

Concomitant airway expression of granulocyte-macrophage colony-stimulating factor and decorin, a natural inhibitor of transforming growth factor- β , breaks established inhalation tolerance

Filip K. Swirski¹, Beata U. Gajewska¹, Clinton S. Robbins¹, Abigail D'Sa¹, Jill R. Johnson¹, Mahmoud A. Pouladi¹, Mark D. Inman² and Martin R. Stämpfli¹

¹ Department of Pathology and Molecular Medicine, Division of Respiratory Diseases and Allergy and Centre for Gene Therapeutics, McMaster University, Hamilton, Canada

² Department of Medicine, McMaster University, Hamilton, Canada

We previously showed that granulocyte-macrophage colony-stimulating factor (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 10 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. 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 transforming growth factor (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 Th2 effector function were dramatically reduced. Concurrent expression of GM-CSF and decorin, 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 that dysregulated expression of an extracellular matrix protein may break established tolerance and lead to eosinophilic airway inflammation.

Key words: Allergy / Tolerance / Lung / TGF- β / GM-CSF

Received	17/2/04
Revised	12/5/04
Accepted	2/6/04

1 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].

Under experimental conditions, intranasal or aerosol exposure to OVA does not lead to inflammation but induces a state of antigen-specific unresponsiveness, termed inhalation tolerance. If these animals are subsequently subjected to protocols of antigen-induced airway inflammation, decreased airway eosinophilia, attenuated airway hyper-responsiveness (AHR) and reduced antigen-specific IgE are observed [5–8]. Studies by Akbari et al. [7] suggest that intranasally induced tolerance is mediated by IL-10-producing regulatory cells that develop via the inducible costimulator (ICOS)-ICOS ligand pathway. It is unclear whether tolerance induced with ten daily OVA aerosol exposures is bound by this mechanism, although other mechanisms are likely, given that neither IL-10 [6] nor IFN- γ [6, 8] are necessary. TGF- β , a molecule important in the regulation of oral tolerance [9, 10] and organ-specific autoimmune disease [10, 11], may be involved, but its role remains largely unexplored [12, 13].

[DOI 10.1002/eji.200425012]

Abbreviations: BAL: Bronchoalveolar lavage Ad: Adenovirus RDA: Replication-deficient adenovirus AHR: Airway hyper-responsiveness RRS: Respiratory system resistance MCh: Methacholine

We previously reported that GM-CSF expression in the airway microenvironment of mice breaks tolerance induction and allows for allergic mucosal sensitization to OVA [14]. 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. GM-CSF acts in an antigen-dependent manner since exposure of mice to GM-CSF alone does not lead to eosinophilic airway inflammation. Furthermore, the inflammatory process critically required the interaction between MHC class II and T cells; we observed no airway eosinophilia in mice that are deficient in MHC class II. 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 [15] and a natural inhibitor of TGF- β [16], 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.

2 Results

2.1 Cellular response in the bronchoalveolar lavage fluid and the peripheral blood of tolerant mice exposed to OVA in a GM-CSF-enriched airway environment

To address whether GM-CSF breaks established inhalation tolerance, BALB/c mice were exposed for 10 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 9 days in a GM-CSF-enriched airway environment. GM-CSF was expressed using an adenovirus (Ad)-mediated gene transfer approach. We previously reported that intranasal delivery of 3×10^7 PFU leads to expression of GM-CSF for 8–10 days, reaching peak levels of 80 pg/ml 7 days post-instillation [17]. Twenty-four hours after the last exposure animals were killed and BAL fluid was collected.

We observed a significant increase in total cell number as well as mononuclear cells and eosinophils in the BAL fluid and peripheral blood of animals initially exposed to saline only and subsequently to OVA with GM-CSF (Fig. 1). Prior

