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REVERSAL OF METHYLPREDNISOLONE EFFECTS IN ALLERGEN-EXPOSED FEMALE BALB/c MICE

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A high percentage of asthma is associated with aeroallergen exposures. Glucocorticoids such as methylprednisolone represent a major method for managing chronic asthma. However, studies suggested that corticosteroid therapy might have the potential to stimulate rather than inhibit adaptive immune inflammatory reactions, raising concerns about possible adverse reactions due to excessive repeated methylprednisolone treatment. Therefore, a murine model of allergen-induced inflammation was characterized and used to investigate the effects of repeated intraperitoneal (ip) and transnasal treatments with methylprednisolone (0–20 mg/kg body weight) and cyclosporin A (20 mg/kg body weight). Sensitized BALB/c female mice were exposed daily to ovalbumin (OVA) aerosols for up to 5 d with 24-h postexposure analyses for airway responses to methacholine aerosols and inflammatory cell recoveries by bronchoalveolar lavage (BAL) and tissue collagenase dispersion. Although increased tissue neutrophils, lymphocytes, monocytes, and macrophages reached maximal levels after 2 daily OVA exposures, recoverable eosinophil numbers continued to rise over the 5-d period. Daily ip treatments with a 5-mg/kg body weight dose of methylprednisolone diminished both OVA-induced airway responses to methacholine and inflammatory-cell accumulations to levels comparable to those observed with cyclosporin A. However, treatments with higher doses of methylprednisolone reversed this anti-inflammatory effect, indicated by a return to untreated levels of OVA-induced eosinophil recovery. A similar biphasic response in eosinophil recoveries was observed using daily transnasal methylprednisolone treatments that correlated with a concomitant fall and rise in BAL interleukin-13. These results supported the hypothesis that repeated high-steroid treatments might activate rather than suppress allergen-induced immune responses.

The prevalence of asthma continues to increase, especially among children. Half of the cases reported in the Third National Health and Nutrition Examination Study (NHANES III) were identified as being associated with allergen exposures in individuals with specific sensitivities mainly to cat, mold, and white oak antigens (Arbes et al., 2007). Increased incidence of asthmatic reactions triggered by environmental

exposures and characterized by airway constriction, cough, and inflammation is being attributed to enhanced aeroallergens exposures possibly resulting from climate change, as well as a result of exposures to such irritant air pollutants as cigarette smoke, ozone, and nitrogen dioxide (Gilmour et al., 2006).

The combined use of bronchodilator and corticosteroid therapy is the standard approach

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in the acute care management of individuals with moderate to severe early- and late-phase asthmatic reactions. Although there is a desire to move away from the systemic administration of corticosteroids such as methylprednisolone, these drugs still represent the most effective method for the management of the inflammatory component of acute asthma. However, the action of such glucocorticoids on antigen-induced inflammatory processes is complex. While glucocorticoids appear to be effective in reducing innate immune inflammatory cell accumulations, under different conditions, these agents were shown to have the potential to enhance adaptive immune mechanisms to promote antigen induced Th2-lymphocyte associated inflammation (Elenkov, 2004; Franchimont, 2004). Previous uses of experimental models based on short-term allergen-exposures of sensitized rats and guinea pigs demonstrated that pretreatment with glucocorticoids including methylprednisolone abrogates subsequent ovalbumin (OVA)-induced eosinophil inflammatory cell airway accumulations (Birrell et al., 2003; Chand et al., 1990; Heuer et al., 1994; Huang et al., 1999; Kassera et al., 2004; Murai et al., 2005). However, as more studies explore the complex immune mechanisms leading to such allergen-induced inflammatory reactions, accumulating evidence suggests that repeated steroid use might actually result in a subsequent enhancement of inflammation (Elenkov, 2004; Franchimont, 2004). Therefore, the main objective of the present study was to establish a murine model of allergen-induced lung inflammation and to use it to explore how different levels of repeated methylprednisolone treatments might affect the development of allergen-induced lung inflammation spread out over a 5-d period of daily allergen exposures. Results were compared with the effects of similar treatment with the immune-suppressive drug cyclosporin A as a positive control. Cyclosporin A has been used to explore the role of T cells in allergen-induced lung inflammation and airway reactivity, as well as being considered as a potential treatment for severe asthma (Ezeamuzie & Nwankwoal, 2000; Khan et al.,

2000; Larche et al., 2003; Underwood et al., 2001).

A murine model based on up to five daily exposures of sensitized female BALB/c mice to the experimental allergen OVA was established for use in this investigation (Birrell et al., 2003; Jaffar et al., 2004). Although studies have employed single acute allergen exposures of guinea pigs or Brown Norway rats to study early- and late-phase reactions, murine allergen exposure models using repeated low levels of OVA exposure are being utilized with increasing frequency to delineate the sequence of events leading to allergen-induced airway inflammation, hyperreactivity (Hahn et al., 2003; Jaffar et al., 2004), and more recently tolerance and airway remodeling (Temelkovski et al., 1998; Yiamouyiannis et al., 1999). Most studies of lung inflammation rely on bronchoalveolar lavage (BAL) cell recoveries to indicate lung inflammation, yet correlation of the time course of inflammatory events with, for example, the onset of airway hyperreactivity required the use of both BAL and collagenase tissue dispersion techniques to assess total lung inflammatory status (Birrell et al., 2003; Delorme et al., 2002; Shultheis & Bassett, 1994). Therefore, in this study, inflammatory cell contents in the airway and the combined tissue marginated and interstitial compartments, respectively, were determined, providing an opportunity to correlate changes in tissue inflammatory cell content with airway responses to methacholine challenge. This approach provided a detailed evaluation of the time course of cellular events and associated physiological responses to methacholine challenge over a 5-d period of repeated daily OVA exposures and a suitable base for exploring the effects of both intraperitoneal (ip) and transnasal repeated treatments with methylprednisolone.

METHODS

Animals

Specific-pathogen-free female BALB/c mice weighing 20–25 g were obtained from Hilltop Laboratory Animals Inc. (Scottsdale,

PA) and maintained in high-efficiency particulate-filtered air with free access to food and water. Following 7–10 d of acclimatization, all animals were sensitized by ip injections of 10 μ g OVA (Grade V, Sigma Chemical Co., St. Louis) and 2 mg aluminum hydroxide suspended in 0.2 ml sterile isotonic saline. Animals were injected on d 1 and d 14. Daily exposures to either saline (control) or OVA aerosols were initiated 21 d from the first ip OVA treatment.

