IL-13 stimulates vascular endothelial cell growth factor and protects against hyperoxic acute lung injury

Jonathan Corne,[1] Geoffrey Chupp,[1] Chun Guen Lee,[1] Robert J. Homer,[2] Zhou Zhu[1] Qingsheng Chen,[1] Bing Ma,[1] Yuefen Du,[1] Francoise Roux,[1] John McArdle,[1] Aaron B. Waxman,[1] and Jack A. Elias[1]

[1]Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, and
[2]Department of Pathology, Yale University School of Medicine, New Haven, Connecticut, USA

Address correspondence to: Jack A. Elias, Section of Pulmonary and Critical Care Medicine, Yale University School of Medicine, Department of Internal Medicine, 333 Cedar Street, 105 LCI, PO Box 208057, New Haven, Connecticut 06520-8057, USA. Phone: (203) 785-4163; Fax: (203) 785-3826; E-mail: jack.elias@yale.edu.

Jonathan Corne's present address is: Department of Respiratory Medicine, D Floor, South Block, University Hospital, Queens Medical Centre, Nottingham, United Kingdom.
Aaron B. Waxman's present address is: Pulmonary and Critical Care Medicine, New England Medical Center, Boston, Massachusetts, USA.

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Hyperoxia is an important cause of acute lung injury. To determine whether IL-13 is protective in hyperoxia, we compared the survival in 100% O_2 of transgenic mice that overexpress IL-13 in the lung and of nontransgenic littermate controls. IL-13 enhanced survival in 100% O_2. One hundred percent of nontransgenic mice died in 4–5 days, whereas 100% of IL-13–overexpressing mice lived for more than 7 days, and many lived 10–14 days. IL-13 also stimulated VEGF accumulation in mice breathing room air, and it interacted with 100% O_2 to increase VEGF accumulation further. The 164–amino acid isoform was the major VEGF moiety in bronchoalveolar lavage from transgenic mice in room air, whereas the 120– and 188–amino acid isoforms accumulated in these mice during hyperoxia. In addition, antibody neutralization of VEGF decreased the survival of IL-13–overexpressing mice in 100% O_2. These studies demonstrate that IL-13 has protective effects in hyperoxic acute lung injury. They also demonstrate that IL-13, alone and in combination with 100% O_2, stimulates pulmonary VEGF accumulation, that this stimulation is isoform-specific, and that the protective effects of IL-13 are mediated, in part, by VEGF.

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Introduction

The administration of supplemental O_2 is an important part of the therapy of patients with a wide variety of cardiopulmonary and other disorders. Unfortunately, when fractional inspired concentrations of O_2 greater than 50% are used, this treatment is associated with significant toxicity. Although these concentrations of O_2 increase alveolar and arterial oxygen tension and augment tissue oxygen delivery, they simultaneously stimulate the generation of toxic reactive oxygen species. When administered for a prolonged period, 100% oxygen causes acute lung injury characterized by severe endothelial damage, alveolar-capillary permeability alterations, and pulmonary edema (1–3).

IL-13 is a pleiotropic 12-kDa protein product of a gene on chromosome 5 at q31 that is produced in large quantities by appropriately stimulated CD4+ Th2 cells and in lesser quantities by Th1 cells (4, 5). It has a variety of proinflammatory effects that are relevant to asthma and other Th2-dominated inflammatory disorders including the ability to induce IgE production (6), CD23 expression (7), and endothelial cell (EC) VCAM-1 expression (8). However, IL-13 is not strictly proinflammatory. In fact, a large body of literature demonstrates that IL-13 also has potent and complex anti-inflammatory effects that include the ability to inhibit TNF, IL-8, and IL-1β elaboration, transcription factor NF-κB activation, and chemokine elaboration, and the ability to induce the production of the IL-1 receptor antagonist (9–13). In keeping with these properties, IL-13 has been demonstrated to have protective effects in a number of animal models including immune complex lung injury (10), ischemia reperfusion liver injury (14), lethal endotoxemia (15), and LPS-induced ocular injury (16). The ability of IL-13 to regulate hyperoxic acute lung injury (HALI), however, has not been investigated, and the relationship(s) between the pro- and anti-inflammatory effects of IL-13 in vivo has not been defined.

Endothelial injury and cell death are major features in the pathogenesis of HALI (17–20). After acute oxygen exposure, pulmonary microvascular ECs rapidly die, leaving areas of denuded capillary basement membrane (1, 21). Recovery from HALI requires EC proliferation to restore normal capillary architecture (1, 22). Studies of this recovery response have suggested that the EC proliferation that is seen is mediated by angiogenic growth factors. VEGF may play a key role in this response, as it

is a powerful endothelial mitogen (23, 24), inhibits EC apoptosis (23–26), and is produced in significant quantities by alveolar type II cells during recovery from hyperoxic exposures (17). To date, however, the ability of EC mitogens and apoptosis regulators to confer protection in the setting of HALI has not been investigated. In addition, although IL-13 has been noted to have angioregulatory properties (27–29), its ability to stimulate VEGF elaboration and the ability of VEGF to mediate protective effects of IL-13 has not been documented.

We hypothesized that IL-13, while inducing tissue inflammation, could ameliorate HALI. We also hypothesized that IL-13, alone or in combination with hyperoxia, could induce VEGF elaboration and that the VEGF would contribute to the protective effects of IL-13 in this setting. To test this hypothesis, we compared the sensitivity to 100% O_2 of wild-type (WT) mice and transgenic mice generated in our laboratory in which the overexpression of IL-13 causes significant tissue inflammation and physiological dysregulation (30). These studies demonstrate that IL-13–overexpressing mice exhibit a remarkable tolerance to the toxic effects of 100% O_2, as they survive for significantly greater periods in 100% O_2 than do WT littermate controls. They also demonstrate that IL-13, alone and in combination with hyperoxia, induces VEGF elaboration; highlight the isoform specificity of these inductive responses; and demonstrate that IL-13–induced protection in 100% O_2 is partially abrogated after in vivo VEGF neutralization.

Methods

Animals. Two types of overexpression transgenic mice were generated in our laboratory and used in these studies. Both were generated using CBAxC57BL/6 zygotes and bred onto a C57BL/6 genetic background. Unless otherwise indicated, WT littermates were used as negative controls.

