Reports

Pairwise agonist scanning-flow cytometry (PAS-FC) measures inside-out signaling and patient-specific response to combinatorial platelet agonists

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Understanding the response of cells to multiple stimuli is vital for predicting donor specific responses and better understanding the signaling pathways involved. This is of particular importance in platelets because exposure of phosphatidylserine (PS) occurs upon costimulation but not with a single agonist. Here, we describe a multiplexed pairwise agonist scanning-flow cytometry (PAS-FC) method of measuring platelet inside-out responses to all pairs of six platelet agonists (convulxin, SFLLRN, AYPGKF, ADP, U46619, and PGE_2) used at their EC_{50} concentrations. These agonists allowed exploration of platelet signaling downstream of GPVI, PAR-1, PAR-4, $P2Y_1$, $P2Y_{12}$, TP, and IP receptors. The three-color flow cytometry method simultaneously measured integrin $\alpha_{IIb}\beta_3$ activation with PAC-1 antibody, P-selectin exposure (via α granule release) with anti-P-selectin, and PS exposure with annexin V. These responses were consistent across a healthy male donor pool. In duplicate measurements with each donor, 4 of the 10 donors had a sufficiently unique 45-parameter (15 pairs × 3 colors) phenotype to self-cluster ($P < 0.001$). This method has the potential for efficiently scanning for patient specific responses across a broad agonist-receptor space.

Cells are subject to numerous dose dependent agonist signals at a given time in a biological system, resulting in a complex integrated response. In many instances, signaling pathways use common second messengers that may result in synergistic or antagonistic effects that could not be predicted from study of the agonists in isolation (1). Platelets respond to multiple signals in vivo in a donor specific manner to halt blood loss (2), a process which is also central to the thrombotic risks associated with the 2 million heart attacks and strokes that kill 800,000 people each year in the United States alone (3). Platelets are an ideal cell type for studying the effects of multiple signaling pathways because they are anucleate, easily obtained from donors, and their responses can be used to make donor specific predictions about thrombosis (4).

In vivo, a platelet can encounter numerous different activating and inhibitory signals at the same time, and the integrated response to all of these determines the prothrombotic state of the platelet. In a thrombotic event, the first signal encountered is often activation of glycoprotein VI (GPVI) ITAM tyrosine kinase signaling upon binding collagen in the exposed sub-endothelial matrix (5). This occurs during platelet rolling, enabled by GPIb-IX-V binding vWF, which gives the platelet time for the GPVI signal to induce calcium mobilization that activates $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins to firmly adhere the platelet to the site of injury (6). In addition to activation of adhesion molecules, ADP is released from the dense granules early during the activation process and signals via platelet P2 receptors in an autocrine and paracrine fashion (7). After activation dependent

synthesis by cyclooxygenase 1 (COX1), thromboxane A_2 (TXA_2) is also released and binds platelets in the lumen and at the site of injury (8). Somewhat independent of the platelets, tissue factor (TF) exposed at the site of the injury initiates the extrinsic pathway of the coagulation cascade leading to the production of thrombin, which very strongly activates platelets through the PAR receptors. In addition to these and other minor activating signals, the endothelium constitutively releases prostacyclin (PGI_2) and nitric oxide (NO) as inhibitory signals to platelet activation.

Platelets can encounter each of these signals at varying doses during the thrombotic process and combinations of four or more simultaneously would be common in the core of a growing thrombus in vivo. However, most in vitro studies of these

Method summary:

