challenge protocol.

Sensitization and antigen challenge

Mice were sensitized and challenged according to a previously described protocol [16]. Briefly, mice were sensitized either with 8μg OVA (Sigma Chemicals, St Louis, MO, USA) or 200μg ragweed (Greer Laboratories, Lenoir, NC, USA), both adsorbed to 4mg aluminium hydroxide (Aldrich Chemicals, Milwaukee, WI, USA) at 4°C overnight in 0.5mL phosphate buffer saline (PBS), twice, 5 days apart, by intraperitoneal (i.p.) injection. Seven days following the second sensitization mice were either placed in a plexiglass chamber (10cm × 15cm × 25cm) and exposed to aerosolized OVA (1% wt/vol. in 0.9% saline) for 1h twice, 4h apart, or received 200μg ragweed in 30μL Coca's buffer (0.085m NaCl, 0.064m $NaHCO_3$; pH = 8.1) intranasally. The aerosol was produced by a Bennet nebulizer at a flow rate of 7L/min. In the experiments involving airway hyper-responsiveness (AHR), mice were exposed to OVA aerosol daily for 1h over a period of 3days.

Collection and measurement from specimens

Bronchoalveolar lavage (BAL) was performed as previously described [16]. In brief, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with PBS (0.25mL followed by 0.2mL). Approximately 0.25mL of the instilled fluid was consistently recovered. Total cell counts were determined using a haemocytometer. After centrifugation, supernatants were stored at −20°C for cytokine measurements by ELISA; cell pellets were resuspended in PBS and slides were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA, USA) at 10*g* for 2min. The HEMA 3 Stain Set (Biochemical Sciences Inc., Swedesboro, NJ, USA) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leucocytes using standard haemocytological procedures to classify the cells as mononuclear cells, neutrophils or eosinophils. Additionally, blood was obtained by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin; 3μm-thick sections were stained with haemotoxylin and eosin.

Cytokine and immunoglobulin measurements

ELISA kits for IL-4, IL-13 and IFN-γ were purchased from R & D Systems (Minneapolis, MN, USA), while kits for IL-5 were obtained from Amersham (Amersham, Buckinghamshire, UK). The threshold of detection for IL-4, IL-13 and IFN-γ was <2pg/mL, and for IL-5 was 5pg/mL.

Levels of OVA-specific IgE were detected with an ELISA that has been described in detail previously [16]. For OVA-specific IgG1 and IgG2a, Maxi-Sorb plates (NUNC Brand Products, Naperville, IL, USA) were coated with 5μg OVA in borate buffer pH8.3–8.5 overnight at 4°C. Subsequently, coated wells were blocked with 1% BSA in PBS for 2h at room temperature (21°C). After washing, serum samples were incubated overnight at 4°C, washed, and developed with biotin-labelled, anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA) overnight at 4°C. Plates were washed and incubated with alkaline-phosphatase streptavidin for 1h at room temperature. The colour reaction was developed with p-Nitrophenyl phosphate tablets. Samples were compared with a standard serum containing OVA-specific IgG1 and IgG2a. Units correspond to maximal dilution that results in an OD that is greater than the blank plus two standard deviations.

Airway hyper-responsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (RRS) to increasing internal jugular vein doses of methacholine (MCh) as previously described [17,18]. Exposed tracheas were cannulated and a constant inspiratory flow was delivered by mechanical ventilation (RV5, Voltek Enterprises Inc., Toronto, Canada). Paralysis was achieved using pancuronium (0.03mg/kg i.v.) to prevent

respiratory effort during measurement. RRS was measured following consecutive i.v. injections of saline, then 10, 33, 100 and 330μg/kg of MCh (ACIC (Can), Brantford, Ontario, Canada), each delivered as a 0.2-mL bolus. Evaluation of airway responsiveness was based on the peak RRS measured in the 30s following the saline and MCh challenges.

Splenocyte, lymph node and lung cell isolation

Spleens and thoracic lymph nodes, including the hilar, mediastinal and tracheobronchial, were removed and adjacent connective tissue was dissected away. They were immediately placed in cold (4°C) HBSS (Gibco BRL, Grand Island, NY, USA). The spleens were triturated between the ends of sterile frosted slides and the resulting suspension was filtered through nylon mesh (BSH Thompson, Scarborough, Ontario, Canada). The cell suspension was centrifuged at 300*g* for 10min at 4°C. Red blood cells were lysed with ACK lysis buffer, and the splenocytes were washed with HBSS and resuspended in RPMI (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine (Sigma Chemicals Co.) and 1% penicillin/streptomycin. The nodes were ground between the frosted ends of slides and filtered through a nylon mesh (BSH Thompson). The cell suspension was centrifuged at 300*g* for 10min at 4°C, and resuspended again in PBS. After this washing step, the cells were resuspended either in flow cytometric analysis buffer (PBS supplemented with 0.2% BSA) or in RPMI (Gibco BRL) supplemented with 10% FBS, 1% l-glutamine (Sigma Chemicals Co.) and 1% penicillin/streptomycin.

For isolation of lung cells, lungs were flushed via the right ventricle of the heart with 10mL of warm (37°C) HBSS (calcium and magnesium free) containing 5% FBS (Sigma Chemicals Co.), 100U/mL penicillin and 100mg/mL streptomycin (Gibco BRL). The lungs were then cut into small (approximately 2mm in diameter) pieces and shaken at 37°C for 1h in 15mL of 150U/mL collagenase III (Worthington Biochemical, Freehold, NJ, USA) in HBSS. Using a plunger from a 5-mL syringe, the lung pieces were triturated through a metal screen into HBSS, and the resulting cell suspension was filtered through nylon mesh. After lysing red blood cells with ACK lysis buffer (0.5m NH_4Cl, 10mm $KHCO_3$ and 0.1nm Na_2EDTA at pH7.2–7.4), cells were washed twice and mononuclear cells were isolated by density centrifugation in 30% Percoll (Pharmacia, Uppsala, Sweden).

Flow cytometry

Panels of monoclonal antibodies were selected to study the phenotype of cells in the lymph nodes. To minimize non-specific binding, 10^6 cells were incubated with 0.5μg Fc Block (CD16/CD32, Pharmingen, Mississauga, Canada) at 0–4°C for 10min and subsequently with first stage monoclonal antibodies at 0–4°C for 30min. Cells were then washed and treated with second stage reagents. Data were collected using a FACScan and analysed using WIN-MDI software (Becton Dickinson, Sunnyvale, CA, USA). The following antibodies and reagents were used: anti-CD3, biotin-conjugated 145–2C11 (Pharmingen); anti-CD4, FITC-conjugated L3T4 (Pharmingen); anti-CD8, FITC-conjugated Ly-2 (Pharmingen); anti-CD69, PE-conjugated H1 2F3 (Pharmingen); anti-CD25, PE-conjugated PC61 (Pharmingen); and Streptavidin PerCP (Becton Dickinson, San Jose, CA, USA). Titration was performed to determine the optimal concentration of each antibody.

Splenocyte and lymph node cell culture

Splenocytes and lymph node cells were cultured in medium alone or with 40μg OVA/well at 8×10^5 cells/well in a 96-well flat-bottom plate (Becton Dickinson, Lincoln Park, NJ, USA). After 5 days of culture, supernatants were harvested for cytokine measurement.

Collection, extraction, separation and isolation of RNA from tissue

Thoracic lymph nodes and lung mononuclear cells were collected and placed in 1mL TriPure Isolation Reagent, a monophasic solution of phenol and guanidine thiocyanate (Boehringer Mannheim Canada, Laval, QC, Canada). The tissues were then homogenized with a Polytron 7mm power homogenizer (Kinematica, Luzerne, Switzerland). The sample homogenates underwent phase separation and RNA was isolated according to the procedure provided with the TriPure Isolation Reagent. RNA pellets were resuspended in 20μL of diethylpyrocarbonate (DEPC)-treated RNase-free water. To determine the concentration of total RNA collected, the optical density (OD) was calculated using an Ultrospec 1000 UV/Visible spectrophotometer (Pharmacia Biotech (Biochrom) Ltd, Cambridge, England). The RNA was stored at −70°C until needed.

TaqMan: real time quantitative polymerase chain reaction

CD28, CTLA-4, B7.1 and B7.2 expression was evaluated using real-time quantitative polymerase chain reaction (PCR) analysis on an ABI PRISM Sequence Detector 7700 (PE Applied Biosystems, Foster City, CA, USA). In brief, an oligonucleotide probe was designed to anneal to the gene of choice between two PCR primers. The probe was fluorescently labelled with FAM (reporter gene) on the 5′ end and with TAMRA (quencher dye) on the 3′ end. A similar probe and PCR primers were purchased for mGAPDH (PE Applied Biosystems). The probe for this gene incorporated VIC as the reporter dye. PCR reactions were run that included the primers and probes for these two genes as well as cDNA made from cells isolated from lymph nodes. As the polymerase moves across the gene during the reaction, it cleaves the dye from one end of each probe, which causes a fluorescent emission that is measured by the Sequence Detector 7700. The emissions recorded for each cDNA can then be converted to determine the level of expression for the genes normalized to the expression of mGAPDH. Expression of CD28, CTLA-4, B7.1 and B7.2 was determined on cells isolated from mononuclear lung cells following OVA challenge.

Data analysis

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

Results

Activation marker expression on $CD4^+$ and $CD8^+$ T cells

Mice were exposed to aerosolized OVA or saline daily for 20 min over a period of 10 days. Subsequently, mice were subjected to two intraperitoneal injections of OVA in conjunction with

aluminium hydroxide as outlined in Materials and methods (sensitization). Twenty-four hours after the second injection, lymph node cells were collected and pooled. In naïve mice, the average total cell number, out of 8–10 animals, was 0.6×10^6 cells/mouse. The cell number of sensitized mice initially exposed to repeated OVA aerosolizations was almost five times greater (2.8×10^6 cells/mouse). This cell number was even greater in mice initially exposed to saline (5.9×10^6 cells/mouse). Next, we performed flow cytometric analyses on these cells. In naïve animals, 42.2% of the total cells obtained stained for CD3/CD4. In animals initially exposed to OVA aerosolizations, this number was lower (29.4%) and similar to that observed in animals initially exposed to saline (28.8%). Hence, the total number of cells staining for CD3/CD4 was 0.25×10^6 cells/mouse for naïve mice, 0.82×10^6 cells/mouse for mice initially exposed to OVA and 1.7×10^6 cells/mouse for mice initially exposed to saline.

A similar pattern was observed for cells staining for CD3/CD8. While in naïve animals 16.5% of the total cells stained for CD3/CD8, in animals initially exposed to OVA this number was lower (12.5%) and similar to that observed in animals initially exposed to saline (11.0%). As a result, the total cell number of cells staining for CD3/CD8 was 0.10×10^6 cells/mouse for naïve mice, 0.35×10^6 cells/mouse for mice initially exposed to OVA and 0.65×10^6 cells/mouse for mice initially exposed to saline. We then evaluated the levels of activation of these cells, as assessed by CD69 and CD25 expression. Figure 1 depicts the levels of CD69 expression on $CD4^+$ and $CD8^+$ T cells in naïve animals, and animals initially exposed to either OVA or saline. In naïve animals, CD69 expression was low on both $CD4^+$ cells (7.9%) and $CD8^+$ cells (5.6%). Animals initially exposed to OVA expressed higher levels of CD69 on both $CD4^+$ cells (15.0%) and $CD8^+$ cells (18.0%). Animals initially exposed to saline expressed similar levels of CD69 on both $CD4^+$ cells (18.2%) and $CD8^+$ cells (23.2%). The experiment was repeated three separate times yielding similar results. Expression of CD25 was similar to that of CD69. Specifically, the proportion of $CD4^+$ T cells that also expressed CD25 was 17.7% in animals initially exposed to OVA and 16.9% in those exposed to saline control.

With respect to antigen-presenting cells (APC), we found that, in naïve mice, 16.5% of the total cells stained for MHC class II. In animals initially exposed to OVA aerosolizations, this number doubled (33.7%), similar to that observed in animals initially exposed to saline (33.4%). In fact, most of the MHC class II cells also stained for B220, a marker of B cells. In naïve mice, 15.9% of the total cells stained for MHCII/B220. This number doubled in animals initially exposed either to OVA (32.5%) or saline (33.0%). The total number of cells staining for MHC class II and B220, then, was 0.095×10^6 cells/mouse in naïve mice, 0.91×10^6 cells/mouse in mice initially exposed to OVA, and 1.9×10^6 cells/mouse in mice initially exposed to saline. The experiment was repeated three separate times yielding similar results.

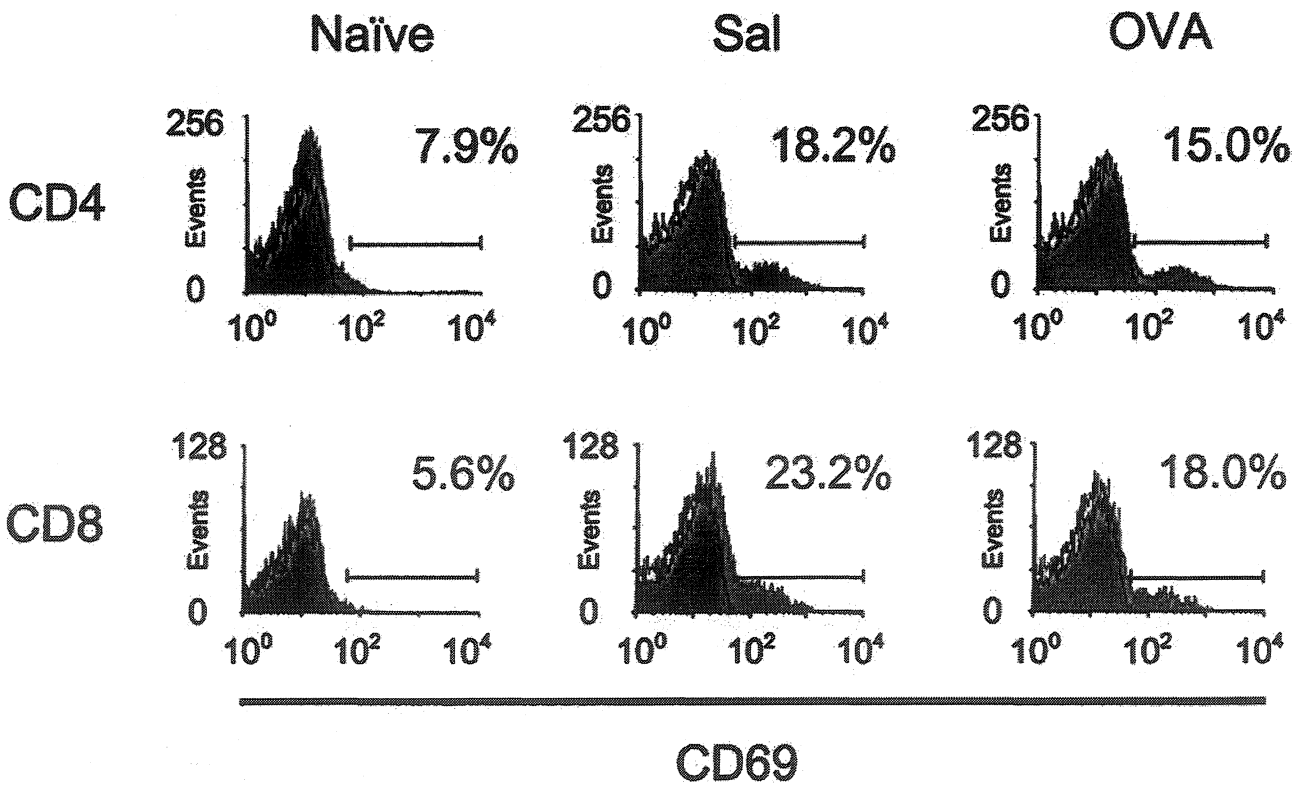

Fig. 1. Effect of passive OVA exposure on the expression of the activation marker CD69 on T cells in the thoracic lymph nodes after sensitization. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Twenty-four hours after the second i.p. sensitization, mice were killed, their thoracic lymph nodes removed and the activation of T cells was evaluated according to CD69 expression gated on either CD4/CD3 or CD8/CD3. Lymph nodes from naïve mice were also characterized (one of four representative experiments is shown; $n = 8$–10 mice/group).

