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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 mediators^{6,13–15} 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).^{4,5,17,18}

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)^{23,24} thereby suppressing macrophage TNF- α production.²³ In human monocyte cells, mature elafin inhibited LPS-induced production of monocyte chemoattractant protein-1 (MCP-1) and activation of both activator protein-1 (AP-1) and nuclear factor κ B (NF- κ B) 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 antiprotease 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

The first three authors contributed equally to this work.

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Table 1 Comparison of the antiprotease activity of elafin variants

	Neutrophil elastase K_i (mol/l)	Proteinase 3 K_i (mol/l)
WT-elafin	8×10^{-11}	1.2×10^{-10}
GG-elafin	2×10^{-11}	5.25×10^{-10}
QQ-elafin	3×10^{-11}	5.1×10^{-10}

WT, wild type.

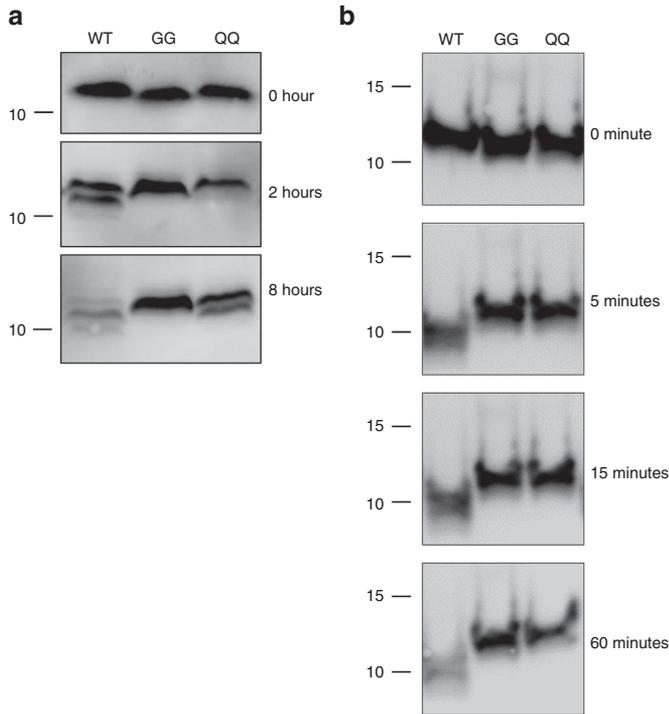


Figure 2 Susceptibility of elafin variants to cystic fibrosis BALF and neutrophil elastase proteolytic cleavage. (a) WT-elafin and elafin variants were incubated with pooled *Pseudomonas*-positive BALF collected from CF patients for 0, 2, and 8 hours at 37 °C. (b) WT-elafin and elafin variants were incubated with a 3:1 molar excess of neutrophil elastase for 0, 5, 15, and 60 minutes. Samples were subjected to SDS-PAGE under nonreducing conditions followed by western blot analysis, and elafin was detected using a biotinylated anti-elafin antibody. BALF, bronchoalveolar lavage fluid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; WT, wild type.

