DEACTIVATION OF THE LPS ANTAGONIST ERITORAN BY HDL-ASSOCIATED APOLIPOPROTEINS

by

JACKLYN GABRIELLA FLEISCHER

B.Sc., Queen's University, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2010

ABSTRACT

Background: Sepsis is a complex and life-threatening infection by gram-negative bacteria that is caused by the endotoxin lipopolysaccharide or LPS. Lipid A, the active moiety of LPS, exerts its effects through interaction with toll-like receptor 4 (TLR4), triggering a signalling cascade that results in the massive release of pro-inflammatory cytokines such as tumour necrosis-α (TNF-α) and interleukin-1 (IL-1). Eritoran is a synthetic lipid A analogue that is a powerful antagonist of LPS, however it undergoes a time-dependent deactivation after administration as a consequence of binding to high-density lipoproteins (HDL). At present, there remains much uncertainty surrounding the site of eritoran association with HDL. Therefore, the objective of this study was to determine if HDL-associated apolipoproteins inhibit eritoran's ability to block LPS-induced TNF-α release from whole blood.

Methods: Assess eritoran activity after LPS stimulation in human whole blood in the presence of free or reconstituted HDL containing apolipoproteins, including a combination rHDL containing all apolipoproteins found on native HDL/septic HDL. Activity will be measured by TNF-α release.

Results: Differences exist in the effects on drug activity when apolipoproteins are free versus lipid-bound. In rHDL, the major apolipoproteins in both the healthy and septic state, A1 and SAA, caused a significant reduction in eritoran antagonistic activity. Additionally, in rHDL, A1 and SAA are superior to minor apolipoproteins A2 and C1 in dampening drug activity, while a native combination rHDL, normal rHDL (NrHDL), deactivated eritoran to a similar degree as rHDL-A1.

Conclusions: Apolipoproteins associated with HDL are likely to facilitate eritoran deactivation. Apo A1 and SAA should be of particular focus as they are the major apolipoproteins found on HDL in both the healthy and septic state and were observed to significantly reduce eritoran activity. Further evaluation of the physical association between apolipoproteins and eritoran, particularly binding mechanisms, should be explored.

Significance: This study will help to elucidate the components of HDL that facilitate loss of eritoran antagonistic activity, illuminating how associations are formed between lipoproteins and xenobiotics. Clinically, these results can be used to strategize novel ways to thwart drug deactivation *in vivo* and extend its therapeutic action in septic patients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
APO	Apolipoprotein
AUC	Area under the plasma concentration-time curve
BSA	Bovine serum albumin
CD14	Cluster of differentiation 14
C_{max}	Maximum plasma concentration of a drug
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E5564	Eritoran
HBSS	Hank's balanced salt solution
HDL	High density lipoprotein
ICU	Intensive care unit
IL-1	Interleukin-1
IL-6	Interleukin-6
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MD-2	Myeloid differentiation protein 2
NFκB	Nuclear factor kappa B
PAMP	Pathogen-associated molecular pattern
rHDL	Reconstituted HDL

SAA	Serum amyloid A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRS	Systemic inflammatory response syndrome
TC	Total cholesterol
TG	Triglycerides
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha
TIR	Toll/interleukin-1 receptor domain
TRL	Triglyceride rich lipoprotein
VLDL	Very low density lipoprotein

ACKNOWLEDGEMENTS

There are several people to thank for their guidance and support that contributed greatly to this dissertation and to my learning experience as a graduate student in the Faculty of Pharmaceutical Sciences at UBC. First and foremost, I would like to extend my sincerest gratitude to my supervisor, Dr. Kishor Wasan, who gave me the opportunity to improve and develop as both a researcher and academic and whose constant encouragement, leadership, and guidance has made this project possible. Additionally, I am incredibly grateful to my committee members, Dr. Gordon Francis, Dr. Marc Levine, Dr. Thomas Chang and Dr. Stelvio Bandiera for all their insightful comments, direction, and the personal time they took to work with me on building this study. It was most helpful throughout my graduate training and I cannot tell you how much I appreciate it.

I would also like to give a warm thanks to Dr. Sheila Thornton and Stephen Lee whose scientific advice, support, and friendship was invaluable. Thank you both. Likewise, I would like to thank all other members of the Wasan Lab. It was always a pleasure to work with you. Of course, a special thanks to my family – Mom, Dad and Alex – you're always there for me and I love you dearly. Similarly, a heartfelt thank you to Katie Sweeney for always being a good listener and outlet, but most importantly a true friend.

Lastly, this project could not have materialized without the generous funding of CIHR and Eisai Pharmaceuticals. In addition, I would like to thank and acknowledge Dan Rossignol from Eisai for your excellent input, enthusiasm and support – I've thoroughly enjoyed our correspondence throughout this project. Of course, thanks to Ted Chan from the Francis lab whose positive attitude and expertise I admire and whose friendship I am glad to have come by.

1. BACKGROUND

1.1 The innate immune response and sepsis

The human body is well equipped with a sophisticated set of barriers to infectious agents that serve to protect the host against illness and disease. Pathogens that successfully cross physical barriers like the skin or mucosal membranes find themselves in direct combat with our innate immune system. The innate or cell-mediated response is produced by leukocytic cells including neutrophils, monocytes and phagocytic cells like macrophages that act to destroy pathogens by phagocytosis and/or the secretion of lytic enzymes. The distinguishing feature of the innate system is that it is non-specific. These cells respond to any type of antigen encountered, eliciting a rapid immune response and coordinating a cross-talk between the innate immunity, whose response is brief, and the humoral immunity, where opsonisation by secreted antigen-specific antibodies target the pathogen for elimination and possess a 'memory' for the infectious agent. During infection, macrophages are stimulated by invading microorganisms to release low molecular weight (<30 kDa) regulatory proteins called pro-inflammatory cytokines, that generally act on surrounding tissue to induce local inflammation [1]. Pro-inflammatory cytokines such as tumour necrosis factor alpha, TNF-α, interleukin-1, IL-1, contribute to the immune response by inducing the transcription of additional cytokines and enzymes required in the regulation of vasoactive lipid mediators (prostaglandins and thromboxanes) and reactive oxygen species such as nitric oxide (NO) [2].

