

# Prolonged Ovalbumin Exposure Attenuates Airway Hyperresponsiveness and T Cell Function in Mice

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## Key Words

Airway hyperresponsiveness • Th2 cells • Mouse • Inflammation • Chronic allergen challenge

## Abstract

**Background:** Continuous exposure of sensitized mice to an innocuous antigen, such as OVA, does not lead to chronic airway eosinophilia, but induces antigen unresponsiveness and resolution of the inflammatory response. In this study we explored mechanisms underlying attenuation of the airway inflammatory response, assessed whether the phenomenon is strain-specific, and determined its consequences to airway physiology. **Methods:** Mice were sensitized and exposed to OVA for two and four weeks. Analysis involved BAL, flow cytometry, adoptive transfer of OVA specific CD4 T cells, ex vivo cytokine expression and response to methacholine challenge. **Results:** Chronic exposure to antigen resulted in decreased eosinophilia in 5 different mouse strains. Likewise, numbers of lung CD4 T cells expressing activation and Th2 markers sharply declined following continuous OVA exposure. Transfer studies using OVA TcR transgenic cells revealed that the contraction of lung T cells included antigen-specific cells. Systemically, we observed a loss of Th2 memory effector function. Finally, we observed significantly attenuated airway hyper-responsiveness (AHR) in chronically exposed animals. **Conclusions:** Attenuation of airway eo-

sinophilia in response to chronic OVA exposure is independent of genetic background. Airway eosinophilia, but not systemic responses, correlates with and is predictive for airway hyperresponsiveness. Our study contributes to the understanding of immune regulatory processes controlling antigen-driven airway inflammatory responses.

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## Introduction

Asthma is a heterogeneous disease characterized by eosinophilic airway inflammation, intermittent reversible airway obstruction and airway hyperresponsiveness [1, 2]. Although each is recognized as an integral component of the asthmatic phenotype, airway inflammation is the central abnormality that leads to airway damage and dysfunction. Allergens are recognized as important inducers of acute exacerbation of asthma and it is argued that they contribute significantly to the initiation and persistence of airway inflammation [3]. The current understanding is that allergen exposure mediates expression of a range of inflammatory mediators, including Th2 cytokines such as IL-4, IL-5 and IL-13, that consequently leads to peribronchial and perivascular eosinophilic airway inflammation, IgE secretion and bronchial hyperresponsiveness.

Allergens, however, are relatively harmless agents; in contrast to viruses, bacteria and parasites, allergens do *not* replicate. Hence, mounting potentially damaging immune-inflammatory responses to eliminate allergens is unnecessary and may even jeopardize lung function, as observed in asthmatic individuals [1, 2]. In spite of universal exposure to allergens, only 15% of the population develop allergic diseases [4, 5], suggesting that the immune system has evolved effective regulatory mechanisms to control responsiveness to these harmless agents [6]. Under experimental conditions, exposure of mice to innocuous antigen does not lead to airway inflammation, but induces a state of antigen-specific unresponsiveness, termed inhalation tolerance [7–10]. Previously regarded as immunologically dormant, we currently understand this type of tolerance to be governed by complex cellular and molecular pathways, likely involving CD4+ [11] and CD8+ T cells [12], expression of regulatory cytokines such as IL-10 [13], IFN $\gamma$  [14, 15], TGF $\beta$  [16] and co-stimulatory molecules such as B7RP1 [17, 18].

We and others recently reported the presence of antigen-dependent immune-regulatory processes that regulate inflammation even in sensitized mice [19–21]. Specifically, continuous exposure of sensitized Balb/c mice to OVA, a surrogate allergen, does not lead to chronic airway inflammation, but attenuation of airway eosinophilia. The objective of the current study was to investigate whether this is a strain specific phenomenon, to study the mechanisms underlying attenuation of the airway inflammatory responses and to assess its consequences to airway physiology. We show that chronic exposure to OVA attenuated airway eosinophilia in 5 different mouse strains, led to loss of antigen-specific T cells in the lungs, decreased Th2 memory effector function, and attenuated airway hyperresponsiveness. Our findings show that inhalation of antigen alone does not sustain inflammation and is associated with a loss of antigen-specific memory function.

## Material and Methods

### Animals

Female Balb/c mice (6–8 weeks old) were obtained from Charles River Laboratories (Ottawa, Ont., Canada). Female DBA/1, A/J, C57BL/6J and 129/SVJ (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, Me., USA). D011.10 T cell receptor transgenic mice (provided by Dr. Ken Croitoru, McMaster University, Hamilton, Ont., Canada) were bred in-house. Mice were housed in an access-restricted area (cages, food and bedding are autoclaved), 12-h light-dark cycle and food and water ad libitum. The Animal Research Ethics Board of

McMaster University approved all experiments described in this study.

### Allergic Mucosal Sensitization

To induce allergic mucosal sensitization, mice were exposed to OVA in the context of a GM-CSF enriched airway micro-environment [22]. GM-CSF was expressed using a replication-deficient E1/E3-deleted human type 5 adenoviral construct carrying the transgene for murine GM-CSF (Ad/GM) [23]. The viral construct ( $3 \times 10^7$  PFU) was delivered intranasally in a total volume of 30  $\mu$ l of phosphate buffered saline (PBS) (two 15- $\mu$ l administrations 5 min apart) into animals anaesthetized with isoflurane (Baxter Corporation, Toronto, Ont., Canada). Three days following delivery of the viral construct, animals were exposed to aerosolized OVA (1% wt/vol in 0.9% saline) (Grade V, >98% pure by agarose gel electrophoresis, Sigma Chemicals, St. Louis, Mo., USA) for five days followed by two days of rest. This exposure protocol was performed for two, three or four weeks. We previously reported that robust eosinophilic airway inflammation is observed following 2 weeks of OVA exposure, while after 4 weeks eosinophilia is ablated [19]. Mice were exposed in a Plexiglas chamber (10  $\times$  15  $\times$  25 cm) for 20 min daily. The aerosol was produced by a Bennet/Twin nebulizer (Puritan-Bennett Corporation, Carlsbad, Calif., USA) at a flow rate of 10 l/min.

