CALL FOR PAPERS | Leukocyte Inflammatory Mediators and Lung Physiology

Thymulin evokes IL-6-C/EBPβ regenerative repair and TNF-α silencing during endotoxin exposure in fetal lung explants

Stephen C. Land and Froogh Darakhshan
Tayside Institute of Child Health, Maternal and Child Health Sciences, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY, United Kingdom

Submitted 20 November 2002; accepted in final form 13 March 2003

Land, Stephen C., and Froogh Darakhshan. Thymulin evokes IL-6-C/EBPβ regenerative repair and TNF-α silencing during endotoxin exposure in fetal lung explants. Am J Physiol Lung Cell Mol Physiol 286: L473–L487, 2004. First published March 14, 2003; 10.1152/ajplung.00401.2002.—Chorioamnionitis is associated with fetal lung disease, and potentiated LPS-evoked TNF-α silencing at either PO2. Thymulin at fetal vs. ambient PO2; however, exposure to 0.1–50 μg/ml LPS were cultured for 96 h at fetal (23 mmHg) or ambient (142 mmHg) mesenchyme-epithelial differentiation during exposure to Escherichia coli lipopolysaccharide (LPS). Gestation day 14 fetal rat lung explants were cultured for 96 h at fetal (23 mmHg) or ambient (142 mmHg) PO2. Airway surface complexity (ASC, perimeter/√area) was greater at fetal vs. ambient PO2; however, exposure to 0.1–50 μg/ml LPS significantly raised ASC at 2 μg/ml in ambient PO2 explants. LPS (50 μg/ml) depressed ASC in both conditions to untreated ambient PO2 control values without changes in necrosis or apoptosis. To manipulate LPS-evoked TNF-α and IL-6 release, we exposed explants and A549 cells to combinations of 50 μg/ml LPS, 10 μM ZnCl2, and 0.1–1,000 ng/ml thymulin at either PO2. Thymulin+Zn2+ suppressed and potentiated LPS-evoked TNF-α and IL-6 release, yielding an IC50(TNF-α) of 0.5 ± 0.01 ng/ml and IC50(IL-6) of 1.4 ± 0.3 ng/ml in A549 cells. This was accompanied by activation of the p38 MAPK-MAPKAP-K2 pathway with sustained expression of TNF-α and IL-6 transcripts at ambient PO2. LPS+thymulin+Zn2+-treated explants showed proliferation of CCAAT-enhancer binding protein-β (C/EBPβ) and fibroblast growth factor-9 immunoreactive mesenchyme, which was abolished by IL-6 antisense oligonucleotides. The post-transcriptional suppression of immunogenic TNF-α synthesis coupled with raised IL-6 and C/EBPβ-dependent mesenchyme proliferation suggests a role for bioactive thymulin in regulating regenerative repair in the fetal lung.

bronchopulmonary dysplasia; lung morphogenesis; mitogen-activated protein kinase; fibroblast growth factor; cytoregulation

WHEREAS AGENTS THAT CAUSE intra-amniotic inflammation and infection increase the risk of preterm labor, aberrant lung development, and subsequent respiratory disorders, an increasing body of evidence points towards an active role for inflammatory acute-phase response proteins in promoting antenatal lung maturation. Models of lipopolysaccharide (LPS)-induced chorioamnionitis, for example, show significant increases in lung volume, compliance, and surfactant protein expression that parallel the accumulation of inflammatory cells in fetal membranes and acute expression of proinflammatory cytokines, notably IL-1, TNF-α, and IL-6 (24, 25, 31). More recent work has highlighted roles for IL-1α and -β in mediating this response (50), whereas other studies demonstrate key roles for TNF-α in potentiating airway bifurcation and IL-6 in hyperoxic cytoprotection of the perinatal lung (22, 49). The clinical appeal of these observations is hampered, however, by conflicting studies that argue roles for the same cytokines in alveolar hypoplasia, pulmonary fibrosis, necrosis, and dysplastic lung development (32, 33, 34, 45). Although experimental approaches differ among these studies, there remains the possibility that systemically expressed immunomodulators, absent from cell culture experiments, set the scope of cytokine signaling responses during an inflammatory stimulus.

Thymulin (facteur thymique sérique) is a nona-peptide neuroendocrine hormone secreted by thymic epithelial cells that regulates systemic immunity in its bioactive, Zn2+-conjugated form by augmenting the expression of factors involved in T-cell development, maturation, and migration in lymphoid tissues (15). Outwith the thymus, bioactive thymulin is consistently reported to silence proinflammatory cytokine and chemokine expression in response to a wide range of inflammatory or autoimmune disease conditions (reviewed in Ref. 40). In the lung, this effect has been observed as a suppression of C-C and C-X-C cytokines coupled with lowered leukocyte infiltration during bleomycin-induced pulmonary fibrosis (31). Additionally, thymulin+Zn2+ evokes a marked cessation in the growth rate of pulmonary metastases, suggesting that the anti-inflammatory effect has the potential to regulate cellular growth and differentiation of the lung (29, 30).

We have recently shown that the anti-inflammatory properties of bioactive thymulin are conserved in fetal distal lung epithelial cell cultures isolated from late gestation (day 19) rat fetuses (18), suggesting that pathways involved in thymulin-cytokine interactions are functional in the fetal lung close to term. In examining whether exogenous thymulin may modulate both form and magnitude of responses to an inflammatory agent (Escherichia coli LPS) our objectives were to 1) establish the relationship between airway surface complexity (ASC) and combinations of culture PO2 and LPS dosage in explants isolated from pseudoglandular-stage fetal rat lung, 2) determine the potential for thymulin+Zn2+ to modulate this relationship under conditions least favorable for explant airway

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

growth, 3) examine candidate cytokine pathways through which thymulin + Zn\(^{2+}\) may mediate its effects, and 4) unite these observations with changes in the expression of growth factors involved in the regulation of lung morphogenesis. Our results show that bioactive thymulin simultaneously suppresses endogenous release of TNF-\(\alpha\) and potentiates IL-6 expression through a CCAAT-enhancer binding protein-\(\beta\) (C/EBP\(\beta\))-dependent pathway, an established route for regenerative repair in other tissues. The hyperexpression of this pathway was dependent on the physiological scope of regenerative repair responses to inflammatory stimuli throughout development.

**EXPERIMENTAL PROCEDURES**

**Materials.** Anti-goat IgG FGF-9 (C-19), anti-goat IgG FGF-10 (C-17), and anti-mouse IgG2a, C/EBP-\(\beta\) (H-7) antibodies and recombinant protein standards were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat IgG-fluorescein isothiocyanate (FITC), anti-mouse IgG-tetramethyl-rhodamine-isothiocyanate (TRITC), and horseradish peroxidase (HRP)-conjugated antibodies were from Diagnostics Scotland (Carluke, Lanarkshire, UK), with chemiluminescent detection using the latter conjugate performed using ECL Western blotting detection reagents from Amersham Pharmacia Biotech (Little Chalfont, UK). 4′,6-Diaminido-2-phenylindole, dihydrochloride (DAPI)-antifade was from Q-Biogene. \(\gamma\)-\(\text{ATP was from Perkin Elmer Life Sciences (Mechelen, Belgium). Recombinant human heat shock protein (HSP}}\) 27 was from Calbiochem-Novabiochem (La Jolla, CA). All species-specific antibodies and recombinant standards used for cytokine ELISA of rat IL-6 and TNF-\(\alpha\) were from R&D Systems (Minneapolis, MN). Control and antisense oligonucleotides against rat IL-6 mRNA were purchased from Biognostik (Göttingen, Germany). Oligonucleosome detection and cell proliferation \[3\text{-}4,5\text{-diamidino-2-yl}-2\text{-y}-2,5\text{-diphenyl tetrazolium bromide (MTT)}\] were conducted with kits from Roche Diagnostics (Mannheim, Germany). Culture solutions, antibiotics, and serum were from Gibco-BRL. Life Technologies (Paisley, UK). Thymulin, \(E\). coli LPS, and other bench chemicals were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

