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Thrombin Differentially Modulates the Acute Inflammatory Response to *Escherichia coli* and *Staphylococcus aureus* in Human Whole Blood

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Thrombin plays a central role in thromboinflammatory responses, but its activity is blocked in the common ex vivo human whole blood models, making an ex vivo study of thrombin effects on thromboinflammatory responses unfeasible. In this study, we exploited the anticoagulant peptide Gly-Pro-Arg-Pro (GPRP) that blocks fibrin polymerization to study the effects of thrombin on acute inflammation in response to *Escherichia coli* and *Staphylococcus aureus*. Human blood was anticoagulated with either GPRP or the thrombin inhibitor lepirudin and incubated with either *E. coli* or *S. aureus* for up to 4 h at 37°C. In GPRP-anticoagulated blood, there were spontaneous elevations in thrombin levels and platelet activation, which further increased in the presence of bacteria. Complement activation and the expression of activation markers on monocytes and granulocytes increased to the same extent in both blood models in response to bacteria. Most cytokines were not elevated in response to thrombin alone, but thrombin presence substantially and heterogeneously modulated several cytokines that increased in response to bacterial incubations. Bacterial-induced releases of IL-8, MIP-1α, and MIP-1β were potentiated in the thrombin-active GPRP model, whereas the levels of IP-10, TNF, IL-6, and IL-1β were elevated in the thrombin-inactive lepirudin model. Complement C5-blockade, combined with CD14 inhibition, reduced the overall cytokine release significantly, both in thrombin-active and thrombin-inactive models. Our data support that thrombin itself marginally induces leukocyte-dependent cytokine release in this isolated human whole blood but is a significant modulator of bacteria-induced inflammation by a differential effect on cytokine patterns. ***The Journal of Immunology*****, 2022, 208: 1–8.**

Thromboinflammation is defined as the interplay of thrombosis and inflammation (1) and is manifested in response to vascular trauma, microbial infection, and biomaterials (2). In thromboinflammatory responses, thrombin plays a central role. On the one hand, it cleaves fibrinopeptides from fibrinogen to form fibrin monomers that polymerize through protofibrils to form a fibrin clot (3); it activates coagulation factors V, VIII, XI, and XIII (4). On the other hand, thrombin can directly mediate cellular responses by activating protease-activated receptors (PARs) 1, 3, and 4 expressed at varying levels on platelets, leukocytes, and endothelial cells (5, 6). To further enhance thromboinflammatory responses, thrombin-activated platelets release numerous mediators that further modulate inflammatory and thrombotic events (7–10).

The complement system and the TLR systems of innate immunity have evolved to sense and respond to the presence of exogenous microbial- and endogenous damage-associated molecular patterns (11–13). On pattern recognition, complement activation leads to the cleavage of C3 and C5, resulting in the formation of C3a and C5a fragments, respectively, as powerful anaphylatoxins that induce inflammation. Thrombin has also been shown to cleave C5, activating the complement system independent of canonical complement recognition (14–16). However, we have recently challenged the physiological role of this observation in a whole blood model, in which thrombin is fully active but was unable to cleave plasma C5 at physiological pH (17). In contrast with circulating recognition molecules of the complement system, the TLRs are both cell surface and endosomal receptors that typically sense extracellular and intracellular microbial ligands (11–13). Several TLRs, including TLR2 and TLR4, use CD14 as a coreceptor (18, 19). Leukocyte activation, particularly monocyte and macrophage activation, through TLRs is a potent stimulus for the synthesis and release of cytokines in the acute inflammatory response. Taken together, the interconnection of these sensing systems and thrombin requires fully active thrombin with its mutual connections with other blood components for thromboinflammatory studies under conditions where the components are preserved and can cross-talk in their physiological forms.

Fresh human whole blood offers an excellent physiological environment to study the mutual interactions of the innate immunity and

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Abbreviations used in this article: FSC, forward scatter; GPRP, Gly-Pro-Arg-Pro; PAR, protease-activated receptor; PF1 + 2, prothrombin fragment 1 + 2; sC5b-9, soluble C5b-9; SSC, side scatter; TAT, thrombinanti–thrombin; βTG, β-thromboglobulin.

hemostatic systems in response to stimulus ex vivo. However, a prerequisite is that the ex vivo whole blood must be anticoagulated with the anticoagulant that exerts minimal effects on other blood components. We originally designed a whole blood model anticoagulated with the thrombin inhibitor lepirudin, which did not affect the complement system (20), and have used this model to study the role of complement in the inflammatory responses induced by both infectious and sterile agents (20–27). However, lepirudin is a direct thrombin inhibitor, making the study of the effects of thrombin on thromboinflammatory responses unfeasible. Therefore, we recently modified this model by replacing lepirudin with the fibrin polymerization inhibitory peptide Gly-Pro-Arg-Pro (GPRP) and used this model to investigate the effects of thrombin on complement activation (17). Because this model exhibits a potent generation of active thrombin, it also enables unlimited platelet activation without forming a blood clot. In this study, we aimed to investigate the role of thrombin in the acute inflammatory reaction induced by Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* in human whole blood.

