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Interleukin-4 suppresses the expression of macrophage NADPH oxidase heavy chain subunit (gp91-phox)

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Abstract

The production of superoxide anion by NADPH oxidase is a principal nonspecific bactericidal activity of macrophages and neutrophils in host defense. However, exuberant production of superoxide anion also damages host tissues. Cloning and DNA sequencing of the 91 kDa subunit (gp91-phox) open reading frame indicated a high degree of sequence conservation, greater than 90% in nucleotide and amino acid sequences, between the porcine and human cDNAs. We show in pigs that interleukin-4 (IL-4), a T lymphocyte cytokine which plays a major role in mediating antibody responses to pathogens, suppresses superoxide anion production in macrophages by specifically reducing the level of mRNA encoding gp91-phox. Messenger RNA levels are suppressed approx. 70% within 4 h and persist for 24 h without any change in the rate of mRNA turnover. Nuclear run-on analysis showed that IL-4 did not alter the rate of gp91-phox gene transcription under conditions in which IL-1β transcription was inhibited. These results indicate that IL-4 suppresses the inflammatory response of macrophages by mechanisms that include post-transcriptional regulation of the 91 kDa catalytic subunit of NADPH oxidase, and transcriptional regulation of inflammatory cytokine expression.

Keywords: NADPH oxidase; gp91-phox; Interleukin-4; Macrophage; (Swine)

1. Introduction

Macrophages play a central role in host protection by initiating inflammatory and immune reactions against bacterial pathogens. They elicit an innate, non-specific inflammatory response by producing inflammatory cytokines and toxic oxygen radicals. The production of toxic oxygen radicals including superoxide anion (O_2^-) is an important mechanism of microbial killing by macrophages.

Formation of superoxide anion is catalyzed by a membrane-associated enzyme system, NADPH oxidase, which is an electron transport, multicomponent complex consisting of several membrane-bound and cytosolic subunits [1–3]. The membrane-bound component of NADPH oxidase, also known as cytochrome *b*, is a heterodimer consisting of 91 kDa glycoprotein heavy chain (gp91-phox) and 22 kDa polypeptide light chain (p22-phox) subunits [4,5]. It has been shown that the membrane components of NADPH oxidase can be activated by chemoattractants, the tumor promoter phorbol myristate acetate (PMA), or the calcium ionophore A23187 [6]. Recent studies revealed that human macrophages and neutrophils treated with interferon (IFN)-γ, tumor necrosis factor-α (TNF) and the bacterial cell wall product, lipopolysaccharide (LPS), can be induced to express increased levels of gp91-phox protein and mRNA [7–12].

Although important for microbicidal activity, an increasing body of evidence indicates that free radicals derived from oxygen, including superoxide anion, play a role in mediating tissue damage and inflammatory diseases either directly or indirectly through the generation of other inflammatory mediators [13–16]. Superoxide anion causes lung tissue damage both in vivo and vitro in human and animal models [17–21]. In addition, various clinical disorders and inflammatory diseases, including inflammatory bowel disease [22], acute pancreatitis [23], renal diseases such as glomerulonephritis and vasculitis [24], skin inflammation [25], acalculous cholecystitis [26] and uveitis [27],

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are associated with aberrant or over-production of superoxide anion or other reactive oxygen intermediates. Moreover, recent studies on HIV induction of superoxide anion generation suggest an important role in AIDS disease progression [28].

Interleukin-4, also known as B cell stimulatory factor-1 (BCSF-1) or B cell growth factor (BCGF), is a 20 kDa protein produced by activated T_H2 cells which activates the resting B cell to proliferate and increases markedly the production of antibodies [29,30]. In addition to its stimulating properties, however, an increasing body of evidence shows that IL-4 possesses anti-inflammatory effects on monocytes and macrophages. It has been shown to down-regulate the expression and secretion of IL-1, IL-6, IL-8 and TNF in human and porcine monocytes and macrophages stimulated with IFN-γ or LPS [31–36].

The observations that IL-4 suppresses the induction of inflammatory cytokine expression and the production of superoxide anion suggests that IL-4 is an important negative regulator of macrophage inflammatory activities. We were therefore interested in determining if IL-4 suppressed superoxide anion production at the transcriptional level similar to its effect on inflammatory cytokine levels. We found that IL-4 suppresses superoxide anion production in porcine alveolar macrophages by reducing the level of gp91-phox mRNA posttranscriptionally without affecting mRNA stability.

2. Materials and methods

2.1. Reagents

Human recombinant IL-4 and porcine recombinant TGFβ1 were obtained from R & D Systems (Minneapolis, MN).

2.2. Alveolar macrophages

Alveolar macrophages were collected by lung lavage from normal 6–8-week-old pigs. Cells were washed with endotoxin-free PBS and cultured in DMEM with 2% pig serum and antibiotics. The cells were allowed to incubated at 37°C with 5% CO_2 overnight before treatment.

