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Massachusetts Institute of Technology

Blood Clotting and Traumatic Injury with Shock Mediates Complement-Dependent Neutrophil Priming for Extracellular ROS, ROS-Dependent Organ Injury and Coagulopathy

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Running title: *C5a primes PMNs after trauma to increase organ injury.*

Keywords: trauma; complement; NADPH oxidase, coagulation; inflammation; PMN; neutrophil; reactive oxygen species; endothelium

Abbreviations: polymorphonuclear leukocyte (or neutrophil), PMN; protease-activated receptor, PAR; human umbilical vein endothelial cells, HUVEC; electric cell-substrate impedance sensing, ECIS; tumor necrosis factor-alpha, TNF-alpha; reactive oxygen species, ROS; activated protein C, aPC; antithrombin-III, ATIII; prothrombin fragment 1+2, PF1+2; nicotinamide adenine dinucleotide phosphate, NADPH; platelet-poor plasma, PPP; heart rate, HR; systolic blood pressure, SBP; mean arterial pressure, MAP; ethylenediaminetetraacetic acid, EDTA; diphenyleneiodium, DPI; complement component 5a, C5a; CD88 (complement component C5a receptor 1), C5aR; N-formylmethionine-leucyl-phenylalanine, fMLP; Dulbecco's phosphate buffered saline with divalent cations, DPBS++ ; Dulbecco's phosphate buffered saline without divalent cations, DPBS- ; Russell Viper Venom factor X activating enzyme, RVVX; Russell Viper Venom factor V activating enzyme, RVV; bovine serum albumin, BSA; acute respiratory distress syndrome, ARDS; superoxide dismutase, SOD; horseradish peroxidase, HRP; neutrophil extracellular trap, NET; emergency department, ED; institutional animal care and use committee, IACUC; trauma-induced coagulopathy, TIC; damage associated molecular patterns, DAMPs; US Department of Defense, DoD; US National Institutes of Health, NIH; Trans-Agency Consortium for Trauma Induced Coagulopathy, TACTIC; Injury Severity Score, ISS; Red Blood Cell, RBC

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SUMMARY

Polymorphonuclear (PMN) leukocytes participate in acute inflammatory pathologies such as ARDS following traumatic injury and shock, which also systemically activates the coagulation system. Trauma can prime the PMN NADPH oxidase complex for an enhanced respiratory burst, but the relative role of various priming agents in this process remains incompletely understood. We therefore set out to identify mediators of PMN priming during coagulation and trauma-shock and determine whether PMN ROS generated in this manner could influence organ injury and coagulation. Initial experiments demonstrated that PMNs are primed for predominantly extracellular ROS production by products of coagulation, which was abrogated by CD88/C5a receptor(C5aR) inhibition. The importance of this was further highlighted by demonstrating that known PMN priming agents result in fractionally different amounts of extracellular versus intracellular ROS release depending on the agent used. Plasma from trauma patients in hemodynamic shock(n=10) also primed PMNs for extracellular ROS in a C5a-dependent manner, which correlated with both complement alternative pathway activation and thrombin generation. Furthermore, PMNs primed by pre-incubation with products of blood coagulation directly caused loss of endothelial barrier function in-vitro that was abrogated by C5aR blockade or NADPH oxidase inhibition. Finally, we show in a murine model of trauma-shock that p47phox KO mice with PMNs incapable of generating ROS were protected from inflammatory end-organ injury and activated protein C-mediated coagulopathy. In summary, we demonstrate that trauma-shock and coagulation primes PMNs for predominantly extracellular ROS production in a C5a-dependent manner that contributes to endothelial barrier loss and organ injury, and potentially enhances traumatic coagulopathy.

INTRODUCTION

Polymorphonuclear (PMN) leukocytes (commonly referred to as neutrophils), the primary innate immunocyte involved in acute responses to infection and other

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pathophysiologic insults, generate large amounts of bactericidal reactive oxygen species (ROS) via the NADPH oxidase complex (1). In addition to ingesting and killing pathogens, PMNs can also undergo a process of 'priming', which results in partial release of proteases and other granule contents into the extracellular space, along with 'pre-activation' of the NADPH oxidase (1-3). Primed PMNs typically produce small to minimal amounts of ROS spontaneously, but upon exposure to an activating stimulus (e.g. N-formyl peptides released by bacteria or from host mitochondria following trauma, or integrin engagement upon adhesion), primed PMNs produce much larger amounts of ROS, with accelerated kinetics, compared to unprimed cells (3-5). PMN priming is thought to play a critical role in the development of organ injury/failure after major pathophysiologic insults such as traumatic shock (trauma-shock). The classic "two-hit hypothesis" of organ failure following trauma hypothesizes that PMN priming from the initial trauma followed by a second stimulus (e.g. surgery) activates the PMN to release excessive extracellular ROS and proteases, damaging adjacent non-injured cells and leading to auto-inflammatory organ injury (2, 6, 7). A significant number of cytokines and related molecules have been identified that are capable of inducing PMN priming, but the relative importance of the different priming agents in inducing organ injury after trauma-shock has not been firmly established.

After major blunt or penetrating trauma, patients suffer from both tissue injury and hypovolemic shock from blood loss. In patients with otherwise survivable injury, early death usually results from uncontrolled hemorrhage in-part due to trauma-induced coagulopathy (TIC), while late death is usually due to inflammatory organ injury, microvascular thrombosis, and/or infection due to the development of a profoundly immunosuppressive state (8, 9). The complex pathophysiology that underlies these clinical observations appears to be initiated by the effects of trauma-shock on pathways that originally evolved to protect the host organism, including the innate immune response (e.g. PMNs) to damage-associated molecular patterns (DAMPs), and activation of both the coagulation and complement systems (10-12). Furthermore, the ability of PMN and the coagulation system to alter each other's behavior is likely paramount in trauma, where both PMN and the coagulation system are activated, but this has only recently received significant attention. Some of these recent findings

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include the observation that platelets are activated and thrombin is generated in response to neutrophil extracellular traps (NETs), and conversely that thrombin can lead to increased levels of the granule marker CD11b on the PMN surface (13-15). To further delineate additional potential crosstalk between PMN inflammatory responses that drive the pathophysiology seen after trauma-shock and the coagulation system, we hypothesized that specific molecules produced during the process of blood coagulation could directly prime PMN for ROS production and that the ROS released by PMN could damage the endothelium, cause organ injury, and potentially alter coagulation after trauma-shock.

MATERIALS AND METHODS

Human Blood Products and Patients

Blood products used in this study for PMN purification and ex-vivo coagulation experiments were obtained (as described in more detail below) with written informed consent and approval from the Institutional Review Board at the Massachusetts institute of Technology. In clinical studies, whole blood was drawn in to 3.2% citrate tubes from trauma patients in the emergency department (ED) and again 24 hours later and platelet-poor plasma generated and frozen -80C, and stored at the NIH/DoD Trans-Agency Consortium for Trauma Induced Coagulopathy (TACTIC) repository for future use. The inclusion criteria of the studies from which the TACTIC samples were obtained were (a) adult trauma patients in shock, as defined by HR >108 and SBP <90, or a SBP <70 without the tachycardia requirement (University of Pittsburgh Medical Center); and (b) adult trauma patients meeting full trauma team activation for blunt or penetrating trauma, excluding patients on anticoagulants, with pre-existing coagulopathies or malignancies, dialysis patients, thermal injury, or patients on immunosuppression (Mayo Clinic). These studies were both performed with approval from the respective Institutional Review Boards at the University of Pittsburgh Medical Center and the Mayo Clinic, and written informed consent for study participation was obtained on all trauma patients.

