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A cluster of immunoresolvents links coagulation to innate host defense in human blood[§]

Paul C. Norris^{1,*}, Stephania Libreros^{1,*}, Nan Chiang¹, and Charles N. Serhan^{1,†}

¹Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, 02115, USA

Abstract

Blood coagulation is a protective response that prevents excessive bleeding upon blood vessel injury. Here, we investigated the relationship between coagulation and the resolution of inflammation and infection by lipid mediators (LMs) through metabololipidomic-based profiling of human whole blood (WB) during coagulation. We identified temporal clusters of endogenously produced pro-thrombotic and proinflammatory LMs (eicosanoids), as well as specialized proresolving mediators (SPMs). In addition to eicosanoids, a specific SPM cluster was identified that consisted of resolvin E1 (RvE1), RvD1, RvD5, lipoxin B₄, and maresin 1, each of which was present at bioactive concentrations (0.1 to 1 nM). Removal of adenosine from the coagulating blood markedly enhanced the amounts of SPMs produced and further increased the biosynthesis of RvD3, RvD4, and RvD6. The cyclooxygenase inhibitors celecoxib and indomethacin, which block the production of thromboxanes and prostanoids, did not block the production of clot-driven SPMs. Unbiased mass cytometry analysis demonstrated that the SPM cluster produced in human blood targeted leukocytes at the single-cell level, directly activating ERK and CREB signaling in neutrophils and CD14⁺ monocytes. Treatment of human whole blood with the components of this SPM cluster enhanced both the phagocytosis and killing of *Escherichia coli* by leukocytes. Together, these data identify a pro-resolving LM circuit, including endogenous molecular brakes and accelerators, which promoted host defense. These temporal LM-SPM clusters can provide accessible metabolomic profiles for precision and personalized medicine.

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[†]Corresponding author. cserhan@bwh.harvard.edu.

*These authors contributed equally to this work.

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Introduction

Barrier breach poses multiple threats to human health in the form of bleeding, infection, and tissue destruction that is countered naturally by the processes of hemostasis, the acute inflammatory response, and tissue regeneration (1–3). Hemostasis (“stopping of blood”) progresses rapidly through initiation of the extrinsic or intrinsic pathways that converge in coagulation and clot formation. These events are accompanied by inflammation through the activation of platelets, neutrophils, and monocytes within hemostatic plugs (4, 5). Specific arachidonic acid–derived eicosanoids play integral roles in hemostasis and inflammation, for example, thromboxane A₂ (TxA₂; see table S1 for abbreviations of other lipid mediators) is a potent prothrombotic mediator, whereas prostaglandins and leukotrienes (6, 7) collectively increase vascular permeability, recruit neutrophils to injury sites, and position neutrophils for lipid mediator (LM) class switching from leukotriene biosynthesis to SPM production (8). This enables the production of specialized pro-resolving mediators (SPMs; see fig. S1), a process that is pivotal for the transition from inflammation to resolution (8–11). SPMs, in turn, counter-regulate proinflammatory mediators (for example, cytokines and LM), accelerate efferocytosis (the phagocytosis of dying and dead cells) and wound healing, as well as reduce antibiotic requirements, in part, by enhancing phagocytosis without immune suppression (8, 12). Hence, SPMs are considered to be immunoresolvents because they are agonists of the resolution of inflammation and infections (13).

Here, we devised an approach coupled with solid-phase extraction (SPE) and LM-SPM liquid chromatography-tandem mass spectrometry (LC-MS/MS)–based metabololipidomics to assess the endogenous mass of LMs and elucidate the temporal production and relationships between specific eicosanoids and SPMs in human tissues and SPM functions. Using this approach, we elucidated the SPM panel (that is, the cluster) of mediators produced during coagulation, including specific modulators of their biosynthesis, and the direct SPM signaling targets in human whole blood that promoted host defense against pathogens.

Results

LM-SPM profiling of human blood coagulation

To assess the relationship between blood coagulation, innate immune phagocytic function, and LMs, it was essential to obtain the complete LM profile by monitoring seven LM metabolomes focused on D-series resolvins, E-series resolvins, protectins, maresins, lipoxins, prostaglandins, and leukotrienes and their biosynthetic pathway markers during the coagulation time course of human blood. To this end, fresh human whole blood was subjected to coagulation through the intrinsic pathway (3) and monitored over time (0 to 24 hours) to confirm clot formation and its contraction. Each sample was rapidly snap-frozen and freeze-thawed (see Materials and Methods) to lyse the cells and extract total eicosanoids and SPMs from the supernatants for SPE-LC-MS/MS metabololipidomics. Blood clots formed between 8 to 15 min after the onset of coagulation, which was followed by clot retraction and serum formation, which increased rapidly between 4 and 8 hours based on increased 750-nm light transmission through the fluid phase that formed above retracted clots (Fig. 1A). The percentages of various leukocyte populations (neutrophils, lymphocytes,

and monocytes) and their viability were determined throughout the time course (tables S2 and S3).

