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RECURRING BALB/C MOUSE LUNG INFLAMMATORY RESPONSES TO EPISODIC ALLERGEN EXPOSURE

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Abstract

This study detailed the sequence of recurring inflammatory events associated with episodic allergen exposures of mice resulting in airway hyperreactivity, sustained inflammation, goblet cell hyperplasia, and fibrogenesis that characterize a lung with chronic asthma. Ovalbumin (OVA)-sensitized female Balb/c mice were exposed to saline-control or OVA aerosols for 1hr per day for episodes of 3 days every week for up to 8 weeks. Lung inflammation was assessed by inflammatory cell recoveries using bronchoalveolar lavages (BAL) and tissue collagenase dispersions. Cell accumulations were observed within airway submucosal and associated perivascular spaces using immunohistochemical and tinctorial staining methods. Airway responsiveness to methacholine aerosols were elevated after 2 weeks and further enhanced to a sustained level after the 4th and 8th weeks. Although by the 8th week, diminished OVA-induced accumulations of eosinophils, neutrophils and monocyte-macrophages were observed, suggesting diminished responsiveness, the BAL recovery of lymphocytes remained elevated. Airway but not perivascular lesions persisted with a proliferating cell population, epithelial goblet cell hyperplasia and evidence of enhanced collagen deposition. Examination of lung inflammatory cell content before the onset of the 1st, 2nd and 4th OVA exposure episodes demonstrated enhancements in residual BAL lymphocyte and BAL and tissue eosinophil recoveries with each exposure episode. Although tissue monocyte-macrophage numbers returned to baseline prior to each exposure episode, the greatest level of accumulation was observed after the 4th week. These results provide the basis for establishing the inflammatory and exposure criteria by which episodic environmental exposures to allergen might result in the development of a remodeled lung in asthma.

Keywords

ovalbumin; airway reactivity; eosinophils; neutrophils; lymphocytes; macrophages

INTRODUCTION

Early investigations into the immunological mechanisms and inflammatory cell recruitments associated with acute asthmatic responses have utilized a range of experimental animal

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model systems based on short-term relatively high level inhalation exposures of sensitized animals to experimental allergens such as ovalbumin (OVA) (Bassett, et al., 2010; Evans et al., 1997; Hamada et al., 1999; Whitehead et al., 2003). Over the last decade there has been considerable interest in using animal allergen exposure models to explore the determinants of the chronic asthmatic condition with emphasis being placed on understanding the interrelationships between allergen-induced inflammation, hyperplasia of mucus-secreting goblet cells, airway extracellular matrix thickening, angiogenesis, and sustained hyperreactivity to bronchoconstrictor challenge (Brodie 2008; Epstein, 2004; Kenyon et al., 2003; Kips et al., 2003; Kumar and Foster, 2002; Locke et al., 2007; Shinagawa and Kojima 2003; Singh et al., 2008; Southam et al., 2007). An understanding of the pathogenesis of asthma is prerequisite to identifying risk factors, improving management and providing a better understanding of how exposures to environmental pollutants such as allergens, viruses, particulates, and irritant gases might cause or exacerbate asthma (Gilmour et al., 2006; Kelly and Fussell, 2011; Holgate, 2011; Ghio et al., 2012; Milligan et al., 1998). The development of suitable experimental animal models to explore how repeated allergen exposures might lead to chronic asthma has been confounded by either the early onset of tolerance or the development of extensive lesions in both airway and lung parenchyma, more characteristic of a lung with allergic alveolitis and related conditions (Kumar and Foster, 2002; Temelkovski et al., 1998). However, these limitations have been shown to be readily overcome by the use of an episodic aerosol allergen exposure regimen that utilizes relatively low levels of allergen exposure of mice 3 days a week for up to 8 weeks (Kumar and Foster, 2002; Temelkovski et al., 1998), an approach that has been utilized effectively by other investigators (Locke et al., 2007; Kenyon et al., 2003; Sakai et al., 2001; Shinagawa and Kojima, 2003). Results demonstrated sustained inflammatory lesions restricted to the airway epithelial sub-mucosa rather than to the peripheral alveolar regions of the lung. The question of whether or not there are recurring and changing patterns of inflammatory cell accumulations with each weekly exposure to allergen over the extended time of airway remodeling, has yet to be extensively explored. Therefore, the present investigation utilized this model to describe the extent and pattern of weekly inflammatory cell accumulations as a first step in improving our understanding of how an episodic allergen exposure regimen that more realistically represents the human experience, might lead to the previously observed remodeling of the airways. Expanding the usefulness of this murine model will enhance the study of the inflammatory and exposure determinants by which episodic allergen and other air pollutant exposures might induce or contribute to development of airway remodeling associated with the chronic asthmatic condition (Holgate, 2011).

Although some investigators utilizing this model system used morphometric methods to correlate allergen-induced lung inflammation with airway remodeling (Temelkovski et al., 1998), others relied on the bronchoalveolar lavage (BAL) recovery of cells (Locke et al., 2007; Kenyon et al., 2003; Sakai et al., 2001; Shinagawa and Kojima, 2003), with less emphasis being placed on quantifying interstitial and marginated inflammatory cell contents and on the qualitative assessment of cell populations within inflammatory lesions. Using this previously reported mouse model of episodic allergen exposure (Temelkovski et al., 1998), the present investigation focused on establishing the inflammatory cell contents before and after weekly episodes of allergen exposures using both BAL and collagenase tissue dispersion techniques (Bassett et al., 2010). Macrophages were shown to be involved in interstitial structural changes (Homer et al. 2011), and both neutrophil and eosinophil infiltrations were implicated as contributing to lung damage and enhanced airway reactivity to bronchoconstrictor challenge (DeLorme et al., 2002; Evans et al., 1997; Schultheis et al., 1994). Therefore by using immunohistochemical staining procedures, this study also focused on identifying the lung sites of accumulation of specific inflammatory cell populations at different stages in the development of a mouse lung with many of the characteristics observed in human chronic asthma.

