PCSK9 REGULATES LDLR-MEDIATED UPTAKE OF BACTERIAL LIPIDS

PCSK9 REGULATES LDLR-MEDIATED UPTAKE OF LIPOPOLYSACCHARIDE AND LIPOTEICHOIC ACID

By

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LAY ABSTRACT

Bacterial compounds stimulate inflammation that can be overwhelming during sepsis. Understanding the processes behind uptake and clearance of these compounds may lead to better sepsis treatments. Therefore, our goal was to understand how uptake of two bacterial compounds, lipopolysaccharide and lipoteichoic acid, occurs by liver cells called hepatocytes. Hepatocytes are naturally equipped to clear foreign compounds, so understanding their role in clearing bacterial compounds is important. Another goal was to identify the role of the protein PCSK9 in this uptake process, as treatments targeting PCSK9 could be applied to sepsis once we understand its role in this disease. Our research demonstrates the negative role of PCSK9 in regulating uptake of lipopolysaccharide and lipoteichoic acid through a lipoprotein receptor called LDLR, and identifies the role of lipoproteins in this process. These findings further our understanding of the hepatocyte response to bacterial compounds in relation to sepsis, and identify PCSK9 as a potential target for new sepsis therapies.

ABSTRACT

The liver regulates inflammation during sepsis, and most liver functions are carried out by hepatocytes. Bacterial lipids, including lipopolysaccharide (LPS) and lipoteichoic acid (LTA), can be cleared by hepatocytes, but the underlying mechanisms are uncertain. Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates uptake of LPS by hepatocytes, but it is unknown whether LTA uptake is similarly regulated. Therefore, our objectives were to characterize the PCSK9-regulated pathway of bacterial lipid uptake by hepatocytes by identifying whether low-density lipoprotein (LDL) receptor (LDLR) and LDLR-related protein 1 (LRP1) are the target receptors, and by determining which lipoproteins are involved.

To study this pathway, we assessed the uptake of fluorescently-labeled LPS or LTA by human HepG2 hepatocytes using flow cytometry. We pre-treated HepG2 cells with PCSK9, alone or in combination with anti-LDLR or anti-LRP1 antibodies, in order to identify the PCSK9-regulated receptors that are involved, and utilized media containing normal serum or lipoprotein-deficient serum to investigate the lipoprotein-dependence of this pathway. We also determined the roles of LDL and HDL in bacterial lipid uptake through a series of add-back experiments to lipoprotein-deficient serum, and blocked LDLR to confirm that LDLR mediates LDL-dependent uptake. The HepG2 cell response to variable degrees of bacterial lipid uptake was also assessed in a subset of experiments by measuring several cytokines and extracellular alanine aminotransferase (ALT) activity in the cell culture supernatant.

We found that PCSK9 regulates LDLR-mediated uptake of both LPS and LTA through an LDL-dependent mechanism, while LRP1 is not involved. Increased bacterial lipid uptake did not result in any hepatocellular injury or cytokine production, as measured by ALT activity and interleukin (IL)-6, IL-8, IL-10, and IL-17 concentrations.

In conclusion, we completed our objective of characterizing the PCSK9-regulated pathway of bacterial lipid uptake, and provide supporting evidence for targeting PCSK9 as a novel therapeutic avenue in sepsis.

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
ANOVA	analysis of variance
ApoA	apolipoprotein A
ApoB	apolipoprotein B
BODIPY-LTA	BODIPY 630/650-labeled lipoteichoic acid
BPI	bactericidal/permeability-increasing protein
CETP	cholesterol ester transfer protein
CLP	cecal ligation and puncture
DMEM	Dulbecco's modified Eagle medium
EGF-A	epidermal growth factor repeat A
FBS	fetal bovine serum
HDL	high-density lipoprotein
ICU	intensive care unit
IDL	intermediate-density lipoprotein
IL	interleukin
JE/MCP-1	murine monocyte chemoattractant protein-1
LBP	lipopolysaccharide binding protein
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LOF	loss-of-function

LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
LRP1	low-density lipoprotein receptor-related protein 1
LRP8/ApoER2	LDL receptor-related protein 8/apolipoprotein E receptor 2
LTA	lipoteichoic acid
MIP-2	macrophage inflammatory protein-2
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen associated molecular pattern
PCSK	proprotein convertase subtilisin/kexin
PCSK9	proprotein convertase subtilisin/kexin type 9
PLTP	phospholipid transfer protein
PRR	pathogen recognition receptor
SR-BI	scavenger receptor class B, type I
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
VLDL	very low-density lipoprotein

INTRODUCTION

Sepsis is a complex disease characterized by dysregulated inflammation and organ dysfunction in response to infection, which is most commonly bacterial in nature. The organs that are particularly affected include the lungs, kidneys, and liver. Current treatments for patients with sepsis include fluid resuscitation to stabilize blood pressure, and antibiotics to target the infection (Dellinger et al., 2013). Although antibiotics help to improve patient outcomes, mortality rates in septic patients still range from 15% to as high as 50% (Mayr et al., 2014; Fleischmann et al., 2016; Stoller et al., 2016), which demonstrates that additional treatment strategies are necessary. One such strategy could involve improving clearance of pro-inflammatory bacterial molecules known as pathogen associated molecular patterns (PAMPs), especially lipopolysaccharide (LPS) and lipoteichoic acid (LTA), which are released upon lysis of bacteria following treatment with microbicidal antibiotics (Holzheimer, 2001). These PAMPs can bind to a class of receptors called pathogen recognition receptors (PRRs) that are expressed by innate immune cells, resulting in an inflammatory response. Although this inflammatory response to LPS and LTA is well characterized, our understanding of the mechanisms involved in uptake and clearance of these bacterial molecules is limited, and thus further study is warranted prior to the development of effective new therapies for sepsis.

The liver plays a central role in the inflammatory response during sepsis through production of complement proteins, acute phase proteins and inflammatory cytokines (Strnad et al., 2017), and is also a key organ for the uptake, metabolism and clearance of toxins, drugs, and other exogenous or endogenous compounds. In addition to these

functions, the liver is involved in secreting non-inflammatory circulating proteins that are important for maintaining homeostasis in the blood, of which a classical example is albumin. Furthermore, the liver plays a key role in nutrient metabolism through its regulation of glucose and lipid homeostasis (Jones, 2016), the latter of which is achieved through production of bile to allow for initial digestion of dietary lipids and through complex processes involving lipid uptake and secretion via lipoproteins. The primary cell type that performs the majority of these functions is the hepatocyte; however, there are a wide variety of other cell types that also contribute to maintaining homeostasis and normal hepatic function, including fenestrated endothelial cells, hepatic stellate cells, and several types of resident innate immune cells, such as natural killer cells, natural killer T cells, and resident liver macrophages named Kupffer cells (Kowalewska et al., 2011). Of these cell types, Kupffer cells and endothelial cells are known to take up LPS and LTA through class A scavenger receptors and other related receptors (van Oosten et al., 2001), which are not expressed by hepatocytes, suggesting that different mechanisms may be involved in LPS and LTA uptake depending on the cell type. Therefore, we focused this study on understanding the mechanisms by which LPS and LTA are taken up by the most predominant cell type in the liver that also functions to metabolize and clear a variety of foreign compounds—the hepatocyte.

In this introduction to my thesis, I will review the inflammatory response to LPS and LTA, as well as the sequestration of these bacterial molecules within lipoproteins. I will also comment on the roles of lipid transfer proteins and discuss the importance of lipoprotein receptors in lipid homeostasis. Furthermore, I will introduce proprotein

convertase subtilisin/kexin type 9 (PCSK9) and discuss its role in regulating lipoprotein receptors expressed by hepatocytes, with a focus on the low-density lipoprotein receptor (LDLR) and LDLR-related protein 1 (LRP1). I will also review the evidence that suggests important roles for PCSK9 and LDLR in regulating inflammation during sepsis. Finally, I will conclude the introduction by explaining the purpose of this study in relation to the existing literature.

BACTERIAL LPS AND LTA

A. STRUCTURE & FUNCTION

LPS and LTA are both large, amphipathic molecules that are anchored into bacterial membranes to provide structural integrity and protection. LPS is normally found anchored into the outer membrane of Gram-negative bacteria whereas LTA is anchored into the cell membrane of Gram-positive bacteria, which do not possess an outer membrane.

The general structure of LPS is composed of three regions (Figure 1A): 1) the outermost polysaccharide chain named the O antigen; 2) a linker region called the core polysaccharide, made up of both inner and outer cores; and 3) the inner-most lipid region, known as Lipid A, which anchors LPS into the outer membrane (Anwar & Choi, 2014). The Lipid A component consists of two phosphorylated glucosamine molecules each containing three fatty acid chains (Anwar & Choi, 2014). Although the molecular weight and size of LPS is mostly dependent on the O antigen region, which varies highly among

Gram-negative bacterial species, the Lipid A region is also important to consider as it plays a major role in the immunogenic characteristics of LPS (Molinaro et al., 2015).

The structure of LTA is composed of two main regions (Figure 1B): a longer repeating glycerophosphate chain, and the shorter glycolipid anchor (Shiraishi et al., 2016). The glycerophosphate chain contains variable side groups, which often include D-alanine, glucose, galactose, or *N*-acetylglucosamine (Shiraishi et al., 2016), and such variability contributes to inter-species differences in the functional and immunogenic characteristics of LTA (Kang et al., 2016). The glycolipid anchor can also vary among species, but is often composed of two to four hexose molecules joined to a diacylglycerol (or less commonly, triacylglycerol) (Shiraishi et al., 2016). Similarly to LPS, studies suggest that the lipid region of LTA is important for stimulation of innate immune cells (Han et al., 2003; Hong et al., 2014).

Although LPS and LTA clearly vary in structure, they also share some key similarities. Both LPS and LTA are cell surface molecules that allow for live bacteria to be recognized by innate immune cells. Importantly, both are also anchored into membranes through lipid regions, and thus will be referred to as "bacterial lipid moieties" or "bacterial lipids" throughout this thesis. The main roles of these bacterial lipids in bacterial physiology are to provide structural support; to separate the intracellular microenvironment from that outside of the bacterium in order to maintain homeostasis; and to protect from environmental stresses, such as physical, chemical, or biological insults (Putker et al., 2015; Shiraishi et al., 2016). Moreover, LTA can act as an adhesion

molecule, and thus may also play a role in host colonization by Gram-positive bacteria (Shiraishi et al., 2016).

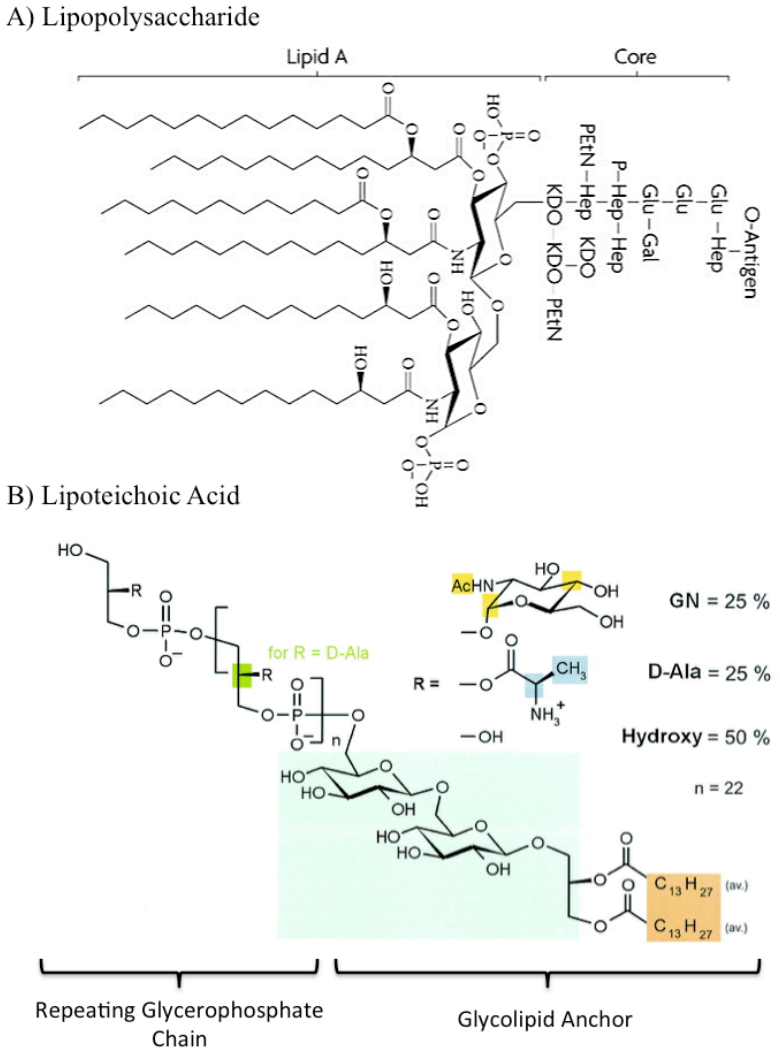

Figure 1. Structural representations of the general chemical structures of LPS (A) and LTA (B). PEtN, phosphoethanolamine; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-manno-oct-2-ulosonic acid; Glu, D-glucose; Gal, D-galactose; GN, glucosamine; D-Ala, D-alanine. Colour legend: Green = R groups; Light blue = dihexose region of glycolipid anchor; Blue = stereochemistry of D-alanine R group; Yellow = stereochemistry of glucosamine R group; Orange = lipophilic region of fatty acid chain. Modified from: (Morath et al., 2002) & (Ruiz et al., 2009).

