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Regulation of Pulmonary and Systemic Bacterial Lipopolysaccharide Responses in Transgenic Mice Expressing Human Elafin

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The control of lung inflammation is of paramount importance in a variety of acute pathologies, such as pneumonia, the acute respiratory distress syndrome, and sepsis. It is becoming increasingly apparent that local innate immune responses in the lung are negatively influenced by systemic inflammation. This is thought to be due to a local deficit in cytokine responses by alveolar macrophages and neutrophils following systemic bacterial infection and the development of a septic response. Recently, using an adenovirus-based strategy which overexpresses the human elastase inhibitor elafin locally in the lung, we showed that elafin is able to prime lung innate immune responses. In this study, we generated a novel transgenic mouse strain expressing human elafin and studied its response to bacterial lipopolysaccharide (LPS) when the LPS was administered locally in the lungs and systemically. When LPS was delivered to the lungs, we found that mice expressing elafin had lower serum-to-bronchoalveolar lavage ratios of proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α), macrophage inflammatory protein 2, and monocyte chemoattractant protein 1, than wild-type mice. There was a concomitant increase in inflammatory cell influx, showing that there was potential priming of innate responses in the lungs. When LPS was given systemically, the mice expressing elafin had reduced levels of serum TNF- α compared to the levels in wild-type mice. These results indicate that elafin may have a dual function, promoting up-regulation of local lung innate immunity while simultaneously down-regulating potentially unwanted systemic inflammatory responses in the circulation.

The regulation of inflammatory cytokines and cell influx of neutrophils and macrophages is important in a variety of lung and systemic pathologies, such as acute respiratory distress syndrome, pneumonia, and sepsis (11, 22, 25, 44). Recent studies have highlighted the importance of cytokine-chemokine gradients between the alveolar space and the blood compartments in influencing the outcome of lung and systemic inflammations (3, 43). Such studies have shown that in rats concomitant lung administration and systemic administration of bacterial lipopolysaccharide (LPS) resulted in a reduction in the inflammatory cell influx in the alveolar space compared to the influx in animals treated only via the pulmonary route because of a reduced lung-blood chemotactic gradient. In related studies, it was shown that endotoxemic rats and mice with experimentally induced bacterial pneumonia have a poor outcome, possibly because of a lack of pulmonary neutrophilic migration and clearance of organisms (8, 28, 46).

Of interest in this context are low-molecular-weight mucosal elastase inhibitors, such as secretory leukocyte protease inhibitor (SLPI) and elafin/elastase-specific inhibitor (34, 35). These agents have been shown to be induced by early wave cytokines,

such as interleukin-1 and tumor necrosis factor (TNF) (32), and to have antimicrobial properties (17, 36, 37, 47). Their concentrations are very low in blood, and they are not expressed in the liver (1, 20, 27, 29).

Recently, it has been shown that a transient overexpression approach in which adenovirus is used as a gene vector is efficient for delivering human elafin (driven by the powerful mouse cytomegalovirus [MCMV] promoter) to mouse lung tissues (37, 38). In addition, it was found that in mice there was an increase in inflammatory cell migration to airways in response to intratracheally instilled bacterial LPS (38), suggesting that this inhibitor may be important as a chemoattractant and in the priming of innate responses in lung cells.

Because lung-targeted adenovirus protocols give rise to lung compartmentalization of transgene expression (49) and hence only local elafin expression in the previously described model (37), we decided to create transgenic mice expressing human elafin more ubiquitously in order to have coexistent local expression and systemic expression of elafin. To do this, a mouse transgenic line expressing human elafin cDNA under control of the MCMV promoter was generated, and its characteristics were assessed in two self-limiting models of inflammation, first by using intratracheally instilled bacterial LPS (akin to the method used in the adenovirus study mentioned above) and second by using a systemic LPS administration protocol.

MATERIALS AND METHODS

Generation of transgenic mice expressing human elafin cDNA under control of the MCMV promoter. A 6.3-kb fragment containing the elafin cDNA fragment under control of the MCMV promoter was isolated from the PDK6 plasmid (33) and used for microinjection. This elafin cDNA codes for the full-length elafin

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molecule, which contains transglutamination sites thought to be important for the binding of the molecule to the intertittium (27, 29, 33).

Transgenic mice were generated by a standard protocol (18) by injecting linear DNA (5 ng/ μ l) into the male pronuclei of fertilized ova derived from C57BL6 \times CBA F1 females. Injected ova at the two-cell stage were transferred to the oviducts of surrogate pseudopregnant CD1 females, where development was allowed to progress to term. One founder line was identified (see below) which transmitted the elafin cDNA transgene under control of the MCMV promoter to its offspring in Mendelian fashion. The transgenic line was then bred to homozygosity. The number of elafin transgene copies was determined by standard methods. Briefly, the intensity (as determined by Phosphorimager analysis) of *Hind*III-digested genomic DNA was compared with the intensities of spiked known amounts of elafin cDNA. The numbers of copies were determined by using the following equation: 1 copy of transgene = $0.538(3.78 \times 10^{-6} \mu\text{g}) = 2$ pg of genomic DNA.

The wild-type controls used had the same mixed background (C57BL6 \times CBA) as the transgenic mice expressing elafin.

All results described for the wild-type and transgenic mice expressing elafin were obtained with F2 generation mice.