When host defences are unable to kill and clear microbes at the site of infection the bacterial load increases significantly in the blood, resulting in an over-production of cytokines and other cellular mediators that are harmful to the host [3]. Sepsis, is clinically

defined when the host experiences the symptoms of a systemic inflammatory response (SIRS) to infection. The manifestation of SIRS and thus, sepsis, can include two or more of the following: (1) temperature is >38ºC or <36ºC, (2) heart rate >90 beats/min, (3) tachypnea - respiratory rate >20 breaths/min or $PaCO_2$ <32mm Hg, (4) white blood cell count >12,000cu mm or <4,000cu mm or >10% immature neutrophils [4]. Since the release of pro-inflammatory cytokines is normally down-regulated by counterbalancing anti-inflammatory cytokines such as IL-10 and IL-4, the massive systemic release of pro-inflammatory cytokines in sepsis results in a loss of regulation causing vascular permeability and leakage (swelling in tissues), microthrombosis (fibrin clots in small blood vessels), hypotension and tissue hypoperfusion ending in organ failure and death [5]. The development of severe sepsis, as briefly described above, is always associated with organ dysfunction and hypotension and includes indicators of hypoperfusion abnormalities such as lactic acidosis, oliguria (decreased urine production) and acute alteration of mental status [4].

1.1.1 Prevalence of sepsis and current mode of treatment

At present, there remains an urgent clinical need to improve current approaches to the treatment of sepsis to address the climbing rate of hospitalization and mortality from this condition seen within the last 20 years. According to a nationwide study conducted in the United States, there was doubling in the rate of those hospitalized for severe sepsis and a 1.7-fold increase in the rate of mortality between 1993 and 2003 [6]. In 2001, the national estimate of sepsis cases based on hospital discharge databases from seven states was 751, 000 cases per annum, with a projected average cost of $22,000 per patient [7]. The national studies conducted by Dombrovskiy and Angus showed that approximately 70% of patients included

developed dysfunction in at least one organ and that the most commonly affected organ systems were respiratory, cardiovascular and renal. Organ failure is seen as a marker for the severity of the condition and duration of organ dysfunction is associated with increased mortality in patients with severe sepsis [8]. Using the US National Hospital Discharge Survey from 1979 to 2000, Martin *et al.* also found an increase in the incidence rate of sepsis (8.7% per year), and propose that the climb in incidence may be attributed to increased use of invasive procedures and immunosuppressants, chemotherapy, transplants, HIV infection and the increase in microbial resistance to antibiotics [9].

In view of the grim statistics of growing disease prevalence in the population, many clinical trials have been conducted to test a variety of drugs designed to target different points in the sepsis cascade. Some of these include human monoclonal antibody against endotoxin (CHESS), anti-TNF-α (INTERSEPT), and tissue factor pathway inhibitor (OPTIMIST) all of which failed to improve patient outcome [10]. Sepsis that cannot be treated surgically (i.e. controlling the source of infection, such as perforations of the GI tract) must be treated symptomatically. This treatment consists of an intravenous infusion of fluids (fluid resuscitation) to restore vascular blood volume and improve tissue perfusion that is typically reduced due to hypotension in septic patients [11]. In more severe cases, sometimes vasoactive drugs like epinephrine and norepinephrine will be used for their vasoconstrictive activity in efforts to achieve sufficient blood pressure and tissue perfusion. Nevertheless, these treatments do not abolish the cause of sepsis but rather act to offset the physiological effects caused by the over-activation of the endotoxin-induced cascade. Additionally, even if the spread of infection is stopped and pathogens are destroyed by antibiotics, the significant release of bacterial membranes into the systemic circulation demands simultaneous inhibition

of the sepsis cascade to protect the patient. Thus, the necessity for new treatments is apparent. With an increasing understanding of the underlying events leading to severe sepsis, a new pharmacological agent has shown to be a promising remedy.

1.2 Cellular activation by lipopolysaccharide (LPS) and toll-like receptor 4 (TLR4)

In order for our immune defences to be effective they need to easily recognize invading microorganisms while being sensitive enough to distinguish between self and non-self. The immune cells of our bodies do this by identifying different types of pathogens by specific conserved motifs called pathogen-associated molecular patterns or PAMPs [12]. The outer leaflet of gram-negative bacteria outer membranes consist primarily of lipopolysaccharide (LPS), the endotoxin believed to be responsible for the activation of immune cells and subsequent release of several lipid and protein mediators seen during normal infection and sepsis. The majority of patients who develop sepsis or severe sepsis will typically have positive blood cultures containing gram-negative bacteria at the time of their diagnosis [13].

1.2.1 Lipopolysaccharide structure

LPS is a relatively large molecule that can be divided into three distinct domains: a membrane-anchoring domain called lipid A, a middle region consisting of a non-repeating oligosaccharide called the core and an outer region consisting of a repeating oligosaccharide called the O-antigen (**Figure 1**) [14]. The conserved lipid A region is the pathogen-associated molecular pattern of LPS and is considered to be its stimulatory and bioactive component [15].