Materials and Methods

Reagents

Lepirudin (Refludan) was obtained from Celgene (Uxbridge, U.K.), and the GPRP peptide (Pefabloc FG [Pefa-6003]) was from Pentapharm (Basel, Switzerland). Complement inhibitors anti-C2 Ab (mAb, clone 175-62) and anti–factor D Ab (mAb, clone 166-32) were provided by Genentech (San Francisco, CA); the anti-C5 Ab eculizumab (Soliris) was obtained from Alexion Pharmaceuticals (Zurich, Switzerland) and the C5aR1 antagonist, PMX53 (sequence Ac-Phe-[Orn-ProdCha-Trp-Arg]), was prepared as previously described (28). The blocking Ab against CD14 (r18D11) was prepared as previously described (29). The PAR-1 inhibitor, ML161, was purchased from R&D Systems (Minneapolis, MN).

Whole blood sampling and incubations

Fresh human whole blood from healthy donors aged 23–60 y of both sexes was sampled from the antecubital vein into 4.5 ml NUNC polypropylene tubes (Nalgene Nunc, Roskilde, Denmark) containing lepirudin (50 μg/ml final concentration) and GPRP (8 mg/ml final concentration), as previously described (17, 20). Aliquots of whole blood were immediately put in sterile polypropylene tubes and preincubated for 5 min in a roller block heater at 37°C with inhibitors or PBS. The inhibitors were used at their final concentrations: anti-C2 (71 μg/ml), anti–factor D (36 μg/ml), eculizumab (100 μg/ml), PMX53 (10 μg/ml), anti-CD14 (15 μg/ml), and PAR-1 inhibitor (ML161, 3 μM). The start of bacterial incubation was referred to the time when either heat-inactivated *E. coli* strain LE392 (ATCC 33572) at a final concentration of 10^7/ml, *S. aureus* Cowan strain 1 (ATCC 12598) with a final concentration of 10^8/ml (both from American Type Culture Collection, Manassas, VA), or PBS (Sigma-Aldrich) was added to the blood. The whole blood was incubated for 15 min for the analysis of complement, coagulation, platelet activation markers, and the early leukocyte activation markers. For transcription-dependent markers, cytokines, and tissue factor, incubations were carried out for up to 4 h. After incubation, EDTA was added at a final concentration of 10 mM. A sample was taken for analysis of flow cytometry markers, and the remaining blood samples were centrifuged at 3000 × *g*, 4°C for 20 min. Plasma was isolated after centrifugation and immediately stored at −80°C until further analyses.

Plasma soluble C5b-9, thrombin–anti-thrombin complex, PF1 + 2, and β-thromboglobulin analysis

Complement activation marker soluble C5b-9 (sC5b-9) was evaluated by ELISA using the mAb aE11 reacting with a neoepitope exposed in C9 when incorporated into the sC5b-9 complex (30), biotinylated anti-C6 mAb (clone 9C4), and performed as previously described (30, 31). Samples were diluted between 1:5 and 1:100 depending on the activation level. Prothrombin activation was measured by the prothrombin fragment 1 + 2 (PF1 + 2) and thrombin activation by the thrombin–anti-thrombin (TAT) complexes in plasma using the Enzygnost commercial kits F1 + 2 and TAT (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) according to the manufacturer's protocols. Samples were diluted between 1:10 and 1:1000 for prothrombin cleavage fragment PF1 + 2 and between 1:20 and 1:5000 for TAT. Platelet activation was also assessed by measuring β-thromboglobulin (βTG) released into plasma by a commercial kit (Asserachrom βTG; Diagnostica Stago, Parsippany, NJ) in accordance with the manufacturer's instructions. Samples were diluted between 1:500 and 1:12,500.

Flow cytometry analysis of extracellular markers

Surface activation markers CD11b and CD35 on granulocytes and monocytes, and CD63 and CD62P on platelets were analyzed after 15-min incubation of whole blood with bacteria or PBS. Cells in whole blood were stained with anti-CD11b allophycocyanin/Fire 750 (BioLegend, San Diego, CA), anti-CD35 Alexa Fluor 647 (Invitrogen, Carlsbad, CA), anti-CD63 PE-Cy7 (Invitrogen), anti-CD62P PE (BD Biosciences, San Jose, CA), anti-CD14 PerCP (BD Biosciences), anti-CD14 V500 (BD Biosciences), anti-CD15 V450 (Invitrogen), anti-CD15 BV605 (BD Biosciences), or anti-CD42a FITC (BD Biosciences) for 30 min. The erythrocytes were subsequently lysed using the high-yield lyse solution (Invitrogen) or BD Pharm Lyse (BD Biosciences). Cells were then fixed by resuspension in 0.1% (v/v) paraformaldehyde in PBS, containing 0.1% BSA. The samples were analyzed on the flow cytometer Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA) with the threshold set at forward scatter (FSC) 25×10^3 to exclude particles smaller than platelets. Single cells were gated using FSC-area against FSC-height. Granulocytes were gated in a side scatter (SSC)/CD15 dot plot; monocytes were gated in an SSC/CD14 dot plot from the $CD15^-$ cells; platelets were gated in an SSC/CD42a dot plot. CD11b and CD35 expressions were analyzed as median fluorescence intensity of each leukocyte population. CD63 and CD62P were analyzed on the $CD42a^+$ populations containing either free platelets or platelets in complex with other cell types. Tissue factor expression on monocytes was analyzed after 4-h incubation of whole blood. In this study, cells were stained with anti-CD15 V450 (Invitrogen), anti-CD42a PE (BD Biosciences), anti-CD14 PerCP (BD Biosciences), and anti-tissue factor FITC (Sekisui Diagnostics, Lexington, MA). Monocytes for tissue factor analysis were gated using SSC/CD14 and then gated further on $CD42a^-$ cells. Tissue factor was analyzed as the median fluorescence intensity of single monocytes.

