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***In Vitro* Methods to Characterize the Effects of Tobacco and Nontobacco Products on Human Platelet Function**

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Abstract

In this document, we describe methods for the isolation, treatment, and functional testing of human blood platelets *in vitro*. Functional assays for inflammatory function include flow cytometry and immunoassays for platelet release of platelet factor 4, soluble CD40L, prostaglandin E₂ and thromboxane. Assays for platelet hemostatic function described here examine platelet spreading, aggregation using platelet-rich plasma, and thromboelastography. Also described here are methods for testing cigarette smoke on primary human platelets *in vitro*, which our lab developed to address a major knowledge gap regarding how cigarette smoke dysregulates platelets and the contribution of this platelet dysregulation to cardiovascular disease. Some of these protocols may be useful and be repurposed for investigation of the toxicity potential of other tobacco products and environmental insults.

Keywords

Platelets; inflammation; tobacco; toxicology testing; cardiovascular; biomarker

Introduction

Platelets are the second most numerous cell in the blood, second only to RBCs, and are responsive to an array of stimuli including hemostatic and inflammatory agents. Platelet function studies are traditionally used as measures of immune dysfunction or inflammation with regard to cardiovascular disease and thrombosis. For example, platelets are the major source of soluble CD40 ligand (CD154, sCD40L), which is accepted as a biomarker for inflammation and thrombosis (*i.e.* thrombus stability). Unlike many primary cells, platelets are easily isolated from the blood and are amenable to several tests for function. Platelets are

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also well-suited for use in high throughput studies to discern effects of singular, as well as combined, treatments or toxic insults. As platelets are a highly understudied cell and adaptable to multiple types of function testing, they are ideal cells to use for drug and toxicological testing. Herein, we describe methods of platelet isolation, treatment, and assays to determine inflammatory and hemostatic function.

Basic Protocol 1: Human platelet isolation and preparation

Platelets are highly suited for both *in vitro* and *ex vivo* research. Platelets can be isolated from human donors via venipuncture to collect whole blood. Differential centrifugation steps yield platelet rich plasma (PRP), platelet poor plasma (PPP), and a platelet pellet. These various derived fractions can then be used for a variety of hemostatic measures and assays to investigate platelet function. Key to the success of these types of analyses is maintaining platelets in a quiescent, unactivated form throughout the isolation process. In this protocol, the steps for platelet isolation and precautions for preventing platelet activation are described.

Donor considerations

All human subjects must give their informed consent to participate in a study, which must first be approved by an appropriate institutional review board. It is the investigators' responsibility to inform the subject of what the study involves, potential side effects, and answer any questions posed by the donor. Additionally, donor confidentiality must be maintained.

Donors must qualify for the study parameters defined by investigators and their institution. These criteria will need to be determined by the investigators and the appropriate review boards at their institutions.

Some studies may require the use of blood from donors whose blood contains known infectious agents. While human blood should always be handled as if it contains infectious agents, additional protective measures may be required. We cannot advise on these precautions and urge investigators to consult with the appropriate review board and safety committee at their institution.

Notes for the handling of platelets

Platelets must be maintained at room temperature (RT) following venipuncture and during subsequent centrifugation procedures. Gentle handling and pipetting is also a requirement. The choice of anticoagulant is an important consideration in platelet studies. For the studies described in the following protocols, it is important to use sodium citrate or acid citrate dextrose (ACD) with addition of prostacyclin I_2 (PGI_2), an endogenous negative regulator of platelet activation, at indicated steps to help to maintain platelet quiescence during isolation.

Reagents and Materials

- Phlebotomy supplies:
 - 18–21-gauge needle (BD Vacutainer Cat # 367281)

- Alcohol wipes (Fisherbrand Cat. # 06-669-62)
- Gauze (VWR Cat. # 93000-314)
- Self-adherent bandages or other bandages (Hartmann Cat. # 25100000)
- Vacutainer one use holder (BD Vacutainer Cat. # 364815)
- EDTA vacutainer tubes, for complete blood counts (BD Cat. # 367841)
- Sodium Citrate vacutainer tubes (BD 369714; Fisher Cat. # 02-688-26)
- ACD (Acid Citrate Dextrose) vacutainer tubes (BD Cat. # 364606)
- Prostacyclin (PGI₂) (Cayman # 18220), stock solution 1 mg/ml in sterile filtered TRIS-Buffer, stored as 20–30 µL aliquots, –80°C for up to 6 month
- Tyrode's Salt solution (TSS), stored at RT (Sigma Cat. # T2397)
- ACD (acid-citrate-dextrose) solution pH = 7.3, sterile filtered and stored at RT
 - 85 mM Sodium Citrate (+ Na₃ • 2H₂O), 111mM D-glucose, 71 mM Citric acid (monohydrate)
- Wide-bore transfer pipettes or P1000 pipette tips (LPS Cat. # L322201 or VWR Cat. #89049-166)
- P200 and P1000 Pipetman
- 15 and 50 mL sterile, disposable conical tubes (VWR Cat. # 82050-276 and Cat. # 82050-346)

Equipment

- Blood Cell Counter, we use a Sysmex automated cell counter
 - (Hemocytometer or Flow cytometer can be substituted)
- Centrifuges, Eppendorf and Swinging bucket style
- Vacuum Aspirator

Protocol Steps

1. Collect whole blood from consenting donor via venipuncture into vacutainer tubes. Each ACD tube will result in collection of 2–3 mLs of platelet rich plasma (donor dependent). It is recommended that at least 2 tubes be collected and PRP pooled, more depending on the amount of platelets required. It is expected to obtain 2×10^8 platelets per mL of PRP after washing.
 - a. The first tube used in collection is not used for platelet isolation, as the venipuncture may cause some platelet activation. As such, the first tube can be an EDTA vacutainer tube that can also be used for cell counts.
 - b. The rest of the whole blood should be collected in sodium citrate or ACD vacutainer tubes. Gently mix tubes at RT via inversion or slowly

rock on a platform rocker to ensure adequate mixing of the blood and anticoagulant.

2. Complete blood cell counts can be obtained with the EDTA tube, using an automated blood counter or hemocytometer.
3. Centrifuge whole blood at $250 \times g$ for 15 min at RT, with moderate acceleration and minimal braking.

Slow acceleration and braking helps prevent unwanted platelet activation and results in better definition of the different blood layers (see Figure 1). Moderate acceleration for a Sorvall Legend X1R centrifuge is 5, and minimal braking is 0 or 1.

4. After centrifugation, remove caps from tubes, being careful not to disrupt the blood layers. Slowly collect the top 2/3rd of the platelet rich plasma (PRP) using a wide-bore pipette, being careful not to disturb the buffy coat layer. Pool PRP into 15 or 50 mL conical tubes, ensuring a slow pipetting technique (see Figure 1).

When possible, all PRP should be pooled together to produce a homogeneous mixture for use in downstream isolations and assays.

5. Add 1 $\mu\text{g/mL}$ of freshly thawed PGI_2 solution to the pooled PRP and mix by gentle inversion. Thaw the PGI_2 briefly at RT just prior to addition, as it is short-lived (12-min half-life).

PGI_2 is a platelet antagonist and is used here to prevent unwanted platelet activation during centrifugation and washing steps. As it is short lived, PGI_2 will not affect platelet activity after isolation, making it an ideal antagonist for this application.

6. To isolate the platelet pellet, centrifuge the PRP for 10 min at $1,000 \times g$, with full acceleration and moderate braking.
7. During the centrifugation step, prepare 28 mL of wash buffer for every 25–40 mL of pooled PRP. Platelet wash buffer contains 3 mL of ACD solution and 25 mL TSS. It is important that both solutions are at RT.
8. Thaw and add 0.1 $\mu\text{g/mL}$ PGI_2 to the prepared wash buffer immediately before use.

Thaw a new aliquot of PGI_2 just prior to use.

9. Carefully remove platelet-poor plasma (PPP) using a vacuum aspirator without disturbing the platelet pellet.

Tilting the conical at a 90 degree angle so it is parallel with the ground and placing the aspirating pipette where the slope of the tube changes approximately 0.5 inches from the pellet will ensure all liquid is removed, without disrupting the fragile platelet pellet.

10. Immediately add ~5mL of PGI₂ containing wash buffer to each platelet pellet slowly, using a wide bore pipette, down the side. Do not dislodge the pellet. Let sit for 5 min.
11. Platelet resuspension: Only the top 2/3rd of the platelet pellet should be resuspended to avoid collection of contaminating cells (leukocytes, red blood cells), which reside under the platelet pellet due to their higher density. Platelets must be very carefully resuspended in buffer to prevent activation. Using a wide bore pipette, withdraw 2–3 mL of wash buffer from the tube containing the platelet pellet, and slowly dispense the buffer back down the side of the tube to resuspend platelets.
- Platelets being resuspended will look like smoke as they are released from the pellet. Platelets should float away from the top layer individually, not in chunks. Any white blood cells (WBCs) and red blood cells (RBCs) present will be on the bottom of the pellet and should be avoided.
12. Repeat the pipetting in step 11 until the solution is cloudy, then transfer most of the solution (washed platelet solution) into a separate 50 mL conical tube, leaving some solution on the platelet pellet to prevent it from drying.
13. Add 10 mL of fresh wash buffer to the platelet pellet as before, slowly with a wide bore pipette. Repeat the washing steps in 11 and 12. Continue resuspending platelets, transferring buffer, and adding fresh buffer until you have used all of the wash buffer.
- The platelet washing step is done in a stepwise fashion, only using a portion of the wash buffer at a time, so that if the red blood cell layer below the platelets is disturbed, the remaining contaminated platelet suspension can be discarded, and only the prior removals of pure platelets are used.
- a. Negative bead selection (CD45) can be performed at this step to ensure that the platelets are free of contaminating white blood cells (See **Alternate and/or Support protocols**).
14. Centrifuge the washed platelet solution at $1,000 \times g$ for 10 min with moderate acceleration and full brake.
15. Carefully aspirate the wash buffer without disturbing the platelet pellet.
16. Resuspend the top 2/3rd of the platelet pellet in a total of 5 mL of TSS, 1 mL at a time, as before. Move washed platelets to a clean tube.
- This step is prone to the most activation as there is no PGI₂ during this step. Thus, it is especially important to be careful during this step.
17. Count platelets using an Automated Blood Counter, hemocytometer, or flow cytometer. Count an undiluted sample as well as a 1:1 dilution in TSS. Dilute

platelets in TSS to a final concentration of 1.1×10^8 platelets / mL or other appropriate dilution, as indicated for specific assays.

Washed platelets can be used for *in vitro* assays detailed in this document.

