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Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: Lymphocytic and eosinophilic inflammation without airway hyperreactivity

(macrophages/neutrophils/epithelial cells)

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ABSTRACT **To investigate the contribution of interleukin-4 (IL-4) to airway inflammation *in vivo* and to explore directly its relationship to airway reactivity, we created transgenic mice in which the murine cDNA for IL-4 was regulated by the rat Clara cell 10 protein promoter. Expression was detected only in the lung and not in thymus, heart, liver, spleen, kidney, or uterus. The expression of IL-4 elicited hypertrophy of epithelial cells of the trachea, bronchi, and bronchioles. Hypertrophy is due, at least in part, to the accumulation of mucus glycoprotein. Histologic examination of parenchyma revealed multinucleated macrophages and occasional islands of cells consisting largely of eosinophils or lymphocytes. Analysis of lung lavage fluid revealed the presence of a leukocytic infiltrate consisting of lymphocytes, neutrophils and eosinophils. Mice expressing IL-4 had greater baseline airway resistance but did not demonstrate hyperreactivity to methacholine. Thus, the expression of IL-4 selectively within the lung elicits an inflammatory response characterized by epithelial cell hypertrophy, and the accumulation of macrophages, lymphocytes, eosinophils, and neutrophils without resulting in an alteration in airway reactivity to inhaled methacholine.**

Interleukin 4 (IL-4) is a cytokine produced in mice by the Th2 subset of T lymphocytes (1, 2), by immature bone marrow-derived mast cells (3), by mast cell lines (4), and probably by basophils (5, 6). While this cytokine possesses a wide range of biologic functions on many cell types (for review, see refs. 7 and 8), many of both the *in vitro* (9–12) and *in vivo* (13, 14) effects of murine IL-4 (mIL-4) demonstrate that this cytokine plays an important role in the regulation of protective immune responses (7, 8) and the regulation of IgE synthesis (15, 16). In addition, data suggest that IL-4 possesses an important *in vivo* role in priming T cells to evolve into IL-4 producing Th2 cells (17).

While a full characterization of the role(s) for Th2 cells in human disease remains far from complete, data does support a pivotal immunologic role for Th2 cells in airway inflammation associated with some forms of human asthma (18). Thus, to investigate the contribution of IL-4 to airway inflammation *in vivo* and to explore directly its relationship to airway hyperreactivity, we created transgenic mice that expressed a single cytokine, murine IL-4, selectively within the lung. In creating this mouse, we capitalized on the fact that approximately 50–70% of epithelial cells in the trachea, bronchi, and bronchioles (pulmonary conducting airways) of mice are Clara cells (19), which produce a 10-kDa protein referred to as CC10. We used the Clara cell CC10 protein promoter to express IL-4 constitutively in the lung.

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MATERIALS AND METHODS

Production of Transgenic Mice. The rat CC10 promoter (2.4 kb) was isolated from a pRT-CAT plasmid as a *Hin*dIII fragment and subcloned into the *Hin*dIII site in pBluescript KS (Stratagene). This plasmid was subsequently linearized with *Sma*I and *Bam*HI and then ligated with a 0.85-kb *Sma*I–*Bam*HI fragment containing the simian virus 40 (SV40) small t intron and polyadenylation sequence (pCC10-SV40). The murine IL-4 cDNA (a kind gift from Dr. Philip Leder) was isolated as a 0.5-kb *Hin*dIII–*Eco*RI fragment and ligated to *Nde*I–*Bgl*II-linearized pCC10-SV40 using *Nde*I–*Hin*dIII and *Bgl*II–*Eco*RI oligonucleotide adapters. The DNA was purified from large scale plasmid preparations through two sequential CsCl gradients. After digestion with *Xho*I and *Sac*II, the construct fragment was separated by electrophoresis through a 1% agarose gel (SeaKem GTG;FMC) and isolated by electroelution. The DNA fragment then was purified through Elutip-D columns following the manufacturer's instructions (Schleicher and Schuell) and dialyzed on filters against injection buffer (0.5 mM Tris·HCl/25 mM EDTA, pH 7.5) prior to injection into CBA × C57BL/6 F1 fertilized eggs as described previously by us (20). Positive founder animals were identified by Southern blot analysis of tail DNA using a ^{32}P-labeled full-length fragment of the murine IL-4 cDNA. Animals were housed in filter-top cages lined with bedding of either pine shavings or corn cob. Mice were provided with standard chow and water *ad libitum*.