Mice were exposed for 1 h/d for up to 5 d either to an OVA aerosol generated from a 1% OVA solution in sterile isotonic saline or to saline alone (control), using a collision nebulizer and a nose-only exposure system that provides a relatively monodispersed aerosol with a mean aerodynamic diameter of 0.25 μ m (CH Technologies, Westwood, NJ). Air flow was adjusted to provide an aerosol concentration of 25 mg OVA/m³. Twenty-four hours following the last exposure, mice were screened in some cases for their responses to methacholine aerosol challenge and then anesthetized (60 mg/kg body weight sodium pentobarbital, ip) prior to assessment of inflammatory status. The protocols used in this project were approved by Wayne State University's Institutional Animal Care and Use Committee (IACUC), with all procedures being consistent with U.S. government guidelines. The facilities, including those for animal exposure, are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Drug Treatments

Intraperitoneal administrations were conducted with either methylprednisolone (Solu-Medrol, Pharmacia & Upjohn Company, Kalamazoo, MI) or cyclosporin A (Sigma) suspended in 0.2 ml sterile isotonic saline containing methylcellulose dispersant (0.2% w/v). Based on previous steroid treatments of mice (Birrell et al., 2003), cohorts of 4–6 mice were ip treated with either 0 (dispersant control), 5, 10, or 20 mg/kg body, 1 d before and 4 h after each daily exposure to either saline or OVA aerosols. Using the same treatment regimen, a separate experiment was conducted using 4 mice under each exposure condition treated

with either dispersant or 20 mg/kg body weight cyclosporin A. Additional cohorts of untreated mice were exposed to saline and OVA aerosols to assess possible effects of the dispersant alone.

Transnasal administrations were conducted on mice anesthetized with isoflurane using 15 μ l of increasing doses of methylprednisolone hemisuccinate (Sigma) solubilized in a carrier containing 200 mg/ml (2-hydroxypropyl)- β -cyclodextrin in sterile isotonic saline, providing stable clear solutions for treatments with 0 (saline carrier), 2.5, 5, 10, or 15 mg/kg body weight. Each administration was followed by an additional 15 μ l of isotonic saline wash. Two separate experiments were conducted using the treatment and exposure regimens described earlier with cohorts of at least two mice under each condition.

Evaluation of Lung Physiology

Awake mice were placed in whole-body plethysmographs, each equipped with a bias flow air supply, a pneumotachograph resistance, and pressure transducers (Buxco Electronics, Inc., Wilmington, NC). Changes in the enhanced pause in breathing (Penh) resulting from exposure to increasing aerosol concentrations of the bronchoconstrictor methacholine were determined as a surrogate indicator of altered airway constriction, as previously investigated and evaluated (Birrell et al., 2003; DeLorme & Moss, 2002; Hamelmann et al., 1997; Whitehead et al., 2003). Following 30 min of acclimatization within the plethysmograph, mice were exposed for 2 min to methacholine aerosols with increasing concentrations from 0 to 100 mg/ml that corresponded to 0 to 6.25 g methacholine/m³. A 5-min recovery period was allowed between methacholine applications, during which time mean Penh values were recorded. An additional series of experiments was conducted with OVA-exposed and ip methylprednisolone-treated mice to correlate these Penh findings with direct measurements of changes in airway resistance when challenged by similar increasing aerosol concentrations of methacholine (Hahn et al., 2003). These assessments were achieved in mice anesthetized using ketamine (80 mg/kg body weight) and xylazine (10 mg/kg

body weight) in combination while being artificially ventilated via a tracheal cannula with a tidal volume of 0.225 ml at 120 breaths/min (Hugo Sachs Electronik-Harvard, Germany), with flow and volume assessed using a whole-body plethysmograph transducer and a second transducer to assess changes in airway pressure (Buxco Electronic, Inc.). Mice were exposed for 30 s to a series of increasing aerosol concentrations of methacholine up to 100 mg/ml, with measurements of enhanced airway resistance recorded and averaged over the following 2-min period. Data obtained for each mouse using both of these methods were subjected to polynomial regression analyses as previously described (DeLorme et al., 2002; DeLorme & Moss, 2002), demonstrating coefficients of greater than .985 in all cases. The resulting quadratic equations were then used to establish single numbers for comparing the effects of different pretreatments and exposures at specific levels of methacholine aerosol challenge (Whitehead et al., 2003).

Determination of Inflammatory State

As previously described in studies conducted with rats and guinea pigs (DeLorme et al., 2002; Shultheis & Bassett, 1994), ventilated lungs of anesthetized mice were first perfused with warm isotonic saline (37°C) via the pulmonary artery to remove blood components, followed by an initial bronchoalveolar lavage (BAL) via the trachea with a 0.8-ml aliquot of phosphate-buffered saline (PBS, pH. 7.4). The BAL fluid supernatants were stored at -80°C for later analyses that in some cases included determinations of interleukin-13 (IL-13) using established kit-based methods (IL-13 Mouse ELISA Kit, KMC2221, InVitrogen, Carlsbad, CA). The recovered cells were combined with recoveries from 4 additional lavages using PBS containing 3 mM ethylenediamine tetraacetic acid (EDTA) to ensure maximal recovery of phagocytic cells. The resulting cell populations were subjected to differential analyses following staining with modified Wright's stain for identification of macrophages, lymphocytes, neutrophils and eosinophils (DeLorme et al., 2002; Shultheis & Bassett, 1994). The remaining

parenchymal tissue was isolated, weighed, sliced, and incubated in Dulbecco's modified Eagle's medium containing collagenase (75 U/ml; Worthington Biochemical Corporation, Lakewood, NJ), fetal calf serum (10% v/v; Gibco, Grand Island, NY), and 50 U/ml DNase (Sigma), as previously described (DeLorme et al., 2002; Holt et al., 1985; Shultheis & Bassett, 1994). A total cell count was conducted and differential cell analyses were performed after separate staining with modified Wright's stain for neutrophils, eosinophils, and lymphocytes, and nonspecific esterase staining for the identification of the combined monocyte and macrophage population (Li et al., 1973). Results were reported as total BAL- and tissue-recovered cells per lung. In separately exposed animals, samples of lung tissue were prepared for light microscopic examination by fixing in acetone containing the protease inhibitors phenylmethylsulphonyl fluoride and iodoacetate followed by infiltration and embedment in glycol methacrylate, as previously described (Britten et al., 1993). Airway structure was maintained by prior intratracheal infusion of 150 µl agarose at 37°C followed by 15 min at 4°C to set the gel (Plopper et al., 1991), followed by isolation of cross-sectional tissue samples for fixation, embedding, sectioning, and staining with toluidine blue.

Statistical Analyses

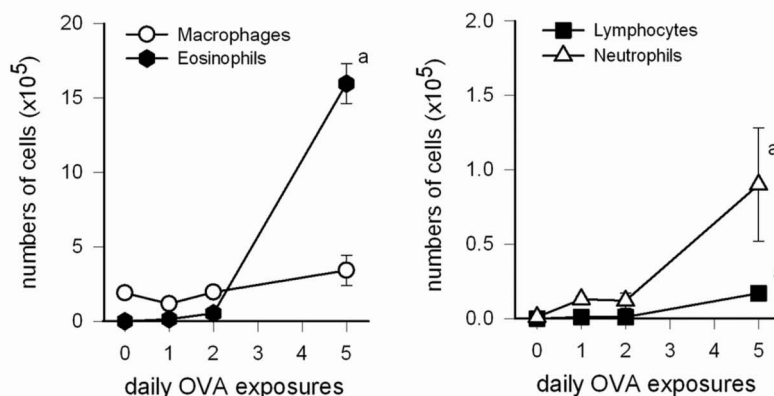
Data from these experiments were analyzed by either one-way or two-way analyses of the variance with comparisons of the means using either Dunnett's or Student-Newman-Keuls multiple-range tests, respectively, with a level of significance of $p < .05$. Logarithmic transformations of the data were conducted when variances were found to be heterogeneous by Hartley's test. Data are presented as means $\pm$ SE.

