Research Paper

A Broad-Spectrum Infection Diagnostic that Detects Pathogen-Associated Molecular Patterns (PAMPs) in Whole Blood

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ABSTRACT

Background: Blood cultures, and molecular diagnostic tests that directly detect pathogen DNA in blood, fail to detect bloodstream infections in most infected patients. Thus, there is a need for a rapid test that can diagnose the presence of infection to triage patients, guide therapy, and decrease the incidence of sepsis.
Methods: An Enzyme-Linked Lectin-Sorbent Assay (ELLecSA) that uses magnetic microbeads coated with an engineered version of the human opsonin, Mannose Binding Lectin, containing the Fc immunoglobulin domain linked to its carbohydrate recognition domain (FcMBL) was developed to quantify pathogen-associated molecular patterns (PAMPs) in whole blood. This assay was tested in rats and pigs to explore whether it can detect infections and monitor disease progression, and in prospectively enrolled, emergency room patients with suspected sepsis. These results were also compared with data obtained from non-infected patients with or without traumatic injuries.
Results: The FcMBL ELLecSA was able to detect PAMPS present on, or released by, 85% of clinical isolates representing 47 of 55 different pathogen species, including the most common causes of sepsis. The PAMP assay rapidly (<1 h) detected the presence of active infection in animals, even when blood cultures were negative and bactericidal antibiotics were administered. In patients with suspected sepsis, the FcMBL ELLecSA detected infection in 55 of 67 patients with high sensitivity (>81%), specificity (>89%), and diagnostic accuracy of 0·87. It also distinguished infection from trauma-related inflammation in the same patient cohorts with a higher specificity than the clinical sepsis biomarker, C-reactive Protein.
Conclusion: The FcMBL ELLecSA-based PAMP assay offers a rapid, simple, sensitive and specific method for diagnosing infections, even when blood cultures are negative and antibiotic therapy has been initiated. It may help to triage patients with suspected systemic infections, and serve as a companion diagnostic to guide administration of emerging dialysis-like sepsis therapies.

1. Introduction

Infections that can trigger a systemic inflammatory response syndrome (SIRS) leading to sepsis due to the presence of pathogens or released toxins (Bone, 1992) are most commonly diagnosed using blood cultures in combination with characteristic physiological alterations (e.g., increased heart and respiratory rates, temperature, white blood cells counts) (Angus and van der Poll, 2013; Yealy et al., 2014; Singer et al., 2016). But only 15 to 30% of sepsis patients with documented infection produce positive blood cultures, (Tsalik et al., 2012; Gille-Johnson et al., 2013; Loonen et al., 2014; Knabl et al., 2016; Bacconi et al., 2014) and even then it can often take days before definitive results are obtained. With the absence of direct biomarkers for sepsis, blood proteins involved in the host inflammatory response, such as C-reactive protein (CRP) and procalcitonin (PCT), have been used to detect infection and predict outcome in septic patients (Pierrakos and Vincent, 2010; Chan and Gu, 2011). However, while rising levels of the CRP and PCT correlate with an increased likelihood of infection and sepsis severity, these tests produce a wide range of sensitivities

and specificities (Pierrakos and Vincent, 2010; Carr, 2015). Moreover, none of these inflammatory biomarkers unequivocally distinguishes infection-induced SIRS from sterile SIRS caused by burn injury (Seoane et al., 2014), trauma (Wojtaszek et al., 2014) or surgery (Battistelli et al., 2014). Other groups (e.g. Spectral Diagnostics) have developed assays that measure pathogen-derived biomarkers (e.g., endotoxin), but they have not gained widespread use (Marshall et al., 2004) possibly because they only detect Gram negative bacteria. More rapid molecular diagnostic assays (e.g., based on PCR and mass spectrometry) can identify specific types of pathogens, but MALDI mass spectrometry requires a culture step (Idelevich et al., 2014) to expand the number of live pathogens to above 10^5 colony forming units (CFUs), which again limits its use to the minority of patients who are culture positive (Tsalik et al., 2012; Gille-Johnson et al., 2013). PCR-based methods, such as SeptiFast (Roche, Diagnostics GmbH), hybcell® Pathogens (Anagnostics Bioanalysis GmbH), and IRIDICA (Abbott Molecular) have been developed that can detect specific types of whole pathogens directly in blood within 4–6 h. However, because they require the presence of the pathogen DNA in blood, they fail to detect suspected bloodstream infections in 60–90% of patients (Knabl et al., 2016; Bacconi et al., 2014). The T2Candida Panel (T2 Biosytems) has demonstrated improved high specificity and sensitivity for fungal infections, and it can identify invasive candidiasis in blood culture negative patients (Mylonakis et al., 2015). However, none of these tests can be used to detect a broad spectrum of pathogens (i.e., Gram negative and positive bacteria, fungi, viruses, and parasites) as well as the toxins they release (e.g., endotoxin, lipoteichoic acid) that are largely responsible for triggering the cytokine cascade that leads to SIRS and sepsis.

To measure the presence of a wide range of pathogens directly from blood samples we leveraged a recently developed engineered version of the natural human blood opsonin, Mannose Binding Lectin, as a broad-spectrum agent that can capture a wide range of pathogens and released toxins. This engineered protein contains the carbohydrate recognition domain of Mannose Binding Lectin linked to the Fc portion of human IgG1, and hence, it is known as FcMBL (Kang et al., 2014). FcMBL binds many Pathogen-Associated Molecular Patterns (PAMPs), (Parlato and Cavaillon, 2015) which are carbohydrate cell wall materials including lipopolysaccharide endotoxin (LPS), lipoteichoic acid and outer membrane vesicles, that are present on, or released by, various types of live and dead pathogens, including more than 90 different Gram negative and positive bacteria, fungi, viruses, and parasites (Kang et al., 2014; Schultz et al., 2008; Dommett et al., 2006). PAMP release can occur spontaneously (e.g., with Escherichia coli), and it increases significantly when pathogens are killed by antibiotics (Didar et al., 2015) and likely by immune cells as well. We previously showed that FcMBL can be used in dialysis-like devices to efficiently remove living Staphylococcus aureus and Escherichia coli bacteria as well as dead pathogens and cell wall-derived LPS from blood in rats based on its carbohydrate binding activity (Kang et al., 2014; Didar et al., 2015). Given its broad spectrum binding capabilities, we set out here to develop a simple and rapid infection diagnostic based on the use of FcMBL to detect the presence of PAMPs in whole blood of infected animals and human patients that might be used in the future to triage patients, accelerate antibiotic administration, and serve as a companion diagnostic to identify patients with high levels of PAMPs for emerging dialysis-like sepsis therapies (Basu et al., 2014; Tullis et al., 2010; Mitaka and Tomita, 2011; McCrea et al., 2014).

2. Methods

2.1. Study design

2.1.1. Detection of blood-borne infections in animals

To determine whether the FcMBL ELLecSA can be used to monitor disease progression in vivo, we used two models of E. coli infection — intravenous bolus injection in Juvenile Yorkshire swine with analysis of serial blood samples and intraperitoneal injection in rats that received antibiotic therapy. Study approvals (pig protocol number 14-03-2519; rat protocol number 13-12-2547R) were obtained from the Institutional Animal Care and Use Committee (IACUC) of Boston Children's Hospital, Harvard Medical School, the Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Material Command (USAMRMC) Office, and Department of Defense (DOD). All studies were conducted in accordance with the guidelines outlined in *The Guide for the Care and Use of Laboratory Animals*, in an AAALAC-accredited, USDA registered facility.

In the pig studies, animals were sedated and maintained on inhaled anesthetics during surgical procedures to place intravenous lines, and they were given appropriate analgesics to last for the entire study duration prior to recovery. For the infection study, E. coli 41949 pathogen obtained from a human clinical isolate was infused via the jugular vein into female Yorkshire Swine, 30–50 kg ($n = 2$ for each time point). Animals were randomly assigned and no blinding was performed. Animals received an initial dose of bacteria ($0·5 \times 10^7$ CFU/kg) at the start of the experiment ($T = 0$ h) followed by a 6 h infusion of $5·0 \times 10^7$ CFU/kg ($T = 3$ to 9 h). Blood samples were drawn at intervals throughout the study for blood culture in a BD™ Bactec™ Media System (Standard/10 Aerobic/F vials), as well as for complete blood count (CBC, Abaxis) and FcMBL ELLecSA. When the study was terminated at 35 h, organ samples (liver, lungs, spleen) were harvested using sterile technique and processed for organ culture. For the vehicle control, swine ($n = 2$) received saline with no E. coli and blood samples were drawn at the indicated time points over a 24 h period.

To determine the efficacy of the ELLecSA in the presence of antibiotic treatments, rats (Wistar male ~ 370 g; Charles River Laboratories, USA) were injected intraperitoneally with 2×10^9 CFU of E. coli (ATCC No. 8739). Rats were randomly assigned to antibiotic treatment ($n = 6$) or saline control ($n = 6$) with no blinding performed. Infection was allowed to proceed for 4 h before treatment with cefepime at 60 mg/kg or saline control. Animals were sacrificed 8 h after infection and a terminal blood draw was performed and analyzed.

2.1.2. Sepsis diagnosis in humans: study design and patient enrollment

To assess the potential value of the FcMBL ELLecSA as a diagnostic assay of infection and sepsis, we analyzed blood samples collected from patients who were clinically suspected to have sepsis in the Beth Israel Deaconess Medical Center (BIDMC) Emergency Department. All patients provided informed consent and approval was granted by the BIDMC Committee on clinical investigations. PAMP levels in the blood of patients were assessed using the FcMBL ELLecSA and results were compared with those from analysis of two control patient cohorts — non-infected healthy blood donors and non-infected hospital patients presenting with traumatic injuries. The patient enrollment is summarized in Fig. 1.