Selective and sensitive targeted metabololipidomics identified increased endogenous amounts of both prothrombotic and proinflammatory mediators [thromboxanes (Tx_s), prostaglandin (PG_s), and leukotriene (LT_s)] and SPMs with different temporal profiles (Fig. 1, B to D, table S4). LC-MS/MS-acquired results gave at least 6 diagnostic ions for each mediator identified together with matched LC retention times with authentic mediators (Fig. 1C and fig. S2). The first cluster to appear included the eicosanoids Tx_{B2} (a marker of Tx_{A2}), leukotriene B₄ (LTB₄), and PGD₂ (Fig. 1D and table S4). A select SPM cluster, consisting of RvE1, RvD1, RvD5, MaR1, and LXB₄, increased throughout this time course, together with PGE₂ and PGF_{2α}. Note that although RvD2 was present in blood (fig. S2), it was not apparently increased in abundance during coagulation. RvD2 is produced by human adipocytes (14) and other human tissues (15–17), prevents secondary thrombosis and necrosis in dermal burn wounds (18), and is protective in abdominal aortic aneurysm (19). Principal component analysis (PCA) confirmed that the production of TXB₂, PGD₂, and LTB₄ were associated with early time points, whereas the specific SPM cluster was associated with later times during coagulation (Fig. 1E). Hence, initial platelet activation led to thromboxane production, as documented previously (20), and ongoing platelet-leukocyte interactions and transcellular biosynthesis contributed to the temporal production of SPMs during coagulation.

Regulation of human SPM production

In healthy donors, the highest concentrations of SPMs generated within 24 hours of coagulation in blood were as follows: RvD1 (549 pM), LXB₄ (303 pM), MaR1 (209 pM), RvD5 (115 pM), and RvE1 (58 pM) [the average values are reported (in pg/ml) in table S4]. These SPMs were markedly reduced in concentration individually and in total 98% in blood containing the anticoagulant heparin (Fig. 2A and fig. S3). The addition of an inhibitor of the platelet integrin α IIb β 3, a blocker of platelet-platelet interactions, reduced the total amount of the SPM cluster by ~50% ($P < 0.01$) and concomitantly increased clot volume by ~20% ($P < 0.01$) (Fig. 2B), without reducing cell viability (table S5). These results suggest that clot formation and platelet integrin-mediated retraction of the clot were both required for SPM production, and that most SPMs were produced from endogenous cellular substrates in whole blood.

Unmasking of further SPM production and specific SPM pathways

Because red blood cells release adenosine deaminase (ADA) to remove excess adenosine, we questioned whether this function affected SPM production during coagulation, and if so, whether removal of accumulated adenosine altered SPM production. Adenosine inhibits neutrophil functions, including the production of LTB₄ (21). We found that ADA statistically significantly increased SPM production in blood (Fig. 2C), which in total was more than 8 times greater at 24 hours than that achieved by coagulation alone (Fig. 2C; see fig. S4A for changes in the bioactive LM profile and pathway markers). Clearance of adenosine with ADA increased the production of specific SPMs from the coagulation cluster. These included RvD1 and RvD5, as well as the lipoxins, that is, LXA₅ produced from EPA, and 15

epi-LXA₄ produced from AA (Fig. 2, C and D and fig. S4A). RvD3, RvD4, and RvD6 were also identified and increased in abundance in the ADA-treated samples (Fig 2C and E). The greatest concentrations of these D-series resolvins were: RvD3 (150 pM), RvD4 (447 pM), and RvD6 (304 pM). ADA cleared adenosine ($P < 0.01$) by 1 hour during coagulation based on the LC-MS/MS-based monitoring of the protonated adenine fragment; MRM transition 268 > 136 (Fig. 2D; see Materials and Methods). In addition, ADA statistically significantly increased the number of platelet-neutrophil aggregates during the time course of coagulation (Fig. 2D and fig. S4B), suggesting that removal of adenosine enhanced SPM production through transcellular biosynthesis (22) involving platelet 12-LOX and neutrophil 5-LOX (23–25). In this context, platelet-neutrophil aggregates and transcellular biosynthesis lead to lipoxin production (23, 26) through LTA₄, as well as maresin production through 14-HpDHA (24, 25). AT-LXA₄ and LXA₅ were also increased in abundance during coagulation (Fig. 2C and fig. S4A). PCA indicated that the amounts of LTB₄ (a potent chemoattractant) were greatest at 1 hour of coagulation (with and without ADA) and the SPMs were present in the greatest amounts at 24 hours of coagulation when ADA was added to clear adenosine (Fig. 2F). Cell viability was not decreased by ADA (table S5). These results suggest that coagulation enables most SPM pathways in human blood to produce proresolving mediators that are substantially regulated by the local adenosine concentration and ADA.

Next, we assessed the effects of therapeutic cyclooxygenase 1 (COX-1) and COX-2 inhibitors on SPM production during coagulation because NSAIDs block the biosynthesis of thromboxanes and prostaglandins, as well as increase bacterial killing in blood (27). We found that total prostaglandin and thromboxane production was blocked by indomethacin (>99% inhibition) compared to that during coagulation alone at 24 hours (fig. S5A). In the presence of indomethacin, total SPM amounts did not substantially change compared to those in the absence of inhibitor (see table S6 for the complete time course). Addition of the COX-2 inhibitor celecoxib did not statistically significantly alter the amounts of SPMs, prostaglandins, or thromboxanes in whole blood (fig. S5B), whereas the addition of the lipoxygenase inhibitor baicalein (BAI), which has greater selectivity toward lipoxygenases than toward cyclooxygenases (28), reduced the total amounts of SPMs (fig. S5B). Hence, these results suggest that the SPMs generated during coagulation were produced through lipoxygenase-initiated pathways and were not affected by NSAIDs.

Excessive inflammation and vascular permeability promote the formation of hemorrhagic exudates that contain increased numbers of red blood cells and microthrombi (5), whereas sterile (29) and purulent exudates (30) contain predominantly leukocytes. To assess SPM production in vivo during coagulation, we used an established sterile zymosan-initiated murine peritonitis model (29) in combination with intraperitoneal (i.p.) administration of thrombin, which increased the numbers of red blood cells and leukocytes in hemorrhagic exudates (fig. S6, A and B). SPMs from the human blood coagulation cluster, namely RvE1, RvD1, RvD5, LXB₄, and MaR1, were also present and statistically significantly increased in abundance in hemorrhagic exudates compared to sham saline alone (fig. S6, C and D). These results indicate that the same human SPM coagulation cluster that we identified in vitro was also produced in vivo in hemorrhagic exudates in mice.