RESULTS

Allergen-Induced Lung Inflammation

Since no significant effects on inflammatory cell recoveries were observed as a result of daily saline aerosol exposures, results obtained

A: Lavage - Recovered Cells



B: Tissue-Recovered Cells

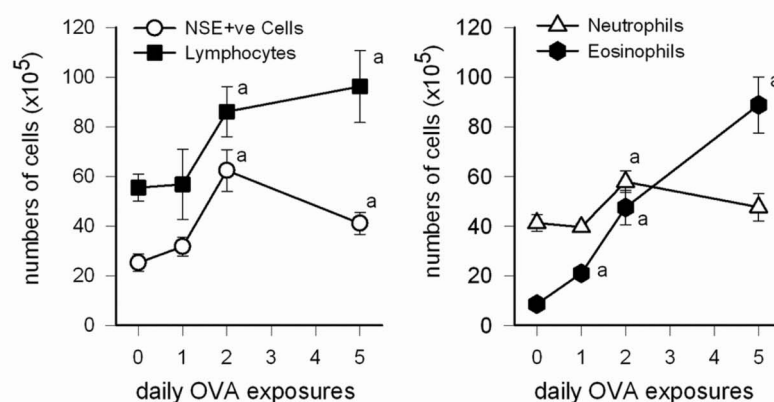


FIGURE 1. Results represent the mean ($\pm$ SE, $n = 4$) recovery of inflammatory cells by (A) bronchoalveolar lavage (BAL) and (B) collagenase lung tissue dispersion from sensitized female BALB/c mice 24 h following 0 (control), 1, 2, and 5 daily exposures to OVA aerosol. NSE+ve cells represent marginated and interstitial monocytes and macrophages. Significantly different from saline-exposed control mice with $p < .05$, indicated by a.

from unexposed and saline-exposed mice were combined for presentation (Figure 1). The lavage of the lungs of unexposed control mice demonstrated a total cell recovery of $1.9 \pm 0.2 \times 10^5$ ($\pm$ SE, $n = 4$) with alveolar macrophages representing greater than 99% of the recovered cells. However, 24 h following the fifth daily exposure to OVA, the total number of recoverable cells increased to $20.4 \pm 2.6 \times 10^5$ ($\pm$ SE, $n = 4$), with significantly elevated numbers of lymphocytes, neutrophils, and eosinophils appearing in the lavage fluid (Figure 1A). Collagenase dispersions of lavaged lungs from control saline-exposed animals demonstrated that 15% of a total tissue cell recovery of $135 \pm 3 \times 10^5$ ($\pm$ SE, $n = 4$) were nonspecific esterase positive

cells (NSE+ve), representing the combined monocyte and macrophage marginated and interstitial cell populations. Thirty-nine percent of cells were identified as lymphocytes, with 30% neutrophils and 10% eosinophils. The remaining 6% of tissue-recovered cells could not be characterized and were assumed to be noninflammatory cells surviving the collagenase dispersion process. Twenty-four hours after d 5 of OVA exposure, total tissue recoverable cells were enhanced to $278 \pm 57 \times 10^5$ ($\pm$ SE, $n = 4$). After 2 daily exposures to OVA the lung lymphocyte population was increased and remained enhanced significantly 24 h following the fifth OVA exposure (Figure 1B). In contrast, tissue-recovered monocyte-macrophage

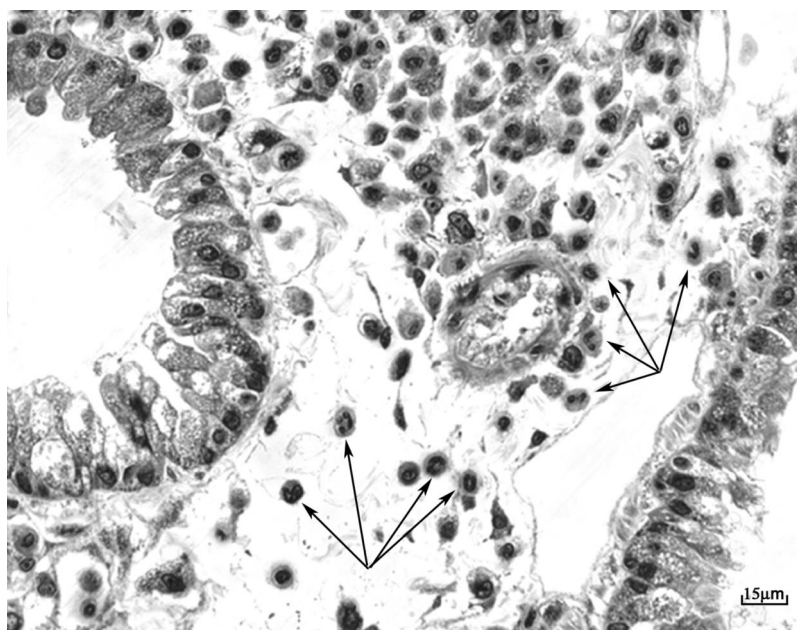


FIGURE 2. Light micrograph of a representative lung lesion obtained from 3 BALB/c mice 24 h following 5 daily exposures to OVA aerosols, indicating accumulation of inflammatory cells in the perivascular and peribronchial spaces with a selection of granulocytes indicated by arrows.

and neutrophil numbers were found to peak significantly after two daily OVA exposures, but returned to baseline levels after the fifth daily exposure (Figure 1B). Tissue eosinophil numbers were found to rise linearly with each subsequent exposure to OVA, beginning 24 h after the first OVA exposure (Figure 1B).

Although sections of mouse lung tissue 24 h following 5 daily OVA exposures demonstrated some granulocytes located within the conducting airway submucosa, the major accumulations of both granulocytes and mononuclear cells were observed in peribronchial and perivascular spaces of the lung (Figure 2). No inflammatory-cell accumulations were observed in the peripheral parenchymal regions of the lung.

Twenty-four hours following one, two, and five daily OVA exposures, mice were studied for their airway responses to methacholine as measured using the Penh index (Figure 3A). No marked changes in the response to increased methacholine were observed 24 h after the first (data not shown) and the second daily exposures to OVA. However, enhanced responses to methacholine were observed in mice 24 h after the fifth daily OVA exposure,

as indicated by greater delayed pauses in the breathing pattern (Penh) for each increasing concentration of methacholine (Figure 3A). In a similar experiment, airway resistance measurements with increasing methacholine aerosol challenges were also shown to be greatly enhanced in mice 24 h following the fifth daily exposure to OVA (Figure 3B). In this latter experiment, OVA-associated BAL eosinophil recoveries were found to be comparable with those demonstrated previously (Figure 1).

