Immunoregulatory effects of regulated, lung-targeted expression of IL-10 in vivo

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Spight, Donn, Bin Zhao, Michael Haas, Susan Wert, Alvin Denenberg, and Thomas P. Shanley. Immunoregulatory effects of regulated, lung-targeted expression of IL-10 in vivo. Am J Physiol Lung Cell Mol Physiol 288:L251–L265, 2005. First published October 1, 2004; doi:10.1152/ajplung.00122.2004.—Regulation of pulmonary inflammation involves an intricate balance of both pro- and anti-inflammatory mediators. Acute lung injury can result from direct pulmonary insults that activate alveolar macrophages to respond with increased cytokine expression. Such cytokine gene expression is mediated in part via NF-κB. IL-10 has been previously identified as an important endogenous anti-inflammatory cytokine in vivo on the basis of inhibiting NF-κB activation; however, the mechanism of this inhibition remains incompletely defined. We hypothesized that IL-10 regulated NF-κB activation in vivo via IkK inhibition. A transgenic mouse that allowed for externally regulated, lung-specific human IL-10 overexpression was generated. In the transgenic mice, introduction of doxycycline induced lung-specific, human IL-10 overexpression. Acute induction of IL-10 resulted in significant decreases in bronchoalveolar lavage fluid neutrophils (48%, P = 0.03) and TNF (62%, P < 0.01) following intratracheal LPS compared with transgenic negative mice. In vitro kinase assays showed this decrease to correlate to diminished lung IkK activity. Furthermore, we also examined the effect of chronic IL-10 overexpression in these transgenic mice. Results show that IL-10 overexpression in lungs of mature mice increased the number of intrapulmonary cells the phenotype of which was skewed toward increased B220+/CD45+ B cells and CD4+ T cells and was associated with increased CC chemokine expression. Thus regulated, lung-specific IL-10 overexpression may have a variety of complex immunologic effects depending on the timing and duration of expression.

interleukin-10; transgenic/knockout; lung; protein kinases; neutrophils; lipopolysaccharide

ONE OF THE HALLMARKS OF INFLAMMATION is the infiltration of leukocytes. In the setting of compartmentalized models of acute lung injury (ALI), proinflammatory cytokines, such as TNF-α and IL-1β, released by activated macrophages upregulate both endothelial cell and leukocyte adhesion molecules (35). In the lung, this causes anchoring of activated neutrophils to the pulmonary vascular endothelium. Subsequent emigration of neutrophils into the alveolar space requires the presence of chemokines (29, 33). The mechanism by which certain stimuli (e.g., endotoxin) initiate lung inflammation has been extensively studied. In the example of intratracheal endotoxin [lipopolysaccharide (LPS)] challenge, activation of the resident macrophage triggers signal transduction pathways that are crucial to the transcriptional regulation of those cytokine, adhesion molecule, and chemokine genes that participate in the lung inflammatory response (29, 33, 42–44). One of the principal pathways described in both experimental models, as well as human studies of ALI is the NF-κB pathway (1, 20, 23, 36). LPS has been demonstrated to activate the NF-κB pathway via phosphorylation of IkB-α via I kappa kinase (IkK). Phosphorylated IkB-α is then polyubiquitinated and targeted for rapid degradation by the 26S proteasome. Degradation of IkB-α unmasks the NF-κB nuclear translocation sequences, allowing NF-κB to enter the nucleus to direct transcription of target genes. This pathway directing proinflammatory cytokine expression can be counterregulated by endogenous, anti-inflammatory cytokines such as IL-10.

We have previously examined the role of IL-10 in regulating acute pulmonary inflammation (20, 34). Exogenous administration of IL-10 in a rat model of immune complex-mediated alveolitis abrogated the inflammatory response in a manner that was linked to decreased expression of TNF expression (26). In the same model, blocking of endogenous IL-10 activity resulted in an augmented inflammatory response as measured by neutrophil influx and TNF expression (32). We and others have also reported the ability of IL-10 to abrogate chemokine expression in lung inflammation thus diminishing neutrophil influx (34). Furthermore, the mechanism by which IL-10 and a related Th2 cytokine, IL-13, attenuated lung inflammation was related to inhibition of NF-κB (20); however, the mechanism of this inhibition in vivo remained incompletely defined. Because we had observed preservation of IkB-α in response to IL-10 in vitro (34), we hypothesized that IL-10 might regulate NF-κB activation in vivo via IkK inhibition.

In contrast to these general “anti-inflammatory” effects, recent reports had suggested that IL-10 (employing a transgenic mouse capable of constitutive, lung-specific IL-10 overexpression) might mediate untoward effects of propagating allergic lung inflammation that were unexpected in light of these previously reported functions (19). It remained unclear as to whether these findings were the result of transgenic expression throughout the course of fetal and postnatal lung development (related to the transgenic promoter used) or specific to IL-10 overexpression. To more fully elucidate regulation of signal transduction as well as examine additional immunomodulating properties of IL-10 in vivo, a bitransgenic mouse that allowed for externally regulated, lung-specific human IL-10 overexpression was generated. The introduction of external regulation of IL-10 overexpression via a tetracycline-responsive transgene afforded the opportunity to further examine this biology in that IL-10 overexpression could be turned on at any time point postnatally after fetal lung development had been completed. Our results suggest that “chronic” IL-10 overexpression (1–3 mo) can alter lung homeostasis with...
regard to the constitutive cellular profile, whereas “acute” IL-10 overexpression modifies the inflammatory response to intratracheal endotoxin challenge in association with in vivo inhibition of ICκB and thus NF-κB in this model of compartmentalized lung injury. As a result, forced, lung-specific IL-10 overexpression may have a variety of complex immunologic effects depending on the timing and duration of expression.

MATERIALS AND METHODS

Except where indicated all reagents were obtained from Sigma (St. Louis, MO).