Plasma cytokines

Plasma samples collected earlier from the whole blood experiments were thawed on wet ice and analyzed for the presence of 27 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, bFGF-2, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF, and VEGF) using the Bio-plex Human Cytokine 27-Plex multiplex assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The measurements were performed in the Bio-Plex 200 system (Bio-Rad Laboratories).

Statistics and data analysis

Statistical comparisons were performed using GraphPad Prism 9. Tests used were paired nonparametric Wilcoxon signed rank test, pairwise nonparametric Friedman test with Dunn correction, and 2 × 3 and 2 × 2 (single versus double) chi-square test, as indicated in the respective figures.

Ethics statement

This study was designed and performed according to the ethical guidelines of the Declaration of Helsinki. Informed written consent was obtained from the blood donors. The study was approved by the ethical committee of the Norwegian Regional Health Authority (ethical permit REK#S-04114, 2010/934).

Results

Activation of complement and coagulation in lepirudin and GPRP whole blood models

Activation of the complement system and cleavage of thrombin were characterized in human whole blood anticoagulated with the thrombin inhibitor lepirudin or the fibrin polymerization inhibitor GPRP. After 15-min incubation, sC5b-9, an endpoint marker of complement activation, was detected at similar levels in the two models, both in the absence of bacteria and on bacterial incubations (Fig. 1A). Complement inhibitors targeting C2 and factor D in combination, or C5 alone, reduced sC5b-9 to background levels in both models for up to 4 h of incubation, confirming a complete inhibition of the complement terminal pathway by these inhibitors (Supplemental Fig. 1).

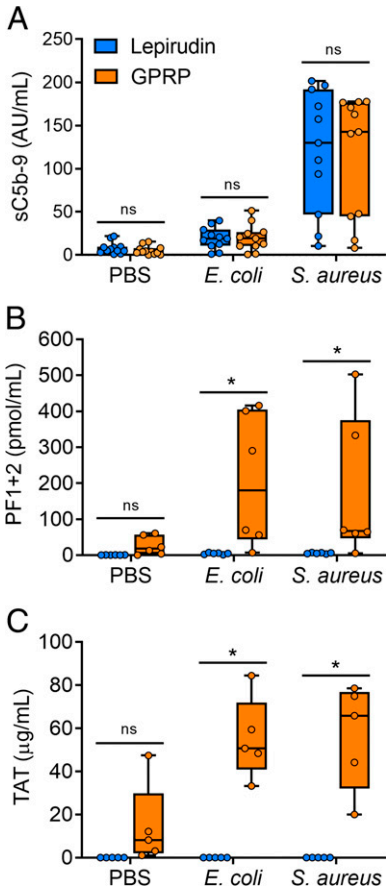

FIGURE 1. Activation of the complement and coagulation systems in lepirudin (thrombin-inactive) and GPRP (thrombin-active) models. Plasma prepared after incubation of whole blood with *E. coli* (10^7/ml), *S. aureus* (10^8/ml), or PBS for 15 min was analyzed using ELISA for measures of (**A**) terminal sC5b-9 complement complex (sC5b-9), coagulation markers (**B**) PF1 + 2, and (**C**) TAT complex. Each dot represents one donor data point (n = 5–11). Significant differences were determined statistically using pairwise nonparametric Friedman test with Dunn correction. *$p < 0.05$. AU, arbitrary unit; ns, nonsignificant.

Meanwhile, thrombin activation was elevated in the GPRP model in comparison with the lepirudin model, evidenced by the generation of PF1 + 2 (Fig. 1B) and TAT (Fig. 1C). PF1 + 2 levels increased in both models on incubation with both bacteria, but the conversion of prothrombin to thrombin in response to the addition of *E. coli* or *S. aureus* was 30–50 times greater in the GPRP model as compared with the lepirudin model (Fig. 1B). TAT remained at baseline in the lepirudin model before and after bacterial incubations (Fig. 1C). In the GPRP model, TAT levels were elevated even with PBS incubation and were further increased on bacterial incubation (Fig. 1C).

Activation of monocytes, granulocytes, and platelets

The activation of leukocytes and the expression of monocyte tissue factor were evaluated in both models after bacterial incubations. After 15-min incubation, monocyte activation, as measured by the expression of surface activation markers CD11b and CD35, increased in response to the presence of either *E. coli* or *S. aureus* in both models (Fig. 2A, 2B). However, only monocyte CD35 showed significant increases in the GPRP model as compared with the lepirudin model when incubated with PBS or *E. coli* (Fig. 2B). In contrast, monocyte tissue factor significantly increased in the lepirudin model compared with the GPRP model in response to bacterial incubations for 4 h (Fig. 2C). Similarly, granulocyte CD11b and CD35 were detected at the same level in the presence of either *E. coli* or *S. aureus* in both models, suggesting a similar degree, and thus thrombin independence, of granulocyte activation in both models (Fig. 2D, 2E).