2.3. Probes

The human gp91-phox cDNA probe used for screening of a cDNA library was a 1.8 kb *Xho*I fragment cloned into pCDM-8 and was kindly provided by Dr. Stephen Chanock (The Children's Hospital, Boston, MA). The porcine p22-phox cDNA was a 786 bp fragment containing the complete 576 bp coding sequence cloned in our laboratory (GenBank accession number UO2477). β-Actin was a 706 bp cDNA cloned by reverse transcriptase-polymerase chain reaction amplification of porcine fibroblast mRNA (GenBank accession number U07786). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was a 1.25 kb *Pst*I fragment of human GAPDH cDNA and was kindly provided by Dr. William A. Toscano (Tulane University, New Orleans, LA). All probes were labeled with [^{32}P]dCTP (Amersham, 3000 Ci/mmol) by random hexanucleotide priming to a specific activity of $0.5–1.0 \cdot 10^9$ cpm/μg.

2.4. cDNA library construction and screening

A porcine macrophage cDNA library was constructed in λgt10 by Clontech (Palo Alto, CA) using mRNA from porcine alveolar macrophages stimulated with LPS. Approximately 100000 plaques were screened with a ^{32}P-labeled human gp91-phox cDNA probe. Two positive clones were identified by screening. One clone (λ90A) was further analyzed by restriction digestion with *Eco*RI. Two restriction fragments of 1.6 kb and 0.7 kb were observed upon digestion. The 1.6 kb *Eco*RI fragment was isolated and subcloned into pGEM3Zf(−) plasmid and subjected to DNA sequencing.

2.5. PCR for 5′-end cloning

PCR primers to amplify the 5′-end of the porcine gp91-phox cDNA coding region were designed based on published human gp91-phox cDNA sequence and synthesized on an Applied Biosystems 391 DNA synthesizer. Reverse transcription-PCR was performed as described in Perkin-Elmer's RNA PCR kit with modifications. Briefly, total cellular RNA was isolated from pig alveolar macrophages stimulated with LPS and reverse transcribed into cDNA with oligo dT priming. The cDNA was denatured at 94°C for 4 min followed by 30 cycles of amplification in a 25 μl volume, with each cycle consisting of 93°C for 30 s, 55°C for 30 s and 72°C for 30 s. The amplified fragment was purified on a SpinBind column (FMC BioProducts, Rockland, ME) and was cloned into pGEM3Zf(−) for sequencing.

2.6. DNA sequencing and computer analysis

DNA sequencing was performed using the dideoxy-termination method [37] with vector-derived sequencing primers and nested internal specific oligonucleotide primers. Double stranded DNA templates were used for all of the reactions and were sequenced in both directions. Sequence analyses were performed with Intelligenetics and Eugene software packages at the Molecular Biology Computing Center and Department of Microbiology, University of Minnesota.

2.7. Superoxide assay

Superoxide production was quantified by measuring the superoxide dismutase (SOD)-inhibitable reduction of ferri-

cytochrome *C* as described by Peterson et al. [38]. Macrophages ($1.5 \cdot 10^5$ cells/well) were plated in 96-well plates and treated with IL-4 at various concentration for 24 h. Medium was removed and 120 μl of Hank's balanced salt solution containing 0.1% gelatin (GHBSS) alone or GHBSS containing 60 U SOD was added. Eighty μl of 80 μM ferricytochrome *C* with or without 200 nM PMA in GHBSS was added and the plates were incubated at 37°C for 30 min. Absorbance was read at A_{550} on a Perkin-Elmer Automated MicroPlate Reader. The amount of O_2^- release was calculated from the formula:

$$O_2^-/\text{well} = \text{absorbance} \cdot 15.87 \cdot 10^7/1.5 \cdot 10^5$$

and expressed as nmol/10^7cells/30 min [39].

2.8. Northern blot

Macrophage total cellular RNA was purified by acid-guanidinium-phenol extraction as described [40]. Northern blot assays were performed according to standard procedures [41] with the following modifications. Briefly, 20 μg of total cellular RNA was electrophoresed in a 1% agarose gel containing formaldehyde and transferred onto Magna-Graph nylon membranes (MSI, Westborough, MA), in 20 × SSC (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate). Membranes were pre-incubated at 42°C for 1 h in a hybridization buffer containing 1 mM EDTA, 0.5 M sodium phosphate (pH 7.2) and 7% SDS, then hybridized for 16–24 h at 42°C with the ^{32}P-labeled cDNA probes. After hybridization, membranes were washed twice for 20 min in 2 × SSC/0.1% SDS at 42°C and once for 20 min with 0.1 × SSC/0.1% SDS at 65°C. The hybridized mRNAs were visualized by autoradiography using Fuji FX film and Lightning-plus intensifiers (Kodak, Rochester, NY). Northern blot bands were quantified by densitometric scanning using a Shimadzu CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan) and normalized to either β-actin or GAPDH mRNA in the same sample.

2.9. Nuclear run-on assay

Alveolar macrophages were cultured for 4 h in the presence or absence of 1 μg/ml of LPS plus 50 ng/ml of recombinant human IL-4. To isolate nuclei, aliquots containing $6 \cdot 10^7$ cells were lysed in 4 ml of a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40. Nuclei were pelleted at 500 × g, resuspended in 200 ml of storage buffer (40% glycerol, 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) and frozen in liquid N_2. Run-on transcription reactions were performed as described [42].