Platelet rich plasma obtained from human blood was stimulated with all pairs of six different agonists. For platelet phenotyping, 96-well plate flow cytometry allowed 3-color detection of integrin activation, granule release, and phosphatidylserine exposure.
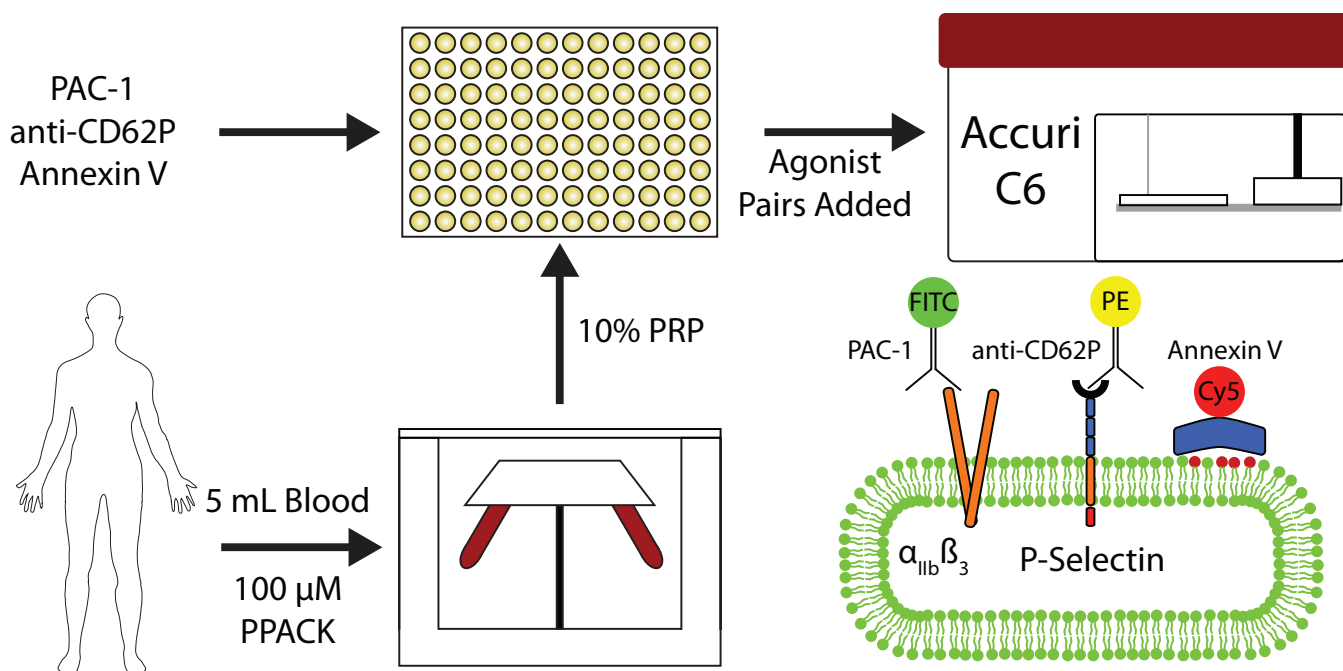
Figure 1. Schematic of pairwise agonist scanning flow cytometry assay. Blood drawn from healthy male donors was centrifuged to isolate platelet rich plasma, which was diluted to 10% v/v and added to a 96 well plate loaded with antibodies. Ten minutes prior to analysis, 10× stocks of agonists were added. PAC-1 measures integrin $\alpha_{IIb}\beta_3$ activation, anti CD62P measures degranulation via P-selectin exposure, and annexin V binds exposed phosphatidylserine (PS; red head group phospholipids).

signaling pathways are done in isolation. The effects of thrombin (9), ADP (10), and collagen (11) are known in great detail, but the previous studies do not address the conditions of the non-isotropic environment of a thrombus with many signaling molecules (12). To investigate the effects of simultaneous addition of agonist pairs, we have extended the previous Ca^{2+} assay that found donor specificity in platelet cytosolic calcium levels to utilize flow cytometry (2). Since integrin activation, degranulation, and phosphatidylserine (PS) exposure are all activation markers downstream of the cytosolic calcium, they may also exhibit donor specificity. Binding of the IgM antibody PAC-1 was used to measure the degree of integrin activation as it only binds the active form of the integrin $\alpha_{IIb}\beta_3$ (13). Secretion was measured using an IgG antibody against P-selectin (CD62P), which is exposed on the platelet surface when α granules fuse with the plasma membrane. This is not a direct measure of dense granule secretion, but dense granules release before

α granules (14), so it is indicative of release of both types. Finally, the exposure of PS to serve as a catalytic surface for the coagulation cascade on the plasma membrane outer leaflet was measured by binding of annexin V (15). These three measures of platelet activation give a fuller representation of platelet behavior upon multiple agonist activation.