Intraperitoneal Drug Treatments

Although baseline lavage recoveries of inflammatory cells were unaffected by the use of ip administration of the methylcellulose dispersant, such treatments significantly enhanced the tissue collagenase lung recoveries of the monocyte-macrophage population (NSE+) from an untreated baseline mean value of $32.8 \pm 4.1 \times 10^5$ ($\pm$ SE, $n = 10$) to $52.1 \pm 5.9 \times 10^5$ cells ($\pm$ SE, $n = 4$) and recoveries of lymphocytes from $64.4 \pm 8.2 \times 10^5$ ($\pm$ SE, $n = 10$) to $85.6 \pm 7.6 \times 10^5$ cells ($\pm$ SE, $n = 4$). Further elevation in these dispersant-related tissue cell recoveries resulting from OVA exposures

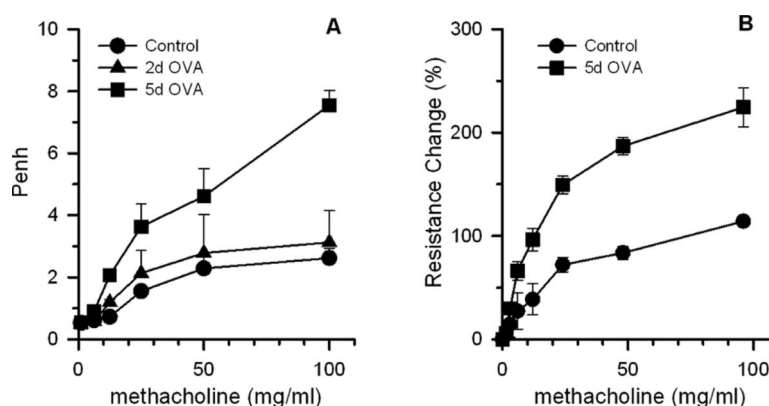


FIGURE 3. Results represent the mean values for: (A) Penh ($\pm$ SE, $n = 4$) obtained on exposing unanesthetized BALB/c mice to aerosols with increasing concentrations of methacholine, 24 h following 0 (control), 2, and 5 daily exposures to OVA aerosol; and (B) percent change in airway resistance ($\pm$ SE, $n = 3$) resulting from exposing ventilated anesthetized mice to similar increasing aerosol concentrations of methacholine, 24 h following 5 daily exposures to either saline or OVA.

TABLE 1. Lavage-Recovered Mononuclear Cells From Intraperitoneally Treated Mice

	Alveolar macrophages ($\times 10^5$ per lung)	Lymphocytes ($\times 10^5$ per lung)
Dispersant + saline ($n = 10$)	1.93 ± 0.21	0.03 ± 0.01
Dispersant + OVA ($n = 10$)	3.13 ± 0.58^a	0.33 ± 0.08^a
5 mg Methylprednisolone + OVA ($n = 7$)	1.72 ± 0.13^b	0.13 ± 0.04^b
10 mg Methylprednisolone + OVA ($n = 4$)	2.05 ± 0.16^b	0.06 ± 0.02^b
20 mg Methylprednisolone + OVA ($n = 4$)	2.32 ± 0.27	0.29 ± 0.18
20 mg Cyclosporin A + OVA ($n = 4$)	1.90 ± 0.19	0.14 ± 0.02

Note. Results represent the mean $\pm$ SE of cells ($\times 10^5$) recovered by bronchoalveolar lavage 24 h following 5 daily exposures to either saline or OVA aerosols, and following daily ip treatments with either dispersant (methylcellulose) alone or dispersant containing either 5, 10, or 20 mg/kg body weight methylprednisolone, or 20 mg/kg body weight cyclosporin A. It should be noted that the addition of either drug did not result in any significant effect on cell recoveries from saline-exposed animals (data not shown).

^aSignificantly different from saline-exposed mice treated with carrier alone with $p < .05$.

^bSignificantly different from dispersant-treated mice exposed to OVA with $p < .05$.

could not be demonstrated. Although tissue recoveries of neutrophils and eosinophils were unaffected by the dispersant treatments alone, the OVA-induced increased recovery of eosinophils was diminished by the dispersant-treatment to a mean value of $40.4 \pm 4 \times 10^5$ ($\pm$ SE, $n = 4$) from $88.5 \pm 12 \times 10^5$ cells ($\pm$ SE, $n = 10$).

The treatment of mice either with increasing doses of methylprednisolone (5, 10, or 20 mg/kg body weight) or with 20 mg/kg body weight cyclosporin A was conducted as two separate experimental series. Since data from dispersant-treated saline- and OVA-exposed mice were not significantly different between these two series, the results were combined for presentation purposes (Table 1 and Figure 4).

These ip drug treatments of saline-exposed mice did not significantly alter lavage and tissue inflammatory-cell recoveries when compared with mice treated with dispersant alone (data not shown). However, 24 h following 5 daily OVA exposures of dispersant-treated mice, lavage-recovered macrophages and lymphocytes demonstrated 1.6- and 10-fold increases, respectively, that were diminished by treatments with 5 or 10, but not 20, mg methylprednisolone (Table 1). OVA-induced increases in lavage recoveries of these cell types were also prevented by treatment with cyclosporin A (Table 1).

Daily ip treatments with 5 mg or 10 mg/kg body weight methylprednisolone and 20 mg/kg body weight cyclosporin significantly diminished

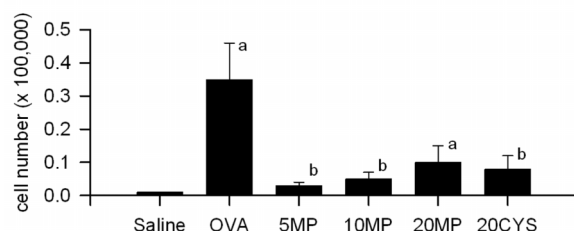
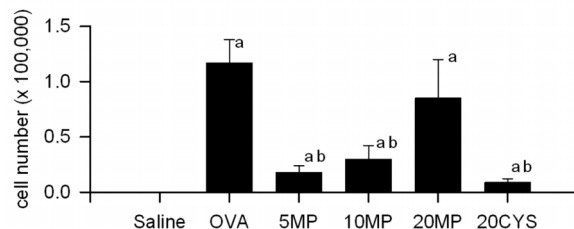
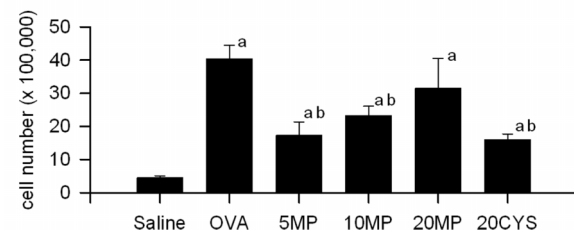
A. BAL-Recovered Neutrophils**B. BAL-Recovered Eosinophils****C. Tissue-Recovered Eosinophils**

FIGURE 4. Results represent the mean ($\pm$ SE, $n = 4-10$) recoveries of (A) neutrophils and (B) eosinophils by bronchoalveolar lavage (BAL), and (C) eosinophils by collagenase lung tissue dispersion, 24 h following 5 daily exposures of sensitized female BALB/c mice to either saline or OVA aerosols and daily ip treatments with dispersant or dispersant containing 5, 10, or 20 mg/kg body weight methylprednisolone (MP) or 20 mg/kg body weight cyclosporin A (CYS). Significantly different from saline-exposed dispersant-treated mice indicated by a and significantly different from corresponding OVA-exposed dispersant-treated mice indicated by b, with $p < .05$.