B. IMMUNOGENICITY

LPS and LTA are both PAMPs, which refers to a class of molecules that can stimulate the innate immune response. Canonical activation of the inflammatory response by PAMPs is initiated through binding to a subset of PRRs, known as Toll-like receptors (TLRs) expressed by neutrophils, monocytes, macrophages, and other cell types (Oviedo-Boyso et al., 2014). LPS mainly binds to TLR4 (Chow et al., 1999), while LTA binds to TLR2 (Schwandner et al., 1999); however, both of these ligand-TLR interactions involve the CD14 co-receptor, and stimulate myeloid differentiation primary response gene 88 (MyD88)-dependent activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Oviedo-Boyso et al., 2014). Such activation of NF-κB results in transcription of a milieu of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, IL-17, and others, which play important roles in regulating inflammation in diseases such as sepsis (Tak & Firestein, 2001). Widespread release of these pro-inflammatory cytokines during sepsis can potentiate systemic leukocyte recruitment to the liver and other organs (Fox-Robichaud & Kubes, 2000; Ondiveeran & Fox-Robichaud, 2004; Kowalewska et al., 2011), and recruitment of activated leukocytes to the hepatic microcirculation during endotoxemia has been associated with hepatocyte apoptosis and resulting liver dysfunction (Eipel et al., 2004). Therefore, the exaggerated inflammatory response that occurs due to circulating PAMPs may contribute to hepatic dysfunction during sepsis.

While there is consensus regarding the pro-inflammatory nature of LPS, and the majority of the literature supports the pro-inflammatory nature of LTA (Kang et al., 2016),

it is worthy to note that some studies have raised questions regarding the physiological response to LTA *in vivo*. For example, intravital microscopy studies have demonstrated that LPS increases leukocyte recruitment in the microcirculation of various tissues following either local or systemic administration, while LTA does not (Yipp et al., 2002). These findings imply that there may be differences in how LPS and LTA activate leukocytes or endothelial cells in preparation for leukocyte recruitment, or differences in how LPS and LTA are handled in the circulation.

BACTERIAL LIPIDS, LIPOPROTEINS, AND THEIR RECEPTORS

In addition to binding to inflammatory cells through specific PRRs, circulating bacterial lipids can bind to a variety of endogenous lipid carrier proteins, which can facilitate receptor binding, or incorporation of LPS and LTA into lipoproteins with subsequent uptake through lipoprotein receptors (Walley et al., 2015).

A. LIPOPROTEINS & APOLIPOPROTEINS

The blood contains a variety of vesicular particles called lipoproteins, which function to carry hydrophobic or amphipathic substances, namely lipids or lipid moieties, within the hydrophilic environment found in blood. Lipoproteins can be classified into four major categories based on density (Feingold & Grunfeld, 2000), presented here in order from highest to lowest density: 1) high-density lipoprotein (HDL); 2) low-density lipoprotein (LDL); 3) very low-density lipoprotein (VLDL); and 4) chylomicrons. Additional subcategories have been derived from remnants or variations of the

lipoproteins in the aforementioned categories, including: intermediate-density lipoprotein (IDL), Lipoprotein(a), and chylomicron remnants (Feingold & Grunfeld, 2000). These lipoprotein groups not only vary in size and density, but more importantly in the types of cargo they carry (i.e. triglycerides, cholesterol, phospholipids, or other lipid moieties), and in the apolipoproteins they possess (see Table 1). The apolipoproteins act as ligands for receptor binding and subsequent endocytosis, and thus play a key role in determining the cell specificity of lipoprotein uptake based on receptor expression. A wide range of apolipoproteins exist and these have been previously described in detail (Feingold & Grunfeld, 2000), however, the noteworthy apolipoprotein groups include Apolipoprotein A (ApoA), Apolipoprotein B (ApoB), and Apolipoprotein E (ApoE). ApoA is subdivided mainly into ApoA-I and ApoA-II, the former of which is the major structural protein of HDL (Zhou et al., 2015), whereas ApoB exists as two main isoforms: ApoB48, which is found on chylomicrons (Kane et al., 1980), and ApoB100, which is a major component of LDL, VLDL, and IDL, and a key ligand for LDLR (Bradley et al., 1984; Yang et al., 1986). ApoE can also bind to LDLR (Brown et al., 1986), and is found on most types of lipoproteins except LDL, which suggests that most lipoproteins can be taken up through LDLR even though LDL is the prototypical ligand.

Both LPS and LTA have been found to distribute into a variety of lipoproteins, including HDL, VLDL, and LDL within human blood (Levels et al., 2001; Levels et al., 2003). Interestingly, the distribution profiles for LPS and LTA were found to be similar, such that ~60-70% of each bacterial lipid distributed into HDL, ~25-30% into LDL, and 4-12% into VLDL, when incubated with human blood *ex vivo* (Levels et al., 2001; Levels

et al., 2003). These findings suggest that HDL, LDL, and to a lesser extent, VLDL, may play important roles in the physiological handling and potential uptake of bacterial lipids by various cell types.

Table 1. Characteristics of major lipoprotein classes

Lipoprotein	Density (g/mL)	Size (nm)	Major Apolipoproteins	Major Cargo	Main Receptors
Chylomicrons & remnants	< 0.930	75–1200	ApoB48, ApoA-I, A-II, A-IV, ApoC, ApoE	Triglycerides, LPS/LTA(?)	LRP1, LDLR, LRP8/ApoER2
VLDL	0.930–1.006	30–80	ApoB100, ApoE, ApoC	Triglycerides, LPS/LTA(?)	VLDLR, LDLR, LRP1, LRP8/ApoER2
LDL	1.019–1.063	18–25	ApoB100	Cholesterol, LPS/LTA(?)	LDLR
HDL	1.063–1.210	5–12	ApoA-I, Apo A-II, ApoC, ApoE	Cholesterol, Phospholipids, LPS/LTA(?)	SR-BI, LDLR

Updated from: (Feingold & Grunfeld, 2000). Additional references: (Brown et al., 1986), (Havel, 1998), (Sasamoto et al., 2017)

B. LIPID TRANSFER PROTEINS

Prior to incorporation into lipoproteins, bacterial lipids and endogenous lipid moieties in the blood may bind to lipid transfer proteins. Lipid transfer proteins are a class of small (typically < 10 kDa), soluble, cysteine-rich proteins that generally contain four or five α-helices stabilized by disulphide bridges, which collectively form a tunnel within the protein that functions to bind lipids and provide shelter from the surrounding aqueous environment (Salminen et al., 2016). Although there are many different lipid transfer proteins that belong to different gene families across various animal species, some of the main lipid transfer proteins found in humans include cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), lipopolysaccharide binding protein (LBP), and bactericidal/permeability-increasing protein (BPI). Of these, CETP and PLTP are known to play roles in the transfer of endogenous lipids between lipoproteins, while LBP, BPI, and PLTP can interact with LPS and possibly other bacterial lipids (Walley et al., 2015). These interactions can include detoxification, by packaging free-LPS into chylomicrons or other lipoproteins (Vreugdenhil et al., 2003); transfer from one type of lipoprotein to another, such as transfer of LPS from HDL to LDL by LBP and PLTP (Levels et al., 2005); or potentially, transfer to a receptor, such as the proposed LBP/CD14 pathway (Steinemann et al., 1994) that may be involved in delivering LPS to TLR4.

C. LIPOPROTEIN RECEPTORS

Classical uptake of lipoproteins and their lipid cargo occurs through a family of structurally related receptors, which include LDLR, VLDL receptor, and several LDL receptor-related proteins (LRPs), such as LRP1 (May et al., 2007), as well as a structurally distinct receptor named scavenger receptor class B, type I (SR-BI) (Pagler et al., 2006). As previously mentioned, LDLR binds to ApoB100 found on the surface of LDL and VLDL particles resulting in their endocytosis, whereas VLDL receptor binds to apolipoprotein E, which is found on chylomicrons, VLDL, and some HDL particles, but not on LDL (Feingold & Grunfeld, 2000). On the contrary, LRP1 and SR-BI can bind to a wide variety of lipoproteins, though SR-BI plays a more prominent role in lipid homeostasis by mediating reverse cholesterol transport through HDL (Pagler et al., 2006). The cell and tissue expression patterns of these receptors, along with the balance of various lipoproteins found in the blood, are both important factors in maintaining lipid homeostasis within the blood and different tissues. Furthermore, a growing body of evidence suggests that dysregulated lipid homeostasis may play a major role in the pathophysiology of sepsis.

Since LPS and LTA have both been shown to distribute into a variety of lipoproteins, and lipoproteins are internalized through the aforementioned lipoprotein receptors by many cell types, including hepatocytes, it has been hypothesized that lipoprotein-bound bacterial lipid moieties can be taken up by hepatocytes similarly to how hepatocytes normally take up cholesterol from circulating LDL (Walley et al., 2015). Indeed, LDLR is known to play a role in LPS uptake by hepatocytes (Topchiy et al.,

2016), and lipoprotein-bound LPS has been shown to induce cytokine tolerance in hepatocytes through LDLR-mediated internalization of LPS by these cells (Harris & Kasravi, 2003; Kasravi et al., 2003). However, no studies have directly investigated whether LDLR-mediated uptake of LPS is lipoprotein-dependent, or whether LDLR or other lipoprotein receptors are involved in LTA uptake by hepatocytes. The importance of LDLR in sepsis is supported by studies in $LDLR^{-/-}$ mice, which demonstrate increased mortality compared to wild-type mice undergoing cecal ligation and puncture (CLP)-induced sepsis (Lanza-Jacoby et al., 2003). Therefore, investigating the mechanisms of bacterial lipid uptake should help to clarify the role of LDLR in the complex pathophysiology of sepsis, and may establish novel therapeutic targets for sepsis.

LIPOPROTEIN RECEPTOR REGULATION BY PCSK9

Proprotein convertase subtilisin/kexin (PCSK) type 9 (PCSK9) is a serine protease that is abundantly expressed in the liver, and to a lesser extent in the intestines and kidneys of mice (Zaid et al., 2008). In contrast to other PCSKs, PCSK9 does not catalyze the conversion of proproteins or zymogens to their mature protein forms, but rather catalyzes its own maturation through autocatalytic cleavage of its pro-domain which inhibits further proteolytic activity (Cunningham et al., 2007). PCSK9 has both chaperone-like (Strom et al., 2014) and receptor regulatory activity (Zaid et al., 2008; Canuel et al., 2013). The circulating form of PCSK9 is produced exclusively by the liver (Zaid et al., 2008), and is found in plasma at average concentrations ranging from 170 – 220 ng/mL in healthy individuals, while concentrations are increased in adults with sepsis

(Boyd et al., 2016). According to current knowledge, the primary functional role of circulating PCSK9 is to negatively regulate cell surface LDLR concentrations (Schulz et al., 2015). Secreted PCSK9 binds the epidermal growth factor repeat A (EGF-A) region of LDLRs located on the cell surface of hepatocytes, and induces clathrin-mediated endocytosis of the PCSK9-LDLR complex, which prevents recycling of LDLR and induces its degradation (Figure 2) (Jeon & Blacklow, 2005; Zhang et al., 2007). Such regulation of LDLR by PCSK9 has important consequences on cholesterol homeostasis, as evidenced by PCSK9 mutations playing a causal role in certain cases of autosomal dominant hypercholesterolemia (Abifadel et al., 2003). In fact, PCSK9 plays such an important role in hypercholesterolemia that two human monoclonal antibodies against PCSK9—alirocumab and evolocumab—are currently FDA-approved for the treatment of patients with heterozygous familial hypercholesterolemia and atherosclerotic cardiovascular disease (Mullard, 2015).

Although the primary hepatic target of PCSK9 is LDLR, PCSK9 can also degrade LRP1 (Canuel et al., 2013), VLDL receptor, and LRP8/apolipoprotein E receptor 2 (ApoER2) (Poirier et al., 2008). Of these targets, LRP1 is expressed in the liver by hepatocytes and contributes to postprandial uptake of chylomicrons (Laatsch et al., 2009). Furthermore, LRP1 has been computationally identified as a candidate receptor for TpeL toxin produced by *Clostridium perfringens* (Schmidt et al., 2015), suggesting a potential role for LRP1 in the immune response against bacteria. Indeed, LRP1 can induce anti-inflammatory signaling by negatively regulating NF-κB activity through downregulation of tumor necrosis factor receptor 1 (Gaultier et al., 2008). Furthermore, since LRP1 is a

promiscuous lipoprotein receptor with capabilities for endocytosis, it is plausible that LRP1 could mediate uptake of LPS and/or LTA. Therefore, we aimed to investigate whether regulation of LRP1 plays a role in bacterial lipid uptake by hepatocytes, as such a role for this receptor has not been previously described to our knowledge.

