Sequence-specific 2'-*O*-methoxyethyl antisense oligonucleotides activate human platelets through glycoprotein VI, triggering formation of platelet-leukocyte aggregates

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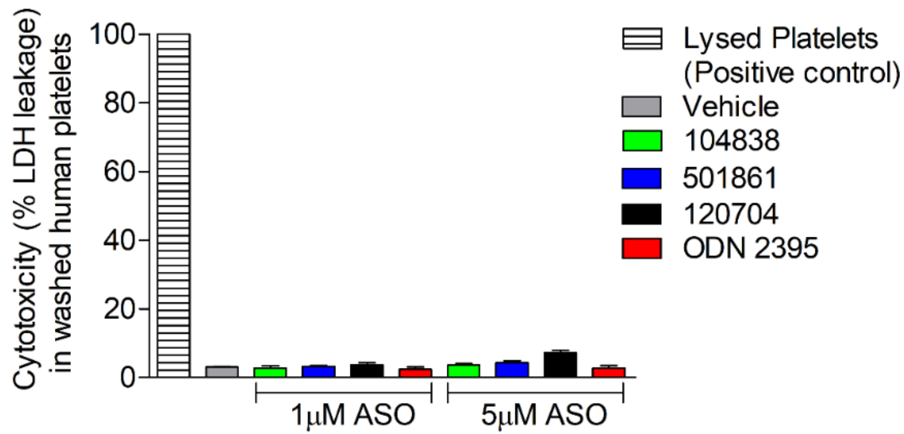
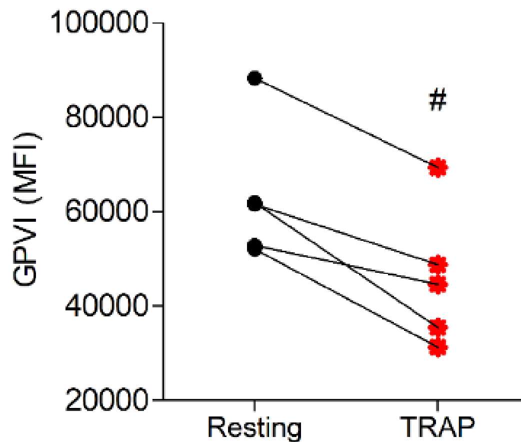
Supplementary Figures

S1



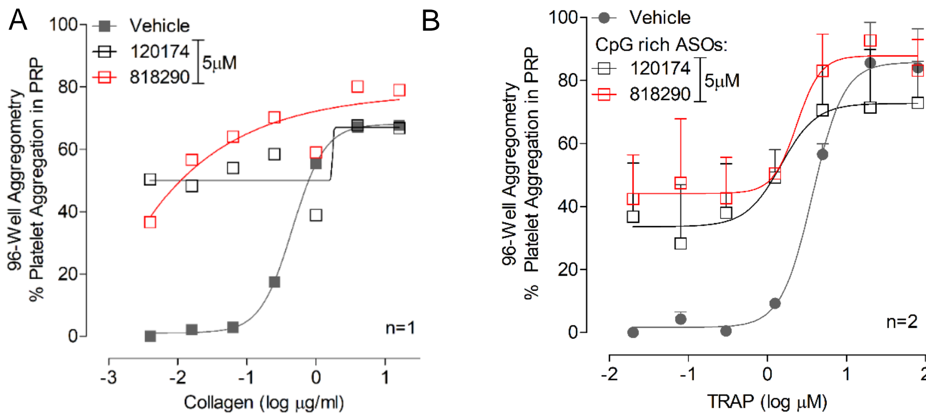
Supplementary Figure 1. Cytotoxicity assay of human platelets. Lactate dehydrogenase (LDH) leakage was measured in washed human platelets (n=3), after treatment with lysis buffer (positive control) or 1 or 5μM of the 2'MOE ASOs 104838 or 501661 or CpG ASOs 120704 or ODN 2395.