To compare the sensitivity of our diagnostic with that of standard blood cultures, minimum patient enrollment was determined using the following assumptions: assuming 60% of patients are discordant on the two tests (positive on one and negative on the other), 40 patients provide 80% power to observe a difference between the tests of 35% (i.e., rate of positive on first, negative on second of 47.5% and rate of negative on first and positive on second of 12.5%) or larger with a two-sided, 0.05 level McNemar's test (of correlated proportions on the same patients).

We recruited the sepsis population from the Beth Israel Deaconess Medical Center Emergency Department. Study inclusion criteria for the sepsis population were: 1) adult patients (age 18 years or older) who exhibited sepsis, as defined by the presence of an infection (determined by the clinical treating team) and the presence of at least two SIRS criteria using a modified definition of the American College of Chest Physicians/Society of Critical Care Medicine.(Bone, 1992; Singer et al., 2016) The SIRS criteria include: temperature > 100.4 °F or <96.8 °F, heart rate > 90 beats/min, respiratory rate > 20 breaths/min or partial pressure of carbon dioxide <32 mm Hg, and white blood cell
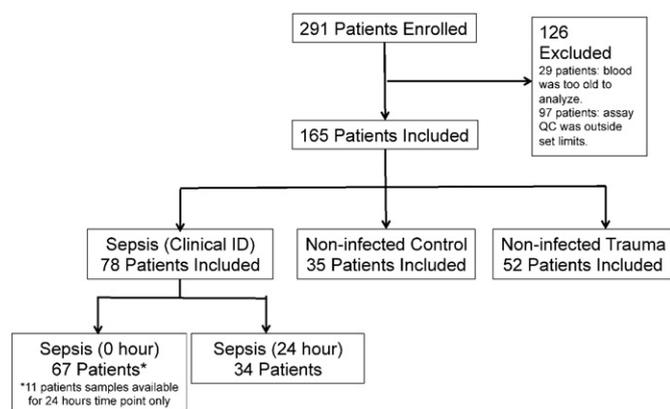
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┌─────────────────────┐      ┌──────────────────┐
│ 291 Patients Enrolled│─────▶│ 126              │
└─────────────────────┘      │ Excluded         │
           │                 │ 29 patients: blood│
           │                 │ was too old to   │
           ▼                 │ analyze.*        │
┌─────────────────────┐      │ 97 patients: assay│
│ 165 Patients Included│      │ QC was outside   │
└─────────────────────┘      │ set limits.      │
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┌──────────────────┐  ┌──────────────────┐  ┌──────────────────┐
│ Sepsis (Clinical ID)│ Non-infected Control│ Non-infected Trauma│
│ 78 Patients Included│ 35 Patients Included│ 52 Patients Included│
└──────────────────┘  └──────────────────┘  └──────────────────┘

┌──────────────────┐  ┌──────────────────┐
│ Sepsis (0 hour)  │  │ Sepsis (24 hour) │
│ 67 Patients*     │  │ 34 Patients      │
│ *11 patients samples available│         │
│ for 24 hours time point only │         │
└──────────────────┘  └──────────────────┘
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Fig. 1. Patient enrollment. Out of the total of 291 patients enrolled, 126 were excluded due to blood samples being stored for longer than 24 h or results of assays not meeting internal validation of 95% confidence interval of slope and background determined from the curve fit analysis of the internal mannan standard (see Supplemental Fig. 1). Of the remaining 165 patients, 78 sepsis patients (67 who entered the study at time 0, and 34 at 24 h [11 patients enrolled at 24 h]), 35 non-infected control patients without evidence of infection or trauma ($n = 23$ hospital controls and $n = 12$ healthy volunteers), and 52 patients with trauma but no clinical suspicion of infection were analyzed.

count > 12.000/µl or <4000/µl or >10% bands. An additional non-infected control population was similarly recruited from the emergency department where patients had to meet the following criteria: 1) adult (>18 years old) 2) absence of infection, and 3) absence of meeting two or more SIRS criteria. We also recruited trauma patients who were adult emergency department patients with a traumatic injury (long bone fracture or hollow viscous injury) without documented infection. Approval for the use of additional non-infected human control blood samples was obtained from Harvard University Faculty of Medicine Committee on Human Studies (protocol number M20403-101). This fourth group was blood donors who were healthy volunteers who had provided informed consent, were nonsmokers, and had not taken aspirin for the 2 weeks before donation.

3. Procedures

3.1. Microbiology

Mannan (Catalogue no. M3640) and LPS from *E. coli* O111:B4 (Cat. no. L2630) were acquired from Sigma Aldrich (Sigma Aldrich, USA). Additional chemicals were purchased from Sigma Aldrich (MN, USA). Clinical bacteria isolates were acquired from Brigham and Women's Hospital Crimson Biorepository, Beth Israel Deaconess Medical Center, and Boston Children's Hospital, Boston, USA; BEI Resources, and ATCC Bethesda, USA; and Hospital Joseph-Ducuing, Toulouse, France. All strains were reviewed, and research was approved by Harvard's Committee on Microbiological Safety (COMS). Bacteria were cultured in RPMI media (ThermoFisher, USA) supplemented with 10 mM Glucose (Sigma Aldrich, USA) to a 0.5 McFarland (McF) standard (Becton Dickinson, USA). RPMI 10 mM glucose was chosen because MBL binds the yeast extract present in traditional bacterial culture media (data not shown).

For screening, bacteria were fragmented by either bead mill treatment at 30 Hz for 10 min using 0.1 mm zirconia/silica beads (BioSpec Products, USA) in a Mixer Mill MM 400 machine (Verder Scientific, Inc., USA) or by treatment with antibiotic (100 µg/ml cefepime, 250 µg/ml amikacin, or 50 µg/ml meropenem) for a minimum of 4 h at 37 °C. FcMBL ELLecSA detection was performed on bead-milled or antibiotic-treated bacteria titered in TBST 5 mM Ca^{2+} (Boston Bioproducts, USA).

In animal infection models, *E. coli* bacteria were diluted to desired concentration in saline/dextrose, and the viability/quantity of the bacteria confirmed by plating, BacLight (Thermo Fisher, USA) live dead stain,

and Bactiter™ (Promega, MI, USA). Endotoxin content of injectable bacteria was determined by Endoscan™ (Charles River Labs, USA).

3.2. Enzyme-Linked Lectin-Sorbent Assay (ELLecSA)

The FcMBL-based ELLecSA leverages the engineered FcMBL previously described (Kang et al., 2014). A diagrammatical representation of the FcMBL-ELLecSA detection of PAMPs from whole blood is outlined in Fig. 2a. In brief, 200 µl of sample is added to 800 µl assay solution containing 5 µg FcMBL-couple MyOne Dynabeads (Invitrogen, USA) at 5 mg/ml with 10 mM glucose, 50 µl heparin sulfate and TBST 5 mM Ca^{2+}. Samples are incubated for 20 min at 25 °C and the FcMBL-beads with bound PAMPs are collected using a KingFisher Flex™ (Thermo Fisher Scientific, Waltham, MA. USA) automated magnetic collection device. Captured PAMPs are washed three times in TBST Ca^{2+} and detected with rhMBL-horse radish peroxidase (HRP) kindly provided by K. Takahashi. The unbound rhMBL-HRP is removed by 4 washes with TBST 5 mM Ca^{2+} and PAMP detection is visualized by incubation in tetramethylbenzidine (1-step™ Ultra TMB-ELISA, Cat no. 34028; Thermo Fisher, Waltham MA, USA). TMB development is terminated with 1 M sulphuric acid, and the optical density is determined at 450 nm wavelength. PAMP levels (PAMP units) are presented as FcMBL binding units, which are determined by comparing optical density (450 nm) measurements of FcMBL bound materials and comparing them to results obtained by carrying out an internal mannan standard curve (Fig. 2b); 1 PAMP unit was determined as being equivalent to 1 ng/ml mannan binding.

3.3. Analysis of CRP as a comparator biomarker

CRP levels were measured in plasma using the Human SimpleStep ELISA (Abcam, UK). Sensitivity and specificity were determined using the manufacturer's criteria for elevated blood CRP levels (>5 µg/ml); each sample was run in duplicate.

3.4. Statistical analysis

All measured values are reported as the average of at least two samples ± standard deviation (SD) or means ± standard error of the mean (SEM) as indicated in the figure legends. Significant differences between groups with equivalent n values were determined using an unpaired *t*-test, with $p < 0.05$ being considered to be significant. Significant differences between groups of unpaired sample were calculated by Wilcoxon Rank Sum analysis and defined as p values < 0.05. Significant differences between groups of paired samples were calculated by Wilcoxon Signed Rank Test analysis with $p < 0.05$. Sensitivity and specificity of each assay were determined by Receiver Operator Characteristics (ROC); area under the curve and standard error of the mean with $p < 0.05$. The ROC area under the curve was used to report diagnostic accuracy. Intra-assay coefficients of variation in blood were determined for low (negative < 0.45 PAMP units) samples ($n = 21$) and high (positive > 2.25 PAMP units) samples ($n = 25$). Inter-assay coefficients of variation of low (negative = 0 PAMP units) and high (positive = 4 PAMP units) signals were determined from standard curve data ($n = 20$).