Animals. All in vivo studies were done under the auspices of Institutional Animal Care and Use Committee (IACUC)-approved protocols at the Cincinnati Children’s Hospital Research Foundation. All animal facilities are accredited by and in compliance with The Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Plasmid construction and oocyte microinjection. The reverse tetracycline transactivator (rtTA) construct was a gift of Dr. Herman Bujard (ZMBH, Heidelberg, Germany) (10), and the 1-kb rtTA coding sequence was placed under the control of the 2.3-kb rat Clara cell secretory protein (CCSP) promoter that selectively directs expression of the transgene in respiratory epithelial cells of the lung (38, 41). Polyadenylation sequences from the human growth hormone gene were used to ensure transcript termination as previously reported (41). The IL-10 cDNA was a gift from Dr. Robert Monford (UT-Southwestern) and was subcloned into the tetracycline on (tetO) transgene human IL-10-tetra-cycline responsive element (huIL-10-TRE) consisting of seven copies of the tet operator DNA binding sequence linked to a minimal cytomegalovirus (CMV) promoter and an SV40 polyadenylation signal (Fig. 1) (10). Plasmid constructs were verified initially by sequencing and subsequently by transient transfection into murine lung epithelial cells stably transfected with constitutively active rtTA plasmid such that doxycycline addition to culture media induced human IL-10 expression. The huIL-10-TRE was then microinjected into FVB/n mouse oocytes by standard transgenic procedures as previously described (8). Founder transgenic mice were identified by Southern blotting of tail DNA cut BamHI restriction digestion followed by probing with huIL-10 cDNA.

PCR genotyping and double transgenic breeding. Subsequent transgenic mice bred from founder parents were identified using PCR primers specific for each transgene as follows: 5′ primer in CCSP-rtTA, 5′-ACT GCC CAT TGC CCA AAC AC-3′; 3′ primer, 5′-AAA ATC TTG CCA GCT TTC CCC-3′. Primers used to identify the huIL-10-TRE were 5′-cag tct gtc gcc tgg tc; 3′, 5′-etc cct ctc aac ctt aca a. Amplification of the PCR product for both CCSP-rtTA and huIL-10-TRE was performed by denaturation at 94°C for 5 min, then 30 cycles of amplification at 94°C for 30 s, 57°C for 60 s, and elongation at 72°C for 60 s, followed by a 10-min extension at 72°C. PCR products were visualized under UV light following electrophoresis in a 0.9% agarose gel.

Heterozygous huIL-10-TRE mice were viable without observable abnormalities and were bred through eight generations to obtain homozygosity. Four separate lines bearing the huIL-10-TRE transgene were chosen for breeding to the previously established CCSP-rtTA activator mice (10, 41) transmitting the genes with Mendelian inheritance to obtain double transgenic mice as determined by PCR screening.

Ribonuclease protection assay and IL-10 ELISA. To confirm organ-specific insertion and expression of the huIL-10 transgene under doxycycline regulation, bitransgenic animals were fed on doxy-chow (0.0625% doxycycline, TestDiet, Precision Diet Formulas; Purina, Richmond, IN) for 2–5 days and then killed for RNA extraction from multiple organs. Transgenic negative mice on doxycycline and bi- or transgenic positive mice off doxycycline were similarly examined. Whole lung, liver, heart, kidney, intestine, and spleen were homogenized in Trizol reagent (GIBCO-BRL). Total RNA was extracted with chloroform and precipitated with isopropanol as previously reported (31). RNA was analyzed by ribonuclease protection assay (RPA) assays using the RiboQuant system purchased from BD Pharmingen (San Diego, CA) that included a probe for human IL-10, or, in separate experiments, a limited mouse chemokine library that included lymphotactin; regulated on activation, normal T-cell expressed and presumably secreted (RANTES); macrophage inflammatory protein (MIP)-1β; MIP-1α; MIP-2; interferon-γ-inducible protein 10; monocyte chemoattractant protein (MCP)-1; T-cell activator-3; eotaxin; and two housekeeping genes: L32 and GAPDH. We confirmed transcription of the human IL-10 transgene by measuring human IL-10 content of bronchoalveolar lavage (BAL) fluid (BALF) obtained from transgenic mice with standard ELISA-based kits in accordance with the manufacturer’s instructions (Biosource International, Camarillo, CA).

IL-10 null mutant mice. In additional studies, mice in which IL-10 was deleted by homologous recombination and backcrossed to the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). IL-10 knockout mice (IL-10 KO) were used at similar ages (8–12 wk of age) and weights (20–25 g body wt) as FVB/n mice for all in vivo experiments. Wild-type (WT) C57BL/6 mice purchased from Jackson Laboratories were used as age- and weight-matched controls for these studies. Animals were housed in IACUC-approved, reverse-flow rooms under sterile conditions with ad libitum access to food and water and under 14:10-h day-night cycles.

ALL. Anesthesia was initiated with isoflurane and maintained with ketamine. Mice were suspended at 60°, and the ventral neck was cleaned with ethanol. The trachea was exposed by a midline surgical incision followed by blunt dissection though ventral strap muscles. To initiate lung injury, LPS (Escherichia coli, strain O55:B5) was administrated intratracheally (it) via a 27-gauge needle in a manner similar to previous reports (29, 33). The LPS dose ranged from 0.05 to 50 μg/mouse in a volume of 100 μl of sterile phosphate-buffered saline (PBS). Negative control animals received 100 μl of PBS it. Unless otherwise noted, for all in vivo experiments, singly transgenic,
homozygous hU-L-10-TRE mice served as age- and weight-matched control mice. Animals were killed with ketamine overdose at the times indicated following initiation of injury. BAL was performed with two aliquots of 2 ml of PBS instilled and withdrawn two times each.

