Supplementary Information

Methods

Preparation of human platelet-rich plasma and isolated platelets

The University of Reading Research Ethics Committee approved all the experimental procedures using human blood from healthy volunteers. The blood samples were collected from healthy, aspirin-free volunteers after obtaining written informed consent. The blood was collected into VACUETTE® blood collection tubes containing 3.2% (w/v) sodium citrate. The blood samples were then centrifuged at 102*g* for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). The PRP was rested at 30°C for 30 minutes prior to use. For the preparation of isolated platelets, the blood was mixed with ACD (2.5% sodium citrate, 2% D-glucose and 1.5% citric acid) at 1 (ACD): 9 (blood) ratio and centrifuged at 102*g* for 20 minutes. The PRP was collected, mixed with appropriate volume of ACD, and centrifuged at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM $Na_2HPO_4.12H_2O$, 12mM $NaHCO_3$, 20mM HEPES and 1mM $MgCl_2$, pH 7.3) with appropriate volume of ACD, and centrifuged once again at 1413*g* for 10 minutes at room temperature. The resultant platelet pellet was resuspended to a final density of 4×10^8 cells/mL in modified Tyrode's-HEPES buffer and allowed to rest for 30 minutes at 30ºC prior to use.

Mouse blood collection and platelet preparation

The mouse strains of *Fpr1*$^{-/-1}$ and *Fpr2/3*$^{-/-2}$ on a C57BL/6 background obtained from William Harvey Research Institute, London, UK and wild type C57BL/6 mice from Envigo, UK were used in this study. The mice were sacrificed with CO_2 and the blood was directly collected by cardiac puncture into a syringe containing 3.2% (w/v) sodium citrate at 1 (citrate):9 (blood) ratio. The blood was then centrifuged at 203*g* for 8 minutes at room temperature and the PRP was collected. The remaining blood was resuspended in 500 µL of modified Tyrode's-HEPES buffer and centrifuged once again at 203*g* for 5 minutes. The resultant PRP then centrifuged at 1028*g* for 5 minutes. The resultant platelet pellet was resuspended in modified Tyrode's-HEPES buffer at a density of $2x10^8$ cells/mL.

Enzyme-linked immunosorbent assay (ELISA) for the detection of LL37

To investigate the presence of LL37 in platelets, a direct ELISA was performed using LL37-selective antibody (sc-166770, Santa Cruz Biotechnology, USA). Human isolated platelets were treated with a vehicle (modified Tyrode's-HEPES buffer) or CRP-XL (1 µg/mL) for five minutes under stirring conditions to obtain resting or activated platelets, respectively. The platelets were centrifuged at 1413*g* for 10 minutes at room temperature. The supernatant was collected, stored at -80°C, lyophilised, and resuspended in Nonidet P40 (NP40) buffer. The resultant pellet was also resuspended in NP40 buffer. Briefly, a 96 well plate was coated with 50 µL of various concentrations of LL37 (for the standard curve), or the resting or activated platelet samples (pellets or supernatants), and incubated at 4ºC overnight. The plate was blocked with 150 µL of assay buffer (0.5% BSA in PBS) for one hour at room temperate. Following washing three times with a wash buffer (0.1% Triton X-100 in PBS), 50 µL of anti-LL37 antibody was added and the plate was then incubated for 4 hours at room temperature. Following incubation, the plates were washed with the wash buffer before the addition of goat anti-mouse horseradish peroxidase-conjugated IgG (Life technologies, UK) and incubation for one hour at room temperate. The plates were then washed three times, and 100 µl 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and allowed to incubate at room temperature until the development of a colour. The reaction was stopped by the addition of 100 µL stop solution (0.5M HCl). The level of absorbance was measured at 450nm using an ELISA microplate reader (EMax precision plate reader, Molecular Devices, UK).

