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High Vascular Endothelial Growth Factor Levels in NZW Mice Do Not Correlate with Collagen Deposition in Allergic Asthma

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Key Words
Airway remodeling · Lipid body · Eosinophil peroxidase deficiency · Transforming growth factor-β · Vascular endothelial growth factor

Abstract
Background: Eosinophils contribute to the early features of allergic lung inflammation through the generation and release of a plethora of mediators. Eosinophil peroxidase (EPO) is one of the eosinophil granule proteins involved in the early response, but its participation in airway remodeling is not established. The present study addressed this question comparing an EPO-deficient mouse strain (NZW) with BALB/c and C57Bl/c strains. ***Methods:*** Mice were immunized with ovalbumin/alum, challenged twice with ovalbumin aerosol, and lung responses were measured at day 22 or 28. Collagen, mucus and eosinophils were determined in lung sections stained with picrosirius, periodic acid-Schiff or hematoxylin-eosin; transforming growth factor-β and vascular endothelial growth factor were determined by ELISA, lipid bodies by enumeration in osmium-stained eosinophils, and airway reactivity to methacholine in isolated lung preparations. ***Results:*** NZW mice showed significantly less collagen around bronchi and blood vessels, less mucus and less eosinophils around bronchi. Eosinophil lipid body formation and airway hyperreactivity were comparable among strains. Levels of transforming growth factor-β were also comparable; however, the NZW mice showed much higher levels of vascular endothelial growth factor, even under basal conditions. ***Conclusions:*** In allergic lung inflammation, the combination of EPO deficiency and overexpression of VEGF found in NZW mice is associated with less collagen deposition, less mucus and reduced tissue eosinophilia. Eosinophil activation and airway hyperreactivity in NZW mice were similar to the other strains.
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Introduction

Asthma is a chronic inflammatory disease of the airways characterized by eosinophil infiltration into the airways under the direction of Th2 lymphocytes. Eosinophils are considered a hallmark of allergic lung inflammation. They proliferate in bone marrow from CD34+ progenitors and differentiate in the presence of interleukin (IL)-5. Complex interactions between cytokines (including IL-4, IL-5 and IL-13), chemokines (eotaxin and RANTES), lipid mediators (platelet-activating factor)

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and adhesion molecules (selectins and VLA4) regulate the trafficking of eosinophils from the bone marrow to the lung [1].

The persistence of eosinophilic lung inflammation can induce structural changes in the airway wall, such as increased thickness of the basement membrane, increased collagen deposition, subepithelial fibrosis, changes in bronchial microcirculation, and smooth muscle hypertrophy and hyperplasia. Some of these changes can occur as soon as the airway obstruction is resolved, generally 24 h after the antigen exposure [2, 3]. Although the mechanisms underlying remodeling are not completely understood, both transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) are involved [4, 5]. VEGF is a dimorphic glycoprotein of 34,000–42,000 Da, consisting of two disulfide-linked peptide chains with identical N termini [6]. VEGF plays several roles both in the development of vasculature and in the maintenance of vascular structure and function. Emerging evidence indicates that VEGF is also implicated in the morphological alterations observed in asthma [4, 5, 7]. TGF-β stimulates the synthesis of several extracellular matrix proteins including collagen, and evidence from animal models suggests that airway remodeling may be prevented using agents which target TGF-β [8, 9].

Eosinophil involvement in acute inflammatory events in asthma is well established. They contribute to the early features of allergic inflammation through the generation of lipid mediators and release of granule contents [1]. Eosinophils store cationic and cytotoxic proteins in their secondary granules, among them the eosinophil peroxidase (EPO), a cationic heme-containing protein that corresponds to nearly 25% of the total protein mass in eosinophil secondary granules [10]. EPO was shown to be cytotoxic to the epithelial cells of the bronchi [11], and in consequence, the airway smooth muscle becomes more reactive to various stimuli. This bronchial hyperreactivity (BHR) is one of the characteristic features of asthma. Studies in humans have shown that there was a correlation between serum levels of EPO and BHR in atopic subjects [12].

The involvement of eosinophils in airway remodeling in asthma was only recently demonstrated by Humbles et al. [13] who showed that transgenic mice whose eosinophil lineage was ablated had less airway remodeling compared with the wild type. However, the role of EPO in airway remodeling was not determined. The present study investigated the contribution of EPO to some aspects of the remodeling process in experimental asthma. To this purpose, we induced an allergic lung inflammation in a mouse strain (NZW) which exhibits EPO deficiency due to a spontaneous mutation [10] and measured collagen deposition around the bronchi and vessels, mucus formation, airway VEGF and TGF-β levels and airway hyperreactivity. We also examined eosinophil numbers in bronchoalveolar lavage and around bronchi, as well as their lipid body formation, indicative of activation. Results found in NZW mice were compared with those found in BALB/c and C56Bl/6 mice, two strains widely used in experimental asthma research.

