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

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 (51). 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 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 (51). 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).

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, 34, 35, 42). 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.


Address for reprint requests and other correspondence: S. C. Land, Tayside Inst. of Child Health, Maternal and Child Health Sciences, Ninewells Hospital and Medical School, Univ. of Dundee, Dundee, DD1 9SY, UK (E-mail: s.c.land@dundee.ac.uk).
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-α and potentiates IL-6 expression through a CCAAT-enhancer binding protein-β (C/EBPβ)-dependent pathway, an established route for regenerative repair in other tissues. The hyperexpression of this pathway was associated with a mass proliferation of undifferentiated mesenchyme tissue involving raised expression of fibroblast growth factor 9 (FGF-9), which was matched by a loss of differentiated space encapsulating structures in the fetal lung. These results demonstrate the presence of an intact thymulin-signaling pathway in the fetal lung that may actively determine 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 IgGα, C/EBPβ (H-7) antibodies plus 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-Diamidino-2-phenylindole, dihydrochloride (DAPI)-antifade was from Sigma-Aldrich (Gillingham, Dorset, UK). Recombinant human heat shock protein (HSP) 70 (C-17), and 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-Diamidino-2-phenylindole, dihydrochloride (DAPI)-antifade was 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 excised and washed free of erythrocytes in ice-cold, sterile Dulbecco’s modified Eagle’s medium (DMEM). Single lung lobes were then mounted on 13-mm diameter Whatman Nucleopore Track Etch membranes (pore diameter of 8 μm) 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 PO₂ of 142 (ambient) or 23 mmHg (fetal) plus 5% CO₂ 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-cm² flasks (Greiner Bio-one, Frechhausen, Germany) in DMEM supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin at either fetal or ambient PO₂. 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 μg/ml of E. coli LPS at fetal or ambient PO₂. 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 L7Z 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 μg/ml LPS, 10 μM ZnCl₂, or LPS together with ZnCl₂. Thymulin was administered to LPS- and ZnCl₂-exposed explants at concentrations of 0, 0.1, 10, or 1,000 ng/ml. Sulfasalazine (SSA, 1 mM), a blocker of nuclear factor (NF)-κB 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, electronmicroscopic mobility shift assay (EMSA), oligonucleosome release, and immunoprecipitation.

The dose-response relationship between thymulin and IL-6 or TNF-α secretion in the presence and absence of LPS and ZnCl₂ 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 × 10⁶/ml in Costar six-well culture flasks (Corning) and were maintained at fetal or ambient PO₂ 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 μM ZnCl₂ for 2 h either alone or in combination with 10 μg/ml LPS. TNF-α and IL-6 release was measured by enzyme-linked immunosorbent sandwich assay (ELISA) as detailed under Cytokine ELISA. Fifty-percent inhibitor constant (IC₅₀) and effector constant (EC₅₀) values were calculated using four-parameter sigmoidal curve regression based on the equation y = a + (Y range/1 + 10^((x-y)/s)), where a is the background Y value and s is the Hill slope. Note that 10 μg/ml LPS was the minimum dose to produce a maximal sustained release of TNF-α 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) MTI. The rate of MTI 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 mM/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 μg/ml LPS together with 10 μM ZnCl₂ and 0.1, 10, or 1,000 ng/ml thymulin. An additional experimental set was conducted in the presence of thymulin plus 10 μM 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)/√area (mm²) using Scion Image 4.0.2 software (Scion, Frederick, MD) as described previously (16). Calibration was achieved using a 5 × 2-mm grid removed from a Fast-Read 102 disposable cell-counting grid (Nunc, Naperville, IL, USA) that was placed onto the meniscus of the medium in each well.

Immunohistochemistry was performed on PBS-rinsed, filter-attatched 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 CEBPb (H-7) antibodies each at 1:200 dilution followed by 5 μl of DAPI-antifade to each section before mounting. Images were obtained under a Zeiss Axioskop fluorescence 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 spectrophotometrically using a Beckman Coulter (Fullerton, CA) DU 650 spectrophotometer at 340 nm by adding 20 or 100 μl of medium to 980 or 900 μl of 50 mM imidazole buffer (pH 7.0) containing 200 μM NADH at 37°C. After measurement of any endogenous rate, we started the reaction by adding 1 mM Na+-pyruvate. Units of LDH activity as an index of necrotic cell lysis were expressed as mM NADH oxidized/min 1 mg secreted protein−1.

