Organ-on-Chip Recapitulates Thrombosis Induced by an anti-CD154 Monoclonal Antibody: Translational Potential of Advanced Microengineered Systems

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Clinical development of Hu5c8, a monoclonal antibody against CD40L intended for treatment of autoimmune disorders, was terminated due to unexpected thrombotic complications. These life-threatening side effects were not discovered during preclinical testing due to the lack of predictive models. In the present study, we describe the development of a microengineered system lined by human endothelium perfused with human whole blood, a “Vessel-Chip.” The Vessel-Chip allowed us to evaluate key parameters in thrombosis, such as endothelial activation, platelet adhesion, platelet aggregation, fibrin clot formation, and thrombin anti-thrombin complexes in the Chip-effluent in response to Hu5c8 in the presence of soluble CD40L. Importantly, the observed prothrombotic effects were not observed with Hu5c8-IgG2α designed with an Fc domain that does not bind the FcγRIIa receptor, suggesting that this approach may have a low potential risk for thrombosis. Our results demonstrate the translational potential of Organs-on-Chips, as advanced microengineered systems to better predict human response.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
Although thrombosis can manifest clinically as a disease, it is becoming more apparent that some drugs also contribute to development of thrombosis, resulting in serious adverse events not always detected during preclinical in vitro testing or animal models.

WHAT QUESTION DID THIS STUDY ADDRESS?
The current study explored the possibility of using Organ-on-Chip technology to recapitulate and potentially predict the risk for thrombotic events induced by novel drug candidates.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
The current study describes a new system and experimental methodology for carrying out human studies on drug toxicity and thrombosis in vitro as well as dissecting the molecular mechanisms that mediate this process.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
The data generated in this study are consistent with clinical findings, highlighting the future potential of Organ-on-Chip technology to study and predict the risk of thrombotic side effects of drug candidates.

Activation of T cells via binding of the CD40 ligand (CD40L/CD154) to its receptor CD40 is a critical step in the initiation of the adaptive immune response. Blocking of CD40L-mediated signaling represents a validated therapeutic strategy for treatment of autoimmune disorders and for preventing organ transplant rejection. Preclinical studies have demonstrated that monoclonal antibodies (mAbs) against CD40L can be used to suppress organ transplant rejection or autoimmunity. However, the development of anti-CD40L mAbs was halted for several years because of incidents of thromboembolism and cardiovascular events during clinical trials with the drug candidates Hu5c8 and IDEC-131, which were under development for the treatment of lupus and Crohn’s disease. Specifically, Hu5c8 caused myocardial infarction in 2/28 patients in a lupus clinical study; cases of

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pulmonary embolism (two patients with one fatality), central nervous system thrombosis (three patients), and deep vein thrombosis (one patient) were reported in other clinical trials; however, the total number of patients in those trials were not disclosed.

Platelets represent the major source of circulating sCD40L, with high concentrations reported in patients with inflammatory diseases. It is believed that thrombosis by Hu5c8 is mediated by formation of a high-ordered immune complex (IC) of Hu5c8 with sCD40L and its binding to the FcγRIIa receptor that is specifically expressed on human platelets. The new generation of anti-CD40L mAbs under development need to provide a great degree of confidence that they have a low risk for thrombosis in patients. While platelet aggregation and activation assays have historically been the gold standards for in vitro assessment of risk for thrombosis, an in vitro model that captures the complexity of thrombosis, including human endothelial–platelet interactions and fibrin clot formation, would increase confidence as a useful tool for selecting compounds with reduced risk for use in human patients.

Advances in microengineering have recently made it possible to create miniaturized in vitro cell culture systems in which human cells and tissues are subjected to fluid flow and mechanical stress in well-controlled microenvironments, known as Organs-on-Chips. Organs-on-Chips allow for controlled studies of organ-level aspects of human physiology and disease, and have been successfully applied in the preclinical testing of therapeutics. We and others have recently described microengineered on-Chip systems containing human endothelium perfused with human whole blood at physiologically relevant shear rates to recapitulate many of the key aspects of thrombosis.

