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A Functional Variant of Elafin With Improved Anti-inflammatory Activity for Pulmonary Inflammation

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Elafin is a serine protease inhibitor produced by epithelial and immune cells with anti-inflammatory properties. Research has shown that dysregulated protease activity may elicit proteolytic cleavage of elafin, thereby impairing the innate immune function of the protein. The aim of this study was to generate variants of elafin (GG- and QQ-elafin) that exhibit increased protease resistance while retaining the biological properties of wild-type (WT) elafin. Similar to WT-elafin, GG- and QQ-elafin variants retained antiprotease activity and susceptibility to transglutaminase-mediated fibronectin cross-linking. However, in contrast to WT-elafin, GG- and QQ-elafin displayed significantly enhanced resistance to degradation when incubated with bronchoalveolar lavage fluid from patients with cystic fibrosis. Intriguingly, both variants, particularly GG-elafin, demonstrated improved lipopolysaccharide (LPS) neutralization properties in vitro. In addition, GG-elafin showed improved anti-inflammatory activity in a mouse model of LPS-induced acute lung inflammation. Inflammatory cell infiltration into the lung was reduced in lungs of mice treated with GG-elafin, predominantly neutrophilic infiltration. A reduction in MCP-1 levels in GG-elafin treated mice compared to the LPS alone treatment group was also demonstrated. GG-elafin showed increased functionality when compared to WT-elafin and may be of future therapeutic relevance in the treatment of lung diseases characterized by a protease burden.

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INTRODUCTION

Mature elafin is a 6kDa serine protease inhibitor largely generated via proteolytic processing of trappin-2 (pre-elafin), primarily by the mast cell-derived protease tryptase.1,2 Elafin was originally isolated from human bronchial secretions in 1985 by Kramps and Klasen3 under the name of LMI-5000 and subsequently by Schalkwijk et al.4 and Wiedow et al.5 in 1990 from human psoriatic skin under the name SKALP and elafin, respectively. In addition, elafin has also been reported to be expressed by macrophages and neutrophils.6 Work to date has shown that elafin acts as a multifunctional host defence protein with antimicrobial, antiprotease, and immunomodulatory properties.7-12 Elafin expression is upregulated at various sites of inflammation by a range of proinflammatory mediators6,13-15 and may provide protection against neutrophilic proteases.16 Elafin is recognized as a potent inhibitor of a restricted set of serine proteases, namely neutrophil elastase (NE) and proteinase-3 (PR3).4,17,18 Trappin-2 and elafin have a number of transglutaminase (TG) reactive residues and therefore can be linked covalently to various extracellular matrix proteins such as fibronectin by tissue transglutaminases and retain potent antiprotease activity.19-22 Due to its cationic nature, it is postulated that trappin-2 displays antibacterial properties via disruption of bacterial cell membranes.7,10 In addition, it has been demonstrated that trappin-2 and mature elafin can bind and neutralize lipopolysaccharide (LPS)12,24 thereby suppressing macrophage TNF-α production.21 In human monocytic cells, mature elafin inhibited LPS-induced production of monocyte chemotactic protein-1 (MCP-1) and activation of both activator protein-1 (AP-1) and nuclear factor κB (NF-κB) via disruptions to the ubiquitin proteasome pathway.9

In the healthy lung, antiproteases such as trappin-2/elafin are present providing the lung with a powerful anti-inflammatory screen. However, in diseases such as adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF), this protease–antiprotease balance is tipped in favor of proteases leading to dysregulated extracellular protease activity resulting in lung damage. A number of pathogen and host proteases have the ability to cleave elafin.25-28 Increased NE activity is present in various respiratory diseases including ARDS, COPD, and CF and may overwhelm the protective anti-protease levels within the lung. Indeed, we have found evidence of proteolytic cleavage of elafin by NE in patients with CF with established Pseudomonas aeruginosa infection.24 Elevated NE levels during Pseudomonas infection in the CF lung also leads to...
cleavage and inactivation of the related antiprotease secretory leucoprotease inhibitor. An imbalance in NE and trappin-2/elafin levels has also been reported in ARDS and COPD patients with secondary bacterial infections.