Immunoglobulin levels in the serum

In the light of the considerable lymphocyte expansion that we documented in the lymph nodes, we investigated whether this led to a humoral response and, if so, the nature of that response. To this end, we measured the immunoglobulin levels in the sera of mice following *in vivo* OVA challenge. Table 1 shows a robust OVA-specific IgE response after OVA challenge in mice initially exposed to saline. This response was diminished by over 70% in mice initially exposed to OVA. Notably, the observed reduction was isotype-specific since both OVA-specific IgG1 and IgG2a were readily detectable in the sera of mice initially exposed to either saline or OVA. Indeed, for IgG1, we observed significantly higher levels of the immunoglobulin in mice initially

Table 1. Serum levels of immunoglobulins

	Naïve	Sal	OVA
IgE	ND	274 ± 50#	77 ± 26*#
IgG1	49 ± 22	368000 ± 54200	1360000 ± 28000*#
IgG2a	5 ± 3	616 ± 452	1260 ± 295

Mice were exposed daily to aerosolized OVA or saline for 10 days. Two days after the last exposure, both groups were then sensitized i.p. and challenged with OVA aerosol. Three hours after challenge serum was collected. Serum was also collected from naïve age-matched mice. Results are expressed as mean ± SE (U/mL). Statistical analysis was performed using anova with the Fisher LSD method. *$P < 0.05$ compared with mice exposed to saline, #$P < 0.05$ compared with naïve mice, $n=3$ for naive, 5 for other groups. ND=not detectable.

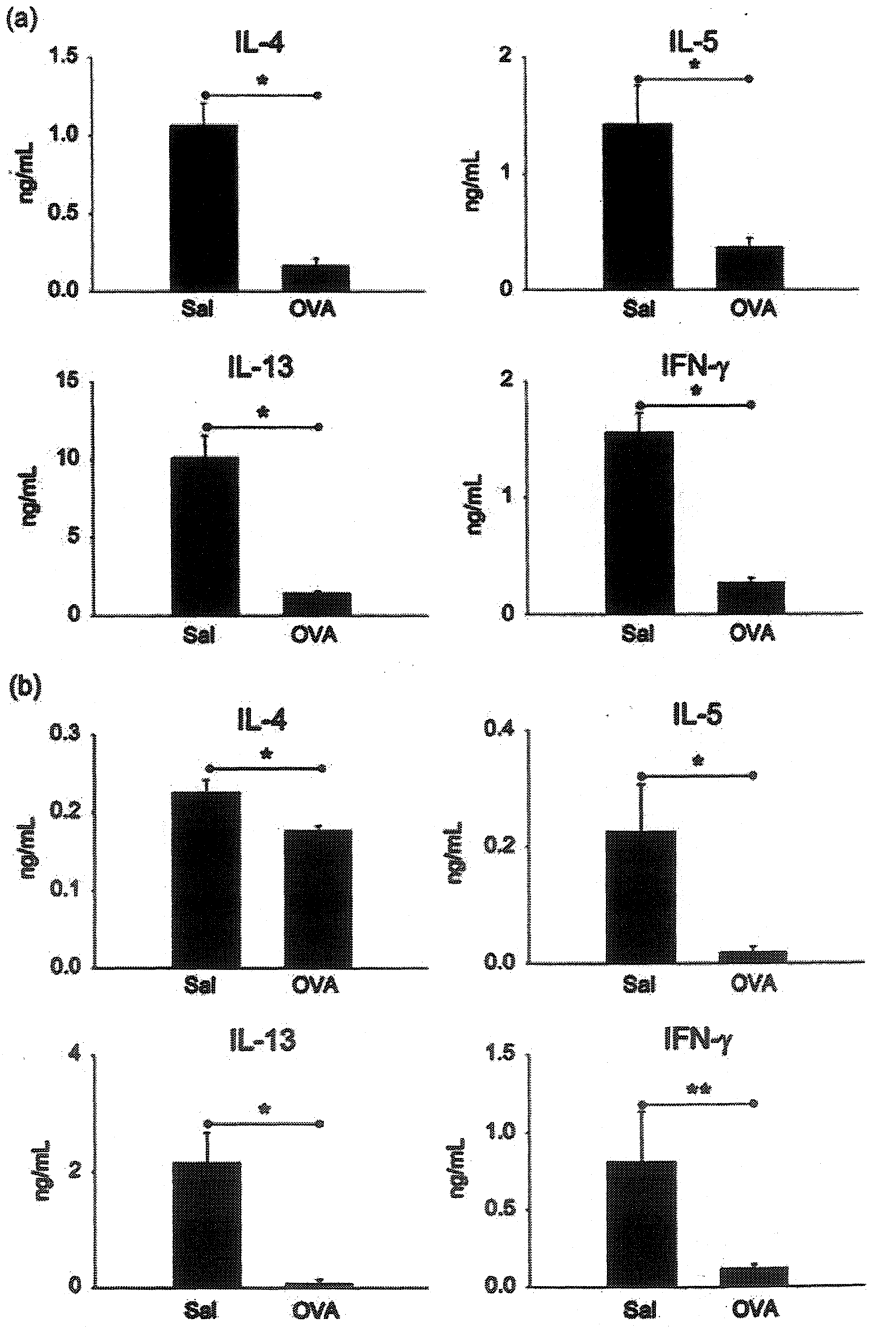

Fig. 2. Cytokine expression from lymph node and spleen cells recalled in culture with OVA. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Twenty-four hours after the second i.p. sensitization, mice were killed and their thoracic lymph nodes (a) and spleens (b) were removed. Cells derived from pooled lymph nodes or individual spleens were placed into culture for 5 days with OVA. No cytokines were produced in medium alone, in the absence of OVA. Cytokines were measured with a sandwich ELISA. For splenocyte cultures, data represent mean ± SEM from four individual spleens. For lymph node cells, data represent mean ± SEM from four individual culture wells. Statistical analysis was performed using a Student's *t*-test; *$P < 0.05$; **$P=0.077$.

exposed to OVA. For IgG2a the levels were not significantly different. The levels in naïve mice were either undetectable, or very low.

Ex vivo Th2 and Th1 cytokine production

Given the evidence of T cell activation, as assessed by levels of CD69 and CD25 expression in mice initially exposed to OVA or saline, we investigated the effector function of these cells. To this end, lymph node cells and splenocytes were removed, placed into culture and exposed to OVA or medium alone for 5 days. The supernatants were then harvested, and the levels of IL-4, IL-5, IL-13 and IFN-γ were measured. Splenocytes were included to gain insight into effector activity in the systemic compartment. While the lymph node cells and splenocytes from animals initially exposed to saline produced high levels of IL-4, IL-5, IL-13 and IFN-γ, animals initially exposed to OVA produced very small amounts of these cytokines (Fig. 2). We detected only negligible levels of cytokine expression in medium alone or in splenocytes and lymph nodes cells from naïve mice cultured with either medium or OVA (data not shown), indicating that the observed response was OVA-specific.

Costimulatory molecule expression in lung mononuclear cells

Having observed a decreased ability of both lymph node and spleen cells from mice initially exposed to OVA, but not saline, to carry out an effector programme upon antigen recall *in vitro*, we proceeded to examine the expression of costimulatory molecules, known to be important in generating effector responses, upon antigen recall *in vivo*. To this end, 24 h after challenge lung mononuclear cells were isolated and total RNA was extracted. CD28, CTLA-4, B7.1 and B7.2 mRNA expression was assessed with real time quantitative PCR (TaqMan). Compared to naïve animals, we observed an up-regulation of CTLA-4, B7.1 and B7.2 mRNA expression in animals initially exposed to saline (Fig. 3). In animals initially exposed to OVA, the levels of these molecules were low, and similar to those observed in naïve animals. Interestingly, the level of CD28 expression in naïve mice was relatively high and similar to that detected in mice initially exposed to saline. Remarkably, the levels of CD28 expression in mice initially exposed to OVA were clearly lower than, even, in naïve mice. Figure 3 depicts a representative of two separate experiments.

Impact of aerosol exposure to OVA on lung/airway responses

Next, we investigated the impact of such lung compartmentalized immune hyporesponsiveness at the cellular, histological and physiological levels. Figure 4 depicts lung histology and the cellular profile in the BAL after *in vivo* OVA challenge. At the tissue level (Fig. 4a), mice initially exposed to saline developed extensive peribronchial and perivascular inflammation. Note the evidence of goblet cell hyperplasia and mucus in the airway. This inflammatory response was eosinophilic in nature (Fig. 4a(ii)). In contrast, inflammation was markedly reduced in animals that were passively exposed to OVA prior to sensitization and challenge (Fig. 4a(iii)). The few focal areas that showed an inflammatory infiltrate were largely devoid of eosinophils (Fig. 4a(iv)). In addition, goblet cell hyperplasia was absent in these mice.

Figure 4(b) shows that initial exposure to OVA markedly decreased inflammation in the BAL. Most notably, the number of eosinophils was reduced by >95%. Indeed, statistical analysis revealed that the cellular profile of mice initially exposed to OVA was not significantly different to that observed in naïve animals. No neutrophils were observed in any of the groups at this time point (data not shown).

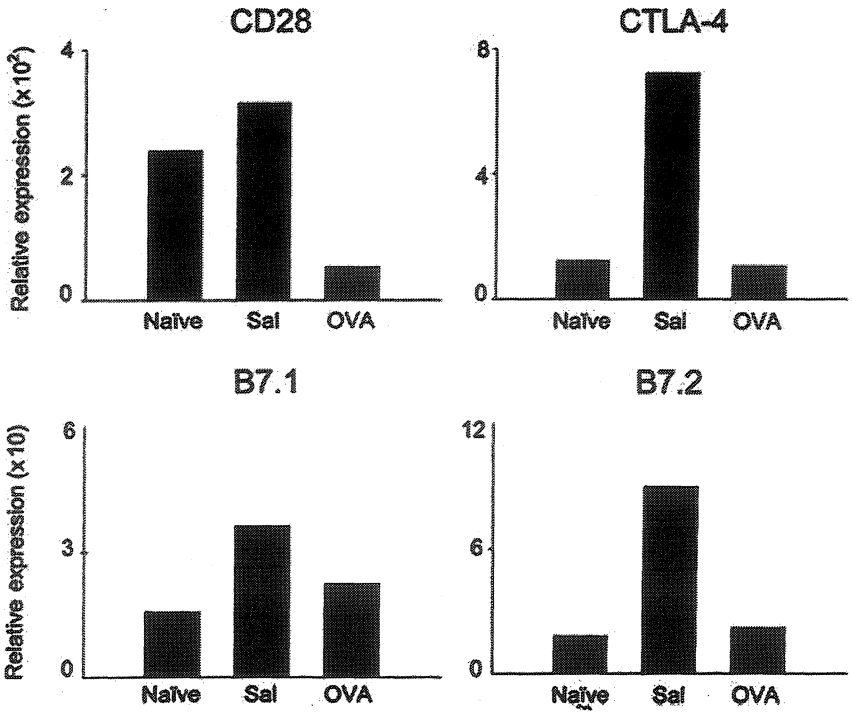

Fig. 3. Effect of initial OVA exposure on the costimulatory molecule profile. Mice were initially exposed to either 1% OVA or saline via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Twelve hours after the last OVA challenge, mice were killed and their lungs removed. Lung mononuclear cells were isolated and total RNA was isolated. Real time quantitative PCR (TaqMan) was run. Panels depict the relative expression, to mGAPDH, of costimulatory molecule mRNA from the lung mononuclear cells of naïve animals and animals initially exposed either to saline (Sal) or OVA (one of two representative experiments is shown; $n=6$ mice/group).

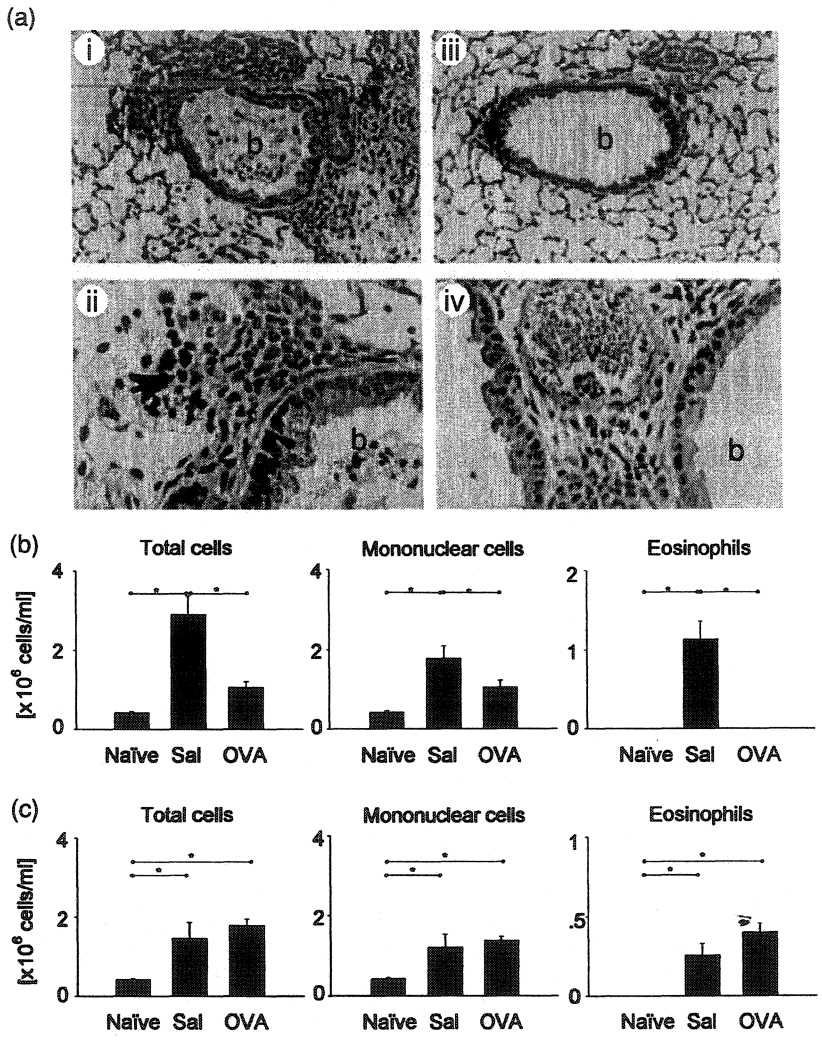

Fig. 4. Light photomicrograph of paraffin-embedded sections of lung tissues and BAL cellular profile after aerosol challenge. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. In (a) and (b), both groups were then sensitized i.p. and challenged with OVA aerosol. In (c) , both groups were then sensitized i.p. and challenged intranasally with ragweed. Animals were killed 72 h after the last challenge. In (a), panels depict tissues from mice initially exposed to either saline (i and ii) or OVA (iii and iv). All sections were stained with haematoxylin and eosin. Note the magnitude of eosinophilia in (iii) and (iv) as compared with (i) and (ii). Magnification of panels: (i) × 250; (ii) × 640; (iii) × 250; (iv) × 640. In (b) and (c), data show total cell number, mononuclear cells and eosinophils in the BAL. Mean ± SEM; $n = 4$–5; statistical analysis was performed using one way anova with Fisher LSD method; $^*P < 0.05$.

Figure 4(c) shows that inhibition of airway inflammation was antigen specific. Mice initially exposed to OVA and then sensitized and challenged with ragweed developed robust eosinophilic inflammation.

We have previously documented the Th2 cytokine profile in the BAL associated with airway inflammation [16]. Based on this information, cytokine levels in the BAL were measured 24 h after OVA aerosol challenge. Figure 5 demonstrates dramatically decreased levels of IL-4, IL-5 and IL-13 in mice that were initially exposed to OVA. For IL-4 we excluded one sample since it was identified as an outlier (t-test, 99% confidence)

To assess whether decreased inflammation was mediated by IL-10 or IFN-γ, we conducted the protocol in IL-10 and IFN-γ KO mice. Figure 6 shows that eosinophilic airways inflammation was similarly inhibited in both KO strains.

To link the cellular response in the lung to airway physiology, we assessed airway responsiveness to increasing intravenous (i.v.) doses of MCh 72 h after antigen challenge. Figure 7 demonstrates significantly increased airway responsiveness in mice that were initially exposed to saline, as compared to naïve controls. This responsiveness was significantly reduced in mice that were initially exposed to OVA. Nevertheless, these mice showed significantly greater responsiveness compared with naïve controls at the higher (100 and 300 μg/kg) concentrations of MCh.