Clara cell 10-kDa protein promoter–IL-13 mice. These mice use the Clara cell 10-kDa (CC-10) promoter to target the expression of murine IL-13 to the lung/airway. In these animals, IL-13 is constitutively expressed with 1.5–3.0 ng/mL levels of IL-13 being detectable in the bronchoalveolar lavage (BAL) fluid. The methods used to generate these mice, the organ specificity of transgene expression, and the phenotype of these animals have been described previously (30).

CC-10–reverse tetracycline transactivator cDNA–IL-13 mice. In these mice, IL-13 is overexpressed in an externally regulable fashion. The approach used to generate these animals has been described by our laboratory (31). It entails the generation of mice with two transgenic constructs. The first (tet-O-CMV-IL-13-hGH) consists of multimers of the tetracycline operator (tet-O), linked to the minimal cytomegalovirus promoter, murine IL-13 cDNA, and human growth hormone (hGH) intronic and polyadenylation sequences. The second construct (CC10–rtTA–hGH) contains the CC10 promoter driving the expression of the reverse tetracycline transactivator cDNA (rtTA) linked to hGH intronic and polyadenylation sequences. rtTA is a fusion construct that contains the tetracycline repressor (tet-R) protein and the powerful herpes virus VP-16 transactivator. It has been mutated such that the tet-R only binds to tet-O in the presence of doxycycline (dox). In dual animals, CC10 constitutively targets rtTA to the lung/airway. In the presence of dox (which is added to the animal's drinking water), rtTA binds in trans to the tet-O and the VP-16 transactivator activates IL-13 gene transcription. In the absence of dox, rtTA does not bind or binds weakly to the tet-O, and low levels or no transgene expression can be appreciated. The details of the preparation of the CC10-rtTA-hGH construct have been described elsewhere (31). The tet-O-CMV-IL-13-hGH construct was prepared by replacing the IL-11 cDNA in the construct tet-O-CMV-IL-11-hGH described previously by our laboratory (31), with the murine IL-13 cDNA. The fidelity of the junction areas of these constructs were confirmed by sequencing, and the constructs were isolated and purified through Elutip-D columns (Schleicher and Schuell, Keane, New Hampshire, USA). Transgenic mice were generated in (CBA x C57BL/6) F_2 eggs by simultaneously microinjecting both constructs. Founders were identified by Southern analysis using radiolabeled cDNA encoding IL-13 and rtTA and bred with WT C57BL/6 animals. The function of this system was evaluated by quantitating the levels of BAL IL-13 before and/or intervals after dox (0.5 mg/mL) administration. At baseline, lungs appeared normal on hematoxylin and eosin (H&E) stains, and levels of BAL IL-13 less than or equal to 75 pg/mL were noted. Increased levels of BAL IL-13 were noted within 24 hours, and plateau levels between 0.3 and 1.2 ng/mL were noted 48–96 hours after dox administration. The full-blown IL-13 phenotype, as described by our laboratory (30), was seen after approximately 6 weeks of dox ingestion. In all lines, RT-PCR analysis of RNA from lungs and other tissues demonstrated that IL-13 was produced in a lung-specific fashion.

Recombinant murine IL-13 administration. In selected experiments, the effects of recombinant (r) IL-13 were evaluated. In these experiments, rIL-13 (500 ng; R&D Systems, Minneapolis, Minnesota, USA) or its saline vehicle control was applied to the nostrils of sedated mice and then aspirated into their lungs. Doses were given 24 hours and 4 hours before the initiation of the hyperoxic exposure.

Oxygen exposure. One- to 2-month-old mice were placed in cages in an airtight Plexiglass chamber (55 × 40 × 50 cm). Throughout the experiment, they were given free access to food and water. Oxygen levels were constantly monitored by an oxygen sensor that was connected to a relay switch incorporated into the oxygen supply circuit. The inside of the chamber was kept at atmospheric pressure, and mice were exposed to a 12-hour light-dark cycle.

BAL. After anesthesia, a median sternotomy was performed and the trachea was exposed by blunt dissec-

tion. A 22-gauge catheter was inserted into the trachea, and BAL was performed by instilling 0.6 mL of PBS into the trachea and gently aspirating back. This was repeated twice. The samples from each animal were pooled and centrifuged, and the supernatant stored at –20°C.

Serum collection. After anesthesia, a median sternotomy incision was performed and extended into the abdomen. The inferior vena cavae was then dissected, and 1 mL of blood was collected and allowed to clot at room temperature for 2 hours. The serum was aspirated after centrifugation at 2,000*g* for 20 minutes and was stored at –20° C until used.

Measurements of VEGF and keratinocyte growth factor in BAL fluids and serum. The levels of VEGF or keratinocyte growth factor (KGF) in BAL fluids and or serum were measured using commercial ELISAs (R&D Systems) specific for each cytokine. Each assay used a polyclonal antibody specific for VEGF or KGF as the capture antibody and a horseradish peroxidase–conjugated (HRD-conjugated) antibody specific for the cytokine being assayed as the detector. The protocols were followed according to the manufacturer's instructions. These assays detected as little as 9 pg/mL of VEGF and 15 pg/mL of KGF.

Immunohistochemical localization of VEGF. Staining was performed on a DAKO autostainer (DAKO Corp., Carpinteria, California, USA). Paraffin-embedded tissues were cut, exposed to three changes of xylene, rehydrated in a series of graded alcohols, rinsed, blocked with avidin and biotin (Biotin Blocking Kit; DAKO Corp.), and endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 5 minutes. The slides were incubated for 1 hour at room temperature with either a 1:200 dilution of a murine IgG_2 mAb that recognized all isoforms of VEGF (Santa Cruz Biotechnology, Santa-Cruz, California, USA) or a 1:200 dilution of a nonspecific anti-mouse IgG_2 control antibody (R&D Systems). To prevent nonspecific binding to mouse tissue, the antibodies were previously biotinylated and blocked with nonspecific mouse serum using a commercially available kit (Animal Research Kit; DAKO Corp.). After incubation with antibody, the slides were incubated with a streptavidin-peroxidase enzyme conjugate (DAKO Corp.) for 15 minutes followed by 3, 3′-diaminobenzidien-tetrahydrochloride (DAB; DAKO Corp.) for 7 minutes. The slides were counterstained with hematoxylin, dehydrated in a series of graded alcohols, and cleared with xylene.

