

Environmental Tobacco Smoke and Progesterone Alter Lung Inflammation and Mucous Metaplasia in a Mouse Model of Allergic Airway Disease

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Abstract The prevalence and severity of asthma is sexually dimorphic. Adult women have a higher incidence of asthma than men. This suggests that this disease may have a hormonal component. Progesterone has been shown to elicit an immune response similar to that seen in allergic asthma and previous studies have shown that progesterone increases total IgE levels in the peripheral blood. In the current study, we examine the effect of environmental tobacco smoke (ETS) and progesterone on hallmarks of asthma pathology in lung tissue with the goal of defining whether progesterone can also exacerbate two key features of airway remodeling: accumulation of eosinophils and increased mucous. We used a mouse model of allergic asthma that includes house dust mite allergen (HDMA). Adult female BALB/c mice were ovariectomized and implanted with time-release progesterone pellets. Mice were housed in filtered air or ETS for 6 weeks (1 mg/m³ total suspended particulate) and exposed to HDMA by inhalation. Progesterone alone did not increase mucous cell mass or the abundance of eosinophils but ETS coupled with progesterone exposure resulted in a significant increase in

mucous cell metaplasia and increased accumulation of eosinophils in the asthma model. Levels of cytokines in the bronchoalveolar lavage fluid, measured using a multiplex cytokine assay, revealed elevated levels of both interleukin (IL)-5 and IL-12(p40) in HDMA-exposed animals. The addition of progesterone further exacerbated this response. We conclude that progesterone, in the absence of estrogen, exacerbates airway inflammation and airway remodeling induced by the toxicant ETS.

Keywords Environmental tobacco smoke ·
Mouse model of asthma · Progesterone

Introduction

Asthma affects males and females differently. Males are disproportionately affected as young children, the gender difference diminishes in adolescence and females have a higher incidence after puberty [4, 5, 34, 45]. Adult women have a 35% greater prevalence of asthma than men [1]. This suggests a hormonal role in the development of asthma. However, there is conflicting data on the impact of sex hormones on asthma. A phenomenon known as premenstrual asthma affects up to 40% of asthmatic women and results in an exacerbation of asthmatic symptoms in the days leading up to menstruation [19, 27, 29]. When the role of exogenously administered hormones and the development of asthma [16] were studied, there was no association between oral contraceptive use and the development of asthma. However, there was a small but significant increase in asthma risk in women who had been on hormone replacement therapy (HRT). Smoking status was strongly related to all asthma indicators. Similarly, women on HRT for at least 10 years have twice the age-related risk of

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asthma as those who had never been on HRT [42]. There are numerous studies in both humans and mice that show an increase in airways hyperreactivity and/or bronchoconstriction when the highest levels of progesterone are present [15, 36, 39]. However, the effect on lung tissue has not been studied.

There have been many studies of tobacco smoke exposure and its relationship to allergy/inflammation. Environmental tobacco smoke (ETS) has been shown to exacerbate allergic responses in ragweed allergic people [7]. The allergic responses promoted by ETS included increased T-helper 2 (TH2) cytokines interleukin (IL)-4, IL-5, and IL-13 and a decrease in interferon gamma in nasal lavage. Combined exposure to ovalbumin (OVA) and ETS in a mouse model upregulates allergic responses to inhaled allergen including peripheral IgE, IgG1, and eosinophils [32], although airway remodeling was not examined. Several other groups have subsequently shown that either sidestream or mainstream tobacco smoke is capable of acting as an adjuvant for a TH2 response in the absence of parenteral sensitization to ovalbumin [25, 30, 41, 43]. ETS enhancement of allergic asthma (IgE and IgG1 in serum) from birth in OVA-sensitized mice is greater in female mice than in male mice, providing an additional impetus to understand the underlying pathology of this response [31]. While OVA sensitized rodent models have been used to great effect to investigate mechanisms of asthma and allergy, they use an allergen that is not normally associated with human allergies. In contrast, house dust mite is a prominent human allergen that has effects on the airway epithelium and the surface lining layer of the airways [6] due to its complement of active proteolytic enzymes [13]. A recent review of such models emphasized the importance of the use of clinically relevant allergens [12].

ETS has both toxic and conditioning effects on lung tissues and also affects hormonal responses. The dose of ETS used in this study does not have many morphologic effects in the lungs of adult mice [44] but has been shown to predispose to abnormal epithelial repair. The dose, 1 mg/m³, is equivalent to levels attained in a car with a smoker with the windows rolled up and is thus an environmentally relevant level within the lower end of the range of tobacco smoke levels used to expose rodent models. Others have also shown that tobacco smoke changes mechanisms that control hormone levels in the body. Indeed, the hypothesis has been advanced that tobacco smoke is an endocrine disrupter [20]. The interaction of tobacco smoke with hormones is complex. To circumvent varying hormone levels and the potential for ETS interaction, in this study we used ovariectomized female mice. We also examined the effect of ETS on the lungs ability to respond to hormones by examining progesterone receptor expression in the lungs of exposed animals.

A number of studies have demonstrated that increased levels of progesterone induce a TH2 response. During pregnancy, there is an increase in immunoglobulins and a decrease in cell-mediated immunity [38]; the immune response during the luteal phase of the ovarian cycle is also skewed to TH2 [10]. We previously demonstrated that progesterone resulted in an increase in total serum IgE and increased IL-4 production in stimulated cells from house dust mite allergen (HDMA)-exposed whole mouse lung [24], although the basal level of expression in the lung lining fluid was not defined. However, the severity of asthma is often determined by what is occurring at the tissue level including local changes in lung immune cells as well as airway remodeling. Localization of eosinophils and IgE-positive cells to the lung correlate to clinical signs of allergic asthma [2] and are major factors in airway inflammation. The histopathology of the lung tissue specific responses has not been previously examined in the HDMA mouse asthma model utilized in this study.

We defined key histopathologic markers of development and exacerbation of allergic asthma in the airways: mucous cell metaplasia, accumulation of peribronchiolar tissue eosinophils and IgE-positive cells, and bronchoalveolar lavage fluid (BALF) cytokine levels. The goal of this study was threefold: (1) to define the histopathology of the airway response in the allergic mouse model, (2) to determine if ETS exposure exacerbates allergic airway tissue remodeling, and (3) to define the effect of progesterone on the airway response.

Methods

Animals Four-week-old female BALB/c mice were obtained from Charles River Laboratories, Inc (Wilmington, MA, USA). Animals were housed in filtered air upon arrival and allowed to acclimate for 1 week prior to ovariectomy. Ovariectomies were performed as previously described [24]. Animals recovered for 3 weeks prior to the start of the experimental protocol. All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Experimental Protocol The experimental protocol is diagrammed in timeline form in Fig. 1.

