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Procoagulant Microparticles Promote Coagulation in a Factor XI-Dependent Manner in Human Endotoxemia

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Summary

Background—Human endotoxemia is characterized by acute inflammation and activation of coagulation, as well as increased numbers of circulating microparticles (MPs). Whether these MPs directly promote coagulation and through which pathway their actions are mediated, however, has not been fully explored.

Objectives—In this study, we aimed to further characterize endotoxin-induced MPs and their procoagulant properties using several approaches.

Methods—Enumeration and characterization of MPs were performed using a new generation flow cytometer. Relative contributions of the extrinsic and intrinsic pathways in MP-mediated procoagulant activity were assessed using plasmas deficient in FVII or FXI or with blocking antibodies to tissue factor (TF) or FXIa.

Results—Total MPs and platelet MPs were significantly elevated in plasma at 6 hours after infusion of endotoxin in healthy human subjects. MPs isolated from plasma following endotoxin infusion also demonstrated increased TF activity in a re-constituted buffer system. When added to re-calcified platelet poor plasma, these MPs also promoted coagulation, as judged by a decreased clotting time with shortening of the lag time and time to peak thrombin using calibrated automated thrombography (CAT). However, the use of FVII deficient plasma or blocking antibody to TF did not inhibit these procoagulant effects. In contrast, plasma clotting time was prolonged in FXI deficient plasma, and a blocking antibody to FXIa inhibited all MP-mediated parameters in the CAT assay.

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Addendum: M. J. Mooberry designed and performed the experiments, analyzed data and wrote the manuscript. R. Bradford performed experiments and analyzed data. E. L. Hobl provided subject samples and helped write the manuscript. F. C. Lin analyzed data and performed statistical analysis. B. Jilma was involved in study concept and design, provided subject samples and critically revised the manuscript. N. S. Key designed experiments, obtained funding, supervised the laboratory work and critically revised the manuscript.

Conclusions—The initiation of coagulation by cellular TF in endotoxemia is in contrast to (and presumably complemented by) the intrinsic pathway-mediated procoagulant effects of circulating MPs.

Keywords

blood coagulation; endotoxins; Factor XI; cell-derived microparticles; tissue factor

Introduction

The human endotoxemia model, in which purified lipopolysaccharide (LPS) from *Escherichia coli* is infused into healthy individuals is a model of acute systemic inflammation, which partially mimics the initial inflammatory response in the early stages of sepsis and other acute inflammatory disorders [1, 2]. Given the close association and interplay between inflammation and coagulation, this model has also been very informative in the study of acute activation of coagulation [3, 4].

Microparticles (MPs) are membrane vesicles (0.1 to 1 μm) released from cells upon activation or apoptosis. The hemostatic and/or thrombotic function(s) of MPs may be related to the presence of phosphatidylserine (PS) on the outer membrane leaflet, as well as the presence of tissue factor (TF) on some MPs [5]. PS exposure allows for docking of calcium-dependent coagulation enzymatic complexes and provides a catalytic surface for the tenase and/or prothrombinase complexes that promote thrombin generation [6]. TF, the transmembrane receptor for Factor VII/VIIa, is believed to be the principal initiator of coagulation *in vivo* [7]. Blood-borne TF resides primarily in the cellular fraction, with monocytes being the dominant source. In certain situations, TF may also be expressed by other cells, such as endothelial cells and platelets, although this is actively debated [8, 9]. Although MP-associated TF represents only a small fraction of TF in blood, it has been shown to contribute to thrombus formation *in vivo* in animal models [10].

Prior investigations into the activation of coagulation in endotoxemia have provided evidence for both intrinsic and extrinsic pathway participation. DeLa Cadena and colleagues demonstrated activation of the contact pathway in a human model of endotoxemia by showing decreased FXI functional activity, decreased prekallikrein (PK) antigen and activity, and increased levels of α2-macroglobulin-kallikrein complexes after endotoxin administration [11]. Similar findings were seen with *ex vivo* endotoxin treatment of whole blood in a time- and dose-dependent manner [12]. Animal models of endotoxemia have also provided evidence for contact pathway activation, with decreases in FXII, and in PK and high molecular weight kininogen, in rabbits [13], and dogs [14], respectively. In other studies, however, evidence for activation of the contact pathway has not been confirmed [15, 16].

Conversely, there is ample evidence for a role of the extrinsic pathway in the activation of coagulation in endotoxemia. Increased intracellular and surface expression of TF on circulating monocytes is detectable after *ex vivo* LPS stimulation [17]. Similarly, total whole blood TF mRNA is increased in human endotoxemia [18, 19], in addition to MP-TF and whole blood TF activity [20]. Perhaps most convincingly, it has been shown that inhibition

of the extrinsic pathway, using either anti-TF antibodies [21–23], active site-inactivated FVIIa [24], tissue factor pathway inhibitor (TFPI) [25], or genetically altered animals with either low TF [26] or FVII levels [27], have attenuated coagulopathy and improved survival in animal models of endotoxemia and sepsis.

Circulating MPs, including those bearing tissue factor (TF+-MPs), have been demonstrated to increase after endotoxin administration [20, 28] and are presumed to contribute to activation of coagulation. However, whether MPs contribute directly to coagulation activation in endotoxemia, and whether their effects are mediated preferentially through one of the pathways has not been well studied. Therefore, in this study, we used a new generation flow cytometer to characterize the circulating MP profile by number and phenotype. MP-dependent procoagulant activity was then interrogated to determine the relative contributions of the intrinsic and extrinsic pathways. Together these data help to further characterize the cellular origin of MPs and their role in the activation of coagulation in human endotoxemia.

Materials and Methods

Study Design and Sample Processing

The study was approved by the Ethics Committee at the Medical University of Vienna. Seventeen healthy subjects were enrolled in a trial using an established model of low grade endotoxemia [29]; subjects were infused with 2 ng/kg LPS (CCRE lot kindly provided by the NIH), with blood samples obtained at 4 time points (baseline [BL], 3, 6, and 24 hours post-LPS infusion). Samples from the 24-hour time point were available in only12 subjects.

