Improved treatment of systemic blood infections using antibiotics with extracorporeal opsonin hemoadsorption

Tohid F. Didar, Mark J. Cartwright, Martin Rottman, Amanda R. Graveline, Nazita Gamini, Alexander L. Watters, Daniel C. Leslie, Tadanori Mammo, Melissa J. Rodas, Joo H. Kang, Anna Waterhouse, Benjamin T. Seiler, Patrick Lombardo, Elisabet I. Qendro, Michael Super, Donald E. Ingber. *Corresponding author. Wyss Institute for Biologically Inspired Engineering at Harvard University, CLSB5, 3 Blackfan Circle, Boston, MA 02115, USA. E-mail address: don.ingber@wyss.harvard.edu (D.E. Ingber).

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form 23 July 2015
Accepted 23 July 2015
Available online 26 July 2015

Keywords:
Sytematic blood infections
Bio-functional hollow fibers
Dialysis like treatment (DLT) of sepsis
Combined drug–device therapy for sepsis
Pathogen and LPS-endotoxin cleansgin

ABSTRACT

Here we describe development of an extracorporeal hemoadsorption device for sepsis therapy that employs commercially available polysulfone or polyethersulfone hollow fiber filters similar to those used clinically for hemodialysis, covalently coated with a genetically engineered form of the human opsonin Mannose Binding Lectin linked to an Fc domain (FcMBL) that can cleanse a broad range of pathogens and endotoxin from flowing blood without having to first determine their identity. When tested with human whole blood in vitro, the FcMBL hemoadsorption filter (FcMBL-HF) produced efficient (90–99%) removal of Gram negative (Escherichia coli) and positive (Staphylococcus aureus) bacteria, fungi (Candida albicans) and lipopolysaccharide (LPS)-endotoxin. When tested in rats, extracorporeal therapy with the FcMBL-HF device reduced circulating pathogen and endotoxin levels by more than 99%, and prevented pathogen engraftment and inflammatory cell recruitment in the spleen, lung, liver and kidney when compared to controls. Studies in rats revealed that treatment with bacteriocidal antibiotics resulted in a major increase in the release of microbial fragments or ‘pathogen-associated molecular patterns’ (PAMPs) in vivo, and that these PAMPs were efficiently removed from blood within 2 h using the FcMBL-HF; in contrast, they remained at high levels in animals treated with antibiotics alone. Importantly, cleansing of PAMPs from the blood of antibiotic-treated animals with the FcMBL-hemoadsorbent device resulted in reduced organ pathogen and endotoxin loads, suppressed inflammatory responses, and resulted in more stable vital signs compared to treatment with antibiotics alone. As PAMPs trigger the cytokine cascades that lead to development of systemic inflammatory response syndrome and contribute to septic shock and death, co-administration of FcMBL-hemoadsorption with antibiotics could offer a more effective approach to sepsis therapy.

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1. Introduction

Sepsis is caused by uncontrolled spread of infectious pathogens and release of toxins that leads to development of a systemic inflammatory response syndrome (SIRS) [1–3]. Worldwide, 18 million cases of sepsis are reported each year and one in three septic patients ultimately die from complications [4]. Unfortunately, identification of the causative pathogens takes days using state-of-the-art microbiology tools, and blood cultures are negative in more than 50% of patients, even in those with fulminant sepsis [5,6]. Current sepsis therapies rely on the administration of broad-spectrum antibiotics before the causative pathogen is identified, and the delay in providing active therapy is associated with increased mortality [5,7–9]. Sepsis treatment is complicated by the release of toxins and bacterial agonists to the immune system.
receptors (pathogen associated molecular patterns, “PAMPs”) from pathogens upon lysis by immune cells or antibiotic therapy [10]. Even effective antibiotic treatments that successfully reduce the load of living pathogens release PAMPs into the blood, which contribute to the development of septic shock and death [11–16]. This observation raised the possibility that removing PAMPs from the circulation might enhance the effectiveness of conventional antibiotic therapy.

Extracorporeal blood purification systems that cleanse blood of lipopolysaccharide (LPS)-endotoxin and cytokines including hemofiltration [17–19], hemoadsorption [20–24], and coupled plasma filtration adsorption (CPFA) [25] have been explored as alternative approaches for sepsis treatment [26,27]. These devices either remove target molecules below a specific size, such as inflammatory cytokines [28], or are coated with ligands that bind and remove a specific type of PAMP, such as the use of Polyoxynin B immobilized on hemofilters to remove endotoxin [20,29]. We recently reported the development of a microfluidic, dialysis-like therapeutic device for sepsis therapy or ‘biospleen’ that removes living pathogens and endotoxin from blood using magnetic nanoparticles coated with a genetically engineered form of the human opsonin, Mannose Binding Lectin, that lacks its complement fixation and coagulation domains, and is linked to an antibody Fc-domain (FcMBL) [30]. MBL binds to carbohydrate components found in the cell walls of more than 90 Gram negative and positive bacteria and in fungi, viruses and parasites, as well as lipopolysaccharide (LPS-endotoxin), but not mammalian cells; hence, this protein can be used to remove pathogens or LPS without prior knowledge of the microbial etiology of the infection. While the biospleen performed well, the complexity of the microfluidic system including 20 feet of tubing per minute to provide incubation time with the nanobeads, use of avidin-biotin linkage chemistry, and high cost of the magnetic nanobeads represent major obstacles for clinical applications of this device.

