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C5a peptidase (ScpA) activity towards human type II and type III interferons

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A B S T R A C T

C5a peptidase, also known as ScpA, is a surface associated serine protease derived from *Streptococcus pyogenes* and has been described as an important factor in streptococcus virulence, capable of cleaving complement components C5a, C3 and C3a. Although the interactions of ScpA with complement components is well studied, extensive screening of ScpA activity against other pro-inflammatory cytokines is lacking. Here, ScpA's ability to cleave human pro-inflammatory cytokines was tested, revealing its ability to cleave human IFNγ, IFNλ1, IFNλ2, C5, IL-37 but with significantly reduced activities. The functional consequence of ScpA's cleavage of IFNγ in its signalling through the Jak-Stat pathway has also been evaluated in an *in vitro* RPE1 cell model. These newly identified targets for ScpA highlight the complexity of streptococcus infections and indeed, the potential for ScpA to have a therapeutic role in the progression of inflammatory diseases involving these cytokines.

1. Introduction

Group A streptococcus is an important species of bacterial pathogens, being the most common cause of bacterial pharyngitis and the cause of scarlet fever and impetigo. As a pathogen, it has developed important mechanisms to avoid host defences [1]. C5a peptidase (ScpA) is a surface associated subtilisin-like serine protease present in Group A streptococcus and, along with other surface proteins, it has been established as an important factor in streptococcus virulence [2,3]. ScpA's primary substrate is the human complement anaphylatoxin C5a, which is cleaved at the C-terminus between H^{67}-K^{68} releasing 6 amino acids [2]. This C-terminal "tail" removal renders the chemokine inactive, significantly dampening C5a driven monocyte recruitment to the infected area aiding streptococcus survival in early infection [3]. ScpA is a multidomain protease comprised of a signalling peptide, a pro-peptide, catalytical domain, PA domain and three fibronectin type III domains (Fn1 – 3). After expression the pro-peptide is autocatalytically removed [4]. In contrast with most proteases of its class, ScpA was initially thought to target only human C5a, being able to discriminate between C5a from different species and displaying little to no activity towards mouse and rat C5a [5]. More recent studies have shown that ScpA also can cleave C3a and complement component C3 [6]. Additionally, human complement C5, human serum albumin, myosin, ovalbumin, and cytochrome *c* have also been screened as potential substrates for ScpA, but no activity was observed [7]. Due to its ability to modulate the complement response, ScpA has been a topic of interest in complement therapeutics and recently it has been proposed as an intervention in sepsis [8]. Autoimmune inflammatory diseases such as psoriasis, have a complex pathology often involving the deregulation of several inflammation pathways, such as the Th1/Th2/Th17 cytokine pathway (IFNγ, TNFα, IL-17, IL-22) and the complement system by upregulation of complement components C3, C5 and C5a [9–12]. Complement factor C5a's role in inflammatory diseases has been established, with studies showing that a deficiency of C5a receptor is correlated with a decrease in disease severity in psoriatic skin and inflammatory bowels disease [13,14]. Currently there are antibody-based therapeutics that have been shown to be effective in inflammatory diseases by targeting C5 and blocking the formation of C5a and antibodies targeting the C5a/C5aR complex are also being developed as potential therapeutics [15,16]. The use of proteases as a therapeutic option has been expanding over the last several decades. Due to their high substrate affinity, specificity, and ability to target multiple molecules, proteases are considered a compelling therapeutic agent [17].

The idea of using ScpA as a potential therapeutic has been previously discussed [18] but to develop ScpA as a therapeutic, a fuller understanding of the protease's role in immune-mediated inflammatory diseases is needed. In addition to the efficient production and stability characterization, understanding of ScpA substrate specificity and site of

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action is an important aspect that requires detailed investigation. Recombinant production and stability studies of ScpA were previously carried out by our group [19]. A panel of cytokines comprised of representative members of the interleukin family, the three interferon families (Type I, Type II and Type III), the XCX chemokine family, and TNFα, which play an important role in both the fight against infection and in the pathology of autoimmune inflammatory diseases [20–35], was compiled and tested for susceptibility to degradation by ScpA. Three versions of ScpA, mimicking the reported end points for the protease after progression of its natural auto-proteolysis pathway, were explored during this work.

2. Methods and materials

2.1. Materials

Human C5, C3, C3a, IFN-α2a and IFN-α2b were purchased from Sigma. Human C5a was purchased from VWR. IL-37, IL-22, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-1α, IP-10, ENA-78, TNF-α, MIG, IFNβ, IFNω, IFNγ, IFNλ1, IFNλ2 were purchased from Peprotech. All chromatography resins and consumables were purchased from Cytiva unless otherwise stated. MALDI matrices, 2,5-dihydroxyacetophenon (2,5-DHAP) and super 2-hydroxy-5-methoxybenzoic acid (sDHB), and protein calibration standards were purchased from Bruker and 1,5-diaminonaphthalene (1,5-DAN) was purchased from Sigma. Myoglobin used as a MALDI-ISD calibrant was purchased from Sigma. For cell culture, retinal pigment epithelial hTERT RPE1 cells were obtained from ATCC (UK) and were confirmed as mycoplasma free by the supplier (https://www.atcc.org/products/crl-4000). Dulbecco's Modified Eagle Medium F12 (DMEM F-12) media, Trypsin-EDTA solution and hygromycin B were purchased from Sigma. Fetal bovine serum (FBS), antibiotic-antimyotic (100X), glutamax (100X) and sodium bicarbonate (7.5 %) were purchased from BioScience. Phospho-Stat1 (Tyr701) (D4A7) rabbit mAb primary antibody was obtained from Cell Signalling and goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody was purchased from Abcam.