**BALF cell counts and differentials.** For determinations of BALF neutrophils, total white blood cell (WBC) counts were performed with a Beckman Coulter counter. Neutrophil numbers were derived from the differential counts determined on the same BALF samples subjected to centrifugation (700 g, 5 min) with a Cytospin centrifuge (Shandon, Shandon, PA) and stained with Diff-Quick products (Baxter, Miami, FL) as previously reported (34). To characterize the phenotype of the lung cellular content, fluorescence-activated cell sorting (FACS) was performed on cells isolated from whole lung homogenates. Briefly, each isolated lung was minced into fine particles with dissection scissors in a 70-μm cell strainer set on one well of a six-well plate. Tissue fragments were incubated at 37°C for 30 min. The cell strainer was transferred to a sterile 50-ml tube, and the crude homogenate was washed with PBS, 1 g BSA, and 0.5% sodium azide. Cells were centrifuged (1,200 rpm, 5 min) at 4°C and then resuspended in 5 ml of FACS buffer solution (1× PBS, 1 g BSA, and 0.5% sodium azide) before staining with CD3, CD4, CD8, B220 (B cell-specific isoform of CD45), CD14 (to identify monocytes), major histocompatibility complex (MHC II), or isotype-matched anti-IgG antibodies as indicated by the source of the primary antibody (also 1 μg/ml, Caltag). Stained cells were incubated on ice for 1 h, then centrifuged and washed twice with FACS buffer solution in new tubes. Cells were then resuspended in 5 ml of complete RPMI/10% BSA and counted with a Coulter counter for the total cell number recovered per whole lung. Cells were then split for subsequent phenotyping by FACS analysis. Cells were treated with 10 mM EDTA in PBS and collected in falcon 2050 tubes (Becton Dickinson, Franklin Lakes, NJ) with FACS buffer solution (1× PBS, 1 g BSA, and 0.5% sodium azide). Cells were centrifuged (1,200 rpm, 5 min) at 4°C and then resuspended in 0.5 ml of FACS buffer solution in new tubes. Cells were washed with either 100 μl of mouse monoclonal anti-CD3 (for T cell identification), CD4 (double-stained with CD3), CD8 (double-stained with CD3), B220 (B cell-specific isoform of CD45), CD14 (to isolate monocytes), major histocompatibility complex (MHC II), or CD80 antibody (1 μg/ml; Caltag, Burlingame, CA) in FACS buffer or an isotype-matched anti-IgG antibodies as indicated by the source of the primary antibody (also 1 μg/ml, Caltag). Stained cells were incubated on ice for 1 h and then washed with FACS buffer and centrifuged (1,200 rpm, 10 min, 4°C). Various color-conjugated secondary anti-mouse antibodies (Caltag) at 1:100 dilutions were added, and cells were incubated on ice for 1 h, then centrifuged and washed twice, and resuspended in 0.2 ml of FACS buffer. Cell surface staining, recorded as mean channel fluorescence (MCF) was measured on a FACSScan flow cytometer (Becton Dickinson, San Jose, CA). Values are reported as means ± SE of percent expression after controlling for MCF from isotype antibody control conditions.

**Western blot analysis.** Whole cell lysates were extracted from confluent six-well plates and subjected to SDS/PAGE (10%) according to the method of Laemmli. The separated proteins were transblotted to nitrocellulose (0.45 μm; Bio-Rad, Richmond, CA) for 2 h at 12 V. After transfer, the membrane was blocked with 20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20 (T-TBS) (vol/vol) containing 5% dry milk for 2 h at room temperature. The blot was incubated for 45 min with 10 μg/ml primary antibody, anti-IκB-α (#C-21 Santa Cruz Biotechnology, Santa Cruz, CA). After washing, secondary antibody (goat anti-rabbit IgG alkaline phosphatase, Bio-Rad) was added at a final dilution of 1:5000 in T-TBS and incubated for 30 min. After washing, the membrane was developed by the addition of chemiluminescent substrate (ECL Product, Amersham). Rainbow molecular mass markers (Amersham) were used to estimate the size of the immunoreactive bands (2,350–46,000 Da).

**In vitro kinase assay.** To determine whole lung IκK activity, in vitro kinase assays were performed on whole lung extracts as previously reported (12). For immunoprecipitation, we precleared cell lysates by combining 400 μg of sample protein with 30 μg protein G sepharose (Amersham-Pharmacia, Piscataway, NJ) adjusted to a final volume of 700 μl with lysis buffer and mixed them on a rocker at 4°C for 20 min. Samples were briefly centrifuged, and the supernatants were transferred to a new Eppendorf tube, incubated with 5 μl of the immunoprecipitating, polyclonal anti-IκB-α and 30 μl of fresh protein-G-sepharose beads equilibrated with lysis buffer, and rocked overnight at 4°C. The pellet was washed twice with lysis buffer containing 0.1 mM Na2VO4, 1 mM PMSF, 0.3 units/ml aprotinin, and 2 mM p-nitrophenyl phosphate (PNPP) and then resuspended in 30 μl of 2× Laemmli buffer before being boiled for 5 min; pull-down was confirmed by Western blot analysis.

**After immunoprecipitation, the NaCl concentration was adjusted to 400 mM with 1 M NaCl, and 30 μl of protein G-sepharose beads (Amersham-Pharmacia), equilibrated with lysis buffer, were mixed at 4°C overnight. The pellet was washed twice with the lysis buffer, followed by washing with a kinase assay buffer containing 25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na2VO4, 2 μg/ml leupeptin, 1 mM PMSF, 0.3 units/ml aprotinin, and 2 mM PNPP, and the pellet was resuspended in kinase assay buffer. The resuspended pellet was mixed with 20 μg of the fusion protein, IκB-α-glutathione S-transferase (IκK substrate), 5 μCi of [32P]ATP