In early 2013, the US Food and Drug Administration (FDA) granted elafin orphan drug designation for the prevention of inflammatory complications associated with transthoracic esophagectomy. However, proteolytic cleavage of elafin could attenuate its anti-inflammatory and antiprotease functions and limit the efficacy of elafin in clinical trials in conditions such as ARDS, CF, and COPD. To address this, we have synthesized novel variants of elafin, and we hypothesize that these variants will be more resistant to NE cleavage and therefore of significant utility in the treatment of pulmonary inflammation in diseases characterized by a NE burden.

RESULTS

Recombinant synthesis of elafin variants

As shown in Figure 1, mutations to the coding sequence for mature elafin, as denoted by the arrows positioned on the WT-elafin sequence, were made at the two previously described NE cleavage sites in order to generate the GG- and QQ-elafin variants. For GG-elafin, the codons coding for valines at position 5 and 9 of the elafin amino acid sequence were mutated to generate glycine residues. For QQ-elafin, the codons coding for valines at position 5 and 9 of the elafin amino acid sequence were mutated to generate glutamine residues. These residues were selected as elastase cleavage after glycine and glutamine is rare (MEROPS database). Neither amino acid substitution had any effect on the isoelectric point and net charge of the protein.

Determination of protease inhibition properties of the elafin variants

As shown in Table 1, the $K_{c}$ of both mutated elafin variants (GG and QQ) against human NE and PR3 were very closely matched to that of WT-elafin. Similarly, addition of equimolar amounts of each elafin molecule (WT, GG, and QQ) to NE resulted in a significant reduction ($P < 0.01$) in the turnover of NE substrate compared to NE alone (data not shown) indicating effective 1:1 stoichiometric inhibition of NE by all three elafin proteins.

Western blot analysis of recombinant elafin incubated with CF bronchoalveolar lavage fluid and NE

To compare the proteolytic susceptibility of GG- and QQ-elafin to WT-elafin, we incubated the three elafin proteins with pooled Pseudomonas-positive CF bronchoalveolar lavage fluid (BALF) over a time period of 0, 2, and 8 hours, and the cleavage products were assessed by western blot analysis (Figure 2a). WT-elafin was rapidly cleaved by CF BALF as denoted by the presence of a double band at 2 hours which was almost completely degraded by 8 hours. In contrast, GG-elafin was completely resistant to cleavage by Pseudomonas-positive CF BALF even after 8 hours of incubation. There was some evidence of cleavage of QQ-elafin by CF BALF, although there was still a significant portion of intact QQ-elafin present after 8 hours. The susceptibility of the elafin variants to proteolysis by NE was also compared (Figure 2b). Similar to the BALF results in Figure 2a, WT-elafin was rapidly cleaved by NE, whereas GG- and QQ-elafin remain relatively intact over the course of the experiment. However, following the 60 minutes of incubation, GG-elafin exhibited enhanced resistance to cleavage when compared to QQ- and WT-elafin. Overall, these data indicate that the GG-elafin variant is more resistant to proteolytic cleavage when compared to QQ- and WT-elafin.

LPS binding and transglutaminase-mediated cross-linking activity of the elafin variants

Elafin can interact with the LPS of Gram-negative bacteria and modulate cellular responses after LPS stimulation. Therefore, the ability of the elafin variants to bind and neutralize LPS were investigated. As illustrated in Figure 3a, QQ-elafin variant retained the highest LPS-binding ability when compared to the WT- and GG-elafin. GG-elafin also bound to LPS substantially more than the WT-elafin, although not to the same extent as the QQ-elafin variant. These findings suggest that the introduction of mutations in the GG- and QQ-elafin variants enhances the LPS binding activity of both molecules compared to WT-elafin. As mentioned previously, elafin can be cross-linked to extracellular matrix proteins such as fibronectin via the action of TG. GG-elafin exhibited comparable binding to fibronectin when compared to WT-elafin in the presence of the TG (Figure 3b).