Discussion

It has been known for a number of years that passive exposure to the soluble antigen OVA, used as an allergen surrogate, prevents the production of OVA-specific IgE, that would otherwise occur in animals subjected to an experimental protocol of allergic airways inflammation [9]. More recently, it has been documented that such passive exposure is also associated with decreased eosinophilia in both peripheral blood [11] and BAL [12]. Against this background of 'anti-inflammatory' outcomes, the objective of our study was to investigate the state of immune responsiveness elicited by passive antigen exposure in both the primary site of sensitization, i.e. the thoracic lymph nodes, and the site of immune-effector activity, i.e. the lung/airway compartment. In brief, our data provide evidence that initial

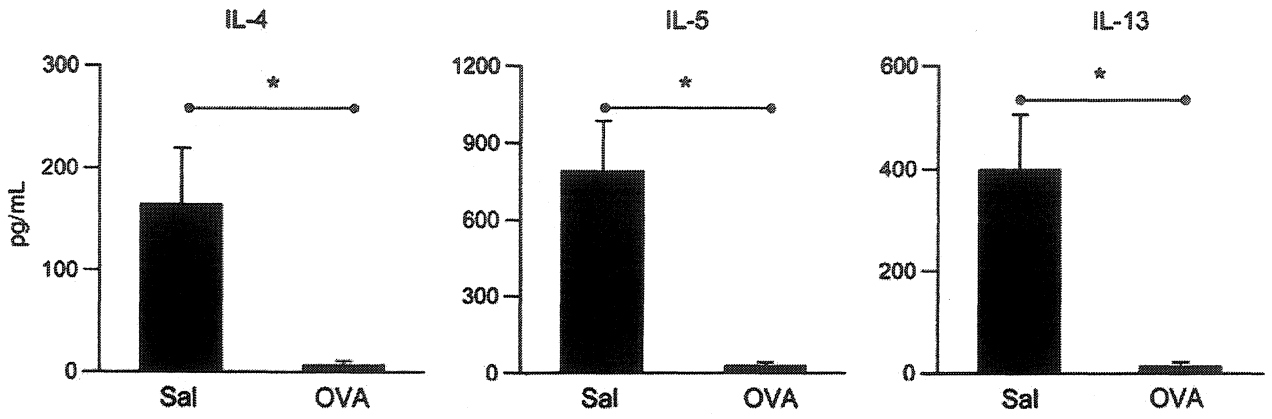

Fig. 5. Cytokine levels in the BAL. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Mice were killed 24h after the last challenge. Cytokines were measured by ELISA. Mean ± SEM; $n=5$–9; statistical analysis was performed using Student's *t*-test; $^*P < 0.05$.

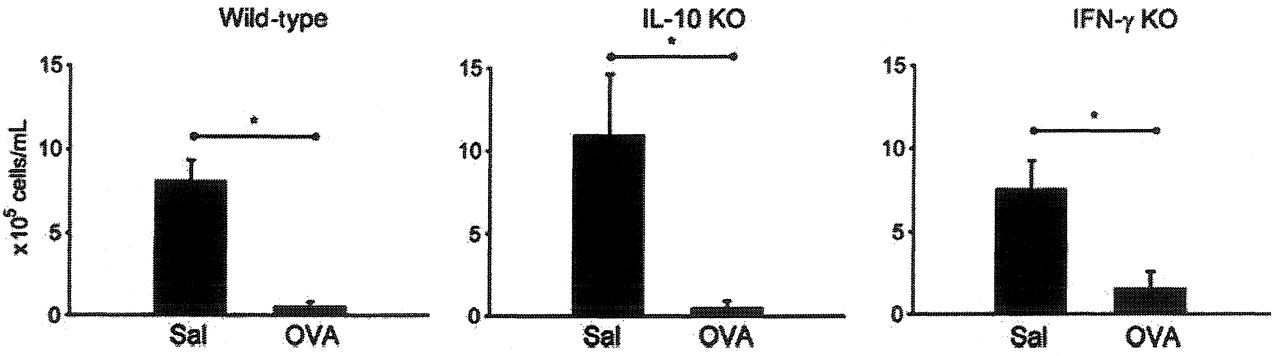

Fig. 6. Cellular profile in IL-10 and IFN-γ knock-out mice. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Animals were killed 72h after the last OVA challenge. The wild-type controls are C57BL/6 mice. Mean ± SEM; $n=3$–4; statistical analysis was performed using anova with Tukey post-hoc test; $^*P < 0.05$.

exposure to OVA leads to immune activation in the thoracic lymph nodes that is, however, associated with divergent effector activities in the immunoglobulin and cytokine profiles.

The protocol that we used to elicit allergic airway inflammation involves two distinct phases: a sensitization procedure carried out intraperitoneally (i.p.), followed by aerosol challenge [16,19,20]. We have established such a model in our laboratory and have previously detailed cellular changes in the BAL, lung tissue and peripheral blood compartments as well as serum levels of immunoglobulins and cytokine profiles in serum and BAL [16]. First, we investigated the phenotypic changes in the lymphocyte population in the lymph nodes in mice that had been initially exposed to repeated aerosolizations of either OVA or saline control. We selected 24h after the second i.p injection because this is the time point of maximal T and B cell expansion in the lymph nodes (Gajewska et al., personal communication). Our data provide three lines of evidence indicating that initial repeated OVA aerosolization was associated with an immune response at this site. First, we documented a considerable expansion in the T cell compartment, regardless of whether mice had been initially exposed to OVA or saline, when compared with naïve controls. Secondly, we observed high expression of T cell activation markers in mice initially exposed to either OVA or saline, specifically CD69 [21,22] and CD25 [23]. Thirdly, mice initially exposed to OVA or saline had a considerable expansion of APC, particularly B cells, as assessed by MHC class II and B220 expression. Notably, the proportion of T cells in OVA- and saline-exposed animals was lower than that observed in naïve mice. This is because of the comparatively greater expansion of MHC class II cells vs. that of T cells. It is more noteworthy, however, that the APC:T cell ratio was almost identical between the animals initially exposed to either saline or OVA.

The considerable expansion in the B cell compartment in animals initially exposed to OVA led us to investigate the functional significance of this event. Upon *in vivo* antigen challenge, animals initially exposed to OVA expressed low levels of OVA-specific IgE, but significantly higher levels of OVA-specific IgG1, when compared with animals initially exposed to saline (Table 1). While several independent groups have shown isotype-specific inhibition of OVA-specific IgE in mice initially exposed to OVA [10,12,24], our data, specifically the elevated levels of the Th2-associated IgG1 [25] in mice initially exposed to OVA, demonstrate isotype switching, rather than isotype inhibition. Similar levels of IgG2a were observed in mice initially exposed to saline or OVA. Therefore, initial exposure to OVA did not categorically inhibit the effector function of B cells, but rather, led to an altered, but still Th2-predisposed, immunoglobulin profile.

That CD69 and CD25 expression was greater in mice initially exposed to OVA or saline indicates T cell activation.

Furthermore, since isotype switching requires T cell help [26], the changes in the immunoglobulin profile discussed above provide additional independent evidence of T cell activation. Therefore, we next assessed another important effector function of these lymphocytes *in vitro*, namely, cytokine production. Interestingly, when lymph node cells of OVA-exposed animals were placed into culture and re-stimulated with OVA, they were unable to produce either Th2- or Th1-associated cytokines (Fig. 2). That we observed minimal levels of IFN-γ argues against immune deviation being an important mechanism in inhibiting IgE responses, as has been suggested in the past [10]. That we observed decreased cytokine production by splenocytes, indicates that such aborted effector function was manifested systemically.

Ultimately, the impact of an immune response should be assessed in the target organ, the site of antigen exposure. Hence, it was important to investigate the activation state of immune cells infiltrating the lung. Here, we focused our analysis on the expression of a number of molecules that have been reported to play critical roles in immune-inflammatory responses, including allergic responses [27–32]. Specifically, we looked at costimulatory molecules expressed on T cells (CD28 and CTLA-4) and APC (B7.1 and B7.2) [33]. CD28, expressed on all T lymphocytes, binds to B7.1 and B7.2. The interaction serves as a second signal in initiating productive immune responses. Upon activation, T cells up-regulate their CTLA-4 expression which, by binding with higher affinity to the APC costimulatory molecules, serves to down-regulate further T cell activation and expansion [33,34].

Our rationale for isolating lung mononuclear cells was to focus on cells present in the lung at the time of challenge. As Fig. 3 attests, CD28, CTLA-4, B7.1 and B7.2 were highly expressed in the lungs of animals initially exposed to saline. In contrast, animals initially exposed to OVA expressed very low levels of these molecules. Indeed, the levels were either similar to, or, in the case of CD28, even lower than, those observed in naïve animals. While much of the work investigating the impact of initial exposure to antigen has focused on T cells and their function [10,13], our data introduce costimulation as a marker and, possibly, as a mechanism by which T cell effector function may be controlled. Indeed, the steady discovery of novel costimulatory molecules maintains the utmost interest in the regulatory potential of costimulation [35].

That initial OVA exposure resulted in such dramatic lack of up-regulation of costimulatory molecule expression in the effector organ, at the time of *in vivo* OVA recall, led us to investigate the cells, molecules and physiological changes associated with airway inflammation. In animals initially exposed to OVA, we observed a dramatic reduction of airway inflammation characterized by decreased numbers of mononuclear cells and eosinophils, compared with mice initially exposed to saline (Fig. 4). At the tissue level, we observed a reduction of eosinophilia in the peribronchial and perivascular regions of the lung, as well as a marked reduction of goblet cell hyperplasia. Furthermore, the levels of Th2 cytokines such as IL-4, IL-5 and IL-13 were considerably decreased in these mice (Fig. 5). This airway unresponsiveness was antigen specific as we observed marked inflammation in animals initially exposed to OVA and then sensitized and challenged with ragweed extract.

IL-10 and IFN-γ have previously been shown to play an important role in tolerance [10,17,18]. Our studies in IL-10 and IFN-γ KO mice indicate that these cytokines are not necessary to the induction and maintenance of this process (Fig. 6). That we were not able to detect IL-10 or IFN-γ in the BAL of mice initially exposed to either saline or OVA further substantiates our findings (data not shown).

We also observed a significantly decreased airway response to methacholine in OVA-exposed mice compared with animals

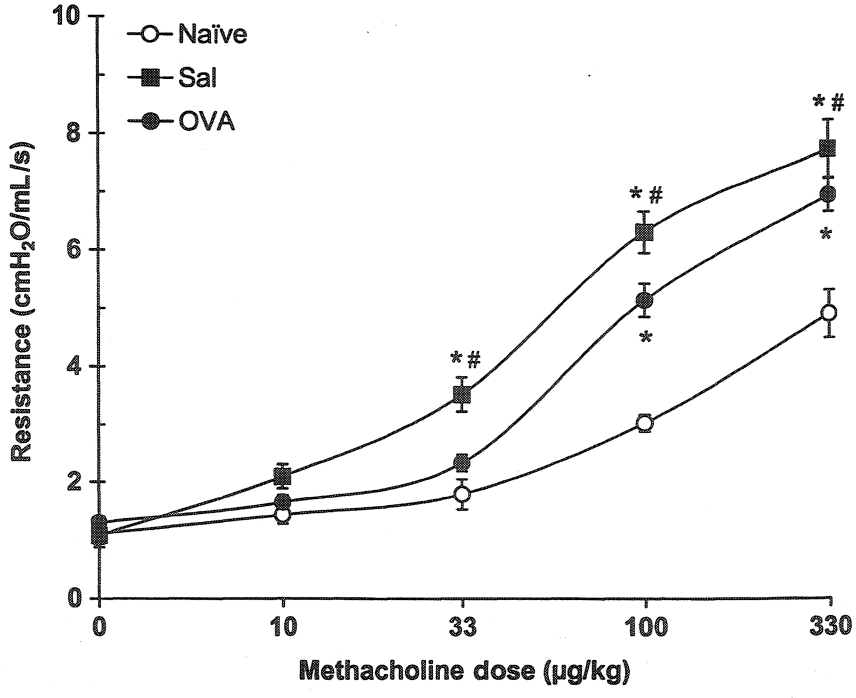

Fig. 7. Effect of passive OVA exposure on MCh-induced increases in RRS. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Seventy-two hours after challenge airway function was assessed. Mean ± SEM; $n=3$ for naïve; $n=7$ for other groups; statistical analysis was performed using anova with Newman-Keuls post-hoc test; *RRS was significantly greater compared with naïve control mice ($P < 0.05$), #RRS was significantly greater compared with sensitized and challenged mice initially exposed to OVA ($P < 0.05$).

initially exposed to saline (Fig. 7). Interestingly, RRS was increased in mice initially exposed to OVA at 100 and 300μg/kg methacholine doses when compared with naïve controls. This incomplete reduction may be attributed to the increased levels of IgG1 in the serum. In this regard, Oshiba et al. demonstrated that passive transfer of either allergen-specific IgE or IgG1 mediates immediate hypersensitivity and airway hyperresponsiveness [36]. That we observed increased AHR in mice initially exposed to OVA is therefore consistent with the presence of OVA-specific IgG1. Together these data suggest that initial aerosol exposure to OVA induced inhalation tolerance insofar as subsequent subjection of mice to a protocol of antigen-induced airways inflammation, did not result in the expected up-regulation of molecules, the infiltration of cells, or the physiological conditions, associated with this phenomenon.

In conclusion, our data indicate that passive exposure to an innocuous antigen such as OVA does not elicit unconditional unresponsiveness but, rather, an active immune response. The response is unique insofar as it is characterized by an altered and compromized effector programme. It is an antigen-specific programme that manifests itself with a distinct immunoglobulin profile, an aborted production of pro-inflammatory Th2 and Th1 cytokines, as well as an unengaged CD28/CTLA-4/B7 costimulatory pathway. That this immune response is ultimately not associated with inflammation in the target organ is what makes it homeostatic, a state that, at least intuitively, is to be expected given the harmless nature of the antigen.

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Chapter 3: Concomitant Airway Expression of GM-CSF and Decorin, a Natural Inhibitor of TGF-β, Breaks Established Inhalation Tolerance

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We previously showed that GM-CSF breaks tolerance induction. The objective of this study was to determine whether GM-CSF breaks established inhalation tolerance. Using a model of mucosal allergic sensitization, we show that GM-CSF alone cannot break established tolerance, but that concomitant expression of GM-CSF and decorin, a natural inhibitor of TGF-β breaks tolerance in the airway. These findings lead to the generation of the hypothesis that at least two signals are required to break established tolerance: one that leads to de novo sensitization (GM-CSF) and one that interferes with regulatory mechanisms (decorin). This is the first observation that established tolerance can be broken *in vivo*. I generated the data and wrote the manuscript. B.U. Gajewska, C.S. Robbins and J.R. Johnson helped with Flow Cytometry; A. D'Sa and M.A. Pouladi helped with experiments and reviewed the manuscript; M.D. Inman helped with AHR; and M.R. Stampfli supervised the project.

Concomitant Airway Expression of GM-CSF and Decorin, a Natural Inhibitor of TGF-β, Breaks Established Inhalation Tolerance

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Running Title: GM-CSF with decorin breaks airway unresponsiveness
Keywords: Allergy, Tolerance, Lung, TGF-β, GM-CSF, decorin

ABBREVIATIONS
BAL – Bronchoalveolar lavage
Ad – Adenovirus
RDA – Replication Deficient Adenovirus
AHR – Airway Hyperresponsiveness
HCl – Hydrogen Chloride
RRS – Respiratory System Resistance
MCh – Methacholine
LSD – least significant difference

SUMMARY

We previously showed that GM-CSF breaks tolerance induction. The objective of this study was to determine whether GM-CSF breaks established inhalation tolerance. To induce tolerance, Balb/c mice were exposed to aerosolized ovalbumin (OVA) for ten consecutive days. A control group was exposed to saline. Subsequently, tolerant and control animals were exposed to OVA in a GM-CSF-enriched airway microenvironment. We found that tolerant animals, unlike control animals, did not develop airway and peripheral blood eosinophilia, had diminished levels of OVA-specific IgE, and reduced airway hyper-responsiveness. While tolerant animals did not express IL-4, IL-5 and IL-13, levels of the regulatory cytokines IL-10, IFN-γ and TGF-β were similar between tolerant and non-tolerant animals. Lung $CD4^+$ T cells were activated according to CD69, CD25 and T1/ST2 expression, but systemic responses characterized by splenocyte proliferation and ex vivo Th2 effector function were dramatically reduced. Concurrent expression of GM-CSF and decorin, a small proteoglycan and a natural inhibitor of TGF-β, reversed eosinophilic unresponsiveness. Our study suggests that the breakdown of tolerance and, by extension, the emergence of eosinophilic inflammation, requires two signals: one that triggers sensitization and one that interferes with negative regulation. Moreover, our study shows for the first time that dysregulated expression of an extracellular matrix protein may break established tolerance and lead to eosinophilic airway inflammation.

INTRODUCTION

Accumulating evidence suggests that the immune system has evolved powerful regulatory mechanisms that control responsiveness to innocuous agents (1, 2). Such regulatory mechanisms are crucial for survival since unwarranted inflammation may compromise the primary function of a specific organ. The effectiveness of these mechanisms is underscored by the observation that, in spite of universal exposure to aeroallergens, the majority of the population does not develop allergic disease (3, 4).

Animal studies have shown that intra-nasal or aerosol exposure to harmless antigen such as OVA induces a state of antigen-specific unresponsiveness, referred to as intra-nasal or inhalation tolerance. Previously regarded as immunologically dormant, we currently understand this type of tolerance to be governed by increasingly complex cellular and molecular pathways, likely involving co-stimulation (5), functional inactivation by macrophages (6), T cell activation (7) and suppression (8), expression of several regulatory cytokines such as IL-10 (9) and IFN-γ (10), and other related mechanisms (1). The role of TGF-β, a molecule critical in the regulation of oral tolerance (11, 12) and organ-specific autoimmune disease (12, 13), remains largely unexplored in inhalation tolerance (14).