### Collection of Specimens

Bronchoalveolar lavage (BAL) was performed using a protocol described in detail previously [24]. Lungs were lavaged with two aliquots of PBS (250  $\mu$ l, followed by 200  $\mu$ l). Total cell counts were determined using a hemocytometer. Cytospins were stained using HEMA 3 Stain Set (Biochemical Sciences Inc., Swedesboro, N.J., USA). BAL differential cell counts were determined from at least 500 leukocytes using standard hemocytological procedures to classify the cells as mononuclear cells, neutrophils or eosinophils.

Prior to isolation of lung mononuclear cells, lungs were perfused through the right ventricle with 10 ml of warm Hank's buffered salt solution (HBSS) and stored on ice. Lung mononuclear cells were isolated according to a standard protocol [19] and resuspended in a final concentration of 1 million cells per 50  $\mu$ l of PBS/0.2% BSA (Sigma Chemicals Co.) for flow cytometric analysis.

Spleens were excised and stored on ice in 15 ml HBSS. Splenocytes were isolated [16] and red blood cells were lysed using ACK lysis buffer (0.5 M NH $_4$ Cl, 10 mM KHCO $_3$ , and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.2 to 7.4) for 1 min. Cells were washed and re-suspended in complete RPMI (Gibco, BRL, Gaithersburg, Md., USA) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Ont., Canada), 1% L-glutamine (Sigma Chemicals Co.), 1% penicillin/streptomycin (Gibco BRL), and 0.5% 2-mercaptoethanol (Sigma Chemicals Co.). Cells were cultured at  $8 \times 10^5$  cells/well in a flat-bottom, 96-well plate (Becton Dickinson, Franklin Lakes, N.J., USA) in medium or 40  $\mu$ g OVA/well. After 5 days of culture, plates were stored at  $-20^\circ\text{C}$  for cytokine analysis.

### Flow Cytometric Analysis

$10^6$  cells were incubated with 0.5  $\mu$ g of Fc Block (CD16/CD32) (Pharmingen, Mississauga, Ont., Canada) at  $0-4^\circ\text{C}$  for 15 min. Cells were then incubated with first stage antibodies at  $0-4^\circ\text{C}$  for

30 min. Each sample was washed with 500  $\mu$ l of PBS/0.2% BSA (Sigma Chemicals Co). Cells were incubated with second stage antibodies at 0–4°C for 30 min. Samples were washed and resuspended in 1% paraformaldehyde. Data were collected using a FACScan and analyzed using Win MDI 2.8 software (Becton Dickinson, Sunnyvale, Calif., USA). The following mAbs from Pharmingen were utilized: anti-CD3, PE- and cy-Chrome-conjugated 145-2C11; anti-CD4, FITC-conjugated L3T4 and cy-Chrome-conjugated RM4-5; anti-CD69, PE-conjugated H1 2F3; anti-CD25, PE-conjugated PC61; anti-DO11.10 TcR, PE-conjugated KJ126 and anti-T1/ST2, FITC-conjugated 3E10 (provided by A.J. Coyle, Millennium Pharmaceuticals, Inc., Cambridge, Mass., USA) labeled in-house according to a standard protocol. PerCP, and streptavidin PerCP were purchased from Becton Dickinson (San Jose, Calif., USA). All experiments were controlled with appropriate isotypes (Pharmingen). Titration was performed to determine the optimal concentration of each antibody.

#### *Respiratory System Resistance*

Respiratory system resistance was assessed according to a protocol described in detail previously [25]. Mice were anesthetized with tribromoethanol (287 mg/kg, i.p.) and paralyzed by administering pancuronium (0.03 mg/kg i.v.). Tracheas were cannulated. A constant inspiratory flow of air was delivered using a mechanical ventilator (RV5, Voltek Enterprises Inc., Toronto, Ont., Canada). Total respiratory system resistance (RRS) was measured in response to consecutive intravenous injections of 10, 33, and 100  $\mu$ g/kg of MCh (ACIC [Can], Brantford, Ont., Canada), each delivered as a 200  $\mu$ l bolus. Evaluation of airway responsiveness was based on the peak RRS measured in the 30 s following saline and MCh challenges.

#### *Cytokine Measurements*

IL-4, IL-5, IL-13, IL-10, and IFN $\gamma$  were measured using enzyme-linked immunosorbent assays (ELISA) from R & D Systems (Minneapolis, Minn., USA). The sensitivity threshold for detection was 2 pg/ml for IL-4, 7 pg/ml for IL-5, 1.5 pg/ml for IL-13, 4 pg/ml for IL-10 and 2 pg/ml for IFN $\gamma$ .

#### *Data Analysis*

Statistical analysis was performed using Sigma Stat version 2.03. Statistical methods and interpretations are indicated in the figure legends. Differences were considered statistically significant when  $p < 0.05$ .

## **Results**

### *BAL Cellular Profile following Prolonged OVA Exposure.*

Balb/c mice were inoculated intranasally with  $3 \times 10^7$  PFU Ad/GM-CSF. We previously reported that this protocol results in GM-CSF expression in the airways for approximately 10 days, with peak expression of  $\sim 80$ –100 pg/ml in the BAL at day 7 [22]. Three days later, animals were exposed to aerosolized OVA or saline, for 2, 3

and 4 weeks. A separate group of animals was not inoculated with Ad/GM-CSF and was exposed to OVA. Figure 1a shows that exposure to OVA in the context of a GM-CSF-enriched airway environment led to significant airway eosinophilia after 2 weeks of OVA exposure ( $85 \pm 9 \times 10^4$  cells/ml). Eosinophilia was diminished following 3 weeks ( $6.5 \pm 2.3 \times 10^4$  cells/ml) and had largely resolved following 4 weeks of OVA exposure ( $0.4 \pm 0.1 \times 10^4$  cells/ml) despite continued antigenic challenge. No airway eosinophilia was observed in animals exposed to OVA, demonstrating that OVA was not contaminated by adjuvants such as endotoxin. Likewise, no eosinophilia was observed when mice were exposed to GM-CSF alone, demonstrating that antigen was necessary.