3.5. Funding

4. Results

4.1. FcMBL ELLecSA

To directly quantify cell wall-derived PAMPs from both live and dead pathogens in whole blood, we developed a modified version of a sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) in which the PAMP-binding lectin FcMBL(Kang et al., 2014) is used instead of an antibody immunosorbent. In this Enzyme-Linked Lectin-Sorbent Assay (ELLecSA), superparamagnetic microbeads (1 μm diameter) coated with FcMBL are used to capture and magnetically collect PAMPs from a small volume (200 μl) of whole human blood; the captured PAMPs are then detected within 1 h after sample collection by adding horseradish peroxidase (HRP)-labeled recombinant human MBL (rhMBL) (Fig. 2a) using an automated, high throughput (96 well), Kingfisher analytical platform (Thermo Fisher Scientific, Waltham, MA. USA). Bound PAMPs were quantified by comparing results to an internal standard curve generated using the purified PAMP, yeast mannan, which is a prototypic carbohydrate ligand for MBL (Super et al., 1989). The FcMBL ELLecSA revealed a 1 ng/ml sensitivity for purified mannan in buffer that was used to generate a standard PAMP binding curve (Fig. 2b).

The limit of detection of the FcMBL ELLecSA for LPS spiked into human whole blood was determined to be 0.3 PAMP units, which corresponds to 15 ng/ml LPS (Fig. 2c). Thus, the FcMBL ELLecSA can rapidly detect PAMPs in blood using small volumes in a high throughput format.

4.2. Broad spectrum detection of bacterial PAMPs

Native MBL has been reported to bind to PAMPs present on the surfaces of a wide variety of microbes,(Dommett et al., 2006) yet various laboratories claim different binding affinities to even common bacteria, such as *Escherichia coli* (Jack and Turner, 2003). To assess the FcMBL ELLecSA's ability to directly detect membrane PAMPs released into patient blood from a broad range of pathogenic bacteria, we first screened multiple pathogens, both when living and after they were destroyed using either bead milling or antibiotics that are often administered to patients as soon as a systemic infection is suspected based on clinical presentation. The FcMBL ELLecSA detected over 85% (47 out of 55) of the living pathogen species tested in the 1 h assay. Past studies suggested that intact MBL showed variable binding to certain Gram negative pathogens,(Jack et al., 1998; Jack and Turner, 2003) and similarly, we found that only 61% (98 out of 163) of intact bacterial isolates
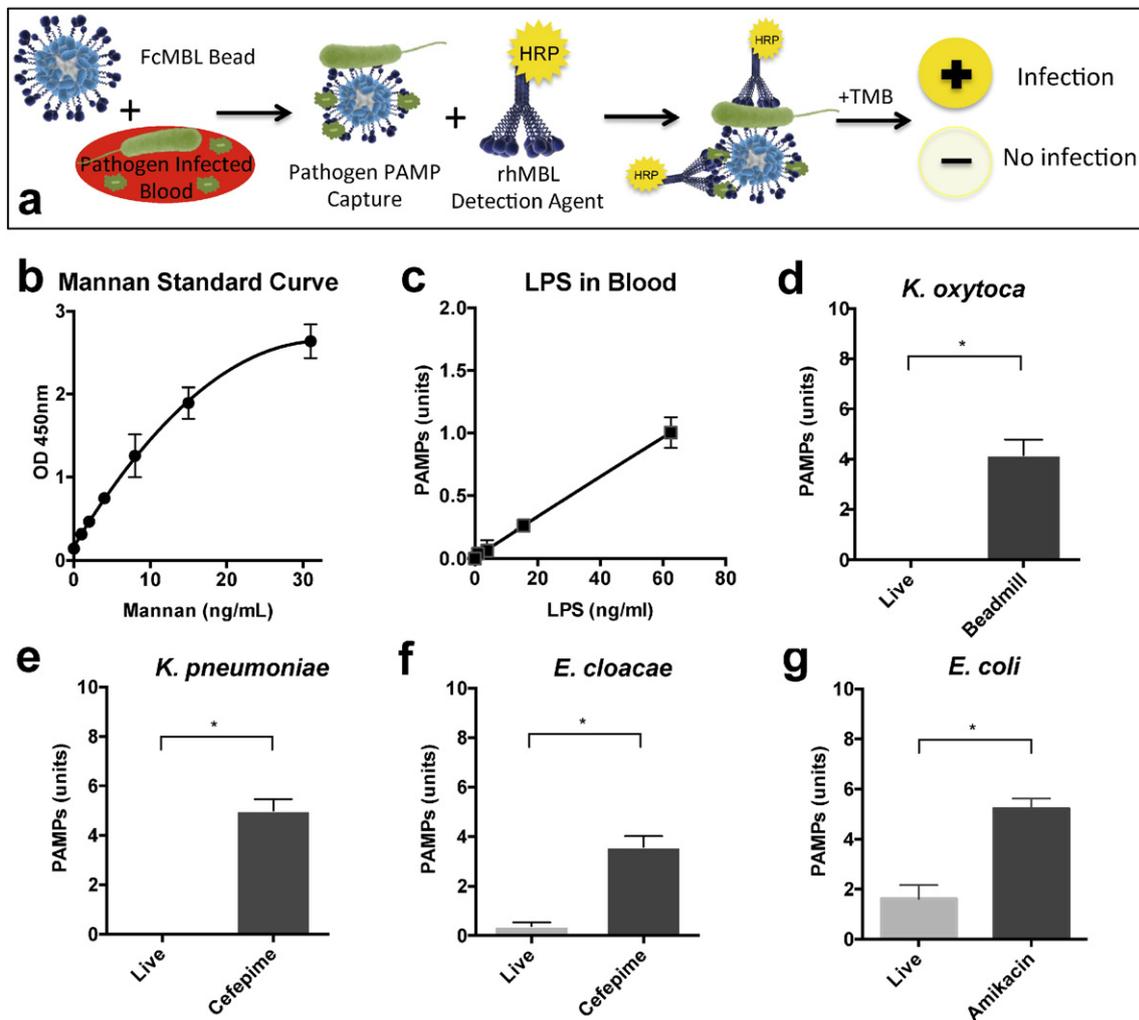

Fig. 2. Development of the FcMBL-based infection diagnostic. (a) Schematic representation of the FcMBL ELLecSA methodology in which PAMPs or whole pathogens contained in blood are captured using FcMBL-coated magnetic microbeads (FcMBL-beads) in combination with applied magnetic fields, and detection of bound material by application of recombinant human MBL linked to horse radish peroxidase (rhMBL-HRP), followed by addition of the chromogenic substrate, tetramethylbenzidine (TMB), which generates a positive signal in infected blood samples. (b) Mannan standard curve showing FcMBL binding to mannan measured as optical density at 450 nm, which was used to determine PAMP units (1 PAMP unit is equivalent to FcMBL binding to 1 ng/ ml mannan). Graph showing detection of the carbohydrate PAMP LPS (c) when diluted in whole blood using the FcMBL ELLecSA. Graphs showing enhanced detection of *Klebsiella oxytoca* (d), *Klebsiella pneumonia* (e), *Enterobacter cloacae* (f), and *Escherichia coli* (g) bacteria using the FcMBL ELLecSA in combination with (dark grey) or without (light grey) mechanical disruption (30 Hz for 10 min in a beadmill) or treatment with the antibiotic cefepime (100 μg/ml) (f) or amikacin (250 μg/ml) for 4 h (g) (* *p* < 0.05; Unpaired *t*-test); data are presented as PAMP units. Bacteria were cultured in RPMI/10% Glucose from a single colony and harvested at log phase (to 0.5 McF; 10^8 CFU/ml).

Table 1

Binding of clinical pathogen isolates by FcMBL. Testing of FcMBL binding was carried out using either intact living bacteria (1 × 10⁷ CFU; live) or fungi (1 × 10⁶ CFU) or a similar number of bacteria that were treated with either a bacteriocidal antibiotic (cefepime 100 μg/ml or meropenem 50 μg/ml for 4 h; ABX) or mechanical disruption using a beadmill (30 Hz, 10 min) to release PAMP-containing fragments. The genus, species, total number of clinical isolates screened for each bacterial species (# of isolates), and the number of live isolates (live detected) and fragmented isolates (fragmented detected) identified by FcMBL ELLecSA are shown. The inset shows the total number of *Escherichia coli* isolates that were detected with the FcMBL ELLecSA, and the number and percent of these isolates that were detected as living organisms (live) or fragmented. NT indicates not tested.