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PMN Preparation

Human PMN were prepared from healthy volunteer blood collected in 10mL vacutainer tubes containing 18mg K_2EDTA (Becton Dickinson) via venipuncture of the antecubital veins. PMN were purified by centrifuging EDTA anticoagulated whole blood through a Ficoll-Paque density gradient (GE Healthcare) followed by RBC sedimentation with 2% dextran w/v (M_r 450,000 – 650,000) (Sigma) in Dulbecco's PBS without divalent cations (DPBS-) (Thermo Fisher Scientific Inc.) followed by hypotonic lysis as described previously (21).

Coagulation and Blood Product Preparation

Plasma was generated from whole blood collected in either EDTA or heparin tubes (final heparin concentration 15.8 units/mL) (Becton Dickinson) by centrifugation at 2000 x g for 10 min with collection of the straw-colored liquid fraction on top as plasma. Platelet-poor plasma (PPP) was generated from whole blood collected in EDTA, heparin, or 3.2% sodium citrate tubes and centrifuged at 3000 x g for 15 min, the top ¾ fraction of plasma was collected and then spun again at 3000 x g for an additional 15 min, with collection of the top ¾ fraction of this as PPP. Platelet depletion to $<10x10^3$ platelets/uL was confirmed by manual counting with a hemocytometer.

Serum was generated by collection of healthy volunteer whole blood into uncoated 10mL glass vacutainer tubes (Becton Dickinson) and clotted by incubation for 2 hrs at 37^oC, then a 2 hr incubation on ice followed by centrifugation at 2000 x g for 10 min with recovery of the liquid product as serum.

Clotted plasma/PPP was generated by reversing EDTA anticoagulant with 15uL of 9% $CaCl_2$ (w/v in H_2O) per mL plasma/PPP or by reversing heparin anticoagulant with 25uL of 1% (w/v) protamine sulfate (Sigma) in Dulbecco's PBS with divalent cations (DPBS++) per mL plasma/PPP, and thereafter was incubated and the liquid product collected in the same manner as for serum. All clotted products (serum, clotted plasma, clotted PPP) were controlled for anticoagulant by adding EDTA or heparin back to the collected liquid products of coagulation to allow for more appropriate comparison with unclotted plasma/PPP products.

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Purified coagulation factors, Russell Viper Venom Factor X Activating Enzyme (RVVX) and Russell Viper Venom Factor V Activating Enzyme (RVV) were all obtained from Haematologic Technologies, Inc.. Factors Xa and Va were generated by incubation of Factor X with RVVX and Factor V with RVV for 2 hrs in sterile-filtered DPBS++ with 0.01% BSA at 37°C in a 500:1 factor:activator ratio according to supplier instructions, where 0.12µg/mL $ZnCl_2$ was supplemented in to the Factor X + RVVX reaction. Factor IXa was generated by incubation of Factor IX with activated Factor XIa for 2 hrs in sterile-filtered DPBS++ with 0.01% BSA at 37°C in a 500:1 Factor IX:Factor XIa ratio. Generation of activated factors and their corresponding activation peptides was confirmed by separating proteins via 10% SDS-PAGE under non-reducing conditions and staining with Coomassie SimplyBlue™ SafeStain (Thermo Fisher Scientific Inc.). All purified and activated coagulation factors were used in PMN priming assays at concentrations equivalent to physiologic levels of the pre-activated factors (i.e. maximum physiologic levels) (16).

Chemiluminescence Measurements of Reactive Oxygen Species Production

For measurements of PMN ROS production, PMN were resuspended in DPBS++ (for heparin-based, purified priming agent-based, and purified coagulation factor experiments) or DPBS- (for EDTA and citrate-based experiments) to 4x10^6 PMN/mL and were incubated with an equal volume of the designated blood product being tested (to give 50% (v/v) blood product concentration during the PMN priming step/incubation) or DPBS++ (for priming agent and coagulation factor experiments) for 30 min at 37°C. PMN were then centrifuged for 5 min at 250 x g at 4°C and washed with DPBS++ (for heparin-based, priming agents, and purified coagulation factors) or DPBS- (for EDTA and citrate) x2, followed by a final resuspension in DPBS++ (for all) equal to the initial volume. 75µL of the resulting PMN solutions were added to a 96-well plate in duplicate and an equal volume of luminol solution (Sigma) in DPBS++ (final assay concentration of 150uM luminol) was then added containing either horseradish peroxidase (HRP, final assay concentration 10u/mL) (Sigma) to specifically measure total ROS production (17), or containing a combination of superoxide dismutase (SOD, final assay concentration

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25U/mL) (Sigma) and catalase (final assay concentration 2,000U/mL) (Sigma) to specifically measure intracellular ROS by quenching the extracellular ROS fraction as has been previously described (18, 19). After a 5 min incubation at 37°C with the designated luminol solution, either vehicle control or N-formyl-methionyl-leucyl-phenylalanine (fMLP, final assay concentration 100nM) (Sigma) were added and chemiluminescence events were measured over 10 minutes. Quantification of ROS was performed by integrating the area under the curve of chemiluminescence events over time as previously described (20).

For experiments using healthy volunteers to measure the effects of different blood products on PMN priming, PMN were used with autologous blood products, and an internal positive priming control with the well-known priming agent TNF-alpha (20ng/mL, R&D Systems) was used to set the reference of normalization such that comparison of priming was feasible across multiple different types of clotted and unclotted blood products. For experiments with the C5a receptor (C5aR) antagonist W-54011 (Tocris), PMN were pre-incubated for 10 min with 5uM W-54011 or vehicle control and then PMN were mixed with the designated blood products and experiments performed as already described. For experiments using a mouse anti-human CD88 (C5a receptor 1) monoclonal antibody (clone S5/1, Bio-Rad Laboratories) to block C5aR1, PMN were pre-incubated with 1uM anti-C5aR1 antibody or 1uM IgG2a isotype control antibody (Bio-Rad Laboratories) (21) for 30 min at 37°C prior to performing the experiments in the fashion already described.

For experiments using traumatic shock patient citrate PPP (n=10 patients) and comparison to healthy volunteer citrate PPP (n=16), all PPP was heterologous to the PMN source including the healthy PPP in order to control for the fact that trauma PPP could not be tested with autologous PMN. When there was adequate trauma patient sample volume and available volunteers, each trauma sample underwent testing with two volunteer's PMN and all ROS data was included from both. In these trauma versus healthy PPP experiments the reference point was set as the uninhibited (i.e. vehicle) +fMLP group and comparison made to each sample's respective W-54011/+fMLP

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group, with results reported as percent change in ROS production due to C5aR inhibition with W-54011 to reflect the degree of priming due to C5a in the samples.

Electric Cell-Substrate Impedance Sensing Co-Incubation Assays for Measurement of PMN Effects on Human Umbilical Vein Endothelial Cell Monolayers

Pooled-donor human umbilical vein endothelial cells (HUVEC) were obtained (Lonza Biologics, Inc.) and grown to confluence at passage 4 on 96-well tissue culture plates containing interdigitated gold electrodes on the basal surface of each well underneath the cell layer (96W20idf, Applied BioPhysics, Inc.). Resistance across the HUVEC monolayer was then continuously measured over time using an Electric Cell-Substrate Impedance Sensing (ECIS) device (model Z-Theta, Applied BioPhysics, Inc.). EGM-2 media (Lonza Biologics, Inc.) was used to grow the HUVEC to confluence, at which point the media was switched to EBM-2 basal media (Lonza Biologics, Inc.) with no serum or growth factors added to the media and the cells were allowed to acclimate and reach steady-state resistance.

Once the HUVEC were stabilized in serum-free growth factor-free media, PMN and blood products from healthy volunteers were made as already described and PMN were pre-incubated with one of the following: (a) 5uM W-54011 to inhibit C5aR signaling; (b) 10uM diphenyleneiodium (DPI) (Sigma) to inhibit ROS production via the NADPH oxidase complex; or (c) vehicle control. After this pre-incubation with inhibitor or control, the PMN were incubated for 30 min at 37°C in an equal volume of heparin plasma or serum (heparinized after clotting to control for heparin in the plasma) (final blood product concentration during incubation of 50% (v/v)). The PMN were then centrifuged at 250 x g for 5 min at 4°C and washed x2 with DPBS-, resuspended in serum-free growth factor-free EBM-2 media, and placed in a Corning HTS Trans-well 96 well permeable support with a 0.4μm pore size polycarbonate membrane (Corning Inc.) to a final concentration of 1x10^{7} PMN/mL and resistance changes in the HUVEC monolayer were measured over time. This trans-well co-incubation method where PMN and HUVEC were not in physical contact with one another allowed for evaluation of changes in endothelial barrier function resulting from any soluble PMN products (i.e.