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

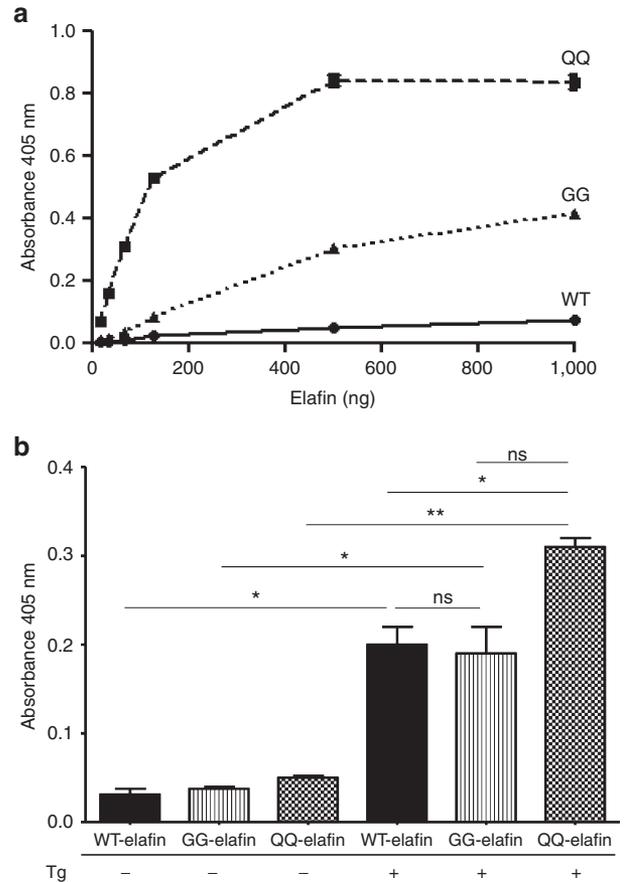


Figure 3 Relative LPS binding and transglutaminase-mediated cross-linking properties of elafin variants. (a) Increasing concentrations of elafin were analyzed via ELISA to determine the relative LPS-binding properties of the elafin recombinant variants. Bound elafin was calculated as the increase in absorbance at 405 nm ($n = 3$). (b) The ability of elafin variants to cross-link to fibronectin in the presence of guinea pig liver transglutaminase was investigated by ELISA. The absorbance read at 405 nm reflects the cross-linking of elafin to fibronectin ($n = 3$). * $P < 0.05$; ** $P < 0.01$. ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

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

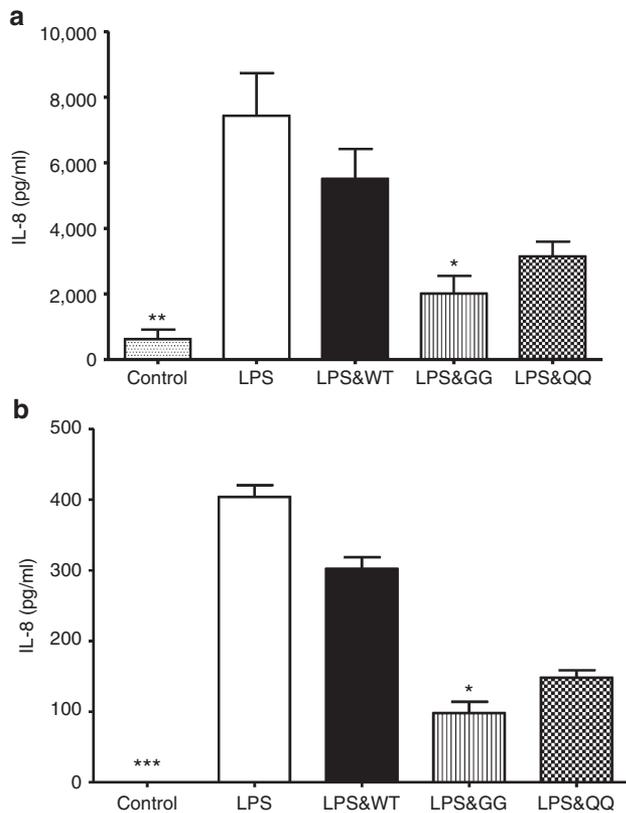


Figure 4 Effect of WT-elafin and elafin variants on LPS-induced IL-8 production by human monocytic cells. **(a)** PBMs ($n = 4$ donors) and **(b)** U937s ($n = 4$) were preincubated with elafin variants followed by incubation with *Pseudomonas* LPS for 16 hours and IL-8 measured in supernatants by ELISA. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ versus LPS. ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; 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 ($112.5 \text{ pmol } 7\text{-amino-4-methylcoumarin (AMC)}/\mu\text{g protein} \pm 42.7 \text{ pmol AMC}/\mu\text{g protein}$), 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