Figure 1 Mutation of neutrophil elastase cleavage sites in recombinant mature elafin. Neutrophil elastase (NE) cleavage sites were previously identified in human mature elafin, and the positions are indicated by the arrows at Val5-Lys6 and Val9-Ser10 (ref. 24). The cleavage sites were mutated to Gly5-Lys6 and Gly9-Ser10 for the GG-elafin variant and to Gln5-Lys6 and Gln9-Ser10 for the QQ-elafin variant. The mutations are indicated by underlined bold type letters.
Furthermore, the QQ-elafin variant demonstrated a significant increase in binding to fibronectin when compared to WT-elafin ($P < 0.05$). A similar trend was also observed when compared to GG-elafin; however, this was found to be nonsignificant.

**Effect of elafin variants on LPS-challenged U937 monocytic cells**

Peripheral blood monocytes (PBMs) and U937 monocytic cells were pretreated with WT-elafin and each elafin variant (10 μg/ml) prior to LPS stimulation. Secreted IL-8 levels in cell-free supernatants were quantified by enzyme-linked immunosorbent assay (ELISA). PBMs (Figure 4a) and U937s (Figure 4b) pretreated with GG-elafin secreted significantly lower levels of IL-8 compared to LPS alone stimulated controls. Furthermore, although WT-elafin and QQ-elafin decreased LPS-induced IL-8 release from PBMs and U937s, this was not significant suggesting that the GG-elafin variant has augmented anti-inflammatory properties over the parental molecule. Given the preservation of binding capabilities to extracellular matrix proteins and LPS, and also the increased resistance to proteolytic cleavage, GG-elafin was selected for further validation experiments in vivo.

**Effect of GG-elafin on acute pulmonary inflammation in vivo**

Leading on from the in vitro studies which demonstrated significant anti-inflammatory properties of GG-elafin compared to WT-elafin, the effects of WT- and GG-elafin in an in vivo model of LPS-induced acute lung inflammation were investigated (Figure 5). Treatment of mice with WT-elafin resulted in a nonsignificant decrease in inflammatory cell infiltration in response to LPS (Figure 5a,b). However, treatment of mice with GG-elafin resulted in a significant reduction in LPS-induced neutrophil infiltration into the lung when compared to mice treated LPS alone (Figure 5a; $P < 0.01$). In order to assess alveolar-capillary barrier permeability induced by LPS, total protein concentrations in BALF were determined.

| Table 1 Comparison of the antiprotease activity of elafin variants |
|-------------------|------------------|------------------|
|                   | Neutrophil elastase $K_i$ (mol/l) | Proteinase 3 $K_i$ (mol/l) |
| WT-elafin         | $8 \times 10^{-11}$ | $1.2 \times 10^{-10}$ |
| GG-elafin         | $2 \times 10^{-11}$ | $5.25 \times 10^{-10}$ |
| QQ-elafin         | $3 \times 10^{-11}$ | $5.1 \times 10^{-10}$ |

WT, wild type.
quantified. BALF protein levels were decreased in the WT-elafin (not significant) and GG-elafin (Figure 5c; \( P < 0.01 \)) treated mice when compared to that of the LPS alone treated mice. In agreement with in vitro observations (Figure 4), these findings suggest that GG-elafin has augmented anti-inflammatory activity over the parental WT-elafin molecule.