Methods

Animals

Male NZW, Balb/c or C57Bl/6 mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with a 12-hour light/dark cycle with water and food ad libitum. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation and approved by the Biomedical Sciences Institute/USP-Ethical Committee for Animal Research.

Induction of Allergic Inflammation

Mice were sensitized on days 0 and 7 by an intraperitoneal injection of a mixture containing 50 μg of ovalbumin (OVA) and 1 mg of Al_2O_3 in saline. At 14 and 21 days after first immunization, the animals were challenged by exposure to an aerosol of OVA (Sigma grade III, 2.5% wt/vol in saline) generated by an ultrasonic nebulizer (ICEL-US800, São Paulo, Brazil) delivering particles of 0.5–10 μm in diameter at approximately 0.75 cm^3/min for 30 min. The variables were measured at day 22 or 28. The control group consisted of 6 animals immunized as described and exposed to aerosols of isotonic saline solution. The experimental groups consisted of 5 animals. Measurements of responses were made at day 22 or 28.

Harvesting of Bronchoalveolar Cells

The animals were killed by an intraperitoneal injection of ketamine/xylazine (50 μl of a 100-mg/ml solution) at day 22 or 28. A tracheal cannula was inserted via a midcervical incision, and the airways were lavaged three times with 1 ml of phosphate-buffered saline (PBS; 10 mM, pH 7.4, at 4°C).

Total and Differential Cell Counts

The bronchoalveolar lavage fluid (BALF) was centrifuged at 170 *g* for 10 min at 4°C, the supernatant was removed and the cell pellet resuspended in 0.5 ml PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hematocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin-eosin (Hema 3).

Assay of EPO Activity

The EPO activity present in BALF cells was determined by a colorimetric assay as described by Strath et al. [14], with slight modifications. In brief, the cell suspensions were collected as de-

scribed above, centrifuged and the cells exposed to Tris-NH_4Cl (0.05 M, pH 8.0) buffer to lyse erythrocytes. The cells were then washed once with PBS and adjusted to 10^5 cells/ml. Aliquots of 100 μl of the cell suspension were transferred to 96-well microplates which were then centrifuged at 150 *g* at 4°C for 10 min. The supernatants were carefully withdrawn, and 100 μl of substrate solution containing *o*-phenylenediamine (2.4 mM) in Tris-HCl (50 mM, pH 8.0) and H_2O_2 (6.6 mM) was added to each well. The plates were incubated at room temperature for 15 min. The reaction was stopped by addition of 50 μl of 4 M H_2SO_4 and the absorbance of the samples determined at 490 nm.

Lipid Body Analysis

While still moist, leukocytes on cytospin slides were fixed in 3.7% formaldehyde in Ca^{2+} Mg^{2+}-free Hanks balanced salt solution (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 (30 min), rinsed in distilled water, immersed in 1% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, stained again with 1.5% OsO_4 (3 min), rinsed in distilled water and then dried and mounted. The morphology of fixed cells was observed, and lipid bodies were enumerated by light microscopy with a 100× magnification in 25 consecutively scanned eosinophils [15].

Evaluation of Airway Reactivity

The airway reactivity to methacholine was measured in isolated lung preparations as previously described [16]. Briefly, lungs were removed, blood was washed out via the pulmonary artery, cannulated through the trachea and perfused (5 ml/min) with Krebs (37°C, 95% O_2 and 5% CO_2) solution. A small incision was made into the lower end of each lobe to permit outflow of the solution. The perfusion pressure (cm H_2O) was recorded in a Beckman R511A using Gould P23DB pressure transducers. Increases over basal levels of perfusion pressure following bolus injection of methacholine into the perfusion solution were taken as a measure of constriction of the airways. The pressure was allowed to return to baseline before the next dose of methacholine was added. The response of each lung to the entire set of methacholine doses was recorded.

TGF-β and VEGF Analysis

The TGF-β_1 and $VEGF_{121–189}$ contents of BALF supernatants were quantified by specific enzyme-linked immunoassay (ELISA) kits from R&D Systems (USA), according to the manufacturer's instructions. For TGF-β, the immunoassay detects only the activated isoform, thus a prior step of sample activation by 1 N HCl is required by the manufacturer.