Sensitized adult Balb/c female mice were exposed for 1hr a day, 3 days a week for up to 8 weeks to OVA aerosol. A combination of BAL and collagenase tissue dispersions was used to assess both airway and tissue inflammatory cell accumulations that together with immunohistochemical and tinctorial staining of tissue sections, provided results that correlated changes in airway structure and responsiveness to bronchoconstrictor challenge with the presence and location of macrophages, lymphocytes, neutrophils and eosinophils. These cells were previously implicated in airway epithelial damage and proliferate repair processes, fibrogenesis, and non-specific airway hyperreactivity.

METHODS

Animals

The exposure model on which this study was based used female rather than male Balb/c mice (Temelkovski et al., 1998). Therefore, specific pathogen free female Balb/c mice weighing 20–25g, obtained from Hilltop Laboratory Animals Inc. (Scottsdale, PA) were used in this study. Mice were maintained in high efficiency particulate filtered air on low dust corn cob bedding with free access to food and water. After 7 days of acclimatization within the animal facilities, all mice were sensitized by intraperitoneal injections of 10 μ g OVA (Grade V, Sigma Chemical Co., St. Louis) and 2 mg aluminum hydroxide suspended in 0.2 ml of sterile isotonic saline (Bassett et al., 2010). Animals were injected on day 1 and day 14. Inhalation exposure experiments were initiated after day 21 from the first OVA treatment. Mice were exposed to sterile isotonic saline or OVA (1% w/v in sterile isotonic saline) aerosols in nose-only exposure systems (CH Systems, New York) as previously described (Bassett et al., 2010). Three overlapping experimental series were conducted with episodic exposures for periods of 1hr per day 3 days per week for up to 8 weeks with analyses conducted after 1, 2, 4, and 8 weeks (Figure 1). Analyses were conducted either before weekly exposure episodes or 24hr following the last exposure of a weekly episode. The first series of experiments examined the effects of 4 and 8 weekly cycles of exposure with a second series conducted to examine lungs after 2 weekly episodes of saline or OVA exposures, providing evaluation of airway responses to bronchoconstrictor challenge, inflammatory cell content and histopathology (Figure 1). A third series of exposures were conducted that examined lung inflammatory cell contents after the first weekly exposure episode and before and after the second and fourth episodic exposures (Figure 1). The protocols for this project were approved by the Institutional Animal Care and Use Committee (IACUC) and all procedures meet 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).

Airway Responsiveness

Prior to evaluation of inflammatory cell contents and histopathology, mice were screened for their breathing responses to increasing aerosol concentrations to the bronchoconstrictor methacholine using whole body plethysmographs, equipped with pneumotachographs, pressure transducers, and a computer data acquisition system (Buxco Research Systems, Wilmington, NC). Airway responsiveness to methacholine concentration was indicated by changes in the breathing pattern as determined by analysis of the plethysmograph pressure waveform that generates an empirical measure of the enhanced pause in expiration (Penh), previously used as a surrogate measure of changes in airway resistance (DeLorme and Moss, 2002; Hamelmann et al. 1997; Sakai et al., 2001; Whitehead et al. 2003). Following 30 min of acclimatization, mice were exposed for 2 min to aerosols with increasing concentrations of methacholine (0–200mg/ml w/v in isotonic sterile saline) with 5 min recovery between each concentration during which time Penh measurements were averaged. After a minimum

of 30 min for recovery, animals were anesthetized (60 mg/kg bdy. wt. sodium pentobarbital) and then analyzed for lung inflammatory cell content.

Assessment of Lung Inflammation

Ventilated lungs of anesthetized mice were first perfused with warm isotonic saline (37°C) via the pulmonary artery to remove blood components prior to extensive BAL with an initial 1ml phosphate-buffered saline (PBS) (pH 7.4) and then with 5 additional lavages with 1ml PBS containing 3 mM EDTA to ensure maximal recovery of phagocytic cells (Bassett et al., 2010). The combined cell populations were then subjected to differential counting using modified Wright's staining for the identification of macrophages, lymphocytes, neutrophils and eosinophils. Results were reported as total BAL-recovered cells. The remaining parenchymal tissue was then subjected to collagenase dispersion as previously described (Bassett et al., 2010; Holt et al., 1985; Schultheis et al., 1994). A total cell count was conducted and differential cell analyses performed after separate staining with modified Wright's stain for neutrophils, eosinophils and lymphocytes, and non-specific esterase (NSE) staining for the identification of the combined monocyte and macrophage population (Li et al., 1973). More detailed analysis of collagenase tissue-recovered lymphocytes was achieved by flow cytometric methods following pooling and purification using a single step Percoll[®] gradient of the remaining tissue-recovered cells. Lymphocytes were single- or double-stained using FITC- and/or PE-stained mouse monoclonal antibodies for B-cells CD19-FITC (1D3, rat Ig2a), and for T-cells with antibodies to CD3e-FITC (2C11, hamster IgG), with either CD4-PE (GK1.5, rat IgG2b) or CD8a(Ly-2)-PE (53-6.7, rat IgG2a) (Becton Dickinson, PharMingen, CA). Analyses were conducted in triplicate using a flow cytometer (FACSCaliber, Becton Dickinson) counting a minimum of 20,000 events with CellQuest[®] software. The lymphocyte cell population was isolated using electronic gating based on forward and side light scattering plots with identification confirmed by examination of stained cells isolated by the cell sorter feature of the flow cytometer.