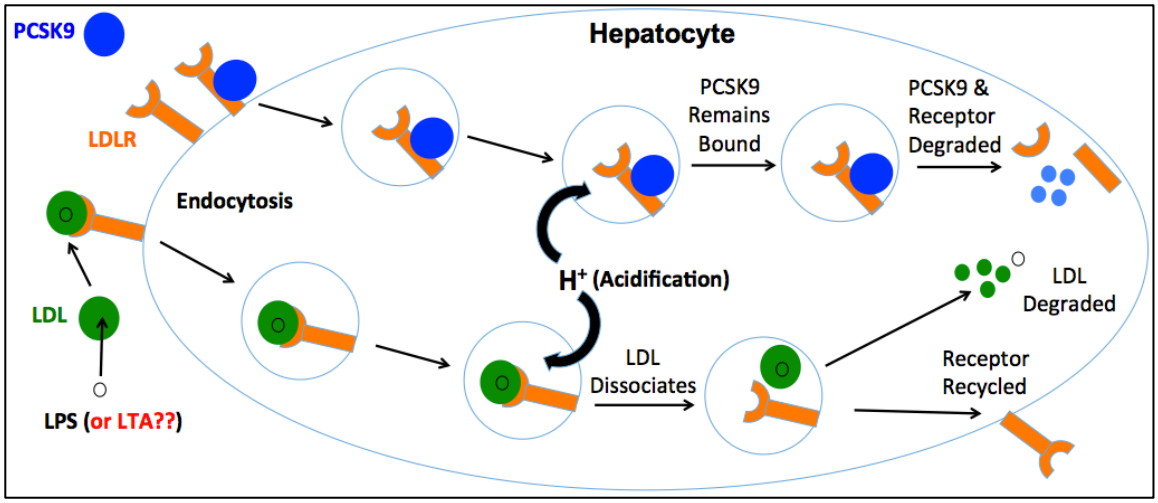

Figure 2. Mechanism of LDLR degradation by PCSK9, and proposed mechanism of LPS/LTA uptake by hepatocytes. Circulating PCSK9 binds to LDLR on the cell surface of hepatocytes. After internalization, PCSK9 remains bound to LDLR in acidified endosomes, thus targeting the complex for lysosomal degradation. By decreasing LDLR concentrations on the cell surface through this mechanism, PCSK9 may reduce LDLR-mediated uptake of LPS and LTA by hepatocytes.

ROLE OF PCSK9 IN SEPSIS & ENDOTOXEMIA

In an elegant translational study, PCSK9 has recently been found to regulate the innate immune response during sepsis (Walley et al., 2014). In this study, septic shock patients with loss-of-function (LOF) mutations in PCSK9 had higher survival rates than patients with normal or gain-of-function mutations in PCSK9 (Walley et al., 2014). Furthermore, this effect was LDLR-dependent, as patients carrying LOF PCSK9 mutations together with homozygous LOF LDLR mutations did not receive such survival benefit. Reductions in mortality, as well as decreased plasma concentrations of TNF-α, murine monocyte chemoattractant protein-1 (JE/MCP-1), and macrophage inflammatory protein-2 (MIP-2), were also observed in CLP mice treated with PCSK9-blocking antibody, compared to isotype controls after 72 hours (Walley et al., 2014). $PCSK9^{-/-}$ mice injected with LPS also had significant reductions in cytokines, such as TNF-α, IL-6 and IL-10, when compared to wild-type mice at 6 hours after LPS administration, indicating that PCSK9 deficiency ameliorates the inflammatory response to bacterial endotoxin (Walley et al., 2014). Furthermore, PCSK9 inhibition reduces the effects of LPS on body temperature and activity in wild-type mice, but not $LDLR^{-/-}$ mice, which again suggests downstream involvement of LDLR.

In another study, plasma PCSK9 concentrations were found to be increased in patients with early sepsis, and such increases correlated with increased odds of developing more than one organ failure (Boyd et al., 2016). More specifically, patients with respiratory failure or septic shock had increased PCSK9 levels compared to septic patients without these complications (Boyd et al., 2016). Previous findings from our

group also support an important role for PCSK9 in the pathophysiology of sepsis, as we observed that differential PCSK9 expression modulates morbidity, infectious burden, coagulation markers, and lung and liver pathology, in addition to inflammatory cytokines, in a mouse model of CLP-induced sepsis (Dwivedi et al., 2016). We found that transgenic mice overexpressing PCSK9 had increased hepatic inflammation and injury in conjunction with increased plasma IL-6, as well as increased inflammation in the lungs, whereas $PCSK9^{-/-}$ mice had reduced inflammation and injury along with reduced bacterial dissemination into the blood (Dwivedi et al., 2016). Collectively, these findings support a key role for PCSK9 in regulating inflammation during sepsis, and suggest that this role has important negative implications in the pathophysiology of sepsis and organ dysfunction.

In vitro, exogenous administration of recombinant human PCSK9 reduced uptake of LPS by HepG2 cells (Walley et al., 2014; Boyd et al., 2016). Since PCSK9 is known to target hepatic LDLR for degradation (Zaid et al., 2008), and LDLR can mediate uptake of lipoprotein-bound LPS (Harris & Kasravi, 2003), it has been hypothesized that depletion of PCSK9 decreases the pro-inflammatory response in sepsis by increasing LDLR-mediated uptake of pathogen lipids, such as LPS and LTA (Walley et al., 2015). Although much is known about the role of PCSK9 in regulating LDLR, there are missing links in proving how this mechanism is responsible for regulating cytokine production during sepsis. Furthermore, little is known about the physiological uptake and clearance of Gram-positive LTA, and its relevance to the inflammatory response during sepsis.

PURPOSE OF THIS STUDY

The purpose of this study was to characterize the PCSK9-regulated pathway of LPS and LTA uptake by hepatocytes, and to assess whether such uptake is associated with cytokine production by these cells. In order to accomplish this, we used the well-studied HepG2 hepatocyte cell line that expresses many of the same receptors as those expressed by primary hepatocytes, including LDLR and LRP1 (Canuel et al., 2013). We hypothesized that LPS and LTA uptake occurs through both LDLR and LRP1, and is regulated by PCSK9. We further hypothesized that lipoproteins are required for these lipoprotein receptor-dependent uptake mechanisms, and went on to characterize which exact lipoproteins are necessary. In an effort to extend our findings *in vivo,* we also performed pilot studies to determine which techniques might be used to further study the physiological relevance of the LPS and LTA uptake mechanisms that we established *in vitro*.

METHODS AND MATERIALS

Preparation of BODIPY 630/650-LTA and Lipoprotein-Deficient Human Serum

LTA from *Enterococcus hirae* was fluorescently labeled using the BODIPY 630/650-X NHS Ester according to manufacturer's protocols (Life Technologies). BODIPY-LTA was separated from unbound fluorophore by filtration through a Sephadex G25 column, and the absorbance of each eluted fraction was measured through spectrophotometry at the peak excitation wavelength of 632 nm. Concentrated fractions were pooled, and absorbance was measured to calculate the final concentration of fluorescently labeled LTA using the known extinction coefficient of the fluorophore.

Lipoprotein-deficient serum was prepared through ultracentrifugation of pooled human serum, collected from seven healthy donors, at 298,000 × g for 48 h at 4 °C, after increasing serum density to 1.215 g/mL with KBr as previously described (Krieger et al., 1981). Following extraction of the lipoprotein-deficient serum fraction, dialysis was performed nine times against 4 L of normal saline over a total of 96 hours at 4 °C through a SpectraPor 4 dialysis membrane to remove any residual KBr. Total protein concentration in the lipoprotein-deficient serum was measured with a Pierce BCA Protein Assay (ThermoFisher), and sterile normal saline was used to adjust the protein concentration to 70 mg/mL.

HepG2 Cell Culture & LTA Uptake Time Course and Dose-Response

HepG2 immortalized human hepatocytes, which express many of the same receptors as primary hepatocytes, including LDLR and LRP1 (Canuel et al., 2013), were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% Fetal Bovine Serum (FBS), and 1% penicillin-streptomycin (100 U/mL of penicillin and 100 μg/mL of streptomycin). Time course experiments of LTA uptake by HepG2 cells were performed by seeding 3×10^5 cells per well into 6-well plates containing sterilized coverslips, and treating cells with 10 μg/mL of BODIPY-LTA for up to 24 hours in media containing 80% DMEM (with 1% penicillin-streptomycin) and 20% normal human serum. After 24 hours, cells were fixed with 4% paraformaldehyde and the coverslips were mounted with DAPI-containing medium onto microscope slides for imaging. Dose-response experiments were performed in 24-well plates by treating HepG2 cells with 1, 2.5, 5, 10, 25, 50, or 100 μg/mL concentrations of BODIPY-LTA over 24 hours in media containing 80% DMEM (with 1% Penicillin-Streptomycin) and 20% normal human serum. Cells were harvested with trypsin/EDTA, and flow cytometry was used to measure the geometric mean fluorescence intensity of 10,000 cells per treatment group.

Effects of PCSK9, anti-LDLR and anti-LRP1 on LPS and LTA Uptake by HepG2 Cells

HepG2 cells were seeded overnight into 24-well plates at a density of 5×10^4 cells per well, after which media was changed to 80% DMEM (containing 1% penicillin-streptomycin), and 20% normal human serum or lipoprotein-deficient human serum

(EMD Millipore). Cells were then pretreated with 2.5 μg/mL of recombinant human PCSK9 (AcroBiosystems) or vehicle control at 6 hours before LPS or LTA treatment. Two hours prior to LPS or LTA treatment, cells were pre-treated with 5 μg/mL of anti-human LDLR antibody (R&D Systems), anti-human LRP1 antibody (ThermoFisher), or IgG isotype control (Jackson ImmunoResearch Laboratories). Cells were then treated with 2.5 μg/mL of AlexaFluor 488-labeled *Escherichia coli* LPS (Life Technologies) or control non-fluorescent *E. coli* LPS (Sigma-Aldrich), or 10 μg/mL of BODIPY 630/650-labeled *E. hirae* LTA or control non-fluorescent *E. hirae* LTA (Sigma-Aldrich) for 24 hours. Cells were harvested with trypsin/EDTA, and the geometric mean fluorescence intensity of 10,000 cells per treatment group was measured through flow cytometry with results normalized by subtracting background auto-fluorescence of controls. Cell culture supernatant was collected from these experiments and frozen at −80 °C for multiplex cytokine assays to measure IL-6, IL-8, IL-10, and IL-17 (Luminex, R&D Systems), and for measurement of extracellular alanine aminotransferase (ALT) activity (Cayman Chemical Company). To obtain HepG2 cell lysate as a positive control for ALT activity assays, HepG2 cells were grown to confluence in 24-well plates and lysed with RIPA buffer containing 1% protease inhibitor cocktail (Sigma) at 4°C for 1 h on a plate shaker. THP-1 monocytes were cultured in RPMI-1640 complete medium containing 10% FBS, and treated with 2.5 μg/mL of non-fluorescent *E. coli* LPS or 10 μg/mL of non-fluorescent *E. hirae* LTA for 24 h to obtain cell conditioned media as a positive control for the multiplex cytokine assay.

Effects of LDL or HDL Add-back to Lipoprotein-Deficient Serum on LPS & LTA Uptake by HepG2 Cells

Cells were seeded into 24-well plates at a density of 5×10^4 cells per well and allowed to adhere overnight. Next day, media was changed to 80% DMEM (containing 1% penicillin-streptomycin) and 20% lipoprotein-deficient human serum. Cells were then treated with increasing concentrations of LDL or HDL (0, 5, 10, 25, 50, 100, 200, or 300 μg/mL), and either 2.5 μg/mL of AlexaFluor 488-LPS, or 2.5 μg/mL or 10 μg/mL of BODIPY 630/650-LTA for 24 hours. Flow cytometry was used to measure the geometric mean fluorescence intensity of 10,000 cells per group.

Effects of blocking LDLR on uptake of LPS & LTA in normal serum, or lipoprotein-deficient serum with or without add-back of LDL or HDL

HepG2 cells were seeded into 24-well plates as described above. The following morning, media was changed to 80% DMEM (enriched with 1% penicillin-streptomycin) and 20% human serum, which was either pooled normal serum (control) from healthy donors, or donor-matched lipoprotein-deficient serum with or without add-back of 100 μg/mL of purified human LDL or HDL. One hour later, cells were treated with anti-LDLR antibody or control IgG for two hours prior to treatment with 2.5 μg/mL of AlexaFluor-488 labeled LPS or BODIPY 630/650-labeled LTA. After 24 hours, cells were harvested and flow cytometry was performed to assess uptake of the fluorescent bacterial lipids by measuring the geometric mean fluorescence intensity of 10,000 cells per treatment group.

Western Blot for LDLR

HepG2 cells were grown to confluence in 12-well plates containing DMEM supplemented with 20% normal human serum. These cells were treated for 6 hours with 2.5, 5 or 10 μg/mL of PCSK9, or left untreated. As a positive control for LDLR expression, CHO 13-5-1 cells were also grown to confluence in DMEM/F-12 media supplemented with 10% FBS, which is a standard choice of media for this cell type. Total protein was harvested by lysing cells on ice in RIPA buffer containing 1% protease-inhibitor cocktail (Sigma-Aldrich). Cell lysates were then cleared through centrifugation at 15,000 × g for 12 min, and total protein was measured using a Pierce BCA Protein Assay (ThermoFisher). Fifty micrograms of protein were separated using SDS-PAGE on a 4–15 % Tris glycine gel, and transferred to a nitrocellulose membrane over 1.5 h at 80V. After blocking, the membrane was incubated with primary antibodies against LDLR (goat anti-human LDLR, 1:1000, R&D Systems) and β-actin (rabbit anti-β actin, 1:5000, Proteintech) overnight at 4°C. Following three washes, the membrane was incubated with AlexaFluor-488 labeled donkey anti-goat and donkey anti-rabbit antibodies (1:1000, Life Technologies) for 2 h at room temperature. After washing over 30 min, fluorescence imaging was performed using the BioRad ChemiDoc MP imaging system.