We previously reported that GM-CSF expression in the airway microenvironment of mice breaks tolerance induction and allows for allergic mucosal sensitization to OVA (15). The ensuing inflammatory response is characterized by a Th2-associated cytokine profile, airway eosinophilia, goblet cell hyperplasia and bronchial hyper-responsiveness, all of which are hallmarks of human asthma. Hence, the development of allergies may reflect in part the failure to induce tolerance; antigen is initially encountered in the context of a signal that triggers allergic sensitization. However, the development of allergies may also reflect a breakdown of *established* tolerance.

The objective of this study was to determine under what conditions established tolerance is broken. While we were unable to break tolerance with GM-CSF alone, concurrent expression of GM-CSF and decorin, a proteoglycan associated with human asthma (16) and a natural inhibitor of TGF-β (17), broke tolerance in the airway. Our study shows for the first time that increased expression of decorin is associated with dysregulated airway responses that lead to eosinophilic

inflammation. Although tolerance has been transferred before, this study is the first to show, to our knowledge, that established inhalation tolerance can be broken in vivo. Furthermore, we provide evidence that two signals are required to break established tolerance: one that allows for sensitization and one that interferes with regulatory pathways.

RESULTS

Cellular response in the BAL and the peripheral blood of tolerant mice exposed to OVA in the context of a GM-CSF-enriched airway environment

To address whether GM-CSF breaks established inhalation tolerance, Balb/c mice were exposed for ten consecutive days to either OVA (tolerance induction) or saline aerosol (control). After 2 days of rest, both groups were then exposed to aerosolized OVA for a period of nine days in the context of a GM-CSF-enriched airway environment. GM-CSF was expressed using an adenovirus mediated gene transfer approach. We previously reported that intra-nasal delivery of $3x10^7$ pfu leads to expression of GM-CSF for 8-10 days, reaching peak levels of 80pg/ml seven days post instillation (18). 24 h after the last exposure animals were killed and BAL was collected. We observed a significant increase in total cell number as well as mononuclear cells and eosinophils in the BAL and peripheral blood of animals initially exposed to saline only and subsequently to OVA in the context of GM-CSF (Fig. 1). Prior exposure to OVA alone resulted in striking reduction of total cells in the BAL and peripheral blood when compared to animals initially exposed to saline. In both compartments, the difference was owing to a significant reduction of eosinophils (>80%), with only a modest and statistically insignificant reduction of the mononuclear cell population in the BAL. These findings suggest that expression of GM-CSF did not break established airway tolerance.

Cytokine Expression in the BAL

Given the preeminent status of cytokines in orchestrating and regulating the central features of allergic airway inflammation, we measured expression levels of Th2-associated (IL-4, IL-5, IL-13) and regulatory (IL-10, IFN-γ, TGF-β) cytokines in the BAL. Balb/c mice were exposed for ten consecutive days to either OVA (tolerance induction) or saline aerosol. Both groups were then exposed to aerosolized OVA for a period of nine days in the context of a GM-CSF enriched airway environment. 24 h after the last exposure mice were killed and BAL was collected. Cytokines were measured by ELISA. While IL-4, IL-5 and IL-13 were secreted at high concentrations in animals initially exposed to saline and then to OVA in the context of a GM-CSF-

enriched airway environment, expression of these cytokines was significantly decreased in animals initially exposed to OVA, reaching levels comparable to those observed in naïve mice (Fig. 2). Conversely, the expression of IL-10, IFN-γ and TGF-β was unaffected by initial exposure to OVA, since levels of these cytokines were similar in both groups.

Impact of initial OVA exposure on humoral responses in animals exposed to OVA in context of GM-CSF

To assess whether expression of GM-CSF breaks IgE unresponsiveness in tolerant mice, we measured serum levels of OVA-specific IgE and IgG1. Balb/c mice were exposed for ten consecutive days to either OVA (tolerance induction) or saline aerosol. Both groups were then exposed to aerosolized OVA for a period of nine days in the context of a GM-CSF enriched airway environment. 28 d after the last exposure, both groups were exposed for three consecutive days to OVA (in vivo recall challenge) and killed two days later. Their serum was collected and antibody titers were assessed. Our results show a divergent expression pattern of these Th2-associated immunoglobulins; whereas animals initially exposed to OVA and then to OVA in the context of GM-CSF secreted nearly undetectable levels of IgE when compared to mice initially exposed to saline and then to OVA in the context of GM-CSF, IgG1 expression was similar in both groups and significantly higher than in naïve mice (Fig. 3).

Impact of initial OVA exposure on lung physiology in animals exposed to OVA in context of GM-CSF

We next assessed airway responsiveness to increasing intravenous (i.v.) doses of MCh during in vivo recall challenge, as described above. Fig. 4 demonstrates significantly increased airway responsiveness in mice that were initially exposed to saline and then to OVA in the context of GM-CSF expression, as compared to naïve controls. This responsiveness was significantly reduced in mice initially exposed to OVA. At no MCh dose measured did we observe differences in airway responsiveness between naïve animals and animals initially exposed to OVA. BAL cellular data obtained at this time point was similar to findings observed during the acute phase of

OVA exposure in the context of GM-CSF expression, as documented in Fig. 1 (unpublished data).

Activation status of $CD4^+$ T cells in the lung

Because $CD4^+$ T cells are indispensable to the development of allergic airway inflammation their phenotypic profile was assessed. $CD4^+CD3^+$ T cells from animals subjected to two sets of daily OVA exposures 10 days each, both times in the absence of GM-CSF, expressed low levels of the activation markers CD69 (9.8%) (19) and CD25 (6.5%) (20, 21), and negligible levels of the Th2-effector marker T1/ST2 (4.4%) (22-24) (Fig. 5). In contrast, animals initially exposed to saline and then subjected to OVA in the context of GM-CSF had dramatically increased levels of CD69 (28.4%), CD25 (13.0%), and T1/ST2 (13.8%). In animals initially exposed to OVA and then subjected to OVA in the context of GM-CSF the expression of CD69 (26.3%), CD25 (13.1%), and T1/ST2 (10.7%), remained high and similar to levels observed in animals initially exposed to saline.

Ex vivo T cell effector function

To gain insight into effector activity of T cells in the systemic compartment, mice were initially exposed either to OVA (tolerance induction) or saline, and then subjected to OVA in the context of GM-CSF. 24 h after the last OVA exposure, mice were killed, and splenocytes were isolated and placed in culture. Fig. 6A shows significantly increased proliferation of splenocytes from animals initially exposed to saline compared to the other groups. In contrast, modest proliferation was observed in animals initially exposed to OVA, not differing significantly from those of naïve animals.

Fig. 6B shows expression of the Th2-associated cytokines IL-4, IL-5 and IL-13 and the regulatory cytokines IL-10, IFN-γ and TGF-β by splenocytes cultured for five days in the presence of OVA. IL-4, IL-5, IL-13, and IL-10 levels were significantly reduced in animals initially exposed to OVA and then to OVA in the context of GM-CSF when compared to animals initially exposed to saline and then to OVA in the context of GM-CSF, while the levels of IFN-γ and TGF-β remained similar between the groups. Although splenocytes from naïve animals cultured with medium alone

did not express any cytokines measured (unpublished data), some expression of IFN-γ and TGF-β was observed in splenocytes from naïve animals cultured with OVA (1615 ± 218 and 640 ± 229 pg/ml, respectively). Splenocytes from animals initially exposed either to OVA or saline and then cultured in medium alone did not express IL-4 or IL-5 but expressed ~60 pg/ml IL-13, ~25 pg/ml IL-10, ~2 • ng/ml IFN-γ and ~100 pg/ml TGF-β.

Impact of concomitant expression of decorin and GM-CSF in the lungs of tolerant mice on airway and systemic responses

To mechanistically address how unresponsiveness is maintained, mice were initially exposed to saline or OVA; both groups were then exposed either to adenovirally encoded GM-CSF and decorin, or GM-CSF and empty control virus (RDA), one day prior to nine daily and consecutive OVA aerosolizations (GM-CSF/Decorin/OVA and GM-CSF/RDA/OVA, respectively). Decorin, a proteoglycan and a component of the extracellular matrix, has previously been shown to sequester TGF-β in the airways (25). Fig. 7A shows that animals initially exposed to OVA and then exposed to OVA in the context of GM-CSF *and* decorin, mount an eosinophilic response that is comparable to animals initially exposed to saline and then subjected to OVA in the context of GM-CSF and RDA. As a control, tolerant mice exposed to OVA in the context of GM-CSF and RDA maintained a state of airway unresponsiveness. Additionally, tolerant mice exposed to GM-CSF and decorin in the absence of OVA, or tolerant mice exposed to decorin and RDA in the absence of GM-CSF, did not develop airway eosinophilia (Fig. 7B). Moreover, mice initially exposed to saline and then to OVA in the context of GM-CSF and decorin mounted an eosinophilic response that was comparable to animals initially exposed to OVA and then to OVA in the context of GM-CSF and decorin (Fig. 7B). To address whether this reversal of tolerance was accompanied by a systemic effect, splenocyte proliferation and IL-5 production to OVA was assessed. Fig. 8 shows that splenocytes proliferated poorly in animals initially exposed to OVA, regardless of the presence of decorin. This is in contrast to the robust proliferation of splenocytes from animals initially exposed to saline and then to OVA in the context of GM-CSF and RDA. Low levels and insignificant differences were observed when splenocytes were cultured in medium

alone. Expression of IL-5 remained low in animals initially exposed to OVA and then exposed to OVA in the context of GM-CSF and decorin, compared to animals initially exposed to saline and then exposed to OVA in the context of GM-CSF and RDA. To ensure that administration of decorin sequestered TGF-β, we measured TGF-β in the BAL. In accordance with previous findings, neither active nor total TGF-β was detected in decorin treated animals ((25) and unpublished data).

DISCUSSION

Allergic diseases likely arise because mechanisms governing homeostasis break down or are subverted. Therefore, a comprehensive understanding of allergic diseases requires the elucidation of mechanisms that not only lead to allergic sensitization but also break tolerance. To date, investigators have identified a number of ways by which the immune system maintains apparent unresponsiveness to a harmless antigen, but many questions remain. Within this framework, our study addressed the regulatory potential of GM-CSF, a growth factor and pro-inflammatory cytokine associated with human asthma and experimental airways inflammation, and decorin, a small leucine-rich proteoglycan of the extracellular matrix and a natural TGF-β inhibitor (17).

An extensive body of work in human asthma and experimental allergic inflammation demonstrates increased production of GM-CSF at both the RNA and protein levels in allergic asthma. Initially described as a hematopoietic growth factor, GM-CSF is currently known to enhance antigen presentation by inducing proliferation, activation and maturation of DC (reviewed in (26)). Hence, GM-CSF may play a more prevalent role in the expression of the allergic phenotype than previously assumed. Indeed, there is a close association between GM-CSF expression and the breakdown of tolerance induction to innocuous antigen. We have previously reported that sustained but transient expression of GM-CSF in the respiratory mucosa breaks induction of inhalation tolerance. The ensuing inflammatory response encompasses all the hallmarks of allergic inflammation, including airway eosinophilia and bronchial hyper-reactivity. Therefore, GM-CSF provides a context that privileges Th2 sensitization, thereby subverting tolerance induction (27).

In this study, we asked whether GM-CSF breaks established tolerance. We exposed animals to OVA alone, and subsequently exposed animals to OVA in the context of a GM-CSF-enriched airway environment. We show diminished airway and blood eosinophilia and negligible levels of Th-2 associated cytokines. During in vivo long-term recall, we observe diminished OVA-specific IgE expression and reduced responsiveness to methacholine challenge. We argue,

therefore, that GM-CSF is unable to break inhalation tolerance, and that context of initial exposure is remarkably important, especially given the pre-eminent role of GM-CSF in inducing allergic sensitization. However, that a small population of eosinophils is observed suggests that Ad/GM-CSF may partially reverse tolerance, especially given that exposure to OVA alone does not lead to eosinophilia (25). This may be indicative of the plasticity or 'leakiness' of the immune regulatory system. Our data also show that $CD4^+$ T cells are activated in the lungs, as assessed by the early activation marker CD69 (19), the IL-2 receptor CD25 (20, 21) and T1/ST2, a marker of Th-2 effector cells (22-24). The high titres of Th-2 associated IgG1 point to a T-cell dependent and OVA-specific isotype switch, and argue that T cells of tolerant mice retain some activity related to humoral immunity. The attenuation of ex vivo effector function in tolerant mice, as documented with proliferation and cell culture experiments, suggests that surface expression of activation markers observed in the lung is not synonymous with inflammation, a discrepancy on which we have previously reported (27). Alternatively, the observation that $CD4^+$ T cells of tolerant mice express markers of Th-2 activation and effector function but remain refractive to OVA-specific proliferation and Th2-associated cytokine expression may be reflective of a regulatory moiety actively suppressing OVA-specific effector responses. In this regard, it has been proposed in a protocol utilizing intra-nasal exposure to OVA that IL-10 plays a vital role in suppressing airway inflammation (9). However, previous studies from our laboratory in KO mice with inhaled OVA argued IL-10 was redundant (27). Although IFN-γ has long been associated with inhalation tolerance, several relatively recent studies, including our own, have shown intact tolerance in the absence of this cytokine (10, 27, 28). Nonetheless, these data do not negate a role for IL-10 or IFN-γ in tolerance but draw attention to the redundancy of the immune system, and suggest that other regulatory mechanisms may be compensating.

Studied as much for its fibrogenic as for its anti-inflammatory properties, TGF-β is an important, if not defining, cytokine in Tr1 and Th3 regulatory cell-dependent suppression (29) (30). Although TGF-β dependent regulation has been described in oral tolerance and organ-specific autoimmune disease (11-13), its role in inhalation tolerance is still largely unknown (14). In this study, we exposed tolerant animals to OVA in the context of concomitant expression of

decorin and GM-CSF. Our rationale for utilizing a viral vector carrying the decorin transgene is two-fold. First, it is well established that decorin binds to, and inactivates the activity of, TGF-β in murine and human airways (31-33). Moreover, administration of a replication-deficient adenovirus carrying the decorin transgene introduces an attractive alternative to mouse transgenics and protein-based interventions: TGF-β null mice die early of multiple causes (34), Smad knockouts may not exhaust complete TGF-β-mediated activity (35, 36), and antibody treatments may lead to serum sickness and are expensive, especially when multiple administrations are required. A single delivery of adenovirally-encoded decorin, on the other hand, ensures continuous expression of the protein for the entire exposure period to antigen and, by extension, disruption of TGF-β-dependent activity in the airways (25).

Our data show that exposure of tolerant animals to OVA in a GM-CSF and decorin-rich airway environment reverses eosinophilic unresponsiveness and leads to robust airway inflammation. The event is localized to the lung compartment because OVA-specific proliferation of splenocytes and the associated IL-5 production remains ablated. Despite the observation that TGF-β was undetectable in decorin-treated animals, it is yet to be determined whether this reversal of airway tolerance is TGF-β sufficient or whether TGF-β independent mechanisms are also involved. The observation that decorin reduces experimental pulmonary fibrosis by a TGF-β-mediated mechanism (25, 33) combined with recent studies in patients with rhinitis and asthma depicting that unresponsiveness to house dust mite and birch pollen allergens can be reversed by neutralizing TGF-β (37), is compelling corroborative evidence that the molecule is critical to the airway changes we observe following decorin administration, and further supports the notion that fibrosis and inflammation may be inversely related. Not unlike IL-10 or IFN-γ, the similar expression of TGF-β in BAL and culture supernatants in both tolerant and non-tolerant animals may be indicative of immune plasticity; TGF-β is known to be vastly pleiotropic (38). Alternatively, the similar levels may be indicative of a decorin-dependent, TGF-β-independent mechanism. Indeed, decorin has recently been shown to affect expression of several molecules in cultured human gingival fibroblasts, including metalloproteinases, tissue inhibitors of metalloproteinases,

IL-1β, IL-4 and TNF-α (39), while human glioma cells engineered to express the proteoglycan have been shown to stimulate alloreactive immune responses independently of TGF-β (40). That increased expression of decorin has been observed in human asthma further solidifies a role of this molecule in allergic diseases (16). We propose that the combined expression of GM-CSF and decorin generates an airway microenvironment with compromised regulatory activity likely by disrupting TGF-β-mediated suppression. That OVA was necessary to establish robust eosinophilia argues for antigen dependence. That decorin alone was unable to break tolerance argues that a pro-inflammatory environment must accompany decorin expression if these changes are to take place. Together, these data suggest a broader context in which decorin contributes to biological processes. The data also suggest that airway tolerance is overcome by two interventions: one that allows for allergic sensitization (GM-CSF) and one that inhibits regulatory activity (decorin). It remains to be elucidated whether such intervention permanently changes the phenotype of the response to OVA.