Cell targets of SPMs in whole blood

To assess whether the SPMs produced during coagulation were active signal transducers in whole blood, we used time-of-flight mass cytometry (CyTOF) (31) to identify specific SPM cell targets. For example, RvE1 in whole blood reduces the abundances of L-selectin and CD18 in neutrophils and monocytes, diminishing leukocyte rolling (32). We incubated fresh peripheral human blood with RvE1, RvD1, RvD5, LXB₄, or MaR1 (the constituents of the coagulation SPM cluster) at 37°C and then stained the samples with a panel of mass tag antibodies specific for 19 cell surface proteins and the phosphoepitopes of eight intracellular proteins (table S7). We found that the abundances of phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK1/2) and phosphorylated the cAMP response element binding protein (CREB) were increased in individual CD15⁺ neutrophils and CD14⁺ monocytes within whole blood as visualized by t-Stochastic Neighbor Embedding (viSNE) unsupervised clustering analysis (33) of each SPM (Fig. 3 A and B and fig. S7).

Each SPM led to a statistically significant increase in the abundances of pERK1/2 ($P < 0.01$) and pCREB ($P < 0.0001$) in neutrophils and CD14⁺ monocytes (Fig. 3, C and D). The abundances of phosphorylated p38 mitogen-activated protein kinase (MAPK), S6, and AKT in CD14⁺ monocytes were statistically significantly increased by each SPM (Fig. 3 C and D, Fig. S8 $P < 0.001$). In CD20⁺ B cells, only RvD5 and LXB₄ substantially increased the abundance of pS6 ($P < 0.001$). The SPMs, specifically RvE1, RvD1, RvD5, MaR1, and LXB₄ (at 50 nM), did not stimulate the phosphorylation of NF- κ B or the signal transducer-activator of transcription (STAT) family members STAT3 and STAT5. SPADE analysis of mononuclear cells (CD45⁺CD41⁻CD15⁻) demonstrated increases in pERK and pCREB abundances within myeloid cell subsets by these SPMs (Fig. S8C). Together, these results established a rank order of SPM-activated intracellular signaling in whole blood that showed the most substantial increases in phosphoprotein abundances mostly in neutrophils and monocytes.

SPM host defense actions in human whole blood and phagocytes

Because RvE1, RvD1, RvD5, LXB₄, and MaR1 each activated intracellular signaling in phagocytes (monocytes and neutrophils) within whole blood (Fig. 3 and fig. S8), we investigated the specific and combined host defense actions of these SPMs. Within human whole blood, this SPM panel (used together at 1 to 50 nM each) statistically significantly reduced *E. coli* survival obtained at concentrations as low as 1 nM (see Fig. 4A) and enhanced the phagocytosis of *E. coli* by neutrophils (CD66b⁺ cells) at concentrations as low as 100 pM (Fig. 4B). The SPM panel also enhanced the phagocytosis of *E. coli* by monocytes (CD14⁺ cells) in whole blood at 1 nM ($p < 0.01$) as measured by flow cytometry. Imaging flow cytometry of whole blood neutrophils (CD66b⁺) and monocytes (CD14⁺) showed an increased fluorescence intensity caused by the phagocytosis of *E. coli* (BacLight) in samples incubated with the SPM cluster panel compared to that in control samples incubated with vehicle (Fig. 4C). We then assessed the effects of endogenous SPMs produced during coagulation on bacterial killing. Human blood was incubated with *E. coli* in the presence or absence of a lipoxygenase inhibitor. Bacterial counts were statistically significantly greater (>10 -fold; $P < 0.0001$) in the presence of the lipoxygenase inhibitor (fig. S9A), which coincided with a $>80\%$ reduction in the abundances of members of this SPM

cluster from coagulation (fig. S9B). Thus, blockade of endogenous SPM production impaired bacterial killing by peripheral blood phagocytes

Thrombus formation compartmentalizes systemic bacteria within microvessels to minimize bacterial tissue invasion, which in part promotes the intravascular association between bacteria and macrophages (34). We therefore questioned whether clot-derived SPMs specifically enhanced the phagocytosis of bacteria by macrophages in addition to their clearance by blood neutrophils and monocytes. We found that the extent of phagocytosis of *E. coli* by human macrophages was statistically significantly enhanced by individual SPMs of the coagulation cluster, namely RvD1, RvE1, LXB₄, and MaR1 (Fig. 4, D and E). Individually, LXB₄ and RvE1 evoked the greatest increases in macrophage phagocytosis. Members of the SPM panel, each at 1 nM when used together, resulted in enhanced phagocytosis by macrophages when compared to that by macrophages treated with select SPMs alone (Fig. 4E). These results suggest that the SPMs produced during blood coagulation potentially enhance bacterial killing and containment by the predominant leukocytes (that is, neutrophils and monocytes) in human blood. To further demonstrate the utility of this approach with diseased tissues versus healthy tissues, we obtained solid tissue tumors and healthy human testis, which is a known location for DHA enrichment in human organs, and malignant testis for direct comparisons. Each tissue gave clearly distinct LM-SPM profiles. For example, these tissues had both prostaglandins and thromboxanes, whereas healthy testis showed increased amounts of SPMs (fig. S10). These data suggest that diseased and healthy tissues can be profiled and compared through our approach.

