Published in final edited form as:

Thromb Haemost. 2017 January 26; 117(2): 339–348. doi:10.1160/TH16-03-0218.

Endotoxaemia-augmented murine venous thrombosis is dependent on TLR-4 and ICAM-1, and potentiated by neutropenia*

Andrea T. Obi¹, Elizabeth Andraska^{1,2}, Yogendra Kanthi³, Chase W. Kessinger⁴, Megan Elffline¹, Cathy Luke¹, Teruna J. Siahaan⁵, Farouc A. Jaffer⁴, Thomas W. Wakefield¹, and Peter K. Henke¹

¹Conrad Jobst Vascular Research Laboratory, Section of Vascular Surgery, Department of Surgery, University of Michigan, Ann Arbor, Michigan, USA

²University of Michigan Medical School, Ann Arbor, Michigan, USA

³Department of Cardiology, University of Michigan, Ann Arbor, Michigan, USA

⁴Cardiovascular Research Center, Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

⁵School of Pharmacology, University of Kansas, Lawrence, Kansas, USA

Summary

Venous thromboembolism is a major cause of death during and immediately post-sepsis. Venous thrombosis (VT) is mediated by cell adhesion molecules and leukocytes, including neutrophil extracellular traps (NETs). Sepsis, or experimentally, endotoxaemia, shares similar characteristics and is modulated via toll like receptor 4 (TLR4). This study was undertaken to determine if endotoxaemia potentiates early stasis thrombogenesis, and secondarily to determine the role of VT TLR4, ICAM-1 and neutrophils (PMNs). Wild-type (WT), ICAM-1^{-/-} and TLR4^{-/-} mice underwent treatment with saline or LPS (10 mg/kg i. p.) alone, or followed by inferior vena cava (IVC) ligation to generate stasis VT. *In vivo* microscopy of leukocyte trafficking was performed in non-thrombosed mice, and tissue and plasma were harvested during early VT formation. Pre-thrombosis, circulating ICAM-1 was elevated and increased leukocyte adhesion and rolling occurred on the IVC of LPS-treated mice. Post-thrombosis, endotoxaemic mice formed larger, platelet-poor thrombi. Endotoxaemic TLR4^{-/-} mice did not have an augmented thrombotic response and exhibited significantly decreased circulating ICAM-1 compared to endotoxaemic WT controls. Endotoxaemic ICAM-1^{-/-} mice had significantly smaller thrombi compared to controls. Hypothesising that PMNs localised to the inflamed endothelium were promoting thrombosis, PMN depletion using anti-Ly6G antibody was performed. Paradoxically, VT formed without PMNs was amplified, potentially related to endotoxaemia induced elevation of PAI-1 and

*Presented in part at the 2014 Vascular Research Initiatives Conference and Arteriosclerosis, Vascular Biology and Thrombosis 2014 Annual Scientific Sessions.

Correspondence to: Peter K. Henke, MD, University of Michigan Health System, 1500 E. Medical Center Drive, Cardiovascular Center – 5463, Ann Arbor, MI 48109–5867, USA, Tel.: +1 734 763 0250, Fax: +1 734 647 9867, henke@umich.edu.

Conflicts of interest: None declared.

circulating FXIII, and decreased uPA. Endotoxaemia enhanced early VT occurs in a TLR-4 and ICAM-1 dependent fashion, and is potentiated by neutropenia. ICAM-1 and/or TLR-4 inhibition may be a unique strategy to prevent sepsis-associated V T.

Keywords

TLR4; cellular adhesion molecules; ICAM-1; sepsis; LPS; venous thrombosis; venous thromboembolism

Introduction

Venous thromboembolism (VTE) and sepsis represent a major threat to hospitalised patients, each accounting for 200,000 to 300,000 deaths in the United States per year (1, 2). The development of sepsis substantially increases the risk of VTE: in two large inpatient studies from the National Surgical Quality Improvement Program, sepsis was associated with a significant 1.7-to 3.9-fold increased risk of VTE (3, 4). A Denmark population study of 15,009 first time hospital diagnosed VTEs over a 10-year period demonstrated an adjusted 3.6 fold (odds ratio [OR] 2.3–5.8) increase in VTE in setting of septicemia (5). In a recent report of 113 consecutive patients with severe sepsis, the rate of development of VTE was 37.2 % despite adherence to thromboprophylaxis guidelines, highlighting the need for improved prophylactic measures in this patient population (6). The surgeon general (7), Surviving Sepsis Campaign guidelines (8) and Agency for Healthcare Research and Quality (9) have all identified VTE prevention and treatment as a national healthcare priority. Improved understanding of the underlying biologic mechanisms that pre-dispose patients to increased VTE risk is essential to decreasing sepsis related morbidity and mortality.

Recent studies suggest a major role for leukocyte interaction with the endothelium and activation in the initiation of venous thrombosis (VT) (10, 11). Early VT is dependent upon endothelial recruitment of leukocytes by endothelial cell adhesion molecules (CAMs) (12–14). Analogous to mechanisms governing early VT, the acute sepsis response is characterised by elevated circulating CAMs (15) and early polymorphonuclear cells (PMNs) activation (16). However, despite apparent similarities in underlying processes of CAM shedding and PMN activation, mechanisms by which sepsis predisposes to development of acute VT remain to be defined.

Toll-like receptor 4 (TLR4) mediates the host response system to endotoxaemia, and has been pursued as a potential therapeutic target in the treatment of overwhelming sepsis (17). Endotoxin (lipopolysaccharide, LPS) stimulates up-regulation of soluble ICAM-1 (sICAM-1) in humans (18) and mouse myocardium (19). ICAM-1 has recently been demonstrated to mediate early VT in non-septic animals (20). In other vascular systems, ICAM-1 mediates endotoxaemic organ dysfunction in a TLR-4 dependent, PMN-independent fashion (21). However, activated PMNs in VT and sepsis have a variety of pro-coagulant functions: release of elastase and cathepsin G which degrade tissue factor pathway inhibitor (TFPI) (11), release of DNA and histones to form neutrophil extracellular traps (NETs) (22), and anti-fibrinolytic activities (23). Currently, it is believed that PMNs are both central and necessary in mediating the innate immune response and coagulation (24).

Conversely, other studies suggest that thrombosis occurs via a PMN-independent mechanism during endotoxaemia (25, 26).