Genus	Species	# of isolates	Live detected	Fragmented detected
Acinetobacter	*baumannii*	3	1	2
Aeromonas	*sobria*	1	1	1
Bacillus	*subtilis*	1	1	1
Bacteriodes	*fragilis*	1	1	1
Burkholderia	*cepacia*	1	1	1
Clostridium	*difficile*	1	1	1
Clostridium	*perfringens*	1	1	1
Citrobacter	*freundii*	1	1	NT
Enterobacter	*aerogenes*	4	2	4
Enterobacter	*cloacae*	8	5	8
Enterococcus	*faecalis*	5	0	0
Enterococcus	*faecium*	1	0	0
Enterococcus	*gallinarum*	1	0	0
Escherichia	*coli*	26	10	24
Klebsiella	*oxytoca*	7	4	7
Klebsiella	*pneumoniae*	9	6	9
Listeria	*monocytogenes*	1	1	1
Micrococcus	*sp.*	1	1	1
Morganella	*morganii*	1	1	1
Mycobacterium	*leprae*	2	NT	2
Mycobacterium	*tuberculosis*	2	NT	2
Neisseria	*meningitidis*	1	1	1
Proteus	*acnes*	1	1	1
Proteus	*mirabilis*	4	0	0
Proteus	*vulgaris*	1	0	0
Providencia	*rettgeri*	1	0	0
Pseudomonas	*aeruginosa*	1	1	1
Salmonella	*enteriditis*	2	1	2
Salmonella	*paratyphi A*	2	1	2
Salmonella	*typhimurium*	6	5	6
Serratia	*marcescens*	5	3	3
Shigella	*flexneri*	1	1	1
Staphylococcus	*aureus*	17	15	16
Staphylococcus	*aureus (MRSA)*	2	2	2
Staphylococcus	*epidermidis*	4	2	2
Staphylococcus	*hominis*	2	0	1
Staphylococcus	*lugdunensis*	2	1	NT
Staphylococcus	*simulans*	1	1	NT
Stenotrophomonas	*maltophilia*	1	0	0
Streptococcus	*agalactiae*	2	0	2
Streptococcus	*Group A*	1	1	1
Streptococcus	*mitis*	1	0	0
Streptococcus	*pneumoniae*	5	2	2
Streptococcus	*pyogenes*	1	1	1
Streptococcus	*viridans*	2	1	2
Yersinia	*pseudotuberculosis*	6	6	6

E. coli isolates	Number detected	Percent detected
Total	26	
Live	10	38
ABX	24	92

Genus	Species	# of isolates	Detected
Aspergillus	*fumigatas*	1	1
Candida	*albicans*	2	2
Candida	*glabrata*	1	1
Candida	*guilliermondii*	1	1
Candida	*krusei*	1	1
Candida	*tropicalis*	1	1
Candida	*parapsilosis*	1	1
Fusarium	*sp.*	1	1
Saccharomyces	*cerevisiae*	1	1

produced a positive signature (Table 1). However, when we mechanically disrupted the pathogens, or treated them with a bacteriocidal beta-lactam antibiotic (cefepime) that disrupts the cell wall and thereby exposes or releases previously hidden carbohydrate PAMPs,(Hurley, 1992) we were then able to measure membrane PAMPs released by *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Enterobacter cloacae* that displayed only weak FcMBL binding when intact (Fig. 2d–f). The levels of FcMBL-bound PAMP materials released by similarly treated clinical isolates of *Escherichia coli* also enabled us to increase detection of the presence of the pathogen from 38% to 92% (Table 1). Similar responses were obtained with another class of antibiotic that acts by inhibiting protein synthesis (the aminoglycoside, amikacin; Fig. 2g), showing that the FcMBL ELLecSA's can detect an increase in exposed or released wall-associated PAMPs even when a bacteriocidal antibiotic does not act by directly targeting the bacterial cell wall. Importantly, the FcMBL

ELLecSA was able to detect PAMPs released from 85% of all clinical isolates tested encompassing 14 of 16 bacterial species that have been found to be most commonly associated with sepsis based on positive blood cultures at the Brigham and Women's Hospital in Boston and Johns Hopkins School of Medicine in Baltimore (Supplemental Table 1).

4.3. Detection of blood-borne infection in vivo using the FcMBL ELLecSA

To determine whether the FcMBL ELLecSA can be used to monitor clinical disease progression, we infected pigs with an intravenous bolus of a clinical isolate of *Escherichia coli*. Serial blood samples were drawn, and results from the FcMBL ELLecSA were compared with those of traditional blood culture, post-mortem organ cultures, and total white blood cell (WBC) count. A seen in human sepsis, the pig blood cultures were initially positive, but rapidly became negative as
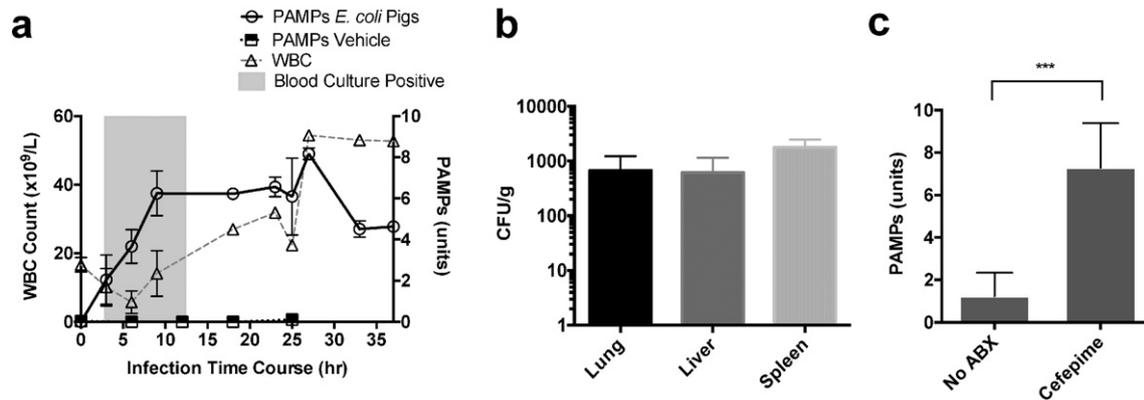
Fig. 3. Detection of infection in animals by measuring PAMPs in blood using the FcMBL ELLecSA. a) Graph showing PAMP levels measured with the FcMBL ELLecSA (open circles) versus white blood cell (WBC) counts (open triangles) and positive blood culture results (grey shaded area) and in samples obtained from pigs injected with *Escherichia coli* (0.5×10^7 CFU/kg) and then infused with E. coli (5×10^7 CFU/kg) over 6 h or vehicle (no *E. coli*) at the start of the 36 h experiment. b) Pathogen loads measured in cultures of lung, liver, and spleen after the organs were removed at the end of the study and bead-milled to release harbored bacteria (4 different regions of each organ were processed, and pathogen loads are normalized per gram of organ tissue). c) Rats were infected intraperitoneally with *Escherichia coli* (2×10^9 CFU) and then treated with the antibiotic cefepime (60 mg/kg) starting 4 h after pathogen injection. The FcMBL ELLecSA was used to measure released PAMPs at the conclusion of the experiment (8 h) and compared to control rats that were infected but received no antibiotic (no ABX) (*** $p < 0.0001$; unpaired *t*-test).

the bacteria were cleared from the blood (Fig. 3a), although the animals remained infected as viable pathogens were detectable in the organs at the end of the 36 h experiment (Fig. 3b). In contrast, the released membrane PAMPs detected by the FcMBL ELLecSA steadily increased during infusion, and remained elevated during the course of the infection mirroring the increase in WBC count (Fig. 3a). No increase in PAMPs was observed in control animals. As the blood cultures are negative after 12 h, the PAMPs detected with the FcMBL ELLecSA after this time in this model must result from cell wall fragments (e.g., blebs, LPS) that are released by pathogens growing within solid organs. Thus, in this large animal model, the PAMP levels detected in blood by the FcMBL ELLecSA provided a determination of infection at distant sites, even when live pathogens were not present within blood.

We also explored whether we could detect PAMPs in infected animals receiving antibiotic therapy using a rat infection model (Didar et al., 2015). This model and dose were chosen because infected rats are highly resistant to *Escherichia coli*, and thus, only exhibit low levels of bacteria and PAMPs in blood when analyzed over a short time course (<8 h) (Didar et al., 2015). When rats were injected with *Escherichia coli* intraperitoneally and treated with either cefepime (100 mg/kg) or saline 4 h later, we detected more than a 6-fold increase in release of bacterial PAMPs into the blood from the localized nidus of infection within the peritoneum in antibiotic-treated rats using the FcMBL ELLecSA ($p < 0.0001$) (Fig. 3c), even though blood cultures from these infected animals were negative. Thus, these findings demonstrate that the FcMBL ELLecSA can be used with standard of care antibiotic treatment, whereas this is not possible using conventional blood culture assays or molecular diagnostic assays that require the presence of live organisms.

4.4. Detection of infection in human subjects

To assess the potential value of the FcMBL ELLecSA as an infection diagnostic for patients at risk for developing sepsis, we analyzed blood samples collected from prospectively enrolled patients at the BIDMC Emergency Department, who were suspected to have an infection based on their initial clinical presentation. Their blood PAMP values detected by the FcMBL ELLecSA were compared with results obtained from two other patient cohorts — non-infected patients with non-infectious etiologies of illness and non-infected patients presenting with traumatic injuries. 291 patients were enrolled in the study using medical criteria outlined in the Methods section (Fig. 1). We imposed additional internal assay validation limits as inclusion criteria: The ELLecSA had to be run within 24 h of the blood draw (to ensure the sample was fresh), and assays were excluded if the internal standard mannan curve failed to meet

the 95% confidence interval for the slope and background (Supplemental Fig. 1). FcMBL ELLecSA data from 164 patients were included for analysis: 78 patients who presented with suspected infection, 35 non-infected controls, and 52 non-infected trauma patients (Table 2). Patient blood was assayed by culture and ELLecSA at study entry (0 h) and 24 h later. The analysis revealed that the FcMBL ELLecSA detected significantly higher PAMPs in the clinically identified infection patient cohort than the non-septic control cohort at both time points (Wilcoxon Rank Sum test; $p < 0.001$ at 0 and 24 h) (Fig. 4a). When paired samples of the same patients were analyzed at 0 and 24 h, there was much broader range of values in the septic patients, however, the difference between the mean values measured at the different time points were found not to be significant (Wilcoxon Signed Rank test; $p < 0.13$) (Fig. 4b).