Approximately 5 μg of linearized recombinant plasmid DNA for porcine NADPH-91 kDa subunit, NADPH-22 kDa subunit, NADPH-47 kDa subunit, IL-1β, TGFβ1, human GAPDH cDNA and pGEM3Zf(+) plasmid were applied to nylon filters using a slot-blot apparatus (Gibco BRL). Replicate filters were fixed by ultraviolet light and prehybridized for at least 4 h at 42°C in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt's solution, and 0.1% SDS. The ^{32}P-labeled RNA derived from each sample was adjusted to equivalent levels of radioactivity, added to the filters in fresh hybridization solution at a concentration of $1 \cdot 10^5$ cpm/ml, and incubated at 42°C for 40 h. The filters were washed in 2 × SSC and 0.1% SDS at 42°C for 30 min, and 0.1 × SSC and 0.5% SDS at 60°C for 30 min. Filters were then exposed to X-ray film and quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Suppression of porcine macrophage superoxide production by IL-4

Interleukin-4 inhibits the expression of a variety of inflammatory cytokines, including IL-1β, IL-8, and TNF, in porcine alveolar macrophages [36]. In order to determine whether IL-4 also suppressed other macrophage inflammatory activities and thus plays a general down regulatory role on macrophage functions during inflammation, we examined the effect of IL-4 on macrophage superoxide production. Alveolar macrophages were isolated and cultured for 24 h in the presence or absence of various concentrations of IL-4 from 0 to 20 ng/ml and superoxide production was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome *C*. The level of superoxide production in untreated cells was approximately 35 nmol/10^7 cells per 30 min, or 30 nmol/mg protein per h. This value is equivalent to the level of superoxide production previously described in porcine alveolar macrophages [43]. As shown in Fig. 1, IL-4 suppressed macrophage superoxide production in a dose-dependent manner with a 50% reduction occurring at a concentration of 10 ng/ml. Maximal suppression of 70% was achieved at 20 ng/ml of IL-4. Thus, in addition to its

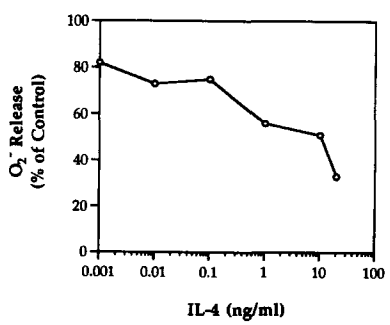

Fig. 1. Suppression of macrophage superoxide production by IL-4. Macrophages were cultured in 96-well plate with 10^5 cells/well. Cells were treated with various concentrations of IL-4 (0–20 ng/ml) for 24 h. Superoxide production was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*.

suppressive effect on inflammatory cytokine expression, IL-4 also suppressed superoxide production in porcine macrophages.

3.2. Cloning of porcine gp91-phox cDNA

A 1483 bp fragment of porcine gp91-phox cDNA was cloned by screening the porcine macrophage library with human gp91-phox cDNA. Sequence analysis showed that the first 1381 bp of this fragment corresponds to the 3′-terminal part of the human gp91-phox cDNA coding region. An additional 376 bp fragment corresponding to the 5′-terminal part of human gp91-phox cDNA coding region was cloned by PCR. Fig. 2 shows the nucleotide sequence and deduced amino acid sequence of porcine gp91-phox cDNA (GenBank accession number UO2476). The open reading frame extending from position 50 to 1456 predicts a primary translation product of 468 amino acids with the estimated relative molecular mass of 54 000 Da. Another in-frame potential translation initiator ATG exists at nucleotide 62. However, only the sequence flanking the ATG at position 50 resembles the consensus