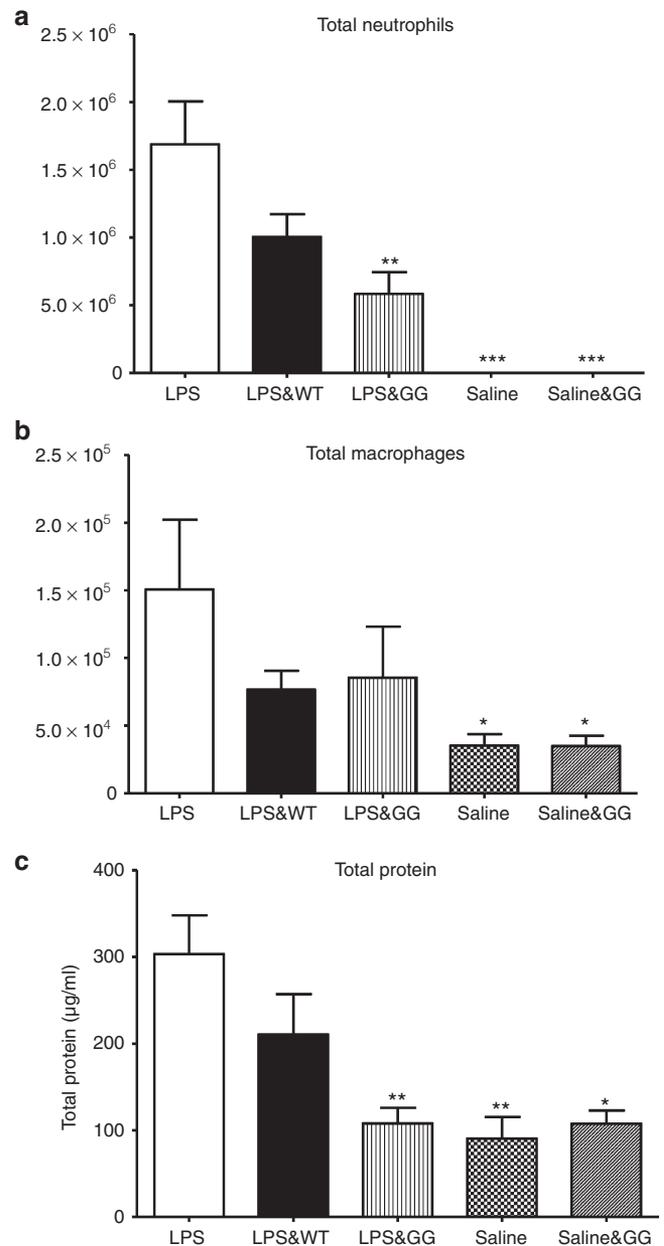


Figure 5 Elafin-GG attenuates inflammatory cell infiltration in a LPS-induced mouse model of acute lung inflammation. **(a)** Differential cell counts demonstrated that the decrease in total cell numbers in the LPS-treated mice with GG-elafin was attributed to a decrease in neutrophil infiltration in the lung. **(b)** Macrophage cell counts demonstrated a decrease in the cell population in mice inoculated with LPS and treated with both WT- and GG-elafin. **(c)** Protein analysis revealed a decrease in the presence of protein in BALF collected from lung tissue in mice inoculated with LPS and GG-elafin compared to LPS alone treated mice and the mice treated with LPS and WT-elafin ($n = 4-6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus LPS. BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; WT, wild type.