Materials and methods

Platelet Preparation
Whole blood was drawn from healthy male volunteers, according to the University of Pennsylvania Institutional Review Board guidelines with informed consent, into Phe-Pro-Arg-chloromethylketone (PPACK; Haematologic Technologies, Essex Junction, VT) with a final anticoagulant concentration of 100 µM. All donors affirmed not taking any medication for 10 days prior to donation and not consuming alcohol for 3 days prior to donation. The whole blood samples were centrifuged at 120× *g* for 10 min to obtain

platelet rich plasma (PRP), which was diluted to 10% v/v in HEPES buffered saline (HBS; 20 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ at pH 7.4). Calcium was added to the buffer to facilitate proper formation and activation of the $\alpha_{IIb}\beta_3$ integrin as well as binding of annexin V to exposed PS.

Agonist Selection
The agonists in this assay were chosen as representative of the major signaling cues a platelet will encounter during a thrombotic event. They are also the same agonists used in the calcium assay previously developed by our lab to allow for direct comparison of results (2). Convulxin (CVX; Centerchem, Norwalk, CT) is a rattlesnake venom protein that directly binds and activates glycoprotein VI (GPVI), the primary collagen signaling receptor on platelets (16). This is used because soluble monomeric collagen only binds to the integrin $\alpha_2\beta_1$, which is an adhesive receptor and has little direct effect on signaling (17), and fibrillar collagen is not soluble, making it unsuitable for use in flow cytometry (18). Thrombin acts on the two G_q coupled protease activated receptors (PARs) in humans, PAR1 and PAR4, which signal differentially (19, 20). As such, we used the individual PAR agonist peptides, SFLLRN and AYPGKF (Bachem, King of Prussia, PA), to investigate PAR1 and PAR4 signaling individually. This also removes the need

Table 1. Comparison of agonist EC$_{50}$ values for different measures of platelet activation

Measure of activation	Convulxin	SFLLRN	AYPGKF	ADP	U46619	PGE$_2$
Cytosolic Ca^{2+}	5.3 nM	15.2 µM	112.4 µM	1.17 µM	1.19 µM	24.6 µM
PAC-1	1.6 nM	4.4 µM	267 µM	595 nM	n/a	11.5 µM
anti-CD62P	2.4 nM	6.6 µM	223 µM	371 nM	n/a	49.4 µM

