SUPPLEMENTAL MATERIAL

Methods

Transcriptomics analysis

Preparation of human cell cultures and blood cells followed by transcriptomics analysis were performed by the Bloodomics Consortium as described previously.[1] Blood was obtained from 7 healthy volunteer donors and the blood cells were isolated using an automated magnetic labelling method as described previously.[1] Briefly, the red blood cells (RBCs) were first removed from the whole blood using gravity sedimentation through HetaSep™ starch solution (StemCell Technologies, Canada) following manufacturer's instructions. The resulted nucleated cell-rich fraction was used to isolate granulocytes by magnetic separation using the CD66 positive selection kit (StemCell Technologies, Canada). $CD4^+$ Th cells were isolated from peripheral blood mononuclear cells after treating the whole blood Human Monocyte Depletion cocktail RosetteSep™ (StemCell Technologies, Canada) following manufacturer's instructions. Similarly, $CD8^+$ Tc cells, $CD14^+$ monocytes, $CD19^+$ B cells and $CD56^+$ natural killer (NK) cells were isolated from peripheral blood mononuclear cells (after removing the platelet-rich plasma) using Histopaque-1077 in an Accuspin tube (Sigma Aldrich, UK). All the cells were isolated using positive selection kits using the markers mentioned above (StemCell Technologies, Canada) to ensure high purity. To generate human megakaryocytes, $CD34^+$ cells were isolated from umbilical cord blood and cultured for 7 days in serum-free medium supplemented with rhTpo and interleukin-1β.[2] Gene expression analysis was performed using Illumina Human WG-6 V2 Expression BeadChips (Illumina Inc, USA) and the data were analysed using Illumina Bead-Studio software after normalising the values to the background signals. The expression levels of connexins in different cell types were clustered using CIMminer software.[3]

Real-time quantitative PCR analysis

Peripheral blood was obtained from normal healthy volunteers with informed consent. Blood was collected by venepuncture, without application of a tourniquet. Platelet activation was inhibited by addition of acid-citrate dextrose (ACD) (100mM disodium citrate, 128mM D-glucose, pH 5), PGE_1 (0.1µM), aspirin (0.3µM), and EDTA (2µM). Platelet rich plasma (PRP) was prepared by centrifugation of anticoagulated blood for 20min at 180g at RT. PRP was incubated with CD45 beads Dynabeads® (Invitrogen, UK) for 15 minutes at RT with rotation to deplete residual leukocytes. Leukocyte-depleted PRP was then incubated with IgG Dynabeads® (Invitrogen, UK) bound with anti-CD42b (BD Pharmingen, UK), for 15 minutes at RT with rotation. Positively-selected platelets were washed in PBS and lysed in Lithium Dodecyl-Sulphate (LiDS) buffer (10mM Tris HCl (pH 7.5); 0.15M LiCl; 1mM EDTA; 0.1% LiDS). The mRNA was directly isolated using oligo(dT) Dynabeads® (Invitrogen, UK), and cDNA synthesis performed using cloned Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Invitrogen, UK) according to the manufacturer's instructions. Real-time PCR was performed using QuantiTect primer assays (Qiagen, UK) with QuantiFast SYBR green master mix (Invitrogen, UK). PCR cycling (ABI PRISM 7900HT Sequence detection System; Applied Biosystems) was performed using the parameters: 5 minutes at 95^{o}C, 2 minutes at 50^{o}C, followed by 40 cycles of 10s at 95^{o}C and 1 minute at 60^{o}C. Melting curves were performed to verify the specificity of the PCR product. Cycle threshold (C_T) values were normalised to expression of β-actin (ACTB).

Platelet preparation, aggregation, dense granule secretion and immunoblotting

Blood was obtained from healthy, aspirin-free, human volunteers with informed consent. Platelets were prepared and re-suspended 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) to the final density of $4x10^8$ cells/ml for aggregation assays as described previously.[4] Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leucocytes were rarely encountered. Total cell contaminant level was <1 per 13000 platelets. Aggregation assays were performed (as described previously[5]) using collagen-related peptide [(CRP-XL), a potent GPVI-selective agonist, from Prof R Farndale (University of Cambridge, UK)], thrombin (Sigma Aldrich, UK), collagen (Nycomed, Austria) or ADP (Sigma Aldrich, UK) in the presence or absence of gap junction blockers such as ^{37,43}Gap27 (SRPTEKTIFII) (a selective mimetic peptide which specifically blocks the extracellular loop region of Cx37 and Cx43), carbenoxolone (a synthetic derivative of glycyrrhetinic acid that non-selectively blocks gap junctions) and 18β-GA (a plant derived non-selective gap junction blocker) (Sigma Aldrich, UK). ^{37,43}Gap27 and carbenoxolone were dissolved in modified tyrodes-HEPES buffer and 18β-GA was dissolved in DMSO. A scrambled peptide (REKIITSFIPT) purchased from Anaspec, USA was used as a control in selected experiments where ^{37,43}Gap27 was used. Similarly, appropriate concentrations of tyrodes or DMSO were used as vehicle controls for carbenoxolone or 18β-GA, respectively. The concentrations of these inhibitors were chosen in the current study based on the concentration ranges used in other cell types previously.[6-8] The final concentration of DMSO used in experiments was 0.01% (v/v) which is well tolerated in platelets.

