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IgE mediates broncho-vascular remodeling after neonatal sensitization in mice

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1. ABSTRACT

The temporal origins of childhood asthma are incompletely understood. We hypothesize that allergen sensitization which begins in early infancy causes IgE-mediated airway and vascular remodeling, and airway hyper-responsiveness. Mice were sensitized with ovalbumin (OVA) without or with anti-IgE antibody from postnatal day (P) 10 through P42. We studied airway resistance in response to Methacholine (MCh) challenge, bronchoalveolar lavage fluid (BAL) inflammatory cell content, immunohistochemistry for inflammation, alpha-smooth muscle actin (alpha-SMA) and platelet/endothelial cell adhesion molecule (PECAM) proteins, and Western blotting for vascular endothelial growth factor (VEGF) protein. Compared to controls, mice treated with OVA had increased airway resistance (baseline: 192% of control; MCH 12 mg/mL 170% of control; P less than 0.0.5). OVA treatment also increased lung alpha-SMA, VEGF and PECAM compared to controls. Inflammatory cells in the BAL and perivascular and peribronchiolar inflammatory cell infiltrates increased over controls with OVA exposure. These changes were counteracted by anti-IgE treatment. We conclude that mice sensitized in early infancy develop an IgE-mediated hyper-reactive airway disease with airway and vascular remodeling. Preventive approaches in early infancy of at-risk individuals may reduce childhood asthma.

2. INTRODUCTION

Asthma is commonly first diagnosed in childhood with symptoms of atopy, episodic dyspnea and wheezing (1). Children with asthma exhibit reduced airflow in baseline spirometry and increased airway responses to histamine. It is known that exposure to allergens early in life may lead to sensitization, wheezing, and asthma in later life (2,3). However, the mechanism behind this early life susceptibility is not fully understood. Thickening of the reticular basement membrane, and eosinophilic inflammation characteristic of asthma in older children and adults are not present in young symptomatic children with reversible airflow obstruction (4). However, these observations do not preclude the possibility of immunoglobulin-mediated pathogenesis of asthma beginning in very early infancy.

The pathophysiology of allergic asthma includes airway hyper-responsiveness, airway inflammation, and tissue remodeling. The airway remodeling includes thickened basement membrane, increased smooth muscle

mass, and changes in airway mucosal vascularity (5, 6). Importantly, inflammation of the pulmonary vasculature is also associated with airway inflammation. The contribution of the vascular bed to airway wall remodeling has not been fully elucidated. Adult asthmatics have significant increases in airway vascularity associated with increased airflow obstruction (7). Bronchoscopic biopsies from the major airways of subjects with mild asthma exhibited increased bronchial vessel numbers and size and increased type IV collagen in vessel walls (8). A current hypothesis suggests that the vascular engorgement of bronchial and pulmonary vessels and consequent thickening of the airway mucosa leads to narrowing of the bronchial lumen, increased airway resistance, and decreased forced expiratory flow rates. Vascular endothelial growth factor (VEGF) and other endothelial cell markers are increased in the sputum of asthmatic patients (8), (9). However, little is known about pulmonary vascular remodeling caused by sensitization in very early infancy that leads to asthma. Asthma symptoms are not uncommon in early infancy, suggesting the likelihood of very early sensitization, perhaps as early as the first few months of life, leading to significant vascular remodeling.

IgE plays a major role in allergic disease by causing mast cells to release histamine and other inflammatory mediators which may induce α-smooth muscle proliferation. Studies show that neonatal rodents can mount a vigorous IgE response to allergen challenge. Ovalbumin exposure begun at birth significantly increased serum IgE at 7 days of age, with increased airway smooth muscle vimentin (guinea pigs) and smooth muscle actin (mice) (10, 11). These studies suggest the possibility that allergen-induced changes in IgE may underlie asthma arising in very early infancy.