Lung Lavage and Preparation of Tissue for Histologic Examination. Mice were sacrificed by the intraperitoneal injection of 825 μg of Avertin (Sigma) per kg of mouse. Lung lavage and enumeration of cells were performed as described previously (21).

The lungs next were excised completely from the chest, inflated with 1 ml of 10% formalin, immersed in 10% formalin, and processed and stained by the clinical Histopathology laboratory of the Yale New Haven Hospital.

ELISA. Quantitation of IL-4 or IL-5 in the lavage fluid and serum was performed using assays for the respective murine cytokine according to the manufacturer's instructions (Endogen, Boston, MA). Antibody to IL-4 does not cross-react with murine IL-2, IL-3, IL-5, IL-6, IL-7, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, or inter-

Abbreviations: mIL-4, murine interleukin-4; SV40, simian virus 40; MCh, methacholine.

feron-γ. Antibody to IL-5 does not cross-react with these same cytokines or with IL-4.

RNase Protection Assay. A 500-bp cDNA coding for the murine IL-4 was cloned into the *Sma*I site of vector pGEM-7Zf (Promega). The plasmid was restricted either with *Pvu*II and *Hin*dIII to isolate a 670-bp template DNA carrying the T7 promoter and cDNA sequences for the generation of antisense RNA or with *Pvu*II and *Eco*RI to provide a 760-bp template carrying the SP6 promoter and cDNA sequences for sense RNA synthesis. Appropriate restriction fragments were isolated from 1% agarose gels and purified using the Geneclean II kit (Bio 101) according to the manufacturer's instructions.

RNA synthesis was performed using the MAXIscript *In Vitro* transcription kit, (Ambion) with T7 or SP6 RNA polymerase and [α-^{32}P] UTP (800 Ci/mmol, Amersham). Full-length transcripts were isolated by gel purification in 4% acrylamide/8 M urea gels.

Tissues from different organs were isolated and frozen in liquid N_2. Lysate RNase protection assays were performed using the ribonuclease protection kit II (RNA Lab) according to the manufactuer's instructions. RNA–RNA duplexes were analyzed by electrophoresis in 5% acrylamide gels and visualized by autoradiography.

Physiologic Assessment. Mice were assessed physiologically by an investigator blinded to the genotype of the mice, using methods modified from Martin *et al.* (22) and Amdur and Mead (23) as described by us previously (24).

Statistical Analysis. Data are represented as the mean±SD. Statistical analyses were performed using the Mann-Whitney U test or Spearman rank correlation analysis, as indicated (25) and were corrected for multiple comparisons. P values < 0.05 were considered significant. Statistical analyses were performed using the SAS system 6.10 (SAS Institute, Cary, NC).

RESULTS

Generation of Transgenic Mice. We constructed several lines of transgenic mice in which the expression of murine IL-4 was regulated by the rat Clara cell protein, CC10 promoter (Fig. 1). Of 77 original progeny screened by Southern blot analysis of tail DNA, 25 (32%) were positive for the CC10-IL-4 transgene with copy numbers ranging from 1 to >100 copies per genome (data not shown). Nineteen of these founders were backcrossed with CBA mice.

Lung Selectivity of IL-4 Expression. To assess the selectivity of the expression of mRNA for the lung, we performed RNase protection assays on seven separate organs using an IL-4-specific-antisense probe. The results demonstrate that mRNA for IL-4 was found exclusively in lung tissue from animals expressing IL-4 protein and not in the six other organs tested (Fig. 2*A*) nor in lung tissue from transgene negative animals (Fig. 2*B*). In addition, autoradiographs from RNase protection assays using sense RNA also were negative (data not shown).