OVA-induced enhancements in neutrophils and eosinophils obtained by lavage (Figure 4, A and B). However, statistical analysis failed to demonstrate a significant decrease in lavage recovery of these granulocytes after administration with the higher 20-mg/kg body weight methylprednisolone dose. In contrast, there were no changes in tissue-recovered neutrophil numbers as a result of either OVA exposure or drug treatments when compared with a saline-exposed mean value of $51.1 \pm 4.0 \times 10^5$ ($\pm$ SE, $n = 10$) (data not shown). OVA-enhanced tissue recoveries of eosinophils were

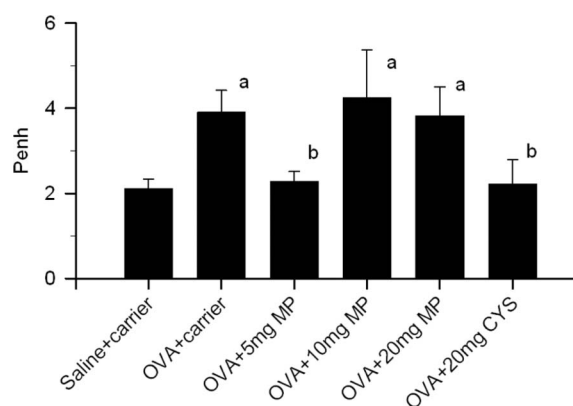


FIGURE 5. Results represents the mean ($\pm$ SE, $n = 4$) values for Penh obtained on exposure to 100 mg/ml methacholine of sensitized female BALB/c mice 24 h following 5 daily exposures to either saline or OVA aerosols and daily ip treatments with dispersant or dispersant containing 5, 10, or 20 mg/kg body weight methylprednisolone (MP) or 20 mg/kg body weight cyclosporin A (CYS). Significantly different from saline-exposed dispersant-treated mice indicated by a, and significantly different from corresponding OVA-exposed dispersant-treated mice indicated by b, with $p < .05$.

significantly reduced by 5 and 10 mg/kg body weight methylprednisolone and cyclosporin at 20 mg/kg body weight, but not by treatment with methylprednisolone at the 20-mg/kg body weight level (Figure 4C).

Some of the animals in this study were also screened for their responses to increasing concentrations of methacholine aerosols as illustrated by the mean Penh values obtained on exposure to 100 mg/ml methacholine (Figure 5). The twofold rise in Penh values resulting from OVA exposure of dispersant-alone-treated mice were significantly diminished in mice treated with 5 mg methylprednisolone and 20 mg cyclosporin A, but not in those mice treated with ip injection of 10 or 20 mg methylprednisolone (Figure 5). An additional experiment using direct methods to assess airway resistance in ventilated mice was also conducted to further demonstrate the effects of methylprednisolone treatment on OVA-associated enhanced airway reactivity to methacholine. Results demonstrated that as a result of exposure to a methacholine aerosol concentration of 100 mg/ml, saline-exposed control mice exhibited a mean rise in airway resistance of $126 \pm 6\%$ ($\pm$ SE, $n = 3$) and that in OVA-exposed carrier-treated mice

TABLE 2. Lavage-Recovered Mononuclear Cells from Transnasally Treated Mice

	Alveolar macrophages ($\times 10^5$ per lung)	Lymphocytes ($\times 10^5$ per lung)
Carrier + saline ($n = 8$)	2.83 ± 0.23	0.01 ± 0.01
Carrier + OVA ($n = 8$)	3.29 ± 0.30	1.27 ± 0.28^a
2.5 mg Methylprednisolone + OVA ($n = 6$)	3.51 ± 0.73	0.50 ± 0.25^a
5 mg Methylprednisolone + OVA ($n = 6$)	4.25 ± 0.59	0.68 ± 0.32^a
10 mg Methylprednisolone + OVA ($n = 4$)	3.64 ± 0.28	0.43 ± 0.18^a
15 mg Methylprednisolone + OVA ($n = 5$)	2.20 ± 0.41	0.68 ± 0.27^a

Note. Results represent the mean $\pm$ SE of cells ($\times 10^5$) recovered by bronchoalveolar lavage 24 h following 5 daily exposures to either saline or OVA aerosols, and following daily trans-nasal treatments with either carrier alone or carrier containing either 2.5, 5, 10, or 15 mg/kg body weight methylprednisolone. It should be noted that the addition of methylprednisolone did not result in any significant effect on cell recoveries from saline-exposed animals (data not shown).

^aSignificantly different carrier-treated mice exposed to saline with $p < .05$.

was elevated to a value of $263 \pm 18\%$ ($\pm$ SE, $n = 3$). This latter OVA-associated value was significantly reduced to a value of $188 \pm 18\%$ ($\pm$ SE, $n = 3$) by daily ip treatments of OVA-exposed mice with 5 mg/kg body weight methylprednisolone.

Transnasal Drug Treatments

Increases in cell recoveries as a result of the trans-nasal administration of the sterile isotonic saline carrier alone were observed only in alveolar macrophage and neutrophils, with mean values of $3.0 \pm 0.2 \times 10^5$ cells and $0.08 \pm 0.02 \times 10^5$ cells ($\pm$ SE, $n = 6$), respectively. No significant effects were observed in the tissue recoveries of inflammatory cells. Consistent with the preceding experiments, there were no additional effects on the lavage and tissue recoveries of inflammatory cells from the lungs as a result of the methylprednisolone treatments of saline-exposed mice (data not shown).

No OVA-induced increases were observed in lavage recoveries of alveolar macrophages beyond those associated with transnasal treatment with the carrier alone. In contrast, lavage recoveries of lymphocytes were enhanced by OVA to $1.3 \pm 0.3 \times 10^5$ cells ($\pm$ SE, $n = 8$), but not significantly diminished by any of the methylprednisolone treatments (Table 2). Lavage recoveries of neutrophils and eosinophils were increased by OVA exposures and diminished by transnasal treatments with methylprednisolone at 5 mg/kg body weight, but not at the higher dose of 15mg/kg body weight (Figure 6, A and B). As described earlier, no

methylprednisolone-induced changes could be observed in the tissue recovery of neutrophils, but eosinophil numbers were significantly diminished after 5- and 10-mg doses but not after 15 mg/kg body weight (Figure 6C).

In order to explore possible immune mechanisms to account for the reversal of methylprednisolone inhibition of allergen-induced eosinophilia at the higher repeated dose, lavage fluid samples from the transnasal experiments were analyzed for levels of IL-13 (Figure 7). Results demonstrated OVA-induced enhancement in IL-13 BAL-recovery was only abrogated following treatment with methylprednisolone at 5 mg/kg body weight, but not with higher doses.