Because of the central role that IgE plays in the pathology of asthma in older children and adults, anti-IgE antibody is a mainstay of asthma maintenance therapy in these patients. Anti-IgE antibody neutralizes circulating IgE, preventing its binding to its high-affinity mast cell receptor. Inhibition of IgE activity reduces allergen-induced eosinophilic inflammation (3). The role of IgE in development of asthma beginning in very early infancy, including airway smooth muscle hypertrophy, vascular remodeling and airway inflammation is not known. We hypothesized that allergen sensitization in very early infancy is mechanistically associated with both vascular and airway remodeling and increased airway hyper-responsiveness. We further hypothesized that this response is mediated by IgE.

3. MATERIALS AND METHODS

3.1. Materials

Reagents were obtained as follows: Rat Anti-PECAM1 (Platelet endothelial cell adhesion molecule, also known as CD31) monoclonal antibody was from Millipore (Billerica, MA). Antibodies to rabbit polyclonal alpha smooth muscle actin (α-SMA) and vascular endothelial growth factor (VEGF), and ImmunoCruz™ rabbit ABC Staining System were from Santa Cruz Biotechnology. Anti-rabbit IgG and anti-mouse IgG antibodies were from Cell Signaling Technology (Danvers, MA). Mayer's hematoxylin was from Dako, (Carpinteria, CA). Rat anti-mouse IgE and IgG isotype antibodies were from Pharmingen, (San Diego CA). Alum precipitated OVA (grade III) leupeptin, aprotenin, phenylmethyl-sulfonylfluoride (PMSF) and antipain were from Sigma (St Louis, MO). Protein bichinchonic acid (BCA) microassay kit was from Pierce (Rockford, IL).

3.2. Animals

Neonatal mice (BALB\c) and their dams were purchased from Taconic Farms, NY and housed at the Tufts Medical School laboratory animal facility. This facility confirms strictly to the current National Institutes of Health guidelines for animal care. The animal use protocol was approved by the Tufts University and Tufts Medical Center Institutional Animal Care and Use Committee.

3.3. Allergen sensitization

Ten-day-old (P10) BALB/c mice received 25 µg OVA with 1 mg of aluminum hydroxide as adjuvant, or an equal volume of saline intra-peritoneally (IP). Then beginning on P15, mice were given 100 µg OVA (in 50 µL saline) by intranasal administration three days a week for four weeks under Isoflurane sedation (3.5.%). At P45 (24 hours after the last OVA exposure) some mice were deeply sedated with ketamine and xylazine for pulmonary function measurements. Others were sacrificed, bronchoalveolar lavage (BAL) fluid obtained and the lungs processed for western blot analysis or immunostaining.

3.4. Anti-IgE treatment

Additional groups of mice exposed to OVA or saline as above were also treated with anti-IgE or isotype IgG antibody (antibody control). Monoclonal rat anti-mouse IgE or isotype IgG antibody (50 µg IP) was given at age P8 and P9 prior to initiating OVA exposure on P10. Anti-IgE or isotype IgG injections were continued weekly along with OVA or saline until the OVA or saline treatments were concluded.

3.5. Airway resistance

Airway resistance was measured at age P45. After Ketamine (90 µg/gm body weight) and xylazine (10 µg/gm body weight) sedation the trachea was cannulated. The cannula was connected to a computer-controlled small animal ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada), and ventilation delivered at a frequency of 150 breaths/minute and tidal volume of 5 µL/kg (12). Airway resistance in response to inhalation of aerosolized methacholine (MCh) in increasing doses (0-12 mg/mL