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ROS) released in response to pre-incubation with various blood products, where the blood products themselves were washed away prior to PMN addition to the trans-well to prevent any confounding effect of the blood products directly on the HUVECs.

Animal Trauma-Hemorrhage Model of Trauma-Induced Coagulopathy and End-Organ Injury

Murine experiments were performed with IACUC approval and in accordance with NIH guidelines using a literature-established murine trauma-hemorrhage model that is known to cause organ injury, activate the coagulation system and cause trauma-induced coagulopathy (22, 23). Adult (8-12 week old) male WT and p47phox KO mice (Taconic BioSciences) on a C57Bl/6 background were compared, where p47phox KO mice have PMN that are unable to generate ROS via the NADPH oxidase complex due to the absence of the p47phox protein.

Anesthesia was induced in an isoflurane chamber and the mice were then positioned supine on a plexiglass board and restrained with soft tape tethers to the limbs and tail with ongoing isoflurane anesthesia via nose cone. Normothermia was maintained using a thermal control unit and rectal temperature probe. Upon satisfactory positioning and anesthesia, the abdomen and bilateral groins were shaved and prepped with betadine scrub x3 and a 2cm midline laparotomy and bilateral groin incisions were made. The intraabdominal organs were inspected to confirm no inadvertent organ injuries, and then the laparotomy was closed with 5-0 prolene suture (Ethicon Inc.) and the incision bathed in 1% lidocaine. The left femoral artery and right femoral vein were then cannulated with sterile PE-10 tubing and the left femoral artery tubing was connected to a pressure transducer (TSD104A, Biopac Systems) and amplifier (MP1004-CE, Biopac Systems), and the groin cannulation sites were then bathed in 1% lidocaine solution (Hospira Inc.). Anesthesia was then stopped and a baseline mean arterial pressure (MAP) > 90mmHg was confirmed, at which point shock was induced by withdrawing an estimated 25% of total blood volume (calculated by multiplying body weight by 0.077, in mL) using a pipette to withdraw blood from the left femoral arterial catheter. The MAP was continuously monitored and aliquots of 60-70uL of blood

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withdrawn as needed to maintain a goal MAP of 35 +/-5mmHg for 60 min. Sham mice were treated the same, except they did not have any blood withdrawn to induce shock and thus underwent 60 min of board stress. Half-way through the 60 minute shock (or board stress) period, 1mg/kg of filter-sterilized ^{125}I-labeled albumin (albumin and ^{125}I labeling kit from Thermo Fisher Scientific Inc. and labeled according to manufacturer's instructions) was administered via the right femoral vein catheter.

After the 60-minute shock (or board stress) period was complete, the mice were euthanized by isoflurane overdose and the laparotomy reopened for an immediate blood draw from the IVC in a 9:1 ratio with 3.2% citrate (for thrombin-anti-thrombin complex measurements) or 3.2% citrate containing 1mM benzamidine (for activated protein C analysis), centrifuged to obtain PPP, and stored at -80 until use on ELISA. The lungs were then harvested, vasculature flushed with saline, and lung tissue homogenized in saline for measurement of lung permeability to ^{125}I-labeled albumin using a gamma counter and taking in to account the plasma ^{125}I-labeled albumin levels (counts/minute).

ELISA measurement of complement fragments, thrombin generation (PF 1+2 and thrombin-antithrombin), and aPC levels

ELISA was performed according to manufacturer instructions for the following: human C5a, Bb, and C4d levels (Quidel Corporation); total thrombin generation from human samples by measuring PF1+2 levels (Lifespan Biosciences, Inc.); total thrombin generation in mouse samples by measuring thrombin-antithrombin complex levels (Enzyme Research Laboratories Inc.). Mouse aPC levels were measured using a custom ELISA-type assay developed in the laboratory of Dr. Charles Esmon that is specific for the activated form of Protein C as previously described (23, 24).

Statistical Analysis

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Statistical analysis was performed using GraphPad Prism Version 6.07 (GraphPad Software Inc, San Diego, CA). Data are expressed as mean +/- SEM. Significant differences between groups were determined using ANOVA with Holm-Sidak's correction for multiple comparisons, except for the C5a, Bb, and C4d ELISA data for trauma patient PPP which was not normally distributed and therefore compared using Kruskal-Wallis tests with Dunn's correction for multiple comparisons. Spearman's correlations were performed to determine relationships between complement fragment levels, PF1+2 levels, and the ability to inhibit extracellular ROS using a C5aR inhibitor. Alpha was set at the 0.05 level.

RESULTS

Products of coagulation prime PMNs for early extracellular ROS generation in a cell- and platelet-independent manner.

Agents that prime the PMN NADPH oxidase for enhanced ROS production relative to agonists (e.g. fMLP) alone (Figure 1a) vary considerably in the extent to which they enhance intracellular versus extracellular ROS following exposure to a second activating stimulus, with some priming agents leading to significantly larger proportions of extracellular ROS than others (Figure 1b). To investigate a potential relationship between PMN priming and coagulation such as what is seen in trauma, as well as the fractional distribution of extracellular versus intracellular ROS generated in response to any observed priming, we first explored whether the products of blood clotting ex-vivo were sufficient to induce priming of PMNs for enhanced ROS production. As shown in Figure 1c and d, fMLP stimulation of resting human PMNs incubated with unclotted blood plasma obtained from healthy volunteers showed minimal ROS production. In marked contrast, PMNs incubated in serum from whole blood from the same donors that had been clotted ex-vivo were primed for significant ROS production in response to fMLP. Furthermore, PMN priming similar to what was seen by incubation of PMN with serum was also observed when PMNs were incubated

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in supernatants from previously isolated plasma that was then clotted ex-vivo (i.e. RBC's and leukocytes removed prior to clotting) (Figure 1e). When platelets were also depleted from plasma to make PPP followed by clotting, the supernatants from clotted PPP also primed PMN in a similar fashion to serum (Figure 1f). These results indicate that: (a) agents capable of priming PMNs for large amounts of ROS can be generated by production of 'serum' from plasma upon blood clotting; and (b) the priming activity generated following ex-vivo blood clotting does not require the presence of leukocytes, red blood cells, or platelets during the clotting process. Similar results were obtained regardless of the anticoagulant method used to prepare the plasma, as quantified in Figures 1g and h. Furthermore, with respect to the fractional distribution of ROS, PMN priming by the products of coagulation predominantly led to generation of extracellular ROS, particularly at early times after stimulation (compare red and blue shaded curves in Figures 1c-f), quantified at the 10 minute marker in Figure 1i. Notably, even in the absence of fMLP, PMNs incubated with clotted blood products generated more ROS over time compared to PMN incubated in unclotted blood products (cyan line in Figure 1c-f), although this was significantly less than that seen following fMLP stimulation.

Complement component C5a, but not coagulation factors or their activation peptides, is the principle PMN priming agent following ex-vivo blood clotting.