In the present work, we explored whether this on-Chip model of human umbilical vein endothelial cells (HUVECs) can be used as a preclinical tool to retrospectively predict the prothrombotic effects of Hu5c8-IgG1 (Hu5c8) in vitro and to prospectively compare the outcome with the modified mAb Hu5c8-IgG2. It is believed that thrombosis by Hu5c8 was mediated by binding of IgG1 to its Fc receptor on platelets, and that thrombosis by Hu5c8 was mediated by binding of IgG1 to its Fc receptor on platelets, and the new generation anti-CD40L mAbs have been designed to prevent this interaction. Evaluation of Hu5c8-IgG2 receptor, formatted not to bind FcγRIIa receptor. These results provide confidence that the newer-generation anti-CD40L mAbs in the clinic that have been designed not to bind FcγRIIa receptors have a potential low risk for thrombosis. The ability of the model to provide reliable measurements of clinically relevant endpoints makes it a suitable platform to assess risk for thrombosis for a broad class of molecules developed for therapeutic applications.

RESULTS
A microengineered blood Vessel-on-Chip
The design of our Vessel-Chip is based on a previously described two-channel microengineered chip design used to create a human Lung-Chip, which we have already demonstrated to be suitable for in vitro drug testing. Briefly, the Vessel-Chip is made of a transparent elastomer (PDMS) with two main fluidic chambers separated by a thin porous membrane (Figure 1a). The geometry of the lower vascular microchannel was slightly modified to incorporate an additional anticoagulant port (Figure 1a), which is a critical element for blood sampling and downstream analysis of soluble biomarkers, as described below. Notably, while in the original Lung-Chip human umbilical vein endothelial cells lined only the lower surface of the porous extracellular matrix-coated membrane that separates the two microchannels; here we applied a recently described protocol to ensure full surface coverage of all four walls of the vascular channel, which resulted in the formation of a hollow endothelium-lined vessel within the lower compartment of the chip (Figure 1a). Within 48 hours postseeding, the endothelial cells formed a confluent monolayer, as indicated by a continuous pattern of intercellular staining for the junctional protein, VE-Cadherin, when analyzed by immunofluorescence microscopy (Figure 1b; Figure S1, Movie S1). When perfused with cell culture medium, the endothelium remained stable for at least 6 days postseeding, as confirmed by phase contrast microscopy (Figure S2c) and by quantifying vascular permeability using fluorescent dextran as previously described (Figure S2a,d).

Analysis of Thrombosis-on-Chip
To test the thrombotic modulating activities of our Vessel-Chip, we perfused freshly collected human whole blood through the lumen of the Vessel-Chip. Because baseline platelet activation can occur due to prolonged storage (data not shown) or donor characteristics, blood samples were assessed for activation prior to compound testing. Approximately one in five donors exhibited significant platelet adhesion under these conditions, and these samples were not used for additional testing. Platelets were stained using fluorescently labeled nonblocking antibodies for the platelet surface marker CD41 and low concentrations of fluorescent fibrinogen were added to enable visualization of fibrin deposition in real-time. Importantly, the blood was recalibrated to reestablish its full coagulation potential immediately prior to being introduced into the main inlet of the vascular channel
Figure 1. On-Chip reconstitution of thrombosis. (a) Left, schematic representation of the Vessel-Chip showing the main features: inlet port, main channel, and imaged area, outlet port and the on-Chip anticoagulant port. Right, schematic cross-section of the chip showing all four walls of the vascular channel lined with endothelial cells. (b) Top, endothelial morphology by fluorescent VE-cadherin staining (green) and nuclei staining (blue). Bottom, high magnification section of endothelial VE-cadherin staining (bar 20 μm). (c) Under control conditions (“Blood”) platelets (red) and fibrin (cyan) are sparse and detectable at the edge of the imaged areas. Endothelial exposure to TNF-α (“TNF-α”) or preincubation of blood with soluble collagen (“sCollagen”) led to formation of a high number of larger aggregates containing a platelet-rich core decorated with fibrin (bar 100 μm). (d) Typical ultrastructure of blood clots formed on-Chip; in control conditions (“Blood”) the sparse platelets are dispersed on an endothelial surface. In stimulated conditions (“TNF-α” and “sCollagen”) activated platelet aggregates and fibrin networks with trapped red blood cells are attached to an endothelial cell surface (bar 5 μm). Methods for sampling, processing, and imaging for scanning electron microscopy are described in the Supplementary Information. (e) Platelet coverage and fibrin deposition were significantly increased in stimulated, prothrombotic conditions in multiple donors (n = 4, error bars denote SEM; *P < 0.05), and the effects were suppressed by adding the drug eptifibatide. Preparation of the Vessel-Chip and imaging of fixed samples are described in the Supplementary Information.