Histological Analysis

Lungs were immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced in 5-μm sections and stained with periodic acid-Schiff (PAS)/hematoxylin for the evaluation of mucus-producing cells or with picrosirius for the evaluation of collagen fibers. Collagen (picrosirius polarization method) and mucus production (PAS/alcian blue) were quantified in the airways with the aid of a digital analysis system (Image Pro® Plus for Windows®, Media Cybernetics, USA) under 200× magnification. The images were generated by a microscope (Axioplan, Zeiss, Germany) connected to a camera (Sony Triniton CCD, Sony, Japan) fed into a computer through a frame grabber (Oculus TCX, Coreco Inc., St. Laurent, Canada) for off-line processing. The thresholds for collagen and mucus were established after enhancing the contrast to a point at which the structures were easily identified as black (mucus) or birefringent (collagen) and kept constant during measurements. The area occupied by fibers was determined by digital densitometric recognition. To avoid bias, the basal membrane perimeter was measured and results were expressed as the percentage of the area stained for collagen fiber or mucus per total tissue area.

The density of eosinophils in lung parenchyma was assessed by conventional morphometry. Using a 100-point grid with a known area (10,000 μm^2 at a 1,000× magnification) attached to the ocular of the microscope, we counted the number of points hitting the outer area of the airway wall and the number of points hitting the adventitia of vessel walls. The tissue area in each microscopic field was calculated according to the number of points hitting the tissue, as a proportion of the total grid area. We then counted the number of eosinophils within that tissue area. Eosinophil density was determined as the number of eosinophils in each field divided by tissue area. Measurements were expressed as cells per square millimeter [17–19]. Counting was performed in 20 fields in each animal, at a 1,000× magnification.

Statistical Analysis

Data are expressed as mean ± SEM of 5–6 determinations. Statistical differences between groups were determined by analysis of variance (ANOVA), complemented with Neuman-Keul's test. Values of $p < 0.05$ were considered indicative of a significant difference between means.

Results

Eosinophils in BALF and Lung Tissue

Mice were immunized with OVA and submitted to two OVA aerosol challenges (experimental group) or saline aerosol (control group). In the BALF (fig. 1a), antigen challenge in immunized mice induced a significant increase in eosinophil number at day 22 in all three strains. This increase was 2-fold higher in NZW than in Balb/c or C57Bl/6 mice. By day 28, BALF eosinophilia was reduced in all three strains.

We also measured the eosinophil infiltrated in the lung tissue by conventional morphometry expressed as the number of eosinophils divided by tissue area (cells/μm^2). Antigen challenge in immunized mice also induced a significant increase in eosinophil numbers in lung tissue at day 22 (fig. 1b). At this time, both Balb/c and C57Bl/6 mice had 2.5- and 4-fold more eosinophils around the bronchi than NZW mice. By day 28, tissue eosinophilia was reduced only in the NZW strain.

BALF cell suspensions collected at day 22 were assayed for EPO activity. Figure 1c shows that in the Balb/c and C57Bl/6 mice, there was a significant increase in EPO