After 15-min incubation with bacteria, platelet activation was evaluated by measuring the expression of surface activation markers CD62P and CD63 by flow cytometry and the level of βTG released by ELISA in both blood models (Fig. 2F–H). The α-granule–originated CD62P increased nearly 100 times in the GPRP model as compared with that in the lepirudin model (Fig. 2F). The dense granule-derived CD63 detected in the GPRP model was 20-fold higher than that in the lepirudin model (Fig. 2G). Similarly, the soluble α-granule–derived βTG was significantly increased in the GPRP model compared with the lepirudin model (Fig. 2H). Collectively, the data support a mainly thrombin-dependent activation of platelets in whole blood, in contrast with the leukocytes.

Plasma cytokine release in the lepirudin and GPRP whole blood models

Cytokine response was analyzed in the two whole blood models after incubation for 4 h in the absence or presence of bacteria. Of the 27 cytokines analyzed, 10 cytokines were detected at background levels in the absence of bacteria and increased to >100 pg/ml in response to the presence of either *E. coli* or *S. aureus* (Table I, Fig. 3). Except for IL-1ra that responded similarly in both models, the other nine cytokines showed significantly different responses between the two blood models on bacterial incubation (Fig. 3). Interestingly, the difference between the two models varied among the cytokines, but the response was virtually identical between *E. coli* and *S. aureus* for all the cytokines (Fig. 3). The chemokines IL-8, MIP-1α, and MIP-1β were significantly increased in the presence of bacteria in the GPRP model compared with the lepirudin model (Fig. 3), suggesting a thrombin-dependent elevation of these cytokines. In contrast, IP-10 and the typically proinflammatory cytokines TNF, IL-6, and IL-1β were significantly enhanced in the lepirudin model (Fig. 3). MCP-1 and IFN-γ were also significantly higher in the lepirudin model; however, the difference in absolute values was marginal (Fig. 3).

Role of complement and CD14 on thrombin-dependent cytokine release

To determine the involvement of complement and the TLR coreceptor CD14 in inducing the cytokine response, inhibitors targeting C2 and factor D in combination, C5, C5aR1, CD14, or C5, and CD14 in combination were applied. On incubation with *E. coli*, the combined inhibition of C5 and CD14 was most efficient, resulting in statistically significant reductions of the cytokine levels in either one or both blood models for 9 of the 10 cytokines (Fig. 4). TNF, IL-6, IP-10, MIP-1β, and MCP-1 were significantly reduced in both models (Fig. 4A–E), while a significant reduction of IL-1β was observed only in the lepirudin model (Fig. 4F); the levels of IL-8, MIP-1α, and IFN-γ decreased only in the GPRP model (Fig. 4G–I). Interestingly, IL-1ra was not reduced in either of the models (Fig. 4J). Similarly, the combined inhibition of C5 and CD14 led to statistically significant reductions of TNF, IL-6, IL-8, and IFN-γ in both blood models incubated with *S. aureus* (Fig. 5A, 5B, 5G, 5I). Significant reductions of IL-1β (Fig. 5F) and MIP-1α (Fig. 5H) were observed only in lepirudin blood incubated with *S. aureus*, while the levels of MIP-1β (Fig. 5D) and MCP-1 (Fig. 5E) decreased under the combined inhibition of C5 and CD14 in GPRP blood incubated with *S. aureus*.

Complement inhibition at the C3 level by combining anti-C2 and anti–factor D Abs did not result in significant decreases of any of the analyzed cytokines in any of the two blood models incubated with *E. coli* (Supplemental Fig. 2) or *S. aureus* (Supplemental

