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Nonhematopoietic NADPH oxidase regulation of lung eosinophilia and airway hyperresponsiveness in experimentally induced asthma

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Abstract

Pulmonary eosinophilia is one of the most consistent hallmarks of asthma. Infiltration of eosinophils into the lung in experimental asthma is dependent on the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells. Ligation of VCAM-1 activates endothelial cell NADPH oxidase, which is required for VCAM-1-dependent leukocyte migration in vitro. To examine whether endothelial-derived NADPH oxidase modulates eosinophil recruitment in vivo, mice deficient in NADPH oxidase (CYBB mice) were irradiated and received wild-type hematopoietic cells to generate chimeric CYBB mice. In response to ovalbumin (OVA) challenge, the chimeric CYBB mice had increased numbers of eosinophils bound to the endothelium as well as reduced eosinophilia in the lung tissue and bronchoalveolar lavage. This occurred independent of changes in VCAM-1 expression, cytokine/chemokine levels (IL-5, IL-10, IL-13, IFN γ , or eotaxin), or numbers of T cells, neutrophils, or mononuclear cells in the lavage fluids or lung tissue of OVA-challenged mice. Importantly, the OVA-challenged chimeric CYBB mice had reduced airway hyperresponsiveness (AHR). The AHR in OVA-challenged chimeric CYBB mice was restored by bypassing the endothelium with intratracheal administration of eosinophils. These data suggest that VCAM-1 induction of NADPH oxidase in the endothelium is necessary for the eosinophil recruitment during allergic inflammation. Moreover, these studies provide a basis for targeting VCAM-1-dependent signaling pathways in asthma therapies.

Keywords

endothelium; gp91 phox; eosinophils; VCAM-1

Pulmonary Eosinophilia is a consistent feature of asthma. The mechanisms underlying eosinophil recruitment to the lung following allergen exposure are complex, involving the coordinate actions of adhesion molecules, the chemokine eotaxin, and T-cell-derived cytokines such as IL-5 (41,73). In particular, it has been shown that eosinophil recruitment to the lung in murine models of allergic inflammation is dependent upon eosinophil binding to the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (12,25,49).

Although it has traditionally been thought that VCAM-1 simply serves as a scaffold for leukocyte binding to endothelium, we have reported that it activates endothelial cell intracellular signal transduction pathways, which result in endothelial cell shape changes and leukocyte passage (30,37). Specifically, we have shown that ligand binding to VCAM-1 activates the flavoprotein NADPH oxidase in endothelial cells in vitro (37). NADPH oxidase catalyzes the production of superoxide from oxygen. Then, superoxide dismutates to hydrogen peroxide. VCAM-1 stimulates endothelial cell production of nontoxic levels of H₂O₂ (1 μM H₂O₂) that is dependent on endothelial cell NADPH oxidase but not other reactive oxygen species (ROS)-generating enzymes (15,17,18,63). This H₂O₂ is required for VCAM-1-dependent changes in actin localization in endothelial cells and for VCAM-1-dependent leukocyte migration in vitro (37). VCAM-1-stimulated ROS oxidize and activate endothelial cell-associated matrix metalloproteinases (18), which regulate cell shape. Whether NADPH oxidase in nonhematopoietic cells is involved in VCAM-1-dependent eosinophil migration has not been demonstrated. We suggest that, in addition to ROS modulation of lung tissue responses (11,43,59,61,66), ROS are required for VCAM-1 signals and thus VCAM-1-dependent infiltration of eosinophils into the lung in response to ovalbumin (OVA) inhalation. We have reported that when we elevate the levels of the antioxidant bilirubin, it inhibits OVA-induced lung eosinophilia and blocks VCAM-1 signaling in vitro (30). However, it has not been investigated whether NADPH oxidase in nonhematopoietic cells in vivo contributes to lung eosinophilia or airway hyperresponsiveness (AHR). For studies on NADPH oxidase function during inflammation, there are several NADPH oxidase-deficient mouse models. These models include mice deficient in the catalytic subunit of NADPH oxidase or mice deficient in one of the regulatory subunits of NADPH oxidase. In mice deficient in one of the regulatory subunits of NADPH oxidase (p47 phox or p67 phox), there is inhibition of ROS generation from multiple isoforms of NADPH oxidase that differ in their catalytic subunit but use common regulatory subunits. We have reported that the form of NADPH oxidase containing the catalytic subunit gp91 phox (Nox2) is critical for VCAM-1 signaling in endothelial cells in vitro (15,17,18,63). Therefore, we focused on the function of gp91 phox using the gp91 phox-deficient mice (CYBB mice). Other considerations when using gp91 phox-deficient mice are that 1) many cell types express gp91 phox including hematopoietic cells and nonhematopoietic cells (1,32,39,46,57,66,70,74) and 2) gp91 phox in hematopoietic cells is critical to their function (47). To focus our studies on the function of gp91 phox in nonhematopoietic cells, chimeric CYBB mice were used in which the hematopoietic cells were wild type for gp91 phox and the nonhematopoietic cells were gp91 phox deficient.

In this report, it is demonstrated that chimeric mice with NADPH oxidase-deficient nonhematopoietic cells and wild-type leukocytes have reduced lung eosinophilia, increased eosinophil accumulation on the luminal surface of endothelial cells, and reduced AHR in experimentally induced asthma. This occurred without altering expression of VCAM-1, without altering several chemokines or cytokines that regulate eosinophilia, and without altering infiltration of T cells, mononuclear cells, or neutrophils. Furthermore, bypassing the endothelium by intratracheal administration of eosinophils restored the AHR in the chimeric NADPH oxidase-deficient mice. The study of VCAM-1 signals in experimental asthma will result in a better understanding of the basic mechanism(s) for VCAM-1-dependent eosinophil

transendothelial migration in asthma and provide potential novel targets for therapeutic intervention in the eosinophil component of asthma.

MATERIALS AND METHODS

Animals

CYBB mice (gp91 phox deficient, C57BL/6 background), CD45.1 mice (C57BL/6 background), green fluorescent protein (GFP) mice (C57BL/6 background), and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). NJ.1638 mice were obtained from James Lee. The mice were housed in barrier cages and were specific pathogen-free as determined in sentinel mice. The procedures were reviewed and approved by the Animal Care and Use Committees at Northwestern University, the University of Cincinnati, and Cincinnati Children's Hospital.

Adoptive transfer

Bone marrow cells, 2.5×10^6 , collected from femurs and tibias of 6- to 8-wk-old GFP mice (5) or CD45.1 mice, were injected into the tail vein or orbital sinus of whole body lethally irradiated wild-type C57BL/6 mice and CYBB mice (cesium-137, 11 Gy split dose with 3 h between doses) as previously reported (5). The mice were used in the studies 12 wk after bone marrow reconstitution. To examine the reconstitution of hematopoietic cells after bone marrow transplant with bone marrow from either GFP mice or CD45.1 mice, blood leukocytes or spleen cells were examined by flow cytometry of unlabeled cells or cells labeled with FITC-conjugated mouse anti-mouse CD45.1 (clone A20, BD PharMingen).