Given the observed effects of elafin on neutrophilic infiltration into the lung, chemokine levels in BALF were investigated (Figure 6). There was no difference in the levels of KC and MIP-2 in either the GG-elafin or the WT-elafin treated mice when compared to the LPS alone treated mice (Figure 6a, b). However, there was a significant difference in BALF MCP-1 levels from mice treated with the GG-elafin compared to those which received the LPS treatment alone as shown in Figure 6c (\( P < 0.01 \)). In contrast, mice treated with WT-elafin did not exhibit a significant reduction in MCP-1 compared to mice treated with LPS alone (Figure 6c). To investigate the effects of elafin on LPS-induced protease burden, we measured NE activity in BALF. Overall, NE activity was undetectable in the majority of samples. Low turnover of substrate was detected only in a number of the LPS samples (112.5 pmol 7-amino-4-methylcoumarin (AMC)/μg protein ± 42.7 pmol AMC/μg protein), and no activity was detected in the LPS+WT or LPS+GG BAL samples, which suggests that both forms of elafin are able to inhibit elastase activity in vivo. As a surrogate marker of protease activity, we measured levels of endostatin in the BALF from our study by ELISA. As illustrated in Figure 6d, LPS challenge upregulated the levels of endostatin in BALF compared to the saline groups. Although both WT- and GG-elafin inhibited the generation of endostatin in comparison to the LPS alone group, only the GG-elafin was significantly...
lower versus LPS alone, which correlates well with our neutrophil counts in Figure 6a.

DISCUSSION

Elafin has been previously shown to possess potent inhibitory properties toward the neutrophil serine proteases, NE and PR3. Given its low molecular weight and potent antiprotease activity, it has been postulated that elafin could have potential therapeutic relevance in a number of diseases characterized by a protease burden. However, it has previously been demonstrated by our group that elafin is susceptible to proteolysis by NE in the lungs of CF patients chronically infected with P. aeruginosa. Therefore, the development of a more cleavage-resistant elafin molecule using directed mutagenesis may offer an attractive strategy for the development of elafin as a therapy. The generation of the QQ- and GG-elafin variants in this study confirmed enhanced cleavage resistance over the native WT-elafin molecule when exposed to Pseudomonas-infected CF BALF. Furthermore, the QQ-elafin variant demonstrated a significantly increased affinity for fibronectin in the presence of transglutaminase when compared to WT-elafin. The increased binding affinity of the QQ-elafin is likely due to the introduction of glutamine residues, but this requires further validation. In addition, we have demonstrated increased LPS binding and a reduction in LPS-induced IL-8 production in monocytic cells preincubated with GG-elafin compared to WT-elafin. These data led us to consider if GG-elafin may have additional anti-inflammatory effects in vivo compared to WT-elafin.

With the use of the LPS-induced mouse model of acute lung inflammation, we have demonstrated that the GG-elafin variant possessed enhanced anti-inflammatory characteristics when compared to the parent WT-elafin molecule as demonstrated by the noted reduction in inflammatory cells within the lungs, in particular, neutrophils. It is acknowledged that neutrophilic influx characterizes the early stages of acute pulmonary inflammation and is associated with disruption of the alveolar-capillary barrier and lung tissue damage. Although a near onefold reduction in neutrophilia was observed in the mice receiving LPS+GG elafin compared to those receiving LPS alone, this may not be sufficient to reduce lung damage in the host. Further studies are required to more carefully evaluate the effect of a onefold reduction perhaps using more advanced in vivo models of lung damage. NE activity as measured using the substrate AAPV-AMC was only detected in a number of the LPS alone BALF samples. No turnover of substrate was detected in the LPS+WT or LPS+GG BAL samples, which suggests that both forms of elafin are able to inhibit elastase activity in vivo. However, given that the turnover of substrate was very low, we suspect that the majority of elastase may be present on the surface of airway neutrophils. We also quantified levels of the endostatin which is a COOH-terminal fragment of collagen XVIII that can be generated by elastase and is upregulated in the BALF from a human in vivo LPS challenge model and acute

![Figure 6](image-url)
MCP-1, known to regulate the expression of NF-κB. We have previously reported that elafin can inhibit LPS-induced production of MCP-1 by monocytic cells via inhibition of LPS-mediated activation of the transcription factors AP-1 and NF-κB. Thus, it is more likely that the greater stability of GG-elafin compared to WT-elafin may be beneficial in the progression of elafin in various clinical trials, we have shown that GG-elafin is capable of diminishing the combined problem of an excessive neutrophil protease burden as well as increased neutrophilic inflammation, both of which are characteristic features of a number of acute and chronic inflammatory lung conditions.