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

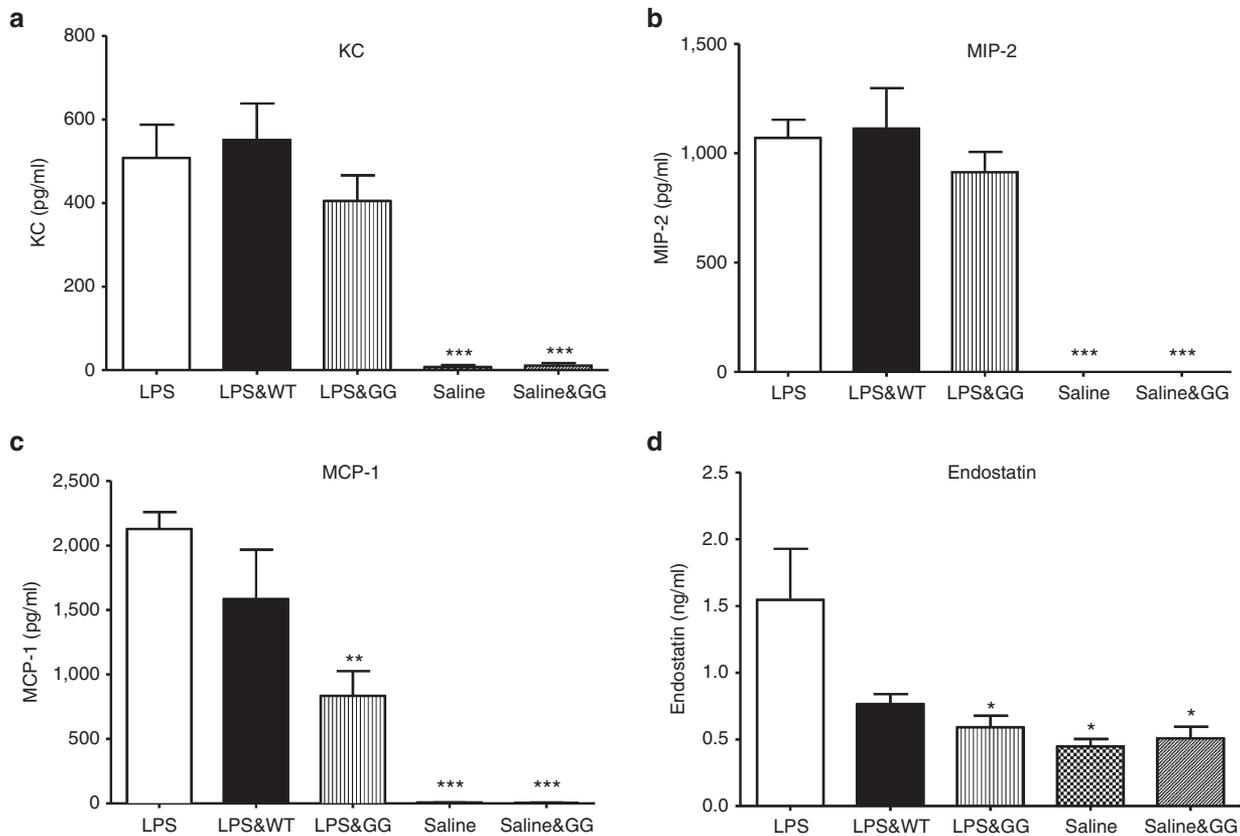


Figure 6 Effects of elafin variants on murine pulmonary cytokine levels. Levels of (a) KC, (b) MIP-2, (c) MCP-1, and (d) endostatin in BALF from mice treated with LPS alone, LPS and WT-elafin, LPS and GG-elafin, saline alone and saline plus GG elafin were determined by ELISA ($n = 4-6$). * $P < 0.05$; *** $P < 0.001$ versus LPS. BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; WT, wild type.

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.^{16,32} 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*.²⁴ 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,²² 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.^{33,34} 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.³⁵ 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

lung injury patients.^{36,37} 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 compared to LPS alone, which correlates well with our neutrophil counts.

It is well documented that the chemokines, KC and MIP-2, play key roles in the regulation of neutrophil infiltration into the lung, especially in response to various mediators, such as TNF- α and LPS. However, in this current investigation, we noted that KC and MIP-2 levels remained unaffected in response to elafin treatment. However, we have shown that *in vivo* administration of GG-elafin in mice receiving LPS resulted in a significant reduction in MCP-1 levels compared to LPS alone and mice receiving LPS and WT-elafin. MCP-1, known to regulate the expression of β_2 integrin, is classically associated as a chemoattractant for monocytes/macrophages, and it has been shown to trigger the adhesion of monocytes to inflamed pulmonary epithelium.^{34,38} Additionally, MCP-1 has been shown to act as a neutrophil chemoattractant in a number of acute and chronic inflammatory models, with recent data suggesting that MCP-1 may have a significant and key role to play in neutrophil recruitment and infiltration in the lung.^{38–41} It has also been proposed to indirectly regulate KC and MIP-2 expression during pulmonary *Escherichia coli* infection.³⁸ Furthermore, MCP-1 has been shown to play an important role in the bacterial clearance, and a deficiency of MCP-1 resulted in attenuation of immune cell influx as neutrophil and macrophages were reduced in MCP-1^{-/-} mice.^{38,41}

