Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Efficient chemical synthesis of human complement protein C3a

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METHODS & MATERIALS

Peptide synthesis

Reduced C3a was manually synthesized step-wise on a 0.5 mmol scale in three segments consisting of C3a(1-22)-α-thioester, C3a(23-48)-α-thioester and C3a(49-77). C3a(1-22)-α-thioester and C3a(23-48)-α-thioester were synthesized on trityl-associated mercaptopropionic acid leucine and trityl-associated mercaptopropionic acid arginine resins respectively to yield activated C-terminal α-thioesters upon HF cleavage.[1] S-trityl was removed with two 1 minute washes of 2.5% (v/v) triisopropylsilane and 2.5% (v/v) water in TFA. C3a(49-77) was synthesized on a Boc-Arg-Pam resin. Peptide coupling was carried out according to *in situ* neutralization protocols.[2] Coupling efficiencies were measured using the quantitative ninhydrin test.[3] Couplings with yields of lower than 99.5% were double coupled. Boc-protected amino acids were used with the following side chain protecting groups: D(Chxl); E(Chxl); H(DNP); K(Clz); N(Xan); Q(Xan); R(Tos); S(Bzl); T(Bzl); Y(BrZ). All cysteine residues were protected with MeBzl except for C23, which was incorporated into the peptide as 1,3-thiazolidine-4-carboxylic acid (Thz).[4,5] Thz was used in place of cysteine due to unwanted reactivity during ligation and easy conversion to cysteine using methoxyamine.[5]

After chain assembly, DNP groups of C3a(49-77) were removed by stirring the resin twice with 10 mL of 20% 2-mercaptoethanol:10% DIEA in DMF for 30 minutes each time, draining the resin and flow washing with DMF in between. Resins were flow washed with DCM for 1 min and dried under vacuum prior to HF cleavage. Simultaneous side chain removal and cleavage of peptides from the dried resins were carried out with 9 mL of HF and1 mL p-cresol as scavenger for 1 hour at 0°C. Cleaved peptides were precipitated and washed twice with cold diethyl ether. The peptides were then dissolved in 50% acetonitrile, 0.1% TFA (v/v) in water and lyophilized. C3a(1-22) was dissolved in 10% acetonitrile/0.1% TFA in water and purified by preparative reverse-phase (RP) HPLC, while C3a(23-48) and C3a(49-77) were dissolved in 6M guanidine(Gdn)•HCl and purified. HPLC fractions were characterized by analytical HPLC-UV and

electrospray ionization MS (ESI-MS). Fractions containing the desired peptide and similar purity were pooled, lyophilized and stored at -20ºC.