Multiple components of the coagulation cascade are known to modulate PMN functions, including fibrinopeptide B, a chemotactic agent (25) and thrombin via protease-activated receptors (26). We therefore investigated whether any of the major activated coagulation factors, or their activation peptides, that are produced during the clotting process could prime PMNs for ROS generation in response to fMLP stimulation. In these experiments, ROS production was assayed using luminol-dependent chemiluminescence as described above, with TNF-alpha (20 ng/ml) serving as an internal positive control for priming. As shown in Figure 2a, no PMN priming was observed in response to maximum physiologic concentrations of activated coagulation factors, their activation peptides, or fibrinopeptides resulting from thrombin-mediated fibrinogen cleavage. Another major protease system known to be present in the plasma

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component of blood is the complement system, which when activated has known inflammatory signaling functions via its anaphylatoxins C3a and C5a (reviewed in (27)). As seen in Figure 2a, C3a was unable to prime PMN for ROS generation in response to fMLP, while C5a was a potent priming agent, consistent with numerous previous reports in the literature (28-31). To determine whether C5a was the relevant priming agent following ex-vivo blood coagulation, we investigated whether inhibition of CD88 (C5a Receptor-1, C5aR) signaling using a specific C5aR-blocking monoclonal antibody, or the C5aR antagonist W-54011 could abrogate PMN priming for enhanced ROS production by products of coagulation (Figure 2b-f). As seen in Figure 2b-e, PMN priming by serum or clotted plasma was significantly inhibited by the C5aR blocking antibody (compare blue and red curves in panels b-d). Similar results were obtained using the C5aR antagonist W-54011 (Figure 2f). Both the C5aR blocking antibody and W-54011 had no significant effect on PMN priming in the plasma controls, although very small amounts of non-significant partial agonism was observed. To validate the potential role of C5a as the major PMN priming agent by products of coagulation, an ELISA was performed (Figure 2g), confirming the presence of elevated C5a levels in ex vivo clotted blood and clotted plasma compared to unclotted plasma (Figure 2g). Importantly, both clotted plasma and serum had significant elevations in the complement component Bb relative to plasma (Fig. 2h), but not C4d (Fig. 2i), indicating activation of the alternative pathway during coagulation, but not the classical or lectin pathway.

Traumatic shock patient plasma primes PMNs for extracellular ROS production in a C5a/alternative pathway-dependent manner that correlates with thrombin generation.

The observation that C5a generated during *ex-vivo* coagulation primed PMN for extracellular ROS release led us to explore its potential clinical relevance in trauma patients with shock in whom the coagulation system is known to be activated systemically (11, 32). To accomplish this, citrate PPP from trauma patients in shock (n=10, Table 1) was obtained upon patient arrival to the ED and again 24 hours later and evaluated for its ability to prime resting PMNs for ROS production in a C5a-

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dependent manner by comparing vehicle controls with the C5aR antagonist W-54011. Kinetic ROS production curves from an example patient and corresponding control are shown in Figure 3a-c, demonstrating that while the healthy control PPP did not prime PMN, this traumatic shock patient's PPP from the ED primed PMNs for substantial extracellular ROS release, but by 24 hours after admission this particular patient's PPP no longer did. The amount of total, extracellular and intracellular ROS inhibited by W-54011 treatment was quantified for all healthy and traumatic shock patient PPP groups and compared to determine the extent to which C5a was responsible for any observed PMN ROS production after incubation in the traumatic shock patients' PPP (Figure 3d-f). Overall (Figure 3e), we found that traumatic shock patient PPP obtained from the ED and 24 hours later both primed healthy resting PMNs for extracellular ROS production in a manner that was significantly inhibited by C5aR antagonism with W-54011, with somewhat less priming observed at the later (24hr) time point (ED vs. healthy control PPP: 25.4 +/- 5.6% reduction in ROS, p=0.0001. 24hr vs. healthy: 16.8 +/- 5.5% reduction in ROS, p=0.0074). In addition, an ELISA performed on traumatic shock patient PPP from the ED and 24-hour time points demonstrated significant elevations in C5a and Bb levels relative to healthy control PPP, but no change in C4d levels (Figure 3h-j). PF1+2, a marker of total thrombin generation and activation of coagulation, demonstrated a trend towards elevated PF1+2 levels in trauma patient PPP relative the healthy control PPP that did not reach significance when comparing absolute values (ED vs. healthy: p=0.054. 24hr vs. healthy: p=0.070). Importantly, however, there was a significant correlation between the amount of PMN extracellular ROS that could be inhibited by W-54011 treatment and the levels of C5a, Bb, and PF1+2 in the PPP. No such correlation was observed with C4d levels (Table 2). Furthermore, there was also a significant correlation between C5a levels and both Bb and PF1+2 levels (Table 2).

PMNs primed by products of coagulation are sufficiently active to cause loss of endothelial barrier function in vitro via C5a-dependent ROS release.

The potential importance of PMN priming by C5a present in products of coagulation was next evaluated using electrical cell-substrate impedance sensing

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(ECIS) assays to evaluate whether soluble products released by PMNs in response to C5a levels seen in clotted blood products were sufficient to compromise endothelial barrier function in culture. In these experiments, PMNs were pre-incubated in a given blood product, washed with HUVEC culture media (serum-free, growth hormone-free) to remove the blood product, and then added to a trans-well filter placed over a layer of confluent HUVEC cells on an ECIS plate that electrically measures electrical impedance and resistance across the monolayer. This setup allows evaluation of the effects of PMN release products on the endothelium, since no direct contact can occur between PMNs and HUVEC due to the presence of the trans-well filter. As seen in Figure 4a and b, PMNs that were pre-incubated in serum led to significant losses of endothelial barrier function, while PMN pre-incubated in plasma did not cause any changes in endothelial resistance over a 12 hour time course (compare red and black traces). Resistance just prior to PMN addition and then at the 6 and 12 hour time points after PMN addition are quantified in Figure 4b. Importantly, either blockade of PMN C5aR signaling with W-54011 during serum incubation, or inhibition of ROS generation by the PMN NADPH oxidase complex with DPI, led to abrogation of the observed endothelial barrier loss caused by PMN priming.

PMN ROS generated in response to trauma-shock is a critical mediator of end-organ injury and coagulopathy in-vivo.

Given our finding that traumatic shock patient PPP primes resting PMNs for extracellular ROS, we next asked whether PMN ROS generated during trauma-shock had a role in end-organ injury and coagulation *in vivo*, using a well-established mouse model of trauma-hemorrhagic shock that causes lung injury and trauma-induced coagulopathy (TIC) (23, 31). To specifically elucidate the contribution of PMN-derived ROS, these experiments were conducted comparing wild-type control mice to p47phox knock-out mice that lack a functional NADPH oxidase, resulting in an absence of PMN ROS production (33).

As shown in Figure 5a, p47phox KO mice subjected to the trauma-hemorrhagic shock model were significantly protected from lung injury as measured by the ^{125}[I]-

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radiolabeled albumin lung permeability assay, when compared to WT mice (p<0.001), where lung capillary permeability to proteins is a principle component of ARDS, a severe form of inflammatory lung injury and failure (34). In contrast, the lung permeability of the p47phox KO mice was essentially identical to that of the mock-injured controls. Furthermore, with respect to effects of PMN ROS on coagulation, p47phox KO mice had significantly lower aPC levels compared to WT mice (p=0.026), suggesting protection from traumatic coagulopathy in this TIC model (Figure 5b). Finally, while a non-significant trend towards lower total thrombin generation was also observed in p47phox KO mice compared to WT mice (p=0.078) (Figure 5c), it is noteworthy that the WT mice had a significant elevation in total thrombin generation compared to sham treated controls (p=0.015) while the p47phox KO mice did not (p=0.153) (Figure 5c).

DISCUSSION

Trauma is the leading cause of death in the United States from ages 1-44, responsible for over 180,000 deaths and $406 billion in healthcare costs annually (35, 36). Death after traumatic injury often involves organ failure and sepsis resulting from inflammatory organ injury and immune dysfunction, and in many cases surgical bleeding and coagulopathy is also a major contributor to death (8, 9, 37). While the importance of PMN priming and activation in contributing to end-organ injury after trauma-shock and other illnesses (e.g. sepsis) has been recognized previously, a complete understanding of the underlying mechanisms responsible for priming after trauma-shock is lacking. A plethora of cytokines, chemokines, and DAMPs are released into the circulation after trauma (38), but the relative importance of different cytokines in the PMN priming process after trauma-shock and hemorrhage is unclear. In the current study, we explored possible links between the activation of blood clotting after trauma-shock and subsequent inflammatory tissue injury mediated though PMN priming, where priming specifically refers to a state where PMN have an enhanced respiratory burst after exposure to a second activating stimulus (3-5, 39).