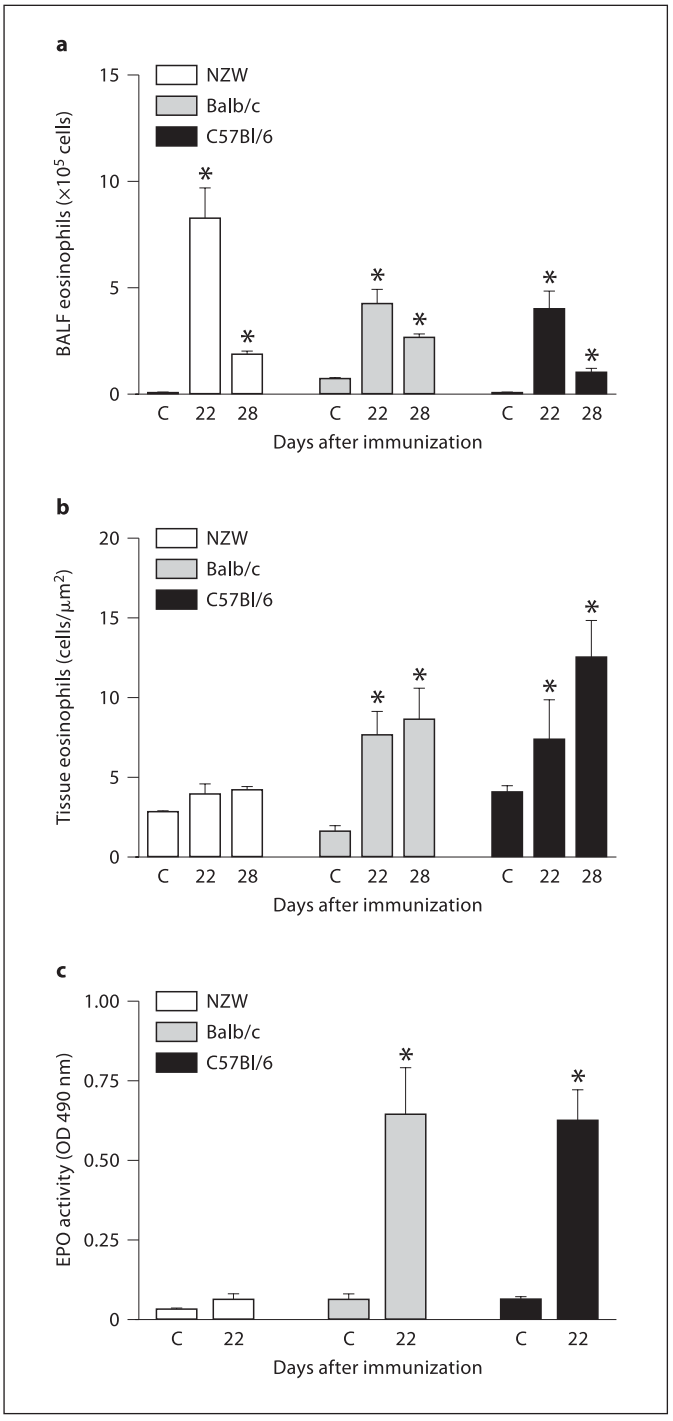

Fig. 1. Eosinophils in the BALF and in lung tissue. Mice were immunized with an intraperitoneal injection of OVA/alumen, one booster injection 7 days later and two antigen aerosol challenges on days 14 and 21 after immunization. Eosinophils were counted in hematoxylin-eosin-stained cytocentrifuge preparations of BALF cells (**a**) or in lung slices (**b**). **c** Eosinophil peroxidase activity in bronchoalveolar lavage cells. Results shown are the mean ± SEM of 5–6 animals per group. * $p < 0.05$ in comparison with the control (C) group (immunized and saline challenged).

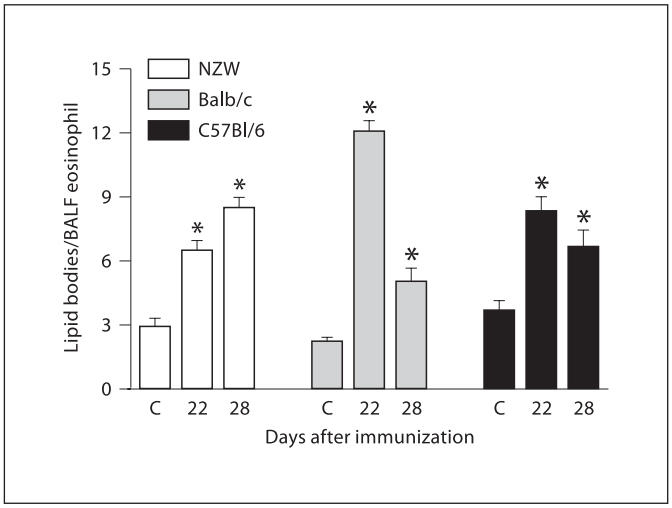

Fig. 2. Lipid body formation. Mice were immunized and challenged as described above. BALF cells were harvested at day 22 or 28. Lipid bodies present in eosinophils were enumerated under light microscopy after osmium staining. Results shown are the mean ± SEM of lipid bodies per eosinophil from 25 eosinophils from each of 5–6 animals per group. *$p < 0.05$ in comparison with the control group (C).

activity relative to control samples at this time point, whereas in the NZW mice, EPO activity was undetectable.

Eosinophil Lipid Body Formation

Lipid bodies are intracellular lipid-rich organelles that contribute to enhance eicosanoid production by inflammatory cells [15]. As shown in figure 2, BALF eosinophils from all three strains showed a significant increase in lipid bodies in the experimental group at days 22 and 28. The number of lipid bodies in the control and the naive groups did not differ significantly between the strains studied (data not shown).

Reactivity of the Airways to Methacholine

The basal perfusion pressure was the same for lungs from NZW, Balb/c or C57Bl/6 strains (9.9 ± 0.6, 10.3 ± 0.6 and 10.8 ± 0.3 cm H_2O, respectively). Bolus injections of methacholine (0.1–100 μg) into the perfusing solution caused bronchoconstriction measured as an increase in perfusion pressure. The experimental groups of both strains exhibited greater responses to methacholine than those of their corresponding control group (fig. 3). For instance, in NZW mice, a perfusion pressure increase of 10 cm H_2O was induced by 6.5 μg of methacholine in