Maus *et al.*³⁴ reported that the delivery of exogenous MCP-1 did not lead to alteration of neutrophil influx to the lung. However, the coadministration of MCP-1 and low-dose LPS lead to an excessive recruitment of neutrophils, with an approximate 22-fold increase compared to the 8-fold increase of monocytes.³⁴ The reason for decreased MCP-1 levels in the lungs of mice treated with GG-elafin in our study is not clear, but it may be due to the enhanced stability of GG-elafin as an NE inhibitor compared to WT elafin. NE has previously been shown to upregulate MCP-1 production by macrophages.⁴² In addition, small molecule inhibitors of NE have been shown to significantly reduce MCP-1 levels in the lungs of mice treated with LPS.⁴³ Therefore, the enhanced stability of GG-elafin compared to WT-elafin may help reduce the NE burden more effectively and impact on NE's ability to upregulate MCP-1 production in target cells. Given their similar K_i values, we would expect both elafin variants to reduce the activity of their target proteases such as elastase; however, as indicated by the neutrophil counts and the ELISA results, it appears that the GG-elafin variant possesses a more potent anti-inflammatory activity compared to the WT-elafin due to its increased stability. We have previously reported that elafin can inhibit LPS-induced production of MCP-1 by monocytic cells via inhibition of LPS-induced activation of the transcription factors AP-1 and NF- κ B.⁹ Thus, it is more likely that the greater stability of GG-elafin compared to WT had a more significant effect on LPS-induced expression of MCP-1 via reduced activation of AP-1 and/or NF- κ B.

In conclusion, our demonstration of increased proteolytic resistance of GG-elafin compared to WT-elafin may be beneficial in future therapeutic regimes. In late 2013, Phase 2 clinical trials were completed for the prevention of myocardial injury upon coronary artery bypass surgery within the National Health Service Lothian's Edinburgh Heart Centre with reports expected in early 2014. Given

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.^{8,44} 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 CTC GAG AAA AGA GCG CAA GAG CCA GGC AAA GGT CCA GGC TCC ACT AAG CC-3') or QQ-elafin (5' ATC CTC GAG AAA AGA GCG CAA GAG CCA CAA AAA GGT CCA CAA TCC ACT AAG CC-3') and a reverse primer (5'-CGA-GCGGCCGC-CCCTCTCACTGGGGAAC-3') (Eurofins Operon), and a blend of PCR enzymes (Expand High Fidelity PCR System; Roche, Meylan, France). The cDNA was then cut with *Xho*I and *Not*I (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 competent *E. 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 *Sal*I digestion, the recombinant pPIC9 vectors were electroporated 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 BMMY medium (methanol 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 \times 15 cm) equilibrated with equilibrium buffer using an AKTA chromatographic system. 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 electrophoresis⁴⁵ under nonreducing conditions and Coomassie staining (Supplementary Figure S1).

Protease activity assays. The K_i of each elafin (WT, GG, and QQ) was evaluated as previously described using human NE and PR3 as the target proteases.^{8,44} 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 μ mol/l NE substrate N-Methoxy-Succinyl-Pro-Ala-Ala-Val-7-amino-4-methylcoumarin (AAPV-AMC) for 30 minutes. Changes in fluorescence were monitored 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^a

	CF BALF (n = 5)
Age at BAL, years	14.63 (1.788)
Neutrophil elastase activity, $\mu\text{mol/l}$	18.00 (3.334)
FEV1	37.6 (6.153)
Total cells/ml	1.308×10^7 (1.213×10^6)
Neutrophils/ml	1.229×10^7 (1.351×10^6)
Macrophage/ml	5.745×10^5 (3.031×10^5)

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

^aValues 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.²⁴ 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 antielafin antibody (1:500, overnight at 4 °C; R&D Biosystems, 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.^{24,46} 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, Paisley, UK) and reading the absorbance at 405 nm in a microplate reader (Synergy HT using Gen5 software; BioTek, Bedfordshire, UK).