ATP secretion assays were performed using luciferin-luciferase luminescence substrate (Chrono-log, USA) as described previously.[9] SDS-PAGE and immunoblotting were performed using standard protocols as described previously[10,11] using connexin specific antibodies [rabbit anti-human Cx32, 37 & 43 (In house antibodies,[12,13] data not shown), mouse anti-human Cx43 & 32 (Invitrogen, UK), and rabbit anti-human Cx37 (Epitomics, USA & Invitrogen, UK)]. Rabbit anti-human GAPDH or 14-3-3ζ (Santacruz Biotechnology, USA) was used as a loading control in all the western blots. Human liver and heart lysates were obtained from Abcam, UK and HUVECs and HeLa cell lysates were prepared in house. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against various signalling proteins were obtained from Epitomics, USA. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK.

Mouse blood collection and platelet preparation

The *Cx37*$^{+/+}$ and *Cx37*$^{-/-}$ mice were from the original colony on a C57BL6 background[14] and the genotyping was performed as described previously.[15] Mice were sacrificed and blood was collected immediately by cardiac puncture into a syringe containing ACD (2.5% Sodium citrate, 2% D-glucose and 1.5% citric acid) [at 1 (ACD): 9 (blood) ratio] for aggregation assays. Similarly the blood was collected into syringe containing 4% citrate [at 1 (citrate): 9 (blood) ratio] for flow cytometry and clot retraction assays. The blood was centrifuged at 203g for 8 minutes and PRP collected into fresh tubes. After addition of PGI_2 (12.5ng/ml), the PRP was further centrifuged at 1028g for 5 minutes. The resultant platelet pellet was resuspended in modified tyrodes-HEPES buffer. The cell count was measured using

cellometer Auto T4 (Nexcelom Bioscience, USA). The platelets were rested for 30 minutes before aggregation assays were performed.

Immunohistochemistry

Washed human platelets in suspension ($1x10^7$ cells/ml) were allowed to spread over fibrinogen (100µg/ml) coated cover slips for 1 hour. Unbound platelets were washed and spread platelets were fixed with 3.7% (v/v) formaldehyde in modified Tyrodes-HEPES buffer for 20 minutes. After washing, cells were incubated with rabbit antibody[12] raised against the homologous region which covers the extracellular regions of Cx32, Cx37, Cx40 and Cx43 for 1 hour followed by washing unbound antibodies 5 times with modified Tyrodes-HEPES buffer. The cells were then incubated with Alexa Fluor®-488 labelled goat anti-rabbit IgG (Invitrogen, UK) for 1 hour before washing and imaging using fluorescence microscopy.

Transmission electron microscopy

Human washed platelets were prepared as described previously, aggregated with 1U/ml of thrombin for 90 seconds and pelleted using a micro-centrifuge. After removing the buffer carefully, the platelets were fixed in 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 25mM HEPES (pH 7.3) overnight at 4°C. The platelet pellet was then washed twice with 25mM HEPES before secondary fixation in 1% (v/v) osmium tetroxide for 1 hour at room temperature. The platelet pellet was then washed twice with 25mM HEPES before being dehydrated through a graded acetone series. The dehydrated pellet was embedded in EPON resin according to manufacturer protocols (Agar Scientific, UK). Sections of approximately 90nm thickness were cut with a diamond knife and mounted on

formvar/ carbon TEM grids. Sections were stained with lead citrate for 10 minutes, washed in water, and observed on a Philips CM20 TEM at 80 kV and 25 K true magnification. The imaging medium was Kodak Electron image film, SO-163.

Calcium flux

An equal volume of PRP and Fluo-4 NW dye (Invitrogen, UK) were mixed and incubated for 30 minutes at 37°C. The platelets were then stimulated with different concentrations of CRP-XL in the absence or presence of gap junction blockers ([37,43]Gap27 and carbenoxolone). The intensity of fluorescence was measured at 37°C for 120 seconds using an excitation wavelength of 485nm and emission at 510nm by Fluostar Optima (BMG Labtech, Germany) spectrofluorimeter. Similar experiments were performed in the presence of 1mM EGTA, 10µM indomethacin and 2U/ml apyrase to analyse the effect of gap junctions under the conditions where aggregation was disfavoured. Data were analysed by calculating the percentage of calcium released at 90 seconds.

Flow cytometry

Fibrinogen binding was measured using FITC labelled rabbit anti-human fibrinogen antibody (Dako UK Ltd). PRP was diluted 100-fold using HEPES buffered saline, and this (205µl) was mixed with 5µl of antibodies in the presence or absence of 15µl of gap junction blockers [[37,43]Gap27 (100µg/ml), carbenoxolone or 18β-GA (100µM)] in a micro titre plate. Platelets were then stimulated with 75µl of CRP-XL to the final concentration of 1µg/ml and the data was collected at a medium flow rate (30µl/min) for 5 minutes using accuri® C6 flow

cytometer (BD Biosciences, UK). Data were analysed by calculating the median fluorescence intensity at 90seconds.