Assessment of IL-4 Expression within Lung and Serum. The expression of IL-4 in lung lavage fluid was assessed for the presence of mIL-4 by ELISA. As shown in Fig. 3, lung lavage fluid from one representative mouse from each of three mouse lines contained quantities of IL-4 ranging from 0.148 to 5.9 ng/ml. Serum from mouse 14-5-6 contained much less IL-4, 0.539 ng/ml, than found in lung lavage from the same animal, 5.9 ng/ml. Lavage fluid from mouse 29-3.1 contained 0.781 ng/ml of IL-4, while serum contained the minimal amount of IL-4 detectable, 0.058 ng/ml. Lavage fluid from mouse 73-3 contained 0.148 ng/ml of IL-4, while serum contained only 0.045 ng/ml of IL-4. Only minimally quantifiable levels of IL-4 or undetectable levels of IL-4 (<0.015 ng/ml) were found within either lung lavage fluid or within serum from transgene negative animals. There was a statistically significant correlation between lavage and serum IL-4 levels, ($R = 0.59, P = 0.05$) from a combination of 22 IL-4-expressing and transgene negative mice. Lavage of all animals utilized the same total input volume and the volume retrieved in all cases was greater than 2 ml. No IL-5 (<0.015 ng/ml) was detected in lavage fluid of mice expressing IL-4.

Histologic Assessment of Conducting Airways. Examination of animals for the phenotypic expression of IL-4 was by the light microscopic examination of histologic sections of lung tissue stained with hematoxylin and eosin. Mice were euthanized for study at approximately 2 months of age. In three of the 19 separate mouse lines, impressive histologic changes were observed in epithelial cells of the conducting airways in transgene positive mice (Fig. 4*A*) compared with sections from transgene negative mice (Fig. 4*B*). The three mouse lines demonstrating phenotypic changes were from the same lines that expressed IL-4 in the lavage fluid. Transgene positive mice that did not express IL-4 did not demonstrate these histologic changes. In mice that expressed IL-4, epithelial cells from the trachea down to small peripheral airways were hypertrophic. This hypertrophy was due to the presence of a homogenous-appearing material within the cytoplasm of many of these cells (Fig. 4*A*), which stained positively with Alcian blue/Periodic acid Schiff (AB/PAS) (not shown).

Histologic Assessment of Lung Parenchyma. Histologic assessment of lung parenchyma from transgene positive IL-4 mice revealed the presence of occasional islands of cells (Fig. 4*C*) that were not seen in the lungs of mice that did not express IL-4 (Fig. 4*D*). By H&E staining, many of the cells present in some of these islands had horseshoe-shaped nuclei and orange staining granules in their cytoplasm. Staining with Giemsa confirmed that many of the cells were eosinophils (Fig. 4*E*). Examination of lung parenchyma also revealed the presence of many macrophages within alveolar spaces of mice that expressed IL-4 (Fig. 4*F*). Many of these macrophages were multinucleated. The nuclei of these cells were morphologically identical. No cells stained positively with Giemsa, also suggesting that these were not simply macrophages that had ingested apoptotic eosinophils. Only rarely were macrophages visualized in alveoli from control mice (Fig. 4*D*), and no multinucleated macrophages were ever observed.

Cellular Constituents of Lung Lavage Fluid. The total number of cells retrieved by lung lavage was increased in mice that expressed IL-4, $1.1 \pm 0.7 \times 10^6$, $n = 17$, compared with the number retrieved from mice that did not express IL-4, $0.2 \pm 0.2 \times 10^6$, $P < 0.001$, $n = 11$. In the IL-4-expressing mice differential cell counts revealed 43.7 ± 18.3% macrophages, 20.6 ± 10.3% lymphocytes, 19 ± 10.8% eosinophils, and 16.6 ± 8.7% neutrophils (Fig. 5). In transgene negative control mice, 99.2 ± 1.7% were macrophages and 0.8 ± 1.5% were lymphocytes (Fig. 5). While the percentage of macrophages

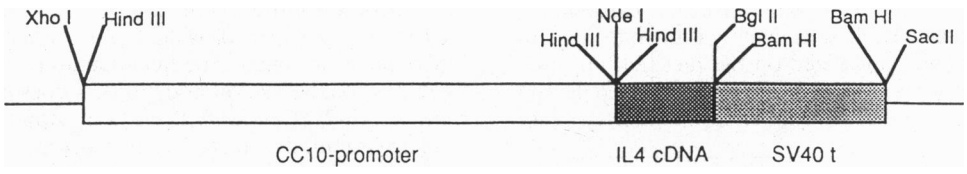

FIG. 1. CC10-IL-4-SV40 transgene. The CC10 promoter was fused 5′ of the murine cDNA for IL-4, and a fragment containing the SV40 small t intron and polyadenylation site was fused 3′ to the cDNA. Important restriction sites are marked.