We determined apoptosis in lysed explants using a oligonucleosome detection ELISA according to the manufacturer’s instructions. Briefly, intact nuclei were removed from the lysate by centrifugation, and 20 μl of the cleared supernatant were placed into each well of a 96-well plate previously coated with an antihistone antibody. After incubation for 30 min, the plates were washed three times and a peroxidase-conjugated anti-DNA antibody was added to each well. After a further 30 min, the wells were washed as before, and the density of oligonucleosomes was captured by the anti-histone/anti-DNA complex was determined by the addition of 2′-azino-di-[3-ethylbenzthiazoline sulfonate] chromagenic substrate. Incidence of apoptosis was expressed as the fractional difference between the background-corrected spectrophotometric absorbance at 490 nm of controls vs. experimental groups.

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 125–8,000 pg/ml (IL-6 and IL-1β) and were used on condition that a R2 value >0.90 was obtained.

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 extract 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 32P-oligonucleotides (1 ng) and nuclear extracts (1 μg) were incubated and samples were then incubated on ice for 30 min. Supershift reactions were done by incorporating 1 μl of monoclonal antibody to C/EBPb or NF-κB p65 for 30 min before the addition of labeledprobe. 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 extract and serum-starved A549 cultures by following the phosphorylation of its specific substrate, HSP27. Reactions were carried out with 20 μg of protein per sample at 37°C in a buffer containing 100 mM Tris-acetate (pH 7.4), 50 mM β-glycerophosphate, 2.5 mM MgCl₂, 1.5 mM EGTA, 1 μM DTT, 0.5 mM Na+-orthovanadate, 0.25 mM ATP, 2 μCi [γ-32P]ATP, 2 μM microcystin, and 1 tablet per 25 ml of Complete proteinase inhibitor cocktail (Roche). Reactions were initiated by addition of 1 μg of recombinant human HSP27. After 30 min, we stopped the reaction by adding SDS to a final concentration of 1% and denatured the samples by heating them for 5 min to 95°C. Proteins were 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 Transcriptionase 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′-CTTCCCTACTTCAAGAGGCC-3′ (sense) and 5′-GCCACAGTGAAGATGCT-3′ (antisense), respectively. A 540-bp TNF-α product was generated with primers derived from nucleotides 4,568–4,577 and 5,939–5,958 of rat TNF-α cDNA (GenBank accession no. L00981) and were 5′-CGCTTCTTCTGCTACTGAAC-3′ (sense) and 5′-TTTCCACTCTGAGAAGACCT-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 ng/ml of each primer to a final Tris-X-100, 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

Downloaded from http://ajplung.physiology.org/ by 10.22.32.247 on October 28, 2017
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 precleared in 10 μl of BSA-blocked protein A-Sepharose and incubated overnight at 4°C with gentle agitation. Immunoprecipitated 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 MX 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). Note that this approach controls for the permeability of the porous filter support and accounts for the generally high concentrations of LPS (and presumably thymulin) required to produce an immunogenic effect in this study. Thymulin dosage extended over the range of TNF-α and IL-6 inhibitory constant (IC₅₀) and EC₅₀ values for A549 cells derived from Fig. 4, A and B.

**Characterization of explant ASC responses to culture P O₂ and LPS.** As native Po₂ 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. *P < 0.05 relative to control (0 h) 23 mmHg; †P < 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 (ASC₉₆–ASC₀/ASC₀ h) at fetal and ambient P O₂: • with solid line, explants maintained at 142 mmHg; ○ with dotted line, explants at 23 mmHg. Lines were fitted using log normal 4-parameter regression. *P < 0.05 relative to 142 mmHg control for 96 h; †P < 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 PO2 were conserved over a
range of 0.01–10 μg/ml LPS, beyond which the fractional
change in ASC (ASC0–96h/ASC0h) fell significantly
to 0.8 ± 0.2 (Fig. 1, B and C). Mean epithelial thickness for
cultures maintained at fetal PO2 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 PO2 or
exposed to 50 μg/ml LPS at either PO2.

Explants maintained at ambient PO2 showed a dynamic
growth response to LPS. Despite a significant reduction in
ASC0–96h 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 ASC0–96h
value of 1.7 ± 0.3 at 2 μg/ml LPS. As with the fetal PO2
explants, LPS concentrations at 50 μg/ml reduced the ASC0–96h
to 0.7 ± 0.1; therefore, these conditions were selected to test
the cytoprotective potential of thymulin+Zn2+ as being the
most disruptive to airway growth.