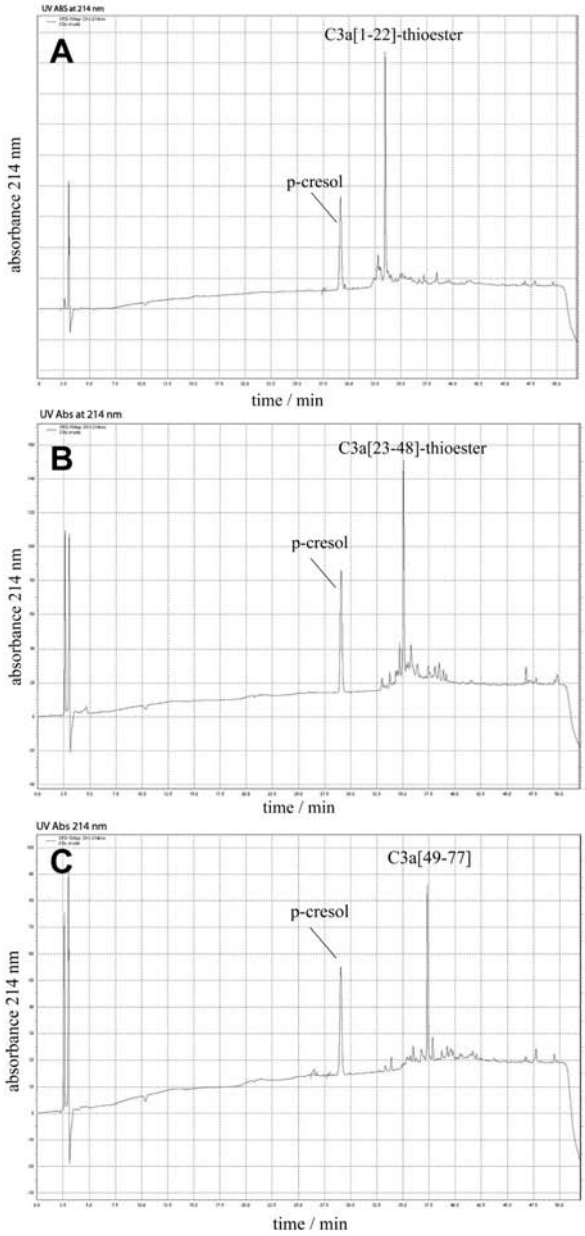

Figure 1: RP**–**HPLC analysis of crude C3a peptides after HF cleavage. **A**: C3a(1-22)-α-thioester **B**: C3a(23-48)-α-thioester and **C**: C3a(49-77). HPLC elution was achieved using a linear gradient of 0-45% of solvent B in solvent A over 45 mins at 40°C with a flow rate of 1.0 mL/min (A – 0.05% TFA in water, B – 90% acetonitrile/0.043% TFA in water). Column: Agilent Zorbax 300SB, C18 (4.6 x 250 mm, 5 μm).

ESI-MS data:

C3a(1-22)-α-thioester

SVQLTEKRMDKVGKYPKELRKC-[COS-CH_2-CH_2-CO]-Leu

M_{found}: 2838.5 ± 0.3 Da; M_{calc}: 2838.3 Da (average isotope composition)

C3a(23-48)-α-thioester [Cys23Thz]

Thz-EDGMRENPMRFSCQRRTRFISLGEA-[COS-CH_2-CH_2-CO]-Arg

M_{found}: 3347.1 ± 0.3 Da; M_{calc}: 3346.8 Da (average isotope composition)

C3a(49-77)

CKKVFLDCCNYITELRRQHARASHLGLAR

M_{found}: 3402.9 ± 0.3 Da; M_{calc}: 3403.0 Da (average isotope composition)

Native Chemical Ligation

Ligations were done in a one-pot approach as previously described.[5] 5.9 µmol (20.0 mg) of peptide C3a(49-77) and 5.9 µmol (19.7 mg) of C3a(23-48) were combined and dissolved in 3.0 mL of ligation buffer consisting of 6M guanidine hydrochloride (Gdn•HCl), 200 mM Na_2HPO_4, 50 mM Tris(2-carboxyethyl)phosphine•HCl (TCEP) and 50 mM 4-mercaptophenylacetic acid (MPAA), pH 7.1. The solution was stirred under argon and the reaction was monitored by HPLC-UV. The first ligation was found to be quantitatively complete after 6 hours. The ligation product of 6488.2 ± 1 Da was observed in the ESI-spectrum (expected average MW: 6487.6 Da).

754 µmol (63.0 mg) of methoxyamine•HCl was added to the ligation mixture for a final concentration of 250 mM. pH was adjusted to 3.7 with 5M HCl and the solution was stirred under argon overnight for conversion of Thz to cysteine. High resolution ESI-MS was performed the following morning to ensure a loss of 12 Da corresponding to quantitative conversion of Thz to cysteine (observed MW: 6475.2 ± 0.8 Da, expected average MW: 6475.6 Da).

After readjusting to pH 7.1 with 5M NaOH, 7.7 µmol (21.9 mg) of C3a(1-22) was added to the ligation mixture and the solution was stirred under argon. Ligation progress was monitored by HPLC-UV and found to be quantitative after 6 hours. After 6 hours, 3.0 mL of a 6M Gdn•HCl, 200 mM Na_2HPO_4, 2% (v/v) 2-

mercaptoethanol solution, pH 8.1 was then added to the ligation mixture and the solution was stirred under argon for 20 minutes. Fresh TCEP was then added to a concentration of 20 mM and stirred for a further 20 minutes under argon. 60 µL of TFA was added to the mixture for a 1% (v/v) TFA solution. The solution was then syringe filtered and analyzed by ESI-MS for reduced C3a (observed MW: 9095.4±1.8 Da, expected average MW: 9094.8 Da,). Reduced C3a was purified by RP-HPLC on a Agilent Zorbax 300SB, C3 column (9.4 x 250 mm, 5 µm). Fractions containing the desired product were lyophilized and stored at -20ºC. Yield: 41% (2.4 µmol/22.2 mg).