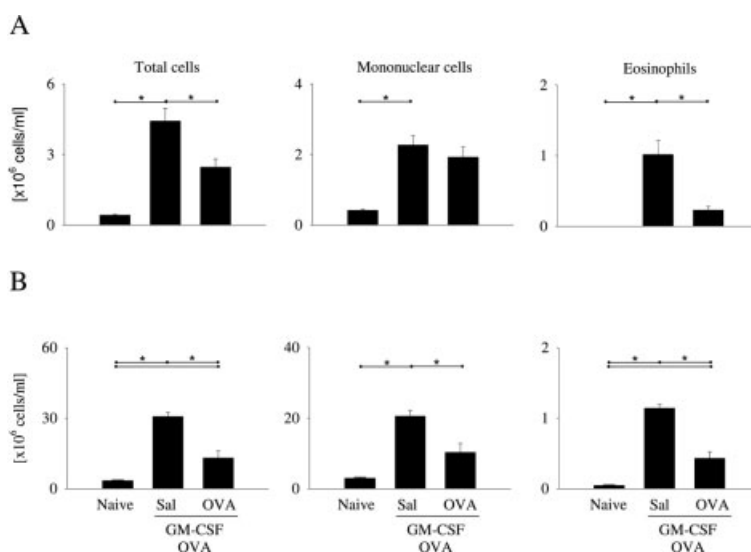


Fig. 1. BAL fluid and peripheral blood cellular response in tolerant mice exposed to OVA in the context of GM-CSF. BALB/c mice were exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA in the context of GM-CSF. Panel (A) depicts BAL fluid cellular data; panel (B) shows peripheral blood. Data represent means $\pm$ SEM; $n=8$ for BAL fluid, $n=4$ for peripheral blood; * $p<0.05$.

exposure to OVA alone resulted in striking reduction of total cells in the BAL fluid 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 fluid. These findings suggest that expression of GM-CSF did not break established airway tolerance.

2.2 Cytokine expression in the bronchoalveolar lavage fluid

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 fluid. BALB/c mice were exposed for 10 consecutive days to either OVA (tolerance induction) or saline aerosol. Both groups were then exposed to aerosolized OVA for a period of 9 days in a GM-CSF-enriched airway environment. Twenty-four hours after the last exposure mice were killed and BAL fluid 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 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 naive 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.

2.3 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 10 consecutive days to either OVA (tolerance induction) or saline aerosol. Both groups were then exposed to aerosolized OVA for a period of 9 days in a GM-CSF-enriched airway environment. Twenty-eight days after the last exposure, both groups were exposed for 3 consecutive days to OVA (*in vivo* recall challenge) and killed 2 days later. Their serum was collected and antibody titers were assessed. Our results show a divergent expression pattern of these Th2-associated Ig, 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 with GM-CSF; IgG1 expression was similar in both groups and significantly higher than in naive mice (Fig. 3). Negligible levels of both Ig were detected immediately following initial OVA exposure.

2.4 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 doses of methacholine (MCh) during *in vivo* recall challenge, as described above. Fig. 4 demon-

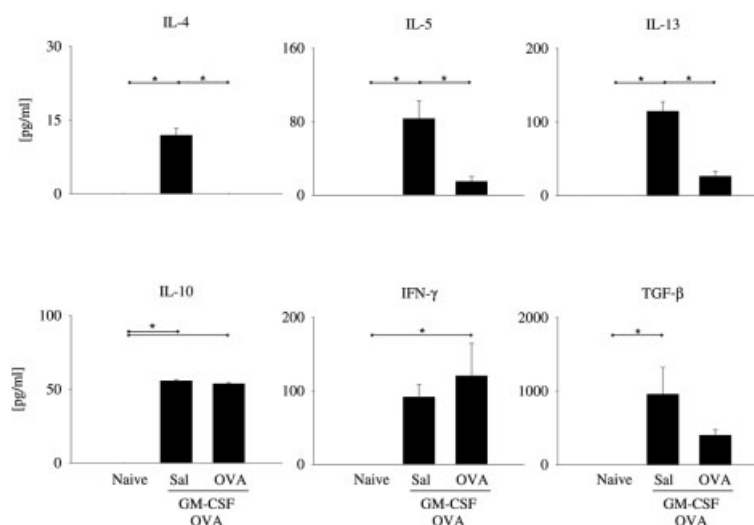


Fig. 2. Cytokine expression in the BAL fluid of tolerant mice exposed to OVA in context of GM-CSF. BALB/c mice were exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Cytokine expression was assessed in the BAL fluid. Data represent means $\pm$ SEM, $n=4-9$, * $p<0.05$.

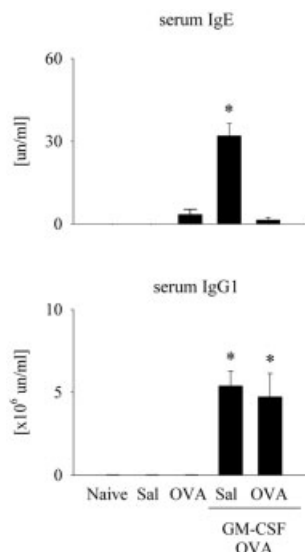


Fig. 3. OVA-specific IgE and IgG1 levels in the serum of tolerant mice exposed to OVA in context of GM-CSF. BALB/c mice were exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Twenty-eight days after the last exposure, mice were challenged with aerosolized OVA. OVA-specific IgE and IgG1 levels were measured in the serum following the initial 10 days of OVA exposure and after challenge. Data displayed represent means $\pm$ SEM; $n=5$ for naive, $n=8$ for all other groups; * $p<0.05$.

strates 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 naive controls. This responsiveness was significantly reduced in mice initially exposed to OVA. At no MCh dose measured we did observe differences in airway responsiveness between naive animals and animals initially exposed to OVA. BAL fluid cellular data obtained at this time point were 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).