Nitroblue tetrazolium slide assay for leukocyte NADPH oxidase activity

Details of this assay are available in Ref. 47. Briefly, glass slides were coated with 10 μ l of 20 μ g/ml PMA and dried. Blood, 50 μ l, was placed on the glass slides with 25 μ l of 1.2 mg/ml nitroblue tetrazolium (NBT) and 2 μ g PMA for 45 min at 37°C. The clot was gently peeled off. The cells on the slide were fixed for 1 min in 100% methanol, counterstained with safranin, and scored by light microscopy for labeling with NBT.

Ovalbumin response

OVA fraction V (catalog no. A5503) was from Sigma. We used two protocols for OVA sensitization and challenge as indicated (Fig. 1). For both protocols, 12 wk after the bone marrow adoptive transfer (5), the chimeric mice were injected intraperitoneally with 200 μ l aluminum hydroxide-adsorbed ova (50 μ g ova, 1 mg alum) or control aluminum hydroxide in saline ($n = 6-8$ mice/group) (12). For *protocol 1*, after only one sensitization with OVA, the mice were intranasally challenged on *days 14* and *15* with 150 μ g OVA in 50 μ l saline, and the lungs were lavaged on *day 16* (Fig. 1A). This protocol results in infiltration of approximately equal numbers of eosinophils, neutrophils, mononuclear cells, and lymphocytes. For *protocol 2*, which yields a predominant eosinophilia, the mice received a second sensitization with OVA-alum or alum-saline on *day 7* and then intranasally challenged on *days 14, 16, and 19* with 150 μ g ova in 50 μ l saline and lavaged on *day 20* (Fig. 1B). Lungs were lavaged twice with 0.5 ml ice-cold PBS and frozen in OCT compound. Tissue sections were fixed and stained with hematoxylin, phloxine, and eosin. Lavage cell numbers were determined with a hemocytometer. Differential counts of cytocentrifuged and Diff Quik-stained cells were made on at least 200 cells according to standard morphological criteria.

Eosinophil isolation and transfer

Three sets of CD45.1 mice were sensitized twice with OVA-alum and challenged three times with OVA as in the timeline in Fig. 1C. On the day of eosinophil transfer, the bronchoalveolar

lavage (BAL) was collected from OVA-challenged CD45.1 mice (Fig. 1C). In addition, for transfer of blood eosinophils, blood eosinophils were isolated from nonchallenged NJ.1638 mice, which express an IL-5 transgene (36). Red blood cells were removed by hypotonic lysis (6). The cells from the BAL or the blood were separated on a single-step Percoll gradient (60% Percoll, density = 1.084; 1× HBSS, 15 mM HEPES, pH 7.4) (6). Mononuclear cells were removed by adherence to plastic for 1 h in RPMI-1640 with 5% heat-inactivated FCS (53). The nonadherent cells were negatively immunomagnetically selected to remove lymphocytes, neutrophils, and monocyte/macrophages (23,48,52) using anti-B220 (clone RA3-6B2; BD PharMingen, San Diego, CA), anti-CD90.2 (Thy1.2, antibody clone 35-2.1; eBioscience, San Diego, CA), and rat anti-mouse CD14 (clone RMC5-3, BD PharMingen) (3 µg of each antibody/tube with cells from 4 BALs in 200 µl or 1 µg of each antibody/tube of 200 µl of 10⁶ blood cells for 20 min at 4°C). The cells were washed, incubated with 10 µl of goat anti-rat IgG microbeads (Miltenyi Biotec, Auburn, CA), and passed through a MACS MS⁺ separation column (Miltenyi). Cytospins were stained with hematoxylin and eosin (H&E) to determine the percent eosinophils, and cell viability was determined by trypan blue exclusion (40). This isolation generated a preparation of >96% viable eosinophils. The isolated eosinophils were transferred intratracheally to OVA-sensitized chimeric CYBB mice on the days of OVA challenge as in the timeline in Fig. 1C.

Airway hyperresponsiveness

AHR to intravenous acetylcholine was measured as previously described (20,29). Mice were anesthetized, intubated, and ventilated. Airway pressure was measured with a pressure transducer in the tracheal cannula. Acetylcholine (50 or 75 µg/kg, as indicated) was injected intravenously, and airway pressure was recorded. Change in peak airway pressure was reported as the airway pressure time index (cmH₂O · s).

Expression of VCAM-1

VCAM-1 expression in frozen lung tissue was examined by immunofluorescence labeling and confocal microscopy as we have previously described (30). The fluorescence of anti-VCAM-1-labeled cells was quantified by calculating the sum of the pixel intensities per 100 µm² for the vessel and lumen minus the sum for the lumen (16,30,37).

T cells and eosinophils in lung tissue sections

Frozen lung tissue sections were examined by immunohistochemistry using anti-MBP (kind gift from Dr. James Lee), rat anti-mouse CD4 (clone RM4-5, BD PharMingen), or rat anti-mouse CD8 (clone 53-6.7, BD PharMingen), or control rat IgG_{2a} (clone R35-95, BD PharMingen) followed by labeling with rabbit anti-rat IgG biotin (catalog no. 6180-08; Southern Biotech, Birmingham, AL), horseradish peroxidase-conjugated donkey anti-rabbit IgG (catalog no. NA9340; Amersham Biosciences, Piscataway, NJ), and the 3,3'-diaminobenzidine (DAB) substrate kit (catalog no. SK4100; Vector Laboratories, Burlingame, CA). The slides were lightly counterstained with 0.5% methyl green, dehydrated, and coverslipped. The cells were quantified by counting the number of major basic protein-positive (MBP⁺) cells per millimeter luminal surface of the endothelium or by number of MBP⁺ cells on the endothelial luminal surface/vessel. In addition, the number of perivascular and peribronchial MBP⁺ cells were counted in five ×40 high-powered fields. Frozen tissue sections were also stained with H&E.

Lung digests

Lung lobes were excised, minced, and digested in RPMI-1640 media supplemented with 2% FCS, 1 mg/ml collagenase A, and 50 U/ml DNase for 1 h at 37°C. Digested lung fragments were pushed through mesh screens to obtain single cell suspensions. The CD4⁺ cells, CD8⁺

cells, or CD4⁺CD25⁺TCR β ⁺ cells were examined in lung digests of chimeric CYBB mice and chimeric wild-type mice with bone marrow from GFP mice. The lung digests were incubated with Fc block and then labeled with the following fluorochrome-labeled antibodies: Alexa Fluor 647-conjugated anti-mouse CD4 (clone RM4-5, BD PharMingen), PE-conjugated anti-mouse CD25 (clone PC61, BD PharMingen) and PE-Cy5-conjugated anti-mouse TCR β (clone H57-597, BD PharMingen). The cells were then analyzed by flow cytometry using a BD LSR1 flow cytometer and Cell Quest software (Becton-Dickinson Bioscience, San Jose, CA).

