Clots Are Potent Triggers of Inflammatory Cell Gene Expression

Indications for Timely Fibrinolysis


Objective—Blood vessel wall damage often results in the formation of a fibrin clot that traps inflammatory cells, including monocytes. The effect of clot formation and subsequent lysis on the expression of monocyte-derived genes involved in the development and progression of ischemic stroke and other vascular diseases, however, is unknown. Determine whether clot formation and lysis regulates the expression of human monocyte-derived genes that modulate vascular diseases.

Approach and Results—We performed next-generation RNA sequencing on monocytes extracted from whole blood clots and using a purified plasma clot system. Numerous mRNAs were differentially expressed by monocytes embedded in clots compared with unclotted controls, and IL-8 (interleukin 8) and MCP-1 (monocyte chemoattractant protein-1) were among the upregulated transcripts in both models. Clotted plasma also increased expression of IL-8 and MCP-1, which far exceeded responses observed in lipopolysaccharide-stimulated monocytes. Upregulation of IL-8 and MCP-1 occurred in a thrombin-independent but fibrin-dependent manner. Fibrinolysis initiated shortly after plasma clot formation (ie, 1–2 hours) reduced the synthesis of IL-8 and MCP-1, whereas delayed fibrinolysis was far less effective. Consistent with these in vitro models, monocytes embedded in unresolved thrombi from patients undergoing thrombectomy stained positively for IL-8 and MCP-1.

Conclusions—These findings demonstrate that clots are potent inducers of monocyte gene expression and that timely fibrinolysis attenuates inflammatory responses, specifically IL-8 and MCP-1. Dampening of inflammatory gene expression by timely clot lysis may contribute to the clinically proven efficacy of fibrinolytic drug treatment within hours of stroke onset.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1819-1827. DOI: 10.1161/ATVBAHA.117.309794.)

Key Words: fibrin ♦ fibrinogen ♦ inflammation ♦ monocytes ♦ thrombin

Coagulation contributes to the pathogenesis of many disease processes, including sepsis, myocardial infarction, and ischemic stroke.1,2 On vessel injury, blood is exposed to tissue factor, resulting in the formation of thrombin that stems blood loss by activating coagulation proteases and platelets.3,4 Thrombin also induces the conversion of fibrinogen to fibrin, resulting in the formation of clots that seal wounds.5,6

When clots form, they trap numerous blood cells, including monocytes. Monocytes play an important role in bridging hemostasis and inflammation because they generate tissue factor and inflammatory cytokines in response to external stimuli.5,9 Perhaps the most well-known stimulus of monocyte gene expression is lipopolysaccharide (LPS).1,2 However, LPS is not present in most clinical situations where clots form, and individual clotting factors, such as tissue factor, thrombin,13,14 and fibrin,15–17 have variable and generally mild stimulatory effects on monocyte gene expression. This suggests that clots are ineffective triggers of gene expression by monocytes, but this assumption has not been rigorously tested. Accordingly, we determined whether intact clots alter gene expression patterns in monocytes using human models of whole blood and plasma clot formation. Our data demonstrate that clots induce marked changes in the monocyte transcriptome, including increased expression of IL-8 (interleukin-8) and MCP-1 (monocyte chemoattractant protein-1). Remarkably, these shifts were more intense than changes in gene expression induced by LPS.

After fibrin formation occurs under physiological conditions, tPA (tissue-type plasminogen activator) is generated and immediately begins breaking down the fibrin matrix.18 Inflammatory fibrinolysis is used as a therapeutic intervention in pathological thrombosis.19,20 In the setting of stroke, tPA is administered at high levels to quickly dissolve the clot and improve blood flow to ischemic brain. In either setting, the effect of fibrinolysis on cytokine expression has not been
examined. Our results demonstrate that fibrinolysis significantly downregulates IL-8 and MCP-1 synthesis by monocytes, depending on when lysis was initiated. These findings have significant implications in ischemic stroke because timely administration of tPA after stroke dictates outcomes.

Materials and Methods
Materials and Methods is available in the online-only Data Supplement.

Results
Whole Blood Clots Trigger Inflammatory Gene Expression in Monocytes
To test whether clots trigger inflammatory gene expression, whole blood was clotted with recombinant tissue factor in the presence of calcium for 2 hours, and gene expression patterns were examined in purified monocytes. Isolated monocytes were mostly CD14+C16− (>90%), and this population did change significantly after whole blood clot formation (Figure I in the online-only Data Supplement). RNA-seq revealed that monocytes expressed over 10,000 transcripts and nearly 1000 were differentially expressed (ie, >4-fold) in clot-retrieved monocytes compared with baseline monocytes (Figure 1A). Among the differentially expressed transcripts were IL-8 and MCP-1, which were increased by >6- and 48-fold, respectively, in clot-retrieved monocytes (Figure 1A and 1B). Increased mRNA expression was confirmed by real-time polymerase chain reaction (Figure II in the online-only Data Supplement) and was associated with elevated levels of IL-8 and MCP-1 protein, which accumulated in a time-dependent fashion (Figure 1C). In addition to IL-8 and MCP-1, 13 other proteins involved in inflammation were increased after whole blood clot formation, and mRNA for these genes correlated with their protein expression levels (Figure III in the online-only Data Supplement). These cytokines were also expressed in thrombi extracted from patients (Figure IV in the online-only Data Supplement), indicating that in vivo clot formation is also associated with the generation of inflammatory proteins.