Histopathology

Non-lavaged lungs for paraffin wax embedding were fixed using a constant volume method by intratracheal instillation of 1ml paraformaldehyde (2% w/v) in 0.1M cacodylate buffer pH 7.4 prior to incubation overnight in fixative. Whole mouse lungs were then embedded in paraffin wax by standard dehydration and infiltration procedures. Groups of 15 4µm thick serial sections were cut at 500µm intervals throughout the whole lung. One section from each group was stained with hematoxylin and eosin. Immunohistochemical staining for different mouse inflammatory cell populations was conducted using the streptavidin biotin-peroxidase (SABP) technique. See table 1 for details of the primary antibodies and antigen retrieval methods used. Prior to incubation with the primary antibody, endogenous peroxidase was blocked with 0.03% hydrogen peroxide in methanol, endogenous avidin/biotin interactions were blocked using avidin biotin blocking kits (Vector Labs, Peterborough, UK), and non-specific protein interactions were blocked using a solution of Dulbecco's essential medium (DMEM) containing 20% fetal calf serum and 1% bovine serum albumin. Following overnight incubation with the primary antibodies at 4°C, sections were incubated with an appropriate biotinylated secondary antibody and the SABP complexes. Finally, sections were incubated with the peroxidase substrate diaminobenzidine (DAB) to yield a brown reaction product at the site of antigen:antibody interactions within a background counter stained with Mayers hematoxylin. In addition, sections were stained with periodic acid Schiff (PAS) technique to identify airway epithelial goblet cell mucins, and with martius scarlet blue (MSB) trichrome staining for extracellular matrix collagen and fibrin. Collagen was indicated in non-lavaged lungs by measurements of whole lung hydroxyproline (OHproline) content using previously described methods (Choi et al., 1994).

Statistical Analyses

Data from these experiments were analyzed by either Student's *t* test for comparison of two unpaired means or by one way analyses of the variances for comparisons of several means with controls using Dunnett's test and a level of significance of $p < 0.05$. Logarithmic transformations of the data were conducted on a routine basis when variances were found to be heterogeneous by Hartley's test.

RESULTS

Airway Responsiveness to Methacholine

Saline- and OVA-exposed mice were screened for their responsiveness to aerosol inhalation of the bronchoconstrictor methacholine, as determined by measurements of delayed pause in breathing (Penh). No significant differences were observed in saline-exposed control mice from different series of experiments conducted over the 8 week period and thus data were combined in Figure 2. Results demonstrated that 24hr following the 2nd weekly episode of OVA exposures, enhanced responses to methacholine challenge were observed, as indicated by significant increases in the mean Penh values at methacholine aerosol concentrations of greater than 50mg/ml, when compared with corresponding values obtained from saline-exposed control mice (Figure 2). A greater response to methacholine challenge was observed after 4 weekly cycles of OVA that was sustained in mice exposed for 8 weekly episodes of OVA exposure (Figure 2).

Inflammatory Cell Accumulations

The inflammatory state of lungs was indicated in this study by the recovery of inflammatory cells from both extensive bronchoalveolar lavage (BAL) and collagenase dispersion of the remaining parenchymal tissue (Table 2, Figure 3 and 4). The mean BAL-recovery of cells from the lungs of saline control mice were identified as being predominantly alveolar macrophages with the occasional recovery of lymphocytes and neutrophils that represented no more than 0.5% of the total BAL-recovered cell populations. Eosinophils and epithelial cells were not observed in the lavages of these control mice (Table 2). In contrast, cells recovered by collagenase dispersions of control mice demonstrated that 20% could be identified as non-specific esterase positive monocytes and macrophages, 50% as lymphocytes, 26% as neutrophils and 3% as eosinophils (Table 2). Unidentifiable cell numbers ranged from 3 to 8% of the total recovered tissue cells and were assumed to be either epithelial or mesenchymal cells that survived the collagenase tissue dispersion treatment. BAL recoveries of total inflammatory cells were lower in saline-exposed control mice after the 2nd and 4th weekly episodic exposures to saline (Table 2). Total tissue recoveries were also observed to be reduced after 4 weekly episodic cycles (Table 2), accounted for by decreased numbers of recoverable neutrophils and lymphocytes (Table 2). These differences most likely reflected minor baseline differences between the mouse cohorts utilized in this study as opposed to time dependent changes over the 8 weeks of repeated exposures to saline.

Although BAL and tissue recoveries of inflammatory cells 24h following the 1st, 2nd and 4th weekly episodes of OVA exposure were significantly enhanced when compared with corresponding saline-exposed control levels, these enhancements were no longer evident by the end of the 8th weekly episode (Table 2). The measurements of cell recoveries from mouse lungs prior to the 2nd and 4th weekly exposures to OVA demonstrated that BAL inflammatory cell numbers did not return to saline control levels prior to subsequent 3 days of OVA exposure. This observation contrasted with the return to saline control levels in the tissue recoverable inflammatory cell pools between weekly exposures to OVA (Table 2), indicating some inflammatory cell persistence within the airways prior to the next weekly

OVA exposure episode. Differential analyses of these BAL and tissue recovered cell populations provided a more detailed picture of the relative changes in the individual inflammatory cell populations over the 8 weeks of the study (Figures 3 and 4).