OVA restimulation of cells from lung digests

As described in Ref. 22, the lung digest cells were plated at 1×10^6 cells/200 μ l in a 96-well plate in lymphocyte culture medium (RPMI-1640 supplemented with 1 mM HEPES, pH 7.2, 10 mM sodium bicarbonate, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamicin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 5% heat-inactivated fetal calf serum). The cells were stimulated with 500 μ g/ml OVA for 48 h at 37°C in 6% CO₂. The medium was collected and centrifuged at 4°C, and then the supernatants were collected and stored at -80°C.

Expression of cytokines and chemokines

As described in Ref. 29, supernatants from 48 h cultures of lung digests were tested for eotaxin, IL-4, IL-5, IL-10, IL-13, and IFN γ by ELISA (PharMingen and Endogen). The limit of detection was 10 pg/ml for each assay.

Serum immunoglobulins

Sera of the blood of exsanguinated mice after airway measurements were examined for total IgE, OVA-specific IgE, OVA-specific IgG₁, and OVA-specific IgG_{2a} by ELISAs. Measurement of total serum IgE levels was conducted using an IgE-specific ELISA according to manufacturer's instructions (PharMingen) with antibody clones R35-72 and R35-118. Optical density readings were made at 405 nm (29). The data are presented as ng/ml for total IgE. For OVA-specific immunoglobulins, 96-well plates were coated overnight with 50 μ l OVA (10 μ g/ml) in PBS at 4°C, washed with PBS/Tween-20 and then blocked with 200 μ l 10% FBS/PBS for 2 h at room temperature. The plates were washed with PBS/Tween-20 and then 50 μ l/well of diluted sera was added (1/10 dilution in 10% FBS/PBS for IgE, 1/500 dilution for IgG₁ or 1/100 dilution for IgG_{2a}). The plates were incubated overnight at 4°C, washed with PBS/Tween, and incubated with a 1/250 dilution in 10% FBS/PBS of biotin-conjugated anti-mouse IgG₁ (clone A85-1, PharMingen), biotin-conjugated anti-mouse IgG_{2a} (clone R19-15, PharMingen), or biotin-conjugated anti-mouse IgE (clone R35-118, PharMingen) for 45 min at room temperature. The plates were washed, and 50 μ l/well of a 1/400 dilution of avidin peroxidase was added at room temperature for 30 min. The plates were washed and incubated with the peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline)sulfonic acid (ABTS; Kirkgaard and Perry, Gaithersburg, MD) for 15-30 min at room temperature. Plates were read at 405 nm, and background absorbance was determined at 595 nm. Data are presented as fold change in optical density at 405 nm compared with the OVA-stimulated chimeric wild-type animals.

Statistical analysis

Data are presented as means $\pm$ SE. Results were analyzed by a completely random ANOVA followed by a multiple comparison test (Sigma Stat, Jandel Scientific).

RESULTS

Chimeric NADPH oxidase-deficient mice have reduced OVA-stimulated eosinophilia

We have reported that VCAM-1 activation of endothelial cell NADPH oxidase is required for VCAM-1-dependent leukocyte migration in vitro (17,18,37). However, it is not known whether VCAM-1-dependent leukocyte migration requires nonhematopoietic NADPH oxidase in vivo. To address this issue, we examined the effects of a deficiency in the gp91 phox catalytic subunit of NADPH oxidase (CYBB mice) on eosinophilic migration in an experimental model of allergic inflammation as eosinophilia in this model is VCAM-1 dependent (12,49). To examine the effects of NADPH oxidase deficiency in the resident lung cells rather than leukocytes, we generated bone marrow chimeric CYBB mice. To generate these mice, irradiated CYBB mice and C57BL/6 wild-type mice received a tail vein injection of 2×10^6 bone marrow cells from GFP mice. At 9–12 wk post bone marrow transfer, >97% of the leukocytes from tail vein bleeds expressed GFP as determined by flow cytometry (data not shown). In addition, we collected blood and tested PMA-stimulated granulocyte NADPH oxidase activity by the NBT slide assay (47). NBT labeling was detected in cells from all animals (data not shown). The GFP expression and the results from the NBT slide assay confirm the generation of chimeras. We hypothesized that the VCAM-1-dependent eosinophilic response to OVA would be reduced in the chimeric CYBB mice but that the VCAM-1-independent infiltration of neutrophils and lymphocytes would not be altered in the chimeric CYBB mice. During responses to OVA, there is an initial infiltration of neutrophils followed by a reduction in neutrophils and accumulation of eosinophils (2,33,50,58,60,64,75). To determine whether the chimeric CYBB mice had reduced VCAM-1-dependent eosinophil migration but not altered VCAM-1-independent leukocyte migration, *protocol 1* for OVA stimulation (Fig. 1A) was chosen for the initial studies, since it results in infiltration of approximately equal numbers of eosinophils, neutrophils, and lymphocytes.

For the studies in Figs. 2–4, the mice were sensitized only once and challenged only twice with OVA (*protocol 1*). Specifically, at 10 wk after the bone marrow transfer, the chimeric mice were primed intraperitoneally with 50 μ g OVA in alum or alum in saline. These mice were challenged intranasally on *days 14* and *15* with 150 μ g OVA in saline or saline. The BAL and lung tissues were collected on *day 16*. OVA challenge stimulated a significant increase in the infiltration of total leukocytes in the BAL fluid compared with saline controls (Fig. 1B) as previously described (12). Interestingly, the number of eosinophils in the BAL was significantly lower in the OVA-challenged CYBB chimeric mice compared with the OVA-challenged wild-type chimeric mice (Fig. 2, C and D). Eosinophil numbers in the CYBB chimera BAL were 62% less than that of wild-type chimeric mice, which is consistent with reports that 60–75% of eosinophilia is blocked by intraperitoneal administration of anti-VCAM-1 or intratracheal administration of small molecule $\alpha 4\beta 1$ antagonists in OVA-challenged wild-type mice (12,31,51). However, there was no significant change in the number of eosinophils free in the blood available for migration for chimeric wild-type vs. chimeric CYBB mice (Fig. 2A). This is in contrast to reports that inhibition of eosinophil adhesion to endothelium with anti- $\alpha 4$ -integrin increases the number of eosinophils free in the blood and decreases tissue eosinophils (7). In Fig. 2, C and D, the numbers of neutrophils, lymphocytes, and monocytes in the BAL from OVA-challenged chimeric CYBB mice were not different than OVA-challenged chimeric wild-type mice. This is consistent with a report indicating that 200 μ g anti-VCAM-1 blocks eosinophilia but higher doses of this antibody are necessary to induce a small inhibition of lymphocyte infiltration (12), as well as a report that inhibition of VCAM-1 expression reduces recruitment of eosinophils but not lymphocytes to the lung in response to OVA (71). In summary, a deficiency of gp91 phox in nonhematopoietic cells blocked infiltration of eosinophils in response to OVA sensitization and challenge, suggesting

that NADPH oxidase in resident lung cells is required for the majority of the eosinophil recruitment during the allergic response.