**Electrophoretic mobility shift assay.** Nuclear extracts from whole lungs were prepared as previously described (20) at the times indicated after stimulation with LPS (E. coli O55:B5, 100 ng/ml medium). We confirmed purity of nuclear extracts by assessing the relative level of lactate dehydrogenase in nuclear versus cytoplasmic extracts. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA), and samples were stored at −70°C until used for electrophoretic mobility shift assay (EMSA). Double-stranded NF-κB consensus oligonucleotide: 5′-AGTGGGGGAC TTTCCCAGGC-3′ (Promega, Madison, WI) was end-labeled with γ[32P]ATP (3,000 Ci/mmole at 10 μCi/ml; Amersham, Arlington Heights, IL) and purified by Bio-Spin chromatography columns (Bio-Rad). For EMSA, 10 μg of nuclear proteins were incubated in EMSA binding buffer [12 mM HEPES, pH 7.9; 4 mM Tris-HCl, pH 7.9; 25 mM KCl; 5 mM MgCl2; 1 mM EDTA; 1 mM DTT; 50 ng/ml poly(di-dC); 1% glycerol (vol/vol); 0.2 mM PMSP] on ice, with radiolabeled oligonucleotide for 20–30 min. The specificity of the reaction was determined by co-incubating duplicate samples with either 100-fold molar excess of unlabeled oligonucleotide or 100-fold molar excess of an irrelevant oligonucleotide (Oct-1, Promega). Binding reactions were resolved using a 4%, nondenaturing polyacrylamide gel run in 0.5% TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA) for 1 h at constant current (30 mA). Gels were dried to Whatman 3M paper (Whatman, Clifton, NJ), dried under vacuum at 80°C for 1 h, and developed with autoradiography done at room temperature on Kodak X-OMAT-AR film for 3–8 h.
Offspring were genotyped via PCR and subsequently phenotypically analyzed, transgenic mice that were subsequently crossed with the previously established CCSP-rtTA line of FVB/n mice (41). This expression was induced to a greater degree in the presence of doxycycline (1 μM) to culture media resulted in a further increase in IL-10 expression at both time points compared with control media. Data are reported as mean values ± SE. Values represent determinations from 1 to 8 wells for each condition. ND, not detected. *P < 0.01 compared with 24 h, control medium value; †P < 0.05 compared with control media values at 48 and 72 h, respectively.

RESULTS

Generation of bitransgenic mice. Sequencing of the human IL-10-TRE vector confirmed the IL-10 cDNA to be in proper reading frame. Transient transfection of the plasmid into an established line of murine lung epithelial cells that were stably transfected with a surfactant protein C-rtTA construct demonstrated that antigenically reactive IL-10 was expressed and that this expression was induced to a greater degree in the presence of doxycycline (Fig. 2). Thus this TRE-human IL-10 transgene was injected into pseudopregnant FVB/n female mice. Founder mice were initially identified by Southern blots probed with the human IL-10 cDNA. Subsequent mating generated homozygous, transgenic mice that were subsequently crossed with the previously established CCSP-rtTA line of FVB/n mice (41). Offspring were genotyped via PCR and subsequently phenotyped by the expression of human IL-10 as determined by RPA analysis of whole lung mRNA (Fig. 3A). Of note, no human IL-10 transgene expression was found in any other organs (heart, liver, kidney, and intestine) examined as had been observed with previous use of the lung-specific CCSP promoter (data not shown). Four separate lines of bitransgenic positive (TG+) mice were established and bred to homozygosity. For the purposes of these studies the kinetics of expression was determined on the single line (F6DH2) of mice that was used in all experiments. Mice containing only the hIL-10-TRE transgene (i.e., singly transgenic) were denoted as TG− mice and provided either standard chow (TG− dox−) or doxycycline (TG− dox+). As shown in Fig. 3B, rapid induction of IL-10, as measured in BALF, was observed within 24 h of providing access to doxycycline. Peak expression was observed by 48–72 h and could be maintained from 1 wk to 3 mo (the longest period examined). After removal of doxycycline the “off” kinetics was determined, and rapid decline of IL-10 expression was seen over the initial 48 h with total loss of detectable expression by 72 h (Fig. 3B). Use of the CCSP-rtTA transgene was not associated with any detectable “leak” of either human IL-10 mRNA or protein in TG+ mice not provided doxycycline (dox−), at least to the level of detection by RPA and ELISA, respectively. Immunostaining for human IL-10 was performed using an antiserum against the human IL-10 transgene expression was found in any other organs

Histological analysis. Mice were killed by ketamine overdose while under isoflurane anesthesia. Whole lungs were filled with 10% buffered formalin, removed en bloc, and placed in 10% buffered formalin. Whole lungs were processed through staining and embedding in paraffin for subsequent sectioning. Lung sections (2 μm) were stained with hematoxylin and eosin and examined by routine light microscopy. Immunostaining for human IL-10 was performed using a mouse monoclonal antibody to human IL-10 (catalog no. SC-8438, Santa Cruz Biotechnology) at a dilution of 1:10 and a streptavidin-biotin-peroxidase (ABC) detection system (Vector Laboratories). Antigen retrieval by heating the tissue sections/slides in a 0.01 M sodium citrate buffer (pH 6.0) for 15 min at 90°C was required to maximize the immunoreaction as previously reported (37). The precipitation product was enhanced with nickel-cobalt to give a black reaction product, and the tissue was then counterstained with nuclear fast red. Controls for background staining included the elimination of the primary antibody, as well as inclusion of transgenic negative mouse lung and bitransgenic, doxycycline-negative mouse lung.