Basic Protocol 2: High Throughput Screening (HTS) of platelet function *in vitro*

To screen compounds for effects on platelets, a high-throughput format is advantageous. In the method described here, platelets from one individual are treated in 96-well plates, enabling efficient screening of different compounds at varying concentrations on cells isolated from the same donor, limiting variability between samples. While HTS is advantageous in that more compounds and doses can be used on cells from the same donor, each sample uses a reduced volume compared to a low-throughput method; the maximum volume per sample in a 96-well plate is 350 μ L. There are times when higher volumes studies are necessary and traditional testing (*i.e.* in Eppendorf tubes) may be more practical. These methods are also described below. When deciding whether to test platelets in a traditional or high-throughput manner, volume required per sample should be considered in addition to time constraints, as pipetting in a 96 well plate will be significantly faster than in tubes.

Reagents and Materials

- Washed platelets (Basic Protocol 1) – 35 mL of 1.1×10^8 /mL per 96 treatments
- Sealable adhesive film for microplates (VWR Cat. # 60941-062)
- Conical 96-well plates for treating platelets (plates may be polystyrene or polypropylene, we use ThermoScientific Cat. # 249944)
- U-bottom 96-well plates for -20°C storage (plates may be (polystyrene or polypropylene, we use VWR Cat. # 82050-633)
- 96-well plate compatible with LSRII HTS system (refer to manufacturer's instructions)
- Wide-orifice pipette tips for P1000 and P200 (VWR Cat. #89049-166 and Cat. # 53503-610)
- Multichannel pipetman capable of dispensing 50-1000 μ L
- P1000 pipetman
- P200 pipetman
- TSS, stored at RT (Sigma Cat. # T2397)
- Refer to protocols on platelet spreading and flow cytometry for required reagents for those assays

Equipment

- 37°C incubator

- LSRII with HTS capabilities

Protocol Steps

1. Design layout for 96-well plate treatments. Label the microplate lid with designations, and ensure the lid is marked to match the plate when running more than one plate to ensure no contamination occurs. We recommend three replicates per treatment.
2. Pipette at least 5 μL of the desired treatment or vehicle into the appropriate wells of a labeled conical 96-well plate.
3. Gently mix the $1.1 \times 10^8/\text{mL}$ diluted washed platelets by inversion, then carefully pour into a reagent reservoir 30 mL of $1.1 \times 10^8/\text{mL}$ washed platelets for each 96-wells needed for treatment.
4. Pipette 300 μL of $1.1 \times 10^8/\text{mL}$ of platelets into each well of the conical 96-well plate using a multichannel pipettor fitted with wide-orifice pipette tips. Pipette up and down gently 3 times upon addition of platelets.

Alternatively, Seal the 96-well plates with adhesive film. It is critical to ensure a good seal. Mix the platelets with the reagents by slowly inverting the 96-well plates 3 times each.

5. Place the plates in a 37°C incubator, and incubate for 30 min or other desired amount of time.

We suggest 30 min such that the platelets have time to respond, but also so that further assays can be performed and completed within 5 hr of the blood draw. It is also suggested that for each treatment a time course be completed, to determine the peak response time.

6. After incubation, carefully remove the seal and dispense the desired amount of platelets for the following assays:
 - a. Flow cytometry:
 - i. Prepare a separate conical 96-well plate with 90 μL TSS and 100 μL of 2 % Paraformaldehyde (PFA) for a total of 1% PFA in each well.
 - ii. Using a multichannel pipette fitted with wide-orifice tips, remove 10 μL from each well into the plate prepared above with fix and TSS. Ensure platelets are mixed first, as they will settle to the bottom over time.
 - iii. Incubate at RT for 15 min.
 - iv. After fixing, add 150 μL of TSSs. Centrifuge at $1,200 \times g$ for 10 min at RT.
 - v. Remove the solution by gently “flicking” the plate into an appropriate sink or disposal container.

This step should be done gently, with the plate held in the palm of the hand and a gentle flick of the wrist down into the sink, resulting in removal of solution out of the plate. The platelet pellet will not be visible, so it is critical to be careful not to dislodge it.

- vi. Resuspend the platelet pellet in Fc Block + PAB. Block for 15 min.
- vii. Stain with desired antibody. The amount of antibody used must be determined for each individual antibody to ensure saturation. Incubate in dark at RT.
- viii. Move 100 μ L of fixed, blocked, and stained platelets into a 96-well plate appropriate for use in the LSRII HTS system (as per manufacturer's recommendation).

b. Platelet spreading:

- i. Using a P1000 fitted with a wide-orifice pipette tip, add 150 μ L of 1.1×10^8 /mL platelets to 300 μ L of TSS or media in a separate 96-well plate.
- ii. After moving all samples, seal the plate and mix gently by inversion as before.
- iii. Add 300 μ L of these platelets to prepared coverslips, and perform assay as described in **Basic Protocol 4**.

7. Centrifuge the remaining volume in the plate at 1,200 $\times$ g for 10 min at RT.

8. Remove platelet supernatant to a new, labeled 96-well plate. Place on ice or freeze at -20°C immediately.

Platelet supernatants can be used to determine platelet secretion, as described in **Basic Protocol 4**.

9. If desired, lyse the platelet pellet with 50 μ L of preferred lysing buffer. Place on ice or freeze at -20°C immediately.

Platelet lysate can be used to determine protein concentration and western blot.

10. Store the supernatants and lysates at -20°C or below. Limit freeze-thaw cycles.

Basic Protocol 3: Cigarette Smoke Extract (CSE) treatment of platelets

Cigarette smoke is reported to contain in excess of 4000 compounds (Borgerding & Klus, 2005). Cigarette smoke extract is a solution generated from the soluble components of main-stream and side-stream cigarette smoke, using reference cigarettes (Kentucky Tobacco Research & Development Center), which serve as an international standard for cigarette research. Two methods of CSE generation are described in **Supporting Protocol 1**. Platelets can be treated with CSE in a standard or high-throughput manner (**Basic Protocol 2**),

depending on the needs of the experiment. Here, we describe the standard treatment of platelets with CSE.

Reagents and Materials

- CSE (**Supporting Protocol 1**), made same day
- Washed human platelets (**Basic Protocol 1**)
- Eppendorf tubes
- P10, P200, P1000 pipetman
- Pipetman tips for P10, P200, P1000 (wide-orifice and standard)

Equipment

- Incubator at 37°C

Protocol Steps

1. Dilute washed platelets to 1.1×10^8 platelets/mL. Keep at RT.
2. Make dilutions of CSE such that 5 μ L of the dilution added to the final volume of platelets to be tested will result in the desired concentration to be tested.
3. Using the P10, pipette 5 μ L of the appropriate concentration of CSE into the corresponding Eppendorf tube.

To ensure accurate pipetting, wipe the pipette tip with a Kim wipe prior to expressing the CSE. Be careful not to touch the Kim wipe to the opening of the pipette tip, as the CSE will be drawn onto the Kim wipe, making the pipetted volume inaccurate.

4. Before moving to the Eppendorf tubes, mix the platelet solution gently by inversion to ensure a homogenous population of platelets are added to each treatment.
5. Using a P1000 fitted with wide-orifice tips, move the desired volume of platelets to each Eppendorf tube. Mix by gently pipetting twice.
6. Incubate the treated platelets for the desired time.

It is advisable to perform a time-course to ensure the platelet response is accurately captured.

7. After incubation, platelets and platelet supernatants may be assayed using the methods described below.

Basic Protocol 4: Platelet supernatant immunoassays

Platelets are sentinel cells in the blood and contain molecules that enable them to respond to hemostatic or inflammatory insults. In response to insult (*e.g.* pathogens) or injury (via exposure of the extracellular matrix), platelets are activated and release alpha-granules, which contain pre-made inflammatory molecules including sCD40L and Platelet Factor 4

(PF4); platelets also synthesize inflammatory lipid mediators, including thromboxane A₂ (TXA₂) and prostaglandin E₂ (PGE₂). These inflammatory mediators are present in the serum *in vivo* and act on platelets, as well as endothelial cells, monocytes, and neutrophils. These inflammatory molecules are easily detected in platelet supernatant *in vitro* via immunoassay. Concentrations of PF4, PGE₂, and thromboxane B₂ (TXB₂) (a stable metabolite of TXA₂) were detected using commercially available immunoassays. For these assays, we briefly discuss steps we take for assay optimization. The assay for sCD40L was developed by our lab and will be described in detail.

Reagents, Materials, and Equipment common to all immunoassays

- Immulon-4 microtiter 96-well plates (Dynex Technologies, Inc., Chantilly, VA, Cat.#: 3855)
- Plate washer
- Multichannel pipetman
- Reagent reservoirs (LPS Cat. # M100520)
- Microplate reader capable of reading wavelengths at 610nm, 540nm, 450nm, and 405nm

Thromboxane and PGE₂ immunoassays—We detect TXB₂ and PGE₂ using a modified version of commercially available immunoassays; we purchase the individual components of this kit and prepare the ELISA plates, as follows:

Reagents and Materials

- **EIA Wash buffer:** LPS-free water, 10 mM Potassium Phosphate, 0.05% Tween 20
- **EIA Buffer:** 100mM Potassium phosphate (pH 7.4), 0.1% BSA, 400 mM Sodium Chloride, 1.0 mM EDTA, 0.01% Sodium Azide.
- **PGE₂ plate coating antibody solution:** 2 µg/mL in 1× PBS of Goat anti-Mouse IgG antibody (Sigma Cat. # M-2650, RRID:AB_260486)
- **TXB₂ plate coating antibody solution:** 2 µg/mL in 1× PBS of Goat anti-Rabbit IgG (Fc Region Specific – Human Serum Protein Adsorbed) (Jackson ImmunoResearch Laboratories Cat.#: 111-005-046, RRID:AB_2337917)
- **Saturation buffer:** 1 X EIA Buffer, 0.2% BSA, 0.02% Sodium Azide
- **PGE₂ kit:** Cayman chemical (Ann Arbor, MI), Cat. # 514010
 If preparing the plates as described, only the PGE₂ monoclonal antibody (Cayman Cat. # 414013, RRID:AB_327973), PGE₂ AChE Tracer (Cayman Cat. # 414010), PGE₂ standard (Cayman Cat. # 414014), and Ellman's reagent (Cayman Cat. # 400050) must be purchased
- **TXB₂ kit:** Cayman Chemical (Ann Arbor, MI), Cat. #: 501020

If preparing the plates as described, only the TXB₂ antiserum (Cayman Cat. # 401022), TXB₂ AChE Tracer (Cayman Cat. # 401020), TXB₂ standard (Cayman Cat. # 419034), and Ellman's reagent (Cayman Cat. # 400050) must be purchased

Plate preparation steps

1. Coat a 96 well Immulon-4 microtiter plate with 200 μ L per well of Plate Coating Antibody Solution. Cover with parafilm and store overnight at 4°C without shaking. Alternatively, the solution may be applied for 3 hr at RT. Washing is not required before the next step.
2. Block non-specific binding by adding 100 μ L per well of Saturation Buffer. Cover with parafilm and store overnight at 4°C. Alternatively, the solution may be applied for 2 hr at RT. Coated and blocked plates may be stored at 4°C for up to one week.
3. Immediately before use, wash plates with EIA wash buffer 3 times. After last wash, ensure all EIA wash buffer is removed from wells.