VCAM-1 expression was not altered in the OVA-challenged chimeric CYBB mice

As it has been shown that VCAM-1 expression can be modulated by cytokines and ROS, we determined whether VCAM-1 expression was altered in the lungs of OVA-challenged CYBB chimeric mice. To do this, frozen tissues from the mice in Fig. 2 were fluorescently labeled with anti-VCAM-1 or isotype control antibodies followed by a secondary antibody. The labeled tissues were examined by confocal microscopy to quantify the sum of the fluorescence pixel intensity per square micrometer of endothelial cells. OVA stimulated the expression of VCAM-1 in both the CYBB chimeras and C57BL/6 chimeras, and there was no difference in their VCAM-1 expression (Fig. 3). These results suggest that the signals in the CYBB chimeras were sufficient for induction of VCAM-1 expression.

Eosinophils accumulated on the endothelial luminal surface in the OVA-challenged chimeric CYBB mice

As there were fewer eosinophils in the BAL and a lack of accumulation of eosinophils in the blood, we examined localization of eosinophils in lung tissue sections. Eosinophils had accumulated on the luminal surface of the endothelium in the OVA-stimulated CYBB chimeras but not the OVA-stimulated C57BL/6 chimeras or the saline-treated controls (Fig. 4). Specific labeling and quantitation of eosinophils in the tissue was done using anti-MBP antibody as MBP is an eosinophil-specific granule protein. There was an increase in MBP⁺ cells on the endothelial luminal surface (Fig. 4) and a decrease in extravascular MBP⁺ eosinophils (data not shown). A decrease in extravascular MBP⁺ cells in chimeric CYBB mice was also confirmed in a later study (Figs. 8F and 9F) using OVA *protocol 2* (Fig. 1B). Taken together, the data is consistent with OVA-induced eosinophil binding to endothelium and a loss of signals to induce eosinophil migration across the endothelium, resulting in an accumulation of eosinophils bound to the endothelium.

The chimeric NADPH oxidase-deficient mice had reduced AHR

We determined whether gp91 phox deficiency in nonhematopoietic cells alters AHR in the chimeric CYBB. To induce AHR and predominant eosinophilia, the number of OVA sensitizations and challenges were increased (OVA *protocol 2*) (Fig. 1B). The optimal protocol for OVA-induced AHR in the C57BL/6 mice was intraperitoneal sensitization with OVA-alum on *day 0* and *day 7* followed by intranasal challenge with OVA on *days 14, 16, and 19* and then testing for AHR on *day 20* (data not shown). For these studies, we used chimeric CYBB mice and chimeric wild-type C57BL/6 mice that were irradiated and reconstituted with CD45.1 bone marrow 12 wk before OVA challenge. The leukocytes were CD45.1⁺ as determined by flow cytometry (data not shown). The OVA-challenged chimeric CYBB mice had significantly less AHR after stimulation with 50 µg acetylcholine/kg (Fig. 5) and reduced eosinophilia in the BAL (data not shown).

Expression of several cytokines that regulate eosinophilia was not altered in the OVA-challenged chimeric CYBB mice

As cytokines stimulate eosinophil infiltration into tissue, we examined whether CYBB chimeric mice had altered expression of relevant cytokines. Lung digests from the saline and OVA-challenged mice in Fig. 5 were restimulated *in vitro* with OVA for 48 h, and the supernatants were examined by ELISA for IL-4, IL-5, IL-10, and IL-13. There was OVA-stimulated production of IL-5, IL-10, and IL-13 (Fig. 6, B–D). Furthermore, there was no significant difference in these cytokines between the OVA-challenged chimeric CYBB mice and the OVA-challenged chimeric C57BL/6 wild-type mice. Although OVA-stimulated IL-4

levels were moderate in the wild-type mice, there was a significant reduction in IL-4 by OVA-restimulated lung digests from the chimeric CYBB mice (Fig. 6A). The level of IL-4 in the saline groups is near the detection limit for the ELISA. These results suggest that although there was a reduction in IL-4 expression, the expression of other Th2 cytokines that regulate eosinophilia was not altered in the chimeric CYBB mice.

Bypassing the endothelium by intratracheal eosinophils resulted in recovery of acetylcholine-stimulated AHR

As non-hematopoietic NADPH oxidase was required for the infiltration of eosinophils, the question remained whether loss of NADPH oxidase function in endothelial cells or other resident cells of the lung was critical for the reduction in OVA-stimulated eosinophilia and AHR. The gp91 phox isoform is expressed in endothelial cells, fibroblasts, and epithelial cells and at very low levels in smooth muscle cells (1,32,39,46,57,66,70,74). Therefore, if gp91 phox in the lung resident cells other than the endothelial cells is necessary for the AHR, then intratracheal administration of eosinophils would not recover the AHR. However, if wild-type eosinophils were sufficient for the AHR in these mice but the eosinophils could not migrate across the endothelium, then AHR would be recovered by bypassing the endothelium with intratracheal administration of eosinophils. In addition, it has been reported that eosinophils administered intratracheally migrate from the alveolar space into the tissue and draining lymph nodes (53). Therefore, it was determined whether intratracheal administration of eosinophils in the chimeric CYBB mice recovers the AHR. For these studies, *protocol 2* for OVA sensitization/challenge was used (Fig. 1C). Chimeric CYBB mice and chimeric wild-type mice were sensitized with OVA on *days 0* and *7* and then challenged with OVA on *days 14, 16, and 19*. At the time of each intranasal OVA challenge, additional groups of chimeric CYBB mice received purified eosinophils intratracheally (Fig. 1C). Chimeric CYBB mice received either eosinophils from BAL of OVA-challenged CD45.1 mice or eosinophils from the blood of IL-5 transgenic mice, since eosinophils from BAL can differ from blood eosinophils with regard to adhesion molecule expression and cell function (26,44,45). Eosinophils were isolated from the BAL of OVA-stimulated C57BL/6 mice that were prepared by challenging these mice with OVA 24 h before the OVA challenges in the chimeric mice as indicated in the timeline in Fig. 1C. Eosinophils prepared by panning and negative immunomagnetic selection were identified as >96% viable eosinophils as determined by H&E staining of cytopins and trypan blue exclusion (data not shown). In a control experiment, we had determined that when eosinophils were purified from BAL from C57BL/6 mice that received two OVA sensitizations and three OVA challenges, there were 2×10^4 , 2.4×10^5 , and 7.7×10^5 BAL eosinophils recovered/mouse on *days 14, 16, and 19*, respectively. Therefore, 1.5–4 times this number of eosinophils was administered (Fig. 7A) to provide a reasonable number of eosinophils to reconstitute the BAL and lung tissue after intratracheal administration.