To address whether the ability to resolve airway eosinophilia, despite continuous antigen exposure, is strain-dependent, DBA/1, A/J, C57BL/6J and 129/SVJ mice were inoculated intranasally with  $3 \times 10^7$  PFU Ad/GM-CSF and exposed to aerosolized OVA for 2 or 4 weeks. An additional group of animals was exposed to OVA for two weeks followed by two weeks of exposure to aerosolized saline (2/4 weeks). Figure 1b shows that after 2 weeks of OVA exposure, all mouse strains developed an eosinophilic response in the BAL. Despite continued exposure to OVA, airway eosinophilia resolved by 95% after 4 weeks of OVA exposure regardless of the genetic background of the experimental animals. BAL eosinophilia following 4 weeks of continuous OVA exposure was similar to that observed in animals exposed to 2 weeks of OVA followed by 2 weeks of saline exposure.

### *Flow Cytometric Analysis of Lung CD4 T Cells*

Given the central role of T cells in orchestrating eosinophilic airway inflammation [26], we investigated whether attenuated airway eosinophilia following prolonged OVA exposure was associated with a contraction of CD4 (CD3+CD4+) T cells in the lungs. Balb/c mice were inoculated intranasally with Ad/GM-CSF and exposed to aerosolized OVA for 2 or 4 weeks. An additional group of animals was exposed to OVA for two weeks followed by two weeks of exposure to aerosolized saline. Lung mononuclear cells were isolated and number and activation status of CD4 T cells was assessed using flow cytometry. We observed a marked expansion of mononuclear cells and CD4 T cells in the lungs following 2 weeks of OVA exposure (fig. 2). MNC and CD4 T cell numbers decreased markedly after 4 weeks of continuous OVA exposure and were similar to those observed in naive mice [19], or mice exposed to OVA for 2 weeks followed by exposure to saline for 2 weeks. It is noteworthy