2.5 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 for 10 days each, both times in the absence of GM-CSF, expressed low levels of the activation markers CD69 (9.8%) [18] and CD25 (6.5%) [19, 20], and negligible levels of the Th2 effector marker T1/ST2 (4.4%) [21–23] (Fig. 5). In contrast, animals initially exposed to saline and then

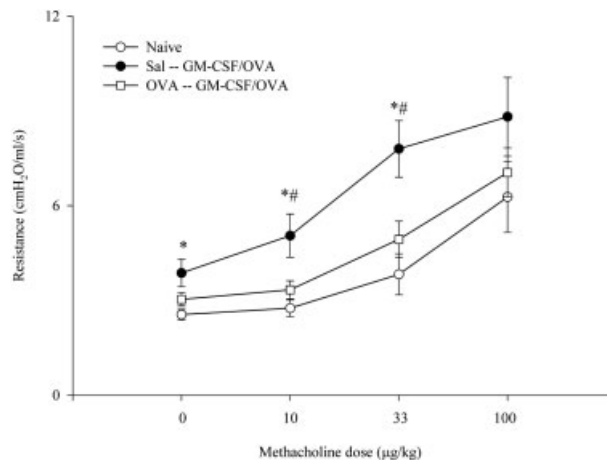


Fig. 4. Impact of GM-CSF expression on AHR in tolerant mice. Mice were exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Twenty-eight days after the last exposure, mice were challenged with aerosolized OVA and airway function was assessed. Data represent means $\pm$ SEM; $n=5$ for naive; $n=8$ for all other groups. Statistical analysis was performed using ANOVA with Newman-Keuls post-hoc test. *RRS was significantly greater compared to naive control mice ($p<0.05$), #RRS was significantly greater compared to sensitized and challenged mice initially exposed to OVA ($p<0.05$).

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 with 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.

2.6 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. Twenty-four hours 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 naive 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

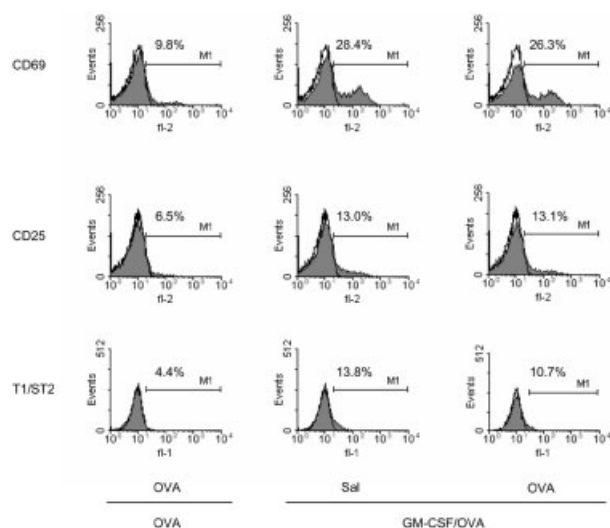


Fig. 5. T cell activation status in the lungs of tolerant mice exposed to OVA in context of GM-CSF. Mice were exposed to OVA (tolerance induction) or saline aerosol on 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Another group of animals was subjected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

cultured for 5 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 naive 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 naive animals cultured with OVA (1,615 $\pm$ 218 and 640 $\pm$ 229 pg/ml, respectively). Splenocytes from animals initially exposed to either OVA or saline and then cultured in medium alone did not express IL-4 or IL-5 but expressed $\sim$ 60 pg/ml IL-13, $\sim$ 25 pg/ml IL-10, $\sim$ 2 ng/ml IFN- γ and $\sim$ 100 pg/ml TGF- β .

2.7 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 to either adenovirally encoded GM-CSF and decorin, or GM-CSF and empty

control virus [replication-deficient Ad (RDA)], 1 day prior to nine daily and consecutive OVA aerosol exposures (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 [24]. 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 fluid. In accordance with previous findings, neither active nor total TGF- β was detected in decorin-treated animals ([24] and unpublished data).