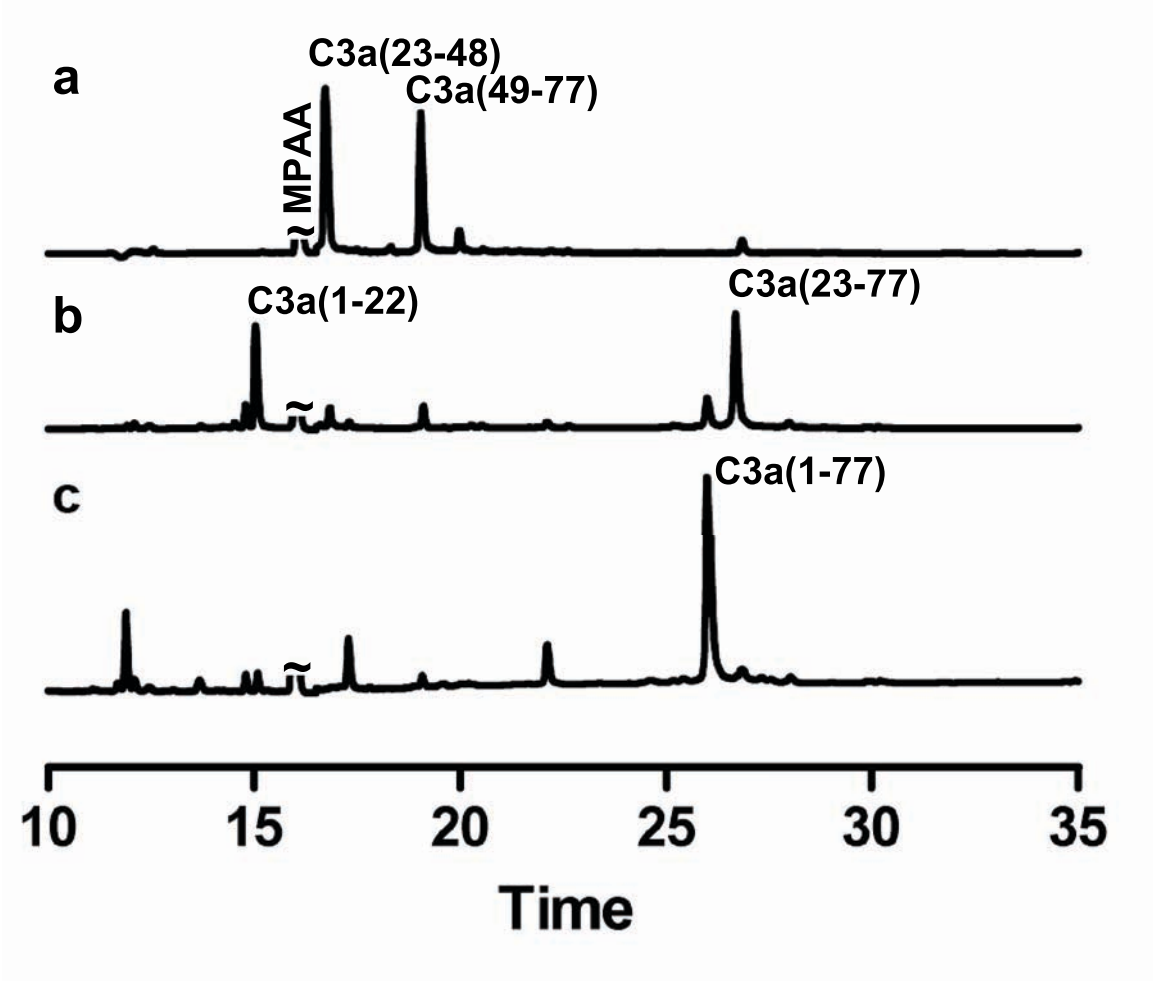

Figure 2: RP–HPLC analysis of C3a ligations observed at 214 nm. **a**: Ligation of C3a(23-48) and C3a(49-77) shortly after mixing (t=0 hrs). The reaction is complete after 6 hrs (not shown). Following Thz→Cys conversion, C3a(1-22)-α-

thioester was added and the pH readjusted to pH 7.1 **b:** Ligation shortly after addition of C3a(1-22)**. c:** The ligation of C3a(1-22) and C3a(23-77) reaches completion after 6 hours. HPLC elution was achieved using a linear gradient of 10-55% of solvent B in solvent A over 32 mins at 40°C with a flow rate of 1.0 mL/min (A – 0.05% TFA in water, B – 90% acetonitrile/0.043% TFA in water). Column: Agilent Zorbax 300SB, C18 (4.6 x 250 mm, 5 μm).