Endotoxaemia-induced potentiation of thrombosis is well-described in arteries, venules and microvasculature, but little is known about its effect on large veins. It is currently unknown whether this process is mediated by TLR4 and whether it is dependent upon ICAM-1 or PMNs. In this experimental investigation, we first characterise the endothelial response to endotoxaemia and VT formation under these circumstances. Intravital microscopy was applied to directly visualise and quantify leukocyte interaction with endothelium under conditions of endotoxaemia. Next, we assessed the effect of TLR4 and ICAM-1 deletion on VT formation under endotoxaemic conditions. Lastly, PMN depletion was performed to delineate the role of PMNs in endotoxaemic driven VT. We show for the first time endotoxaemia-enhanced stasis thrombosis of large veins occurs via a TLR-4 and ICAM-1 dependent fashion, with a paradoxical increase in thrombosis with neutropenia.

Materials and methods

Animals

Male mice (C57/BL6, 20–25 gm, wild types [WT]) were used for all studies. TLR4^{-/-} mice on C57 background and ICAM-1^{-/-} (B6.129S4-Icam1^{tm1Jcgr/J}, not expressing functional isoform) on C57 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were bought directly from Jackson Laboratory and underwent standard PCR genotyping. For all procedures, mice underwent general anesthesia with isoflurane and O₂ with continuous monitoring. All animal studies were done with University of Michigan Animal Use Committee approval.

Lipopolysacchride (LPS) sepsis mouse model

WT C57/BL6 mice were subjected to intraperitoneal (i. p.) LPS (sub-lethal dose: 10 mg/kg in 0.2 ml saline, *E. coli* 0111: B4 strain, Sigma Aldrich, St. Louis, MO, USA) or equal volume of vehicle (normal saline) (27, 28). Vein wall and serum were harvested at 2 hours (h) post-injection to correspond with circulating cytokine response (29).

Intravital microscopy

Leukocyte trafficking to the vein wall was measured using intravital microscopy (IVM) of the jugular and femoral veins under endotoxaemic or resting conditions. Mice were injected with LPS or normal saline 2 h prior to imaging (n=3 mice/group). Rhodamine 6G (R6G, 0.15 mg/kg, i. v., ex/em 526/555 nm) was injected 20 minutes (min) prior to imaging to visualise leukocytes. Immediately prior to imaging, the vessel was surgically exposed and fluorescent isothiocyanate-conjugated dextran (FITC-dextran, MW: 2,000,000, ex/em 490/520 nm, 0.5 mg in 100 µl PBS, Sigma) was injected intravenously to identify the vessel lumen.

Jugular vein images of leukocyte adhesion were captured using a custom-built confocal intravital microscope (30). R6G was excited with a 561 nm laser (Jive, Cobolt AB, Solna, Sweden) and detected with a PMT through a 593 ± 20 nm bandpass filter (FF01–593/40–25,

Semrock, Rochester, NY, USA). Second harmonic generation signals of the jugular vein wall collagen were obtained using a Mai Tai HP femtosecond pulsed laser (840 nm irradiation light, 200 mW power, Spectra-Physics, Irvine, CA, USA). SHG signals from collagen were detected through a 432–482 nm bandpass filter on a non-descanned PMT. The vein was imaged with a 60x, N. A.=1.0, water-immersion microscope objective lens (LUMPLFLN 60XW, Olympus, Center Valley, PA, USA), yielding a field of view of 476 μm by 481 μm . A Z-stack was acquired using a 2 μm step-size. Images were acquired at 30 fps and each image was an average of 10 frames.

Intravital microscopy of leukocyte adhesion and flux were collected utilising an epifluorescence microscope (Nikon 90i, Tokyo, Japan) equipped with a monochromatic cooled CCD camera and matched filter sets. Epifluorescence microscope permitted lower magnification images that could interrogate a larger area of the vein wall to allow for quantification of leukocyte-endothelial interactions. NIH ImageJ (Bethesda, MD, USA) was utilised for all image analysis. Leukocyte adherent and rolling populations were calculated per unit time. Adherent cells were defined as cells interacting with the venous wall in a stationary position for over 30 seconds (s), and rolling cells were defined as cells rolling on the wall for over 5 s (31). Leukocytes were distinguished from R6G-stained platelets by their larger size. Leukocyte adhesion and rolling was assessed in a 0.04 mm² area of the femoral vein wall at 9 frames/s. Rolling leukocyte velocities were assessed over a 5 s period.

VT mouse model

VT was formed via generation of stasis by inferior vena cava (IVC) ligation (32). Mice were anaesthetised via 2 % inhaled isoflurane and midline laparotomy performed. If treatment was administered (saline or LPS), IVC ligation was performed 2 h post injection. Venous side branches and dorsal branches were interrupted and infrarenal IVC was ligated with a 7–0 prolene suture (Ethicon Inc, Somerville, NJ, USA) to generate stasis thrombosis. Fascia and skin were secured with 5–0 vicryl suture and skin glue, and mice were recovered under a warming lamp. Mice were euthanised at 2 h post thrombosis to coincide with initial solid VT formation (10). Prior to processing, the IVC and thrombus were measured (cm) and weighed (grams) en bloc and separately (32, 33). Serum or plasma as appropriate for assay was collected and snap frozen for later analysis.

Neutrophil depletion

Neutrophils were depleted with a single IP injection (250 μg) of Purified Rat anti-Mouse Ly6G clone: 1A8 (BD Pharmingen, #551459, San Jose, CA, USA) given one day prior to sepsis and venous thrombosis induction. Control groups received Purified Rat IgG2b, k Isotype Control (250ug IP, BD Pharmingen, #553986) at the same frequency.

Enzyme linked immunosorbance assays (ELISAs)

Thrombi and vein wall were homogenized and sonicated and then centrifuged at 13,200g for 30 min. The supernatant was collected for ELISA analysis. ELISA for vein wall P selectin, E selectin, ICAM-1, VCAM-1 (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA), circulating IL-1 β , IL-6, sICAM-1 (Quantikine ELISA, R&D Systems), and TLR4 (My BioSource, San Diego, CA, USA) were determined by commercially available kits

according to the manufacturer's instructions. Plates were read on a Synergy 2 Plate Reader (Biotek, Winooski, VT, USA) at 450nm.