S2



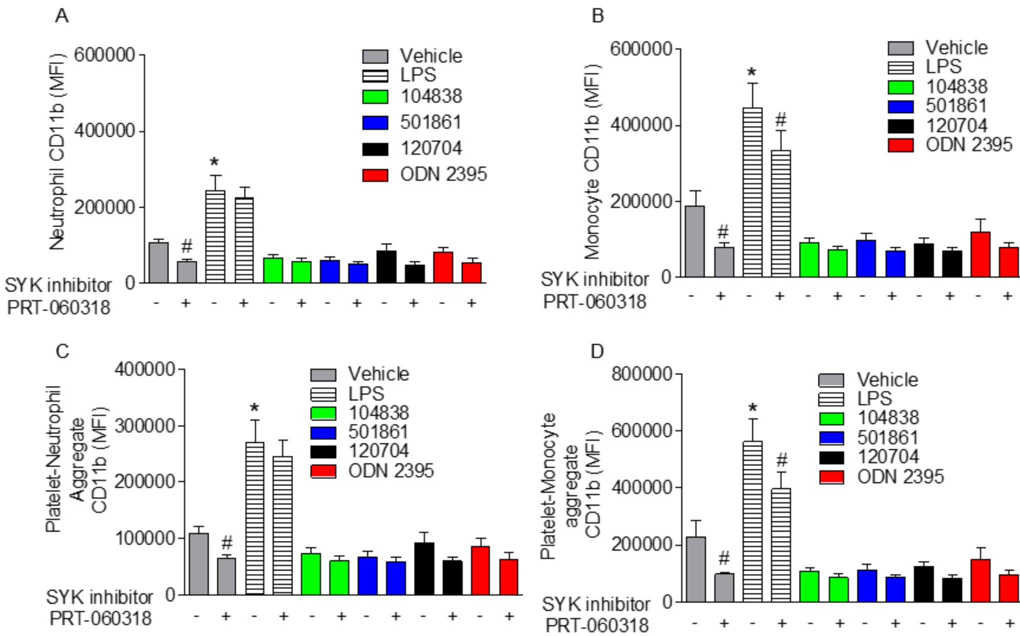
Supplementary Figure 2. **Influence of TRAP stimulation on platelet GPVI levels.** A) Individual donor platelet GPVI receptor levels (median fluorescence intensity, MFI) were identified in resting and the platelet agonist thrombin receptor activating peptide (TRAP, 25μM) stimulated PRP (n=5). # P<0.05 by Student's paired t-test.

S3



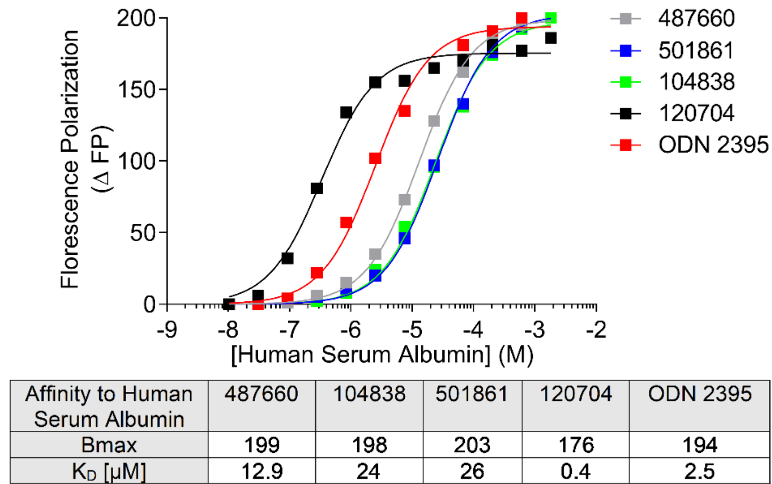
Supplementary Figure 3. CpG ASOs 120704 and ODN 2395 induces spontaneous aggregation in PRP in two donors with high GPVI levels. 96-well platelet aggregometry was used to generate full concentration response curves to A) the platelet agonist collagen and B) the platelet agonist thrombin receptor activating peptide (TRAP) after incubating platelet rich plasma (PRP) with 5µM of the CpG ASOs 120704 or ODN 2395. Spontaneous aggregation was observed in the point furthest to the left which represent unstimulated aggregation in a well that has not been treated with agonist. These two donors had higher platelet GPVI levels compared to other donors tested.

S4



Supplementary Figure 4. Effects of ASOs on neutrophil and monocyte activation. A) Neutrophil (CD66b+) and B) monocyte (CD14+) activation (surface CD11b) after human whole blood was pretreated (for 10 min) with or without a SYK inhibitor (PRT-060318, 10µM) followed by 30 min incubation with either vehicle (10mM HEPES), lipopolysaccharide (LPS 0.01mg/ml), 2'MOE ASOs 104838 or 501661, CpG ASOs 120704 or ODN 2395 (all at 5µM). The extent of neutrophil and monocyte activation was assessed according to CD11b expression (a broad immune cell activation marker). C) Platelet-neutrophil aggregates, (CD41/61+, CD66b+, CD11b+) and D) platelet-monocyte aggregates (CD41/61+, CD14+, CD11b+) were recorded. * P<0.05 compared to Vehicle by One-Way ANOVA, Dunnett's Post-test, # P<0.05 Paired students t-test for the effect of the SYK inhibitor, n=9 human donors.