Blood was collected into 3.8% citrated tubes (Vacutainer®; Becton Dickinson), with the first 2–3 mLs discarded. Platelet free plasma (PFP) was obtained immediately by two sequential centrifugations, each at 2,500g for 15 minutes, as per the International Society on Thrombosis and Haemostasis pre-analytical protocol for MP analysis by flow cytometry [30], and stored at −80°C until analysis.

Materials

TAT ELISA kit (Enzygnost® TAT micro kit) and human recombinant relipidated TF (Innovin®) were purchased from Siemens (Muenchen, Germany). Zymuphen MP-activity kit was from Aniara (West Chester, OH, USA). Control monoclonal antibody (mouse IgG) was from Sigma-Aldrich (St. Louis, MO, USA). FX and FVIIa were from Enzyme Research Laboratories (South Bend, IN, USA). FXa chromogenic substrate (Pefachrome® FXa8595) was from Pentapharm (Basel, Switzerland). Mouse anti-human TF monoclonal antibody (HTF-1) was provided by Dr. Ronald Bach (VA Medical Center; Minneapolis, MN, USA). AnnexinV-FITC, APC-labeled anti-CD41a, and FITC-labeled anti-CD31 were from BD Pharmingen (San Jose, CA, USA). PE-labeled anti-human CD235a was from Biolegend (San Diego, CA, USA). PE-labeled anti-CD14 was from eBioscience (San Diego, CA, USA). Gigamix beads were a gift from Philippe Poncelet (Biocytex; Marseille, France). CytoCount™ beads were from Dako (Carpinteria, CA, USA). FVII-deficient and FXI-deficient plasmas were from diaPharma (West Chester, OH, USA). Lipid vesicles were from

Avanti Polar Lipids (Alabaster, AL, USA), and large unilamellar vesicles containing 15% phosphatidylserine (PS) were prepared by extrusion as previously described [31, 32]. Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calibrator (α2-macroglobulin/thrombin) were from Diagnostica Stago (Parsippany, NJ, USA). Mouse anti-human Factor XIa antibody (aXI) and corn trypsin inhibitor (CTI) were from Haematologic Technologies, Inc. (Essex Junction, VT, USA).

MP Enumeration and Characterization by Flow Cytometry

PFP aliquots (30 μL) were stained with different combinations of the following markers/antibodies: 10 μL AnnexinV-FITC mixed 1:1 with Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$), 10 μL APC-labeled anti-CD41a, 10 μL PE-labeled anti-CD14, 10 μL FITC-labeled anti-CD31, and 2 μL PE-labeled anti-CD235a. All markers/antibodies were centrifuged at 20,000g x 20 min prior to use. Platelet-derived MP (PMP), monocyte-derived MP (MMP), endothelial-derived MP (EMP), and red cell-derived MP (RMP) subsets were identified (Figure 1C–F). Total MPs (TMPs) were defined as all AnnexinV+ events falling within the MP gate. MP size gate was set between 200–1,000 nm utilizing Gigamix® beads. Calcium-free samples and single-stained MP-free plasma (supernatant from normal, healthy donor PFP centrifuged at 100,000g x 60 minutes) were used as negative controls. Stained samples were incubated in the dark for 20 minutes and then diluted with Annexin binding buffer (500 μL) prior to analysis. All buffers were 0.1 μm filtered. CytoCount® beads (30 μL) were added to each sample immediately prior to analysis. MP concentration was calculated as follows: (MP counted x concentration of beads)/number of beads counted. All samples were analyzed on a Stratedigm S1000Ex (Stratedigm; San Jose, CA, USA) new generation flow cytometer equipped with a forward scatter photomultiplier tube using a side scatter trigger and with 0.1 μm filtered sheath fluid. Event rates were maintained at < 8,000 events/second to minimize coincident events, with more highly concentrated samples requiring further dilution prior to enumeration.

Microparticle Tissue Factor (MP-TF) Activity in Isolated MPs

MP-TF activity in isolated MPs was measured using a two stage chromogenic assay as previously described [33]. MP-TF activity was run on a random subset of patients (BL, 3 hr, 6 hr = 5 subjects; BL, 3 hr, 6 hr, 24 hr = 8 subjects). Since previous studies have also shown a reproducible peak in plasma TAT complexes at 3 hours [34], TATs were only measured at this time point.

MP Procoagulant Activity

MP prothrombinase activity was assayed using the Zymuphen™ MP-Activity kit, according to manufacturer's instructions. Notably, the functional part of this assay is not performed in a plasma milieu. Therefore, using a more physiologically relevant endpoint of clot formation, MP procoagulant activity was also measured in a plasma milieu using an in-house clotting assay (re-calcification time). Briefly, MPs from baseline and 3 hour time points were isolated from subject plasma by high-speed centrifugation (20,000 g x 30 min) and re-suspended in an equal volume of HBSA. Re-suspended MPs (50 μL) were incubated with various factor-deficient and control plasmas (50 μL) at 37°C for 2 minutes. After re-calcification with 25 mM $CaCl_2$ (50 μL), clotting times were measured using a STart® 4

Hemostasis Analyzer (Diagnostica Stago; Parsippany, NJ, USA). HBSA alone incubated with normal control plasma was used as a negative control. Plasmas used included: 1) normal, pooled PFP from citrated whole blood (prepared identically to endotoxemia samples as described in Study Design section); 2) FVII-deficient plasma; and 3) FXI-deficient plasma. Exogenous phospholipid (4 μM final) was added to all plasma preparations prior to incubation with MPs.