Do not allow the plates to “dry out”, add sample immediately after washing plates. In order to facilitate this, we suggest preparing the samples and required reagents before washing the plates.

4. Refer to manufacturer's protocol for instructions for sample preparation, the addition of PGE₂ or TXB₂ antibody and tracer, and how to read and interpret the plates.

Platelet Factor 4 (PF4) immunoassay—PF4 ELISA was performed using a PF4 ELISA (R&D systems, Cat. #DPF40), as per manufacturer's protocols. PF4 is present in high concentrations in washed platelet supernatants. We have found that diluting samples from 1:1000 to 1:10,000 results in an absorbance reading within the range of the standard curve. We suggest creating a series of dilutions and freezing the more concentrated dilutions, such that if the samples are too dilute, the more concentrated dilutions are available for testing.

sCD40L Immunoassay—sCD40L can be detected using any number of commercially available ELISA kits. Here, we describe our method for sCD40L detection. We find that diluting samples 1:5 is usually within the generated standard curves.

Reagents and Materials

- Mouse-anti-human sCD40L antibody (clone MK13A4 at 3 μ g/mL; ThermoFisher Cat. # BMS153; RRID: AB_10597130)
- Blocking buffer (1 $\times$ PBS, 1% BSA, 0.1% NaN₃)
- Washing buffer (1 $\times$ PBS, 0.05% Tween 20, 0.1% NaN₃)
- Recombinant human sCD40L standard (Bender Med Systems Cat. # BMS239S)

- Biotinylated mouse-anti-human sCD40L antibody (Ancell Cat. # 353-030)
- Streptavidin-alkaline phosphatase (BioRad Cat. # 170-3554)
- Phosphatase substrate kit (BioRad Cat. # 172-1063)

Protocol Steps

1. Coat 96-well plates with 100 μ L mouse anti-human sCD40L antibody (3 μ g/mL in PBS).
2. Incubate plates overnight at RT.
3. Add 200 μ L of blocking buffer to each well, incubate for 1 hr at RT.
4. While the plates are blocking, prepare a dilution plate of the standards and samples. Prepare 1 dilution plate for each ELISA plate to be run; these should be identical, as the samples will be moved directly from the dilution plate to the ELISA plate. The diluent for standards and samples is blocking buffer.
 - a. Standard dilutions: The standard for this ELISA is recombinant human sCD40L (rhCD40L).
 - i. Create two identical standard curves with 7 or 11 points, using a 1:2 dilution series (standard: total volume). Dilute with blocking buffer. The highest dilution will be 400 pg/mL rhCD40L.
 - ii. Fill the last wells with blocking buffer; these will be used to determine background signal when reading the plates.
 - iii. Each standard will require 100 μ L plated onto the ELISA. For each standard curve, start with 250 μ L of 400 pg/mL standard. Dilute 125 μ L of 400 pg/mL recombinant human sCD40L into 125 μ L pf blocking buffer. Repeat down to the last dilution.
 - b. Sample Dilutions: Start with a dilution of 1:5 (sample: total volume) in blocking buffer.
 - i. Each sample will require 100 μ L/well for the ELISA plate, aim to dilute 120 μ L of sample for each of three technical replicates.
5. Wash plates 3 times with wash buffer (using a plate washer, aspirating after each wash, or manually by “flicking” the wash buffer into the sink after each wash and blotting on bench paper after the last wash).
 - a. Leave the wash buffer from the last wash in the wells until the dilution plates are complete. This is to limit the possibility of the ELISA plates ‘drying out’.
6. After the last wash and aspiration, move 100 μ L of sample or standard from the dilution plates to the appropriate location in the ELISA plates. Incubate for 2 hr at RT.

7. Wash plates 3 times with wash buffer.
8. Add 100 μ L/well of 2 μ g/mL biotinylated mouse-anti-human sCD40L antibody in blocking buffer and incubate at RT for 2 hrs.
9. Wash plates 3 times with wash buffer.
10. Add 100 μ L of Streptavidin alkaline phosphatase to each well. Incubate for 20 min.
11. Wash plates 3 times with wash buffer.
12. Add 100 μ L/well of pNPP substrate solution (prepared as per manufacturer instructions).
13. Keep the developing plates in the dark or cover with aluminum foil.
14. Read plates (absorbance at 405nm, reference at 610nm), subtracting the reference wavelength.
 - a. Read the plates every 5–10 min for the first 30 min, to ensure that plates do not develop too quickly (changing to a bright yellow) and that the highest standard is not overdeveloped.
15. Plot the standard curve as a scatterplot using excel or other appropriate program.
16. Calculate the equation of the line from the plotted data using the computer program.
17. Using this equation, calculate the concentration of sCD40L in each sample.

Basic Protocol 5: Platelet spreading assay

Platelet spreading is a surrogate measure of platelet hemostatic function in regard to vascular injury and wound healing. Our protocol is an adaptation from McCarty *et al.*, 2005. Resting platelets have a discoid morphology, and do not bind other platelets or cells. Platelets are activated upon exposure to agonists, including ADP, thrombin, thromboxane, and extracellular matrix components. This activation elicits major cytoskeletal rearrangements, resulting in shape change; platelets round up, then extend filopodia and lamellipodia, spreading into a thin, flat disk. To measure the ability of platelets to spread under different conditions, platelets should be treated with CSE just prior to the spreading assay (see **Supporting Protocol 1**) and applied to prepared glass coverslips coated with fibrinogen. The extent of spreading is monitored and quantified visually by microscopy.

Reagents and Materials

- Washed human platelets (**Basic Protocol 1**)
- 1 $\times$ Phosphate buffered Saline (PBS; ThermoFisher Cat. # 14200-075)
- Fibrinogen (Sigma Cat. # F-3879)
 - Stock solution: Dilute to 2.5 mg/ml in PBS. Store at -80°C for up to 6 months.

- Dilute to 250 µg/mL in PBS for use (prepare fresh)
- Bovine Serum Albumin (BSA) (Sigma Cat. # A4503) for blocking coverslips
 - 0.5% in PBS, prepared fresh
- Paraformaldehyde (PFA) – diluted to 4%
- Fluoromount G (Southern Biotech Cat. # 0100-01)
- Microscope lens immersion oil (VWR Cat. # 48218-061)
- Wide-bore pipettes (LPS Cat. # L322201)
- Wide bore pipet tips for Pipetman
- 24-well tissue culture dish (VWR Cat. # 82050-892)
- 12 mm round #1 microscope cover glass (Carolina Biologicals, Deckgläser Cat. No 1001/12)
- 70% ethanol (EtOH)
- Superfrost slides (VWR Cat. # 48311-703)
- Wooden toothpicks

Equipment

- Vacuum aspirator
- Incubator at 37°C
- 4°C storage
- Forceps, fine-pointed
- Microscope with differential interference contrast capabilities (DIC), and 40 and 100× powered lens. (Olympus BX51 light microscope (Olympus, Melville, NY))
- Camera attached to microscope (SPOT Pursuit digital camera)
- Computer capable of running the appropriate imaging software (see manufacturer's suggestions)
- Microscope Imaging Software (SPOT RT software (New Hyde Park, NY))

Protocol Steps

1. Prepare coated coverslips in advance of performing the platelet spreading assay.
 - a. Using a 24 well cell culture plate, carefully place 1 cover glass in each well (or as many wells as needed), using forceps. If possible, plan to do each treatment type in duplicate (2 cover glass/per treatment).
 - b. Wash glass coverslips with 1× PBS; fill each well with 1× PBS, then remove PBS wash by vacuum aspiration. Repeat 3 times. Ensure that coverslips are completely dry before moving to step 1c.

This step is to clean off any debris from the coverslips.

Coverslips should be flush at the bottom of the well.

- c. Using a P200 pipetman fitted with a wide bore tip, carefully apply 50 μL of 250 $\mu\text{g/mL}$ fibrinogen to the center of each cover glass. Do **not** spread the drop to the edges.

If coverslips are still wet, the fibrinogen will wick under them.

- d. Carefully cover the plate with a microplate lid and seal the plate by wrapping it with parafilm. Place on a level, horizontal surface and incubate at 4°C overnight (preferable) or at RT for approximately 2 hrs. Take care not to disturb the drops.
- e. After incubation, wash the cover glasses as before, ensuring the coverslip does not flip. Repeat 3 times.
- f. To block coverslips, add 300 μL of freshly prepared block solution (0.5% BSA in PBS) to each well. Cover and incubate at RT for 45 min.
- g. Aspirate the block solution and wash coverslips 3 times with 1× PBS. Take care not to touch the surface of the cover glass.
- h. Add 500 μL of 1× PBS to each well and cover and wrap in parafilm. Leave the plate at RT while platelets are prepared (not longer than 2 hrs) or coated cover glass can be stored for up to 24 hrs at 4°C; allow plates to equilibrate to RT before performing the spreading assay.
- i. Prepare platelets:
 - i. Isolate and wash platelets as described in Basic Protocol 1, and dilute platelets to $1.1 \times 10^8/\text{mL}$ in TSS or media.
 - ii. Treat platelets with CSE (**Basic Protocol 2**) or other desired solutions for up to 1 hr at 37°C.
 - iii. Using wide-bore pipette tips, dilute platelets to $3.3 \times 10^7/\text{mL}$ in TSS or media, for a total volume of at least 650 μL .

2. Application of treated and control platelets to cover glass.

- a. Design a layout of platelet placements in the 24 well plate.
- b. Aspirate PBS from cover glass.
- c. Using a P1000 pipetman fitted with a wide-bore tip, gently mix the platelet suspensions via pipetting then slowly add 300–500 μL of platelet suspensions to each well (as indicated by the template). Take care not to disturb or scratch the cover glass.
- d. Cover and carefully place the plate at 37°C for 45 min.
- e. After incubation, carefully remove the platelet suspension by vacuum aspiration and gently wash the coverslips with 300 – 500 μL of 1× PBS for a total of 2 washes.