In this study, we set out to develop a more robust, simplified and clinically relevant extracorporeal device for sepsis therapy by leveraging well-proven hollow fibers to streamline our device design and remove the requirement for magnetic beads or microfluidics, while retaining the power of the broad-spectrum pathogen and toxin capture capabilities of FcMBL. In addition, we explored if this extracorporeal device could be used to remove PAMPs in combination with antibiotic therapy. Here we show that the FcMBL-HF device we developed, which employs commercially available dialysis filters containing hollow fibers covalently coated with FcMBL, efficiently cleanses pathogens and endotoxin from flowing human blood in vitro and from blood of living rats flowing through an extracorporeal circuit. Importantly, by simultaneously leveraging of our ability to detect live and dead pathogens in blood based on FcMBL binding, we discovered that antibiotics produce a rapid rise in release of PAMPs into blood, and that these inflammatory pathogen fragments can be effectively cleared from blood by simultaneous use of the FcMBL hemoadsorption filter (FcMBL-HF) device. Moreover, combined therapy with antibiotics and the FcMBL-HF produced a significant reduction in pathogen load in lung, liver and spleen compared to antibiotic therapy alone, as well as stabilization of vital signs in the animal sepsis model. Thus, FcMBL hemoadsorption may represent a powerful adjuvant to antibiotics for sepsis therapy.

2. Materials and methods

2.1. Fabrication of the FcMBL-HF device

FcMBL protein was expressed and purified from a stable transfection of CHO-DG44 cells (Invitrogen, Carlsbad, CA). The Purified protein was dialyzed into PBS (Life Technologies) and purity and functionability were confirmed as previously described [30]. The hollow fibers were treated with oxygen plasma (1 min, O2, 100 W, 200 mTorr) using a PE-100 plasma system (PlasmaEtch) to activate the surface for amino-silanization, followed by injecting 5% v/v, 3-amino propyltrimethoxysilane solution (APTMS, Sigma, St. Louis, MO) in anhydrous ethanol and incubating for 1 h. Hollow fibers were then rinsed with anhydrous ethanol (Sigma, St. Louis, MO), distilled deionized water and ethanol in sequence, and dried by blowing nitrogen gas through them for 5 min. The APTMS-functionalized hollow fibers were then placed in an oven at 60°C for 5 days. FcMBL was covalently attached to amino-silanized hollow fibers using EDC ((1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), Sigma, St. Louis, MO) chemistry. For this purpose EDC (20 mg/mL) was prepared in PBS and was mixed (1:1 volume ratio) with a 256 µg/mL solution of FcMBL and immediately incubated with the hollow fibers for two hours at room temperature, then incubated overnight at 4°C. The FcMBL-functionalized hollow fibers were extensively washed with PBS solution and stored at 4°C prior to use. The functionality of the FcMBL-HF device was maintained 4 months after functionalization when stored in PBS with 10 mM EDTA, indicated by its efficiency to cleanse LPS-endotoxin spiked into saline.

Small volume MicroKros polysulfone-based hollow fiber filters (500 µm in diameter and 50 kD porosity, 10 hollow fibers in each filter) were purchased from SPECTRUM LABS. MicroKros hemofilters were used in the rat studies due to their small volume. To demonstrate the pathogen/endotoxin cleansing in high flow rates (50–200 mL/min), Nk25-0238 (a kind gift from NxStage Inc.) hemofilters (polysulfone hollow fibers, 200 µm in diameter and 50 kD porosity, over 5000 hollow fibers in each filter) from NxStage were used.

2.2. Characterization and optimization of the functionalization process

X-ray photoelectron spectroscopy (XPS), was performed on a Thermo Scientific K-Alpha X-Ray Photoelectron Spectrometer (Thermo Scientific) at different stages of the functionalization process to characterize and confirm covalent surface functionalization. Polysulfone and polyethersulfone surfaces were oxygen plasma treated and amino-silanized as described above. XPS scans were analyzed using the Thermo Scientific Avantage Data System v5.915 (Thermo Scientific).

In addition, applying an amine-reactive fluorescent succinimidyl ester (CF™647 SE, Biotium, Hayward, CA) confirmed the presence of amine groups on the surface after APTMS coating. For this purpose, SE was diluted in PBS (1:1000 v/v) and incubated with the APTMS-coated samples for 1 h. Samples were then rinsed with PBS and analyzed using fluorescence microscopy (Supplementary Fig. 1a). Samples without APTMS coating that were incubated with SE were used as control.