Calibrated Automated Thrombogram (CAT)

MP contribution to thrombin generation in plasma was measured using CAT as previously described [35], with minor modifications. Briefly, MPs were isolated from the plasma of eight additional subjects using high-speed centrifugation (20,000 g x 30 min). The MP pellet was then re-suspended in an equal volume of MP-depleted normal pooled plasma (NPP). NPP was prepared from multi-donor whole blood collected in citrate and CTI (18 μg/mL) and initially centrifuged to yield platelet free plasma, with a second high speed centrifugation (20,000 g x 20 min) to remove residual MPs. NPP was collected in citrate plus CTI to prevent *ex vivo* contact pathway activation in the substrate plasma that could serve as a stimulus for thrombin generation [36]. Next, 80 μL of re-suspended MPs were incubated with or without blocking antibodies to TF or FXIa, respectively (HTF-1 or anti-FXIa; 40 μg/mL final), for 10 minutes. MP reagent supplied by the manufacturer (20 μL), and consisting of phospholipid (4 μM final), was then added and allowed to incubate for 10 minutes. Finally, thrombin generation was initiated by dispensing fluorogenic substrate in $CaCl_2$ (416 μM and 16 mM final, respectively), calibrated against wells containing α2-macroglobulin/thrombin and plasma, and analyzed with Thrombinoscope software v3.0.0.29 (Thrombinoscope BV, Maastricht, the Netherlands). To ensure the absence of *ex-vivo* generated FXIa bound to MPs that could influence thrombin generation, PFP from one subject at the 3 hour time point and from whole blood treated *ex-vivo* with LPS was treated with PPACK 1 μM for 10 minutes prior to MP isolation. MPs were then washed once before CAT analysis. Additional aliquots of each PFP sample were also run in parallel without PPACK treatment. Parallel samples of PFP from *ex-vivo* LPS treated whole blood were also assessed for residual FXIa activity using a chromogenic substrate for FXIa with either no treatment, a single wash, or PPACK treatment followed by a single wash.

Statistics

We report descriptive statistics, including mean ± standard deviation (SD) or median [interquartile range (IQR)] as indicated, depending upon the distribution of the data analyzed. Normality was assessed using the Shapiro-Wilk normality test. Changes over time in MP-TF activity, clotting times and thrombin generation parameters (peak thrombin, time to peak thrombin, lag time, and endogenous thrombin potential [ETP]) were analyzed using one-way repeated measurements ANOVA with Tukey's multiple comparisons test. For ETP analysis, a value of zero was assigned to all samples that had such low levels of thrombin generation that area under the curve could not be calculated. Zymuphen MP Activity and MP flow cytometry data were analyzed using the Friedman test with Dunn's multiple comparisons test. TAT complexes were analyzed using the paired Student's t-test. Pearson and Spearman correlation coefficients were used according to the distribution of data being analyzed. P values < 0.05 were considered significant.

Results

Total and Platelet MP Numbers in Plasma are Increased in Endotoxemia

Utilizing a new generation flow cytometer, we can reliably resolve polystyrene particles as small as 200 nm by scatter detection alone (Figure 1A,B). In endotoxemia plasmas, significant increases were noted over time for both TMPs and PMPs (Figure 2A,B). TMPs peaked at 6 hours ($p<0.05$; baseline: 708 TMPs/μL plasma [330, 1,336]; 6 hr: 1,301 TMPs/μL plasma [732, 2,757]; median [IQR]), as was the case for PMPs ($p<0.01$; baseline: 92 PMPs/μL plasma [65, 183]; 6 hr: 246 PMPs/μL plasma [157, 765]) with a trend towards significance at 3 hours ($p=0.09$; 3 hr: 232 PMPs/uL plasma [109, 597]). RMP enumeration revealed a similar time course with a non-significant increase in peak numbers at 6 hours ($p=0.09$; 3 hr: 190 RMPs/μL plasma [90, 484]; 6 hr: 263 RMPs/μL plasma [133, 718]) (Figure 2C). No significant changes were noted for either MMP or EMP subsets.

Coagulation is Activated in Endotoxemia

Plasma TAT levels were significantly elevated at 3 hours compared to baseline (Figure 3B), with an almost 15-fold increase ($p<0.0001$; baseline: 4.5 ± 3.4 μg/mL vs. 3 hr: 65.6 ± 23.9 μg/mL).

MP-TF Activity on Isolated MPs is Increased in Endotoxemia

As previously described [33], there was a time-dependent increase in MP-TF activity on isolated MPs that peaked at 3 hours post LPS infusion (Figure 3A). MP-TF activity increased almost 10-fold at 3 hours ($p<0.0001$) and remained significantly elevated at 6 hours ($p<0.001$; [baseline: 0.06 ± 0.05 pg/mL; 3hr: 0.51 ± 0.18 pg/mL; 6hr: 0.47 ± 0.23 pg/mL; mean ± SD]). However, there was no correlation between MP-TF activity and TAT levels at 3 hours (Pearson $r=0.206$, $p=0.50$).

MPs in Endotoxemia Display Increased Prothrombinase Activity and Shorten MP-Dependent Clotting Times

Changes over time in MP prothrombinase activity were similar to those observed with flow cytometric analysis of MP numbers (Figure 4), with a significant increase at 3 hours ($p<0.05$; baseline: 1.85 nM PS equivalents [0.89, 2.98]; 3 hr: 5.73 nM PS equivalents [3.87, 7.04]; median [IQR]) and a peak activity at 6 hours ($p<0.0001$; 6 hr: 7.74 nM PS equivalents [5.11, 11.06]). There were no significant correlations between MP prothrombinase activity and TMPs by flow cytometry at any time point. Interestingly, however, there was a strong correlation between MP prothrombinase activity and RMPs at 6 hours (Spearman $r=0.721$; $p<0.01$), with a trend towards moderate correlation at baseline ($r=0.475$; $p=0.056$) and at 3 hours ($r=0.471$; $p=0.059$). Additionally, there was a significant correlation between MP prothrombinase activity and TAT complexes at 3 hours ($r=0.487$; $p<0.05$).

Endotoxin-induced increases in MP procoagulant activity were also observed in plasma in the clotting assay. When re-suspended in control PFP, MP-dependent procoagulant activity was increased at 3 hours (Figure 5) as evidenced by a significant reduction in clotting time compared to baseline ($p<0.0001$; baseline: 246 ± 46 seconds [s]; 3 hr: 186 ± 39 s; mean ± SD).