Southern genomic analysis. Transgenic mice were identified by Southern blot analysis of DNA samples isolated from tail biopsies by using standard procedures. Briefly, tail DNA samples were extracted with phenol-chloroform following proteinase K digestion overnight. The resulting DNA was subsequently precipitated with ethanol. Genomic DNA samples (10 μ g) were digested with *Hind*III, resolved on a 1% agarose gel, and transferred in 0.4 N NaOH to a GeneScreen Plus nylon membrane (NEN-Dupont, Stevenage, Hertfordshire, United Kingdom). The filter was then hybridized with a 32 P-labeled random primer-labeled human elafin cDNA probe. Prehybridization (1 h) and hybridization (overnight) were performed in 0.5 M Na₂HPO₄ (pH 7)–1 mM EDTA–7% sodium dodecyl sulfate at 65°C. The filter was washed twice in 0.04 M Na₂HPO₄–1% sodium dodecyl sulfate at 65°C for 30 min and exposed for autoradiography, typically overnight.

RNA preparation and Northern blot analysis. Tissues were harvested from adult (8-week-old) female mice. Total RNA from various murine tissues was prepared with the Trizol reagent used according to the manufacturer's instructions (Gibco BRL, Paisley, United Kingdom). Total RNA (20 μ g) was resolved on a 1% agarose–1.7 M formaldehyde gel, transferred overnight in 20 \times SSC to a Zetaprobe membrane (Bio-Rad, Hemel Hempstead, Hertfordshire, United Kingdom), and then baked at 80°C for 1 to 2 h (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filter was prehybridized, hybridized, and washed as described above for the Southern analysis but at 55°C instead of 65°C. The filter was exposed for autoradiography for 1 week.

DNase treatment of RNA templates. To eliminate the possibility of genomic DNA contamination, RNA samples prepared from murine tissues or macrophages were digested with RQ1 RNase-free DNase I (Promega, Southampton, United Kingdom) used in accordance with the manufacturer's instructions prior to use in a reverse transcription-PCR (RT-PCR) (see below).

RT-PCR and Southern analysis. Total RNA isolated from a variety of mouse tissues (0.35 μ g) and peritoneal macrophages was used as the template for first-strand cDNA synthesis. Oligonucleotide primers (MWG-Biotech, Milton Keynes, United Kingdom) were designed to amplify a 477-bp fragment of the elafin transcript. The elafin primers were sense primer 5' GCAGTCTCTTGAT CGTGGTG 3' (starting at nucleotide 11 after the ATG start codon) and antisense primer 5' GCCGTGGGCATCCTGAATGG 3' (nucleotides 467 to 486).

Primers were also designed to amplify a 258-bp fragment of murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense primer, 487 to 506 bp; antisense primer, 726 to 745 bp). Total RNA was incubated with 0.25 μ g of oligo(dT) (Promega), 1 μ l of 10 \times PCR buffer (Promega), 100 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL), 7 U of RNasin (Gibco BRL), and 125 μ l of each deoxynucleoside triphosphate (Pharmacia, Buckingham, Hertfordshire, United Kingdom) in a 10- μ l (total volume) reaction mixture. The reaction mixture was incubated at 37°C for 45 min and then heated at 90°C for 5 min. Then 4 μ l of 10 \times PCR buffer, 80 pmol (each) of the forward and reverse elafin primers, 8 pmol (each) of the forward and reverse GAPDH primers, 2.5 U of *Taq* DNA polymerase (Promega), and enough H₂O to bring the total volume to 50 μ l were added to the RT reaction mixture. The reaction mixture was subjected to 35 cycles of DNA amplification (94°C for 30 s, 60°C for 30 s, 70°C for 1 min) in a MJ Research thermocycler. The reaction mixture was then held for 5 min at 70°C. When necessary, Southern blot analysis was performed by using a standard method, when the concentrations of RT-PCR products were at the limit of detection by ethidium bromide gel staining.

In vivo experiments. (i) Intratracheal instillation of LPS. Female transgenic mice expressing elafin and wild-type (WT) mice (ages, 8 to 10 weeks) were

instilled intratracheally with 50 μ g of *Escherichia coli* O127:B8 LPS, as described previously (37). Twenty-four hours later, mice were sacrificed under anesthesia by transection of the abdominal aorta, blood was retrieved for further serum preparation, and the lungs and trachea were removed en bloc. A bronchoalveolar lavage (BAL) was immediately performed with two serial instillations of 250 and 200 μ l of phosphate-buffered saline (PBS). Recovered BAL fluid (BALF) (typically 350 to 450 μ l) was centrifuged at 2,000 \times g for 5 min. Supernatants were frozen at –20°C until they were used, while each cell pellet was resuspended in 100 μ l of PBS. Cell counting (with a hemacytometer) and cytospinning (300 rpm for 3 min at room temperature) were performed. After cytospinning preparations were stained with Diff-quick (Dade Diagnostika, GmbH, Germany) for analysis of differential cell counts.

(ii) Intraperitoneal instillation of LPS. Female transgenic mice expressing elafin and WT mice (ages, 8 to 10 weeks) each received one intraperitoneal dose of 120 μ g of LPS (as described above) in 100 μ l of PBS. Control mice were injected with PBS alone. Serum samples were collected after 1.5 h and 24 h by tail bleeding.

Thioglycolate challenge and culture of peritoneal macrophages. Female transgenic mice expressing elafin and WT mice (ages, 8 to 10 weeks) were injected intraperitoneally with 1 ml of 4% thioglycolate broth to induce peritonitis. The mice were sacrificed after 120 h, and peritoneal lavage performed with 5 ml of cold PBS. The cells were pooled and washed with PBS. A total of 5×10^5 cells were then cultured in 48-well plates in 1 ml of RPMI 1640 (Gibco BRL) medium supplemented with 25 mM HEPES, 2 mM glutamine, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 10% fetal bovine serum. After overnight incubation, nonadherent cells were removed by washing with serum-free medium, and all of the adherent cells were macrophages. LPS was then added at different concentrations (0 to 5 ng/ml), and the cells were cultured for 48 h in serum-containing medium. Each supernatant was removed and stored at –20°C until it was used for enzyme-linked immunosorbent assay (ELISA) analysis.