For P-selectin exposure, washed human platelets at a density of $5x10^7$ were incubated with PE-CY^{TM}5 labelled mouse anti-human CD62P antibody (BD Biosciences, UK) for 5 minutes before stimulating with different concentrations of CRP-XL in an aggregometer in the presence or absence of gap junction blockers [^{37,43}Gap27 (100µg/ml), carbenoxolone or 18β-GA (100µM)] for 90 seconds. The reactions were stopped by adding double the volume of 0.2% (v/v) formyl saline and the cells were analysed using flow cytometry. A total of 5000 gated events were collected and data was analysed by calculating the median fluorescence intensity (For simple comparison between vehicle and treated samples, data were converted into percentage of vehicle) of gated cells. Similar experiments were performed in the presence of 1mM EGTA, 10µM indomethacin and 2U/ml apyrase to analyse the effect of gap junctions under the conditions where aggregation disfavoured.

For identification of connexin expression on the surface of platelets, PRP diluted 10 fold in HEPES-buffered saline was incubated with various dilutions of rabbit anti-connexin antibody[12] raised against extracellular loop regions and Cy5® labelled goat anti-rabbit IgG antibody in a total of 50µl volume for 30 minutes at RT. The cells were then fixed with 450µl of 0.2% (v/v) formyl saline followed by another 10 fold dilution in 0.2% (v/v) fomyl saline before analysing in the flow cytometer. A total of 5000 gated events were collected and data analysed by calculating the median fluorescence of gated cells.

Clot retraction

Human PRP was obtained as described above and rested at 30°C for 30 minutes. PRP (200µl) was mixed with 5µl of red blood cells and vehicle or gap junction blockers [^{37,43}Gap27 (100µg/ml); 100µM of carbenoxolone or 18β-GA]. The final volume of this mix was made to 1ml with modified Tyrodes-HEPES buffer and incubated for 5 minutes at room temperature. The clot generation was initiated by adding thrombin (1U/ml) and a glass capillary was placed at the centre of the glass test tube, around which the clot formed. Clot weight was measured as a marker for clot retraction at different time points.

***In vitro* thrombus formation**

In vitro thrombus formation was performed as described previously.[9] Briefly, the $DIOC_6$ (Sigma Aldrich, UK) labelled human citrated blood was pre-incubated with vehicle or gap junction blockers [^{37,43}Gap27 (100µg/ml); 100µM of carbenoxolone or 18β-GA] and perfused over a collagen coated Vena8 BioChip (Cellix Ltd, Ireland) at a shear rate of 20 dynes/ cm^2. Z-stack images of thrombi were obtained for every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The fluorescence intensity and thrombus volume were calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

***In vivo* thrombus formation**

In vivo thrombus formation was analysed as described previously.[16] Briefly, C57BL/6 mice were anesthetised by intraperitoneal injection of ketamine (125mg/kg), xylazine (12.5mg/kg)

and atropine (0.25mg/kg) and maintained with 5mg/kg pentobarbital as required through a jugular vein cannula. The control or ^{37,43}Gap27 (100µg/ml of blood) was infused into the mouse circulation 5 minutes before the injury. The cremaster muscle was exteriorized, connective tissue removed, and an incision was made to allow the muscle to be affixed as a single sheet over a glass slide. During preparation and throughout the experiment the muscle preparation was hydrated with buffer (135mM NaCl, 4.7mM KCl, 2.7mM $CaCl_2$, 18mM $NaHCO_3$, pH 7.4). Platelets were labelled with Alexa fluor®488 conjugated anti-mouse GPIbβ antibody (0.2µg/gram of mouse weight) (Emfret Analytics, Germany). The injury on the cremaster arteriole wall was induced with a Micropoint® Ablation Laser Unit (Andor technology plc, UK). Thrombus formation was observed using an Olympus BX61W1 microscope (Olympus Imaging Ltd, UK). Images were captured prior and after the injury by a Hamamatsu digital camera C9300 (Hamamatsu Photonics UK Ltd) charge-coupled device (CCD) camera in 640 x 480 format and analyzed using Slidebook5 software (Intelligent Imaging Innovations, USA).