Transglutaminase mediated cross-linking of elafin variants to fibronectin. Transglutamination reactions were performed as previously described²² with minor modifications. Briefly, Greiner high binding 96-well plates were coated with 1 mg/ml of human fibronectin (Sigma-Aldrich) in 0.1 mol/l sodium carbonate buffer (pH 9.5) overnight at 4 °C. The wells were washed with PBS, and the free sites were blocked by incubation in block buffer (50 mmol/l Tris, 150 mmol/l NaCl, and 2% TWEEN 20) for 1 hour at 37 °C. After washing, elafin variants (8.5×10^{-7} mol/l) were added with or without guinea pig liver transglutaminase (0.33 U/well; Sigma-Aldrich) in 50 mmol/l Tris–HCL buffer (pH 7.5), 2 mmol/l CaCl₂, and 0.5 mmol/l dithiothreitol and incubated for 1 hour 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 $\mu\text{g}/\text{ml}$) 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 blunted 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 a small incision in the upper trachea and tied in place with 3.0 surgical mersilk (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 cytospun onto coated cytoslides (Shandon; Thermo Scientific) and stained with the Giemsa and May-Grünwald stain (VWR, Lutterworth, UK). Briefly, cells were fixed in methanol for 20 minutes and allowed to air dry. Slides were immersed in May-Grünwald 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 media (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 $\times 40$ 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 \pm 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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REFERENCES