Discussion

Thromboxanes and prostaglandins are well-established modulators of coagulation (6); however, the roles of lipoxygenase-derived mediators, such as the resolvins and other SPMs produced in human whole blood, are unclear. Our findings suggest that a specific cluster of SPMs is formed during coagulation and their actions target phagocytes in the surrounding milieu functioning in whole blood. RvE1 increases phagocytosis by isolated macrophages through GPCR-mediated pathways (that is, the ChemR23 receptor) that activate ribosomal S6 (32). RvD1 and MaR1 each increase the abundance of pCREB in human monocytes (35). Our single-cell analysis demonstrated that RvE1, RvD1, RvD5, LXB₄, and MaR1 (constituents of the same clot-SPM cluster) each activated CREB and S6 in neutrophils and monocytes, which led to enhanced phagocytosis by these leukocytes in blood, accelerating the first line of defense against pathogens. Another SPM-activated pathway in human whole blood that we identified was the phosphorylation of S6 in B cells (Fig. 3, C and D). This may play a role in B cell responses given that SPMs are also present in lymphoid organs (15), and that RvD1, RvD2 and MaR1 regulate the adaptive immune responses mediated by T cells (36). Of interest, supplementation of the diet with omega-3 fatty acids attenuates the production of proinflammatory cytokines (37) and increases the amounts of SPMs in peripheral blood (15, 38). A study identified that omega-3 essential fatty acids, the precursors of SPMs, were biomarkers of a lower risk for fatal coronary heart disease (39, 40). Hence, our results suggest a potential physiologic mechanism by which coagulation initiates the endogenous production of functional n-3-derived SPMs that affect innate immune cells. Because the physiologic coagulation of blood is protective in humans, SPM

production by clots may be of direct relevance to pathophysiology events in surgery (41), infection (11, 27, 42, 43), vascular inflammation (44, 45), stroke, and cancer (46, 47).

Resolvins and protectin D1 are present in plasma and serum (15, 38). Specifically, RvD1, RvD2, PD1, and 17*R*-RvD1 were identified in human plasma (38) before it was possible to identify RvD4 with a matched synthetic standard (48). Thus, in view of the present results indicating that coagulation produces the SPM cluster (RvE1, RvD1, RvD5, LXB₄, and MaR1), this did not appear to involve increases in the amounts of PD1, MaR2, RvD2, RvE2, or RvE3. Hence, the plasma SPM quantities and those of the specific members of the identified clot-driven SPM cluster may reflect blood-borne production of SPMs, the release of SPMs into circulation from tissues, or both. Although the SPMs identified herein were not studied under blood flow conditions, note that COX-2 is increased in both abundance and activity by laminar shear stress (49) and hypoxia (50). COX-2 can also contribute to the production of SPMs, including RvE1, by cell-cell interactions between blood leukocytes and vascular endothelial cells (8).

To illustrate and demonstrate the utility of our profiling system, we also determined whether differences in lipid mediator-SPM profiles between healthy and diseased tissues could be discerned with this LC-MS/MS-based metabololipidomic approach. As an example of this, healthy human testis tissue, which is rich in DHA that is essential for fertility and spermatogenesis (51), and malignant testis tissue each gave distinct profiles. The seminoma tissue had both prostaglandins and thromboxanes and the normal testis tissue had increased amounts of SPMs (fig. S10). Normal testis tissue contained statistically significantly greater amounts of SPMs and LTB₄ (fig. S10B), illustrating the potential diagnostic capacity of our metabololipidomic approach with human whole blood and solid tissue tumors as well as suggesting a function for SPMs in this tissue.

Our LM-metabololipidomics results demonstrated a temporal sequence in the synthesis of the families of lipid mediators that was initiated through the coagulation of human blood. The prothrombotic TxA₂ and the inflammatory eicosanoids (prostaglandins and leukotrienes) were rapidly produced by platelets, as anticipated, as well as by platelet-leukocyte aggregates, with a peak in the formation of specific proresolving mediators of inflammation. These resolution mediators included a distinct SPM cluster, consisting of RvD1, RvD5, RvE1, LXB₄, and MaR1, each member of which activated distinct intracellular signaling pathways in single leukocytes within the whole blood matrix, involving ERK1/2, CREB, p38 MAPK, S6, and AKT. These SPMs produced through clot formation enhanced both the phagocytosis and killing of *E. coli* by human neutrophils, monocytes, and macrophages.

The full functional potential of the biosynthesized SPMs was obscured by the accumulation of local adenosine, which inhibited the production of SPMs, thus providing additional evidence for resolution-toxic agents that disrupt SPM production at sites of inflammation (8). Artifactual red cell hemolysis occasionally occurs during blood collection, which leads to an increase in the amount of adenosine, which reduces platelet-neutrophil interactions (52). Thus, clearing of adenosine increased platelet-neutrophil aggregation and the biosynthesis of RvD3, RvD4, and RvD6, which enabled quantitation of the full spectrum of

D-series resolvins, except for RvD2, which was not increased during whole-blood coagulation (Fig. 1), yet is protective against abdominal aortic aneurysm (19) and secondary thrombosis and necrosis in thermal burn wounds (18). SPMs also stimulate macrophages to phagocytize thrombi in the form of fibrin clots (53), which may facilitate the remodeling of clots or their removal. The isolation and workup procedures reported herein could enable identification of the spectrum of SPMs produced by human tissue and their function in this milieu. Together, these results demonstrated previously uncharacterized links mediated by SPMs between the coagulation of blood and innate host defense mechanisms activated by specialized proresolving mediators in human tissues. This approach and new procedures for SPM profiling in human tissues opens new avenues for both personalized and precision medicine given that the substrates for LMs and SPMs are essential and are acquired by the host through nutrients.