Neutrophils were not significantly recoverable by BAL in saline-exposed mice, indicating that saline exposures alone did not result in any inflammation of the airways (Figure 3). In contrast, relatively high numbers were recovered by tissue collagenase dispersion at all time points, reflecting the previously observed high levels of mouse lung interstitial and marginated neutrophils that are not detectable by BAL-sampling alone (Figure 3)(Bassett et al., 2010). Similar numbers of OVA exposure-induced neutrophils were consistently recovered by BAL in mice after 1, 2, and 8 weekly episodes as well as prior to the 2nd and 4th weekly OVA exposures. However, the 1st weekly exposure resulted in a significant decrease in tissue recoverable neutrophils. After the 4th weekly exposure, additional increases in neutrophil recoveries by both BAL and tissue collagenase dispersion were observed, indicating an enhanced level of OVA-induced neutrophil recruitment at this time point that was no longer evident after the 8th week (Figure 3).

The major feature of weekly OVA-induced lung inflammation was the enhanced recoveries of both BAL and tissue recoverable eosinophils observed after the first 4 weekly OVA exposure episode. During this time period, these eosinophil levels did not return to saline control levels prior to the following OVA exposure episode (Figure 3). Although after the 8th weekly episode eosinophil recoveries were markedly reduced compared with the 1st weekly episode to OVA, the levels of BAL and tissue-recovered eosinophils remained significantly elevated when compared with corresponding saline control values (Figure 3).

BAL-recoveries of alveolar macrophages were found to be significantly enhanced after the 1st episodic exposure to OVA with elevated levels remaining significantly increased prior to the 2nd week of exposures when compared with recoveries from corresponding saline-exposed control mice (Figure 4). Subsequent weekly exposure episodes demonstrated reduced rather than elevated BAL recoveries (Figure 4). However, the 1st, 2nd and 4th weekly episodes of OVA exposure significantly enhanced the tissue-recoverable non-specific esterase positive (NSE +ve) cell population, representing the combined tissue monocytes and macrophage populations. These values were observed to be greatest after the 4th weekly OVA exposure episode, representing a 3.5- fold enhancement over saline control values that returned to below saline-control values following the 8th weekly episode (Figure 4). It is interesting to note that unlike the above granulocytes, the tissue monocyte/macrophage pools were found not to have accumulated above baseline control values prior to each weekly exposure to OVA prior to the 2nd and 4th weekly exposures (Figure 4).

Following the 2nd weekly cycle of OVA exposures, mouse lungs demonstrated a consistent increase in BAL recoverable lymphocytes that was also observed to be significantly elevated between the onset of the 2nd and the 4th weekly exposures (Figure 4). This appearance of lymphocytes in the BAL fluid recoveries was still elevated after 8 weekly episodes of OVA exposure (Figure 4). Significant changes in the relatively high baseline level of tissue-recoverable lymphocytes could only be detected after the 2nd and 4th weekly exposure episodes (Figure 4). In contrast, flow cytometric analyses of lymphocytes recovered from collagenase tissue dispersions after the 4th and 8th weekly episodes of OVA exposure, indicated relative changes in lymphocyte subpopulations. The % of total lymphocytes that stained positively for CD19⁺ (B cells) was found to be unchanged after the 4th episode of ovalbumin exposure when compared with a mean saline control value of 22.9 ± 2.4 % ($\pm$ SEM, n=4), but was found to be substantially enhanced to 31.6 ± 0.2 % after the 8th week of exposure. In contrast, after the 4th week of OVA exposure, the relative number of tissue-recovered lymphocytes identified as being CD3⁺ (T cells) was enhanced from a saline

control value of 33.3 ± 1.9 to 45.9 ± 1.3 % ($\pm$ SEM, $n=4$). This enhanced value was reduced to the lower level of 39.8 ± 0.6 % ($\pm$ SEM, $n=4$) after 8 weeks. The proportion of recovered $CD3^+$ lymphocytes identified as being $CD4^+$ was greatly enhanced after 4 weeks with a rise in the $CD4^+/CD8^+$ ratio to 7.5 from a saline control value of 3.3 that after 8 weeks of episodic OVA exposures returned to a value of 3.9. Data suggested that after 4 weeks OVA exposure, the major changes in $CD3^+$ T helper cells observed were accounted for by a relative increase in $CD4^+$ rather than $CD8^+$ cells; and although the relative level of $CD4^+$ T-lymphocytes was returning to normal levels by 8 weeks, the relative number of $CD19^+$ B-lymphocytes remained elevated.