Statistics. All values were expressed as means ± SE. Data sets were examined with one- and two-way analysis of variance, and individual group means were then compared with two-tailed t-tests. Significance was defined by P value <0.05.
low as 5 ng of LPS was associated with a modest degree of neutrophil influx that was significant compared with controls challenged with PBS (Fig. 4B). Subsequent studies were performed with the dose of 5,000 ng/mouse. To determine the effect of IL-10 overexpression on early neutrophil accumulation, TG+ mice on doxy-chow for 5 days were challenged with intratracheal LPS and compared with age- and weight-matched huIL-10-TRE single transgenic (TG−) mice also given doxy-chow. Mice that underwent induction of anesthesia and had intratracheal inoculation with PBS served as negative controls. BALF recovered from PBS challenged mice (both TG+ and TG−) demonstrated no neutrophil influx at 6 or 24 h (Fig. 5, A and C, respectively). In TG− dox+ or TG+ dox− mice, intratracheal administration of 5,000 ng of LPS resulted in significant increase in both total WBC counts and neutrophil-specific counts at 6 h compared with negative controls (Fig. 5A). The overexpression of human IL-10 (mean 710 ± 86 pg/ml) in TG+ dox+ mice was associated with a significant reduction in total BALF WBCs at 6 h (48% decrease compared with TG− dox+ mice, P = 0.03, Fig. 5A) that could be almost

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TNF was detected in TG mice. LPS challenge in TG mice caused a substantial increase in BALF TNF at 6 h (Fig. 5B) that was significantly attenuated by IL-10 overexpression in TG mice (62% decrease compared with TG− dox− mice, P < 0.01). In a similar manner, IL-10 overexpression was associated with significant reductions in BALF neutrophil influx, as well as TNF expression, compared with TG− dox− mice challenged with LPS at the 24-h time point (Fig. 5C). At this time point, neither neutrophils nor TNF were detected in either of the PBS-challenged groups. Of note, TG− dox− and TG+ dox− mice did not express IL-10 and had LPS-induced PMN and TNF values that were not statistically different from TG− mice groups reported above (data not shown). These data demonstrated that the regulated, overexpressed hIL-10 attenuated cytokine and cellular inflammation following LPS.

Effect of IL-10 overexpression on LPS-induced activation of the NF-κB pathway. Because of the role of NF-κB in transcriptionally regulating proinflammatory gene expression in acute lung inflammation and the previous observation that IL-10 abrogated its activation, we sought to identify whether the upstream kinase, IkK, was inhibited in the presence of IL-10 overexpression. In vitro kinase assays for IkK were performed on whole lung homogenates from either PBS (negative) or LPS-challenged mice from the same groups described above. As shown in Fig. 6, lungs from negative control mice contained little IkK activity at either the 30- or 60-min time point. In contrast, LPS challenge to TG− dox+ mice caused a significant increase in the signal for IkK at both 30 (18-fold increase vs. negative) and 60 (20-fold increase vs. negative) min. LPS-induced activation of IkK was significantly attenuated in TG+ dox+ mice at both 30 (60% reduction, P < 0.001) and 60 min (50% reduction, P < 0.01; Fig. 6). These data corroborate a recent report in which IL-10 attenuated IkK activation (14). To correlate this effect on IkK to activation of NF-κB, we performed EMSA on nuclear protein extracts from whole lungs from the same groups of mice. Intratracheal LPS in TG− dox+ mice significantly increased the signal for NF-κB compared with PBS challenge at 60 min (Fig. 7A). IL-10 overexpression (TG+ dox+ mice) decreased this signal in a manner consistent with inhibition of IkK. As further confirmation of the key role of endogenous IL-10 in regulating NF-κB, IL-10 null mutant mice were similarly challenged with intratracheal LPS. In these experiments, there was a significant increase in the nuclear signal for NF-κB in IL-10 KO mice compared with WT mice challenged with LPS that similarly correlated to a significant increase in IkK activity (Fig. 7B). These data support the hypothesis that IL-10 is a key regulator of NF-κB activity in vivo via regulation of the upstream kinase IkK.

Effects of long-term IL-10 overexpression on the constitutive lung phenotype. As previous investigators had suggested an altered lung phenotype associated with constitutively active, lung-specific human IL-10 overexpression, we sought to characterize the effect of chronic IL-10 overexpression in this transgenic mouse model (19). Given the dependence of doxycycline administration for persistent overexpression of IL-10, several important observations regarding the use of this transgenic model were made in the course of these long-term studies.

Fig. 4. BALF cell counts in FVB/n mice. A: time-course response. BALF cells were measured by Coulter counter at 6, 12, 18, and 24 h following intratracheal PBS (negative control mice) or LPS (5,000 ng/mouse). Neutrophil (PMN) values were determined from differential counts on cytospin samples. All values were reported as means (×1,000) ± SE with n = 8 mice per group. *P < 0.01 compared with PBS-challenged FVB/n mice for total white blood cell (WBC) counts; †P < 0.01 compared with PBS-challenged FVB/n mice for PMN counts. B: LPS dose-response effect. BALF cells were measured by Coulter counter at 24 h, and PMN values were determined from differential counts on cytospin samples. All values were reported as means (×1,000) ± SE with n = 8 mice per group. *P < 0.01 compared with PBS-challenged FVB/n mice for total WBC counts; †P < 0.001 compared with PBS-challenged FVB/n mice for PMN counts.