Oxidative Folding

1.7 μmol (15.2 mg) reduced C3a was dissolved in 12.6 mL 6M Gdn•HCl (pH ~ 5) for a 1.2 mg/mL peptide solution. The peptide solution was then added to 150 mL of oxidation buffer consisting of 50 mM Na_2HPO_4, 8 mM reduced L-glutathione (GSH), 1mM oxidized L-glutathione (GSSG), pH 7.5, degassed by helium sparging for 30 mins. Oxidation was performed at 22°C and monitored by HPLC and found to be quantitatively complete after 2 hours (Figure 2). The oxidation product was analyzed by HPLC-UV and compared to 0.1 mg/mL native C3a (Merck BioSciences) run with an identical program. After 2 hours, 0.813 mL of TFA was added to the oxidation mixture (final concentration: 0.5% TFA). Oxidized C3a was then purified by RP-HPLC on a Agilent Zorbax 300SB, C3 column (9.4 x 250 mm, 5 μm) and isolated in high yield of 65% (1.1 μmol, 9.9 mg). Purity was determined by RP-HPLC-UV. A high-resolution ESI-MS spectrum of oxidized C3a (Figure 3) was obtained on a AB SCIEX tripleTOF 5600 system. Observed MW: 9088.71 ± 0.2 Da; calculated MW: 9088.65 Da (average isotope composition).

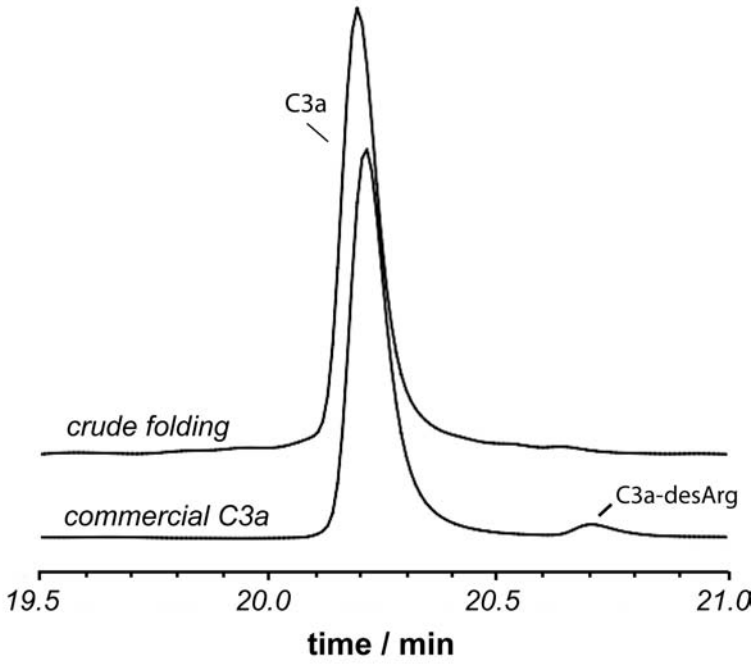

Figure 3: RP-HPLC analysis of crude folding mixture (upper trace) and commercially available C3a.