Briefly, ovariectomized animals were implanted with either progesterone (PG) or placebo (PL) pellets, placed into either ETS chambers or housed in filtered air (FA), and sensitized i.p. to either HDMA or a sham of phosphate-buffered saline (PBS) in aluminum hydroxide. Animals sensitized to HDMA also received whole body exposure to aerosolized HDMA. Animals were euthanized 48 h following their last HDMA aerosol. Bronchoalveolar lavage was performed on half of the animals [24] while the lungs were

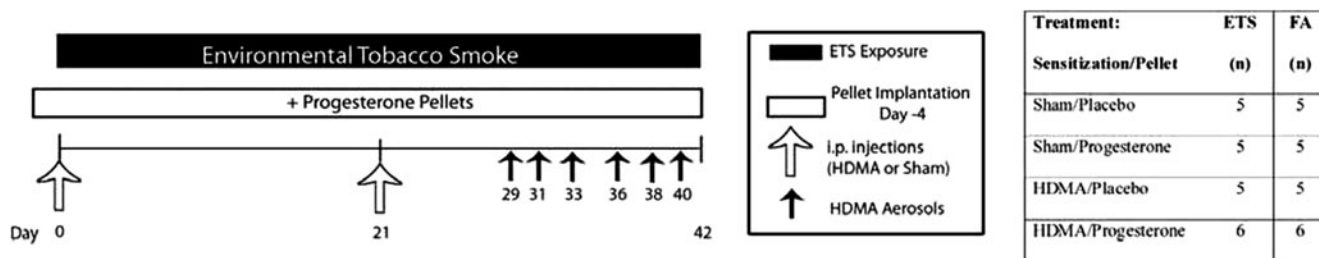


Fig. 1 Timeline and organization of experiment. Ovariectomized female mice were exposed to the experimental protocol outlined above

harvested for histologic evaluation from the other half. There were four treatment groups and each was further divided for FA or ETS exposure resulting in a total of eight groups, see Table 1 for descriptions. The *n* for each group was five except for the HDMA/PG-treated groups, which had an *n* of six.

Environmental Tobacco Smoke Exposure Mice were exposed to ETS at the University of California, Davis Center for Health and the Environment in chambers designed by Teague et al. [40]. The smoke was generated using a Model TE-10 smoke machine (Teague Enterprises, Davis, CA, USA) and type 2R4F research-reference cigarettes. The cigarettes were produced by the Kentucky Tobacco Research & Development Center (University of Kentucky, Lexington, KY, USA). Each exposure chamber has a measurement of 69×69×61 cm. Mice were exposed for 6 h a day Monday through Friday for a total of 6 weeks. The animals remained in these chambers after the smoke generation ceased and were supplied with filtered air. Once allergen exposure began, the HDMA animals were housed in a separate ETS exposure chamber (chamber #2). ETS exposures were carefully monitored and the chamber levels of key components were measured. The total suspended particulates (TSP) was 1.02 ± 0.07 mg/m³ for both chambers. Nicotine concentration in chamber 1 was 217.5 ± 81.4 and 232.5 ± 34.65 µg/m³ in chamber 2. Carbon monoxide levels for the two chambers were 2.31 ± 0.77 and 2.71 ± 0.57 ppm, respectively.

House Dust Mite Aerosolization Mice were exposed to *Dermatophagoides farinae* (Greer Laboratories, Lenoir,

NC, USA) using a Tri-R Airborne Infection Apparatus (Tri-R Instruments, New York, NY, USA) first described by Middlebrook [22]. Briefly, the mice were placed in a divided wire cage within the exposure unit. A Heart™ nebulizer (Westmed, Inc, Tucson, AZ, USA) was used to aerosolize a 1 mg/ml solution of HDMA, which then passed through a venturi to introduce more air flow before entering the exposure chamber. Animals were exposed for 30 min. The amount of HDMA aerosolized was 8.6 ± 1.62 ml for an average aerosol output of 0.29 ± 0.054 ml/min. The chamber was exhausted for 20 min following exposure. Animals were then removed from the chamber and returned to filtered air.

Pellet Implantation Pellets were obtained from Innovative Research of America (Sarasota, FL, USA). Each pellet contained 12.5 mg of product (Progesterone or Placebo) and had a diameter of 3 mm. The pellets were designed to release their contents over a 60-day period and result in peak physiologic levels of progesterone. Implanted pellets resulted in sustained physiologic levels of progesterone 28.3 ± 8.42 vs. 13.5 ± 7.22 ng/ml in the placebo-treated animals, *p* value <0.0001 and were implanted as previously described [24]. There was no difference in progesterone levels between the ETS-exposed and ETS non-exposed animals.

Lung Preparation The lungs of the animals were tied off at the hilus following cannulation. The right lobes were harvested for cell culture [24]. The left lobes were fixed with 4% paraformaldehyde at 30 cm pressure for 1 h and stored in 4% paraformaldehyde until they were embedded into paraffin. The lobe was divided into four pieces approximately 4–5 mm thick. The four pieces consisted of the cranial slice that was embedded caudal side down; the slice containing the hilum was embedded cranial side down; the third slice, caudal to the hilum slice was embedded cranial side down; and finally, the caudal slice was embedded cranial side down. Embedded tissues were cut into 5-µm sections using a microtome and placed on precleaned Gold Seal micro slides (Becton, Dickinson and Company, Portsmouth, NH, USA). Slides were examined

Table 1 Treatment group assignments

Treatment: sensitization/pellet	ETS (n)	FA (n)
Sham/placebo	5	5
Sham/progesterone	5	5
HDMA/placebo	5	5
HDMA/progesterone	6	6

using high-resolution bright field light microscopy on an Olympus BH2 microscope.

Histologic Staining of Lung Sections The lung was examined for eosinophilia and mucous cell metaplasia. A combined eosinophil and mast cell staining kit (American Master Tech Scientific, Lodi, CA, USA) was used on slides prepared by deparaffinizing the lung sections in three changes of toluene and rehydrating them through a graded series of ethanol. Slides were placed in Astra Blue stain for 30 min to stain mast cells. Following a brief rinse in tap water, slides were placed in Vital New Red to stain eosinophils for 30 min. Sections were dehydrated in ethanol and cleared in toluene prior to being mounted using Clearmount mounting solution (Zymed, South San Francisco, CA, USA). Mucous cells were detected using an Alcian blue/periodic acid Schiff's (AB/PAS) stain. Following deparaffinization and rehydration, slides were placed in the alcian blue stain for 30 min, rinsed briefly in tap water, placed in the 1% periodic acid for 7 min, rinsed in distilled water, and soaked in Schiff's for 15 min. Slides were rinsed in tap water for 5 min prior to dehydrating in ethanol and clearing in toluene. Slides were mounted using Clearmount mounting solution (Zymed, South San Francisco, CA, USA).