Plasma Clot Formation Induces Inflammatory Gene Expression in Monocytes
Next we determined whether clotted plasma triggers gene expression in a manner similar to whole blood. For these studies, recombinant tissue factor was added to recalcified, cell-free plasma and allowed to clot before addition of purified autologous monocytes (Figure V in the online-only Data Supplement). Changes in monocyte gene expression in response to clot formation were then assessed. RNA-seq analyses revealed that mRNAs were differentially expressed

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Nonstandard Abbreviation</th>
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<tr>
<td>FXIII</td>
<td>factor XIII</td>
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<td>IL-8</td>
<td>interleukin-8</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MCP-1</td>
<td>monocyte chemotactrant protein-1</td>
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<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<td>TLR4</td>
<td>toll-like receptor-4</td>
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<td>tPA</td>
<td>tissue-type plasminogen activator</td>
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Figure 1. Whole blood clot formation markedly alters gene expression patterns in monocytes. A, RNA sequencing was performed in baseline or clot-retrieved monocytes, and relative expression of individual genes were plotted against each other. Red and green indicate increased and decreased gene expression, respectively, between clotted compared with baseline (4-fold difference or greater). IL-8 (interleukin 8) and MCP-1 (monocyte chemotactrant protein-1) mRNA expression compared with other genes is highlighted by arrows. B, Distribution of RNA-seq reads across MCP-1 and IL-8 transcripts (with intron/exon structures depicted below each plot) in monocytes from unclotted (baseline) blood and clotted whole blood. C, Protein for IL-8 and MCP-1 protein was measured in the plasma of clotted whole blood at specific times postclot formation. The bars in this graph indicate means±SEM of 3 independent experiments (n=3). The asterisk indicates *P<0.05 compared with freshly isolated monocytes that were processed immediately. BL indicates baseline; and NT, not treated.
in monocytes embedded within clots compared with freshly isolated monocytes (baseline), unstimulated monocytes (untreated) that were left in suspension culture for equivalent time periods, or thrombin-stimulated monocytes (Figure 2A and B). Similarity matrices demonstrated that RNA patterns for freshly isolated, untreated and thrombin-stimulated, and clot-retrieved monocytes clustered in distinct nodes (Figure 2A and B). More than 1100 transcripts were differentially expressed by >4-fold between the clotted and control groups (Figure 2C). In addition, patterns of gene expression in monocytes isolated from whole blood and plasma clots were moderately correlated with one another (Figure VI in the online-only Data Supplement; R=0.364 and P=0.0008), demonstrating that the reduced plasma model clot system mimics responses observed in a whole blood clotting model.

Monocytes embedded in plasma clots displayed increased expression for IL-8 and MCP-1 mRNA compared with all control groups as measured by RNA-seq (Figure 2D). Quantitative real-time polymerase chain reaction confirmed increases in IL-8 and MCP-1 (Figure 2E) and demonstrated a time-dependent change in mRNA expression (Figure VII in the online-only Data Supplement). Consistent with changes in mRNA, high levels of IL-8 and MCP-1 protein were detected in plasma clot samples (Figure 2F). In contrast, protein for IL-8 and MCP-1 was not observed in untreated or thrombin-stimulated samples resuspended in M199 (Figure 2F). Monocytes embedded within clots also produced more IL-8 and MCP-1 protein than LPS-stimulated monocytes (Figure 2F), and monocytes stained similarly for IL-8 and MCP-1 as compared with thrombi isolated from human patients (Figure VIII in the
Global protein synthesis was also markedly higher in clotted samples when compared with not treated and LPS-treated samples (Figure 2G and Figure IX in the online-only Data Supplement).

**MCP-1 and IL-8 Expression Are Regulated at the Transcriptional Level**

Protein for IL-8 and MCP-1 was markedly increased after 18 hours but not before then (Figure 3A). Accumulation of IL-8 and MCP-1 protein was completely blocked in the presence of cycloheximide, a global inhibitor of protein synthesis (Figure 3B; Figure X in the online-only Data Supplement). Actinomycin D also prevented monocytes from generating IL-8 and MCP-1 protein (Figure 3C). To further examine transcriptional regulation of IL-8 and MCP-1 induced by plasma clots, we treated monocytes with a NF-κB (nuclear factor kappa B) inhibitor. Inhibition of NF-κB blocked IL-8 and MCP-1 synthesis (Figure XI in the online-only Data Supplement). Taken together, these results demonstrate that the synthetic events were primarily controlled at the transcriptional level.

**Fibrin Formation Is Required for Cytokine Synthesis**

Results displayed in Figure 2E and 2F demonstrate that treatment of purified monocytes (ie, not in plasma) with low concentrations of thrombin (0.1 U/mL) did not have appreciable effects on IL-8 and MCP-1 mRNA or protein levels, and thrombin did not increase global protein synthesis (Figure IX in the online-only Data Supplement). We also found that increasing concentrations of purified thrombin had no appreciable effect on IL-8 or MCP-1 synthesis (Figure 4A). However, pretreatment of plasma with heparin, which blunts thrombin generation and, thereby, clot formation, significantly reduced IL-8 and MCP-1 synthesis (Figure 4B). The specific thrombin inhibitor lepirudin also reduced cytokine synthesis in a dose-dependent manner (Figure 4C). These studies indicated that thrombin generation, but not thrombin itself, is critical for clot-dependent cytokine production.