Histopathology

Having established quantitative measures of OVA-induced lung inflammation, it was important to ascertain the location and characterization of inflammatory lesions. Serial sections of paraffin-wax embedded mouse lungs following 2, 4 and 8 weekly episodes OVA exposure were compared with those from saline-exposed control mice. The above whole lung evaluations of inflammatory cell accumulations were consistent with focal inflammatory cell accumulations within the lungs of mice exposed for 2 and 4 weekly episodes of OVA exposure. Lesions were only located in OVA-exposed mice in bronchial-associated lymphoid tissue (BALT) connected to perivascular spaces in close association with major conducting airways as demonstrated by representative photomicrographs in both Figures 5 and 6. No consolidated lesions were observed in the distal parenchymal regions of the lung. Although the overall extent of these airway associated inflammatory lesions was diminished after 8 weekly episodes of OVA exposure, airway sub-mucosal lesions were still observable (Figures 5C, 6D and 6H). However, the extensive inflammatory cell accumulations within perivascular spaces observed after 2 and 4 weekly episodes of OVA exposure (Figures 5B, 6C and 6G) had mainly disappeared after 8 weeks (Figures 6D and 6H).

At all time points, inflammatory lesions demonstrated cells undergoing proliferation as indicated by positive staining with an antibody against Ki67 (Figure 5A–C). $CD3^+$ T-lymphocytes populations were also observed within these accumulations (Figure 5 D–F). Positive immunostaining for these lymphocytes was also seen throughout the alveolar regions of the lung in both control and OVA-exposed mouse lungs, consistent with the substantial parenchymal pool of lymphocytes recovered by collagenase dispersion of lavaged lungs (Figure 4).

Staining for the tissue macrophage population using the CD68 antibody demonstrated their alveolar location in both control and OVA-exposed lungs (Figure 5G). Major concentrations of $CD68^+$ staining cells were observed within the perivascular inflammatory lesions after both the 2nd and 4th weekly episodes of OVA exposure (Figure 5H). A few $CD68^+$ staining cells were also observable within the airway sub-mucosal lesions at all time points. However, inflammatory cell accumulations within perivascular spaces were no longer prominent after 8 weeks of OVA exposures (Figures 5C, 6D and 6H). The previously noted rise in tissue recoverable monocytes and macrophages over the 4 weekly exposures to OVA with a subsequent return to control levels after 8 weeks (Figure 4), were consistent with the perivascular spaces being a major lung tissue site of their accumulation (Figure 6H).

The observed parenchymal staining for neutrophils based on the use of an antibody against myeloperoxidase (MPO), was seen throughout the parenchyma of both saline and OVA-exposed mouse lungs (Figure 5K), consistent with the relatively high number of neutrophils recovered by tissue collagenase dispersion (Figure 3). However, at all time points from the onset of OVA exposure, no MPO-positive staining cells were observed within the airway sub-mucosal or perivascular inflammatory lesions (Figures 5J and 5L). In contrast, staining

for eosinophils using an antibody against major basic protein (EMBP) demonstrated major accumulations within both airway mucosal and perivascular inflammatory lesions after 2 and 4 weekly exposures to OVA (Figures 5M and 5N). After 8 weeks, the density of eosinophil staining within the remaining airway lesions was markedly diminished, consistent with the return towards saline-control levels in the number of eosinophils recovered by tissue collagenase dispersion (Figures 3 and 5O).

PAS-positive staining of airway epithelium indicated the presence of mucin-containing goblet cells that were not readily observable in control lungs (Figure 6A), but found in the epithelium of major conducting airways following both 2 and 4 weekly OVA exposure cycles (Figures 6B and 6C). Although such staining was still evident in lungs following the 8th OVA exposure episode, the density and number of airways affected appeared diminished (Figure 6D). At all time points, although PAS-positive stained cells from OVA-exposed mice were predominantly associated with airway regions of inflammatory cell accumulation, staining in non-affected epithelial regions were also observed.

Examinations of comparable sized airways were unable to demonstrate any major thickening of the underlying epithelial MSB-stained basement membrane collagen following 2 and 4 weeks of OVA exposure compared to saline-exposed controls (Figures 6E and 6F). However, increased staining for collagen was observable within the perivascular spaces at these two time points, associated with accumulations of inflammatory cells (Figure 6G). Following 8 weeks of OVA exposure, areas of collagen staining were observed within the airway walls, but mainly associated with perivascular spaces that were relatively free from the inflammatory cell accumulations observed at earlier time points (Figure 6H). OVA-exposure associated accumulation of collagen was further demonstrated by measurements of whole lung hydroxyproline (OHproline) that progressively increased from a saline control mean value of 21.7 ± 1.7 to values of 26.6 ± 1.8 and 28.6 ± 0.7 mg OHproline per lung ($\pm$ SE, $n=4$) after the 2nd and 4th weeks of OVA exposure, respectively, and reaching a statistically significant elevated value of 29.1 ± 1.93 ($\pm$ SE, $n=4$) by week 8.

DISCUSSION

The main focus of this study was to characterize the inflammatory responses and consequences of repeated three days per week episodes of allergen exposure of sensitized mice over a period of 8 weeks. By employing both traditional BAL and collagenase dispersion techniques in combination with immunopathological evaluations of inflammatory lesions, it was possible to map the extent and location of inflammatory cell accumulations in the lung prior to and after weekly episodic exposures. As demonstrated by others employing this or similar exposure regimens, inflammatory cell accumulations were restricted to airway submucosal and associated perivascular spaces with no major accumulations in the distal parenchymal lung (Collins et al., 2003; Locke et al., 2007; Kenyon et al., 2003; Hamada et al., 1999; Temelkovski et al., 1998; Sakai et al., 2001; Shinagawa and Kojima, 2003; Van Hove et al., 2009). Although weekly cycles of OVA aerosol exposure resulted in eosinophil, lymphocyte and monocyte/macrophage accumulations after the 2nd and 4th weekly cycles, by the 8th week, these inflammatory cell populations were diminished, suggesting the onset of unresponsiveness to the effects of repeated allergen exposure by this latter time point. However, residual inflammatory cell accumulations were observed within the airway submucosa but no longer observed in the perivascular spaces. Surface airway epithelial goblet cell hyperplasia was still evident and the airways demonstrated sustained elevated reactivity to bronchoconstrictor challenge, consistent with previous reports (Kenyon et al., 2003; Temelkovski et al., 1998), but inconsistent with a study of Sakai et al. (2001) which reported similar airway remodeling but no sustained elevated levels of Penh response to methacholine. Since these latter authors used an initial 6 days of daily OVA exposure before

