ONLINE SUPPLEMENT

A role for Whey acidic protein Four-Disulphide-Core 12 (WFDC12) in the regulation of the inflammatory response in the lung

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METHODS

Recombinant WFDC12 expression

For recombinant expression, the plasmid was transformed into *E.coli* M15 (Qiagen) and transformed cells grown overnight at 37°C on LB agar plates supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin (Sigma-Aldrich, Dorset, UK). Liquid cultures in LB broth (+ 100 μg/ml ampicillin and 25 μg/ml kanamycin) were started from a single colony and grown at 37°C with shaking until an OD_{600} of approximately 0.6. Cultures were then induced by addition of isopropyl thio-β-D-1-galactopyranoside (Sigma-Aldrich) to a final concentration of 0.1 mM and grown for a further 4 hr. Bacterial cells were pelleted by centrifugation at 4200 x g for 20 min at 4°C and stored at -80°C until required.

Recombinant WFDC12 purification

Bacterial pellets were lysed in a denaturing buffer (6 M guanidine hydrochloride, 50 mM Hepes pH 7.8, 150 mM sodium chloride, 10% glycerol and 10 mM β-mercaptoethanol) and incubated on ice for 30 min. The lysate was subjected to six 10 s sonication pulses at 100 W, on ice, with 30 s intervals between pulses. The lysate was then cleared by centrifugation at 17, 000 x g for 20 min at 4°C, and the cleared portion (supernatant) incubated with nickel-nitrilotriacetic acid agarose (Qiagen) for 2 hr at 4°C with agitation. This mixture was loaded into a 1 ml diameter affinity column (Qiagen) and allowed to pass through under gravity. The agarose bed was washed extensively with 6 M guanidine hydrochloride, 50 mM Hepes pH 7.8, 500 mM sodium chloride, 10% glycerol and 10 mM β-mercaptoethanol. Histidine-tagged protein was then eluted using wash buffer supplemented with 250 mM imidazole.

Elution fractions were analysed by SDS-PAGE, as detailed below, to determine those with maximum protein yield.

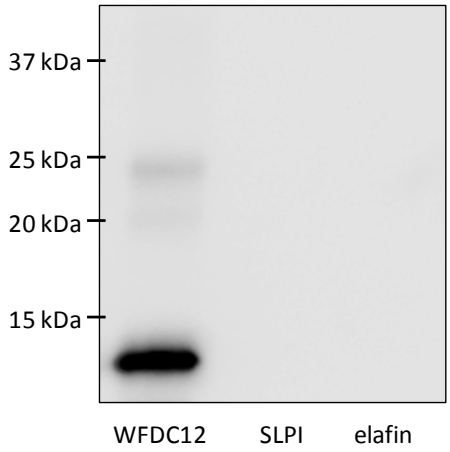
Prior to refold, residual endotoxin was removed from denatured protein preparations using Polymyxin B agarose beads (Sigma-Aldrich). Protein samples were incubated with the suspension (100 µl per ml of protein) for 1 hr at room temperature with gentle agitation. The beads were pelleted by centrifugation for 10 min at 17, 000 x g after which the protein supernatant was carefully removed. The denatured protein was then refolded via step-wise dialysis into buffers containing oxidised glutathione to aid disulphide bond formation. All dialysis steps were carried out at 4°C.

The His-tag is small, poorly immunogenic and uncharged at pH 8 therefore unlikely to affect conformational or functional properties of the protein to which it is attached. For these reasons the His-tag was allowed to remain on the purified protein.

One dimensional nano-liquid chromatography coupled to ESI-MS-MS (1D nLC-MS-MS, reverse phase chromatography) was performed by Fingerprints Proteomics Facility (College of Life Sciences, University of Dundee, UK) to confirm the identity of rWFDC12 using a 4000 QTRAP tandem MS system (Applied Biosystems, Life Technologies).

SDS-PAGE and Western blotting

To assess purity of recombinant WFDC12 protein, samples were separated in 15% gels then stained with Coomassie stain (0.25% w/v Coomassie brilliant blue, 40% methanol, 10% acetic acid) for 1 hr at room temperature, and subsequently destained (40% methanol, 10% acetic acid) until an optimal contrast between protein bands and the gel was reached. For Western blotting, samples were separated by SDS-PAGE (15% gels unless otherwise stated) and then transferred to 0.1 µm nitrocellulose membrane (Sigma-Aldrich). Free sites were blocked with 5% Marvel in PBS containing 0.1% Tween overnight at 4°C. Membranes were incubated with a polyclonal rabbit anti-WFDC12 antibody (generated for this study by GenScript, New Jersey, USA). The WFDC12 antibody was specific to a sequence in the C-terminus of the protein (DVSRPYPEPGWEAK) and showed no cross-reactivity with SLPI or elafin (Supplementary Figure A). Proteins were then detected using anti-rabbit HRP-conjugated secondary antibody (Thermo Fisher Scientific, Leicestershire, UK) and visualised by chemiluminescence (GE Healthcare, Buckinghamshire, UK). Images of gels and blots were captured using a Syngene G:Box and GeneSnap software (Syngene UK, Cambridge).