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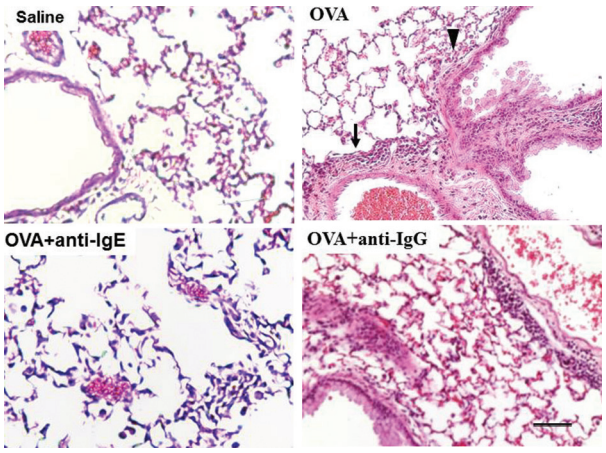

Figure 1. Anti-IgE reduces lung inflammatory cell infiltrates in sensitized mice. Representative photomicrographs of 5-micron sections from paraffin embedded lung from P45 mice (H&E stain; 20X). Increased inflammatory cells were observed surrounding blood vessels and airways in OVA-exposed mice compared to saline controls; anti-IgE ameliorated this increase in inflammatory cells. Isotype IgG had no effect. Arrows indicate blood vessels; arrow heads indicate airways. Bar represents 50 µm.

saline) was measured. The frequency-independent airway resistance (RRS) in Cm of H_2O/ml/s was determined using the Scireq software as described (13).

3.6. Bronchoalveolar lavage

Mice were tracheotomized in the supine position; 0.5. mL of PBS was instilled and withdrawn three times, and the aliquots pooled. The total cell count in the BAL fluid was measured using a hemocytometer after gentle vortexing of the sample. The BAL protein concentration was measured using the BCA microassay after low speed centrifugation to remove cells.

3.7. Western blot

Lung tissue was harvested into an ice-cold solution of PBS containing protease inhibitors (leupeptin (2 mg/mL), aprotenin (1 mg/mL), 1mM PMSF and antipain (2mg/mL). Proteins (30mg per sample) were separated by electrophoresis on a 10% acrylamide-SDS gel, then transferred to a nitrocellulose membrane and probed overnight at 4ºC using a mouse monoclonal VEGF antibody (1:200) followed by goat anti-mouse IgG HRP secondary antibody (1:4000) for two hours at room temperature. The resulting signal was identified using chemiluminescence (Amersham, Bio-Rad) and exposure to x-ray film. Blots were stripped and reprobed with anti-actin antibody as an internal control. Signal intensity was measured using densitometry scanning.

3.8. Immunostaining

Lungs were inflated to 20 mm H_2O with 4% paraformaldehyde, fixed overnight and embedded in paraffin. Five-micron sections were deparaffinized in xylene, rehydrated and washed in PBS. Inflammatory cell infiltration was identified by staining with hematoxylin and eosin and viewing by light microscopy. For α-SMA, endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide (in PBS) for 10 minutes. Sections were fixed and blocked with PBS containing 0.1.% Triton X-100 and 1% BSA at room temperature for 45 minutes. PECAM expression was identified by incubating with an anti-PECAM antibody (1:50) overnight at 4^0C followed by FITC anti-rat IgG (1:2000) for 2 hours at room temperature. 4', 6-diamidino-2-phenylindole (DAPI) dye was used to stain the nuclei. Immunohistochemistry for α-SMA was performed after antigen retrieval in sodium citrate buffer at 95ºC for 30 minutes. The sections were blocked with blocking serum (Santa Cruz) and incubated at room temperature for 2 hours, treated overnight with primary antibody (1:200), followed by biotinylated secondary antibody for 1hour, then incubated with AB enzyme reagent and counterstained with hematoxylin. Slides from at least three different animals were reviewed for each stain.

3.9. Statistical analysis

Results were expressed as mean ± SEM of N=4-6 mice. Statistical analyses were done by ANOVA with post-hoc multiple comparison testing with either the Dunnett or the Bonferroni post-hoc test using Graph Pad InStat software (GraphPad Prism Inc., La Jolla CA).