In Fig. 7, *B* and *C*, there was a 63% reduction in eosinophilia and a 66% reduction in AHR in the OVA-challenged chimeric CYBB mice compared with OVA-challenged chimeric wild-type mice. Importantly, in Fig. 7C, the AHR in OVA-challenged chimeric CYBB mice was rescued by intratracheal administration of BAL eosinophils isolated from OVA-challenged wild-type mice but not by blood eosinophils from IL-5 transgenic mice. This recovery of AHR suggests that gp91 phox in lung fibroblasts and epithelial cells was not essential for the AHR. The number of eosinophils in the BAL and tissues at the time of AHR testing was determined. Intratracheal administration of eosinophils recovered eosinophils in the BAL (Fig. 7B), demonstrating successful intratracheal administration of eosinophils that would have then migrated into tissues and to draining lymph nodes (53). We examined localization of eosinophils in the lung tissue from mice in Fig. 7. The intratracheal administration of BAL eosinophils recovered the perivascular and peribronchial eosinophils in the OVA-challenged CYBB chimeras (Figs. 8 and 9). Although the number of perivascular eosinophils increased

after intratracheal administration of eosinophils, there was still an accumulation of eosinophils attached to the luminal surface of the endothelium (Fig. 8, *E* and *G*). Thus, as there were eosinophils in both the tissue and attached to the endothelium in Fig. 8, *E* and *G*, it is consistent with the postulate that the intratracheally administered eosinophils migrated across the epithelium to the leukocyte-rich perivascular areas but the blood eosinophils in the OVA-challenged chimeric CYBB mice were still prevented from migrating across the endothelium. Compared with Fig. 2, which used *protocol 1* for OVA administration, the increased number of OVA administrations in *protocol 2* resulted in increased eosinophilia in wild-type mice (Fig. 7*B*) and fewer neutrophils and lymphocytes, as we previously reported with this protocol using the same lot of OVA (30). Furthermore, there were no differences in the numbers of BAL lymphocytes, monocytes, or neutrophils for the OVA-stimulated chimeric CYBB and wild-type groups (data not shown).

Although there was no change in total lymphocyte numbers, an alteration in T cell subsets or cytokine production could modulate eosinophilia. Infiltration of eosinophils is stimulated by T cell-derived cytokines such as IL-4 and IL-5 (41,73). Furthermore, depletion of CD4⁺CD25^{high} immunoregulatory T cells increases lymphocyte recruitment and decreases IL-4 and IL-5 production and eosinophil recruitment in response to antigen challenge (56). Therefore, we determined whether there was a difference in lung T cell subsets, cytokines, or chemokines in the mice from Fig. 7. Interestingly, when we examined T cell subsets in these lung digests, there was no difference among the OVA-challenged groups for number of CD4⁺ cells, CD8⁺ cells, TCRβ⁺ cells, or TCRβ⁺CD4⁺CD25^{high} cells (Fig. 10, *A–C*). There was also no difference among the OVA-challenged groups in localization of CD4⁺ and CD8⁺ cells around the vessels or bronchial airways (Fig. 10*D*). The OVA-challenged groups did not display differences in total serum IgE (Fig. 10*E*) or serum OVA-specific Igs (Fig. 10*F*). Furthermore, as indicated in Table 1, there was no significant difference in OVA-stimulated groups for the cytokines IL-5, IL-10, IL-13, or IFNγ or the chemokine eotaxin in OVA-restimulated lung digests from mice in Fig. 7. The levels of IL-4 were significantly reduced in OVA-challenged CYBB groups, although the levels of IL-4 production were modest for all groups (Table 1). Intratracheal administration of eosinophils did not recover the reduced IL-4 in the OVA-stimulated chimeric CYBB mice (Table 1) but did recover the AHR (Fig. 7*C*). In summary, these data and the data indicating accumulation of eosinophils on the endothelial cell luminal surface are consistent with an endothelial gp91 phox requirement for VCAM-1-dependent eosinophilia and AHR.

DISCUSSION

In this study, we demonstrate that gp91 phox deficiency in the nonhematopoietic compartment of chimeric CYBB mice blocks eosinophil infiltration and AHR in OVA-challenged lungs. AHR is recovered by bypassing migration of eosinophils across the endothelium via intratracheal delivery of eosinophils at the time of OVA challenge. As the intratracheal delivery of eosinophils recovered the AHR, it indicates that the gp91 phox in cells other than endothelial cells such as fibroblasts or epithelial cells was not required for the AHR. These data are consistent with our previous reports that endothelial cell NADPH oxidase is a mediator of VCAM-1 signaling in vitro (17,37) and reports that VCAM-1 is required for infiltration of eosinophils in response to OVA challenge to the lung (12,31,49,51,71). This is the first report on a nonhematopoietic gp91 phox requirement for VCAM-1-dependent lung eosinophilia.

We and others (15,17,37,67) have shown that leukocyte binding to VCAM-1 or antibody cross-linking of VCAM-1 stimulates endothelial cell NADPH oxidase in vitro. Inhibition of endothelial cell NADPH oxidase by pharmacological agents or by gp91 phox antisense blocks VCAM-1-dependent leukocyte migration in vitro (17,37). This NADPH oxidase generation of ROS activates endothelial cell signals in vitro for the opening of an “endothelial cell

gate” (15,18). In the in vivo studies herein, the knockout of gp91 phox in nonhematopoietic cells appears to be responsible for the accumulation of eosinophils attached to the luminal surface of lung endothelium after OVA challenge and responsible for the reduction in tissue eosinophilia. In these chimeric CYBB mice, there were sufficient blood eosinophils for infiltration into the tissues, since numbers of eosinophils free in the blood were not altered. Moreover, there was sufficient VCAM-1 expression for adhesion of eosinophils and there was sufficient eotaxin for the stimulation of eosinophil chemotaxis in the chimeric CYBB mice. The NADPH oxidase activity in the endothelial cells of CYBB mice is deficient, as demonstrated in response to hypoxia (1). Thus, with the loss of nonhematopoietic gp91 phox signals in chimeric CYBB mice, there is inhibition of transendothelial promotion of eosinophil migration in vivo. This is consistent with the in vitro inability of VCAM-1 to stimulate outside-in endothelial cell signals in gp91 phox antisensetreated endothelial cells (15,17,18).