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Our initial studies showed that products produced by blood coagulation ex-vivo led to robust priming of the PMN NADPH oxidase complex predominantly for early extracellular ROS release in response to fMLP. This observation was independent of the presence or absence of platelets and blood cells during the coagulation process, and led to priming and total ROS release approaching 60% of that observed in the internal positive control (20ng/mL TNF-alpha), which corresponds to a level of TNF-alpha approximately 100-fold higher than levels typically seen in septic shock (40).

Surprisingly, a focused screen of activated coagulation factors and peptides relevant to in-vivo clotting, including fibrinopeptides, failed to demonstrate priming of PMNs for enhanced ROS production (Fig 2). This was somewhat unexpected since: (a) many known PMN chemotactic agents also prime PMNs for increased ROS production, yet no priming was observed for the known PMN chemotactic protein fibrinopeptide B (25); and (b) protease-activated receptors (e.g. PAR-2) on PMNs, which are activated by thrombin cleavage, have been shown to elevate levels of the granule marker CD11b on the PMN surface (15). Instead, the only signaling molecule we identified in our targeted screen of plasma proteases, cofactors and activation peptides that could specifically prime PMNs for ROS release was C5a, which arose, at least in part, through activation of the complement alternative pathway.

Loss of endothelial barrier function is a key component of organ injury after physiologic insults such as trauma and sepsis (reviewed in (41)), and complement has been implicated in organ injury/failure after trauma in several context-dependent manners (12, 42-46). Building on this, we attempted to show that C5a generated during the process of unregulated blood clotting could cause PMN to release soluble mediators that provoke endothelial barrier loss as an ex-vivo surrogate for organ injury. This is highly relevant to major trauma where coagulation is no longer locally regulated but instead systemically active (11), and is also particularly relevant to surgical and traumatic bleeding into body cavities/potential spaces where large collections of blood undergo clotting in the extravascular space (e.g. traumatic hemothorax, hemoperitoneum, etc.) much as they would ex-vivo in a tube. Using a trans-well filter ECIS assay to measure endothelial barrier resistance while preventing physical contact

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between PMN and confluent HUVEC during co-incubation, we found that C5a generated during unregulated clotting of whole blood caused PMN to release extracellular ROS that, in turn, directly caused a loss in endothelial barrier function. These observations linking PMN ROS to endothelial barrier loss were further supported by our finding of a decrease in lung capillary permeability to albumin in vivo in p47phox-null mice subjected to trauma with hemorrhagic shock.

The specific importance of C5a in trauma-shock induced PMN priming was then investigated using platelet-poor plasma from trauma patients who presented in shock (Table 1), where we found that C5a levels were sufficiently elevated in these patients to prime PMN for extracellular ROS generation (Fig. 3) that correlated with both thrombin generation and alternative complement pathway activity (Table 2). Reciprocally, we also observed, using an in-vivo murine model of trauma-shock (22, 23), that the ROS released from PMNs following trauma and hemorrhagic shock directly contributed to lung injury and coagulopathy (Fig. 5). These findings suggest that trauma-shock primes PMN for extracellular ROS release, likely at least in part due to C5a generation, and that this may be an important mechanism involved in development of inflammatory organ injury and possibly also coagulopathy, as illustrated in Fig 5d.

The existence of such a link is supported by several other studies, particularly those from Huber-Lang and colleagues (42, 43, 47). Links between complement pathway activation and C5a generation with modulation of PMN-mediated inflammation and dysfunction of hemostasis had been previously elucidated in a variety of models of sepsis, with early PMN activation and late PMN dysfunction (48-50). The extent to which different pathways contribute to C5a production after trauma, however, remains an area for additional investigation. In a murine study of immune-mediated lung injury, for example, Huber-Lang et al. showed that C5a was generated in both a C3-dependent and C3-independent manner (51). These authors further suggested that the C3-independent component of C5 convertase activity, which was observed primarily in C3 null animals, arose from thrombin cleavage of C5, since the extent of lung damage that they observed was significantly reduced by ATIII or hirudin addition. Further work by Amara et al. has clearly established a potential role for coagulation and fibrinolytic (e.g.

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plasmin) proteases in the activation of complement proteins (47), and alternative complement pathway activation has previously been reported in trauma and burn patients (12, 52). These findings are consistent with ours, where we found that elevated C5a levels in plasma from our trauma-shock patients significantly correlated with both alternative pathway activation (Bb levels) and thrombin generation (PF1+2 levels), and importantly, had the ability to prime naive PMNs for extracellular ROS production.

There are several limitations to our study. First, the majority of our experiments were conducted ex-vivo in idealized systems that do not necessarily directly extrapolate to in-vivo findings, indicating that further in-vivo studies will be needed in the future. Second, while we focused on PMN-derived ROS, a variety of other bioactive mediators are released from primed and activated PMNs that could further contribute to tissue injury and coagulation changes, including proteases and other hydrolases. Third, while our murine model used trauma-shock to study in-vivo importance of PMN ROS for development of inflammatory organ injury and effects on the coagulation system (where the mouse trauma-shock model was specifically chosen because our trauma patients were in shock), we were limited in our sample measurements and were unable to measure C5a levels to further corroborate our C5a findings in the human trauma samples. Finally, we acknowledge that our trauma samples were from a limited number of patients and the results must therefore be interpreted with caution and replicated in larger studies.

In summary, we have shown that trauma-shock and products of coagulation prime PMN for pathologic extracellular ROS release in a C5a-dependent manner that, in trauma patients in shock, correlates with both thrombin generation and alternative pathway activation. Using a mouse model of trauma and hemorrhagic shock to mimic our patients' clinical state, we showed that ROS released by PMN directly contributes to lung injury and aPC-mediated traumatic coagulopathy. These findings, in combination with the work of others, suggest that a critical link exists between complement and PMN ROS in development of organ injury and coagulation changes after trauma. Future studies and computational models are needed to further elucidate the connections between complement, coagulation, and inflammation at the systems biology level.

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Conflicts of Interest

The authors have no conflicts of interest to report for this manuscript.

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Figure 1. PMNs are primed for early extracellular ROS generation by products of coagulation.

(a,b) PMN incubated with vehicle control versus the designated known priming agents were measured for priming/ROS generation with addition of 100nM fMLP by luminol chemiluminescence with horseradish peroxidase (HRP) (total ROS generation) or superoxide dismutase (SOD) + catalase (intracellular ROS generation). Total and intracellular ROS generated over 10 minutes was then quantified by integrating the area under the curve (AUC) from 0-10 minutes (total ROS/luminescence generated from 0-10 minutes is shown in panel a to demonstrate the effect of fMLP alone versus priming agent + fMLP on total ROS generation), and extracellular and intracellular ROS was then expressed as a fraction (%) of total ROS generated (where extracellular ROS was indirectly measured by subtracting the intracellular fraction from the total ROS). PMN incubated in 50% (v/v) (c) Serum, (d) Plasma, (e) Clotted Plasma, and (f) Clotted Platelet-Poor Plasma (Clotted PPP) were measured for priming/ROS generation without or with addition of 100nM fMLP by luminol chemiluminescence with HRP (total ROS generation) or SOD + catalase (intracellular ROS generation). Each condition was normalized to an internal positive PMN priming control (20ng/mL TNF-α + 100nM fMLP) to allow for comparison between multiple different blood products. Total amounts of ROS generated for each condition were quantified by integrating the area under the curve (AUC) from 0-10 min for experiments performed using (g) heparin and (h) EDTA as the initial anticoagulant and compared to their respective plasma group.