Animals

Male and female mice (20–30 g weight) were utilized for all animal experiments, which have been approved by the Animal Research Ethics Board at McMaster University and meet the criteria for ethical treatment of animals established by the Canadian Council on

Animal Care. $PCSK9^{+/-}$ and PCSK9 Tg mice were generously provided by Dr. Nabil G. Seidah, whose lab previously generated these strains (Zaid et al., 2008). $PCSK9^{-/-}$ and wild-type mice were obtained by breeding heterozygous mice, while female PCSK9 $Tg^{+/0}$ (Tg) mice were bred with wild-type male mice to produce $Tg^{+/0}$ and $Tg^{0/0}$ (wild-type) mice. All mice are on a C57BL/6J background, are housed in standard clean environments with regulated 12 h light/dark cycles, and have access to food and water *ad libitum*.

Pilot Experiments assessing Effects of Differential PCSK9 Expression on LPS or LTA Uptake in the Mouse Liver

Mice were anesthetized using Isoflurane and injected with 5 mg/kg of BODIPY 630/650-LTA or FITC-LPS (Sigma-Aldrich), either intravenously through the tail vein or jugular vein, or through the intraperitoneal route of injection. In a subset of experiments, mice were sacrificed after 6 hours, and the liver was harvested and snap-frozen in OCT for frozen sectioning. Frozen sections of 5 μm thickness were fixed in acetone, stained with DAPI and mounted on slides for fluorescence microscopy (Olympus DP72 Microscope). Images were captured using Slidebook 5.0 Software, and images from the DAPI filter were overlaid onto images taken under the FITC filter for LPS experiments or TRITC filter for LTA experiments. In another subset of experiments, mice were anesthetized using ketamine-xylazine and the liver was surgically prepared for intravital microscopy as previously described (Patrick et al., 2017). At 5 hours after injection of BODIPY-LTA or control, intravital confocal microscopy of the liver was performed. Vascular beds

(sinusoids vs. venules) were first identified using transillumination intravital microscopy, after which confocal microscopy was utilized to measure the maximum fluorescence intensity of 3-5 independent fields of view per mouse. Data were analyzed using Slidebook 6.0 Software, and the mean of the maximum fluorescence intensity from all fields of view was calculated for each animal.

Plasma PCSK9 Concentrations in Septic ICU Patients

PCSK9 concentrations were measured in plasma collected from intensive care unit (ICU) patients, who were enrolled in the DYNAMICS study (ClinicalTrials.gov identifier: NCT01355042) between November 2010 and January 2013 from nine tertiary care centers across Canada. Patients for this study were recruited according to previously described inclusion and exclusion criteria (Dwivedi et al., 2012). PCSK9 concentrations were measured, using ELISA as previously described (Dubuc et al., 2010), in plasma samples from 39 patients with documented Gram-positive or Gram-negative bacterial sepsis, and in plasma collected from 14 healthy volunteers.

Statistical Analyses

Student's unpaired t-test or analysis of variance (ANOVA) were used for all statistical analyses with a significance threshold of $p < 0.05$ (GraphPad Prism 5.0).

RESULTS

Preliminary Dose-Response & Time-Course Experiments in HepG2 Cells

To determine whether LTA uptake by hepatocytes occurs similarly to previously documented LPS uptake (Topchiy et al., 2016), we examined the time- and dose-dependence of this process in human HepG2 cells, an immortalized hepatocyte cell line. Over the course of 24 h, HepG2 cells progressively internalized BODIPY-LTA in a time-dependent (Figure 3, A–D) and dose-dependent manner that did not reach saturation even at high concentrations of 100 μg/mL (Figure 4A). In a preliminary dose-response experiment to determine effective concentrations of recombinant human PCSK9, and anti-LDLR or anti-LRP1 antibodies, we observed that PCSK9 and anti-LDLR, but not anti-LRP1, dose-dependently inhibited uptake of LPS by HepG2 cells (Figure 4B). The saturating effects at PCSK9 concentrations approaching 10 μg/mL on LPS uptake are consistent with previous findings (Boyd et al., 2016). From this preliminary experiment, we chose fixed concentrations of 2.5 μg/mL of PCSK9 and 5 μg/mL of anti-LDLR or anti-LRP1 antibodies for further experiments. Importantly, these particular concentrations were chosen to investigate whether there is an additive effect of treatment with PCSK9 in conjunction with either of these antibodies, as none of these concentrations resulted in maximal effect in our preliminary dose-response experiment (Figure 4B).

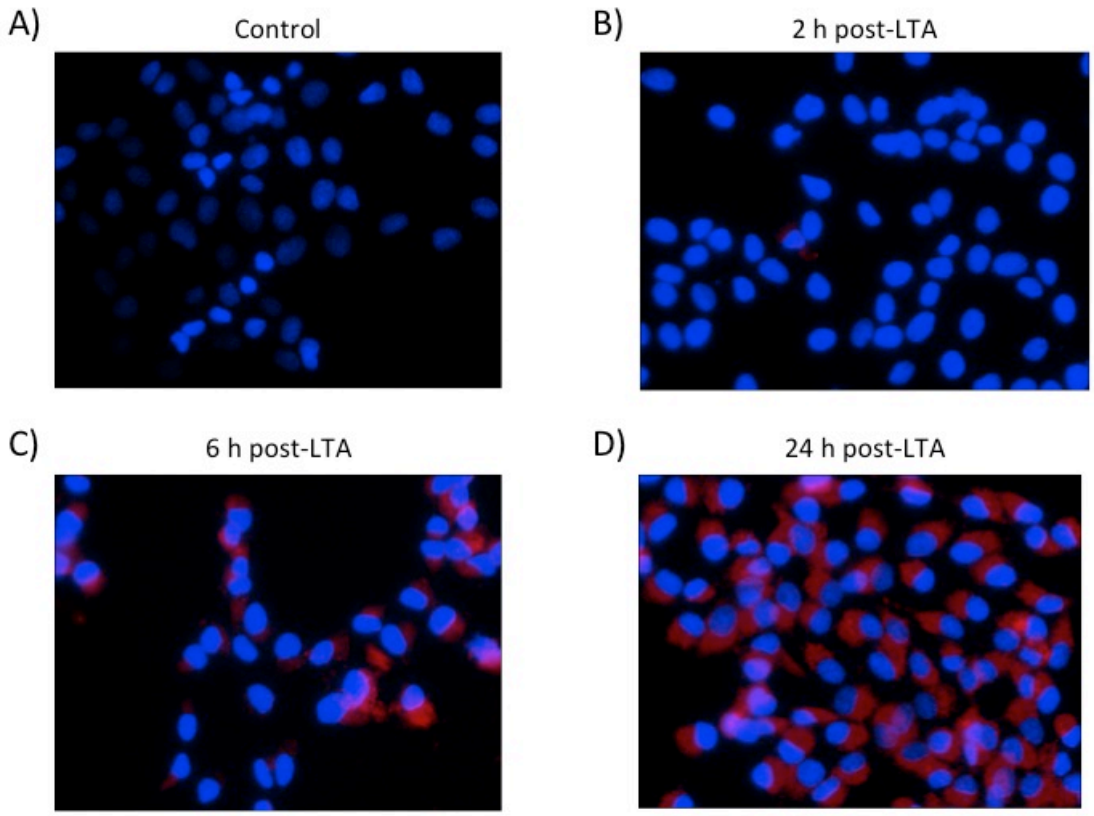

Figure 3. Time course of LTA uptake by HepG2 cells over 24 h. Cells were cultured in DMEM supplemented with 20% normal human serum, and uptake of fluorescent LTA was visualized through fluorescence microscopy in untreated cells (A) or in cells treated with 10 μg/mL of BODIPY 630/650-LTA for 2 h (B), 6 h (C), or 24 h (D). Nuclei are stained in blue with DAPI and BODIPY 630/650-LTA appears red. Data are representative of 3 independent experiments.

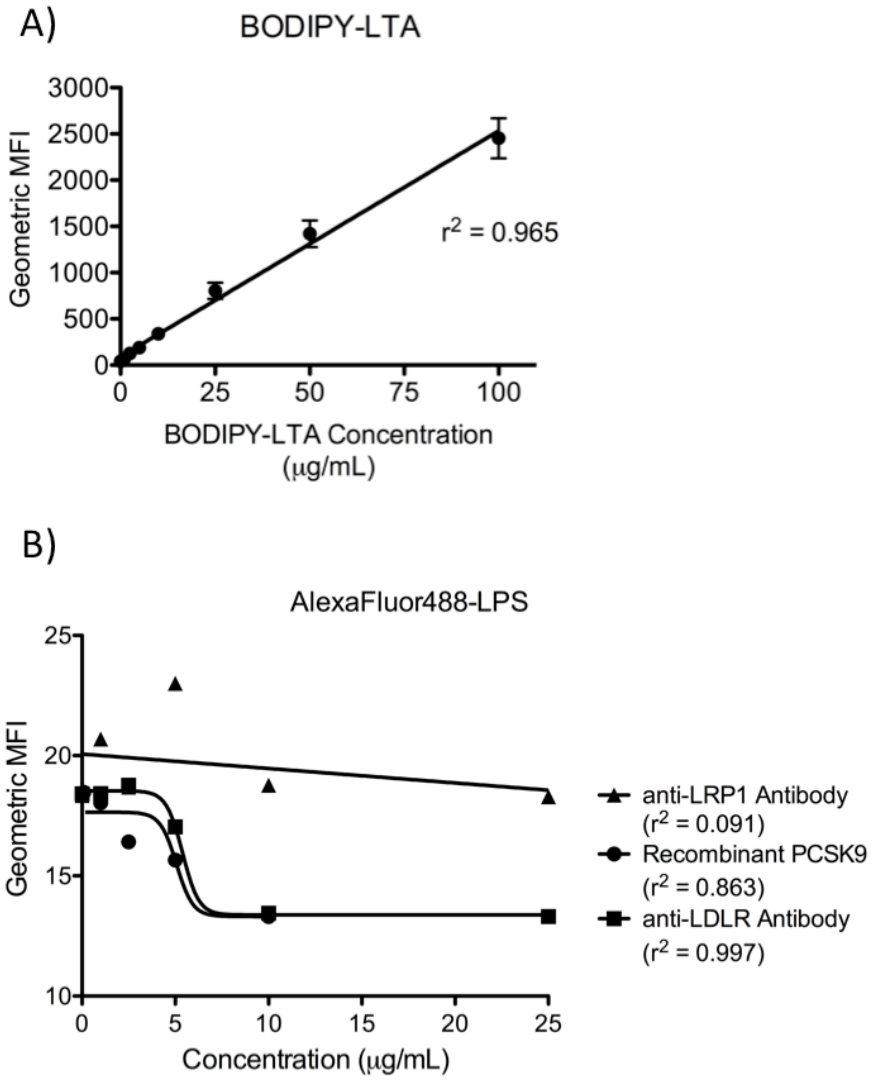

Figure 4. Dose-response curves of LTA (A) and LPS (B) uptake by HepG2 cells. BODIPY 630/650-LTA uptake at increasing concentrations (A), and uptake of 2.5 μg/mL AlexaFluor 488-LPS by HepG2 cells in response to increasing concentrations of recombinant human PCSK9, anti-LDLR antibody, or anti-LRP1 antibody (B) were measured after 24 h. Data are presented as geometric mean ± SEM obtained from 3 independent experiments (A), or as individual data points from a single experiment (B).

Determining which Lipoprotein Receptors targeted by PCSK9 are involved in Bacterial Lipid Uptake

To determine which lipoprotein receptors regulated by PCSK9 are involved in uptake of bacterial lipids, we treated human HepG2 cells with LDLR-blocking antibody, LRP1-blocking antibody, or control IgG, with or without PCSK9 pre-treatment, and measured uptake of fluorescently labeled LTA or LPS. For experiments conducted in normal serum, we observed that PCSK9 and anti-LDLR treatments significantly reduced uptake of LTA and LPS compared to controls, while blocking LRP1 had no effect (Figure 5A,C). Furthermore, treating cells with both PCSK9 and LDLR-blocking antibody did not have an additive effect on uptake of LPS or LTA, which suggests that the negative regulatory effects of PCSK9 on bacterial lipid uptake by HepG2 cells occur solely through LDLR. This mechanism was also found to be lipoprotein-dependent, as PCSK9 and anti-LDLR treatments had no effect on LTA or LPS uptake by HepG2 cells when cultured in lipoprotein-deficient serum (Figure 5B,D).

Assessing the Physiological Response of HepG2 Cells to Variable Uptake of LPS and LTA

In order to assess if differential uptake of LPS or LTA results in an inflammatory response or injury to hepatocytes, we measured inflammatory cytokines including IL-6, IL-8, IL-10, and IL-17, as well as alanine aminotransferase (ALT) activity in the cell culture supernatant obtained from these experiments. There were no significant differences between experimental groups for any of the measured cytokines, which were

expressed at low concentrations ≤ 10 pg/mL, whereas THP-1 monocytes produced significantly increased concentrations of IL-6 in response to LPS, and IL-8 in response to both LPS and LTA (Figure 6, A–D). Differential uptake of LPS or LTA also resulted in no significant differences in hepatocellular injury as measured by extracellular ALT activity in the cell culture supernatant, whereas ALT activity was significantly increased in HepG2 cell lysate that served as a positive control for this assay (Figure 6E,F). Collectively these findings suggest that differential uptake of LPS or LTA does not result in a pro-inflammatory response or hepatocellular injury in HepG2 hepatocytes.

