Figure 2

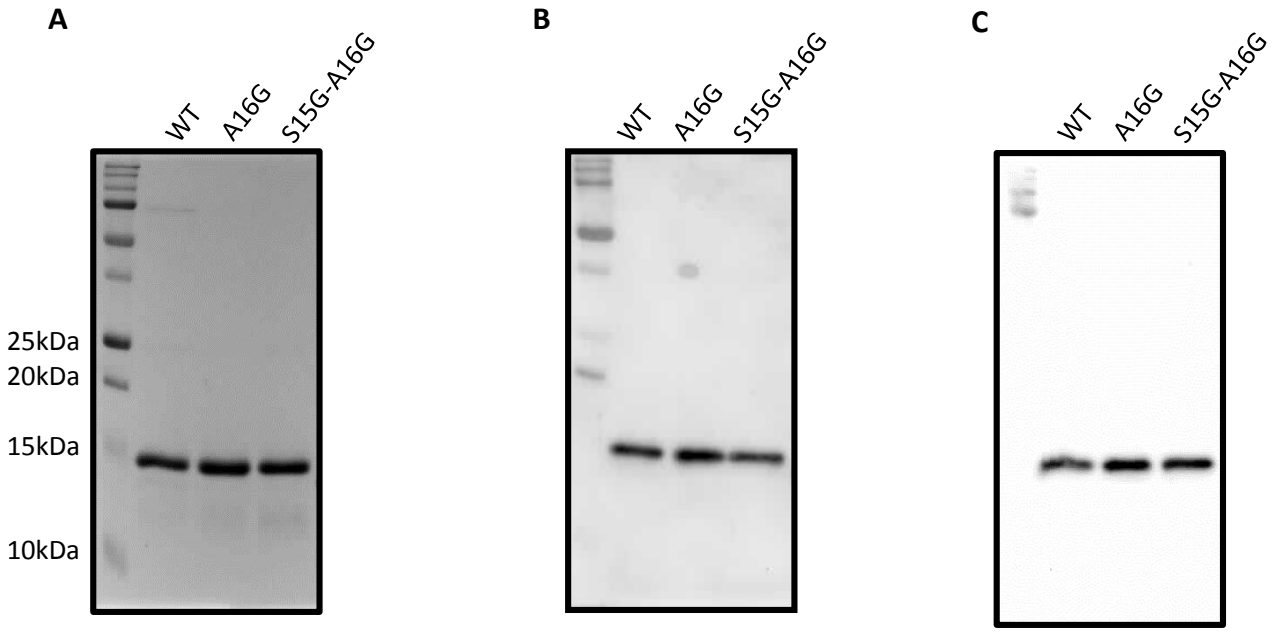

Figure 2. SDS-PAGE analysis of purified SLPI-WT, SLPI-A16G and SLPI-S15G-A16G. SLPI variants were expressed as N-terminal His_6-tagged recombinant proteins within the pQE30 expression vector in M15 [pREP4] *E. coli* and purified by IMAC.Purified SLPI-WT, SLPI-A16G and SLPI-S15G-A16G variants were analysed by SDS-PAGE on 15% (w/v) SDS-PAGE gels under reducing Laemmli conditions. **(A)** Gels were stained with Coomassie Brilliant blue for total protein analysis. **(B)** Proteins were transferred onto nitrocellulose membrane and SLPI detected with a biotinylated anti-SLPI antibody. **(C)** Proteins were transferred onto nitrocellulose membrane and SLPI detected with a rabbit anti-His tag antibody.

Methods

Cloning, expression and purification of SLPI variants

pET32c-SLPI was a kind gift from Dr. André Cantin (University of Sherbrook, Canada). The DNA sequence encoding for human wild-type SLPI was cloned into the KpnI and HindIII restriction sites of the pQE30 expression vector (Qiagen, Manchester, UK) and this plasmid is referred to as pQE30-SLPI-WT. For each SLPI variant, the synthesis of the mutant strand was performed by PCR using 25 ng of pQE30-SLPI-WT plasmid, 125 ng of the relevant forward and reverse primers (Table 1), 2.5 U of *PfuTurbo* DNA polymerase and 1 µl of dNTP mix and 5 µl of 10 x reaction buffer as required in a final volume of 50 µl as per (Quik-site Directed Mutagenesis kit, Agilent Technologies LDA UK Limited,

Stockport, UK). This mix was then subjected to PCR under the conditions of 30 s at 95°C; 16 cycles of 30 s at 95°C, 1 min at 55°C, and 4 min 30 s at 68°C. After cooling down the reaction to 37°C, 10 U of DpnI (New England Biolabs, Hitchin, UK) were added to the PCR mix and the parental strands were digested at 37°C for 1 h. The digestion products were then used to transform *E. coli* XL1-Blue supercompetent cells (Agilent Technologies LDA UK Limited, Stockport, UK). The success of the site-directed mutagenesis was verified by DNA sequencing and, for each SLPI variant, a single clone was used for all subsequent experiments. The plasmids encoding for the SLPI variants SLPI-A16G and SLPI-S15G-A16G are referred to as pQE30-SLPI-A16G and pQE30-SLPI-S15G-A16G, respectively.