(Figure S2a,b). After 14 minutes of continuous perfusion, the flow of blood was interrupted and the chips were immediately imaged using fluorescence microscopy. Under normal healthy conditions, the endothelium provided an antithrombotic surface where blood flowed smoothly, and indeed we detected minimal platelet adhesion or fibrin deposition under control conditions (Figure 1c). The vascular endothelium can also act as a signaling platform that mediates the recruitment of different blood cells under conditions of tissue inflammation.30 We recently showed that human endothelium grown under similar conditions on-Chip and treated with the inflammatory cytokine, tumor necrosis factor-α (TNF-α), demonstrated enhanced expression of factors such as tissue factor, von Willebrand factor, and adhesion molecules that are key mediators of thrombosis.31 In the present study, to explore how the system specifically responds to activation of endothelium vs. platelets, we pretreated the endothelium with TNF-α (50 ng/mL) for 6 hours to mimic tissue inflammation and compared these results with those obtained by administering soluble collagen (sCollagen; 10 μg/mL), which is a standard platelet activator frequently used in vitro and in vivo.32 Treatment with either TNF-α or sCollagen led to significant stimulation of platelet aggregation and fibrin deposition on the surface of the endothelium, as demonstrated by increased areas of platelet coverage and fibrin deposition (Figure 1c). Scanning electron microscopic (SEM) analysis also revealed that TNF-α pretreatment of the vascular endothelium induced formation of compact clots composed primarily of erythrocytes and platelets surrounded by a fibrin network, whereas blood treated with sCollagen formed a meshwork of complex fibrin-rich clots that contained mostly red blood cells with altered stellate morphology, which is known to be associated with the retraction of fibrin during the later stages of blood clotting33,34 and with release of inflammatory cytokines (Figure 1d).35 These differences in clot composition are consistent with the mechanisms of thrombosis by both agents. Specifically, thrombosis by TNF-α is primarily driven by activation of the endothelium and release of factors that promote adhesion and platelet-to-platelet interactions which then lead to local thrombin activation, fibrin formation, and clot stabilization.36 In contrast, thrombosis by sCollagen involves direct activation of the classic intrinsic coagulation pathway, which induces fibrin formation and activation of platelets in parallel via binding of sCollagen to their integrin α2β1 receptors.37 Furthermore, we confirmed that the ability of both these prothrombotic treatments (TNF-α and sCollagen) to promote platelet aggregation and fibrin clot formation could be suppressed by coadministering a clinically relevant concentration of eptifibatide (2 μg/mL) (Figure 1e; Figure S2c) that inhibits the endogenous platelet integrin αIIB/βIII receptor,
which mediates fibrinogen binding. Together, these data provide convincing evidence of de novo formation of fibrin-rich clots in vitro, a relevant pathophysiological endpoint for thrombosis.

**Hu5c8 induces Thrombosis-on-Chip**

Having demonstrated that key endpoints for thrombosis could be studied in the microengineered Vessel-Chip, we next investigated whether the system could be used retrospectively to recapitulate thrombosis associated with Hu5c8. Platelet activation assays conducted to study thrombotic risk for this molecule typically use optimized, but not clinically relevant, stoichiometric ratios of Hu5c8 and sCD40L to generate high-ordered immune complexes. In our studies, we tested Hu5c8 at 240 μg/mL, which was benchmarked to plasma concentrations at 20 mg/kg in cynomolgus monkeys, which is the same dose that caused thrombosis in human; it was presumed that the concentrations in monkeys would approximate human plasma levels at 20 mg/kg which have not been published. We also used disease-relevant concentrations of sCD40L (10 ng/mL), benchmarked to serum concentrations reported in lupus patients (1.5–15 ng/mL). Blood from four donors treated with or without Hu5c8, sCD40L, or both (Hu5c8/sCD40L) was perfused through the lumen of the microengineered vessel at a flow rate of 60 μL/min, yielding a wall shear stress of 5 dyne/cm², which is comparable to values found in veins under physiological conditions. There were no significant treatment-related effects when the endothelium was exposed to either sCD40L or Hu5c8 alone compared to untreated blood, whereas treatment with Hu5c8/sCD40L induced a large increase in platelet aggregate formation and fibrin deposition on the endothelium (Figure 2a). Quantification of platelet coverage conducted on four different donors, all tested in duplicate using computerized image analysis, confirmed that only the combination of Hu5c8 and sCD40L, and not either Hu5c8 or sCD40L alone, significantly promoted clot formation within the Vessel-Chip (Figure 2b). Additionally, in line with the hypothesis that binding of Hu5c8 to sCD40L promotes platelet activation and aggregation, ultimately causing thrombosis in vivo, SEM analysis of the microengineered vessels perfused with blood containing the combination of Hu5c8/sCD40L revealed the presence of micro-thrombi that were rich in fibrin (Figure 2c). Modest, but statistically significant, increases in expression of von Willebrand factor (vWF), platelet-endothelial adhesion molecule-1 (PECAM-1, CD31), and CD40 were observed only in samples treated with Hu5c8/sCD40L, suggesting that the endothelium becomes activated under these conditions (Figure 2d). We conclude that our Vessel-Chip, perfused with recalcified human blood, is capable of recapitulating Hu5c8-mediated thrombosis at clinically relevant concentrations of Hu5c8 and sCD40L.