Because thrombin converts fibrinogen to fibrin, we determined whether mediators released into the clotted milieu or fibrin formation are required for cytokine synthesis. The addition of fibrin-free serum did not induce IL-8 or MCP-1 synthesis (Figure 5A), suggesting that fibrin formation is the primary instigator of these synthetic events. To examine this postulate in more detail, we added Gly-Pro-Arg-Pro to thrombin-treated plasma to see if inhibition of fibrin polymerization prevents MCP-1 and IL-8 synthesis. As shown in Figure 5B, the addition of Gly-Pro-Arg-Pro blocked IL-8 and MCP-1 synthesis. The concentration of Gly-Pro-Arg-Pro to inhibit fibrin formation had no effect on LPS-stimulated monocytes (Figure XIII in the online-only Data Supplement). We also found that the addition of tissue factor and calcium to fibrinogen-deficient plasma did not induce a robust cytokine response as compared with the presence of fibrinogen (Figure 5C). In addition, tissue factor, alone, had little effect on cytokine production (Figure XIII in the online-only Data Supplement). Reintroduction of fibrinogen to
fibrinogen-deficient plasma, however, triggered IL-8 and MCP-1 synthesis in a concentration-dependent manner (Figure 5D). When thrombin generation was blocked by the presence of heparin, but fibrin formation was allowed to proceed by the addition of batroxobin—a snake venom capable of cleaving fibrinogen—monocytes synthesized IL-8 and MCP-1 (Figure 5E). Fibrin stabilization through factor (F) XIII may play a critical role inducing monocyte-derived IL-8 and MCP-1. To address the role of FXIII, we formed plasma clots in the presence and absence of a specific FXIII inhibitor, T101. Plasma clots formed in the presence of T101 had reduced fibrin cross-linking but had no effect on cytokine production (Figure XV in the online-only Data Supplement). In addition, plasma clots were generated with recombinant tissue factor and calcium. After 2 hours, serum was removed from the plasma clots by centrifuging the sample at 12,000g for 20 minutes. Monocytes were then incubated with plasma clots or in residual serum. B, Monocytes were embedded into plasma fibrin clots formed in the presence of Gly-Pro-Arg-Pro (GPRP), a fibrin polymerization inhibitor or vehicle (water). C, Monocytes were embedded in plasma clots that were generated from normal plasma or fibrinogen-deficient plasma. D, Monocytes were embedded in fibrinogen-deficient plasma that was reconstituted with increasing concentrations of fibrinogen. E, Plasma fibrin clots were formed in the presence of heparin to prevent thrombin generation and in the presence or absence of batroxobin (1 U/mL), which generates fibrin in a thrombin-independent manner. As a control, monocytes were resuspended in M199 and stimulated with batroxobin. In all treatment conditions, cell-free supernatants were harvested after 18 hours, and IL-8 (interleukin-8) and MCP-1 (monocyte chemoattractant protein-1) levels were assessed. The bars in the panels represent the means±SEM of 3 independent experiments for each group. The asterisk and hashtag indicates P>0.05 in clot samples versus their comparative controls.

Fibrinolysis Blunts Clot-Induced Cytokine Synthesis in a Time-Dependent Manner

Next we determined whether fibrinolysis (breakdown of the fibrin clot) affects clot-induced cytokine synthesis. Monocyte mRNA expression patterns were examined in plasma clots that were lysed with tPA 30 minutes after clot formation and the addition of monocytes. As shown in Figure 6A, mRNA expression patterns were markedly different in clot-retrieved monocytes compared with untreated monocytes, and these changes were blunted in the presence of tPA. Furthermore, patterns of gene expression in monocytes isolated from plasma clots and plasma clots plus tPA demonstrated a negative correlation with one another (R=0.807,
Monocytes play prominent roles in driving coagulation and inflammation, in part, through de novo synthesis of thrombin inflammatory proteins on stimulation. Here, we determined for the first time intact clots trigger robust, global expression of inflammatory genes in monocytes, including IL-8 and MCP-1. Thrombi extracted from patients demonstrated similar patterns of cytokine expression, suggesting that in vivo clot formation can also induce the generation of IL-8 and MCP-1 (Figure IV in the online-only Data Supplement). The ability of the clot to activate multiple inflammatory cytokines...
(IL-8, MCP-1, and others; Figure XVII in the online-only Data Supplement) and pathways underscores the complexity of the interaction between coagulation reactions and inflammation. Furthermore, these data suggest that dysregulation of clot formation may significantly impact downstream processes, such as wound healing, and could contribute to ongoing coagulation responses because genes, such as tissue factor display, increased expression in clot-retrieved monocytes (Figure XVIII in the online-only Data Supplement).

RNA-seq analysis of monocytes derived from whole blood clots demonstrated robust differential gene expression compared with monocytes isolated immediately after whole blood was drawn. To further verify specific changes in the monocyte transcriptome because of plasma clot formation, we performed additional RNA-seq analysis from monocytes embedded in a purified plasma clot system. RNA-seq analysis revealed that monocytes embedded in plasma clots mounted a robust gene expression response, and differentially expressed mRNAs resembled observed changes in the more complex whole blood clot (Figure VI in the online-only Data Supplement; \( R=0.364; P \) value =0.0008). Plasma clots induced robust synthesis of IL-8 and MCP-1 mRNA and protein and a marked increase in global protein synthesis. Consistent with an increase in global protein synthesis, ribosomes attached to endoplasmic reticulum were more frequently observed in clot-embedded monocytes compared with unstimulated monocytes (Figure IX in the online-only Data Supplement). The upregulation of mRNAs followed by subsequent synthesis of their corresponding proteins (Figure XVII in the online-only Data Supplement) in clot-retrieved monocytes suggests that the synthesis of numerous genes is regulated at the transcriptional level. In this regard, transcriptional inhibitors completely abrogated the synthesis of IL-8 and MCP-1, demonstrating that clots induce the transcription and then translation of both mRNAs.

The data indicate that generation of fibrin is essential for plasma clot–induced changes in gene expression. We first demonstrated using heparin, a clinically useful anticoagulant, a significant reduction in cytokine protein expression. To focus on the role of thrombin, we next used the direct thrombin inhibitor lepirudin,\(^{21}\) which also blocked the production of cytokines. We did not observe IL-8 and MCP-1 synthesis when increasing concentrations of thrombin were introduced to purified monocytes in the absence of plasma, despite the fact that the higher doses used exceeded previously reported peak levels of thrombin generation.\(^{22,23}\) We also found that thrombin did not increase global protein synthesis by monocytes, and mRNA patterns in thrombin-stimulated monocytes clustered with mRNA expression in unstimulated monocytes. These results are similar to those produced by Nieuwenhuizen et al\(^{13}\) who demonstrated that high concentrations of thrombin did not induce cytokine synthesis but contrast a report showing that thrombin regulates the expression of inflammatory mRNAs.\(^{14}\) The reasons for these differences are not obvious but may be because of the techniques (RNA-sequencing versus microarray analysis) and the experimental milieu used. Others have used genetics models to examine the role of thrombin in regulating inflammation. For example, deletion of protease activator receptor 1, the major receptor for thrombin signaling, had no effect on macrophage cytokine synthesis.\(^{24}\) Taken together, these findings indicate only a small role for thrombin in regulating these inflammatory responses.