MP-Dependent Clotting Occurs in a FXI-Dependent Manner

To evaluate the relative contributions of the extrinsic and intrinsic pathways in MP-mediated clot formation, MPs were added to FVII-deficient and FXI-deficient plasma, respectively. With both baseline and 3 hour MP samples, there were no significant prolongations of clotting times in FVII deficient plasma, indicating that MP-dependent initiation of clot formation was not dependent on FVII. In contrast, there was a significant prolongation of clotting times observed in FXI deficient plasma at both time points (Figure 5). Using MPs obtained from baseline plasma, clotting time increased from 246 ± 46 s (mean ± SD) in control PFP to 345 ± 86 s in FXI deficient plasma ($p<0.001$). Similarly, with MPs obtained 3 hours after LPS infusion, clotting time increased from 186 ± 39 s in control PFP to 265 ± 63 s in FXI deficient plasma ($p<0.0001$).

MPs in Endotoxemia Enhance Thrombin Generation in a FXI-Dependent Manner

Utilizing CAT, MPs generated during endotoxemia shortened the lag time by almost half and the time to peak thrombin by one-third compared to baseline MPs, with no significant differences in other parameters of thrombin generation (Figure 6A). Using baseline MPs, lag time was 37.7 ± 11.4 min (mean ± SD) compared to 22.9 ± 5.7 min using MPs generated after 3 hours ($p<0.05$), and time to peak thrombin decreased from 42.7 ± 11.9 min at baseline to 27.8 ± 5.3 min at 3 hours ($p<0.05$).

When the relative contributions of the intrinsic and extrinsic pathways on MP-initiated thrombin generation were examined, observations were consistent with the clotting assay results. That is, FXIa inhibition had a significant impact on MP-mediated thrombin generation, but no significant changes were observed following TF inhibition (Figure 6B-D). Peak thrombin levels were significantly decreased in both baseline and 3-hour MP samples with FXIa antibody treatment; for baseline MPs, peak thrombin decreased from 26.6 ± 14.2 nM (mean ± SD) to 1.3 ± 0.9 nM ($p<0.01$) with FXIa inhibition, and for 3-hour MPs, peak thrombin decreased from 34.7 ± 7.34 nM to 2.5 ± 1.5 nM ($p<0.0001$). Inhibition of FXIa also significantly prolonged lag time and time to peak thrombin, although this was only observed with the 3-hour MPs. Lag time increased from 22.9 ± 5.7 min (mean ± SD) to 72.4 ± 42.7 min ($p<0.01$), and time to peak thrombin increased from 27.8 ± 5.3 min to 85.0 ± 22.6 min ($p<0.001$). As demonstrated in the representative thrombin generation curve in Figure 6A, endogenous thrombin potential (ETP) was also significantly reduced with FXIa inhibition, with near complete blunting of thrombin generation at baseline and 3 hours. At baseline, ETP decreased from 388.3 nM [206.9, 479.4] (median [IQR]) to 0 nM [0, 10.25] ($p<0.05$), and at 3 hours from 394.8 nM [301.9, 463.0] to 0 nM [0, 20] ($p<0.05$) with FXIa blockade.

No significant difference was noted in thrombin generation profiles with or without PPACK treatment of MPs in the endotoxemia subject sample or the *ex-vivo* LPS stimulated blood (Figure 6E–F). In general, MPs were able to promote thrombin generation in all samples. TF inhibition had no effect on thrombin generation other than mild prolongation of lag time in 3 of the samples, whereas inhibition of FXIa again caused nearly complete abrogation of thrombin generation. Absence of residual FXIa activity on MPs was also confirmed in parallel samples incubated with a chromogenic substrate for FXIa (Figure 6G).

Discussion

In this study, we sought to characterize circulating MP numbers and phenotype, as well as the procoagulant properties of MPs in the human endotoxemia model. Flow cytometry only detects the largest MPs, in contrast to functional assays that measure the effects of the entire MP population. This discrepancy is worth mentioning, as the smallest MPs may provide a procoagulant surface, and in fact the fraction of MPs < 200 nm has been shown to account for at least 50% of thrombin generating capacity [37]. We found that MPs induced by endotoxin infusion were more procoagulant than baseline (pre-endotoxin) MPs, as evidenced by shortened clotting times, shortened lag times and time to peak thrombin with CAT, as well as increased MP-associated prothrombinase activity. The latter increased with a time course that mirrored increases in MPs detected by flow cytometry with peak values at 6 hours. Unexpectedly, however, the procoagulant effect of MPs was dependent on FXI, but not FVII or TF. Indeed, ETP was almost completely abrogated by an anti-FXIa antibody, which was observed both at baseline and at 3 hours. It is notable that others have reported similar findings regarding MP-initiated thrombin generation in sepsis, where eight of nine patients exhibited strong inhibition of thrombin generation with a monoclonal anti-FXI antibody, as compared to only four of nine and two of nine with anti-TF and anti-FXII antibodies, respectively [38].

It should be noted that MP-TF activity was not measured in a plasma milieu, and thus potentially important natural regulators, such as TFPI, were absent. This limitation presumably explains the discrepancy between the MP-TF increase on isolated MPs (Figure 3A) and the absence of demonstrable TF/FVII(a) dependent activity in plasma-based assays (Figures 5 & 6). It may also explain the lack of correlation between MP-TF activity and TAT complexes. Others have also shown that increases in a similar MP-TF activity assay in cancer patients do not necessarily predict increased microparticle TF-dependent procoagulant activity in plasma [39]. Our observations are in contrast to what has been reported in a murine model of endotoxemia, where MP-TF activity and TAT levels correlated strongly [40]. Although our data indicate that MP-associated TF does not play a role in activation of coagulation in human endotoxemia, they do not exclude the possibility that other circulating sources of TF – such as that associated with monocytes – may play a central role in this regard. This possibility is supported by the fact that TFPI or active site inhibited FVIIa largely abrogate coagulation activation in human endotoxemia [41, 42]. Thus, although MPs can potentially promote coagulation activation through different pathways in endotoxemia (Figure 7), TF-dependent mechanisms do not appear to play a significant role in thrombin generation (Table 1).