Thymulin+Zn2+ is cytoprotective during exposure to high
LPS concentrations at fetal and ambient PO2. The cytoprotective
potential of thymulin-Zn2+ was tested in explants exposed to
concentrations of LPS that exceeded the range shown to
promote airway bifurcation at fetal or ambient PO2 (Fig. 1).
ASC0–96h was related to the incidence of necrosis (LDH
leakage) or apoptosis (oligonucleosome release) in the pres-
ence of 50 μg/ml LPS; 10 μM ZnCl2; or 0.1, 10, or 1,000
ng/ml thymulin (Fig. 2). At fetal PO2, none of the treatments
altered the incidence of apoptosis significantly from the con-
trols; however, administration of Zn2+ or LPS+Zn2+ signifi-
cantly raised LDH leakage over control values. Each dose of
thymulin lowered the incidence of LPS+Zn2+-induced necro-
sis to a level that was not statistically significant from controls.

This was not associated, however, with any recovery of ASC0–96h
to control values.

At ambient PO2, tissue damage patterns evoked by moderate
hyperoxia or LPS followed a different pattern. Necrotic LDH

![Fig. 2. Thymulin+Zn2+ is cytoprotective but does not evoke airway proliferation during exposure to lethal LPS+Zn2+ at fetal and ambient PO2. Explants were main-
tained for 96 h under the conditions indicated. Release of lactate dehydrogenase (LDH, A) and oligonucleosomes (B)
were taken as indexes of necrosis and apoptosis, respec-
tively, and are presented compared with the fractional
change in airway surface complexity (ASC0–96h; C) at fetal
or ambient PO2 for 96 h. *P < 0.05 or **P < 0.01 relative
to control group at either PO2; means ± SE, n = 8.](http://ajplung.physiology.org/)
leakage remained relatively constant among all treatment groups; however, LPS+Zn²⁺ evoked a marked increase in apoptotic oligonucleosome release that was abolished by administration of thymulin at each concentration. ASCP₀⁻⁹₆h was modestly raised by thymulin+Zn²⁺ treatment becoming statistically significant at the highest dosage of thymulin.

Thymulin+Zn²⁺ evokes IL-6, and suppresses TNF-α, release at ambient, but not fetal P O₂. To determine whether thymulin+Zn²⁺ 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 Zn²⁺-treated explants showed any change in the spontaneous release of IL-6 at either P O₂ (Fig. 3A); however, thymulin administered with Zn²⁺ increased IL-6 secretion under both conditions, becoming statistically significant at ambient P O₂. Likewise, LPS evoked a significant increase in IL-6 release, which was diminished in combination with Zn²⁺, an effect that may stem from the increased rate of apoptosis observed under these conditions in Fig. 2. Notably, administration of thymulin at 10 and 1,000 ng/ml conserved the release of IL-6 in LPS+Zn²⁺-treated explants at levels that were significantly greater than controls but did not differ statistically from LPS or thymulin+Zn²⁺-treated explants.

TNF-α release did not change significantly in control explants maintained under either P O₂ or in the presence of Zn²⁺ or thymulin+Zn²⁺ (Fig. 3B). In keeping with the key role of this cytokine in the immunogenic acute-phase response, LPS significantly raised the expression of TNF-α at ambient P O₂, which was sustained on addition of Zn²⁺; as with the IL-6 response under these conditions, statistical significance beyond control levels remained elusive, presumably due to high rates of apoptosis. Nevertheless, thymulin administered at 0.1 and 1,000 ng/ml significantly lowered the release of TNF-α at ambient P O₂ below the levels observed with LPS+Zn²⁺.

As differences in size, cellular differentiation, and development presumably mask the kinetics of thymulin+Zn²⁺-evoked cytokine responses in explants, dose-response relationships were determined with a human pulmonary adenocarcinoma cell line (A549) that displays alveolar type II pneumocyte-like characteristics. We then correlated these results with the change in cellular proliferation by following the rate of MTT reduction in the presence of LPS+thymulin+Zn²⁺. As with the rat lung explants, thymulin+Zn²⁺ evoked a spontaneous, statistically significant rise in IL-6 release at ambient P O₂ (EC₅₀ = 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+Zn²⁺; however, incremental dosage with thymulin evoked a fivefold increase in the scope for IL-6 release at both P O₂ (EC₅₀ = 0.64 ± 0.34 (23 mmHg) and 1.43 ± 0.29 (142 mmHg)) over control cultures that had been similarly treated with thymulin+Zn²⁺ alone. In hand with the observed suppression of LPS+Zn²⁺-evoked TNF-α release in explants, incremental dosage with thymulin significantly inhibited the release of this cytokine from A549 cultures at either P O₂ over a dose range that showed similar kinetics for IL-6 release (IC₅₀ = 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+Zn²⁺ in the absence of thymulin showed high 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 P O₂.