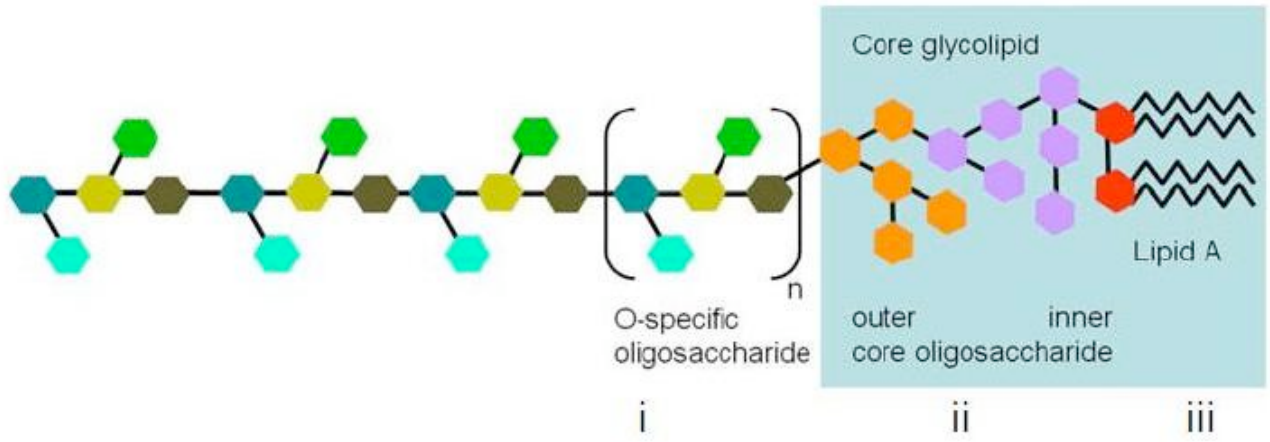


Figure 1. The general structure of gram-negative bacterial lipopolysaccharide (LPS)/ endotoxin. Lipopolysaccharide (LPS), structural components of outer cell membrane of gram-negative bacteria, are amphipathic molecules that are responsible for the inflammatory response to infection. LPS can be broken down into three distinctive regions: (i) variable O-antigen (consisting of a repeating oligosaccharide unit), (ii) conserved core region (consisting of short sugar chains) and (iii) the highly conserved lipid A region (a highly acylated phosphorylated diglucosamine). Lipid A is the membrane-anchoring portion of the molecule and is also the bioactive and pyrogenic moiety of the LPS molecule. LPS molecules are negatively charged, have a molecular weight of 10-20 kDa, and have a relatively low isoelectric point (pI = 2) causing them to form large aggregates (≥ 1000 kDa) in an aqueous solution. Figure taken from *Endotoxin Compendium V 2.6 © Hylos GmbH*.

1.2.2 Toll-like receptor 4 (TLR4) is the primary receptor for LPS

Only within the last 10 years or so have researchers been able to identify the long sought LPS receptor in immune cells. These receptors, referred to as toll-like receptors (TLRs), are a family of type I transmembrane proteins that include an extracellular domain containing multiple leucine-rich repeats (LRRs), a single α-helix membrane-spanning domain, and a cytosolic domain [16]. Their intracellular signalling domain called the toll/interleukin-1 receptor (TIR) domain, based on its homology to the IL-1 receptor, is a distinguishing feature of this family of receptors and each of its four main adaptor proteins [17]. The original toll receptor was discovered in *Drosophila* for its ability to control the dorsal-ventral patterning in developing fly embryos upon binding its ligand spatzle [18]. In flies, the toll signalling pathway was found to be involved in the production of antifungal peptides and has considerable homology to the IL-1 receptor signalling pathway that involves the expression of genes promoted by NFκB during an immune response [19, 20].

The major breakthrough in understanding the molecular mechanism behind the LPS-induced cellular response came with the discovery of the human homologue of toll, now known as toll-like receptor 4 (TLR4). Positional cloning studies of C3H/HeJ and C57BL/10ScCr mice, shown to have a defective or completely abolished response to bacterial endotoxin, identified the gene responsible [5, 20, 21]. C3H/HeJ mice had a point mutation in *TLR4* which led to an amino acid change (P712H) in the respective protein while C57BL/10ScCr mice were carriers of a null allele and had complete absence of the receptor protein, demonstrating that TLR4 is an essential component for LPS signalling.

1.2.3 The details of LPS recognition

As with most signalling cascades, the recognition of ligand by receptor is usually a collaborative effort involving several accessory components and LPS is no exception. Recognition of LPS by monocytes/macrophages begins with the binding of serum lipopolysaccharide binding protein (LBP) to LPS from either a micellar aggregate or gram-negative bacterium and the transfer of monomeric LPS to membrane-bound CD14 (mCD14) on the cell surface. As mCD14 is a glycosylphosphatidylinositol (GPI)-linked protein that lacks transmembrane or cytosolic domains, its role is not to transduce signals across the membrane. Rather, it functions to present the LPS monomer to TLR4 and another essential accessory protein called myeloid differentiation protein-2 (MD-2) that associates with the ectodomain of TLR4. These three proteins, CD14, TLR4 and MD-2 form what is referred to as the LPS receptor complex. As it is understood, LPS binds directly to MD-2 and it is through non-covalent bonding (hydrogen bonding and electrostatic interactions) that MD-2 associates with the ectodomain of TLR4, inducing a homodimerization event between TLR monomers. This homodimer ultimately serves as a scaffold for the recruitment of intracellular adaptor proteins and finally, signal transduction (**Figure 2**) [22-25].