By carrying out a receiver operating characteristic (ROC) comparison of each sepsis blood draw against non-infected controls, we determined that the FcMBL ELLecSA threshold of >0.45 PAMP units was the optimal threshold to distinguish infection from non-specific signals (Fig. 4c). Based on this threshold, the accuracies of the FcMBL ELLecSA diagnostic were determined to be 0.87 and 0.89 at 0 and 24 h, respectively (Fig 4d) with an inter-assay coefficient of variation of 12.9% (low 11.4%; high 14.4%) and an intra-assay coefficient of variation of 8.3% (low 9.7%; high 6.9%). At the initial blood draw, the ELLecSA sensitivity was 81% and specificity was 89% (55/67 patients), and these increased to 85% and 89% one day later (29/34 patients) when compared to blood samples from non-infected controls (Fig. 4d). In contrast, when we compared these results to blood cultures as a method of direct detection of bacteria, and for which molecular diagnostics have similar detection rates (Bacconi et al., 2014; Knabl et al., 2016), the blood cultures only

Table 2
Baseline characteristics of patients.

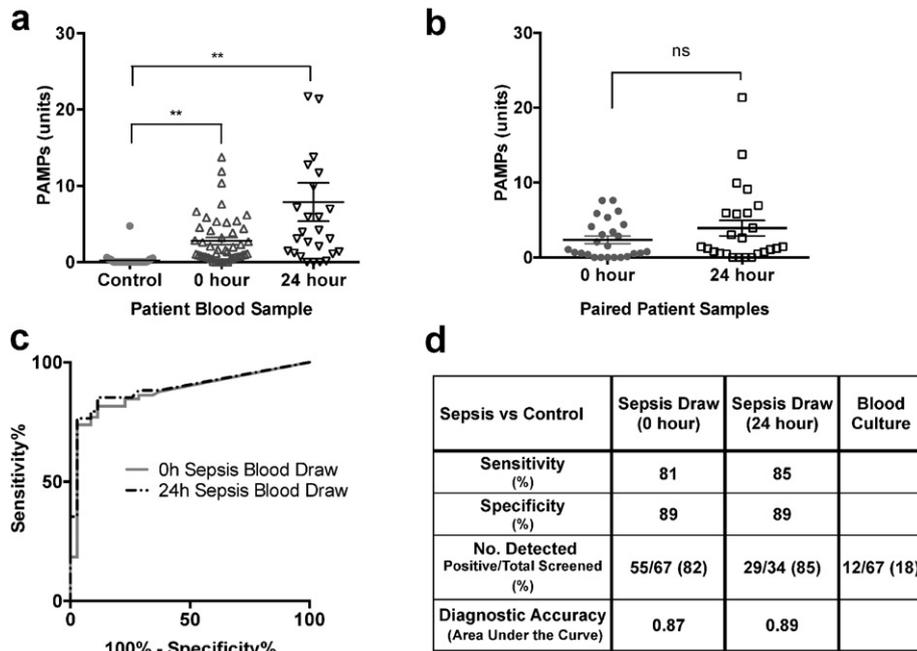| Baseline patient characteristics			
	Sepsis	Non-infected control	Non-infected trauma
Number of patients	78	35	52
Age	61.5 (STD15.7)	35.7 (STD19.2)	61.8 (20.2)
Female gender (%)	44%	62%	67%
Severe sepsis	78%	NA	NA
Septic shock	29%	NA	NA
Blood culture positive	12	0	2
Sofa score (range)	3.1 (STD2.8)	NA	NA
Mortality (%)	6.8%	0%	1.9%

Fig. 4. The FcMBL ELLecSA enables detection of patients with sepsis compared to healthy controls based on higher PAMP levels. (a) PAMP levels were significantly increased in blood samples from patients with suspected sepsis at both study entry (0 h) and 1 day later (24 h) compared with non-infected control donor samples (**, $p < 0.001$; Wilcoxon Rank Sum test). (b) Change in PAMP scores for paired sequential blood samples at 0 and 24 h for sepsis patients (ns, no significant difference; Wilcoxon Signed Rank Test). (c) Receiver operating characteristic (ROC) comparison of the FcMBL ELLecSA data for sepsis patients versus non-infected controls. Areas under the ROC curve for sepsis patient bloods at 0 and 24 h were = $0.870 + 0.041$ (mean + SEM; $p < 0.0001$) and $0.889 + 0.050$ ($p < 0.0001$), respectively. (d) Performance characteristics of the FcMBL ELLecSA for detection of infection in patients suspected of sepsis versus healthy non-infected controls using a PAMP threshold of >0.45 units, as determined by ROC analysis.

detected 18% of infections (12/67) in the sepsis patient cohort (Fig. 4d). The FcMBL ELLecSA detected infection in 11/12 of the blood culture positive samples and 7/8 patients with positive bacterial identification cultured from other (non-blood) biological samples (Supplemental Table 2) and the remaining 47 patients were culture negative. These results demonstrate the FcMBL ELLecSA can detect infection in patients whether they are blood culture positive or negative.

4.5. Performance of the FcMBL ELLecSA compared to the CRP assay

Current blood protein biomarker assays used for detection of sepsis (e.g. CRP, PCT) are indirect in that they measure increases in the inflammatory response to infection, rather than the pathogens or toxins themselves (Chan and Gu, 2011). To determine if the FcMBL-based PAMP assay offers greater specificity, we compared our ELLecSA with the
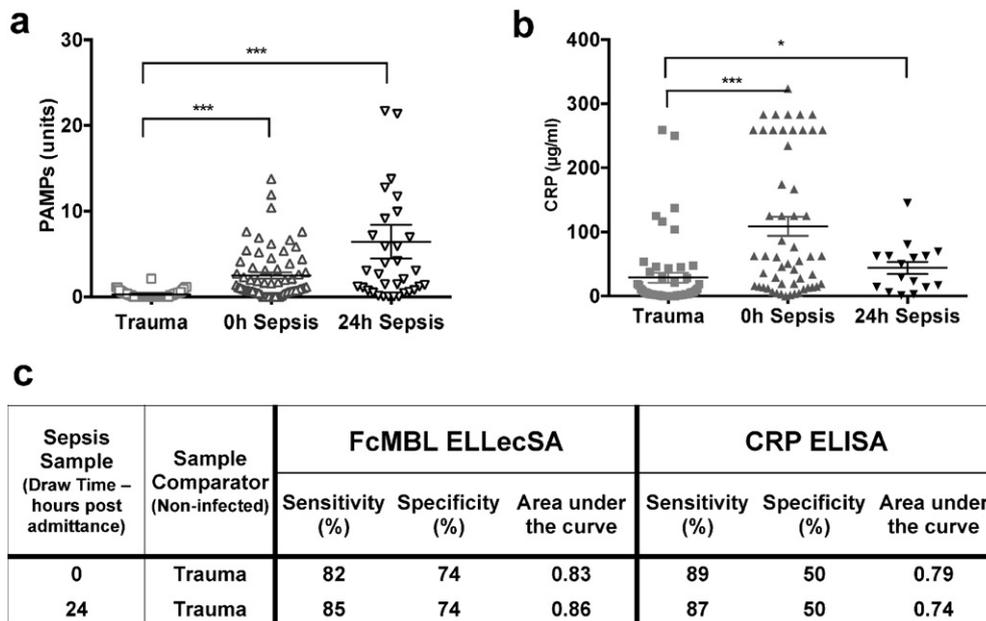

