

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 (10 cm $\times$ 15 cm $\times$ 25 cm) and exposed for 20 min 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 10 L/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 8 μ g OVA (Sigma Chemicals, St Louis, MO, USA) or 200 μ g ragweed (Greer Laboratories, Lenoir, NC, USA), both adsorbed to 4 mg aluminium hydroxide (Aldrich Chemicals, Milwaukee, WI, USA) at 4°C overnight in 0.5 mL 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 (10 cm $\times$ 15 cm $\times$ 25 cm) and

exposed to aerosolized OVA (1% wt/vol. in 0.9% saline) for 1 h twice, 4 h apart, or received 200 μ g ragweed in 30 μ L Coca's buffer (0.085 M NaCl, 0.064 M NaHCO₃; pH = 8.1) intranasally. The aerosol was produced by a Bennet nebulizer at a flow rate of 7 L/min. In the experiments involving airway hyper-responsiveness (AHR), mice were exposed to OVA aerosol daily for 1 h over a period of 3 days.

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 (0.25 mL followed by 0.2 mL). Approximately 0.25 mL of the instilled fluid was consistently recovered. Total cell counts were determined using a 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 2 min. 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 30 min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin; 3 μ m-thick sections were stained with haematoxylin 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 <2 pg/mL, and for IL-5 was 5 pg/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 5 μ g 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 (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 1 h 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 (0.03 mg/kg i.v.) to prevent

respiratory effort during measurement. RRS was measured following consecutive i.v. injections of saline, then 10, 33, 100 and 330 µg/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 30 s 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 300g for 10 min 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 300g for 10 min 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 10 mL of warm (37°C) HBSS (calcium and magnesium free) containing 5% FBS (Sigma Chemicals Co.), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco BRL). 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 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 (0.5 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂EDTA at pH 7.2–7.4), 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, 10⁶ cells were incubated with 0.5 µg Fc Block (CD16/CD32, Pharmingen, Mississauga, Canada) at 0–4°C for 10 min and subsequently with first stage monoclonal antibodies at 0–4°C for 30 min. 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 40 µg 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 1 mL 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 7 mm 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 20 µL 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.