ELISA. ELISA analyses to detect murine TNF alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2) were performed by using commercially available ELISA kits (R & D Systems, Abingdon, Oxon, United Kingdom) in accordance with the manufacturer's instructions. Human elafin was measured by using an ELISA available in-house (37). Culture supernatants from peritoneal macrophages were diluted 1:20 before ELISA analysis of cytokines. Serum samples (collected either 1.5 or 24 h after treatment) and BALFs were either not diluted or diluted fivefold in PBS.

Statistical analysis. Normally distributed data were analyzed by the Student unpaired *t* test. When data were not normally distributed, nonparametric tests (Kruskal-Wallis and Mann-Whitney tests) were employed. Data were considered to be statistically significantly different when the *P* value was <0.05.

RESULTS

Genotype analysis of transgenic mice expressing elafin cDNA. The genotypes of mice were determined in order to determine whether elafin cDNA was present. Tail biopsy DNA was prepared and digested with *Hind*III (generation of a 562-bp fragment showed that the transgene was intact), as described in Materials and Methods. Heterozygous F1 mice were selected by Southern analysis and PCR. Male and female mice heterozygous for the elafin cDNA were crossed to generate homozygous F2 animals (Fig. 1). Homozygous (+/+) mice expressing elafin cDNA (referred to as elafin mice below) were crossed to establish the mouse line expressing elafin. Analysis (see Materials and Methods) revealed that the elafin mice had three to five copies per genome. The WT mouse controls used had the same mixed background (C57BL6 \times CBA) as the elafin mice.

Northern blot and RT-PCR analyses of elafin mice. Figure 2 shows the tissue distribution of human elafin in elafin mice as determined by Northern blot analysis (Fig. 2A) and RT-PCR (Fig. 2B and C).

This analysis showed that although most tissues tested were positive for the transgene (Fig. 2B), stomach, small intestine, skeletal muscle, and bone tissues were the tissues in which

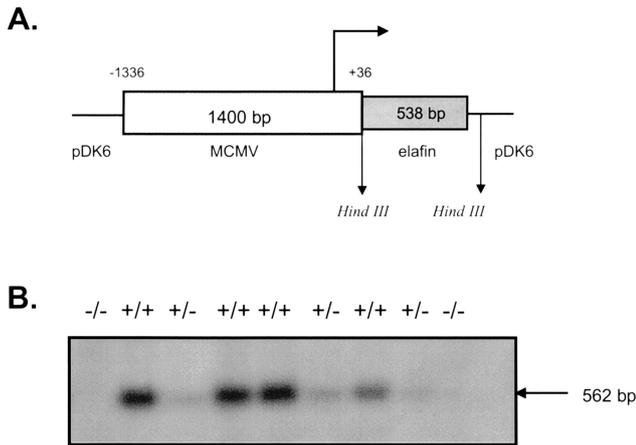


FIG. 1. Construction and identification of the elafin cDNA transgene under control of the MCMV promoter. (A) Construct used to generate transgenic mice expressing human elafin cDNA under control of the MCMV promoter. A 1,400-bp fragment of the MCMV promoter (nucleotides -1336 to 36) drives expression of the elafin cDNA (538 bp). Restriction sites for *Hind*III, which were used to identify the presence of the transgene, are indicated. (B) Genomic DNA (10 μ g) isolated from tail biopsies was digested with *Hind*III and resolved on a 1% agarose gel. The gel was transferred to a nylon membrane, and the filter was hybridized with radiolabeled human elafin cDNA. The offspring from a heterozygous intercross included wild-type ($-/-$) mice and mice heterozygous ($+/-$) and homozygous ($+/+$) for the human elafin cDNA transgene. The position of the 562-bp human elafin cDNA fragment is indicated by an arrow.

elafin was most abundant (Fig. 2A). Due to their lower level of elafin expression, blood and bone marrow samples were treated separately (Fig. 2C). Compared to the intensity of the elafin bands in the stomach sample (Fig. 2C, lane 5), the elafin bands in the blood and bone marrow samples were much less intense. In addition, PCR gave rise to at least two bands in the bone marrow samples and eight bands in the blood sample. The specificity of these products was therefore checked by Southern analysis (by using elafin cDNA as a probe), and only the 477-bp expected elafin product hybridized strongly, showing that both blood and bone marrow cells expressed the elafin cDNA transgene.

When tissues were analyzed for protein production (by ELISA), the elafin levels were not always reliably measured, which probably reflected the relative lack of sensitivity of the assay (data not shown).

Inflammatory responses to intratracheally instilled bacterial LPS. (i) Cytokines. In order to assess whether elafin mice responded differently to LPS than WT mice responded, both groups of mice were instilled intratracheally with 50 μ g of LPS (or PBS). Because of the importance of TNF- α as an early mediator of inflammation, we first measured the TNF- α levels in BALF and serum. As shown in Table 1, as expected, both groups of LPS-treated mice had higher BALF TNF- α levels than their PBS-treated counterparts. It should be noted that the response to LPS was largely (but not totally) compartmentalized in the lungs in WT mice (there was a 22-fold increase in the TNF- α level in BALF and a 1.3-fold increase in serum after LPS treatment). There was also a trend (not statistically significant) toward increased levels of the cytokines in BALF from LPS-treated elafin mice compared to the levels in LPS-

treated WT mice. When the systemic levels of TNF- α in serum were examined, the elafin mice had lower levels than the WT mice after PBS or LPS treatment, suggesting that elafin may have a stronger systemic anti-inflammatory activity than a local lung proinflammatory activity in these mice.