beginning a three days per week regimen for 6 weeks, an earlier onset of diminished inflammatory response to allergen might have accounted for this difference in outcome (Sakai et al., 2001). The use of higher levels of allergen with each exposure might also account for other differences between studies as discussed by Temelkovski et al., (1998) and observed for Balb/c mice by Shinagawa and Kojima (2003).

Increased airway collagen and smooth muscle resulting from enhanced activities of mesenchymal fibroblast and myofibroblast are a feature of asthma pathogenesis (Holgate, 2011). An observed elevated collagen content in the present study further indicated that airway remodeling occurred as reported by others (Kenyon et al., 2003; Shinagawa and Kojima, 2003; Van Hove et al., 2009). Kenyon et al. (2003) using the same exposure regimen demonstrated significant enhanced airway submucosal collagen deposition after 4 weeks of mouse allergen-exposure, although Sakai et al. (2001) failed to note any marked changes in basement membrane thickness in their model of prolonged allergen exposure.

The recovery of eosinophils by BAL was previously shown to follow earlier tissue accumulations during the first 3 days of allergen exposure as the major outcome of a Th2 lymphocyte mediated immune response (Bassett et al., 2010). Enhanced BAL eosinophil recoveries correlated with enhanced tissue recoveries and their accumulation within lung inflammatory lesions after 1, 2 and 4 weeks of episodic OVA exposure. Although the elevated numbers of eosinophils remaining in airway and lung tissue prior to each weekly episode of OVA exposure increased between the 2nd and 4th week, the subsequent additional eosinophil accumulation over the following 3 exposure days appeared to be similar to that observed after the first weekly exposure episode. However, following the 8th weekly episode of OVA exposure, tissue-recoverable eosinophils could only be detected at a lower level although significantly greater than their recovery from corresponding saline-control lungs. These observations are consistent with the possible onset of unresponsiveness to the weekly effects of allergen exposure noted previously (Kumar and Foster, 2002; Locke et al., 2007; Sakai et al., 2001; Singh et al., 2008; Van Hove et al., 2009; Yiamouyiannis et al., 1999).

The observed persistent airway sub-mucosal lesions noted in the present study are consistent with previously reported morphometric analyses of airway mucosal inflammatory cell contents and concomitant sustained airway hyperreactivity at this later time point (Temelkovski et al., 1998). In the present study, major basic protein positive eosinophils and CD3⁺ lymphocytes were observed within these lesions after 8 weeks with continued extensive Ki67⁺ immune-staining, suggesting an allergen responsive airway with a possible continuing expanding lymphocyte population. However, the extensive perivascular inflammatory cell accumulations triggered by allergen exposures observed during the first 4 weeks of episodic OVA exposures were clearly diminished by this 8th week. After each weekly allergen episode it might be expected that the extent of the response be modulated by the subsequent down-regulation of the ongoing Th2 lymphocyte response as a result of regulatory T helper cells (Jaffar et al., 2004; Lloyd and Hawrylowicz, 2009) and/or airway epithelial located IL17 generating $\gamma\delta$ T cells (Murdoch and Lloyd, 2010). These proposed down regulatory pathways might therefore have only begun to overwhelm the influence of repeated cycles of OVA Th2 lymphocyte-mediated infiltration of eosinophils between the 4th and 8th weekly exposure episodes in the present study. The need for further evaluation of lung tissue lymphocyte subpopulations and their respective activities indicated by BAL fluid cytokine networks between the 4th and 8th week is clearly indicated for the use of this model in the exploration of such hypotheses. Based on previous reports, a down-regulation of the Th2 lymphocyte driven process would be indicated by the presence of T regulatory CD4⁺CD25⁺ cells and decreases in IL4, IL5 and IL13 (Jaffar et al., 2004; Lloyd and Hawrylowicz, 2009), with possible enhancement in the activity of IL17⁺ $\gamma\delta$ T cells (Lloyd and Murdoch, 2010; Murdoch and Lloyd, 2010). It is interesting to note that in the present

study, although evaluation of tissue-recovered lymphocyte populations indicated a relative enhancement in CD3⁺CD4⁺ lymphocytes by the 4th week, an enhanced proportion of lymphocytes was identified as being CD19⁺ B lymphocytes by the 8th week. These observations might reflect a release of IL-17 associated with the development of local immunity associated with elevated IgA or IgM (Jaffar et al., 2009). In contrast, in this model system, Kumar et al., 2000) demonstrated an elevated airway IgG-mediated response with no elevation in IgA and no IgM or IgE synthesizing cells. Alternatively a relative elevation of B lymphocytes would be consistent with a proposed role for a subpopulation of B lymphocytes in the suppression of the allergen-induced Th2 pathway in response to repeated allergen exposures (Singh et al., 2008; Yiamouyiannis et al., 1999).