Characterization of VEGF isoforms in BAL. To characterize the isoforms of BAL VEGF, Western blot analysis was performed. BAL fluids were concentrated tenfold by centrifugation (Centricon concentrator; Amicon Inc., Beverly, Massachusetts, USA) and, along with recombinant human VEGF protein controls (R&D Systems), were resolved on 12% acrylamide reducing gels and transferred to PDVF membranes (Immobilin-P; Millipore Corp., Bedford, Massachusetts, USA). The membranes were blocked with 5% nonfat milk, washed with 0.1% Tween 20, and incubated for 2 hours at room temperature in 2.5% milk in a 1:10,000 dilution of a rabbit polyclonal antibody that recognized all isoforms of mouse VEGF (Santa Cruz Biotechnology). After washing with 0.1% Tween 20, they were incubated for 1 hour with a biotinylated goat anti-rabbit antibody (Amersham Life Science, Bucks, United Kingdom) and washed in 0.1% Tween 20. Protein bands were detected using the ECL Western blot detection system (Amersham Life Science).

In vivo neutralization experiments. To determine whether VEGF mediated the enhanced resistance to 100% oxygen seen in the IL-13–overexpressing mice, neutralizing antibodies to VEGF and antibody controls were administered to transgenic and WT littermate control mice and their survival in 100% oxygen was assessed. The goat polyclonal anti-VEGF and polyclonal control antisera were administered (30–100 μg intraperitoneally) just before the initiation of the 100% oxygen exposure. WT littermate mice given intraperitoneal PBS were included as additional controls.

Statistics. Normally distributed data are expressed as means ± SD and assessed for significance by Student's *t* test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test. Differences in survival between transgenic and WT animals were assessed using χ^2 analysis.

Results

CC10–IL-13 transgene–positive mice demonstrate increased survival in hyperoxia. To determine whether the overexpression of IL-13 led to increased survival in hyperoxia, CC10–IL-13 mice and their WT littermate controls were exposed to 100% oxygen, and their survival was assessed (Figure 1). Seven of the WT mice died by day 4, and all died by day 5. In contrast, all the CC10–IL-13 transgenic mice lived for 7 days. Four were still alive at 10 days, and two did not die until 14

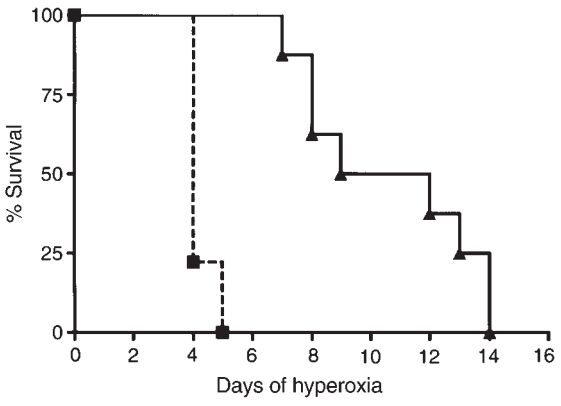


Figure 1
Survival of CC10–IL-13 mice and their littermate controls in 100% oxygen. CC10–IL-13 transgenic mice (triangles/solid line) and WT littermate controls (squares/dashed line) (n = 9 for each group) were exposed to 100% O_2 and survival was assessed.

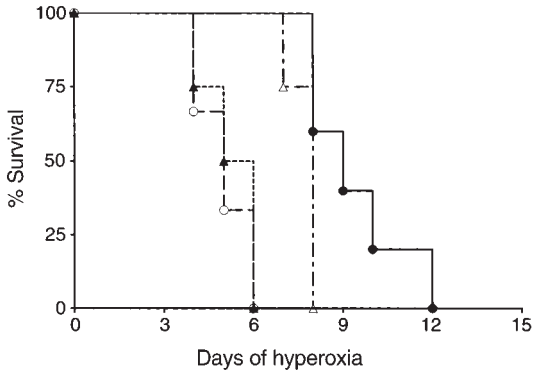


Figure 2
Survival in 100% oxygen of CC10–rtTA–IL-13 mice on normal water and dox water. The survival of CC10–rtTA–IL-13 transgenic mice on normal water (open triangles) and dox water (solid circles) and of WT littermate control mice on normal water (open circles) and dox water (solid triangles) was assessed as described in Methods. In these experiments, dox was added to the animal's drinking water 2 weeks before the initiation of the hyperoxic exposure.

days after the start of the hyperoxic exposure. The difference in survival between the transgenic and WT mice was statistically significant ($P < 0.0001$).

CC10–rtTA-IL-13 inducible transgenic mice demonstrate increased survival in hyperoxia. As described previously (30), CC10–IL-13 transgenic mice have prominent anatomical alterations including inflammation and airway fibrosis. In an attempt to determine whether the IL-13 or the structural alterations it induced were responsible for the enhanced survival of the CC10–IL-13 transgenic animals, we determined if a similar protective effect was seen in transgenic mice that expressed significant quantities of IL-13 for shorter intervals. In the first series of experiments, we compared the survival in 100% oxygen of CC10–rtTA–IL-13 mice on dox for 2 weeks ($n = 5$), CC10–rtTA–IL-13 mice on normal water ($n = 4$), and WT littermate controls fed normal water ($n = 4$) and dox water ($n = 4$). At this 2-week time point, the transgenic mice on dox water have modest pulmonary inflammation but no subepithelial airway fibrosis. As expected, the WT mice both on and off dox all died within 4–6 days. The CC10–rtTA–IL-13 transgenic mice on dox showed enhanced survival with 100% of the animals living for 8 days and others living for 9, 10, and 12 days, respectively (Figure 2). CC10–rtTA–IL-13 mice on normal water showed a survival that was intermediate between the WT mice and the dox-treated CC10–rtTA–IL-13 mice dying after 7–8 days of 100% oxygen exposure (Figure 2). The difference in survival between the CC10–rtTA–IL-13 mice on dox and their WT littermate controls was statistically significant ($P < 0.0001$).