Recent reports in other clinical settings also support the notion that MPs can promote activation of coagulation through the intrinsic pathway. Both PMPs and RMPs generated *ex vivo* were shown to initiate thrombin generation in a FXII-dependent mechanism [43]. In sickle cell disease, MP-mediated thrombin generation was reduced 2-fold with an anti-FXI antibody but was unaffected by an anti-human TF antibody [44]. The extent of this inhibition by the FXI antibody was also significantly correlated with the number of RMPs detected by flow cytometry. In a similar study, thrombin generation initiated by RMPs from stored red cell units was decreased by approximately 50% in the presence of a FXI

inhibitory antibody, whereas no inhibition was noted with an antibody to TF [45]. The findings from the latter two studies may be pertinent to our study, in that RMPs were the only MP subset to correlate with total PS equivalents at multiple time points, and MP prothrombinase activity was the only parameter that correlated with TAT complexes. In aggregate, these data suggest a role for RMPs in promoting coagulation via the intrinsic pathway.

Enumeration and characterization of MPs was performed using a new generation flow cytometer, which allows for detection of smaller MPs [46]. We determined that total MPs and PMPs peaked at 6 hours, with a similar trend noted for RMPs. Although the time course for these increases differed from that seen with MP-TF activity and TAT complexes [4], they were congruous with previously published reports of MP numbers detected by flow cytometry in endotoxemia [47].

A potential limitation of our study is the fact that blood samples from endotoxemia subjects were not collected in citrate plus CTI to prevent *ex vivo* activation of the contact pathway. Our results, however, support the case that artifactual *ex vivo* contact activation was not the cause for MP-dependent coagulation activation occurring in a FXI-dependent manner. Specifically, MPs isolated from endotoxemia subjects were added to pooled, donor PFP collected in citrate plus CTI to prevent contact activation. Additionally, treatment of subject plasma with PPACK prior to MP isolation did not affect FXI-dependent thrombin generation, and these data were also confirmed using MPs generated by *ex-vivo* LPS treatment of whole blood. Further confirmation of the absence of residual *ex-vivo* generated FXIa on MPs was also performed by analyzing samples with a chromogenic substrate for FXIa. These data confirm that FXI-dependent thrombin generation was wholly dependent on added MPs rather than pre-formed circulating activated contact factors in the substrate or test plasmas. Another potential limitation was the fact that we focused exclusively on MPs, and did not concurrently measure cell-associated procoagulant activity. However, we [28] and others [18] have previously shown that TF mRNA and activity [48] in the mononuclear cell fraction increase dramatically in the human endotoxemia model.

In summary, we have found that MPs are increased in human endotoxemia and are procoagulant in nature. The largest increases in MP numbers occurred with PMP and RMP subsets, both of which peaked at 6 hours post LPS infusion. Our data further demonstrate a potentially important role for FXI and the intrinsic pathway in MP-mediated effects on coagulation activation in endotoxemia. This effect may not be specific to MPs generated in endotoxemia, but rather a property of MPs in general. The mechanism(s) by which MPs promote coagulation through the intrinsic pathway, and whether different MP subsets activate coagulation through different pathways is not clear. Direct activation of the contact pathway, enhancement of thrombin-mediated activation of FXI, or some other unknown mechanism may be responsible. Further studies are warranted to evaluate these possibilities.

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Essentials

- The procoagulant effects of microparticles (MPs) on coagulation in endotoxemia are not known.
- MPs from endotoxemia volunteers were evaluated for procoagulant activity in a plasma milieu.
- MPs from endotoxemia volunteers shortened clotting times and enhanced thrombin generation.
- MP procoagulant effects were mediated in a Factor XI dependent manner.