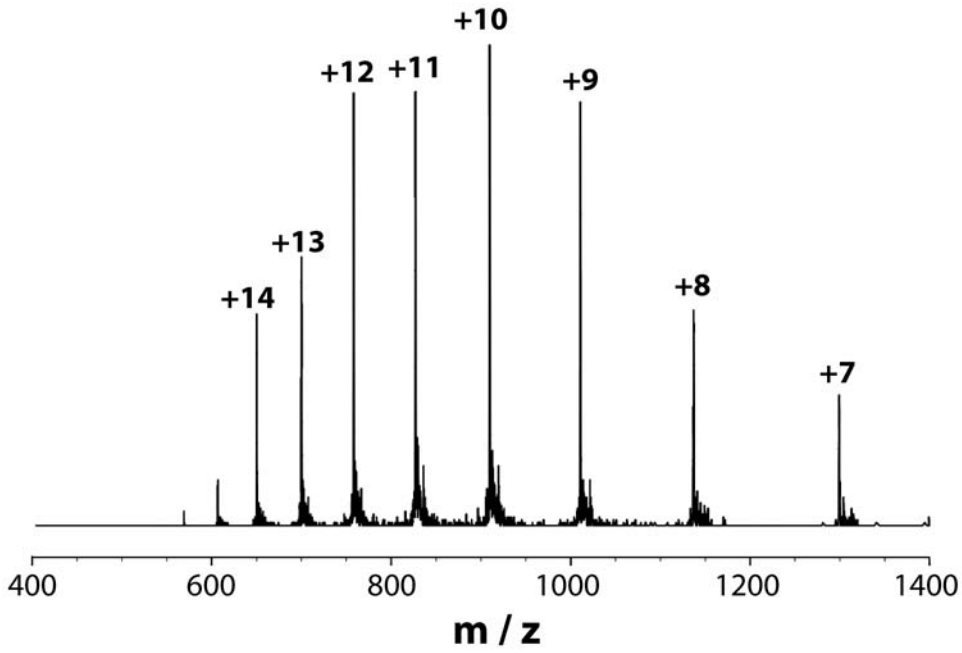

Figure 4: ESI-MS spectrum of synthetic oxidized C3a. Observed MW: 9088.71 ± 0.2 Da; calculated MW: 9088.65 Da (average isotope composition).

Crystallization

Crystals of synthetic C3a were obtained by using the hanging drop, vapour diffusion technique. Lyophilized C3a was dissolved in 2mM HCl at a concentration of 5 mg/mL. The hanging drop consisted of 1 µL of protein solution mixed with 1 µL of reservoir solution (0.1 M TRIS-HCL, 0.2 M $(NH_4)_2HPO_4$, 49–51% (v/v) 2-methyl-2,4-pentanediol (MPD), pH 8.5) and was equilibrated against 500 µL of reservoir solution. Crystals were setup at 4ºC and incubated at 8ºC for 4 days (96h) after which the plates were moved to a 4ºC cold room. Rhomboid-shaped crystals appeared approximately 6 days after setting up the drops and grew to dimensions of about 0.5 x 0.2 x 0.2 mm.

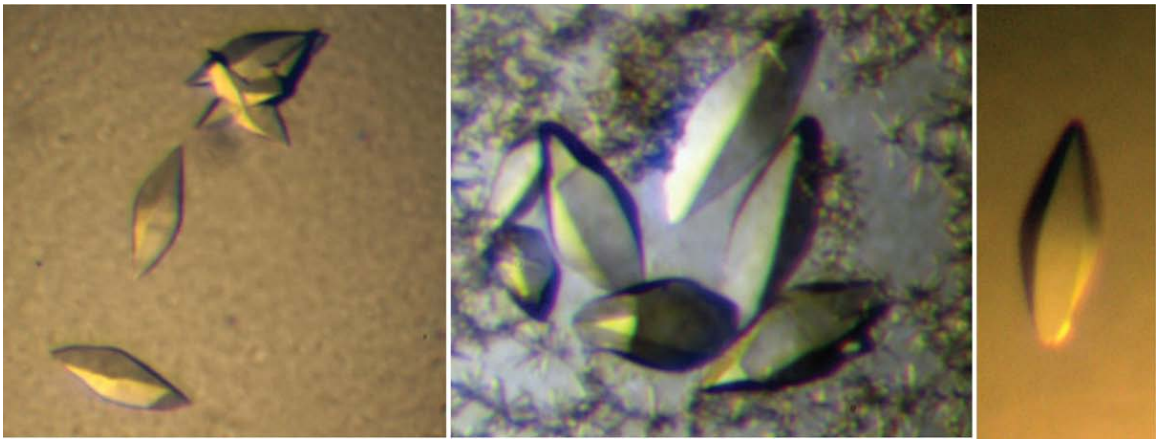

Figure 5: Crystals of synthetic C3a.