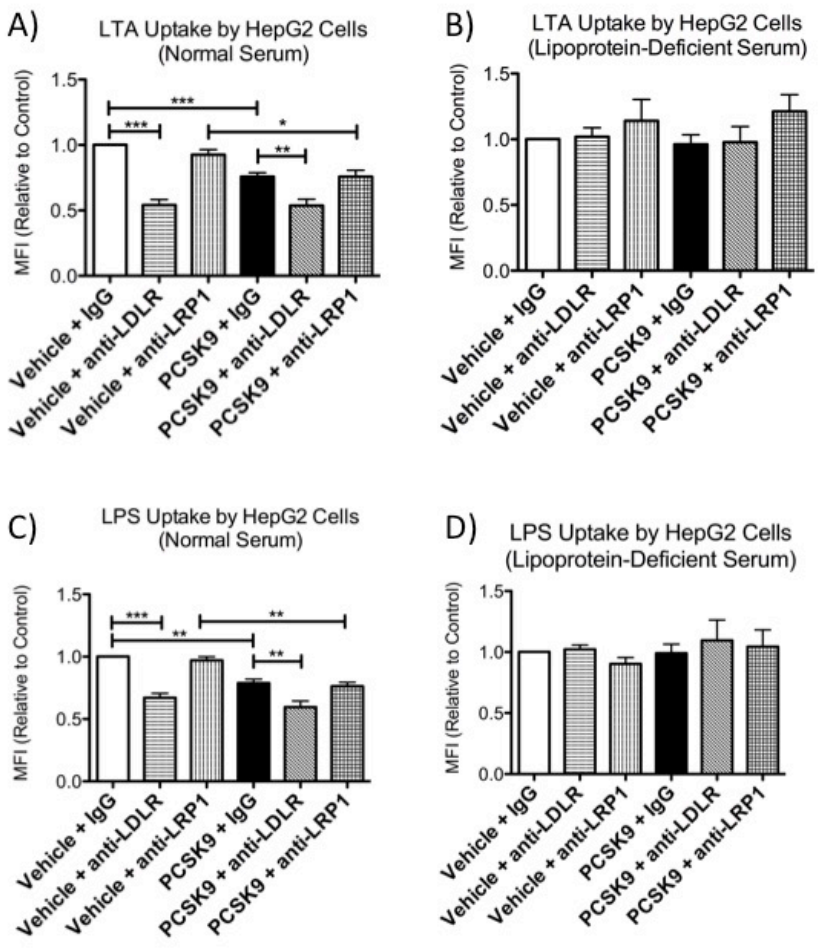

Figure 5. PCSK9 regulates lipoprotein-dependent uptake of LTA (A&B) and LPS (C&D) by HepG2 cells through LDLR, not LRP1. HepG2 cells were cultured in 20% normal human serum (A&C) or lipoprotein-deficient human serum (B&D) and were pre-treated with 2.5 μg/mL of recombinant human PCSK9 or vehicle control at 6 h before, as well as anti-LDLR, anti-LRP1 or control IgG antibodies at 2 h before treatment with BODIPY 630/650-LTA (10 μg/mL; A&B) or AlexaFluor 488-LPS (2.5 μg/mL; C&D) for 24 h. Data were collected from 4–5 independent experiments at 24 hours after LPS or LTA treatment using flow cytometry, and are expressed as geometric mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by one-way ANOVA.

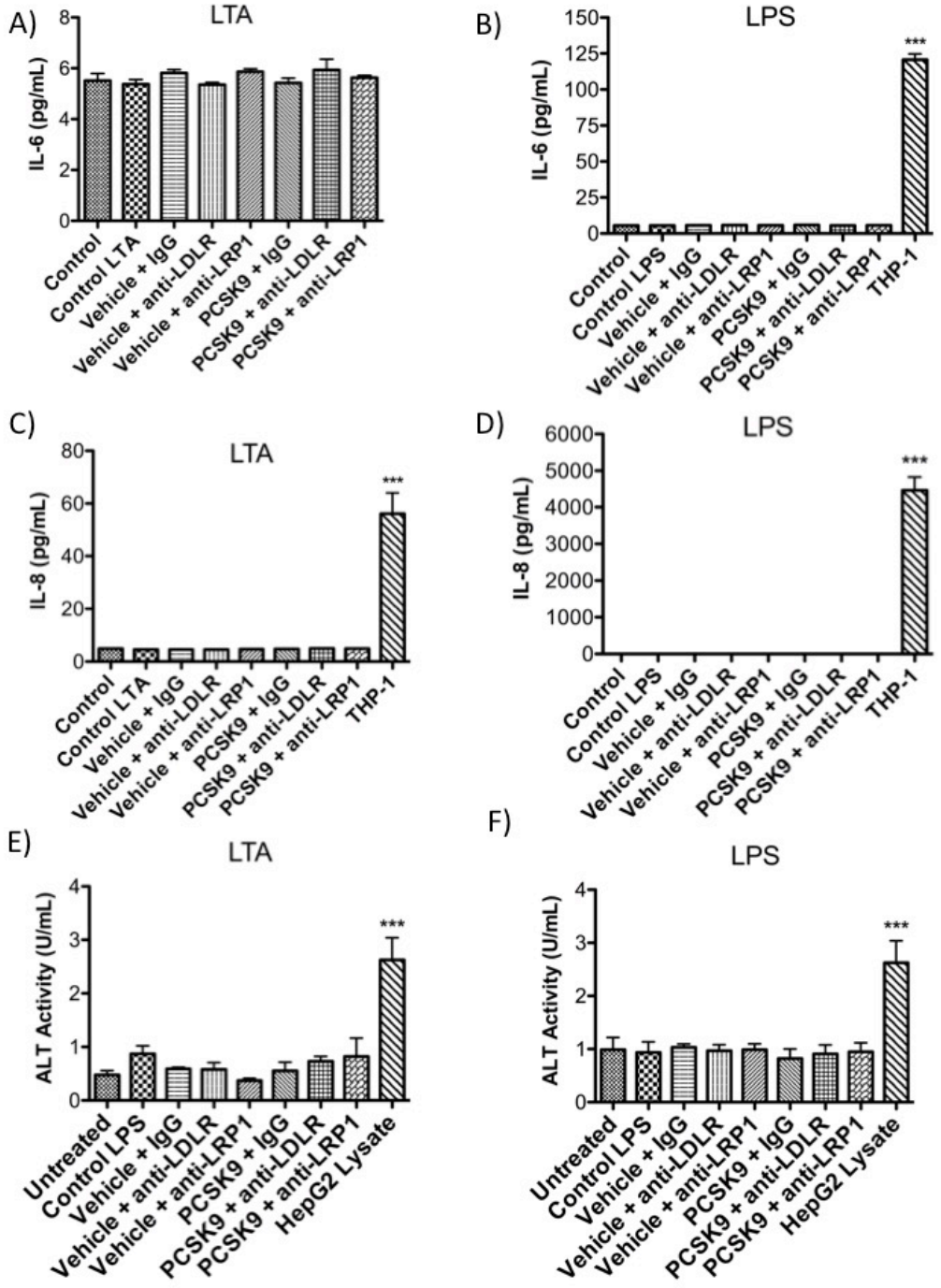

Figure 6. Cytokine concentrations (A-D) and ALT activity (E&F) in cell culture supernatant collected from HepG2 cells with differential uptake of LTA and LPS following pre-treatment with PCSK9, anti-LDLR and/or anti-LRP1. HepG2 cells

were cultured in 20% normal human serum, and were treated with 10 μg/mL of BODIPY 630/650-LTA (A,C,E) or 2.5 μg/mL of AlexaFluor 488-LPS (B,D,F) for 24 h. Data are expressed as mean ± SEM from 4–5 independent experiments. IL-10 and IL-17 concentrations were below the detectable limit, thus data are not shown. THP-1 monocytes were treated over 24 h with 2.5 μg/mL of *E. coli* LPS or 10 μg/mL of *E. hirae* LTA as a positive control for cytokine secretion, and HepG2 cell lysate was used as a positive control for ALT activity. Note: THP-1 monocytes did not produce IL-6 in response to LTA treatment. *** $p<0.001$ by one-way ANOVA.

Determining which Lipoproteins are required for LDLR-mediated Uptake of Bacterial Lipids

To determine which lipoproteins are required for LDLR-mediated uptake of LPS and LTA by HepG2 cells, we first performed dose-response experiments to assess the effects of increasing LDL or HDL concentrations on bacterial lipid uptake. Adding LDL back into lipoprotein-deficient serum at increasing concentrations resulted in dose-dependent increases in LTA and LPS uptake by HepG2 cells, whereas add-back of HDL demonstrated no such effect (Figure 7, A-C). Interestingly, LDL add-back demonstrated a similar saturating effect on LPS and LTA uptake at ≥50 μg/mL LDL concentrations in the presence of 2.5 μg/mL concentrations of these bacterial lipids (Figure 7A,B), whereas saturation did not occur until ≥200 μg/mL LDL concentrations for 10 μg/mL of LTA (Figure 7C). This suggests that there is a linear relationship between the LDL concentrations in serum and its capacity to bind and facilitate uptake of bacterial lipids, as a 4-fold increase in LDL is required to achieve saturation of uptake following a 4-fold increase in bacterial lipid concentration. Collectively, these data suggest that LDL may be required for LDLR-mediated uptake of bacterial lipids, while HDL is not, and that LDL concentrations appear to be a limiting factor in determining the extent of bacterial lipid uptake by HepG2 cells.

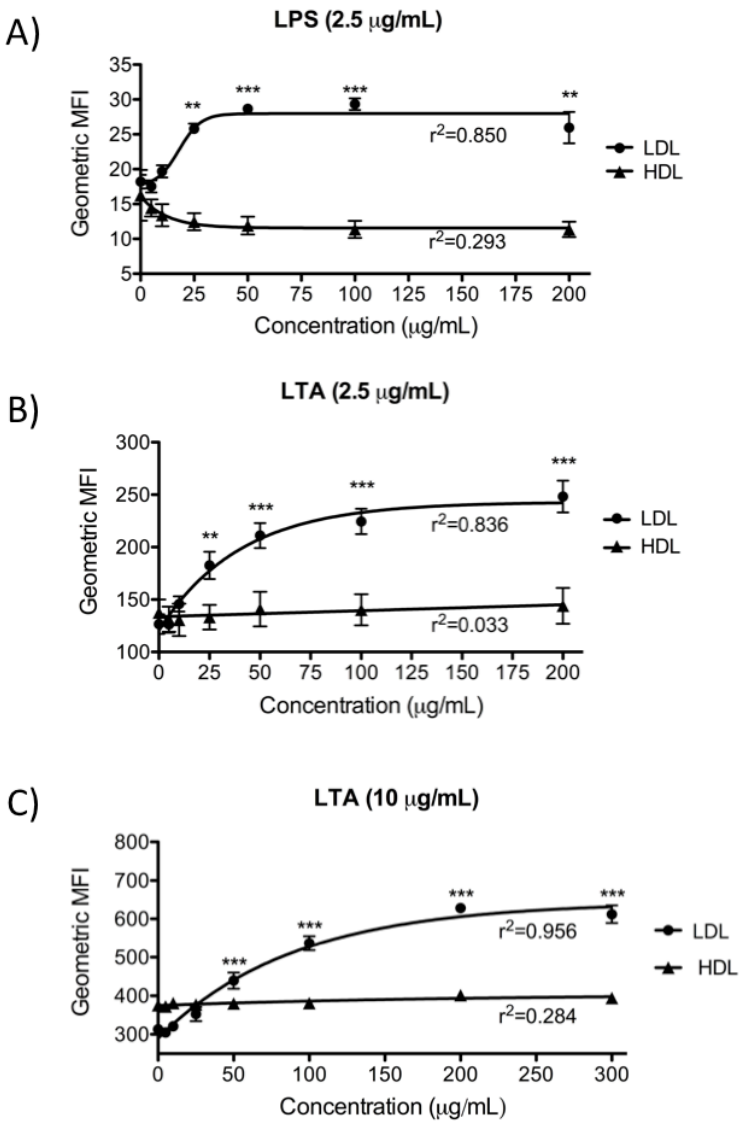

Figure 7. Dose-response of LDL or HDL add-back to lipoprotein-deficient serum on the uptake of LPS (A) and LTA (B,C) by HepG2 cells. Cells were cultured in 20% lipoprotein-deficient serum and treated with 2.5 of AlexaFluor 488-LPS (A), or 2.5 μg/mL (B) or 10 μg/mL (C) of BODIPY 630/650-LTA for 24 h. Data are shown as mean ± SEM from 3–4 experiments. **p<0.01, ***p<0.001 vs. 0 μg/mL of LDL by one-way ANOVA.

Confirming that LDL-dependent uptake of LPS & LTA occurs through LDLR

To confirm that the effects of LDL on bacterial lipid uptake occur through LDLR, we performed experiments involving add-back of LDL or HDL to lipoprotein-deficient serum with or without blockade of the LDLR. Consistent with previous experiments, blocking LDLR significantly decreased BODIPY-LTA uptake when cells were cultured in 20% normal serum, but not lipoprotein-deficient serum (Figure 8A). Furthermore, addition of 100 μg/mL of LDL to lipoprotein-deficient serum increased LTA uptake compared to lipoprotein-deficient serum alone, but this effect was abolished by anti-LDLR treatment. On the contrary, addition of 100 μg/mL of HDL to lipoprotein-deficient serum had no effect on BODIPY-LTA uptake compared to normal serum or lipoprotein-deficient serum alone, irrespective of whether LDLR was blocked or not (Figure 8A).