4. RESULTS

4.1. Lung inflammation

Hematoxylin and eosin stained slides were examined for peribronchiolar and perivascular inflammatory cell infiltration. Lungs from the saline treatment group had very few macrophages and neutrophils in the peribronchiolar and perivascular regions. OVA-treated animals had severe dense perivascular and peribronchiolar inflammatory cell infiltration. Lungs from the OVA plus anti-IgE treatment showed a marked decrease in inflammatory cells around both airways and blood vessels. This decrease was not seen in mice exposed to OVA plus isotype IgG (Figure 1).

4.2. BAL cell and protein content

Overall, 50-75% of the instilled volume was recovered as BAL fluid. The recovery volume did not differ among the various groups. OVA-exposed mice had significantly more cells in the BAL fluid compared to saline-exposed and anti-IgE-treated OVA-exposed mice (Figure 2A). The protein concentration was also significantly increased in the BAL fluid from OVA-exposed mice compared to animals exposed only to saline (Figure 2B). Anti-IgE treatment counteracted the protein increase induced by OVA.

4.3. Airway remodeling

Airway remodeling was assessed by examination of airway α-SMA expression using

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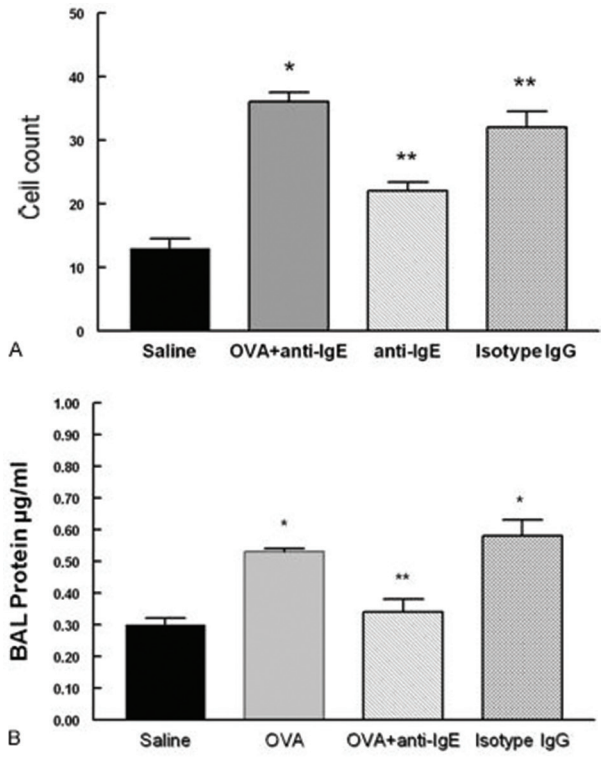

Figure 2. Anti-IgE reduces BAL fluid cell and protein content in OVA-exposed mouse lungs. Isotype IgG had no effect on OVA-induced increases. A: Cell counts in BAL fluid from mice exposed to saline, OVA or OVA with either anti-IgE or isotype IgG conditions. Data represent means ± SEM; N=4-6; *: P<0.0.5 compared to saline controls; **: P < 0.0.5. compared to OVA. B: BAL total protein content from mice exposed to saline, OVA, OVA + anti-IgE or OVA + isotype IgG. X-axis labels: Anti IgE = OVA + anti-IgE; Isotype IgG = OVA + isotype IgG.

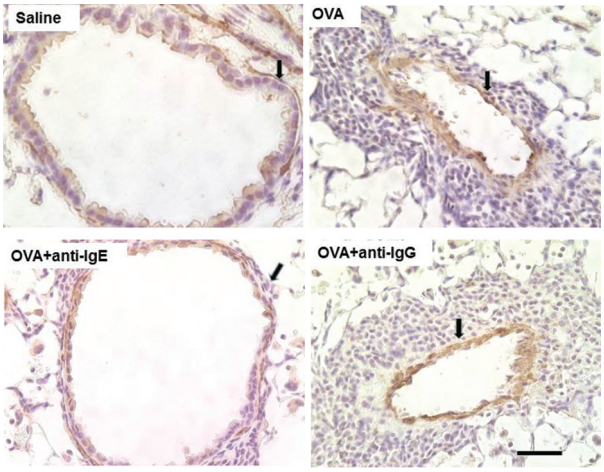

Figure 3. Immunohistochemistry of α-SMA. Representative photomicrographs of 5-micron lung sections from mice exposed to saline, OVA, OVA + anti-IgE or OVA + isotype IgG stained for α-SMA (40X magnifications) are shown. Arrows point to positive stain for α-SMA around bronchioles and blood vessels. Bar represents 50 µm.