Immunofluorescence microscopy

Human platelets were stimulated with modified Tyrode's-HEPES buffer (resting platelets) or 3 µM U46619 (activated platelets) and were left to settle on poly-L-lysine coverslips for 1 hour at 37ºC. The platelets were then fixed with 0.2% formyl saline and then permeablised with 0.2% Triton in PBS. Following washing with PBS, the coverslips were incubated with primary antibodies for LL37 (Novus Biological, UK) and phalloidin (Invitrogen, UK) overnight at 4ºC and washed with PBS prior to staining with Alexa 488 and Alexa 647-conjugated secondary antibodies for phalloidin and LL37, respectively, and incubated for one hour at room temperature in the dark. Coverslips were extensively washed and mounted onto slides. Platelets were imaged with a magnification of 100x using a Nikon A1-R confocal microscope (Nikon Instruments, UK).

In vitro thrombus formation assay

In vitro thrombus formation was performed as described previously[3, 4]. In brief, human $DiOC_6$-labelled (Sigma Aldrich, UK) human whole blood was pre-incubated with a vehicle or different concentrations of LL37 (10, 20 and 50 µM) for 10 minutes before perfusion over collagen (400µg/mL)-coated Vena8™ Biochips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm^2. Z-stack fluorescence images of thrombi were obtained every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The fluorescence intensity was calculated by analysing the data using ImageJ software (National Institutes of Health, USA).

Tail bleeding assay

Tail bleeding assay was performed as described previously[5]. The British Home Office approved the experimental procedures. In brief, C57BL/6 mice (10-12 weeks old; Envigo, UK) were anaesthetised using ketamine (80 mg/kg) and xylazine (5 mg/kg) administered via the intraperitoneal route for 20 minutes before the experiment and were then placed on a heated mat (37°C). Vehicle or LL37 (20 µM) (synthetic; purity: 96.6% from Tocris, UK) was infused via femoral artery 5 minutes before the dissection of 1mm of tail tip, and the tail was immersed in sterile saline. The time to cessation of bleeding was measured and the assay was terminated at 20 minutes.

Platelet aggregation assay

In vitro platelet aggregation assays were performed by optical aggregometry using a two-channel platelet aggregometer (Chrono-Log Corporation, USA). Isolated platelets (270 µL) were added into a siliconised cuvette and pre-warmed at 37°C for 90 seconds. Upon the addition of an agonist, the platelets were allowed to aggregate under continuous stirring at 1200rpm for 5 minutes at 37ºC and the level of aggregation was monitored. To analyse the effects of FPR2/ALX on LL37-induced platelet aggregation, FPR2/ALX-selective inhibitor, WRW_4, used. The platelets were pre-treated with WRW_4 (5 µM) for 5 minutes before the addition of LL37 (20 µM) and the level of aggregation was monitored. Data were analysed by calculating the percentage of maximum platelet aggregation obtained at 5 minutes.

ATP secretion assay

To assess the level of dense granule secretion in platelets, ATP secretion assays were performed using a luciferin–luciferase luminescence substrate by lumi-aggregometry (Chrono-log, USA). The level of ATP released from platelets upon stimulation with a platelet agonist, CRP-XL (0.25 µg/mL), in the presence and absence of different concentrations of WRW_4 was measured by observing the level of luminescence released.