Morphometry Mucous cells within stained lung sections were quantified using a previously described method [23]. All airways with a minimum diameter of 480 μm from the four sections of lung from each animal were imaged using an Olympus BH-2 microscope. Airways were numbered consecutively as they were imaged. Quantitative analysis was performed on eight airways from each animal. A random number table was used to determine which airways would be quantified. Points of a 140 point cycloid grid that fell on epithelial cells staining positive for mucosubstances were counted and those points that fell on the epithelium were counted as the reference volume. The mass of mucous cells within the epithelium (volume per surface area of basal lamina; V_s , $\mu\text{m}^3/\mu\text{m}^2$) was then calculated as described by Miller et al. [23].

Immunohistochemistry Immunohistochemistry was performed using Vectastain peroxidase kits (Vector Laboratories, Burlingame, CA, USA). The optimal dilution which gave positive tissue staining with minimal background staining was determined for each primary antibody using a dilution series. All tissue for immunohistochemistry was fixed and processed the same length of time after necropsy and a slide from every animal was present in each immunochemical assay. Antigen retrieval was performed using hot citrate buffer (pH 6.0) for 30 min, endogenous peroxidase was blocked using a 3% solution of hydrogen peroxide and a nonspecific protein block

was performed using 1% bovine serum albumin. Slides were washed in PBS between steps. For the PGR receptor (PGR), a rabbit anti-human PGR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:100 dilution. IgE cells were detected using a goat anti-mouse IgE antibody (Bethyl Laboratories, Inc, Montgomery, TX, USA) at 1:1,000. Antibodies were added directly to the slides and incubated overnight in at 4°C. On the second day, the appropriate Vectastain kit was used to detect primary antibody binding. For PGR, biotinylated rabbit IgG was used and for IgE, a biotinylated goat IgG was added to slide and incubated at room temperature for 30 min. Following a wash with PBS, avidin–HRP antibody was incubated for 30 min. Protein localization was detected using DAB enhanced with nickel chloride to give a black precipitate. Slides were dehydrated through a series of ethanol, cleared in xylene, and mounted with non-aqueous mounting media (CytoSeal, Stephens Scientific, Riverdale, NJ, USA). Controls included the addition of a slide that did not receive the primary antibody and an additional slide that did not receive the secondary antibody. These controls assured that the staining was not due to background or nonspecific staining and were negative for DAB precipitate.

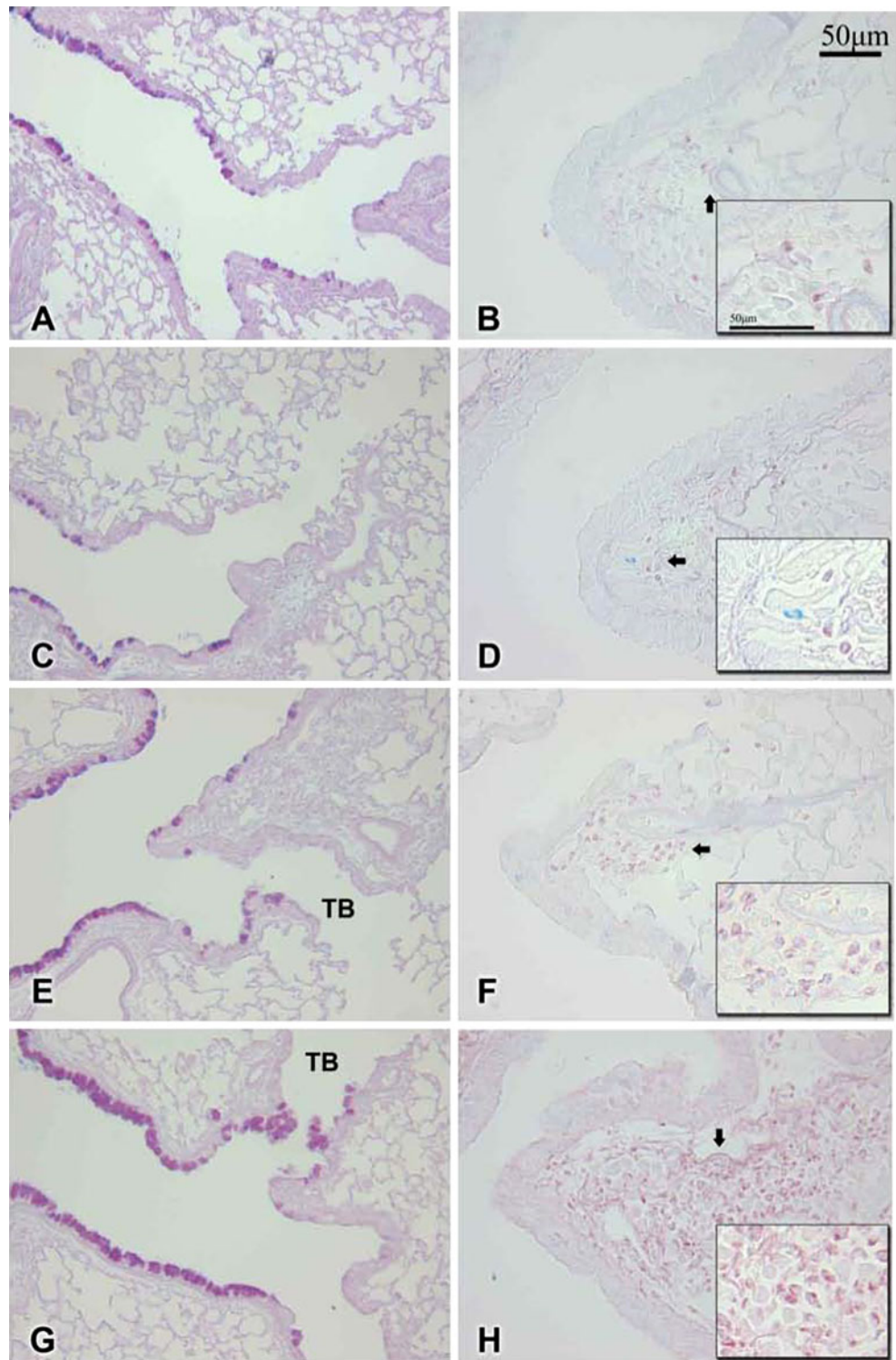
Bronchoalveolar Lavage for Cytokine Determination Bronchoalveolar lavage was performed as previously described [24]. Briefly, three 1 ml instillations of Hanks balanced salt solution were used to lavage the lungs. Following centrifugation, the supernatants were analyzed for multiple cytokines including IL-4, IL-5, IL-12 (p40), IL-12 (p70), IL-18, and IFN- γ using a fluorescent bead based assay (Bio-Rad, Inc, Hercules, CA, USA). A standard curve for each cytokine was run with all samples. Data is presented as mean concentration in picograms/milliliter $\pm$ standard deviation.