MATERIALS AND METHODS

Cloning and recombinant synthesis of elafin proteins. Recombinant synthesis of mature human WT-elafin was carried out in Pichia pastoris as previously described.44,45 In order to generate GG-elafin (V5G/V9G) and QQ-elafin (V5Q/V9Q), site-specific mutations were introduced to the elafin cDNA by PCR. First, the elafin cDNA template was amplified by PCR using forward primers for GG-elafin (5′-ATC TTC CAG AAA AGA GGC CAA GAG CCA GGC TTC ACT AAG CC-3′) or QQ-elafin (5′-ATC TTC CAG AAA AGA GGC CAA GAG CCA AAA AAA GTT CCA TCC ACT AAG CC-3′) and a reverse primer (5′-CGA-GCCGCGCC-CCCTCTCACTGGAAGAC-3′) (Eurofins Operon) and a blend of PCR enzymes (Expand High Fidelity PCR System; Roche, Meylan, France). The cDNA was then cut with XhoI and NotI (THERMO Fisher Scientific Biosciences, Villebon sur Yvette, France) and ligated into the pPIC9 vector (Invitrogen, Groningen, The Netherlands) linearized with the same enzymes. After transformation of Escherichia coli XL1 blue (Agilent Technologies, Waldbronn, Germany), positive clones were screened by PCR, and DNA sequencing was performed to be sure that only the two desired mutations were correctly introduced. After Salt digestion, the recombinant pPIC9 vectors were electroplated in the GS115 P. pastoris strain, and transformants screened by their capacity to grow on histidine-free regeneration dextrose medium. Finally, elafin production was performed in BMGY medium (methyl 1%) to select the clones having the higher level of elafin secretion.

Purification method. Concentrated supernatants of elafin secreting P. pastoris cultures were dialysed against 25 mmol/l sodium phosphate, pH 6.0 (equilibrium buffer) and loaded onto a Source 15S (GE Healthcare Life Sciences, Buckinghamshire, UK) cation-exchange column (1.6×15 cm). The column was washed exhaustively with equilibrium buffer to remove unbound proteins, and the bound inhibitors were eluted at a flow rate of 1 ml/minute with a linear NaCl gradient (0–1 mol/l) in equilibration buffer for 40 minutes. The purity of each elafin preparation was assessed by high-resolution Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions and Coomassie staining (Supplementary Figure S1).

Protease activity assays. The K of each elafin (WT, GG, and QQ) was evaluated as previously described using human NE and PR3 as the target proteases.44 To determine stoichiometric inhibition of NE by the elafin proteins, equal concentrations of each elafin variant (WT, GG, and QQ) were incubated with NE (8.5×10−7 mol/l) in incubation buffer (0.1 mol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.5; 0.5 mol/l NaCl) for 30 minutes at room temperature and then incubated with 50 μmol/l NE substrate N-Methoxy-Succinyl-Pro-Ala-Ala-Val-7-amino-4-methylcoumarin (AAPV-AMC) for 30 minutes. Changes in fluorescence were measured at 365/460 nm for excitation/emission and plotted as relative fluorescence units per minute. BALF NE activity was determined using AAPV-AMC (Enzo Life Sciences, Exeter, UK) as described previously.29

Cystic fibrosis bronchoalveolar lavage fluid samples and study approval. BALF samples were obtained from five CF patients with chronic P. aeruginosa infection (Ps+) as described previously.29 Clinical information for patients is depicted in Table 2. Ethical approval was obtained from the institutional review board of the Adelaide and Meath Hospital incorporating the National Children’s Hospital with all parents providing written informed consent prior to participation.
Table 2 Cystic fibrosis bronchoalveolar lavage fluid patient data