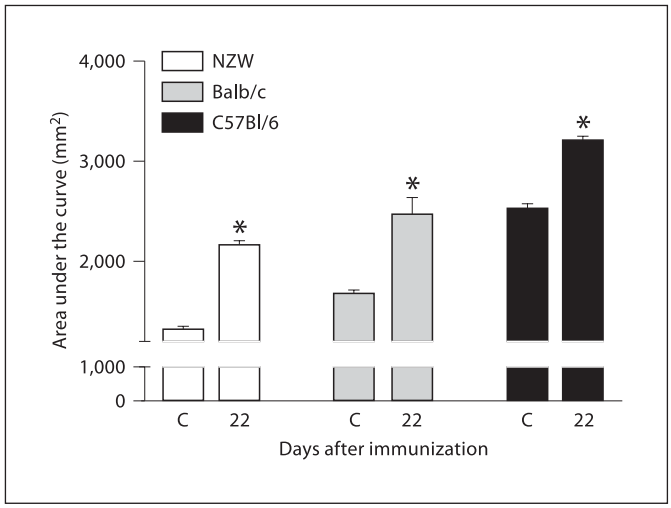

Fig. 3. Airway reactivity to methacholine. Mice were immunized and challenged as above. At day 22, the lungs were removed, perfused via the trachea, and increases in perfusion pressure to bolus injection of methacholine were recorded. The dose response curves to methacholine are expressed as the area under the curve. Results are expressed as mean ± SEM of 7–8 animals per group. * $p < 0.01$ in comparison with the control group (C).

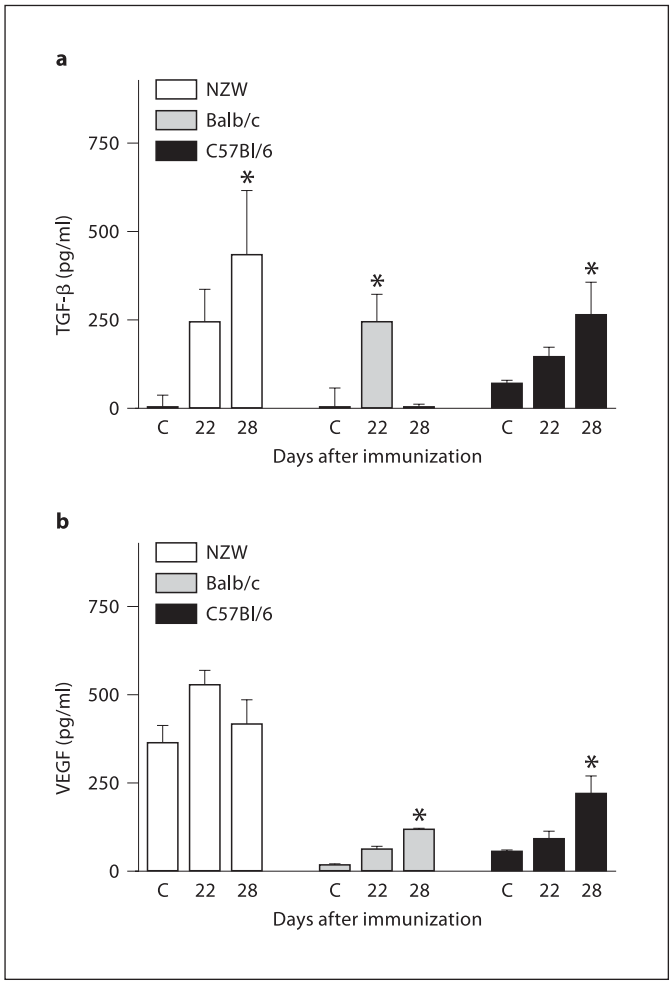

Fig. 4. Concentrations of cytokines in BALF supernatant. Mice were immunized and challenged as described above. BALF was harvested at days 22 and 28, centrifuged, and the supernatant removed. TGF-β (**a**) and VEGF (**b**) content was measured by specific enzyme-linked immunoassay according to the manufacturer's instructions. Results shown are the mean ± SEM of 5–6 animals per group. * $p < 0.05$ in comparison with the control group (C).

the control mice but by only 1.7 μg methacholine in the experimental group, showing an almost 4-fold increase in responsiveness. Comparable increases in response following antigen challenge were observed in the lungs isolated from the Balb/c mice. It can be seen that the phenomenon of hyperreactivity occurred in the three strains.

TGF-β and VEGF Levels in BALF

As shown in Figure 4a, antigen challenge in NZW and C57Bl/6 mice induced an increase in TGF-β at day 22 that was sustained until day 28. In contrast, levels of this cytokine were increased only at day 22 in Balb/c mice.

For VEGF, levels in the BALF of NZW mice were not significantly increased by antigen challenge (fig. 4b), whereas for Balb/c and C57Bl/6 mice, a small increase occurred only at day 28. The most striking difference between the strains was that VEGF was always at a much higher concentration in NZW than in either Balb/c or C57Bl/6 mice, even in nonmanipulated mice (320 ± 25, 13 ± 2 and 20 ± 3 pg/ml, respectively).