2.2. ScpA expression and purification

All ScpA clones were gifted to the group by Dr. Jakki Cooney, University of Limerick. ScpA residues 31–1032 cloned into a pGEX-6P1 vector as a fusion protein with an N-terminal GST tag were recombinantly expressed in *E. coli* DH5α cells. This version of ScpA corresponds to the form of ScpA anchored to the surface of *Streptococcus pyogenes* which includes the presence of the pro-peptide [7]. Another version of ScpA comprising residues 79–1032 cloned in the same vector and expressed in *E. coli* DH5 α cells was also used. This version would imitate ScpA after auto-proteolysis of the pro-peptide according to previously published results [7]. For the purpose of this work $ScpA_{31\text{-}1032}$ is called ScpA and $ScpA_{79\text{-}1032}$ is called ScpA79ΔPro. Transformed cells were incubated in LB broth with 100 μg/mL ampicillin, 2 % glucose at 37°C and 250 rpm until OD reached 0.6, after which expression was induced with 0.1 mM IPTG and incubated for a further 3 hrs. Cells were lysed by the addition of 25 mg/L of lysozyme and incubated for 1 hr. Cells were harvested by centrifugation at 6000 rpm for 20 min and cell pellets were stored at −70°C. ScpA was purified using a three-step chromatography process. Cell pellets were dissolved in 100 mL PBS with 1 mg of DNase and ScpA was isolated from *E. coli* proteins by overnight incubation with 10 mL GST-Sepharose affinity resin. To remove the GST tag, the resin containing bound ScpA was re-suspended in 50 mL PreScission protease buffer (50 mM Tris-HCl, pH 80.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) and incubated with 125 units of PreScission protease for 72 hrs at 4°C, DTT concentration was adjusted every 24 hrs by addition of 50 μL of 1 M DTT. ScpA was further purified by anion exchange chromatography in a HiTrap Q FF 5 mL column and by size exclusion chromatography in a 180 mL XK16/100 sepharose S200 HR column. Sample purity was evaluated between each purification step by SDS-PAGE and ScpA was stored in PBS at −70°C. A mature form of ScpA starting at Ala^{92} [19], was also produced with a method closely following the above-described methods, but alterations were introduced. ScpA was separated from *E. coli* protein by GST-Affinity chromatography and eluted with 10 mM reduced glutathione. The eluted protein was diluted to a concentration of 1 mg/mL and incubated at 37 °C for 72 hrs to allow for the auto-proteolysis of the pro-peptide. After incubation, cleaved ScpA was then separated from the cleaved tag/pro-peptide complex by additional GST affinity chromatography. ScpA was further purified using anion exchange chromatography and size exclusion chromatography as previously described. Additionally, an active site mutated versions of ScpA ($ScpA_{S512A}$) was produced according to Tecza et al. [18].

2.3. Screening of ScpA activity against human proteins

Initial activity testing of the three ScpA variants against various human proteins were carried out by applying the following conditions: 10 μg of C5a/C3a/cytokine (IL-37, IL-22, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-1α, IP-10, ENA-78, TNF-α, MIG, IFN-α2a, IFN-α2b, IFNβ, IFNω, IFNγ, IFNλ1, IFNλ2) was incubated with 400 nM of ScpA in PBS, in a total volume of 10 μL, at 37°C for 6 h. 1 mg of C3/C5 was incubated with 400 nM of ScpA in PBS, in a total volume of 10 μL, at 37°C for 6 h. Control proteins were incubated without ScpA. Additionally, the assay was repeated using an inactive version of ScpA (ScpAS512A) against IFNγ, IFNλ1 and IFNλ2. Intact mass of the control proteins and the proteins incubated with ScpA were analysed by mass spectrometry and SDS-PAGE.

For ion source decay (ISD) analysis, the protein cleaved product was obtained by incubation of 10 μg of protein with 800 nM of ScpA in a total volume of 10 μL in PBS at 37 °C. Cleavage reactions were monitored by MALDI intact mass analysis and stopped when the target product reached ~ 90 % of total peaks by the addition of 0.1 % TFA at a 1:1 ratio. All experiments were performed in triplicate.

2.4. MALDI-TOF intact mass analysis

All samples were prediluted at a 1:1 dilution with 0.1 % trifluoroacetic acid (TFA) prior to mixing with the relevant matrix preparations. For intact mass analysis, 2,5-DHAP was chosen as the matrix and prepared as a 15.2 mg/mL solution in 75:25 ethanol, 18 mg/mL diammonium hydrogen citrate in water. Sample was mixed with 2 % TFA and matrix solution at a 1:1:1 vol ratio and 1 μL of the sample/matrix solution was deposited on a ground steel MTP target plate and allowed to dry at room temperature. All spectra were obtained using an UltrafleXtreme TOF/TOF device from Bruker equipped with a SmartBeam2 laser. Intact mass spectra were obtained in linear positive mode with an acceleration voltage of 20 kV, 250 ns ion extraction delay, 2777 V reflector detector gain, a 100-mV digitizer sensitivity and a low mass suppression up to 3000 Da. The centroid algorithm was used to assign peak masses in intact mass analysis and all spectra were externally calibrated using Protein Standard I, the samples of which were prepared following the described procedure. All data was acquired and analysed using the Compass 1.4 software suit (Bruker).

2.5. MALDI-TOF top-down sequencing

sDHB was used as the matrix for all samples except for C3a, C5 and IFNλ1 where a matrix solution composed of 1,5-DAN and picolinic acid (PA) was used. A 50 mg/mL sDHB solution was prepared in 50:50 acetonitrile: 0.1 % TFA solution. 1,5-DAN was prepared as an 8 mg/mL solution in 50:50 acetonitrile: 0.1 % TFA and mixed 75:25 with a 20 mg/mL solution of picolinic acid in a 50:50 acetonitrile: 0.1 % TFA solution. Sample was mixed at a 1:1 vol ratio with sDHB solution or with 1,5-DAN/PA solution and 1 μL of sample/matrix solution was deposited on a polished steel MTP target plate and allowed to dry at room temperature. All spectra were obtained using an UltrafleXtreme TOF/TOF device from Bruker equipped with a SmartBeam2 laser. In-source decay (ISD) spectra was obtained in positive reflector mode with an acceleration voltage of 20 kV, 280 ns ion extraction delay, 2752 V linear detector gain, a 100-mV digitizer sensitivity and a low mass suppression up to 700 Da. The SNAP peak picking algorithm was used to assign monoisotopic masses in ISD spectra which was externally calibrated using myoglobin as standard prepared in the relevant matrix. All data was acquired and analysed using the compass 1.4 software suit from Bruker.