<table>
<thead>
<tr>
<th>CF BALF (n = 5)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at BAL, years</td>
<td>14.63 (1.788)</td>
</tr>
<tr>
<td>Neutrophil elastase activity, μmol/l</td>
<td>18.00 (3.334)</td>
</tr>
<tr>
<td>FEV1</td>
<td>37.6 (6.153)</td>
</tr>
<tr>
<td>Total cells/ml</td>
<td>1.308 × 10^6 (1.213 × 10^6)</td>
</tr>
<tr>
<td>Neutrophils/ml</td>
<td>1.229 × 10^6 (1.351 × 10^6)</td>
</tr>
<tr>
<td>Macrophage/ml</td>
<td>5.745 × 10^5 (3.031 × 10^5)</td>
</tr>
</tbody>
</table>

BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; FEV1, forced expiratory volume in 1 second.

Values represent mean (SEM).

**Western blot analysis of recombinant elafin incubated with CF BALF and neutrophil elastase.** Each elafin variant (WT, GG, and QQ; 50 ng) was incubated with 5 μl of pooled Pseudomonas-positive CF BALF in TBS in a final volume of 20 μl for 0, 2, and 8 hours at 37 °C as previously described. Alternatively, the elafin variants (100 ng) were incubated with a 3:1 molar excess of neutrophil elastase (Elastin Products Company, Owensville, MO) in 0.1 mol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 mol/l NaCl, pH 7.5, in a total volume of 20 μl for 0, 5, 15, and 60 minutes at 37 °C. The reactions were terminated by addition of nonreducing sample treatment buffer and boiling at 99 °C for 10 minutes. Samples were separated by Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (17.5%) under nonreducing conditions and transferred onto 0.1 μm nitrocellulose membrane (Sigma-Aldrich, Dorset, UK). The membrane was blocked for 1 hour at room temperature in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20. Elafin was detected using a biotinylated anti-elafin antibody (1:500, overnight at 4 °C; R&D Systems, Abingdon, UK). After washing, the membrane was incubated with streptavidin–HRP (1:2,500, 20 minutes at room temperature; BioLegend, London, UK), and elafin visualized by chemiluminescence (GE Healthcare). Images were captured using the Syngene G:Box and GeneSnap software (SynGene, Cambridge, UK).

**LPS-binding ELISA.** The ability of elafin variants to bind LPS was analyzed by ELISA as described previously. Briefly, Greiner high binding 96-well plates were coated with 100 ng/well of P. aeruginosa LPS (Sigma-Aldrich, Dorset, UK) for at least 3 hours at 37 °C. The wells were washed three times with distilled water before air-drying overnight at room temperature. Plates were blocked with 1% BSA in PBS containing 0.1% (v/v) Tween 20 for at least 1 hour at room temperature. The wells were blocked with 200 μl/well 1% BSA in PBS for 1 hour at 37 °C. Elafin variants (0–1,000 ng) were made up to 100 μl and incubated on the plate for 2 hours at 37 °C. After washing, biotinylated anti-elafin antibody was added to the plate for 2 hours at room temperature (100 μl/well; 1:100). Plates were again washed, and 100 μl of streptavidin–HRP added per well for 30 minutes at room temperature. After washing, peroxidase activity was measured by the addition of ABTS substrate (Life Technologies) and reading the absorbance at 405 nm in a microplate reader (Synergy HT using Gen5 software; BioTek).

**Cell culture and ELISA.** Ethical approval for use of PBMs from buffy coat was given by Northern Ireland Blood Transfusion Service. Human myelomonocytic U937s were purchased from the American Type Culture Collection (Manassas, VA). Both PBMs and U937s were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Life Technologies), 2 mmol/l L-glutamine, and 1% (v/v) penicillin/streptomycin (PAA laboratories, Pasching, Austria). Cells for experiments were seeded at 5 × 10^5/ml and were preincubated with elafin variants (WT, GG, and QQ; 10 μg/ml) for 1 hour followed by incubation with Pseudomonas LPS (Sigma–Aldrich) for 16 hours. Cell-free supernatants were stored at −80 °C until required. Levels of human IL-8 in PBM and U937 supernatants were quantified using ELISA kits from R&D Systems according to manufacturer’s instructions.