Histology of the Lungs

By image analysis, as described in Methods, we determined the fraction of the total lung area positively stained for mucus. In control groups of either NZW, Balb/c or C57Bl/6 mice, none of the bronchi contained mucus in contrast to the experimental groups. Figure 5a shows that in lungs from NZW mice, only a few bronchi exhibited mucus, whereas in Balb/c and C57Bl/6 mice, 15% of the lung parenchyma was covered with mucus. Figures 6a–c illustrate mucus deposition in the bronchi of NZW, Balb/c and C57Bl/6 mice, respectively.

With picrosirius staining, we were able to visualize the collagen fibers around the bronchi and vessels and to measure the area positively stained by image analysis.

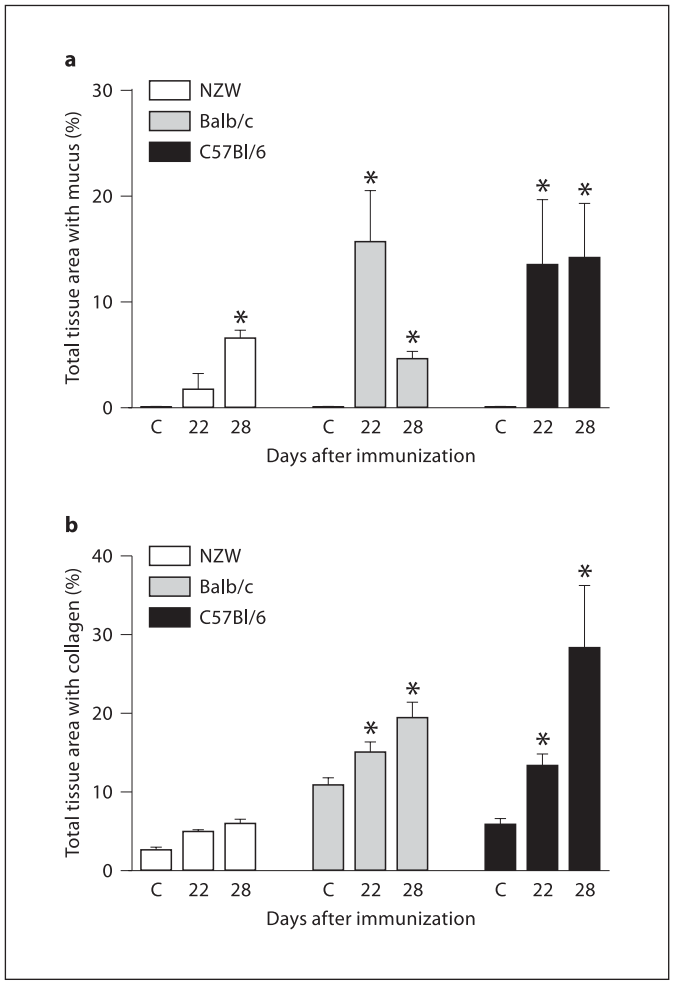

Fig. 5. Mucus production and collagen formation. Mice were immunized and challenged as described above. Lungs were removed at day 22 or 28 and stained with PAS/hematoxylin or with picrosirius for mucus (**a**) or collagen (**b**) stereological evaluation, respectively. Briefly, parallel sections from a block of material were sampled using an image analysis software, and the area positively stained was represented as percentage of the total tissue area. Results are the mean ± SEM of 5–6 animals per group. * $p < 0.05$ in comparison with the control group (C).

Figures 6d–f show that after antigen challenge, the area of peribronchial collagen increased in a time-dependent manner in both Balb/c and C57Bl/6 mice. However, in NZW mice, the collagen area measured in the experimental group was not different from the control group. No differences in staining were seen between the naïve and control group (sensitized and saline challenged).

Discussion

Our results showed that in a model of allergic lung inflammation, the mouse strain NZW was able to present some early phase features, like BHR and airway eosinophilia, but not later features, like lung tissue remodeling, in spite of the amounts of VEGF and TGF-β they produced. We were unable to detect EPO activity in BALF cells of NZW mice, confirming the deficiency of this enzyme in this strain. Concerning the early events of allergic lung inflammation, our results are compatible with those reported by Denzler et al. [20] who used an EPO knockout strain and found lung eosinophil infiltration as well as BHR after immunization and antigen challenge.