Expression of wild-type SLPI,SLPI-A16G and SLPI-S15G-A16G variants in M15 [pREP4] E. coli

M15 [pREP4] *E. coli* cells (Qiagen, Manchester, UK) were transformed with the pQE30-SLPI-WT, pQE30-SLPI-A16G and pQE30-SLPI-S15G-A16G plasmids and grown overnight with shaking at 37°C in Luria-Bertani (LB) medium (5 ml) supplemented with 100 µg/ml Ampicillin and 25 µg/ml Kanamycin (Sigma-Aldrich, Dorset, UK). These overnight cultures (2.5 ml) were used to inoculate LB (500 ml) supplemented with 100 µg/ml Ampicillin and 25 µg/ml Kanamycin and pre-warmed to 37°C. The cultures were grown with shaking at 37°C until OD_{600} reached 0.6. Expression of the recombinant proteins was then induced with IPTG (1 mM final concentration). The cultures were incubated at 37°C with shaking for another 4 h before the cells were harvested by centrifugation (4,000 rpm for 30 min at 4°C). Cells pellets awaiting purification were stored at - 80°C.

Refold and IMAC purification of wild-type SLPI and its SLPI-A16G and SLPI-S15G-A16G variants

Cell pellets were thawed on ice then resuspended in lysis buffer (40 ml) containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The cells were lysed for 2 h at r.t with shaking. The cell lysates were clarified by centrifugation (4,500 x g, 1 h, r.t.) followed by filtration of the supernatants through 0.2 µm filter disks. HiTrap™ Chelating HP columns (1 ml) (GE Healthcare Life Sciences, Little Chalfont, UK) were mounted on AKTAprime™ chromatography systems (GE Healthcare Life Sciences, Little Chalfont, UK), charged with Ni^{2+} ions and equilibrated with a buffer containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The clarified cell lysates were loaded onto the columns at 1 ml/min. Non-specifically bound material was washed off the column at 1 ml/min with 10 column volumes of buffer containing 8 M urea, 1 mM β-mercaptoethanol, 500 mM NaCl, 5 mM imidazole and 20 mM sodium phosphate, pH 8.0. The denatured recombinant proteins bound onto the column were refolded on-column at a flow rate of 0.5 ml/min by gradually removing the urea from the buffer over 15 column volumes. The refolded recombinant proteins bound to the column were then washed at 1 ml/min with 10 column volumes of buffer containing 500 mM NaCl, 5 mM imidazole, 20 mM sodium phosphate, pH 8.0 and 1 mM β-mercaptoethanol. Refolded recombinant proteins were eluted off the column at 1 ml/min by increasing the concentration of imidazole from 5 mM to 500 mM in 20 column volumes. Elution fractions (1 ml) were collected and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing SLPI-WT or its SLPI-A16G and SLPI-S15G-A16G variants were pooled and dialysed at 4°C with gentle agitation against 10 volumes of PBS pH 7.4 with two changes of dialysis buffer. The concentrations of the purified recombinant proteins

were determined by BCA assay according to the manufacturer's instructions (Pierce BCA Assay, Fisher Scientific UK, Leicestershire).

Characterisation of the purified SLPI-WT and its SLPI-A16G and SLPI-S15G-A16G variants by SDS-PAGE

Purified dialysed SLPI-WT and its SLPI-A16G and SLPI-S15G-A16G variants were analysed by SDS-PAGE on 15% (w/v) polyacrylamide gels under reducing Laemmli conditions. Gels were stained with Coomassie Brilliant blue for total protein analysis. For Western blotting, gels were transferred onto nitrocellulose membrane and the membranes blocked with 3% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20 for 1 h at r.t. Probing was carried out either with biotinylated anti-SLPI antibody (1:500 dilution in 3% BSA (w/v) PBS containing 0.1% Tween 20 for 1 h at r.t.; R&D Systems, Abingdon, UK) followed by incubation with streptavidin conjugated horseradish peroxidase (1:2,500 dilution in 3% BSA (w/v) PBS containing 0.1% Tween 20 for 20 min at r.t.; BioLegend, London UK) or with rabbit anti-His antibody (1:1,000 dilution in 3% BSA (w/v) PBS containing 0.1% Tween 20 for 1 h at r.t.; (Insight Biotechnology Ltd., Wembley, UK) followed by incubation with HRP conjugated goat anti-rabbit antibody (1:10,000 dilution in 3% BSA (w/v) PBS containing 0.1% Tween 20 for 1 h at r.t.; (Insight Biotechnology Ltd., Wembley, UK). Peroxidase activity was detected using the chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK) and analysed using the Syngene G:Box and GeneSnap software (SynGene UK, Cambridge).