**Mice.** C57BL/6 female mice were purchased from Charles Rivers Laboratories and also bred in-house. Mice aged between 10 and 12 weeks were used for in vivo experiments. Housing and experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen’s University Ethical Review Committee. The animals were maintained on a 12 hour cycle of light followed by 12 hour cycle of darkness with free access to chow and water.

**Effect of elafin variants on pulmonary inflammation in a LPS-induced acute lung injury mouse model.** Under anesthesia, Pseudomonas LPS (0.4 mg/ml; Sigma–Aldrich, Serotype 10, Source strain ATCC 27316) in saline and saline alone were intratracheally instilled into the mice (n = 4–6/group) with the aid of a blunt 24g IV catheter (BD Biosciences, Oxford, UK) into the lungs to a final volume of 50 μl. Pseudomonas LPS and saline alone were combined with 100 μg of WT- or GG-elafin and instilled as described above. After 4 hours, the mice were sacrificed and BALF collected. Briefly, a blunt needle (23 gauge) was inserted through the incision in the upper trachea and tied in place with 3.0 surgical monofil (Ethicon, Livingston, UK). BALF collection was performed by instilling a total of 0.8 ml of sterile ice-cold PBS gently into the lungs of each mouse with careful aspiration of the fluid. BALF cells were pelleted by centrifugation at 2,200 rpm for 10 minutes at 4 °C, and cell-free BALF was collected and stored at −80 °C until required. BALF cells were resuspended in 100 μl of PBS, and total cells counts were performed using a hemocytometer.

**Differential cells staining and cell counts.** Differential cell staining was performed on the cells collected and counted from the BALF. Approximately 5 × 10^5 cells were cytopsIn onto coated cytoslides (Shandon; Thermo Scientific) and stained with the Giemsa and May–Grünnwald stain (VWR, Lutterworth, UK). Briefly, cells were fixed in methanol for 20 minutes and allowed to air dry. Slides were immersed in May–Grünnwald stain for 8 minutes and transferred into PBS for a quick and gentle wash. Slides were then immersed into the Giemsa stain for 8 minutes and washed briefly under tap water. Excess water was removed, and the slides were allowed to air dry. Slides were mounted with aqueous VectaMount medium (Vectorlabs, UK), sealed with clear nail polish and allow to dry. Cells were visualized using a Leica DM5500B microscope and images captured using the image analysis Leica AL software (version 3.7, Leica Microsystems, Milton Keynes, UK) under ×40 objective lens. Histological cell counts were conducted on each slide where at least 400 cells per slide were counted.

**BALF protein and cytokine analyses.** BALF total protein concentrations were determined using the BCA method (Pierce BCA Assay; Fisher Scientific UK, Leicestershire, UK). Relative cytokine profiles in BALF (n = 4–6/group) from mice treated with LPS alone, LPS + GG-elafin, and...
LPS + WT-elafin were analyzed for levels of CXCL1/KC, CXCL2/MIP-2, and CCL2/MCP-1 using ELISA kits from R&D Systems. Endostatin levels were quantified by ELISA (USCN Life Science, Hubei, China). All kits were used according to the manufacturer’s instructions.

**Statistical analysis.** All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and are reported as mean ± SEM. Results are representative of at least n = 3 unless otherwise indicated. Means were compared by unpaired t-test, one-way analysis of variance (ANOVA with Tukey’s multiple comparison test), or Kruskal–Wallis test (with Dunn’s multiple comparison test) as appropriate. P < 0.05 was accepted to indicate statistical significance.

**SUPPLEMENTARY MATERIAL**

**Figure S1.** Purity of recombinant protein preparations.

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