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CTCAACAAGA GTTCGAAGAC AGCTGGACAG GAACCTCACC TTTCATAAA ATG GTG GCG TGG      61
                                                        M   V   A   W
ATG ATT GCA CTT CAC GCC ACG ATT CAC ACC ATT GCC CAT CTG TTT AAT GTG GAA    115
M   I   A   L   H   A   T   I   H   T   I   A   H   L   F   N   V   E
TGG TGT GTG AAC GCC CGA GTC AAC AAT TCT GAC CCG TAC TCA ATA GCA CTC TCT    169
W   C   V   N   A   R   V  *N   N   S   D   P   Y   S   I   A   L   S
GAC ATA GGA GAT AAG CCT AAT GAA ACG TAC CTC AAT TTC GTT CGA CAG AGA ATC    223
D   I   G   D   K   P  *N   E   T   Y   L   N   F   V   R   Q   R   I
AAG AAT CCT GAA GGA GGC CTC TAC GTG GCT GTG ACT CGG CTG GCA GGC ATC ACA    277
K   N   P   E   G   G   L   Y   V   A   V   T   R   L   A   G   I   T
GGC GTC GTC ATC ACG CTG TGT CTC ATA TTA ATT ATC ACC TCC TCC ACC AAG ACC    331
G   V   V   I   T   L   C   L   I   L   I   I   T   S   S   T   K   T
ATC CGG AGG TCT TAC TTC GAG GTG TTC TGG TAC ACA CAC CAT CTC TTT GTG ATC    385
I   R   R   S   Y   F   E   V   F   W   Y   T   H   H   L   F   V   I
TTC TTC ATT GGC CTT GCC ATC CAT GGA GCC GAG CGA ATT GTA CGT AGG CAG ACC    439
F   F   I   G   L   A   I   H   G   A   E   R   I   V   R   R   Q   T
CCA AAG AGT TTG TTG GTC CAC GAT CCA AAG GCA TGT GCT CAA AAC ATC TCA CAA    493
P   K   S   L   L   V   H   D   P   K   A   C   A   Q  *N   I   S   Q
TGG GGA AAA ATA AAG GAC TGC CCG ATC CCG GAG TTC GCA GGG AAC CCT CCT ATG    547
W   G   K   I   K   D   C   P   I   P   E   F   A   G   N   P   P   M
ACT TGG AAA TGG ATC GTG GGT CCC ATG TTC CTG TAT CTG TGT GAG AGG CTG GTG    601
T   W   K   W   I   V   G   P   M   F   L   Y   L   C   E   R   L   V
CGG TTT TGG CGA TCT CAG CAG AAG GTG GTC ATT ACC AAG GTG GTC ACT CAT CCT    655
R   F   W   R   S   Q   Q   K   V   V   I   T   K   V   V   T   H   P
TTC AAA ACC ATC GAG CTA CAG ATG AAG AAG AAG GGA TTC AGG ATG GAG GTG GGA    709
F   K   T   I   E   L   Q   M   K   K   K   G   F   R   M   E   V   G
CAA TAC ATT TTC GTC AAG CGT CCC GCA GTG TCC AAG CTG GAG TGG CAC CCT TTC    763
Q   Y   I   F   V   K   R   P   A   V   S   K   L   E   W   H   P   F
ACC CTG ACC TCT GCC CCT GAG GAA GAC TTC TTT AGC ATC CAT ATC CGC ATT GTT    817
T   L   T   S   A   P   E   E   D   F   F   S   I   H   I   R   I   V
GGC GAC TGG ACA GAG GGA CTG TTC AAA GCC TGT GGC TGT GAT AAG CAG GAG TTT    871
G   D   W   T   E   G   L   F   K   A   C   G   C   D   K   Q   E   F
CAA GAT GCC TGG AAA CTA CCT AAG ATC GCT GTG GAC GGG CCC TTC GGC ACC GCC    925
Q   D   A   W   K   L   P   K   I   A   V   D   G   P   F   G   T   A
AGT GAA GAC GTG TTC AGT TAC CAG GTG GTC ATG TTA GTG GGA GCA GGG ATT GGG    979
S   E   D   V   F   S   Y   Q   V   V   M   L   V   G   A   G   I   G
GTC ACG CCC TTC GCC TCC ATT CTC AAG TCG GTC TGG TAC AAG TAT TGC AAT AAT   1033
V   T   P   F   A   S   I   L   K   S   V   W   Y   K   Y   C   N  *N
GCC ACC AAT CTG AGG CTC AAA AAG ATC TAC TTC TAC TGG CTG TGC CGG GAC ACA   1087
A   T   N   L   R   L   K   K   I   Y   F   Y   W   L   C   R   D   T
CAT GCC TTC GAG TGG TTT GCC GAC CTG CTG CAG CTG CTG GAG ACC CAG ATG CAG   1141
H   A   F   E   W   F   A   D   L   L   Q   L   L   E   T   Q   M   Q
GAG AGG AAC AAC GCC GGC TTC CTC AGC TAC AAC ATC TAC CTC ACT GGC TGG GAT   1195
E   R   N   N   A   G   F   L   S   Y   N   I   Y   L   T   G   W   D
GAG TCA CAG GCC AAT CAC TTT GCC GTG CAC CAT GAT GAG GAG AAA GAT GTG ATC   1249
E   S   Q   A   N   H   F   A   V   H   H   D   E   E   K   D   V   I
ACA GGC CTG AAG CAA AAG ACC TTG TAC GGA CGG CCT AAC TGG GAT AAC GAG TTC   1303
T   G   L   K   Q   K   T   L   Y   G   R   P   N   W   D   N   E   F
AAG ACC ATT GCA AGT CAA CAC CCC ACT ACC AGA ATA GGA GTT TTC CTC TGC GGA   1357
K   T   I   A   S   Q   H   P   T   T   R   I   G   V   F   L   C   G
CCG GAA GCC TTG GCT GAA ACC CTC AAT AAG CAG TGC ATC TCC AAC TCC GAC TCC   1411
P   E   A   L   A   E   T   L   N   K   Q   C   I   S   N   S   D   S
AGC CCG AGG GGA GTG CAT TTC ATT TTC AAT AAG GAA AAC TTC TAA CCGGTCTCAT   1466
S   P   R   G   V   H   F   I   F   N   K   E   N   F  Stop
CCACGAGGAA ATAAATGTGG GTCGTACTGC CAAATGCCCA GATAATGCCA GTTGATAATA GAAGT   1531
TCCTT CCTTAAAAAA AAAAAAAAAA CG                                           1558
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Fig. 2. Nucleotide and predicted amino acid sequence of porcine gp91-phox. The nucleotide sequence was obtained from cDNA clone λ90A and a 5′-end PCR fragment. The potential translation initiators ATG^{50} and ATG^{62} are underlined. Four potential *N*-linked glycosylation sites are indicated by asterisks. The sequence data are deposited in GenBank (accession number UO2476).