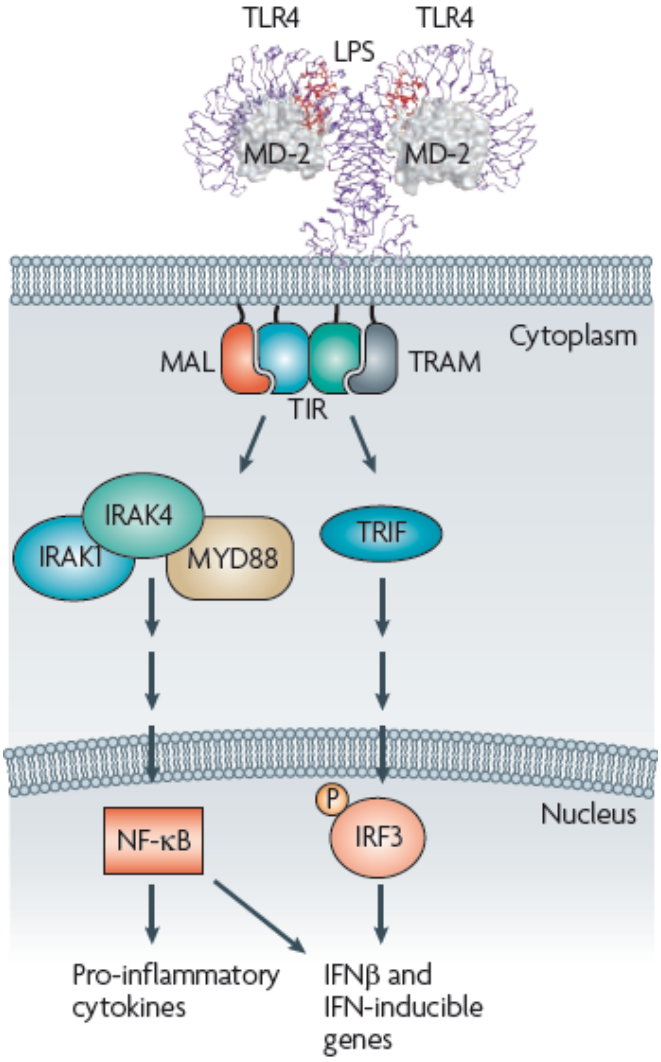


Figure 2. Monocyte/macrophage activation by LPS at TLR4 initiates an intracellular signalling cascade that results in the expression of pro-inflammatory cytokines via NFκB. The LPS receptor complex consists of CD14 (not shown) and a heterotetramer of MD-2 /TLR4. Mammalian TLRs are type I transmembrane proteins, containing an extracellular domain, a transmembrane domain, and an intracellular domain referred to as 'TIR' or toll/IL-1 receptor homology domain. The recognition of LPS can activate two separate intracellular pathways in immune cells: (1) Myeloid differentiation factor 88 (MyD88)-dependent and (2) MyD88-independent (TRIF/TRAM) signalling. In the former, MyD88 or Mal (MyD88-adaptor-like) interacts with IL-1 receptor-associated kinases (IRAK) 1 and 4 causing IRAK phosphorylation and subsequent interaction with another adaptor molecule called TRAF6 (not shown). TRAF6 will eventually activate the IKK complex (inhibitors of κB kinase) inducing phosphorylation of IκB and the release of transcription factor NFκB into the nucleus. Alternatively, LPS recognition can cause recruitment of adaptor proteins TRIF (TIR-domain containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule) activating the transcription factor IF3 and thereby inducing the expression of IFN-β (interferon β) and other IFN-inducible genes. Figure taken from Hennessey *et al. Nature Reviews: Drug Discovery,* 2010 [98]

1.3 Eritoran is a competitive inhibitor of LPS

Eritoran tetrasodium, whose chemical name is [α-D-glucopyranose, 3-O-decyl-2-deoxy-6-O-[2-deoxy-3-O-[(3R)-3-methoxydecyl]-6-O-methyl-2-[(11Z)-1-oxo-11-octadecenyl)amino]-4-O-phosphono-β-D-glucopyranosyl]-2-[(1,3-dioxotetradecyl)amino]-1-(dihydrogen phosphate) is a second-generation lipid A analogue whose structure is similar to that of the nontoxic *Rhodobacter sphaeroides* lipid A (**Figure 3**) [23]. The antagonism of LPS by eritoran is the result of its structural likeness to endotoxin and its ability to compete with LPS at the cell-surface receptor complex containing TLR4 and MD-2, thus preventing the release of pro-inflammatory cytokines and other inflammatory mediators. In HEK293 cells expressing human TLR4 and MD-2, eritoran was shown to inhibit LPS from binding MD-2, which in turn prevented the precipitation of MD-2 bound-TLR4 [26]. Furthermore, a recent study addressing the mode of ligand recognition by TLR4 found that when LPS was added to TLR4-MD2 complexes it caused the aggregation of the complexes consistent with the size of a TLR4-MD-2 heterotetramer, whereas the addition of eritoran thwarts this dimerization event and thus, the subsequent downstream response [27]. Additionally, crystal structures of eritoran bound to the TLR4-MD-2 complex show that it binds within the hydrophobic pocket of MD-2, occupying 90% of the available volume with no direct interaction with TLR4 (**Figure 4**) [27].

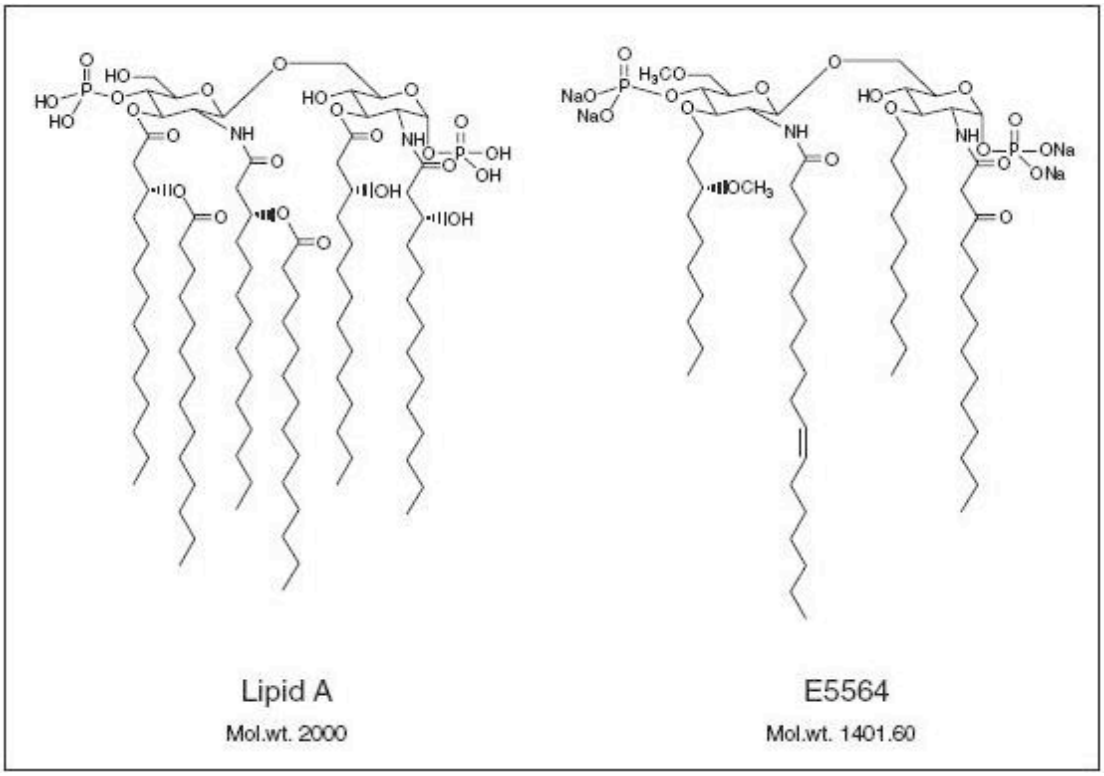


Figure 3. Chemical structures of *E.coli* lipid A and eritoran (E5564). Eritoran is a second-generation LPS antagonist and a structural analogue of the lipid A moiety of LPS from the non-toxic bacterium *Rhodobacter sphaeroides*. Figure taken from Wong *et al. Journal of Clinical Pharmacology*, 2003 [30]