Our study is the first to document that GM-CSF alone cannot break already established tolerance, a process that may be governed by TGF-β and other decorin-related mechanisms. It is also the first observation that expression of decorin affects airway inflammation. Typically a negative regulator of TGF-β-dependent fibrosis, this new role for decorin cautions against narrowly defined intervention strategies. Indeed, while decorin plays an important role in regulating fibrosis, it may be effective at eliciting airway inflammation. The inability to break tolerance with GM-CSF, a cytokine with well-defined pro-inflammatory properties, argues that context of initial exposure to antigen has significant and remarkably persistent influence on subsequent intervention, a notion we have previously explored in a model of chronic exposure (41). The concomitant expression of decorin, however, shows that airway unresponsiveness breaks under conditions where regulatory activity is suppressed. These findings serve to further situate tolerance as an active and resilient process governed by a network of diverse mediators.

MATERIALS AND METHODS

Animals

Female Balb/c mice (6-8 weeks old) were purchased from Harlan (Indianapolis, IN). Mice were kept in a 12 h light-dark cycle with unlimited access to food and water. Cages, food and bedding were autoclaved, and gloved, gowned and masked personnel carried out all animal manipulations in a laminar flow hood. The Animal Research Ethics Board of McMaster University approved all experiments.

Tolerance induction

Mice were placed in a plexiglass chamber (10cm X 15cm X 25cm) and exposed for 20 min daily over a period of 10 consecutive days to aerosolized OVA (1% wt/vol in 0.9% saline) (Sigma Chemicals, St. Louis, MO) (27). Control mice were exposed to saline only. The aerosol was produced by a Bennet/Twin nebulizer (Puritan-Bennett Corporation, Carlsbad, CA) at a flow rate of 10 litres/min.

Delivery of Adenoviral Constructs

We utilized replication-deficient E1/E3-deleted human type 5 adenoviral (Ad) constructs carrying the transgenes for murine GM-CSF (Ad/GM) (42) or human decorin (Ad/Dec) (33) in the E1 region of the viral genome. As control, we included an E1-deleted replication-deficient human type 5 adenoviral (RDA) construct carrying no transgene (43). All adenoviral constructs were delivered intra-nasally in a total volume of 30: l of PBS vehicle (two 15: l administrations 5 min. apart) into animals anaesthetized with isoflurane (Baxter Corporation, Toronto, ON, Canada).

Allergic Mucosal Sensitization

Two days after tolerance induction, mice were exposed to OVA in the context of a GM-CSF-enriched airway microenvironment as previously described in detail (15). GM-CSF was expressed using an adenovirus-mediated gene transfer approach. A dose of $3x10^7$ pfu Ad/GM-CSF construct was delivered intra-nasally one day prior to OVA exposure. In some experiments, a

concurrent dose of $3x10^7$ pfu Ad/Dec or $3x10^7$ pfu RDA was delivered. Mice were exposed to OVA aerosol (1% wt/vol in 0.9% saline) for 20 min daily for nine days, and killed 24 h later. A nine-day protocol is superior to ten days in that it allows for concurrent measurement of cytokines. In experiments involving airway hyperresponsiveness (AHR) and immunoglobulin measurements, mice were rested for a period of 28 days, re-exposed to OVA aerosol daily for 20 min over a period of three days, and killed two days later.

Collection and Measurement from Specimens

Bronchoalveolar lavage (BAL) was performed as previously described (44). In brief, lungs were dissected and the trachea was 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 0.25 ml of the instilled fluid was consistently recovered. Total cell counts were determined using a hemocytometer. After centrifugation cell pellets were resuspended in PBS and slides were prepared by cytocentrifugation (Shandon Inc., Pittsburgh, PA) at 300 rpm for 2 min. HEMA 3 Stain Set (Biochemical Sciences Inc., Swedesboro, NJ) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological procedures to classify cells as mononuclear or eosinophils. Peripheral blood was obtained utilizing heparin-coated capillaries (Fisher Scientific Pittsburgh, PA). Total white blood cell counts were determined after lysing red blood cells, and cell differentials were assessed on smears stained with the HEMA 3 Stain set. For serum, animals were bled with non-heparin capillary tubes. Serum was prepared by incubating whole blood for 30 min at 37ºC and spun for 15 min at 4ºC.

Cytokine and Immunoglobulin Measurements

ELISA kits for IL-4, IL-5, IL-13, IL-10, IFN-γ, and TGF-β were purchased from R&D Systems (Minneapolis, MN). The threshold of detection for IL-4, IL-13, and IFN-γ was < 2pg/ml; IL-10 was < 4pg/ml; TGF-β was < 7 pg/ml; IL-5 was < 5pg/ml. To obtain total TGF-β levels samples were incubated with HCl prior to ELISA, as directed by the manufacturer.

Levels of OVA-specific IgE were detected with an ELISA that has been described in detail previously (44). For OVA-specific IgG1, Maxi-Sorb plates (NUNC Brand Products, Denmark) were coated with 5µg OVA in borate buffer pH 8.3-8.5 overnight at 4°C. Subsequently, coated wells were blocked with 1% BSA in PBS for 2 h at room temperature. After washing, serum samples were incubated overnight at 4°C, washed, and developed with biotin-labeled, anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) overnight at 4°C. Plates were washed and incubated with alkaline-phosphatase streptavidin for 1 h at room temperature. The color reaction was developed with p-Nitrophenyl phosphate tablets. Samples were compared to a standard serum containing OVA-specific IgG1. Units correspond to maximal dilution that results in an OD that is greater than the blank plus two standard deviations.

Airway Hyperresponsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (RRS) to increasing internal jugular vein doses of methacholine (MCh), as previously described (45, 46). Exposed tracheas were cannulated and a flow interrupter technique was applied by mechanical ventilation (RV5, Voltek Enterprises Inc., Toronto, ON, Canada). Paralysis was achieved using pancuronium (0.03 mg/kg i.v.) to prevent respiratory effort during measurement. RRS was measured following consecutive i.v. injection of saline, then 10, 33, and 100 µg/kg of MCh (ACIC (Can), Brantford, ON, Canada), each delivered as a 0.2 ml bolus. Evaluation of airway responsiveness was based on the peak RRS measured in the 30 s following the saline and MCh challenges.

Lung Cell and Splenocyte Isolation

Spleens were ground and filtered through a nylon mesh (BSH Thompson, Scarborough, ON, Canada). The cell suspension was centrifuged at 1200 rpm for 10 min at 4º C. Red blood cells were lysed with ACK lysis buffer, and the splenocytes were washed with HBSS and resuspended in RPMI (Gibco BRL) supplemented with 10% FBS, 1% L-glutamine (Sigma Chemicals Co.), 1% penicillin/streptomycin (Bibco, BRL), and 0.5% 2-mercaptoethanol (Sigma Chemicals Co.).

For isolation of lung cells, lungs were flushed via the right ventricle of the heart with 10 ml of warm (37°C) HBSS. The lungs were then cut into small (approximately 2 mm in diameter) pieces and shaken at 37°C for 1 h in 15 ml of 150 U/ml collagenase I (Worthington Biochemical, Freehold, NJ) in HBSS. Using a plunger from a 3 ml syringe, the lung pieces were triturated and filtered through a nylon mesh into HBSS. Cells were washed twice and mononuclear cells were isolated by density centrifugation over a 30% and 60% Percoll gradient (Pharmacia, Uppsala, Sweden).

Splenocyte Cell Culture

Splenocytes were cultured in medium alone or with 80 µg OVA/ml at $8X10^5$ cells/well in a 96-well flat-bottom plate (Becton Dickinson, Lincoln Park, NJ). After 5 days of culture, supernatants were harvested for cytokine measurement. For proliferation, splenocytes were cultured in medium alone or with 40, 80 and 160 µg OVA/ml at $8X10^5$ cells/well in a 96-well round-bottom plate (Becton Dickinson). After two days of culture 1µCi of ^{3}H-thymidine (Perkin Elmer Life Sciences, Boston, MA) in 20µl of RPMI was added to each well. 24 h later, ^{3}H-thymidine incorporation was assessed with TopCount NXT microplate scintillation & luminescence counter (Packard BioScience Company, Meridan, CT).

Flow Cytometry

Panels of monoclonal antibodies were selected to study the phenotype of cells in the lung. To minimize non-specific binding, 10^6 cells were incubated with 0.5 µg Fc Block (CD16/CD32; Pharmingen, Mississauga, ON, Canada) at 0-4ºC for 15 min and subsequently with first stage monoclonal antibodies at 0-4ºC for 30 min. Cells were then washed and treated with second stage reagents. Data were collected using a FACScan and analyzed using WIN-MDI software (The Scripps Research Institute, La Jolla, CA). The following antibodies and reagents were used: anti-CD3, PE-conjugated 145-2C11 (Pharmingen); anti-CD3, cy-Chrome-conjugated 145-2C11 (Pharmingen); anti-CD4, FITC-conjugated L3T4 (Pharmingen); anti-CD4, cy-Chrome-conjugated RM4-5 (Pharmingen); anti-CD69, PE-conjugated H1 2F3 (Pharmingen); anti-CD25, PE-conjugated PC61 (Pharmingen); anti-T1/ST2, FITC-conjugated 3E10 (kindly provided by A.J.

Coyle, Millenium Pharmaceuticals, Inc., Cambridge, MA) labeled in-house according to a standard protocol (17); and Streptavidin Cy-Chrome (Pharmingen). The experiment was controlled with appropriate isotypes (Pharmingen). Titration was performed to determine the optimal concentration of each antibody.

Data analysis

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

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FIGURE LEGENDS

Figure 1. *BAL and peripheral blood cellular response in tolerant mice following exposure to OVA in context of GM-CSF.* Balb/c mice were initially exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA in the context of GM-CSF. Panel **A** depicts BAL cellular data; panel **B** shows peripheral blood. Data represent mean ± SEM. Statistical analysis was performed using one-way ANOVA with the Fisher least significant difference (LSD) post hoc test (*, $p<0.05$). n = 8 mice/group for BAL; n = 4 mice/group for peripheral blood.

Figure 2. *Cytokine expression in the BAL of tolerant mice following exposure to OVA in context of GM-CSF.* Balb/c mice were initially exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Cytokine expression was assessed in the BAL. Data represent mean ± SEM. Statistical analysis was performed using one-way ANOVA with the Fisher LSD post hoc test (*, $p<0.05$). n = 4-9 mice/group.

Figure 3. *OVA-specific IgE and IgG1 levels in the serum of tolerant mice following exposure to OVA in context of GM-CSF.* Balb/c mice were initially exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. 28 d after the last exposure, mice were challenged with aerosolized OVA for 20 min on 3 consecutive days and killed two days later. OVA-specific IgE and IgG1 levels were measured in the serum. Data displayed represent mean ± SEM. Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$). n = 5 mice/group for naïve; n = 8 mice/group for other groups.

Figure 4. *Impact of GM-CSF expression on airway hyperresponsiveness in tolerant mice.* Mice were initially exposed to either OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. 28 d after the

last exposure, mice were challenged with aerosolized OVA for 20 min on 3 consecutive days and airway function was assessed two days later. Data displayed represent mean ± SEM. Statistical analysis was performed using ANOVA with Newman-Keuls post hoc test (*RRS was significantly greater compared to naïve control mice ($p<0.05$), #RRS was significantly greater compared to sensitized and challenged mice initially exposed to OVA ($p<0.05$)). n = 5 mice/group for naïve; n = 8 mice/group for other groups.

Figure 5. *T cell activation status in the lungs of tolerant mice following exposure to OVA in context of GM-CSF.* Mice were initially exposed to either OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Another group of animals was subjected to two blocks of OVA exposure only. 24 h after the last exposure, mice were killed. Data show the expression profile of CD69, CD25, and T1/ST2 on $CD3^+CD4^+$ T cells. Five animals were pooled per experimental group. One of two representative experiments is shown.

Figure 6. *Splenocyte proliferation and cytokine production.* Mice were initially exposed to either OVA or saline aerosol for 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. 24 h after the last exposure, mice were killed, their splenocytes isolated and placed into culture. In **A**, data show ^{3}H-Thymidine incorporation to increasing doses of OVA in vitro after three days of culture (mean ± SEM). Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$ compared to all other groups). In **B**, data show expression of cytokines measured by ELISA after five days of culture with 80μg/ml OVA (mean ± SEM). Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$). n = 3-5 mice/group.

Figure 7. *BAL cellular profile in tolerant mice exposed to OVA in the context of GM-CSF and decorin expression in the airway.* Mice were initially exposed to either OVA or saline aerosol for 10 consecutive days. **A.** Mice exposed to OVA were then subjected to OVA aerosol in the context

of GM-CSF and Decorin or OVA in the context of GM-CSF and empty control virus (RDA). Mice initially exposed to saline were subjected to OVA in the context of GM-CSF and RDA. Animals were killed 24 h after the last of nine consecutive daily exposures to OVA. Data represent the cellular profile in the BAL (mean ± SEM). Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$). n = 6-8 mice/group. **B.** Mice initially exposed to saline were then subjected to OVA in the context of GM-CSF and decorin while mice initially exposed to OVA were then subjected either to GM-CSF and decorin in the absence of OVA or decorin and RDA in the presence of OVA. Animals were killed 24 h after the last of nine consecutive daily exposures to OVA. Data represent the cellular profile in the BAL (mean ± SEM). Statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$ compared to all other groups). n = 3 mice/group.

Figure 8. *In vitro proliferation and IL-5 production in tolerant mice exposed to OVA in the context of GM-CSF and decorin expression in the airway.* Mice were initially exposed to either OVA or saline aerosol for 10 consecutive days. Mice exposed to OVA were then subjected to OVA aerosol in the context of GM-CSF and decorin or OVA in the context of GM-CSF and empty control virus (RDA). Mice initially exposed to saline were subjected to OVA in the context of GM-CSF and RDA. Animals were killed 24 h after the last of nine consecutive daily exposures to OVA. Data represent proliferation of splenocytes and IL-5 production from individual animals cultured for three days (proliferation) or five days (IL-5 production) in medium (gray bars) or OVA (black bars) (mean ± SEM). For proliferation, statistical analysis was performed using one-way ANOVA with Fisher LSD post hoc test (*, $p<0.05$). For IL-5 production, statistical analysis was performed using Kruskal-Wallis one-way ANOVA on ranks (*, $p<0.05$). n = 6-8 mice/group.

Figure 1

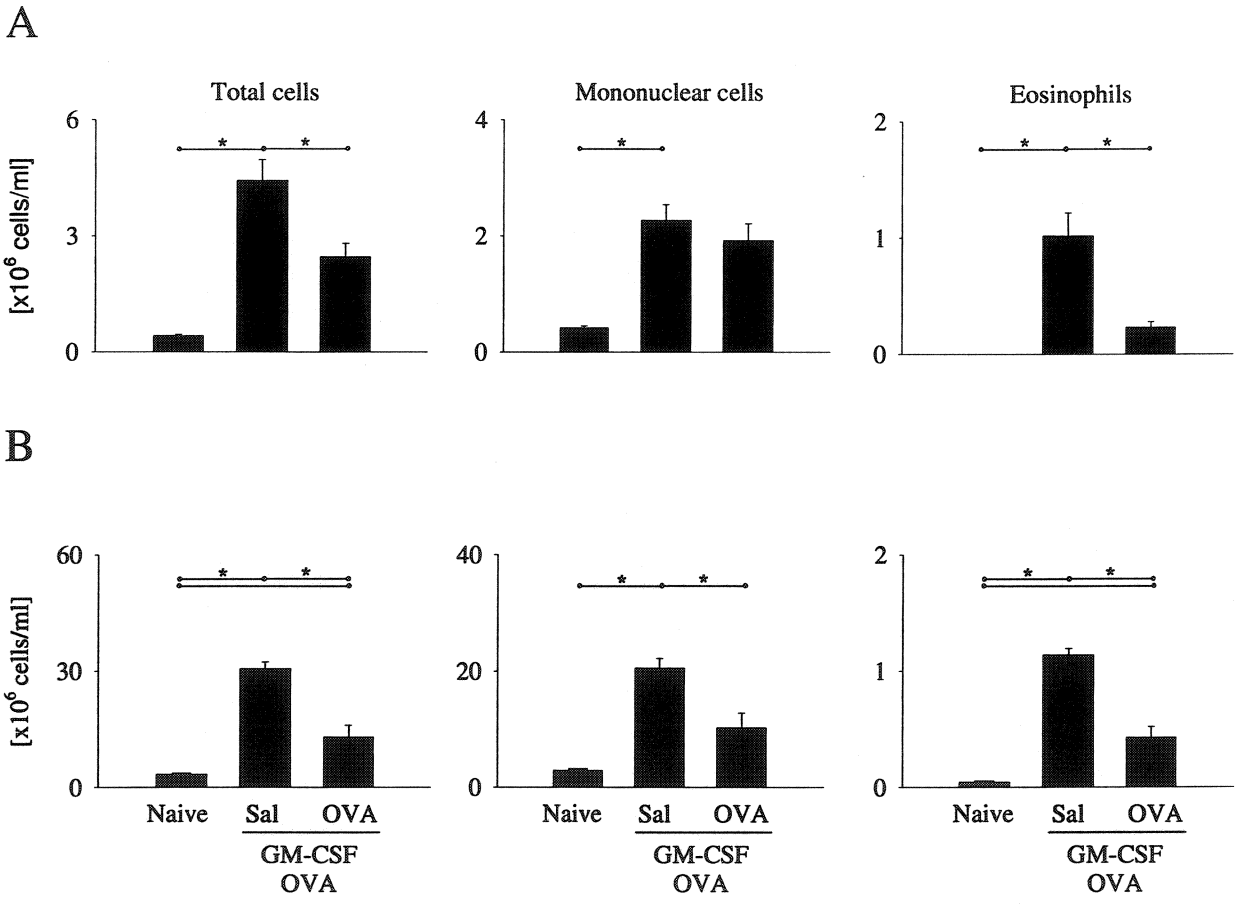

A
Total cells
Mononuclear cells
Eosinophils
[x10^6 cells/ml]
6
3
0
4
2
0
2
1
0
B
60
30
0
40
20
0
2
1
0
Naive
Sal
OVA
GM-CSF
OVA