entirely attributable to the neutrophil component (51% decrease, P = 0.026). These results are consistent with prior reports of IL-10’s effect on LPS-induced neutrophil influx and support the conclusion that the transgenically overexpressed IL-10 was biologically active in vivo. BALF from this experiment were also examined for TNF content by ELISA, as IL-10 inhibition of neutrophil influx was previously associated with decreased cytokine expression (25, 26). Although very little TNF was detected in TG− dox− mice challenged with PBS, no TNF was detectable in negative control, TG+ dox+ mice at 6 h (Fig. 5B). We observed that in TG− dox+ or TG+ dox− mice caused a substantial increase in BALF TNF at 6 h (Fig. 5B) that was significantly attenuated by IL-10 overexpression in TG+ dox+ mice (62% decrease compared with TG− dox+ mice, P < 0.01). In a similar manner, IL-10 overexpression was associated with significant reductions in BALF neutrophil influx, as well as TNF expression, compared with TG− dox+ mice challenged with LPS at the 24-h time point (Fig. 5C). At this time point, neither neutrophils nor TNF were detected in either of the PBS-challenged groups. Of note, TG− dox− and TG+ dox− mice did not express IL-10 and had LPS-induced PMN and TNF values that were not statistically different from TG− mice groups reported above (data not shown). These data demonstrated that the regulated, overexpressed hIL-10 attenuated cytokine and cellular inflammation following LPS.
To determine the effect of long-term IL-10 overexpression on baseline cell counts in the lungs of mature mice, we studied four groups of animals at two different time points. The experimental groups consisted of age- and weight-matched TG/H11001 and TG/H11002 mice provided access to either standard or doxy-chow for 1 and 3 mo followed by BAL (performed at 12 and 20 wk of age, respectively). Identical experimental groups were studied in immature mice (doxy-chow started at birth with BAL performed at 1 mo of age). Summary results are shown in Table 1 with the following observations. Compared with TG/H11002 dox/H11001 mice incapable of IL-10 overexpression, a significant increase in BALF cell counts was noted in TG/H11001 dox/H11001 mice at all time points tested. Importantly, an effect of doxycycline alone was noted as reflected by significantly decreased BALF cell counts in both doxycycline-treated groups compared with both groups off doxycycline for the duration of the experiments (Table 1). Thus, although there was a significant increase in BALF cell counts related to IL-10 overexpression, controlling for doxycycline exposure was critical. Furthermore, no differences in BALF cell counts were observed with short-term doxycycline exposure (3–15 days). These data led us to speculate that the doxycycline-dependent effect may have been related to a persistent level of antibiotic in the mice that resulted in a suppression of a “chronic” low level of lung infection despite the filtered-air, barrier conditions employed for animal housing. To test this hypothesis, TG/H11001 littermates were randomly separated to receive either doxy- or standard chow for 1 mo (n/H1005 6 each group). At death, BALF cell counts and PMN accumulation at 6 h. LPS induced a significant increase in BALF cells predominantly comprising PMNs in both TG– dox+ and TG+ dox– mice. IL-10 overexpression (TG+ dox+/LPS group) resulted in a significant decrease (48% decrease, P = 0.03) in BALF cells that was attributable to decreased PMNs (51% decrease, P = 0.026). B: BALF TNF levels measured 6 h following intratracheal PBS or LPS. Although LPS induced a significant increase in TG– dox+ and TG+ dox–/LPS groups, IL-10 overexpression significantly attenuated this response (62% decrease, P < 0.01). C: effect of IL-10 overexpression on BALF PMN accumulation and TNF levels 24 h after LPS challenge. IL-10 overexpression was associated with significant decreases in both PMN (31% decrease, P = 0.01) and TNF (61% decrease, P = 0.007) compared with TG– dox+, LPS-challenged mice. All values in A–C are reported as means ± SE with n = 6 for each PBS-challenged group and n = 15 for each LPS-challenged group.

To determine the effect of long-term IL-10 overexpression on baseline cell counts in the lungs of mature mice, we studied four groups of animals at two different time points. The experimental groups consisted of age- and weight-matched TG+ and TG– mice provided access to either standard or doxy-chow for 1 and 3 mo followed by BAL (performed at 12 and 20 wk of age, respectively). Identical experimental groups were studied in immature mice (doxy-chow started at birth with BAL performed at 1 mo of age). Summary results are shown in Table 1 with the following observations. Compared with TG– dox+ mice incapable of IL-10 overexpression, a significant increase in BALF cell counts was noted in TG+ dox+ mice at all time points tested. Importantly, an effect of doxycycline alone was noted as reflected by significantly decreased BALF cell counts in both doxycycline-treated groups compared with both groups off doxycycline for the duration of the experiments (Table 1). Thus, although there was a significant increase in BALF cell counts related to IL-10 overexpression, controlling for doxycycline exposure was critical. Furthermore, no differences in BALF cell counts were observed with short-term doxycycline exposure (3–15 days). These data led us to speculate that the doxycycline-dependent effect may have been related to a persistent level of antibiotic in the mice that resulted in a suppression of a “chronic” low level of lung infection despite the filtered-air, barrier conditions employed for animal housing. To test this hypothesis, TG+ littermates were randomly separated to receive either doxy- or standard chow for 1 mo (n = 6 each group). At death,
the number of CFU from the lungs was determined as outlined in MATERIALS AND METHODS. Lungs of TG− dox+ mice averaged 11 ± 6 CFU compared with TG+ dox+ mice, in which no bacterial growth was observed (CFU = 0).

Together, these results show that doxycycline had an independent, long-term, yet relevant effect on the constitutive lung cell counts. The presence of “low level” bacterial colonization may have contributed to the difference in baseline cell counts noted...
Differential cell counts were performed on all lavage samples. On average, >98% of the recovered cells were of a mononuclear cell phenotype with only a rare neutrophil or eosinophil observed (<1%). However, the histological appearance of the recovered cells changed over time in the IL-10-overexpressing mice. As shown in Fig. 8, TG− dox− mice had typical appearing alveolar macrophages with the normal cytoplasmic to nuclear ratio at both 1 (Fig. 8A) and 3 mo (Fig. 8C) that were not different from TG− dox− mice. In contrast, TG+ dox+ mice had larger-appearing macrophages with an increased cytoplasmic to nuclear ratio at 1 mo (Fig. 8B) that increased more strikingly by 3 mo (Fig. 8D). Higher power examination of some of these cells revealed pseudopodia-like extensions (Fig. 8E). In contrast, TG+ dox− mice never demonstrated these changes even at 20 wk of age (Fig. 8F).

Because of the striking appearance of these recovered macrophages, as well as the increased cell counts, histological examination of lungs was performed. TG− dox− mice fed doxy-chow for 3 mo demonstrated normal lung histology (Fig. 9, A and B). In contrast, IL-10 overexpression for 3 mo in TG+ dox+ mice was
associated with substantial peribronchiolar, mononuclear cell infiltration (Fig. 9, C and D). In some areas this included the presence of large intra-alveolar macrophages similar to those recovered by BAL (Fig. 9E). TG+ dox− mice did not show these histological changes (Fig. 9F). This histology suggest that the BAL data may underrepresent the number and types of cells localizing in the lung in response to chronic IL-10 overexpression.