Since TNF- α is an early cytokine involved in initiation of the inflammatory response, we extended our study to two other cytokines, MCP-1 and MIP-2, which are chemokines for monocytes and neutrophils, respectively. Because of the potential dual activity of elafin in altering the TNF- α response (weakly proinflammatory in the lung and strongly anti-inflammatory systemically), we expressed the data as serum/BAL cytokine ratios (Fig. 3). In accordance with the TNF- α data, elafin mice

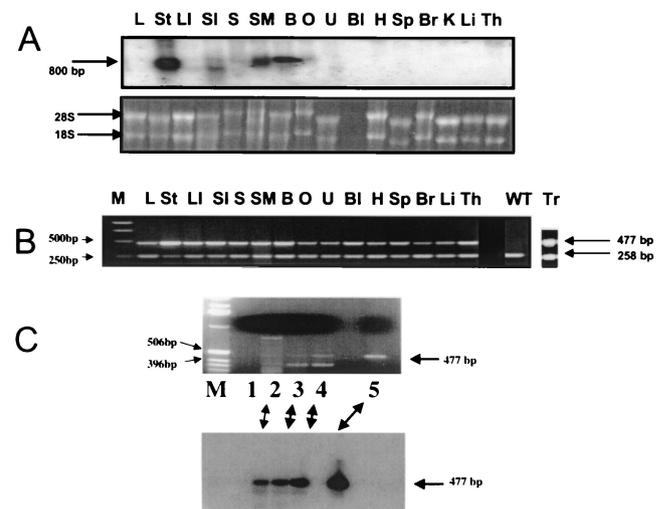


FIG. 2. Expression of the human elafin cDNA transgene in female murine tissues. (A) Northern analysis of total RNA (20 μ g) from lung (L), stomach (St), large intestine (LI), small intestine (SI), skin (S), skeletal muscle (SM), bone (B), ovary (O), uterus (U), bladder (Bl), heart (H), spleen (Sp), brain (Br), kidney (K), liver (Li), and thymus (Th) tissues. Ethidium bromide staining of gels prior to transfer (lower panel) was used to check equivalent sample loading. The positions of the 28S and 18S rRNA are indicated on the left. The filter was hybridized with the radiolabeled human elafin cDNA probe. The position of the 800-bp band representing human elafin mRNA is indicated on the left. Wild-type mice showed no elafin signal (data not shown), confirming the results of previous studies with rats (33) showing that the human elafin probe does not cross-react with any rodent message. (B and C) RT-PCR analysis of elafin performed with RNA (0.35 μ g) from various murine tissues (B) or from blood cells and bone marrow (C). The message for GAPDH was coamplified with elafin to standardize its expression. A 20- μ l aliquot of each RT-PCR product was resolved on a 1.5% agarose gel. The positions of the 477-bp elafin PCR and 258-bp GAPDH PCR products are indicated on the right. The positions of molecular weight markers are indicated on the left (lane M) (1.0-kb ladder; catalog number G 5711 for panel B; Promega). The abbreviations for the organs are the same as those described above; trachea (Tr) and wild-type liver RNA (WT) samples also were analyzed. (C) Total blood cells from two elafin mice were obtained by exsanguination and subsequently pooled. Bone marrow was obtained from the same mice, and samples were treated separately for RT-PCR. The specificity of the 477-bp elafin cDNA band (top panel) was verified by Southern blot analysis (bottom panel). Lane M, molecular weight markers (catalog number 15615-016; Promega); lane 1, negative control in which water was used in place of DNA; lane 2, total blood cell elafin cDNA; lanes 3 and 4, bone marrow elafin cDNA (independent samples); lane 5, stomach elafin cDNA.

TABLE 1. BALF and serum TNF- α levels after intratracheal LPS administration^a

Mice	Treatment	No. of mice	Fold increase in TNF- α in:	
			BALF	Serum
WT	PBS	3	1	1
	LPS	5	22.2 ^b	1.29
Elafin	PBS	3	0.85	0.45 ^b
	LPS	7	39.1 ^b	0.07 ^{b,c}

^a BALF and sera from elafin mice and WT mice were obtained 24 h after intratracheal administration of PBS or LPS, and TNF- α levels were measured by ELISA (see Materials and Methods). Nonparametric data were expressed as fold increases compared with the values for WT mice that received PBS (median). Statistical significance was assessed by using the Kruskal-Wallis and Mann-Whitney tests.

^b Significantly different from the value obtained for WT mice that received PBS ($P < 0.05$).

^c Significantly different from the value obtained for WT mice that received LPS ($P < 0.05$).

exhibited the same decrease in serum/BALF MCP-1 and MIP-2 ratios as those WT mice. It should be noted that as shown in Table 1, although a trend was observed, the transgene did not have a significant effect on the absolute cytokine levels in the BALF.