In the second series of experiments, we compared the survival in 100% O_2 of CC10–rtTA–IL-13 mice on dox for 24 hours and appropriate controls. At this time point, lungs from transgenic CC10–rtTA–IL-13 mice are normal on H&E evaluation. This time point was also chosen because it took approximately 24 hours for the induction of IL-13 by dox to be appreciated. This enabled us to initiate the 100% O_2 exposure at the time IL-13 production was being induced. In these experiments, all the WT mice died in 5–6 days. In contrast, 75% of the IL-13–producing animals lived 7 days or more, with 25% surviving for 10- to 14-day intervals ($P < 0.05$) (data not shown). Thus, IL-13–induced protection was readily appreciated in this short-term IL-13 exposure system.

rIL-13 confers protection in 100% O_2. Studies were also undertaken to determine whether rIL-13 could protect WT mice from the toxicities of 100% O_2. As illustrated in Figure 3, 25% of vehicle control treated WT mice died after 4 days, 50% died after 4–5 days, and 100% died after 5 days of 100% O_2 exposure. In contrast, the administration of as little as 1 μg of rIL-13 over the 24-hour interval before the institution of the 100% O_2 exposure caused 80% and 30% of the animals to live for more than 5 and 5.5 days, respectively. The differences between the survival of these mice were highly significant ($P < 0.0002$). Thus, rIL-13 can protect lungs from WT mice from the damaging effects of 100% O_2. When these studies and the studies with the CC10-rtTA-IL-13 mice on dox water for 24 hours are viewed in combination, they demonstrate clearly that the protective effects of IL-13 can be seen in anatomically normal lungs and cannot be attributed solely to the structural alterations that are induced by the cytokine.

CC10–IL-13 transgenic mice have increased levels of BAL fluid VEGF, but not KGF. Epithelial and endothelial injury and proliferation are important components of the damage and repair responses, respectively, seen after hyperoxic exposures. As a result, we hypothesized that factors that promote epithelial and endothelial proliferation could lead to enhanced repair and

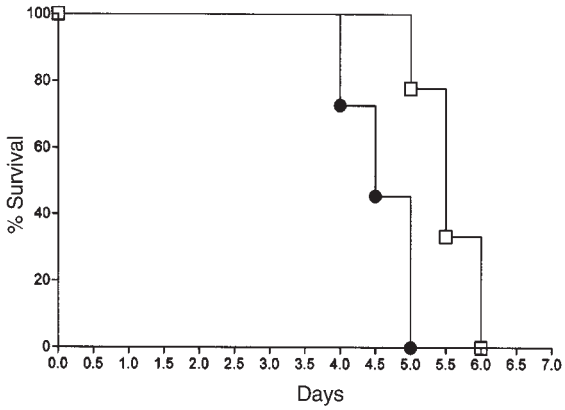
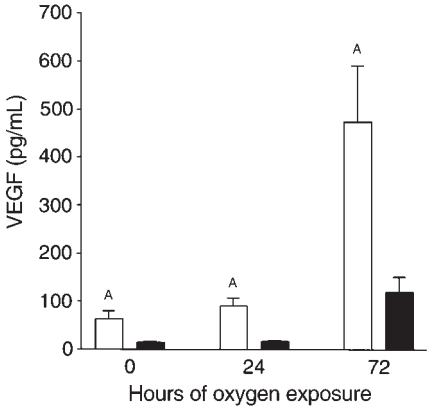


Figure 3
Survival of rIL-13–pretreated WT mice in 100% O_2. WT mice were randomized to receive rIL-13 (500 ng) (squares) or vehicle control (circles) 24 hours and 4 hours before the initiation of 100% O_2 exposure. Survival was then assessed as described in Methods (n = a minimum of 8 for each group).

Figure 4
Levels of BAL fluid VEGF in CC10–IL-13 mice in room air and after exposure to 100% oxygen. BAL fluids were obtained from CC10–IL-13 mice (open bars) and their WT littermate controls (solid bars) before and at intervals after exposure to 100% oxygen. The levels of VEGF in these fluids were quantitated using a commercial ELISA that detects all isoforms of VEGF. The noted values represent the mean ± SD of a minimum of nine animals at each time point. ($^{A}P < 0.05$, comparing CC10–IL-13 transgenic and WT nontransgenic animals at each time point).



increased survival after hyperoxic insults. KGF stimulates type II alveolar epithelial cell proliferation and confers protection in 100% oxygen (32). VEGF is a powerful EC mitogen (23, 24). We, therefore, quantitated the levels of BAL fluid KGF and VEGF in transgenic and nontransgenic mice before and after exposure to 100% oxygen.

The levels of KGF in BAL fluids from CC10–IL-13 transgenic and WT nontransgenic mice in room air were at or below the limits of detection of our assay. In addition, these levels did not change in a significant fashion in nontransgenic or animals after 100% oxygen exposure (data not shown).

In contrast to KGF, IL-13 and hyperoxia, alone and in combination, caused significant alterations in the levels of BAL VEGF. IL-13 alone was a modest stimulator of VEGF, inducing significantly higher levels of BAL fluid VEGF in CC10–IL-13 transgenic mice than in their WT littermate controls (63.4 ± 16 vs. 14.2 ± 3 pg/mL; $P < 0.0001$). Hyperoxia alone also augmented VEGF accumulation with levels of BAL fluid VEGF in WT littermate control mice of 120 ± 30 pg/mL after 72 hours of 100% O_2 exposure ($P < 0.05$) (Figure 4). Interestingly, the highest levels of VEGF were found in transgenic animals exposed to 100% oxygen. These levels peaked after 72 hours of 100% oxygen exposure (473 ± 119 pg/mL;. $P < 0.0001$ vs. littermate control mice exposed to 100% O_2 for 72 hours; and $P < 0.005$ compared with transgenic mice in room air) (Figure 4).

Serum levels of VEGF are lower than levels in BAL. To determine whether the increased levels of BAL VEGF in transgenic and WT mice, before and or after 100% oxygen exposure, were due to leakage of VEGF from the circulation, the levels of serum VEGF in these animals were quantified. Serum levels of VEGF in WT animals breathing room air were consistently at or just above the limits of detection of our assay. In addition, these levels were not significantly different than the