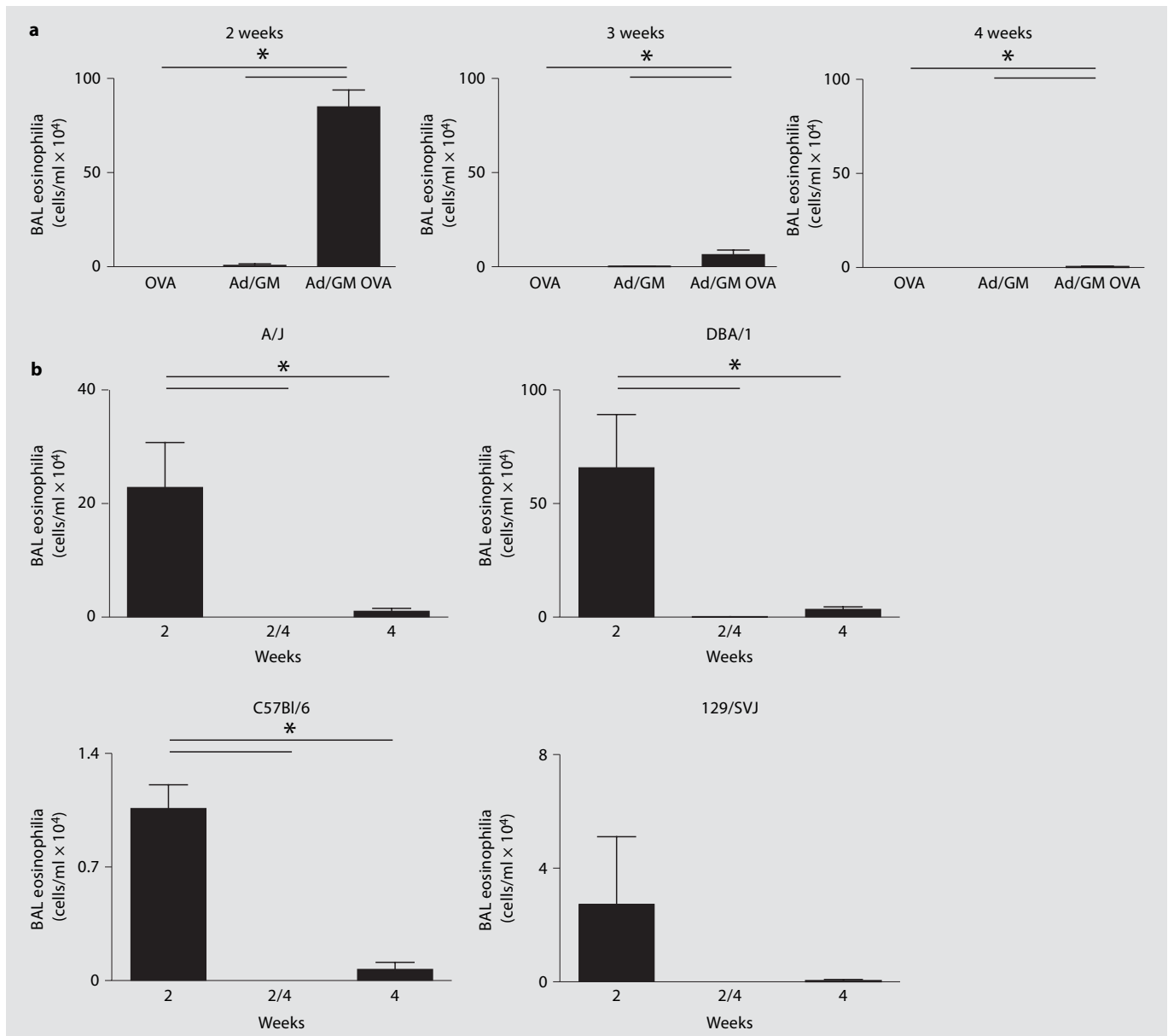


**Fig. 1.** BAL cellular profile. **a** Balb/c mice were inoculated with Ad/GM-CSF and exposed to OVA or saline for 2, 3 and 4 weeks. A separate group was exposed to OVA only. **b** DBA/1, A/J, C57BL/6J and 129/SVJ mice were inoculated with Ad/GM-CSF and exposed to aerosolized OVA for either 2 or 4 weeks. A separate group of animals was inoculated with Ad/GM-CSF and exposed to 2 weeks of OVA followed by 2 weeks of saline exposure (2/4 weeks). Data represent BAL eosinophilia (mean  $\pm$  SEM, **a**  $n = 3-4$ , **b**  $n = 4-5$ ). Statistical analysis was performed using one-way ANOVA with Tukey post hoc test, \*  $p < 0.05$ .

that the decline in mononuclear cells and CD4 T cells was similar between mice continuously exposed to OVA and mice exposed to saline only in weeks 3 and 4.

Next, we assessed expression of activation (CD69 and CD25) and Th2 (T1/ST2) markers on CD4 T cells. Fig-

ure 3 shows a significant increase in T cells expressing the activation markers CD69 following 2 weeks of OVA exposure. A similar trend was also observed for the activation marker CD25, although it did not reach statistical significance. Despite continuous exposure to OVA, the

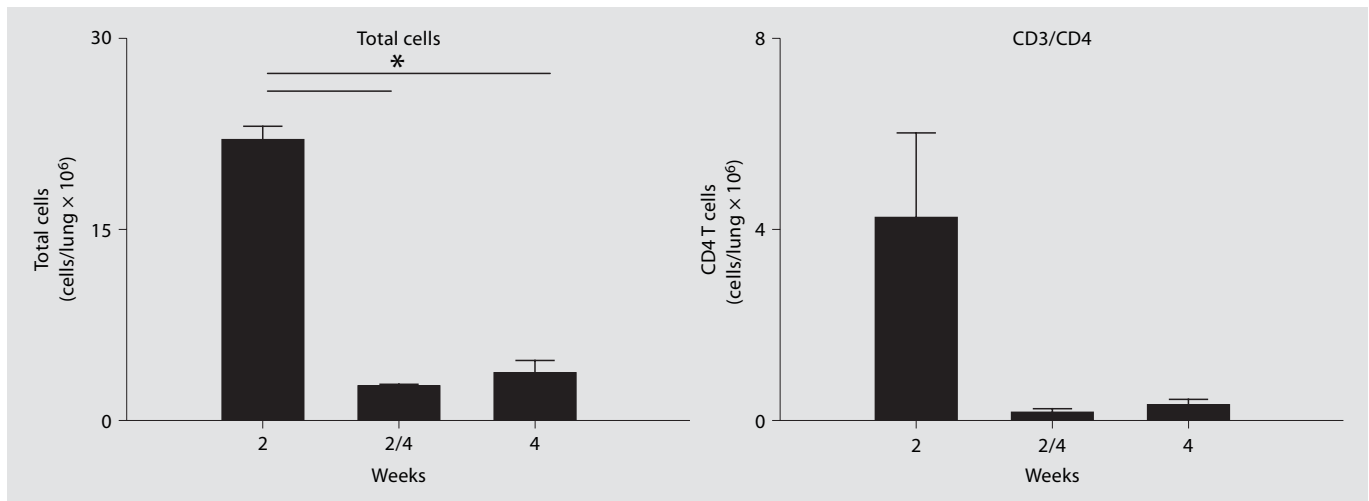


**Fig. 2.** Lung mononuclear and CD4 T cells. Three days following delivery of Ad/GM-CSF, Balb/c mice were exposed to aerosolized OVA for either 2 or 4 weeks. A separate group of animals was exposed to 2 weeks of OVA followed by 2 weeks of saline exposure (2/4 weeks). Data show total number of lung mononuclear cells and CD4 (CD3+/CD4+) T cells (mean  $\pm$  SEM;  $n = 3$  (4–5 animals were pooled in each experiment)). Statistical analysis was performed using one way ANOVA with a Fisher LSD post-hoc test, \*  $p < 0.05$  compared to the mice exposed to OVA for 2 weeks.