**Biomarker assessment on-Chip**

As blood eventually coagulates inside the components and tubing of the microengineered system, continuous blood sampling from the on-Chip vessel becomes virtually impossible over time. To overcome this limitation, we complemented the outflow port of our Chip with a third port where anticoagulants, such as sodium citrate, can be introduced into the flow and mixed with blood immediately before exiting the outlet of the vascular channel (Figure 3a). To functionally test the system, recalcified blood was perfused through the vascular channel, while citrate was introduced on-Chip via the port situated next to the outlet of the vascular channel (Figure 3a), and effective anticoagulation could be detected at both the macro scale and micro scale (Figure S3). Blood sampled from the chip was analyzed for TAT complex, an accepted clinical blood biomarker for procoagulation. Levels of TAT (Figure 3b) were significantly increased following treatment with TNF-α or Hu5c8/sCD40L, and minimally increased with sCollagen, demonstrating a good correlation with the imaging endpoints described above. Thrombin converts fibrinogen into fibrin during clot formation, and antithrombin plays an important role in maintaining homeostasis by inhibiting the effect of thrombin. Formation of TAT in the Vessel-Chip therefore confirms that local and intrinsic generation of thrombin, a potent platelet agonist, occurs in the microengineered vessel and that counter-regulatory mechanisms for coagulation are retained. In addition to anti-thrombin evidenced by TAT formation, mRNA levels of the SERPINE class of inhibitors of blood coagulation proteases, plasminogen activator inhibitor-1 (PAI-1) and SERPINE-2, increased 8- and 2-fold, respectively (Figure S4). In contrast, there were no changes in the concentration of D-dimer in effluents from chips treated simultaneously with Hu5c8 and sCD40L (data not shown), suggesting that the rate of coagulation exceeded fibrinolysis under these assay conditions, or that longer incubation times may be required to observe formation of fibrinolytic products. We conclude that the Vessel-Chip allows for both qualitative and quantitative assessment of critical events characterizing blood clotting. The system is indeed able to recapitulate some of the key aspects of thrombosis, including platelet adhesion, aggregation, fibrin deposition, and release of clinical biomarkers related to clotting, such as TAT.

**Hu5c8-IgG2, an mAb designed not to bind FcγRIIa, does not induce Thrombosis-on-Chip**

Mechanistic studies using platelet assays suggest that high-ordered immune complexes of Hu5c8 and sCD40L activate platelets via interaction of the Hu5c8 IgG1 domain with platelet FcγRIIa receptors. This has influenced the design of anti-C40L mAbs with formats that do not bind to FcγRIIa receptors as a strategy for reducing risk for thrombosis. To investigate this hypothesis in our Vessel-Chip, which has incorporated additional complexity compared to platelet assays, experiments with Hu5c8-IgG1 were conducted in the presence of the FcγRIIa blocking antibody IV.3. In addition, we prospectively tested the IgG2 variant of Hu5c8, designed not to bind FcγRIIa receptors (Figure 4a). Blood samples from human donors were aliquoted and either treated with sCD40L, Hu5c8/sCD40L, Hu5c8-IgG2/3/sCD40L, Hu5c8/sCD40L/IV.3, or remained untreated (control). Each condition was tested with samples from 3 to 15 donors (for demographic information, see Table S1), and all conditions were tested in duplicate and analyzed for platelet coverage (Figure 4b), fibrin deposition (Figure 4c),...
or increased formation of TAT measured in the effluent (Figure 4d). Platelet coverage and fibrin deposition following treatment were normalized and reported as fold-increase relative to untreated control values for each donor.