S5



Affinity to Human Serum Albumin	487660	104838	501861	120704	ODN 2395
Bmax	199	198	203	176	194
K_D [μM]	12.9	24	26	0.4	2.5

Supplementary Figure 5. Binding affinity of ASOs to human serum albumin. A florescence polarization assay was used to measure binding of Alexa647-labeled ASOs to human serum albumin. Bmax is the total density of receptors in a sample and K_D is the equilibrium dissociation constant. The smaller the K_D, the greater binding affinity of the ASO to the human serum albumin.

Supplementary Methods

Human and mouse megakaryocyte cultures and proplatelet analysis

Human cord blood-derived primary CD34+ cells were cultured in serum-free stem cell medium (with 70ng/mL thrombopoietin TPO13), and mature megakaryocytes (MKs) were purified by magnetic bead separation on day 11 as previously described.[1] Mouse MKs were derived from fetal liver cultures extracted from CD-1 pregnant mice at day 13.5 of gestation, as reported previously.[2] Primary mouse fetal liver cells were homogenized and cultured in Dulbecco's Modified Eagle Media (DMEM) in the presence of recombinant murine thrombopoietin (TPO; 70ng/mL). Mature MKs were purified by bovine serum albumin density gradient on day 4. Human-derived, or mouse-derived MKs were plated on a 96-well half area plate (Greiner Bio one 675101), dosed with 5µM of the ASOs and imaged at hourly intervals for 24 hours via IncuCyte Live Cell Analysis System (IncuCyte Zoom). These images were analyzed for the percentage of MKs producing proplatelets, as well as the area containing proplatelets using Ilastik (version 1.3.0) and Cell Profiler (version 3.0.0) as described previously.[3]

Blood collection

Blood was collected via venipuncture into citrated tubes (Sarstedt S-Monovette 02.1067.001) and centrifuged at 177 x g for 20 min to isolate platelet-rich plasma (PRP). PRP or whole blood (WB) was treated with ASO drugs (1µM, 5µM or 10µM, see figure legends), and incubated for 30 min at 37°C. To generate washed platelets, 0.28µM of prostaglandin E_1 (PGE_1, P5515 Sigma) was added to the PRP prior to centrifugation (at 1000 x g for 5 min). The supernatant was aspirated and platelet wash buffer (140 mM NaCl, 5 mM KCL, 12 mM sodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0) was added at an equal volume to original PRP volume. PGE_1 was again added to avoid platelet activation. Platelets were gently resuspended and then centrifuged (at 1000 x g for 5 min). The supernatant was aspirated off and platelets were resuspended in a resuspension buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, pH 7.4) and allowed to rest for at least 30 min before further treatment (to ensure that the inhibitory effect of the PGE_1 had worn off).

Immunogold Electron Microscopy

Electron microscopy imaging, consultation, and services were performed in the Harvard Medical School (HMS) Electron Microscopy Facility. Washed platelet (WP) samples (from n=3 donors) were incubated with 5µM of ASOs or vehicle (10mM HEPES) control for 1 hour at 37°C. Samples were fixed 1:1 by volume with 4% paraformaldehyde in 0.2M Millonig's phosphate buffer and centrifuged (at 1000 x g for 5 min) and were then frozen and sectioned. A rabbit polyclonal anti-ASO was obtained from Ionis to probe the ASO drugs. Samples were labeled with protein A-Gold (British Biocell International). Grids were examined with a G2 Spirit Bio Twin; Tecnai electron microscope at an accelerating voltage of 80kV and 15000x magnification. Images were recorded with a 2k CCD camera (Tecnai G^2 Spirit BioTWIN).

Cytotoxicity LDH leakage assay in human WP

Lactate dehydrogenase (LDH) leakage was measured using the Thermo Scientific Pierce LDH cytotoxicity kit (according to the manufacturer's instructions) in human WP (n=3), after treatment with vehicle or 1µM or 5µM of the 2'MOE ASOs 104838 or 501661 or CpG ASOs 120704 or ODN 2395 for 30min at 37°C. The treated WP were centrifuged at 1000xg for 15 min and supernatants were saved for analysis. The extent of cytotoxicity (LDH leakage) was expressed as the

percentage of total enzyme activity obtained from a control incubation of WP lysed with 0.3% Triton X-100 lysis buffer.