Although thrombin did not directly modulate gene expression in purified, plasma-free monocytes, our data demonstrate that thrombin is still indirectly necessary for cytokine responses by acting on fibrinogen. Our data suggest that thrombin exerts its effect by inducing fibrin formation, which directly triggers gene expression pathways in monocytes. Indeed, pretreatment of plasma with the peptide Gly-Pro-Arg-Pro, which inhibits fibrin polymerization by blocking A:a interactions, significantly reduced cytokine synthesis.\(^{25,26}\) Likewise, fibrinogen-deficient plasma failed to support cytokine production, but reintroduction of fibrinogen to plasma restored clot formation and cytokine synthesis. Batroxobin,\(^{27}\) which cleaves fibrinogen into fibrin independent of thrombin, also restored clot formation and supported IL-8 and MCP-1 production in heparinized plasma. These data demonstrate that fibrin formation elicits gene expression responses in monocytes. However, the absence of fibrinogen did not completely abolish cytokine formation (Figure 5C), and purified fibrin generated by thrombin was unable to induce IL-8 and MCP-1 synthesis (Figure XV in the online-only Data Supplement). These findings suggest that additional factors circulating in the plasma or generated during clot formation are necessary in addition to fibrin to induce robust cytokine response in monocytes. While the current study focused on the role of fibrin in the setting of plasma, additional studies are warranted to determine other critical factors important in regulating this response.

The exact mechanism by which fibrinogen/fibrin drive IL-8, MCP-1, and other inflammatory gene expression is not known. Previous studies have demonstrated that fibrinogen is capable of inducing synthesis of IL-8, MCP-1, IL-6, IL-1β, and other cytokines in immune cells and vascular endothelial cell in vitro and in vivo.\(^{24,28–31}\) The mechanism behind fibrinogen- or fibrin-driven cytokine responses was thought to be through TLR4 (toll-like receptor-4) on macrophages.\(^{32,33}\) However, inhibition of TLR4\(^{34,35}\) had no effect on cytokine production (Figure XIX in the online-only Data Supplement), suggesting that ligation of TLR4 by fibrinogen is not the mechanism for induction of cytokine synthesis in our experiments.\(^{29}\) In addition, these data indicate that low levels of LPS in clots are not responsible for increased IL-8 and MCP-1 synthesis. The generation of FXIIIa and its role in stabilizing fibrin through cross-linking may also play a role in driving the cytokine response in monocytes. Previous studies have demonstrated that FXIIIa is capable of binding to \(\alpha_\text{IIb}\beta_3\) on monocytes and upregulating proliferation and migration while preventing apoptosis.\(^{36}\) Using pharmacological inhibition and FXIII-deficient plasma, we observed minimal changes in IL-8 and MCP-1 synthesis (Figure XIV in the online-only Data Supplement). Another possibility is CD11b/CD18, which binds fibrinogen/fibrin with high affinity through the c-terminal region of fibrinogen’s gamma chain.\(^{37}\) It has been shown that this site becomes exposed on conversion of fibrinogen to fibrin,\(^{38,39}\) and previous publications have shown that interactions of fibrin with CD11b/CD18 regulate NF-κB-dependent cytokine production. Interestingly, pharmacological inhibition of NF-κB blunts clot-induced synthesis of IL-8 and MCP-1.
(Figure XI in the online-only Data Supplement). However, an inhibitory antibody against CD11b blocked fibrinogen binding but had little effect on cytokine production (Figure XX in the online-only Data Supplement). Additional studies using mice deficient in CD11b/CD18 are necessary to fully examine the role CD11b/CD18 in plasma clot-mediated IL-8 and MCP-1 synthesis because studies using CD11b/CD18 knockout mice demonstrated that they are protected from cerebral ischemia reperfusion injury, suggesting that interactions of fibrin with CD11b/CD18 may regulate inflammatory processes during stroke.

While the ability of fibrin to interact with vessels and cells is important in stemming blood loss, fibrin deposition and its prompt removal are necessary in wound repair. The removal of fibrin from a thrombus is regulated by the generation of plasmin from its proform, plasminogen. Plasminogen binding to fibrin in conjunction with tPA results in plasmin generation and the subsequent degradation of the fibrin clot; therefore, a delicate balance is needed under physiological conditions between procoagulant and fibrinolytic processes to prevent bleeding or thrombosis and to ensure proper wound repair. Previous studies in plasminogen-deficient mice have demonstrated exacerbated inflammatory diseases associated with arthritis, nerve damage, and osteoporosis. Genetic or pharmacological depletion of fibrinogen rescued the associated phenotype, suggesting that fibrin and its subsequent lysis modulates the disease process. Additionally, Cole et al demonstrated that plasminogen-deficient animals have elevated levels of IL-6. These studies, together with our findings, demonstrate that the balance between fibrin formation and lysis critically regulates IL-8, MCP-1, and other inflammatory cytokine production by monocytes.

As many as 795,000 individuals experience a new or recurrent stroke in the United States each year. Intravenous fibrinolytic therapy involving the use of tPA for acute stroke is widely agreed on to be beneficial if administered within 3 to 4.5 hours of symptom onset. This demonstrates that timely administration of tPA is necessary for maximal benefit to the patients. While fibrinolytics allow for reperfusion in the ischemic area, our data suggest an added benefit of timely fibrinolysis is modulation of cytokine production in the ischemic area. Specifically, addition of tPA to clots within 2 hours significantly reduces the synthesis of IL-8 and MCP-1. Increased inflammation is a hallmark finding in stroke, as evidenced by increased levels of IL-8, MCP-1, IL-1β, and other cytokines in the blood—which associate with poor outcomes. While these studies suggest certain cytokines are important in regulating outcomes in stroke, they have not examined the role of fibrinolytic therapy in regulating cytokine expression after stroke. Our findings demonstrate for the first time and strongly suggest that the positive benefits of timely tPA administration in stroke is due, in part, to an attenuated inflammatory response.

Acknowledgments

We thank Dr Tammy Smith for input on experimental design and data collection in reference to Figure 1 and Figures III and IX in the online-only Data Supplement, as well as critically reviewing the article. We thank Ms Diana Lim for her excellent preparation of the figures. We thank Drs Tim LaPine and Tom Martins at ARUP for their help with the multiplex analysis. In addition, we thank the High-Throughput Genomics and Bioinformatics Analysis and the Electron Microscopy cores at the University of Utah.