2.6. Maintenance of cells

Retinal pigment epithelial RPE1 cells were cultured in DMEM F-12 media supplemented with 10 % FBS, 1 % antibiotic-antimyotic, 1 % glutamax, 2.6 mg/mL sodium bicarbonate and 1 ng/mL hygromycin B in a T75 flask. The cells were maintained at 37 °C in a 5 % CO_2 humidified incubator.

2.7. pSTAT1 induction with IFNγ

Prior to seeding, hTERT RPE1 cells were grown under normal cell culture conditions at 37 °C and 5 % CO_2 in DMEM supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin and 1 % GlutaMAX until they reached 80 % confluency. Cells were trypsinized, counted, and diluted to a concentration of 20,000 cells/mL and with 200 μl per well seeded in a 96 well black walled Screenstar imaging cell culture plate (655866, Greiner). Seeded cells were further cultured for 48 h at 37 °C and 5 % CO_2 in DMEM normal culture media. Cells were then either left untreated or treated with mature ScpA (20 nM), IFNγ (100 ng/mL) or cleaved IFNγ (100 ng/mL) for 45 min at 37 °C in a 5 % CO_2 humidified incubator. Cleaved IFNγ (100 μg/mL) was generated by incubating IFNγ with 20 nM of mature ScpA, in PBS, for 6 hrs at 37 °C. No subsequent purification was performed, and the solution was diluted in RPE1 media as indicated for the assay.

2.8. Immunofluorescence staining

After incubation, cells were washed with sterile PBS and fixed with 4 % paraformaldehyde overnight at 4 °C. Cells were permeabilized by incubation with 0.1 % Triton X for 15 min at room temperature and blocked with 10 % FBS in PBS for 1 hr at room temperature. Anti pSTAT1 primary antibody was diluted 1:100 in blocking buffer and cells were incubated with antibody solution overnight at 4 °C. Cells were washed with PBS and incubated with anti-goat Alexa Flour secondary antibody diluted 1:1000, DAPI dye diluted 1:500 and phalloidin dye diluted 1:1000 in blocking buffer for 1hr at room temperature. Cells were washed and stored in PBS. Experimental plates were imaged with an ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices). Overall, 9 fields of view per well were acquired at 10x magnification with triplicate wells being quantified per condition per experiment (equating to a minimum of 4000 cells per condition per experiment) being quantified. Cell nuclear pSTAT1 fluorescence (Alexa fluor 488) was quantified using acquired image sets by CellProfiler standard segmentation pipelines referencing cell identification marker phalloidin (555 nm) and nuclear identification marker DAPI (405 nm). The histograms representing the mean fluorescence of pSTAT1 are depicted in Fig. 4B with the mean fluorescence intensity derived from three independent experimental replicates. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test for post hoc comparisons. Representative image composites were rendered using ImageJ from the images acquired as above.

3. Results

3.1. ScpA activity screening against a panel of pro-inflammatory cytokines

The activity of ScpA against a panel of immune response mediators was evaluated using SDS-PAGE and mass spectrometry analysis. ScpA was incubated with the cytokines at 37 °C and the reaction was evaluated after 6 hrs. The activity of three different versions of ScpA against these cytokines were evaluated. A version of ScpA was expressed with the pro-peptide (ScpA) and another ScpA version was expressed with starting amino acid Ala^{79} (ScpA79ΔPro), corresponding to the previously reported end point of autoproteolysis [7]. Finally a mature version of ScpA (mature ScpA) was produced by allowing the natural autoproteolysis of the enzyme at 37 °C. With this we aim to identify any role of ScpA's pro-peptide in the enzymes activity. In total 19 proteins were tested as substrates, with all three versions of ScpA showing activity towards the single member of Type II IFN (IFNγ), the two tested members of Type III IFN (IFNλ1 and IFNλ2), C5, IL-37 and the previously reported C3, C3a and C5a (Fig. 1 and Figure S1).

No activity was observed towards all other tested interleukins (IL-22, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-1α), IP-10, ENA-78, TNF-α, MIG or towards any of the Type I IFNs tested (IFN-α1, IFN-α2, IFNβ, IFNω) (Figures S1 and S2).

All three versions of ScpA successfully cleaved IFNγ into two products with masses of 15834.4 Da and 13469.6 Da (Fig. 1A). In the conditions described above, IFNγ was fully converted into the 15.8 kDa product in approximately 2.5 hrs. Only once most of the cytokine was converted did the 13.5 kDa peak start to emerge (Fig. 2). The full conversion to the 13.5 kDa product took place over 48 hrs, during which some precipitation was observed. In the initial screening, which took place over 6 hrs, IFNλ1 appeared to be converted into a single product with mass of 19141.4 Da (Fig. 1C) but during sample preparation for ion source decay analysis, where the cytokines are incubated for longer periods of time to achieve full cleavage, several other products were identified with masses of 18586.1 Da, 15458.4 Da and 13741.1 Da (Fig. 2). Like IFNγ, IFNλ1 appears to be cleaved at different sites although in contrast with other samples, there wasn't a point at which there was a one product majority. IFNλ2, IL37, C3a and C5a were all cleaved by ScpA resulting in a single product with masses of 19091.3 Da, 19095.7 Da, 8354.6 Da and 7742.5 Da respectively (Fig. 1B, D, I and J). C3 and C5 were also susceptible to ScpA cleavage releasing 9764.5 Da and 8356.5 Da products from C5 and C3, respectively (Fig. 1E–H). In addition, the processed cytokines were analysed by SDS-PAGE to confirm the results and these images can be found in the supplementary information (Figure S5). Furthermore, IFNγ, IFNλ1 and IFNλ2 were incubated with an inactive form of ScpA ($ScpA_{S512A}$) and no activity was observed (Figure S4).