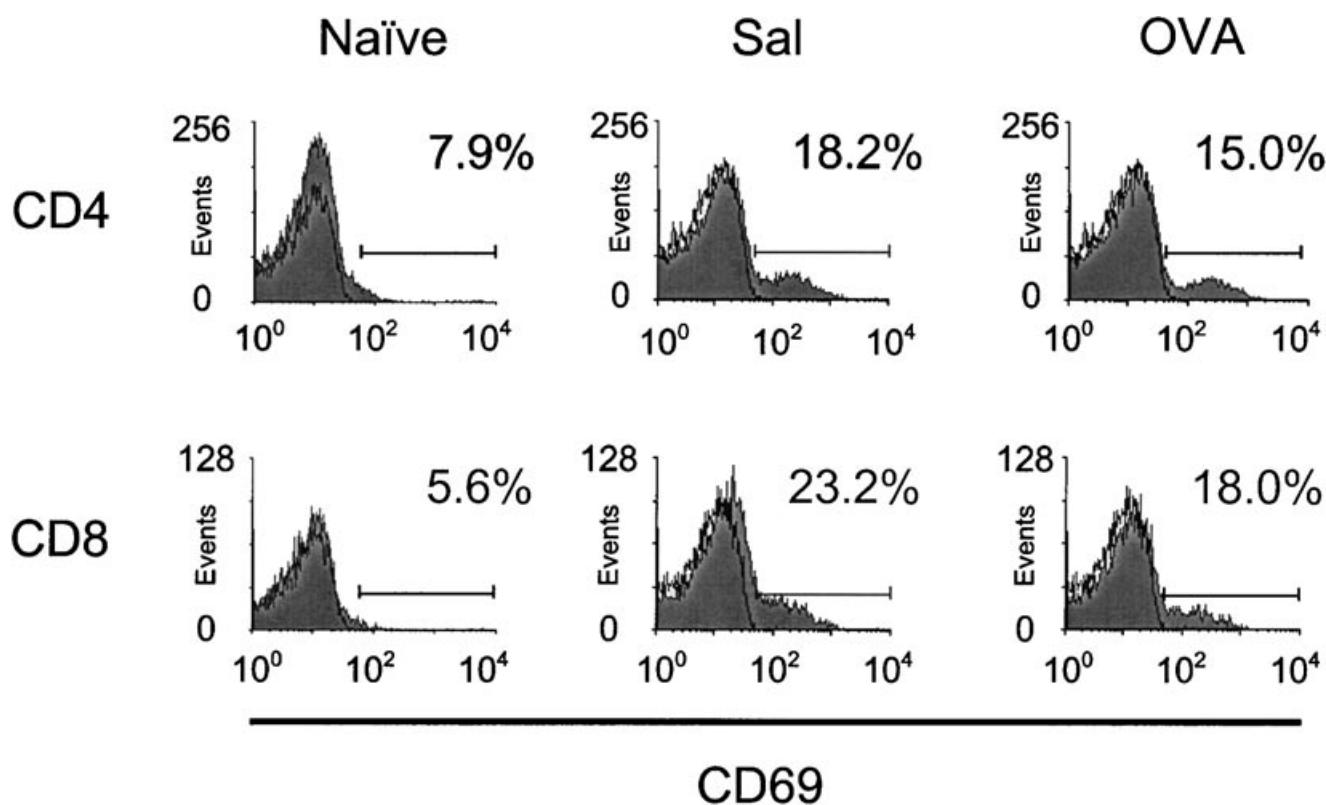


Fig. 1. Effect of passive OVA exposure on the expression of the activation marker CD69 on T cells in the thoracic lymph nodes after sensitization. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Twenty-four hours after the second i.p. sensitization, 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 naïve, 5 for other groups. ND=not detectable.