Calculated EC$_{50}$ values for platelet activation measured by binding of PAC-1 and anti-CD62P antibodies to integrin $\alpha_{IIb}\beta_3$ and P-selectin respectively and cytosolic Ca^{2+} values that were obtained previously. Platelet rich plasma was incubated with each of the five activating agonists, while PGE$_2$ was coincubated with 15 µM SFLLRN. U46619 did not elicit significant antibody binding.
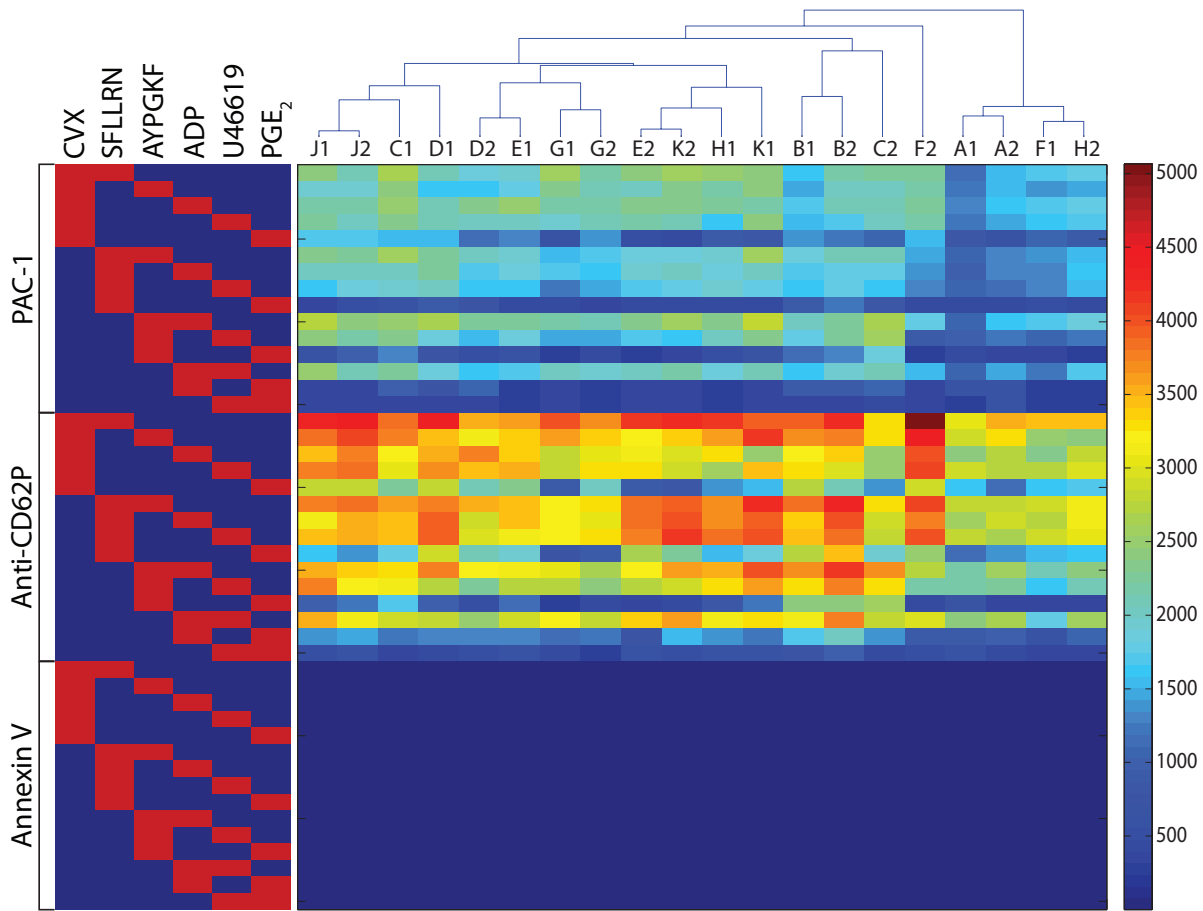
Figure 2. Clustering of donor pairwise activation responses. The matrix shows the 45 responses for each donor's pair of experiments. The clustering tree above the matrix shows that 4 of 10 donors had results that were able to self-cluster. On the left, red squares indicate the pair of agonists used in that row and brackets indicate the fluorescent probe.
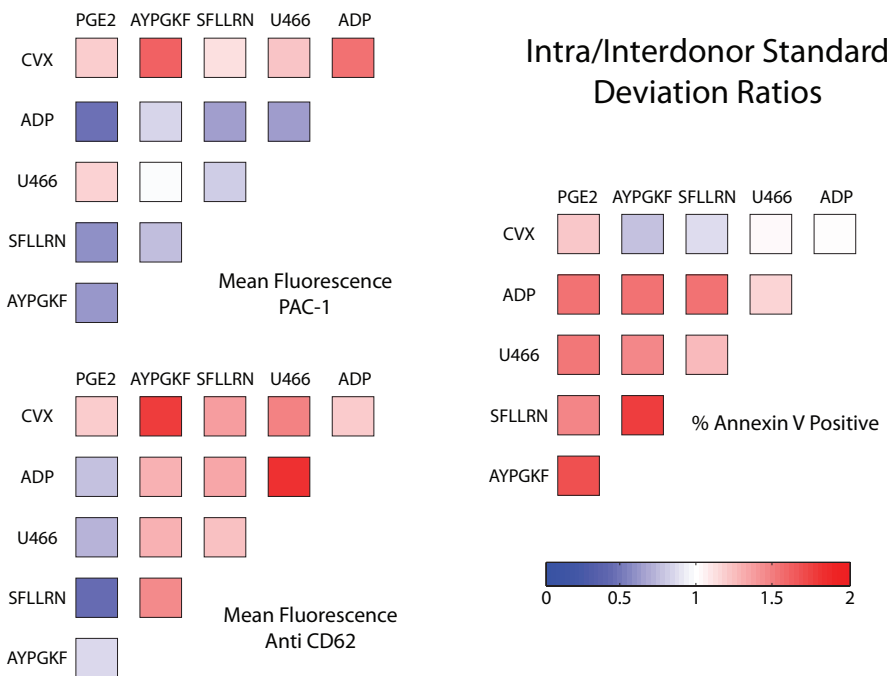