3.2. Identification of ScpA cleavage sites in cleaved cytokines

To identify ScpA cleavage sites in the cytokines, a top-down sequencing approach, using a MALDI TOF instrument, was taken. For the purpose of this work, we focused on the characterization of the ScpA cleavage site in human interferons, identified as novel targets, Table 1. The characterization of the cleavage site in IL-37 is out of scope for this paper and is subject to further investigation. Cytokines were incubated at 37 °C with ScpA79ΔPro and the reaction was monitored using intact mass analysis. C3a, IFNγ and IFNλ2 were also incubated with ScpA and mature ScpA to identify potential cleavage site variations using different ScpA versions but no difference was observed in cleavage between all three versions. The reaction was stopped with TFA to a final concentration of 0.05 % once the amount of cleaved product reached over 90 %. All products were analysed without subsequent purification steps.

The C3a cleavage site was confirmed to be between A^{71}-S^{72}, releasing 7 C-terminal amino acids, which corroborates the results published by

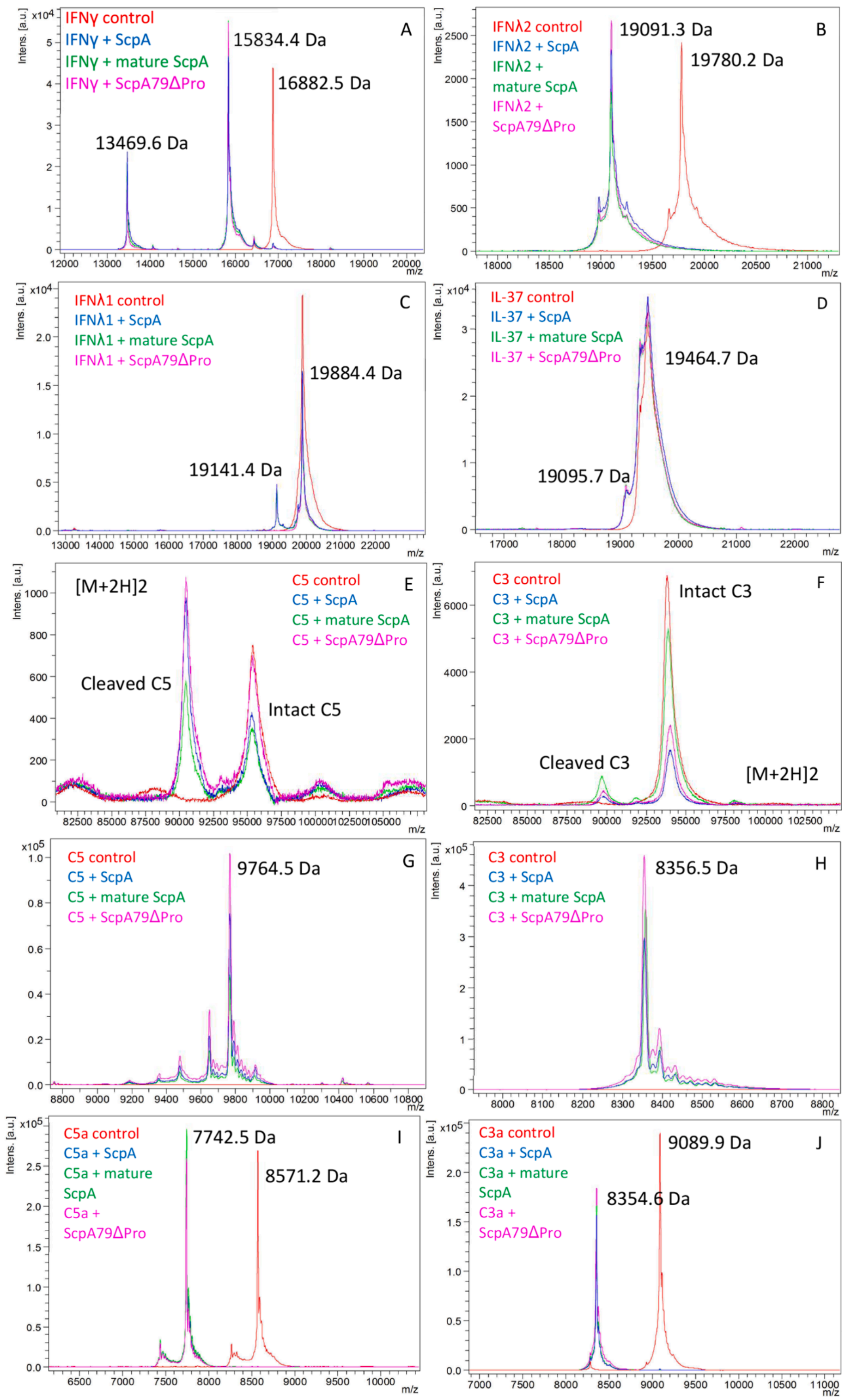

Fig. 1. ScpA activity against cytokines assessed by mass spectrometry analysis of reaction mixtures at the following conditions: 10 μg of cytokine/chemokine or 1 mg (C3 and C5) was incubated with 400 nM of ScpA in PBS, in total volume of 10 μL, at 37 °C for 6 h. Reactions were stopped by addition of TFA to a final concentration of 0.05 % TFA. Panels E and F correspond to the double charged peaks of C5 and C3 respectively and panels G and H correspond to the released product from C5 and C3 respectively. Samples were prepared following the dried droplet method using 2,5 DHAP as MALDI matrix. Spectra were obtained in positive linear mode. (n = 3).