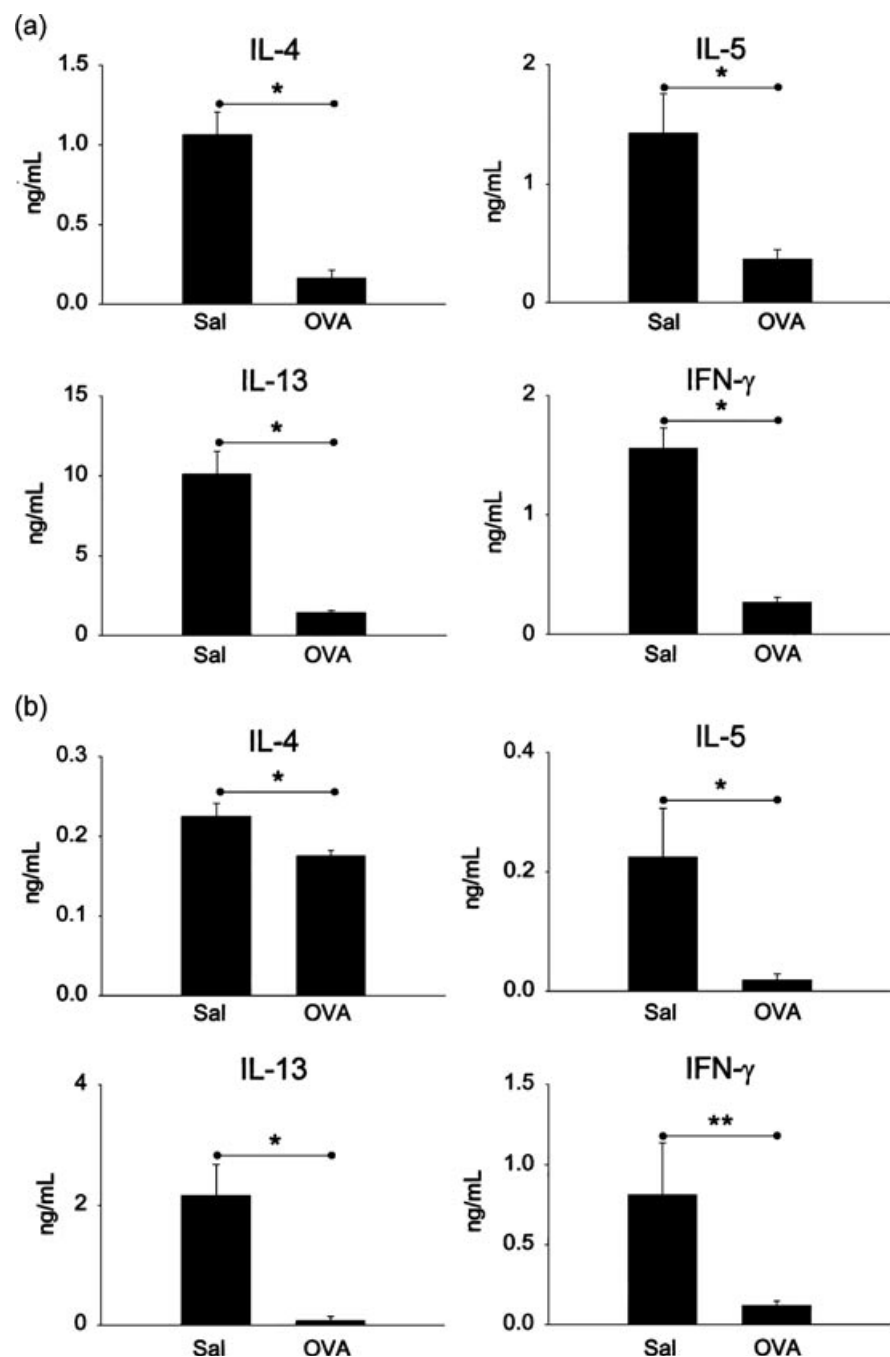


Fig. 2. Cytokine expression from lymph node and spleen cells recalled in culture with OVA. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Twenty-four hours after the second i.p. sensitization, mice were killed and their thoracic lymph nodes (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, 24h 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).

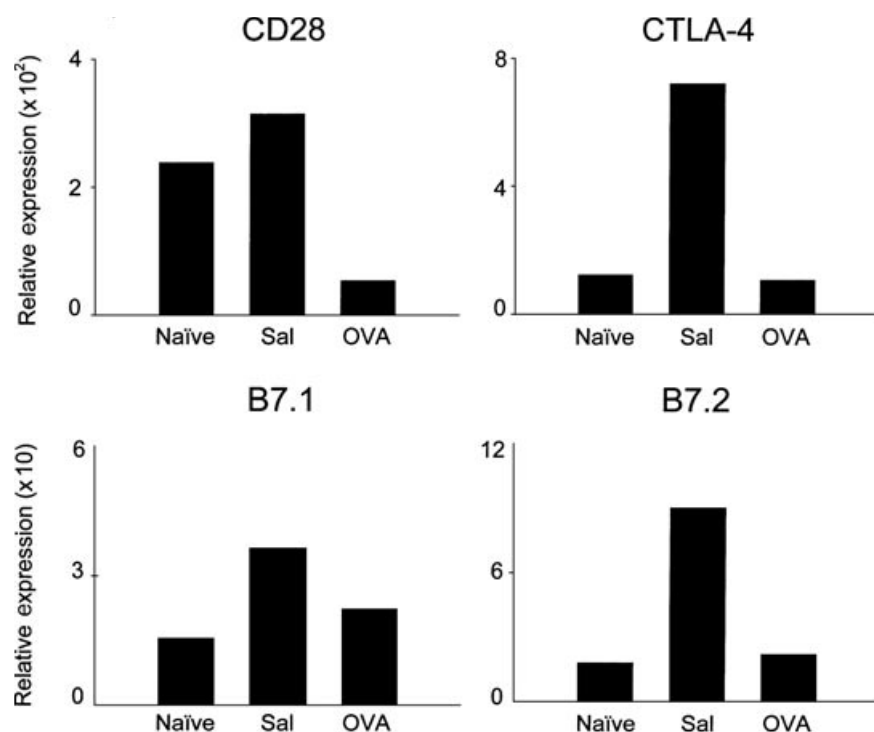


Fig. 3. Effect of initial OVA exposure on the costimulatory molecule profile. Mice were initially exposed to either 1% OVA or saline via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Twelve hours after the last OVA challenge, mice were 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).