(ii) Inflammatory cells. The finding that elafin mice had lower serum/BALF ratios of inflammatory cell cytokines suggested that the chemokine gradient generated may have resulted in an increase in the number of inflammatory cells in BALF from elafin mice compared to the number of inflammatory cells in BALF from WT mice. Figure 4 shows that as expected, LPS induced an influx of inflammatory cells in BALF from the two groups of mice compared to the number of cells

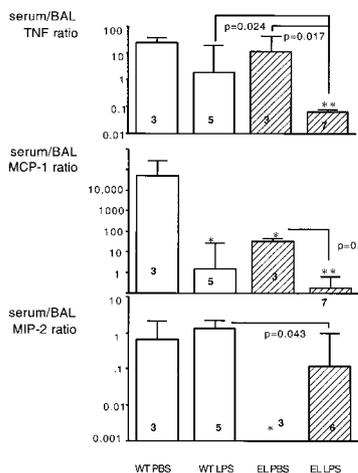


FIG. 3. Intratracheal administration of LPS decreases serum/BALF cytokine ratios in elafin mice compared to the ratios in WT mice. BALF and sera from elafin mice (EL) and WT mice were obtained 24 h after PBS administration or after intratracheal administration of 50 μ g of LPS (see Table 1). Serum/BALF ratios were calculated for each cytokine (TNF- α , MCP-1, MIP-2) and are expressed as the median value (plus the upper interquartile). Statistical significance is indicated as follows: one asterisk, $P < 0.05$ compared to the value for WT PBS; two asterisks, $P < 0.05$ compared to the values for WT PBS and the other linked groups. The numbers in the bars indicate the number of animals in each group.

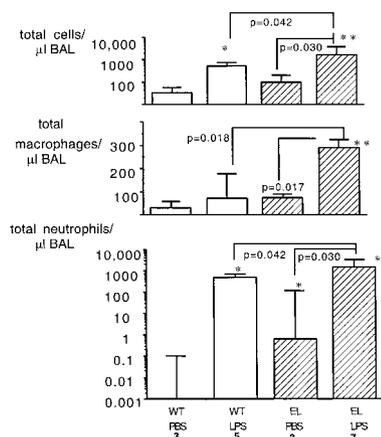


FIG. 4. Intratracheal administration of LPS increases the number of inflammatory cells in BALF from elafin mice compared to the number of inflammatory cells in BALF from WT mice. Transgenic mice expressing elafin (EL) and WT mice received either PBS or 50 μ g of LPS intratracheally (as described in the legend to Fig. 3). After 24 h, BALF was obtained, and differential cell counting was performed by cytopinning. The results are expressed as the median value (plus the upper interquartile). One asterisk, $P < 0.05$ compared to the value for WT PBS; two asterisks, $P < 0.05$ compared to the values for WT PBS and the other linked groups. The numbers at the bottom indicate the number of animals in each group.

after the PBS treatment. In WT mice the increase consisted mostly of neutrophils, whereas in elafin mice both the number of neutrophils and the number of macrophages were elevated. Indeed, when the LPS-treated groups were considered separately, BALF from elafin mice contained more inflammatory cells than BALF from WT mice contained.

Interestingly, although the levels of human elafin protein in sera and BALF were very low (consistently less than 50 pg/ml) in elafin mice and zero in WT mice, in elafin mice treated with PBS and LPS positive correlations were found between the levels of elafin in BALF and the levels of TNF- α , MCP-1, and MIP-2 (Fig. 5).

When serum levels of cytokines and elafin were analyzed, only the relationship between TNF- α and elafin levels approached statistical significance ($P = 0.081$).

Serum TNF- α levels after intraperitoneal LPS administration. The in vivo experiments whose results are shown in Table 1 and Fig. 3 showed that intratracheal instillation of 50 μ g of LPS did not lead to total lung compartmentalization of the LPS stimulus in WT mice since the TNF- α levels were increased in the serum of these mice. Since elafin mice had lower levels of circulating TNF- α (Table 1), we hypothesized that if elafin could down-regulate the systemic effect of LPS when LPS is administered intratracheally, it may dampen the responses when LPS is injected systemically. Since the level of TNF- α in serum is known to increase transiently after systemic LPS administration and TNF- α is considered a major initiator of the septic response (4–6, 13, 39, 42), we used previously established protocols to measure TNF- α immunoreactivity in serum 1.5 and 24 h after intraperitoneal LPS injection.

Figure 6 shows that as expected, the serum TNF- α levels in WT mice were greater 1.5 h after LPS administration and

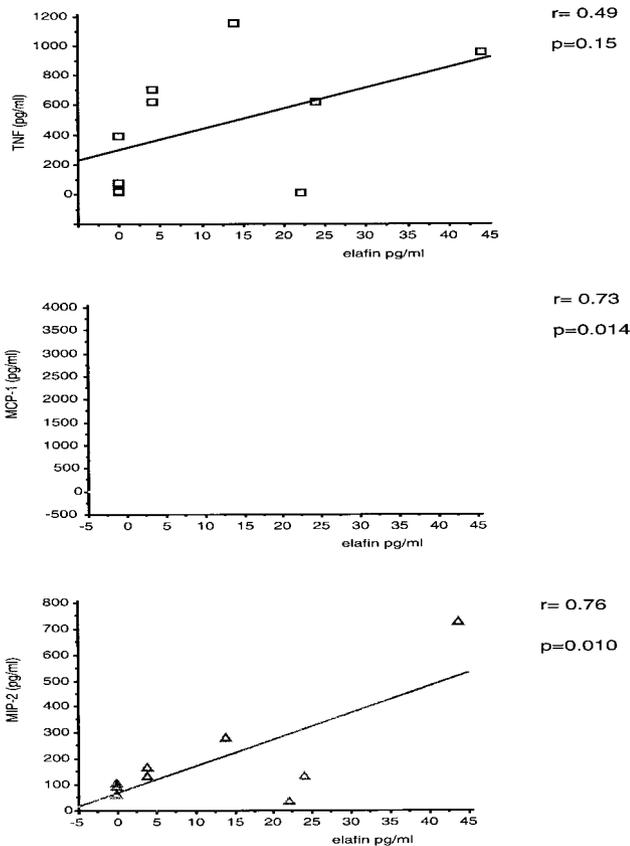


FIG. 5. Correlation between BALF elafin and cytokine levels. BALF from elafin mice instilled intratracheally with PBS or LPS (see the legends to Fig. 3 and 4) were analyzed for elafin and cytokine (TNF- α , MCP-1, MIP-2) levels.

returned to the baseline value within 24 h. In sera from elafin mice, the TNF- α levels were significantly lower than those in WT mice at 1.5 h, suggesting that elafin is indeed involved in down-regulating systemic endotoxin responses.