These studies revealed that there were slight (up to ~2-fold) increases in platelet coverage in some of the donors treated with sCD40L; however, there was no significant increase in the mean value compared to controls.47,48 As expected, perfusion of Vessel-Chips with the combination Hu5c8/sCD40L resulted in a statistically significant increase in platelet aggregation and fibrin clot formation, as well as increased levels of TAT (Figure 4b–d). Blocking FcγRIIa with IV.3 blocking antibody prevented increases in platelet coverage, fibrin deposition, and TAT release mediated by treatment of Hu5c8 with sCD40L. More important, treatment with Hu5c8-IgG2α and sCD40L did not induce these endpoints. Collectively, these studies show that the prothrombotic endpoints induced by Hu5c8 in the presence of disease-relevant concentrations of sCD40L in our model also relies on FcγRIIa binding.20,47

**DISCUSSION**

Thrombosis can manifest clinically as acute coronary diseases, ischemic stroke, or deep vein thrombosis secondary to formation of arteriole or venous clots. Some therapeutics, such as the anti-CD40L mAb Hu5c8, can also cause thrombosis, resulting in serious adverse events. Given the continued interest in developing therapies against CD40L coupled with ethical concerns and expense associated with the conduct of primate studies, a predictive human-relevant system is needed. The system needs to recapitulate the major mechanisms associated with thrombosis in order to be useful for a derisking potential for thrombosis by new anti-CD40L mAbs as well as other targets where thrombosis may be of concern. Simple *in vitro* tests, such as light transmission platelet aggregometry and flow cytometry of platelet activation,
Figure 3  On-Chip anticoagulation of outflowing blood allows sampling and analysis of effluent. (a) Citrate solution is actively pushed into the outflow stream of blood as it leaves the vascular channel. This prevents clotting inside connectors and tubing, allowing for longer experiments as well as conventional analysis of the outflowing blood samples. (b) Thrombin antithrombin (TAT) levels can be analyzed in plasma from blood flowing out of the Vessel-Chip. TNF-α pretreatment of the endothelium, as well as sCollagen or the combination of sCD40L and Hu5c8 dosed in the blood before perfusion, leads to elevated levels of TAT in the outflow samples (n = 4, error bars denote SEM; *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant).

Figure 4  Mechanistic insights on the thrombosis induced by combination of Hu5c8 and sCD40L on-Chip. (a) Schematic representations of how antibodies with different structures modulate the interaction of platelets with immune complexes. By using antibodies with modified Fc regions (IgG2α, middle), or by using anti-Fcγ receptor blocking antibodies (IV.3, bottom), interactions between immune complex and platelets can be prevented. Only conditions with Hu5c8 containing a normal Fc region, and incubated in blood in the absence of blocking antibodies induced (b) platelet adhesion, (c) fibrin formation, and (d) TAT release on-Chip (n = 15; n = 5; n = 7, respectively, vertical bars denote mean, error bars denote SEM; *P < 0.05, ***P < 0.001). Information of blood donors can be found in Table S1.
can be used in candidate drug testing to signal a hazard for thrombosis, but such tests, although valuable, do not capture other contributory aspects of thrombosis, such as the mechanical forces of flowing blood, interaction of platelets with vessel walls, and crosstalk with the coagulation cascade. Therefore, there is a need for in vitro translational models that capture the relevant biology contributing to thrombosis.

In this study we describe a microengineered Vessel-Chip that contains a confluent endothelial layer that is perfused with human whole blood at physiologically relevant shear stress, thus incorporating critical elements that are relevant for studying human blood–endothelial interactions in vitro. It has also been designed to allow collection of effluent for biomarkers and we demonstrated that the Vessel-Chip can be used to analyze multiple in vitro-relevant endpoints for assessing risk for thrombosis, including platelet–endothelial interactions, platelet aggregation, fibrin clot formation, and release of markers of coagulation, such as TAT. These endpoints are more comprehensive and more physiologically relevant compared to platelet aggregation or activation assays that, albeit important, monitor one contributory aspect towards clot formation. The potential application of the Vessel-Chip is different from other systems, such as the Badimon chamber used in the clinical setting. A key difference is that the Badimon chamber assesses for thrombosis ex vivo by direct flow of blood from a treated clinical subject over exposed porcine aorta as the thrombogenic substrate, while the Vessel-Chip is suited for studying intrinsic clot formation caused by drug candidates in vitro and designed to provide mechanistic insights, making it more suitable for preclinical and laboratory translational research.