Light transmission aggregometry in PRP

PRP was transferred into the cuvette with a stir bar and treated with vehicle (10mM HEPES), or 5μM ASOs, for 30 min at 37°C, under stirring (1200 rotations per minute, rpm) conditions. Platelet-poor plasma (PPP) was obtained by centrifugation of remaining WB at 15000 x g for 5 min. PPP was used as a control to represent 0% aggregation (100% light transmission). Thrombin Receptor Activating Peptide-6 (25μM TRAP6, Sigma-Aldrich T1573) was added to the four channels, and platelet aggregation traces were recorded for 10 min using a light-transmission aggregometer (Chrono-Log, model 700). Data are presented as % maximum platelet aggregation.

96-well platelet aggregation in PRP

This method was adapted from previous studies.[4, 5] 96-well plates (Greiner Bio one 675101) were coated with 5μL of agonist at increasing concentrations: TRAP-6 (Sigma-Aldrich T1573, 0.08-80μM,) or Type 1 Collagen (Chrono-par NC9533954, 0.016-16μM) and stored at -80°C until use. PRP and PPP were collected as described above. Vehicle or drug-treated PRP (40μL) was added to each well of the agonist coated 96-well plate, and the plate was placed on a BioShake IQ plate shaker at 1200 rpm for 5 min at 37°C. Plates were then analyzed using a plate reader (Biotek) at 595nm absorbance. Percent aggregation was established based on PPP and PRP light absorbance values and concentration-response curves were generated using non-linear regression with a four-parameter variable slope in Graph Prism 5 (Graphpad Software).

Whole blood impedance aggregometry

WB was diluted 1:1 with saline and treated with HEPES (vehicle), 1 or 5μM ASOs, for 30 min at 37°C. TRAP (25μM) induced aggregation and traces were measured for 10 min using a WB lumi-aggregometer (Chrono-Log, model 700). Data are presented as maximum platelet aggregation.

P-selectin surface expression

PRP and WB were treated with vehicle (HEPES,10mM), 5μM ASO treatment or TRAP-6 (25μM) for 30 min at 37°C and then stained with PE anti-human CD62P (Biolegend INC, 304906) in the dark for 20 min at room temperature. Samples were fixed (1:25 BD Biosciences FACS lysing solution, 349202) and incubated in the dark for 10 min at room temperature. Samples were stored at 4°C until flow cytometry analysis was performed on the same day.

Glycoprotein VI surface expression

PRP was generated from blood samples taken on the same day, from the same donors as the P-selectin and platelet-leukocyte flow cytometry experiments, to do a matched correlation analysis. Untreated and TRAP-treated (25μM) PRP were incubated at 37°C for 30 minutes and then stained with GPVI antibody (PE-HY101, 1:100, BD Biosciences, 565241) in the dark for 20 min at room temperature, then fixed (BD Biosciences FACS lysing solution, 349202) in the dark for 10 min at room temperature. Samples were stored at 4°C until flow cytometry analysis was performed on the same day.

Platelet-leukocyte aggregates

WB was pretreated with the SYK inhibitor (PRT-060318, 10μM, Abmole M5252) or vehicle (0.1% DMSO) for 10 min at 37°C, followed by treatment with vehicle (HEPES 10mM), ASOs (5μM), TRAP (25μM), Collagen (20μg/ml) or LPS (0.01mg/ml Sigma-Aldrich, L2630) for 30 min at 37°C. WB was co-stained with anti-human CD41/61-PE (BioLegend 359806), used as a platelet marker, and anti-human CD14-APC antibody (Beckman Coulter IM258OU), used as a monocyte marker, and incubated in the dark for 15 min. When staining was completed, samples were fixed (1:25 BD Biosciences FACS lysing solution, 349202) for 10 min at room temperature in the dark. Fixed samples were stored at 4°C prior to being run on a FACS Canto II flow cytometer. 2,500 CD14+ events were recorded for each sample and platelet-leukocyte aggregates were identified and gated using FlowJo 7.6.5 software.