Figure 3. Comparison of variation within a donor to variation across donors. The mean of the standard deviations from each donor's 2 experiments is divided by the standard deviation for the mean value of each donor for all 45 data points. Some conditions vary more for a single donor (red) while others vary more across the cohort (blue).

for inhibitors of fibrin polymerization such as Gly-Pro-Arg-Pro, which would be required in the presence of thrombin to maintain sample viscosity. The use of U46619 (Sigma-Aldrich, St. Louis, MO) in place of the physiological thromboxane A_2 (TXA_2) was required due to the short (~30 s) half-life of TXA_2 in solution (21). Similarly, prostacyclin is a very short-lived molecule, so the more chemically stable prostaglandin E_2 (PGE_2; Sigma-Aldrich, St. Louis, MO) was chosen. In both cases, the more stable analog signals through the same receptor as the physiological ligand (8, 22). The only physiologic agonist that could be directly utilized in this assay was adenosine diphosphate (ADP; Sigma-Aldrich, St. Louis, MO).

96 well plate preparation

Each sample well of a white, flat-bottomed 96 well plate (Corning, Corning, NY) was loaded with 10 µL 10% v/v PRP, as well as 2 µL each FITC PAC-1, PE anti-CD62P (AK-4 clone), and Cy5 annexin V (BD Biosciences, San Jose, CA) as shown in Figure 1. In addition, 64 µL HBS was added to wells that would receive a pair of agonists, while
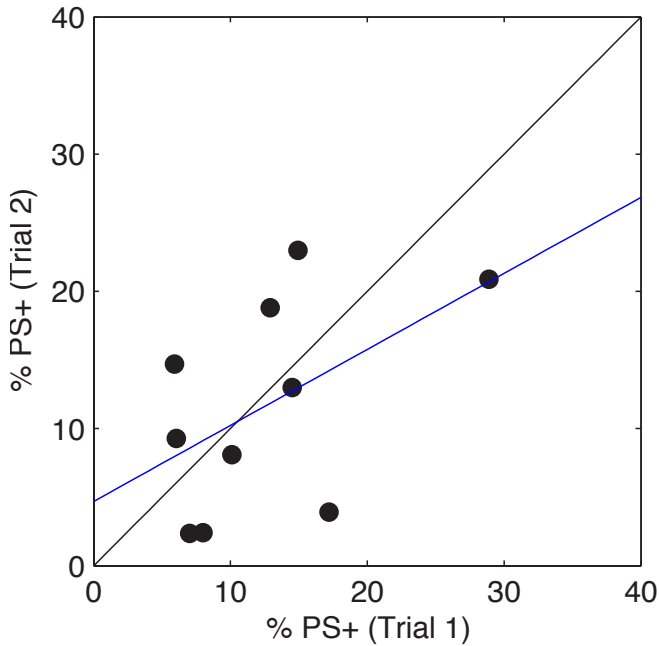
Figure 4. CVX-SFLLRN induced phosphatidylserine (PS) exposure correlation. The percentage of cells exposing PS in each donor's first sample (abscissa) is compared with the percentage measured in the second sample (ordinate) for the addition of convulxin and SFLLRN. The linear regression (blue line) shows correlation between trials ($R^2 = 0.2577$).