**Culture of rat explants and human adenocarcinoma A549 cell line.** All procedures accorded with the Animals (Scientific procedures) Act 1986, UK. Pregnant Sprague-Dawley rats were killed by cervical dislocation on day 14 of gestation (term is 22 days), and the fetuses were decapitated. Right and left lung lobes were then mounted on 13-mm diameter Whatman Nucleopore Track Ech membranes (pore diameter of 8 \(\mu\)m) suspended on the surface of 2 ml of serum-free DMEM containing Ham’s F-12 nutrient mix (DMEM/F-12) with 100 U/ml penicillin/streptomycin, each within a single well of a 12-well Costar cluster dish (Corning, NY). Explants were then placed into humidified 37°C incubators set to maintain either P\(_{02}\) of 142 (ambient) or 23 mmHg (fetal) plus 5% CO\(_2\) and were then left to equilibrate for 12 h before the beginning of each experiment. For all experimental manipulations, care was taken to ensure that medium was pre-equilibrated to the appropriate oxygen tension and temperature. To avoid hypoxia-reoxygenation effects, explants maintained at 23 mmHg were cultured in a temperature-, gas-, and humidity-controlled MACS VA500 environmental workstation (Don Whitley Scientific, Shipston, UK).

A human lung epithelial A549 cell line was routinely maintained in filter capped Cellstar 75-cm\(^2\) flasks (Greiner Bio-one, Frickhausen, Germany) in DMEM supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin at either fetal or ambient P\(_{02}\). Cultures were routinely passaged within 90% confluence.

**LPS- thymulin + Zn\(^{2+}\), and IL-6-dependent effects.** After the 12-h preincubation period, explants were washed once in phosphate-buffered saline (PBS) and then incubated in the presence of DMEM/F-12 containing 0, 0.5, 2, 10, or 50 \(\mu\)g/ml of \(E\). coli LPS at fetal or ambient P\(_{02}\). Digital images were captured at 0, 24, and 96 h with a DIC-HR digital camera (World Precision Instruments, Sarasota, FL) mounted on a Leica LZ7 binocular microscope under identical magnification and gain settings. At the 96-h time point, the medium was removed and frozen for determination of lactate dehydrogenase (LDH) leakage and protein and cytokine secretion.

To examine the effect of thymulin on LPS-evoked effects, we maintained explants over an identical time course without treatment or in the presence of 50 \(\mu\)g/ml LPS, 10 \(\mu\)M ZnCl\(_2\), or LPS together with ZnCl\(_2\). Thymulin was administered to LPS- and ZnCl\(_2\)-exposed explants at concentrations of 0, 0.1, 10, or 1,000 ng/ml. Sulfasalazine (SSA, 1 mM), a blocker of nuclear factor (NF)-\(\kappa\)B transcriptional activation, was included with each treatment to distinguish between transcription-dependent and -independent components of the cytokine response. After 96 h, explants were digitally imaged, and the medium was retained for assessment of LDH leakage and protein and cytokine secretion. Explant tissue was divided into groups to be processed for immunohistochemistry, electrophoretic mobility shift assay (EMSA), oligonucleosome release, and immunoprecipitation.

The dose-response relationship between thymulin and IL-6 or TNF-\(\alpha\) secretion in the presence and absence of LPS and ZnCl\(_2\) was assessed in a human lung A549 cell line, which shares phenotypic characteristics common to alveolar type II epithelial cells. Cells were seeded at a density of 5 \(\times\) 10\(^5\)/ml in Costar six-well culture flasks (Corning) and were maintained at fetal or ambient P\(_{02}\) in 2 ml/well DMEM with 10% fetal calf serum and antibiotics. At 80% confluence, the medium was replaced with phenol red-, serum-, and antibiotic-free DMEM, and the cells were exposed to 0.01, 1, 10, 100, or 1,000 ng/ml thymulin plus 10 \(\mu\)M ZnCl\(_2\) for 2 h either alone or in combination with 10 \(\mu\)g/ml LPS. TNF-\(\alpha\) and IL-6 release was measured by enzyme-linked immunosorbent sandwich assay (ELISA) as detailed under Cytokine ELISA.

Fifty-percent inhibitor constant (IC\(_{50}\)) and effector constant (EC\(_{50}\)) values were calculated using four-parameter sigmoidal curve regression based on the equation \(Y = a + (Y\text{ range})/1 + 10^{(\text{EC}_{50} - s)/x}\), where \(a\) is the background Y value and \(s\) is the Hill slope. Note that 10 \(\mu\)g/ml LPS was the minimum dose to produce a maximal sustained release of TNF-\(\alpha\) and IL-6 without significant loss of cells. In an identical experiment, A549 cell proliferation was determined by supplementation of culture with 10% (vol/vol) MTT. The rate of MTT reduction by cellular oxidative phosphorylation was determined by solubilization of its formazan salt followed by spectrophotometric detection at 550 nm with background correction at 690 nm.

To establish IL-6 dependency of thymulin + Zn\(^{2+}\) cytoprotection, we preincubated explants for 12 h in the presence of 10 mmol/ml of control or antisense oligonucleotide targeted against rat IL-6 mRNA and then exposed them for a further 96 h to 0 or 50 \(\mu\)g/ml LPS together with 10 \(\mu\)M ZnCl\(_2\) and 0.1, 10, or 1,000 ng/ml thymulin. An additional experimental set was conducted in the presence of thymulin plus 10 \(\mu\)M Zn\(^{2+}\) in the absence of LPS. At the end of the experiment, explants were digitally imaged, and the medium plus explant tissue were processed as before.

**Morphometry and immunohistochemistry.** Explant surface complexity was determined from digital images as perimeter (mm)/\(\sqrt{area (mm^2)}\) using Scion Image 4.0.2 software (Scion, Frederick, MD) as described previously (16). Calibration was achieved using a 20 \(\times\) 2-mm grid removed from a Fast-Read 102 disposable cell-counting chamber (ISL, Paignton, UK) that was placed onto the meniscus of the medium in each well.

Immunohistochemistry was performed on PBS-rinsed, filter-attached explants fixed for 8 h in a solution of PBS containing 10%
formalin (pH 7.2). After processing, explants were embedded in paraffin, and 3-μm sections were mounted on Histogrip-coated slides (Zymed, San Francisco, CA), dried, deparaffinized, and then gradually rehydrated. We performed antigen retrieval by microwaving slides for three bursts of 7 min at 800 W in 750 ml of Antigen Unmasking Buffer (Vector Laboratories, Burlingame, CA). Sections were then blocked in 10% preimmune goat serum in Tris-buffered saline [TBS; in mM: 50 Tris (pH 8.0), 138 NaCl, and 2.7 KCl] for 1 h and then incubated overnight with anti-goat FGF-9, anti-goat FGF-10, or anti-mouse CEBPβ (H-7) antibodies each at 1:200 dilution followed by 5 μl of DAPI-antidote to each section before mounting. Images were obtained under a Zeiss Axioskop fluorescent microscope equipped with a Hamamatsu C4742–95 color digital camera using Openlab (Improvision, Coventry, UK) software. The contrast properties were optimized for each section and then referenced against the respective negative control image using identical camera and microscope settings.