Fig. 5. The FcMBL ELLecSA enables detection of patients with sepsis compared to non-infected trauma patients. (a) PAMP levels were extremely low in trauma patients, and significantly increased in blood samples from patients with suspected sepsis at both study entry (0 h) and 1 day later (24 h) compared with non-infected trauma patient samples (***, $p < 0.0001$; Wilcoxon Rank Sum test). (b) CRP levels measured in the same sepsis samples using a CRP ELISA also were increased in blood samples from patients with suspected sepsis at both study entry (0 h) and 1 day later (24 h) compared with non-infected trauma patient samples (***, $p < 0.0001$; *, $p < 0.003$; Wilcoxon Rank Sum test). (c) Comparison of the performance characteristics of the FcMBL ELLecSA versus the CRP ELISA revealed that while both assays have similar high sensitivity, the ELLecSA has a much higher specificity. CRP performance characteristics were determined by ROC analysis of CRP ELISA data using a 5 mg/ml cutoff value (Fig. 6).
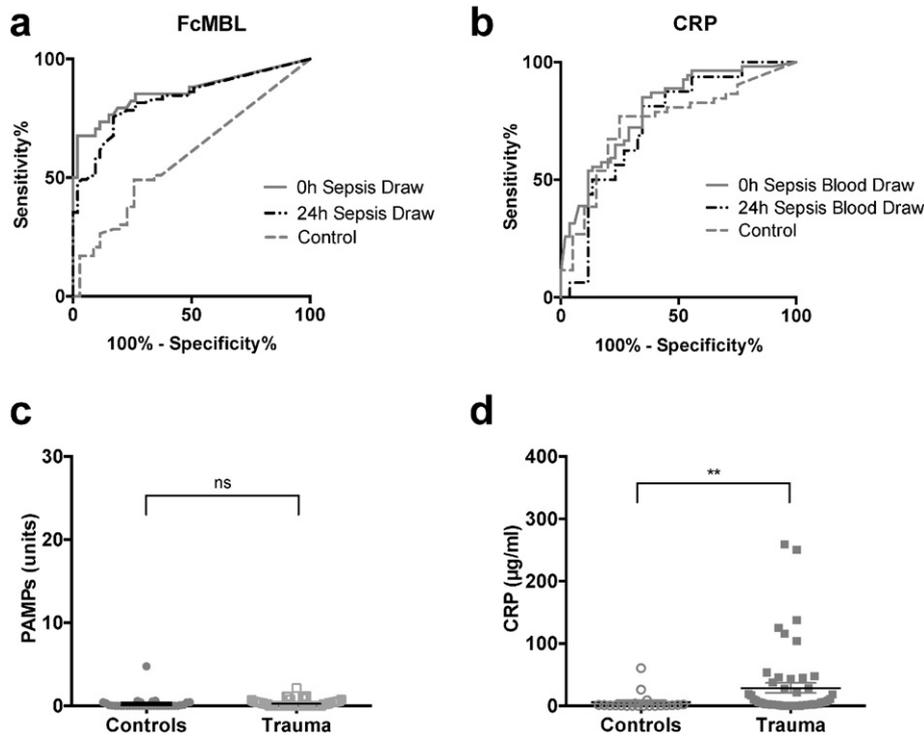
Fig. 6. Receiver operating characteristic (ROC) curves used to compare the ability of the FcMBL ELLecSA and CRP assays to differentiate patients with systemic infection or sepsis from those with sterile trauma. Plasma extracted from the same patient samples analyzed by the FcMBL ELLecSA were then diluted and analyzed using the CRP ELISA. (a) FcMBL ELLecSA. Areas under the ROC curves at 0 and 24 h for the septic patient samples compared to those with trauma were $0.831 + 0.038$ ($p < 0.0001$) and $0.867 + 0.045$ ($p < 0.0001$); area under the ROC curve for the non-infected control versus trauma was $0.589 + 0.061$ ($p < 0.156$). (b): CRP ELISA. Areas under the ROC curves at 0 and 24 h for the septic patient samples compared to those with trauma were $0.793 + 0.045$ ($p < 0.0001$) and $0.740 + 0.063$ ($p < 0.003$); area under the ROC curve for the non-infected control versus trauma was $0.741 + 0.064$ ($p < 0.0016$). (c) FcMBL ELLecSA PAMP levels were not significantly increased in blood samples from patients with non-infected trauma compared with non-infected control (Wilcoxon Rank Sum test). (d) CRP levels were significantly increased in blood samples from patients with non-infected trauma compared with non-infected control ($p < 0.0013$; Wilcoxon Rank Sum test).

inflammatory sepsis biomarker CRP by screening additional blood samples from 52 non-infected trauma patients. The PAMP levels in blood samples from this non-infected patient cohort measured using the FcMBL ELLecSA were significantly lower than those in patients with clinically documented infection ($p < 0.0001$), with 25% (13/52) false positives using the same threshold described above (>0.45 PAMP units) (Figs. 5a; 6c), while the specificity of the FcMBL ELLecSA for patients with infection compared to those with non-infected trauma was 74% (Figs. 5c; 6a). The observed drop in sensitivity from 81% (versus control) down to 74% (versus trauma) for detecting infection may result from undocumented infection detected by FcMBL, as two of the non-infected trauma patients later became blood culture positive (Table 2). In contrast, while CRP levels also were higher in blood from infected patients ($p < 0.0001$ and <0.003 at 0 and 24 h, respectively) (Fig. 5b; 6d), use of the CRP assay resulted in a false positive rate that was almost 2 times higher (49%; 25/52) resulting in a low specificity of only 50% in non-infected trauma patients (Figs. 5c; 6b). Taken together, these results confirm that compared to the conventional CRP assay currently used clinically, the FcMBL ELLecSA provides significantly greater specificity (74% versus 50%) to distinguish patients with infection from those with trauma.

5. Discussion

The need for rapid and accurate diagnosis of infection is great as delayed administration of antimicrobial therapy increases mortality by the hour as the infection rapidly evolves from local infection to sepsis, and eventually, to severe sepsis and septic shock (Martin et al., 2003; Kumar et al., 2006; Ferrer et al., 2014; Liu et al., 2014). A positive blood culture remains the current gold standard for laboratory diagnosis of systemic infection; unfortunately, most (>70%) septic patients

have negative blood cultures (Tsalik et al., 2012; Gille-Johnson et al., 2013) and the sepsis and infection biomarkers currently in clinical use that measure the patient's inflammatory status, such as CRP, are of limited value in patient populations with inflammation induced by non-infectious illnesses or trauma. Thus, to address the critical need for an infection-specific biomarker that can assist physicians to decide on whether hospitalization is critical, antibiotics should be administered, or if recently developed dialysis-like sepsis therapies (Basu et al., 2014; Tullis et al., 2010; Mitaka and Tomita, 2011; McCrea et al., 2014) are appropriate, we leveraged the wide pathogen- and toxin-binding capabilities of the engineered FcMBL opsonin (Kang et al., 2014) to develop a broad-spectrum rapid diagnostic that directly detects the presence of pathogen-released PAMPs in whole blood. The FcMBL ELLecSA detects the cell wall materials of a broad range of living and dead pathogens, including negative and positive bacteria, fungi, viruses and parasites. Importantly, in our studies with human subjects, the FcMBL-based ELLecSA proved to be a sensitive and specific systemic biomarker for infections, detecting PAMPs in >80% of blood samples from emergency department patients with infection-related disease aetiologies, even though <20% of these patients had positive blood cultures. The FcMBL ELLecSA's ability to detect PAMPs directly also can be leveraged to distinguish patients with infection from those with non-infection related inflammatory conditions. Our ability to use this PAMP assay to discriminate between patients with infection and those with trauma is in line with the new Sepsis 3 guidelines in which SIRS criteria have been questioned because of issues related to determining the source of infection (Singer et al., 2016). The PAMP infection diagnostic also can be implemented in settings outside of central hospitals where qSOFA/SOFA scores are more challenging to determine, and it potentially can enable clinicians to identify patients with infection even when they have low qSOFA or SOFA scores in the hospital setting (Singer et

al., 2016). Thus, the FcMBL-based PAMP assay may serve as a new rapid diagnostic for infection in patients who are suspected to have a blood-borne infection or early-stage sepsis, and thereby help to reduce unnecessary use of antibiotics.

One of the key findings of this study is that the FcMBL ELLecSA detected PAMPs released from 47 of 55 (92%) clinically relevant pathogen species screened, including 14 of 16 of the most frequent bacterial species associated with sepsis at two major academic clinical medical centers (Brigham and Women's Hospital in Boston and Johns Hopkins School of Medicine in Baltimore); the only exception was *Enterococci*, which was responsible for less than 5% of these infections. The broad spectrum of PAMP detection by the FcMBL ELLecSA was reflected in our ability to rapidly (<1 h) detect >80% of infections in human patients in our study whether or not they were receiving antibiotic therapy. This is in contrast to blood cultures that were positive in only 18% of these same patients, which is similar to levels of detection reported in past clinical studies (Tsalik et al., 2012; Gille-Johnson et al., 2013; Loonen et al., 2014; Knabl et al., 2016; Bacconi et al., 2014). Indeed, the FcMBL ELLecSA was not only able to detect infection, but to track infection in near real time in our pig infection model regardless of blood culture results. Therefore, the FcMBL ELLecSA-based PAMP detection method potentially offers a promising new way to assist physicians as a rapid infection-specific diagnostic. In fact, based on animal studies (Didar et al., 2015), it may even be possible to determine when bacteriocidal antibiotics are effective in vivo by detecting an abrupt increase in PAMPs.

Two other infection biomarkers, the Limulus Amebocyte Lysate (LAL) and Endotoxin Activity Assay (EAA), also detect one particular type of PAMP (LPS) directly; however, they are not widely used because they can only diagnose Gram negative infections (Marshall et al., 2004). Indeed, the LAL Assay performed poorly as a diagnostic tool for patients with suspected bacteremia (Hurley et al., 2015), likely due to assay enhancement or interference by components in complex biological samples, including blood (Bates et al., 1998). The EAA sepsis diagnostic is also limited in its use because it must be run immediately following the blood draw (Marshall et al., 2004). It can detect elevated signals in septic patients with Gram negative infections compared to non-septic patients (Mignon et al., 2014), but it is not often used clinically because it lacks specificity (Marshall et al., 2004) and has limited effectiveness in neutropenic patients (Romaschin et al., 2012). In addition, the EAA diagnostic has been shown to detect elevated levels of LPS in trauma patients resulting from gut leakage and antibiotic therapy (Charbonney et al., 2016). Importantly, the levels of endogenous LPS released from the gut that were reported in that study are below the detection limit of our FcMBL ELLecSA (negative in the trauma cohort screened) and thus, it should not complicate our assay. Further clinical testing of specific patient populations (e.g. post-surgery, and burn) will be required to further optimize the assay threshold, and possibly identify specific threshold subsets for each patient population. In addition, the FcMBL ELLecSA diagnostic described here can detect a wide variety membrane-associated PAMPs (not just LPS) that are released into blood by Gram positive bacteria (e.g., lipotechoic acid), fungi, viruses and parasites, as well as by Gram negative bacteria. The FcMBL ELLecSA is also much more resistant to interference by components of biological fluids than either the LAL and EAA assays, and so it should be more valuable for clinical use.