Hyperreactivity of airway smooth muscle has been linked to the damage of airway epithelium [21]. Therefore, it was surprising to find that the airways in lungs isolated from either strain were equally hyperreactive, when the levels of EPO were so strikingly different. Clearly, in this model, the effects of EPO were not crucial in determining the airway constrictor response. It is also relevant to point out that although Takeda et al. [22] found a correlation between eosinophil numbers in BALF and the degree of hyperreactivity, in our model, the correlation does not apply, suggesting that BALF eosinophilia alone is not sufficient to induce BHR. Our results do not rule out the participation of eosinophils as effector cells of allergen-induced hyperreactivity. Indeed, PHIL mice, which are devoid of eosinophils but otherwise have a full complement of hematopoietically derived cells, did not developed BHR when sensitized and challenged with OVA [23].

In addition to EPO, activated eosinophils can release a number of other cytotoxic proteins, cytokines and lipid mediators. In particular, cysteinyl leukotrienes are thought to play an important role in the development of BHR [24]. The lipid bodies of eosinophils are key sites for leukotriene C_4 production, since they are rich in triglycerides and surrounded by a monolayer of phospholipids and have all the enzymatic machinery needed. Besides, their numbers are modulated by eosinophil priming [25–27]. Indeed, after the antigen challenge of sensitized mice, we observed BALF eosinophil activation, as attested by increased lipid body formation in all three strains. Of note, lipid bodies are solubilized in cells subjected to fixation and staining with alcohol-based reagents. Therefore, we could not analyze the lipid body formation in tissue eosinophils.

In addition, our results show that in NZW mice, the homing of eosinophils in response to allergic inflamma-

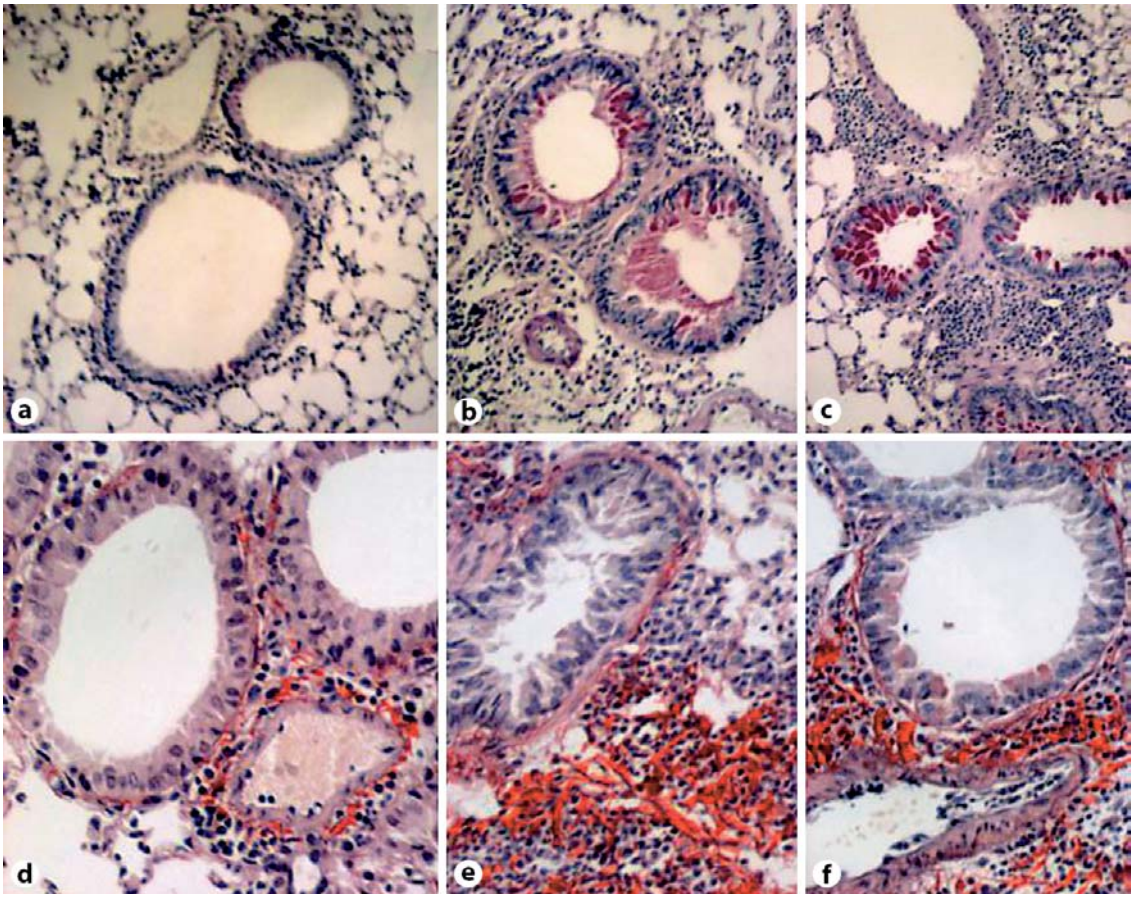

Fig. 6. Photomicrography of pulmonary parenchyma showing mucus and collagen formation after allergic lung inflammation. PAS-stained lungs from sensitized NZW (**a**), Balb/c (**b**) and C57Bl/6 (**c**) mice that received two OVA aerosol challenges and were killed at day 22, showing mucus formation in pink. Picrosirius-stained lungs from sensitized NZW (**d**), Balb/c (**e**) and C57Bl/6 (**f**) mice that received two OVA aerosol challenges and were killed at day 22, showing collagen deposition in red. Magnification of all panels ×400.