- f. Following the last wash, carefully add 300–500 µL of freshly prepared 4% PFA to each well to fix the platelets. Incubate at RT for 15 min, undisturbed.
 - g. Aspirate the 4% PFA and wash one time with 1×PBS.
 - h. Fill each well with 1 × PBS.
3. Mounting the cover glass for microscopy.
 - a. Prepare microscope slides during the last incubation.
 - b. Label each slide (each slide can accommodate two to three cover glasses). Wipe clean the slide with 70% EtOH and allow to dry.
 - c. Place 1–3 small drops of Fluoromount G using a small pipet tip or toothpick.
 - d. Using a toothpick and forceps, carefully lift the cover glass. Grasp the edge with the forceps. Place the cover glass coated side down on a drop of Fluoromount on the labeled slide.

Take care not to drop the cover glass, as it is difficult to determine which side of the glass is the coated or to break the glass. This is why it is recommended to have a duplicate.
 - e. Allow the Fluoromount G and cover glass to set for at least 1 hr at RT or overnight at 4°C. Once set, the cover glass can be secured to the slide with clear nail polish on the edge.
4. Microscopy and Quantification
 - a. Set up the microscope for differential interference contrast (DIC). This will be dependent on the microscope being used.
 - b. At 40× magnification, confirm that platelet distribution is homogenous on the cover slip. The edges of the coverslip may be uneven, as fibrinogen may not have coated evenly. In this case, confirm that the center of the coverslip is covered evenly with platelets.
 - c. Using 60× or 100× magnification, take pictures of at least 5 separate fields of view. It is suggested that these fields be close to the middle of the coverslip, as the edges are more likely to be unevenly coated with fibrinogen.
 - d. Image enhancement:
 - i. Images may be edited to increase contrast, enabling visualization of the platelets.
 - ii. Make copies of original images, making note in the file name that these are edited. All original files of unedited images should be retained.
 - iii. Using Spot software, edit image and adjust the histogram.

- e. Spreading can be quantified by counting fully spread platelets in 5 separate fields of view or choosing to count different spreading categories, such as unspread (round), partially spread (filopodia present), or fully spread (flat, lamellapodia present) (Figure 2).

Basic Protocol 6: Flow cytometry assay for platelet surface markers

Platelets contain the marker CD62P (P-selectin) within the membrane of alpha granules. Upon activation, platelets release alpha granules, whose membranes fuse with the platelet membrane, increasing CD62P on the platelet surface. Platelet activation and alpha-granule release is often characterized by CD62P surface expression. Flow cytometry is an efficient, accurate method to measure platelet surface CD62P. See Figure 4 for gating schematic. As we use washed platelets, the flow gating is fairly simple.

Reagents and Materials

- FC Block (Fisher-Miltenyi Biotec Cat.# 130-059-901)
- Tyrode's salt solution (TSS)
- Paraformaldehyde (PFA) – 2% in PBS
- Mega Mix Sizing Beads 3.0, 0.9, and 0.5 μm (we use BioCytex Cat. # 7801)
- Antibodies to CD62p or other desired markers
 - We use mouse-anti human CD62P conjugated to AF647 (Biolegend Cat. # 304918; RRID: AB_2185110)
 - We use Invitrogen Cat. # MG121 for the isotype control
- Eppendorf tubes (1.5 mL)
- PAB (1 $\times$ PBS, 1% BSA, 0.1% Sodium Azide, 0.2 μm sterile filtered), stored at RT

Equipment

- BD Accuri or other flow cytometer
- Vacuum aspirator
- Flow cytometry analysis software (we use FlowJo)

Protocol steps

1. Prepare Fc receptor block according to manufacturer protocols, in PAB.
2. Isolate and prepare washed human platelets as in **Basic Protocol 1**.
3. After fixing, add 1 mL TSS to each tube, Centrifuge at 1200 $\times$ g for 10 min to remove fix.
4. Aspirate fix carefully.
5. Resuspend platelets 200 μL FC receptor block prepared above.

6. Incubate at RT in dark for 15 min.
7. Separate fixed platelets into two 100 μ L aliquots.
Treat fixed platelets with either the desired antibody or isotype control mix, and incubate for 15 min in dark.
In our assays, we add 5 μ L of the antibody or isotype control.
8. Run flow on at medium flow rate, 30 μ L per sample.
9. Run PAB and Mega Mix sizing beads as controls.
Use sizing beads that include 1 μ m and 3 μ m.
10. Save FCS file.

Basic Protocol 7: Flow cytometry assay for platelet monocyte complexes

This procedure describes a method for the identification and enumeration of platelet monocyte complex formation from peripheral whole blood using differential staining and flow cytometry. This protocol is an adaptation of Singh *et al* (Singh, Davidson, Kiebalá, & Maggirwar, 2012).

Reagents and Materials

- Sodium Citrate vacutainer tubes (BD Cat. # 369714; Fisher Cat. # 02-688-26)
- 4% Paraformaldehyde in PBS
- 2% BSA in PBS
- PAB (1 $\times$ PBS, 1% BSA, 0.1% Sodium Azide, 0.2 μ m sterile filtered), stored at RT
- ACK RBC Lysis Solution
- Anti-human CD14 PE (BDB Cat. # 555398; RRID:AB_395799)
- Anti-human CD16 PE/Cy7 (Biolegend Cat. # 302016; RRID:AB_314216)
- Anti-human CD41 AF647 (Biolegend Cat. # 303726; RRID:AB_2566537)
- Anti-human CD62P FITC (BD Cat. # B555523; RRID:AB_395909)
- BD Anti-mouse Ig Positive and Negative Compensation Beads (BD Biosciences Cat. # 552843)
- 1.5 and 2 mL microfuge tubes
- Sterile, disposable serological pipets

Equipment

- BD Accuri C6 flow cytometer, or equivalent instrument
- Refrigerator for reagent storage

- –20°C Freezer, for reagent storage
- Ducted Biosafety cabinet
- Pipet aid, such as Drummond
- Pipetman, or equivalent pipettor and filter tips
- Igloo cooler for sample transport
- Vacuum aspirator
- High Speed Microfuge Centrifuge, such as Beckman Coulter Microfuge 20 with fixed angle rotor
- Mini vortexer

Protocol Steps

- 1** Be sure all reagents and centrifuges are at RT before beginning. Collect 1 sodium citrate vacutainer tube (2 mL) and keep at RT.
- 2** Remove 500 μ L of whole blood from the sodium citrate vacutainer tube and place in a 1.5 mL microfuge tube, containing 500 μ L of 4% PFA/PBS.
- 3** Incubate at RT for 15 min.
- 4** Divide into 3 $\times$ 200 μ L aliquots and add 1 mL of 2% BSA/PBS to each tube (Label #1, 2 & 3).
- 5** Centrifuge at 350 $\times$ g for 6 min., discard BSA/PBS. **If platelet analysis separate from the PMC analysis is desired, increase the centrifugation to 1000 $\times$ g to retain the platelets.*
- 6** Resuspend each pellet in 1 mL of ACK solution. Incubate at RT in the dark for 10 min.
- 7** Centrifuge at 350 $\times$ g (*1000 $\times$ g) at RT for 6 min and discard ACK solution.
- 8** Resuspend each pellet in 1 mL 2% BSA to wash. Centrifuge at 350 $\times$ g (*1000 $\times$ g) at RT for 6 min.
- 9** Discard wash and resuspend each pellet in 50 μ L BSA/PBS for antibody staining.
- 10** To prepare for staining, vortex positive and negative comp beads and mix 4 drops of each in a microfuge tube.
- 11** Divide mixture between 4 tubes labeled Comp 1–4.
- 12** Add the following antibodies to the resuspended cells and Comp tubes:
 - i.** Tube 1: unstained control
 - ii.** Tube 2: 10 μ L CD14 PE; 5 μ L CD16 PE Cy7
 - iii.** Tube 3: 10 μ L CD14 PE; 5 μ L CD16 PE Cy7; 5 μ L CD41 AF647; 20 μ L CD62P FITC

- iv. Comp 1: 10 μ L CD14 PE
 - v. Comp 2: 5 μ L CD16 PE Cy7
 - vi. Comp 3: 5 μ L CD41 AF647
 - vii. Comp 4: 20 μ L CD62P FITC
- 13 Mix well and incubate all the tubes in the dark at RT for 25–30 min.
 - 14 Add 1 mL of PAB to each tube and centrifuge all tubes at 1000 $\times$ g for 6 min.
 - 15 Resuspend each pellet in 300 μ L PAB.
 - 16 Place tubes on ice and keep dark until acquisition on Accuri.

Acquisition on Accuri

- 17 Follow the start-up guidelines for the Accuri C6 or cytometer being used
- 18 Following the recommendations for the Accuri or other cytometer, begin compensation procedures. Vortex sample briefly, then run without limits and establish a gate for beads. Stop acquiring, delete events and set instrument to count 50,000 events in the established gate. Repeat for remaining compensation controls and “Save File”.
- 19 Set gates for WBC acquisition. Acquire 50,000 events at slow speed with FSC-H threshold at 160,000.
- 20 Vortex each sample briefly just prior to measurement and acquire samples 1–3. “Save File”
- 21 *If analyzing platelets, set the platelet gate and acquire 100,000 events at slow speed with FSC-H threshold at 20,000 and SSC-H at 12,000 and repeat acquisition with samples 1–3. Save File.
- 22 Analyze using Flow Jo or other appropriate software.

Basic Protocol 8: Aggregometry assay

Aggregometry is a measure of platelet hemostatic function in response to an agonist. It is a measure that is used clinically to measure how well platelets “stick together” or aggregate. For aggregometry, platelet aggregation is initiated by the addition of an agonist (*i.e.* arachidonic acid, thrombin, ADP, epinephrine, collagen) to whole blood or PRP. Dynamic platelet clumping in response to the agonist is monitored over time. It is advised to measure aggregation in response to several different agonists (separately), as each agonist binds different receptors; compounds may alter one pathway but not another. Testing different agonists creates a more comprehensive view of platelet function.

In this protocol we discuss the use of a chemiluminescent reagent, Chrono-lume, which reacts with ATP, releasing light. The aggregometer records the emission, and using an ATP standard the amount of ATP released may be calculated. In the use of toxicological

screening, it is important to determine if ATP release is altered by treatment, though the exact concentrations may not need to be calculated.

This protocol describes aggregation studies using PRP and a Chrono-log series 560VS Aggregometer with a Chrono-log model 810 Aggrolink. If using other types of aggregometers, some of the following may provide insights on setup and process.