Figures

Figure S1

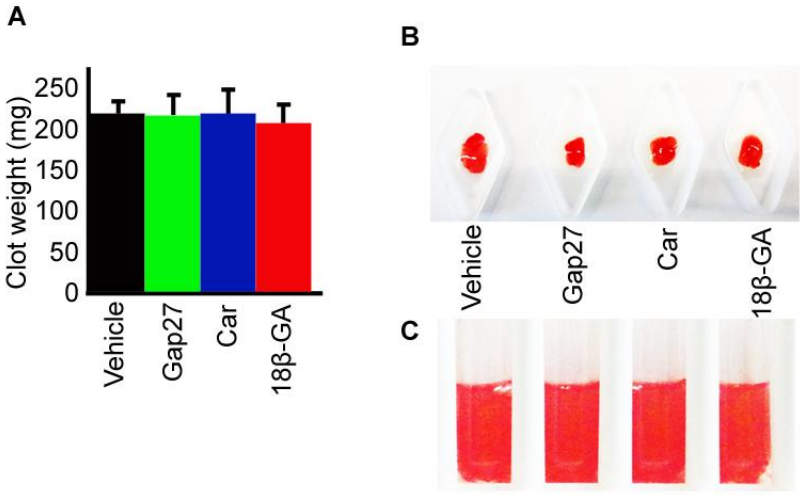

Figure S2

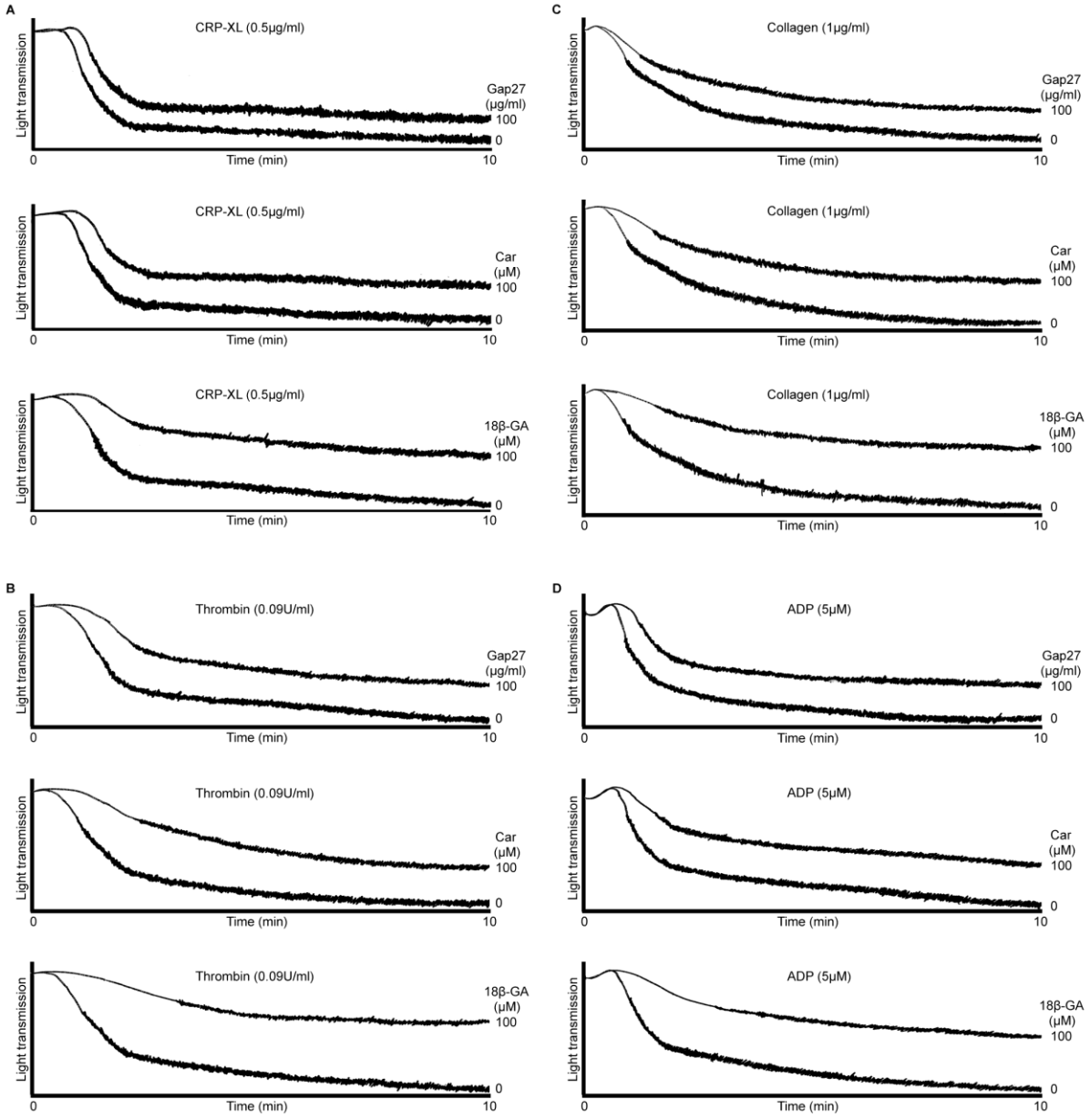
A
CRP-XL (0.5µg/ml)
Gap27 (µg/ml)
100
0
Car (µM)
100
0
18β-GA (µM)
100
0
Light transmission
Time (min)
0
10
C
Collagen (1µg/ml)
Gap27 (µg/ml)
100
0
Car (µM)
100
0
18β-GA (µM)
100
0
B
Thrombin (0.09U/ml)
Gap27 (µg/ml)
100
0
Car (µM)
100
0
18β-GA (µM)
100
0
D
ADP (5µM)
Gap27 (µg/ml)
100
0
Car (µM)
100
0
18β-GA (µM)
100
0