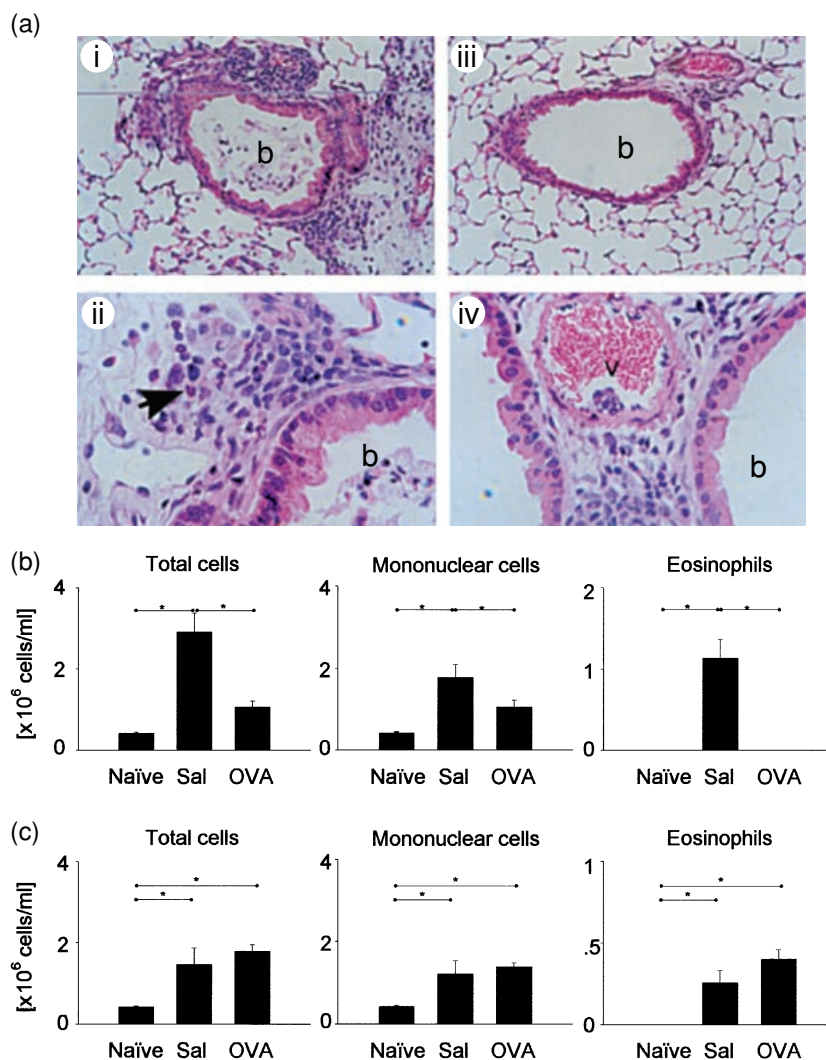


Fig. 4. Light photomicrograph of paraffin-embedded sections of lung tissues and BAL cellular profile after aerosol challenge. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. In (a) and (b), both groups were then sensitized i.p. and challenged 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) $\times 250$; (ii) $\times 640$; (iii) $\times 250$; (iv) $\times 640$. In (b) and (c), data show total cell number, mononuclear cells and eosinophils in the BAL. Mean $\pm$ 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 μ g/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

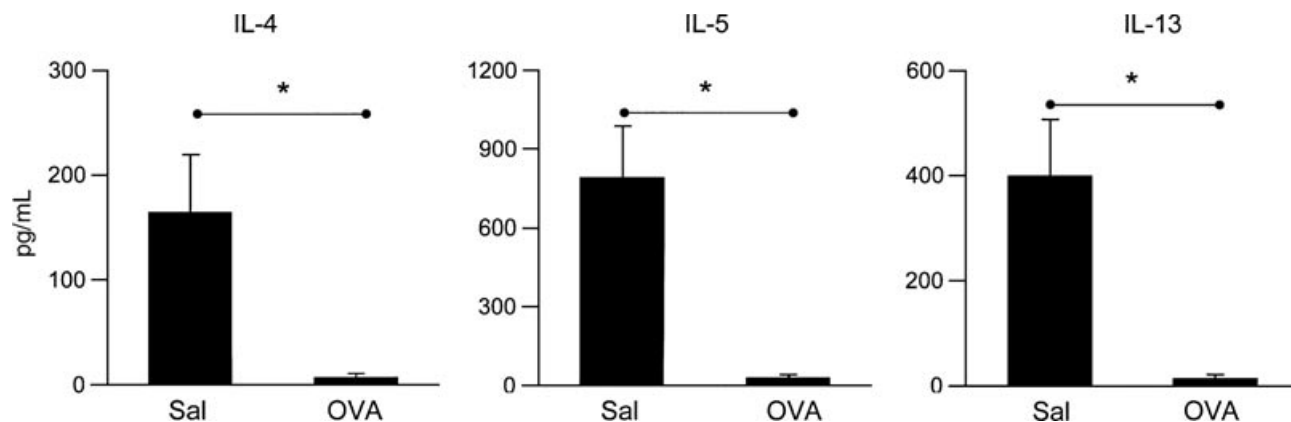


Fig. 5. Cytokine levels in the BAL. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Mice were killed 24 h after the last challenge. Cytokines were measured by ELISA. Mean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