Western immunoblotting

Protein levels of Cathepsin G, MPO, Fibrin, GAPDH, Factor XIIIa, CD41, elastase, citrullinated histone-3 (cit-H3), urokinase type plasminogen activator (uPA), and plasminogen activator inhibitor 1 (PAI-1) were measured by Western blot from vein wall or thrombus harvested tissue. Protein was isolated using RIPA buffer (ThermoScientific, Rockford, IL, USA) with dissolved complete ULTRA mini tablets (Roche, Mannheim, Germany). Proteins were electrophoretically separated on NuPAGE 4–12 % Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Nonspecific binding was blocked with starting block (TBS) blocking buffer (ThermoScientific). Antibodies used included: anti-H3 (1/500 dil., Abcam, Cambridge, MA, USA), anti-elastase (1/1000 dil., Abcam, Cambridge, MA, USA), anti-Cathepsin-G (1/1000 dil., Novus, Littleton, CO, USA), anti-TFPI (2ug/mL dil., Novus), GAPDH (1/2000 dil., Santa Cruz, Dallas, TX, USA), anti- fibrin (clone 59d8, 1/1000 dil., gift of Dr. Charles Esmon, Oklahoma City, OK, USA), anti-MPO(1/2000 dil., Upstate, Lake Placid, N Y, USA), CD41(1/1000 dil., Santa Cruz, Dallas, TX, USA), anti-Factor XIIIa (1/1000 dil., Enzyme Research Laboratories, South Bend, IN), anti-uPA (1/500 dil., active enzyme, Santa Cruz), and anti-PAI-1 (1/1000 dil., Abcam, Cambridge, MA, USA). Immunoreactive bands were visualised with supersignal west pico chemiluminescent substrate (ThermoScientific), and densitometry was performed using Image J software. Optical densities were summed and normalised to total protein.

Thrombus erythrocyte (hemoglobin) content

Thrombus lysates were diluted in sodium phosphate (20 mM, pH 7.4) and NaCl (150 mM, PBS) by 10 fold. Absorbance was measured at 575 nm (34).

Histology

Fresh tissue (IVC with thrombus intact) was fixed in 10 % buffered formaldehyde for 2 h, transferred to 70 % ethanol, and subsequently embedded in paraffin for immunohistochemistry (5 µm tissue sections, 3 sections per mouse). After blocking non-specific sites with species-specific serum, sections were stained for activated PMNs by Ly6G (BD Pharmingen, San Diego, CA, USA), ICAM-1(R&D Systems, Minneapolis, MN, USA), TLR4 (Thermo-Fisher Scientific, Invitrogen, Waltham, MA, USA)staining. For the next steps, a species-specific kit was used. DAB (diaminobenzidine) was used to develop the colour and then the slides were counterstained with hematoxylin and cover slipped (Vector Laboratories, Inc., Burlingame, CA, USA). Cells were quantified in a blinded fashion, with positive cells in five high-power fields (magnification, 1000x) radially around the IVC wall were counted and totaled.

Exogenous CAM inhibitor studies

ICAM-1 interactions with the activated lymphocyte associated antigen-1 (LFA-1) receptor were inhibited using the cIBR peptide, a cyclic peptide inhibitor derived from residues 1–21

of the Domain I N-terminus of ICAM-1. WT C57/BL6 mice were subjected to i. p. cIBR 12 h before surgery and re-dosed at completion of surgery (150 µg/150 µl, Siahaan lab, University of Kansas). Selectins were blocked with GMI-1271 or GMI-1070 (10 mg/kg administered 12 h prior at time of surgery completion). GMI-1271 is a small molecule E selectin antagonist. It is designed to mimic the conformation of the sialyl-Lex carbohydrate ligand, which functions in early recognition and binding events of cell extravasation from the bloodstream. GMI-1070 is a pan-selectin inhibitor and has primary activity against E-selectin, with second and tertiary activity against P- and L-selectin (35).

Statistical analysis

All data are represented as mean $\pm$ SD as indicated. Unpaired Student's two tailed t-test and one-way ANOVA with Bonferroni post-hoc comparison were used for comparison between the experimental groups and controls as appropriate (Graph Pad, Prism, San Diego, CA, USA). A p-value <0.05 was assigned significance.

Results

Endotoxaemia produces venous inflammation and enhances leukocyte-endothelial interactions *in vivo*

Injection with sub-lethal dose of LPS (10 mg/kg, i. p.) resulted in up-regulation of cellular adhesion molecules ICAM-1, VCAM-1 (► Figure 1 A, B), E and P-selectin (Suppl. Figure IA, B, available online at www.thrombosis-online.com) locally on the vein wall at 2 h post injection. Simultaneously, serum markers of inflammation, IL-1 β and IL-6 were upregulated (► Figure 1 C, D) and circulating leukocytes and platelets were decreased (Suppl. Figure IC-F, available online at www.thrombosis-online.com), consistent with endotoxaemic response (36).

Leukocyte-endothelial interactions were visualised and quantified *in vivo* utilising intravital fluorescence microscopy. High-resolution confocal imaging of the jugular vein in both LPS and control-treated mice showed leukocytes interacting with the vein wall (► Figure 1 E). Compared to control mice, mice with LPS-induced endotoxaemia showed an increased number of rolling leukocytes (► Figure 1 E and G) with decreased leukocyte rolling velocities (► Figure 1 F and H) compared to WT control mice. Endotoxaemic mice also showed an increased adherent leukocyte population (► Figure 1 I), along with an increased leukocyte flux (► Figure 1 J).

Venous thrombogenesis in endotoxaemic mice is characterised by formation of platelet and neutrophil-poor thrombi and activation of the factor XIII- fibrinogen axis

A paucity of platelets was noted in the thrombi of endotoxaemic mice (► Figure 2 A-C). Circulating platelet counts were also decreased in endotoxaemic mice (Suppl. Figure IF, available online at www.thrombosis-online.com), but were not appreciably altered by the thrombosis insult (580.8 ± 41.7 K/ul LPS without thrombosis vs 581.8 ± 40.4 K/ul LPS with thrombosis, $n=4-6$, $p=0.99$). Endotoxaemia moderately decreased the number of circulating PMNs in non-thrombosed mice (Suppl. Figure ID, available online at www.thrombosis-online.com), whereas those that underwent VT had a marked decrease in circulating PMNs

compared to controls (2365 ± 125.5 vs 306.5 ± 97.5 per ul, $n=5$, $p=0.0010$). Venous thrombi in the setting of endoxaemia had decreased PMN content compared to saline controls (► Figure 2 D-F). Neutrophil enzymes MPO and cathepsin G were decreased in the LPS treated animals compared to controls (► Figure 2 G, H). The haemostatic marker TFPI (control = 0.720 ± 0.089 vs LPS = 0.724 ± 0.042 ; $n=5$, $p=0.97$) and extracellular histone marker cit-H₃ (control = 2.18 ± 0.27 vs LPS = 1.87 ± 0.23 ; $n=5$, $p=0.40$) did not differ significantly between treatment groups.