Therefore, we proceeded to further characterize these cells by performing FACS analysis on cells recovered from whole lung homogenates. As shown in Table 2, IL-10 overexpression was associated with a significant increase in total cells that were principally comprised of CD3+/CD4+ T cells and B220+ B cells. Despite the finding that CD14+ cells were not different between groups, we remained impressed with the altered histological appearance of the alveolar macrophages. To characterize their receptor cell surface expression, we measured MHC II and CD80 expression by FACS analysis. A significant increase in CD80 (Fig. 10A) and MHC II (Fig. 10B), consistent with a mature macrophage phenotype, was observed only in overexpressing mice compared with all other experimental mouse groups. Representative FACS histograms for CD80 and MHC II (Fig. 10, C–F) are provided. Together these

Table 2. Percentage of cells isolated from whole lung homogenates with positive expression of cell surface markers as measured by FACS analysis

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Total Cells</th>
<th>%CD3</th>
<th>%CD4</th>
<th>%CD8</th>
<th>%B220</th>
<th>%CD14</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG− Dox+</td>
<td>14.0±0.91×10⁶</td>
<td>15.9±1.2</td>
<td>10.6±0.4</td>
<td>2.4±0.5</td>
<td>9.4±1.6</td>
<td>4.6±1.4</td>
</tr>
<tr>
<td>TG+ Dox+</td>
<td>22.2±2.33×10⁶</td>
<td>26.5±2.1</td>
<td>19.6±1.4</td>
<td>2.4±0.4</td>
<td>17.0±2.2</td>
<td>3.1±0.8</td>
</tr>
<tr>
<td>P</td>
<td>0.02</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.94</td>
<td>0.03</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Values are means ± SE. FACS, fluorescence-activated cell sorting.
data suggest that long-term overexpression of huIL-10 in the lung is associated with recruitment to the lung of an increased number of mononuclear cells comprising CD4+ T cells and B cells and a more mature population of tissue macrophages.

**Effect of long-term IL-10 overexpression on the chemokine expression.** As previous investigators had identified MCP-1 as being important for recruiting mononuclear cells to sites of inflammation (9, 45), we hypothesized that long-term IL-10 overexpression might be driving CC chemokine expression. To test this hypothesis, whole lungs from mice overexpressing IL-10 for 1–3 mo (TG+ dox+) were harvested for chemokine RNA determination (by RPA) and chemokine protein expression in whole lung homogenates (by ELISA) and compared with results from control mice groups (TG+ dox−, TG− dox+, or TG− dox−). By RPA, only TG+ dox+ mice demonstrated an increased signal for the CC chemokines: MCP-1, RANTES, and MIP-1α mRNA (Fig. 11), though not the CXC chemokine MIP-2. Consistent with the mRNA expression, significant increases in MCP-1, RANTES, and MIP-1α were observed in whole lung homogenates only from TG+ dox+ mice (Fig. 12, A–C, respectively), but not for the CXC chemokines MIP-2 or KC (data not shown). Interestingly, despite significant and substantial increases in the whole lung amount, little MCP-1 was consistently measured in BALF from TG+ dox+ mice (data not shown). These data suggest that long-term overexpression of IL-10 is associated with increased CC chemokine expression that is possibly responsible for the recruitment of the increased mononuclear cell population.

**DISCUSSION**

IL-10 remains one of the most studied anti-inflammatory cytokines because of its numerous immunity-modulating prop-
properties and the increasing recognition of its role in communication and regulation between the innate and adaptive immune responses. IL-10 was initially identified from activated Th2 helper T cells on the basis of its ability to inhibit IFN production from Th1 T cell clones (40). Multiple anti-inflammatory properties have been attributed to IL-10 (reviewed in Refs. 24, 30). In various model systems, IL-10 has been shown to inhibit production of numerous proinflammatory cytokines and chemokines that are known to contribute to the development of acute inflammatory states, including ALI. The mechanism by which IL-10 mediates these various effects has been the target of much investigation over the past decade. To gain further insight into the role that IL-10 played in modulating in vivo immune responses specifically in the lung, we developed a transgenic mouse capable of regulatable, lung-specific human IL-10 overexpression. Regulation of the lung-specific overexpression was achieved using a tetO responsive element so that addition of doxycycline to the chow resulted in rapid (within 24 h) IL-10 transgene expression.

The ability to regulate expression of the transgene afforded the unique opportunity to study the effect of IL-10 overexpression at various ages of mice in a manner not achievable with a constitutively active transgene strategy. In doing so, we were able to investigate the possible confounding effect of transgenic overexpression during the course of fetal lung development, when either the surfactant protein or Clara cell promoters that are commonly used to promote lung specificity are endogenously turned on. Although the short-term application of doxy-chow did not appear to alter the milieu of the lung in vivo, long-term studies similar to those reported previously (19) identified the confounding factor of a doxycycline-dependent, IL-10-independent effect on modification of what we interpret to be a low-level bacterial colonization in lungs of housed mice not previously appreciated. Although this transgenic strategy can be criticized on the basis on needing doxycycline to induce transgene overexpression, it is likely that the opposite strategy of using a TRE-off system would be similarly plagued with an alteration of the host’s endogenous flora.

Fig. 11. Effect of long-term IL-10 overexpression on CC chemokine mRNA expression. A: RPA including probes for monocyte chemoattractant protein (MCP)-1; regulated on activation, normal T cell expressed, and presumably secreted (RANTES); and macrophage inflammatory protein (MIP)-1α was performed on RNA isolated from whole lungs of designated mouse groups. Only TG+ dox+ mice demonstrated significant increases in the signal intensity for MCP-1, RANTES, and MIP-1α RNA. As a control for specificity of the RPA probe, mouse RAW 264.7 cells were transiently transfected with either a human IL-10 or empty overexpression construct for 24 h, and the RNA was isolated and analyzed by RPA as shown. Only the IL-10-transfected cells had increased mouse MCP-1 RNA. Further specificity of the RANTES and MIP-1α probe was confirmed using control RNA (right side). RPA is representative of 3 separate determinations. TRF, transfection.