sequence for functional initiation codons defined by Kozak [44]. Thus the ATG at position 50 is likely the translation initiation codon. These two initiator ATGs are also found at the same positions in human gp91-phox cDNA sequence [45]. The deduced porcine gp91-phox contains four potential *N*-linked glycosylation sites of the canonical form Asn-X-(Thr/Ser). Comparison of porcine and human gp91-phox cDNA coding regions showed a high homology for both nucleotide sequence (90.4%) and amino acid sequence (92.1%) between the two species. The calculated p*I* of the predicted porcine gp91-phox is 8.7. Fig. 3 shows the comparison of the hydropathy profiles of the predicted porcine and human gp91-phox. Both proteins showed nearly identical profiles in which one extensive hydrophobic segment is obvious. This segment spans amino acids 65–92 and may represent a transmembrane domain of the protein.

3.3. Suppression of gp91-phox mRNA expression by IL-4

To examine the molecular basis for IL-4 suppression of macrophage superoxide production, we determined the effect of IL-4 on the steady-state levels of mRNA encoding gp91-phox and p22-phox by Northern blot analysis. Using the cloned porcine gp91-phox cDNA as a probe, we detected a single mRNA species of approximately 4.5 kb in macrophages with or without LPS stimulation. In these experiments, macrophages were cultured for 4 h with various concentrations of IL-4 from 0 to 100 ng/ml. The results showed that IL-4 suppressed the steady-state levels of gp91-phox mRNA (Fig. 4). The suppression, consistent

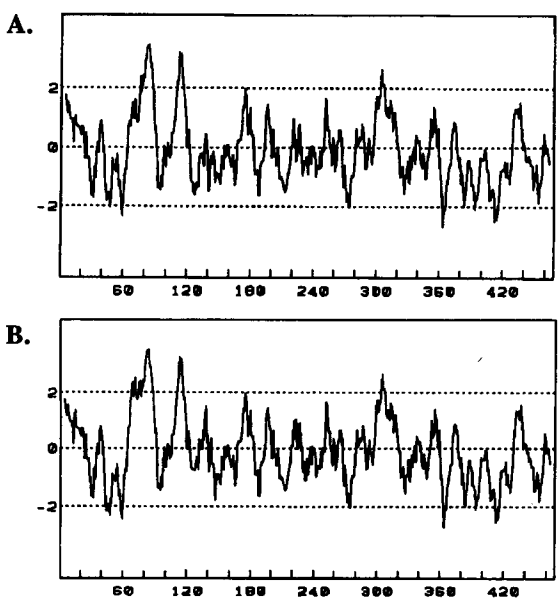

Fig. 3. Comparison of porcine (A) and human (B) gp91-phox hydrophobicity profiles. Hydrophobicities were determined from predicted amino acid sequences by Kyte and Doolittle hydropathy analysis. The upward deflections indicate hydrophobic regions.

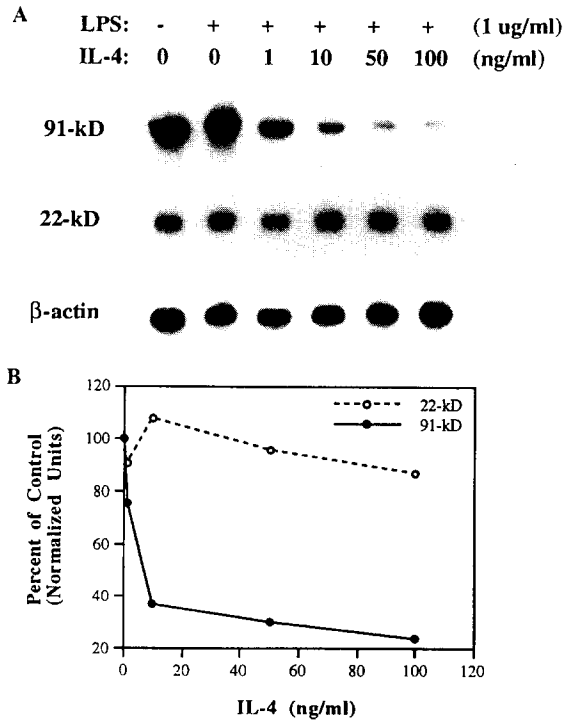

Fig. 4. Effects of IL-4 on gp91-phox and p22-phox mRNA levels. Macrophages were cultured for 4 h in the presence or absence of LPS (1 μg/ml) and various concentrations of IL-4 from 0 to 100 ng/ml. Total cellular RNA was extracted and hybridized sequentially with cDNA probes for gp91-phox, p22-phox, β-actin mRNAs (A). The gp91-phox and p22-phox mRNA levels were quantitated by densitometric scanning of hybridized bands and normalized to β-actin mRNA levels (B).

with the effect of IL-4 on superoxide activity, was dose-dependent, and maximal suppression of approximately 75% was detected at about 10 ng/ml (Fig. 4B). By contrast, p22-phox mRNA levels were not suppressed by IL-4. Thus, the expression of gp91-phox and p22-phox mRNAs is independently regulated in macrophages and the effect of IL-4 is specific for gp91-phox. In addition, the mRNA levels of another immunosuppressive cytokine, TGFβ1, as well as housekeeping genes including β-actin and GAPDH, were not decreased by IL-4 (data not shown), indicating that the suppressive effect of IL-4 on gp91-phox mRNA levels was not due to a cytotoxic effect.