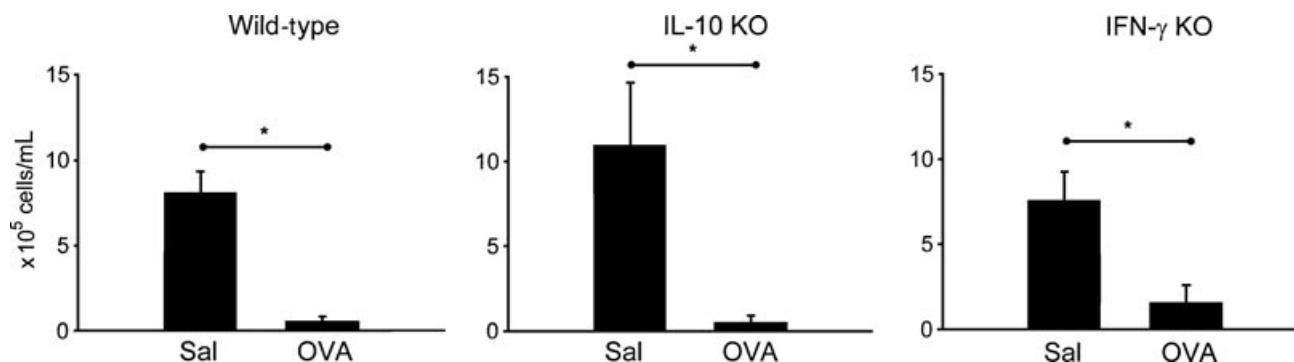


Fig. 6. Cellular profile in IL-10 and IFN- γ knock-out mice. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Animals were killed 72 h after the last OVA challenge. The wild-type controls are C57BL/6 mice. Mean $\pm$ 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 24 h 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

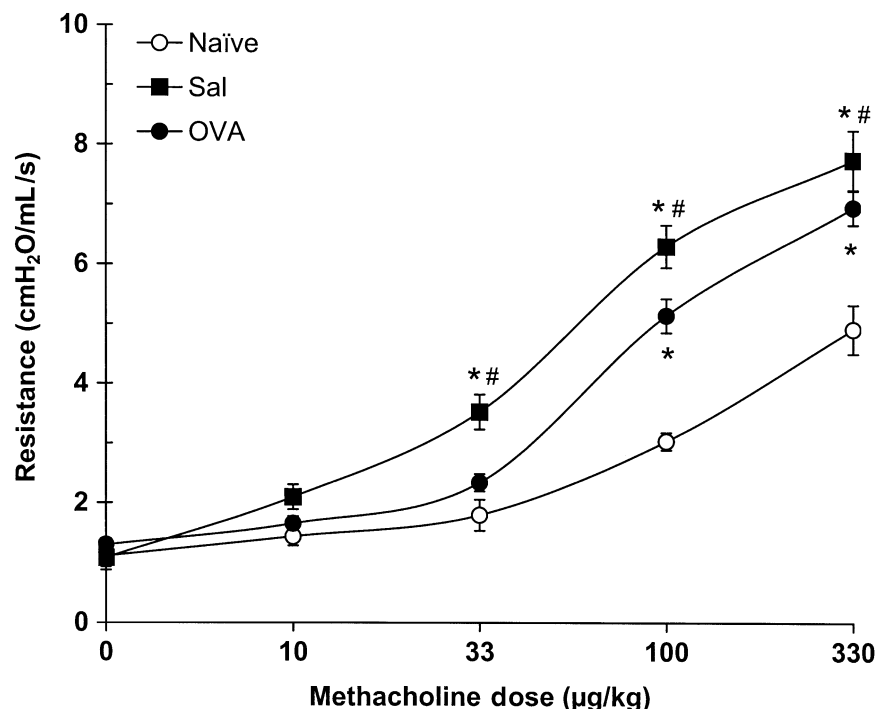


Fig. 7. Effect of passive OVA exposure on MCh-induced increases in RRS. Mice were initially exposed to either 1% OVA or saline (Sal) via aerosol for 20 min on 10 consecutive days. Both groups were then sensitized i.p. and challenged with OVA aerosol. Seventy-two hours after challenge airway function was assessed. Mean $\pm$ 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 300 µg/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 hyper-responsiveness [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 compromised 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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