A systematic approach was taken in characterizing the Vessel-Chip to control for potential variability in the system. Assay and detection conditions for measurement of endpoints such as platelet aggregation, fibrin clot formation, and measurement of TAT were optimized using TNF-α as a positive control. Sources of potential variability were identified and controlled; for instance, blood was used within 5 hours of arrival to prevent spontaneous platelet activation, which is known to occur with prolonged storage. In addition, untreated donor blood was perfused through the chamber and donor samples that caused high background platelet adhesion were discarded; approximately one of five samples were excluded from use with this approach. Donor variability in platelet response is a well-known phenomenon, and this was minimized by controlling technical variability. We used these parameters to generate the standards for acceptance criteria of the data and ensure reproducibility. The conditions described were sufficient for conducting of fit-for-purpose proof-of-concept studies, but a more comprehensive validation of the Vessel-Chip will be required for it to meet qualification standards. This would include further characterization of inter- and intrassay variability, reproducibility, and chip storage stability using a larger donor pool and tool test compounds, e.g., TNF-α and a negative control that does not cause thrombosis. Inclusion of untreated controls will inform on incidence of background platelet activation. A confirmatory set of defined test compounds with varied mechanisms of thrombosis will then be used to challenge the robustness of the model further. Inclusion of positive and negative controls during routine testing would also be recommended, given the known donor variability in platelet response. Genetic analysis of the donor pool may also provide insight to the donor variability in response.

Apart from demonstrating the utility of the Vessel-Chip for drug discovery and development applications, we made a number of significant contributions to understanding the mechanism of thrombosis induced by Hu5c8. We used clinically relevant concentrations of Hu5c8 and sCD40L in human blood samples to get a thrombogenic effect in contrast to platelet assays that used supraphysiological concentrations. The mechanism for the increased sensitivity of our system is currently unknown and may be related to the completeness of the model in recapitulating key attributes of the coagulation cascade that is absent in platelet activation assays.

We observed a consistent donor variability in platelet aggregation, TAT secretion, and clot formation following perfusion of Hu5c8/sCD40L in the Vessel-Chip. Coincidentally, thrombosis induced by Hu5c8 and IDEC-131 was also noted only in a few patients: 2/28 participants in a lupus-nephritis clinical study with Hu5c8, and a single incident of thromboembolism produced by the IDEC-131 antibody in a Crohn’s disease clinical trial. Apart from demonstrating the utility of the Vessel-Chip for translational models that capture the relevant aspects of the coagulation cascade in response.

In summary, our results clearly demonstrate that the Vessel-Chip could become a useful system for prospective assessment of thrombosis-related issues during drug discovery, to derisk issues related to thrombosis during the drug development process, and to potentially serve as a model for evaluating anti-coagulant or antiplatelet agents.

**METHODS**

**Blood pretreatment and perfusion**

Fresh citrated blood samples from donors without medication prior to donation were purchased from Research Blood Components (Cambridge, MA). The donor population consisted of all ethnic backgrounds and the age ranged from 20–65 years old. Most of the samples were from male (75%) and 25% were from female (Supplemental Table S1). Cloning, expression, and purification methods used for obtaining Hu5c8 and Hu5c8 (IgG2σ) are described in the Supplementary Information. Hu5c8 or Hu5c8 (IgG2σ) were combined with sCD40L (Tonbo) in
phosphate-buffered saline (PBS) incubated for 20 minutes at room temperature. The solution was then diluted in a blood sample to reach the final concentration of 240 μg/mL (Hu5c8) and 10 ng/mL (sCD40L). Citrated human blood was used within 4 hours of a blood draw in order to minimize preanalytical effects on platelet function. Blood was incubated with antibodies, sCD40L, or 10 μg/mL of soluble collagen (BIODATA) for 20 minutes at room temperature. Platelets were labeled with human CD41-PE antibody (10 μL/mL, Invitrogen, La Jolla, CA) directly added to the blood and incubated at room temperature for 5 minutes. Cell culture methods and preparation of the Vessel-Chip is described in the Supplementary Information. The outlet of the chip was connected to a syringe pump with a system of tubing and connectors made of medical grade silicon that excludes any metal components or potential causes of platelet activation. Fluorescence image analysis is described in the Supplementary Information. Before every experiment, each Vessel-Chip was mounted on the chip, and 800 μL/mL of a solution containing 100 mM calcium chloride and 75 mM magnesium chloride to the blood to permit calcium- and magnesium-dependent platelet function and coagulation.

Additional Supporting Information may be found in the online version of this article.

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CONFLICT OF INTEREST
D.E.I. holds equity in Emulate Inc. and chairs its scientific advisory board. A.D.v.d.M. serves as a scientific consultant to the company.

AUTHOR CONTRIBUTIONS

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