Experiments investigating the effects of LDL or HDL on uptake of LPS showed similar trends overall, with a few notable exceptions (Figure 8B). For instance, blocking LDLR appeared to decrease LPS uptake by HepG2 cells cultured in normal serum, and this difference was significant by Student's t-test but not by ANOVA. Furthermore, HepG2 cells cultured in lipoprotein-deficient serum had significantly increased uptake of LPS compared to cells cultured in normal serum (Figure 8B), an effect which was not observed with LTA. Although seemingly paradoxical, this may be explained as a sequestration effect, which is supported by the observation that LPS uptake following HDL add-back to lipoprotein-deficient serum is similar to that observed in normal serum that naturally contains HDL (Figure 8B).

These data collectively demonstrate that LDL-dependent LTA and LPS uptake is mediated through LDLR, and that HDL may sequester LPS thereby reducing its uptake from the serum.

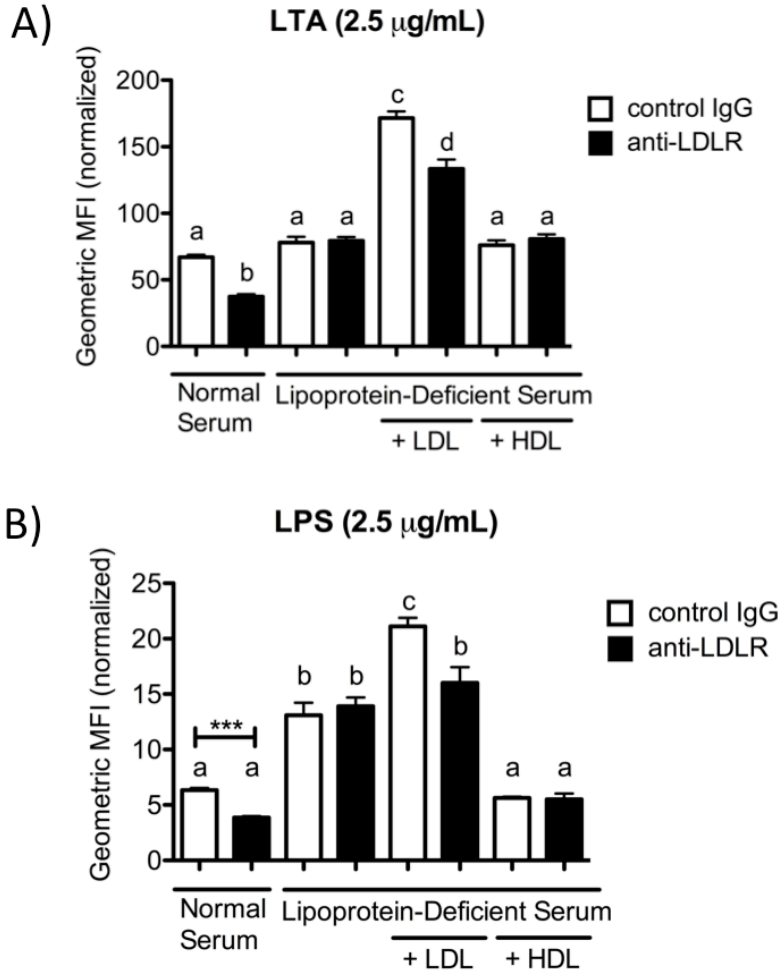

Figure 8. LDL-dependent uptake of LTA (A) and LPS (B) by HepG2 cells is mediated by LDLR. Cells were cultured in 20% normal human serum or donor-matched lipoprotein-deficient serum, with or without 100 μg/mL of LDL or HDL, and treated with anti-LDLR antibody or control IgG for two hours prior to 24 h treatment with 2.5 μg/mL of BODIPY 630/650-LTA (A) or AlexaFluor 488-LPS (B). Data are presented as geometric mean fluorescence intensity ± SEM, from 3–4 experiments. Different letters indicate significant differences between groups with $p < 0.01$ by ANOVA; *** $p < 0.001$ by Student's t-test.

Troubleshooting with recent PCSK9 Lots

Given the results in Figure 8, we wanted to replicate this set of experiments using PCSK9 in place of LDLR-blocking antibody to strengthen the evidence for the regulatory role of PCSK9 in the LDL-dependent, LDLR-mediated bacterial lipid uptake mechanism. However, our initial two lots of recombinant human PCSK9 (AcroBiosystems) were depleted, so we ordered a new lot (AcroBiosystems) in addition to receiving a separate multi-freeze-thawed lot from the lab of Dr. Nabil G. Seidah. These new lots of recombinant PCSK9 produced no observable effect in two pilot experiments, which were intended to replicate our initial set of experiments involving PCSK9 (Figure 5) with a lower LTA concentration of 2.5 μg/mL (Figure 9A). Although it is possible that other factors may be responsible for differences between results in these two sets of experiments (Figure 5 vs. 9A), the comparable effect of anti-LDLR antibody on LTA uptake in both sets of experiments excludes the potential influence of most other variables, such as differences in cell responsiveness or LTA concentration. As an initial troubleshooting measure, we performed dose-response experiments to assess whether a higher PCSK9 concentration would be able to compensate for this apparent lack of activity at 2.5 μg/mL concentrations, but there was no dose-dependent effect on either LPS or LTA uptake (Figure 9B), in contrast to our previous observations with past PCSK9 lots (Figure 4C). These results suggested that the two new lots of PCSK9 may be non-functional. As another troubleshooting step, we decided to use the same PCSK9 concentration and dosing intervals as those previously demonstrated to have a significant and reproducible effect on LPS uptake (Walley et al., 2014; Boyd et al., 2016; Topchiy et

al., 2016), but again found that there was no observable difference in uptake of AlexaFluor 488-LPS in PCSK9-treated cells compared to AlexaFluor 488-LPS control (Figure 9C). In order to confirm that the recent lot of PCSK9 was non-functional, we performed a Western blot for LDLR expression following multiple PCSK9 treatments. In these experiments, we treated cells with increasing concentrations of PCSK9 and compared LDLR expression with that of untreated controls. The results from these experiments confirm that the new lot of PCSK9 is non-functional, as there was no decrease in LDLR expression even at high PCSK9 concentrations of 10 μg/mL (Figure 9D). In addition to testing the new lots at 5 & 10 μg/mL concentrations, we used a freeze-thawed aliquot of previously functional PCSK9 for the 2.5 μg/mL treatment group as a control to test for potential loss of functionality due to freeze-thaw and/or long-term storage. Indeed this control demonstrated no effect on LDLR expression, which suggests that PCSK9 function is sensitive to multiple freeze-thaw cycles and/or long-term storage. This may partly explain the lack of functionality in the PCSK9 received from Dr. Seidah, but does not account for similar observations in the commercially purchased lot from AcroBiosystems.

Therefore, we were unable to replicate the experiments performed in Figures 5 & 8 with new conditions due to the non-functional lot of PCSK9 that was recently obtained. Confirmation of the lack of effect on LDLR expression suggests that these results should be interpreted as a lack of biological function with this particular lot of recombinant PCSK9 protein, rather than bringing into question the reproducibility of our previous findings that PCSK9 plays a regulatory role in bacterial lipid uptake.

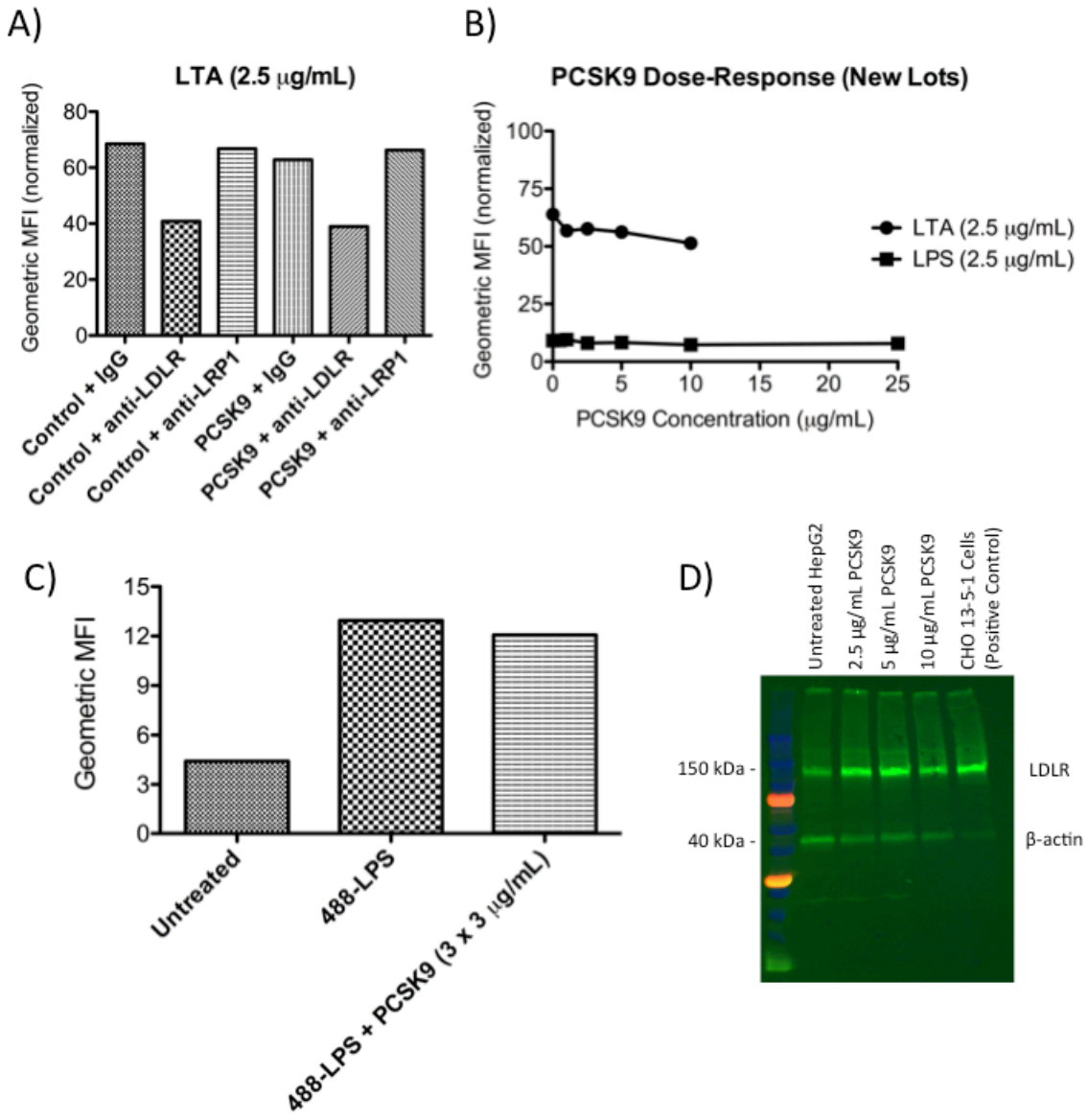

Figure 9. Troubleshooting PCSK9 activity from recent lots. (A) Pooled data from pilot experiments using two new & different (2.5 μg/mL) PCSK9 lots to replicate the findings from Figure 5 with the 2.5 μg/mL concentration of LTA (n=2). (B) Dose-response experiments (n=1) testing the effects of increasing PCSK9 concentrations on uptake of 2.5 μg/mL of BODIPY-LTA (filled circles) or AlexaFluor 488-LPS (filled squares) by HepG2 cells cultured in DMEM + 20% normal serum. (C) Pilot experiment (n=1) testing

the effects of multiple 3 μg/mL PCSK9 doses at 3 h before, 4 h after, and 19 h after treatment of HepG2 cells with 2.5 μg/mL of AlexaFluor 488-LPS, in accordance with previously published methods (Walley et al., 2014). (D) Western blot demonstrating effects of increasing PCSK9 concentration (from the new lots) on LDLR expression by HepG2 cells following 6 h of treatment. Western blot data are representative of 3 biological replicates utilizing both new lots of PCSK9, as well as freeze-thawed PCSK9 from one past functional lot for 2.5 μg/mL concentrations as a control.

Pilot Animal Experiments

In a preliminary set of animal experiments, we set out to identify a suitable experimental technique to investigate the effects of differential PCSK9 expression and LDLR deficiency on hepatic uptake of LPS and LTA. We attempted to visualize hepatic uptake of fluorescently labeled LTA at 6 hours after intravenous injection of 5 mg/kg through the tail vein in C57Bl/6J mice, using fluorescence microscopy to image frozen sections of the livers obtained from these mice. As shown in Figure 10, there was no detectable fluorescence under the TRITC filter corresponding to the BODIPY-630/650 Fluorophore in any of the groups. We then decided to perform another preliminary set of experiments with FITC-LPS obtained from Sigma-Aldrich, since published reports have demonstrated use of this commercially available preparation to investigate hepatic LPS uptake (Scott et al., 2009). In this set of experiments, we injected wild-type C57Bl/6J mice with 5 mg/kg FITC-LPS either intravenously through the tail vein or jugular vein, or through the intraperitoneal route. As seen in Figure 11, similar fluorescence intensity was visualized in controls and mice injected with FITC-LPS through the jugular vein or intraperitoneal routes, whereas mice injected through the tail vein showed slightly increased fluorescence. However, due to the inconsistency in clearly discernable signals resulting from relatively high background auto-fluorescence in controls, we deemed this technique as unsuitable to adequately test our hypothesis of differential uptake between different genotypes of mice based on PCSK9 expression. Therefore, we pursued the use of confocal microscopy to image the liver *in vivo*, as this technique is more sensitive and robust for visualizing and quantifying fluorescence. The pilot data from these experiments show consistent

increases in fluorescence intensity in mice injected with fluorescent LTA compared with phosphate-buffered saline treated controls (Figure 12 A,B). Furthermore, this preliminary data suggests that there may be a trend towards increased LTA uptake in peri-sinusoidal hepatocytes of PCSK9$^{-/-}$ mice compared to wild-type controls (Figure 12A), although additional experiments are needed for verification.