The surface area of mesenchyme, airway cuboidal epithelium, and airway space was determined from immunohistochemical sections as detailed by Bolender et al. (6). Briefly, a 196-cm² isometric grid sectioned into 1-cm² units was superimposed onto each image, and the number of grid intersections that coincided with each of the three dominant structural features structure (mesenchyme, differentiated epithelium, and luminal airway space) was counted. We determined the fractional surface area for each by expressing these counts as a fraction of the whole. We determined the thickness of the epithelial compartment from digital images of the sections at each intersecting point using the camera-calibrated micrometer function supplied with Openlab software.

**Determination of necrosis and apoptosis.** The rate of necrosis was determined by the rate of appearance of LDH activity in the culture medium supporting each explant. We determined LDH activity specified by the Bio-Rad protein assay.

Nuclear extraction and EMSA. Pooled explants were mechanically disrupted in a buffer containing (in mM): 20 HEPES (pH 7.9), 1 EDTA, and 10 NaCl, plus 0.1% (vol/vol) Nonidet P-40 and 1 Complete (Roche) protease inhibitor tablet per 25 ml of lysis solution. Nuclei were collected by cold centrifugation at 5,000 g for 10 min and disrupted by a single 15-s burst of sonication in a phospho-protein lysis buffer containing (in mM): 150 NaCl, 20 Tris-HCl (pH 7.5), 2.5 Na⁺ pyrophosphate, 1 EDTA, 1 EGTA, 1 β-glycerophosphate, and 1 NaVO₄, plus 1 tablet per 25 ml of Complete (Roche) protease inhibitor. After centrifugation at 10,000 g for 10 min at 4°C, the supernatant was retained and the protein content was determined by the Bio-Rad protein assay.

Oligonucleotides were end-labeled with [γ-³²P]ATP with T4 polynucleotide kinase and then purified from unincorporated radioactivity with ProbeQuant G-50 microcolumns (Amersham Pharmacia, St. Albans, UK). We set up binding reactions using 20 μg of explan nuclear protein retained as the pelleted fraction from preceding apoptosis determinations in a buffer containing (in mM): 20 Na⁺ HEPES (pH 7.9), 25 KCl, 5 MgCl₂, 1 EDTA, and 1 diethiothreitol (DTT) plus 0.05 μg/μl poly(dI-dC). Labeled oligonucleotide probes (1 μg) were mixed with 2 μl of nuclear extracts and samples were then incubated on ice for 30 min. Supershift reactions were done by incorporating 1 μl of monoclonal antibody to CEBPβ or NF-κB p65 for 30 min before the addition of labeled probe. Samples were electrophoresed through a nondenaturing 6% polyacrylamide gel at 10 mA in 0.5× TBE buffer ([1× TBE = 90 mM Tris-borate, 2 mM EDTA, pH 8.3). Dried gels were analyzed with a Canberra-Packard (Pangbourne, UK) Instant Imager, then subsequently exposed to film.

Mitogen-activated protein kinase-activated protein kinase 2 activity. Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) activity was determined in both explant and serum-supplemented explant cell lysates by exposure to 10% SDS-polyacrylamide gel and then stained with Coomassie blue to reveal the HSP27 band. The gel was then dried, fractionated on a 15% SDS-polyacrylamide gel and then stained with Coomassie blue to reveal the HSP27 band. The gel was then dried, and radioactivity corresponding to phosphorylated HSP27 was observed by exposure to film.

**RNA isolation and RT-PCR.** Total RNA was extracted from pooled explants using TRIzol reagent (Invitrogen, Paisley, UK). Random-primed, first-strand cDNA synthesis was generated from 1 μg of total RNA per treated sample with the AMV Reverse Transcriptase System (Promega, Madison, WI) and was subsequently used for PCR. PCR primers were derived from nucleotides 3,206–3,225 and 7,187–7,206 of rat IL-6 cDNA to yield a 650-bp product (GenBank accession no. M26745) and were 5'-CTCCCTCTTACCAAGTC-3' (sense) and 5'-GCCACAGTGGAATGTC-3' (antisense), respectively. A 540-bp TNF-α product was generated with primers derived from nucleotides 4,568–4,577 and 5,399–5,398 of rat TNF-α cDNA (GenBank accession no. L00981) and were 5'-CGCCCTGTCGTGAC-3' (sense) and 5'-TTCCTTTCAGTTGAAGTCC-3' (antisense). PCR reactions were constructed using 20 ng of each cDNA in a buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 100 μM of each primer to 25 μl of cDNA, and 2.5 units Taq DNA polymerase. Polymerization was continued for 30 cycles (30 s at 94°C, 60 s at 57°C, and 120 s at 72°C) in a Techne Touchgene Gradient PCR machine, after which PCR products were

**Cytokine ELISA.** We determined TNF-α and IL-6 secretion in duplicate samples of the medium supporting the explant using R&D Systems rat antibodies and standards by following the recommended ELISA procedure for each cytokine. We performed assays using Nunc-Immuno MaxiSorp ELISA plates (Nunc) with the optical density of each well determined at 450 nm with wavelength correction at 595 nm using a Dynatech Laboratories (Guernsey, UK) MRX microplate reader. Assays were linear from 62.5–1,000 pg/ml (TNF-α) or 12.5–8,000 pg/ml (IL-6 and IL-1β) and were used on condition that a R² value >0.90 was obtained.
fractionated on a 1% agarose gel, and the identity of each PCR product was confirmed by sequencing. Preliminary experiments demonstrated that 30 cycles were sufficient to resolve each product within the log phase of the PCR reaction.

Immunoprecipitation of FGF-9 and FGF-10. Pooled explants were sonicated by three 10-s bursts at 4°C phospho-protein lysis buffer. Explant protein (50 μg) was preblocked with 10 μl of BSA-blocked protein A-Sepharose and incubated overnight at 4°C with gentle agitation. Immunoabsorbed proteins were separated by SDS-polyacrylamide electrophoresis on a 12% gel and transferred onto nitrocellulose by Western blotting. Blots were visualized following overnight incubation with IgG FGF-9 antibody at 1:1,000 dilution using an anti-goat IgG HRP-conjugated secondary antibody and chemiluminescent detection on Kodak MXB film. Images were digitally scanned, and band intensities as pixels per cm² were determined with Un-Scan-It software (Silk Scientific, Orem, UT).

Data handling and statistics. Data are presented as means ± SE with the number of independent repetitions provided in the legend to each figure. Statistical significance was assessed relative to control groups using one- or two-way analysis of variance with the level of significance determined using the post hoc Tukey’s honestly significant difference. Where data are displayed as a fractional difference relative to control, the reported statistics were performed on the original nonreferenced data.

RESULTS

Choice of LPS and thymulin dose range. Pilot experiments in which IL-6 and TNF-α release was titrated against LPS concentration yielded EC₅₀ values of 0.79 ± 0.46 μg/ml (IL-6) and 0.86 ± 0.92 μg/ml (TNF-α). Experiments were conducted over a range of LPS dosage up to ~50 times these values (0–50 μg/ml).