74 µL was added to wells for single agonist controls. Ten minutes prior to flow cytometry analysis, 10 µL of a 10× stock of the appropriate agonist was added, giving a final volume of 100 µL in each well. This gives a final concentration of 1% v/v PRP, which eliminates autocrine and paracrine signaling that could affect responses in a platelet concentration dependent manner (23).

Flow Cytometry

This assay utilized an Accuri C6 flow cytometer with CSampler (BD Biosciences, San Jose, CA) to automate well plate handling. The sample flow rate was set to low (14 µL/min with a 10 µm core), and samples were analyzed for 60 s following 10 min of incubation with agonist(s). The time required for movement of the CSampler arm and suction of each sample into the flow cytometer meant it was possible to analyze one sample every two minutes. Compensation was set such that 7.5% of FL1 was subtracted from FL2 and 4.0% FL2 from FL1 to account for emission spectra overlap between FITC and PE.

Results and discussion

Determining the dynamic range of each agonist

In order to determine the concentration range over which each of the six agonists affects integrin activation, degranulation, and PS exposure, each one was tested individually. No single agonist led to significant PS exposure, so EC_{50}s could not be calculated for that response. Sigmoidal dose-response curves were constructed based on the mean fluorescence of PAC-1 and anti-CD62P binding at each dose. The inhibitory effects of PGE_2 were studied by simultaneous stimulation with 15 µM SFLLRN. This allowed us to define an EC_{50} for each agonist for both integrin activation and degranulation (Table 1). Addition of U46619 did not elicit an increase in either signal,
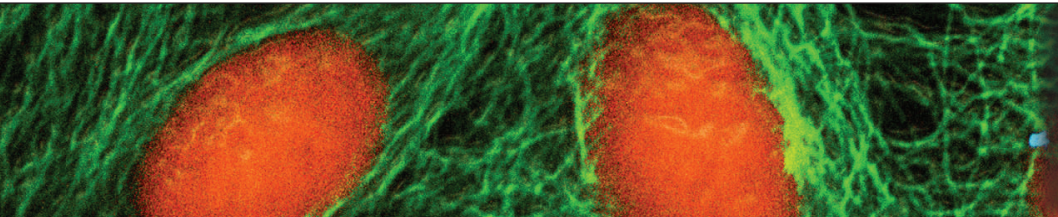
but it does exhibit signaling in the pairwise conditions. The EC_{50} concentrations of each agonist calculated for PAC-1 and anti-CD62P binding are within a factor of three of those seen in the previous calcium assay (2). Therefore, we chose to use the concentrations from the calcium assay so that data from both assays would be directly comparable.

Pairwise agonist combinations and donor specificity

Each of the 15 pairwise combinations of the 6 agonists at their EC_{50} concentrations was tested. For each sample, the mean fluorescence of PAC-1 and anti-CD62P binding was determined, as well as the percentage of PS exposing platelets. To determine whether the donor specificity seen in the pairwise calcium responses was maintained through the signaling cascades to integrin activation, degranulation, and PS exposure, PAS-FC was carried out on blood draws no more than 2 weeks apart for each of 10 healthy male donors. The 45 data points from each experiment (15 agonist pairs × 3 colors for $\alpha_{IIb}\beta_3$ activation, P-selectin display, and PS exposure) were converted into a vector that became a column in the pairwise response matrix (Figure 2). Donor specificity was determined by generating a hierarchical cluster tree based on the Euclidean distance between each possible pair of vectors as calculated by the linkage function in MATLAB. Of the 10 donors, 4 exhibited self-clustering, where their 2 vectors clustered together, indicating the possibility of donor specificity. To determine the significance of 4 out of 10 donors self-clustering, in silico cohorts of 10 hypothetical donors were constructed by randomly choosing values from the 10 original donors for each of the 45 data points. Simulating 10 million such cohorts showed that 4 self-clustering donors is highly significant ($P < 0.001$), and no cohorts with 8 or more self-clustering donors ever occurred.