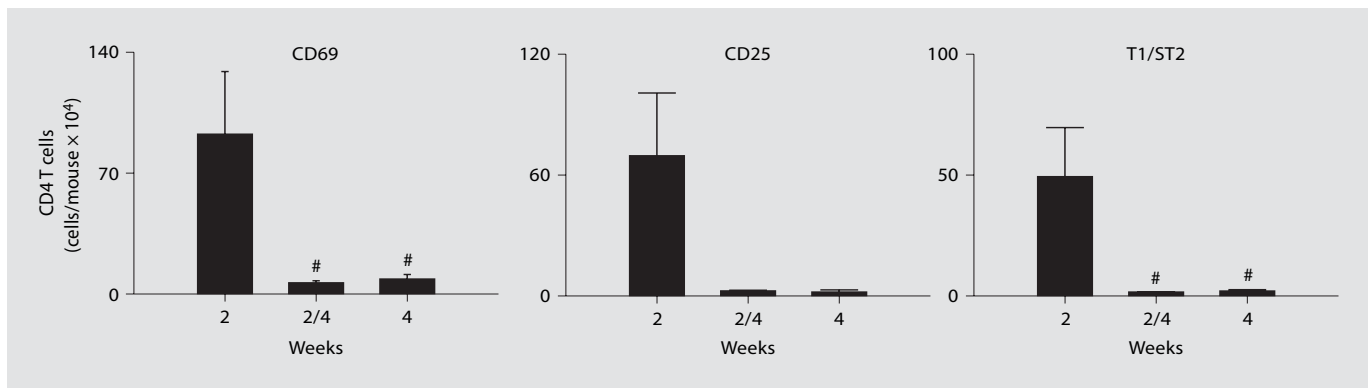


**Fig. 3.** Flow cytometric analysis of CD69, CD25, and T1/ST2 expression on lung CD4+ T cells. Three days following delivery of an adenoviral construct expressing GM-CSF, Balb/c mice were exposed to OVA for 2 weeks or 4 weeks. A separate group of animals was exposed to 2 weeks of OVA followed by 2 weeks of saline exposure (2/4 weeks). CD69, CD25, and T1/ST2 were assessed on CD3+/CD4+ cells (mean  $\pm$  SEM;  $n = 3$  (4–5 animals were pooled in each experiment)). Statistical analysis was performed using one way ANOVA with a Fisher LSD post-hoc test; #  $p < 0.05$  compared to the mice exposed to OVA for 2 weeks.

number of CD4 T cells expressing CD69 and CD25 was dramatically reduced after 4 weeks and similar to the numbers observed in mice that were allowed to resolve the inflammatory response following 2 weeks of OVA exposure. Similarly, we observed a significant increase in CD4 T cells expressing the Th2 marker T1/ST2 following

2 weeks of OVA exposure. The number of T1/ST2-expressing CD4 T cells was significantly reduced following 4 weeks of OVA exposure. The decrease in number of T1/ST2+ CD4 T cells was due to the overall decline of lung CD4 T cells and a significantly lower percentage of T cells expressing this marker (2 weeks:  $11.8 \pm 0.4\%$ , 2/4 weeks:

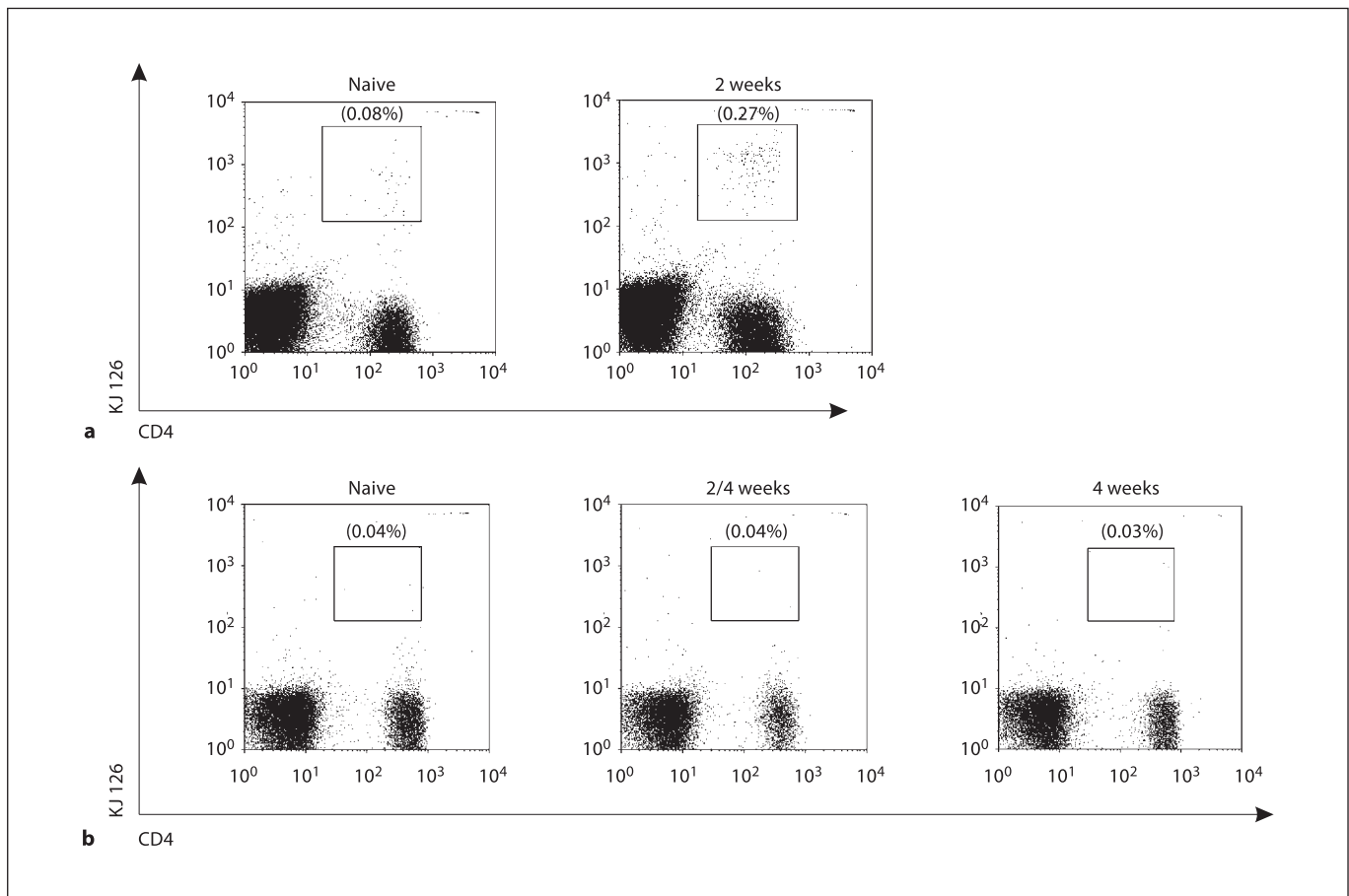


**Fig. 4.** Adoptive transfer of OVA-specific TcR transgenic splenocytes. DO11.10 splenocytes were adoptively transferred into naive Balb/c mice one day prior to delivery of Ad/GM-CSF. Subsequently, animals were exposed to OVA for 2 or 4 weeks. **a** Percentage of KJ126+CD4+ cells in naive lungs and in lungs following 2 weeks of OVA exposure (2 weeks). **b** Percentage of KJ126+CD4+ cells following either 2 weeks of OVA exposure followed by 2 weeks of saline exposure (2/4 weeks) or 4 weeks of continuous OVA exposure (4 weeks). Naive animals received DO11.10 splenocytes but were not exposed to OVA. Data shown are representative of 2 experiments.