3.4. Kinetics of IL-4 suppression of macrophage gp91-phox mRNA expression

To determine the time course of IL-4 suppression of macrophage gp91-phox mRNA expression, macrophages were cultured for 0–24 h with no treatment, LPS (1 μg/ml) alone, or with LPS and IL-4 (10 ng/ml) added simultaneously. The cells were harvested at the times indicated in Fig. 5 for Northern blot analysis. All mRNA levels were normalized to GAPDH mRNA levels. Cells without treatment expressed readily detectable amounts of gp91-phox mRNA throughout the entire period of 0–24 h

(Fig. 5). The reduced amount at 24 h was due to less total RNA on the gel, as determined by staining with ethidium bromide and hybridization to GAPDH cDNA. LPS alone induced gp91-phox mRNA expression at all time points. Addition of IL-4, however, markedly decreased gp91-phox mRNA levels. The suppression occurred rapidly and was detected at 2 h after IL-4 addition. Maximal suppression was observed at 4–6 h after IL-4 addition and persisted throughout the entire period of 24 h. By contrast, the expression of p22-phox, TGFβ1 and GAPDH mRNAs was not suppressed by IL-4 at all time points [46].

3.5. Interleukin-4 does not change the stability of gp91-phox mRNA

In order to determine whether the decreased gp91-phox mRNA levels were due to a decrease in mRNA stability, the rate of gp91-phox mRNA degradation in macrophages was examined in the presence and absence of IL-4. Macrophages were treated with or without IL-4 (10 ng/ml) for 4 h. Following the addition of 3 μg/ml of actinomycin D to block de novo synthesis of mRNA, cells were harvested every hour afterwards up to 6 h. The rate of gp91-phox mRNA degradation was measured by determining the amount of remaining mRNA in cells as detected by Northern blot analysis and densitometric quantitation (Fig. 6). The half-life of gp91-phox mRNA in cells treated with or without IL-4 was the same, at approximately 4 h, indicating that IL-4 did not affect its stability in macrophages.

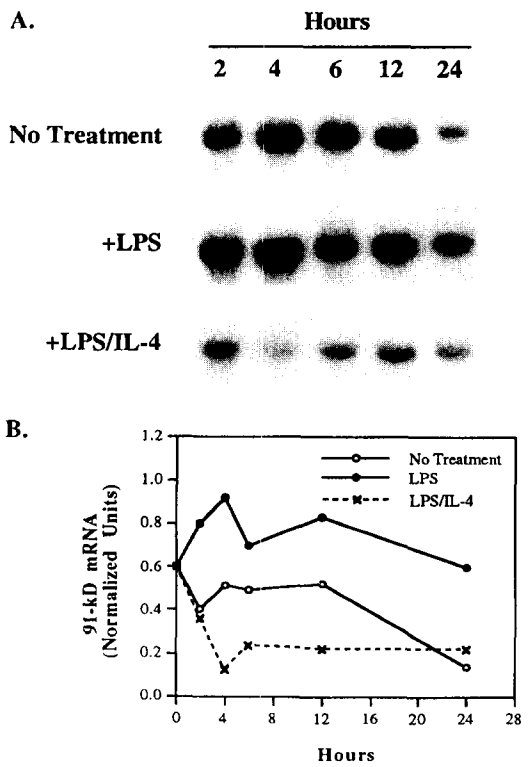

Fig. 5. Time-course of IL-4 suppression of gp91-phox mRNA expression. Macrophages were cultured for 0–24 h in the presence or absence of LPS (1 μg/ml), or LPS and IL-4 (10 ng/ml). Cells were harvested at the time indicated. Total cellular RNA was extracted and hybridized with gp91-phox and GAPDH cDNA probes sequentially (A) and gp91-phox mRNA levels were quantitated by densitometric scanning of hybridized bands and normalized to GAPDH mRNA levels (B).

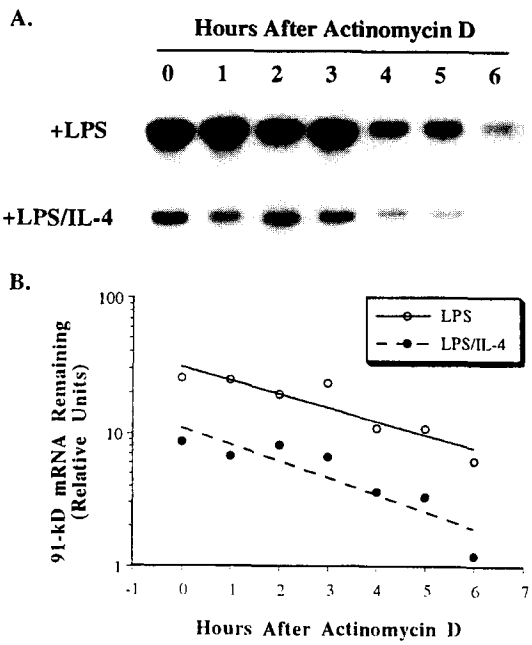

Fig. 6. Effect of IL-4 on gp91-phox mRNA stability. Macrophages were treated with LPS (1 μg/ml) or LPS/IL-4 (10 ng/ml) for 4 h. Actinomycin D (3 μg/ml) was added and the cells were harvested at hourly intervals. Total cellular RNA was hybridized with gp91-phox cDNA and the mRNA levels were quantitated by densitometric scanning.