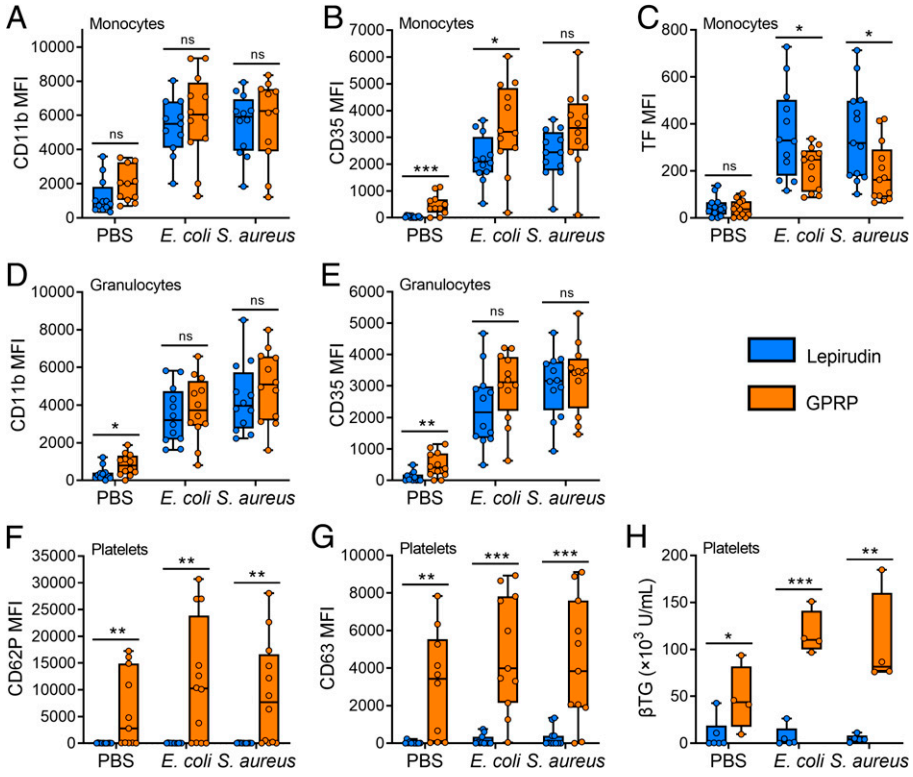

FIGURE 2. Leukocyte and platelet activation in lepirudin (thrombin-inactive) and GPRP (thrombin-active) models. Whole blood was incubated with either *E. coli* (10^7/ml), *S. aureus* (10^8/ml), or PBS for 15 min (**A**, **B**, and **D**–**H**) or 4 h (**C**). Monocyte activation was evaluated by the surface activation markers CD11b, CD35, and tissue factor (A–C) and the granulocyte activation by CD11b and CD35 using flow cytometry (D and E). Platelet activation was evaluated by the surface markers CD62P (F) and CD63 (G) with flow cytometry, as well as the release of the soluble βTG using ELISA (H). Each dot represents one donor data point (n = 5–13). Significant differences were statistically determined using pairwise nonparametric Friedman test with Dunn correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. MFI, median fluorescence intensity; U, international units.

Fig. 3). In a similar manner, inhibition of the C5aR1 receptor by PMX53 did not lead to significant reductions of almost any analyzed cytokines (Supplemental Figs. 2, 3). Significant effects of PMX53 were observed only on MIP-1β (Supplemental Fig. 2D) in the lepirudin model and on IFN-γ (Supplemental Fig. 2I) in the GPRP model incubated with *E. coli*. Inhibition of PAR-1 by ML161 did not significantly reduce the levels of any cytokines in either lepirudin- or GPRP-anticoagulated blood when incubated with *E. coli* (Supplemental Fig. 4).

Discussion

Since we developed the whole blood model anticoagulated with the thrombin-specific inhibitor lepirudin (20), a considerable understanding of the acute inflammatory response to bacterial and sterile agents under close-to-physiological conditions has been obtained (20–27, 32–34). Although this blood model is efficiently anticoagulated without influencing the complement system, it shades the contribution of thrombin in shaping acute inflammatory responses. In contrast, the fibrin polymerization peptide GPRP blocks the coagulation system downstream of thrombin, enabling a study on the contribution of thrombin in the acute phase of thromboinflammatory responses (35). In this study, we compared thromboinflammatory responses in whole blood anticoagulated with lepirudin or GPRP in response to the incubation with Gram-negative and Gram-positive bacteria. The only difference between the two blood models is the activity of thrombin, in which thrombin is fully active in the GPRP model, but completely blocked in the lepirudin model. By comparing the activation of monocytes, granulocytes, platelets, and the release of cytokines in the two models, we revealed differential effects of thrombin on bacterial-induced inflammation. To the best of our knowledge, this is the first model used to specifically study the functions of native thrombin acting in a highly relevant physiological system ex vivo without any known interventions with the proteins and cells involved.

Table I. Levels of plasma cytokines released in whole blood after incubation with buffer control (PBS)

Cytokine	Lepirudin (pg/ml)	GPRP (pg/ml)
TNF	374 ± 344	90 ± 52**
IL-1β	3 ± 2	3 ± 2
IL-6	8 ± 5	15 ± 14
IL-8	305 ± 213	394 ± 384
IP-10	919 ± 326	635 ± 419*
MIP-1α	36 ± 27	5 ± 3***
MIP-1β	803 ± 528	269 ± 236***
MCP-1	48 ± 28	88 ± 66*
IFN-γ	16 ± 5	53 ± 53*
IL-1ra	611 ± 428	389 ± 274

All data are given as mean ± SD (n = 19).
*$p < 0.05$, statistically different from lepirudin.
**$p < 0.01$, statistically different from lepirudin.
***$p < 0.001$, statistically different from lepirudin.

Consistent with our previous observation (17), there was no significant difference in complement activation between the two models in both sterile and bacterial incubations (Fig. 1A). Obviously, a major difference observed between the two models was the thrombotic response in the GPRP model. The elevations of thrombin activation markers (Fig. 1B, 1C) and platelet activation markers (Fig. 2F–H) in both the absence and the presence of bacteria illustrate the instant hemostatic response in whole blood on extravasation into a sampling tube. Active thrombin, as in the GPRP model, can self-amplify its further production via feedback activation of coagulation factors V and VIII (36), and mediate platelet activation by PAR-1 and PAR-4 activation (37, 38). Together, this may explain the elevated activation of platelets observed in the GPRP model. Because platelet activation is a hub in the acute vascular response to trauma (39), this observation suggests that inhibition of platelet activation would be a potential strategy in the treatment of vascular trauma.