To demonstrate covalent attachment of FcMBL onto the hollow fiber surfaces, FcMBL coupling onto APTMS coated surfaces was performed with and without EDC (Supplementary Fig. 1b). Presence of FcMBL on the functionalized surfaces was measured using fluorescently labeled anti-Fc human IgG antibody (100 µg/mL in PBS, Abcam, Cambridge, MA). This confirmed covalent attachment of FcMBL using EDC. To find the optimal concentration of FcMBL, different FcMBL concentrations were used to covalently coat polysulfone surfaces and the FcMBL concentration on the surface was measured using fluorescently labeled anti-Fc human IgG (Abcam, Cambridge, MA) as described above (Supplementary Fig. 1c).

Fluorescence microscopy was carried out using a Zeiss Axioc Observer Z1 3 (AXIO3) inverted fluorescence microscope. Three separate samples for each experiment were imaged. Analysis of
fluorescence images was carried out on five images taken from separate areas of each sample using ImageJ software.

2.3. Operation of the FcMBL-HF device

We used two different hollow fiber hemofilters for in vitro blood cleansing. We mainly focused on MicroKros polysulfone based hollow fibers applying a flow rate of 0.2 mL/min because they were a suitable option for our animal studies due to their small volume. In addition we demonstrated efficient cleansing of pathogens and endotoxins using medical grade larger hemofilters. For in vitro experiments human blood was obtained from healthy donors in accordance with protocols of Harvard University Faculty of Medicine Committee on Human Studies (protocol number M20403-101) and the Defense Advanced Research Projects Agency (DARPA). All experiments were performed in a hemoperfusion configuration where the blood passed through the inner lumen space of the hollow fibers and the outer space was filled with saline. Pathogens (Candida albicans, Staphylococcus aureus and Escherichia coli) with known concentrations were stored at −80 °C and diluted to desired concentration prior to use. For in vitro studies, bacteria were spiked into TBST with 5 mM Ca2+ (50 mM Tris–HCL, 150 mM NaCl, 0.05% Tween–20, 5 mM CaCl2, Boston BioProducts) or into human whole blood and flowed through the FcMBL-HF. After passing through the device, the cleansed blood was recycled back to the inlet, producing a closed blood circulation loop. During the 5-h blood cleansing samples were collected (200 μL) from the main sample reservoir and were plated using a spiral planer (Eddy jet 2 NEUTEK group Inc.) to quantify pathogen colony forming units (CFUs). As control hollow fibers without FcMBL coating were used in each experiment and sample collection and analysis was performed simultaneously for both FcMBL-HF device and control hollow fibers. For cleansing bacterial fragments produced as a result of antibiotics treatment, different E. coli strains (ATCC8739, RS218 (a kind gift from James Johnson MD, University of Minnesota) and ATCC700928 (CFT073)) were cultured, grown to a concentration of 10^8 CFU/mL and treated with cefepime (100 μg/mL) for 4 h. Antibiotic-treated pathogens were cultured to confirm that no live pathogens remained after antibiotic treatment. Samples were then spiked into saline or blood and were passed through the FcMBL-HF and control devices to investigate the cleansing efficiency using the FcMBL-HF device.

Fungi were cultured on dextrose agar plates and incubated at 30 °C; bacteria were cultured on LB agar (or blood agar) plates and incubated at 37 °C. Colonies were counted after 36 h (Fungi) and 24 h (bacteria) to determine the pathogen concentration in samples from FcMBL-HFs and control hollow fibers. Cleansing efficiency was calculated based on the pathogen concentration in the FcMBL-HF device compared to the control hemofilter (Equation (1)). C_{FcMBL} and C_{control} are the concentrations of bacteria (CFU/mL) remaining in the main sample reservoir (Fig. 2a) of the FcMBL-HF and control devices respectively.

\[
\text{Eff.} = \left(1 - \frac{C_{\text{FcMBL}}}{C_{\text{control}}} \right) \times 100
\]  

(1)

2.4. FcMBL-ELISA assay

We used a FcMBL-ELISA to measure the level of pathogen associated molecular patterns (PAMPs) in blood. FcMBL-coated magnetic beads (1 µm beads from Dynabeads) were loaded into a 96-well plate. 100 μL of the sample volume was added to the desired wells containing the FcMBL-beads (25 μg) in TBST 5 mM Ca2+, heparin (for blood sample assays), and 10 mM glucose. The sample and the beads were shaken for 20 min. The beads were then washed and assayed using an automated KingFisher Flex magnetic particles processor (Thermo Scientific) device. The captured PAMPs were detected using horseradish peroxidase (HRP)-labeled rh-MBL (manufactured by Enzon Pharmaceuticals Inc. and provided by K. Takahashi, Massachusetts General Hospital). 3′,5′-Tetramethylbenzidine (TMB, Thermo Scientific) was added for colorimetric quantification and optical density was measured in duplicate at 450 nm wavelength. Each assay contained a mannan standard curve; LPS standard curve was used when quantification of LPS concentration in the samples was desired (Supplementary Fig. 5a,b).