Figure 2

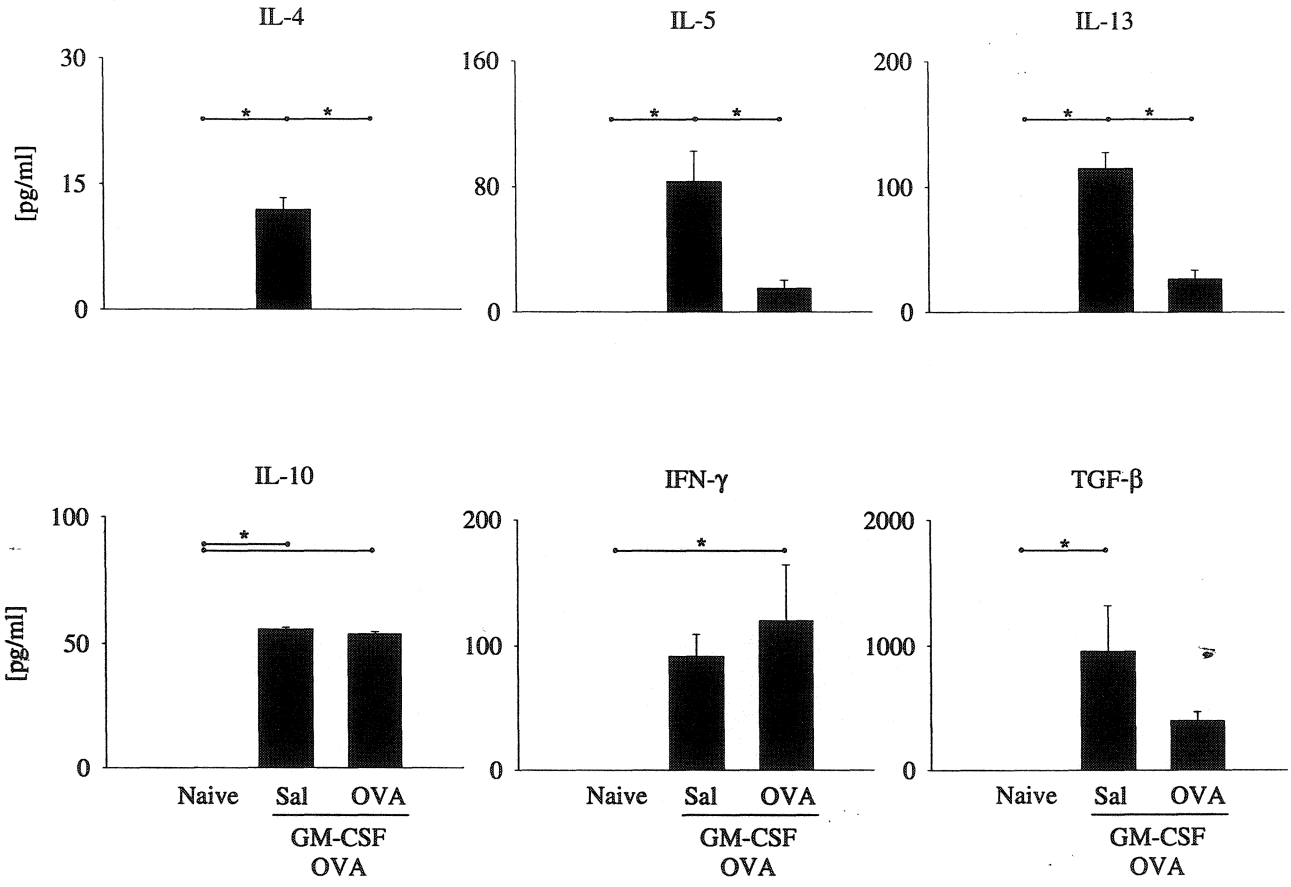

IL-4
IL-5
IL-13
IL-10
IFN-γ
TGF-β
[pg/ml]
30
15
0
160
80
200
100
100
50
200
100
2000
1000
*
Naive
Sal
OVA
GM-CSF
OVA

Figure 3

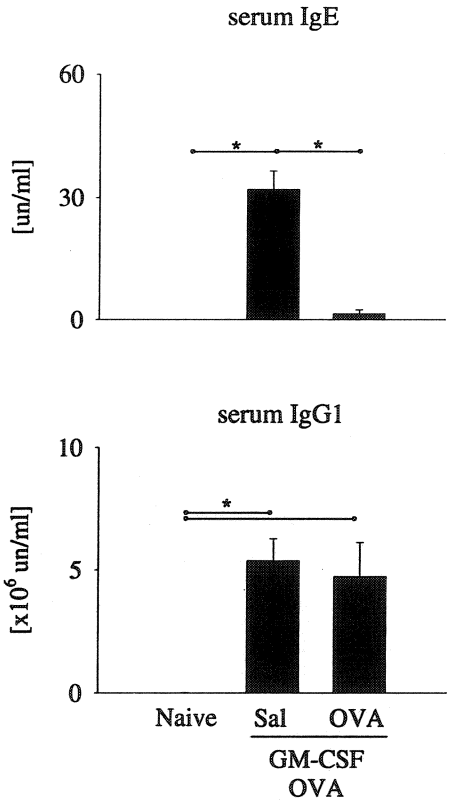

serum IgE
60
30
0
[un/ml]
*
*
serum IgG1
10
5
0
[x10^6 un/ml]
*
Naive
Sal
OVA
GM-CSF
OVA

Figure 4

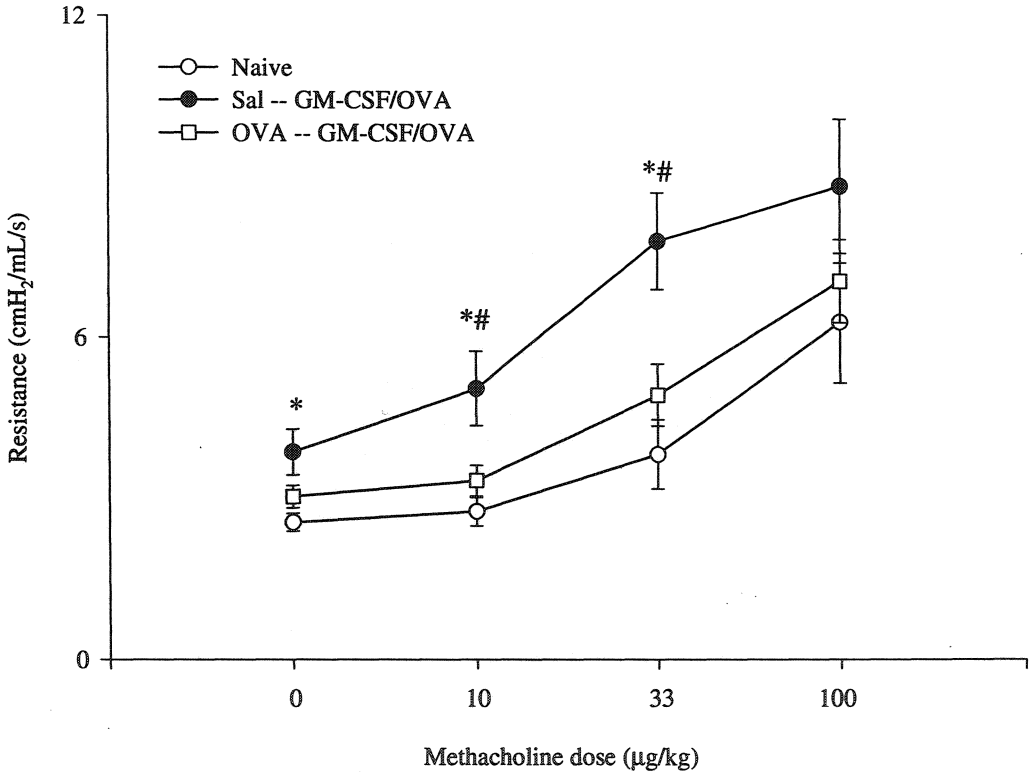

12
6
0
Naive
Sal -- GM-CSF/OVA
OVA -- GM-CSF/OVA
*
*#
*#
Resistance (cmH2/mL/s)
0
10
33
100
Methacholine dose (µg/kg)

Figure 5

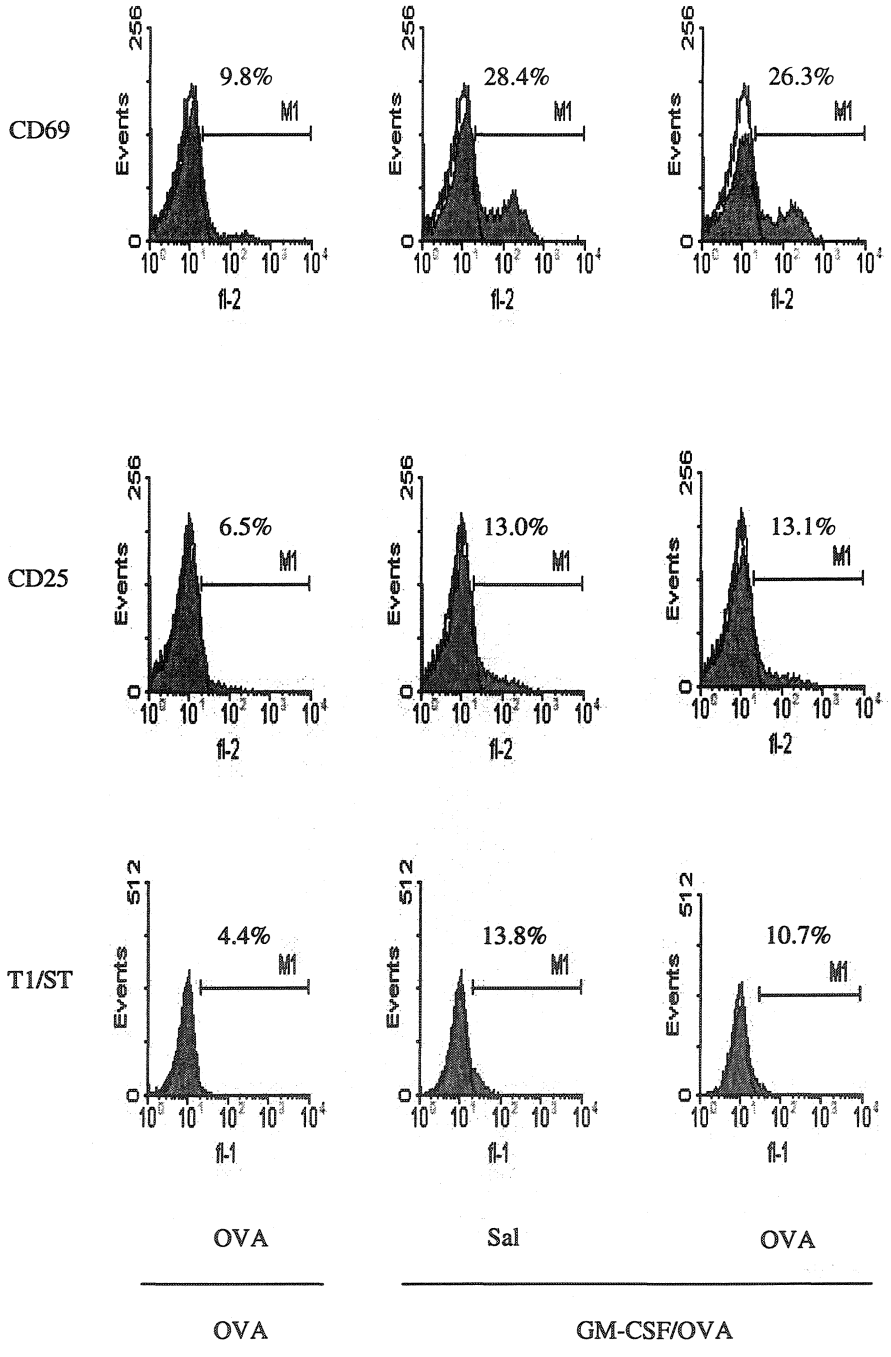
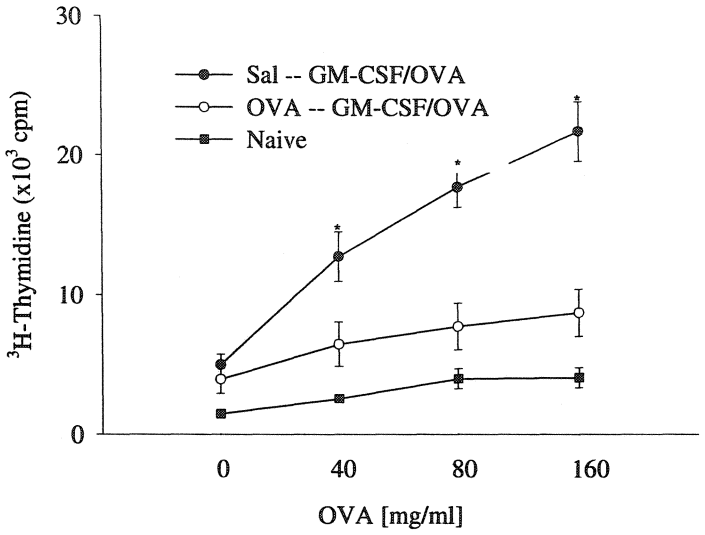
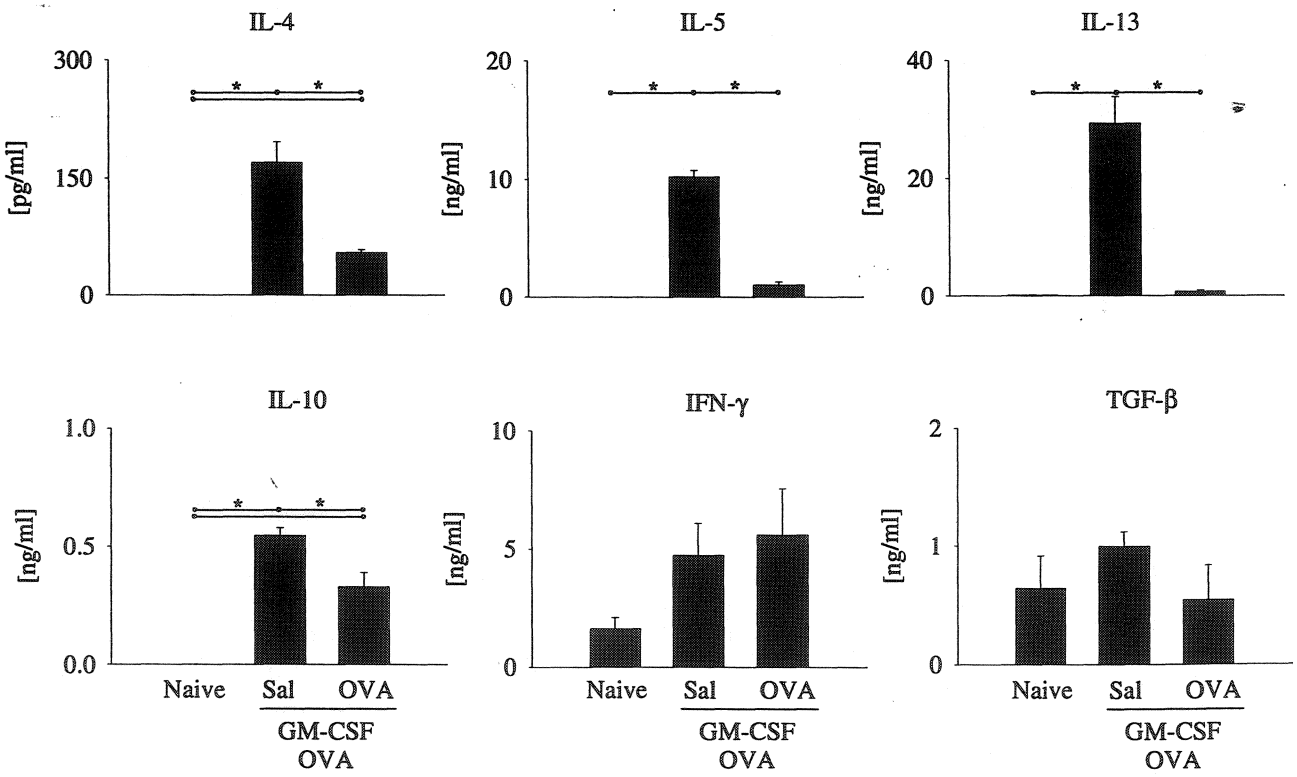