Figure S3

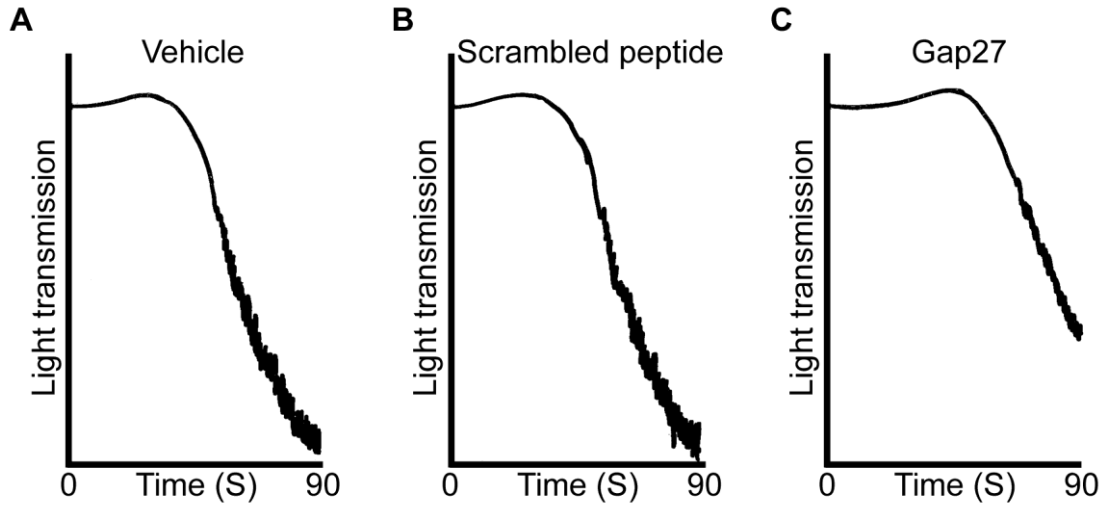
A
Vehicle
Light transmission
0
Time (S)
90
B
Scrambled peptide
Light transmission
0
Time (S)
90
C
Gap27
Light transmission
0
Time (S)
90

Figure S4

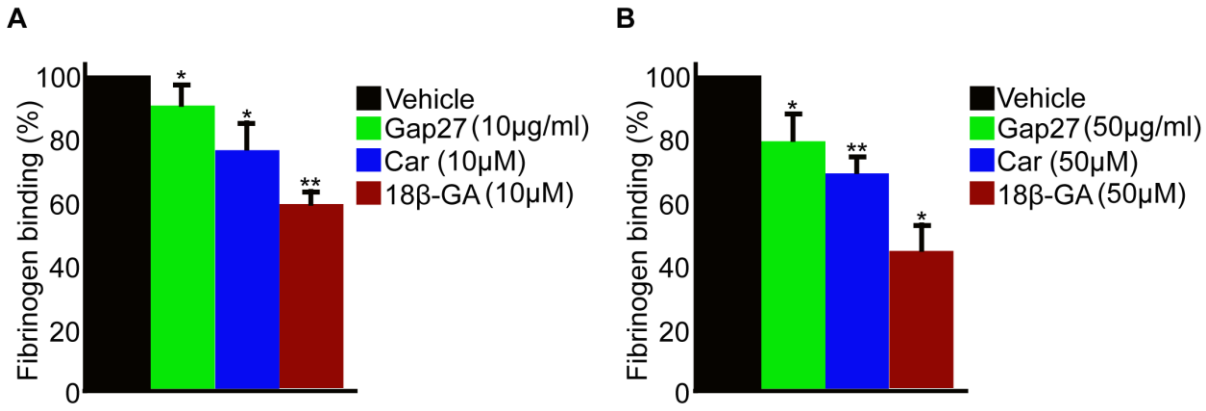
A
Fibrinogen binding (%)
100
80
60
40
20
0
*
*
**
Vehicle
Gap27 (10μg/ml)
Car (10μM)
18β-GA (10μM)
B
Fibrinogen binding (%)
100
80
60
40
20
0
*
**
*
Vehicle
Gap27 (50μg/ml)
Car (50μM)
18β-GA (50μM)