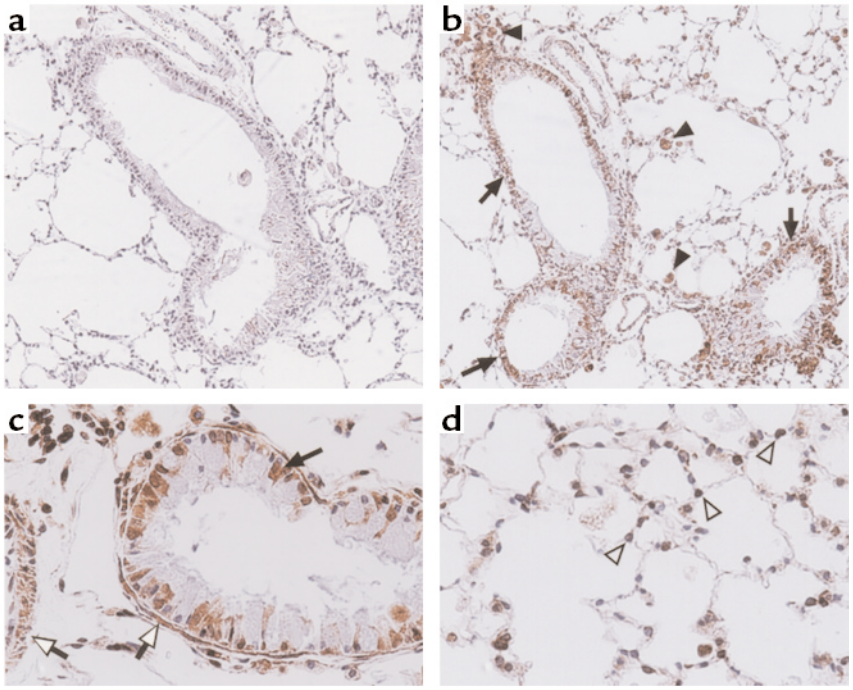


Figure 5
Immunohistochemical localization of VEGF. Sections were obtained from lungs from CC10–IL-13 transgenic mice breathing room air, and immunohistochemical approaches were used to define the localization of VEGF as described in Methods. (**a**) The staining that is seen with the control antibody. ×25 (all photomicrographs are original magnification). Panel **b** contains a low-power photomicrograph illustrating the staining in pulmonary parenchyma, airways (solid arrows), blood vessels, and alveolar macrophages (solid arrowheads). ×25. High-power photomicrographs illustrating airway epithelium (solid arrows) and smooth muscle (open arrows) staining are illustrated in **c**. ×100. Alveolar type II epithelial cell (open arrowheads) staining is highlighted in **d**. ×100.

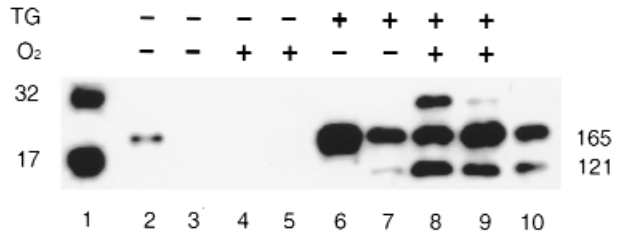


Figure 6
Western blot characterization of isoforms of VEGF in BAL fluids. BAL fluids were obtained from CC10–IL-13 transgenic (TG) mice and WT littermate controls (–) before (O_2^-) and after (O_2^+) exposure to 100% oxygen for 72 hours. The isoforms of VEGF in these fluids were analyzed by Western blotting as described in Methods. Lane 1 contains molecular weight markers, and lane 10, recombinant human $VEGF_{121}$ and $VEGF_{165}$ controls.

levels of VEGF in serum from CC10–IL-13 transgenic mice breathing room air or transgenic or WT mice exposed to 100% oxygen (data not shown). This demonstrates that the increased levels of VEGF in the BAL fluids from our transgenic and WT mice were due to increases in local VEGF production/elaboration.

VEGF is localized to airway epithelial cells, smooth muscle cells, type II alveolar epithelial cells and macrophages. To localize the VEGF in our animals, immunohistochemistry was performed on lung sections prepared from animals breathing room air and animals on 100% oxygen for 24, 48, and 72 hours. The patterns of VEGF expression were similar at all time points with staining localized predominately to selected airway epithelial cells, type II alveolar epithelial cells, airway and vascular smooth muscle cells and macrophages (Figure 5). There were no obvious differences in the localization of staining in the CC10–IL-13 transgenic and WT nontransgenic animals.

IL-13 and hyperoxia stimulate VEGF in an isotype-specific fashion. Multiple isoforms of VEGF are produced as a result of alternative splicing from a single gene (23, 24, 33). To determine whether IL-13, alone or in combination with 100% oxygen, stimulates the production of specific isoforms of VEGF, Western blot analysis of BAL fluid was undertaken. VEGF was detectable in the BAL fluids from some but not all WT mice breathing room air. When it was detected, it migrated in a fashion compatible with the 164–amino acid isoform of murine VEGF (Figure 6, lane 2). VEGF was induced and readily detected in the BAL fluids from CC10–IL-13 transgenic mice breathing room air. The major VEGF moiety in these BAL fluids also migrated in a fashion consistent with murine $VEGF_{164}$ (Figure 6, lanes 6 and 7). Bands compatible with other VEGF isoforms were not consistently noted (Figure 6). In CC10–IL-13 transgenic mice exposed to 100% oxygen for 72 hours, the migration and intensity of the $VEGF_{164}$ band did not change. However, there was an impressive increase in moieties compatible with the 120 and 188 amino acid isoforms of murine VEGF (Figure 6). When viewed in combination, these studies demonstrate that IL-13 selectively stimulates $VEGF_{164}$, whereas IL-13 plus hyperoxia selectively stimulate moieties compatible with $VEGF_{120}$ and $VEGF_{188}$.

In vivo neutralization of VEGF decreases the survival of CC10–IL-13 mice in 100% oxygen. To determine whether VEGF contributed to the enhanced survival of CC10–IL-13 transgenic mice in hyperoxia, we compared the survival in 100% oxygen of transgenic mice and their WT littermate controls after administration of neutralizing antiserum to VEGF or a matched antiserum control. Neither antibody preparation altered the survival of WT mice in 100% O_2. These animals and saline-treated animals all died after 4–5 days of hyperoxic exposure. In contrast, CC10–IL-13 transgenic mice given IgG control antibody lived for 9–17 days with one mouse dying at day 9, five at day 11, and two at day 17. The anti-VEGF antibody decreased the survival of these transgenic animals with 6 mice dying by day 10 and the remaining dying by day 12 ($P < 0.0001$ versus control antibody treated animals) (Figure 7).

Discussion

In these studies, we test the hypothesis that IL-13 is protective in the setting of HALI and that this protection is mediated, in part, by the ability of IL-13 to stimulate the accumulation of EC-regulating mediators. In these studies, we focused on the ability of IL-13 to induce VEGF because VEGF is a well-documented endothelial mitogen and regulator of EC apoptosis (23–26). Our studies demonstrate that IL-13 has remarkable protective effects in 100% oxygen.