2.5. LPS-endotoxin binding assay

Lipopolysaccharide (LPS) endotoxin extracted from E. coli (Sigma Aldrich) was spiked into TBST 5 mM Ca2+ buffer or human whole blood and the cleansing efficiency of the FcMBL-HF device was assessed. LPS concentration in saline was measured using an FDA approved Limulus Amoebocyte Lysate (LAL) assay (Endosafe, Charles River Inc.). LPS concentration in blood was measured using the FcMBL-ELISA as explained above. A calibration curve with known concentrations of LPS spiked into blood was used to convert the OD signal from ELISA to ng/mL of LPS concentration (Supplementary Fig. 5a).

2.6. Animal studies

Animal protocols were reviewed and approved by 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 Materiel Command (USAMRMC) Office, and Department of Defense (DOD). Rats (Wistar, 14 weeks old, male ~370 g) were purchased with double catheters placed in their jugular veins from Charles River Laboratories (Wilmington, MA, USA). FcMBL-HFs and control hollow fibers (without FcMBL coating) were connected to the living rat through an extracorporeal circuit using the jugular vein catheters. Rats were anesthetized with isoflurane using a nose cone. To construct the extracorporeal blood cleansing circuit; 23G blunt needles were inserted into the catheters and then connected to male luer-barb fitting connectors (EW-45504-00, Cole-Parmer, IL, USA) at each end of the circuit (Tygon® 1/16” ID, PVC) (Supplementary Fig. 4). Using a syringe pump, saline (90 ml kg^-1 day^-1) containing heparin (50 unit kg^-1 h^-1) was continuously injected (required by IACUC protocols) to prevent dehydration and coagulation of blood while circulating blood through the FcMBL-HF similar to standard medical practice in humans receiving extracorporeal hemodialysis. To study the biocompatibility of the device, healthy rats (n = 3) were connected to the FcMBL-HF device and the animal’s blood was circulated through the extracorporeal circuit for 5 h. Rats were then taken off anesthesia and monitored for 24 h.

We used E. coli and S. aureus bacteremia models by injecting a bolus of saline containing 5 × 10^8 CFU of E. coli (ATCC No. 8739) or S. aureus (ATCC 12598), followed by continuously infusing 5 × 10^8 CFU of E. coli or S. aureus for 5 h while treating the rats with the FcMBL-HF (n = 3). In control experiments, we treated the rats (n = 3) with the hemofilter without FcMBL coating. The experimental and control animals received the same continuously infused volume of saline and heparin.

For endotoxin cleansing, LPS-endotoxin (100 mg/kg body weight) extracted from E. coli (0111:B4) was injected intravenously (n = 3), followed by extracorporeal blood cleansing using the FcMBL-HF device. Fluid administration, blood sample collection and analysis were similar to that described above. For all the pathogen and endotoxin studies, blood samples (300 μL) were
collected every hour using a 3-way valve connected to the FcMBL-HF device in the circuit. Blood samples were then used to perform blood analysis and blood-culture assays. At the end of each experiment rats were euthanized and the major organs were harvested. Organs were bead-milled using a RETSCH Mixer Mill MM 400 (Verder Scientific, France) and concentration of live pathogens in the spleens, lungs, livers and kidneys were measured via plating [31]. Furthermore the organs were fixed in 10% formalin, cryosectioned, and processed for immunohistochemistry and H&E staining. Anti- E. coli, anti-LPS and anti-CD45 antibodies (diluted 1:100 in PBS, from Abcam) were used to stain against E. coli, LPS and inflammatory cells. All tissues were also stained with DAPI (Vector Labs, Burlingame, CA) in VECTASHIELD mounting medium (Vector Labs, Burlingame, CA). Tissue samples were imaged using a Zeiss Axio Observer Z1 3 (AXIO3) inverted fluorescence microscope.

2.7. Combinatorial FcMBL-HF and antibiotic treatment

To demonstrate the performance of the developed technology in combination with conventional antibiotics treatment for septic patients, we designed an experiment with three groups of rats in which all three groups were intraperitonealy infected with $5 \times 10^8$ CFU of E. coli (ATCC No. 8739). Group i were used as control with no treatment, group ii were treated with an optimal dosage of antibiotics (cefepime, 100 mg/kg) intraperitonealy 4 h after E. coli injection, and group iii were treated with antibiotics (cefepime, 100 mg/kg) 4 h after infection and were further treated with the FcMBL-HF 4 h post antibiotics treatment for 2 h. Blood samples (500 μL) were collected after 0,4,8 and 10 h using the jugular catheters. Blood samples were used to measure both live pathogen and pathogen-associated molecular pattern (PAMP) levels in each group at different time points. Complete blood analysis was performed as well as analysis of major animal organs for live pathogen levels, LPS-endotoxin and inflammatory cells (CD45) as described above. Due to the animal use regulation’s requirement that use of animals be minimized, and our finding that uncoated hollow fiber devices do not result in effective removal of live bacteria or LPS-endotoxin from flowing blood in vitro or in vivo (Figs. 2 and 3), we did not perform sham experiments with uncoated hollow fiber devices in studies with antibiotic-treated rats.