immunohistochemistry. Figure 3 shows increased amounts of α-SMA in large bronchi and bronchioles. This increased expression was counteracted by anti-IgE treatment but not by isotype IgG.

4.4. Airway resistance

OVA exposure beginning at P10 caused increased airway resistance at baseline and in response to increasing doses of aerosolized MCh. Figure 4 shows that the baseline RRS was increased in OVA-treated mice (192% of control), and continued to be increased in response to MCH (170% of control at 6 and 12 mg/mL of MCH). Anti-IgE reduced the airway resistance to near-control levels, especially at 6 and 12 mg/mL MCh. Isotype IgG treatment did not reduce the airway resistance.

4.5. Vascular remodeling

VEGF expression and imaging of PECAM-expressing cells were used as indices of vascular remodeling. Chronic allergen exposure of adult mice increases VEGF expression (14). To determine whether initiating allergen exposure in early infancy increased lung VEGF levels, Western blot analysis was performed. VEGF was increased in OVA-sensitized mice (126.4.% ± 6 of the saline control). Anti-IgE, but not isotype IgG blocked this increase (Figure 5). PECAM immunostaining identified increased PECAM-positive cells in OVA-exposed mice compared to controls (Figure 6). This increase was counteracted with anti-IgE treatment but not with isotype IgG.

5. DISCUSSION

The purposes of our study were to determine if IgE mediates asthmatic changes induced by allergen exposure in very early infancy and to determine if the IgE-mediated changes resulted in both vascular and airway remodeling. This neonatal mouse model of asthma differs from other mouse models in that it shows the effects of intermittent allergen exposure during the developmental period of lung alveolarization and subsequent early airway and vascular growth. Murine alveolar and accompanying vascular development are at their peak P10. We found that allergen exposure of mice beginning in very early infancy increases RRS accompanied by remodeling of the airway and pulmonary vascular components, and that these effects are mediated by IgE. Because of the age during allergen exposure this is new information.

Elevated IgE levels and eosinophilic inflammation are associated with bronchial asthma and allergic rhinitis in human children and adults (2). Adult mice with asthma have inflammation with high IgE levels in the serum (15, 16). Aeroallergen exposure of preschoolers leads to IgE-mediated hyper-reactive airway disease later in life (2) (3). Additional studies showed that solid food exposure within

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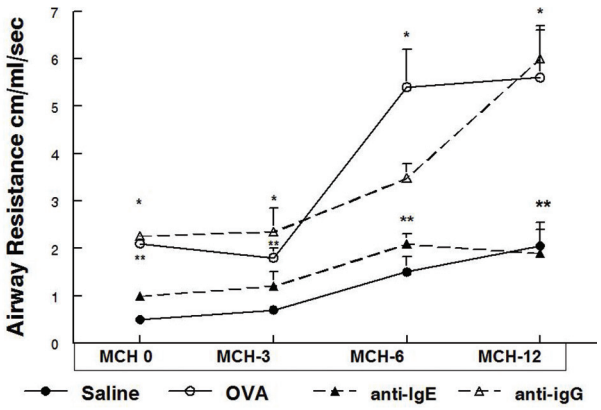

Figure 4. Airway resistance (RRS) in response to increasing doses of methacholine. RRS was measured using the forced oscillation technique. Figure shows the dose-response results from saline controls (closed circles), OVA sensitization (open circles), OVA sensitization with anti-IgE (closed triangles, dotted line) and OVA sensitization with isotype IgG treatment (open triangles, dotted line). Data represent means ± SEM; N=4-5; *: P<0.0.5 compared to saline controls (**: P<0.0.5 compared to OVA at MCH 6 and 12 mg/ml.