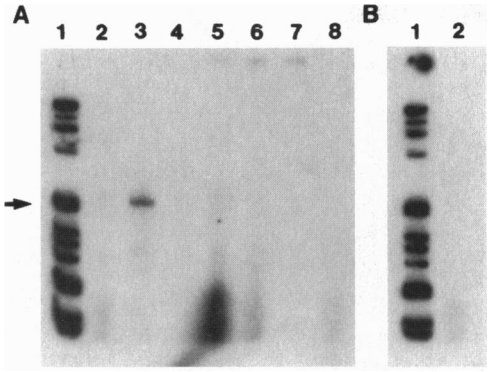

FIG. 2. Lung selectivity of mRNA expression. Results from RNase protection assay performed on tissues from seven different organs isolated from a transgene positive mouse (*A*) and a transgene negative littermate (*B*). The arrow indicates the size of the protected fragment (500 bp). (*A*) Lane 1, 1-kb ladder; lane 2, liver; lane 3, lung; lane 4, heart; lane 5, kidney; lane 6, spleen; lane 7, thymus; lane 8, uterus. (*B*) Lane 1, 1-kb ladder; lane 2, lung.

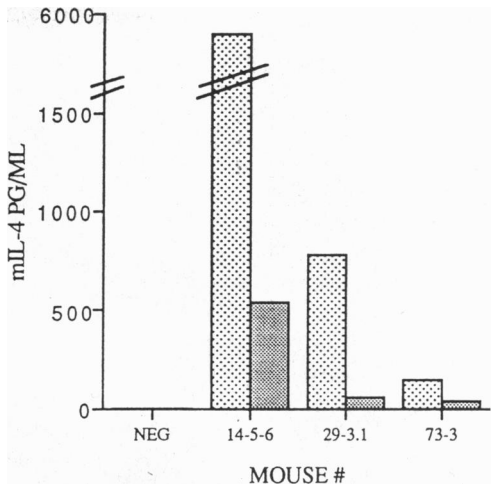

FIG. 3. Lung lavage and serum IL-4 levels. Lavage (lightly spotted columns) and serum (darkly spotted columns) levels are shown from individual, representative mice from a transgene negative line and from the three separate transgene positive lines that expressed IL-4. The minimum detectable level of IL-4 in the ELISA was 15 pg/ml.

retrieved from lung lavage fluid decreased in the mice expressing IL-4, there was, nevertheless, an absolute increase in the total number of these cells, from a mean of 208,110 in transgene negative mice to a mean of 476,900 in mice expressing IL-4. Thus, as assessed by total lung lavage, the lungs were the site of an impressive accumulation of a variety of inflammatory effector cells. Toluidine blue staining of formalin-fixed lung tissue did not reveal the presence of mast cells in our IL-4-expressing mice. Interestingly, BAL IL-4 levels correlated with total macrophages, $R = 0.65$ $P \leq 0.03$; total lymphocytes, $R = 0.67$, $P \leq 0.02$; total neutrophils, $R = 0.79$, $P \leq 0.001$; and total eosinophils, $R = 0.73$, $P \leq 0.004$ retrieved by lung lavage.

Physiologic Assessment. To assess the physiologic changes associated with targeted expression of IL-4 in the pulmonary conducting airways of our mice, we determined baseline airway resistance and bronchial responsiveness to inhaled methacholine via plethysmography as described previously (24). Baseline airway resistance in 11 mice expressing IL-4, 0.325 ± 0.073 cm H_2O/ml/sec was significantly greater than that in 12 age- and sex-matched transgene-negative littermates, 0.224 ± 0.037 cm H_20/ml/sec, $P < 0.0008$ (Fig. 6*A*). This observation corresponds nicely to the hypertrophy of epithelial cells lining the pulmonary conducting airways (Fig. 4*A*), which would be expected to result in significant narrowing of airways and therefore, an increase in baseline resistance. Interestingly, when data from IL-4 expressing and transgene negative mice were assessed together, there was a statistically significant positive correlation between lavage IL-4 levels and baseline resistance ($R = 0.73$, $P = 0.001$). However, while the log dose of methacholine necessary to achieve a 100% increase (PC100) in respiratory resistance in the mice expressing IL-4, 0.781 ± 0.955 mg/ml, was greater than results obtained in the transgene negative littermates, 0.152 ± 0.297 mg/ml, this difference approached, but did not reach, statistical difference $P = 0.07$ (Fig. 6*B*). In addition, we found no significant correlation between lavage IL-4 and PC100 nor between the PC100 values and total eosinophils found in lavage fluid from all mice.