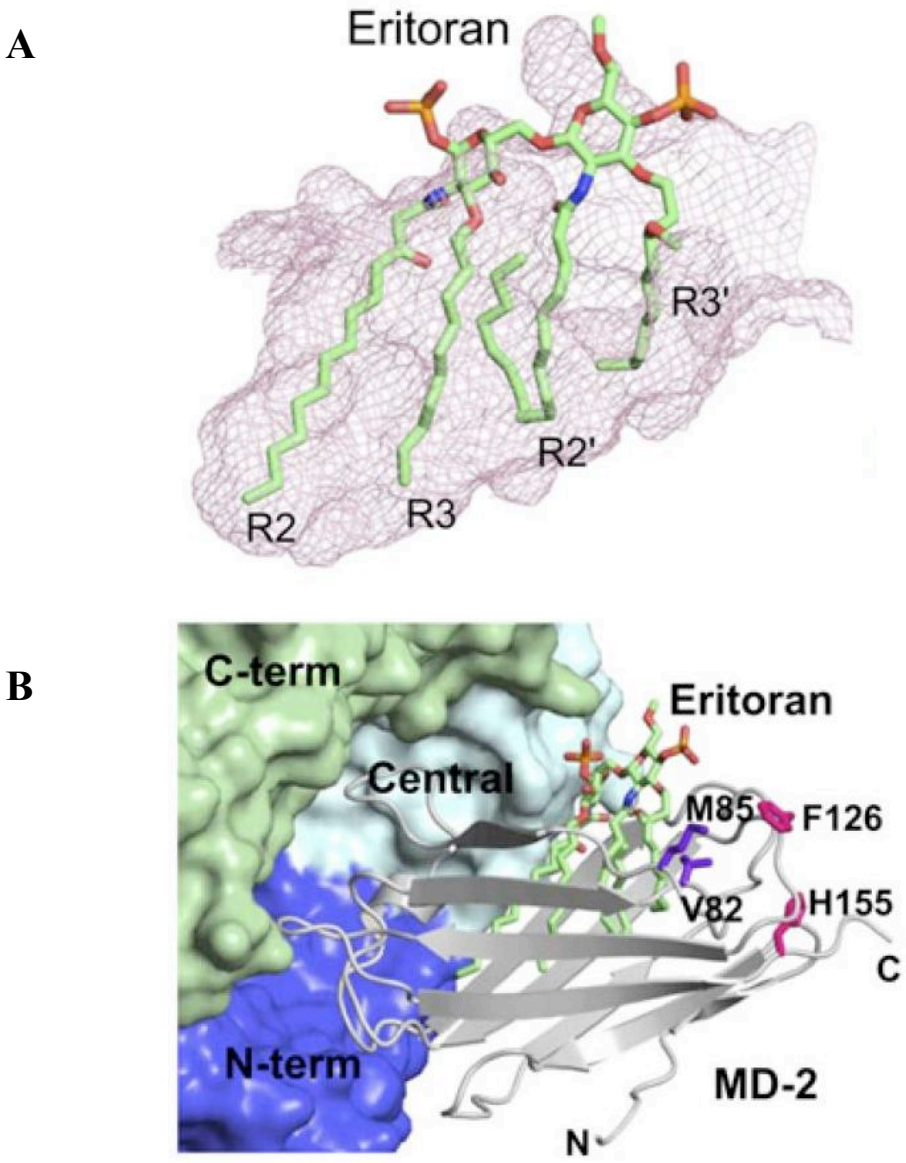


Figure 4. Structural models depicting eritoran association with toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2). (A) Eritoran binds within the hydrophobic pocket of MD-2 in which all four acyl chains occupy ~90% of the available volume. MD-2 surface is represented by the purple mesh and the acyl chains of eritoran are labelled. The negatively charged phosphates (orange) attached to the diglucosamine portion of eritoran are exposed to solvent at the neck of the pocket and further associate with positively charged residues at the opening through ionic bonds. (B) A schematic diagram illustrating the direct interaction between the ectodomain of TLR4 (divided into three surfaces) to an MD-2 molecule after eritoran binding (whose acyl chains are depicted in green). Typically, LPS bound to MD-2 will induce the formation of a heterotetramer between TLR4/MD-2 dimers in which residues F126 and H155 on MD-2 (shown in pink) are essential. However, eritoran thwarts this formation preventing signal transduction and the expression of pro-inflammatory cytokines. Figure taken from Kim *et al. Cell,* 2007 [27]

1.3.1 Eritoran is highly effective at inhibiting cytokine release and the effects of endotoxemia

The direct evidence that eritoran can block LPS signalling via the TLR4-MD-2 receptor complex and inhibit the release of pro-inflammatory cytokines is illustrated by the effectiveness of this drug when tested *in vitro* and *in vivo*. In a series of preliminary studies assessing the efficacy of the drug it was established that 10nM (14ng/mL) of eritoran can inhibit TNF-α response by 100% in human whole blood after stimulation with 10ng/mL LPS *ex vivo*, while itself possessing no LPS-like agonist activity. In addition, the drug demonstrated a similar inhibitory effect on other measures of the LPS-induced cellular response such as interleukin-6 (IL-6), nitric oxide production and NFκB promoter activity [28]. Furthermore, when bacillus Calmette-Guerin (BCG)-primed mice were concomitantly injected with a lethal dose of LPS (100μg/kg) and eritoran, there was a reduction in mortality after 72-hours dependent on increases in the dose administered [28]. A complete reduction in the incidence of mortality was reached at an equalizing dose of 100μg/kg eritoran. What is fascinating is that when BCG-primed mice were injected with whole *E.coli* bacteria one hour prior to drug treatment there was only a moderate reduction in the incidence of mortality at 72-hours when treated with eritoran, or the antibiotic latamoxef, alone. However, co-injection of both eritoran and latamoxef reduced mortality up to 80% demonstrating the effectiveness of their joint administration, a combination generally employed in treating humans with sepsis [28].

Moreover, in clinical studies healthy volunteers who were administered an IV bolus of 4ng/kg LPS, experienced chills, fever, tachycardia, myalgia, headache, slight changes in WBC counts and increased levels of cytokines and acute phase proteins such as C-reactive

protein. However, pre-treatment with an infusion of 100μg of eritoran (the expected human dose to have the same cytokine blocking effect as 10nM in blood) fifteen minutes prior to receiving LPS almost completely eliminated all symptoms associated with endotoxin, confirming its efficacy in blocking cellular activation by LPS and its ability to ameliorate physiological effects in a human endotoxemia model [29].