Activation of the factor XIII-fibrinogen axis is a central determinant of venous thrombogenesis, entrapping erythrocytes and forming larger thrombi (34). Early thrombi 2 h post-IVC ligation in WT mice subjected to endotoxaemia exhibited a non-significant trend in fibrin content (0.5229 ± 0.03 vs 0.6557 ± 0.07 , ratio to GADPH; $n=5-9$, $p=0.108$) and erythrocytes (0.0480 ± 0.01238 vs 0.0820 ± 0.0109 corrected abs at 575 nm; $n=6-10$, $p=0.063$). However, circulating XIII was elevated in endotoxaemic mice (► Figure 2 I).

Endotoxaemia potentiates venous thrombosis in a TLR-4, ICAM-1 dependent manner

Endotoxaemia enhanced the magnitude of thrombosis in WT mice, but not TLR4^{-/-} or ICAM-1^{-/-} mice (► Figure 3 A). Endotoxaemia induced an increase in circulating sICAM-1 in TLR4^{-/-} mice that was significantly attenuated compared to WT (► Figure 3B). ICAM-1^{-/-} mice demonstrated no significant change in thrombus size (► Figure 3 A) with LPS treatment, despite an increase in nonfunctional sICAM-1 (► Figure 3 B). This finding was also observed in WT mice given an exogenous ICAM-1 peptide inhibitor (Suppl. Figure II, available online at www.thrombosis-online.com), but was not significantly reduced with P/E selectin inhibition. TLR4 was significantly elevated in the thrombi (► Figure 3 C) and slightly increased in vein wall (► Figure 3 D) and located within the thrombus proper.

Endotoxaemia venous thrombosis is further potentiated by neutropenia

To determine whether endotoxaemia-driven thrombosis depends upon PMNs, LPS treated mice underwent treatment with IgG control or anti-Ly6G. Mice undergoing PMN depletion had significantly lower circulating PMNs compared to IgG treated controls (► Figure 4 A). Thrombi in these mice were larger than those in mice treated with control antibody (► Figure 4 B). Vein wall PAI-1 levels increased in response to LPS, and were significantly elevated in animals undergoing PMN depletion (► Figure 4 C). Neutrophil elastase, an enzyme responsible for breakdown of PAI-1, was 3.3-fold lower in the PMN depleted group compared to IgG controls (0.87 ± 0.03 vs 2.90 ± 0.73 , $n=4-6$, $p=0.007$). Consistent with an antifibrinolytic milieu, vein wall uPA was significantly decreased amongst the PMN depleted mice (► Figure 4 D). The haemostatic marker TFPI (IgG 2.39 ± 0.48 , Ly6G 1.21 ± 0.04 , $n=4-6$, $p=0.09$) was non-significantly decreased in the PMN depleted mice. As PMN antibody depletion may stimulate PMNs to release NETs, we assessed for extracellular histones cit-H₃ and no significant difference was found in thrombus content (IgG control = 0.923 ± 0.24 vs anti-PMN = 0.67 ± 0.04 , $n=5-6$, $p=.35$).

Discussion

Clinically, VTE is a major source of in-hospital morbidity and mortality, with sepsis a major predisposing factor (37). The use of appropriate chemical thromboprophylaxis does not necessarily offset the increased risk of thrombosis in this high risk cohort (38), suggesting that further elucidation of sepsis-driven VT mechanisms is necessary. In this murine study of endotoxaemia and stasis VT we demonstrate for the first time: 1) endotoxaemia induces large vein wall inflammation and amplifies leukocyte-endothelial interactions *in vivo*; 2) endotoxaemia-enhanced large vein thrombosis depends upon TLR-4 and ICAM-1 upregulation and; 3) unexpectedly, endotoxaemia-enhanced large vein thrombosis is augmented during neutropenia, possibly as a result of impaired fibrinolysis.

Venous inflammation, thrombosis and resolution are dependent upon the presence of CAMs, as inhibitor studies and P and E selectin genetically altered mice have demonstrated (13, 39–41). ICAM-1 has been less well studied in VT, with one laser-induced thrombosis model suggesting that microvascular thrombosis is dependent upon LFA-ICAM-1 interactions (20) and another study demonstrating ICAM-1 inhibition inhibits late leukocyte vein wall infiltration (42). In a TNF- α sepsis model, CAMs (ICAM-1, VCAM-1, P selectin, E selectin) were elevated in mouse IVC, consistent with our results (43). Correlating with elevated vein wall CAMs, *in vivo* microscopy demonstrated increased leukocyte-endothelial interactions in our endotoxaemic mice, with slower leukocyte rolling and greater adhesion. Consistently, larger thrombi formed in endotoxaemic mice. Surprisingly, these very early thrombi were relatively acellular with decreased PMNs and their products suggesting that PMN activation was not the primary mediator of augmented thrombosis at this time point.

Given the elevation of all CAMs, we attempted pharmacologic inhibition of selectins (P and E) and ICAM-1. The greatest reduction of thrombogenic response occurred with ICAM-1 inhibition in WT mice (Suppl. Figure II, available online at www.thrombosis-online.com), suggesting that at least in this sepsis-like model, ICAM-1 interactions are predominant. It could be that a different physiologic stress, such as trauma or non-stasis associated VT would result in an altered CAM profile. Data regarding P selectin contribution to augmented thrombogenesis seen under conditions of endotoxaemia is mixed (44, 45). In preliminary P/E selectin inhibition experiments, we experienced a relatively high mortality rate (> 50 %) and therefore we did not further pursue this avenue.

The host response to endotoxaemia is mediated by TLR4, a transmembrane receptor responsible for pathogen recognition and activation of innate immune and systemic inflammatory response (46–48). Others have shown that LPS enhanced microvascular thrombosis is dependent upon the presence of TLR4 (49, 50), similar to our observations in this large vein study. In a mouse cremaster microvascular thrombosis model, restoration of pro-thrombotic phenotype was achieved with platelet derived TLR4 (49). In our model, venous thrombi were formed in a significantly larger vascular bed and were found to be platelet-poor. This is more consistent with data from carotid artery thrombosis model suggesting that endothelial cells, and not platelets, were the source of TLR4 responsible for augmented thrombosis (50). TLR4 content in the mouse IVC was higher than in the thrombi, supporting this notion. However, intrathrombus TLR4 significantly increased under

conditions of endotoxaemia, suggesting that perhaps another cellular source such as monocytes (50), were involved.