DISCUSSION

The second major new finding of our work is that the marked inflammatory response characterized by hypertrophy of conducting epithelial cells and the accumulation of macrophages, lymphocytes, eosinophils, and neutrophils in the lung of our animal resulted in an increase in baseline airway resistance (Fig. 6*A*). The results of this work establish for the first time that using the rat Clara cell CC10 promoter fused to the cDNA for mIL-4 and a fragment containing the SV40 small t intron and polyadenylation site, transgenic mice could be created that expressed the single gene product, IL-4, constitutively in the lung. The creation of these unique animals enabled us to study *in vivo* the biologic and physiologic consequences of the local expression of this cytokine. RNase protection assays on seven different organs from our mice confirmed that mRNA was detected only in the homogenates of lungs from animals that expressed IL-4 protein. Because of these observations, because we detected much more IL-4 in lung lavage fluid of our mice than in serum (Fig. 3), and because we found a statistically significant positive correlation between lavage and serum IL-4 levels, we conclude that expression of IL-4 is selective for the lung and most likely occurs by epithelial cells of the conducting airways.

At least four different groups of investigators previously have made transgenic mice expressing IL-4 (26–29), but none of these mice expressed IL-4 selectively within the lung. Whereas many lung diseases are associated with marked inflammatory reactions predominantly within the conducting airways [asthma is but one example of this (30)], and whereas epithelial cells situated in these conducting airways release numerous mediators with putative mechanistic roles in these inflammatory responses (31), we utilized a lung selective promoter (32) that would drive IL-4 protein expression in lung epithelial cells of the trachea, bronchi, and bronchioles.

The results of our work with these mice reveal several new findings. First, they demonstrate that the expession of a single gene product, IL-4, in the lungs of our mice elicits, directly and/or indirectly, phenotypic changes of an intense inflammatory response in the lung (Figs. 4 and 5). This is supported by the following observations. (*i*) From the level of the trachea down to the bronchioles, the cytoplasmic portions of the epithelial cells were markedly hypertrophic (Fig. 4*A*). Positive staining with AB/PAS suggests this material is mucus glycoprotein (ref. 33 and unpublished data). (*ii*) We also observed an approximate 2-fold increase in the absolute number of alveolar macrophages present in lung lavage fluid of mice expressing IL-4, compared with controls. Many of the alveolar macrophages contained more than one nucleus, something we never observed in our transgene negative mice. Murine IL-4 induces the formation of multinucleated, alveolar macrophages *in vitro* (34). Thus, we conclude that the presence of multinucleated alveolar macrophages in our IL-4 expressing mice is the result of the direct action of IL-4 on this cell population. This finding also confirms that the expressed IL-4