2.8. Hematology

During the extracorporeal blood cleansing on living animals blood samples were collected from rats at different times during treatment and complete blood count was performed using a CBC machine (Hemavet HV950, Drew Scientific Group).
2.9. Statistical analysis

Results are expressed as average values ± s.d. for all reported data. In vitro blood cleansing assays were performed using blood from three different donors. Statistical analysis was performed using two-tailed student’s t-test and significant differences were defined by p values < 0.05.

3. Results

3.1. In vitro pathogen and endotoxin cleansing using the FcMBL-HF device

FcMBL that was expressed in CHO DG44 cells and purified as described in Methods was covalently coupled to the surface of the inner lumen of commercially available polysulfone (MicroKros from SPECTRUM LABS) and polyethersulfone (Nx25-0238 from NxStage) hollow fiber dialysis filters (Fig. 1a,b) by first functionalizing them with amine-groups and then using EDC coupling (Fig. 1c). Two different techniques, X-ray photoelectron spectroscopy (XPS) (Fig. 1d) and binding of an amine-reactive fluorescent dye (Supplementary Fig. 1a,b), were used at different stages of the functionalization process to confirm efficient covalent surface coating. Optimal surface coating was obtained using ~80 μg/mL FcMBL (Fig. 1e, Supplementary Fig. 1c), and 128 μg/mL FcMBL concentration was used in all subsequent studies.

To test the FcMBL-HF’s ability for pathogen and LPS cleansing, infected samples were re-circulated through the device in a
Fig. 3. In vivo pathogen cleansing using the FcMBL-HF device. (a) Schematic showing the experimental set-up for blood cleansing with the FcMBL-HF device in a rat animal model. (b) Blood analysis results from biocompatibility studies in which rats were connected to the FcMBL-HF for 5 h and were monitored for 24 h (mean ± s.d., n = 3). Note that white blood cells (WBC, 10^3/µL), red blood cells (RBC, 10^6/µL) and platelets (PLT, 10^5/µL) all remained in the normal range (dashed boxes) at the end of 5 h. (c) E. coli concentration in the blood of rats treated with the FcMBL-HF (n = 3) or a control untreated hollow fiber (n = 3). In less than 3 h, a 2 Log reduction in E. coli concentration was achieved in the animals treated with the FcMBL-HF compared to the controls (mean ± s.d., n = 3, P < 0.01). (d) S. aureus concentration in the blood of rats treated with the FcMBL-HF (n = 3) or a control hollow fiber (n = 3). In less than 3 h, FcMBL-HF treatment resulted in a 1 Log reduction in S. aureus concentration compared to treatment with the control device (mean ± s.d., n = 3, P < 0.05). (e) Distribution of E. coli in the major organs of rats after treatment with the FcMBL-HF device compared to the uncoated hollow fibers. FcMBL-HF treatment resulted in a reduction in E. coli concentration by over 99% in the spleen and 90% in the liver, lung and kidney of the treated rats compared to the controls (mean ± s.d., n = 3, P < 0.01). (f) Concentration of S. aureus in the major organs of rats after treatment compared to the control rats. FcMBL-HF treatment resulted in over 90% reduction in S. aureus concentration in these organs compared to the controls (mean ± s.d., n = 3, P < 0.05). (g) Immunofluorescence images of major organs of healthy rats, or rats infected with E. coli that were either untreated or treated with FcMBL-HF device. Tissues were stained for E. coli (anti-E. coli antibody; red) and immune cells (CD45⁺; red) as well as nuclei (DAPI; blue). Rats treated with FcMBL-HF had significantly lower levels of E. coli and immune cells in all of the major organs (bar, 20 µm). (h) Blood analysis of E. coli infected rats after 5 h revealed levels of white blood cells (WBC, 10^3/µL) that were below normal levels; however, these levels recovered to the normal range in rats treated with the FcMBL-HF device. Platelet (PLT, 10^5/µL) counts were also significantly lower than the normal range in the control rats, while it remained in the normal range in FcMBL-HF treated animals after 5 h of treatment (mean ± s.d., n = 3). (i) White blood cell (WBC, 10^3/µL) counts remained in the normal range for both control and treated animals infected with S. aureus; however, control rats had lower white blood cell counts compared to the treated animals. Dashed boxes show the normal range in h and i (mean ± s.d., n = 3).
dialysis-like hemoadsorption flow circuit (Fig. 2a). The cleansing efficiency of the FcMBL-HF was calculated as the percentage of added pathogens, normalized for non-specific binding and bacterial growth over the 5-h study by comparison with an uncoated HF. When tested in vitro using saline or human whole blood, over 90% of representative Gram negative (E. coli) bacteria, Gram positive (S. aureus) bacteria, and fungi (C. albicans) were removed from both saline buffer and human whole blood within 5 h at a flow rate of 0.2 mL/min (Fig. 2b). Importantly, significant (p < 0.05) reductions in pathogen numbers could be detected within 2 h after initiation of therapy (Fig. 2c and Supplementary Fig. 2a,b).