In contrast, monocytes and granulocytes were activated similarly in both models (Fig. 2A–E), suggesting that the activation of these

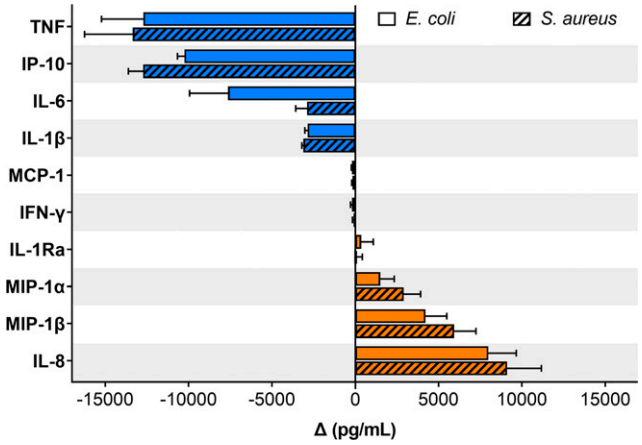

FIGURE 3. Comparison of cytokine release in lepirudin (thrombin-inactive) and GPRP (thrombin-active) model in response to incubation with *E. coli* and *S. aureus*. Cytokines were analyzed for the quantitative differences in the response between the lepirudin and GPRP whole blood models ($n = 19$). All values are presented as difference data [Δ (pg/ml)] after subtracting the cytokine levels detected in GPRP whole blood by those detected in lepirudin whole blood, respectively. Cytokines shown in blue upregulate in the lepirudin model (thrombin independent) in response to bacterial incubation and hence have negative Delta (Δ) values. Cytokines shown in orange upregulate in the GPRP model (thrombin dependent) and hence have positive Delta (Δ) values. Solid bars denote values from *E. coli* incubations, whereas hatched bars are from *S. aureus* incubations.

cells was not significantly affected by the availability of thrombin. In fact, monocytes express thrombin receptors PAR-1 and PAR-3 (40), but monocyte activation was not significantly enhanced by the presence of thrombin as in the GPRP blood model (Fig. 2A–C). Leukocyte activation is enhanced in response to complement activation (20, 41), and the C5a-C5aR1 activation is a particular proinflammatory signal pathway after complement activation (42). Hence similar thrombin-independent complement activation, as observed in this study and the previous one (17), may explain a similar activation of both monocytes and granulocytes.

The hemostatic response alone, i.e., in whole blood incubated at 37°C without bacteria, was not a robust stimulus to trigger the release of most of the analyzed cytokines. However, thrombin had a significant modulatory effect on the cytokine release if the response was initially triggered by innate immune activation induced by the presence of bacteria (Fig. 3). The effects of thrombin on the release of cytokines were differential for each cytokine and could be categorized into three groups: negatively regulated, positively regulated, and indifferently regulated (Fig. 3). Many of the cytokines that increased in either of the blood models, i.e., the thrombin-enhanced cytokines in the GPRP model and thrombin-inhibited cytokines in the lepirudin model, are typically elevated in the acute response to trauma (43, 44). TNF, IL-6, and IL-1β are signature proinflammatory cytokines. We found that the inhibition of thrombin, as in the lepirudin model, markedly increased the concentration of these cytokines in response to the bacteria (Fig. 3). In contrast, the three main