Lynskey et al [6]. The C5a cleavage site was also observed to match that of previously published results with ScpA cleaving between H^{67}-K^{68} removing 8 C-terminal amino acids [36]. IFNλ2 is cleaved by ScpA between H^{6}-G^{7} removing 6 N-terminal amino acids.

To identify the first point of cleavage in IFNγ, the reaction was stopped after 2.5 hrs when most of the product present in the sample was the one with a mass of 15.8 kDa. Top-down sequencing confirmed that ScpA cleaves IFNγ at the C-terminus between Q^{156}-M^{157}, removing 10 terminal amino acids, whilst no N-term modifications were observed. To identify the second point of cleavage the reaction was continued until most of the product in sample was the 13.5 kDa product. Top-down sequencing confirmed that ScpA cleaves IFNγ between H^{20}-S^{21}

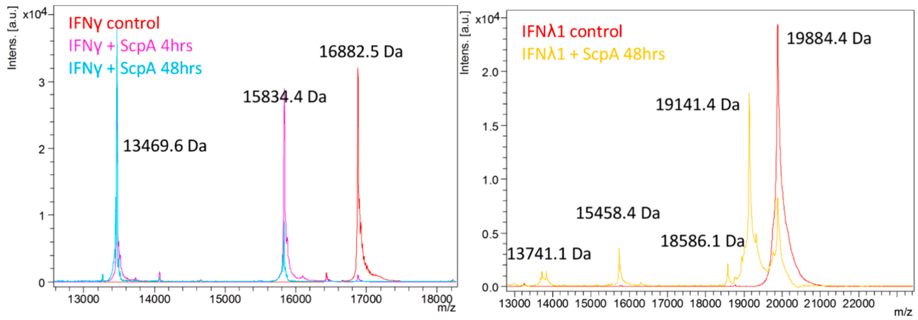

Fig. 2. ScpA activity against IFNγ (left) and IFNλ1 (right) assessed by mass spectrometry analysis of reaction mixtures of 10 μg of protein incubated with 800 nM of ScpA in a total volume of 10 μL in PBS at 37 °C. After incubation samples were mixed 1:1 with 0.1 % TFA. Samples were prepared following the dried drop method using 2,5 DHAP for MALDI-TOF intact mass analysis in linear positive mode. (n = 3).

Table 1
Amino acid sequences of C5a, C3a, IFNγ, IFNλ1 and IFNλ2 with identified ScpA cleavage sites highlighted in red. Samples were prepared following the dried droplet method for MALDI-TOF top-down sequencing. C5a, C3a and IFNγ, were prepared using 1,5DAN as MALDI matrix and IFNλ1 and IFNλ2 were prepared using sDHB as MALDI matrix.

Substrates	Sequences
C5a	TLQKKIEEIA AKYKHSVVKK CCYDGACVNN DETCEQRAAR ISLGPRCIKA FTECCVVASQ LRANIS**HK**DM QLGRL
C3a	SVQLTEKRMD KVGKYPKELR KCCEDGMREN PMRFSCQRRT RFISLGEACK KVFLDCCNYI TELRRQHAR**A** **S**HLGLAR
IFNγ	MQDPYVKEAE NLKKYFNAG**H** **S**DVADNGTLF LGILKNWKEE SDRKIMQSQI VSFYFKLFKN FKDDQSIQKS VETIKEDMNV KFFNSNKKKR DDFEKLTNYS VTDLNVQRKA IHELIQVMAE LSPAAKTGKR KRS**QM**LFQGR RASQ
IFNλ1	PTSKPTTTGK GCHIGRFKSL SPQELASFKK ARDALEESLK LKNWSCSSPV FPGNWDLRLL QVRERPVALE AELALTLKVL EAAAGPALED VLDQPLHTLH HILSQLQACI QPQPTAGPRP RGRLHHHWL**HR** LQEAPKKESA GCLEASVTFN LFRLLTRDLK YVADGNLCLR **TS**THPEST
IFNλ2	PVARL**HG**ALP DARGCHIAQF KSLSPQELQA FKRAKDALEE SLLLKDCRCH SRLFPRTWDL RQLQVRERPM ALEAELALTL KVLEATADTD PALVDVLDQP LHTLHHILSQ FRACIQPQPT AGPRTRGRLH HWLYRLQEAP KKESPGCLEA SVTFNLFRLL TRDLNCVASG DLCV

removing 20 amino acids at the N-terminus.

The ISD spectra from the IFNλ1 sample was extremely complex due to the presence of several products and peptides. From the obtained spectra, two sequences were able to be extracted. No N-terminal modifications were observed, however, two cleavage sites were observed, one between $T^{171} - S^{172}$ corresponding to the 19.1 kDa product and another between $H^{125} - H^{126}$ corresponding to the 13.7 kDa product. Due to sample availability, it was not possible at this time to effectively separate the different products from each other to better elucidate ScpA's mechanism of action against IFNλ1. The ion source decay spectra for identification of the cleavage sites are provided in the supplementary information, Figures S6-S10.

3.3. Comparative activity study

Measured activities of ScpA, mature ScpA and ScpA79ΔPro towards human C5a and newly identified human protein substrates: hC3a, IFNγ, IFNλ1 and IFNλ2 revealed that the 3 versions of the enzyme showed reduced activities towards hC3a, IFNγ, IFNλ1 and IFNλ2 when compared to their activities towards hC5a (Fig. 3). ScpA, mature ScpA and ScpA79ΔPro displayed 63 % ± 160.4, 75 % ± 14.8 and 62 % ± 9 activity against hC3a, compared to their activities for hC5a. IFNλ2 was processed at 6 % ± 0.7 activity by ScpA, at 7 % ± 0.005 activity by ScpA79ΔPro and at 2 % ± 0.42 activity by mature ScpA. In comparison with hC5a, all three different versions of ScpA processed IFNy and IFNλ1 with 98 % ± 0.3, and ≥ 99.2 % ± 0.02 reduced activities, respectively.