Data Analysis All data analysis was done using StatView (SAS Institute, Cary, NC, USA) using an analysis of variance (ANOVA) and followed by Fisher's post hoc comparison test. Data is presented as means $\pm$ standard deviation. Differences were considered significant if the p value was 0.05 or lower.

Results

Mucous Cells and Eosinophils All HDMA-treated animals had mucous cell metaplasia as indicated by AB/PAS staining in the airway epithelium (Fig. 2a, c, e, and g). No mucous cells were detected in samples that were not exposed to HDMA (not shown). Likewise, the distribution and abundance of peribronchiolar eosinophils varied greatly

Fig. 2 Mucous cells and eosinophils in mouse lung tissue. Images of paraffin sections from the left lobe of the mouse lung tissue stained with AB/PAS to detect mucous containing cells (**a**, **c**, **e**, **g**) or Vital New Red/Astra Blue dye to stain eosinophils/mast cells (**b**, **d**, **f** and **h**). **a**, **b** HDMA/placebo/FA; **c**, **d** HDMA/placebo/ETS; **e**, **f** HDMA/progesterone/FA; **g**, **h** HDMA/progesterone/ETS. Mucous cells extended farther into terminal bronchioles (TB) and were more abundant in animals exposed to progesterone. Peribronchial accumulations of eosinophils (arrows) were detected in all HDMA exposed groups but these accumulations were especially large in the HDMA/progesterone/ETS group (**h**). Higher magnification insets are used to show cellular detail of the accumulated cells. Bars 50 μ m



among the groups exposed to HDMA (Fig. 2b, d, f, h) but were not present in non-HDMA-exposed animals (not shown). The distribution and abundance of mucous metaplasia differed between groups with only the most severely affected groups, those that were treated with progesterone-containing pellets (HDMA/PG/FA and HDMA/PG/ETS; Fig. 2e, g) having more mucous cells in

the most distal airways, terminal bronchioles. Further, the mucous cell composition was more magenta in these groups. The increase in magenta staining in the mucous cells indicates a shift in the nature of the mucosubstances. The HDMA/PG/ETS group (Fig. 2g) had the largest amount of mucous metaplasia both in the number of airways it covered and the degree to which it occurred.

The cells were hyperplastic and extended to the terminal bronchioles.

Vital New Red dye was used to stain eosinophils in mouse lung tissue. The non-HDMA-exposed animals did not have tissue eosinophilia (data not shown). Accumulations of eosinophils were visible in all HDMA-exposed groups (Fig. 2b, d, f, and h) but varied in their distribution and abundance within tissue compartments by group. HDMA/ETS-treated animals had the greatest abundance of eosinophils (Fig. 2f and h). Eosinophils were localized in the submucosa of the airways with large accumulations around the blood vessel side of the airway (arrows in Fig. 2). Eosinophils were also present in the parenchyma. The same pattern of eosinophil distribution was seen in the animals but eosinophils were less abundant in the HDMA/PG/FA group compared to the HDMA/PG/ETS group (compare Fig. 2f with h). The majority of eosinophils seen in the HDMA/PL-treated animals were distributed around blood vessels (Fig. 2b and d) with very few in the parenchyma. As with the PG-treated animals, ETS exposure increased the abundance of eosinophils (Fig. 2d). Astra Blue dye was used to visualize mast cells. Mast cells, in general, were very rare across all groups. When present, mast cells were found in both the airway epithelium or in the submucosa of the HDMA-treated animals (Fig. 2d and f).

The changes in mucous cells were confirmed by quantitative morphometry (Fig. 3). All HDMA-exposed groups had a significant increase in mucous cell mass

compared to sham-exposed groups. The mass of mucous cells was greatest in the HDMA/PG/ETS group with V_s of $18.9 \pm 2.80 \mu\text{m}^3/\mu\text{m}^2$. This was significantly greater than all other treatment groups and indicates a synergistic effect of PG and ETS on mucous metaplasia. Analysis of the interaction of PG and ETS revealed that there was a very significant interaction between the two ($p=0.0023$ with a power of 0.942). However, the addition of either ETS or PG did not change mucous cell mass over the increase created by HDMA exposure alone (see HDMA/PL/FA group in Fig. 3).

Cytokines in Bronchoalveolar Lavage BALF was analyzed for the presence of TH2-associated cytokines (IL-4 and IL-5) and TH1 cytokines (IFN- γ , IL-18, and IL-12 (p70)) using a multiplex bead-based assay (Fig. 4). IL-12 contains two subunits (p35 and p40) to form the heterodimeric p70, which is a strong inducer of TH1 type responses. However, the p40 subunit is a known antagonist to IL-12 p70 and has been shown to favor a TH2 response [49]. Both IL-12 p40 and p70 were measured in the BALF. Of the six cytokines analyzed in the BALF, half were upregulated (Fig. 4a–c) in allergic animals (IL-4, IL-5, and IL-12 (p40)). The other half was downregulated (Fig. 4d–f) in those same animals (IFN- γ , IL-18, and IL-12 (p70)). All of the TH2 associated cytokines were significantly increased in the allergic “asthmatic” animals. IL-4 was significantly elevated in all of the HDMA-treated animals when compared to the sham-treated animals (Fig. 4a). The addition of ETS or progesterone increased the level of IL-4 in the HDMA-exposed animals although the combination of these two treatments did not increase IL-4 further. The HDMA/PG/ETS group had the highest mean concentration of IL-4 (3.21 ± 0.589 pg/ml); nearly a 15-fold increase over the sham/PL/FA group. This was also significantly greater than the HDMA/PL/FA group ($p<0.05$). The allergic “asthmatic” animals had significantly more IL-5 in the BALF than all of the sham-treated animals, $p<0.0001$ (Fig. 4b). The addition of progesterone resulted in a significant elevation of IL-5 ($p=0.0001$) as did ETS with progesterone ($p=0.0290$). The mean IL-5 concentration in the HDMA/PG/FA-treated group (3.71 ± 0.329 pg/ml) was significantly greater than that of the HDMA/PL/FA group (2.31 ± 0.734 pg/ml), $p<0.05$. The HDMA/PG/ETS co-treated animals had the highest concentration of IL-5 in the BALF (4.73 ± 1.177 pg/ml); a tenfold increase over the sham/PL/FA group ($p<0.001$) and a twofold increase over the HDMA/PL/FA group ($p<0.001$). This was also significantly greater than the HDMA/PL/ETS group ($p<0.01$). The mean concentration of the IL-12 (p40) subunit was significantly increased in HDMA vs. all of the sham-treated animals, $p<0.0001$ (Fig. 4c). Progesterone alone as well as the addition of progesterone to ETS in the allergic model significantly enhanced the concentration of IL-12 (p40) in the lavage. The HDMA/PG/ETS group had the highest concentration of