- Guyot, N, Zani, ML, Berger, P, Dallet-Choisy, S and Moreau, T (2005). Proteolytic susceptibility of the serine protease inhibitor trappin-2 (pre-elafin): evidence for trypsin-mediated generation of elafin. *Biol Chem* **386**: 391–399.
- Schalkwijk, J, Wiedow, O and Hirose, S (1999). The trappin gene family: proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. *Biochem J* **340** (Pt 3): 569–577.
- Kramps, JA and Klasen, EC (1985). Characterization of a low molecular weight anti-elastase isolated from human bronchial secretion. *Exp Lung Res* **9**: 151–165.
- Schalkwijk, J, Chang, A, Janssen, P, De Jongh, GJ and Mier, PD (1990). Skin-derived antileucoproteases (SKALPs): characterization of two new elastase inhibitors from psoriatic epidermis. *Br J Dermatol* **122**: 631–641.
- Wiedow, O, Schröder, JM, Gregory, H, Young, JA and Christophers, E (1990). Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. *J Biol Chem* **265**: 14791–14795.
- Mihaila, A and Tremblay, GM (2001). Human alveolar macrophages express elafin and secretory leukocyte protease inhibitor. *Z Naturforsch C* **56**: 291–297.
- Simpson, AJ, Maxwell, AI, Govan, JR, Haslett, C and Sallenave, JM (1999). Elafin (elastase-specific inhibitor) has anti-microbial activity against gram-positive and gram-negative respiratory pathogens. *FEBS Lett* **452**: 309–313.
- Zani, ML, Baranger, K, Guyot, N, Dallet-Choisy, S and Moreau, T (2009). Protease inhibitors derived from elafin and SLPI and engineered to have enhanced specificity towards neutrophil serine proteases. *Protein Sci* **18**: 579–594.
- Butler, MW, Robertson, I, Greene, CM, O'Neill, SJ, Taggart, CC and McElvaney, NG (2006). Elafin prevents lipopolysaccharide-induced AP-1 and NF-kappaB activation via an effect on the ubiquitin-proteasome pathway. *J Biol Chem* **281**: 34730–34735.
- Baranger, K, Zani, ML, Chandenier, J, Dallet-Choisy, S and Moreau, T (2008). The antibacterial and antifungal properties of trappin-2 (pre-elafin) do not depend on its protease inhibitory function. *FEBS J* **275**: 2008–2020.
- Williams, SE, Brown, TI, Roghanian, A and Sallenave, JM (2006). SLPI and elafin: one glove, many fingers. *Clin Sci (Lond)* **110**: 21–35.
- Scott, A, Weldon, S and Taggart, CC (2011). SLPI and elafin: multifunctional antiproteases of the WFDC family. *Biochem Soc Trans* **39**: 1437–1440.
- Pfundt, R, Wiggins, M, Bergers, M, Zweers, M, Frenken, M and Schalkwijk, J (2000). TNF-alpha and serum induce SKALP/elafin gene expression in human keratinocytes by a p38 MAP kinase-dependent pathway. *Arch Dermatol Res* **292**: 180–187.
- Sallenave, JM, Shulmann, J, Crossley, J, Jordana, M and Gaudie, J (1994). Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol* **11**: 733–741.
- Bingle, L, Tetley, TD and Bingle, CD (2001). Cytokine-mediated induction of the human elafin gene in pulmonary epithelial cells is regulated by nuclear factor-kappaB. *Am J Respir Cell Mol Biol* **25**: 84–91.
- Pfundt, R, van Ruissen, F, van Vlijmen-Willems, IM, Alkemade, HA, Zeeuwen, PL, Jap, PH *et al.* (1996). Constitutive and inducible expression of SKALP/elafin provides anti-elastase defense in human epithelia. *J Clin Invest* **98**: 1389–1399.
- Schalkwijk, J, de Roo, C and de Jongh, GJ (1991). Skin-derived antileucoprotease (SKALP), an elastase inhibitor from human keratinocytes. Purification and biochemical properties. *Biochim Biophys Acta* **1096**: 148–154.
- Wiedow, O, Lüdemann, J and Utecht, B (1991). Elafin is a potent inhibitor of proteinase 3. *Biochem Biophys Res Commun* **174**: 6–10.
- Guyot, N, Zani, ML, Maurel, MC, Dallet-Choisy, S and Moreau, T (2005). Elafin and its precursor trappin-2 still inhibit neutrophil serine proteinases when they are covalently bound to extracellular matrix proteins by tissue transglutaminase. *Biochemistry* **44**: 15610–15618.
- Nara, K, Ito, S, Ito, T, Suzuki, Y, Ghoneim, MA, Tachibana, S *et al.* (1994). Elastase inhibitor elafin is a new type of proteinase inhibitor which has a transglutaminase-mediated anchoring sequence termed "cementin". *J Biochem* **115**: 441–448.
- Molhuizen, HO, Alkemade, HA, Zeeuwen, PL, de Jongh, GJ, Wieringa, B and Schalkwijk, J (1993). SKALP/elafin: an elastase inhibitor from cultured human keratinocytes. Purification, cDNA sequence, and evidence for transglutaminase cross-linking. *J Biol Chem* **268**: 12028–12032.
- Baranger, K, Zani, ML, Labas, V, Dallet-Choisy, S and Moreau, T (2011). Secretory leukocyte protease inhibitor (SLPI) is, like its homologue trappin-2 (pre-elafin), a transglutaminase substrate. *PLoS One* **6**: e20976.
- McMichael, JW, Roghanian, A, Jiang, L, Ramage, R and Sallenave, JM (2005). The antimicrobial antiproteinase elafin binds to lipopolysaccharide and modulates macrophage responses. *Am J Respir Cell Mol Biol* **32**: 443–452.