Characterization of explant ASC responses to culture P O₂ and LPS. As native P O₂ in the midgestation lung averages ~23 mmHg (midpoint between the P O₂ of the amniotic/lung luminal fluid and umbilical vein P O₂), experiments were conducted in explants cultured either at fetal or at hyperoxic ambient P O₂ to control for oxygen-dependent effects within each experimental regime (Fig. 1, A and C). At both P O₂s, explants developed

Fig. 1. Characterization of explant airway growth responses to culture P O₂ and LPS. A: airway surface complexity [ASC: airway perimeter (mm)/√airway area (mm²)] in explants maintained at fetal (23 mmHg) or ambient (142 mmHg) P O₂ at 0 and 96 h. αP < 0.05 relative to control (0 h) 23 mmHg. βP < 0.01 relative to 142 mmHg for 96 h; means ± SE, n = 10. B: effect of LPS on the fractional change in ASC from 0 to 96 h (ASC_f0-96h: ASC 96 h/ASC 0 h) at fetal and ambient P O₂. α with solid line, explants maintained at 142 mmHg; β with dotted line, explants at 23 mmHg. Lines were fitted using log normal 4-parameter regression. αP < 0.05 relative to 142 mmHg control for 96 h; βP < 0.01 relative to 23 mmHg control. Means ± SE, n = 6. C: representative images of single explants at 0 and 96 h of culture at fetal or ambient P O₂ in the presence of 0, 2, or 50 μg/ml LPS. Images have been optically inverted to emphasize airway space volume. Bar = 1 mm.
fluid-filled cyst-like structures coupled with a loss of contiguous airway; however, explants cultured at fetal PO_2 displayed pockets of dense airway bifurcation that maintained overall ASC statistically greater than ambient PO_2 cultured explants. This regionalized hyperplasia at fetal PO_2 was conserved over a range of 0.01–10 μg/ml LPS, beyond which the fractional change in ASC (ASC_{0–96h}; ASC_{96h}/ASC_{0h}) fell significantly to 0.8 ± 0.2 (Fig. 1, B and C). Mean epithelial thickness for cultures maintained at fetal PO_2 was 11.2 ± 0.6 μm, which thinned significantly (P < 0.05, n = 6) to 7.1 ± 0.3 and 4.2 ± 0.3 μm in explants maintained respectively at ambient PO_2 or exposed to 50 μg/ml LPS at either PO_2.

Explants maintained at ambient PO_2 showed a dynamic growth response to LPS. Despite a significant reduction in ASC_{0–96h} in control explants, those exposed to 0.5–10 μg/ml LPS displayed an increase in contiguous airway bifurcation and an absence of cystic structures, peaking at an ASC_{0–96h} value of 1.7 ± 0.3 at 2 μg/ml LPS. As with the fetal PO_2 explants, LPS concentrations at 50 μg/ml reduced the ASC_{0–96h} to 0.7 ± 0.1; therefore, these conditions were selected to test the cytoprotective potential of thymulin+Zn^{2+} as being the most disruptive to airway growth.

Thymulin+Zn^{2+} is cytoprotective during exposure to high LPS concentrations at fetal and ambient PO_2. The cytoprotective potential of thymulin-Zn^{2+} was tested in explants exposed to concentrations of LPS that exceeded the range shown to promote airway bifurcation at fetal or ambient PO_2 (Fig. 1). ASC_{0–96h} was related to the incidence of necrosis (LDH leakage) or apoptosis (oligonucleosome release) in the presence of 50 μg/ml LPS; 10 μM ZnCl_2; or 0.1, 10, or 1,000 ng/ml thymulin (Fig. 2). At fetal PO_2, none of the treatments altered the incidence of apoptosis significantly from the controls; however, administration of Zn^{2+} or LPS+Zn^{2+} significantly raised LDH leakage over control values. Each dose of thymulin lowered the incidence of LPS+Zn^{2+}-induced necrosis to a level that was not statistically significant from controls. This was not associated, however, with any recovery of ASC_{0–96h} to control values.

At ambient PO_2, tissue damage patterns evoked by moderate hyperoxia or LPS followed a different pattern. Necrotic LDH FIG. 2. Thymulin+Zn^{2+} is cytoprotective but does not evoke airway proliferation during exposure to lethal LPS+Zn^{2+} at fetal and ambient PO_2. Explants were maintained for 96 h under the conditions indicated. Release of lactate dehydrogenase (LDH; A) and oligonucleosomes (B) were taken as indexes of necrosis and apoptosis, respectively, and are presented compared with the fractional change in airway surface complexity (ASC_{0–96h}; C) at fetal or ambient PO_2 for 96 h. *P < 0.05 or **P < 0.01 relative to control group at either PO_2; means ± SE, n = 8.
leakage remained relatively constant among all treatment groups; however, LPS+Zn\(^{2+}\) evoked a marked increase in apoptotic oligonucleosome release that was abolished by administration of thymulin at each concentration. ASC0.96b was modestly raised by thymulin+Zn\(^{2+}\) treatment becoming statistically significant at the highest dosage of thymulin.

Thymulin+Zn\(^{2+}\) evokes IL-6, and suppresses TNF-α, release at ambient, but not fetal, PO\(_{2}\). To determine whether thymulin+Zn\(^{2+}\) cytoprotection involved modulation of the LPS acute-phase response, we examined the release of two rapidly expressed cytokines, TNF-α and IL-6, which are, respectively, proapoptotic/necrotic and antiapoptotic/proliferative. Neither control nor Zn\(^{2+}\)-treated explants showed any change in the spontaneous release of IL-6 at either PO\(_{2}\) (Fig. 3A); however, thymulin administered with Zn\(^{2+}\) increased IL-6 secretion under both conditions, becoming statistically significant at ambient PO\(_{2}\). Likewise, LPS evoked a modestly raised by thymulin/Zn\(^{2+}\) concentration. LPS+thymulin+Zn\(^{2+}\) evoked a spontaneous, statistically significant rise in IL-6 release at ambient PO\(_{2}\) (EC\(_{50}\) = 0.54 ± 0.16) without detectable change in basal TNF-α release (Fig. 4, A and B). In contrast to our explant observations, there was little IL-6 release in cultures incubated solely in the presence of LPS+Zn\(^{2+}\): however, incremental dosage with thymulin evoked a fivefold increase in the scope for IL-6 release at both PO\(_{2}\) (EC\(_{50}\) = 0.64 ± 0.34 (23 mmHg) and 1.43 ± 0.29 (142 mmHg)) over control cultures that had been similarly treated with thymulin+Zn\(^{2+}\) alone. In hand with the observed suppression of LPS+Zn\(^{2+}\)-evoked TNF-α release in explants, incremental dosage with thymulin significantly inhibited the release of this cytokine from A549 cultures at either PO\(_{2}\) over a dose range that showed similar kinetics for IL-6 release (IC\(_{50}\) = 0.42 ± 0.01 (23 mmHg) and 0.53 ± 0.01 (142 mmHg)).

The amplification of IL-6 and coordinated suppression of TNF-α release correlate with a significant increase in metabolic oxidative activity, interpreted as cellular proliferation, in A549 cells (Fig. 4, C and D). Cultures treated with LPS+Zn\(^{2+}\) in the absence of thymulin showed spontaneous release of TNF-α with little detectable IL-6, which was coupled with a modest rate of MTT reduction. Addition of 1–100 ng/ml thymulin synergistically raised IL-6 release, suppressed TNF-α expression, and significantly raised the rate of MTT reduction, which was greatest in cells maintained at fetal PO\(_{2}\).