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Disclosures

None.

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Clots Trigger Inflammatory Cell Gene Expression

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Highlights

- Clot formation induces a robust inflammatory response in monocytes specifically through fibrin-dependent mechanisms.
- Timely fibrinolysis blunts monocyte inflammatory responses, revealing a potential novel mechanism of fibrinolytic therapy in stroke.
Clots Are Potent Triggers of Inflammatory Cell Gene Expression: Indications for Timely Fibrinolysis


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Supplemental Figure I. Whole blood clot formation does not alter CD14+CD16− and CD14+CD16+ populations. Whole blood (10 mLs) was left untreated or allowed to clot (20 mM CaCl₂ final and 1:20000 Innovin, final) for two hours. Monocytes were isolated using CD14+ microbeads from miltenyi biotec as described in the material and methods. Roughly 2 million monocytes were isolated from untreated blood and clotted blood (data not shown). Monocytes were then stained for CD14 and CD16. CD14 and CD16 positivity was determined by flow cytometry compared to IgG controls. CD14++CD16−, CD14++CD16+, and CD14+CD16++ populations were unchanged between the two groups (N=3).
Supplemental Figure II. IL-8 and MCP-1 mRNA increase compared to untreated whole blood. Monocytes were isolated from untreated whole blood (NT) or whole blood clots (WB Clot) after 2 hours at 37°C at 5% CO₂ and mRNA isolated. Real-time PCR to measure IL-8 and MCP-1 levels was then performed (n>3, *p<0.05).
Supplemental Figure III. Clot induced changes in the expression of mRNAs and their corresponding proteins correlate with one another. (A, B) Whole blood clots were formed as described in the Supplemental Methods. Proteins were assessed by a dot blot cytokine protein array in freshly-isolated whole blood (BL- baseline) and compared to a whole blood
clot after 18 hours. IL-8 (red) and MCP-1 (blue) are indicated on the actual dot blot and the relative levels of proteins that were increased in response to clotting are shown in panel B. (C) Proteins with increased expression (Panel B) were correlated to their corresponding mRNA expression levels, as measured by RNA-sequencing.
Supplemental Figure IV. IL-8 and MCP-1 are expressed in clots extracted from patients with renal disease. Thrombi were harvested from the iliac artery of a patient who developed thrombosis after undergoing renal transplantation. The clots were stained for CD14 (green), IL-8 (red), or MCP-1 (red). IgG using isotype controls at similar concentrations are shown to assess background stain. Nucleated cells were stained with DAPI (blue). Differential contrast is shown in the far-right panels. The images are representative of three independent samples taken from patients with varying degrees of renal disease.
Supplemental Figure V. Tissue factor driven thrombin generation induces greater levels of thrombin and clot formation compared to the contact pathway. Plasma was isolated as described in the material and methods. Plasma was recalcified (20 mM final) in the presence (TF pathway) or absence (contact pathway) of recombinant tissue factor (1:20000 final) and thrombin generation measured using a fluoregenic substrate as previously described (A). Clot formation was measured in parallel using a thermomax plate reader that monitored changes in optical density at 405 nm (B). Fibrinolysis was also measured with the addition tissue factor plasminogen (tPA) in parallel clot formation assays (C). All assays were monitored for 2 hours at 37°C. Tracings are from representative experiments (n=3).
Supplemental Figure VI. Gene expression changes observed in whole blood and plasma fibrin clots correlate with one another. Inflammatory mRNA expression fold changes in monocytes from whole blood clots versus BL whole blood compared to fold changes in monocytes from plasma clot versus NT. Inflammatory genes selected according to the inflammation Gene Ontology (GO) (GO term: inflammation) Genes that did not significantly change (p<0.05) in response to clotting in either group were excluded from the analysis. A Pearson correlation revealed a positive correlation (p<0.05) between whole blood and plasma clots.
Supplemental Figure VII. IL-8 and MCP-1 mRNA increase over time in plasma clots. Monocytes were embedded into plasma clots or stimulated with 0.1 U/mL thrombin and lysed in Trizol. IL-8 and MCP-1 mRNA was measured at various time point (n>3, *p<0.05).
Supplemental Figure VIII. IL-8 and MCP-1 is expressed by monocytes embedded into plasma clots. Monocytes embedded into plasma clots after 18 hours were processed similar to human thrombi isolation for cytokine staining (see material and methods). Cryosectioned plasma clots were stained for IL-8 (red), MCP-1 (red) and CD14 (green). DAPI was used to stain the nucleus. IgGs for IL-8 and MCP-1 are the same. Light transmission images were taken to examine plasma clot morphology (DIC, far right). IL-8 and MCP-1 staining co-localized with CD14, a specific monocyte marker. The images are representative of three independent experiments.
Supplemental Figure IX. Monocytes embedded in plasma fibrin clots have increased frequency of ribosomes tracking along endoplasmic reticulum. (A)

Electron micrographs depicting monocytes at baseline compared to monocytes embedded in plasma fibrin clots. Monocytes embedded in plasma fibrin clots have increased cytoplasm to nuclear ratio as well as increased frequency of ribosomes attached to endoplasmic reticulum (white arrow). These images are representative of 3 independent experiments.
Supplemental Figure X. Production of IL-8 and MCP-1 is transcriptionally and translationally controlled. Monocytes were treated with vehicle, actinomycin D (ActD; 5 µg/mL), or cycloheximide (5 µg/mL) for one hour before being embedded into plasma clots. Supernatant were harvested at the indicated time points and IL-8 and MCP-1 were measured (N>3, *p<0.05).
Supplemental Figure XI. Production of IL-8 and MCP-1 is NFκB-dependent.