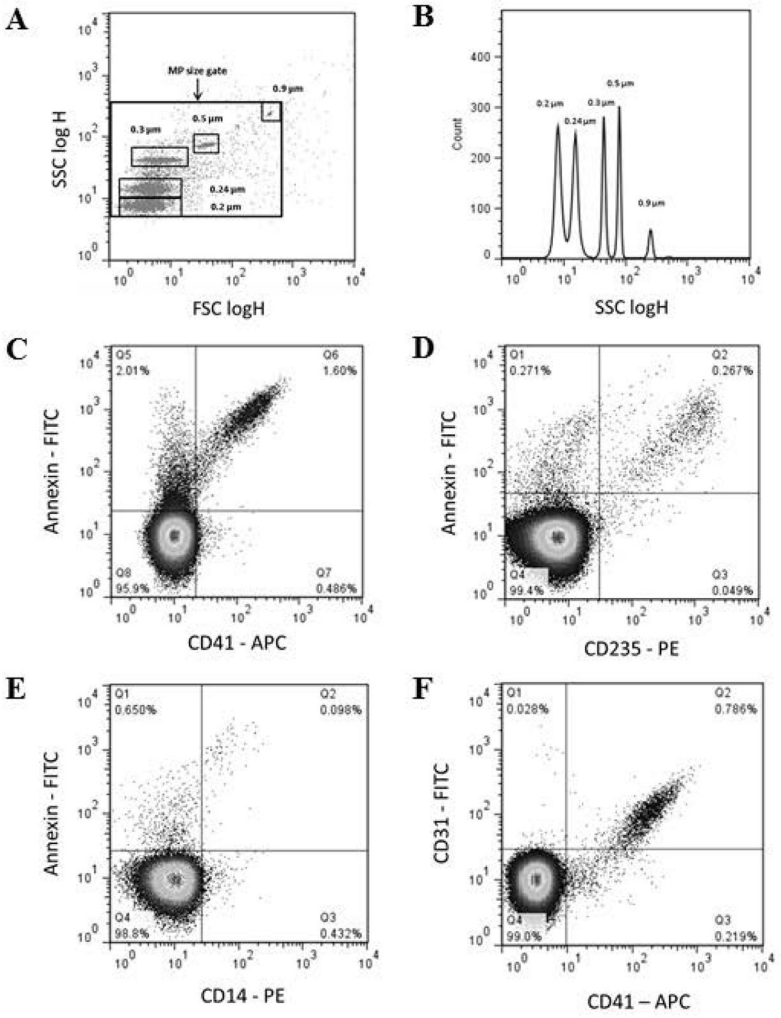
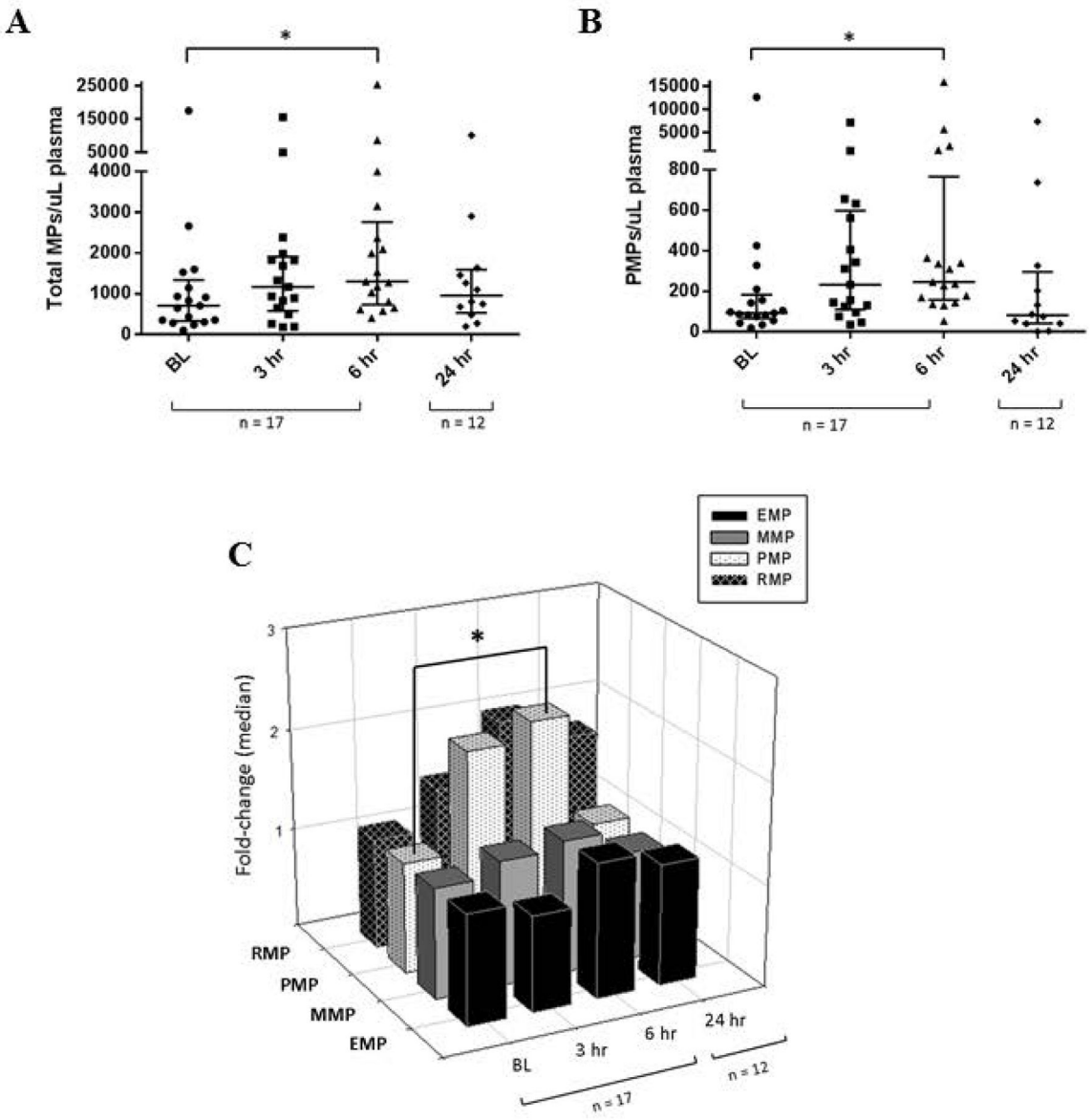


Figure 1.