CD69
CD25
T1/ST
9.8%
28.4%
26.3%
6.5%
13.0%
13.1%
4.4%
13.8%
10.7%
M1
Events
fl-2
fl-1
OVA
Sal
OVA
OVA
GM-CSF/OVA

Figure 6

A


30
20
10
0
^{3}H-Thymidine (x10^3 cpm)
Sal -- GM-CSF/OVA
OVA -- GM-CSF/OVA
Naive
0
40
80
160
OVA [mg/ml]

B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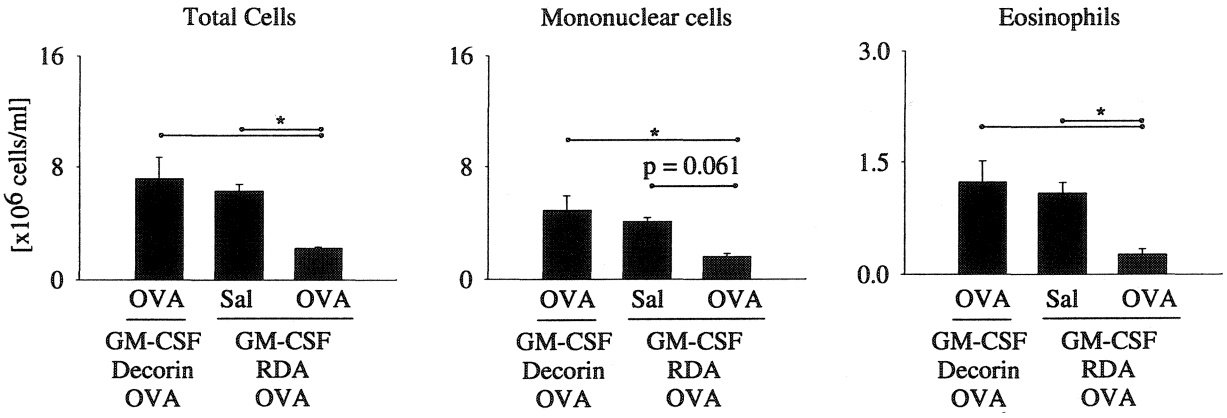
IL-13
40
20
0
[ng/ml]
IL-10
1.0
0.5
0.0
[ng/ml]
IFN-γ
10
5
0
[ng/ml]
TGF-β
2
1
0
[ng/ml]
Naive
Sal
OVA
GM-CSF
OVA

Figure 7

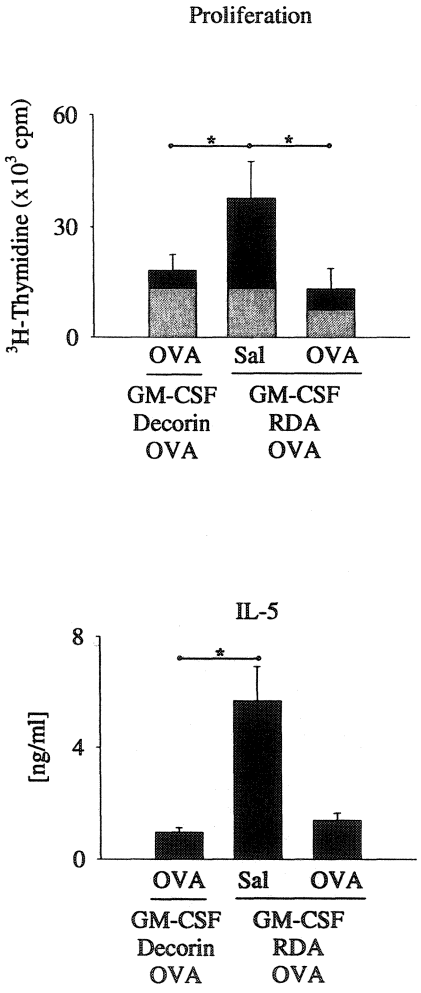
A



B

	Sal GM-CSF Decorin OVA	OVA GM-CSF Decorin --	OVA Decorin RDA OVA
Total cells ($x10^6$)	7.4 ± 1.1*	3.8 ± 1.1	2.2 ± 0.3
Eosinophils ($x10^6$)	1.8 ± 0.3*	0.54 ± 0.22	0.01 ± 0.01

Figure 8


Proliferation
^{3}H-Thymidine (x10^3 cpm)
60
30
0
*
*
OVA
Sal
OVA
GM-CSF
Decorin
OVA
GM-CSF
RDA
OVA
IL-5
[ng/ml]
8
4
0
*
OVA
Sal
OVA
GM-CSF
Decorin
OVA
GM-CSF
RDA
OVA

Chapter 4: Chronic Exposure to Innocuous Antigen in Sensitized Mice Leads to Suppressed Airway Eosinophilia That Is Reversed by Granulocyte Macrophage Colony-Stimulating Factor.

This article appeared in the *Journal of Immunology* (2002); 169: 3499-3506..

In this study we investigated the impact of chronic allergen exposure on airway inflammation and humoral responses in sensitized mice. We show that continued exposure to OVA does not lead to persistent inflammation but to abrogated eosinophilia. Delivery of recombinant GM-CSF during long term re-challenge fully restored airway eosinophilia. This study shows that a unique tolerant state may be generated in sensitized mice. It suggests that understanding principles that lead to or prevent chronic inflammation elicited by innocuous antigen is key to our understanding of allergic diseases. I generated the data and wrote the manuscript. D. Sajic helped with experiments; C.S. Robbins and B.U. Gajewska helped with Flow Cytometry; M. Jordana was involved in the conceptualization of the project; and M.R. Stampfli supervised the project.

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

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

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

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

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

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

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

0022-1767/02/$02.00

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

Materials and Methods

Animals

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

Allergic mucosal sensitization

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

Delivery of rGM-CSF

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

Collection and measurement from specimens

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

Cytokine and Ig measurements

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

Lymph node and lung cell isolation

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

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

Flow cytometry

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

Data analysis

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

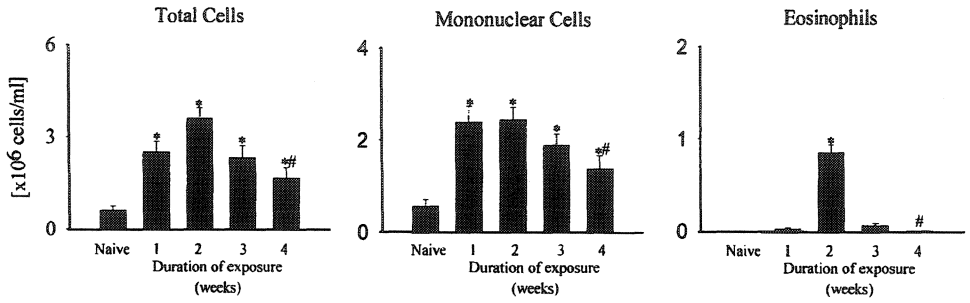
Results

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

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

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

A



B

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

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

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

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

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

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

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

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

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

*, $p < 0.05$ when compared to naive.

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

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

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

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

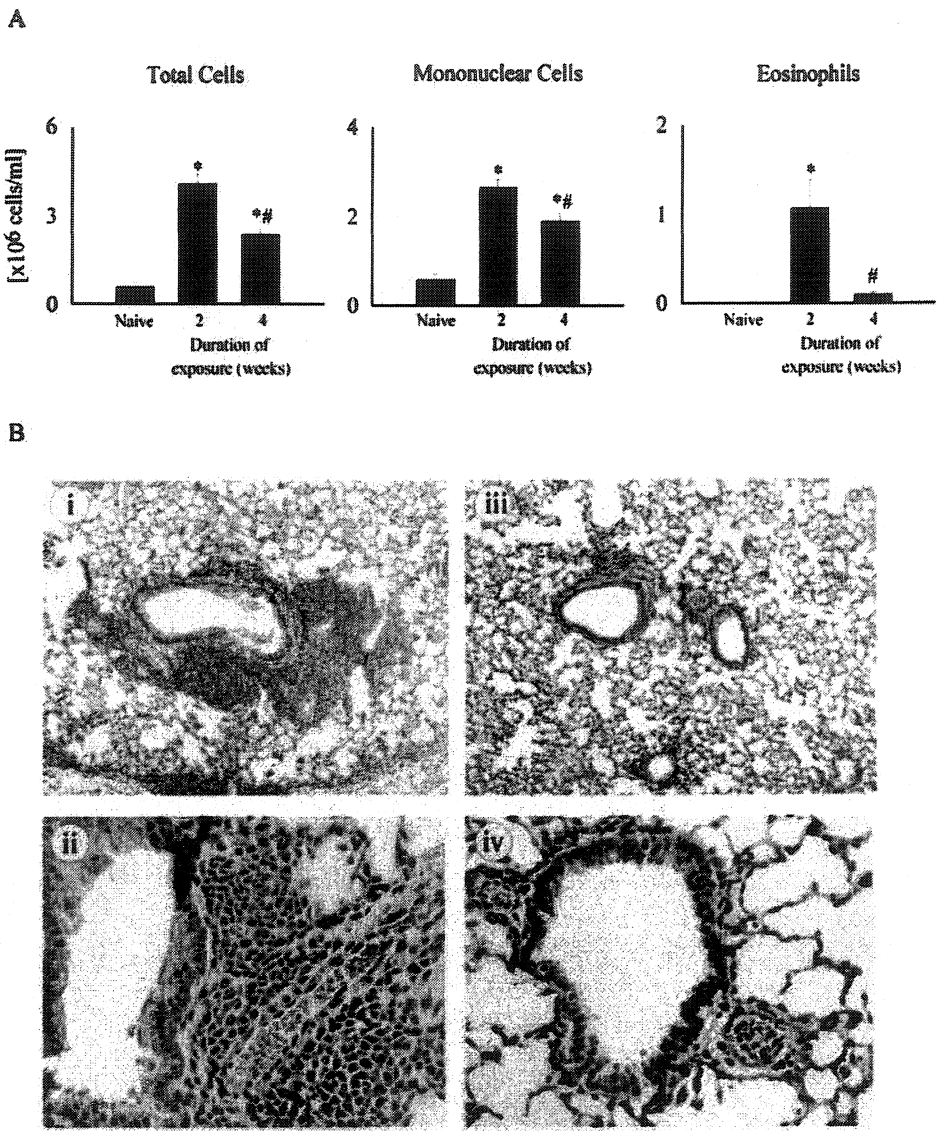


FIGURE 2. Inflammatory response during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on three consecutive days and killed 2 days later. *A*, The BAL cellular profile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

T cell and APC phenotype during in vivo Ag recall

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

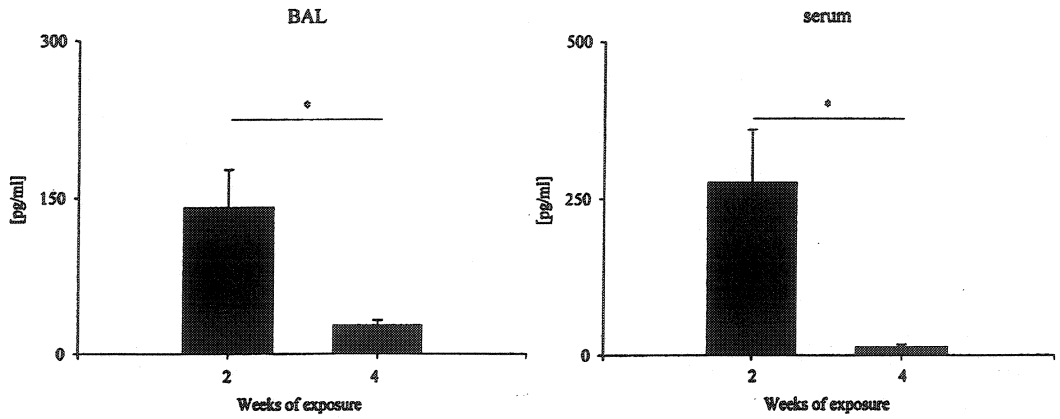


FIGURE 3. Expression of IL-5 in the BAL and serum during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest after the exposure protocol, both groups were challenged with OVA and killed 1 day later. Data show the expression of IL-5 in the BAL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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

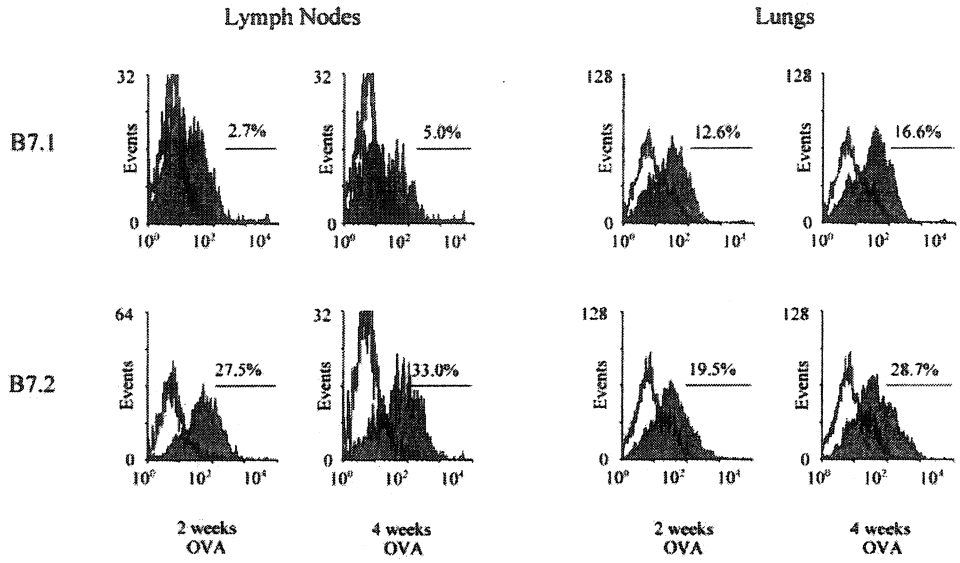


FIGURE 4. Costimulatory molecule expression on DCs during long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on two consecutive days and killed 1 day later. Data show the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

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

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

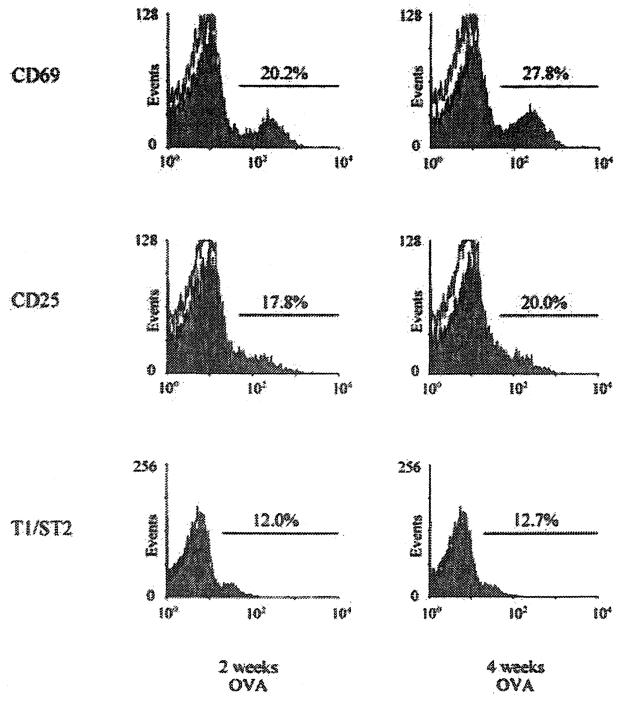


FIGURE 5. Activation of T cells in the lungs after long-term in vivo recall. Mice were exposed to 2 and 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, both groups were challenged with OVA on three consecutive days and killed 2 days later. Data show the expression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

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

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

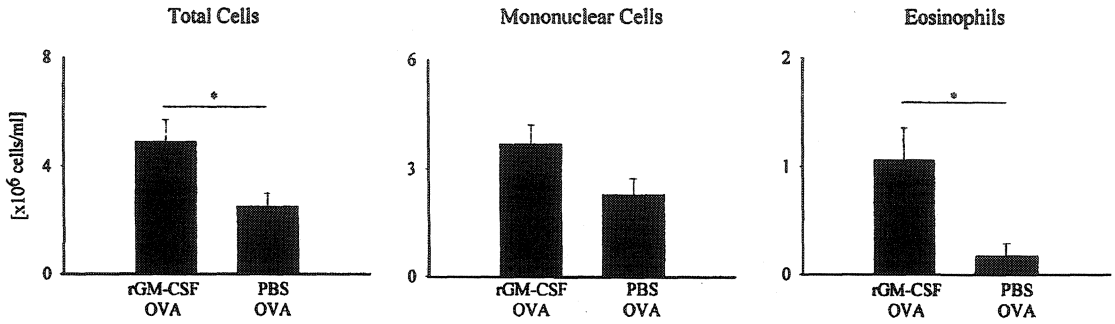


FIGURE 6. Exposure to OVA in the context of rGM-CSF during long-term in vivo recall. Mice were exposed to 4 wk of OVA in the context of GM-CSF. After 28 days of rest following the exposure protocol, mice were challenged with OVA on nine consecutive days and killed 1 day later. One group of mice received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

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

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

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

Acknowledgments

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Chapter 5: Discussion

An understanding of mechanisms behind immune tolerance is likely to lead to novel treatment strategies for a myriad of pathologies, among them allergic diseases. Until the late 1990s immune tolerance was defined as failure to respond to antigen. Studies on central tolerance identified apoptosis in the thymus as the predominant mechanism by which potentially autoreactive T cells are prevented from responding, while generation of anergy, defined as the inability of T cells to respond to stimulation, was deemed the main mechanism in the periphery. In themselves active processes, the consequence of deletion and anergy is a passive state; the associated absence of T cells, or their dormant phenotype, precludes the existence of a dominant, and therefore transferable, component. Epidemiological evidence suggested, however, that other mechanisms are involved. In spite of universal exposure to common environmental antigens such as house dust mite, a relatively small proportion of the population suffers from allergic asthma, suggesting the presence of robust and long lasting mechanisms that maintain homeostasis. Given that T cells bearing a vast diversity of receptor affinities to antigen exit the thymus continually, it may be argued that the healthy immune system is in a state of constant readiness. It is likely then, that passive mechanisms of tolerance co-evolved with dominant mechanisms and these, together, maintain homeostasis.