Thymulin+Zn²⁺ augments activity of the nuclear factors of IL-6 transcription. The preceding results suggest that thymulin+Zn²⁺ 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+Zn²⁺, LPS+Zn²⁺, and LPS+thymulin+Zn²⁺ at ambient P O₂ (Fig. 5A). In the absence of SSA, fetal P O₂ 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 P O₂ (Fig. 5, B and C). Independent administration of thymulin or Zn²⁺ 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 P O₂ control. Notably, the C/EBP activation by thymulin+Zn²⁺ and LPS+thymulin+Zn²⁺ diminished to control levels on inclusion of an antisense oligonucleotide.

Fig. 3. Effect of thymulin+Zn²⁺ on LPS-evoked secretion of TNF-α (B) and IL-6 (A) in explants maintained at fetal or ambient P O₂. Explants were maintained for 96 h either under control conditions or in the presence of LPS (50 μg/ml), Zn²⁺ (10 μM), thymulin (1,000 ng/ml + ZnCl₂), or LPS + Zn²⁺ in combination with 0, 0.1, 10, or 1,000 ng/ml thymulin. *P < 0.05 relative to control at each P O₂. †P < 0.05 relative to LPS+Zn²⁺ + 0 ng/ml thymulin; n = 4 (IL-6) and n = 5 (TNF-α); nd, not detectable.
served with thymulin

Zn\(^{2+}/H11001\) as a negative control, and two doses of thymulin exposed for 60 min to LPS as a positive control, LPS together

tative experiment conducted in serum-starved A549 cultures

activate MAPKAP-K2 via the phosphorylation of a speci

cleotide against IL-6, suggesting a positive feedback relationship between IL-6 and C/EBP expression and activation. Additionally, SSA abrogated C/EBP and NF-κB DNA binding activity in a manner that was consistent with the blockade of IL-6 release in Fig. 5A. Supershift experiments identified the C/EBP\(^\beta\) isoform as the predominantly active species of this transcription factor in explants at either fetal PO\(_2\) or ambient PO\(_2\) in the presence of thymulin+Zn\(^{2+}\) (Fig. 5D).

MAPKAP-K2 activity and mRNA abundance of IL-6 and TNF-α are sustained by thymulin+Zn\(^{2+}\). 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+Zn\(^{2+}\) 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+Zn\(^{2+}\). HSP phosphorylation was weakly evident in A549 cells at ambient PO\(_2\) 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+Zn\(^{2+}\) alone. The activation observed with thymulin+Zn\(^{2+}\), 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, PO\(_2\). Expression was potently induced at ambient PO\(_2\) 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 PO\(_2\), but potently expressed at ambient PO\(_2\). This expression was not altered significantly by treatment with LPS or thymulin but was abolished by SSA.

Thymulin+Zn\(^{2+}\) evokes mesenchyme hyperplasia in the fetal lung. To determine whether the cytoprotective effects of thymulin+Zn\(^{2+}\) 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 PO\(_2\) observed in Fig. 2. The differentiated epithelial fraction of each explant was calculated from histological sections as described in experimental procedures (Fig. 7).

Explant IL-6 secretion evoked by LPS+thymulin+Zn\(^{2+}\) was abolished in the presence of IL-6 antisense oligonucleotide but remained unaltered by addition of a random sequence control oligonucleotide (Fig. 7A). Significantly, antisense treatment did not abolish the suppressive effect of thymulin+Zn\(^{2+}\) on LPS-stimulated TNF-α release, suggesting that the TNF-α silencing effect we observed with this hormone does not stem from a suppressive IL-6 feedback effect.

Figure 7B 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 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 PO\(_2\) cultured explants to LPS+thymulin+Zn\(^{2+}\) 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 Po2 explants. IL-6 antisense oligonucleotide applied under the same conditions as the control oligonucleotide raised the differentiated epithelial fraction to ~30% of total explant surface area but significantly increased LDH leakage and apoptosis beyond that observed with any other treatment.