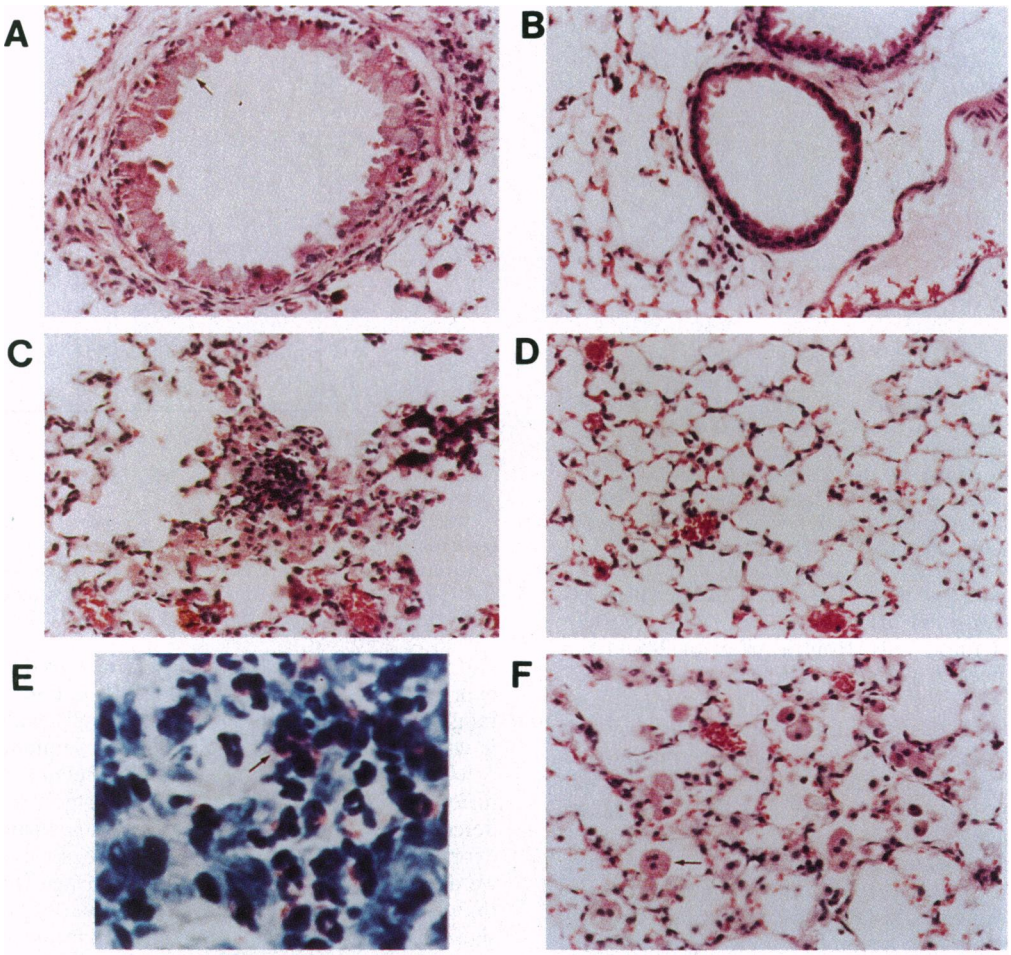

FIG. 4. Lung histology—conducting airways and parenchyma. Sections of formalin-fixed and paraffin-embedded lung tissue from an IL-4-expressing mouse (*A*, *C*, *E*, and *F*) and from a transgene negative mouse (*B* and *D*) were stained with H&E and viewed by light microscopy. The conducting airways from mice that expressed IL-4 revealed hypertrophy of airway epithelial cell cytoplasm by a homogeneous appearing and gray staining material (*A*, arrow) (200×) are compared with cells from airways in the transgene negative mouse (*B*) (200×). H&E staining of parenchymal sections revealed occasional islands of cells (*C*), which appeared to contain eosinophils (200×) not seen in parenchyma from a nonexpressing mouse (*D*) (200×). High power (1000×) view of one island (*E*) stained with Giemsa confirms the presence of many eosinophils (arrow). Another area of lung parenchyma (*F*) (200×) demonstrates many macrophages, several of which are multinucleated (arrow).

is biologically active *in vivo*. (*iii*) In our IL-4-expressing animals, there was an impressive increase in the percentage of cells that were lymphocytes (Fig. 5) representing an approximate 100× increase in the absolute number of these cells in lung lavage fluid. One possibility is that many of these cells are mature $CD8^+$ lymphocytes since Tepper and colleagues found a substantial increase in thymocytes with this phenotype in their IL-4 mice (26). (*iv*) Approximately 20% of all lavage cells from our IL-4 expressing mice were eosinophils (Fig. 5). Eosinophils also were conspicuously present within some of the cellular islands in the lung parenchyma of our mice. No eosinophils were seen in our transgen-negative mice. At this time we do not know whether or not the eosinophils in the lungs of our mice are actively releasing cellular constituents or mediators. The precise reason(s) for the presence of eosinophils is also not known. It does not appear to be due to the presence of IL-5 (35) within the lung since we were unable to detect IL-5 in the lavage fluid of several of our animals. (*v*) Neutrophils constituted approximately 17% of the cells present in lavage fluid of our IL-4 expressing mice. Neutrophils are seen frequently in the lungs of patients with any of a myriad of lung diseases. The reason(s) for their presence in the lungs of our mice remains to be elucidated. These data and the statistically significant positive correlations between lavage IL-4 levels and each cell type in lavage fluid are consistent with (but do not prove) a cause-and-effect relationship between IL-4 expression and lung hypercellularity.