**Thymulin+Zn\(^{2+}\) augments activity of the nuclear factors of IL-6 transcription.** The preceding results suggest that thymulin+Zn\(^{2+}\) acts as a specific agonist of IL-6 expression in lung explants and may functionally regulate the activity of nuclear factors involved in IL-6 gene expression. As IL-6 transcription is augmented by the synergistic interaction between the nuclear factor of IL-6, C/EBPβ, and NF-κB (1, 20, 37), we examined the effect of the transcriptional blocker SSA on IL-6 release together with C/EBPβ and NF-κB DNA binding activity. SSA consistently abolished the release of IL-6 protein from explants treated with thymulin+Zn\(^{2+}\), LPS+Zn\(^{2+}\), and LPS+thymulin+Zn\(^{2+}\) at ambient PO\(_{2}\) (Fig. 5A). In the absence of SSA, fetal PO\(_{2}\) raised the consensus DNA binding activity of C/EBP, but not NF-κB, whereas C/EBP binding was low, and NF-κB raised, at ambient PO\(_{2}\) (Fig. 5, B and C). Independent administration of thymulin or Zn\(^{2+}\) did not result in a statistically significant effect on the activity of either transcription factor. When administered together, however, the binding of both factors displayed an increasing trend that was 4.7 ± 2.1 (C/EBP)- and 2.3 ± 0.8 (NF-κB)-fold above the ambient PO\(_{2}\) control. Notably, the C/EBP activation by thymulin+Zn\(^{2+}\) and LPS+thymulin+Zn\(^{2+}\) diminished to control levels on inclusion of an antisense oligonu-
served with thymulin. A and B: IL-6 and TNF-α release in response to thymulin+Zn²⁺ (squares) and LPS + thymulin+Zn²⁺ (circles) at fetal (open symbols) or ambient (closed symbols) P0₂. Concentrations of thymulin, Zn²⁺, and LPS are provided in EXPERIMENTAL PROCEDURES. Values are means ± SE, n = 4. *P < 0.05 relative to 0.1 ng/ml thymulin within each series; **P < 0.05 relative to LPS + 0.1 ng/ml thymulin+Zn²⁺. C and D: correlation of LPS+thymulin+Zn²⁺-evoked changes in IL-6 and TNF-α release with 3,4-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT) reduction at fetal (open symbols) or ambient (closed symbols) P0₂. LPS and Zn²⁺ were maintained at 100 and 10 μM, respectively; each symbol represents an incremental change in thymulin concentration as follows (in ng/ml): circles, 0; squares, 1; triangles, 10; diamonds, 100. Values are plotted as bidirectional means ± SE, n = 4 in each case. Note that in some cases, error bars are within symbols. **P < 0.05 relative to cytokine control (LPS+Zn²⁺ + 0.0 ng/ml thymulin); *P < 0.05 relative to MTT control (LPS+Zn²⁺ + 0 ng/ml thymulin).

MAPKAP-K2 activity and mRNA abundance of IL-6 and TNF-α are sustained by thymulin+Zn²⁺. MAPKAP-K2 is the terminal kinase involved in regulating the expression of TNF-α mRNA and posttranslational stabilization of IL-6 (33, 38); therefore, we examined the potential for thymulin+Zn²⁺ to activate MAPKAP-K2 via the phosphorylation of a specific target substrate, HSP27 (Fig. 6). Figure 6A shows a representative experiment conducted in serum-starved A549 cultures exposed for 60 min to LPS as a positive control, LPS together with an inhibitor of the upstream kinase p38 MAPK (SB-203580) as a negative control, and two doses of thymulin+Zn²⁺. HSP phosphorylation was weakly evident in A549 cells at ambient P0₂ and was potently induced in the presence of 100 and 1,000 ng/ml thymulin. In explants that had been exposed to each treatment regimen for 60 min, we observed a constitutive activation of HSP27 phosphorylation (Fig. 6B) that masked any specific effect thymulin+Zn²⁺ alone. The activation observed with thymulin+Zn²⁺, with or without LPS, was diminished by SB-203580.

Figure 6C shows the overall expression of IL-6 and TNF-α mRNA detected following 30 cycles of RT-PCR under a similar treatment regimen as for Fig. 5A. IL-6 mRNA was readily detected in explants cultured at fetal, but not ambient, P0₂. Expression was potently induced at ambient P0₂ by LPS and thymulin, administered separately or together, and was abolished in each case by SSA. TNF-α expression showed a converse oxygen sensitivity to IL-6, being weakly detected at fetal P0₂, but potently expressed at ambient P0₂. This expression was not altered significantly by treatment with LPS or thymulin but was abolished by SSA.

Thymulin+Zn²⁺ evokes mesenchyme hyperplasia in the fetal lung. To determine whether the cytoprotective effects of thymulin+Zn²⁺ during LPS treatment bore consequences for lung morphology, we used random sequence control and antisense oligonucleotides derived against rat IL-6 to manipulate the patterns of apoptosis and necrosis at ambient P0₂ observed in Fig. 2. The differentiated epithelial fraction of each explant was calculated from histological sections as described in EXPERIMENTAL PROCEDURES (Fig. 7).

Fig. 4. IL-6 and TNF-α release kinetics and mitogenic response of A549 cells to thymulin+Zn²⁺. A and B: IL-6 and TNF-α release in response to thymulin+Zn²⁺ (squares) and LPS + thymulin+Zn²⁺ (circles) at fetal (open symbols) or ambient (closed symbols) P0₂. Concentrations of thymulin, Zn²⁺, and LPS are provided in EXPERIMENTAL PROCEDURES. Values are means ± SE, n = 4. *P < 0.05 relative to 0.1 ng/ml thymulin within each series; **P < 0.05 relative to LPS + 0.1 ng/ml thymulin+Zn²⁺. C and D: correlation of LPS+thymulin+Zn²⁺-evoked changes in IL-6 and TNF-α release with 3,4-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT) reduction at fetal (open symbols) or ambient (closed symbols) P0₂. LPS and Zn²⁺ were maintained at 100 and 10 μM, respectively; each symbol represents an incremental change in thymulin concentration as follows (in ng/ml): circles, 0; squares, 1; triangles, 10; diamonds, 100. Values are plotted as bidirectional means ± SE, n = 4 in each case. Note that in some cases, error bars are within symbols. **P < 0.05 relative to cytokine control (LPS+Zn²⁺ + 0.0 ng/ml thymulin); *P < 0.05 relative to MTT control (LPS+Zn²⁺ + 0 ng/ml thymulin).

Figure 6B shows the effect of IL-6 antisense treatment on the distribution of differentiated epithelial structures as a correlate of necrosis (LDH leakage) or apoptosis (oligonucleosome release). Untreated explants maintained at 23 mmHg exhibited the highest proportional fraction of differentiated epithelium together with unperturbed fractional rates (values of 1.0 = no change) of LDH leakage and apoptosis over 96 h of culture. Exposure of ambient P0₂ cultured explants to LPS + thymulin+Zn²⁺ in the presence of the control oligonucleotide resulted in a significant decrease in the proportion of differentiated epithelium to ~8% of the total explant surface area. This was accompanied by negligible perturbation in LDH leakage, but significant inhibition of apoptosis. Similarly, exogenously
applied recombinant rat (r) IL-6 significantly reduced the rate of apoptosis but did not alter the differentiated epithelial fraction from that observed with untreated fetal or ambient P\(_{O_2}\) explants. IL-6 antisense oligonucleotide applied under the same conditions as the control oligonucleotide raised the differentiated epithelial fraction to \(\sim 30\%\) of total explant surface area but significantly increased LDH leakage and apoptosis beyond that observed with any other treatment.