Supplementary Figure A. Recombinant WFDC12, SLPI and elafin (50 ng each) were subjected to SDS-PAGE and assessed by Western blot using a polyclonal rabbit anti-WFDC12 antibody.

Protease activity assays

Cathepsin G (213 nM), elastase (16.9 nM) and proteinase-3 (86.2 nM) (Elastin Products Company, Missouri, USA) were incubated alone or with various amounts of rWFDC12 (or SLPI/elafin as positive control) for 15 min at room temperature before addition of the appropriate substrate. A Biotek Synergy HT microplate reader (Biotek, Bedfordshire, UK) was used to measure relative fluorescence units every minute for up to 1 hr. Results were first expressed as the change in relative fluorescence units (dRFU) over a fixed period of time. This was then used to express activity as percentage of control (i.e. protease alone).

Radial diffusion assays

Bacteria were grown aerobically to mid-logarithmic phase in Mueller Hinton broth (Oxoid, Thermo Fisher Scientific) at 37°C. The cultures were pelleted and washed three times in 10 mM sodium phosphate buffer (pH 7.4), then resuspended in the same buffer. Approximately 5 x 10^6 bacterial cells were mixed thoroughly with sodium phosphate buffer, 1% (w/v) agarose and minimal Mueller Hinton broth to form an underlay gel (total volume 10 ml). This was poured into a square Petri dish (10 cm x 10 cm) and allowed to solidify. Wells of 2.5 mm diameter were punched in the agar and 3 µl of various WFDC12 test concentrations added. Cecropin A (25 µM, Sigma-Aldrich) was included as a positive control and sterile water as a negative control. Plates were then incubated for 3 hr at 37°C to allow diffusion of the protein through the agarose gel, after which an overlay gel consisting of 1% (w/v) agarose in Mueller Hinton broth (total volume 10 ml) was poured over the base and allowed

to solidify. The plates were incubated overnight at 37°C, treated with conditioning media (10% acetic acid, 2% DMSO) and stained using a dilute solution of Coomassie brilliant blue.

THP-1 cell culture experiments

Human acute monocytic leukaemia cells (THP-1) were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (Gibco, Life Technologies), 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin (PAA laboratories GmbH, Austria). For experiments testing anti-inflammatory activity of rWFDC12, cells were resuspended at 1 x 10^6/ml and incubated with a range of rWFDC12 concentrations for 1 hr, followed by stimulation with 100 ng/ml *E. coli* LPS (Alexis, Enzo Life Sciences) for 16 hr. Control cells were incubated for 16 hr with media, LPS or rWFDC12 alone. Cell-free supernatants were collected for measurement of cytokines (see below). In experiments measuring WFDC12 secretion, cells were stimulated with LPS for 4 hr and cell-free supernatants collected for use in a WFDC12 indirect ELISA as described below.

Cytokine ELISAs

Levels of the human cytokines IL-8, MCP-1 and IP-10 in cell-free supernatants were quantified using ELISA kits from R&D Systems (Abingdon, UK). All kits were used according to the manufacturer's instructions.

Cross-linking of rWFDC12 to fibronectin

Transglutamination reactions were based on previously described methods [1,2], with minor modifications. Recombinant WFDC12 (5 µg) was incubated with 20 µg fibronectin (Sigma-Aldrich) and 1 mU guinea pig liver transglutaminase (Sigma-Aldrich) in 200 mM Tris HCl pH 7.5, 20 mM $CaCl_2$ and 0.1 mM DTT for 2 hr at 37°C (total volume 50 µl). A reaction using heat-inactivated (99°C for 15 min) transglutaminase was also included as a negative control. Reactions were terminated upon addition of sample treatment buffer without reducing agent, boiled and subsequently analysed by SDS-PAGE as described above. Samples were separated in 7.5% and 15% gels, transferred to nitrocellulose membrane and immunoblotted with polyclonal rabbit anti-WFDC12 antibody to allow detection of WFDC12-fibronectin complexes (7.5% gel) and unbound WFDC12 (15% gel). Additionally, the samples were separated in a 7.5% gel and stained using a Colloidal blue staining kit

(Novex, Invitrogen) to allow visualisation of unbound fibronectin and WFDC12-fibronectin complexes. Images of gels and blots were captured using a Syngene G:Box and GeneSnap software.