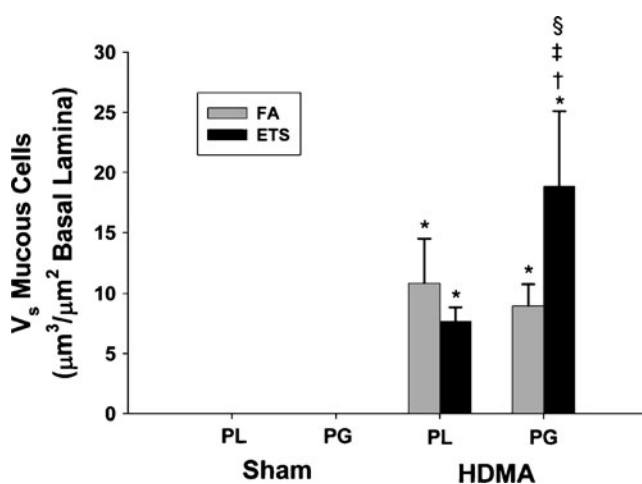


Fig. 3 The mass of stained mucosubstance in the airway epithelium determined using stereology. Animals were exposed to HDMA or a sham HDMA exposure and then implanted with either progesterone (PG) or placebo (PL) pellets and then also exposed to either environmental tobacco smoke (ETS) or filtered air (FA). The group that was exposed to HDMA/Progesterone/ETS had significantly more mucosubstances in the airway epithelium than any other group. Asterisk significant from all of the sham, non-HDMA, exposed. Dagger significant from HDMA/Placebo/ETS. Double dagger significant from HDMA/placebo/FA. Section sign Significant from HDMA/ progesterone/FA. Significance at $P<0.05$

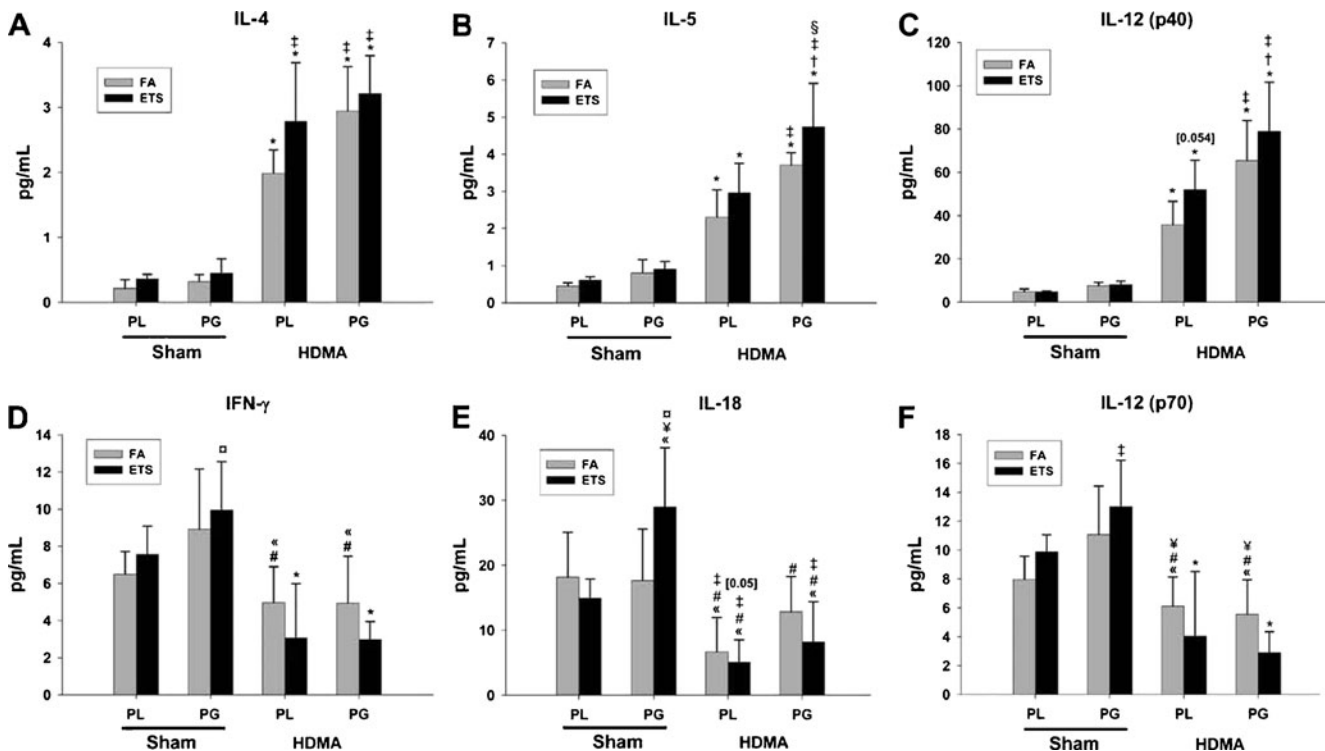


Fig. 4 Cytokine protein levels in bronchoalveolar lavage fluid (BALF) measured using a bead-based multiplex assay. **a–c** Cytokines upregulated in the allergic HDMA exposed animals (IL-4, IL-5, and IL-12 (p40)). **d–f** Cytokines downregulated in the allergic asthmatic animals (IFN- γ , IL-18, and IL-12 (p70)). Concentrations of the given cytokines are expressed in mean picograms per milliliter $\pm$ SD. Asterisk significant from all of the sham, non HDMA, exposed. Dagger significant from HDMA/Placebo/ETS. Double dagger significant from HDMA/placebo/FA. Section sign significant from HDMA/Progesterone/FA. Currency sign Significant from sham/placebo/ETS.