Figure S5

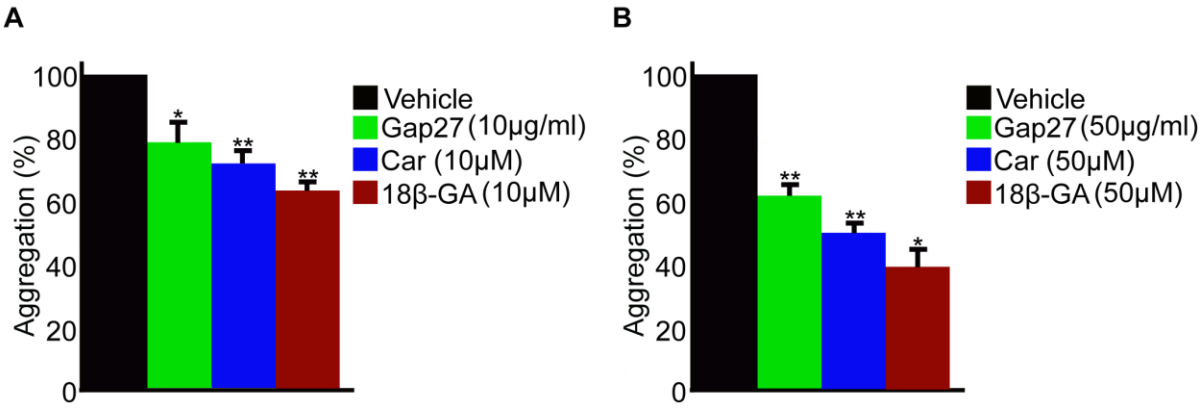
A
100
80
60
40
20
0
Aggregation (%)
*
**
**
Vehicle
Gap27 (10μg/ml)
Car (10μM)
18β-GA (10μM)
B
100
80
60
40
20
0
Aggregation (%)
**
**
*
Vehicle
Gap27 (50μg/ml)
Car (50μM)
18β-GA (50μM)

Figure S6

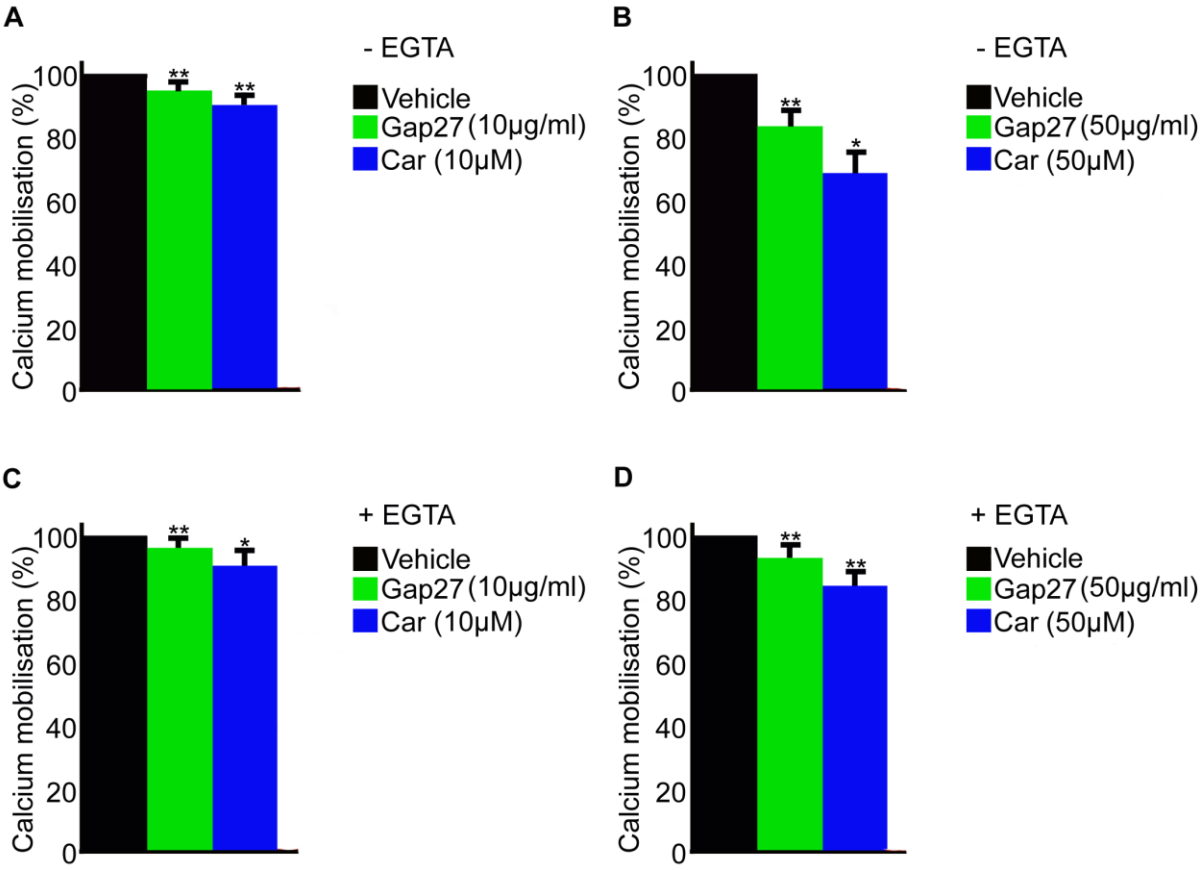
A
Calcium mobilisation (%)
100
80
60
40
20
0
**
**
- EGTA
Vehicle
Gap27 (10µg/ml)
Car (10µM)
B
Calcium mobilisation (%)
100
80
60
40
20
0
**
*
- EGTA
Vehicle
Gap27 (50µg/ml)
Car (50µM)
C
Calcium mobilisation (%)
100
80
60
40
20
0
**
*
+ EGTA
Vehicle
Gap27 (10µg/ml)
Car (10µM)
D
Calcium mobilisation (%)
100
80
60
40
20
0
**
**
+ EGTA
Vehicle
Gap27 (50µg/ml)
Car (50µM)