3.6. Effect of IL-4 on the rate of gp91-phox gene transcription

Nuclear run-on assays were performed to determine if gp91-phox expression was induced at the transcriptional level. Nuclei were isolated from resting macrophages and from cells treated with LPS (1 μg/ml), IL-4 (50 ng/ml) or both for 4 h. Transcripts were extended in vitro with the incorporation of [^{32}P]UTP and hybridized to a panel of plasmid clones that included gp91-phox, p22-phox, p47-phox, IL-1β, TGFβ1, GAPDH, and pGEM3Zf(+) vector alone. The results show that LPS and IL-4 neither increased nor decreased the rate of transcription of any of the genes analyzed except for IL-1β (Fig. 7). Phosphorimager analysis showed less than a 2.5-fold difference in the amount of radioactivity in samples from the NADPH oxidase subunit genes, TGFβ1 and GAPDH regardless of treatment. The only effect of IL-4 on transcription was to suppress by more than 80% the induction of IL-1β by LPS. Hence, the effect of IL-4 on mRNA levels of gp91-phox appears to be post-transcriptional, perhaps at the level of mRNA processing.

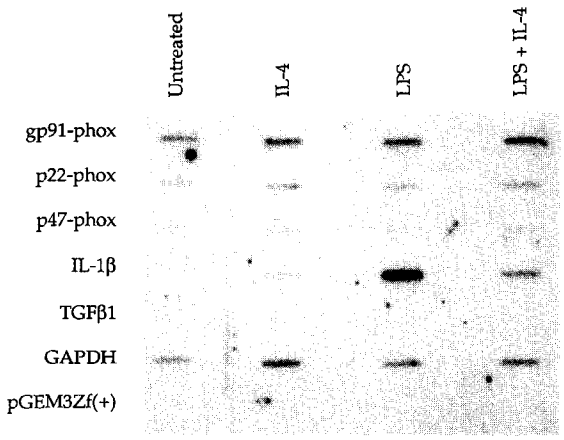

Fig. 7. Effect of IL-4 on gp91-phox transcription. Nuclei were isolated from cells treated with LPS (1 μg/ml), IL-4 (50 ng/ml), both or neither for 4 h. Nuclear run-on assays were performed as described in Section 2. Labeled transcripts were hybridized to the indicated cDNAs and visualized by autoradiography.

3.7. Effect of TGFβ1 on porcine macrophage superoxide production and NAPDH oxidase gene expression

To investigate whether the suppression of superoxide production and NADPH oxidase expression was a specific response of macrophages to IL-4, the effect of IL-4 was compared with another immunosuppressive cytokine, TGFβ1, on macrophage superoxide production and NADPH oxidase gene expression. Macrophages were cultured for 6 h with 10 ng/ml of TGFβ1, 10 ng/ml of IL-4, or neither. Superoxide production and gp91-phox mRNA levels were determined by superoxide assay and Northern blot, respectively, and expressed as percent of control for comparison. Superoxide anion production in untreated cells varied from 30 to 50 nmol $O_2^-/10^7$ cells/30 min. As shown in Table 1, IL-4 significantly decreased superoxide production and gp91-phox mRNA levels to $32 \pm 2\%$ and $26 \pm 6\%$, respectively. However, superoxide production and gp91-phox mRNA levels remained unchanged at $101 \pm 3\%$ and $102 \pm 10\%$, respectively, in cells treated with TGFβ1.

Table 1
Comparison of the effect of TGFβ1 and IL-4 on suppression of superoxide production and gp91-phox gene expression

Treatment	Superoxide anion [a]	gp91-phox mRNA [b]
None	100	100
TGFβ1 (10 ng/ml)	101±3	102±11
IL-4 (10 ng/ml)	32±2	26±6

[a] Cells were cultured for 24 h with or without TGFβ1 or IL-4. The superoxide production was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome C. Results are expressed as the percent of control.

[b] Cells were cultured in the presence or absence of TGFβ1 or IL4 for 6 h. Total cellular RNA was hybridized sequentially with probes for gp91-phox, GAPDH or β-actin. The gp91-phox mRNA levels were determined by densitometric scanning, normalized to GAPDH or β-actin and expressed as percent of control.