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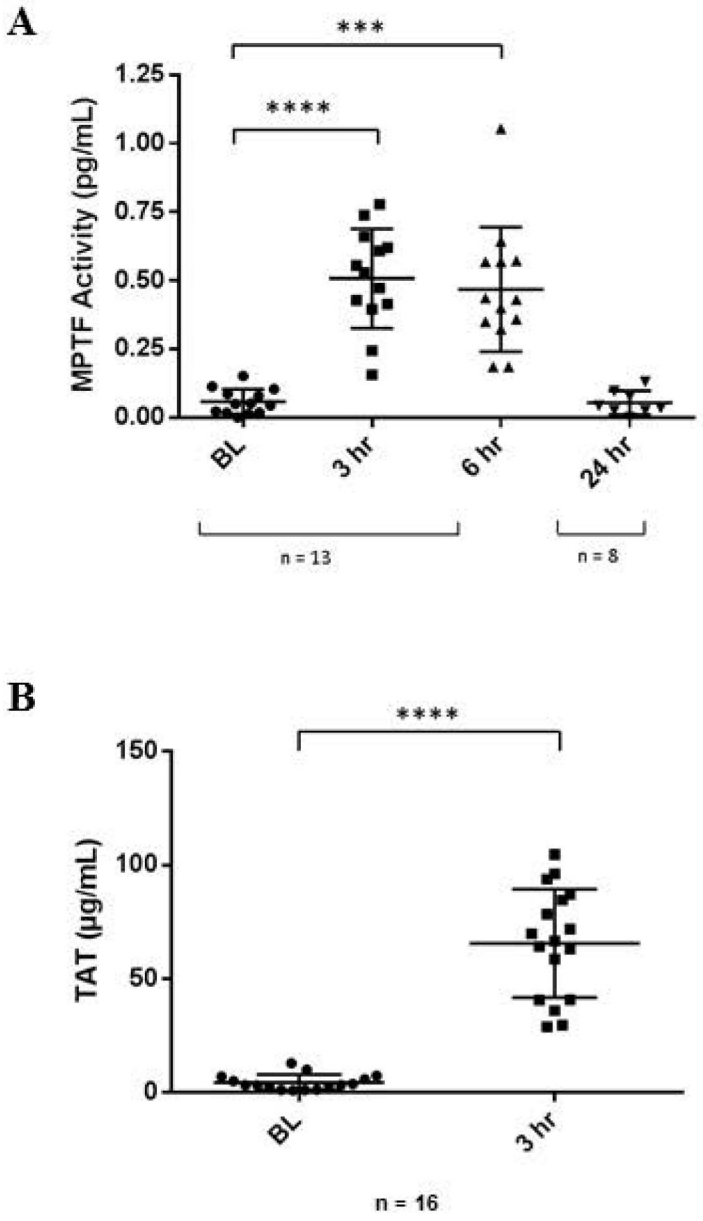

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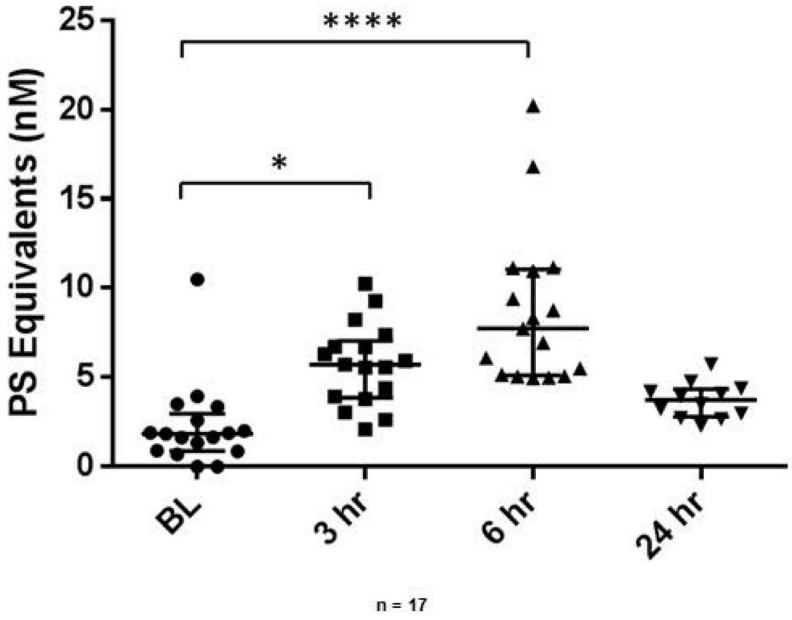

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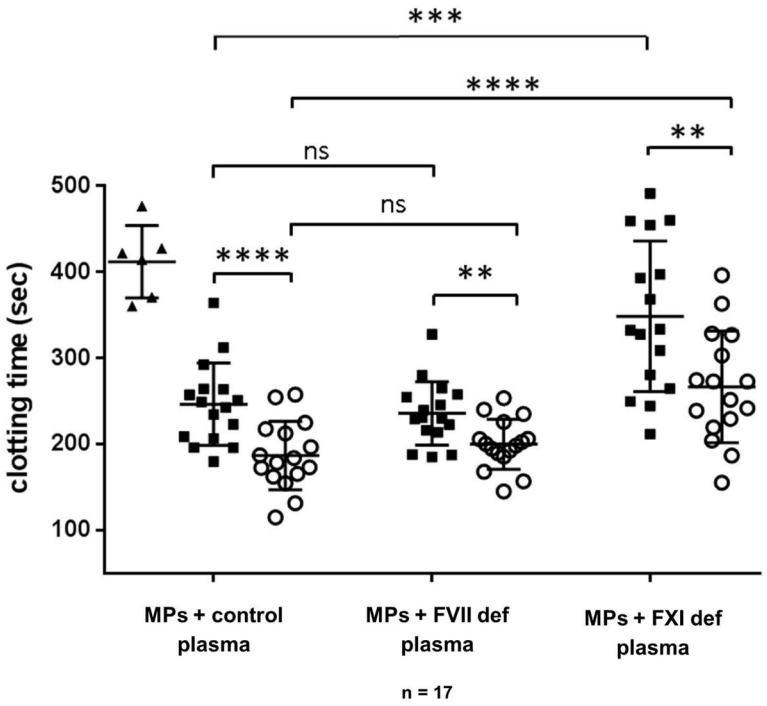