Ex vivo stimulation of peritoneal macrophages with LPS. As shown in Fig. 3, the serum/BAL cytokine ratios were lower in elafin mice than in WT mice. In addition, we showed that bone marrow cells and blood cells were positive for the transgene (Fig. 2C). Given the reported anti-inflammatory properties of the related molecule SLPI in monocytes and macrophages (10, 21, 23, 26), we hypothesized that monocytes derived from elafin mice would be less responsive to LPS, thereby explaining the reduced systemic TNF- α expression in vivo (Fig. 6). To further investigate this, we isolated monocyte-derived peritoneal macrophages 4 days after intraperitoneal thioglycolate injection and stimulated the cells in vitro with different levels of LPS. Figure 7 shows that peritoneal macrophages from elafin mice produced less TNF- α , MCP-1, and MIP-2 than peritoneal macrophages from WT mice produced, and Fig. 8 shows that these cells produced elafin mRNA, as determined by RT-PCR (the protein level was below the threshold of detection) (data not shown).

DISCUSSION

Human neutrophil elastase inhibitors, such as alpha-1 proteinase inhibitor and SLPI, have recently been used in in vivo models as purified or recombinant proteins to modulate lung and systemic inflammation (24, 30, 31, 40). Workers in our laboratory are particularly interested in elafin, a more specific neutrophil elastase inhibitor which exhibits 40% homology with SLPI. This 10-kDa molecule is part of a four-disulfide-core protein family which has recently been named the trappin family (35). In addition, it has been shown that in vitro and in vivo elafin is a cationic molecule with activity against gram-positive and gram-negative pathogens (36, 37) and can therefore be considered a defensin-like molecule with a role in innate immunity.

Recently, the use of adenovirus as a lung vector for transient gene expression of potentially therapeutic transgenes under control of the strong MCMV promoter allowed us to discover

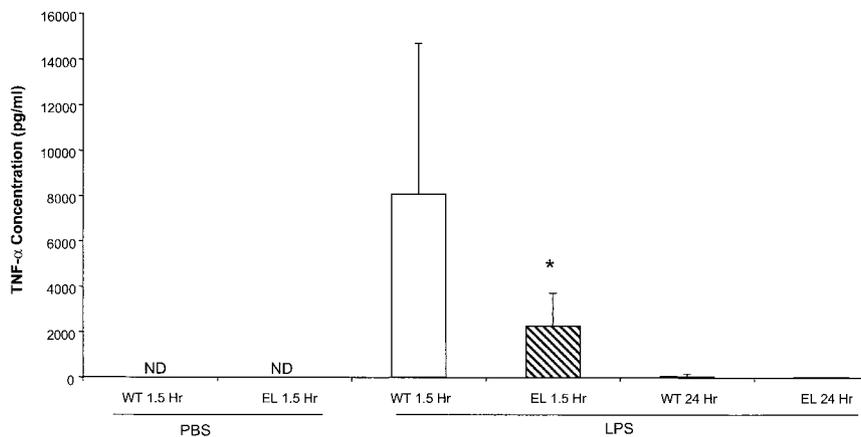


FIG. 6. Serum TNF- α levels are lower in elafin mice following intraperitoneal LPS injection. A TNF- α ELISA analysis was performed with serum samples obtained from WT mice and elafin mice (EL) (ages, 8 to 10 weeks) either 1.5 or 24 h after intraperitoneal administration of 120 μ g of LPS or PBS. The numbers of mice in the groups were as follows: WT mice treated with LPS, 10 mice; elafin mice treated with LPS, 10 mice; WT mice treated with PBS, 5 mice; and elafin mice treated with PBS, 5 mice. All data are expressed as the mean \pm standard deviation. An unpaired two-tailed Student's *t* test was used to establish statistical significance between groups. The levels of TNF- α in the serum of elafin mice 1.5 h after LPS treatment were significantly less than the levels in LPS-treated WT mice ($P = 0.02$), as indicated by the asterisk. ND, not detected.