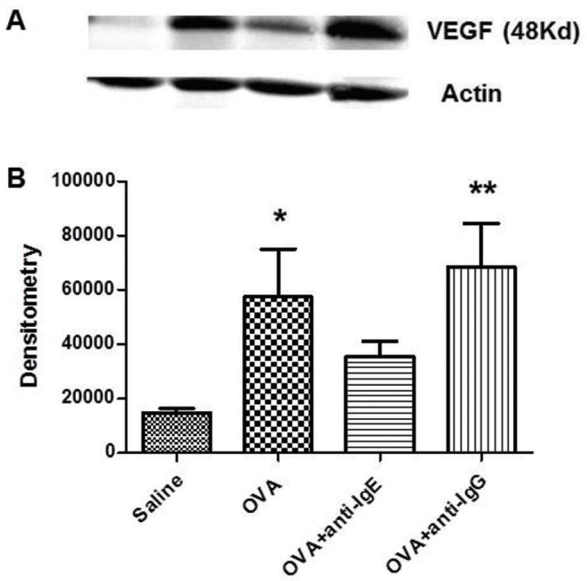

Figure 5. VEGF protein expression. A: Representative Western blot for VEGF mouse lungs following exposure to saline, OVA, OVA + anti-IgE or OVA + isotype IgG. B: Densitometry of western blots for VEGF. The signals were normalized to actin. Data represent means ± SEM; N=3; *: P=0.0.5 compared to saline control. **: P=0.0.5 compared to OVA-exposed mouse lungs.

a specific window in the first year of life is associated with elevated IgE levels at age 5, but asthmatic symptoms were not reported (17). Our mouse model of antigen exposure beginning at P10 indicates that IgE-mediated asthma can be initiated by allergen exposure in very early infancy and suggests that anti-IgE antibody may have therapeutic and preventive effects very early infancy.

Although the systemic blood vessels that supply the tracheal and main stem bronchi in the mouse lung do not penetrate into the intraparenchymal airways as they do in larger mammals, the mouse lung remains a useful model for studying pulmonary vascular remodeling in asthma. New blood vessels enter the lung directly via a new vascular network that develops between the visceral and parietal pleurae, supplied by several intercostal blood vessels (18). Thus, while our findings do not implicate vascular remodeling causing airway restriction as seen in humans, our data do indicate that very early onset of allergen sensitization causes remodeling of the pulmonary vascular bed. Whether this remodeling leads to significant ventilation-perfusion mismatches as a component of asthma remains to be determined.

Allergic asthma involves airway hyper-responsiveness with fixed airway obstruction due to airway remodeling, including changes in airway mucosal vascularity. Increased vessel size and number, and increased expression of VEGF have been documented in asthmatic airways (14). Increased airway smooth muscle cells and vascular network density are more pronounced in children with obstructive airway disease (19). We found that anti-IgE reduced OVA-induced airway and vascular α-SMA expression, implying reduced airway and vascular remodeling. In addition to its neutralizing effect on IgE, this antibody also stabilizes mast cells in allergic rhinitis and asthma (20). These effects could prevent the vascular remodeling that we observed.