$6.4 \pm 0.8\%$ , 4 weeks:  $6.4 \pm 1.0\%$ , mean  $\pm$  SEM,  $n = 3$ ,  $p = 0.004$ , one way ANOVA with Fisher LSD post-hoc test). In contrast, the proportion of CD4 T cells expressing CD69 and CD25 was similar in all groups tested.

#### *Impact of Continuous OVA Exposure on OVA-Specific T Cells*

To assess the impact of continuous OVA exposure on OVA-specific T cells, we adoptively transferred  $2 \times 10^7$  splenocytes from OVA-specific T cell receptor transgenic mice (DO11.10) into naive Balb/c recipients. DO11.10 splenocytes were transferred intraperitoneally 24 h prior to delivery of  $3 \times 10^7$  PFU Ad/GM-CSF. Three days later, animals were exposed to aerosolized OVA for 2 or 4 weeks.

A group of animals was exposed to OVA for 2 weeks followed by 2 weeks of saline exposure. Finally, a group of animals received DO11.10 splenocytes only, and was not exposed to OVA. The number of total cells, mononuclear cells, neutrophils and eosinophils was significantly increased following 2 weeks of OVA exposure compared to mice exposed to 2 weeks of OVA followed by 2 weeks of saline and animals exposed to OVA for 4 weeks (data not shown). Levels of total cells, mononuclear cells, neutrophils, and eosinophils in the latter two groups were similar to that observed in naive animals receiving DO11.10 cells only. These findings are in agreement with the data shown in figure 1 and demonstrate that adoptive transfer of DO11.10 cells did not affect the response to OVA. Fig-

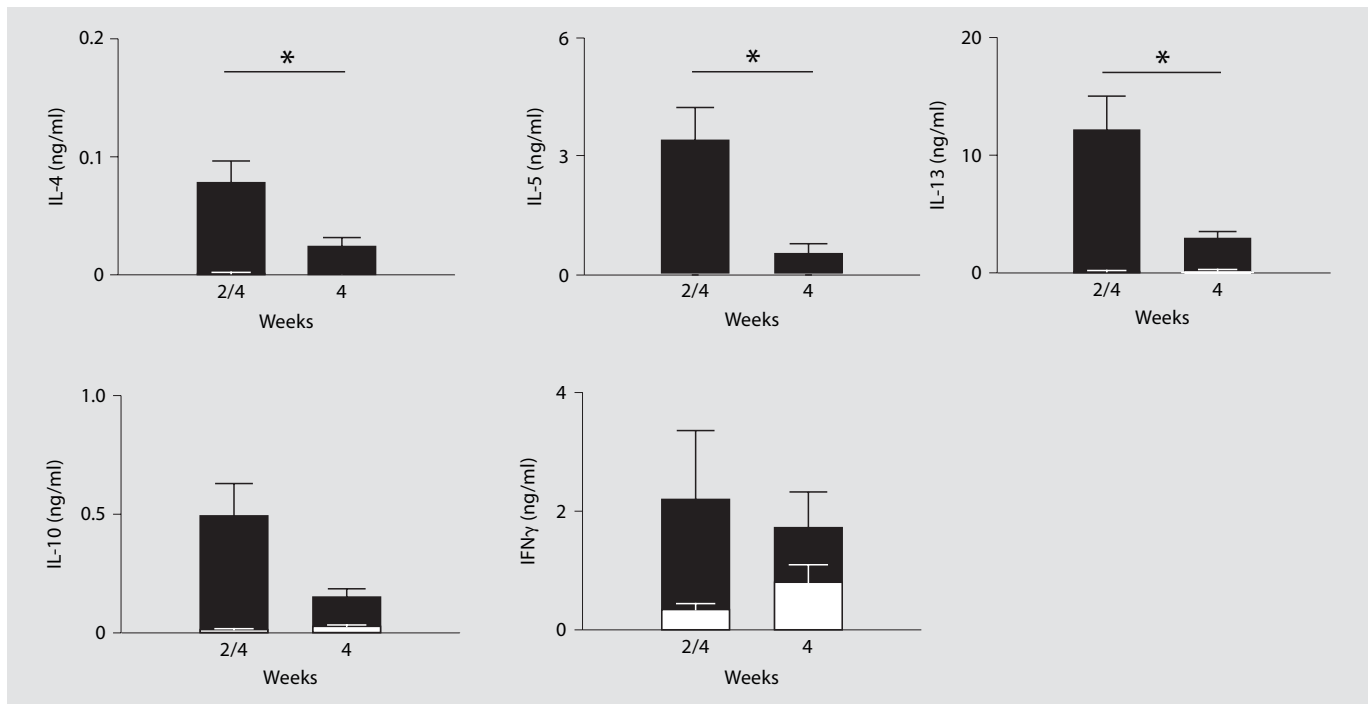


**Fig. 5.** Cytokine production by splenocytes. Three days following delivery of an adenoviral construct expressing GM-CSF, Balb/c mice were exposed to OVA for 2 weeks followed by 2 weeks of saline or 4 weeks of continuous OVA exposure. Splenocytes were cultured in medium (white bars) or stimulated with OVA (black bars) for 5 days (mean  $\pm$  SEM;  $n = 3$  (4–5 animals were pooled in each experiment)). Statistical analysis was performed by t test; \*  $p < 0.05$ .

ure 4a shows that following 2 weeks of OVA exposure we observed a marked increase in CD4<sup>+</sup> T cells (0.27%) staining positive with KJ126, a DO11.10 TcR-specific antibody. After 4 weeks, 0.04% of KJ126<sup>+</sup> T cells remained in the lungs regardless of whether mice were exposed to OVA for 2 weeks or 4 weeks (fig. 4b). This represents a 85% reduction compared to the 2 weeks time point and shows that antigen-specific T cells decrease within the lung in the context of prolonged OVA exposure.