3 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

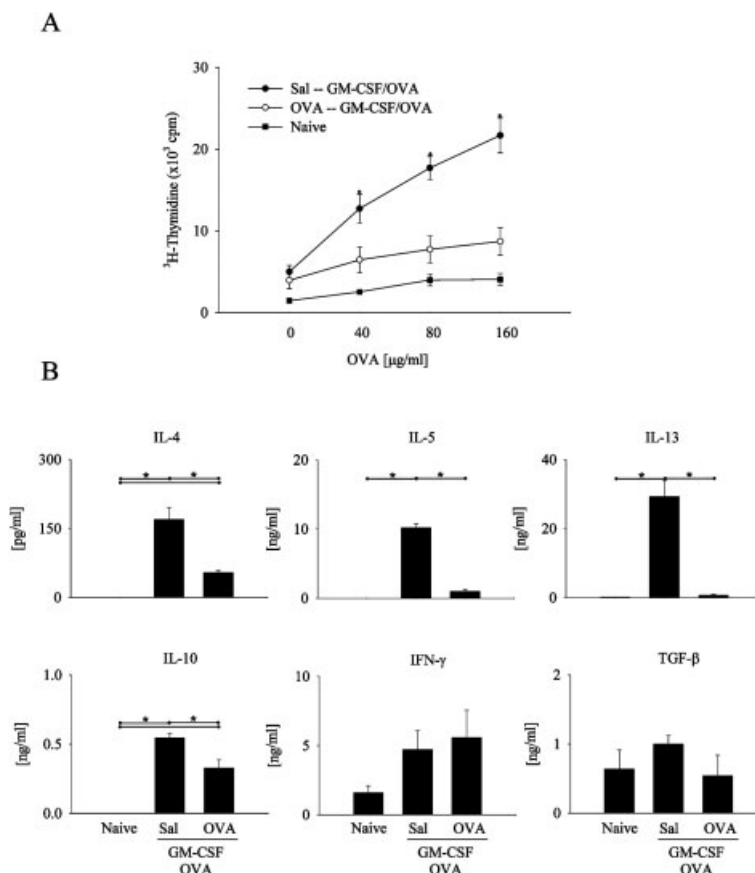


Fig. 6. Splenocyte proliferation and cytokine production. Mice were exposed to OVA or saline aerosol for 10 consecutive days. Both groups were then subjected to OVA aerosol in the context of GM-CSF. Twenty-four hours after the last exposure, splenocytes were isolated. (A) Data show [³H]thymidine incorporation to increasing doses of OVA after three days of culture. (B) Data show expression of cytokines measured by ELISA after 5 days of culture with 80 μg/ml OVA (means ± SEM, *n*=3–5).

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 [16].

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 [25]). 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 [6].

In this study, we asked whether GM-CSF breaks established tolerance. We exposed animals to OVA alone, and subsequently exposed animals to OVA in a GM-CSF-enriched airway environment. We show diminished airway and blood eosinophilia and negligible levels of Th2-associated cytokines. During *in vivo* long-term recall, we observed diminished OVA-specific IgE expression and reduced responsiveness to methacholine challenge. We argue, therefore, that the context of initial exposure is remarkably important, especially given that GM-CSF breaks tolerance induction [14]. 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 [14]. This may be indicative of the plasticity or 'leakiness' of the immune regulatory system.

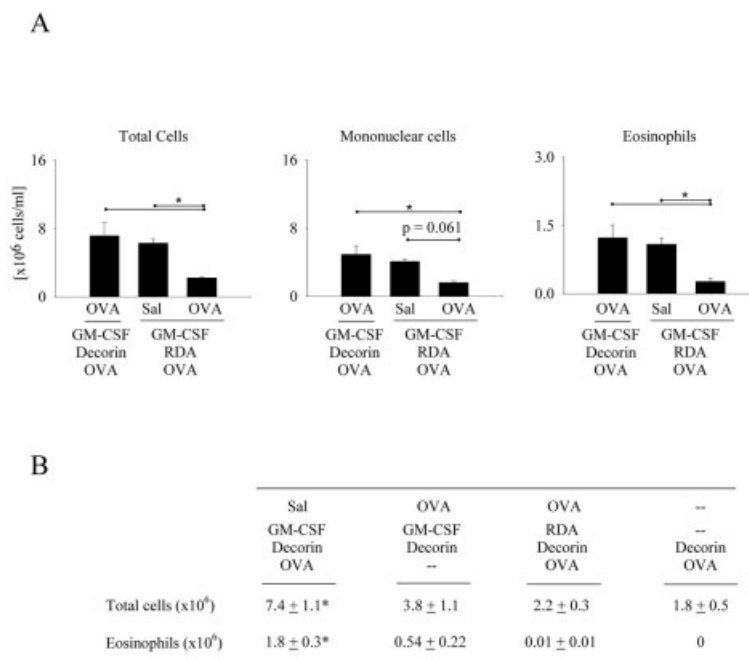


Fig. 7. BAL fluid cellular profile in tolerant mice exposed to OVA in the context of GM-CSF and decorin. Mice were exposed to OVA or saline aerosol for 10 consecutive days. (A) Mice exposed to OVA were then subjected to OVA aerosol in the context of GM-CSF and decorin or OVA in the context of GM-CSF 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 9 consecutive daily exposures to OVA. Data represent the cellular profile in the BAL fluid (means ± SEM, $n=6-8$, $*p<0.05$). (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 subjected either to GM-CSF and decorin in the absence of OVA or decorin and RDA in the presence of OVA. Additionally, naive mice were exposed to decorin and OVA. Animals were killed 24 h after the last of 9 consecutive daily exposures to OVA. Data represent the cellular profile in the BAL fluid (means ± SEM, $n=3$, $*p<0.05$ as compared to all other groups).