An attempt at generating binding data between ScpA and its novel substrate was made using surface plasmon resonance. Binding kinetics between ScpA/C5a and ScpA79ΔPro/C5a were obtained with a K_D value of 34.5 and 25 nM respectively [19] and are closely related to previously published data [18]. No favourable assay conditions were found where accurate binding data could be generated between the ScpAs and IFNγ, IFNλ1, IFNλ2 using this method. Due to high background signal from the high concentrations of ScpA used no binding data was able to be extracted from the sensor grams leading to inconclusive results (Figures S17 and S18).

3.4. ScpA effect on the IFNγ signalling pathway

Interferon gamma (IFNγ) activates the JAK/STAT signaling pathway, leading to the phosphorylation and subsequent nuclear translocation of phosphorylated STAT1 (pSTAT1), which acts as quantifiable indicator of pathway activity [37,38]. Here we evaluated the biological impact of ScpA-mediated cleavage of IFNγ using a human retinal pigment epithelial (hTert-RPE1) cell line. RPE1 cells have a normal karyotype [39] and have been widely used to study physiological events making them suitable epithelial cells for this evaluation [40,41]. Cells were left untreated, incubated with ScpA alone, with intact IFNγ alone or with IFNγ pre cleaved by ScpA. Results depicted in Fig. 4a demonstrate a significant elevation in nuclear pSTAT1 (visualized as green) in cells treated with intact IFNγ compared to untreated controls. Conversely, cells exposed to IFNγ cleaved by ScpA exhibited pSTAT1 levels similar to control groups. These data were quantified through high content imaging, assessing the average nuclear signal of pSTAT1 using automated segmentation and intensity measurement through CellProfiller analysis (Fig. 4b), confirming that treatment with intact IFNγ significantly enhances nuclear pSTAT1 accumulation relative to controls (either untreated or treated with ScpA).

4. Discussion

C5a peptidase is a serine protease present on Group A Streptococcus cell surfaces and has been established as an important virulence factor in early infection [2,3]. Up to this point, ScpA was known to be a highly specific enzyme capable of cleaving human complement components C5a, C3 and C3a, even being able to discriminate between complement components from other species [4–7]. The data presented in this paper expands the current roster of ScpA's substrates by showing the capability of ScpA to process other pro-inflammatory cytokines. From the panel tested type II interferon, IFNγ, and the type III interferons tested IFNλ1 and IFNλ2 were shown to be cleaved by ScpA whilst none of the Type I interferons tested were susceptible to cleavage, Table 1. Taking into consideration the similarity in sequence and structure between the members of the interferon families, it could be assumed that the remaining untested IFNs belonging to Type I IFN family may not be

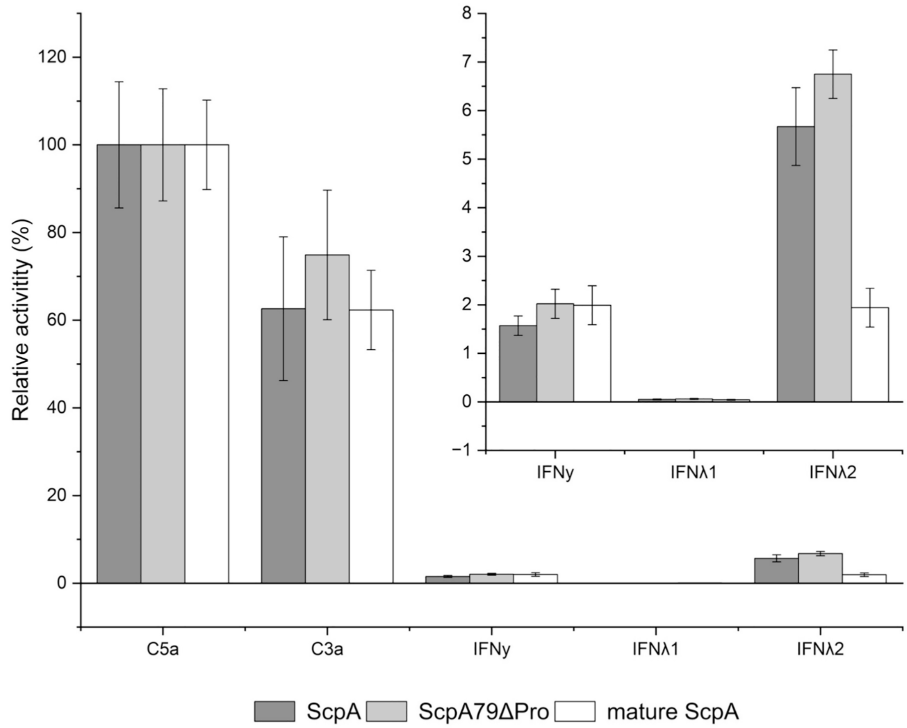

Fig. 3. Relative activities of ScpA, ScpA79ΔPro and mature ScpA towards hC5a, hC3a, IFNγ, IFNλ1 and IFNλ2. The reactions were performed with enzyme to substrate molar ratio of 1:100 in PBS and inhibited by TFA at final concentration of 0.05 %. Samples were prepared following the dried drop method using 2,5 DHAP as matrix for MALDI-TOF intact mass analysis in linear positive mode. Relative activity was calculated in relation to the slope of the reaction between C5a and ScpA. Data represents the average of 3 independent experiments and related standard deviations (n = 3).

susceptible to ScpA either, while IFNλ3 and IFNλ4 belonging to Type III IFNs could be vulnerable to being processed by the enzyme. Further testing would be required to verify this hypothesis. IL37 was also shown to be cleaved by ScpA and, in contrast to results published by Anderson et al. [7], we have also clearly observed digestion of complement component C5 after incubation with ScpA.