tion in the lungs was affected: eosinophil infiltration into airways was higher in NZW, but only few cells were found in lung tissue in contrast to the intense infiltration around the bronchi and vessels found in Balb/c and C57Bl/6 mice. This differential localization might be due to different profiles of chemokines produced after challenge of sensitized mice, according to their genetic background. Alternatively, NZW eosinophils may lack or express different types of receptors for chemokines or matrix components. Despite of the differential localization of eosinophils in NZW, they developed similar levels of BHR. Thus, BHR may be related with BALF but not tissue eosinophil numbers.

Interestingly, our results also showed that compared with Balb/c and C57Bl/6 strains, the NZW strain had higher basal levels of VEGF in the airways and that antigen challenge did not further increase the levels of this cytokine. VEGF is thought to contribute to airway remodeling by promoting hypervascularity and mucosal edema [28]. Besides, in humans, VEGF seems to play a role in the thickening of the basal membrane [15]. Changes in the airway tissue involve a delicate balance between synthesis and degradation of extracellular matrix. Based on the histological analysis of the lungs with picrosirius staining, we saw that after immunization and antigen challenge, the NZW mice showed reduced levels of collagen compared with both Balb/c and C57Bl/6 strains which, in turn, had a time-dependent increase in collagen. Such collagen deposition was most marked in the peribronchovascular regions of the lung. Although ac-

tual remodeling is expected to occur in more chronic models of asthma, Leigh et al. [29] showed that collagen deposition starts soon after the number of inflammatory cells decreases. In NZW mice, we observed a time-dependent increase in TGF-β levels in BALF after the antigen challenge, but a very low collagen deposition. Collagen deposition can be correlated, to a certain extent, with TGF-β levels [30, 31].

Such dissociation between TGF-β production and collagen deposition in NZW mice is intriguing. However, as pointed out in Methods, the method used in this study measures only the activated TGF-β isoform, and thus, we had submitted all samples to previous activation. It is then possible to speculate that the NZW strain is producing TGF-β that for some reason is not appropriately activated in the extracellular millieu and thus cannot signal for collagen production.

Lungs from the NZW mice produced less mucus than those from the Balb/c and C57Bl/6 mice. This difference cannot be attributed to the EPO deficiency of the NZW strain because previous work in EPO knockout mice has shown that mucus production was quantitatively similar to the wildtype mice [20]. Further, the lower production of mucus in NZW mice cannot be attributed to the overexpression of VEGF that we have discovered in this strain, because Lee et al. [4] showed that transgenic C57Bl/6 mice overexpressing VEGF have increased the production of mucus and collagen. VEGF was shown to induce globlet cell metaplasia and to increase *Muc5ac* and epithelial *gob5* gene expression through a mechanism dependent on IL-13 [4]. Impaired expression of the genes related to IL-13 would explain the low production of mucus in NZW mice despite the high amount of VEGF. Further studies are required to check this hypothesis.

Eosinophil infiltration in the lung tissue seems to affect the extracellular matrix in the airways during allergic inflammation, since a reduction in collagen and fibrin deposition was observed in mice that had their eosinophilic lineage ablated [13]. Since we have observed that NZW mice have fewer eosinophils infiltrated into the lung parenchyma than Balb/c and C57Bl/6 mice, this could explain the reduced collagen found in this strain. In summary, the results presented confirmed the EPO deficiency of NZW mice, which has been previously described, and demonstrated for the first time that this strain presents high levels of VEGF in the BALF. These characteristics of NZW strains make it difficult to discriminate the role of EPO and VEGF in allergic lung remodeling. However, it is clear that they affect the allergic inflammation. Moreover, it is clear that collagen deposition is not associated with VEGF in this mouse strain. Another interesting observation was that in NZW mice, the infiltration of eosinophils around bronchi and vessels was negligible compared with the other strains. Thus, it seems that collagen deposition is somehow related to the number of eosinophils in lung tissue. These results raise new questions in the complex field of allergic asthma, particularly concerning the role of VEGF and eosinophils in airway hyperreactivity and tissue remodeling.

Acknowledgments

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