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(i) Extracellular versus intracellular ROS at 10 min was then quantified and expressed as a fraction (%) of total ROS generated, where extracellular ROS was indirectly measured by subtracting the intracellular fraction from the total ROS, and the difference between extracellular and intracellular amounts for each blood product were then compared within each group. Results reported as mean ± SEM. Significance set at $p \leq 0.05$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Figure 2. C5a generated by the alternative pathway of complement during coagulation is responsible for priming of PMNs by products of coagulation.

(a) Screen of potential PMN priming agents that could be responsible for the observed PMN priming by products of coagulation. PMN were pre-incubated with the specified agent(s), followed by addition of luminol + HRP and then treatment with 100nM fMLP. Total ROS production over 10 min was measured, with results normalized to an internal positive priming control (20ng/mL TNF-α) and compared with fMLP alone (i.e. no priming agent, "None") for reference. (b-f) PMN priming by products of coagulation (50% (v/v)) measured by total ROS generation in response to fMLP in the presence or absence of C5aR blocking antibody (versus IgG Control) or the C5aR antagonist W-54011 (versus vehicle control). Results normalized to an internal positive priming control (20ng/mL TNF-α). (g-i) ELISA for complement fragments C5a, Bb, and C4d from n = 4 healthy volunteers. Results reported as mean +/- SEM. Significance was set at $p \leq 0.05$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: FP = fibrinopeptides; PF1+2 = prothrombin fragment 1+2; AP = activation peptide.

Figure 3. Trauma patient plasma primes PMN for extracellular ROS production in a C5a-dependent manner via complement alternative pathway activity.

Kinetic curves of total and intracellular ROS generation from a representative experiment where healthy human PMN were pre-treated with the C5aR antagonist W-

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54011 or vehicle control and then incubated in 50% (v/v) (a) heterologous healthy citrate PPP, (b) trauma patient citrate PPP drawn in the ED, or (c) trauma patient citrate PPP drawn 24 hrs after traumatic injury, washed, and then stimulated with fMLP. (d-f) The % inhibition of total, extracellular, and intracellular ROS generation over 10 minutes by the C5aR antagonist W-54011 compared to vehicle controls for healthy and trauma patient citrate PPP. (h-i) ELISA for levels of C5a, Bb, C4d, and PF1+2 in healthy control and trauma patient citrate PPP. Results reported as mean +/- SEM. Significance was set at $p \leq 0.05$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Figure 4. PMN ROS released in response to C5a generated by the complement alternative pathway during coagulation degrades endothelial barrier function.
(a) Kinetic curves and (b) multiple comparisons at discrete time points of Electric Cell-Substrate Impedance Sensing (ECIS) assays. Human PMN were first incubated in 50% (v/v) plasma or serum without or with pre-treatment of PMN with W-54011 or DPI, then washed with cell culture media, and subsequently placed in a trans-well filter over confluent human umbilical vein endothelial cell (HUVEC) monolayers grown on ECIS plates to measure endothelial barrier function. Results reported as mean +/- SEM. Significance was set at $p \leq 0.05$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Figure 5. p47phox KO mice with PMN unable to generate ROS via the NADPH oxidase complex are protected from inflammatory lung injury and coagulopathy in a trauma-hemorrhage (T/H) model.

C57Bl/6 WT and p47phox KO mice underwent laparotomy and hemorrhagic shock via a controlled femoral arterial bleed to a MAP of 35 +/-5mmHg for 60 minutes, followed by euthanasia, IVC blood draw, and organ harvest. Sham mice underwent all the same procedures including femoral artery cannulation, however no shock was induced during the 60 minutes prior to euthanasia. Mice were then evaluated for organ injury via (a) ^{125}I-labeled albumin lung permeability assay, and coagulopathy via ELISA for (b)

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activated Protein C levels and (c) thrombin-antithrombin complex levels as a surrogate for total thrombin generation. Results reported as mean +/- SEM. Significance was set at $p \leq 0.05$. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. (Modified from original work by Iris Fung, and reproduced from (50)).

Supplemental Figure 1. Media, treatment, and blood product controls for ECIS PMN-HUVEC trans-well experiments.

Vehicle (DMSO), W-54011, and DPI effects on HUVEC monolayer barrier function compared to EBM2 alone, as measured by both capacitance (a,b) and resistance (c,d). Effects of plasma or serum when added to EBM2 media (no PMNs) (e).

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Table 1. Trauma patient demographics.

Variable	N (%), or median (IQR)
Total Patients	N=10
Male	6 (60%)
Female	4 (40%)
Age	54 (37-67)
ISS	23 (21-26)
Mortality at 30 Days	2 (20%)
Penetrating Trauma	1 (10%)
Blunt Trauma	9 (90%)
HR on Scene	118 (89-143)
HR in ED	111 (77-147)
SBP on Scene (mmHg)	83 (64-97)
SBP in ED (mmHg)	87 (76-105)
Shock Index (HR/SBP) on Scene	1.4 (1.1-1.6)
Shock Index (HR/SBP) in ED	1.1 (0.9-2.0)
INR in ED	1.2 (1.0-1.4)
Peak INR w/in 24hrs	1.3 (1.1-1.5)
Platelet Count in ED	211 (157-255)
RBC units transfused w/in 24hrs	5.5 (0.75-8.75)
Plasma units transfused w/in 24hrs	0.5 (0-4.5)
Platelet units transfused w/in 24hrs	0.5 (0-2)

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Table 2. Correlation of trauma patient complement levels, thrombin generation, and inhibition of PMN extracellular ROS production by C5aR antagonism.

	|ΔExtracellular ROS|		C5a level		C4d level		Bb level		PF1+2 level	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
|ΔExtracellular ROS|			0.309	0.027	0.033	0.815	0.464	<0.001	0.371	0.006
C5a level	0.309	0.027			0.198	0.254	0.626	<0.001	0.350	0.039
C4d level	0.033	0.815	0.198	0.254			0.185	0.278	0.063	0.714
Bb level	0.464	<0.001	0.626	<0.001	0.185	0.278			0.271	0.109
PF1+2 level	0.371	0.006	0.350	0.039	0.063	0.714	0.271	0.109		

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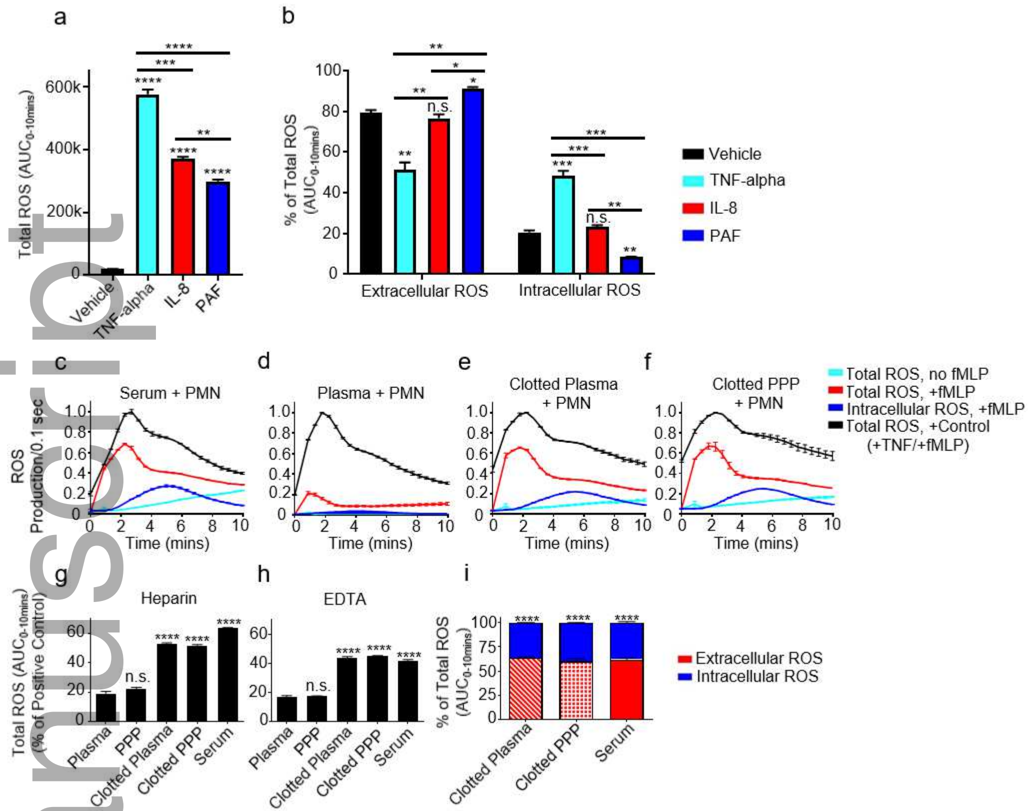
Figure 1