1.3.2 Eritoran loses activity in a time-dependent manner

Although eritoran appears as a very promising drug for the treatment of severe sepsis caused by LPS, there remains one concern that still poses a major problem for its use in the clinic. Wong *et al.* evaluated the pharmacokinetic and pharmacodynamic parameters of four doses of eritoran in healthy volunteers and found that increases in AUC_{0-oo} were dose-dependent, clearance was slow (0.67-0.95 mL/h/kg), the drug had a small volume of distribution (41-54 mL/kg) and a fairly long half-life (42-51 hrs) [30]. What was surprising about this study was that when 10ng/mL LPS was added to whole blood *ex vivo* from subjects previously infused with the varying doses of drug (350, 1000, 2000, 3500 μg), there was a dose dependent loss of drug activity in the blood samples drawn over an 8-hour period. The highest dosing group, 3500 μg, which had 95% inhibitory activity against LPS-induced TNF-α release at the end of infusion, had only 45% inhibitory activity 8-hours post-infusion (**Figure 5**). Circulating levels of eritoran in the plasma with a half-life of approximately 50 hours were disparate with the pharmacodynamics of the drug that showed a clear time-dependent deactivation after drug administration. This loss of activity has been attributed to the propensity of eritoran to bind rapidly to lipoproteins in plasma, predominantly high-density lipoproteins (HDL).

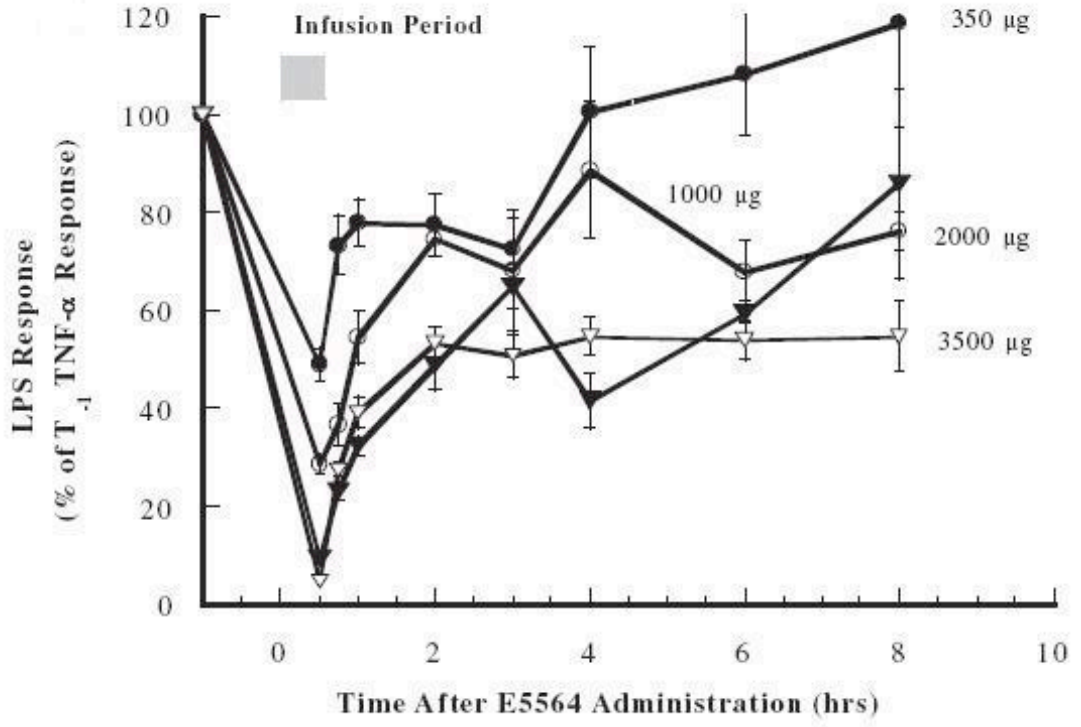


Figure 5. Average inhibition of LPS-induced TNF-α release ex vivo in blood samples from subjects infused with increasing doses of eritoran (E5564). Healthy male volunteers were split into four dosing groups and given a 30-min infusion of 350 µg (○), 1000 µg (▼), 2000 µg (▽), 3500 µg (●) eritoran, respectively. Blood samples were collected at pre-dose and up to 8 hours post-infusion and tested *ex vivo* in 48-well plates against stimulation with 10ng/mL LPS for 3 hours at 37ºC. Eritoran antagonistic activity was assessed by TNF-α release, as compared to release measured 1 hour prior to drug administration. Values are mean ± SEM. Figure taken from Wong *et al. Journal of Clinical Pharmacology*, 2003 [30]

1.3.3 Plasma high-density lipoprotein (HDL) sequesters eritoran and prevents drug activity

The role of lipoproteins in the body is to serve as transport vehicles for hydrophobic lipids in the blood, an aqueous milieu. The general structure of a lipoprotein is a phospholipid monolayer intercalated by solubilizing apolipoproteins and cholesterol that enclose a core of neutral lipids, which include triglycerides and cholesterol esters (**Figure 6**). Time course distribution studies of radiolabeled eritoran in normal plasma showed that 60% of spiked drug associated with HDL within five minutes and that there was no re-distribution among lipoprotein classes (**Figure 7a**) [31]. Additionally, a similar lipoprotein distribution profile was observed after 72-hour infusion of varying doses of drug into human volunteers (**Figure 7b**) [32].