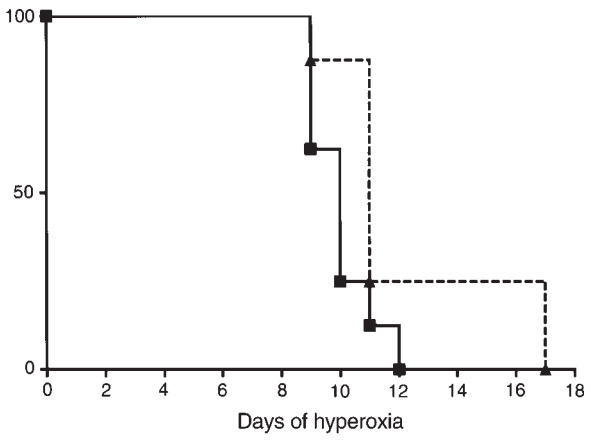


Figure 7
Effect of neutralization of VEGF on the survival of CC10–IL-13 mice in 100% oxygen. To determine whether VEGF contributed to the enhanced survival of CC10–IL-13 mice, transgenic mice were exposed to 100% oxygen after the intraperitoneal administration of either nonspecific IgG (triangles, dashed line) or an anti-VEGF antibody (squares, solid line) as described in Methods ($n = 8$ for each group). There was a significant reduction in the survival of the group given anti-VEGF antibody compared with the mice given the control antibody ($P < 0.0001$).

They also demonstrate that IL-13 selectively stimulates the accumulation of the 164–amino acid isoform and that IL-13 and 100% oxygen interact to further increase the accumulation of VEGF moieties compatible with the 120– and 188–amino acid isoforms of murine VEGF. Last, they demonstrate that IL-13–induced protection in HALI is mediated, at least in part, by VEGF, as in vivo neutralization of VEGF decreased IL-13–induced protection in this setting.

The gas exchange function of the lung requires an extensive capillary network of ECs in close proximity to thin alveoli. During HALI, the excessive production of reactive oxygen intermediates leads to membrane and DNA injury and eventually a cell death response with features of early apoptosis and necrosis (3, 34). ECs are a major site of this injury response, and the loss of and/or impaired function of pulmonary capillary endothelial cells contributes to the fluid and protein leak and pulmonary edema seen in this disorder. During HALI, little EC proliferation occurs (1). In contrast, recovery from HALI requires EC proliferation to restore the integrity of the vascular endothelium (1, 17, 22). In these studies, we reasoned that stimuli that decrease EC death responses and/or stimulate EC proliferation, if activated and/or induced before or during HALI, would be protective in this setting. To assess the validity of this reasoning, we determined if IL-13, alone or in combination with 100% oxygen, regulated VEGF accumulation; characterized the localization of VEGF in the lung; and determined whether VEGF contributed to the protective effects of IL-13 in this setting. These studies demonstrate for the first time that IL-13 stimulates VEGF when expressed in a transgenic fashion in the lung and that IL-13 and 100% oxygen interact to augment VEGF accumulation further. In keeping with reports from others (17, 35, 36), they also demonstrate that pulmonary VEGF appears in airway epithelial cells, alveolar type II epithelial cells, and macrophages. Importantly, these studies also demonstrate that VEGF contributes to the protective effects of IL-13 in HALI. When viewed in combination, these studies support the concept that stimuli that induce and/or recapitulate selected features of the repair/recovery response of HALI can confer protection in this injury state. They also raise the exciting possibility that some, and/or all, of the large number of other angiogenic factors that have been described can confer protection in a similar and/or related fashion.

IL-13 is a potent stimulator of tissue inflammation, airway fibrosis, and alveolar remodeling in our transgenic mice and other in vivo systems (30, 37, 38). In keeping with the importance of angiogenesis in these responses (23, 35, 39), IL-13 has been shown to have endothelial regulatory properties. They include the ability to stimulate and inhibit EC migration (28) and chemotaxis (29) and to inhibit the production of hyaluronectin, an inhibitor of angiogenesis (27). Our studies add to our knowledge of the angioregulatory properties of IL-13 by demonstrating that IL-13 stimulates the in vivo accumulation of VEGF. These findings differ from those from Matsumoto et al. who demonstrated that IL-13 inhibits macrophage VEGF elaboration (40). The basis for this difference is not fully understood. It is likely, however, to be due to the different experimental systems that were used. Matsumoto al. used an in vitro culture system in which cells from patients with renal diseases were studied (40). We used a normal whole murine lung and transgenic cytokine. Regardless of the mechanism, our studies suggest that VEGF is a major mediator of the effects of IL-13 on local vascular structures. If true, this has impressive implications for the pathogenesis of the vascular alterations seen at sites of Th2-dominated tissue inflammation as seen in diseases such as asthma (41) and schistosomiasis (42).

VEGF exists in at least five isoforms, formed by alternative splicing of a single VEGF gene. These isoforms differ in their solubility, receptor affinity, and mitogenic potency (18, 23, 24). The human 121– and 165 –amino acid isoforms (analogous to the murine 120– and 164–amino acid isoforms, respectively) do not bind and bind heparin and heparan-sulfate weakly, respectively. As a result, they are soluble moieties in cell supernatants and biologic fluids. In contrast, larger isoforms such as human $VEGF_{189}$ and its murine analog $VEGF_{188}$ bind with a high specificity and tend to remain cell- and/or matrix-associated in vitro and in vivo. The importance and distribution of VEGF isoforms in the normal and injured lung have not received significant attention. It has been demonstrated, however, that the relative proportion of $VEGF_{189}$ is greater in the adult lung than in other tissues and that during development, the levels of $VEGF_{165}$ mRNA decrease while the levels of $VEGF_{189}$ mRNA increase (18). In HALI, in adult and newborn rabbits, the relative proportion of $VEGF_{189}$ mRNA also declines but returns to normal during lung recovery (18). Our studies demonstrate that IL-13 selectively stimulates $VEGF_{164}$ in the murine lung. Since, in humans, $VEGF_{165}$ is the most mitogenic of the isoforms of VEGF (18, 43), it appears that IL-13 interacts with the VEGF system in a fashion that maximizes its angiogenic effect. In contrast, $VEGF_{188}$ and $VEGF_{120}$ were augmented by IL-13 plus 100% oxygen. The analogous human isoform of $VEGF_{188}$,$VEGF_{189}$, does not bind to the Flk-1 VEGF receptor and may be a matrix storage form of the molecule (18). Thus, the physiologic consequences of the induction of $VEGF_{120}$ and $VEGF_{188}$ by IL-13 and hyperoxia are not clear. In addition, the mechanisms responsible for the isoform specificity of these inductive events will require additional investigation because the different isoforms of VEGF can be produced in a primary fashion or generated via post-translational cleavage of larger VEGF isoforms by enzymes like urokinase-type plasminogen activator and plasmin (18, 44).