Our data also show that CD4⁺ T cells are activated in the lungs, as assessed by the early activation marker CD69 [18], the IL-2 receptor CD25 [19, 20] and T1/ST2, a marker of Th2 effector cells [21–23]. The high titers of Th2-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. Alternatively, the differential effect on IgE and IgG1 may be a consequence of different half-lives. Specifically, reduced IgE levels may result from the short half-life of this isotype, whereas IgG1 remains enhanced due to a half-life of about 21–25 days. This scenario implies that the initial 10 days of OVA exposure induced IgG1 and IgE levels, while the following treatment may have shut off OVA-specific antibody production. This, however, is not supported by our data, since we did not observe elevated levels of OVA-specific IgG1 and IgE following initial OVA exposure.

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 synon-

ymous with inflammation, a discrepancy on which we have previously reported [6]. Alternatively, the observation that CD4⁺ T cells of tolerant mice express markers of Th2 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 intranasal exposure to OVA that IL-10 plays a vital role in suppressing airway inflammation [26]. However, previous studies from our laboratory in knockout mice with inhaled OVA argued that IL-10 was redundant [6]. 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 [6, 8, 27]. 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-

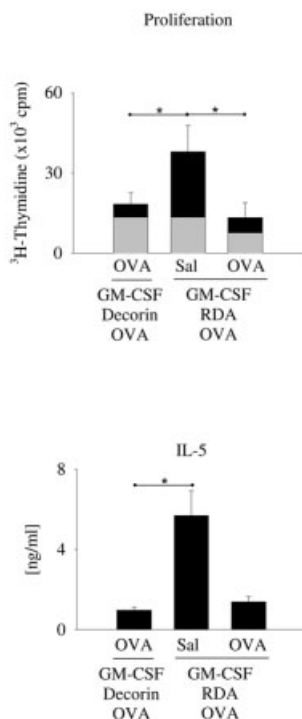


Fig. 8. *In vitro* proliferation and IL-5 production in tolerant mice exposed to OVA in the context of GM-CSF and decorin. Mice were exposed to OVA or saline aerosol for 10 consecutive days. Mice exposed to OVA were then subjected to OVA aerosol in the context of GM-CSF and decorin or OVA in the context 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 splenocytes cultured in medium (gray bars) or OVA (black bars) (means $\pm$ 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.

dependent suppression [28, 29]. Although TGF- β -dependent regulation has been described in oral tolerance and organ-specific autoimmune disease [9–11], its role in inhalation tolerance is still largely unknown [12]. 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 twofold. First, it is well established that decorin binds to and inactivates the activity of TGF- β in murine and human airways [30–32]. Moreover, administration of an RDA carrying the decorin transgene introduces an attractive alternative to mouse transgenics and protein-based interventions: TGF- β -null mice die early of multiple causes [33], Smad knockouts may not exhaust complete TGF- β -mediated activity [34, 35], 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 [24].

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 [24, 32], 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 [36], 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 fluid and culture supernatants in both tolerant and non-tolerant animals may be indicative of immune plasticity; TGF- β is known to be vastly pleiotropic [37]. 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-1 β , IL-4 and TNF- α [38], while human glioma cells engineered to express the proteoglycan have been shown to stimulate alloreactive immune responses independently of TGF- β [39]. That increased expression of decorin has been observed in human asthma further solidifies a role of this molecule in allergic diseases [15]. 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. GM-CSF may not be unique in its ability to break established tolerance in the presence of reduced immune suppression. It remains to be elucidated whether other immune-stimulatory molecules would suffice, such as LPS or heat-labile toxins. 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. As decorin typically is 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 the 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 [40]. 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.

4 Materials and methods

4.1 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.

4.2 Tolerance induction

Mice were 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) [6]. Control mice were exposed to saline only.

4.3 Delivery of adenoviral constructs

Adenoviral constructs carrying the transgenes for mouse GM-CSF (Ad/GM) [41] or human decorin (Ad/Dec) [32] were delivered intranasally in a total volume of 30:1 of PBS vehicle (two 15:1 administrations 5 min apart) into animals anaesthetized with isoflurane (Baxter Corporation, Toronto, Canada). As control, we utilized an RDA carrying no transgene [42].