Fig. 12. MCP-1, RANTES, and MIP-1α levels on whole lung homogenates from same groups as denoted in Fig. 11. Only TG+ dox+ mice showed significant increases in the expression of these CC chemokines compared with all other groups. Values are reported as means ± SE from n = 10–12 mice per group. *P < 0.001.
IL-10 to inhibit activation of the NF-κB, we focused the current studies on the ability of IL-10 to inhibit activation of the NF-κB signaling pathway. Our previous in vivo studies showed that pretreatment with IL-10 abrogated NF-κB activation in a manner associated with impaired phosphorylation and, thus, degradation of the inhibitory protein IkB-α (20). Therefore, we postulated that the inhibition of NF-κB by IL-10 in vivo may include attenuation of IkK activity as was demonstrated in these studies. The precise molecular mechanism by which this inhibition occurs remains to be elucidated and is the focus of ongoing studies. IL-10 signals through its receptor complex, which is structurally related to the type II cytokine receptor family similar to the IFN-γ receptor. Although two subunits have been identified, IL-10 receptor 1 (IL-10R1) is principally responsible for IL-10 binding and is necessary for IL-10 signaling (18). Ligation of the IL-10 receptor activates JAK1 and TYK2 tyrosine kinases, members of the JAK-STAT family of signal transduction proteins, and results in phosphorylation and activation of STAT1α and STAT3 (11, 28). IL-10 activation of STAT1α and STAT3 has been associated with induction of the suppressor of cytokine synthesis-3 protein (6, 17) and inhibition of LPS-induced macrophage activation (3); however, whether this effect involves modulation of NF-κB and IkK activity remains controversial. Whether IL-10-induced activation of these proteins might lead to transcriptional activation of a specific inhibitor of IkK or alter key protein-protein interactions necessary for IkK activation remains unknown. As a result, the links between these IL-10 signaling and attenuation of NF-κB-driven inflammatory gene expression remains incompletely understood.

The data derived from work with the constitutively active, lung-specific IL-10-overexpressing mice suggested that IL-10 overexpression is associated increased tissue inflammation and fibrosis in a manner independent of an IL-13/IL-4Ra/STAT6 activation pathway (19). Because the transgene used in that study was constitutively turned on during fetal lung development, it remained unclear as to whether the effect observed was related to IL-10 overexpression or some alteration of the lung during fetal and early postnatal development. Consistent with these findings, our studies involving long-term overexpression showed an increased number of lung cells that display an altered phenotype both histologically and by cell surface expression. The appearance of an increased number of mononuclear cells comprising principally CD4+ T cells and B220+ B cells, as well as a change in the cell surface expression of mature macrophage markers MHC II and CD80 (2), suggests that IL-10 can modulate the cellular constitution of the lung. Thus, while short-term expression of IL-10 may be beneficial in regulating an induced proinflammatory response, long-term expression might alter the cellular milieu such that the subsequent host response to an immune challenge in this state may be harmful. This hypothesis is being examined in ongoing studies.

This finding of an altered number and phenotype of baseline lung mononuclear cells raised the possibility the IL-10 overexpression results in increased chemokine expression. Previous reports had identified MCP-1 as a monocyte-specific chemokine-attractant for T cells, NK cells, basophils, and monocytes (13). More recently it has been suggested that MCP-1 also displays multiple immunoregulatory functions including both T cell differentiation and monocyte maturation (27). Furthermore, a link between IL-10 and MCP-1 expression had been established previously. Ikeda et al. (16) observed that IL-10 differentially regulates MCP-1 expression depending on the inflammatory milieu of the cell culture system, though additional chemokines were incompletely studied. These investigators demonstrated that in resting monocytes/macrophages, IL-10 induces MCP-1 expression. In contrast, IL-10 inhibited MCP-1 expression when these same cells were stimulated with LPS (16). Together these data imply that under nonstimulated, basal conditions, as were examined in the chronic IL-10 model employed in the current studies, IL-10 appears capable of driving not just MCP-1 but also other CC chemokine expression. In turn, this increased CC chemokine expression may be responsible for the long-term effects on the cell number and phenotype changes reported above in a manner similar to what has been reported with the intratracheal inoculation of MCP-1 alone (21, 22). This mechanism might also explain the reason these observations were only made with long-term IL-10 overexpression (>2 wk). An alternative explanation is that whereas MCP-1 mediates monocyte cell influx to the lung, IL-10 itself may alter the cell phenotype. For example, it has been demonstrated that IL-10 can modify the cell surface marker expression of monocytes by increasing CD16 expression (5) and Fc gamma receptor expression (4, 39). However, it is unlikely that IL-10 is directly responsible for the increased MHC II or CD80 expression as these have been shown to be decreased by IL-10 in some in vitro models (7, 46). Unfortunately, we have been unable to devise a technically feasible strategy for long-term neutralization of the various CC chemokines in the IL-10-overexpressing mice to either confirm or refute this hypothesis by abrogating the cellular changes observed. Perhaps crossbreeding of the bitransgenic line onto the existing CC chemokine null mutant mice (e.g., MCP-1 KO mice) may afford the ability to test this hypothesis directly.

In summary, IL-10 continues to be pursued as a potential therapeutic option in numerous inflammatory disease states on the basis of its potent anti-inflammatory properties. However, as has been observed for several cytokines, the immunity-modulating properties of IL-10 appear more diverse than simply anti-inflammatory. We have succeeded in generating transgenic mice capable of regulatable, lung-specific IL-10 overexpression to further our understanding of the role of IL-10 in modulating lung inflammation. Although we have confirmed IkK as being attenuated by IL-10 in the NF-κB signaling pathway following induction of acute lung inflammation, long-term overexpression studies revealed additional immunity-regulating properties. These observations appear to be related to IL-10 driving CC chemokine expression, which may mediate an increased number of mononuclear cells to the lung. The ability to regulate IL-10 overexpression should provide a novel
and unique tool for further examining the role of IL-10 in lung immunobiology.

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