Thymulin +Zn\(^{2+}\) modulates FGF-9 and -10 expression to cause mesenchyme hyperplasia. As airway complexity rests on the coordination of signals among regionally expressed growth factors and repressors, we sought to determine whether the observed changes in airway complexity could be accounted for by modulation of FGF-9 and -10 expression. Figure 8A shows representative cross sections of gestation day 16 fetal lungs immunostained for FGF-9, FGF-10, and C/EBP\(\beta\) protein. Although a focal staining of FGF-9 and -10 was observed throughout lung sections, FGF-9 protein showed a rather more diffuse pattern of staining, whereas FGF-10 was predominantly localized in the differentiated epithelial compartment of the airways. C/EBP\(\beta\) immunoreactivity was exclusively mesenchymal and exhibited focal pockets of intense nuclear staining around airway structures. Figure 8B demonstrates FGF-9, FGF-10, C/EBP\(\beta\), and DAPI staining in explants following 96 h of culture at either 23 or 142 mmHg. Explants cultured at 142 mmHg were additionally treated with 50 \(\mu\)g/ml LPS, LPS and 10 \(\mu\)mol ZnCl\(_2\) + 1,000 ng/ml thymulin, thymulin + Zn\(^{2+}\) + 10 nmol/ml IL-6 antisense oligonucleotides or 10 ng/ml rrIL-6. Mesenchyme FGF-9 staining was evident in explants maintained at 23 mmHg but was largely absent from those at 142 mmHg or exposed to LPS treatment. LPS plus thymulin + Zn\(^{2+}\) resulted in homogeneous FGF-9 expression, mesenchymal proliferation, and a loss of differentiated epithelial structures but little fragmentation of nuclear DNA. Addition of IL-6 antisense oligonucleotides muted the mesenchymal hyperplasia but also resulted in distinct DNA fragmentation in the mesenchymal compartment. Exogenous rrIL-6 noticeably increased the proportion of FGF-9 immunoreactive tissue and significantly diminished the overall proportion of airway space without loss of epithelial structures relative to ambient P\(_{O_2}\) controls. FGF-10 immunostaining showed regionally intense pockets of staining under all conditions but became diffuse in LPS + thymulin + Zn\(^{2+}\)-treated explants. C/EBP\(\beta\) showed the same exclusively mesenchymal distribution as in gestation day 16 rat lung but became predominantly nuclear in location in explants exposed to LPS and LPS + thymulin + Zn\(^{2+}\). Addition of an IL-6 antisense oligonucleotide entirely abolished this effect, whereas rrIL-6 evoked strong nuclear accumulation of C/EBP\(\beta\) in all tissue compartments.

Fig. 6. Effect of thymulin+Zn\(^{2+}\) on MAPKAP-K2 activity and stability of IL-6 and TNF-\(\alpha\) mRNA. A: phosphorylation of the MAPKAP-K2 substrate heat shock protein (HSP) 27 in A549 cultures maintained in serum-free DMEM containing 10 \(\mu\)M ZnCl\(_2\) at ambient P\(_{O_2}\) for 60 min in the presence of LPS (10 \(\mu\)g/ml), SB-203580 (20 \(\mu\)M), or thymulin at the concentrations shown. Figure is representative of \(n = 3\) independent experiments. B: MAPKAP-K2 activity in pooled explant lysates maintained for 24 h at each culture P\(_{O_2}\) followed by addition of each combination of Zn\(^{2+}\), LPS, thymulin, and SB-203580 (20 \(\mu\)M) for 60 min. Concentrations for each as given in Fig. 5; representative of \(n = 4\) experiments. C: RT-PCR experiment demonstrating the expression of transcripts for IL-6 (650 bp) and TNF-\(\alpha\) (500 bp) after 30 reaction cycles. Under the conditions used, the PCR reaction was maximal at 50 cycles (IL-6) and 60 cycles (TNF-\(\alpha\)). Concentrations of reagents are as for Fig. 5B; representative of 4 independent experiments.

Fig. 5. Transcriptional response of the nuclear factors of IL-6 transcription to thymulin+Zn\(^{2+}\) in fetal rat lung explants. A: effect of sulfasalazine (SSA) on explant IL-6 release. Explants were maintained for 96 h at ambient P\(_{O_2}\) either without treatment or in the presence of combinations of Zn\(^{2+}\) (10 \(\mu\)M), thymulin (1,000 ng/ml) + Zn\(^{2+}\), LPS (50 \(\mu\)g/ml), or SSA (1 mM). \(^*P < 0.05\) relative to control, \(n = 6\). B: EMSA for CCAAT enhancer binding protein (C/EBP, top) and NF-\(\kappa\)B (bottom) in explants exposed to fetal or ambient P\(_{O_2}\) for 96 h or ambient P\(_{O_2}\) together with LPS (50 \(\mu\)g/ml), thymulin (1,000 ng/ml), ZnCl\(_2\) (10 \(\mu\)M), 10 nmol/ml of rat IL-6 antisense oligonucleotide, SSA (1 mM), or combinations of each treatment as indicated. The loading of nuclear protein and oligonucleotide probe concentration was equal throughout. C: compiled data from 4 independent EMSA experiments showing the fold change in band pixel density relative to 142 mmHg control. \(^*P < 0.05\) relative to 142 mmHg control, \(n = 4\). D: representative EMSA from a pool of 3 independent experiments demonstrating a supershifted band for the \(\beta\)-isoform of C/EBP in explants maintained either at fetal P\(_{O_2}\) or at ambient P\(_{O_2}\) together with thymulin + Zn\(^{2+}\).
A: effect of IL-6 antisense oligonucleotide on IL-6 and TNF-α protein release from explants. Experiments were incubated for 96 h at ambient PO2 in the presence of LPS (50 μg/ml), LPS + thymulin (1,000 ng/ml) + ZnCl2 (10 μM) with either 10 nmol/ml random sequence control oligonucleotide (As control) or 10 nmol/ml rat IL-6 antisense oligonucleotide (IL6 As). *P < 0.05 relative to LPS + thymulin+Zn2+ treatment in the presence of a random sequence control oligonucleotide, **P < 0.05 relative to 142 mmHg control, ***P < 0.05 relative to LPS; n = 4 in each case. B: effect of IL-6 antisense oligonucleotide on the relationship between the differentiation of epithelia-lined structures and necrosis (measured as LDH release, top) or apoptosis (measured as oligonucleosome release, bottom). Explants were maintained as in A with an additional group exposed to recombinant rat IL-6 (rrIL-6, 10 ng/ml) at ambient PO2. **P < 0.05 relative to LPS + thymulin+Zn2+ treatment in the presence of a random sequence control oligonucleotide; where each data point represents the mean ± SE of 4 independent experiments.

Figure 9A shows a stacked histogram detailing changes in the fractional surface area of each compartment as calculated from six independent experiments. The surface area fraction of the mesenchyme compartment was significantly raised relative to fetal and ambient PO2 explants in both LPS+thymulin+Zn2+ treated explants in the presence of control or antisense oligonucleotides. Whereas mesenchyme proliferation was not observed as such with rrIL-6, the overall mesenchyme fraction was raised (P = 0.09), and total airway space was significantly (P < 0.01) diminished relative to explants maintained at ambient PO2. Notably, ambient PO2 treatment of explants with rrIL6 resulted in a distribution of airway space, mesenchyme, and epithelium that was not significantly different from explants maintained at fetal PO2. These data are complemented by mass-specific differences in FGF-9 rather than FGF-10 protein abundance (Fig. 9B).

DISCUSSION

These results demonstrate that lung morphogenesis is responsive to components of the inflammatory response and that, consequently, immunomodulators may significantly alter the course of lung damage during infection. Although none of our experiments restored a normal pattern of lung morphogenesis per se, we show that the balance of mesoepithelial differentiation can be tipped substantially in favor of mesenchyme proliferation by thymulin under conditions that otherwise favor
necrotic or apoptotic tissue loss. As mesenchyme mass ultimately determines lung volume and alveolar surface area (19) and as IL-6-evoked mesenchyme proliferation is integral to the regenerative repair process (37), manipulation of serum thymulin titers may present a means for promoting lung morphogenic potential during perinatal lung inflammatory diseases.