Structure determination

X-ray diffraction data sets were collected in a cryostream (100 K) with a Rigaku Saturn 944 CCD detector and CuKα radiation from a Rigaku FR-E+ SuperBright generator (Rigaku/MSC) at the University of Queensland, Australia. The raw data sets were auto-indexed, integrated and scaled using the CrystalClear 2.0 package (Rigaku/MSC) and iMOSFLM [6]. The C3a structure (residues 14-72) was first determined by molecular replacement with the program Phaser [7], using the crystal structure of human anaphylatoxin desArg C5a (PDB ID: 3HQA,[8]) as a search model. The structure of C3a was then further extended at the N- and C-termini (residues 10-77) using the dataset collected at the MX2 micro

crystallography beamline at the Australian Synchrotron. Buccaneer [9], Coot [10] and Arp/Warp [11] were used for model building and manual adjustment, and REFMAC5 [12] was used for refinement until satisfactory model statistics were obtained (Table 1). The final C3a model consists of 68 residues, one MPD and 19 water molecules. The structure quality analysis was done using Molprobity [13] and SFcheck [14] in the CCP4i program suite [15].

Statistics for data collection and refinement		
Data collection	***Rigaku Saturn944***	***MX2 Beamline***
Space group	$P3_121$	P63
Cell dimensions (Å)		
a, *b*, *c*	39.2, 39.2, 80.66	68.29, 68.29, 39.27
α, ß, γ	90.0, 90.0, 120.0	90.0, 90.0, 120.0
Resolution (Å)	33.79-2.10 (2.18-2.10)	39.27-2.14
Reflections		
Observations	15,526	129,557
Unique reflections	4,478	5,870
Redundancy	3.47 (3.35)	22.1 (22.0)
Completeness (%)	99.7 (99.3)	100.0 (96.4)
R_{merge}	0.046 (0.446)	0.106 (0.787)
I/σ (I)	15.5 (1.9)	15.9 (3.4)
Refinement		
Resolution (Å)		19.42-2.14
R_{work} / R_{free}		0.208 / 0.267
No. atoms		
Protein		557
Water		19
Ligand		8
B-factor (Å^2)		
Protein		60.82
Water		57.09
Ligand		72.95
R.m.s deviations		
Bond lengths (Å)		0.017
Bond angles (°)		1.907
Ramachandran plot:		
Most favored regions (%)		95.5

Table 1: Statistics for X-ray data collection and structure refinement

Isolation of Human Monocyte-derived Macrophages (HMDM)

Human monocyte-derived macrophages (HMDM) were isolated using Ficoll-paque density centrifugation (GE healthcare Bio-Science, Uppsala, Sweden) from buffy coat of anonymous human donors provided by Australian Red Cross Blood Service, Brisbane. $CD14^+$ monocytes were positively selected using $CD14^+$ MACS magnetic beads (Miltenyi Biotech, Auburn, CA, USA) after successive magnetic sorting and washings. The $CD14^+$ monocytes were then cultured at 37°C, with 5% CO_2 and differentiated to HMDM using 100 ng/mL of recombinant human macrophage colony stimulating factor (M-CSF) (PeptroTech Inc, Rocky Hill, New Jersey, USA) at 1.5×10^6 cells/mL. HMDM were kept in IMDM supplemented with 10% FBS, penicillin (10 U/mL), streptomycin (10 U/mL) and L-glutamine (2 mM) (Invitrogen). HMDM were supplemented after 5 days with fresh medium containing 100 ng/mL M-CSF. HMDM were harvested by gentle scraping in saline solution on day 7.

Intracellular Calcium Release Assay

HMDM were plated at 5×10^4 cells/well in a 96-well cleared-bottom black-wall assay plate (Corning) and incubated overnight at 37°C. Before assay, the medium was removed and cells were incubated with dye-loading buffer (12 mL assay buffer, 4 μM Fluo-3 AM, 25 μL Pluronic acid F-127 and 1% FBS) for an hour at 37°C. After an hour, cells were washed once with assay buffer (HBSS supplemented with 2.5 mM probenecid and 20 mM HEPES, pH 7.4). To determine the agonist activity of C3a, the synthesised and the commercial C3a protein (57 μM stock in PBS) were further diluted with HBSS buffer to the desired concentrations for intracellular calcium release assay. For antagonist assay, the C3a antagonist (SB290157)[16] was diluted with HBSS buffer to give a range of concentrations and then was pre-incubated with the cells for 15 min before the addition of C3a agonist. FLIPR was used to monitor the intracellular release of Ca^{2+} via fluorescence measurement for 5 min (excitation 495 nm, emission 520 nm). The intracellular Ca^{2+} release was monitored immediately for 5 min after the injection of the desired concentration of the C3a. Duplicate measurements were

made for each data point, mean ± SEM are reported from experiments as indicated. Net changes in fluorescence were calculated as a percentage relative to the maximum response given by the test ligand. Changes in fluorescence (% response) were plotted against logarithmic C3a concentrations. The half maximal effective concentration (EC_{50}) values were derived from concentration response curves using a nonlinear regression curve in Graphpad Prism v5.