Reagents and Materials

- ChronoLume reagent (ChronoLog Corp Cat. # 395)
- ADP (ChronoLog Corp Cat. # 384)
- ATP Standard (ChronoLog Corp Cat. # 387)
- Disposable siliconized magnetic stir bars (ChronoLog Corp Cat# 311)
- Aggregation cuvettes (ChronoLog Corp Cat# 312)
- Pipetman (various volumes)

Equipment

- Chronolog Lumi-Aggregometer
- Aggro-link
- Computer capable of running the AGRO/LINK software (see manufacturer's suggestions)
- Microfuge Centrifuge
- Centrifuge, such as Beckman AllegraX-15

Protocol Steps

Sample Preparation

- 1 Bring ATP standard and luminescence reagents to RT. **Adding cold reagent to platelets will activate the platelets**
- 2 Process whole blood as described in Basic Protocol 1, steps 3 and 4 only (Do not add PGI₂).
- 3 Count the platelets in PRP using the same method as in **Basic Protocol 1**, then adjust with PPP to a set concentration. Prepare PPP by centrifuging PRP at 1,200×g for 10 min at RT.
- 4 Set aside 3 mL of PRP, for use in setting the baseline of the aggregometer and determining donor sensitivity to platelet agonist.
- 5 Treat PRP with test compounds and vehicles in a staggered manner. As each aggregation is 6 min long, the administration of treatments must be offset by at least 6, preferably 8, min, such that platelets are exposed to the different treatments for the same amount of time. It is important to set up beforehand the

order in which treatments will be administered. Incubate PRP for 30 min at 37°C.

- i. *For each treatment, use a minimum of 600 µL PRP.*
 - ii. *It is important to note that PRP may not respond identically to washed platelets. Therefore, the compound may be needed at a higher concentration than with washed platelets. A preliminary dose-response is suggested for several donors to confirm the doses to be tested.*
- 6 Pipette 700 µL of PRP into a 1.5 mL Eppendorf tube and centrifuge at 1200×g for 10 min at RT to remove platelets. *This will be the platelet-poor plasma (PPP) control.*
- 7 Set up cuvettes, one per treatment, placing a stir bar into all of them. Set up a separate cuvette without a stir bar, which will be used for a blank. Check the tubes for cracks, label tubes.

It is important to place stir bars into the cuvettes before addition of PRP, as addition of a stir bar to PRP could splash and create a bubble.
- 8 Remove the PPP from the centrifuged fraction, avoiding the platelet pellet and add 500 µL PPP to the aggregometry cuvette without a stir bar.
- 9 Pipette 450 µL from the PRP set aside in Step 3 into each of two prepared cuvettes containing stir bars.

These will be used to set up baseline and run the ATP standard.
- 10 Wipe the cuvettes with a clean KimWipe and place PPP in reference well (“poor”).

Instrument Setup

- 11 Allow the aggregometer to warm up, the heater block should be at 37°C before use.
- 12 Set stir on instrument panel go 1200 rpm and set the luminescence gain at 0.005.
- 13 Aggrolink program set up:
 - i. Set the aggregation test procedure to 6:00 min duration.
 - ii. For lumi-aggregation, set the input channels “Trace 1” to “Optical” and “Trace 2” to “Luminescence.”
 - iii. Leave the gain blank for both traces.
- 14 While the treated platelets are incubating, you may set the baseline as well as run the ATP standard.
- 15 Baseline set up and Data Collection for ATP standard:
 - i. Place one of the cuvettes from “Sample Preparation” step 8–9 in test well (“Rich”). Be sure the stir bar is spinning in the PRP tube.

If the bar is not spinning, ensure the unit is seated correctly and that the tubes are fully inserted.

- ii. Click on the “Run New” button.
- iii. You should see blue line begin to track on the chart. If it is not at 0% aggregation (left axis), press and hold the “set baselines” button on the aggregometer until the blue line reaches 100% and then release.

The black line for luminescence will not be visible at the bottom of the graph until ChronoLume reagent is added.

- iv. Once the aggregation baseline is set, click “stop” and then “rerun”. Add 50 μ L of RT Chrono-Lume Reagent to the PRP tube, then click “okay”.
- v. Run sample for 2 min to achieve stable baselines: The blue line should remain close to 0% (left axis) and black line MAY be around **10–20%, or at 0%** (right axis).

If tracking is erratic or you can’t see the black line, twist the PRP cuvette as there may be imperfections in tube that prevent proper light transmission.

- vi. Once both baselines stabilize, press and hold the “set baselines” button again as above; and release when blue line is at 100% (left axis). Blue line should then be back at 0% (left axis) and black line should also be at 0% (right axis).
- vii. Click “stop” and then “rerun”.
- viii. **Prior to starting the test**, withdraw 5 μ L of ATP standard into the pipette, **THEN click “okay” and immediately** add the standard to the cuvette.
- ix. At completion of the test, press “Stop” and save.

- 16** You may use the second cuvette of PRP to determine if the patient is responsive to the agonist to be used.

- i. Repeat step 14 above, but at 14.viii, instead of ATP standard, withdraw the appropriate volume of agonist into the pipette prior to starting the test, then click “okay” and immediately add the agonist to the PRP.

We commonly use 5 μ M ADP.

- ii. If the patient is not sensitive to this agonist (*i.e.* there is no aggregation), a higher dose of agonist may be tested. If appropriate, a different agonist such as collagen or arachidonic acid, may be used.

Sample data collection

- 17** After the treated PRP has incubated for 30 min, repeat step 14, again using ADP instead of ATP standard at step 14.viii. Repeat for each sample.

- i. To move to next sample, click on “RUN NEXT” button, type in test identification information, edit test procedure information (*i.e.* reagent and concentration) and click “Okay.”

18 Analysis

- i. From the “Edit” pull down, click on “Calculate results” and adjust the start and duration times for trace 2 to match the times you set for trace 1. Click “Okay.”
- ii. Once calculations have finished, BE SURE TO SAVE.
- iii. For test samples: Remove the PRP tube from test well and visually inspect for aggregates Add observations to the “test procedure” information.

Basic Protocol 9: Thromboelastography

Thromboelastography (TEG) is a robust measure of platelet function, as it measures clotting parameters including time to clot initiation (R), clot strength (Maximum Amplitude; MA), and fibrinolysis. TEG is used clinically to predict thrombotic events during surgery and thus, is a very translational measure of human platelet function. While not appropriate for high-throughput screening, TEG is a powerful tool to determine the effects of an identified compound on clotting.

While TEG gives a comprehensive view of clot formation in whole blood, it has several limitations. As TEG involves whole blood, cells other than platelets may respond to the compound being tested, and in turn affect platelet function and clotting.

Another limitation is that the thromboelastograph has limited sampling ability; the machine described in this protocol only has the ability to measure 2 samples at a time. As the blood must be used within 2 hours of drawing, and as TEG requires an hr or more to run, researchers are limited to testing one control and one test compound. If more than one machine is available, additional conditions may be tested simultaneously.

TEG should be performed as per manufacturer’s instructions. Here, we describe TEG using a Thromboelastograph Hemostasis System 5000 series machine.

Reagents and Materials

- Sodium citrate vacutainer tubes (BD 369714; Fisher Cat. # 02-688-26)
- Kaolin (Fisher Cat. #. NC0369158)
- Disposable cups + pin (Haemonetics Corporation Cat. # NC0063549)
- 0.2 M CaCl₂
- Eppendorf tubes

Equipment

- Thromboelastograph Hemostasis Analyzer 5000 series (Haemoscope Corporation, Niles, IL, USA)
- Computer capable of running the appropriate TEG software (see manufacturer's suggestions)

Protocol Steps

Sample preparation

- 1 Collect whole blood into sodium citrate vacutainer tubes. Mix by gentle inversion.
- 2 Pipette at least 500 μL of whole blood into an Eppendorf tube with the desired reagents. Incubate for up to 30 min at 37°C.

Instrument set-up—Set-up the instrument using the manufacturer's instructions. The following steps are appropriate for the Hemostasis System 5000 series thromboelastograph using TEG RemoteViewing™ software (Haemoscope Corporation, Niles, IL, USA).

- 3 Place the disposable TEG cups with pins in the thromboelastograph.
- 4 Ensure TEG cups are firmly in place by bringing the container holding the cup toward the lever-arm at the top of the thromboelastograph; using the index finger, push the button on the bottom of the cup container. Move the container with the cup back to the starting position, and ensure the cups are flush with the container.
- 5 Pipette 20 μL of 0.2M CaCl_2 into each cup

Running TEG

- 6 Pipette 20 μL of Kaolin into separate 1.5 mL Eppendorf tubes, one for each treatment. Label the tubes.
- 7 When the samples are done incubating, pipette 500 μL of the whole blood into the corresponding kaolin-containing Eppendorf tube. Mix gently by pipetting up and down. Let sit for 5 min.
- 8 On the TEG program, make sure the samples to be tested are labeled and the appropriate testing conditions are selected (*i.e.* specify Kaolin treated whole blood).
- 9 Before starting, make sure the first treatment is selected in the TEG program. When you start, speed is critical. Perform steps 7–9 one at a time for each sample; for sample one perform steps 7–9 completely, then perform steps 7–9 for the second sample.
- 10 Pipette 340 μL of the Kaolin-treated whole blood into TEG cup, and gently mix with the CaCl_2 by pipetting.

- 11 As soon as the blood is added, move the cup container to the lever arm, and move the arm to “test”.
- 12 On the TEG software, select the next sample to be tested.
- 13 Immediately repeat steps 7–9 for the remaining samples, one sample at a time.

The testing will occur for one hr.

Support Protocol 1: Generation of cigarette smoke extract (CSE)

Cigarette smoke extract (CSE) is an aqueous solution generated from the soluble components of main-stream and side-stream cigarette smoke; CSE is made by bubbling cigarette smoke into media. CSE has been produced in a variety of ways in the past, but there is increasing attempts at using a standard method for CSE generation. The smoke generation parameters (puffs/min, seconds per puff, etc.) used here follow the standards described in ISO 3308. The method described limits variability between preparations of CSE, increasing reproducibility of results.

Media used in the generation of CSE should be at RT, as differences in temperature changes the solubility of cigarette smoke components. Thus, using media at the same temperature is important for the consistency of the constituents of CSE. The following protocol describes steps to produce CSE, using a Jaeger-Baumgartner CSM2072i cigarette smoking machine (CH Technologies, Westwood, NJ).

Our lab has previously characterized CSE as having the highest absorbance at 320nm. Thus, we normalize our CSE preparations to one another by measuring the absorbance at 320nm. For different reference cigarettes, the peak absorbance should be determined and preparations normalized using the absorbance at that wavelength.

This protocol details the generation of CSE using a Jaeger-Baumgartner CSM2072i. Please refer to the manufacturer’s manual on the machine for general set-up. This protocol only details the set-up for CSE generation, not general instructions for machine set-up or maintenance.

It should be noted that other types of tobacco and nontobacco products can be used to generate smoke extracts, smoke condensates and other formulations. This requires adaptation of the equipment and protocols, specific to the agent.