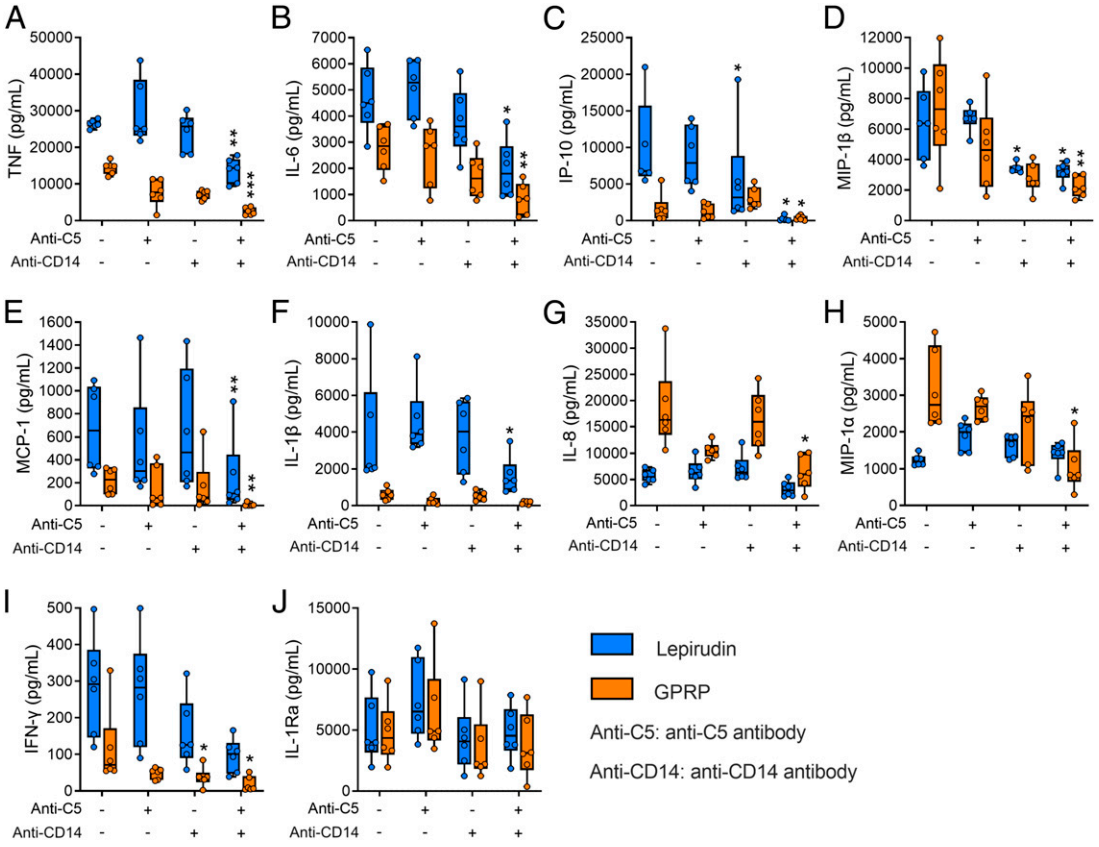

FIGURE 4. Effects of complement and CD14 inhibition on the cytokine response in the lepirudin (thrombin-inactive) and GPRP (thrombin-active) blood models incubated with *E. coli*. Lepirudin and GPRP anticoagulated whole blood were incubated for 4 h with *E. coli* with complement C5 inhibition and/or CD14 inhibition. Cytokines, TNF (**A**), IL-6 (**B**), IP-10 (**C**), MIP-1β (**D**), MCP-1 (**E**), IL-1β (**F**), IL-8 (**G**), MIP-1α (**H**), IFN-γ (**I**), and IL-1Ra (**J**), were analyzed in plasma ($n = 6$). Plus (+) and minus (−) signs under the *x*-axis in each figure indicate the addition, or not, of inhibitors, respectively. Each dot represents one donor data point. Differences were statistically tested by paired, nonparametric Wilcoxon test. Asterisks above each box indicate a significant reduction of cytokine levels on the addition of inhibitors: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

chemoattractants, IL-8, MIP-1α, and MIP-1β, substantially increased by thrombin activation in the GPRP model (Fig. 3).

Thrombin could influence the cytokine response observed in this study by different routes. Thrombin-dependent activation of platelets via PAR-1 and PAR-4 would cause an immediate release of any of the intracellularly preformed mediators, including IL-1β (45), which were found elevated in our study (Fig. 3). However, this immediate release is triggered in direct response to thrombin and is not dependent on any innate immunity mechanisms initiated by the bacteria. Instead, the alteration in cytokine profiles in response to bacteria was likely reliant on reactions from other blood cells. Monocytes and granulocytes were activated in response to innate immune activation primarily via C5aR1 and CD14-dependent TLR activation (41). In addition, monocytes express PAR-1 and PAR-3, so alternatively to activating these cells, thrombin could directly stimulate them to release cytokines, which has been shown for IL-8 and IL-6 in response to thrombin (46). However, stimulating monocytes for cytokine releases via PAR-1 receptor was unlikely the case in this study because PAR-1 blockade did not significantly change the level of cytokines in plasma (Supplemental Fig. 4). This also indicates that PAR-1 activation by thrombin on platelets is not the determining factor for the cytokine expression. However, PAR-4 activation may be as important as PAR-1 for thrombin to activate platelets (38).

Mediators released from activated platelets or cell–cell interaction between activated platelets and monocytes or granulocytes could shape the cytokine response in these cells. Platelet activation in lepirudin anticoagulated blood was close to baseline levels. Xiang et al. (47) showed that PGE_2 released from activated platelets reduced the production of TNF and IL-6 from macrophages. This mechanism was recently confirmed for TNF in both macrophages and monocytes by Linke et al. (48). In contrast, platelets express P-selectin and CD40L, by which they interact with leukocytes via their counteracting ligands P-selectin glycoprotein-1 and CD40. Platelet interaction via CD40L to CD40 has been shown to reduce the production of TNF from monocytes (49), but platelet interaction with monocytes has also been shown to induce a proinflammatory phenotype, with altered production of cytokines and prostanoids (50). Interaction between activated platelets and monocytes via P-selectin and P-selectin glycoprotein-1 and simultaneous stimulation by platelet-released RANTES induce monocytic release of IL-8 and MCP-1 (51). Another possible explanation of how thrombin could influence inflammatory responses is via activation of complement C5 and generation of C5a, but this is a less likely explanation after the inefficient cleavage of C5 by thrombin in whole blood at physiological conditions (17).

Our data demonstrate that the acute cellular response in whole blood in response to bacterial stimuli is differentially dependent on thrombin, further shaped by multiple factors, including complement activation fragments and TLRs. Nine of the 10 cytokines we found elevated in response to bacteria were modulated, either downregulated or upregulated, by thrombin. At the same time, all cytokines,