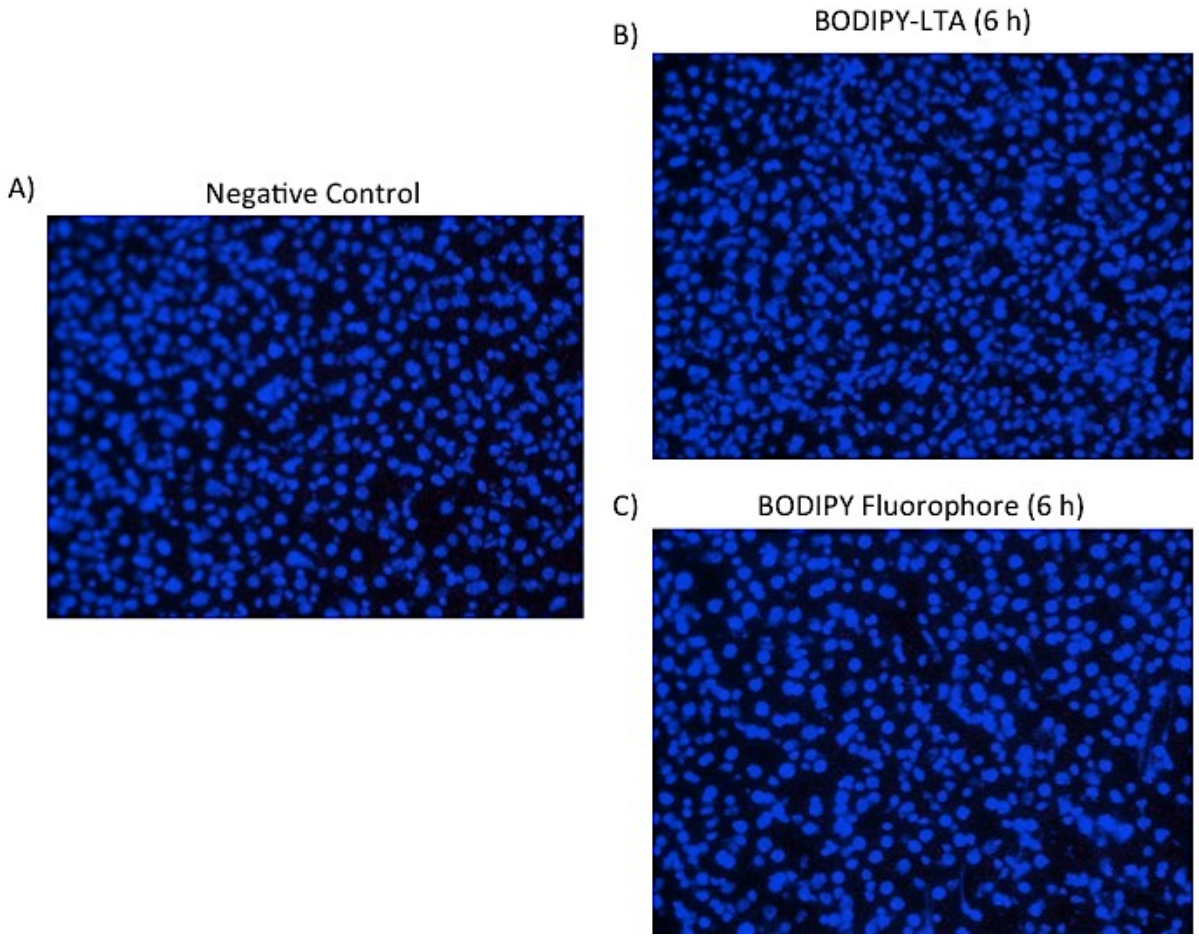

Figure 10. Pilot data from LTA injection experiments in wild-type C57Bl/6J mice. Control phosphate-buffered saline (A), 5 mg/kg of BODIPY 630/650-LTA (B), or equivalent fluorescence units of unbound BODIPY Fluorophore (C) were injected intravenously through the tail vein. After 6 h, mice were sacrificed, and the liver was harvested and snap-frozen in OCT for frozen sectioning. Frozen sections were fixed in acetone, stained with DAPI and mounted on slides for fluorescence microscopy under DAPI & TRITC filters. n = 1 mouse per group.

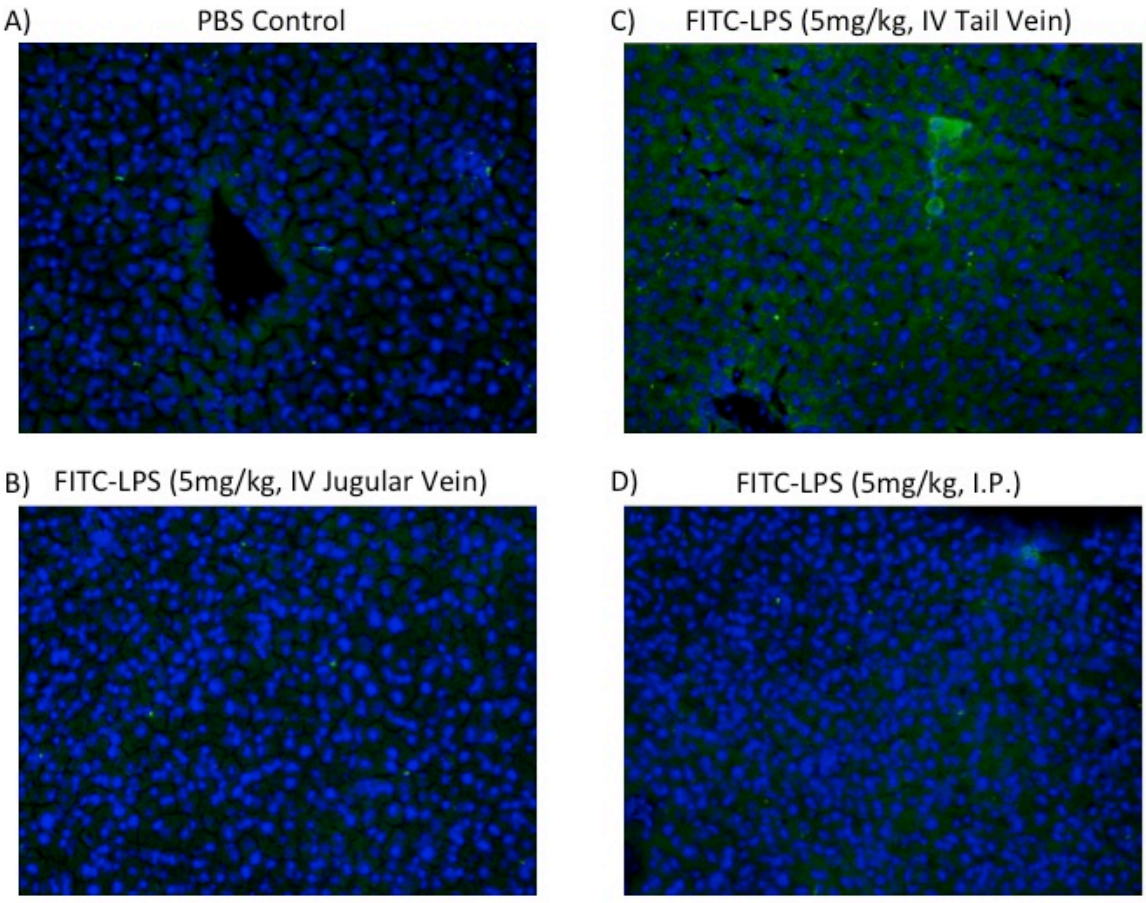

Figure 11. Pilot data from LPS injection experiments in wild-type C57Bl/6J mice. Mice were injected with control phosphate-buffered saline (PBS) (A), or 5 mg/kg of FITC-LPS intravenously (IV) through the jugular vein (B), IV through the tail vein (C), or through the intraperitoneal (I.P.) route (D). After 6 h, mice were sacrificed, and the liver was harvested and snap-frozen in OCT for frozen sectioning. Sections were fixed in acetone, and mounted in media containing DAPI for fluorescence microscopy. n = 1–2 mice per group.

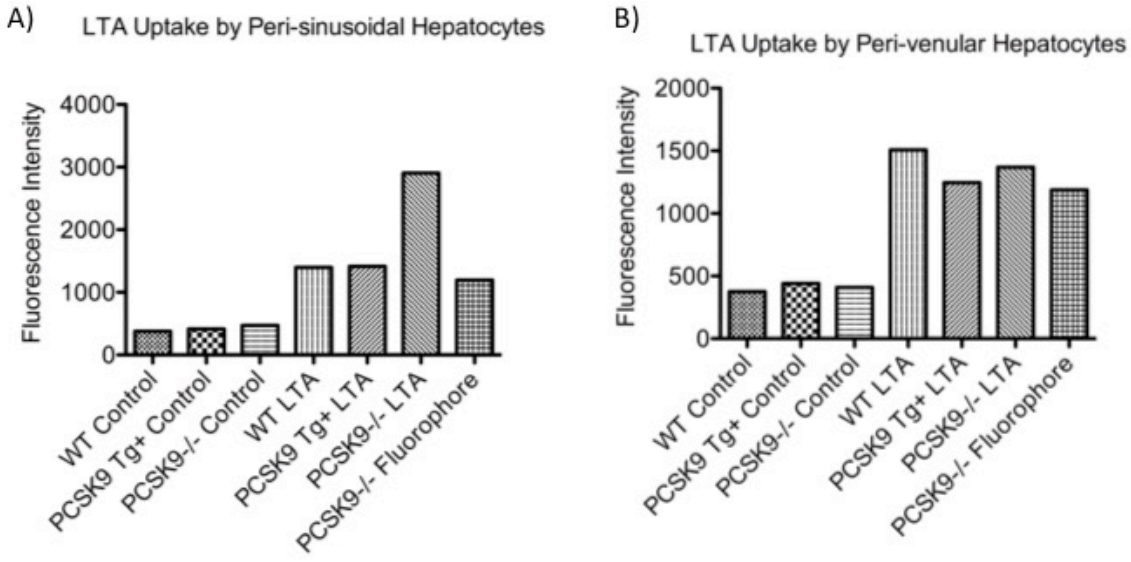

Figure 12. Preliminary *in vivo* uptake of LTA by peri-sinusoidal (A) and peri-venular (B) hepatocytes in wild-type C57Bl/6J, PCSK9$^{-/-}$ and PCSK9 Tg^{+} mice as measured through confocal microscopy. Mice were intravenously injected through the tail vein with 5 mg/kg of BODIPY 630/650-LTA, unbound Fluorophore control, or equal volumes of control PBS at 5 h before imaging. Data represent the mean maximum fluorescence intensity of 3–5 fields of view per mouse, with n = 1 – 2 mice per group. WT, wild-type; Tg^{+}, transgenic PCSK9 overexpressing.

Plasma PCSK9 concentrations in ICU patients with sepsis

As a translational component to our study, we measured plasma concentrations of PCSK9 in a cohort of septic ICU patients from the DYNAMICS study. Plasma PCSK9 concentrations of septic ICU patients started off at baseline levels comparable to healthy controls, then increased over the first 5 days in the study, and returned to baseline by Day 7 in the study (Figure 13). Peak plasma PCSK9 concentrations were observed at Day 5 in the study, and these concentrations were significantly greater than plasma PCSK9 concentrations measured in healthy volunteers. Data obtained after Day 7 suggests that plasma PCSK9 concentrations continue to transiently increase and return to baseline over the course of an ICU stay over 4–5 weeks, however the number of observations at these later time-points are insufficient for drawing firm conclusions.

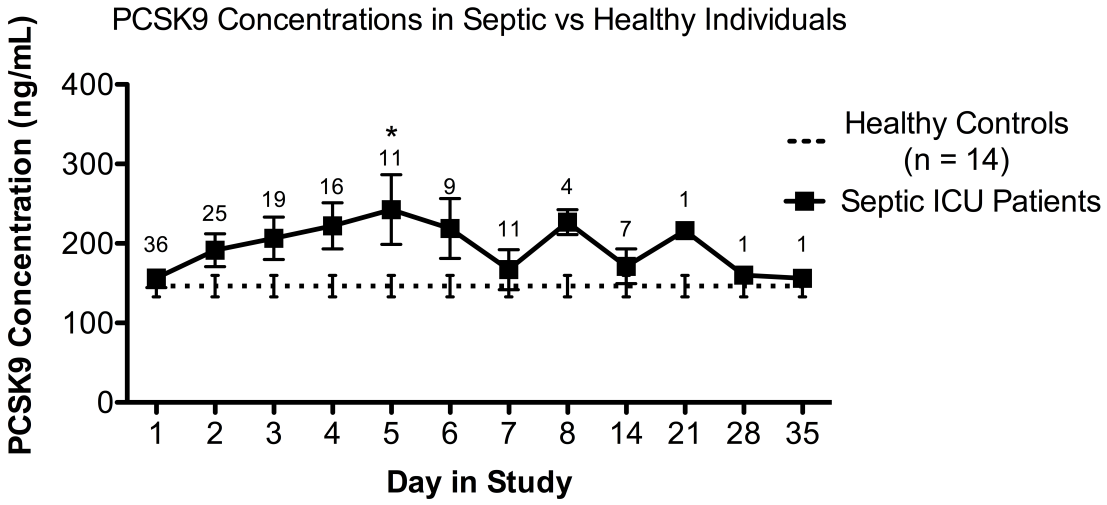

Figure 13. Plasma PCSK9 concentrations in septic ICU patients compared to healthy controls. Data are expressed as mean ± SEM, with 'n' for each 'Day in Study' listed over each data point. *$p<0.05$ by two-way ANOVA; time-points with less than 3 observations were not statistically analyzed.