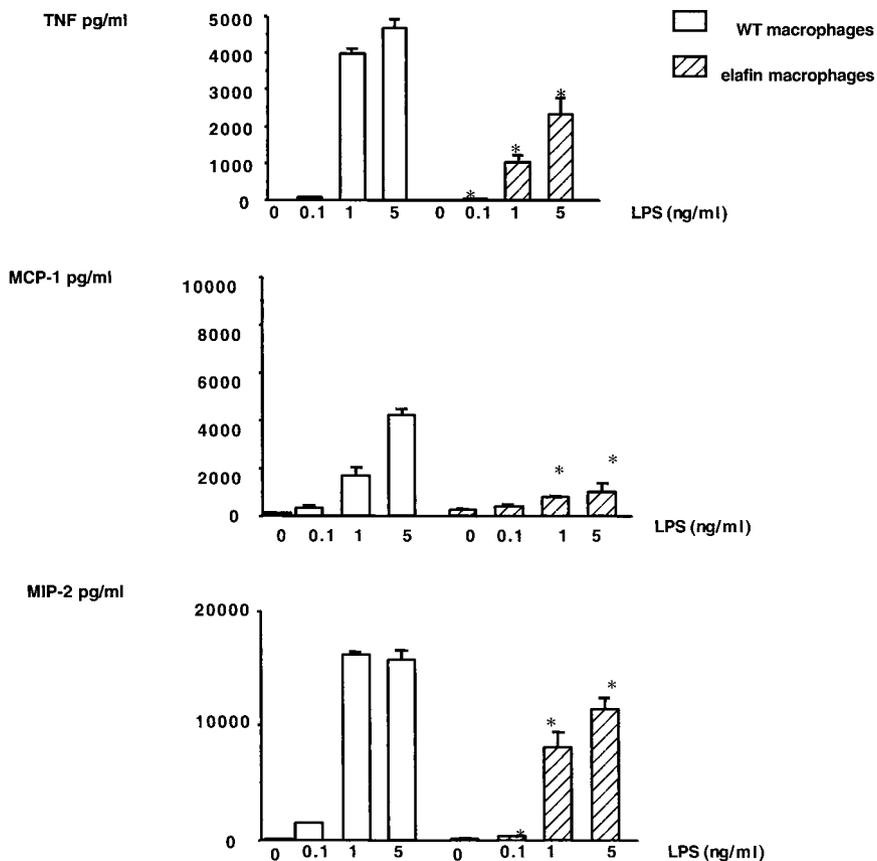


FIG. 7. Peritoneal macrophages from elafin mice are hyporesponsive to LPS. Peritoneal macrophages from elafin mice and WT mice were obtained as described in Materials and Methods. Cells (5×10^5 cells) were then cultured in 48-well plates in 1 ml of RPMI 1640 containing 10% fetal bovine serum with or without different LPS concentrations (0 to 5 ng/ml). After 48 h, the medium was recovered, and the concentrations of TNF- α , MCP-1, and MIP-2 were determined by ELISA. An unpaired two-tailed Student's *t* test was used to establish statistical significance between groups ($n = 3$). An asterisk indicates that a value is statistically significantly different ($P < 0.05$) than the value obtained with the equivalent LPS dose in WT mice

a new property for elafin, its chemotactic activity for lung inflammatory cells (38).

In order to determine thoroughly the role of this molecule in local and systemic inflammation, in the present study we used transgenic mice that were engineered to express human elafin constitutively under control of the same MCMV promoter.

Importantly, our elafin construct codes for the full-length protein, which contains transglutamination-cementoin sites (27, 29, 34, 35) thought to be important in binding of the molecule to the interstitium. Recently, Aiba-Masago et al. studied expression in transgenic mice of bacterial β -galactosidase driven by an MCMV promoter similar to the one used in our study (2). These authors found that the brain, stomach, kidney, and skeletal muscle were the major organs which expressed the transgene. Our study (Fig. 2) showed that the stomach, small intestine, skeletal muscle, and bone are major sites of expression. The reasons for the slight difference in the spectrum of organs in which the transgene is expressed between the two studies are unclear, but the difference could reflect differences in the read-outs (i.e., mRNA levels in our study and protein levels in the study of Aiba-Masago et al.) or differences in the chromosomal location of the transgenes. Alternatively, our human elafin cDNA construct has a 158-bp 3' untranslated region which may contain positive or negative regulatory binding sites which could influence the MCMV-

associated specificity of expression. Interestingly, when studying endogenous expression of human elafin RNA, Nara et al. found that in accordance with our results, stomach and small intestine tissues were positive as determined by an RNase

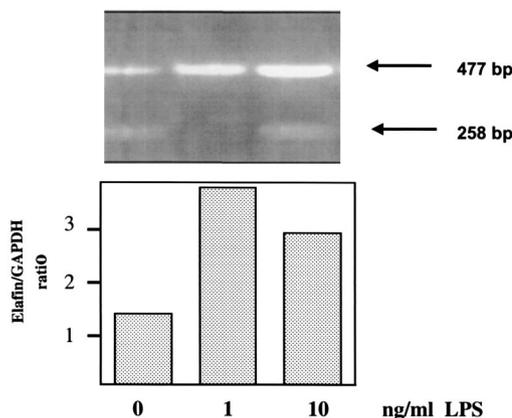


FIG. 8. Elafin mRNA production by peritoneal macrophages. Peritoneal macrophages from elafin mice were obtained and cultured as described in the legend to Fig. 7, except that the LPS doses were 0, 1, and 10 ng/ml. RT-PCR for the elafin and GAPDH transcripts was performed as described in Materials and Methods.

protection assay (27). Using Northern blot analysis, Pfundt et al. showed that the human tongue and pharynx (organs not assessed in the other studies mentioned here) are strong expressers of elafin, although these workers did not study the stomach, small intestine, skeletal muscle, and bone (29).

Although some organs expressed elafin more strongly than other organs, it was demonstrated by using RT-PCR that all the organs tested here were positive for expression of human elafin in the transgenic mice. The results of this study also confirmed the results of a previous study (33) showing that the human elafin cDNA probe does not cross-react with any murine message.

In the second part of our study we tested whether the elafin mice generated here were able to modulate the activity of intratracheally instilled LPS to the same extent that was observed in the adenovirus-elafin model (38).