All steps involving CSE (after generation) are performed in a chemical or tissue culture hood.

Reagents and Materials

- Serum free media (*e.g.* RPMI)
- 6 reference cigarettes (3R4F or current standard) from the Kentucky Tobacco Research Council (Lexington, KY)
- 50 mL conical tubes

- Rubber stopper
- Ring stand and clamp
- Masterflex Tubing (see manufacturer's instructions to determine appropriate tubing)
- 5 ¾" glass Pasteur pipets (VWR Cat. # 14672-200)
- 0.45 µM Syringe filter (VWR Cat. # 28137-938)
- 25 mL Syringe
- Glass cuvettes
- Eppendorf tubes
- pH strips

Equipment

- Jaeger-Baumgartner CSM2072i cigarette smoking machine (CH Technologies, Westwood, NJ)
- Peristaltic pump (Masterflex L/S, Cole Parmer) with a Masterflex L/S standard pump head
- Spectrophotometer that reads at the wavelength 320nm

Protocol steps

1. The night before CSE generation, sterily add 40 mL serum free medium to a 50 mL conical tubs. This is to ensure the medium is at RT for CSE preparation the next day.
2. The morning of CSE generation, move 20 mL of the RT medium to a separate 50 mL conical. This medium will be used as the control for CSE in treatments. Transfer ~1.25 mL of this medium into a 1.5 mL Eppendorf tube, to be used to blank in the spectrophotometer.
3. Assemble ring stand in the hood, attaching the 50 mL tube containing 20 mL media to the clamp. Once assembled, remove the lid of the 50 mL tube and place the rubber stopper on top of the tube.
4. Add tubing adapter to tube connected to the peristaltic pump.
5. Connect one end of a separate piece of tubing to the tubing adaptor (connecting this tube to the peristaltic pump) and the other end to a glass Pasteur pipet.
6. Place the pipet into the opening of the rubber stopper such that tip is submerged in the media but does not touch the bottom of the tube.
7. Fill up ash tray with water and attach it to the cigarette smoke machine.
8. Turn on valves for air.

9. Turn on peristaltic pump to 240 rpm. At this point, air will be pumped through the media. As such, if the set-up is correct, the media will be bubbling due to the airflow. If there is no bubbling, check the tubing for leaks; often the tubing in the peristaltic pump will become worn down and break. Additionally, ensure all tubing connections are secure. If needed, use tape to seal the connections between tubing.
10. Set the Jaeger- Baumgartner CSM2072i cigarette smoking machine to automatic set-up that smokes 6 cigarettes per cycle for 8 min, with one puff per min. Each puff should be set to 2 seconds with a 35 mL draw, as suggested by the Federal Trade Commission. Therefore, there will be 8 puffs per cigarette.
 - a. If this set up is not pre-programmed, follow the manufacturer's instructions and set up this protocol.
11. Cigarettes should be lit by the electrical lighter shortly after the cycle begins. If lighting fails, the cigarette(s) may need to be manually lit.
12. Set timer for ~ 7 min.
13. When timer goes off and you hear the first cigarette ejection, hit stop on the machine to prevent another cycle from starting.
14. Once all of the cigarettes have been ejected, turn off the pump and cap the cigarette smoke extract.
15. Disassemble the set-up, and clean the cigarette smoke machine:
 - a. Turn off the valves.
 - b. Switch the cigarette machine to the manual mode and press the eject button to blow out any air from the machine.
 - c. Dispose of the cigarette ashes/waste in the ash tray into a designated cigarette waste container with a lid. The container should be filled with an absorbent material such as pig litter to absorb the waste-water. If needed, vacuum out the machine.
16. Check the CSE pH with a pH strip (it should be ~7.4).
 - a. If the pH is not 7.4, adjust the pH. Often, the pH will become acidic when CSE is generated.
17. Filter the CSE in the cell culture hood.
 - a. Using a 25 mL Syringe, withdraw the CSE from the tube.
 - b. On a fresh 50 mL conical, place a 1.2 µm luer-lock filter.
 - c. Attach the syringe with CSE to the luer-lock filter and express CSE through the filter.
18. Pipette 1.25 mL of CSE to an Eppendorf tube. Use this CSE for spectrophotometry.

19. Determine the absorbance at 320nm of the CSE to determine its concentration:
 - a. Add 1mL of each RPMI and CSE to separate cuvettes. Use the RPMI as a blank.
 - b. Measure the absorbance of CSE at 320 nm. The concentration of CSE will be reported in Units per mL based on the absorbance; an absorbance at 320nm of 0.650 is 650 Units/mL.
 - c. Typically, this protocol yields concentrations of 400–700 Units/mL.

Dilute CSE to desired concentration.

Manual Method of CSE generation—This method of CSE generation is a modification of the method developed by Carp and Janoff (Carp & Janoff, 1978). If a smoking machine is not available, CSE can be generated manually. This method is also widely used in the toxicology field for *in vitro* studies of CSE exposure.

Reagents and Materials

- Research grade cigarettes (Kentucky Tobacco Research Council (Lexington, KY)) (3R4F)
- Serum-free MEM (Gibco Cat. # 11095-098)
- 0.45-µm filter (25-mm Acrodisc; Pall Corp., Ann Arbor, MI)
- 10 mL filtered disposable pipette
- Pasteur pipettes
- 50 mL conical tube
- 3 hole stopper
- Ring stand and tube holder

Equipment

- Fume Hood
- Spectrophotometer
- pH meter

Protocol steps

1. Assemble the smoking apparatus shown in Figure 3, in a fume hood.
2. Briefly CSE is prepared by bubbling smoke from two cigarettes into 20 mL of serum-free MEM. Each cigarette should be smoked to 0.5 cm above the filter.
3. The pH of the CSE extract is adjusted to 7.4, and sterile filtered with a 0.45 µm filter.
4. To ensure consistency in the CSE between experiments, measurements of optical density are taken at a wavelength of 320 nm immediately following preparation

of the CSE (above steps). An optical density of 0.65 is considered to represent 650 U/mL CSE.

5. Dilute the CSE preparation to the desired concentrations in serum-free MEM. Each CSE preparation is used immediately and prepared fresh for each experiment.
6. Apply CSE to platelets as indicated above in **Basic Protocol 3**.

Alternative Protocol 1: Method for negative selection of platelets

Platelet isolation issues can include WBC contamination. Normal centrifugation protocols as described above in Basic Protocol 1 usually generate platelets with less than 0.05% contamination of other blood cells. Considerations such as RNA studies in platelets may require a more stringent preparation to ensure that platelets are free of nucleated leukocytes.

Reagents and Materials

- CD45 Dynabeads (human) (ThermoFisher, cat #: 11153D)
- PBS (ThermoFisher Cat. # 14200-075)
- Bovine serum albumen (BSA) (Sigma Cat. # A7906-500G)
- EDTA, pH 7.4 (Invitrogen Cat. # 15575020)
- 15 mL conical tube

Equipment

- Table Top and Microfuge Centrifuges
- Rocker
- Magnet Stand

Protocol steps

1. Prepare isolation buffer (PBS supplemented with 0.1% BSA and 2 mM EDTA).
2. **Be sure magnet stand is at RT as platelets are cold sensitive.**
3. Use 100 μ L CD45 beads for up to 12 mL of PRP, as prepared in **Basic Protocol 1**.
4. Mix CD45 beads well/vortex briefly. Wash CD45 beads in isolation buffer (2 $\times$ 10 mL) at RT.
5. Place the tube on the magnet stand and remove wash buffer.
6. Add PRP and rotate gently for 15 min at RT.
7. Place tube on magnet stand for 3–5' to ensue capture of all beads. Carefully remove CD45-depleted PRP to a new 15 mL conical tube without disturbing beads.

8. Continue with platelet washing or perform PRP specific assays.

Commentary

Background information

Human blood platelets are increasingly being recognized as complex cells that, despite lacking a nucleus, are poised to respond to a wide variety of stimuli, including environmental and chemical toxicants. Platelets are now appreciated to have critical immunological functions that contribute to inflammation and associated inflammatory diseases (Morrell, Aggrey, Chapman, & Modjeski, 2014). Platelets not only form the thrombus that occludes a vessel in the final stages of cardiovascular disease (*i.e.* heart attack or stroke), but also contribute to the chronic inflammation that drives the development and progression of CVDs. As a testament to the key role platelets play in inflammation, platelet activation is commonly used as a biomarker of inflammation and CVD risk. For example, platelets are hyper-responsive in cigarette smokers, resulting in elevated levels of sCD40L, platelet aggregation, and circulating microparticles - all of which are associated with increased CVD risk (Csordas & Bernhard, 2013).

Despite their critical role in disease progression and use as inflammatory biomarkers, platelets are rarely used in toxicological screens. There are several reasons for this exclusion. Platelets are still often dismissed as “cellular debris”, and so the effects of compounds on platelet function is often not a concern. Additionally, there is no platelet cell line, so only primary human blood platelets are available for testing. There are cell lines for the platelet precursor cell, the megakaryocyte, but these cell lines do not yield large amounts of platelets, and the platelet-like-particles generated are difficult to confirm as bona-fide platelets. Platelets themselves cannot be cultured, and must be used within hours of isolation; the inability to use platelets in chronic *in vitro* models leaves a false impression that platelets are difficult to use *in vitro*, and so they are ignored for toxicological screens. However, platelets are a valuable tool for *in vitro* screening, as they are abundant in the peripheral blood, easily isolated using simple centrifugation steps, and are amenable to multiple assays for functional testing *in vitro*. Screening the effects of environmental toxicants, such as cigarette smoke or other tobacco products, on platelet function *in vitro* could be a powerful tool in the development of less harmful tobacco products (Spinelli, Lannan, Loelius, & Phipps, 2017). While chronic *in vitro* testing is not possible, acute toxicity on platelets is easily assayed, using the methods described here. In describing how to isolate and treat platelets, as well as how to assay for platelet function, we hope to encourage investigators to use this cell in the testing of current and new tobacco and non-tobacco products.

In toxicological screens, it is important to assay a variety of measures of platelet function to discern the full effects of environmental insults on platelets. Below, we describe the theory behind the assays of platelet function described previously.