VEGF was originally described as vascular permeability factor (VPF), a tumor cell–derived molecule that increased vascular permeability (24, 45). When this property of VEGF and its EC protective effects are viewed in combination, it is clear that VEGF can, in theory, contribute to the pathogenesis of injury-induced permeability alterations or confer cytoprotection in this setting. To differentiate among these different effector possibilities, we compared the sensitivity to 100% O_2 of IL-13 transgenic mice treated with neutralizing antibodies to VEGF or control immunoglobulins. In these studies, neutralization of VEGF caused a significant decrease in survival in hyperoxia. This demonstrates that VEGF contributes to IL-13–induced protection in this setting. It is important to point out, however, that neutralization of VEGF only partially abrogated the protective effects of IL-13. This may be due to limitations in our experimental methodology. Specifically, the need to keep animals in 100% O_2 precluded the administration of multiple doses of anti-VEGF and concerns over surgically induced cytokine elaboration (for example, IL-1 and/or TNF) precluded the use of indwelling osmotic pumps. Alternatively, the partial nature of this response may accurately reflect the contributions of VEGF. The rest of the IL-13–induced protective response may be mediated by other properties of IL-13 such as its ability to diminish reactive oxygen intermediate production (46), inhibit apoptosis (12), inhibit NF-κB and AP-1 transcription factor activation (12) or induce alveolar type II–cell hyperplasia and surfactant apoprotein B elaboration (R. Homer and J.A. Elias, unpublished observations) (47). Our inability to detect heightened KGF levels in our 100% O_2 exposed animals, however, suggest that KGF does not contribute in a significant way to this protective response in a fashion analogous to its protective effects in other experimental systems (32).

In summary, these studies demonstrate that the targeted overexpression of IL-13 in the murine lung confers protection in the setting of HALI. They also demonstrate that IL-13 selectively augments the accumulation of $VEGF_{164}$ and that IL-13 plus hyperoxia interact to cause impressive increases in the levels of moieties compatible with $VEGF_{120}$ and $VEGF_{188}$. Lastly, these studies demonstrate that in vivo neutralization of VEGF reduces the protective effects of IL-13, demonstrating that the protective effects of IL-13 are mediated, in part, by this cytokine moiety. Regulation of the IL-13 and/or VEGF cytokine pathways may prove useful in modifying the pulmonary damage caused by hyperoxia and/or other oxidant-induced tissue injuries.

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1. Crapo, J.D. 1986. Morphologic changes in pulmonary oxygen toxicity. *Annu. Rev. Physiol.* **48**:721–731.
2. Crapo, J.D., Barry, B.E., Foscue, H.A., and Shelburne, J. 1980. Structural and biochemical changes in rat lungs occurring during exposure to lethal and adaptive doses of oxygen. *Am. Rev. Respir. Dis.* **122**:123–143.
3. Waxman, A.B., et al. 1998. Targeted lung expression of interleukin-11 enhances murine tolerance of 100% oxygen and diminishes hyperoxia-induced DNA fragmentation. *J. Clin. Invest.* **101**:1970–1982.
4. de Vries, J.E. 1998. The role of IL-13 and its receptor in allergy and inflammatory responses. *J. Allergy Clin. Immunol.* **102**:165–169.
5. Minty, A., et al. 1997. The related cytokines interleukin-13 and interleukin-4 are distinguished by differential production and differential effects on T lymphocytes. *Eur. Cytokine Netw.* **8**:203–213.
6. Emson, C.L., Bell, S.E., Jones, A., Wisden, W., and McKenzie, A.N.J. 1998. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J. Exp. Med.* **188**:399–404.
7. Zurawski, G., and de Vries, J.E. 1994. Interleukin-13, an interleukin-4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today*. **15**:19–26.
8. Bochner, B.S., Klunk, D.A., Sterbinsky, S.A., Coffman, R.L., and Schleimer, R.P. 1995. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J. Immunol.* **154**:799–803.
9. Lentsch, A.B., Shanley, T.P., Sarma, V., and Ward, P.A. 1997. In vivo suppression of NF-κB and preservation of IkBα by interleukin-10 and interleukin-13. *J. Clin. Invest.* **100**:2443–2448.
10. Lentsch, A.B., Czermak, B.J., Jordan, P.A., and Ward, P.A. 1999. Regulation of acute lung inflammatory injury by endogenous IL-13. *J. Immunol.* **162**:1071–1076.
11. Watson, M.L., et al. 1999. Anti-inflammatory actions of interleukin-13: Suppression of tumor necrosis factor-α and antigen-induced leukocyte accumulation in the guinea pig lung. *Am. J. Respir. Cell Mol. Biol.* **20**:1007–1012.
12. Manna, S.K., and Aggarwal, B.B. 1998. IL-13 suppresses TNF-induced activation of NF-κB, activation protein-1, and apoptosis. *J. Immunol.* **161**:2863–2872.
13. Vannier, E., deWaal, M.R., Salazar-Montes, A., de Vries, J.E., and Dinarello, C.A. 1996. Interleukin-13 (IL-13) induces receptor antagonist gene expression and protein synthesis in peripheral blood mononuclear cells: inhibition by an IL-4 mutant protein. *Blood*. **87**:3307–3315.
14. Yoshidome, H., Kato, A., Miyazaki, M., Edwards, M.J., and Lentsch, A.B. 1999. IL-13 activates STAT6 and inhibits liver injury induced by ischemia / reperfusion. *Am. J. Pathol.* **155**:1059–1064.
15. Muchamuel, T., Menon, S., Pisacane, P., Howard, M.C., and Cockayne, D.A. 1997. IL-13 protects mice from lipopolysaccharide-induced lethal endotoxemia: correlation with down-modulation of TNF-α, IFN-γ, and IL-12 production. *J. Immunol.* **158**:2898–2903.
16. Marie, O., Thillaye-Goldenberg, B., Naud, M.D., and de Kozak, Y. 1999. Inhibition of endotoxin-induced uveitis and potentiation of local TNF-α and interleukin-6 mRNA expression by interleukin-13. *Invest. Ophthalmol. Vis. Sci.* **40**:2275–2282.
17. Maniscalco, W.M., Watkins, R.H., Finkelstein, J.N., and Campbell, M.H. 1995. Vascular endothelial growth factor mRNA increases in alveolar epithelial cells during recovery from oxygen injury. *Am. J. Respir. Cell Mol. Biol.* **13**:377–386.
18. Watkins, R.H., D'Angio, C.T., Ryan, R.M., Patel, A., and Maniscalco, W.M. 1999. Differential expression of VEGF mRNA splice variants in newborn and adult hyperoxic lung injury. *Am. J. Physiol.* **276**:L858–L867.
19. Deneke, S.M., and Fanburg, B.L. 1982. Oxygen toxicity of the lung: an update. *Br. J. Anaesth.* **54**:737–749.
20. Adamson, I.Y.R., Bowden, D.H., and Wyatt, J.P. 1970. Oxygen poisoning in mice. Ultrastructural and surfactant studies during exposure and recovery. *Arch. Pathol.* **90**:463–472.
21. Smith, L.J. 1985. Hyperoxic lung injury: biochemical, cellular, and morphologic characterization in the mouse. *J. Lab. Clin. Med.* **106**:269–278.
22. Durr, R.A., Dubaybo, B.A., and Thet, L.A. 1987. Repair of chronic hyperoxic lung injury: changes in lung ultrastructure and matrix. *Exp. Mol. Pathol.* **47**:219–240.
23. Ferrara, N., and Davis-Smyth, T. 1997. The biology of vascular endothelial growth factor. *Endocr. Rev*. **18**:4–25.
24. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. 1999. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* **13**:9–22.
25. Alon, T., et al. 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med.* **1**:1024–1028.
26. Gerber, H.-P., et al. 1999. VEGF is required for growth and survival in