Other biomarkers that measure the immune response to infection, such as CRP and PCT, are increasingly being implemented for rapid detection of sepsis (Chan and Gu, 2011; Carr, 2015; Albrich and Harbarth, 2015; Prucha et al., 2015). CRP and PCT values have been shown to be elevated in infections, with increasing levels correlating with likelihood of infection and sepsis severity (Tsalik et al., 2012; Loonen et al., 2014; Carr, 2015). However, it is difficult to use these assays to distinguish between patients with sepsis versus sterile SIRS in subpopulations of patients who have experienced surgery (Battistelli et al., 2014; Stoppekamp et al., 2015; Uzzan et al., 2006; Kaukonen et al., 2015), burn injury (Seoane et al., 2014), trauma(Wojtaszek et al., 2014) or stroke (Li and Liu, 2015). In contrast, the unique clinical utility

of the FcMBL-enabled PAMP assay presented here is that in addition to being able to rapidly and specifically detect the presence of infection in whole blood samples, the PAMP assay can reliably distinguish patients with infection from those with trauma-associated inflammation, thereby giving physicians clinically valuable, actionable information. The FcMBL ELLecSA could be used to direct therapy and determine the need for further testing using molecular diagnostics in patient populations where risk of infection is elevated, but detection using these existing inflammation-related biomarkers is ineffective or non-specific. More accurate detection of the specific presence of infection using this PAMP assay can further reduce the inappropriate use of antibiotics as well. It also would help guide physicians as to when to initiate or stop treatment with dialysis-like sepsis therapies that are specifically designed to cleanse patient blood of pathogens and PAMPs, which either have been recently approved for clinical use or are currently in development (Didar et al., 2015; Basu et al., 2014; Mitaka and Tomita, 2011). Future studies will help elucidate the sensitivity and specificity of the FcMBL ELLecSA for these diverse applications and patient populations.

In the present study, the sensitivity of detection of the clinical isolates in vitro by the FcMBL ELLecSA was enhanced upon release of PAMPs by either mechanical disruption or treatment with bacteriocidal antibiotics, and this correlated with the detection of PAMPs in blood samples in both our studies with animals and human patients when blood cultures were negative but infections remained present at distant sites. These observations demonstrate that PAMPs released as a result of immune cell killing reactions or antibiotic therapy can be detected within blood samples using the FcMBL ELLecSA assay. We could detect PAMPs in blood samples of animals that were injected intravenously with live pathogen, in animals that were injected *locally* in the peritoneum with pathogens, as well as in blood culture-negative animals that were later shown to have local infection sites within internal organs (e.g., liver, spleen and lung). More importantly, we showed that we could detect infections in blood culture-negative humans who demonstrated clinical symptoms of sepsis. Thus, the FcMBL-based PAMP blood assay clearly can be used to detect the presence of infections where living pathogens are localized to specific organ sites as well as blood-borne infections. While the assay cannot currently discriminate between these types of infection, this could be explored in future studies (e.g., based on PAMP levels or analysis of their molecular composition). Also, although we did not detect a significant increase in PAMP signal in paired infection samples of patients between time zero (when patients first received broad spectrum antibiotics) and 24 h post-admittance, the overall mean PAMP level appeared to increase between these time points, but a more targeted trial is needed to determine significance. This raises the possibility that the FcMBL ELLecSA could be used to track the efficacy of antibiotic treatments in infected patients in vivo, either alone or in combination with other biomarkers, such as PCT, which are currently being explored as a means to monitor antibiotic therapy for sepsis in the ICU (Carr, 2015; Schuetz et al., 2013; Jensen et al., 2011).

Molecular diagnostics that purport to detect and identify specific pathogens directly in blood are currently in development or entering the clinic. For example, the molecular diagnostic T2Candida Panel (T2 Biosytems) has demonstrated improved specificity, sensitivity and speed of detection (~4 h) for identifying invasive candidiasis in blood culture negative patients (Mylonakis et al., 2015). But due to the low frequency of fungal infections in septic patients, the assay faces challenges for adaptation into the clinic as a general screening test. While the multiplexed PCR methods, SeptiFast (Roche, Diagnostics GmbH), and hybcell® Pathogens (Anagnostics Bioanalysis GmbH), and the PCR-ESI-MS method, IRIDICA (Abbott Molecular), can detect and identify bacteria and fungi in blood within 4–6 h with >90% sensitivity and specificity in patients identified as positive by either blood culture or PCR (Knabl et al., 2016; Bacconi et al., 2014), the sensitivity of these assays drops dramatically to 10–40% for all patients with suspected bloodstream infections (Knabl et al., 2016; Bacconi et al., 2014)

(Supplemental Fig. 2). In contrast, the FcMBL ELLecSA along with immunoassays such as PCT, offer an even more rapid (<1 h) and generic test for identification of infection by a broad range of different types of pathogens. The FcMBL ELLecSA does not identify the specific type of pathogen; however, one can envision combining molecular diagnostic tools, such as mass spectrometry, to distinguish specific pathogen types based on the molecular composition of the captured PAMPs. This assay is also unique in that quantification of PAMP levels over time also might be used to track antibiotic efficacy in vivo without the need for pathogen identification (Carr, 2015; Schuetz et al., 2013; Jensen et al., 2011), and thus, rapidly assist physicians in their selection of an optimal therapy.

In summary, the FcMBL-based ELLecSA offers a rapid, sensitive and specific method for diagnosing a broad range of infections in patients by directly quantifying the levels of PAMPs that initiate and fuel the inflammatory cascade responsible for sepsis, whether present on living pathogens or released as debris or toxins. Based on the promising sensitivity and specificity the FcMBL ELLecSA for recognizing sepsis patients in this pilot study, it is possible that it might be used to identify patients who are not responding to initial antibiotic therapies, and to help reduce inappropriate usage of antibiotics in patient subpopulations (e.g. immune compromised) who are at high risk for infection, but for whom current inflammatory biomarkers are not useful due to their lack of specificity. The FcMBL ELLecSA can be automated in a high-throughput format amenable to pathology laboratories, as well as be adapted for single use point-of-care settings. Most importantly, this simple test for diagnosing systemic infections has the potential to provide clinically actionable information to inform physicians when patients should be hospitalized, antibiotic therapies administered, or dialysis-like therapies initiated, even when blood cultures are negative. It also could potentially be used to detect when bacteriocidal therapies are effective in vivo (i.e., by detecting an abrupt increase in PAMPs immediately following initiation of antibiotic therapy).

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Potential conflicts of interest

D.E.I. and M.S. hold equity in Opsonix Inc., and are members of its scientific advisory board. M.C. is a consultant for Opsonix Inc. D.E.I. also holds equity in Emulate Inc., and chairs its scientific advisory board. D.E.I., M.S., M.C. and M.R. are inventors on patent applications WO2013012924, WO2011090954, WO2015009734, WO2013130875. J.T., and N.G. are inventors on patent application WO2015009734. A.W. is an inventor on patent applications WO2013012924, WO2013130875.

Author contributions

M.C. and M.R. performed literature searches; M.C., M.R., B.S., P.L., N.G., J.T., and D.C. designed, performed and analyzed the ELISA experiments; M.S. and A.L.W. designed, engineered, and produced FcMBL; J.K., A.G., M.R., and T.D. established the rat sepsis models, and J.K., T.D., and A.G. conducted rat studies; A.W., D.L., M.S., and D.B. established and conducted the pig sepsis model studies; N.S. provided human blood samples, patient approvals, and clinical patient data; N.D. and M.C. performed statistical analysis of ELISA data; D.E.I. oversaw the design and analysis of all experiments, and prepared the manuscript with M.C. and M.S., and with input from all authors.

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References

Albrich, W.C., Harbarth, S., 2015. Pros and cons of using biomarkers versus clinical decisions in start and stop decisions for antibiotics in the critical care setting. Intensive Care Med. 41 (10), 1739–1751.

Angus, D.C., van der Poll, T., 2013. Severe sepsis and septic shock. N. Engl. J. Med. 369, 840–851.

Bacconi, A., Richmond, G.S., Baroldi, M.A., et al., 2014. Improved sensitivity for molecular detection of bacterial and Candida infections in blood. J. Clin. Microbiol. 52 (9), 3164–3174.

Basu, R., Pathak, S., Goyal, J., Chaudhry, R., Goel, R.B., Barwal, A., 2014c. Use of a novel hemoadsorption device for cytokine removal as adjuvant therapy in a patient with septic shock with multi-organ dysfunction: a case study. Indian J. Crit. Care Med. 18 (12), 822–824 (Dec).

Bates, D.W., Parsonnet, J., Ketchum, P.A., et al., 1998. Limulus amebocyte lysate assay for detection of endotoxin in patients with sepsis syndrome. AMCC Sepsis Project Working Group. Clin. Infect. Dis. 27, 582–591.

Battistelli, S., Fortina, M., Carta, S., Guerranti, R., Nobile, F., Ferrata, P., 2014. Serum C-reactive protein and procalcitonin kinetics in patients undergoing elective total hip arthroplasty. BioMed Res. Int. 565080.

Bone, R.C., 1992. Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome). J. Am. Med. Assoc. 268 (24), 3452–3455.

Carr, J.A., 2015. Procalcitonin-guided antibiotic therapy for septic patients in the surgical intensive care unit. J. Intensive Care 3 (1), 36.

Chan, T., Gu, F., 2011. Early diagnosis of sepsis using serum biomarkers. Expert. Rev. Mol. Diagn. 11 (5), 487–496.

Charbonney, E., Tsang, J.Y., Li, Y., Klein, D., Duque, P., Romaschin, A., Marshall, J.C., 2016. Endotoxemia following multiple trauma: risk factors and prognostic implications. Crit. Care Med. 44 (2), 335–341.

Didar, T.F., Cartwright, M.J., Rottman, M., et al., 2015. Improved treatment of systemic blood infections using antibiotics with extracorporeal Fc-mannose binding lectin hemoadsorption. Biomaterials 67, 387–392.