Thymulin + Zn2+ 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/EBPβ 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/EBPβ immunoreactivity was exclusively mesenchymal and exhibited focal pockets of intense nuclear staining around airway structures. Figure 8B demonstrates FGF-9, FGF-10, C/EBPβ, 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 µg/ml LPS, LPS and 10 µmol ZnCl2, 1.000 ng/ml thymulin, thymulin + Zn2+ + 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 + Zn2+ resulted in homogeneous FGF-9 expression, mesenchyme proliferation, and a loss of differentiated epithelial structures but little fragmentation of nuclear DNA. Addition of IL-6 antisense oligonucleotides muted the mesenchyme hyperplasia but also resulted in distinct DNA fragmentation in the mesenchyme 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 Po2 controls. FGF-10 immunostaining showed regionally intense pockets of staining under all conditions but became diffuse in LPS + thymulin + Zn2+-treated explants. C/EBPβ 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 + Zn2+. Addition of an IL-6 antisense oligonucleotide entirely abolished this effect, whereas rrIL-6 evoked strong nuclear accumulation of C/EBPβ in all tissue compartments.
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 P O 2 explants in both LPS+/thymulin+/Zn 2+ -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 < 0.05) and total airway space was significantly (P < 0.01) diminished relative to explants maintained at ambient P O 2 . Notably, ambient P O 2 treatment of explants with rrIL-6 resulted in a distribution of airway space, mesenchyme, and epithelium that was not significantly different from explants maintained at fetal P O 2 . 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 mesenchymal 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+/Zn2+ coupled with IL-6 antisense, 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 PO 2s 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 PO 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 atrophy typical of 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 morphogenic 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.

Fig. 8. Distribution of FGF-9, FGF-10, and C/EBPβ expression in native gestation day 16 rat lung and in fetal rat lung explants treated with LPS + thymulin+Zn2+ coupled with IL-6 antisense. A: immunohistochemical detection of FGF-9, FGF-10, and C/EBPβ in native gestation day 16 rat lung. Row a: low-magnification images (×100, bar = 345 μm) of each protein; row b: higher magnification (×400 for FGF-9 and -10, bar = 50 μm; ×200 for C/EBPβ, bar = 175 μm); row c: control staining (×100) where nonimmunoreactive goat serum replaced each antibody. B: immunohistochemical detection of FGF-9, FGF-10, and C/EBPβ together with nuclear 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining in explants maintained at fetal or ambient P O 2 or at ambient P O 2 in the presence of LPS, LPS+thymulin+Zn2+ + random sequence control oligonucleotide (LTZ+CAs), LTZ + IL-6 antisense oligonucleotide (LTZ + IL6As) or recombinant rat IL-6 (rrIL6) at concentrations as indicated in Fig. 7. Scale: FGF-9 and FGF-10, bar = 345 μm; C/EBPβ and DAPI, bar = 50 μm. Images are representative of 4–6 independent experiments.
A molar excess of Zn$^{2+}$ was blocked by thymulin in the presence of a 10- to 1,000-fold under the same conditions as in FGF-9 and -10 mass-specific protein expression in explants maintained in independent experiments were digitized, and significance among the differences is assessed using ANOVA post hoc Tukey significance was assessed using ANOVA post hoc Tukey’s honestly significant difference relative to fetal (23 mmHg, α) or ambient (142 mmHg, β) Po$_2$. 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 rrFGF-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 Po$_2$ 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 Zn$^{2+}$ on the high-dose LPS-evoked decrease in explant ASC. Single administration of Zn$^{2+}$ 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 Po$_2$s. This compound effect was blocked by thymulin in the presence of a 10- to 1,000-fold molar excess of Zn$^{2+}$, suggesting that the cytoprotective effect is linked to thymulin itself as opposed to incidental thymulin-Zn$^{2+}$ chelation or the widely reported antiapoptotic properties of Zn$^{2+}$.

Examination of the physiological mechanism behind the thymulin+Zn$^{2+}$ 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+Zn$^{2+}$ cytoprotection.

To investigate this effect, we examined the capacity for thymulin+Zn$^{2+}$ 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+Zn$^{2+}$ 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 Zn$^{2+}$-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+Zn$^{2+}$ 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 Po$_2$, we found a sustained expression of both mRNAs that, in the case of IL-6 by thymulin+Zn$^{2+}$, occurred irrespective of LPS treatment. Notably, transcriptional blockade using the NF-κB 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+Zn$^{2+}$ 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-
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-isofoms results in breathing difficulties from birth, cyanosis, high postnatal mortality, and, in the case of the α-isofom, hyperproliferation of alveolar type II cells and interstitial thickening (14, 17, 48). More specifically, both the α- and β-isofoms 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-isofom 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 Po₂ 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

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 feedback 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 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 and immunomodulation by thymulin.

ACKNOWLEDGMENTS

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

REFERENCES