IL-12 (p40) in the BALF (78.90 ± 22.670 pg/ml) this is significantly greater than both of the HDMA/PL-treated groups ($p < 0.05$). The addition of ETS to the HDMA-exposed group just missed statistical significance at $P = 0.054$ for the difference between the HDMA/PL/ETS vs. HDMA/PL/FA groups.

The trend was for TH1 to be reduced in the allergic animals. IFN- γ was reduced in the HDMA-treated animals (Fig. 4d). This reduction was significantly enhanced by the addition of ETS exposure to the allergic model. Interestingly, in the absence of HDMA, the groups with the highest concentration of IFN- γ were the sham/PG/ETS-treated group, which were significantly higher than the HDMA/FA-treated groups ($p<0.05$). Similar results were found for IL-18 in the BALF (Fig. 4e). HDMA had a significant negative contribution to IL-18 ($p<0.0001$) and PG had a significant positive impact on IL-18 concentrations in BALF ($p=0.0176$). The sham/PG/ETS group had the highest concentration of IL-18, which was significantly greater than all of the HDMA-treated groups ($p<0.05$). IL-12 (p70) (Fig. 4f) was significantly decreased in all of the HDMA-treated animals

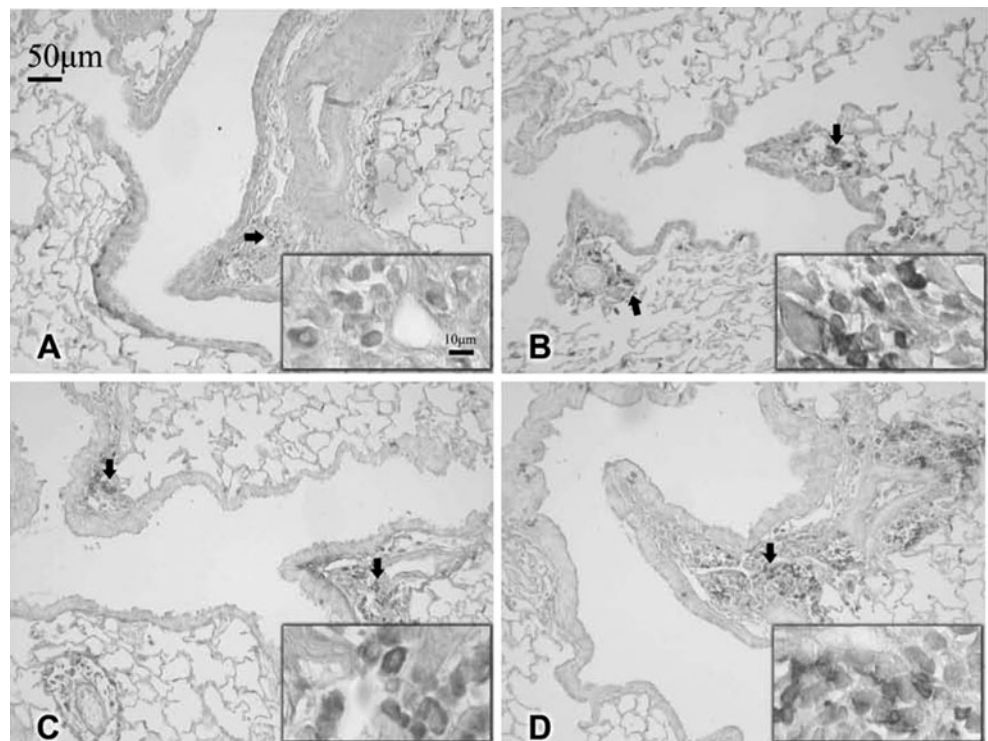
Yen sign significant from sham/placebo/ETS. *Number sign* significant from sham/progesterone/ETS. *Left-pointing double angle quotation mark* significant from sham/progesterone/filtered air. Three-way ANOVA was performed followed by Fisher's post hoc comparison test. Differences with a *p* value <0.05 were considered significant. In **c**, [0.054] indicates the result of the comparison of HDMA/PL/FA with HDMA/PL/ETS that just missed statistical significance. In **e**, [0.05] indicates the result of the comparison of sham/PL/ETS from HDMA/PL/ETS that just missed statistical significance

compared to the sham/PG/ETS group ($P<0.05$). The addition of ETS exposure to the HDMA/PL treatment resulted in the lowest concentrations of IL-12 (p70), which was nearly significantly less than the sham/PL/ETS ($p=0.05$) and was significantly different from both sham/PG-treated groups ($p<0.05$).

IgE-Positive Cell Distribution in the Lung Immunohistochemistry was used to localize IgE-positive cells in the lung tissue. IgE-positive cells were present in the areas of cellular accumulation around the airways in all HDMA-treated animals (Fig. 5). This was most evident in the HDMA/PG/ETS group (Fig. 5d) and that group also had the greatest abundance of IgE-positive cells. Positive cells were also visualized around the blood vessels. The staining was consistent with a membrane bound antigen. IgE-positive cells were present in the alveoli as well; this was most evident in the HDMA/PG-treated animals (Fig. 5c-d).

Progesterone Receptor Distribution in the Lung PGR protein was localized predominantly on the nonciliated