DISCUSSION

The present study examined changes in inflammatory cell populations within the lung over a period of 5 d of repeated OVA exposure. Results were consistent with the present understanding of the sequence of events leading to allergen-induced lung inflammation (Birrell et al., 2003; Elenkov, 2004; Franchimont, 2004; Jaffar et al., 2004; Larche et al., 2003; Roberts et al., 1999; Temelkovski et al., 1998; Umetsu et al., 2003; Yimouyiannis et al., 1999). Although enhanced eosinophil recoveries were not observable in the BAL fluid until after 3 daily OVA exposures, tissue accumulations proceeded in a linear fashion throughout the 5 d of allergen exposure. In contrast, the marginated and interstitial tissue-recoverable pools of monocytes

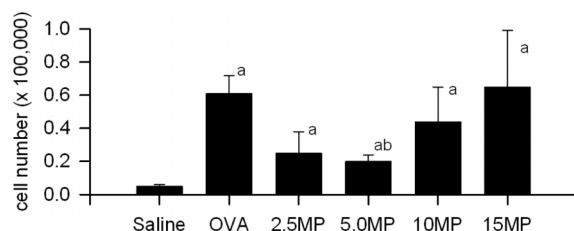
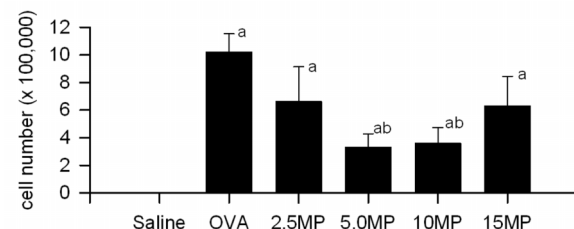
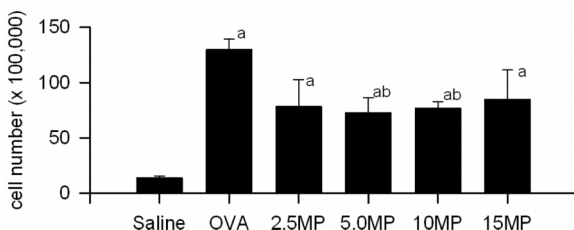
A. BAL-Recovered Neutrophils**B. BAL-Recovered Eosinophils****C. Tissue-Recovered Eosinophils**

FIGURE 6. Results from two experiments represent the mean ($\pm$ SE, $n = 4-8$) recoveries of (A) neutrophils and (B) eosinophils by bronchoalveolar lavage (BAL), and (C) eosinophils by collagenase lung tissue dispersion 24 h following 5 daily exposures of sensitized female BALB/c mice to either saline or OVA aerosols and daily transnasal treatments with carrier (control) or carrier containing 2.5, 5, 10, or 15 mg/kg body weight methylprednisolone (MP). Significantly different from saline-exposed carrier-treated mice indicated by *a*, and significantly different from corresponding OVA-exposed saline carrier-treated mice indicated by *b*, with $p < 0.05$.

and macrophages, neutrophils, and lymphocytes reached their maximum levels after the first 3 d, demonstrating that airway recovered inflammatory cells followed on from their prior accumulations in the combined tissue marginated and interstitial pools.

In this model of repeated allergen exposure, inflammatory-cell accumulations were located in peribronchial and perivascular spaces of the lung, supporting their proposed role in airway hyperreactivity. Consistent with previous studies exploring dexamethasone treatments of allergen exposures of rats (Huang

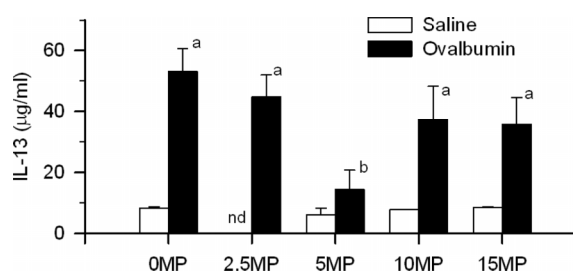


FIGURE 7. Results represent the mean ($\pm$ SE, $n = 3-4$) bronchoalveolar lavage fluid recoveries of IL-13 determined 24 h following 5 daily exposures of sensitized female BALB/c mice to either saline or OVA aerosols and daily transnasal treatments with carrier or carrier containing 2.5, 5, 10, or 15 mg/kg body weight methylprednisolone (MP). Significantly different from OVA-exposed carrier-treated mice indicated by *a* with $p < .05$ (nd, not determined).

et al., 1999) and mice (Birrell et al., 2003), the maximal methylprednisolone and cyclosporin A-induced decreases in BAL and tissue neutrophil and eosinophil recoveries observed in the present study were also associated with concomitant fall in Penh values in response to methacholine aerosol challenges. This delayed pause in breathing (Penh) index has been criticized as it does not provide a direct measure of changes in airway caliber, requiring corresponding measurements of flow and transpulmonary pressure for the assessment of reactivity to methacholine (Bates et al., 2004). However, in the present study, an additional experiment was conducted that utilized changes in airway pressure and flow during forced ventilation confirming the enhanced airway reactivity to methacholine of OVA-exposed mice previously indicated by the Penh method. This elevated airway reactivity to methacholine was similarly demonstrated to be diminished by the ip methylprednisolone treatments associated with a concomitant decrease in lung eosinophil accumulations.

In contrast, several investigations into the mechanisms of acute lung inflammation have failed to correlate enhanced BAL recoveries of neutrophils and eosinophils with concomitant hyperreactivity (Heuer, et al., 1994; Huang et al., 1999; Murlas & Roum, 1985). However, studies using antibody pretreatments against neutrophils- and eosinophils-derived major basic protein in rats and guinea pigs, respectively, where the

time course of tissue rather than BAL inflammatory cell accumulations was determined, have successfully correlated diminished granulocyte accumulations with decreased airway hyperreactivity (DeLorme et al., 2002; Yost et al., 1999; Evans et al., 1997). The possibility that subsets of T but not B lymphocytes might have a direct role in mediating allergen-induced airway hyperreactivity independently of granulocyte infiltration should also not be discounted in these types of investigation (Franchimont, 2004; Hahn et al., 2003; Maclean et al., 1999).

Five daily ip and transnasal treatments with 5 mg/kg body weight of methylprednisolone decreased OVA-induced lavage recoveries of all inflammatory cells, including lymphocytes, macrophages, neutrophils, and eosinophils. These observations are consistent with glucocorticoid-induced reduction in the generation of inflammatory mediators and adhesion molecules responsible for mobilization, endothelial marginalization, and subsequent migration of these cells into the lung. However, the failure to reduce allergen-induced eosinophil-associated lung inflammation following daily treatments with the higher doses of methylprednisolone in this study raises the possibility that other corticosteroid-induced mechanisms might have overridden these anti-inflammatory processes (Franchimont, 2004). Normal glucocorticoid enhancement of eosinophil apoptosis might also have been diminished (Sivertson et al., 2007).