Materials and Methods

Human peripheral blood isolation

Fresh human blood was collected with or without heparin (10 units/ml) from healthy volunteers with specific tubes for collections and 19-gauge butterfly needles with collection syringes to minimize potential cell damage. Each volunteer gave informed consent under protocol # 1999-P-001297, which was approved by the Partners Human Research Committee. All volunteers denied taking nonsteroidal anti-inflammatory drugs for ~2 weeks before donation.

Coagulation of human blood

Human whole blood was transferred in 4-ml aliquots to negatively charged, silicone-coated 10-ml tubes (BD) without anticoagulant. For experiments with heparin, the heparinized blood was placed in 15-ml polypropylene tubes before incubation at 37°C. For LM profiling at designated times, all samples were immediately subjected to a workup procedure by which whole blood was snap-frozen in a dry ice/isopropanol bath and were returned to room temperature ($\times 3$ cycles) before undergoing centrifugation at 100,000g at 4°C. Supernatants (and clots for select experiments; see fig. S3) were collected and were subjected to LC-MS/MS LM metabololipidomics.

LM metabololipidomics

To obtain a complete blood profile of eicosanoids and SPMs each sample was subjected to a procedure involving snap-freezing of whole blood that was then thawed to room temperature three times and centrifuged at 100,000g at 4°C for 30 min before undergoing solid-phase extraction (SPE). Internal standards including d8-5-HETE, d5-RvD2, d5-LXA₄, d4-LTB₄, and d4-PGE₂ (500 pg each; Cayman Chemical) were added together with four volumes of methanol to facilitate protein precipitation. After centrifugation at 1000g at 4°C for 5 min, each sample volume was reduced using a stream of nitrogen gas to 10% methanol and next loaded onto solid-phase extraction (SPE) Isolute C18 SPE 3-mL, 100 mg cartridges (Biotage) after rapid acidification (<30 s) to ~pH 3.5. Before elution, LM bound to the SPE matrix were neutralized with ddH₂O. Methyl formate fractions from the SPE were brought to dryness under a gentle stream of nitrogen and resuspended in 1:1 methanol:water before

injection into a liquid chromatography-tandem mass spectrometry system consisting of a QTrap 5500 (AB Sciex) equipped with a Shimadzu LC-20AD HPLC (Tokyo, Japan). A Poroshell 120 EC-18 column (100 mm × 4.6 mm × 2.7 μm; Agilent Technologies) was kept in a column oven maintained at 50°C, and lipid mediators (LMs) were eluted in a gradient of methanol/water/acetic acid from 55:45:0.01 (v/v/v) to 100:0:0.01 at a flow rate of 0.5 ml/min. To monitor and quantify the amounts of lipid mediators of interest, multiple reaction monitoring (MRM) was used with MS/MS matching signature ion fragments for each molecule (at least six diagnostic ions; ~0.1 pg limits of detection as described previously [15]). PCA was performed as described previously [15] with SIMCA software, version 13.0.3. Calibration curves were obtained daily from authentic (nonsynthetic) standards and matrix suppression for each targeted LM in snap-frozen blood, and supernatants determined and used for recovery and quantitation.

Adenosine quantitation in human blood

Human blood was subjected to the same freeze-thaw procedure as was used for the metabololipidomics. After centrifugation at 1000g at 4°C for 5 min, supernatants were reconstituted with 4 volumes of methanol and kept on ice for 30 min to facilitate protein precipitation. Samples were then subjected to centrifugation at 1000g at 4°C for 5 min. Supernatants were reconstituted to < 1% methanol and then taken to LC-MS/MS for identification and quantitation of adenosine by matched retention time with >99% pure adenosine (Sigma) through MRM transition 268>136 for the protonated adenine fragment (54).

Flow cytometric analysis of clots

Human peripheral blood was collected from healthy individuals without anticoagulant and allowed to coagulate for 24 hours as described earlier. Clots were washed with phosphate-buffered saline (PBS) containing Ca²⁺ (0.9 mM) and Mg²⁺ (0.5 mM) and then gently homogenized and passed through a 70-micron filter. Clot-derived cells were stained for flow cytometric analysis. Cells were stained in FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Fc-receptor-mediated, nonspecific antibody binding was blocked by Human TruStain FcX solution, which was followed by incubation with APC-conjugated anti-human CD14 (clone HCD14), PercP-Cy5.5-conjugated anti-human CD20 (clone 2H7), PE-conjugated anti-human CD66b (clone G10F5), and APC Cy7-conjugated anti-human CD3 (clone HIT3a) (Biolegend). For viability assays, FITC-conjugated annexin V (BD) and propidium iodide (PI) were added to the cells according to the manufacturer's protocol. Samples were analyzed with a FACS Canto II flow cytometer (BD Bioscience) and FlowJo X Software.

Neutrophil-platelet interactions

Whole blood was collected from healthy individuals without anticoagulant and allowed to coagulate for 1, 6, or 24 h with or without ADA. Clots were washed with PBS and then gently homogenized and passed through a 70 micron-filter. Clot derived neutrophil-platelet interactions were analyzed by flow cytometry with FITC-conjugated anti-human CD16 antibody (clone ebio CB16), and PE-conjugated anti-human CD42b (clone HIP1). Neutrophils were identified by their cell surface expression of CD16 and high side and

forward scatter. Platelets were identified based on their low side and forward scatter on a log scale and on their cell surface expression of CD42b. Neutrophil and platelet aggregates were identified as CD14⁺ cells that were double-positive for CD42b and CD16 as CD14⁺,CD42b⁺,CD16⁺,SSC^{high},FSC^{high}.