When septic patients with Gram negative bacterial infections are treated with bactericidal antibiotics, large amount of LPS can be released into their blood [13–16]. When we tested the FcMBL-HF, we found that it removed endotoxin equally well as living E. coli, and it accomplished this more rapidly, resulting in a reduction of LPS levels by more than 80% within 30 min, and over 95% (p < 0.05) within 5 h after initiation of FCMBL-hemoadsorption (Fig. 2d).

Moreover, blood cleansing efficiencies for both E. coli and LPS-endotoxin were greater than 70% even when the flow rates were increased up to 2 mL/min in the SPECTRUM filter and 100 mL/min (6 L/hr) in the larger NxStage dialysis unit (Fig. 2e). This finding has great clinical relevance because the higher flow rate is in the operational range (3–12 L/hr) of FDA approved dialysis systems used in hospital intensive care units [17,32,33].

To demonstrate the robustness of the FcMBL-HF, we tested its ability to remove three different E. coli strains and their released PAMPs from blood with or without antibiotic treatment, including two clinical E. coli strains (RS218 and CFT073) isolated from septic patients that are considered some of the major causes of sepsis [34,35]. We found that the cleansing efficiencies for the two clinical E. coli strains (RS218 and CFT073) were significantly lower (~35%) compared to the >95% efficiency observed with E. coli ATCC 8739 in the absence of antibiotic treatment. Importantly, however, the clearance efficiencies for both the RS218 and CFT073 strains increased significantly (p < 0.05) when antibiotic therapy was combined with extracorporeal FcMBL-based blood cleansing (Fig. 2f). In addition, we found that we could elute the captured pathogens bound to the FcMBL-HFs by flowing calcium-free buffer through the lumen of the hollow fibers at the end of an experiment because FcMBL-mannan binding is calcium-dependent [36] (Supplementary Fig. 3). This unique ability to recover pathogens and PAMPs removed from infected blood could prove invaluable for pathogen identification and antibiotic susceptibility testing in the future.

3.2. Blood cleansing in living animals

We then explored whether the FcMBL-HF can be used to cleanse blood of rats infected with living pathogens or injected with endotoxin in the presence or absence of simultaneous antibiotic therapy. The smaller hollow fiber filters were used to construct the FcMBL-HFs in these studies to accommodate to small total blood volume (~25 mL) of rats. The FcMBL-HF was connected to the jugular veins of living rats using medical grade catheters (Fig. 3a and Supplementary Fig. 4). We performed biocompatibility studies by
flowing the blood of healthy rats (n = 3) through the FcMBL-HF at 0.2 mL/min for 5 h and then monitoring the animals over the following day. During blood circulation, no significant change was detected in the animals' body temperature, breathing rates, hemoglobin levels, blood coagulation properties or levels of white blood cells, erythrocytes and platelets (Fig. 3b).

To determine the ability of the FcMBL-HF to cleanse flowing blood of pathogens in vivo, 5 × 10^8 CFU E. coli or S. aureus were injected intravenously as a bolus in saline, followed by continuous infusion of 1 × 10^8 CFU of bacteria/hour for 5 h while flowing the blood through the FcMBL-HF (Fig. 5).