The consistent appearance of neutrophils in the lavage recoveries from OVA-exposed lungs at all time points and maximal tissue accumulations after the 4th weekly exposure episode also demonstrated their association with allergen-induced lung inflammation. Such enhancement in neutrophil infiltration might be indicative of a change in activity from aTh2 to a Th1 lymphocyte-driven pattern of inflammatory response or perhaps an IL17-associated enhancement in IL8-mediated neutrophil recruitment (Jaffar et al., 2009, 2011). However, immune-staining for neutrophil myeloperoxidase demonstrated that neutrophils were not located within the perivascular or the submucosal lesions. The observed neutrophil enhancements in this model might alternatively be representative of a macrophage-mediated response to increasing allergen-induced epithelial damage rather than representative of a lymphocyte-driven reaction.

Collagenase dispersion recoveries of the non-specific esterase positive monocyte and macrophage combined populations appeared to be a more effective method than BAL for assessing changes in lung content of these important inflammatory cells. Cyclic tissue accumulations with each weekly exposure with the greatest accumulations being observed after the 4th weekly exposure episode correlated with observed accumulations of CD68⁺ macrophages in both airway and perivascular lesions. Their presence at these sites was consistent with a critical role of Th2-polarized macrophages in the deposition and turnover of extracellular matrix components associated with allergen-induced fibrogenesis, angiogenesis and smooth muscle changes previously observed in this model system (Homer et al., 2011; Kenyon et al., 2003; Shinagawa and Kojima, 2003; Van Hove et al., 2009).

Although both neutrophil and eosinophil infiltrations have been implicated in contributing to airway hyperreactivity associated with acute ozone (DeLorme et al. 2002; Schultheis et al., 1994) and allergen-induced inflammations (Evans et al., 1997) by the use of antibodies against neutrophils and eosinophil major basic protein, respectively, their specific roles remain controversial. Although convenient and straightforward, the non-invasive method used in this study for screening airway responses to the bronchoconstrictor methacholine based on changes in breathing pattern using the Penh value is limited by being an empirical measure that does not provide a direct measure of alterations in airway caliber (Bates et al., 2004; Berndt et al., 2011; DeLorme and Moss, 2002; Whitehead et al., 2003). Such airway resistance measurements require invasive methods based on concomitant determinations of airway flow and trans-pulmonary pressure while under anesthesia (Bassett et al., 2010). However, Penh changes have been successfully used as a surrogate for resistance measurements in assessing the effects of bronchoconstrictor challenges in several investigations (DeLorme and Moss, 2002; Kenyon et al., 2003; Kirschvink et al., 2011; Sakai et al., 2001; Whitehead et al., 2003), as well as being correlated with methacholine-induced changes in airway resistance in anesthetized allergen-exposed mice while under conditions of constant volume ventilation (Bassett et al., 2010). The observed enhanced responses to methacholine challenges after 2 and 4 weeks might be attributable to the airway presence of these granulocytes, although neutrophils were not observed to be concentrated

within the airway inflammatory lesions. However, methacholine responsiveness remained high even after 8 weeks when allergen-induced eosinophil accumulation was markedly diminished, suggesting that airway restructuring might be a more predominant factor due to either increasing methacholine access to neuromuscular junctions or enhanced smooth muscle content, as previously suggested by increased α -actin staining even after the 2nd week OVA exposure (Van Hove et al., 2009). The sustained lymphocyte levels observed within allergen-exposed mice in the present study alternatively support the hypothesis that lymphocytes might have a role in contributing to the sustained airway hyper-responsiveness independent of the presence of granulocytes, although T- rather than B-lymphocytes were implicated (Collins et al., 2003; Hahn et al., 2003; MacLean et al., 1999).

The methods used in this study have made it possible to observe a progressive buildup over the first 4 weeks of OVA exposure in the baseline levels of lymphocytes, eosinophils and neutrophils, and although interstitial and marginated monocyte-macrophage levels returned to saline-controls prior to each weekly episode, the responses to OVA increased each week. Such progressive accumulations observed at the onset of weekly OVA exposures are not so readily observable using BAL recoveries alone or by morphological evaluations of tissue inflammatory lesions. Such accumulations might be expected to reflect a range of differing inflammatory cell half-life or transit times in the lung as well as implicating lymphocyte and macrophage involvement in the subsequent down-regulation of allergen-induced inflammatory responses and resultant airway remodeling. Therefore, this allergen exposure model provides a basis to further explore the determinants by which levels of allergen exposure and the time between episodes might affect the long-term development of the chronic asthma.

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Figure 1.

Diagram illustrating the days of analyses in three experimental series involving weekly exposures of mice to either saline or ovalbumin aerosols for 3h per day 3 days per week for 8 weeks.

Figure 2.

Results represent the mean values for Penh ($\pm$ SE) obtained on exposing un-anesthetized female ovalbumin sensitized Balb/c mice to aerosols with increasing concentrations of methacholine (0–200mg/ml), 24h following completion of 2 (n=5), 4 (n=10) and 8 (n=7) weekly episodes of 3 daily 1h exposures of sensitized mice to ovalbumin aerosol (OVA) or the corresponding combined saline aerosol controls (n=16) obtained from the first two series of experiments.

Figure 3.