cei_13166_f1.tif

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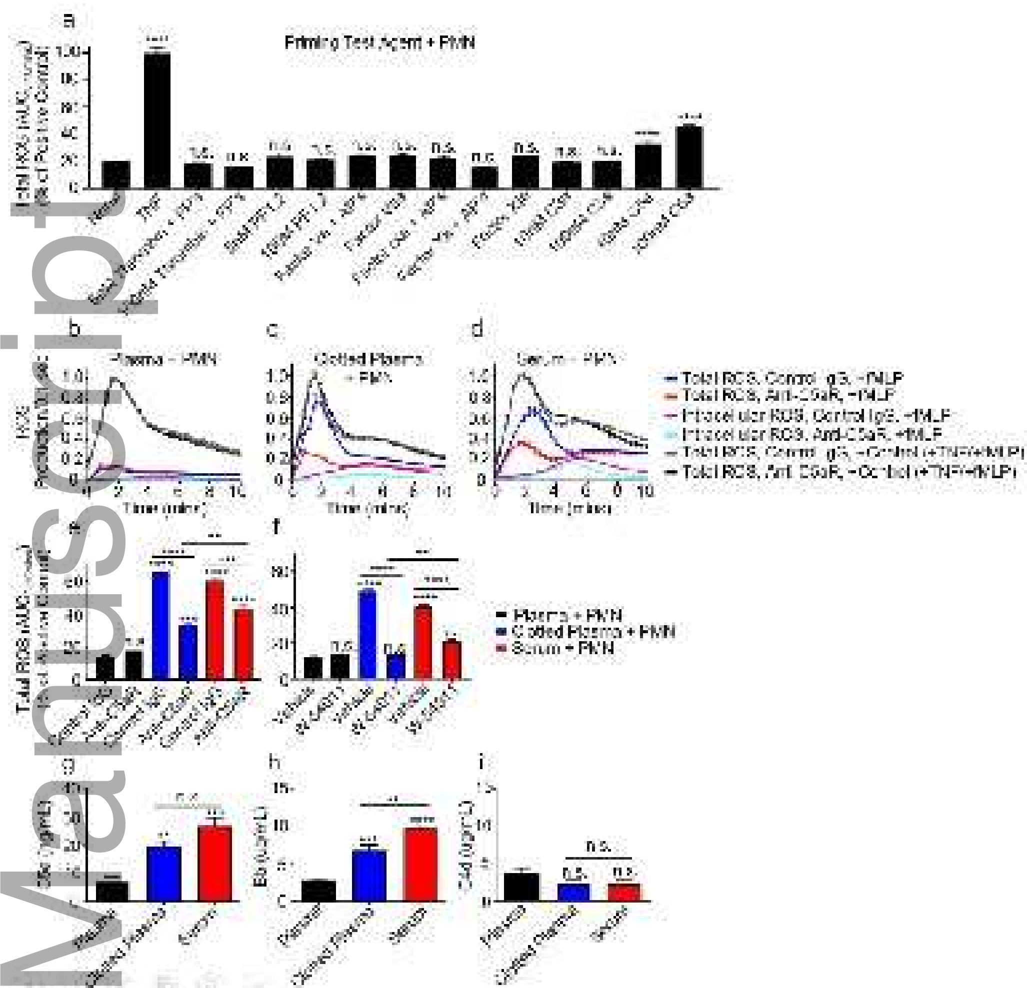
Figure 2



cei_13166_f2.tif

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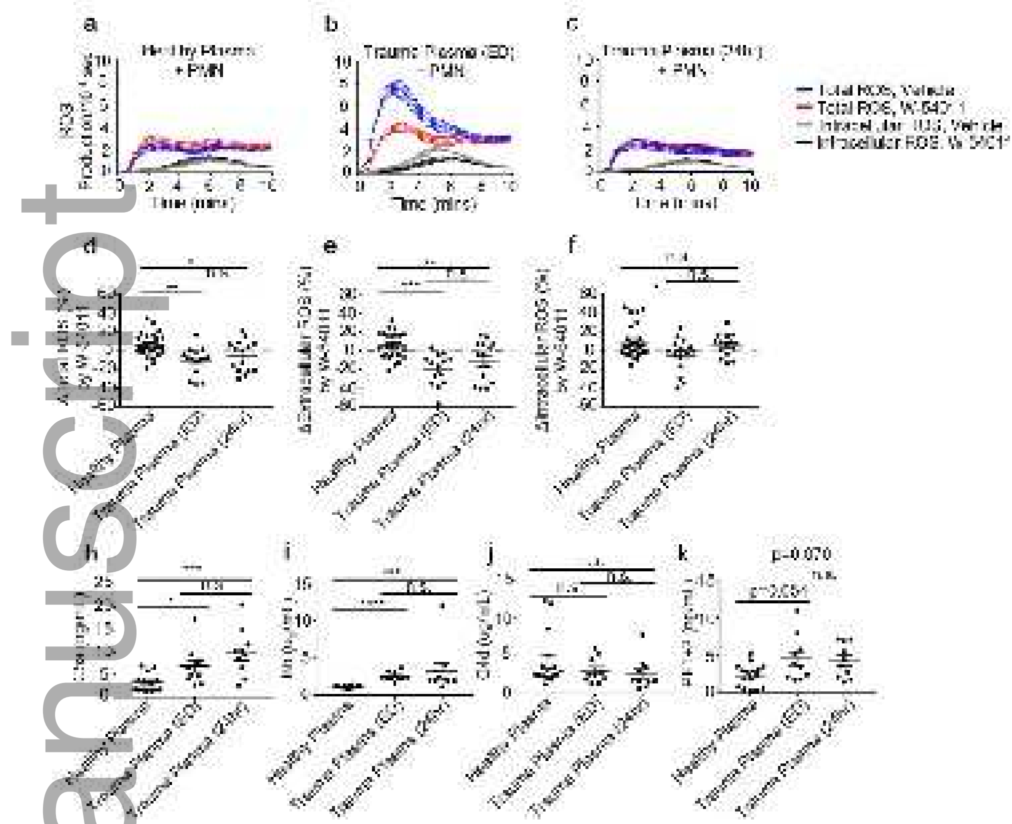
Figure 3