#### *In vitro Cytokine Production by Splenocytes*

Next, we investigated whether continuous OVA exposure decreased antigen-specific memory. Balb/c mice were inoculated with  $3 \times 10^7$  PFU Ad/GM-CSF and exposed to OVA for 4 weeks or 2 weeks followed by 2 weeks of saline (2/4 weeks). Splenocytes were placed in culture with medium alone or stimulated with OVA. Figure 5 shows that splenocytes from animals exposed to 2 weeks of OVA followed by two weeks of saline produced significantly more IL-4, IL-5 and IL-13, compared to animals exposed to 4 weeks of OVA. A similar trend was

observed for IL-10, although it did not reach statistical significance. Negligible levels of IL-4, IL-5, IL-10 and IL-13 production were observed in both groups cultured in medium alone, indicating that the observed response was OVA-specific. While similar levels of IFN $\gamma$  were detected in culture supernatants of OVA-stimulated splenocytes, relative induction of IFN $\gamma$  appeared decreased due to elevated basal expression of the cytokine.

#### *Airway Hyperresponsiveness*

To investigate the physiological consequences of continuous OVA exposure, Balb/c mice were inoculated with Ad/GM-CSF and exposed to either 2 weeks of OVA followed by 2 weeks of saline exposure or 4 weeks of OVA. Both groups were rested for 28 days and challenged with OVA on three consecutive days for 20 min. Airway resistance in response to intravenous injections of MCh was assessed prior to and 48 h after the last challenge. Before OVA recall challenge, airway responsiveness to MCh was similar between naive animals and mice exposed to either 2 or 4 weeks of OVA (data not shown). Following

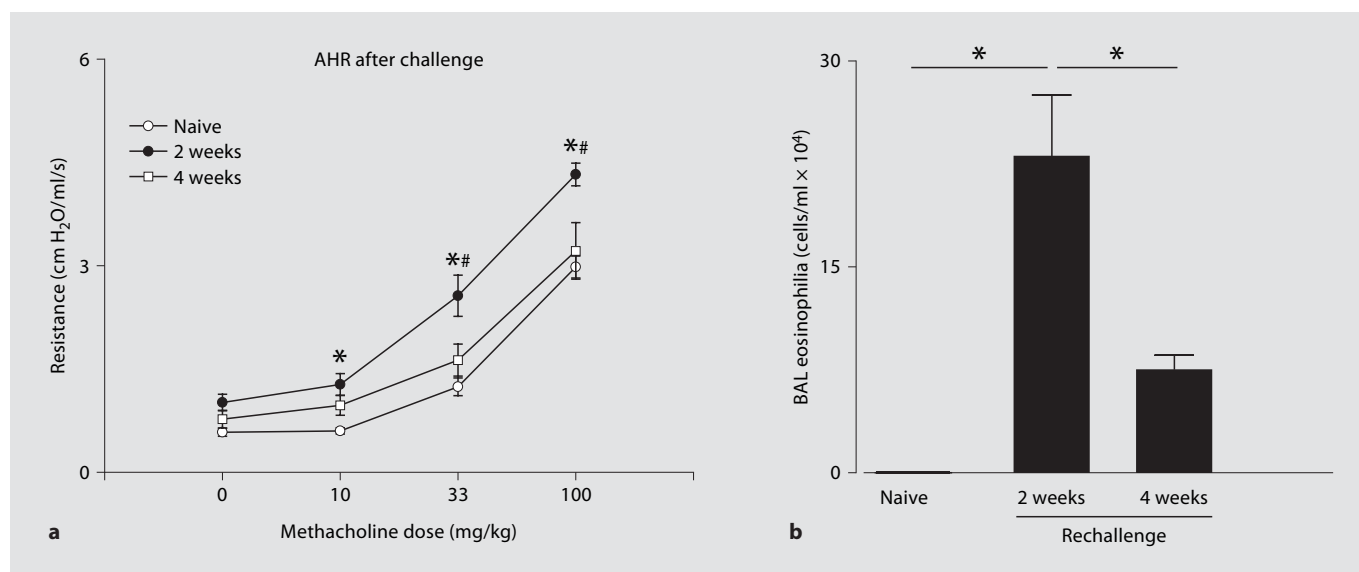


**Fig. 6.** Impact of continuous OVA exposure on respiratory system resistance. Three days following delivery of an adenoviral construct expressing GM-CSF, Balb/c mice were exposed to aerosolized OVA for either 2 or 4 weeks. Animals aerosolized for 2 weeks to OVA were then exposed for another 2 weeks of saline. Subsequently, both groups were rested for 28 days. **a** Respiratory system resistance to increasing intravenous doses of methacholine 48 hours after OVA airway challenge. (mean  $\pm$  SEM;  $n = 5-8$  animals). Statistical analysis was performed with Duncan test, probabilities for post-hoc test; \*  $p < 0.05$  compared to naive; #  $p < 0.05$  compared to 2 weeks. **b** BAL eosinophilia (mean  $\pm$  SEM;  $n = 8$ ). Statistical analysis was performed using one-way ANOVA with Tukey post hoc test; \*  $p < 0.05$ .

recall challenge, we observed a significant increase in airway responsiveness in mice initially exposed to 2 weeks of OVA compared to naive animals and mice exposed to 4 weeks of OVA (fig. 6a). Attenuated airway hyperresponsiveness was associated with significantly decreased BAL eosinophilia in animals initially exposed to 4 weeks of OVA compared to mice exposed to two weeks of OVA only (fig. 6b).