The maintenance of relatively high levels of methylprednisolone throughout the 5 d of allergen-induced lung inflammation might have amplified OVA-induced adaptive immune responses (Elenkov, 2004; Franchimont, 2004). Glucocorticoids block IL-12 production by monocytes and dendritic cells and concomitantly increase macrophage and T-cell generation of IL-10 (Franchimont, 2004). Such a change in cytokine pattern might be expected to reduce T-cell differentiation to Th1 lymphocytes in favor of the generation of additional Th2 cells (Elenkov, 2004; Franchimont, 2004). Therefore, sustained enhancement of the Th2 cell-derived cytokine network

may be responsible for further allergen-induced eosinophil recruitment and retention in the lung.

Measurements of IL-13 have been used as an indicator of heightened Th2 cell lymphocyte activity (Elenkov, 2004; Franchimont, 2004; Padilla et al., 2005). IL-13 has been linked to the production of eotaxin-2 associated eosinophil infiltration, as well as being implicated in hyperreactivity by its possible direct action with smooth muscle (Eum et al., 2005; Pope et al., 2005). The observation that OVA-associated enhancement in BAL recovery of IL-13 was reduced at the daily 5-mg/kg body weight dose, but returned to OVA-stimulated rates at the higher methylprednisolone levels, is consistent with a sustained level of Th2 lymphocyte-driven eosinophil recruitment and retention (Franchimont, 2004). However, more complete analyses of both Th2 and Th1 cytokine networks at different times from the onset of OVA exposure would be needed to confirm this hypothesis, together with the establishment of whether or not methylprednisolone treatments enhance macrophage production of macrophage inhibitory factor (MIF) (Elenkov, 2004; Franchimont, 2004). MIF, previously identified with responses to endotoxin exposure, can exert pro-inflammatory effects on macrophages and T cells, as well as having the potential to override both the anti-inflammatory and immunosuppressive effects of glucocorticoids (Calandra & Roger, 2003; Franchimont, 2004). The possibility that sustained methylprednisolone treatment interferes with T-regulatory-cell activities normally associated with limiting the effects of Th2 cell activities also needs to be considered (Elenkov, 2004; Franchimont, 2004; Jaffar et al., 2004; Umetsu et al., 2003). Glucocorticoids have been shown to downregulate the GR α receptor responsible for mediating their action, providing another possible controlling feedback mechanism to explain the observed insensitivity to the higher levels of methylprednisolone in this study (Pujols et al., 2004, 2007).

In contrast to the complexity of glucocorticoid interactions, high levels of cyclosporin A treatment inhibited the OVA-induced

inflammation with a concomitant abrogation of airway responses to methacholine challenge. These results are consistent with direct downregulation of the adaptive immune system by cyclosporin A and its interference with several signaling pathways that include those responsible for the clone expansion of T lymphocytes and the reduction of the gene expression of pro-inflammatory cytokines (Larche et al., 2003; Khan et al., 2000).

The present study detailed a murine model of allergen-induced lung inflammation that uses relatively low daily exposures to allergen over a 5-d period. By examining both BAL and tissue-recoverable inflammatory cell populations, a more complete picture of lung inflammatory status could be achieved than using BAL alone. Results demonstrated that under conditions of continuing daily allergen exposure, cyclosporin A and low ip doses of the glucocorticoid methylprednisolone are effective in abrogating lung eosinophilia with a concomitant reduction of airway responses to methacholine. The reversal of this anti-inflammatory activity with sustained high doses of ip and transnasal glucocorticoid provides the basis for future investigations into how the anti-inflammatory effects of glucocorticoids might be compromised. Such a model might prove to be useful in further exploring inflammatory-cell dependence of airway hyperresponsiveness to bronchoconstrictor challenge and potential mechanisms to account for glucocorticoid insensitivity in several inflammatory disorders produced by environmental exposures including asthma.

REFERENCES

- Arbes, S. J., Gergen, P. J., Vaughn, B., and Zeldin, D. C. 2007. Asthma cases attributable to atopy: Results from the third National Health and Nutrition Examination Survey. *J. Allergy Clin. Immunol.* 120:1139–1145.
- Bates, J., Irvin, C., Brusasco, V., Drazen, J., Fredberg, J., Loring, S., Eidelman, D., Ludwig, M., Macklem, P., Martin, J., Milic-Emili, J., Hantos, Z., Hyatt, R., Lai-Fook, S., Leff, A., Solway, J., Lutchen, K., Suki, B., Mitzner, W., Paré, P., Pride, N., and Sly, P. 2004. The use and misuse of Penh in animal models of lung disease. *Am. J. Respir. Cell Mol. Biol.* 31:373–374.
- Birrell, M. A., Battram, C. H., Woodman, P., McCluskie, K., and Belvisi, M. G. 2003. Dissociation by steroids of eosinophilic inflammation from airway hyperresponsiveness in murine airways. *Respir. Res.* 4: 3–8.
- Britten, K. M., Howarth, P. H., and Roche, W. R. 1993. Immunohistochemistry on resin samples sections: A comparison of resin embedding techniques for small mucosal biopsies. *Biotechnic Histochem.* 68:271–280.
- Calandra, T., and Roger, T. 2003. Macrophage migration inhibitory factor: A regulator of innate immunity. *Nature Rev.* 3:791–800.
- Chand, N., Hess, F.G., Nolan, K., Diamantis, W., McGee, J., and Sofia, R. D. 1990. Aeroallergen-induced immediate asthmatic responses and late-phase associated pulmonary eosinophilia in the guinea pig: Effect of methylprednisolone and mepyramine. *Int. Arch. Allergy. Appl. Immunol.* 91:311–314.
- DeLorme, M. P., and Moss, O. R. 2002. Pulmonary function assessment by whole body plethysmography in restrained versus unrestrained mice. *J. Pharmacol. Toxicol. Methods* 47:1–10.
- DeLorme, M. P., Yang, H., Elbon-Copp, C., Gao, X., Barraclough-Mitchell, H., and Bassett, D. J. P. 2002. Hyperresponsive airways correlate with lung tissue inflammatory cell changes in ozone-exposed rats. *J. Toxicol. Environ. Health A* 65:1453–1470.
- Elenkov, I. J. 2004. Glucocorticoids and the Th1/Th2 balance. *Ann. NY Acad. Sci.* 1024:138–146.
- Eum, S.-Y., Maghni, K., Tolloczko, B., Eidelman, D. H., and Martin, J. G. 2005. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-15 and eotaxin on airway smooth muscle. *Am. J. Physiol.* 288:L576–L584.
- Evans, C. M., Fryer, A. D., Jacoby, D. B., Gliech, G. J., and Costello, R. W. 1997. Pre-treatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M₂ muscarinic receptors