Donor similarity

Given that this cohort is less diverse in genetic ancestry than the one used for the calcium assay (seven Europeans, one Indian, and two Asians here versus three Europeans, two Asians, two Indians, one Caribbean, one African American, and one African), less donor self-clustering may be a result of donor similarity. To examine this, we calculated the mean of the 10 donor standard deviations (intradonor variation) as well as the standard deviation of the mean value for each donor (interdonor variation) for each of the 45 data points. The ratio of intradonor variation divided by the interdonor variation is shown in Figure 3, where red indicates that variation within the repeats of a donor was larger than variation across donors, while

blue indicates variation across the cohort was more significant. For 17 of the 30 PAC-1 or anti-CD62P binding data points, the intra-donor variation was dominant, which makes it difficult to reliably distinguish donors and contributes to the low self-clustering. Some self-clustering is achieved, however, because of the relative donor specificity of PS exposure, anti-CD62P binding in the presence of PGE_2, and PAC-1 binding in the absence of convulxin. The annexin V positive data for non-CVX conditions can be ignored as there is no PS exposure.

In addition to the genetics of the donors, the range of the experimental space that was explored may contribute to the reduced self-clustering of this assay compared with the previous calcium based assay. Time constraints limited study to only pairwise combinations at EC_{50} concentrations, as completion of the 135 combinations of a pairwise scan at 0.1, 1, and 10× EC_{50} would take 4.5 h to complete. Platelets removed from the body, as well as the agonists, lose reactivity over that amount of time, and fixation is not possible because it artificially induces PS exposure and diminishes PAC-1 binding (24). Given this limited experimental space and our cohort of genetically similar, healthy donors, 4 of 10 self-clustering is likely a typical level of patient specificity that can be achieved currently. Increasing the number of experiments per donor to four or six may increase specificity, but the largest gains to be made are in expanding the concentrations of agonists tested. Testing multiple concentrations of each agonist would provide information about donor-specific sensitivity and maximum response that would allow this assay to provide a more complete picture of platelet activation.

In order to further investigate donor specificity, the percentage of cells exposing PS in response to convulxin and SFLLRN costimulation for each donor's two samples was compared (Figure 4). This response shows correlation between samples ($R^2 = 0.2577$), and a wide range of values are seen. This is consistent with previously reported donor specificity of maximal PS exposure between 20%–70% upon treatment with convulxin and thrombin (25). Such high values are not achieved in this assay due to lower agonist concentrations, but the variable potential for PS exposure among donors is still evident. Expansion of the experimental space to include higher agonist concentrations would provide valuable patient-specific information about maximal responses in PS exposure as well as integrin activation and degranulation, but this requires a flow cytometer that can collect and measure samples more quickly.

There is potential for improvement of this assay, which already gives a wealth of data about the effects of multiple simultaneous signals and significant donor specificity. Currently, there are some flow cytometers that are capable of analyzing a full 384-well plate in 15 min. Design of such a machine that also has the necessary liquid handling capabilities to add agonists at well-defined times before analysis would make full pairwise agonist scanning in flow cytometry possible. At such a point, the ability to test all 135 pairwise conditions for integrin activation, degranulation, PS exposure, and possibly even more measures of activation could provide a tremendous amount of donor specific information that could be utilized in models of thrombosis (4). Any response for which a fluorescently labeled probe exists can easily replace or be added to the three in this assay with the inclusion of a sufficient amount to measure the full dynamic range of the agonists. The number of responses that can be measured simultaneously is limited only by the ability of the flow cytometer to record their emission spectra.

Acknowledgments

Competing Interests

The authors declare no competing interests.

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