4.4 Allergic mucosal sensitization

Mice were exposed to OVA in a GM-CSF-enriched airway microenvironment using an Ad-mediated gene transfer approach (3×10^7 PFU Ad/GM). In some experiments, a concurrent dose of 3×10^7 PFU Ad/Dec or 3×10^7 PFU RDA was delivered. Mice were exposed to OVA aerosol (1% wt/vol in 0.9% saline) for 20 min daily for 9 days. In experiments involving AHR and Ig measurements, mice were rested for a period of 28 days and re-challenged with OVA aerosol daily for 20 min over a period of 3 days.

4.5 Collection and measurement from specimens

For BAL, dissected lungs were cannulated and lavaged twice with PBS (0.25 ml followed by 0.2 ml) [43]. 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. HEMA 3 Stain Set (Biochemical Sciences Inc., Swedesboro, NJ) was used to stain smears. Differential counts were determined from at least 500 leukocytes using standard hemocytological procedures. 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 37°C and spun for 15 min at 4°C.

4.6 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 <2 pg/ml; for IL-10, <4 pg/ml; for TGF- β , <7 pg/ml; for IL-5, <5 pg/ml. To obtain total TGF- β levels, samples were incubated with HCl prior to ELISA, as directed by the manufacturer. Serum levels of OVA-specific IgG1 and IgE were assessed by ELISA [6, 43].

4.7 Airway hyper-responsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (RRS) to increasing internal jugular vein doses of MCh, as described [44]. Evaluation of airway responsiveness was based on the peak RRS measured in the 30 s following the saline and MCh challenges.

4.8 Lung cell and splenocyte isolation

Splenocytes were isolated using a protocol described previously [6]. For isolation of lung cells, lungs were flushed via the right ventricle with 10 ml of warm (37°C) HBSS. The lungs were then cut into small pieces and shaken at 37°C for 1 h in 15 ml of 150 U/ml collagenase I (Worthington Biochemical, Freehold, NJ) in Hanks' balanced salt solution (HBSS). Using a plunger, the lung pieces were triturated and filtered through a nylon mesh. Cells were washed twice and mononuclear cells were isolated by density centrifugation over a 30% and 60% Percoll gradient (Pharmacia, Uppsala, Sweden).

4.9 Splenocyte cell culture

Splenocytes were cultured in medium alone or with 80 µg OVA/ml at 8×10^5 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 8×10^5 cells/well in a 96-well round-bottom plate (Becton Dickinson). After 2 days of culture, 1 µCi of [³H]thymidine (Perkin Elmer Life Sciences, Boston, MA) in 20 µl of RPMI was added to each well. Twenty-four hours later, [³H]thymidine incorporation was assessed with TopCount NXT microplate scintillation and luminescence counter (Packard BioScience Company, Meridan, CT).

4.10 Flow cytometry

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). To minimize nonspecific binding, 10^6 cells were incubated with 0.5 µg Fc Block

(CD16/CD32; PharMingen, Mississauga, Canada) at 4°C for 15 min and subsequently with first-stage monoclonal antibodies at 4°C for 30 min. Cells were 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).

4.11 Data analysis

Data are expressed as mean $\pm$ SEM. Statistical interpretation of results was performed using one-way analysis of variance (ANOVA) with the Fisher least significant difference (LSD) post-hoc test unless stated otherwise in the figure legends. Differences were considered statistically significant when $p < 0.05$.

Acknowledgements: This study was supported in part by the Canadian Institutes of Health Research, the Hamilton Health Sciences Corporation, and St. Joseph's Hospital. F.K.S. is holder of a K. M. Hunter/Canadian Institutes for Health Research (CIHR) Doctoral Research Award. C.S.R. and J.R.J. are holders of an Ontario Graduate Scholarship (OGS).

References

- 1 Hoynes, G. F., Tan, K., Corsin-Jimenez, M., Wahl, K., Stewart, M., Howie, S. E. and Lamb, J. R., Immunological tolerance to inhaled antigen. *Am. J. Respir. Crit. Care Med.* 2000. **162**: S169–S174.
- 2 Strober, W., Kelsall, B. and Marth, T., Oral tolerance. *J. Clin. Immunol.* 1998.; **18**: 1–30.
- 3 Manfreda, J., Becklake, M. R., Sears, M. R., Chan-Yeung, M., Dimich-Ward, H., Siersted, H. C., Ernst, P., Sweet, L., Van Til, L., Bowie, D. M., Anthonisen, N. R. and Tate, R. B., Prevalence of asthma symptoms among adults aged 20–44 years in Canada. *CMAJ* 2001. **164**: 995–1001.
- 4 Farber, H. J., Wattigney, W. and Berenson, G., Trends in asthma prevalence: the Bogalusa Heart Study. *Ann. Allergy Asthma Immunol.* 1997. **78**: 265–269.
- 5 Tsitoura, D. C., Blumenthal, R. L., Berry, G., Dekruyff, R. H. and Umetsu, D. T., Mechanisms preventing allergen-induced airways hyperreactivity: role of tolerance and immune deviation. *J. Allergy Clin. Immunol.* 2000. **106**: 239–246.
- 6 Swirski, F. K., Gajewska, B., Alvarez, D., Ritz, S. A., Cundall, M. J., Cates, E. C., Coyle, A. J., Gutierrez-Ramos, J. C., Inman, M. D., Jordana, M. and Stampfli, M. R., Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin. Exp. Allergy* 2002. **32**: 411–421.
- 7 Akbari, O., Freeman, G. J., Meyer, E. H., Greenfield, E. A., Chang, T. T., Sharpe, A. H., Berry, G., DeKruyff, R. H. and Umetsu, D. T., Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat. Med.* 2002. **8**: 1024–1032.
- 8 Seymour, B. W., Gershwin, L. and Coffman, R. L., Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require CD8⁺ or T cell receptor (TCR)-gamma/delta⁺