Activated PMNs release NETs which are elevated in both sepsis (51, 52) and experimental models of VT (10, 53–55). Given the connection between innate immunity and coagulation (11), we expected endotoxaemia enhanced thrombosis to be neutrophil-dependent. In a landmark article from Massberg et al., microvascular thrombosis via neutrophil serine proteases NE and cathepsin G were implicated as an essential tool for host innate immune response to pathogens. Mice with sepsis secondary to systemic *E. coli* bacteremia had increased microvascular fibrin deposition, which was markedly reduced with gene deletion of NE and cathepsin G (11). von Bruhl et al. demonstrated PMN depletion to protect mice against stenosis induced VT, with little to no thrombus forming in these animals within 48 h of induction (10). In a separate set of experiments, similarly in the stenosis model of VT, infusion of DNase-1 was protective against forming VT (56). However, consistent with observations in rat stasis VT (57), thrombosis in our experiments was amplified during antibody-induced neutropenia. This correlated with lower PMN activation markers of Cathepsin G and MPO in thrombi formed during endotoxaemia. Interestingly, others have shown that low dose LPS stimulation alone is not enough to increase NETs formation and addition of a second stimulus such as malignancy, is required to for an increase in NETs (58). It may also be that the NETs role predominates at a later time point such as 6 or 48 h (53). Moreover, the thrombosis induced by the stasis VT model occurs independent of NETs release as compared to non stasis induced VT (59). This suggests that possibly a second “hit” is necessary to activate PMNs.

Experimentally, endotoxaemia is associated with relative neutropenia (58) and sequestration of the PMNs to tissues such as lung and liver (60), where pathologic microvascular thrombosis can occur. In contrast to these smaller vascular beds, it may be that large vein thrombosis is dependent on the vein wall (61) or acellular components such as microparticles (62). Furthermore, PMNs may play a fibrinolytic role in early VT, as suggested in rat studies where impaired VT resolution in the setting of neutropenia was associated with decreased uPA (63). PAI-1 is elevated in nearly every endothelial bed under conditions of sepsis, consistent with our observations (64). PAI-1 is degraded by neutrophil elastase (65), and this was reduced in the PMN-depleted mice, and consistent with the higher levels of vein wall PAI-1. Although not assessed in the current study, the plasmin inhibitor α 2-antiplasmin is oxidised and inhibited by PMNs, further promoting an antifibrinolytic milieu (66). While we have previously shown that PAI-1 does not affect thrombus size at two days (67, 68), it may be that in very early thrombosis, the VT size is affected by the fibrinolytic/antifibrinolytic balance as others have shown with decreased bleeding times in tumour-bearing mice exposed to low-dose LPS (58).

Transglutaminase factor XIIIa crosslinks fibrin, providing mechanical stability, and is essential for erythrocyte retention in venous thrombi (34). Amongst humans with sepsis, activated factor XIII increases and correlates with mortality (69). In experimental models of VT, factor XIII^{-/-} mice form small VT with fewer erythrocytes (34). In our experiments, we found endotoxaemic mice to form large, erythrocyte-rich, platelet-poor thrombi with significantly elevated circulating factor XIII. Others have found that depletion of factor XIII

under conditions of sepsis prevents coagulation induced organ damage.(70) Under conditions of sepsis, activation of factor XIII may stabilize thrombi that would otherwise be degraded by inherent fibrinolytic mechanisms, such as in the setting of neutropenia, and predispose to pathologic VT.

We acknowledge that several limitations exist in this data set and importantly, several important avenues of research regarding sepsis related venous thrombosis remain unexplored. Although our model of VT is translationally relevant in mimicking clinical DVT (71), our experiments were limited to a single animal model. We have recently discovered that VT formed under conditions of venous stasis is not dependent upon NETs, as gene deletion of PAD4 (necessary for the formation of NETs) or treatment with DNase-I does not reduce thrombus size (59). Tissue factor (TF) has been implicated in formation of VT (72) and is deregulated in sepsis (73) and thus remains an important further avenue of investigation. In a mouse model of microvascular thrombosis, PMNs were the main source of TF at the vein wall and PMN depletion resulted in smaller thrombi (20). In our study, PMN depletion resulted in larger thrombi and thus we chose to explore alternate pathways. Although the data in TLR-4^{-/-} and ICAM-1^{-/-} mice was congruent, further work should characterise cellular location perhaps via tissue specific transgenic mice. Finally, isoflurane, the anesthetic used for brief periods to anesthetise the mice for IVC ligation, has been shown to inactivate LFA-1, and thus represents a potential source of confounding error (74).

In conclusion, we have shown in a translational model of sepsis that endotoxaemia enhances early large vein stasis VT, and that endotoxaemia enhanced DVT is dependent upon TLR4 and ICAM-1 and paradoxically potentiated by neutropenia. Our findings suggest that ICAM-1 and TLR-4 may represent a new and promising target for the prevention and treatment of sepsis related VT in at risk individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This study was supported by: HL092129 (PKH), NIH 5 T32 HL076123-09 (TW and AO), HL089407 (TW), HL122388 (FAJ), K08HL131993 (YK), Elizabeth Anne Baiardi Research Fund, the Conrad and Caroline Jobst Foundation and the Mary Ann Tinker, M. D. Surgical Educational and Research Fund.