Platelet sCD40L and alpha-granule release—Platelet alpha-granules contain inflammatory molecules that include many chemokines and cytokines (*i.e.* CCL5, PF4) that are critical in platelets’ inflammatory function. Additionally, platelets express CD40L on

their surface membrane as well as within alpha granules. Surface bound sCD40L is cleaved into soluble CD40L (sCD40L) upon activation; sCD40L is a marker of inflammation, as it activates leukocytes including neutrophils and monocytes. PF4 is highly present in alpha-granules, and has roles in hemostasis and immunity, potentially impacting CD4 T-cell differentiation (Shi et al., 2014). Additionally, platelet alpha-granules contain membrane-bound CD62P (P-selectin); when alpha-granules are released, P-selectin becomes exposed on the surface of the platelet, increasing surface expression of p-selectin. P-selectin interacts with monocytes and other leukocytes, enabling leukocyte extravasation into damaged tissues. Platelet alpha-granule release is critical for platelet inflammatory function, and so we measure it in several ways. We measure PF4 concentration in the supernatant, as well as surface levels of CD62p, to determine the level of platelet alpha-granule release.

Platelet lipid mediator production—Prostaglandin E_2 and thromboxane A_2 are synthesized from arachidonic acid in response to agonists. PGE_2 is a well-known general inflammatory molecule, which is famously recognized for its pyrogenic function. PGE_2 also sensitizes platelets to agonists, and causes endothelial activation. PGE_2 is detectable in washed platelet supernatant, whereas thromboxane A_2 has a short half-life and is quickly metabolized to the less bioactive TXB_2 . Thromboxane A_2 is thus difficult to detect in washed platelet supernatant; instead, TXB_2 is measured as a surrogate for TXA_2 , as these levels correlate well. These lipid mediators are important for platelet hemostatic and immune function. Measuring lipid-mediator production is important in determining both hemostatic and immune platelet functioning.

Platelet spreading—In addition to ADP, thrombin, and thromboxane agonist, platelets are activated upon exposure to extracellular matrix components (EMC), such as fibrinogen, von Willebrand factor (vWF), and collagen. In resting platelets the fibrinogen receptor $\alpha_{IIb}\beta_3$ is in an inactive state; upon platelet activation, inside-out signaling triggers conformational change and activation of $\alpha_{IIb}\beta_3$ (Bennett, Berger, & Billings, 2009). This signals morphological changes in platelets critical for adhesion and injury repair. Alternatively, other matrix components can be applied to coverslips to discern tobacco effects to platelets that include collagen and vWF, and will aid in the evaluation of the types of signaling that could be dysregulated by these products.

Aggregometry—Aggregometry measures the ability of platelets to undergo shape change and aggregate. It is a common method of measuring platelet function due to its translational potential, as aggregation is a major function of platelets *in vivo*, and its ease of detection via light transmission.

Aggregometry is commonly measured using light transmission, termed light transmission aggregometry (LTA), first described over 50 years ago (Born, 1962; O'Brien, 1961). In brief, LTA measures light transmission through gently stirred platelet rich plasma (PRP), in which the suspended platelets cause PRP to be cloudy and prevent light transmission. As platelets aggregate, increased levels of light passes through and is detected by the photocell in the aggregometer. Thus, aggregation can simply be measured by determining the percentage of light transmitted through PRP. As light transmission aggregation is performed in PRP, it primarily measures platelet function in the absence of other cells, such as leukocytes. LTA

has been modified to include detection of platelet dense granule release using luminescence (Miller, 1984). In luminescence aggregometry, platelet dense granule release is detected through the use of a luminescent reagent that reacts with ATP, a component of dense granules. As dense granules are released, the luminescence increases and is detected by the aggregometer.

A second method of detecting aggregation is impedance. The impedance method of aggregometry uses two electrodes submerged in whole blood. As platelets are activated and aggregate, they bind to the electrodes and impedance between the two electrodes increases. As impedance aggregometry is performed in whole blood, aggregation measured is dependent on not only platelet function but also leukocyte function, as leukocytes are known to interact with platelets.

The method of aggregometry used will depend on the question of interest. If interested in solely platelet function without the effect of other cell types, light transmission would be preferable.

Thromboelastography—Thromboelastography (TEG) measures clot parameters in whole blood, and is used clinically to determine platelet function of patients during surgery. TEG works by measuring the rotation of a pin submerged in whole blood. Briefly, whole blood placed in a turning cup, and the torsion wire (or “pin”) is submerged in the blood. As a clot forms, it binds to the pin and changes the pin’s rotation. From this motion, clotting parameters such as time to clot initiation (R), clot strength (MA), and fibrinolysis are calculated. Each measure is accounted for by separate clotting factors; R measures fibrin formation, MA measures platelet activation and platelet-fibrin bonding, and fibrinolysis.

Critical parameters

There are several important aspects to consider when designing platelet studies. We provide some experimental guidelines and precautions below.

Donor considerations—One critical parameter to consider for the use of human blood platelets *in vitro* is the donor. It is important to consider the demographics of donors when designing experiments. For instance, platelets from smokers respond more readily to cigarette smoke than do platelets from non-smokers; if treating platelets with cigarette smoke, the smoking status (current, former, never) should be known. This requires careful consideration in the creation of the consent forms and screening questions.

However, even knowing these demographics, there is much donor variability. For instance, platelet function may change after removal from the body; this is a highly donor-dependent phenomenon. To combat the time-dependent changes in platelet function, we suggest that platelets should be isolated, treatment completed, and assays requiring live cells be completed within 5 hours.

Another donor-dependent parameter is sensitivity to agonist. For aggregation, some donors’ platelets may not be sensitive to certain agonists. On the other hand, donor platelets may be more sensitive to certain agonists, such that the same dose elicits a much larger response in

one donor than another. Basal platelet function is also highly donor-dependent. For these reasons, we also suggest that in examining data, such as dose responses, the response of each donor be considered separately, as well as in a group.

Platelet handling—Another critical parameter in the use of human platelets *in vitro* is in keeping the platelets quiescent during isolation. Small gauge needles or spurting blood could increase platelet activation. Additionally, a poor “stick” releases factors that activate platelets. Thus, it is important for blood draws to be performed with a wider-gauge needle, and to note any difficulties in a blood draw.

Platelets are exquisitely sensitive to changes in environment, be they jarring movements or changes in temperature. Circulating blood platelets are normally kept quiescent by interactions with molecules released by endothelial cells (PGI₂, nitric oxide (NO)). Thus, to prevent activation of platelets during centrifugation we use PGI₂ and an anticoagulant, ACD. Platelets should never be vortexed, shook, or otherwise jarred. Platelets should also never be subjected too hot or cold temperature extremes, but rather kept at room temperature or 37°C with slow, gentle agitation.

Aggregometry—One confounding factor in aggregometry is donor-to-donor variability. Depending on the donor, platelet reactivity may be stable or may increase or decrease in a time-dependent manner after being drawn from the body. Additionally, donor sensitivity to agonists will also vary, with some donors being unresponsive to agonists, such as ADP.

In regard to time-dependent changes, it is essential that dose-responses be randomized, such that a change due to time is not confused for a dose-dependent response. As a result of the potential for time-dependent changes, it is important to limit the number of treatments to be measured. We also suggest a vehicle control be measured at the start and end of each experiment. This will identify any changes in aggregation over time that are independent of treatment.

To address donors that are insensitive to an agonist, it is important to test the desired agonist before starting aggregation studies. We suggest testing an agonist of interest at a couple of different concentrations to gauge how a particular donor may respond to a given agonist.

Time—Human blood platelets must be isolated and treated, and all assays completed, within 5 hours of the blood draw. When first performing the techniques described here, isolation of human blood platelets is often the most time intensive factor. However, with practice the salient assays can be completed within that time without unwanted platelet activation occurring from poor technique.

Another consideration is in the timing of treatment of PRP in aggregometry studies. As described in critical parameters, once removed from the body platelet function changes over time; in some donors, platelets become hyper-reactive as time passes while in others platelets become hypo-reactive. In aggregometry, this translates to a time-dependent increase or decrease in aggregation. It is thus essential to perform aggregometry efficiently.

TEG—The TEG machines must not be disturbed during testing, as the measurements are sensitive to movement of the machine. Along those lines, keep the area that the thromboelastograph(s) are positioned free of vibration-generating machines (*i.e.* vortex, microcentrifuge).

Troubleshooting

Platelet isolation—The most common problem when first performing these techniques is keeping platelets quiescent during isolation. We have previously described precautions that limit activation. For the experiments described herein, it is important to use unactivated platelets. Therefore, we will describe some issues caused by platelet activation, how to recognize them, and how to prevent the activation in future experiments, summarized in Table 1.

It is of major importance to use agents that reversibly dampen platelet function, such as PGI₂ in the PRP or wash buffer before centrifugation. PGI₂ is a good candidate, as it has a short half-life and functions in the circulation to regulate platelet activity. Prolonged time when resuspending platelets in wash buffer, or platelets coming into contact with something cold during isolation (*i.e.* platelets positions too close to an ice bucket) can lead to unwanted activation. To prevent such issues, be sure to treat platelets with PGI₂ as well as keeping platelets from becoming cold during isolation.

The first sign of platelet activation during isolation is a lack of platelet pellet after spinning down PRP or washed platelets. Platelets undergo shape change when activated, as well as release their granules, resulting in reduced density, causing them to need to be centrifuged at a higher force to pellet. If the platelet pellet is not present, it is not advised to attempt to centrifuge at a higher force, as the platelets are already activated and should not to be used for testing. If the activation of the platelets is less critical, for this step it is possible to isolate the platelets still. Leave the plasma or wash buffer in the tube, add PGI₂ (if originally forgotten), and allow the platelets to rest for 20 minutes. Do not use these platelets for the testing of any compounds on platelet function, or in the characterization of platelets from a specific group, as the results will be inaccurate.

Another sign of platelet activation during the isolation protocol is platelets coming off the pellet in clumps when resuspended, indicating that platelets are activated and sticking together. Platelets should swirl in solution in a manner that resembles a smoke cloud. However, if the platelets are not strongly activated, it is still possible to isolate the platelets and use them for experiments. To do so, let the platelet pellet rest for 10 minutes with the appropriate solution (Wash buffer or TSS) to allow the pellet to soften. Continue the isolation only if the platelets are no longer clumping.

Aggregometry—The major issue that arises in aggregometry is a lack of aggregation in response to a particular agonist. There are several potential causes for a lack of aggregation. The donor may be insensitive to that agonist, the donor's platelets may have stopped functioning, or the agonist used may no longer be active. To determine if the problem is in platelet sensitivity to agonist or platelet dysfunction, we suggest measuring aggregation in response to a strong agonist such as thrombin. If the platelets do not aggregate in response to

the strong agonist, the issue is likely that the platelets have stopped functioning. If aggregation does occur with a strong agonist, it is likely that the donor is insensitive to the previous agonist. This is quite common with weaker platelet agonists, such as ADP.

CSE generation—Over time, particulate matter is deposited from cigarette smoke into the tubing of the cigarette smoke machine, eventually resulting in obstruction of the tubing and interruption of the funneling of smoke into the media. It is important to regularly check the tubing of the machine to ensure no leaks, and that the tubing is not obstructed.