The second major new finding of our work is that the marked inflammatory response characterized by hypertrophy of con-

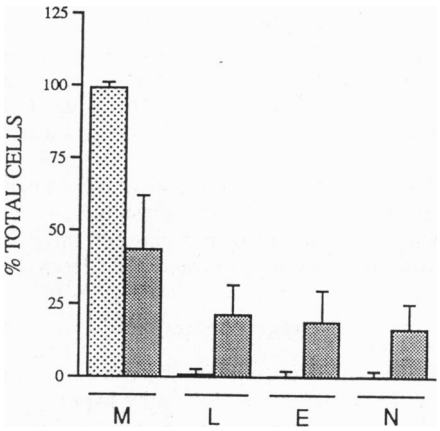

FIG. 5. Inflammatory cells in lung lavage fluid. Differential cell counts of Wright stained cytocentrifuge preparations. Data are the mean ± SD of counts from 14 mice expressing IL-4 (darkly spotted columns) and from eight nonexpressing mice (lightly spotted columns). M, macrophages; L, lymphocytes; E, eosinophils; N, neutrophils.

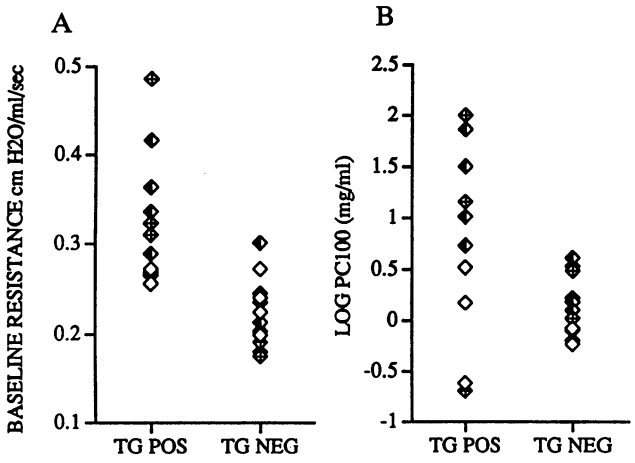

FIG. 6. Respiratory system physiology. Three individual experiments were performed comparing animals expressing IL-4 to transgene negative animals. Baseline airway resistance (*A*) and log PC100 to inhaled methacholine (*B*) are shown for transgene-positive/IL-4-expressing (TG pos) and transgene-negative (TG neg) mice. Each animal is expressed by an individual data point. Open diamonds refer to mice in one experiment, crossed diamonds to mice assessed in a second experiment, and half-shaded diamonds to mice assessed in a third experiment.

ducting airway epithelial cells and the accumulation of macrophages, lymphocytes, eosinophils, and neutrophils in the lungs of our animals resulted in an increase in baseline airway resistence (Fig. 6*A*). Interestingly, lavage IL-4 levels correlated positively with baseline airway resistance. We conclude from these results that the overexpression of IL-4 either directly and/or indirectly results in airway narrowing sufficient to produce an increase in resistance to air flow at baseline. Furthermore, the same IL-4 expressing animals that demonstrated an increase in baseline airway resistance did not demonstrate a statistically significant alteration in airway responsiveness to inhaled methacholine, although there was a trend toward hyporesponsiveness, Fig. 6*B*. This observation coincides nicely with data by others (36), which revealed increased airway responsiveness in a group of IL-4-deficient mice. Importantly, we observed no significant correlation between PC100 and any of the effector cells in lavage fluid. Thus, the expression of the single gene product, IL-4, and its attendant inflammatory reaction were not by themselves sufficient stimuli for the induction of airway hyperresponsiveness to methacholine in this animal model. Therefore, while the presence of IL-4 in the lung may contribute to airway inflammation, other cytokines and mediators, possibly in conjunction with IL-4, probably are necessary for the initiation and maintenance of a unique inflammatory reaction that will result in airway hyperreactivity.

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