Murine peritonitis and hemorrhagic exudates

All experimental procedures were approved by the Standing Committee on Animals of Brigham and Women's Hospital (protocol no. 2016N000145) and complied with institutional and US National Institutes of Health guidelines. Male FVB mice (6- to 8-weeks old) were given zymosan A (1 mg/0.5 ml; Sigma), thrombin (5 units/0.5 mL; Sigma), or both for 4 hours. Mice were then euthanized with isoflurane before peritoneal lavage was performed with 4.0 ml of ice-cold PBS without divalent cations. Lavages were subjected to LC-MS/MS for metabololipidomics analysis and flow cytometric analysis of neutrophil numbers with PE-conjugated anti-mouse Ly6G antibody (clone 1A8). Cells from the lavages were also attached to glass slides by cytospin, and the red blood cells and leukocytes were differentiated from each other with Wright Giemsa stain (Sigma) and enumerated in a minimum of four low-power fields per slide. The cells were also stained with Diff Quick (Electron Microscopy Science) according to the manufacturer's instructions to acquire images with a Keyence BZ-9000 (BIOREVO) inverted fluorescence phase-contrast microscope (40X objective) equipped with a monochrome/color switching camera using BZ-II Viewer software (Keyence).

CyTOF mass cytometry

Fresh blood aliquots (0.5 mL) from healthy donors were incubated with RvE1, RvD1, RvD5, LXB₄ and MaR1 (each at 50 nM) or PBS, 0.1% ethanol as a vehicle control for 1, 5, or 15 min at 37°C and the reactions were stopped by incubation with 1.6% paraformaldehyde for 10 min at room temperature. After hypotonic lysis, the cells were washed twice in CyTOF staining buffer (PBS with 0.5% BSA and 0.1% sodium azide), and transferred into deep well plates for barcoding. Cells were barcoded with Palladium Isotopes (Pd 102, 104, 105, 108, and 110; Fluidigm) according to the manufacturer's instructions. Briefly, cells were washed twice with barcoding permeabilization buffer. Barcodes were transferred to the cells and incubated for 30 min at room temperature. The barcoded cells were washed twice in CyTOF staining buffer and then pooled for staining. Pooled, barcoded cells were incubated for 10 min with FcX block (Biolegend) to block Fc receptor-mediated, nonspecific antibody binding. Cells were then stained for 30 min with 19 metal-label surface antibodies (see table S7) at room temperature to characterize the major immune cell populations in whole blood, and then were washed twice in CyTOF staining buffer. The cells were then permeabilized in 80% ice-cold methanol for 10 min at -20°C. After washing twice, the cells were stained with 8 metal-conjugated antibodies for 30 min at room temperature to measure intracellular functional markers (see table S7). Cells were then washed twice with CyTOF staining buffer and stained in 500 µl of 1:1000 Iridium Intercalator (DVS Science, Toronto) and let stand overnight in PBS at 4°C. Cells were then washed twice with CyTOF staining buffer and twice in MilliQ-filtered deionized water. Cells were reconstituted at 5 × 10⁶ cells/ml containing EQ calibration beads (EQ four elements Calibration Beads; Fluidigm) according to the manufacturer's instructions. Barcoded cells

were analyzed with a Helios CyTOF (Fluidigm) at an event rate of 400 to 500 cells per second and the data were normalized with V6.3.119 Helios Software (Fluidigm) at the LMA CyTOF core facility at the Dana Farber Cancer Institute (Boston, MA USA). Files were debarcoded with the Fluidigm Debarcoder application. Gating was performed with the Cytobank Platform (Cytobank).

CyTOF data analysis

Total leukocytes (CD45⁺CD41⁻ cells; fig. S8A) were analyzed using Cytobank software and equal cell numbers were sampled for unsupervised, high-dimensional visualization t-Stochastic Neighbor Embedding (viSNE) analysis (33). Thirteen parameter viSNE maps were generated from antibodies against the following cell surface markers: CD8, CD4, CD20, CD11c, CD11b, CD123, CD13, CD56, CD33, CD15, CD3, CD14, and HLA-DR. For Spanning-tree Progression Analysis of Density-normalized Events (SPADE) analysis (55), mononuclear cells (CD45⁺CD41⁻CD15⁻ cells; fig. S8A) were analyzed with Cytobank software (Cytobank, Inc.). The down-sampled events were then clustered based on phenotypically similar cells by the expression of CD4, CD8, CD3, CD14, CD16, CD56, CD20, CD123, CD33, CD13, HLADR, CD11c, and CD11b. Because the largest single-cell increases in the abundances of intracellular phosphoproteins occurred at 15 min compared to initial experiments performed at 1 and 5 min after incubation with each SPM (fig. S7), 15-min incubations were thus used throughout. To determine the fold-difference in the abundances of phosphoproteins of interest, vehicle controls were used as reference points and compared to samples incubated with SPMs.

Bacterial killing in human whole blood

E. coli (serotype O6:K2:H1) were cultured in LB broth and washed in sterile saline before being added to blood. Human peripheral blood (45 μ l) was incubated with each member of the SPM panel (RvD1, RvD5, RvE1 MaR1, LXB₄) at 0.1, 1, 10, or 50 nM or with vehicle control (5 μ l of PBS, 0.1% ethanol) for 15 min at 37°C, which was followed by incubation with $\sim 2 \times 10^7$ *E. coli* (5 μ l) for 60 min at 37°C. Samples were then diluted 1:10⁵ in PBS on ice and aliquots were placed on LB agar and incubated overnight in a 37°C incubator. Colonies were enumerated by eye.