Airway hyper-responsiveness is an important physiological consequence of airway remodeling in asthma. An important study showed that while corticosteroid administration improves lung function in children with persistent airway obstruction by reducing inflammation, it does not further enhance the effects of bronchodilator treatment (19). Bronchial biopsies showed that airways of these children had pronounced increases in vascular density and PECAM-positive cells associated with increased airway smooth muscle cells (19). We demonstrated vascular and airway remodeling in association with lung function changes in response to allergen-exposed beginning in the neonatal period of mice was reversed by anti-IgE treatment

Mediators of vascular remodeling may also contribute to airway remodeling. VEGF, an important angiogenic growth factor, also contributes to non-specific airway hyper-responsiveness, has chemotactic effects on eosinophils, and enhances airway smooth muscle cell proliferation (21, 22). VEGF is expressed in human and mouse lungs where it has an important role in lung development. Dysregulated VEGF expression has been implicated as a major contributor to the development of a number of diseases (23). VEGF is increased during lung injury and fibrosis (24). Premature stimulation of VEGF production is a key factor in both the inflammatory

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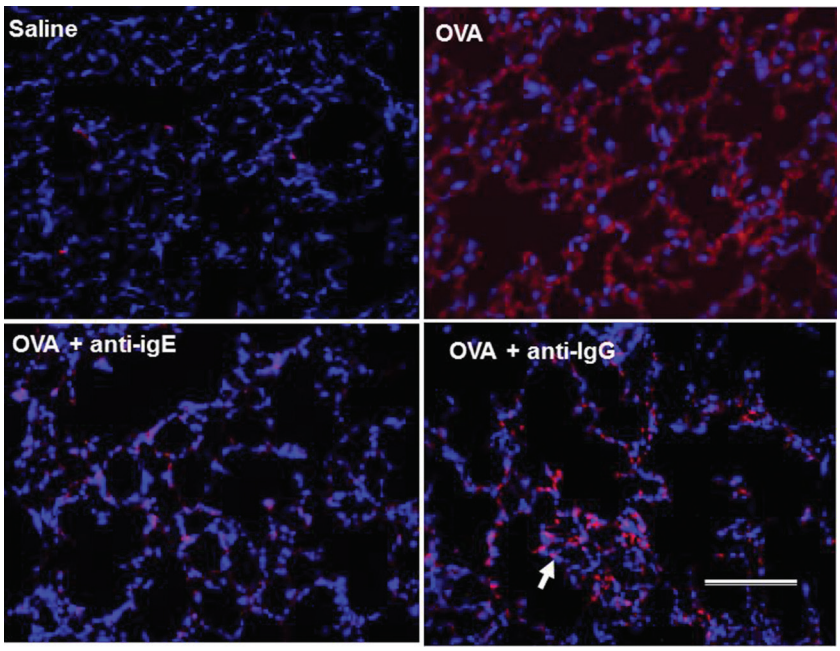

Figure 6. PECAM-expressing cells in mouse lung. Representative photomicrographs of immunofluorescent PECAM staining in 5-micron lung sections from mice treated with saline, OVA, OVA + anti-IgE or OVA + isotype IgG (20X). Arrows indicate positive stain in airways and mesenchyme. PECAM fluorescence in red; DAPI counterstain in blue. Bar represents 100 µm.

and angiogenic processes in these conditions. Cellular sources of VEGF in the airways include epithelial cells, macrophages and airway smooth muscle cells (25).

Increases in the numbers of inflammatory cells in the BAL and in VEGF expression in the lungs of OVA-exposed mice were associated with airway hyper-responsiveness. Previous work showed that anti-IgE antibody treatment of adult mice attenuated both eosinophilic airway inflammation and airway hyper-responsiveness (3). Similarly, we found that anti-IgE antibody reduced the number of inflammatory cells in the BAL fluid and the inflammatory cell infiltration of the perivascular and peribronchiolar regions in early infancy. These changes, which agree with *in vitro* cell studies (26) all occurred in association with improved lung function.

In conclusion, our results indicate that allergen sensitization of mice beginning in very early infancy produces airway hyper-responsiveness with airway and vascular remodeling and inflammation that is mediated by IgE. Importantly, our results indicate that IgE-mediated vascular and airway remodeling is a major contributory factor in the development of allergic asthma in the context of sensitization in very early infancy. Our study provides a potential basis to explore novel therapeutic approaches to prevent childhood asthma.

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