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

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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.

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

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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 Polymyxin 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 functionality were confirmed as previously described [30]. The hollow fibers were treated with oxygen plasma (1 min, O_2, 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-aminoproyltrimethoxysilane 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), Nx25-0238 (a kind gift from NxStage Inc.) hemofilters (polyethersulfone 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 Axio Observer Z1 3 (AXIO3) inverted fluorescence microscope. Three separate samples for each experiment were imaged. Analysis of