To interpret cytoprotective and immunomodulatory responses to thymulin, we began this study by characterizing explant morphogenic responses to culture P O 2 and LPS. Our results revealed a significant increase in ASC in fetal P O 2 cultured explants that was absent from those at ambient P O 2. Whereas several studies have established that the low circulating P O 2 s characteristic of the fetal environment functionally maintain epithelial luminal fluid secretion and lung expansion (3, 4, 26, 36), evidence showing that airway branching is similarly P O 2 dependent is both limited and conflicting. For example, antioxidants have been shown to promote airway proliferation under conditions similar to the mildly hyperoxic ambient P O 2 treatments used here (Ref. 16 and references therein); however, culture of explants in the presence of nitric oxide donors at concentrations sufficient to invoke sustained nitrosative/oxidative stress also raises airway branching morphogenesis (52). Our results with low concentrations of LPS (0.5–2 μg/ml), a proinflammatory agent that promotes release of reactive oxygen and nitrogen species, similarly increased airway morphogenesis irrespective of prevailing P O 2. At higher concentrations (50 μg/ml), this reverted toward a loss of mesenchyme, cystic structure formation, and epithelial attenuating similar to ambient P O 2 controls, suggesting permissive ranges of P O 2 or LPS may facilitate airway morphogenesis. In utero, LPS promotes fetal lung maturation, resulting in improved mass-specific lung volume, alveologenesis, gas exchange surface area, surfactant protein expression, and alveolar type II cell distribution (24, 25, 31, 45, 50). Prolonged exposure to LPS in neonatal or adult lung, however, results in pulmonary fibrosis, reduced epithelial surface area, septal thickening, and enlargement of alveolar space (46). In broad terms, these studies echo the dose-dependent morphogenetic effects evoked by LPS in explants and imply that developmental expression of pathways that govern the immunogenic acute-phase response and thus immune reactivity set the scope for morphogenic responses to inflammation throughout life.
A

![Graph A]

**Fig. 9.** Morphological response and mass-specific change in FGF-9 and FGF-10 protein expression during treatment with LPS + thymulin +Zn2+ coupled with IL-6 translational blockade. A: stacked histogram showing change in surface area fraction of lung compartments in explants exposed to fetal or ambient Po2 or ambient Po2 in the presence of LPS, LPS + thymulin +Zn2+ + control or antisense oligonucleotide or rN-6 at concentrations as detailed in Fig. 7. The height of each stack represents the mean value of 6 independent determinations; the SE has been omitted for clarity. Significance was assessed using ANOVA post hoc Tukey’s honestly significant difference relative to fetal (23 mmHg, α) or ambient (142 mmHg, β) Po2.

B: FGF-9 and -10 mass-specific protein expression in explants maintained under the same conditions as in A. Note that the FGF-10 antibody was not immunoreactive with an nFGF-9 control (lane 1). Immunoblots from 4 independent experiments were digitized, and significance among the differences in relative band density was assessed for each lane relative to fetal Po2 control. *P < 0.05, ns, not significantly different.

To manipulate immune reactivity in pseudoglandular lung explants, we examined the effect of thymulin and its cofactor Zn2+ on the high-dose LPS-evoked decrease in explant ASC. Single administration of Zn2+ at concentrations representative of the umbilical circulation (21) yielded little effect on either ASC or indexes of cell death but, in combination with LPS, augmented the incidence of necrosis/apoptosis beyond other treatments at fetal and ambient Po2s. This compound effect was blocked by thymulin in the presence of a 10- to 1,000-fold molar excess of Zn2+, suggesting that the cytoprotective effect is linked to thymulin itself as opposed to incidental thymulin-Zn2+ chelation or the widely reported antiapoptotic properties of Zn2+.

Examination of the physiological mechanism behind the thymulin+Zn2+ cytoprotective effect revealed a bidirectional regulation of IL-6 and TNF-α expression during an LPS inflammatory stimulus. Bioactive thymulin has previously been observed to inhibit LPS-evoked expression of both TNF-α and IL-6 in peripheral blood mononuclear cells (39, 40); however, to our knowledge, selective (ant)agonistic regulation of these cytokines in fetal lung has not previously been reported. Although TNF-α potently induces genomic expression of IL-6 along with other acute-phase response cytokines, raised IL-6 titers can suppress TNF-α expression and are thought to regulate circulating [TNF-α] during infection (28, 41, 44). IL-6 negative feedback of TNF-α synthesis therefore represents one mechanism that could account for thymulin+Zn2+ cytoprotection.

To investigate this effect, we examined the capacity for thymulin+Zn2+ to alter p38 MAPK-transduced MAPKAP-K2 activity. This terminal kinase plays a pivotal role in the phosphorylation of RNA binding proteins, particularly heterogeneous ribonucleoprotein A0 (38), which govern protein interactions with the AU-rich element (ARE) in the 3′-untranslated region of both TNF-α and IL-6 genes. Whereas MAPKAP-K2 phosphorylation of ARE-binding proteins is necessary for the initiation of TNF-α transcription and nucleo-cytoplasmic mRNA transport, its role in the regulation of IL-6 expression is solely linked to the stabilization of nascent mRNA (33). We reasoned that if thymulin+Zn2+ treatment abolished LPS-evoked MAPKAP-K2 activity, the potential would exist for attenuated IL-6 expression to persist under conditions that completely block TNF-α posttranscriptional processing. Rather than showing an inhibition, our results revealed a potent activation of MAPKAP-K2 in Zn2+-supplemented A549 cells in response to thymulin. Although we failed to demonstrate a similarly concise pattern of MAPKAP-K2 activation in explants, presumably due to our choice of culture conditions, we showed that the activity found in the presence of LPS and thymulin+Zn2+ is largely abolished by the p38 MAPK inhibitor SB-203580. Aside from differences in IL-6 and TNF-α mRNA abundance in control explants at either Po2, we found a sustained expression of both mRNAs that, in the case of IL-6 by thymulin+Zn2+, occurred irrespective of LPS treatment. Notably, transcriptional blockade using the NF-kB inhibitor SSA resulted in the degradation of both transcripts; therefore, this pattern of mRNA expression required continuous transcriptional activity and was not solely due to posttranscriptional mRNA stabilization. Moreover, antisense blockade of thymulin-evoked IL-6 expression did not significantly raise TNF-α mRNA abundance (data not shown) and did not relieve the suppressive effect of this hormone on LPS-evoked TNF-α protein synthesis (Fig. 7A). Our results therefore suggest that the bidirectional change in TNF-α and IL-6 protein synthesis does not stem from IL-6-inhibited TNF-α expression and cannot be accounted for by blockade of the p38 MAPK/MAPKAP-K2 pathway. The silencing of LPS-stimulated TNF-α synthesis by thymulin must occur either by an alternative posttranscriptional mechanism or by increased proteolytic targeting.

In addition to cytoprotection by the thymulin+Zn2+ blockade of TNF-α synthesis, raised expression of IL-6 may directly promote mesenchyme regeneration and repair through a mitogenic response transduced by JAK/STAT, Ras/MAPK, and phosphatidylinositol 3-kinase pathways. Transgenic overex-

AJP-Lung Cell Mol Physiol • VOL 286 • MARCH 2004 • www.ajplung.org
expression of IL-6 in mice markedly extends survival during exposure to 100% O₂, an effect associated with conserved pulmonary expression of antiapoptotic factors such as Bcl-2 and metalloproteinase-1 (49). Moreover, IL-6 genomic knockout illustrates the key role of this cytokine in acute chemotactic signaling via VCAM-1 as well as regulating the expression levels of IL-4, -5, and 13 in bronchiolar lavage (47). Among the identified antiapoptotic properties of IL-6 is a capacity to inhibit transforming growth factor-β (TGF-β)-mediated apoptosis (10, 47), an important regulator of mesoepithelial differentiation and, possibly, alveolarization (8, 23). This suggests that when hyperexpressed, IL-6 regenerative repair may interfere with the normal clearance of tissue necessary for the formation of space encapsulating structures, as well as in promoting fibrosis, and presents one mechanism for the proliferation of mesenchyme induced by thymulin + Zn²⁺ treatment in this study.