## Discussion

We previously reported that continuous exposure of sensitized mice to the surrogate allergen OVA leads to the resolution, rather than persistence, of airway inflammation [19]. The focus of the present study was to investigate underlying immune mechanisms and the physiologic consequences associated with these findings.

Using a well-characterized model of airway inflammation, we show that airway inflammation during chronic exposure to OVA is diminished in five mouse strains tested, strongly arguing that the phenomenon is broadly expressed and independent of genetic background. To ex-

plore possible immune mechanisms, we investigated the lung and peripheral cell compartments during chronic exposure. In the lung, 2 weeks of OVA exposure was associated with a transient increase in CD4 T cells that expressed the activation markers CD69 and CD25, and the Th2 effector marker T1/ST2. These increases coincided with peak airway inflammation. Following four weeks of exposure, CD4 T cell numbers were markedly reduced and similar to mice exposed to OVA for two weeks and subsequently rested. The reduction appeared to be antigen specific, as evidenced by studies utilizing OVA specific DO11.10 T cells. It remains to be determined, however, whether this loss was antigen selective, or whether it represented a general loss of T cell accumulation in the lungs. Systemically, we observed a loss of Th2 memory effector function following 4 weeks of OVA exposure compared to animals exposed for 2 weeks. Stimulated *in vitro* with OVA, splenocytes from 4-week-exposed animals expressed significantly lower levels of IL-4, IL-5 and IL-13 compared to mice exposed for two weeks only, suggesting that prolonged antigen exposure led to deletion of antigen-specific T cells or induction of regulatory moieties.

The immunosuppressive properties of IL-10, as they relate to eosinophilic inflammation, are well known and have been documented extensively [25, 27–30]. In this study, we observed a moderate, albeit not significant, decrease of IL-10 expression after 4 weeks of OVA exposure, suggesting that immune suppression was not achieved through the induction of IL-10-producing regulatory T cells. To assess whether continuous exposure to OVA deviated the immune response from Th2 to Th1, we measured splenocyte IFN $\gamma$  expression. IFN $\gamma$  has been shown to inhibit Th2 cytokine expression, skewing the differentiation of T cells toward Th1 [14, 31]. More recently, it has been shown to inhibit airway hyperreactivity by Th1-like T regulatory cells [15]. That IFN $\gamma$  levels were similar in mice receiving 2 weeks and 4 weeks of antigen exposure provides evidence that attenuation of airway eosinophilia was not dependent on immune deviation toward a Th1 phenotype. Our findings cannot formally rule out IL-10 or IFN $\gamma$  associated immune suppression as a regulatory mechanism, however, as there is sufficient evidence in other studies demonstrating that TR cells mediate their immunosuppressive effects via these pleiotropic cytokines [13, 15].

Consistent with abrogation of airway inflammation, we show that responsiveness to methacholine challenge in Balb/c mice exposed to OVA for 4 weeks is attenuated compared to animals exposed to 2 weeks. Our data emphasize the importance of systemic T cell immunity as a marker of airway reactivity. Importantly, elaboration of Th2 cytokines, but not serum IgE expression [19], is predictive for airway inflammation and hyperresponsiveness.

Understanding immune mechanisms that underlie allergic sensitization and sustain chronic inflammation is pivotal to our understanding of allergic diseases. Allergens are defined as antigens that elicit hypersensitivity or allergic reactions. With exceptions, there are, however, no clear biochemical properties that discriminate allergens from antigens. Abundant experimental evidence demonstrates that exposure to antigen alone (OVA, DerP1) induces inhalation tolerance, while co-administration of the same antigen with an adjuvant such as aluminum hydroxide, GM-CSF or LPS leads to antigen-specific allergic sensitization [6]. On the other hand, exposure of mice to HDM extract is sufficient for allergic sensitization [32, 33]. This is likely due to the protease activity associated with major allergens [34–39]. Our data illustrate that even in a sensitized animal continuous exposure to antigen alone does not lead to persistent inflammation. This again is in sharp contrast to allergen

extracts, since chronic exposure to HDM leads to chronic inflammation [40]. That antigen alone is insufficient at maintaining chronic inflammation emphasizes the importance of adjuvant-like factors for both allergic sensitization and chronic inflammation. Clinically, allergen-derived antigens engineered without protease activity may prove useful as desensitizing or tolerance inducing agents, an approach already in use in allergen immunotherapy [41, 42]. Importantly, our findings are consistent with models of chronic exposure where alum was used as adjuvant [21, 43, 44], indicating that GM-CSF and alum play similar roles as adjuvants, an issue discussed previously by our group [45].

Allergic asthma is a disorder characterized by intermittent physiological symptoms arising in an environment of chronic or sporadic exposure to antigen. Our understanding of how the immune system responds to such stimuli remains incomplete. We provide evidence that chronic exposure to surrogate antigen is associated with attenuated airway eosinophilia in 5 different mouse strains and results in concomitant diminishment of airway hyperresponsiveness and disappearance of antigen-specific T cells from the lungs. While mechanisms that dictate unresponsiveness may differ between the effector and peripheral compartments, peripheral responses are ultimately predictive for clinical manifestations of the disease.

### Acknowledgments

This study was supported in part by the Ontario Thoracic Society and the Canadian Institutes of Health Research (CIHR). F.K.S. is holder of a KM Hunter/CIHR Doctoral Research award, and M.R.S. is holder of a CIHR New Investigator award. We thank Clinton S. Robbins for critically reviewing the manuscript and Mary Kiriakopoulos for secretarial assistance.

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