**Fig. 5.** Combined therapy of living animals with antibiotics and the FcMBL-HF device. (a) Three groups of animals were infected intraperitoneally with 5 × 10^9 CFU of E. coli at time zero (T0) and they were either untreated, treated with antibiotic (ABX, cefepime, 100 mg/kg) four hours after infection (T4), or treated with antibiotic four hours after infection followed by treatment with the FcMBL-HF for two hours (T8). Blood samples were taken from all three groups at times 0, 4, 8 and 10. (b) Pathogen-associated molecular pattern (PAMP) levels did not significantly increase in the untreated group, while they significantly increased in the antibiotic-treated rats at T8 and T10 (mean ± s.d., n = 3, P < 0.01). When we treated the antibiotic-treated animals with the FcMBL-HF device at T8, PAMPs levels significantly decreased (P < 0.01) compared to the rats treated with antibiotics alone. (c) Analysis of living E. coli in the spleen, liver, lung and kidney in each group at the end of the experiment revealed that pathogen concentrations decreased in the major organs of antibiotic-treated animals, and that rats treated with the FcMBL-HF device had significantly lower pathogen levels compared to the two other groups (P < 0.05). These results also show that FcMBL-HF therapy can further decrease the live pathogen levels in the major organs compared to only antibiotic-treated animals. (d) White blood cell (WBC, 10^3/μL) and (e) platelet (PLT, 10^5/μL) counts for each group during the treatment regimen. White blood cell counts significantly decreased below normal range) at T8 for all three groups, while FcMBL-HF treated animals had significantly higher counts compared to only antibiotic-treated animals at the end of the experiment. Platelet counts also significantly decreased at T8 for all three groups as expected; while FcMBL-HF treated animals had higher platelet counts compared to only antibiotic-treated animals at T10. Dashed boxes show the normal range. (f) Immunofluorescence images of spleen, liver, lung and kidney removed from healthy rats, rats without treatment, treated with antibiotics or treated with both antibiotics and the FcMBL-HF device. Tissues were stained for LPS-endotoxin (anti-LPS; red) and immune cells (CD45; red) as well as nuclei (DAPI; blue) (bar, 20 μm).
animal’s blood through the extracorporeal circuit at 0.2 mL/min. Rats were euthanized after 5 h (due to animal protocol restrictions) and their major organs were harvested; control animals were treated identically, except uncoated hollow fiber filters were utilized. The level of living pathogens present in their blood (CFU/mL) and organs (CFU/mg) were quantified. These studies revealed the presence of significantly lower concentrations of both living E. coli (p < 0.01; Fig. 3c) and S. aureus (p < 0.05; Fig. 3d) in blood after 1 h of treatment in rats treated with the FcMBL-HF compared to animals treated with the uncoated device. FcMBL-hemoadsorption reduced the amount of live Gram negative and positive pathogens in the blood by over 2 Logs (>99% efficiency) after 5 h of treatment.

Furthermore, there were major decreases in the level of live Gram negative and positive bacteria present in the spleens, lungs, livers and kidneys of treated rats compared to controls (p < 0.05, Fig. 3e,f). These results were confirmed by histological analysis, which demonstrated significant decreases in the number of both pathogens and inflammatory (CD45+) cells in the major organs (Fig. 3g). Finally, blood analysis confirmed that while erythrocyte levels remained unchanged in rats infected with E. coli, white blood cells and platelets decreased significantly (Fig. 3h), as previously observed in other rat models [37-39]. Likewise, white blood cell levels remained unchanged in rats infected with E. coli- and S. aureus therapy had significantly reduced PAMPs released into the bloodstream by antibiotic therapy that can trigger the inflammatory cascade; it also reduces the levels of pathogens that engraft in the major organs. When we stained the major organs of these animals for LPS (which is a major PAMP molecule) and inflammatory cells, rats treated with the combined antibiotic-FcMBL-HF therapy had significantly lower LPS concentrations and inflammatory cells in all organs compared to antibiotic-treated rats or untreated infected animals (Fig. 5f). We also did not observe significant pathological changes in the major organs of treated animals compared to healthy animal organs. These results indicate that while the antibiotic therapy decreased the levels of live bacteria in the blood, it caused pathogen killing that released LPS-endotoxin which accumulated in the organs. Most importantly, simultaneous treatment with the FcMBL-HF was able to mitigate this potentially damaging effect of antibiotic therapy.

4. Discussion

Sepsis, which results from an inflammatory cascade triggered by systemic microbial infections, is a major cause of death worldwide, and yet there is currently no effective therapy. We recently described a dialysis-like extracorporeal device or ‘biospleen’ which can remove pathogens and endotoxin from flowing infected blood without prior knowledge of the type of the infection [30]. The biospleen is a microfluidic device that captures pathogens using magnetic nanobeads coated with FcMBL – a genetically engineered human blood opsonin with broad pathogen and toxin binding capabilities — and then removes them from blood flowing through the device using applied magnetic forces. While this biospleen technology provided an effective means to cleanse infected blood of septic animals, the number, complexity, use of avidin chemistry for FcMBL conjugation to beads, long incubation circuit for mixing beads and high cost of the system’s components make it difficult to move this device through the regulatory approval process and into the clinic. Thus, the present study was initiated in an attempt to circumvent this limitation by simplifying the design, and covalently immobilizing the key FcMBL capture agent on commercially available hollow fiber filters similar to those currently used for clinical dialysis, hemofiltration and hemoadsorption therapies. For clinical applicability, we also set out to explore if this simplified hemoadsorption device could be used in combination with antibiotic therapy, as virtually all patients with sepsis are treated with broad-spectrum antibiotics, and if there were any synergy in combining these two therapeutic approaches.