To examine this possibility further, we focused on the relationship between IL-6 and its nuclear factor, C/EBPβ, a basic-region leucine zipper transcription factor family member whose activity is potentiated by homo- or heterodimerization with either C/EBPα, -δ, or NF-κB (1, 20, 37). Morphogenic roles have been postulated for C/EBP isoforms in the lung due to their capacity to regulate cellular turnover and differentiation. Genomic knockout of the α- and β-isoforms results in breathing difficulties from birth, cyanosis, high postnatal mortality, and, in the case of the α-isoform, hyperproliferation of alveolar type II cells and interstitial thickening (14, 17, 48). More specifically, both the α- and β-isoforms regulate the expression of Clara cell secretory protein, an important anti-inflammatory and cytoprotective agent of the bronchial airway (9). Our results highlight a specific role for the β-isoform in mediating mesenchyme proliferation in rat lung explants. We show a heterogeneous pattern of C/EBPβ activation in the mesenchyme mass of gestation day 16 fetal rat lung, which was present in explants maintained at 23 mmHg but absent at ambient P0₂ or in the presence of an IL-6 antisense oligonucleotide. Significantly, C/EBPβ and NF-κB, but not C/EBPα or -δ, were synergistically activated under treatment with thymulin+Zn²⁺, either with or without LPS, pointing toward a strong association between thymulin immunomodulation and C/EBPβ regulation of the acute-phase response.

Precise developmental roles for C/EBPβ in the lung are poorly defined, partly because C/EBP family members possess conserved COOH-terminal domains and so may functionally substitute for one another in knockout experiments. In hepatic and colonic tissue, C/EBPβ represents a key component of the proliferative response to oxidative injury and TGF-α-evoked ribosomal protein S-6 kinase activation and is known to mediate mesenchyme proliferation during tissue repair (7, 11). It is also involved in regulating the activation and terminal differentiation of macrophages in lymphatic tissues (43), an observation that brings it into close functional proximity with the established role of bioactive thymulin in the thymus. Our experiments showed that activation of C/EBPβ by LPS and thymulin+Zn²⁺ initiated a hyperexpressed regenerative repair response located in the undifferentiated mesenchyme tissue fraction that, coupled with an inhibition of apoptosis, resulted in the near complete loss of differentiated epithelial structures (Figs. 7–9). Each of the core characteristics of this response (C/EBPβ activation, mesenchyme proliferation, apoptosis blockade, loss of epithelial structures) was reversed by addition of IL-6 antisense oligonucleotides, which muted IL-6-evoked positive feedback of C/EBPβ gene expression, an effect that sustains IL-6 acute-phase response signaling (2). Together, our observations accord with an interpretation of C/EBPβ as a nuclear factor that regulates regional, proportionate, mesenchyme proliferation in the pseudoglandular stage lung and overt proliferation during lung regenerative repair.

As reciprocal signaling between mesenchyme and epithelium drives lung morphogenesis, we examined the association between IL-6- and C/EBPβ-coupled signaling through thymulin+Zn²⁺ on FGF-9 and -10 protein expression. In the pseudoglandular stage, FGF-9 mRNA is expressed in the proximal mesenchyme, and its expression is regulated by IL-6. FGF-9 promotes mesenchyme proliferation and differentiation, and its expression is induced by IL-6. Our results highlight the importance of IL-6 in regulating FGF-9 expression during lung development.

**Fig. 10.** Summary diagram showing the interaction between bioactive thymulin and the immunogenic expression of TNF-α and IL-6 in rat pseudoglandular lung explants. Immunomodulation by thymulin+Zn²⁺ displays 4 distinct features: synergistic activation of C/EBPβ and NF-κB (I) is coupled with an activation of the p38 MAPK pathway resulting in increased MAPKAP-K2 activity (II). This yields a direct increase in the genomic expression and transcript stabilization of IL-6 (III), which may be coupled, either directly or indirectly (via NF-κB activation), with raised TNF-α mRNA expression. Whereas IL-6 becomes fully expressed and mediates a positive feed-back on C/EBP expression/activation, bioactive thymulin silences the expression of TNF-α protein through a posttranscriptional event (IV). As a consequence of dominant IL-6 and FGF-9 expression, rates of necrosis and apoptosis diminish, yielding a proliferation of mesenchyme as a core feature of regenerative repair in the lung.
pleura and transiently in the bronchial epithelium and stimulates proliferation and expansion of the mesenchyme compartment via the receptors FGFR1c and/or FGFR2c (13). FGF-10 is expressed in the mesenchyme adjacent to the distal termini of the developing airways, becoming diffusely expressed in mesenchyme toward the end of this developmental stage (5). Whereas FGF-10 is believed to initiate airway branching by interaction with FGFR2b, FGF-9 regulates the density of this branching through proliferation and expansion of mesenchyme (12, 13); therefore, the proportion of FGF-9 to FGF-10 expression in response to treatments that augmented IL-6 release can be used as an index of airway branching potential. Our immunohistochemical experiments showed that the distribution pattern of both factors responded to changes in culture PO2 (12, 13); therefore, the proportion of FGF-9 to FGF-10 expression in response to treatments that augmented IL-6 release accounted for the immunomodulatory effect of this hormone, probably via C/EBPβ, and showed a mass-specific increase in expression in response to treatments that augmented IL-6 release and mesenchyme proliferation. Given that a putative C/EBPβ-binding element has been reported to reside in the 5′-flanking region of the mouse FGF-9 gene (12) and that IL-6-regulated FGF-9 expression has been shown to trigger proliferation of precursor megakaryocytes in the thrombopoietic response (27), we believe our results highlight the potential for cooperative signaling between IL-6, C/EBPβ, and FGF-9 in initiating the proliferation of mesenchyme as an early phase of the tissue repair response in the lung. Notably, this proliferative effect occurred at the expense of lung mass occupied by airway and vascular structures and highlights the presence of a crucial signaling lesion at the level of mesenchyme redifferentiation.

Overcoming this block will require reinstatement of the pathways that direct epithelial and endothelial redifferentiation with the temporal and spatial signaling pattern that drives lung morphogenesis.

Figure 10 summarizes our studies into the morphogenic response of fetal rat lung explants to treatment with thymulin + Zn2+ and identifies four regulatory components that account for the immunomodulatory effect of this hormone, namely, selective activation of C/EBPβ coupled with stress-evoked NF-κB activity, postranscriptional regulation through p38 MAPK-dependent MAPKAP-K2, increased potential for IL-6 expression, and interference with the turnover pathway for TNF-α protein. Cessation of TNF-α synthesis in association with the antiapoptotic and proliferative properties of IL-6 results in conditions that favor the proliferation of mesenchyme, which may arise as a direct result of IL-6/C/EBPβ positive feedback coupled to FGF-9 signaling. The scope for alveolar development and structural remodeling in newborn infants depends in part on the conservation of mesenchyme tissue from which new pulmonary structures develop (5, 12, 13, 19). Our study illustrates the high potential of bioactive thymulin to initiate mesenchyme proliferation in the fetal lung as a fundamental component of the tissue regenerative repair response. The therapeutic potential of this effect will require tailoring this response to operate with agents and/or conditions that facilitate the regeneration of airway and vascular structures from mesenchymal tissue.

ACKNOWLEDGMENTS

We are grateful to Drs. Kenneth Muir, Scott Nelson, and Tony Davies for help with morphometry and kinase assays and several useful discussions.

Thanks also to Prof. Robert Hume for access to microscopy equipment and Katherine Fyfe for valuable technical assistance.

GRANTS

This work was supported by the Medical Research Council (UK), the Wellcome Trust, Tenovus (Scotland), and the Anonymous Trust.

REFERENCES