In contrast to the VCAM-1 dependence of eosinophil infiltration into OVA-challenged lungs, monocyte and neutrophil infiltration does not require adhesion to VCAM-1 (12). Consistent with this, our data demonstrate that there was no inhibition of monocyte or neutrophil infiltration into OVA-challenged lungs of chimeric CYBB mice. There was significant infiltration of neutrophils with *protocol 1* for OVA sensitization/challenge and some infiltration of neutrophils with *protocol 2* for OVA sensitizations/challenges. It has been reported that OVA induces an initial wave of neutrophilia that is followed by eosinophilia and lymphocyte infiltration (2,33,50,58,60,64,75). In fact, if one intraperitoneal OVA-alum and one intranasal OVA is administered with early collection of the BAL at 8 h, then there is predominant neutrophilia in response to OVA with little infiltration of the other leukocytes. Later in the response to OVA challenges, eosinophils reach peak infiltration at 24–48 h (2,33,50,60,64,75). Neutrophils can also infiltrate into the lung in response to administration of endotoxin-free IL-4 or IL-13 (9.), in response to aerosolized endotoxin-free OVA after adoptive transfer of OVA-specific T cells (10), or in response to endotoxin. Regarding endotoxin contamination, low levels of endotoxin are required for an adequate response to OVA, as previously reported by Eisenbarth et al. (19). In contrast, high levels of endotoxin suppress the OVA response (19). In our studies, OVA fraction V, which is commonly used in this model of asthma, was prepared with fresh saline or fresh alum. The OVA responses in this manuscript and our previous manuscript (30) with the same lot of OVA indicate that with two OVA sensitizations and three OVA challenges there is a predominant eosinophilia and increased IgE, consistent with a low endotoxin level that is necessary for good OVA responses. In addition, all groups receiving OVA were treated with the same preparation of OVA and thus treated equally.

It has been reported that OVA-stimulated lymphocyte infiltration is only marginally VCAM-1-dependent. In OVA-stimulated mice, lymphocyte infiltration is marginally reduced by injection of high levels of anti-VCAM-1 antibody in C57BL/6 mice (12). VCAM-1 function during OVA-stimulated lymphocyte infiltration has not been studied in VCAM-1 knockout mice, since disruption of the VCAM-1 gene is an embryonic lethal (24). In contrast to the marginal effect of anti-VCAM-1 on lymphocyte migration, antibodies to $\alpha 4$ -integrin, a ligand on lymphocytes that binds VCAM-1 or extracellular matrix, significantly block OVA-stimulated lymphocyte migration into lungs of C57BL/6 mice (12). In vitro, in the absence of other endothelial cell adhesion molecules for lymphocytes, lymphocytes do migrate on VCAM-1, and this migration requires endothelial cell gp91 phox (37,62,63). However, when binding to VCAM-1 is blocked in vivo, lymphocytes still migrate because of the redundancy of adhesion molecules that mediate lymphocyte binding to endothelial cells (55). This is also consistent with reports by us and others (15,37,65) that other endothelial cell adhesion molecules for leukocyte binding such as ICAM-1 and PECAM-1 do not signal through endothelial cell ROS generation. In our studies with the chimeric CYBB mice, there was no inhibition of lymphocyte infiltration and no accumulation of lymphocytes on the endothelial

cell luminal surface, suggesting that migrating lymphocytes likely used adhesion molecules other than VCAM-1.

There has been an implication for a role of ROS in OVA-stimulated lung eosinophilia, since studies employing antioxidants demonstrate reduced lung eosinophilia. We have reported that administration of the antioxidant bilirubin blocks OVA-induced eosinophilia in lungs of C57BL/6 mice without altering the OVA-induced expression of several factors regulating eosinophilia such as VCAM-1, eotaxin, or IL-5 (30). Whether the antioxidant bilirubin also modulates AHR was not examined. However, the *in vivo* studies with bilirubin do not directly demonstrate an *in vivo* role for gp91 phox in lung eosinophilia, since there are several forms of NADPH oxidase that generate ROS and these NADPH oxidases are differentially expressed within tissues. Specifically, the gp91 phox (nox2) is expressed by endothelial cells (1,39), fibroblasts (46,57), and airway epithelial cells (66,70,74). Smooth muscle cells express the catalytic subunits nox1, nox4, and very low levels of gp91 phox (nox2) (32). These NADPH oxidases in nonhematopoietic cells generate ROS (1,32,37,39,46,57). The studies in this report focus on gp91 phox function and directly demonstrate an *in vivo* role for nonhematopoietic gp91 phox in eosinophilia. Furthermore, the epithelial and fibroblast gp91 phox were not essential for the AHR, since AHR was restored by intratracheal administration of eosinophils.

In experimental asthma, there is some evidence that AHR is modulated by ROS generation from several lung tissue cells. In tracheal smooth muscle cells, the pharmacological inhibition of NADPH oxidase (nox1 and nox4) reduces their contraction, and this may influence AHR (59). It has been reported that guinea pig tracheal ring contraction is reduced when treated with pharmacological inhibitors of ROS-generating enzymes (59). In addition, ozone-induced AHR is blocked by apocynin, an inhibitor of NADPH oxidase, but, as NADPH oxidase functions in leukocytes and nonhematopoietic cells, the source of NADPH oxidase contributing to this AHR was not determined (61). ROS can also be generated by NADPH oxidases in type II epithelial cells in response to PMA or bacteria (66). The ROS generated in the lung tissue may further affect nitric oxide homeostasis and therefore modulate nitric oxide-stimulated smooth muscle responses in the airway (11,43). In addition, the balance of ROS in the lung tissue is regulated by extracellular antioxidants such as glutathione peroxidase (13,14). This emphasizes that as several ROS-generating enzymes can contribute to AHR, inhibitors and antioxidants used *in vivo* may block ROS from several sources.

The evidence that eosinophils participate in AHR in asthma is debated (72). Eosinophils do release mediators such as ROS, granule proteins, and leukotrienes that modulate lung function (3,34). The eosinophil granule proteins are toxic to bronchial epithelium, and leukotrienes generated by eosinophils act as bronchoconstrictors (72). In intraperitoneal OVA-sensitized CCR3^{-/-} mice, there is reduced lung eosinophilia, but they still exhibit increased AHR in response to OVA challenge (27). This increase in AHR may be related to the increase in lung mast cell numbers after OVA challenge in these CCR3^{-/-} mice (27). In contrast, AHR is reduced in epicutaneous OVA-sensitized CCR3^{-/-} mice, which exhibit reduced eosinophilia in lung parenchyma and BAL (35). In addition, inhibition of eosinophil binding to VCAM-1 with a synthetic peptide blocks AHR in a sheep model of asthma (54). These studies suggest that eosinophils and/or mast cells are a component of AHR. Furthermore, in IL-5^{-/-} mice, there is reduced eosinophil infiltration and reduced AHR (52). The eosinophil infiltration was a significant component of this reduced AHR in the IL-5^{-/-} mice, since intratracheal adoptive transfer of eosinophils into the IL-5^{-/-} mice recovered the AHR (52). In IL-5^{-/-} mice, it has also been reported that eosinophilia but not IL-5 or eotaxin alone are associated with AHR (41). Instead, the combined inhibition of IL-4/IL-13 or IL-5/eotaxin reduce eosinophilia and AHR (21). The cytokines IL-4 and IL-13 can directly stimulate AHR in mast cell ^{-/-} mice and T cell- and B cell-deficient mice (68). However, this does not preclude stimulation of AHR by other mediators in asthma. In contrast to the IL-5^{-/-} mice, in IL-5 transgenic mice,

administration of anti- $\alpha 4$ -integrin antibodies reduces AHR but did not reduce eosinophil infiltration (8). However, increasing IL-5 levels can increase eosinophil CD11/CD18 binding (69), thus generating compensatory mechanisms for eosinophil infiltration independent of VCAM-1. Whether the decrease in AHR in the IL-5 transgenic mice was due to changes in eosinophil function or function of other airway cells in the presence of transgenic IL-5 was not reported (8). In clinical trials, anti-IL-5 did not block AHR (72), suggesting that either eosinophils were not required for AHR or that there were compensatory mechanisms as in the murine models. In summary, AHR is generated by multiple mediators, and when one mediator is altered, other signals may compensate for the generation of AHR.