Flow cytometry based assays

In order to measure the level of fibrinogen binding and P-selectin exposure on the platelet surface, flow cytometry based assays were performed[6]. Five microliters of PRP or isolated platelets were incubated with 1 µl of FITC-conjugated fibrinogen antibody (1:50) and 1 µl of $PECy^5$-conjugated anti-CD62P (P-selectin) (1:50) antibody in the presence and absence of various concentrations of LL37 or platelet agonists. The final volume was made up to 50 µl using HEPES-buffered saline (HBS) (150mM NaCl, 5mM KCl, 1mM $MgSO_4.7H_2O$ and 10mM HEPES, pH 7.4) and the samples were incubated for 20 minutes at room temperature. Following fixation in 0.2% formyl saline, the samples were analysed using an Accuri C6 flow cytometer (BD Biosciences, UK) by counting 5000 events within the gated population for platelets. The median fluorescence intensity was calculated using Accuri C6 software to quantify the levels of fibrinogen binding and P-selectin exposure on the surface of platelets. Similarly, for the analysis of FPR2/ALX expression on platelets, five microliters of PRP were incubated with 1 µl of anti-FPR2/ALX (5µg/mL) and 2 µl of Cy5-conjugated anti-mouse IgG (80 µg/mL) with or without 1 µg/mL CRP-XL. Following 20 minutes incubation at room temperature, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry. For the LL37 binding assay, following the incubation of isolated platelets with 5-FAM-LC-conjugated LL37 or 5-FAM-conjugated scrambled LL37 (20 µM) for 20 minutes, the platelets were fixed in 0.2% formyl saline and analysed by flow cytometry.

Intracellular calcium mobilisation assay

PRP (2mL) was mixed with 2 µL of Fluo-4 AM dye (1 µM) (Life technologies, UK) and incubated for 20 minutes at 30ºC in dark. The PRP was then centrifuged at 1413*g* for 10 minutes at 20ºC. The resultant platelet pellet was suspended in 500 µL modified Tyrode's-HEPES buffer and maintained at 30ºC in dark. The platelets were stimulated with different concentrations of LL37 or platelet agonists and the level of fluorescence intensity was measured by FluoStar Optima Spectrofluorimeter (BMG Labtech, Germany) at 37ºC for 180 seconds using an excitation wavelength of 485nm and emission at 510nm. Data were analysed by calculating the percentage of calcium released at 90 seconds.

SDS-PAGE and immunoblotting analysis

Immunoblot analysis was performed using platelet lysates prepared under reducing conditions. The samples were heated to 90°C for 10 minutes and subjected to SDS-PAGE using 10% resolving gels. The gels were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked by incubation in 5% milk in TBS-T (20mM Tris, 140mM NaCl and Tween-20, pH 7.6). Following an overnight incubation with primary anti-FPR2/ALX antibody (1:500) (Abcam, UK), the blots were washed with TBS-T and incubated with secondary Cy5-conjugated goat anti-rabbit IgG antibodies (1:1000) (Invitrogen, UK) in TBS-T containing 5% milk for one hour at room temperature. Following washing in TBS-T for one hour at room temperature, the blots were analysed using a Typhoon 9400 Variable Mode Imager system (GE Healthcare, UK). Equal loading of proteins in each lane was determined using anti-human 14-3-3ζ antibodies (1:1000) (Santa Cruz Biotechnology, USA).

Lactate dehydrogenase (LDH) Cytotoxicity assay

To test whether LL37 has cytotoxic effects on platelets, the level of LDH released from LL37-treated human isolated platelets was determined using LDH Cytotoxicity Assay Kit (Pierce, UK) according to the manufacturer's instructions. In brief, the platelets were incubated at 37°C under 5% CO_2 for 30 minutes. The vehicle or a range of concentrations of LL37 (1 to 100 µM) was added in duplicates to different wells of a 96 well plate and incubated for 10 minutes. One set of wells were treated with the lysis buffer provided from the kit as a positive control for maximum LDH release, and another set was treated with modified Tyrode's-HEPES buffer for the detection of spontaneous LDH release. Results provided represent mean values from duplicate absorbance

measurements, and are given as fractional LDH release compared to the positive control, which yields maximum LDH release (i.e. 100% cytotoxicity).

Cyclic nucleotide assay

A cAMP ELISA kit (Cambridge Bioscience, UK) was used for the detection of the total cellular levels of cAMP in human and mouse platelets. Human isolated platelets were pre-incubated for 10 minutes with a selective inhibitor for FPR2/ALX, WRW_4. Similarly, platelets obtained from control or *Fpr2/3*$^{-/-}$ mice were also used. The platelets were lysed with 0.1M HCl and the levels of cAMP were calculated according to the manufacturer's protocol.