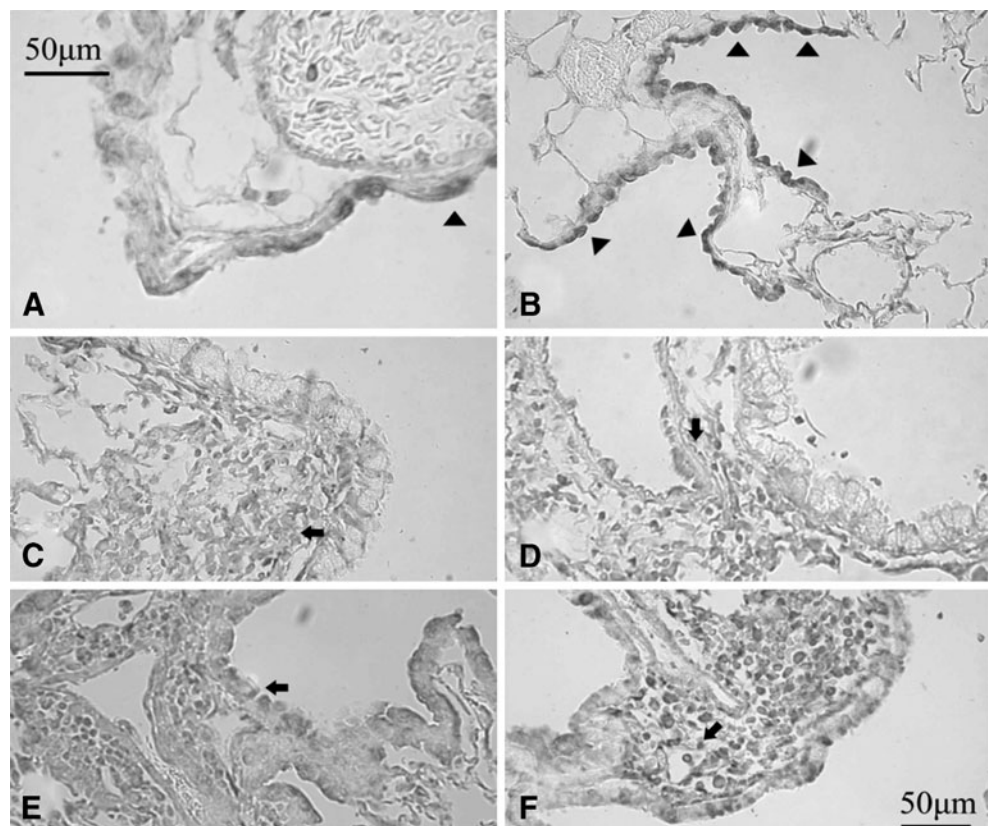
Fig. 5 IgE-positive interstitial cells in HDMA-treated animals. Images of IgE-positive cells detected by immunohistochemistry using nickel enhanced DAB (black staining) to localize IgE-positive cells. **a** HDMA/placebo/FA, **b** HDMA/placebo/ETS, **c** HDMA/progesterone/FA, **d** HDMA/progesterone/ETS. Insets are high magnification images from region indicated by the arrow. Bar 50 μ m



cells of the airway epithelium in the sham-sensitized, non-HDMA-exposed animals (Fig. 6a–b); PG treatment had no apparent effect on receptor density or distribution. ETS exposure increased the intensity and the abundance of the

staining on the epithelium (Fig. 6b). HDMA exposure resulted in a marked decrease in PGR staining on the airway epithelium (Fig. 6c–f). PGRs were present in the alveoli on macrophages as well as type II cells of all

Fig. 6 Progesterone receptor protein expression. Images of progesterone receptor positive cells detected by immunohistochemistry using nickel enhanced DAB (black staining) to localize positive cells: **a–b** progesterone receptor expression on airway epithelial cells in non-HDMA exposed groups show pronounce localization to the distal airway epithelium (arrowheads). **a** Sham/placebo/FA, **b** sham/placebo/ETS. **c–f** Progesterone receptors on inflammatory and airway epithelial cells in HDMA-treated animals. **c** HDMA/placebo/FA, **d** HDMA/placebo/ETS, **e** HDMA/progesterone/FA, **f** HDMA/progesterone/ETS. Insets are high magnification images from region indicated by the arrow. Note also the low level of epithelial cell staining in the HDMA exposed groups (compare c–f with a and b). Bar in f is for b, c, d, e and f. All bars 50 μ m



animals (data not shown). Blood vessels stained positive for the receptors. PGRs were also present on attenuated fibroblasts located on the adluminal side of the basement membrane. Positive staining was present on some infiltrating cells in all HDMA-exposed animals (Fig. 6c–f). The greatest abundance of PGR positive staining cells was seen in the HDMA/PG/ETS group (Fig. 6f).

Discussion

The current study establishes that the HDMA model we employ in the mouse [24] is a robust inducer of mucous cell metaplasia and that this model also increases TH2 cytokines and decreases TH1 cytokines present in the BALF of HDMA-exposed mice. These changes are accompanied by eosinophil recruitment to airway tissue and accumulations of peribronchiolar IgE-positive cells. All of these features correspond to key clinical signs of asthma [2]. ETS exposure alone (HDMA/PL/ETS) in this model had very modest effects on these parameters, although there is a suggestion of accentuation of the HDMA response in BALF cytokines, many of these responses were not significantly changed compared to the HDMA/PL/FA group. This may reflect the modest dose of ETS used in these studies of 1 mg/m³ TSP. Interestingly, progesterone replacement was much stronger at accentuating responses in both the BALF and the tissue than ETS, resulting in a change in mucous composition as well as increased localization of eosinophils. Progesterone plus ETS in the HDMA-exposed animals (HDMA/PG/ETS) had the greatest degree of response for all the parameters tested suggesting that these two exposures to act in concert to exacerbate allergic asthma.

A number of studies have indicated a possible role of hormones on the development and exacerbation of asthma but there has been limited research into the mechanisms behind this phenomenon and much of the research has focused on estrogens. In this study, progesterone pellets inserted into ovariectomized mice resulted in circulating levels consistent with peak progesterone levels seen in both normally cycling female mice [48]. Further, these levels were in the range for women during the luteal phase of their cycle and early pregnancy [35]. PG significantly impacted airway inflammation and airway remodeling in a mouse model of allergic airway inflammation by acting additively with ETS to increase mucous cell mass and the TH2 cytokines IL-4, IL-5, and IL-12p40 in BALF as well as increase the abundance of both tissue eosinophils and IgE-positive cells.

Mucous cell hyperplasia contributes to airway obstruction and is a characteristic phenotype of asthma [17]. Airways of healthy individuals have a limited number of

mucous containing goblet cells and they are not present in the bronchial and bronchiolar airways of normal mice. In the lungs of mice that lacked an HDMA allergen exposure (even ETS-exposed mice), mucous cells were extremely rare. We found mucous cell metaplasia in all of the allergic animals (Figs. 2, 3). ETS exposure did not increase mucous cell abundance. PG had the effect of changing mucous composition and increasing the extent of mucous metaplasia to the most distal airways. Neutral mucins are indicative of hyperviscous mucous and therefore a more advanced disease state [14]. The extent of the PG effect both on small airway involvement and change in mucosubstances is not fully appreciated in the morphometric measurements (Fig. 3) as smaller airways (less than 480 µm in diameter) were not counted and color of mucous staining was not quantified. The addition of ETS to the HDMA/PG-treated animals resulted in a significant increase in the abundance and mass of mucous cells over all other HDMA-treated groups. These cells were hypertrophied and continuous throughout the airways including the most distal bronchioles. An increase in the number of goblet cells is one of the characteristics of mucous metaplasia, a key marker of “airway remodeling” that is associated with number of lung disorders including chronic asthma [8] and chronic obstructive pulmonary disease (COPD) [18]. A recent study of COPD patients suggested that cigarette smoke exposure is related to goblet cell hyperplasia [18]. These observations taken together further suggest an interaction between tobacco smoke in the environment with progesterone in the exacerbation allergic airway disease.