References

1. Heit JA, Silverstein MD, Mohr DN, et al. The epidemiology of venous thromboembolism in the community. *Thromb Haemost.* 2001; 86:452–463. [PubMed: 11487036]
2. Lagu T, Rothberg MB, Shieh MS, et al. Hospitalisations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. *Crit Care Med.* 2012; 40:754–761. [PubMed: 21963582]
3. Bellini G, Teng A, Kotecha N, et al. The identification of risk factors for venous thromboembolism in gastrointestinal oncologic surgery. *J Surg Res.* 2016; 205:279–285. [PubMed: 27664873]
4. Bahl V, Hu HM, Henke PK, et al. A validation study of a retrospective venous thromboembolism risk scoring method. *Ann Surg.* 2010; 251:344–350. [PubMed: 19779324]
5. Schmidt M, Horvath-Puho E, Thomsen RW, et al. Acute infections and venous thromboembolism. *J Intern Med.* 2012; 271:608–618. [PubMed: 22026462]

6. Kaplan D, Casper TC, Elliott CG, et al. VTE Incidence and Risk Factors in Patients With Severe Sepsis and Septic Shock. *Chest*. 2015; 148:1224–1230. [PubMed: 26111103]
7. Bloemenkamp KW, Rosendaal FR, Helmerhorst FM, Buller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep-vein thrombosis associated with oral contraceptives containing a third-generation progestagen. *Lancet*. 1995; 346:1593–1596. [PubMed: 7500751]
8. Dellinger RP, Levy MM, Rhodes A, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit Care Med*. 2013; 41:580–637. [PubMed: 23353941]
9. Shojania KG, Duncan BW, McDonald KM, et al. Making health care safer: a critical analysis of patient safety practices. *Evid Rep Technol Assess*. 2001:i–x. 1–668.
10. von Bruhl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012; 209:819–835. [PubMed: 22451716]
11. Massberg S, Grahl L, von Bruhl ML, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med*. 2010; 16:887–896. [PubMed: 20676107]
12. Zarbock A, Ley K, McEver RP, Hidalgo A. Leukocyte ligands for endothelial selectins: specialised glycoconjugates that mediate rolling and signaling under flow. *Blood*. 2011; 118:6743–6751. [PubMed: 22021370]
13. Myers D Jr, Farris D, Hawley A, et al. Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis. *J Surg Res*. 2002; 108:212–221. [PubMed: 12505044]
14. Wakefield TW, Myers DD, Henke PK. Mechanisms of venous thrombosis and resolution. *Arterioscler Thromb Vasc Biol*. 2008; 28:387–391. [PubMed: 18296594]
15. Zonneveld R, Martinelli R, Shapiro NI, et al. Soluble adhesion molecules as markers for sepsis and the potential pathophysiological discrepancy in neonates, children and adults. *Crit Care*. 2014; 18:204. [PubMed: 24602331]
16. Fijen JW, Kobold AC, de Boer P, et al. Leukocyte activation and cytokine production during experimental human endotoxaemia. *Eur J Intern Med*. 2000; 11:89–95. [PubMed: 10745152]
17. Rossignol DP, Lynn M. TLR4 antagonists for endotoxaemia and beyond. *Curr Opin Investig Drugs*. 2005; 6:496–502.
18. Cox LA, van Eijk LT, Ramakers BP, et al. Inflammation-induced increases in plasma endocan levels are associated with endothelial dysfunction in humans in vivo. *Shock*. 2015; 43:322–326. [PubMed: 25565643]
19. Ao L, Song Y, Fullerton DA, et al. The interaction between myocardial depressant factors in endotoxaemic cardiac dysfunction: role of TNF-alpha in TLR4-mediated ICAM-1 expression. *Cytokine*. 2007; 38:124–129. [PubMed: 17683945]
20. Darbousset R, Thomas GM, Mezouar S, et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood*. 2012; 120:2133–2143. [PubMed: 22837532]
21. Raeburn CD, Calkins CM, Zimmerman MA, et al. ICAM-1 and VCAM-1 mediate endotoxaemic myocardial dysfunction independent of neutrophil accumulation. *Am J Physiol Regul Integr Comp Physiol*. 2002; 283:R477–486. [PubMed: 12121861]
22. Yipp BG, Petri B, Salina D, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med*. 2012; 18:1386–1393. [PubMed: 22922410]
23. Moir E, Greaves M, Adey GD, Bennett B. Polymorphonuclear leukocytes from patients with severe sepsis have lost the ability to degrade fibrin via u-PA. *J Leukoc Biol*. 2004; 76:571–576. [PubMed: 15277568]
24. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol*. 2013; 13:34–45. [PubMed: 23222502]
25. Cicala C, Santacroce C, Itoh H, et al. A study on rat platelet responsiveness following intravenous endotoxin administration. *Life Sci*. 1997; 60:PL31–38. [PubMed: 9000123]
26. Rumbaut RE, Bellera RV, Randhawa JK, et al. Endotoxin enhances microvascular thrombosis in mouse cremaster venules via a TLR4-dependent, neutrophil-independent mechanism. *Am J Physiol Heart Circ Physiol*. 2006; 290:H1671–1679. [PubMed: 16284241]