Our results show that the hollow fiber-based FcMBL-hemoadsorption technology provides multiple advantages compared to the biospleen device. First, the FcMBL-HF obviates the needs for magnetic nanobeads that are costly and that could potentially enter the circulation and cause complications. Second, a single hollow fibercbased device has a significantly enhanced blood flow capacity (up to 200 mL/min compared to ~15 mL/min with a single biospleen unit) that is equivalent to that used in existing hospital intensive care units. This higher flow capacity would permit cleansing of the entire blood volume of a human in less than 30 min, so multiple rounds of blood cleansing could be performed over a period of hours. Third, we fabricated our FcMBL-HF from commercially available, medical grade hollow fiber-based dialysis units that are currently used clinically. These cartridges are also similar to those...
that have been used in patients with other immobilized molecules in the past (e.g., polymyxin-B coated hemoadsorption filters for endotoxin removal [10,41]). The past clinical experience with these dialysis and hemoperfusion units may simplify the regulatory path as we move this technology towards the clinic. As the porous hollow fibers we coat with FcMBL are currently also used for dialysis, it is possible that FcMBL hemoadsorption of microbes, toxins and PAMPs could be carried out simultaneously with dialysis in septic patients using a single multifunctional device in the future, which could have added value for patients with multiple injuries and multi-organ failure.

One of the most important findings in this study is that like many patients with sepsis, infected animals that were blood culture negative contained significantly high levels of pathogen fragments or PAMPs, including LPS-endotoxin, within their blood when antibiotic therapy was administered. As PAMPs can trigger release of inflammatory cytokines that drive the sepsis cascade, this might explain why many patients do not respond to antibiotics alone. Conversely, these results suggested to us that removing PAMPs from blood might enhance the effects of antibiotic therapy, and that is precisely what we observed when we exposed infected animals to antibiotics while simultaneously cleansing their blood with the FcMBL-HF device. One potential caveat for future clinical use, however, is that because FcMBL binding to pathogens is calcium-dependent, this approach might be compromised by use of citrate anticoagulation, and thus, use of heparin would be a much better choice.

Another advance of the combined therapy is that while FcMBL-hemoadsorption therapy effectively removes microbes and inflammatory materials from blood, decreases engraftment of living pathogens, and prevents recruitment of inflammatory cells to the major organs, it does not deal with living pathogens that remain at the nidus of infection. However, co-administration of an appropriate bacteriocidal antibiotic should kill these remaining pathogens that remain localized outside the bloodstream, and hence, produce a synergistic effect on pathogen clearance. It is important to note that as it is critical to identify the specific pathogen type to select the optimal antibiotic therapy, the FcMBL-HF could also provide a way to quickly collect pathogens for diagnostic purposes, for example, by eluting captured microbes bound to the filter when the cleansing treatment is ended. As we demonstrated in this study, elution can be accomplished by washing the contaminated FcMBL-HFs with calcium-free medium or calcium chelators by leveraging the calcium-dependent binding of FcMBL. The same technique could be used to assess the efficacy of blood cleansing, and hence, optimize treatment design as a companion diagnostic in the future.

In summary, these studies show that FcMBL hemoadsorption represents a simple and clinically relevant effective therapy for treatment of systemic blood infections, which can enhance the efficacy of conventional antibiotic therapies. The FcMBL-HF works by rapidly removing live pathogens, endotoxin and other PAMPs from blood that can initiate the sepsis in the extracorporeal circuit results in significantly reduced pathogen loads in the major organs of these animals, as well as decreased inflammatory infiltrates. While antibiotic therapy decreases the numbers of living pathogens in blood, it also releases large amounts of PAMPs that circulate in blood, which might explain why antibiotic therapy often does not suppress the SIRS response in humans. Importantly, we show that antibiotic therapy and FcMBL-hemoadsorption therapy are synergistic because the FcMBL-HF removes circulating pathogens, endotoxin and the released PAMPs, while the antibiotics can kill any live pathogens that remain at distant sites. Although we focus on treatment of sepsis here, the same FcMBL-coated hollow fiber technology could be used for other applications, including removing microbial contaminants from circulating water, food or pharmaceutical products in the future.

Author contributions

Acknowledgments
This work was supported by Defense Advanced Research Projects Agency grant N66001-11-1-4180 and contract HR0011-13-C-0025, and the Wyss Institute for Biologically Inspired Engineering at Harvard University. T.F.D acknowledges National Science and Engineering Research Council of Canada (NSERC) and FQRQS (Fonds de recherche du Québec – Santé) for postdoctoral awards. We thank E. Jiang, A. Mammo and A. Jiang for assistance with histology. Also we would like to thank T. Valentin for help with XPS analysis. XPS analysis was conducted at the Center for Nanoscale Systems at Harvard University, a member of the National Nanotechnology Infrastructure Network, which is supported by the National Science Foundation (ECS-0335765).

Competing financial interests
D. Ingber and M. Super are founders and hold equity in Opsonix, Inc., and Ingber also chairs its scientific advisory board.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.07.046.

References