Whole blood bacterial killing during coagulation

Fresh human blood (2.0 ml) without anticoagulant was incubated with *E. coli* ($\sim 7 \times 10^8$) in the presence or absence of 200 μ M baicalein (a LOX inhibitor; Sigma) and allowed to coagulate at 37°C for 24 hours. Serum from blood were diluted in PBS on ice and aliquots were placed on LB agar and incubated overnight in a 37°C incubator. Colonies were enumerated.

Phagocytosis with human peripheral blood phagocytes—Fresh heparinized whole blood (100 μ l) was collected from healthy donors and incubated with a panel of SPMs (RvE1, RvD1, RvD5, LXB₄, and MaR1; 0.1 to 50 nM each in combination) or vehicle control (0.1% ethanol) for 15 min at 37°C. *E. coli* was labeled with BacLight fluorescent Green dye (Life Technologies) according to the manufacturer's instructions. Labeled *E. coli* was added to samples at a phagocyte:bacterium ratio of 1:50 to initiate phagocytosis at 37°C

for 45 min. Samples were then incubated with APC-conjugated anti-human CD66b antibody (to label neutrophils) and APC-Cy7-conjugated anti-human CD14 antibody (to label monocytes) (Biolegend) for 15 min on ice. Cells were washed twice with 2 ml of ice-cold PBS, which was followed by red blood cell lysis and fixation in 3% paraformaldehyde. Cells were then analyzed either with a BD FACS Canto II flow cytometer (BD Biosciences) or an ImageStream X imaging flow cytometer (Amnis). Fluorescence-associated phagocytes in the neutrophil (CD66b⁺) and monocyte (CD14⁺) populations were subsequently identified with FlowJo software version X.

Imaging flow cytometry

Cells were prepared as described for the phagocytosis assays (see earlier) and were analyzed on an ImageStream X MarkII Flow cytometer (Amnis) with 60x magnification at the Flow and Imaging Cytometry Resources at Boston Children's Hospital. Harvard Medical School (BCH-HMS). The phagocytosis of live *E. coli* by neutrophils (CD66b⁺) and monocytes (CD14⁺) was imaged and analyzed with IDEAS software (Amnis) using bright field focus images to set the cell boundary and gating on CD66⁺, CD14⁺ and bacterial green fluorescence. This permitted visualization of the phagocytosis of *E. coli* by human leukocytes.

Human macrophages

Human peripheral blood mononuclear cells from deidentified healthy human volunteers from the Children's Hospital Boston blood bank were isolated by density-gradient, Ficoll-Histopaque isolation, which was followed by monocyte purification. The monocytes were then cultured for 7 days in RPMI 1640, 10% fetal calf serum (FCS) and were differentiated into macrophages through culturing with granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml).

Real-time analysis of phagocytosis

Real-time imaging of human macrophages was performed by plating the cells (50,000 cells/well in PBS⁺⁺) onto 8-well chamber slides. The chamber slides were kept in a Stage Top Incubation system for microscopes equipped with a built-in digital gas mixer and temperature regulator (TOKAI HIT model INUF-K14). A panel of SPMs (RvE1, RvD1, RvD5, LXB4, and MaR1; 1 nM each or in combination) was added to the macrophages for 15 min, which was followed by the addition of BacLight Green-labeled *E. coli* (at an *E. coli*:phagocyte ratio of 50:1). Images were then acquired every 10 min for 3 hours at 37°C with a Keyence BZ-9000 (BIOREVO) inverted fluorescence phase-contrast microscope (20X objective) equipped with a monochrome-color switching camera using BZ-II Viewer software (Keyence). Mean fluorescence intensity was quantified with a BZ-II Analyzer.

Statistical analysis

Groups were compared by Student's two-tailed t-test (for two groups) or one-way ANOVA with Bonferroni Multiple Comparison Test (for more than two groups) with Prism software version 6 (GraphPad). The criterion for statistical significance was $P < 0.05$. Principal

component analysis (PCA) was performed with SIMCA 13.0.3 software (MKS Data Analytics Solutions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One-sentence summary

Factors generated by blood coagulation that elicit immune responses that protect the host are identified.

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Editor's summary: Linking clots to immunity

The threats to the host posed by barrier breach are counteracted by blood coagulation and the formation of blood clots, processes that are accompanied by inflammation and then tissue regeneration and wound healing. Arachidonic acid–derived prostaglandins and leukotrienes play important prothrombotic and inflammatory roles early in the process, which lead to the generation of other lipids known as specialized proresolving mediators (SPMs), which are important for resolution. Norris *et al.* used metabolipidomics and mass cytometry–based analyses to identify a cluster of SPMs generated at the late stages of human blood coagulation. Treatment of whole blood with these particular SPMs stimulated distinct signaling pathways within specific cell types and enhanced the uptake and killing of bacteria by phagocytic cells. Together, these data connect factors generated by blood coagulation to immune responses that protect the host.

Fig. 1. Human blood coagulation initiates endogenous temporal production of SPMs and eicosanoids

(A to E) Freshly drawn human peripheral blood was incubated in silicone-coated tubes at 37°C for the indicated times and coagulation was stopped by snap-freezing the samples for LC-MS/MS analysis (see Materials and Methods). (A) Time course of the relative changes in the percentage light transmission at 750 nm in the blood samples during clot formation. (B) LC-MS/MS chromatograms of SPMs (top) and eicosanoids (bottom) that were identified in the samples. *a* = 6-trans-LTB₄, *b* = 6-trans, 12-epi-LTB₄, and *c* = 5S,12S-diHETE. (C)

MS/MS spectra with diagnostic ions for the indicated identified mediators. Data in (A) to (C) are representative of 12 donors. (D) Concentrations of the indicated members of the SPM cluster and of eicosanoids in the blood samples over time. Data are means $\pm$ SEM of 12 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs. the zero-hour time point by ratio t-test. (E) Representative PCA score plot (top) and loading plot (bottom) showing temporal clustering of mediators from 12 individual donors. Gray denotes 0 hours and 0.25 hours; blue denotes 0.5 hours and 1 hours; red denotes 3 hours and 6 hours; and green, 24 hours after venipuncture and placement of blood in silicone-coated tubes without anticoagulants.