27. Kanaan Z, Gardner S, Carruba C, et al. Macrophage Genetic Reprogramming During Chronic Peritonitis is Augmented by LPS Pretreatment. *J Surg Res.* 2012; 175:289–297. [PubMed: 21696761]
28. Liu Z, Miner JJ, Yago T, et al. Differential regulation of human and murine P-selectin expression and function in vivo. *J Exp Med.* 2010; 207:2975–2987. [PubMed: 21149548]
29. Parant MA, Pouillart P, Le Contel C, et al. Selective modulation of lipopolysaccharide-induced death and cytokine production by various muramyl peptides. *Infect Immun.* 1995; 63:110–115. [PubMed: 7806345]
30. Runnels JM, Zamiri P, Spencer JA, et al. Imaging molecular expression on vascular endothelial cells by in vivo immunofluorescence microscopy. *Mol Imaging.* 2006; 5:31–40. [PubMed: 16779968]
31. Halin C, Mora JR, Sumen C, von Andrian UH. In vivo imaging of lymphocyte trafficking. *Ann Rev Cell Develop Biol.* 2005; 21:581–603.
32. Wojcik BM, Wroblewski SK, Hawley AE, et al. Interleukin-6: a potential target for post-thrombotic syndrome. *Ann Vasc Surg.* 2011; 25:229–239. [PubMed: 21131172]
33. Henke PK, Varma MR, Moaveni DK, et al. Fibrotic injury after experimental deep vein thrombosis is determined by the mechanism of thrombogenesis. *Thromb Haemost.* 2007; 98:1045–1055. [PubMed: 18000610]
34. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest.* 2014; 124:3590–3600. [PubMed: 24983320]
35. Magnani JL. The discovery, biology, and drug development of sialyl Lea and sialyl Lex. *Arch Biochem Biophys.* 2004; 426:122–131. [PubMed: 15158662]
36. McAvoy EF, McDonald B, Parsons SA, et al. The role of CD14 in neutrophil recruitment within the liver microcirculation during endotoxaemia. *J Immunol.* 2011; 186:2592–2601. [PubMed: 21217012]
37. Pannucci CJ, Laird S, Dimick JB, et al. A Validated Risk Model to Predict 90-Day VTE Events in Postsurgical Patients. *Chest.* 2014; 145:567–573. [PubMed: 24091567]
38. Pannucci CJ, Obi A, Alvarez R, et al. Inadequate Venous Thromboembolism Risk Stratification Predicts Venous Thromboembolic Events in Surgical Intensive Care Unit Patients. *J Am Coll Surg.* 2014; 218:898–904. [PubMed: 24680577]
39. Myers D, Wroblewski S, Londy F, et al. New and effective treatment of experimentally induced venous thrombosis with anti-inflammatory rPSGL-Ig. *Thromb Haemost.* 2002; 87:374–382. [PubMed: 11916067]
40. Myers DD Jr, Henke PK, Wroblewski SK, et al. P-selectin inhibition enhances thrombus resolution and decreases vein wall fibrosis in a rat model. *J Vasc Surg.* 2002; 36:928–938. [PubMed: 12422103]
41. Myers DD Jr, Henke PK, Bedard PW, et al. Treatment with an oral small molecule inhibitor of P selectin (PSI-697) decreases vein wall injury in a rat stenosis model of venous thrombosis. *J Vasc Surg.* 2006; 44:625–632. [PubMed: 16950445]
42. Wakefield TW, Strieter RM, Wilke CA, et al. Venous thrombosis-associated inflammation and attenuation with neutralising antibodies to cytokines and adhesion molecules. *Arterioscler Thromb Vasc Biol.* 1995; 15:258–268. [PubMed: 7749835]
43. Eriksson EE, Karlof E, Lundmark K, et al. Powerful inflammatory properties of large vein endothelium in vivo. *Arterioscler Thromb Vasc Biol.* 2005; 25:723–728. [PubMed: 15681299]
44. Patel KN, Soubra SH, Bellera RV, et al. Differential role of von Willebrand factor and P-selectin on microvascular thrombosis in endotoxaemia. *Arterioscler Thromb Vasc Biol.* 2008; 28:2225–2230. [PubMed: 18802014]
45. Slotta JE, Braun OO, Menger MD, Thorlacius H. Capture of platelets to the endothelium of the femoral vein is mediated by CD62P and CD162. *Platelets.* 2009; 20:505–512. [PubMed: 19852690]
46. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature.* 1997; 388:394–397. [PubMed: 9237759]

47. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol.* 1999; 162:3749–3752. [PubMed: 10201887]
48. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med.* 1999; 189:615–625. [PubMed: 9989976]
49. Stark RJ, Aghakasiri N, Rumbaut RE. Platelet-derived Toll-like receptor 4 (Tlr-4) is sufficient to promote microvascular thrombosis in endotoxaemia. *PLoS One.* 2012; 7:e41254. [PubMed: 22911769]
50. Ren M, Li R, Luo M, et al. Endothelial cells but not platelets are the major source of Toll-like receptor 4 in the arterial thrombosis and tissue factor expression in mice. *Am J Physiol Regul Integr Comp Physiol.* 2014; 307:R901–907. [PubMed: 25275013]
51. Ma AC, Kubes P. Platelets, neutrophils, and neutrophil extracellular traps (NETs) in sepsis. *J Thromb Haemost.* 2008; 6:415–420. [PubMed: 18088344]
52. Margraf S, Logters T, Reipen J, et al. Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. *Shock.* 2008; 30:352–358. [PubMed: 18317404]
53. Brill A, Fuchs TA, Savchenko AS, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost.* 2012; 10:136–144. [PubMed: 22044575]
54. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol.* 2012; 32:1777–1783. [PubMed: 22652600]
55. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA.* 2010; 107:15880–15885. [PubMed: 20798043]
56. Brill A, Fuchs TA, Savchenko A, et al. Neutrophil Extracellular Traps Promote Deep Vein Thrombosis in Mice. *J Thromb Haemost.* 2012; 10:136–144. [PubMed: 22044575]
57. Varma MR, Varga AJ, Knipp BS, et al. Neutropenia impairs venous thrombosis resolution in the rat. *J Vasc Surg.* 2003; 38:1090–1098. [PubMed: 14603221]
58. Demers M, Krause DS, Schatzberg D, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. *Proc Natl Acad Sci USA.* 2012; 109:13076–13081. [PubMed: 22826226]
59. El-Sayed OM, Dewyer NA, Luke CE, et al. Intact Toll-like receptor 9 signaling in neutrophils modulates normal thrombogenesis in mice. *J Vasc Surg.* 2016; 64:1450–1458. [PubMed: 26482993]
60. Wagner JG, Roth RA. Neutrophil migration during endotoxaemia. *J Leukoc Biol.* 1999; 66:10–24. [PubMed: 10410985]
61. Aird WC. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood.* 2003; 101:3765–3777. [PubMed: 12543869]
62. Aleman MM, Gardiner C, Harrison P, Wolberg AS. Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability. *J Thromb Haemost.* 2011; 9:2251–2261. [PubMed: 21883880]
63. Henke PK, Varma MR, Deatrick KB, et al. Neutrophils modulate post-thrombotic vein wall remodeling but not thrombus neovascularisation. *Thromb Haemost.* 2006; 95:272–281. [PubMed: 16493489]
64. Semeraro N, Ammollo CT, Semeraro F, Colucci M. Sepsis, thrombosis and organ dysfunction. *Thromb Res.* 2012; 129:290–295. [PubMed: 22061311]
65. Wu K, Urano T, Ihara H, et al. The cleavage and inactivation of plasminogen activator inhibitor type 1 by neutrophil elastase: the evaluation of its physiologic relevance in fibrinolysis. *Blood.* 1995; 86:1056–1061. [PubMed: 7620159]
66. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med.* 1989; 320:365–376. [PubMed: 2536474]
67. Obi AT, Diaz JA, Ballard-Lipka NL, et al. Low-molecular-weight heparin modulates vein wall fibrotic response in a plasminogen activator inhibitor 1-dependent manner. *J Vasc Surg Ven Lymph Dis.* 2014; 2:441–450e1.