- Guyot, N, Butler, MW, McNally, P, Weldon, S, Greene, CM, Levine, RL *et al.* (2008). Elafin, an elastase-specific inhibitor, is cleaved by its cognate enzyme neutrophil elastase in sputum from individuals with cystic fibrosis. *J Biol Chem* **283**: 32377–32385.
- Guyot, N, Bergsson, G, Butler, MW, Greene, CM, Weldon, S, Kessler, E *et al.* (2010). Functional study of elafin cleaved by Pseudomonas aeruginosa metalloproteinases. *Biol Chem* **391**: 705–716.
- Kerrin, A, Weldon, S, Chung, AH, Craig, T, Simpson, AJ, O'Kane, CM *et al.* (2013). Proteolytic cleavage of elafin by 20S proteasome may contribute to inflammation in acute lung injury. *Thorax* **68**: 315–321.
- Kantyka, T, Latendorf, T, Wiedow, O, Bartels, J, Gläser, R, Dubin, G *et al.* (2009). Elafin is specifically inactivated by RgpB from *Porphyrromonas gingivalis* by distinct proteolytic cleavage. *Biol Chem* **390**: 1313–1320.
- Brown, A, Farmer, K, MacDonald, L, Kalsheker, N, Pritchard, D, Haslett, C *et al.* (2003). House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol* **29** (Pt 1): 381–389.
- Weldon, S, McNally, P, McElvaney, NG, Elborn, JS, McAuley, DF, Wartelle, J *et al.* (2009). Decreased levels of secretory leucoprotease inhibitor in the Pseudomonas-infected cystic fibrosis lung are due to neutrophil elastase degradation. *J Immunol* **183**: 8148–8156.
- Wang, Z, Chen, F, Zhai, R, Zhang, L, Su, L, Lin, X *et al.* (2009). Plasma neutrophil elastase and elafin imbalance is associated with acute respiratory distress syndrome (ARDS) development. *PLoS One* **4**: e4380.
- Mallia, P, Footitt, J, Sotero, R, Jepson, A, Contoli, M, Trujillo-Torralbo, MB *et al.* (2012). Rhinovirus infection induces degradation of antimicrobial peptides and secondary bacterial infection in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **186**: 1117–1124.
- Shaw, L and Wiedow, O (2011). Therapeutic potential of human elafin. *Biochem Soc Trans* **39**: 1450–1454.
- Maus, U, von Grote, K, Kuziel, WA, Mack, M, Miller, EJ, Cihak, J *et al.* (2002). The role of CC chemokine receptor 2 in alveolar monocyte and neutrophil immigration in intact mice. *Am J Respir Crit Care Med* **166**: 268–273.
- Maus, U, Huwe, J, Maus, R, Seeger, W and Lohmeyer, J (2001). Alveolar JE/MCP-1 and endotoxin synergize to provoke lung cytokine upregulation, sequential neutrophil and monocyte influx, and vascular leakage in mice. *Am J Respir Crit Care Med* **164**: 406–411.
- Gehrig, S, Duerr, J, Weitnauer, M, Wagner, CJ, Graeber, SY, Schattner, J *et al.* (2014). Lack of neutrophil elastase reduces inflammation, mucus hypersecretion, and emphysema, but not mucus obstruction, in mice with cystic fibrosis-like lung disease. *Am J Respir Crit Care Med* **189**: 1082–1092.
- Wen, W, Moses, MA, Wiederschain, D, Arbisser, JL and Folkman, J (1999). The generation of endostatin is mediated by elastase. *Cancer Res* **59**: 6052–6056.
- Perkins, GD, Nathani, N, Richter, AG, Park, D, Shyamsundar, M, Heljasaava, R *et al.* (2009). Type XVIII collagen degradation products in acute lung injury. *Crit Care* **13**: R52.
- Balamayooran, G, Batra, S, Balamayooran, T, Cai, S and Jayaseelan, S (2011). Monocyte chemoattractant protein 1 regulates pulmonary host defense via neutrophil recruitment during *Escherichia coli* infection. *Infect Immun* **79**: 2567–2577.
- Ramnath, RD, Ng, SW, Guglielmotti, A and Bhatia, M (2008). Role of MCP-1 in endotoxemia and sepsis. *Int Immunopharmacol* **8**: 810–818.
- Mercer, PF, Williams, AE, Scotton, CJ, José, RJ, Sulikowski, M, Moffatt, JD *et al.* (2014). Proteinase-activated receptor-1, CCL2, and CCL7 regulate acute neutrophilic lung inflammation. *Am J Respir Cell Mol Biol* **50**: 144–157.
- Balamayooran, G, Batra, S, Theivanthiran, B, Cai, S, Pacher, P and Jayaseelan, S (2012). Intrapulmonary G-CSF rescues neutrophil recruitment to the lung and neutrophil release to blood in Gram-negative bacterial infection in MCP-1-/- mice. *J Immunol* **189**: 5849–5859.
- Yamaguchi, Y, Matsumura, F, Liang, J, Okabe, K, Ohshiro, H, Ishihara, K *et al.* (1999). Neutrophil elastase and oxygen radicals enhance monocyte chemoattractant protein expression after ischemia/reperfusion in rat liver. *Transplantation* **68**: 1459–1468.
- Bissonnette, EY, Tremblay, GM, Turmel, V, Piroette, B and Reboud-Ravaux, M (2009). Coumarinic derivatives show anti-inflammatory effects on alveolar macrophages, but their anti-elastase activity is essential to reduce lung inflammation in vivo. *Int Immunopharmacol* **9**: 49–54.
- Zani, ML, Nobar, SM, Lacour, SA, Lemoine, S, Boudier, C, Bieth, JG *et al.* (2004). Kinetics of the inhibition of neutrophil proteinases by recombinant elafin and pre-elafin (trappin-2) expressed in *Pichia pastoris*. *Eur J Biochem* **271**: 2370–2378.
- Schägger, H and von Jagow, G (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**: 368–379.
- Scott, A, Weldon, S, Buchanan, PJ, Schock, B, Ernst, RK, McAuley, DF *et al.* (2011). Evaluation of the ability of LL-37 to neutralise LPS *in vitro* and *ex vivo*. *PLoS One* **6**: e26525.