- T cells or interferon (IFN)-gamma in a murine model of allergen sensitization. *J. Exp. Med.* 1998. **187**: 721–731.
- 9 Perez-Machado, M. A., Ashwood, P., Thomson, M. A., Latcham, F., Sim, R., Walker-Smith, J. A. and Murch, S. H., Reduced transforming growth factor-beta1-producing T cells in the duodenal mucosa of children with food allergy. *Eur. J. Immunol.* 2003. **33**: 2307–2315.
 - 10 Strobel, S., Oral tolerance, systemic immunoregulation, and autoimmunity. *Ann. N. Y. Acad. Sci.* 2002. **958**: 47–58.
 - 11 Belghith, M., Bluestone, J. A., Barriot, S., Megret, J., Bach, J. F. and Chatenoud, L., TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* 2003. **9**: 1202–1208.
 - 12 Haneda, K., Sano, K., Tamura, G., Shiota, H., Ohkawara, Y., Sato, T., Habu, S. and Shirato, K., Transforming growth factor-beta secreted from CD4(+) T cells ameliorates antigen-induced eosinophilic inflammation. A novel high-dose tolerance in the trachea. *Am. J. Respir. Cell Mol. Biol.* 1999. **21**: 268–274.
 - 13 Hansen, G., McIntire, J. J., Yeung, V. P., Berry, G., Thorbecke, G. J., Chen, L., DeKruyff, R. H. and Umetsu, D. T., CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J. Clin. Invest.* 2000. **105**: 61–70.
 - 14 Stampfli, M. R., Wiley, R. E., Neigh, G. S., Gajewska, B. U., Lei, X. F., Snider, D. P., Xing, Z. and Jordana, M., GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest.* 1998. **102**: 1704–1714.
 - 15 Westergren-Thorsson, G., Chakir, J., Lafreniere-Allard, M. J., Boulet, L. P. and Tremblay, G. M., Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. *Int. J. Biochem. Cell. Biol.* 2002. **34**: 1256–1267.
 - 16 Border, W. A., Noble, N. A., Yamamoto, T., Harper, J. R., Yamaguchi, Y., Pierschbacher, M. D. and Ruoslahti, E., Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 1992. **360**: 361–364.
 - 17 Lei, X. F., Ohkawara, Y., Stampfli, M. R., Gaudie, J., Croitoru, K., Jordana, M. and Xing, Z., Compartmentalized transgene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse lung enhances allergic airways inflammation. *Clin. Exp. Immunol.* 1998. **113**: 157–165.
 - 18 Marzio, R., Mauel, J. and Betz-Corradin, S., CD69 and regulation of the immune function. *Immunopharmacol. Immunotoxicol.* 1999. **21**: 565–582.
 - 19 Haczk, A., Macary, P., Huang, T. J., Tsukagoshi, H., Barnes, P. J., Kay, A. B., Kemeny, D. M., Chung, K. F. and Moqbel, R., Adoptive transfer of allergen-specific CD4⁺ T cells induces airway inflammation and hyperresponsiveness in brown-Norway rats. *Immunology* 1997. **91**: 176–185.
 - 20 Kaminuma, O., Fujimura, H., Fushimi, K., Nakata, A., Sakai, A., Chishima, S., Ogawa, K., Kikuchi, M., Kikkawa, H., Akiyama, K. and Mori, A., Dynamics of antigen-specific helper T cells at the initiation of airway eosinophilic inflammation. *Eur. J. Immunol.* 2001. **31**: 2669–2679.
 - 21 Coyle, A. J., Lloyd, C., Tian, J., Nguyen, T., Eriksson, C., Wang, L., Ottoson, P., Persson, P., Delaney, T., Lehar, S., Lin, S., Poisson, L., Meisel, C., Kamradt, T., Bjerke, T., Levinson, D. and Gutierrez-Ramos, J. C., Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med.* 1999. **190**: 895–902.
 - 22 Townsend, M. J., Fallon, P., Matthews, D. J., Jolin, H. E. and McKenzie, A. N., T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J. Exp. Med.* 2000. **191**: 1069–1076.
 - 23 Lambrecht, B. N., De Veerman, M., Coyle, A. J., Gutierrez-Ramos, J. C., Thielemans, K. and Pauwels, R. A., Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest.* 2000. **106**: 551–559.
 - 24 Kolb, M., Margetts, P., Sime, P. J. and Gaudie, J., Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2001. **280**: L1327–L1334.
 - 25 Ritz, S. A., Stampfli, M., Davies, D. E., Holgate, S. T. and Jordana, M., On the generation of allergic airway diseases: from GM-CSF to Kyoto. *Trends Immunol.* 2002. **23**: 396–402.
 - 26 Akbari, O., DeKruyff, R. H. and Umetsu, D. T., Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001. **2**: 725–731.
 - 27 McMenamin, C., Pimm, C., McKersey, M. and Holt, P. G., Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* 1994. **265**: 1869–1871.
 - 28 Weiner, H., Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol. Rev.* 2001. **182**: 207–214.
 - 29 Levings, M. K., Bacchetta, R., Schulz, U. and Roncarolo, M. G., The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int. Arch. Allergy Immunol.* 2002. **129**: 263–276.
 - 30 Schonherr, E., Broszat, M., Brandan, E., Bruckner, P. and Kresse, H., Decorin core protein fragment Leu155-Val260 interacts with TGF-beta but does not compete for decorin binding to type I collagen. *Arch. Biochem. Biophys.* 1998. **355**: 241–248.
 - 31 Redington, A. E., Roche, W., Holgate, S. T. and Howarth, P. H., Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects. *J. Pathol.* 1998. **186**: 410–415.
 - 32 Kolb, M., Margetts, P., Galt, T., Sime, P. J., Xing, Z., Schmidt, M. and Gaudie, J., Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am. J. Respir. Crit. Care Med.* 2001. **163**: 770–777.
 - 33 Kulkarni, A. B., Huh, C., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M. and Karlsson, S., Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 770–774.
 - 34 Dumont, N., Bakin, A. and Arteaga, C. L., Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *J. Biol. Chem.* 2003. **278**: 3275–3285.
 - 35 Shi, Y. and Massague, J., Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003. **113**: 685–700.
 - 36 Jutel, M., Akdis, M., Budak, F., Aebischer-Casaulta, C., Wrzyszc, M., Blaser, K. and Akdis, C. A., IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur. J. Immunol.* 2003. **33**: 1205–1214.
 - 37 Letterio, J. J. and Roberts, A. B., Regulation of immune responses by TGF-beta. *Annu. Rev. Immunol.* 1998. **16**: 137–161.
 - 38 Al Haj Zen, A., Lafont, A., Durand, E., Brasselet, C., Lemarchand, P., Godeau, G. and Gogly, B., Effect of adenovirus-mediated overexpression of decorin on metalloproteinases, tissue inhibitors of metalloproteinases and cytokines secretion by human gingival fibroblasts. *Matrix Biol.* 2003. **22**: 251–258.