CD11b and P-selectin expression of platelet leukocyte aggregates

WB samples were treated with 5μM of ASOs and prepared for flow cytometry, as stated above. The treated WB was then stained with a cocktail of antibodies APC anti-human CD14 (monocytes, BioLegend, 367118), Alexa Fluor 594 anti-human CD66b (neutrophils, BioLegend, 305124), PerCP/Cyanine5.5 anti-human CD45 (leukocytes, BioLegend, 368504), PE anti-human CD41/61 (platelets, BioLegend, 359806), FITC anti-human CD62P (platelet activation, BioLegend, 304904), Brilliant Violet 785™ anti-human CD11b (leukocyte activation, BioLegend, 301346) in the dark for 20 minutes at room temperature and fixed (1:25) with (BD Biosciences FACS lysing solution, 349202) in the dark for 10 min at room temperature. Samples were stored at 4°C, until flow cytometry analysis on a Cytek Aurora flow cytometer the same day.

Confocal microscopy of platelet-leukocyte aggregates

WB samples were treated with 5μM of ASOs and prepared for flow cytometry as stated above. Vehicle, TRAP6 (25μM), 104838, 501861, 120704 and ODN 2395 treated samples set aside and stained with CD41/61 (1:25 dilution, human FITC-conjugated) and CD45 (1:25 dilution, Cy5-conjugated anti-human CD45, 2D1 rabbit anti-human CD45 monoclonal antibody D9M8I, Cell signaling technology) for 20 min dark at 200 rotations per minute rotation. 20μL of the stained blood samples were then transferred to chamber wells (Thermoscientific, 155383) and mixed into 380μL of lysis/fixative buffer (BD Biosciences FACS lysing solution, 349202) for 20 min at room temperature before image acquisition the same day by confocal microscopy. Images were acquired at 60x on Yokogawa spinning-disk confocal on an inverted Nikon Ti fluorescence microscope equipped with a Hamamatsu ORCA-R2 cooled CCD camera. Analysis was conducted using ImageJ software.

IL-8, MCP-1 and SDF1α multiplex immunoassay

PRP or WB (180μl in a round bottomed 96-well plate), was treated with 20μl: vehicle (10mM HEPES), LPS (0.01mg/ml), TRAP (25μM), or 1, 5 or 10μM of the ASOs, for 30 min or 6 hours in a cell culture incubator at 5% CO_2, 37°C. After the 30 min or 6-hour incubation, the 96-well plates were centrifuged at 1000 x g for 15 min and 110μl plasma per well was saved and stored at -80°C until multiplex immunoassay analysis. Interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) or stromal cell derived factor 1α (SDF1α) were measured concomitantly on the same 96-well plate according to the manufacturer's instructions (Mesoscale discovery, U-PLEX Biomarker Group 1 human, 3 Assay plate) and read using a MESO QuickPlex SQ 120 instrument.

Fluorescence Polarization GPVI binding assay

Fluorescence polarization experiments were performed according to a method published previously.[6, 7] Alexa647-labeled ASOs were synthesized at Integrated DNA Technologies. The assay was performed in phosphate buffered saline (PBS) at pH 7.4, in black flat-bottomed non-binding 96-well plates (Costar). Binding of ASOs to recombinant human GPVI protein (R&D Systems, 3627-GP) was evaluated by adding the Alexa647-labeled ASOs to yield 2nM concentration to each well containing 100µL of human GPVI protein from sub nM to low µM concentration. Binding of labeled ASO to Human Serum Albumin (Calbiochem, 126654) was also included as a control experiment. Readings were taken using a Tecan InfiniteM1000 Pro instrument (λ ex =635 nm, λ em =675 nm). Using polarized excitation and emission filters, the instrument measures fluorescence perpendicular to the excitation plane (the 'P-channel') and fluorescence that is parallel to the excitation plane (the 'S-channel'), and then it calculates FP in millipolarization units (mP) as follows: $mP = [(S – P * G) / (S + P * G)] * 1000$. The 'G-factor' is measured by the instrument as a correction for any bias toward the P channel. Polarization values of each Alexa647-labeled ASO in PBS at 2nM concentration were subtracted from each measurement. The equilibrium dissociation constant (K_D) values were calculated with GraphPad Prism 5 software using non-linear regression for curve fit assuming one binding site. The smaller the K_D, the greater binding affinity of the ASO to the human GPVI (or the human serum albumin control).

Statistics

Statistical analysis was performed using GraphPad Prism 5 and was analyzed using One-Way ANOVA, Two-Way ANOVA, Pearson's Correlation analysis or student's t-test as appropriate and indicated in each figure legend. Data are reported as mean ± standard error of the mean (SEM).

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