Monocytes were treated with vehicle, or the NFκB inhibitor (BAY 11-7082, 5 µM final) for one hour before being embedded into plasma clots. Supernatants were harvested at the indicated time points and IL-8 and MCP-1 protein was assessed (N>3, *p<0.05).
Supplemental Figure XII. The fibrin inhibitor GPRP does not affect LPS-induced cytokine synthesis. Monocytes were treated with 40 mg/mL GPRP or vehicle control (water) for 1 hour before addition of LPS (100 ng/mL). Supernatants were collected at 18 hours and cytokine synthesis was measured by ELISA (N>3).
**Supplemental Figure XIII.** Tissue factor does not directly induce MCP-1 synthesis.

Monocytes were left untreated, stimulated with recombinant tissue factor (1:20000 dilution, final) or LPS (100 ng/mL, final). After 18 hours, supernatants were harvested and MCP-1 was measured (n = 3, *p<0.05).
Supplemental Figure XIV. FXIII does not influence IL-8 or MCP-1 production from monocytes embedded in plasma clots. Plasma clots using FXIII deficient plasma were formed in the presence or absence of recombinant FXIIIa (10 µg/mL, final). Plasma clots were also formed in the presence of 5 µM T101, a specific FXIII inhibitor. After 60 minutes, reactions were stopped by the addition of 50 mM DTT, 12.5 mM EDTA, and 8 M urea. Samples were then incubated at 60°C for 1 hour with occasional agitation. Samples were reduced, boiled, and probed with a rabbit anti-human fibrinogen polyclonal antibodies to determine the presence of γ-γ dimers. Monocytes were embedded into clots formed in the presence or absence of FXIII or in the presence of T101 or vehicle and cytokine production was measured after 18 hours.
Supplemental Figure XV. Monocytes embedded in fibrinogen clots combined with serum synthesize IL-8 and MCP-1. Monocytes were embedded in plasma clots as described in the materials and methods or clots formed from purified fibrinogen (2 mg/mL, final) and thrombin (1 U/mL, final). To some purified fibrin clots, serum from autologous donors was added to recapitulate plasma fibrin clots (N=5, p<0.05).
Supplemental Figure XVI. Fibrinolysis blunts clot-induced changes in inflammatory gene expression. Using genes selected according to the inflammation GO term, a correlation analysis was performed between monocyte-derived mRNAs that displayed altered expression (p<0.05) when embedded in plasma clots (compared to NT) versus their expression level when the plasma clots were lysed with tPA (compared to plasma clot) (lysis = 30 minutes post-clot formation). A Pearson correlation revealed a negative correlation (p<0.05) in mRNA expression levels with and without lysis.
Supplemental Figure XVII. Plasma fibrin clots induce robust cytokine protein expression. Baseline plasma and supernatants from monocytes stimulated by plasma clots after 18 hours were harvested for multiplex cytokine analysis that was performed at ARUP Laboratories as described in the Supplemental Methods. N=3 for all experiments. Significance indicated by * with p<0.05.
Supplemental Figure XVIII. Plasma fibrin clots induce tissue factor expression in monocytes. Tissue factor (TF) mRNA expression was assessed in monocytes left untreated (NT) or embedded in plasma clot for 2 hours compared to baseline monocytes. TF express levels were normalized to GADPH. Significance indicated by * with p<0.05 compared to baseline and NT (N=3).
Supplemental Figure XIX. Plasma clots do not induce inflammatory gene expression via toll-like receptor-4 (TLR4). Monocytes were pretreated with the TLR4 inhibitor CLI-095 (1 μM) and then embedded in plasma clots or stimulated with LPS (100 ng/mL). IL-8 (left panel) and MCP-1 (right panel) protein levels were assessed after 18 hours. The bars in this figure represent the mean±SEM of 3 independent experiments. The asterisk indicates p<0.05 in untreated clot or LPS-treated samples versus the TLR4 inhibitor.
Supplemental Figure XX. Inhibition of monocyte CD11b does reduce IL-8 or MCP-1 synthesis in response to plasma clot stimulation. Monocytes were pretreated with 5 µg/mL anti-CD11b antibody (red) or isotype control (blue) before the addition of 10 µg total Alexa-fluor 555 labeled fibrinogen. Fibrinogen binding was assessed by flow cytometry (A). Monocytes were left alone or pretreated with 5 µg/mL anti-CD11b antibody or isotype control before cells were added to plasma clots (B). After 18 hours, supernatants were harvested and IL-8 and MCP-1 were measured (n>3).
Supplemental Methods

Whole blood collection and plasma isolation

The University of Utah Institutional Review Board approved this study and all subjects provided informed consent. Human peripheral venous blood from healthy, medication-free adult subjects was drawn into acid-citrate-dextrose (pH 5.1, 1.4 ml ACD/8.6 ml blood) through standard venipuncture and used immediately upon collection. ACD had no effect on plasma pH level (Supplemental Methods Figure I). Plasma was harvested by sequential centrifugation of the whole blood at 150 x g for 20 minutes (min) followed by another spin at 1500 x g for 20 minutes and then once more at 13,000 x g for 2 min to remove remaining cell contaminants.

Whole Blood Clot Formation

For RNA-seq analysis, 9.5 mL of whole blood was recalcified (20 mM final) by adding whole blood to 500 uL of 400 mM CaCl₂ and gently inverting the tube. Recombinant tissue factor (1:20000, final Dade Innovin, Siemens, Tarrytown, NY) was then added and the whole blood was allowed to clot for 2 hours at 37°C at 5%CO₂. The addition of CaCl₂ did not result in any visual hemolysis after plasma isolation. After two hours, the whole blood clot was dissociated with mechanical shear to remove the monocytes from the clot. Monocytes were isolated with ficoll-paque and positively selected for using CD14 magnetic beads (see below). Purified monocytes were counted and then lysed in Trizol for RNA isolation. Monocytes isolated from whole blood clots were compared to baseline monocytes (i.e. cells immediately isolated after whole blood was drawn) for RNA seq-analysis. For cytokine studies, whole blood was allowed to clot for 18 hours.
before the blood was centrifuged at 13000 x g for 10 minutes. After the initial centrifugation, the supernatant was centrifuged again at 13000 x g for two minutes to remove residual cells. In indicated experiments, tissue-type plasminogen activator (tPA, American Diagnostica, Stamford, CT, USA) was added (1 μg/mL, final) at the initiation of clot formation.