More compelling evidence that tolerance to inhaled antigen depends on dominant mechanisms was generated in animal studies. Holt and colleagues were the first to show in a rat model that initial exposure to OVA compromises expression of OVA-specific IgE upon subsequent immunogenic challenge (184), and demonstrated thirteen years later that a subset of IFN-γ expressing T cells from animals rendered tolerant to OVA prevented IgE expression when adoptively transferred to a non-tolerant host (187). Although it was later

demonstrated by several groups, including our own, that IFN-γ is redundant in maintaining unresponsiveness (141, 142), this observation was nevertheless critical in establishing that inhalation tolerance may be governed by a dominant, T cell mediated mechanism.

Given that IgE is only one of several markers that characterize the asthmatic and allergic phenotype, notable others being airway eosinophilia, expression of Th2-associated cytokines and airway hyper-responsiveness, we (141) and others (183) conducted a comprehensive analysis of immune-inflammatory and physiological processes following immunogenic challenge of tolerant animals. To elicit tolerance in our study, mice were first exposed to ten daily OVA aerosolizations in the absence of adjuvant, according to a protocol established earlier (142). Control mice were exposed to saline. Subsequently, *both* groups were exposed to OVA in the context of aluminum hydroxide (alum), and adjuvant that, when delivered with OVA to naïve mice, leads to the development of OVA-specific inflammation (188, 189). Animals initially exposed to OVA and then to OVA in the context of alum did not develop airway eosinophilia and exhibited dramatically reduced airway inflammation compared to control animals, as described in Chapter 2 and (141). Notable among our findings was that tolerance is induced and maintained in the absence of IL-10 and IFN-γ, molecules previously shown to regulate airway responses (187, 190). Our study was also consistent with findings that costimulation plays an important role in the effector organ during challenge (191, 192).

Since these initial observations were made, work on respiratory tolerance in particular and immune tolerance in general has flourished, largely because of the discovery of regulatory mechanisms involving tolerogenic dendritic cells and T regulatory cells such as CD4+CD25+ T_R, T_R1, T_H3, and T_R. Regulatory cells may develop as part of a homeostatic mechanism designed to shutdown immune responses to infectious agents. The generation

of regulatory cells would depend on the recognition of PAMPs on the surface of microbial agents. Regulatory cells also develop independently of PAMP recognition. These are involved in the induction and maintenance of a state of unresponsiveness to innocuous agents such as allergens. The function of regulatory cells is believed to rely on cell surface or secreted expression of regulatory cytokines such as IL-10 or TGF-β (2). In a series of studies, Umetsu and colleagues have shown that, following respiratory exposure to innocuous antigen, dendritic cells may be used to generate IL-10 producing regulatory T cells, termed T_R. The interaction depends on the ICOS-ICOSL costimulatory dyad. When transferred to a host, these CD4 T cells markedly reduce both lung inflammatory responses and airway hyper-responsiveness. The inhibitory effect is blocked with an antibody specific for IL-10. These findings not only provide a mechanistic framework for respiratory tolerance but also suggest that tolerance and sensitization are closely related.

That development of tolerance or sensitization follows parallel mechanisms (one is not a default response for the other) forces researchers to re-examine one of the central tenets of immunology: the prescient question is no longer whether to respond or not to respond, but *when* and *how* to respond. This seemingly trivial modification of paradigm more fully encompasses the growing sophistication of our understanding of immune system biology. It is well established that the outcome of a specific intervention largely depends on initial exposure: it has been very difficult, for example, to reverse or break established tolerance. While successful at transferring the active component, the studies by Umetsu and others did not attempt to break *established* tolerance. Yet, to break a system *in vivo* is to exhaust that system's capacity to function, while the transfer of an active component identifies one, but not necessarily the most effective, aspect of an immune state.

In an attempt to break established tolerance *in vivo*, we exposed mice to OVA alone (tolerance induction) and then to OVA in the context of GM-CSF. GM-CSF, a growth and maturation factor for DCs, is a natural activator of adaptive immunity, and has been associated with human and experimental asthma (193). We have previously shown that aerosol exposure to OVA in the context of transient but sustained GM-CSF expression in the airway, delivered intra-nasally as an adenovirally-encoded transgene, subverts the establishment of tolerance and leads to airway eosinophilia, IgE expression and airway hyper-responsiveness, all of which are hallmarks of allergic asthma (55, 85, 194). Data presented in Chapter 3 show that established tolerance is not broken when tolerant animals are exposed to OVA in the context of GM-CSF. This is compelling evidence that tolerance is remarkably effective at protecting against development of allergy, even in the presence of a molecule that is as strongly associated with airway inflammation as GM-CSF. Tolerance literature instructs, however, that tolerance induction may generate regulatory T cells that control unresponsiveness by expressing regulatory cytokines (131). This suggests that blockade of a specific regulatory cytokine may be an effective strategy in breaking established tolerance. We therefore exposed tolerant animals to OVA in the context of GM-CSF and decorin. Our rationale for this approach was several-fold. First, decorin is a small leucine-rich proteoglycan of the extra-cellular matrix and a natural inhibitor of TGF-β (195). Not only is it important in suppressing fibrosis but it is also expressed at elevated levels in human asthmatics (196). Secondly, we have shown in knockout animals that other regulatory cytokines, IL-10 and IFN-γ, are redundant in our model, while the role of TGF-β as it pertains to inhalation tolerance is still poorly understood. Finally, for reasons discussed in Chapter 3, delivery of a replication-deficient adenovirus harboring the decorin transgene is an attractive alternative to mouse transgenics and protein-based interventions.

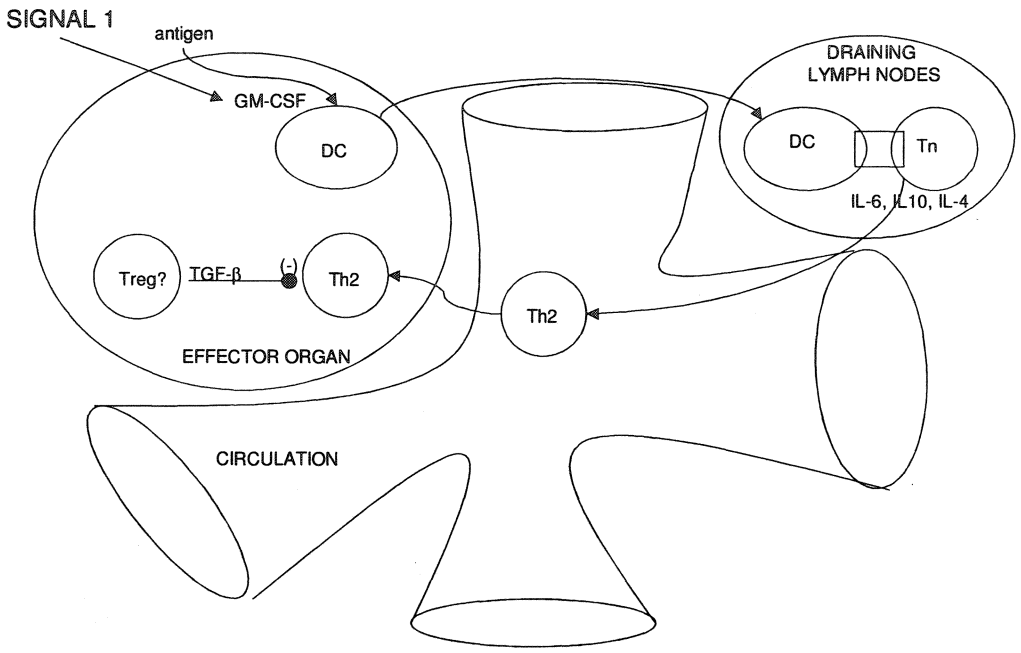

Figure 1A: GM-CSF alone does not break inhalation tolerance. In mice with established tolerance, expression of GM-CSF alone leads to the generation of Th2 cells but not to the breakdown of tolerance. TGF-β, presumably secreted by regulatory cells (Treg) prevents generation of allergic airway inflammation.

We show that expression of GM-CSF and decorin in the airway breaks local but not systemic inhalation tolerance to OVA: while eosinophilic airway inflammation is elevated in animals initially rendered tolerant to OVA and subsequently exposed to OVA in the context of GM-CSF and decorin, systemic responses, notably splenocyte proliferation and IL-5 expression, remain ablated. Importantly, expression of decorin alone does not break established tolerance. Our data may therefore suggest that, in addition to the antigen, two signals are required to break established tolerance: one that leads to *de novo* sensitization (GM-CSF) and one that disrupts regulatory activity (decorin) (Figures 1 & 2). Because of the timing of our experiment, it also suggests that regulation occurs at the effector phase. These data are clinically relevant to the extent that they mimic how allergies may arise. Indeed, that

a significant percentage of allergic diseases develop in the adult population argues that allergies appear because established tolerance to otherwise innocuous yet universally present agents is broken. It is yet to be determined whether viral or bacterial infections lead to dysregulated expression of decorin or decorin-related molecules.

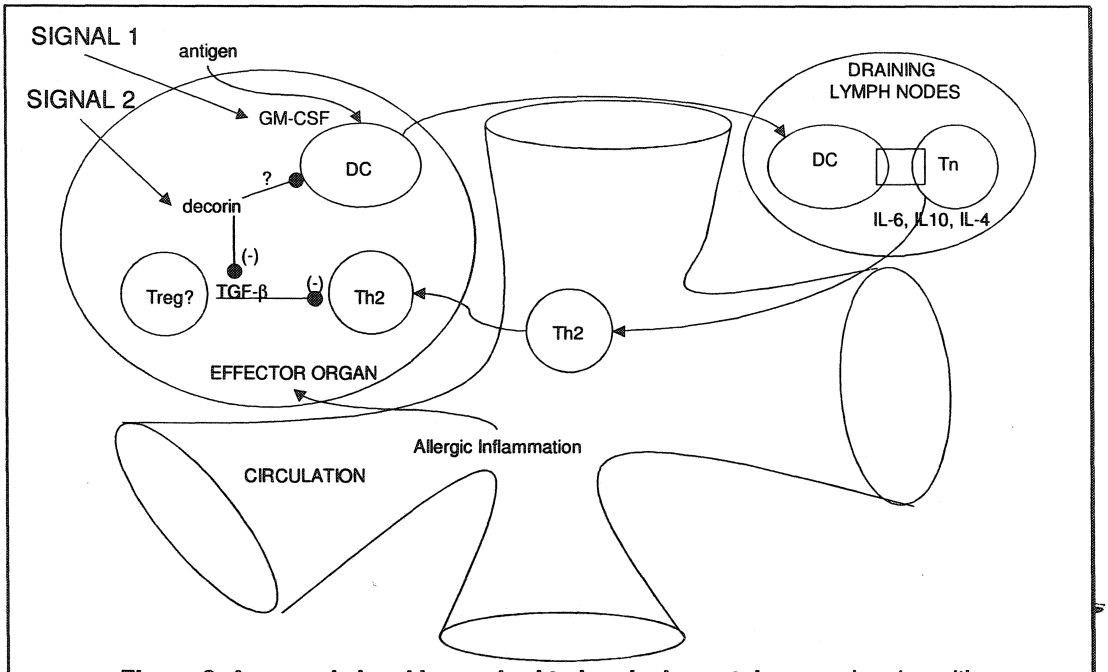

Figure 2: A second signal is required to break airway tolerance. In mice with established tolerance, two signals are required to break airway tolerance: (1) GM-CSF leads to generation of Th2 cells while (2) decorin sequesters TGF-β in the airway, thereby preventing negative regulation in the effector organ.

That at least two additional signals are required to break inhalation tolerance in the airway emphasizes the idea that the immune system is generally committed to respond to antigen according to the context in which the antigen is first encountered. To explore this further, we developed a model of chronic exposure in which GM-CSF was delivered to mice prior to four weeks of daily (Monday to Friday) OVA aerosolizations. Since GM-CSF is expressed in the airway for approximately two weeks, the remaining two weeks consisted of exposure to OVA in the absence of GM-CSF. In addition to exploring the effect of initial exposure on subsequent responses, the model was designed to more closely reflect allergic

asthma, a disease of *chronic* exposure to antigen. To our surprise, while IgE responses remained elevated and T cells expressed markers of activation, continued exposure to OVA did not lead to persistent inflammation but to a tolerant state in which airway and peripheral blood eosinophilia, and Th2 cytokine expression were significantly reduced (Chapter 4, (197)). The observed tolerance of sensitized and chronically exposed animals was broken (eosinophils returned to the airways) on subsequent exposure to OVA in the context of GM-CSF. These data indicate a tolerant phenotype with a distinct regulatory activity from that observed when animals are initially exposed to OVA only. Future studies will explore, for example, whether continued exposure to OVA results in clonal deletion or the generation of a regulatory T cell. Does exposure of sensitized animals preclude the generation of regulatory cells, thus rendering the animals susceptible to the development of allergic inflammation? Importantly, the data may reconcile the apparent discrepancy between persistent exposure to antigen and the intermittent nature of symptoms associated with asthma and other allied allergic diseases: exacerbations among sensitized individuals may depend on a second signal (GM-CSF in our experimental setting), associated either with certain allergens (protease activity) or provided by viral or bacterial infection.

Experimental models have shown that exposure to an innocuous agent such as OVA does not lead to airway inflammation in mice unless the antigen is co-administered with an adjuvant (55, 188). These studies have been instructive to the field of asthma and allergy because they suggest that context in which antigen is encountered is paramount to the ensuing immune response. For example, the failure to generate airway inflammation to OVA, when delivered in the absence of adjuvant, is consistent with epidemiological data in that it argues that innocuous agents are typically non-allergenic. To the extent that OVA is a surrogate allergen, it follows that allergens alone may not be sufficient at eliciting airway

inflammation. On the other hand, that allergic airway inflammation and airway hyper-responsiveness can be generated to OVA in the context of an adjuvant, suggests that a second signal is necessary if sensitization is to take place. Indeed, allergen exposure does not occur in isolation, but likely in the context of immunogenic agents such as viruses, bacteria, and environmental pollutants, agents that generate defined cytokine profiles in the airway microenvironment.

The work presented in this thesis argues that tolerance, a process that until the late 1990s was regarded are immunologically inert, follows parallel mechanisms to those associated with sensitization. It may be argued that the two signals required to break tolerance are akin to the two-signal model of sensitization. That only one signal, GM-CSF, is required to break airway tolerance in chronically exposed but sensitized animals, further re-conceptualizes tolerance as a state governed not by a single mechanism but a more diverse context-inclusive set of immune interactions. This broader view will allow researchers to design novel treatment strategies for allergic diseases and other pathologies.

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