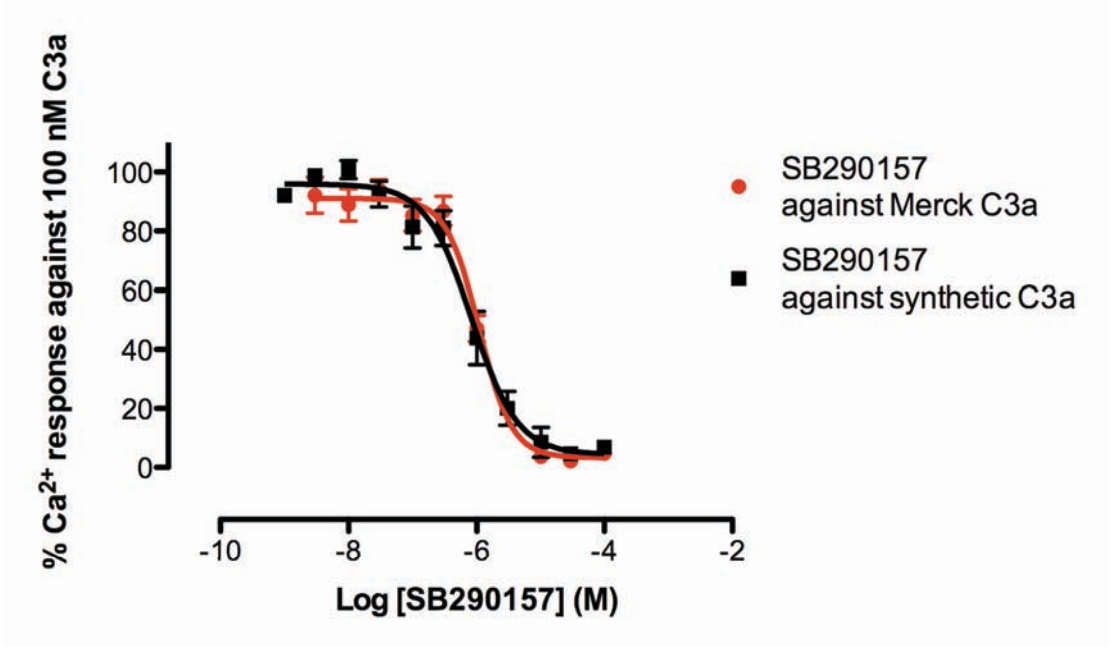

Figure 6: Inhibition of Ca^{2+} release in HMDM by a selective C3a receptor antagonist (SB290157)[16]. The antagonist blocked the calcium response induced by either commercial or synthetic C3a with similar potency (IC_{50} 0.8 ± 0.2 μM (synthetic C3a) and 1.1 ± 0.2 μM (commercial C3a)). Data are means ± SEM of multiple experiments (n = 3 each).

Selectivity assays

To test the selectivity of synthetic C3a, several desensitization assays were carried out. HMDM cells were treated with 300 nM of commercial C3a ligand at t_0 to desensitize the C3a receptor. In the first control assay, 1 μM of commercial C3a was injected at t_{920} (s) to confirm C3aR desensitization (i.e. no calcium release after second injection). Similarly, in the second control assay, 1 μM C5a was injected at t_{920} (s) to show a second calcium response, indicating selective desensitization of C3aR but not C5aR by commercial C3a. In subsequent selectivity assays, 1 μM synthetic C3a was injected at t_{920} (s) and did not induce a second calcium response. Identical results were observed in C3aR desensitization with 300 nM synthetic C3a and injection of 1 μM synthetic C3a at t_{920} (s), indicating selectivity of synthetic C3a to C3aR.