In our studies with the OVA-challenged chimeric CYBB mice, there was reduced eosinophilia and AHR with no effect on IL-5 or IL-13. There was modest production of IL-4 by OVA-challenged chimeric wild-type mice and reduced IL-4 production in the OVA-challenged chimeric CYBB mice. The mechanism for this reduction in IL-4 in the OVA-challenged chimeric CYBB mice (which have wild-type leukocytes) is not known at this point. There was no significant change in background levels of IL-4 in the chimeric CYBB mice, since there was no statistical difference in levels of IL-4 in the saline-treated chimeric CYBB mice vs. the saline-treated chimeric wild-type mice (Table 1, Fig. 6). It is acknowledged that the levels of IL-4 in the saline-treated groups are near the detection limit for the ELISA. Another possibility that was addressed was whether there was an alteration in numbers of CD4⁺CD25^{high} T regulatory cells. Cytokine production can be skewed by CD4⁺CD25^{high} T regulatory cells, since CD4⁺CD25^{high} T cells increase Th1 cell differentiation and reduce Th2 cell differentiation (56). However, nonhematopoietic gp91 phox did not modulate the number of lung CD4⁺CD25^{high} T cells after OVA challenge. Furthermore, in the absence of changes in IL-5, IL-13, IFN γ , eotaxin, and T cells, the intratracheal administration of eosinophils purified from BAL of wild-type mice recovered the AHR in the OVA-challenged chimeric CYBB mice. Despite the recovery of the AHR by the intratracheal administration of the eosinophils, this did not recover the decrease in IL-4 indicating that the eosinophils were necessary for the AHR in these mice.

In our studies, the recovery of AHR in chimeric CYBB mouse by intratracheal transfer of eosinophils isolated from the BAL of OVA-stimulated mice and the lack of recovery of AHR by transfer of similar numbers of eosinophils isolated from blood suggests a functional difference between blood and tissue eosinophils. In contrast to our studies demonstrating insufficient function of blood eosinophils for AHR, it has been reported that intratracheal transfer of blood eosinophils can recover AHR in IL-5^{-/-} mice (52). However, in those studies, the number of transferred blood eosinophils that recovered AHR was 10-fold higher than the number of blood eosinophils used in our studies. Therefore, it could be postulated that the numbers of blood eosinophils used in our studies are below a threshold needed for blood eosinophils to increase the AHR. A functional difference between blood and tissue eosinophils has previously been suggested, since the blood and BAL eosinophils display phenotypic differences in activation markers and adhesion molecules in mice and humans (26,38). It has also been demonstrated that priming of eosinophils by adhesion to VCAM-1 on endothelial cells increases eosinophil function as measured by formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated eosinophil ROS production and neurotoxin release (44,45). Therefore, eosinophils that bind VCAM-1 and migrate across endothelium may become primed, resulting in a functional difference between blood and tissue eosinophils.

In conclusion, nonhematopoietic cell gp91 phox participates in VCAM-1-dependent eosinophilia and AHR in OVA-challenged chimeric CYBB mice. Thus, the vascular generation of ROS by gp91 phox may be a novel target for drug intervention in the limitation of VCAM-1-dependent component of inflammations that have been implicated in diseases such as asthma (12,49), atherosclerosis (28,42), and multiple sclerosis (4). Moreover, limiting VCAM-1

dependent inflammation in chronic disease may be an advantageous target, since it would spare VCAM-1-independent inflammation such as neutrophil clearance of infections.

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Fig. 1.

Timeline for ovalbumin (OVA) sensitization, OVA challenge, and intratracheal transfer of purified eosinophils. Chimeric mice were sensitized and challenged with OVA according to *protocol 1 (A)* or *protocol 2 (B)*. *C*: using *protocol 2*, groups of OVA-challenged chimeric CYBB mice received eosinophils from bronchoalveolar lavage (BAL) of OVA-challenged CD45.1 donor mice or received purified eosinophils from blood of NJ.1638 mice that were not challenged with OVA. AHR, airway hyperresponsiveness.

Fig. 2.

Chimeric CYBB mice have reduced OVA-stimulated lung eosinophilia. Chimeric CYBB mice (CYBB) and chimeric C57BL/6 mice (WT), that had been reconstituted with wild-type (WT) bone marrow from GFP mice, were sensitized once by intraperitoneal on *day 0* and challenged intranasally on *days 14* and *15* with OVA. On *day 16*, blood was collected and lungs lavaged. *A*: blood eosinophils. *B*: total number of BAL leukocytes. *C*: number of mononuclear cells, neutrophils, eosinophils, and lymphocytes in BAL. Bars are very small and near *x*-axis for cells in saline-treated groups. *D*: data are expressed as percent of total leukocytes. *C* and *D*: solid bars, WT saline. Wide-hatched bars, WT OVA. Stippled bars, CYBB saline. Narrow-hatched bars, CYBB OVA. Values are means $\pm$ SE of 6–8 mice per group. * $P < 0.05$ compared with OVA-treated WT mice.

Fig. 3.

Endothelial cell vascular cell adhesion molecule-1 (VCAM-1) expression was not altered in the OVA-stimulated chimeric CYBB mice. Tissue sections from mice in Fig. 2 were indirectly immunofluorescence labeled for VCAM-1 and examined by confocal microscopy. *A* and *C*: chimeric WT C57BL/6 mouse lung. *B* and *D*: chimeric CYBB mouse lung. *A* and *B*: OVA challenged. *C* and *D*: saline challenged. Isotype antibody control labeled sections were negative (data not shown). Shown are representative tissues. *E*: sum of fluorescence intensity per μm^2 of endothelium. Solid bars, CYBB chimera. Hatched bars, WT chimera. Values are means $\pm$ SE for 6–8 mice per group. * $P < 0.05$ compared with saline-treated mice.

Fig. 4.