Evaluation of antiprotease activity of fibronectin-bound rWFDC12

Transglutamination reactions in 96-well plate format were based on previously described methods [1,2], with minor modifications. Briefly, 96-well black high-binding plates were coated with fibronectin (20 µg/well) in 0.1 M sodium carbonate (pH 9.5) overnight at 4°C. The wells were washed the following day with PBS, and then incubated in 50 mM Tris, 150 mM NaCl and 2% Tween 20 for 1 hr at 37°C to block free binding sites. After washing with PBS, rWFDC12 (4.5 µM) was added to the fibronectin coated wells, with or without guinea pig liver transglutaminase (1 mU), in 200 mM Tris HCl pH 7.5, 20 mM $CaCl_2$, and 0.1 mM DTT. The reaction mixture was allowed to incubate for 2 hr at 37°C before removal by extensive washing with PBS. Cathepsin G (213 nM) was then added to the coated wells and incubated for 15 min to allow association of immobilised rWFDC12 with the protease. The fluorogenic substrate Suc-Ala-Ala-Pro-Phe-AMC was added and resulting activity levels recorded and presented as detailed above.

Immunohistochemistry

Tissue was formalin fixed and paraffin-embedded before sectioning (6 µm). Sections were de-paraffinised in two changes of the clearing agent histoclear (10 min each), followed by re-hydration in an ethanol-to-water gradient consisting of absolute ethanol, 95% ethanol and 70% ethanol (5 min each). The tissue sections were then rinsed briefly in water for 5 min. Antigen retrieval for WFDC12, pan cytokeratin and CD68 staining was achieved by boiling for 22 min in 10 mM sodium citrate, 0.05% Tween 20, pH 6. Antigen retrieval for neutrophil elastase (NE) staining required incubation with pepsin for 10 min at 37°C. Endogenous peroxidase activity was limited by treating the sections with 3% hydrogen peroxide (in 50% methanol) for 13 min. The sections were blocked in 10% normal species-specific serum (specific to the species of the secondary antibody) for 1 hr at room temperature, then incubated overnight at 4°C with rabbit anti-WFDC12 antibody (diluted 1:200 in blocking buffer), mouse anti-pan cytokeratin (1:75, Abcam, Cambridge, UK), mouse anti-CD68 (1:300, Abcam), mouse anti-NE (1:300, Santa Cruz) or an equivalent amount of

rabbit/mouse immunoglobulin G. Sections were washed three times (10 min/wash) in PBS + 0.1% Tween (PBST) before incubation with anti-rabbit/anti-mouse secondary antibodies (Vector Laboratories, Peterborough, UK) for 30 min at room temperature. This was followed by further washes in PBST and then incubation with the avidin-biotin complex conjugated with horseradish peroxidise (ABC kit, Vector Laboratories) for 30 min at room temperature. After further PBST washes, the reactions were detected using 3,3-diaminobenzidine (DAB) (Dako, Agilent Technologies) for 30 sec - 1 min, followed by counterstaining with Harris haematoxylin solution for 3 min. The sections were washed in running tap water for 5 min and then blued in 0.2% ammonia water (10 dips). After rinsing in tap water, the sections were de-hydrated through a water-to-ethanol gradient consisting of 70% ethanol, 95% ethanol and absolute ethanol for 5 min each. Following two changes of histoclear (5 min each), the slides were finally mounted in DPX mounting media and visualised using a Leica DM5500B microscope. Images were captured using the Leica AL image software.

Human BALF collection and processing

Bronchoalveolar lavage fluid (BALF) samples from the HARP (ISRCTN70127774) [3], LPS (ISRCTN21056528) [4] and ARENA (NCT01659307) studies were used to quantify WFDC12. Bronchoalveolar lavage was performed according to standard guidelines and as previously described [4,5]. Briefly, three successive 60 ml aliquots of 0.9% saline were instilled into a subsegment of the right middle lobe and each aspirated immediately with low suction. BALF return was measured and immediately placed on ice until transferred to the laboratory for processing. BALF was centrifuged at 900 x g for 5 min at 4°C. The supernatant was removed and stored at -80°C until required.

Measurement of WFDC12 by indirect ELISA

WFDC12 standards, human BALF samples and THP-1 cell-free supernatants were diluted in PBS or RPMI as appropriate and added to wells of a Greiner high binding 96 well plate to coat overnight at 4°C (100 µl/well). Wells were washed three times with PBS + 0.05% Tween (PBST) and blocked with 1% BSA in PBS (200 µl/well) for 1 hr at room temperature with gentle agitation. Wells were washed again with PBST before addition of rabbit anti-WFDC12 antibody (100 µl/well, diluted 1:250 in PBS) and incubation for 2 hr at room temperature. After further washing with PBST, HRP-conjugated goat anti-rabbit antibody (100 µl/well,

diluted 1:5000 in PBS) was added for 1 hr at room temperature. Following a final wash step, the peroxidase substrate ABTS (Invitrogen) was added (100 µl/well) and the absorbance at 405 nm read using a microplate reader.

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