From the mass spectrometry data collected and taking into consideration the progression of product formation during the described conditions from intact IFNγ to the 15.8 kDa product and to the 13.5 kDa product, we can conclude that ScpA is cleaving IFNγ at two different sites with two drastically different levels of affinity. A similar pattern can be observed for IFNλ1 with the appearance of several other peaks in the mass spectrum with increased incubation time, whilst all other substrates were observed to be converted into only one product.

To better understand the mechanism of action of ScpA, a top-down sequencing approach was used to validate and identify the cleavage site in each of its substrates. With this we confirmed that ScpA cleaved IFNγ at the C-terminus removing 10 amino acids and at the N-terminus removing 20 amino acids. Previous studies have been conducted to examine the interactions between IFNγ and the IFNγ receptor where it was found that an 11 amino acid truncation at the C-terminus severely decreasing binding affinity between IFNγ and its receptor [42]. Knowing the points at which ScpA truncates IFNγ at the C-terminus, we can potentially assume that it has a similar effect. IFNγ binds its own receptors IFNGR1 and IFNGR2 who do not have any intrinsic kinase activity but signal through the JAK/STAT pathway [37,38,43]. Therefore monitoring the phosphorylation of the STAT proteins in cells in the presence of IFNγ is a recognised indicator of IFNγ receptor binding and pathway activity, and highlight this pathway as warranting future evaluation of ScpA's effects on interferon mediated pathway regulation. RPE1 cells were incubated with intact IFNγ, IFNγ which had been cleaved by ScpA and with ScpA alone to evaluate ScpA's effects on IFNγ ability to induce STAT1 phosphorylation. Results show an increase in pSTAT1 on the nucleus of cells which have been incubated with intact IFNγ but no significant increase is observed for cells which have been incubated with cleaved IFNγ or ScpA. It was demonstrated that ScpA by itself doesn't appear to have an effect in the cells and when IFNγ is cleaved by ScpA, it loses the ability to induce pSTAT1 phosphorylation leading to the conclusion that ScpA is effective in inactivating IFNγ.

From the ISD spectrum of cleaved IFNλ1, two cleavage sites were confirmed, both at the C-terminus, potentially indicating a progressive digestion of that terminus by ScpA. Intriguingly, although little information is available regarding the relationship between the structure of IFNλ1 and its function, studies have shown that point mutations at the C-terminus of IFNλ3 severely decrease its ability to induce anti-viral activity [44]. Further, the type III interferon family is highly conserved with IFNλ1 sharing 84 % of the amino acid sequence with IFNλ2 and 3. IFNλ2 and 3 are isoforms only differing in 7 amino acids in their sequence. When examining the crystal complex of IFNλ1 and its receptor, these mutations are in a crucial site of interaction between receptor and substrate indicating the importance of the C-terminus in IFN1λR receptor binding [45]. Knowing that ScpA targets IFNλ1 C-terminus removing amino acids corresponding to these sites, it potentially could render the cytokine inactive, though deep molecular understanding on the inhibitory effects of ScpA will be the subject of future investigations.

The screening also showed that ScpA has varying levels of affinity for the different substrates. Whilst C5a and C3a are both fully processed in less than 30 min, all other substrates need considerably more time for full digestion. The relative activity of ScpA against the interferons was measured using MALDI-TOF intact mass analysis, Fig. 3. When using ScpA/C5a activity as a benchmark, ScpA activity towards C3a, IFNγ, λ2 and λ1 was 68 %, 2 %, 5 % and 1 % of the ScpA/C5a activity respectively. In general, the results indicate that both complement factors, hC5a and hC3a could be competing substrates for ScpA in the human body, while IFNγ, IFNλ1 and IFNλ2 would be the secondary targets processed with reduced efficiency.

Three different versions of ScpA were used in these studies to better understand the impact of the pro-peptide on activity. No significant