Dierkes, C., Ehrenstein, B., Siebig, S., Linde, H.J., Reischl, U., Salzberger, B., 2009. Clinical impact of a commercially available multiplex PCR system for rapid detection of pathogens in patients with presumed sepsis. BMC Infect. Dis. 9, 126.

Dommett, R.M., Klein, N., Turner, M.W., 2006. Mannose-binding lectin in innate immunity: past, present and future. Tissue Antigens 68 (3), 193–209.

Ferrer, R., Martin-Loeches, I., Phillips, G., et al., 2014. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. Crit. Care Med. 42 (8), 1749–1755.

Gille-Johnson, P., Hansson, K.E., Gårdlund, B., 2013. Severe sepsis and systemic inflammatory response syndrome in emergency department patients with suspected severe infection. Scand. J. Infect. Dis. 45 (3), 186–193.

Hurley, J.C., 1992. Antibiotic-induced release of endotoxin: a reappraisal. Clin. Infect. Dis. 15 (5), 840–854.

Hurley, J.C., Nowak, P., Öhrmalm, L., Gogos, C., Armaganidis, A., Giamarellos-Bourboulis, E.J., 2015. Endotoxemia as a diagnostic tool for patients with suspected bacteremia caused by Gram-negative organisms: a meta-analysis of 4 decades of studies. J. Clin. Microbiol. 53 (4), 1183–1191.

Idelevich, E.A., Schule, I., Grunastel, B., Wullenweber, J., Peters, G., Becker, K., 2014. Rapid identification of microorganisms from positive blood cultures by MALDI-TOF mass spectrometry subsequent to very short-term incubation on solid medium. Clin. Microbiol. Infect. 20, 1001–1006.

Jack, D.L., Turner, M.W., 2003. Anti-microbial activities of mannose-binding lectin. Biochem. Soc. Trans. 31 (Pt 4), 753–757.

Jack, D.L., Dodds, A.W., Anwar, N., et al., 1998. Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. J. Immunol. 160 (3), 1346–1353.

Jensen, J.U., Hein, L., Lundgren, B., et al., 2011. Procalcitonin-guided interventions against infections to increase early appropriate antibiotics and improve survival in the intensive care unit: a randomized trial. Crit. Care Med. 39, 2048–2058.

Jordana-Lluch, E., Giménez, M., Quesada, M.D., et al., 2015. Evaluation of the broad-range PCR/ESI-MS technology in blood specimens for the molecular diagnosis of bloodstream infections. PLoS One 10 (10).

Kang, J.H., Super, M., Yung, C.W., et al., 2014. An extracorporeal blood-cleansing device for sepsis therapy. Nat. Med. 20 (10), 1211–1216.

Kaukonen, K.M., Bailey, M., Pilcher, D., Cooper, D.J., Bellomo, R., 2015. Systemic inflammatory response syndromecriteria in defining severe sepsis. N. Engl. J. Med. 372 (17), 1629–1638.

Knabl, L., Mutschlechner, W., Orth-Höller, D., 2016. Evaluation of a multiplex OnSpot primer-extension PCR assay in the diagnosis of sepsis. J. Microbiol. Methods 120, 91–93.

Kumar, A., Roberts, D., Wood, K.E., et al., 2006. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit. Care Med. 34, 1589–1596.

Lehmann, L.E., Alvarez, J., Hunfeld, K.P., et al., 2009. Potential clinical utility of polymerase chain reaction in microbiological testing for sepsis. Crit. Care Med. 37 (12), 3085–3090.

Lehmann, L.E., Hunfeld, K.P., Steinbrucker, M., et al., 2010. Improved detection of blood stream pathogens by real-time PCR in severe sepsis. Intensive Care Med. 36 (1), 49–56.

Li, Y.M., Liu, X.Y., 2015. Serum levels of procalcitonin and high sensitivity C-reactive protein are associated with long-term mortality in acute ischemic stroke. J. Neurol. Sci. 15 (352(1–2)), 68–73.

Liu, V., Escobar, G.J., Greene, J.D., et al., 2014. Iwashyna, hospital deaths in patients with sepsis from 2 independent cohorts. J. Am. Med. Assoc. 312 (1), 90–92.

Loonen, A.J., de Jager, C.P., Tosserams, J., et al., 2014. Biomarkers and molecular analysis to improve bloodstream infection diagnostics in an emergency care unit. PLoS One 9 (1), e87315.

Louie, R.F., Tang, Z., Albertson, T.E., Cohen, S., Tran, N.K., Kost, G.J., 2008. Multiplex polymerase chain reaction detection enhancement of bacteremia and fungemia. Crit. Care Med. 36 (5), 1487–1492.

Marshall, J.C., Foster, D., Vincent, J.L., et al., 2004. Diagnostic and prognostic implications of endotoxemia in critical illness: results of the MEDIC study. JID 190, 527–534.

Martin, G.S., Mannino, D.M., Eaton, S., Moss, M., 2003. The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 17 (348(16)), 1546–1554.

McCrea, K., Ward, R., LaRosa, S.P., 2014. Removal of Carbapenem-resistant Enterobacteriaceae (CRE) from blood by heparin-functional hemoperfusion media. PLoS One 9 (12), e114242 (Dec 3).

Mignon, F., Piagnerelli, M., Van Nuffelen, M., Vincent, J.L., 2014. Effect of empiric antibiotic treatment on plasma endotoxin activity in septic patients. Infection 42 (3), 521–528.

Mitaka, C., Tomita, M., 2011t. Polymyxin B-immobilized fiber column hemoperfusion therapy for septic shock. Shock 36 (4), 332–338 (Oct).

Mylonakis, E., Clancy, C.J., Ostrosky-Zeichner, L., et al., 2015. T2 magnetic resonance assay for the rapid diagnosis of candidemia in whole blood: a clinical trial. Clin. Infect. Dis. 15 (60(6)) (892–9).

Parlato, M., Cavaillon, J.M., 2015. Host response biomarkers in the diagnosis of sepsis: a general overview. Methods Mol. Biol. 1237, 149–211.

Pierrakos, C., Vincent, J.L., 2010. Sepsis biomarkers: a review. Crit. Care 14 (1), R15.

Prucha, M., Bellingan, G., Zazula, R., 2015. Sepsis biomarkers. Clin. Chim. Acta 2 (440), 97–103 (Review).

Romaschin, A.D., Klein, D.J., Marshall, J.C., 2012. Bench-to-bedside review: clinical experience with the endotoxin activity assay. Crit. Care 16, 248.

Schuetz, P., Raad, I., Amin, D.N., 2013. Using procalcitonin-guided algorithms to improve antimicrobial therapy in ICU patients with respiratory infections and sepsis. Curr. Opin. Crit. Care 19, 453–460.

Schultz, H., Hume, J., Zhang de, S., Gioannini, T.L., Weiss, J.P., 2008. A novel role for the bactericidal/permeability increasing protein in interactions of Gram-negative bacterial outer membrane blebs with dendritic cells. J. Immunol. 15 (179(4)), 2477–2484.

Seoane, L., Pértega, S., Galeiras, R., Astola, I., Bouza, T., 2014. Procalcitonin in the burn unit and the diagnosis of infection. J. Burns 40, 223–229.

Singer, M., Deutschman, C.S., Seymour, C.W., et al., 2016. The third international consensus definitions for sepsis and septic shock (sepsis-3). J. Am. Med. Assoc. 315 (8), 801–810.

Stoppekamp, S., Veseli, K., Stang, K., Schlensak, C., Wendel, H.P., Walker, T., 2015. Identification of predictive early biomarkers for sterile SIRS after cardiovascular surgery. PLoS One 10 (8), e0135527.

Super, M., Thiel, S., Lu, J., Levinsky, R.J., Turner, M.W., 1989. Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet 2 (8674), 1236–1239.

Tsalik, E.L., Jones, D., Nicholson, B., et al., 2010. Multiplex PCR to diagnose bloodstream infections in patients admitted from the emergency department with sepsis. J. Clin. Microbiol. 48 (1), 26–33.

Tsalik, E.L., Jaggers, L.B., Glickman, S.W., et al., 2012. Discriminative value of inflammatory biomarkers for suspected sepsis. J. Emerg. Med. 43 (1), 97–106.

Tullis, R.H., Duffin, R.P., Ichim, T.E., Joyce, J.A., Levin, N.W., 2010. Modeling hepatitis C virus therapies combining drugs and lectin affinity plasmapheresis. Blood Purif. 29 (2), 210–215.

Uzzan B, Cohen R, Nicolas P, Cucherat M, Perret G.Y. Procalcitonin as a diagnostic test for sepsis in critically ill adults and after surgery or trauma: a systematic review and meta-analysis. Crit. Care Med. 2006; 34(7), 1996–2003.

von Lilienfeld-Toal, M., Lehmann, L.E., et al., 2009. Utility of a commercially available multiplex real-time PCR assay to detect bacterial and fungal pathogens in febrile neutropenia. J. Clin. Microbiol. 47 (8), 2405–2410.

Wojtaszek, M., Staśkiewicz, G., Torres, K., Jakubowski, K., Rácz, O., Cipora, E., 2014. Changes of procalcitonin level in multiple trauma patients. Anaesthesiol. Intensive Ther. 46, 78–82.

Yealy, D.M., Kellum, J.A., Huang, D.T., et al., 2014. A randomized trial of protocol-based care for early septic shock. N. Engl. J. Med. 370, 1683–1693.