68. Obi AT, Diaz JA, Ballard-Lipka NL, et al. Plasminogen activator-1 overexpression decreases experimental postthrombotic vein wall fibrosis by a non-vitronectin-dependent mechanism. *J Thromb Haemost.* 2014; 12:1353–1363. [PubMed: 24943740]
69. Zeerleder S, Schroeder V, Lammle B, et al. Factor XIII in severe sepsis and septic shock. *Thromb Res.* 2007; 119:311–318. [PubMed: 16574200]
70. Lee SY, Chang SK, Lee IH, et al. Depletion of plasma factor XIII prevents disseminated intravascular coagulation-induced organ damage. *Thromb Haemost.* 2001; 85:464–469. [PubMed: 11307816]
71. Diaz JA, Obi AT, Myers DD Jr, et al. Critical review of mouse models of venous thrombosis. *Arterioscler Thromb Vasc Biol.* 2012; 32:556–562. [PubMed: 22345593]
72. Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis. *Ann Rev Physiol.* 2011; 73:515–525. [PubMed: 20690821]
73. Carraway MS, Welty-Wolf KE, Miller DL, et al. Blockade of tissue factor: treatment for organ injury in established sepsis. *Am J Respir Crit Care Med.* 2003; 167:1200–1209. [PubMed: 12714343]
74. Yuki K, Bu W, Xi J, et al. Isoflurane binds and stabilizes a closed conformation of the leukocyte function-associated antigen-1. *FASEB J.* 2012; 26:4408–4417. [PubMed: 22815384]

Abbreviations

VT	venous thrombosis
VTE	venous thromboembolism
CAMs	cellular adhesion molecules
PMNs	polymorphonuclear cells
TLR4	toll like receptor 4
LPS	lipopolysaccharide
NETs	neutrophil extracellular traps
TFPI	tissue factor pathway inhibitor

What is known about this topic?

- Venous thrombosis incidence is increased in those patients with sepsis.
- Small vessel thrombotic potential increases with endoxaemia.
- TLR4 mediates in part the host response to endotoxaemia and thrombosis.

What does this paper add?

- Large vein in vivo responses to endotoxaemia show increased vein wall CAMs and increased leukocyte adhesion.
- Early venous stasis experimental thrombosis is dependent on TLR4 and ICAM-1.
- Neutropenia potentiates experimental venous thrombosis.

Figure 1. Effect of systemic endotoxemia on local and circulating markers of inflammation and thrombosis and leukocyte trafficking

WT mice were treated with i. p. injection of LPS 10 mg/kg or equivalent volume of sterile saline (control) and IVC and plasma or serum collected for analysis after 2 h. ICAM-1 and VCAM-1 protein expression in the IVC was analysed by ELISA (n=5, *p<0.05) (A, B). Serum markers of inflammation, IL-1 β and IL-6 (n=4-5, *p<0.05) (C, D) were analysed at 2 h. Leukocyte interactions with venous endothelium in the jugular veins of LPS and control mice were imaged with high-magnification confocal microscopy (E). Representative axial and coronal confocal images of the jugular vein wall (blue, collagen) and adherent leukocytes (green, R6G) are shown (scale bar=100 μm). For quantification of leukocyte rolling, epifluorescence intravital microscopy of the femoral vein wall and leukocyte rolling was performed at lower magnification (F). The trailing lines indicate the distance that a specific leukocyte traveled over a 5 s period. Quantification of leukocyte rolling (n=6, *p=0.002) (G) and rolling velocities (n=75/group, *p<0.0001) (H), as well as adherent or rolling leukocytes (n=6, *p=0.01, I) and leukocyte flux (n=6, *p=0.001) (J). All data represented as mean ± SD. LPS=lipopolysaccharide, IVC = inferior vena cava, R6G = rhodamine 6G, LPS=lipopolysaccharide, IVC = inferior vena cava.

Figure 2. Effect of systemic endotoxemia on venous thrombi composition

WT mice pre-treated with i. p. injection of LPS 10 mg/kg or equivalent volume of vehicle (control) underwent ligation and thrombi were harvested at 2 h post thrombosis. Platelets were quantified by CD41 western immunohistochemistry (n=6–10, p=0.0010) (A).

Representative photomicrographs of CD41 staining of thrombi of saline treated mice (B) and LPS-treated mice (C) shown at 10x. Neutrophils were counted in the thrombi of control and LPS treated mice utilising Ly6G antibody (n=6, *p=0.003) (D). Representative histology of thrombus stained in IVC is shown in panels E (control) and F (LPS treatment) stained with Ly6G antibody at 10x. PMN markers cathepsin G and MPO were measured via western blot (n=5–6, *p<0.05) (G-H). Circulating factor XIII was measured by western blot (n=4–7, p=0.045) (I).

Figure 3. Effect of systemic endotox -aemia on early venous thrombosis in WT, TLR4^{-/-} and ICAM-1^{-/-} mice

All mice pre-treated with i. p. injection of LPS 10 mg/kg or equivalent volume of sterile saline (control) underwent venous thrombosis and were harvested at 2 h. Thrombus size was measured by weight and length in WT (n=25–30 of LPS and controls, *p=0.001) (A) TLR4^{-/-} (n=5), and ICAM-1^{-/-} (n=6) mice. Circulating sICAM-1 was measured via ELISA (all n=5–10, all *p<0.05) (B). TLR4 protein expression was measured in the thrombi (n=6, p=0.03) (C) and IVC of WT mice (n=6, p=0.23, by ELISA) (D). Photomicrographs of ICAM-1 staining shown for control (C, middle panel) and LPS treated mice (C, right panel) at 100x. TLR-4 staining shown at 200x for both control (D, middle panel) and LPS treated mice (D, right panel) at 1000x. T=thrombus, W=wall, arrows denote examples of positive staining cells.

Figure 4. Neutrophils and Fibrinolytic markers in endotox-aemic VT; effect of neutrophil depletion on venous thrombosis

A select group of mice underwent PMN depletion via a single IP injection (250 ug) of purified rat anti-mouse Ly6G clone antibody or control rat IgG2b. LPS-treated mice undergoing no treatment (true control), IgG (control antibody) or Ly6G (neutrophil depletion) underwent VT and with subsequent measurement of PMNs ($n=4-10$, $p<0.05$) (A). Thrombus size was measured 2 h following induction of VT ($n=5-6$, $*p<0.05$) (B). Enzymes involved in fibrinolysis, PAI-1 ($n=4-6$, $*p<0.05$) (C) and uPA ($n=4-6$, $*p<0.05$) (D), were measured in vein wall in thrombosed animals via western blot. PAI-1 =plasminogen activator inhibitor, uPA=urokinase plasminogen activator.