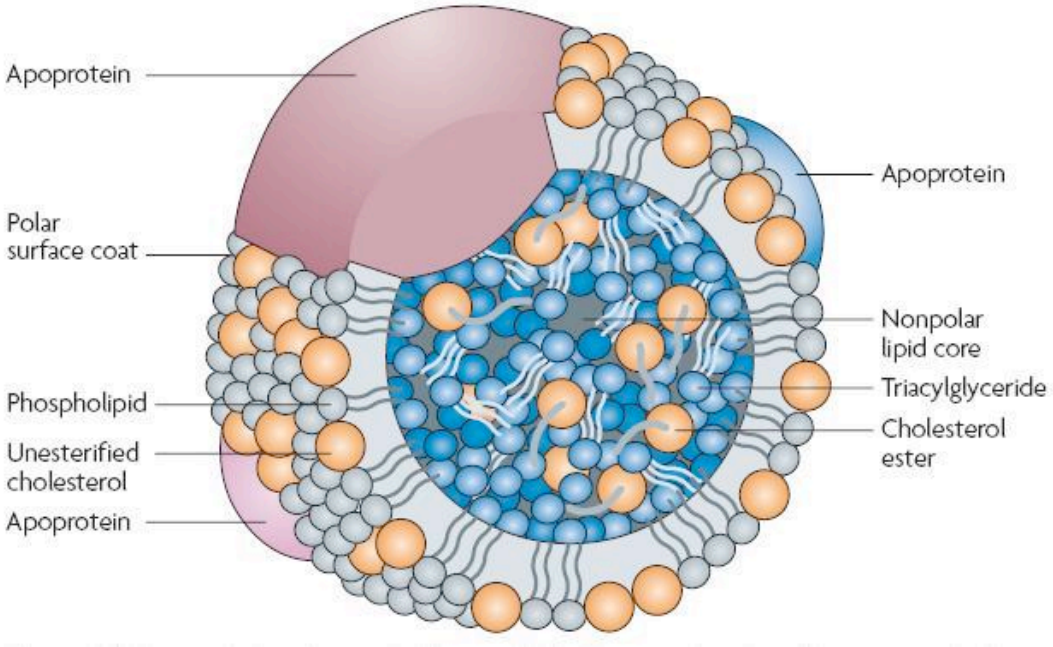


Figure 6. General structure of a lipoprotein. Lipoproteins are soluble complexes of protein and lipid that act as carriers of hydrophobic molecules, namely cholesterol, throughout the blood. They are composed of a phospholipid shell containing unesterified, free cholesterol that is held together by apolipoproteins and enclose a hydrophobic core of neutral lipids including triglycerides and cholesterol esters. There are four main classes of lipoproteins: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Each are distinguished by unique apolipoprotein content, hydrated density and their different mobilities by agarose gel electrophoresis. Figure taken from Wasan *et al. Nature Reviews: Drug Discovery*, 2008 [99]

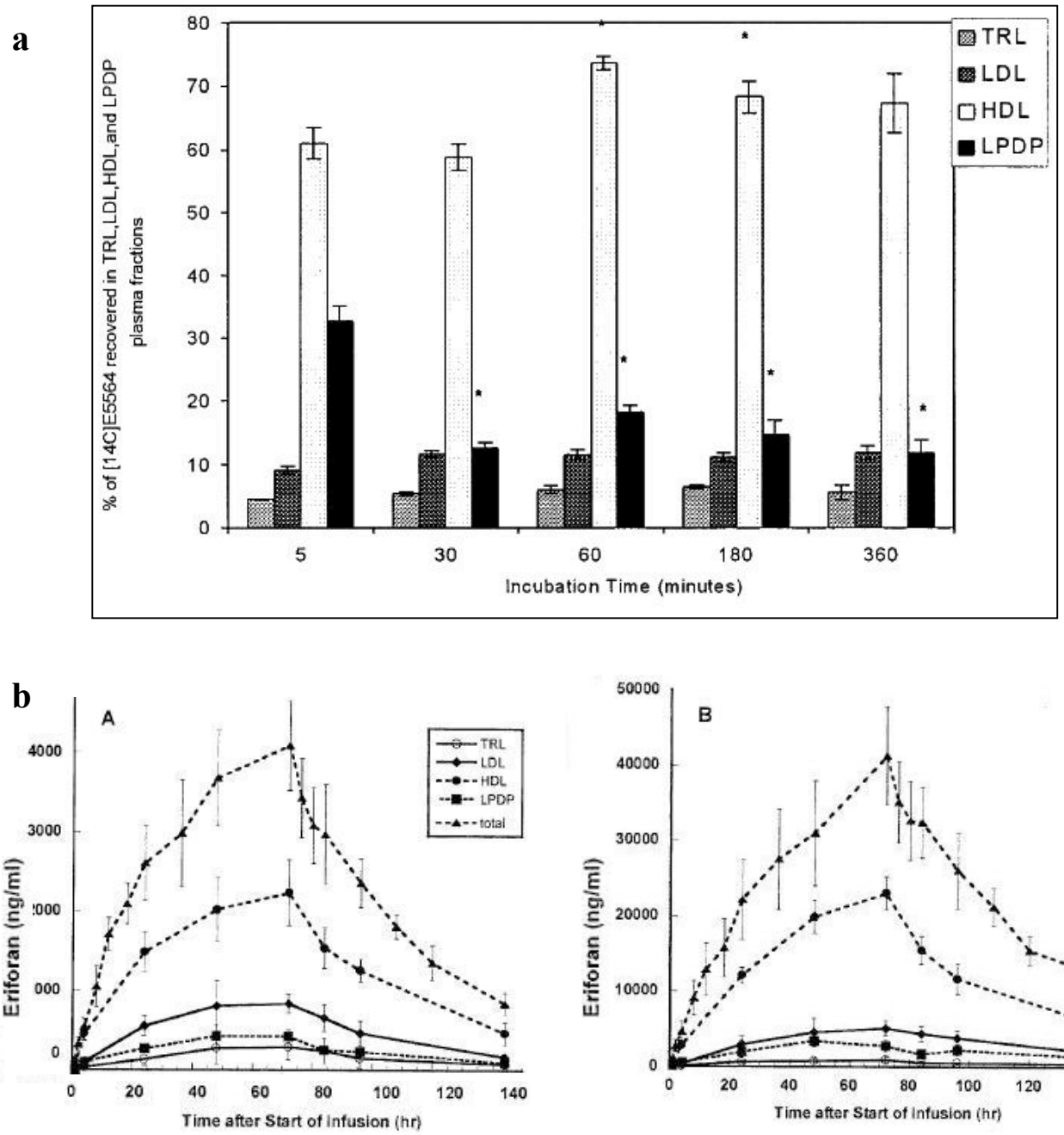


Figure 7. Distribution of eritoran into different lipoprotein fractions. (a) *In vitro* lipoprotein distribution of radiolabeled eritoran in normolipidemic plasma following a range of incubations times at 37ºC .Values shown are means ± SD (n=6). (b) *Ex vivo* lipoprotein distribution of eritoran assessed from plasma samples taken from healthy volunteers during and after a 72-hour infusion. Two dose groups were analyzed: (A) 500 µg/h (36 mg) and (B) 3500 µg/h (252 mg). Plasma at each time point was separated into lipoprotein fractions by density gradient centrifugation and fractions were analyzed for eritoran using LC-MS/MS. Inset denotes symbols for the different fractions. Values shown are the means of each dose group ± SD. TRL, *triglyceride-rich lipoprotein;* LDL, *low-density lipoprotein*; HDL, *high-density lipoprotein*; LPDP, *lipoprotein-deficient plasma*. Figures taken from Wasan *et al. Antimicrobial agents and chemotherapy*, 2003 and Rossignol *et al. Antimicrobial Agents and Chemotherapy*, 2004 [31, 32]