Mass spectrometry analysis for LL37 stability in plasma

Plasma stability of LL37 was determined using human plasma from three individual donors. LL37 was spiked into plasma at a final concentration of 100µg/mL and the solution was subsequently incubated at 37°C for up to 2 hours. Aliquots were removed at 0, 30, 60 and 120 minutes and diluted 1:4 in ice cold methanol. Samples were centrifuged at 2500rpm for 45 min at 4ºC. The supernatant was collected and analysed by mass spectrometry (LC-MS) (Orbitrap, C8 column, Solvent system: 0.1% formic acid in water and 0.1% formic acid in acetonitrile). Stability in plasma was calculated by integrating m/z peak areas of samples using Analyst software (XCalibur, Thermofisher, UK). Similar experiments were also performed in PRP obtained from three separate donors.

Platelet adhesion and spreading on fibrinogen

Isolated human platelets were treated with different concentrations of LL37 or an FPR2/ALX-selective inhibitor, WRW_4, prior to loading onto fibrinogen (100 µg/mL)-coated coverslips and incubation for 30 minutes. The coverslips were then washed with PBS to remove non-adhered platelets. Adhered platelets were fixed with 0.2% formyl saline for 10 minutes prior to permeabilisation with 0.2% Triton X-100 for five minutes at room temperature. Adhered platelets were stained with Alexa 488-conjugated phalloidin for 30 minutes at room temperature. The coverslips were then mounted onto slides and scanned using a Nikon A1-R confocal microscope (60x objective). Ten random fields of view were recorded for each sample. The data were analysed to quantify the number of adhered and spread platelets, and the relative area of spread platelets using ImageJ. The relative surface area of spread platelets was obtained by subtracting the surface area of resting platelets.

Structural modelling and molecular docking analysis

The X-ray crystal structures of FPR2/ALX are not available; hence, homology models of this receptor were developed to determine the plausible interactions between LL37 and this receptor. The amino acid sequence of FPR2/ALX (accession code: P25090) was retrieved from UniProt[7] and submitted to MODELLER-ModWeb server[8] for protein structural modelling. The top three structural models generated were analysed for the sequence similarity, identity and orientation of amino acids. Homology models built on the structural template of human delta opioid 7 transmembrane receptor (PDB code - 4N6H; 1.8 Å) with which FPR2/ALX was found to share 29% identity and was identified as the most appropriate model for FPR2/ALX. This homology model was validated by docking with LL37 using Sybyl-X[9]. The interactions of LL37 with this receptor was identified using PatchDock[10]. The docking results were visualised using the program, PyMOL[11] and the molecular interactions of the docked ligands were analysed by the programme, CONTACTS, as provided in the CCP4 suite of programs[12, 13]. Potential hydrogen bonds were assigned if the distance between two electronegative atoms was less than 3.3Å.

Statistical analysis

Data presented in this study are represented as mean ± SEM. The statistical significance was analysed using two-tailed unpaired Student's *t* test for two-sample comparisons for the data obtained from ELISA for LL37 and quantification, flow cytometric assay for FPR2/ALX expression and platelet receptor characterisation, cAMP assay, LL37 binding assay and platelet aggregometry in the presence of a FPR2/ALX-selective inhibitor. For multiple comparisons, statistical significance was established using one-way or two-way ANOVA followed by Bonferroni's correction for data obtained from *in vitro* thrombus formation, LL37 plasma stability, LL37 binding in mice, ATP release, platelet aggregation and activation, calcium mobilisation, and LDH cytotoxicity assays. Data obtained from the tail bleeding assay were analysed using a non-parametric Mann-Whitney test. All statistical analyses were performed using Graphpad Prism 7 software (GraphPad Software Inc., USA).

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Supplementary Figure 1