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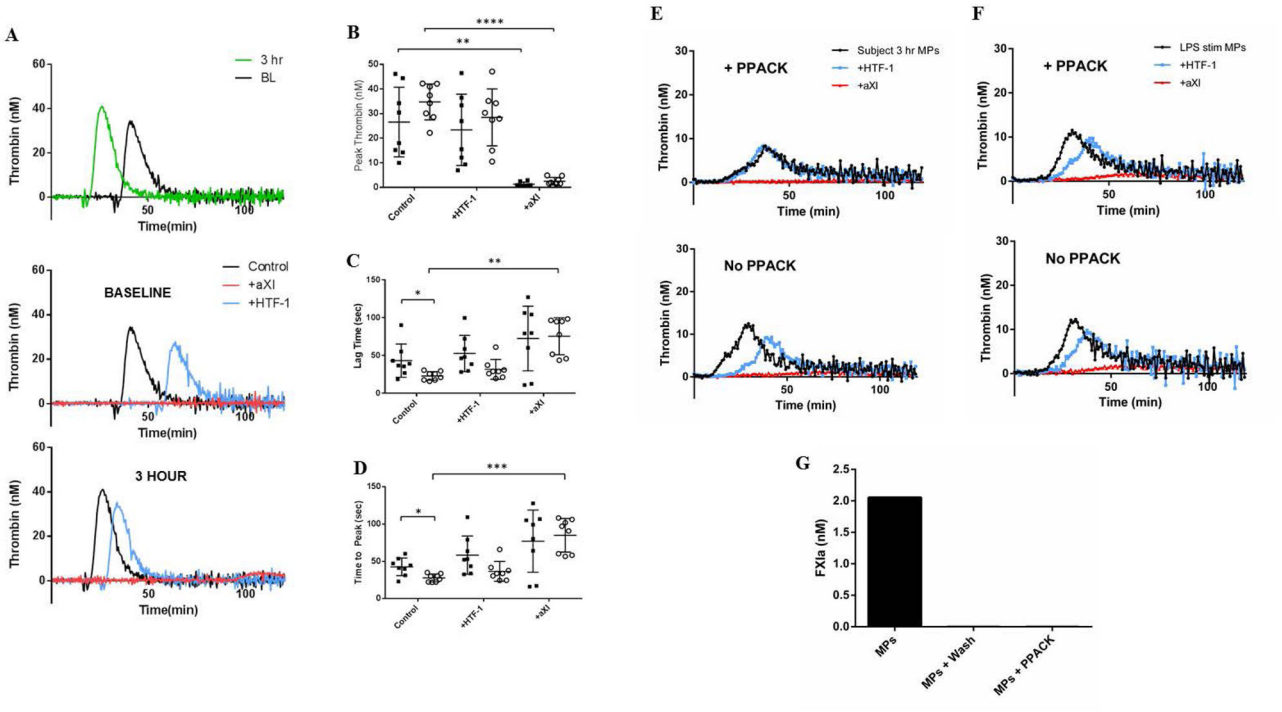

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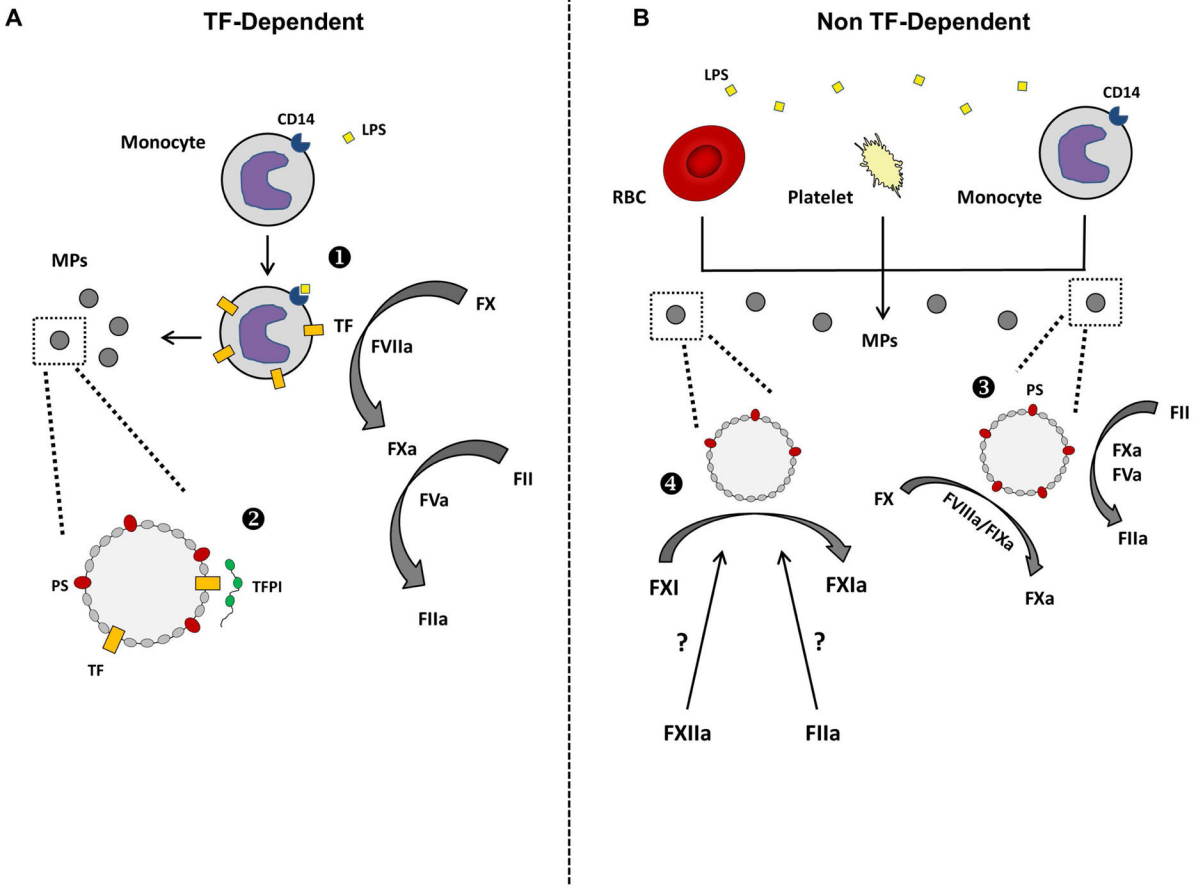

Figure 7.

Table 1

Summary of MP Assays

Assay Environment and Contributors to MP-Mediated Procoagulant Activity	MP-TF Activity	MP Prothrombinase Activity	MP-dependent Clotting Time	MP-mediated Thrombin Generation
Plasma Milieu	−	−	+	+
Buffer System	+	+	−	−
Phosphatidylserine (PS)	+	+	+	+
Tissue Factor (TF)	+	−	−	−
Factor XIa (FXIa)	−	−	+	+