4. Discussion

Interleukin-4, originally characterized as a B-cell growth factor, also possesses anti-inflammatory effects on human and mouse monocytes and macrophages [31–35]. In porcine alveolar macrophages, IL-4 suppressed the production of inflammatory cytokines, including IL-1α, IL-1β, TNFα and IL-8, by suppressing the level of transcription of the genes [36]. Here, we demonstrated that IL-4, in addition to the suppression of macrophage inflammatory cytokine expression, also suppressed superoxide production in porcine alveolar macrophages, but at a posttranscriptional level. The results from porcine alveolar macrophages are consistent with other observations that IL-4 suppressed superoxide production from human and murine macrophages [47–50]. These observations indicate that IL-4 plays an active role in regulating macrophage inflammatory activities.

The effect of IL-4 on production of superoxide anion is due to the suppression of NADPH oxidase activity by IL-4 which is specific for gp91-phox. The p22-phox mRNA levels were not altered by IL-4, indicating that the mRNAs encoding these two subunits are regulated independently in porcine macrophages. Similar observations, in which the 91 kDa mRNA was strikingly regulated but 22 kDa mRNA remained unchanged under the same conditions, was reported previously [7,10,11,51]. This differential regulation, together with the observations that gp91-phox mRNA is only expressed in cells of the phagocytic lineage whereas stable mRNA encoding p22-phox is expressed in all cell types [51], suggests an important catalytic role of gp91-phox in generating superoxide anion in phagocytes.

Comparison of human and porcine gp91-phox cDNA and amino acid sequences showed a very high degree of similarity between these two species in their nucleotide and amino acid sequences as well as their hydropathy profile. The original cloning of human gp91-phox cDNA defined a translation initiator ATG at nucleotide position 322 [45]. However, subsequent purification and sequencing of gp91-phox identified another 43 amino acids whose nucleotide sequence corresponds to the originally designated non-coding region 5′ to the initiator ATG^{322} [52]. The initiation codon ATG at nucleotide position 50 in the cloned porcine gp91-phox cDNA corresponds to the human gp91-phox initiation codon ATG^{322}. Since the predicted molecular mass of the porcine and human gp91-phox are both about 54 kDa, it is possible that cloned cDNAs may not represent the entire coding region of the protein, or that the mature proteins are posttranslationally modified by glycosylation.

The gp91-phox mRNA is present in unstimulated porcine macrophages, and is induced by LPS, similar to other reports [7–9,11,12]. Both constitutive and LPS-in-

duced gp91-phox mRNA expression were suppressed by IL-4, indicating that the IL-4 suppression is a direct effect on gp91-phox mRNA rather than secondary to an effect on LPS responsiveness. The stability of gp91-phox mRNA was not changed by IL-4, similar to the results observed for inflammatory cytokines. Although IL-4 suppression of macrophage inflammatory cytokine mRNA expression occurred at the transcriptional level [36], and others have reported that the regulation of gp91-phox and p47-phox mRNA expression by IFNγ is a transcriptional event [8,10], it appears that IL-4 suppressed gp91-phox mRNA levels posttranscriptionally, perhaps by reducing the efficiency of its transcriptional processing.

Several other cytokines, in addition to IL-4, have also been shown to possess immunosuppressive or anti-inflammatory activities. In particular, TGFβ inhibits the induction of IL-1β and TNFα by LPS in human peripheral blood mononuclear cells [53] and suppresses superoxide production in human macrophages [54]. However, we did not observe the suppressive effect of TGFβ1 on either superoxide production or gp91-phox mRNA expression in porcine alveolar macrophages. One possibility for this discrepancy in TGFβ effect on macrophage superoxide production detected by us and others is due to different experimental conditions and different species. In addition, IL-4 had no effect on TGFβ mRNA expression (data not shown), indicating that IL-4 suppression is not mediated through TGFβ activity. Interleukin-10 and IL-13, cytokines produced by T_H2 cells, are active in suppression of macrophage inflammatory cytokine production, including IL-1α, IL-1β, IL-6, IL-8 and TNFα [55–57] or have activities similar to IL-4 [58], respectively. However, their effects on macrophage superoxide production are not known.

An increasing body of evidence suggests that oxygen metabolites are capable of causing injury to various cells and tissues and mediating inflammatory diseases when overproduced [13–28]. Thus, it appears that, for a productive immune response to acute infection appropriate limitation of inflammatory activity, including superoxide production, is necessary in order to limit host damage after the primary pathogenic insult has been blunted. The limitation of macrophage superoxide production can be achieved through both exogenous treatment with inhibitors of reactive oxygen intermediates and endogenous induction of immunosuppressive modulators. It has been reported that local injection of superoxide dismutase is beneficial in the treatment of rheumatoid arthritis and other inflammatory diseases [19,59–61]. A recent study also showed that intraarticular injection of SOD or catalase significantly reduced the group A streptococcal cell wall-induced inflammatory response and evolution of erosive arthritis in a rat model [15]. In vivo studies with administration of recombinant IL-4 as an immunotherapeutic agent to patients with renal cell carcinoma or malignant melanoma showed that monocytes from post-therapy patients exhibited reduced production of superoxide anion [50]. Although exogenous substances are beneficial in the treatment of inflammatory diseases, a more efficient strategy would be the induction of endogenous immunosuppressive modulators. Our results suggested that IL-4 may be a candidate immunomodulator which acts in vivo to suppress superoxide production and thus prevent undue damage from the inflammatory reaction to pathogens.

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