Spreading—Platelets are challenging to image when fully spread, as upon spreading they become very thin, and occupy a very small focal plane. A common problem when first performing platelet spreading is difficulty in finding platelets on the coverslip, and difficulty in focusing on platelets. It is possible if platelets are not observable on the coverslip that the coverslip was mounted on the wrong side. In this case, it is not possible to recover the coverslip. For this reason, we suggest multiple coverslips be prepared for each treatment.

Another issue that leads to difficulty in imaging platelets is if platelet distribution is not homogenous, causing some areas of the coverslip to contain few platelets. This can be problematic at the edges of the coverslip, as fibrinogen may not have coated evenly to the edge. To prevent this issue, we suggest imaging the center areas of the coverslip after confirming platelet distribution is homogeneous. This issue can also occur due to strong aspiration at one side of the coverslip. To prevent this issue, do not aspirate forcefully from one side. Gently aspirate around the coverslip.

Anticipated results

In general, using a combination of platelet function assays will provide a broad picture of platelet dysregulation in response to toxic insult. These findings can be used to inform as to what changes occur when comparing or screening existing or next-generation tobacco and nontobacco products (*i.e.* cigars, electronic cigarette vapor, snuss, etc.). Platelet dysfunction studies will identify important signaling pathways, mechanistic foundations and new biomarkers associated with CVD risk. Standardization and validation of these types of assays to assess risk and harm are in development. This approach enables researchers to quickly and cost effectively use platelet *in vitro* assessment methods to advise of the potential risks of a given product.

Isolation—For the isolation protocol, great care is necessary in performing each step, and trainees usually require practice before perfecting their technique. We normally anticipate 3 practice isolations, 1 entirely observed, 1 supervised, and 1 unsupervised, though, it is not unusual for trainees to require more practice. The typical results for isolation when first performed are low platelet yields and highly activated platelets. Therefore, we suggest that trainees practice platelet isolation several times before performing experiments.

When trainees are proficient in platelet isolation, it is typical to get a yield of $2 \times 10^8 + 0.5 \times 10^8$ platelets/mL. It is also typical for platelets to have low-levels of activation markers (10–25% CD62P+ in flow cytometry).

CSE treatment—Platelets are sensitive to acute exposure to CSE, with high doses of CSE (32.5 U/mL and higher) decreasing platelet viability. At lower doses of 6.5 U/mL and less, CSE causes a hyper-responsive phenotype in platelets, resulting in increased pro-inflammatory mediator release. Additionally, we have observed that platelets isolated from cigarette smokers or people exposed to second-hand cigarette smoke are more sensitive to CSE than platelets from non-smokers; we anticipate high donor variability in platelet response to CSE, making dose-response testing of CSE on individual donors critically important. Additionally, it is important that dose responses be performed if using a new reference cigarette lot, as variability between lots or types of reference cigarettes could result in significantly different platelet responses.

Platelet spreading—Untreated washed platelets or vehicle treated platelets are anticipated to be 80–100% fully spread. However, platelet spreading is donor dependent with some donors achieving 50% of fully spread platelets. As such, it is of critical importance that a vehicle control is included in each spreading experiment. Exposure to CSE or other tobacco or nontobacco products may produce different outcomes. These effects may range from no effect to reduced or increased spreading. In some instances, there may be problems with adhesion to a particular matrix. When quantifying platelet spreading, it is important to observe whether platelet attachment is different between controls and treatments. These factors will provide information on the receptor types that may be dysregulated and future experiments can be designed to test the hypothesis.

Aggregation—Values for aggregation are donor dependent and will vary depending on the agonist used and concentration used. We have found that 5 μ M ADP typically causes ~50–90% aggregation. To maximize the ability to see changes in aggregation, we use agonist concentrations that elicits a modest response to allow for observation of either reduced or increased aggregation. In the case of CSE, we see an augmentation of aggregation consistent with provoked platelet activation.

Time considerations

Washed human platelets—It is suggested that platelets be isolated, treated, and assayed for function or fixed within 5 hours of whole blood isolation. This is due to limit time-dependent changes in platelet function.

Platelet spreading—Platelet spreading requires an additional hour for completion after the isolation and treatment of washed platelets, 45 minutes for spreading and 15 minutes to fix the cells.

Immunoassays—Immunoassays can be performed on stored platelet supernatants several months after isolation, provided the supernatants are stored properly for the desired assay. Each immunoassay may require 2 days for completion.

Aggregometry—Aggregometry is a time-sensitive assay, and so testing must be completed quickly. It is suggested that from the blood draw, treatment and aggregation testing should be complete within 2 hours.

TEG—It is suggested that TEG be completed within 2 hours from whole blood collection to limit variability between donors. The TEG assay requires one hour to be performed; to complete treatment and the TEG assay within three hours, blood collection and treatment should be completed within one hour.

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References

- Bennett JS, Berger BW, Billings PC. The structure and function of platelet integrins. *J Thromb Haemost.* 2009; 7(Suppl 1):200–205. DOI: 10.1111/j.1538-7836.2009.03378.x [PubMed: 19630800]
- Borgerding M, Klus H. Analysis of complex mixtures – Cigarette smoke. *Experimental and Toxicologic Pathology.* 2005; 57(Supplement 1):43–73. DOI: 10.1016/j.etp.2005.05.010 [PubMed: 16092717]
- Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature.* 1962; 194:927–929. [PubMed: 13871375]
- Carp H, Janoff A. Possible mechanisms of emphysema in smokers. In vitro suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis.* 1978; 118(3):617–621. DOI: 10.1164/arrd.1978.118.3.617 [PubMed: 101105]
- Csordas A, Bernhard D. The biology behind the atherothrombotic effects of cigarette smoke. *Nat Rev Cardiol.* 2013; 10(4):219–230. DOI: 10.1038/nrcardio.2013.8 [PubMed: 23380975]
- McCarty OJT, Larson MK, Auger JM, Kalia N, Atkinson BT, Pearce AC, Watson SP. Rac1 Is Essential for Platelet Lamellipodia Formation and Aggregate Stability under Flow. *The Journal of biological chemistry.* 2005; 280(47):39474–39484. DOI: 10.1074/jbc.M504672200 [PubMed: 16195235]
- Miller JL. Platelet function testing: an improved approach utilizing lumi-aggregation and an interactive computer system. *Am J Clin Pathol.* 1984; 81(4):471–476. [PubMed: 6546638]
- Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. *Blood.* 2014; 123(18):2759–2767. DOI: 10.1182/blood-2013-11-462432 [PubMed: 24585776]
- O'Brien JR. The adhesiveness of native platelets and its prevention. *J Clin Pathol.* 1961; 14:140–149. [PubMed: 13730014]
- Shi G, Field DJ, Ko KA, Ture S, Srivastava K, Levy S, Morrell CN. Platelet factor 4 limits Th17 differentiation and cardiac allograft rejection. *J Clin Invest.* 2014; 124(2):543–552. DOI: 10.1172/jci71858 [PubMed: 24463452]
- Singh MV, Davidson DC, Kiebal M, Maggirwar SB. Detection of Circulating Platelet-Monocyte Complexes in persons infected with Human Immunodeficiency Virus Type-1. *Journal of Virological Methods.* 2012; 181(2):170–176. DOI: 10.1016/j.jviromet.2012.02.005 [PubMed: 22387340]
- Spinelli SL, Lannan KL, Loelius SG, Phipps RP. In Vitro and Ex Vivo Approaches to Evaluate Next-Generation Tobacco and Non-Tobacco Products on Human Blood Platelets. *Applied in Vitro Toxicology.* 2017; 3(1):110–120. DOI: 10.1089/aivt.2016.0034 [PubMed: 28337466]

Significance Statement

Novel *in vitro* methods are essential to characterize the effects of tobacco products on human blood platelets. Platelets are highly understudied vascular cells and their dysregulation is a key element in unremitting inflammation and the development of cardiovascular sequelae. Protocols were developed to evaluate the effects of tobacco and nontobacco products on human blood platelets. This is especially vital as platelets are not a first consideration for toxicology testing and thus, represent a novel and critical area of investigation to assess environmental insults.

Figure 1. PRP isolation from whole blood

Schematic of Steps 3 and 4 in **Basic Protocol 1** above.

Figure 2. Stages of platelet spreading

The image shows platelets imaged using **A.** scanning electron microscopy (SEM) and **B.** DIC at various stages of spreading, from left to right, beginning with adhesion to the matrix through full spreading. The first platelet pictured is an unspread platelet which has just bound to the matrix and rounded up. The following three images are platelets that have extended filopodia and are starting to spread, termed partially spread platelets. The last platelet pictured is a fully spread platelet, which has lamellopodia and flattened into a thin disk.

Apparatus for Cigarette Smoke Extract Preparation

Figure 3. Apparatus Assembly for CSE Preparation

Gather materials and equipment in a fume hood. Assemble as pictured. Light the cigarette and apply vacuum slowly to achieve a consistent bubbling. Burn cigarette #1 to 0.5 cm above the filter. Close the vacuum before removing cigarette #1 and placing cigarette #2 into the apparatus. Repeat with cigarette #2. Continue with step 3 of **Alternative Protocol 1**.

Figure 4.

Flow cytometry gating scheme of washed human platelets. Washed human platelets are a fairly pure population, and as such gating is mostly important to eliminate doublets and small extracellular vesicles. First, singlets are gated on. Next, using sizing beads, platelets are gated on (1–2 μ m). Shown above are the schematics for **A**. Resting platelets and **B**. Activated platelets. Upon activation, platelets become smaller and less granular as well as increase in % CD62p+, as shown above.

Table 1

Commonly Encountered Problems and Solutions.

Protocol	Problem	Possible cause(s)	Solution (current experiment)	Solution (future experiments)
Isolation	No platelet pellet present after wash step	Platelets were activated	--	Ensure PGI ₂ is added during wash/ isolation steps
				Ensure platelets do not contact cold materials
Isolation	Platelets released from pellet in chunks	Platelets were activated	Let the platelet pellet rest for 10 minutes after spin	Ensure PGI ₂ is added to PRP and wash buffer
Aggregation	No aggregation	Reagent bad	Test/use fresh reagent	Do not refreeze agonist once thawed
		Donor insensitive to agonist	Use other agonists	-
Spreading	No platelets on coverslips	Coverslip flipped	-	Care in the future to mount the correct side of the coverslip
CSE Generation	Cigarette smoke not bubbling into media	Clogged tubing	Replace tubing	Regularly check and replace tubing
		Leak in tubing	Check tubing for leaks, replace tubing	Regularly check and replace tubing