- in antigen-challenged guinea pigs. *J. Clin. Invest.* 100:2254–2262.
- Ezeamuzie, C. I., and Nwankwoala, R. N. 2000. Allergen-induced bronchial eosinophilia in guinea-pigs is inhibited by both pre- and post-induction cyclosporin-A treatments. *Int. J. Immunopharmacol.* 22:515–522.
- Franchimont, D. 2004. Overview of the actions of glucocorticoids on the immune response—A good model to characterize new pathways of immunosuppression for new treatment strategies. *Ann. NY Acad. Sci.* 1024:124–137.
- Gilmour, M. I., Jaakkola, M. S., London, S. J., Nel, A. E., and Rogers, C. A. 2006. How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. *Environ. Health Perspect.* 114:627–633.
- Hahn, Y.-S., Taube, C., Jin, N., Takeda, K., Park, J.-W., Wands, J. M., Aydinoglu, M. K., Roark, C. L., Lahn, M., O'Brien, R. L., Gelfand, E. W., and Born, W. K. 2003. $V\gamma^{4+}$ $\gamma\delta$ T cells regulate airway hyperreactivity to methacholine in OVA-sensitized and challenged mice. *J. Immunol.* 171:3170–3178.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, G., Larsen, G. L., and Irvin, C. G. 1997. Noninvasive measurements of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766–775.
- Heuer, H. O., Wenz, B., Jennewein, H. M., and Urich, K. 1994. Dissociation of airway responsiveness and bronchoalveolar lavage (BAL) cell composition in sensitized guinea pigs after daily inhalation of OVA. *Clin. Exp. Allergy* 24:682–689.
- Holt, P. G., Degebrodt, A., Venaille, T., O'Leary, C., Krska, K., Flexman, J., Farrell, H., Shellam, G., Young, P., and Penhale, J. 1985. Preparation of interstitial lung cells by enzymatic digestion of tissue slices: Preliminary characterization by morphology and performance in functional assays. *Immunology* 54:139–147.
- Huang, T. J., Newton, R., Haddad, E. B., and Chung, K. F. 1999. Differential regulation of cytokine expression after allergen exposure of sensitized rats by cyclosporin A and corticosteroids: Relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* 104:644–652.
- Jaffar, Z., Sivakuru, T., and Roberts, K. 2004. $CD4^+CD25^+$ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J. Immunol.* 172:3842–3849.
- Kasserra, C. E., Harris, P., Steton, G. R., Abraham, W., and Langlands, J. M. 2004. IPL576,092, a novel anti-inflammatory compound, inhibits leukocyte infiltration and changes in lung function in response to allergen challenge. *Pulm. Pharmacol. Ther.* 17:309–318.
- Khan, L. N., Kon, O. M., Macfarlane, A. J., Meng, Q., Ying, S., Barnes, N. C., and Kay, A. B. 2000. Attenuation of the allergen-induced late asthmatic reaction by cyclosporin A is associated with inhibition of bronchial eosinophils, interleukin-5, granulocyte macrophage colony-stimulating factor, and eotaxin. *Am. J. Respir. Crit. Care Med.* 162:1377–1382.
- Larche, M., Robinson, D. S., and Kay, A. B. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 111:450–463.
- Li, C. Y., Lam, K. W., and Yam, L. T. 1973. Esterases in human leukocytes. *J. Histochem. Cytochem.* 21:1–12.
- Maclean, J. A., Sauty, A., Luster, A., Drazen, J. M., and DeSanctis, G. T. 1999. Antigen-induced airway hyperresponsiveness, pulmonary eosinophilia, and chemokine expression in B-cell deficient mice. *Am. J. Respir. Cell Mol. Biol.* 20:379–387.
- Murai, A., Abe, M., Hayashi, Y., Sakata, N., Katsuragi, T., and Tanaka, K., 2005. Comparison study between the mechanisms of allergic asthma amelioration by a cysteinyl-leukotriene type 1 receptor antagonist montelukast and methylprednisolone. *J. Pharmacol. Exp. Ther.* 312:432–440.
- Murlas, C. G., and Roum, J. H. 1985. Sequence of pathologic changes in airway mucosa of guinea pigs during ozone-induced bronchial hyperreactivity. *Am. Rev. Respir. Dis.* 131:314–320.

- Padilla, J., Daley, E., Chow, A., Robinson, K., Parthasarathi, K., McKenzie, A. N. J., Tschernig, T., Kurup, V. P., Donaldson, D. D., and Grunig, G. 2005. IL-13 regulates the immune response to inhaled allergens. *J. Immunol.* 174:8097–8105.
- Plopper, C. G., Chang, A. M., Pang, A., and Buckpitt, A. R. 1991. Use of microdissected airways to define metabolism and cytotoxicity in murine bronchiolar epithelium. *Exp. Lung Res.* 17:197–212.
- Pope, S. M., Filkerson, P. C., Blanchard, C., Akei, H. S., Nikolaidis, N. M., Zimmerman, N., Molkentin, J. D., and Rothenberg, M. E. 2005. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. *J. Biol. Chem.* 280:13952–13961.
- Pujols, L., Mullol, J., Torrego, A., and Picado, C. 2004. Glucocorticoid receptors in human airways. *Allergy* 59:1042–1052.
- Pujols, L., Mullol, J., and Picado, C. 2007. Alpha and beta glucocorticoid receptors: relevance to airway disease. *Curr. Allergy Asthma Rep.* 7:93–99.
- Roberts, K., Jaffar, Z. H., Semper, A. E., and Holgate, S. T. 1999. Asthma: Clinical aspects and mucosal immunity. In *Mucosal immunology*, eds. P. L. Ogra, M. E. Lamm, J. Bienenstock, J. Mestecky, W. Strober, and W. McGhee, Jr., pp. 1197–1211. San Diego: Academic Press.
- Schultheis, A. H., and Bassett, D. J. 1994. Guinea pig lung inflammatory cell changes following acute ozone exposure. *Lung* 172:169–181.
- Sivertson, K. K., Seeds, M. C., Long, D. L., Peachman, K. K., and Bass, D. A. 2007. The differential effect of dexamethasone on granulocyte apoptosis involves stabilization of Mcl-1L in neutrophils but not in eosinophils. *Cell Immunol.* 246:34–45.
- Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S., and Kumar, R. K. 1998. An improved murine model of asthma: Selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolized allergen. *Thorax* 53:849–856.
- Umetsu, D. T., Akbari, O., and Dekruyff, R. H. 2003. Regulatory T cells control the development of allergic disease and asthma. *J. Allergy Clin. Immunol.* 112:480–487.
- Underwood, S. L., McMillan, S., Reeves, R., Hunt, J., Brealey, C. J., Webber, S., Foster, M., and Sargent, C. A. 2001. Effects of cyclosporin A administered into the airways against antigen-induced airway inflammation and hyperreactivity in the rat. *Eur. J. Pharmacol.* 420:165–173.
- Whitehead, G. S., Walker, J. K. L., Berman, K. G., Foster, W. M., and Schwartz, D. A. 2003. Allergen-induced airway disease is mouse strain dependent. *Am. J. Physiol.* 285:L32–L42.
- Yiamouyiannis, C. A., Schramm, C. M., Puddington, L., Stengel, P., Baradaran-Hosseini, E., Wolyniec, W. W., Whiteley, H. E., and Thrall, R. S. 1999. Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. *Am. J. Pathol.* 154:1911–1921.
- Yost, B. L., Gleich, G. J., and Fryer, A. D. 1999. Ozone-induced hyperresponsiveness and blockade of M₂ muscarinic receptors by eosinophil major basic protein. *J. Appl. Physiol.* 87:1272–1278.