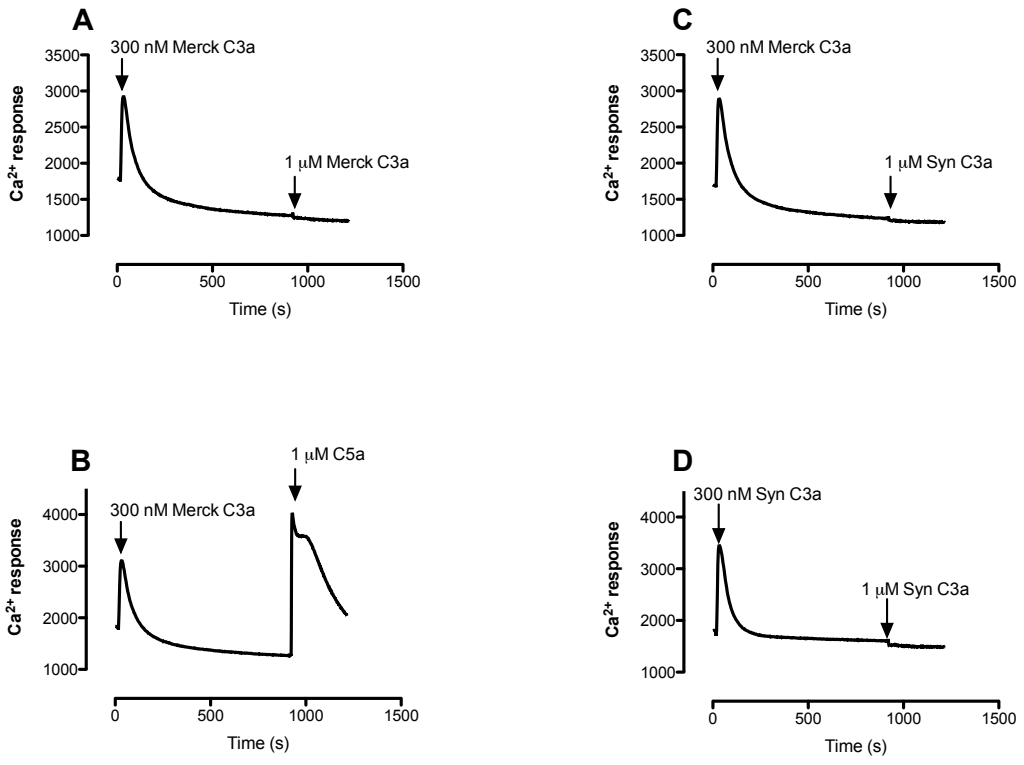

Figure 7: Desensitisation assays for synthetic C3a. (A) HMDM was treated with commercial C3a ligand (300 nM) at t_0 to desensitise the C3a receptor. Commercial C3a at 1 μM was injected at t_{920} (s) to confirm C3aR desensitisation (i.e. no calcium release after 2nd injection). (B) Similar to A, but C5a (1 μM) was

injected at t_{920} (s) showing a second calcium response, indicating that commercial C3a only desensitises the C3a receptor and not the C5a receptor (or other GPCRs). (C) After desensitisation with commerial C3a, the synthetic C3a did not stimulate a second calcium response, suggesting it is selective to C3a. (D) Instead of using commercial C3a for the 1st injection, synthetic C3a (300 nM) was used to desensitise the C3aR. The synthetic C3a has similar effects as the commerical C3a as shown in Fig. A.

[^{125}I]-C3a Radioligand Binding Assay

Receptor binding was performed by ligand competition with 80 pM [^{125}I]-C3a (2200 Ci/mmol; Perkin Elmer, Torrance, CA, USA), HMDM (1.2×10^6 cells/mL), 80 pM [^{125}I]-C3a with/without various concentrations of unlabeled C3a or C3a non-peptidic agonist/antagonist, were mixed with solvent (50 mM Tris, 3 mM MgCl2, 0.1 mM CaCl2, 0.5% (w/v) bovine serum albumin, pH 7.4) for 60 min in a 96-well Nunc round bottom plate at room temperature. Unbound [^{125}I]-C3a was removed by filtration through glass microfiber filter GF/B (Whatman Iner. Ltd, England) which had been soaked in 0.6% polyethyleneimine to reduce non-specific binding. The filter was washed 3 times with cold buffer (50 mM Tris-HCl) pH 7.4 and bound [^{125}I]-C3a was assessed by scintillation counting on a Microbeta counter. Specific [^{125}I]-C3a binding was defined as the difference between total binding and non-specific binding as determined in the presence of 1 μM unlabeled C3a. The IC_{50} value was derived from the concentration of agonist required to inhibit 50% of [^{125}I]-C3a binding.

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