cei_13166_f3.tif

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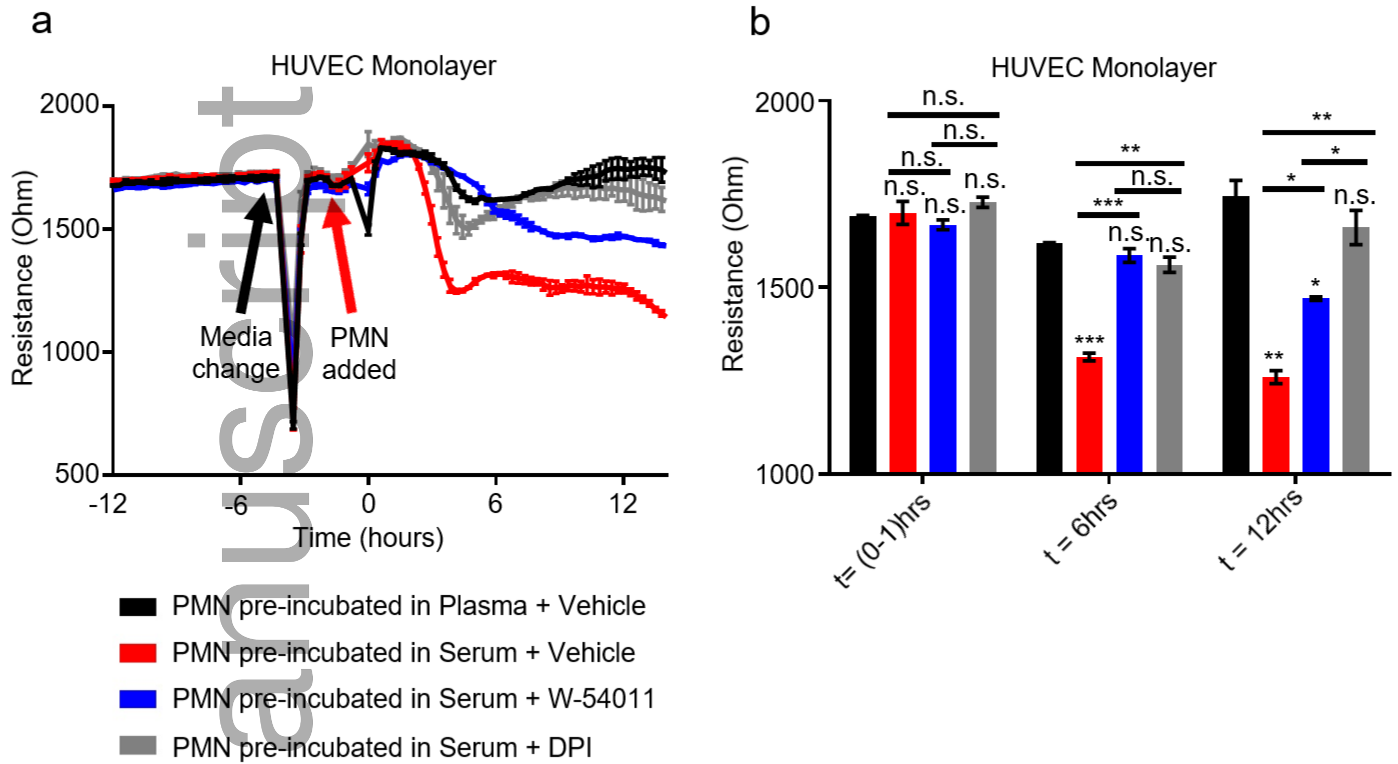
Figure 4



cei_13166_f4.tif

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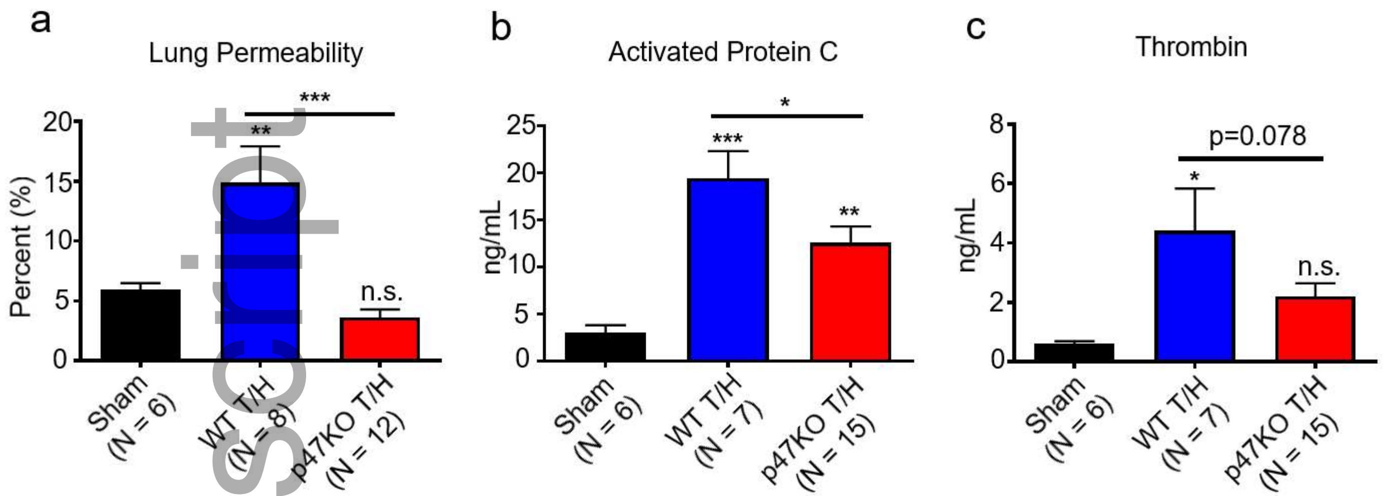
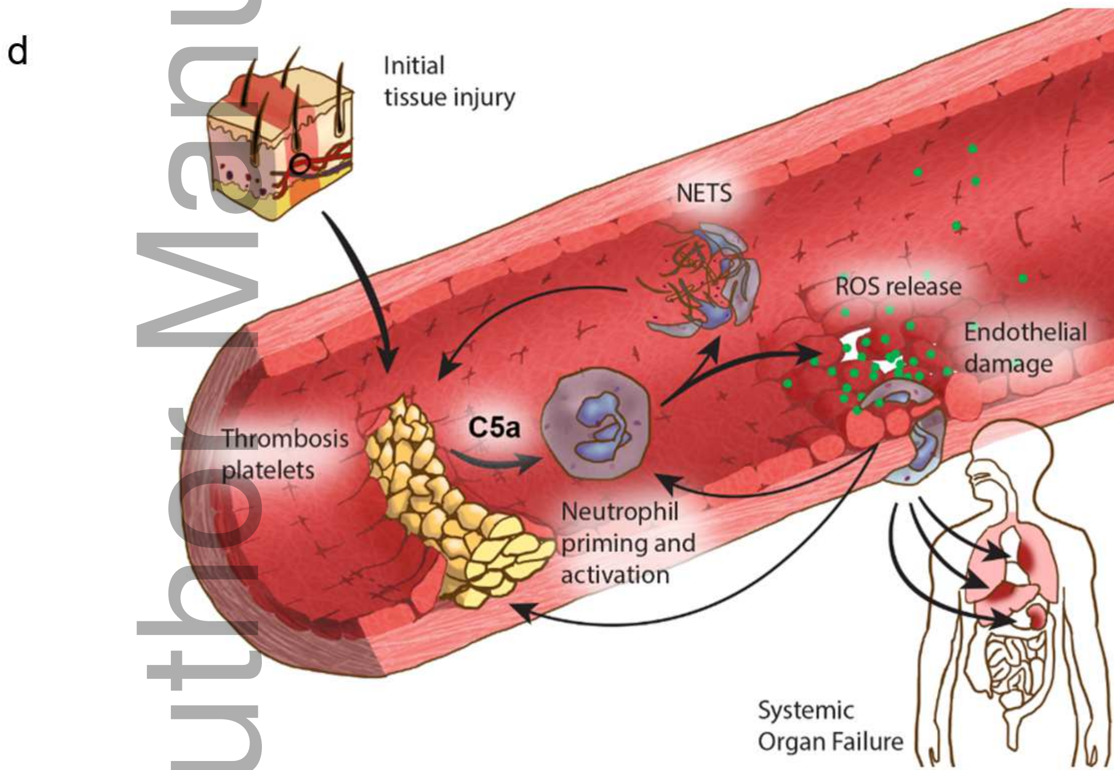
Figure 5





cei_13166_f5.tif

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