neonatal mice. *Development.* **126**:1149–1159.
27. Girard, N., Maingonnat, C., Bertrand, P., Vasse, M., and Delpech, B. 1999. Hyaluronectin secretion by monocytes; downregulation by IL-4 and IL-13, upregulation by IL-10. *Cytokine.* **11**:579–584.
28. Volpert, O.V., et al. 1998. Inhibition of angiogenesis by interleukin-4. *J. Exp. Med.* **188**:1039–1046.
29. Halloran, M.M., Haskell, C.J., Woods, J.M., Hosaka, S., and Koch, A.E. 1997. Interleukin-13 is an endothelial chemotaxin. *Pathobiology.* **65**:287–292.
30. Zhu, Z., et al. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities and eotaxin production. *J. Clin. Invest.* **103**:779–788.
31. Ray, P., et al. 1997. Regulated overexpression of interleukin-11 in the lung: use to dissociate development-dependent and -independent phenotypes. *J. Clin. Invest.* **100**:2501–2511.
32. Panos, R.J., Bak, P.M., Simonet, W.S., Rubin, J.S., and Smith, L.J. 1995. Intratracheal instillation of keratinocyte growth factor decreases hyperoxia-induced mortality in rats. *J. Clin. Invest.* **96**:2026–2033.
33. Tischer, E., et al. 1991. The human gene for vascular endothelial growth factor. *J. Biol. Chem.* **266**:11947–11954.
34. Barazzone, C., Horowitz, S., Donati, Y.R., Rodriguez, I., and Piguet, P.-F. 1998. Oxygen toxicity in mouse lung: pathways to cell death. *Am. J. Respir. Cell Mol. Biol.* **19**:573–581.
35. Fehrenbach, H., Kasper, M., Haase, M., Schuh, D., and Muller, M. 1999. Differential immunolocalization of VEGF in the rat and human adult lung, and in experimental rat lung fibrosis: light, fluorescence, and electron microscopy. *Anat. Rec.* **254**:61–73.
36. Berse, B., Brown, L.F., Van de Water, L., Dvorak, H.F., and Senger, D.R. 1992. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages and tumors. *Mol. Biol. Cell.* **3**:211–220.
37. Chiramonte, M.G., Donaldson, D.D., Cheever, A.W., and Wynn, T.A. 1999. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J. Clin. Invest.* **104**:777–785.
38. Wills-Karp, M., et al. 1998. Interleukin-13: central mediator of allergic asthma. *Science.* **282**:2258–2260.
39. Kikuchi, K., et al. 1998. Serum concentrations of vascular endothelial growth factor in collagen diseases. *Br. J. Dermatol.* **139**:1049–1051.
40. Matsumoto, K., Ohi, H., and Kanmatsuse, K. 1997. Interleukin 10 and interleukin 13 synergize to inhibit vascular permeability factor release by peripheral blood mononuclear cells from patients with lipoid nephrosis. *Nephron.* **77**:212–218.
41. Bradding, P., Redington, A.E., and Holgate, S.T. 1997. Airway wall remodelling in the pathogenesis of asthma: cytokine expression in the airways. In *Airway wall remodelling in asthma.* A.G. Stewart, editor. CRC Press Inc. Boca Raton, Florida, USA. 29–63.
42. Chiaramonte, M.G., et al. 1999. IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by Schistosoma mansoni eggs. *J. Immunol.* **162**:920–930.
43. Keyt, B.A., et al. 1996. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J. Biol. Chem.* **271**:7788–7795.
44. Plouet, J., et al. 1997. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form of urokinase is required for its mitogenic effect. *J. Biol. Chem.* **272**:13390–13396.
45. Roberts, W.G., and Palade, G.E. 1997. Neovasculature induced by vascular endothelial growth factor is fenestrated. *Cancer Res.* **57**:765–772.
46. Sozzani, P., et al. 1998. IL-13 induces tyrosine phosphorylation of phospholipase C gamma-1 following IRS-2 association in human monocytes: relationship with the inhibitory effect of IL-13 on R0I production. *Biochem. Biophys. Res. Commun.* **244**:665–670.
47. Tokieda, K., et al. 1999. Surfactant protein-B-deficient mice are susceptible to hyperoxic lung injury. *Am. J. Respir. Cell Mol. Biol.* **21**:463–472.