Figure S7

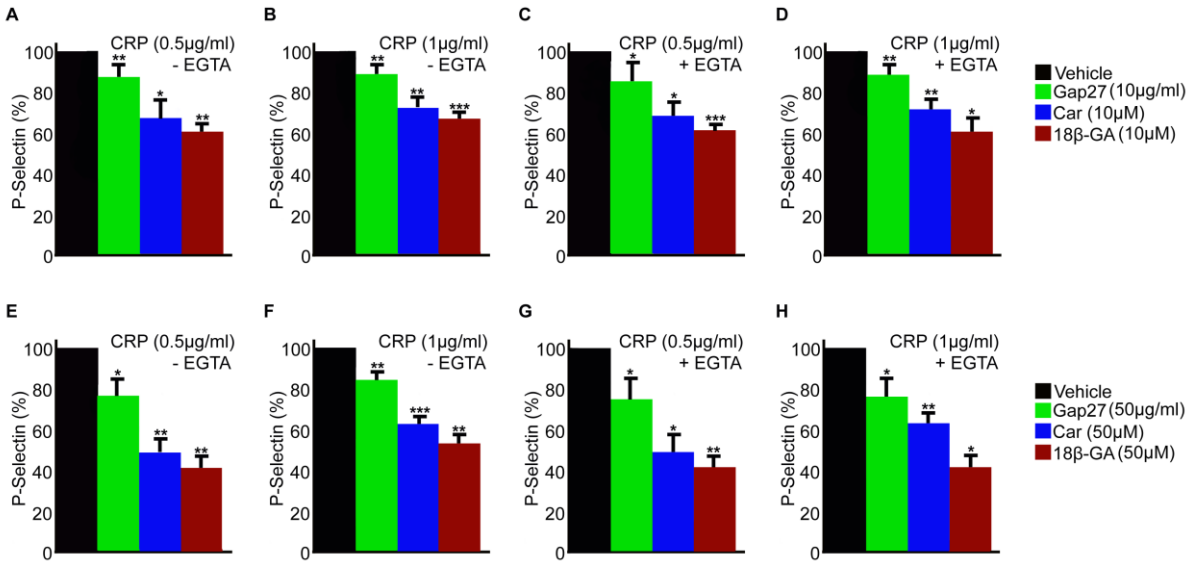
A
CRP (0.5µg/ml)
- EGTA
B
CRP (1µg/ml)
- EGTA
C
CRP (0.5µg/ml)
+ EGTA
D
CRP (1µg/ml)
+ EGTA
P-Selectin (%)
Vehicle
Gap27 (10µg/ml)
Car (10µM)
18β-GA (10µM)
E
CRP (0.5µg/ml)
- EGTA
F
CRP (1µg/ml)
- EGTA
G
CRP (0.5µg/ml)
+ EGTA
H
CRP (1µg/ml)
+ EGTA
Vehicle
Gap27 (50µg/ml)
Car (50µM)
18β-GA (50µM)