Monocyte Isolation

Human monocytes were isolated by drawing human peripheral venous blood (500 ml) from healthy, medication-free donors. Blood was centrifuged at 150 x g for 20 min at 20°C to separate platelet-rich plasma (PRP) from red and white blood cells (RBC/WBC). The RBC/WBC mixture was resuspended with 0.9% sterile saline back to the original volume and layered over an equal volume of Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway, NJ, USA). The layered cells were then centrifuged for 30 min at 400 x g at 20°C. After 30 min, the mononuclear leukocyte layer was removed and washed with Hank’s Balance Salt Solution (Sigma-Aldrich, St. Louis, MO, USA) with 1% human serum albumin (HBSS/A) (University of Utah Hospital, Salt Lake City, UT, USA) and centrifuged for 10 min at 400 x g at 20°C. The cell pellet was then resuspended and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 15 min at 4°C. The cells were then washed with HBSS/A to remove any free CD14 microbeads and then resuspended in HBSS/A. The monocytes were then isolated by running the cell solution through an autoMACs cell separator (Miltenyi Biotec) using the PosselD2 program. Cells were then washed with HBSS/A and resuspended in M199
Purified Plasma Clot Formation

Whole blood was drawn into ACD (pH 5.1) in a ratio of 1:7 (ACD:Blood). Whole blood was then centrifuged for 20 minutes at 150 x g to isolate platelet-rich plasma (PRP). PRP was then centrifuged for 20 minutes at 1500 x g to generate platelet-poor plasma (PPP). Finally, PPP was centrifuged for 2 minutes at 12000 x g to generate cell-free plasma (CFP). CFP (450 µL) was added to a single well of a 24-well tissue culture treated plate and re-calcified with the addition of 25 µL of 400 mM CaCl₂. Recombinant tissue factor (Dade Innovin) was added at the same time (25 µL, 1:20000 dilution final) to trigger tissue factor dependent fibrin clot formation. Clot formation was allowed to occur for 30 minutes at 37°C in humidified incubator with 5% CO₂. Monocytes isolated using CD14 positive selection and resuspended with M199 without supplemental factors were added to the CFP clot at a final concentration of 2 x 10⁶ total cells in a volume of 1000 µL. The final volume of clot and cells was 1500 µL. The cells and clot were allowed to incubate in a humidified incubator for 18 hours at 37°C at 5% CO₂. In experiments with heparin, plasma from the same individual was drawn into heparinized tubes and centrifuged in a similar manner. Heparinized plasma had only Innovin added back. Plasma was sometimes treated with cycloheximide (5 µg/mL, final)(Sigma-Aldrich), actinomycin D (5 µg/mL, final)(Sigma-Aldrich), lepirudin (University of Utah Pharmacy), or GPRP diluted in water (Pentapharm, 40 mg/mL, final). In some assays, tPA (America Diagnostica, 1 µg/ml, final) was added at the indicated time. Fibrinogen
deficient plasma was from Affinity Biologicals and was reconstituted with fibrinogen from Enzyme Research Laboratories at the indicated concentrations. FXIII deficient plasma was from Affinity Biologicals and was reconstituted with FXIIIa from Hematologic Technologies (10 µg/mL). The FXIII inhibitor, 1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride (T101) was from Zedira (San Diego, CA, USA) and was added to plasma clots (5 µM, final) before the addition of tissue factor and calcium. Serum was made by adding thrombin (Sigma-Aldrich, 1.0 U/mL, final) to plasma and allowing the clot to form for an hour. After an hour, the clot was centrifuged for 10 minutes at 13000 x g to remove the clot. Thrombin-independent plasma clots were formed using Batroxobin (Pentapharm, 1 U/mL, final). In some experiments, monocytes were treated with the TLR4 inhibitor, CLI-095 (Invivogen, 1 µM, final), for 1 hour before addition to the plasma fibrin clot. In some experiments, a CD11b monoclonal antibody (M1/70 eBiosciences) or IgG (5 µg/mL, final) was pre-incubated with monocytes before addition to plasma clots. The inhibitory nature of the antibody was tested using Alexa-fluor 555 labeled fibrinogen (eBiosciences) and measuring fibrinogen binding by flow cytometry. In some experiments, monocytes (2 x 10^6, final concentration) resuspended in M199 without supplemental factors^1-5 and in a total volume of 1500 µL were stimulated with thrombin (0.1 U/mL, final) or lipopolysaccharide (100 ng/mL, final Sigma-Aldrich) and IL-8 and MCP-1 measured by ELISA. For purified fibrin clot formation, monocytes (2 x 10^6, final concentration) resuspended in M199 and in a total volume of 1000 µL were added to 500 µL of purified fibrin clots (2 mg/mL, final Enzyme Research Laboratories) generated by the addition of 1.0 U/mL thrombin (final).
FXIII crosslinking experiments

Plasma clots in the absence or presence of FXIII or in the absence or presence of T101 were formed as described above. After 60 minutes, crosslinking and clotting were stopped using a solution containing 50 mM DTT, 12.5 mM EDTA, 8 M urea. Samples were then incubated at 60°C for 1 hour. Samples were reduced, boiled, separated on 10% gels, and transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-human fibrinogen polyclonal antibodies (Dako).

Radiolabeled Protein Synthesis

Monocytes were resuspended in DMEM without methionine or cysteine and allowed to rest for 30 minutes. After 30 minutes, EasyTag™ EXPRESS\textsuperscript{35S} Protein Labeling Mix (0.06 mCi total) (Perkin Elmer) was added to each reaction and allowed to incubate overnight. The next day the monocytes were washed three times in complete media and then lysed in radioimmunoprecipitation assay buffer (RIPA) (1X PBS with 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Lysates were cleared and then Trichloroacetic acid (TCA) precipitated using 20% TCA on ice for 30 minutes. The precipitated proteins were loaded onto a Whatman grade GF/C glass microfiber filter (VWR) and washed five times with 10% TCA and five times with 95% ethanol. Filter papers were then read using a liquid scintillation counter.