- 39 **Munz, C., Naumann, U., Grimm, C., Rammensee, H. G. and Weller, M.**, TGF-beta-independent induction of immunogenicity by decorin gene transfer in human malignant glioma cells. *Eur. J. Immunol.* 1999. **29**: 1032–1040.
- 40 **Swirski, F. K., Sajic, D., Robbins, C. S., Gajewska, B. U., Jordana, M. and Stampfli, M. R.**, Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J. Immunol.* 2002. **169**: 3499–3506.
- 41 **Xing, Z., Ohkawara, Y., Jordana, M., Graham, F. and Gauldie, J.**, Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest.* 1996. **97**: 1102–1110.
- 42 **Bett, A. J., Haddara, W., Prevec, L. and Graham, F. L.**, An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* 1994. **91**: 8802–8806.
- 43 **Ohkawara, Y., Lei, X., Stampfli, M. R., Marshall, J. S., Xing, Z. and Jordana, M.**, Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *Am. J. Respir. Cell Mol. Biol.* 1997. **16**: 510–520.
- 44 **Stampfli, M. R., Cwiartka, M., Gajewska, B. U., Alvarez, D., Ritz, S. A., Inman, M. D., Xing, Z. and Jordana, M.**, Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am. J. Respir. Cell Mol. Biol.* 1999. **21**: 586–596.

Correspondence: Martin R. Stampfli, Department of Pathology and Molecular Medicine, McMaster University, Health Sciences Centre, Room 4H21A, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5
Fax: +1-905-522-6750
e-mail: stampfli@mcmaster.ca