Figure S8

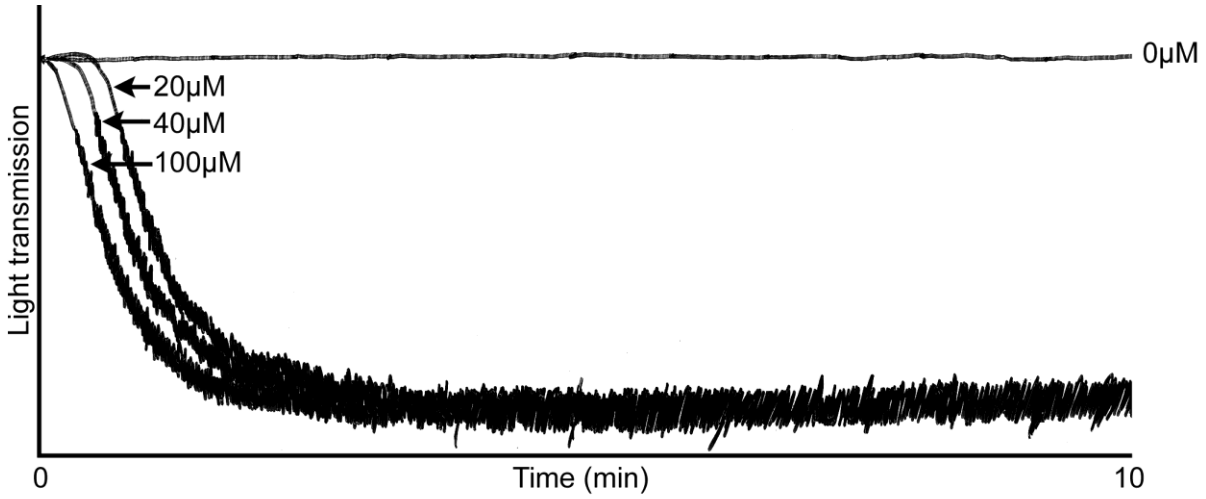

Figure Legends

Figure S1. **Effect of gap junction blockers on initial clot formation. A,** Initial clot weights were measured at 15 minutes after addition of thrombin in the presence or absence of gap junction blockers (100µg/ml ^{37,43}Gap27, 100µM carbenoxolone or 18β-GA). Data represent mean ± S.D (*n*=4). The *p*-values calculated by non-parametric Kruskal-Wallis global statistical method and Dunn's multiple comparison test were >0.05. **B**, representative image of the clots formed in the presence or absence of gap junction blockers. The clots were taken out of the tubes at 15 minutes. **C**, image of clots formed at 15 minutes within the tubes indicating no retraction started at this time point.

Figure S2. **Gap junction blockers inhibit platelet aggregation.** Aggregation performed in the presence or absence of gap junction blockers (100µg/ml ^{37,43}Gap27, 100µM carbenoxolone or 18β-GA) was recorded for 10 minutes following stimulation with 0.5µg/ml CRP-XL (**A**), 0.09U/ml thrombin (**B**), 1µg/ml collagen (**C**) and 5µM ADP (**D**). Traces shown are representative of four separate experiments performed using platelets from four donors.

Figure S3. **Effect of scrambled peptide (control for ^{37,43}Gap27) on platelet aggregation**. Aggregation performed in the absence (**A**) or presence of 100µg/ml scrambled peptide (**B**) or ^{37,43}Gap27 (**C**) was recorded for 90 seconds following stimulation with 0.5µg/ml CRP-XL. Traces shown are representative of four separate experiments performed using platelets from four donors.

Figure S4. **Gap junction blockers affect fibrinogen binding**. The effect of gap junction blockers (**A**, 10µg/ml ^{37,43}Gap27, 10µM carbenoxolone or 18β-GA and **B**, 50µg/ml ^{37,43}Gap27, 50µM carbenoxolone or 18β-GA) on fibrinogen binding was measured by flow cytometry upon stimulation with 1µg/ml CRP-XL. Data represent mean ± S.D (*n*=4). The level of fibrinogen binding obtained with control was taken as 100%. The *p*-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and **p*<0.05, ***p*<0.01 and ****p*<0.001 as calculated by Dunn's multiple comparison test.

Figure S5. **Effect of gap junction blockers on thrombin induced platelet activation**. Aggregation performed in the presence or absence of gap junction blockers (**A**, 10µg/ml ^{37,43}Gap27, 10µM carbenoxolone or 18β-GA and **B**, 50µg/ml ^{37,43}Gap27, 50µM carbenoxolone or 18β-GA) was recorded for 90 seconds following stimulation with thrombin 0.09U/ml. Cumulative data represent mean values ± S.D (*n*=4). The level of aggregation obtained with control was taken as 100%. The *p*-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and **p*<0.05, ***p*<0.01 and ****p*<0.001 as calculated by Dunn's multiple comparison test.

Figure S6. **Effect of connexins in platelet calcium mobilisation**. Calcium mobilisation was measured in Fluo4 NW dye loaded platelets by spectrofluorimetry in the presence or absence of gap junction blockers (**A**, 10µg/ml ^{37,43}Gap27, 10µM carbenoxolone or 18β-GA and **B**, 50µg/ml ^{37,43}Gap27, 50µM carbenoxolone or 18β-GA). Platelets were stimulated with 0.5µg/ml CRP-XL and fluorescence was measured for 90 seconds. Similar experiments were performed in the presence of EGTA (1mM) (**C** and **D**). Data represent mean ± S.D (*n*=4).

The calcium levels obtained at 90 seconds with control was taken as 100%. The *p*-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and *p<0.05, **p<0.01 and ***p<0.001 as calculated by Dunn's multiple comparison test.

Figure S7. **Role of connexins in platelet granule secretion**. Platelets were stimulated with CRP-XL (0.5µg/ml and 1 0.5µg/ml) in the presence or absence of gap junction blockers (**A** & **B**, 10µg/ml ^{37,43}Gap27, 10µM carbenoxolone or 18β-GA and **E** & **F**, 50µg/ml ^{37,43}Gap27, 50µM carbenoxolone or 18β-GA) and the level of P-selectin exposed on surface was measured by flow cytometry. P-selectin exposure was also measured in the presence of EGTA (1mM) (**C** & **D** and **G** & **H**). The level of P-selectin exposure with control was taken as 100%. Data represent mean ± S.D (n=3). The *p*-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and *p<0.05, **p<0.01 and ***p<0.001 as calculated by Dunn's multiple comparison test.

Figure S8. **Effect of 18α-GA on platelet activation**. 18α-GA, a gap junction blocker activates platelets on its own without addition of a platelet agonist. Different concentration of (20µM, 40µM and 100µM) this inhibitor was added to the platelets and the aggregation was monitored for 10 minutes. Indeed, 18α-GA rapidly induces the aggregation of platelets. Traces shown are representative of four separate experiments performed using platelets from four donors.

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