RNA-Seq Analysis

Monocytes were purified using immune-magnetic positive selection as described previously\textsuperscript{6, 7}. For global screening of RNAs (RNA-seq), monocytes were purified from
individual donors. For the RNA-seq and real-time PCR analysis of mRNA expression, washed monocytes were lysed in Trizol and DNase treated total RNA was isolated, as previously described (Rowley et al). An agilent bio-analyzer was used to Quality Control (QC) and quantitate RNA. RNA Integrity Number (RIN) scores were similar between all samples. RNA-seq libraries were prepared with TruSeq V2 with oligo-dT selection (Illumina, San Diego CA). For whole blood clots, 36 base pair (bp) paired end reads were sequenced on an Illumina GAIIx. For all other samples, 50 bp single end reads were sequenced on an Illumina Hiseq 2000. Reads were aligned (Novoalign) to the reference genome GRCh37/hg19 and a pseudotranscriptome containing splice junctions. The USeq analysis package was used to assign reads to composite transcripts (one per gene) and quantitate FPKMs as previously described8-11. Data are deposited in Sequence Read Archive (SRA) through NCBI (SRA number PRJNA397431).

Patient Enrollment and Thrombus Retrieval
This was a prospective cohort study of patients referred to a university tertiary-care referral center with acute thrombosis. The local institutional review board approved this study and all patients provided informed consent (IRB# 42054). Inclusion criteria included patients with acute thrombosis undergoing surgical thrombectomy. Exclusion criteria included inability to provide informed consent or obtain a thrombus specimen. All thrombus specimens were removed by a vascular surgeon, placed immediately into sterile specimen cups, and transported to the laboratory for processing. Demographic data, medical history, and clinical laboratory results were recorded for each patient.
Immunochemistry

Thrombi from patients were fixed with 4% paraformaldehyde for 30 minutes at room temperature before being placed in a 7.5% sucrose/4% paraformaldehyde solution for four hours. After four hours, the thrombi were placed in a 15% sucrose/4% paraformaldehyde solution overnight. The thrombi were then embedded into OCT mounting solution and froze at -80°C. The thrombi were then sectioned in 5-20 µm thick sections and stained. Clots from the in vitro clot system were embedded with OCT mounting solution and then frozen at -80°C. The clots were then sectioned in 5-20 µm thick sections and stained. Sections were permeabilized using 0.1 X Triton followed by blocking with 10% goat serum. Staining was performed using a Rabbit anti-MCP-1 (Abcam 9669), Rabbit anti-IL8 (Abcam 7747), Mouse anti-CD14 (Abcam 63319), Mouse IgG (Santa Cruz 2025), and Rabbit IgG (Abcam 171870) in 10% goat serum overnight. The next day a goat anti-rabbit Alexa Fluor 546 and goat anti-mouse Alexa Fluor 488 were added and the slides were then imaged.

Imaging

High-resolution confocal reflection microscopy was performed with an Olympus IX81, FV300, and a FV1000 (Olympus) confocal-scanning microscope equipped with a 60 × /1.42 NA oil objective for viewing platelets. An Olympus FVS-PSU/IX2-UCB camera and scanning unit and Olympus Fluoview FV 300 and FV1000 Version 5.0 image acquisition software was used for recording. The images were further analyzed with the use of Adobe Photoshop CS Version 8.0, and ImageJ Version 1.50b (National Institutes of Health).
For the ultrastructural analyses, monocytes embedded in clots were fixed in 2.5% glutaraldehyde in PBS buffer. The samples were subsequently washed and postfixed with 2% osmium tetroxide, rewashed, dehydrated by a graded series of acetone concentrations (50%, 70%, 90%, 100%; 2 × 10 minutes each), and embedded in Epon. Thin sections were counterstained (ie, uranyl acetate and lead citrate), viewed with a JEOL JEM-1011 electron microscope (JEOL), and digital images were captured with a side-mounted Advantage HR CCD camera (Advanced Microscopy Techniques).

Quantification of Chemokine and Cytokine Protein Expression
To assess the response of monocytes embedded in plasma clots, we used a multiplexed sandwich capture assay for the quantification of 13 chemokines and cytokines developed at the ARUP Institute for Experimental and Clinical Pathology at the University of Utah as previously described. The chemokines or cytokines assayed included: CD40 ligand, interferon-γ, IL-10, IL-12, IL-13, IL-1β, IL-2, IL-2 receptor, IL-4, IL-5, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). To confirm and extend the chemokine results obtained using the multiplex sandwich capture assay, quantification of IL-8 and MCP-1 in supernatants and cell lysates was performed by ELISA (R&D Systems, Inc.) as per the manufacturer's instructions. Whole blood cytokine analysis was also examined in cell-free supernatants using a Human Cytokine Array Kit (R&D Systems, Inc.)
Statistical methods

For RNA-seq analysis, Deseq2 was used to identify differentially expressed transcripts. To focus on robustly expressed transcripts, only transcripts with per group average > 3 FPKM were included in the analysis. For the plasma clot samples, a q value < .05 and fold change of 4-fold were used as thresholds for differential expression. For the whole blood clot model, assignment of differentially expressed transcripts was based on fold change of 4-fold. For relevant studies, we calculated the mean ± SEM and performed ANOVAs to identify differences among multiple experimental groups. If significant differences existed, a Student Newman-Keuls posthoc procedure was used to determine the location of the difference between groups. When single comparisons were performed, a Student’s t-test was employed. Statistical significance was set at P < .05.
Supplemental Methods Figure I. Plasma isolated from whole blood drawn into ACD has a physiologic pH. Whole blood was drawn into ACD (pH 5.1; ratio 1:7 ACD to blood) and centrifuged for 20 minutes at 150 x g to isolate platelet rich plasma. Cell free plasma was then generated by sequential centrifugation for 20 minutes at 1500 x g followed by an additional 2 minutes at 12,000 x g. The pH of isolated cell free plasma was determined using pH strips. Shown are pH strips (1, 2, 3) from three independent experiments with the reference guide on the right. The color of these three experiment test strips approximated physiologic pH (i.e. pH 7.4, black arrow).
References