Figure 4 shows that there was increased inflammatory cell influx (both neutrophils and macrophages) in BALF from elafin mice compared to that in WT mice following intratracheal LPS administration. A net increase in lung chemotactic activity is caused by chemokine gradients generated either by an increase in the levels of lung chemokines or a decrease in the levels of systemic chemokines (3, 28, 43, 46). We therefore measured serum and BALF chemokine levels and found that neutrophilic, monocytic cytokine, and chemokine (TNF- α , MIP-2, and MCP-1) ratios were lower in the elafin mice (Fig. 3). In addition, the cytokine levels were found to correlate with elafin concentrations (Fig. 5). These results are qualitatively similar to the results observed in our adenovirus study (38). Interestingly, although as indicated above there was a trend toward increased numbers of macrophages in BALF from mice given adenovirus elafin intratracheally, the number of neutrophils prominently increased in that study. In contrast, the numbers of both neutrophils and macrophages were elevated in elafin mice in the present study. The difference between the transient adenovirus and constitutive transgenic models could result from differential chemotactic activity due to a difference in lung elafin expression, which was much higher in the adenovirus study (nanogram levels, compared with the picogram levels in this study), the numbers of potential different receptors (currently uncharacterized), and the affinities on macrophages and neutrophils. Alternatively, a difference in mouse strains (pure C57BL6 mice for the adenovirus-derived experiments and a mixture of CBA and C57BL6 mice for the transgenic mouse experiments) could also be an explanation. Regardless, the results are reminiscent of those obtained with defensins and cathelicidins; indeed, these molecules have also been shown to have cytokine-stimulating activities (7, 41) and chemotactic properties for a variety of cell types in vitro (for a recent review, see reference 52). In some instances, a receptor has been identified; human β -defensin 2 acts via CCR6 on immature dendritic cells and memory T cells (50), and LL37 acts via the formyl peptide receptor-like 1 on human neutrophils, monocytes, and T cells (51).

In addition to the in vitro studies, Zhang et al. recently showed that when administered intratracheally to mouse lungs, human α -defensins (a mixture of α -defensin 1, α -defensin 2, and α -defensin 3) also induced an increased flux of inflammatory cells in the alveolar space (53). Significantly, these authors also reported increased levels of proinflammatory cytokines,

including MCP-1, and a serum-BAL cytokine gradient similar to the one found in our study. Interestingly, chemotactic activity of defensins has also been demonstrated in the peritoneal cavity following *Klebsiella pneumoniae* infection of mice (45).

As mentioned above, the intratracheal LPS data suggested that in elafin mice there is an elevated gradient of cytokines across the alveolus-blood barrier. The reduced serum TNF- α levels in elafin mice after intratracheal LPS administration (Table 1) suggests that elafin may act in this model in part by inhibiting circulating (but not lung) LPS signaling, after partial leakage of LPS from the lung. Indeed, following intratracheal LPS administration, there was a trend toward increased (not decreased) lung TNF- α production in elafin mice, which would argue in our model against spillover of TNF- α from the lung into the circulation.

The cytokine gradient generated by elafin may have a dual function; it may promote up-regulation of innate immunity responses in the lung compartment and simultaneous down-regulation of inflammatory responses in the circulation. The latter down-regulation would be akin to that caused by SLPI, which has been shown to decrease in vitro production of proinflammatory mediators in human monocytes and murine macrophages (10, 21, 26, 54).

In the third part of our study, to test this hypothesis, we injected WT mice and elafin mice intraperitoneally with LPS and measured serum TNF- α levels after treatment. Figure 6 shows that the serum TNF- α levels were indeed reduced in the elafin mice. This systemic hyporesponsiveness of elafin mice was further studied ex vivo by examining macrophage responses to LPS. Following stimulation of peritoneal macrophages with LPS, it was apparent that cells from elafin mice made elafin mRNA and that LPS was able to up-regulate the message. This up-regulation by LPS was probably due to the presence of nuclear factor κ B sites within the MCMV promoter, as observed previously in the adenovirus study (38). In addition, cells from elafin mice produced less TNF- α , MIP-2, and MCP-1 than cells from WT mice produced (Fig. 7). Interestingly, macrophages from elafin mice produced less TNF- α than macrophages from WT mice produced under basal (PBS) conditions, echoing data presented in Table 1, which show that the serum TNF- α levels were lower in the former mice.

In conclusion, these are the three main findings of the present study. (i) The elafin mice that we created expressed human elafin mRNA in a variety of organs (Fig. 1 and 2) and expressed protein levels in the lung and serum, as assessed by ELISA analysis. (ii) After intratracheal LPS administration, increases in numbers of inflammatory cells in BALF and decreases in the serum/BALF cytokine ratios were observed in the elafin mice compared to the values for WT mice (Table 1 and Fig. 3 and 4). In addition, the cytokine levels were correlated with the elafin concentrations in BALF (Fig. 5). (iii) Elafin mice were less responsive to LPS, as assessed in vivo, after intraperitoneal injection of LPS and measurement of circulating TNF- α (Fig. 6) and ex vivo (with peritoneal macrophages) (Fig. 7).

Because of the role of TNF- α in many of the pathophysiologic events in sepsis (14, 15), inhibitors of the TNF- α cascade have been extensively studied (9, 10, 12, 16, 19, 21, 23, 24, 26, 48). We believe that elastase inhibitors, such as elafin, are particularly interesting because of their small size, which allows

better diffusion in tissues. Both in a previous adenovirus study (38) and in the present study a decrease in blood-BALF TNF- α -chemokine gradient in mice overexpressing elafin was found. We believe that the elafin mice should help in assessing which of the two strategies (i.e., local application or systemic application of elastase inhibitors) should be more beneficial in clinical applications. As pointed out above, because of possible differences in site expression, care should be taken to differentiate the physiological effects of an endogenous protein and the potential therapeutic effects of a transgene. We are currently pursuing this line of investigation in our laboratory and with our clinical colleagues.

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