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Fig. 2. Regulators of SPM formation in human blood

(A to E) Freshly drawn human peripheral blood (4 ml) containing heparin (10 U/ml) was placed in polypropylene tubes (A) whereas human peripheral blood without anticoagulant was placed in silicone-coated tubes (B to E) and incubated at 37°C for the time course of 0 to 24 hours (see Materials and Methods). The samples were then subjected to snap-freezing for extraction and LC-MS/MS analysis. (A) Concentrations of the members of the SPM cluster (identified in Fig. 1) in samples treated without (Coag.) or with heparin (10 U/ml) during the incubation period. Data are means $\pm$ SEM of three individual donors. (B) Left:

Combined concentrations of the total SPM cluster, prostaglandins, and thromboxanes after 24 hours of coagulation in the presence or absence of the α IIB β 3 inhibitor eptifibatide (Eptifib.; 20 μ M). Middle: Clot volumes after 24 hours of coagulation of the indicated samples. Right: Representative clot images. Data are means $\pm$ SEM of three individual donors. (C) Concentrations of total SPMs and of the indicated individual SPMs after 24 hours of coagulation in the presence or absence of 200 mU ADA. Data are means $\pm$ SEM of three individual donors. $*P < 0.05$, $**P < 0.01$ by ratio t-test. (D) Top: LC-MS/MS measurements of adenosine concentrations in whole blood at the indicated time points during coagulation in the presence or absence of ADA. One representative of three individual time courses. Bottom: Flow cytometric analysis of the percentage of neutrophil-platelet aggregates, which were defined as being double-positive for CD16 and CD42b, in whole blood allowed to coagulate for the indicated times in the absence (Coag.) or presence of ADA. Data are means $\pm$ SEM of five individual donors. $*P < 0.05$, $**P < 0.01$ by student's t test. For dot plots and gating, see fig. S4B. (E) MS/MS fragmentation profiles with diagnostic ions for the indicated SPMs identified in (C). (F) Left: PCA score plot. Red denotes +ADA for 1 hour; yellow, coagulation for 1 hour; green, coagulation for 24 hours; blue, +ADA for 24 hours. Right: PCA loading plot showing temporal clustering of the indicated mediators from three individual donors. Red denotes LM at 1 hour; blue denotes 24 hours with ADA).

Fig. 3. SPM cellular targets and intracellular signaling in human blood

(A and B) viSNE map representation of (A) cell surface marker expression and localization and (B) changes in intracellular pPERK1/2 and pCREB abundances in the indicated peripheral blood leukocytes incubated at 37°C for 15 min with each SPM (at 50 nM) or vehicle control (PBS). Each dot represents a single cell colored according to the mass tag signal intensity. (C) Heat map representation of the mean fold-changes in the abundances of the indicated intracellular phosphoproteins in the indicated cell types relative to those in the vehicle control (median intensity arcsinh ratio). (D) Changes in the abundances of pPERK1/2,

pCREB, pp38 MAPK, and pS6 in the indicated cell populations after incubation with the indicated SPMs. Data are means $\pm$ SEM of six individual donors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. vehicle control by one-way ANOVA with Bonferroni Multiple Comparison Test.

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Fig. 4. SPMs produced during coagulation of whole blood enhance phagocytosis and bacterial killing. (A)

Analysis of bacterial survival in human peripheral blood incubated for 15 min at 37°C with RvE1, RvD1, RvD5, LXB₄, and MaR1 (from the SPM panel) at the indicated concentrations or with vehicle control, which was followed by the addition of *E. coli* and further incubation for 60 min at 37°C. Data are means $\pm$ SEM of three separate donors. * P < 0.05, ** P < 0.01 vs. vehicle by two-tailed t test. **(B)** Phagocytosis of BacLight Green-labeled *E. coli* by human peripheral blood neutrophils (CD66b⁺) and monocytes (CD14⁺) after incubation for

45 min at 37°C in the absence or presence of members of the SPM panel [as described in (A)]. Top: The MFIs of the indicated cells were determined by flow cytometric analysis. Representative histograms are shown. Bottom: Data are means $\pm$ SEM of four individual donors. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ vs. vehicle control by one-way ANOVA with Bonferroni Multiple Comparison Test. (C) Representative images showing phagocytosis (merge) of *E. coli* (BacLight Green) by neutrophils (CD66b⁺, red) and monocytes (CD14⁺; purple) in response to treatment with vehicle or members of the SPM panel. Data are representative of three separate donors. (D) Real-time analysis of the phagocytosis of BacLight Green-labeled *E. coli* by human macrophages (incubated at a ratio of 50 bacteria per cell) after incubation for 15 min at 37°C with vehicle or the indicated SPMs (1 nM each) separately or in combination (*E. coli* + SPM). Phagocytosis represented by MFI is presented as the mean of 4 fields (20X) per condition (per well). Inset: Representative images from three separate donors. Scale bar, 50 μ m. (E) Analysis of phagocytosis of *E. coli* at 180 min. Data are means $\pm$ SEM of three separate donors. $*P < 0.05$, $**P < 0.01$ vs. SPM panel; $##P < 0.01$, $###P < 0.001$ vs. vehicle by one-way ANOVA with Bonferroni Multiple Comparison Test.