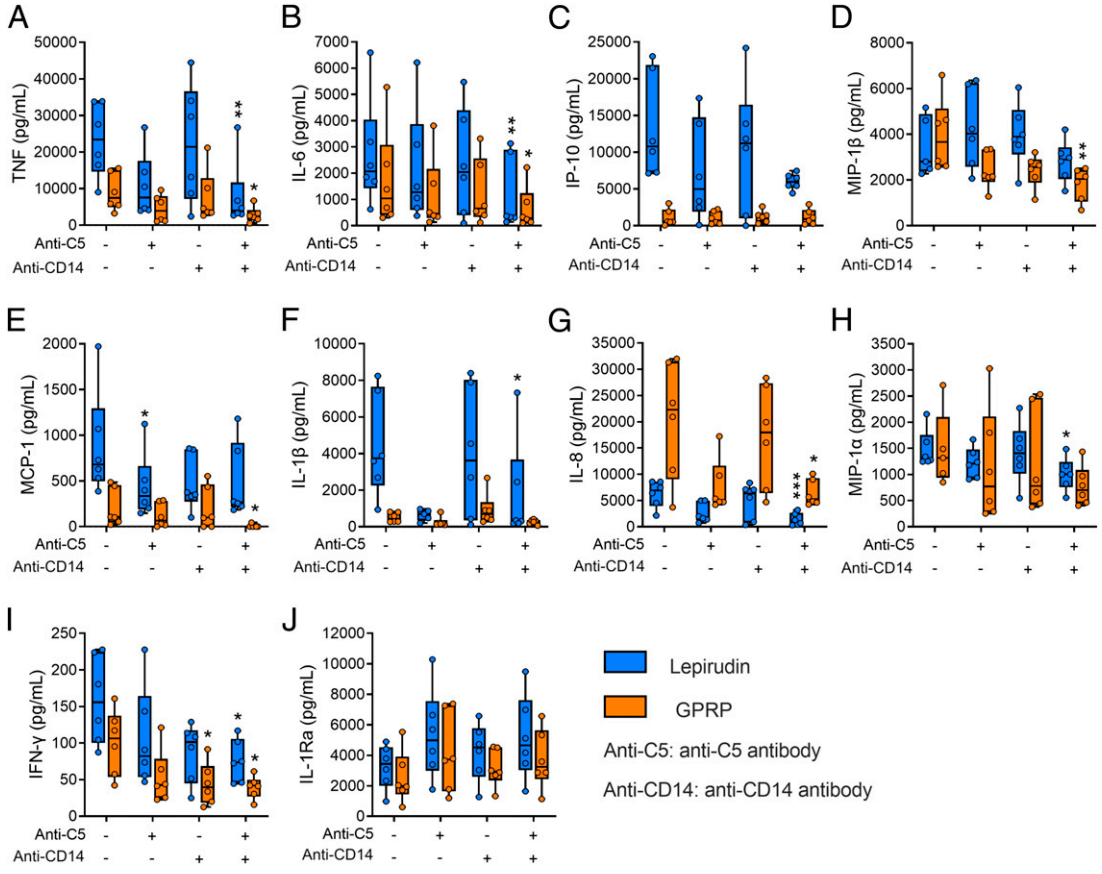

FIGURE 5. Effect of complement and CD14 inhibition on the cytokine response in the lepirudin (thrombin-inactive) and GPRP (thrombin-active) blood models incubated with *S. aureus*. Lepirudin anticoagulated and GPRP anticoagulated whole blood were incubated for 4 h with *S. aureus* with complement C5 inhibition and/or CD14 inhibition. Cytokines, TNF (**A**), IL-6 (**B**), IP-10 (**C**), MIP-1β (**D**), MCP-1 (**E**), IL-1β (**F**), IL-8 (**G**), MIP-1α (**H**), IFN-γ (**I**), and IL-1Ra (**J**), were analyzed in plasma ($n = 6$). Plus (+) and minus (−) signs under the *x*-axis in each figure indicate the addition, or not, of inhibitors, respectively. Each dot represents one donor data point. Differences were statistically tested by paired, nonparametric Wilcoxon test. Asterisks above each box indicate a significant reduction of cytokine levels on the addition of inhibitors: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

except for IL-1ra, in response to *E. coli* and all cytokines, except for IL-1ra and IP-10, in response to *S. aureus* were significantly reduced by the combined inhibition of complement C5aR1 and CD14. The combined inhibition of complement C5 and the TLR coreceptor CD14 has been shown as an efficient approach to attenuate innate immune cell activation in several ex vivo human blood models (52). The combined inhibition of C5 and CD14 was highly effective in both lepirudin and GPRP blood models for both bacteria, supporting the combined inhibition as a therapeutic approach for acute inflammation.

We used GPRP to inhibit the polymerization of soluble fibrin monomers, which enabled the evaluation of thrombin in anti-coagulated human whole blood. Compared with a normal coagulatory response, GPRP anticoagulated blood has increased levels of fibrin monomers and altered levels of fibrin degradation products. Fibrinogen, fibrin, and fibrin degradation fragments have been shown to have proinflammatory and anti-inflammatory effects on blood cells (53) and activate complement (54). Possible effects of soluble fibrin monomers generated in GPRP anticoagulated blood, but not in lepirudin anticoagulated blood, on blood cells and the protein cascades in GPRP anticoagulated blood remain mechanistically unexplored in this study, which is a limitation. In this study, we used dead bacteria to stimulate innate immune activation. We have previously compared live and dead *S. aureus* and *E. coli* for complement- and TLR-dependent phagocytosis and cytokine release in the lepirudin-based whole blood model and found similar responses to live and dead bacteria for both strains of bacteria (21). However, it should be taken into account that live could give a differential effect on innate immune activation, as shown in a heparin anticoagulated porcine whole blood model (55). Although it is interesting to have an insight on these differential effects of live versus inactive bacteria in human whole blood, it is beyond the scope of our study.

In conclusion, we demonstrated in this study that a GPRP-anticoagulated ex vivo human blood model enabled the investigation on the effects of thrombin on thromboinflammatory responses. We found that active thrombin, and the effects thereof, was a potent modulator of the inflammatory responses to bacterial stimulation. Furthermore, although thrombin did not influence the activation of the complement system, monocytes, and granulocytes, it induced elevated activations of platelets and significant alterations of hemostasis and proinflammatory cytokines. Thus, our data highlight the direct dependence of innate immune activation and the hemostatic reaction in shaping thromboinflammatory responses.

Disclosures

The authors have no financial conflicts of interest.

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