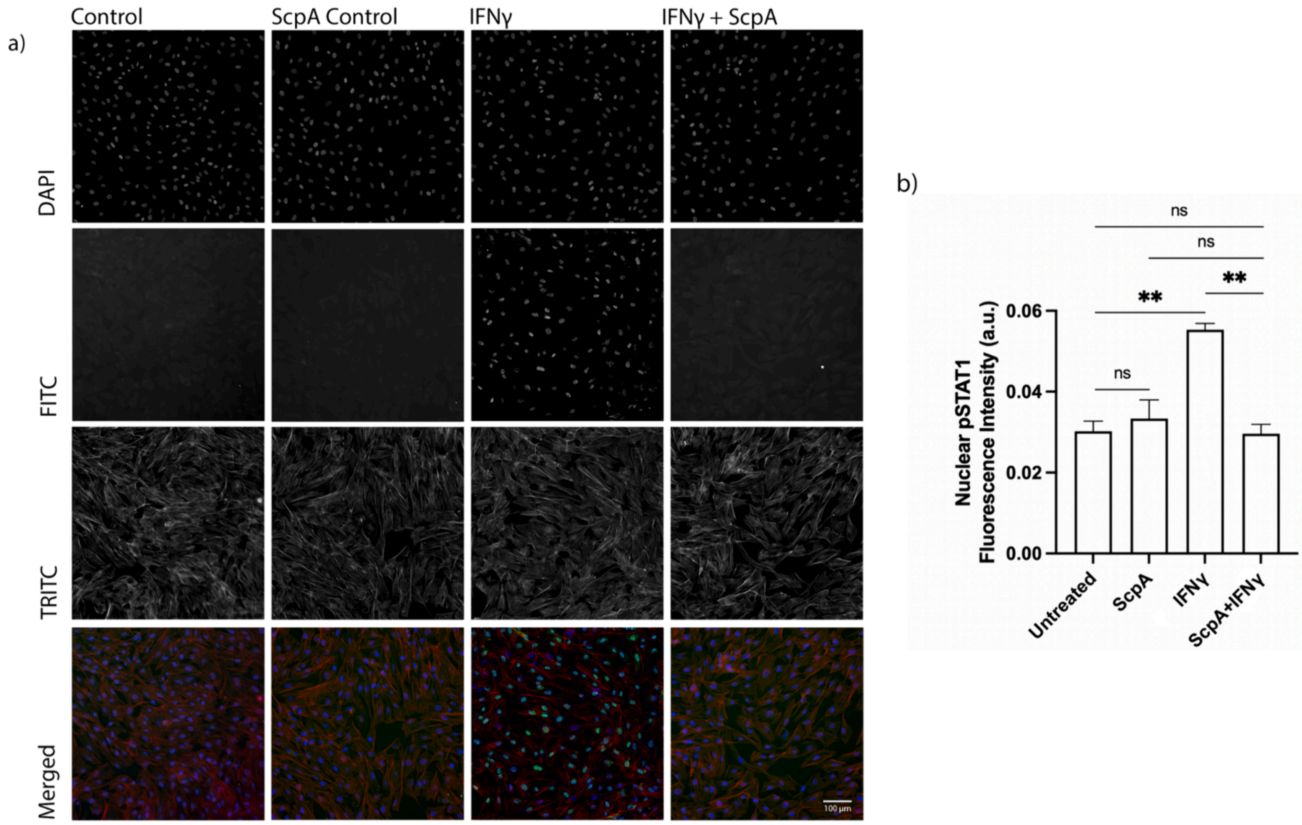

Fig. 4. (a) Expression of phosphorylated STAT1 protein (Green/FITC) in RPE1 cells incubated with only media as a control, ScpA Control (20 nM), IFNy at (100 ng/mL) and ScpA cleaved IFNy. Cell structure is represented in red (phalloidin/TRITC) and nucleus in blue (DAPI). (b) Quantification of mean nuclear fluorescence levels from confocal images of RPE1 cells incubated with only media as a control, ScpA at 20 nM, IFNy at 100 ng/mL and cleaved IFNy. Mean intensity was obtained from the average of a minimum of 4000 cells per well. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test for post hoc comparisons. Experiment was conducted with three biological repeats (n = 3) and three technical repeats (n = 3). Data represents the average of 3 independent experiments and related standard error. ** = $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

difference was observed between ScpA expressed with the pro-peptide (ScpA) and ScpA expressed without the pro-peptide (ScpA79ΔPro) or mature ScpA, except in the case of IFNλ2, where we observed a decrease in activity with mature ScpA. This result supports the recent report from our group that describes an in-depth structural and functional characterization of pro-peptide truncated variants of ScpA expressed in *E. coli* cells which concluded that a version of ScpA without the pro-peptide could be engineered as a potential therapeutic with no significant loss in effectiveness [19].

In conclusion, ScpA has been shown to cleave IFNγ, IFNλ1, IFNλ2, cytokines which are not only important in the immune response to pathogens but are also involved in auto-immune disease pathology. IFNγ plays an important role in cell mediated immunity and modulates several anti-microbial actions [46] including during *Streptococcus pyogenes* infection [47], and Type III Interferons, the most recent members of the interferon family, have been described to modulate anti-viral activity [48]. ScpA potential inactivation of these cytokines better illustrates the role that it might play in early infection in curbing immune system response at the barrier level. This discovery not only gives us further insight into ScpA mechanism of action, but it also highlights the potential use of ScpA as a therapeutic agent. Both type II and members of the type III interferon family have been previously implicated in auto-immune inflammatory disease pathology. Studies have shown that serum levels of IFNγ are linked to disease severity in psoriasis. Injection of IFNγ in non-lesion skin of psoriatic patients resulted in the appearance of lesions [49], implicating IFNγ as a causing agent of psoriatic lesions. Previously published studies have also shown that upregulation of both IFNγ and IFNλ1 causes destruction of the barrier function in patients with inflammatory bowel disease (IBD) [50–52]. ScpA's ability to cleave these cytokines, which are known effectors of diseased states in auto-immune inflammatory diseases, implicate ScpA as a novel therapeutic approach to treat these disorders. Although specific activity of ScpA is shown against these targets, importantly, the kinetic disparity of ScpA activity against the IFNs when compared to C5a raises the possibility that these effects will not translate to a biological impact *in vivo*. Thus, further work is required to fully understand the potential of ScpA as a therapeutic in mitigating IFN driven inflammation in a biological context. Given that IFNs retain some sequence similarity between human and mouse/rat species and C5a and IFNλ1 cleavage sites are conserved between these species (Figures S13-16), administration of ScpA as a treatment in mouse psoriasis or IBD models, where IFNs are reported as important to the pathology, is one interesting area of further exploration. However, it should be noted that ScpA has been previously shown to be able to discriminate between human and mouse C5a (even though the cleavage site is conserved between species) [5], potentially indicating that ScpA's behaviour against the IFNs in a mouse model might not be a good predictor of its therapeutic potential in humans. Therefore, human *in vitro* skin or organoid models mimicking the diseased state of psoriasis or IBD could also be employed to further evaluate ScpA's biological relevance in humans. Further investigations could also examine the impact of ScpA activity on IFN signalling in the context of Group A *Streptococcus pyrogene* (GAS) infection. Examining the levels of IFN or IFN driven signalling through JAK/STAT in cells infected with GAS, ScpA mutant or mutant complemented strains, similar to work performed by Klenk et al. [53], would yield such results. Additionally, efforts could be made to detect ScpA derived interferon cleavage products in samples taken from individuals with an active GAS infection. Together these approaches may yield a better understanding into the mechanisms of action in the context of GAS infection.

CRediT authorship contribution statement

Francisco Duarte: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation. **Malgorzata Teçza:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **Vinayakumar Gedi:** Writing – original draft, Methodology, Investigation. **Kieran McGourty:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Sarah P. Hudson:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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