The HDMA exposure model was associated with pronounced shifts in TH1 (reduced) and TH2 cytokines (increased). The TH1 associated cytokines (IFN-γ, IL-12 (p70), and IL-18) were all reduced in the HDMA-treated groups; they were further diminished in the groups that were also exposed to ETS (Fig. 4). This finding is consistent with the ability of ETS to shift the immune response towards TH2 within the asthmatic phenotype [30, 32]. Figure 2 clearly demonstrates that the most abundant barrier epithelial cell in the allergic animals is the goblet cell. Walter et al. has previously described an increase in the IL-12 p40 subunit in barrier airway epithelial cells during inflammation, including over expression in asthmatic patients [47]. In this mouse model of allergic airway disease, IL-12 p40 is increased in the BALF. The addition of progesterone to the model significantly enhanced IL-12 p40 in the BALF ($p < 0.05$), again consistent with the largest increase mucous barrier epithelial cells being in the HDMA/ETS/PG group. This may indicate a role for progesterone in stimulation of inflammation in airways with abundant mucous cells, such as those found in allergic asthmatics. Further research is needed to define the relationship of mucous-producing cells and IL-12 p40 expression.

Progesterone was associated with increased IL-5 protein in BALF. This effect was enhanced by the addition of ETS. Interleukin 5 is the hematopoietic growth factor responsible for growth and differentiation of eosinophils. This may indicate an interaction of progesterone and ETS in the activation and recruitment of eosinophils into the airways of mice exposed to allergen. IL-5 is critical for eosinophilic inflammation; therefore, it is no surprise that the groups with the greatest tissue eosinophilia were also the groups with the highest IL-5 levels. Not only do these animals have eosinophils present around blood vessels but also in the submucosa of the airways and in alveoli. This alveolar distribution of eosinophils was not present in the PL-treated animals but was found in PG-treated animals. This may be indicative of the effects of circulating PG on eosinophils. A number of studies have correlated tissue eosinophilia with clinical severity of disease [2, 26]. The development of large areas of subepithelial cellular accumulations are often referred to as bronchus-associated lymphoid tissue (BALT) which is not present in healthy lungs of mice or humans. BALT can be induced following infections and other antigenic events [28, 37] such as HDMA exposure. We found large focal areas of lymphomononuclear cells in the submucosa of the airways that match these descriptions in the HDMA-treated animals. Cell accumulations were greatly increased in size and abundance with the addition of PG and ETS, again indicating the most severe disease was found in this combined group (HDMA/PG/ETS).

It has been shown that localized IgE in the lung is closely related to airway inflammation, lung function, and is a better marker than systemic levels of IgE of clinical outcomes in asthma; local IgE has also been shown to be highest in patients with eosinophilic asthma [2]. Our previous study measured circulating levels of IgE in blood, as total serum IgE, and demonstrated that both of the HDMA/PG-treated groups had significantly more than all other groups [24]. The current results detailed in Fig. 5 indicate that there is a correlation between high levels of serum-IgE and tissue eosinophilia. As with the eosinophils, the group with the greatest abundance of IgE-positive cells in the lung was the HDMA/PG/ETS group. The positive cells were most evident in areas of BALT-like formation. There were also IgE-positive cells in the alveolar space. The staining is most pronounced in the HDMA/PG/ETS group. The cytokine responsible for B cell class switching to express IgE is IL-4. The elevated IL-4 levels in the BALF of HDMA-exposed animals are consistent with the immunohistochemical analysis of IgE-positive cells (Fig. 4). All exposure groups that received either ETS or PG in addition to HDMA contained significantly elevated levels of IL-4 compared to the group that only received HDMA (HDMA/PL/FA). This is further evidence of the role of either PG or ETS in the exacerbation of allergic airway disease.

We evaluated the expression of PGRs in the lung tissue. The ability of the hormone to act on a cell is determined by both circulating levels of PG and the concentration of the receptor in the target cell [46]. Our treatment of these animals with exogenous PG resulted in a significant increase in circulating PG that was within normal physiologic limits. However, PG treatment alone did not have an effect on the abundance of PGRs in the lung. It has been previously demonstrated that ovariectomy does not affect PGR levels in rabbit lung [3]. The staining pattern described in the rabbit study included alveolar epithelial cells, fibroblasts, endothelial cells, neutrophils, and eosinophils; the same pattern was seen in the lungs of the mice in the current study. Our data demonstrate that both ETS and HDMA treatment resulted in an increased abundance of cells with PGRs in lung tissue. In the absence of HDMA, ETS increased the intensity of the staining in the airway epithelium indicating an increase in the number of receptors (Fig. 6b). This finding supports the estrogenic properties of tobacco smoke that have been suggested by others whereby tobacco smoke activates estrogen receptors triggering downstream signaling resulting in the induction of progesterone receptor expression [20, 21]. In the HDMA-treated groups, there were fewer PGR staining cells in the airway epithelium. This reduction corresponds to the increase in mucous cell staining, possibly indicating that goblet cells may not have PGRs. In the HDMA animals, the areas of BALT-like formation in the interstitium adjacent to the airways contain many cells that stain positive for PGRs. One possible explanation as to why PGRs would increase in a model of allergic airway disease is the fact that progesterone receptors are often upregulated in proliferating tissues. This has been demonstrated in uterus during the proliferative phase in humans [11] as well as in the developing lungs of mice [33]. The inflammation seen in asthma is a proliferative response and in this model is exacerbated by ETS. ETS also results in an increase in proliferation in human cases of isolated aggregations of lymphoid cells (IALC) [9]. It is therefore possible that the same increase in progesterone receptors would be present in IALC of the allergic asthmatic lung.

In summary, we have demonstrated increased abundance of cell phenotypes typical of human asthma in the airways of a mouse model developed using a known human allergen, HDMA. We found both airway inflammation and mucous cell hyperplasia. Progesterone and ETS separately and together exacerbated mucous cell distribution and abundance as well as inflammation characterized by accumulations of eosinophils and IgE-positive cells. This action may partially be due to an increase in progesterone receptors in the asthmatic lung, an area that merits further study in humans and in animal models to fully establish cause and effect. The current study raises the possibility that

the hormone progesterone may be a factor for the increased incidence of asthma in women, worsening mucous-related obstruction and eosinophilia in the lung tissue. Previous studies have investigated exogenous hormone administration and the risk of asthma, finding that hormone replacement therapy resulted in an increased prevalence of asthma [16]. Our current work demonstrates a potential biologic basis for this increased prevalence; progesterone can increase airway remodeling and inflammation.

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