DISCUSSION

In this study, we establish that Gram-negative LPS and Gram-positive LTA are taken up through similar mechanisms by human HepG2 hepatocytes *in vitro*. Furthermore, we demonstrate that PCSK9 negatively regulates uptake of these bacterial lipids by LDLR, but not LRP1, through a lipoprotein-dependent mechanism. Our findings are consistent with previous studies (Topchiy et al., 2016), which identified the role of LDLR in LPS clearance by hepatocytes, and we have shown that this mechanism also extends to the uptake of LTA. We also identify that the specific lipoprotein required for LDLR-mediated uptake of bacterial lipids is LDL, since LDL add-back to lipoprotein-deficient serum resulted in dose-dependent increases in uptake of both LPS and LTA, while blocking LDLR abolished this effect. We further investigated whether HDL also plays a role, as previous work has documented that LPS and LTA are transferred to HDL prior to incorporation into LDL in the blood (Levels et al., 2003; Levels et al., 2005); our data indicate that there are differences in the effects of HDL on LPS and LTA uptake—namely that HDL reduced uptake of LPS, but had no effect on LTA uptake. These findings are somewhat paradoxical as both LPS and LTA are known to incorporate into HDL (Levels et al., 2001; Levels et al., 2003), and studies have shown that HepG2 cells can internalize HDL particles (Rohrl et al., 2014). Furthermore, since LDLR can bind ApoE (Brown et al., 1986), which is found on a subset of HDL particles (Sasamoto et al., 2017), it was expected that some uptake of HDL and incorporated bacterial lipids might occur through LDLR. Our observation that blocking LDLR has no effect on LPS or LTA uptake in the presence of HDL suggests that either this subset of HDL was present in relatively small

concentrations in the purified HDL samples that we utilized for our experiments, and thus had minimal effect on bacterial lipid uptake, or alternatively, that the ApoE-containing HDL subset does not incorporate bacterial lipids. Future studies could consider investigating the latter hypothesis by comparing LPS and LTA incorporation into different subsets of HDL.

Interestingly, variation in the extent of LPS or LTA uptake by human HepG2 cells did not affect extracellular ALT activity or cytokine concentrations, indicating that this bacterial uptake mechanism may be physiologically natural for hepatocytes. Since HepG2 cells have been documented to produce cytokines in response to various stimuli (Gutierrez-Ruiz et al., 2001; Gomez-Quiroz et al., 2003), our findings cannot be explained by a lack of cytokine production capability in this cell type. Studies have specifically shown that HepG2 cells can secrete cytokines in response to LPS (Gutierrez-Ruiz et al., 2001; Gomez-Quiroz et al., 2003), thus our data contrast with these previously published findings, as we observed no differences between controls and LPS-treated groups using higher LPS concentrations over the same treatment duration. These contrasting observations may be explained by the differences in bacterial origin of the LPS utilized, since we used LPS from *E. coli* rather than from *Salmonella typhimurium* (Gutierrez-Ruiz et al., 2001; Gomez-Quiroz et al., 2003). Indeed, it has been established that LPS from different bacterial sources varies in its ability to induce secretion of pro-inflammatory cytokines, including IL-6 and IL-8 (Agarwal et al., 1995), which we measured. Furthermore, studies have identified that phenotypic differences between Salmonella and Escherichia species result from variable LPS modification downstream of

distinct gene regulation (Winfield & Groisman, 2004), which suggests that differences in the structure of LPS from different species may account for differences in the cell response. Another possibility is that differences in the hepatocyte response to LPS may arise depending on whether the LPS is from an intracellular bacterium, as in the case of Salmonella, or from extracellular bacterial species, although this might also relate to differences in LPS structure.

Our findings that HepG2 cells do not secrete cytokines in response to *E. coli* LPS are also supported by recent unpublished observations from another group (Dr. Keith Walley, personal communication). The ability to take up LPS and LTA without stimulating an inflammatory response may be a homeostatic mechanism that allows hepatocytes in the liver to clear bacterial lipids, such as those that translocate into the portal circulation from the gut lumen by crossing the intestinal barrier (Guerville & Boudry, 2016). Since *E. coli* is an abundant bacterial species in the intestinal microbiota (Herias et al., 1995), an argument can be made that hepatocytes may have evolved the ability to take up and clear bacterial lipids from non-pathogenic bacterial species or serotypes that are part of the natural gut microbiota without generating an inflammatory response, whereas pro-inflammatory cytokines would be produced in response to bacterial lipids from pathogenic serotypes or species, such as *S. typhimurium* (Gutierrez-Ruiz et al., 2001; Gomez-Quiroz et al., 2003).

Uptake of bacterial lipids by hepatocytes might also function to minimize stimulation of other cell types in the liver, such as Kupffer cells, which produce cytokines in response to LPS and LTA (Callery et al., 1990; Overland et al., 2003), and during

sepsis (Koo et al., 1999). It is conceivable that the previously observed reduction of cytokines in the context of PCSK9-deficiency during sepsis (Walley et al., 2014) may result from increases in the LDLR-mediated uptake of bacterial lipids by hepatocytes, thereby reducing the availability of these PAMPs to stimulate PRRs on innate immune cells, such as Kupffer cells, and consequently reducing cytokine-driven inflammation during sepsis. The importance of cross-talk between hepatocytes and other cell types in the liver during sepsis is well established (Strnad et al., 2017). Kupffer cells, for example, have long been known to regulate protein synthesis by hepatocytes in response to *E. coli* LPS (West et al., 1985). Furthermore, processing of LPS by Kupffer cells can improve its binding to hepatocytes while decreasing the pro-inflammatory properties of this modified LPS (Treon et al., 1993). Therefore, it is not a far stretch to suggest that the uptake of LPS by hepatocytes can conversely influence the physiological response of Kupffer cells, given the documented cross-talk between these two cell types in response to bacterial lipids.

One limitation to our study is that human HepG2 cells are transformed hepatocytes, and therefore may not be completely representative of the physiology of primary hepatocytes. Although we were unable to address this limitation by replicating these experiments using primary hepatocytes as-of-yet, it is likely that these findings could be extended to primary hepatocytes as work by other groups has demonstrated that similar LPS uptake occurs in HepG2 cells and primary mouse hepatocytes (Walley et al., 2014; Topchiy et al., 2016). Another limitation related to our work is that physiological processes that occur *in vitro* do not necessarily occur *in vivo*. We have begun to address

this limitation through our pilot experiments investigating hepatic uptake of LPS and LTA in mice. Through our pilot work, it has become apparent that confocal microscopy is a suitable method for evaluating hepatic uptake of fluorescently labeled bacterial lipids *in vivo*. Our preliminary data suggests that PCSK9 deficiency may increase hepatic uptake of LTA in peri-sinusoidal hepatocytes, but not in peri-venular hepatocytes, although these results are very preliminary thus making it unreasonable to interpret conclusively. We speculate that the observed differences in uptake based on microcirculatory location may be explained by the slower velocity of blood flow through the sinusoids when compared to the venules, which would be expected to increase the ability of nearby cells, such as hepatocytes, to take up molecules from the blood. Furthermore, our preliminary data showing increased LTA uptake in PCSK9$^{-/-}$ mice supports the hypothesis that PCSK9 negatively regulates uptake of bacterial lipids by hepatocytes *in vivo*, though these results should be viewed with caution due to their preliminary nature.

Regarding clinical significance, our study is the first to our knowledge to measure circulating PCSK9 concentrations over time in ICU patients, and our findings complement those of Boyd et al., which documented increases in PCSK9 during early sepsis in patients from the Emergency Department (Boyd et al., 2016). Furthermore, our work has elucidated a bacterial lipid uptake pathway with potential therapeutic relevance in sepsis, and also provides several insights into improving our understanding of the complex pathophysiology of sepsis, especially in the contexts of obesity, dyslipidemia, and statin therapy. Obesity is a common risk factor for many diseases, however, there is an 'obesity paradox' in critically ill patients including those with sepsis, which refers to

the protective effect of obesity on survival of ICU patients (Dickerson, 2013). Though this paradox remains poorly understood, recent work from our lab has begun to identify the potential underlying mechanisms by demonstrating that reduced lung inflammation occurs in obese mice subjected to the CLP model of sepsis when compared to their non-obese counterparts (Khan et al., 2014). Furthermore, this effect was related to PCSK9, as obese PCSK9$^{-/-}$ mice did not benefit from reduced lung inflammation following CLP-induced sepsis, while non-obese PCSK9$^{-/-}$ mice did (Khan M et al., manuscript in preparation). While the complete mechanisms underlying the obesity paradox remain unclear, there is evidence suggesting that PCSK9 regulates lung inflammation, and this regulatory role may be related to its role in the bacterial lipid uptake mechanism that we identified in this study.

Dyslipidemia may also be related to clinical outcomes of patients with sepsis, as an observational study found that LDL, HDL, and HDL-associated apolipoproteins were decreased in sepsis non-survivors compared to survivors (Barlage et al., 2009). Furthermore, a recent study found that low LDL-cholesterol levels were associated with increased rates of community-acquired sepsis (Guirgis et al., 2016), which suggests that a reduced ability to maintain lipid homeostasis, and possibly a reduced ability to clear bacterial lipids, in the setting of reduced circulating LDL levels may increase the risk of developing sepsis. Although 'dyslipidemia' is a broad term covering a variety of lipids, lipoproteins and apolipoproteins, the majority of clinical efforts to correct 'dyslipidemia' in sepsis have focused on statin therapy to target LDL-cholesterol. Our findings may help to better understand the physiology of statin therapy in patients with sepsis, and may

provide insight into the conflicting literature on the potential benefits of using statins as an adjunctive therapy in sepsis (Tleyjeh et al., 2009; Janda et al., 2010; Deshpande et al., 2015). Statins function by reducing hepatic cholesterol synthesis through inhibition of the rate-limiting enzyme in this process, HMG-CoA reductase (Alberts et al., 1980). However, the downstream effects of inhibiting hepatic cholesterol synthesis with statins involve upregulation of LDLR (Bilheimer et al., 1983) and PCSK9 (Dubuc et al., 2004), implying that statins may also have effects on the bacterial lipid uptake mechanisms that we have identified. Studies have also demonstrated that statin therapy interacts with PCSK9 genotype, as loss-of-function in PCSK9 improves the LDL-cholesterol lowering effect of statin therapy in patients (Feng et al., 2017), which provides further support for a meaningful interaction between statins and PCSK9, and further suggests that statins may have effects on the LDL-dependent pathway of bacterial lipid uptake. Furthermore, it is possible that the effects of statins on clinical outcomes in sepsis in past clinical trials may have been partially confounded by PCSK9 genotype, which has not been investigated in the majority of the large clinical trials on statins in sepsis. Therefore, similar approaches to those currently used in cardiovascular disease management, namely combining statin therapy with PCSK9 inhibition (Pandey et al., 2017; Sabatine et al., 2017), could prove to be useful for treating sepsis, although this would first need to be studied clinically.

In conclusion, our study directly demonstrates the regulatory role of PCSK9 in bacterial lipid uptake and outlines the detailed pathway by which this occurs via LDLR through an LDL-dependent mechanism, thereby strengthening the evidence in support of an important role for PCSK9 in sepsis. These findings also advance our understanding of

sepsis pathophysiology as it relates to dyslipidemia, obesity, and statin therapy, and highlight another potentially central role for hepatocytes and the liver in sepsis. The implications of our study are that PCSK9 should be considered as a novel therapeutic target for sepsis, and further research should focus on understanding the systemic effects and clinical outcomes of PCSK9 inhibition in patients with sepsis.

FUTURE DIRECTIONS

To expand on our work, future studies should consider replicating our experiments using primary hepatocytes to confirm that the bacterial lipid uptake mechanism we have identified is not restricted to HepG2 cells. Furthermore, completing the confocal microscopy studies that we started, with the addition of $LDLR^{-/-}$ groups, would add *in vivo* evidence to support or refute the physiological importance of the PCSK9-regulated and LDLR-mediated bacterial lipid uptake pathway that we demonstrated *in vitro*; and coupling transillumination intravital microscopy with confocal microscopy in these experiments would allow for investigations into whether hepatic leukocyte recruitment is correlated with bacterial lipid uptake in the liver. Finally, as a prelude to clinical studies of PCSK9 inhibition for treating sepsis, it would be prudent to perform pre-clinical studies of PCSK9 inhibition using animal models of sepsis to better understand the safety profile of these inhibitors in the specific context of the complex pathophysiology of sepsis.

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