Eosinophils accumulated on the luminal surface of the endothelium in the OVA-stimulated CYBB chimeras. Frozen lung tissue sections from mice in Fig. 2 were fixed and stained with hematoxylin and eosin (A and B) or immunolabeled for major basic protein (MBP) (C–F). A and C: WT chimeric mouse lung, OVA challenged. B and D: CYBB chimeric mouse lung, OVA challenged. E: C57BL/6 chimeric mouse lung, saline challenged. F: CYBB chimeric mouse lung, saline challenged. Shown are representative sections. Arrows, eosinophils attached to luminal surface of endothelium. L, vessel lumen. G: number of eosinophils at the luminal surface of the endothelium per mm of the luminal surface of the endothelium in the tissue sections. Values are means $\pm$ SE of 6–8 mice per group. * $P < 0.05$ compared with OVA-treated WT mice.

Fig. 5.

OVA-stimulated chimeric CYBB mice had reduced AHR. Chimeric CYBB mice (CYBB) and chimeric C57BL/6 mice (WT) were generated by reconstitution with CD45.1 bone marrow. The mice were sensitized and challenged with OVA using *protocol 2* (Fig. 1). Briefly, these mice were sensitized intraperitoneally with OVA-alum or saline-alum on *day 0* and *day 7* and challenged intranasally on *days 14, 16, and 19* with OVA or saline. On *day 20*, mice were tested for airway responsiveness to 50 μ g acetylcholine/kg. Airway responsiveness is depicted as airway pressure time index (APTI; cmH₂O · s). Values are means $\pm$ SE of 6–8 mice per group. * $P < 0.05$ compared with the other groups.

Fig. 6.

Expression of several cytokines was not altered in the OVA-stimulated chimeric CYBB mice. Lung digests from mice in Fig. 5 were nontreated or restimulated with OVA for 48 h. The culture supernatants were examined for cytokines by ELISA: IL-4 (A), IL-5 (B), IL-10 (C), and IL-13 (D). Solid bars, nontreated. Hatched bars, OVA challenged. Values are means $\pm$ SE of 6–8 mice per group. There was no statistical difference among the saline groups. * $P < 0.05$ compared with saline-treated mice.

Fig. 7.

Bypassing the endothelium with intratracheal (IT) eosinophils (Eos) resulted in recovery of AHR. **A:** the number of BAL eosinophils or blood eosinophils transferred to each chimeric CYBB mouse on *days 14, 16, and 19*. **B:** the BAL cells were collected after testing for AHR. Shown is the number of eosinophils in the BAL, demonstrating successful intratracheal administration of eosinophils to the chimeric CYBB mice. **P* < 0.05 compared with the other groups. **C:** to examine AHR on *day 20*, 75 μ g acetylcholine/kg was used, since we had determined that the 50 μ g/kg used in Fig. 5 induced about only 30% of the maximum AHR, whereas 75 μ g/kg was at 70% of the maximum AHR (data not shown). In some bars in **C**, the "E" indicates intratracheal administration of eosinophils. **P* < 0.05 compared with saline-treated mice.

Fig. 8.

OVA-stimulated chimeric CYBB mice had reduced perivascular eosinophilia in the lung tissue. The lungs were frozen from mice in Fig. 7, and the tissue sections were stained by immunohistochemistry with anti-MBP. *A* and *B*: WT C57BL/6 chimeric mouse lung. *C* and *D*: CYBB chimeric mouse lung. *E*: CYBB chimeric mouse from the group in Fig. 6 that received 4.1×10^6 total BAL eosinophils (CYBB, OVA, IT BAL eos). *A* and *C*: saline challenged. *B*, *D*, and *E*: OVA challenged. Isotype antibody control labeled sections were negative (data not shown). Shown are representative tissues. *F*: number of perivascular eosinophils per high-powered ($\times 40$) field. * $P < 0.05$ compared with the other OVA-treated groups. *G*: number of eosinophils on the endothelial lumen/vessel. Values are means $\pm$ SE of

6–8 mice per group. * $P < 0.05$ compared with saline-treated mice and OVA-treated WT mice. L, vessel lumen. Arrows, eosinophils attached to luminal surface of endothelium.

Fig. 9.

OVA-stimulated chimeric CYBB mice had reduced peribronchial eosinophilia in the lung tissue. The lungs were frozen from mice in Fig. 7, and the tissue sections were stained by immunohistochemistry with anti-MBP and lightly counterstained with methyl green. *A* and *B*: WT C57BL/6 chimeric mouse lung. *C* and *D*: CYBB chimeric mouse lung. *E*: CYBB chimeric mouse from the group in Fig. 7 that received 4.1×10^6 total BAL eosinophils (CYBB, OVA, IT BAL eos). *A* and *C*: saline-challenged mice. *B*, *D*, and *E*: OVA-challenged mice. Isotype antibody control labeled sections were negative (data not shown). Shown are representative tissues. *F*: number of peribronchial eosinophils per high-powered ($\times 40$) field. * $P < 0.05$ compared with OVA-treated mice. B, bronchial airway.

Fig. 10.

OVA-challenged chimeric CYBB mice did not have altered numbers of T cell subsets, localization of T cells, serum IgE, or serum OVA-specific Igs. From mice in Fig. 7, serum was obtained from the blood, half of the lung was digested, and half of the lung was frozen for tissue sections. *A–C*: the lung digests were immunofluorescence labeled for CD4, CD8, and TCR β and examined by flow cytometry. *B*: open bars, TCR β^+ ; solid bars, CD4 $^+$ TCR β^+ ; hatched bars, CD8 $^+$. *D*: the tissue sections were labeled by immunohistochemistry for CD4 or CD8; HPF, high-powered fields; solid bars, CD4 $^+$ perivascular; open bars, CD4 $^+$ peribronchial; hatched bars, CD8 $^+$ perivascular; stippled bars, CD8 $^+$ peribronchial. *E*: the serum was examined for total IgE by ELISA. *F*: the serum was examined by ELISA for OVA-specific

IgE (solid bars), OVA-specific IgG₁ (open bars), or OVA-specific IgG_{2a} (hatched bars). The OVA-challenged groups were significantly greater than the saline groups in all panels ($P < 0.05$).

Table 1
Cytokine production by OVA-restimulated lung digests from mice in Fig. 7

	WT Saline	CYBB Saline	WT OVA	CYBB OVA	CYBB OVA BAL eos	CYBB OVA Blood eos
IL-4	0	0	40±13*	8±6	5±5	0
IL-5	1,073±156	864±200	2,176±341	2,317±263	2,815±235	2,053±339
IL-10	486±37	412±15	1,079±70	1,024±35	1,004±44	908±74
IL-13	30±9	10±6	1,270±231	917±168	901±91	1,099±198
IFN γ	1.9±1.0	0	0	0	0.2±0.2	0
Eotaxin	49±11	52±8	173±22	178±20	237±17	172±23

Values are means $\pm$ SE, in pg/ml. OVA, ovalbumin; WT, wild type; BAL, bronchoalveolar lavage; eos, eosinophils.

* $P < 0.05$ compared with the other OVA-stimulated groups for IL-4.