Results represent the mean ($\pm$ SE) recoveries of neutrophils and eosinophils obtained by extensive bronchoalveolar lavage (BAL) and by collagenase dispersion of the remaining parenchymal lung tissue from sensitized female Balb/c mice 24hr following episodic exposures to saline (control) (n=4–10), and the 1st (n=4), before (n=4) and after (n=6) the 2nd and 4th, and after the 8th (n=6) weekly episodic exposures to OVA aerosol from all three series of experiments. ^{abcd}Significantly different with $P < 0.05$ from corresponding: ^a saline-exposed control mice; ^b OVA-exposed mice prior to the weekly episodic exposure; ^c OVA-exposed mice following the 1st weekly episodic exposure; and ^d OVA-exposed mice prior to the 2nd weekly episodic exposure.

Figure 4.

Results represent the mean ($\pm$ SE) recoveries of macrophages and lymphocytes obtained by extensive bronchoalveolar lavage (BAL) and non-specific esterase positive (NSE+ve) monocytes and macrophages and lymphocytes obtained by collagenase dispersion of the remaining parenchymal lung tissue from sensitized female Balb/c mice 24hr following episodic exposures to saline (control) (n=4–10), and the 1st (n=4), before (n=4) and after (n=6) the 2nd and 4th, and after the 8th (n=6) weekly episodic exposures to OVA aerosol from all three series of experiments. ^{abcd}Significantly different with $P < 0.05$ from corresponding: ^a saline-exposed control mice; ^b OVA-exposed mice prior to the weekly episodic exposure; ^c OVA-exposed mice following the 1st weekly episodic exposure; and ^d OVA-exposed mice prior to the 2nd weekly episodic exposure.

Figure 5.

Light micrographs of representative lesions obtained from serial sections prepared from lungs from 3–4 Balb/c mice 24h following completion of 2 (G, J, and K), 4 (A, B, D, E, H, M, and N) and 8 (C, F, I, L, and O) weekly episodes of 3 daily 1h exposures of sensitized mice to OVA obtained from the first two series of experiments. Immunohistochemical staining using antibodies against Ki67 (A–C), CD3 (D–F), CD68 (G–I), Myeloperoxidase (MPO)(J–L), and eosinophil major basic protein (EMBP)(M–O). Serial sections were utilized in several cases to demonstrate differential staining of a particular type of lesion.

Figure 6.

Light micrographs of representative lesions obtained from serial sections prepared from the lungs 3–4 Balb/c mice 24h following aerosol exposures to saline (Control)(A and E) or to OVA after 2 (B, C, F and G) and 8 (D and H) weekly episodes of 3 daily 1h exposures of Balb/c sensitized mice obtained from the first two series of experiments. Standard tinctorial staining was achieved using periodic acid Schiff (PAS)(A–D) for mucin and martius scarlet blue (MSB)(E–H) for extracellular matrix proteins.

Table 1

Primary Antibodies

Antibody	Marker Stained	Clone	Antigen retrieval method	Suppliers
CD3	T-Lymphocytes	T3	microwave EDTA buffer	AbDSerotec, Kidlington, UK
CD68	Macrophages	FA-11	pronase	AbDSerotec, Kidlington, UK
ab 9535	Neutrophil Myeloperoxidase	Rabbit Polyclonal	microwave citrate buffer	AbCam, Cambridge, UK
EMBP	Eosinophil Major Basic Protein	Goat Polyclonal	microwave citrate buffer	Biotech Inc., Santa Cruz CA
ki67	Cell Proliferation	TEC-3	microwave citrate buffer	Dako, Ely, UK

Table 2

Total BAL and Tissue Cell Recoveries

	1 week OVA $\times 10^5$ per lung	2 weeks OVA $\times 10^5$ per lung	4 weeks OVA $\times 10^5$ per lung	8 weeks OVA $\times 10^5$ per lung
<u>BAL-Recovered Cells</u>				
Saline-Control	3.8 $\pm$ 0.4	1.9 $\pm$ 0.3	2.3 $\pm$ 0.2	3.3 $\pm$ 0.3
Before OVA	-	5.4 $\pm$ 1.0 ^a	7.8 $\pm$ 1.1 ^a	-
After OVA	10.5 $\pm$ 1.8 ^a	8.6 $\pm$ 1.8 ^a	11.8 $\pm$ 1.1 ^{ab}	2.67 $\pm$ 0.3
<u>Tissue-Recovered Cells</u>				
Saline-Control	193.4 $\pm$ 6.4 ^a	184.8 $\pm$ 18.3	129.3 $\pm$ 8.6	185.9 $\pm$ 9.0
Before OVA	-	142.0 $\pm$ 4.4	158.9 $\pm$ 11.0	-
After OVA	241.0 $\pm$ 14.1	295.7 $\pm$ 38.0 ^{ab}	382.5 $\pm$ 4.3 ^{abc}	215.4 $\pm$ 19.6

Results represent the mean ($\pm$ SE) total recoveries of inflammatory cells obtained by extensive bronchoalveolar lavage (BAL), and by collagenase dispersion of the remaining parenchymal lung tissue from sensitized female Balb/c mice 24 hr following episodic exposures to saline (control) (n=4–10), and the 1st (n=4), before (n=4) and after (n=6) the 2nd and 4th, and after the 8th (n=6) weekly episodic exposures to OVA aerosol from all three series of experiments.

^{abc} Significantly different with $P < 0.05$ from corresponding:

^a saline-exposed control mice;

^b OVA-exposed mice prior to the weekly episodic exposure; and

^c OVA-exposed mice following the 1st weekly episodic exposure