Inhibition of LPS-induced TNF-α release in whole blood was blocked when eritoran was pre-incubated with varying concentrations of HDL, however its activity was retained when it was pre-incubated with the other lipoprotein classes, namely very-low density lipoprotein (VLDL) or low-density lipoprotein (LDL) (**Figure 8a**) [32]. Moreover, increasing levels of total cholesterol (TC) and triglyceride (TG) content in the triglyceride-rich fractions (i.e. VLDL) correlated positively to the amount of drug recovered in this fraction. When eritoran was pre-equilibrated with the TG-rich fraction before being added to plasma approximately 50% of the drug remained amongst the combined VLDL and LDL fractions, as opposed to the 15% distributed into these fractions when eritoran was pre-incubated with saline [31]. With regard to pharmacodynamic activity, three days following a 72-hour infusion of eritoran, the drug still retains some blocking activity against 10ng/mL LPS, suggesting that a sufficient amount of eritoran may accumulate in LDL and VLDL, where drug activity is unaffected (**Figure 8b**) [32]. However, this increasing post-infusion activity is best seen at higher doses (3.5 mg/hr versus 0.5 mg/hr) where enough drug is present in these fractions to be effective. Taken together, the results of the above mentioned studies suggest that varying plasma lipid profiles will have a large effect on the lipoprotein distribution of the drug and its efficacy *in vivo* will depend on which lipoprotein class it associates with.

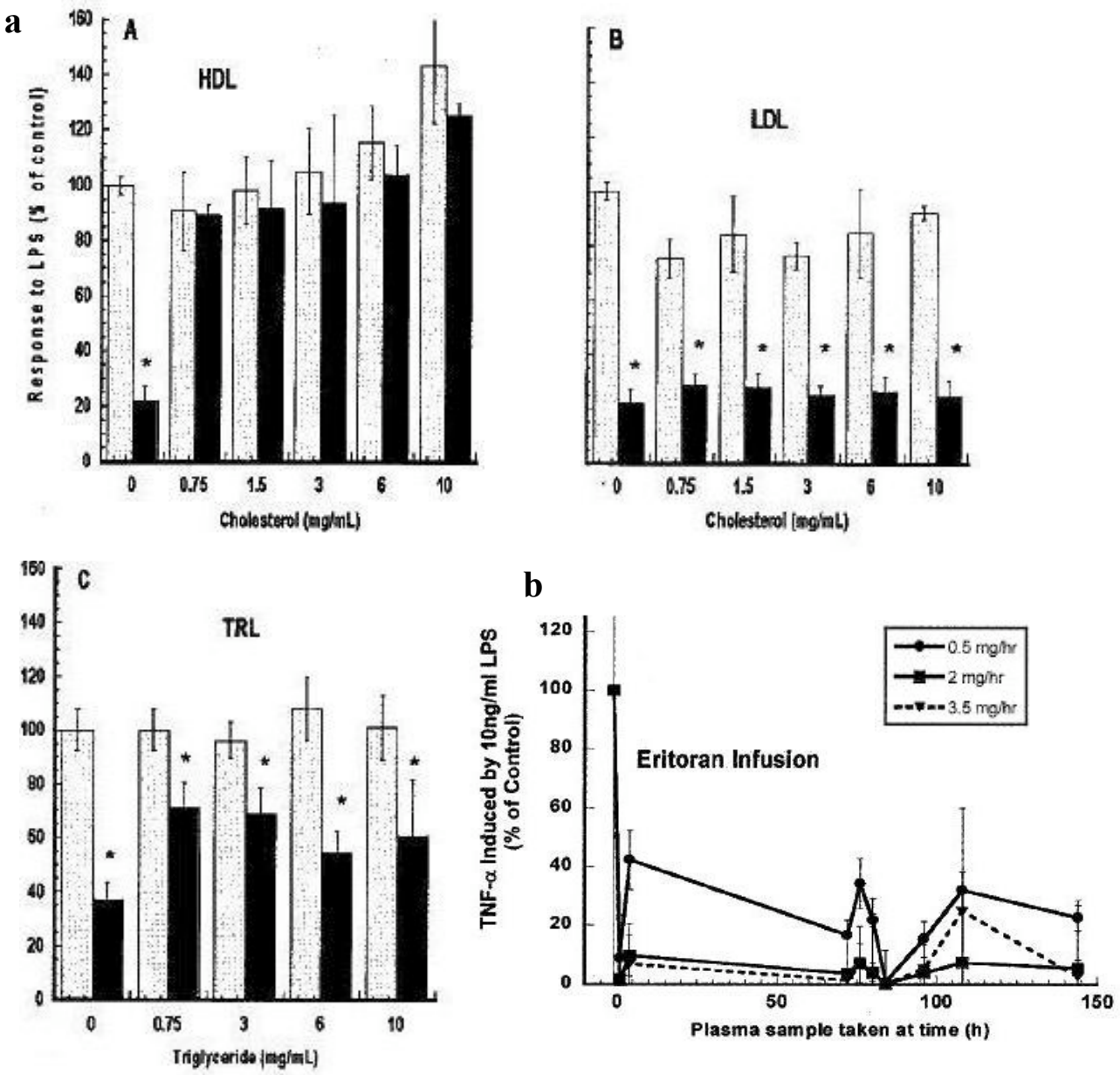


Figure 8. High-density lipoprotein (HDL) blocks the inhibitory activity of eritoran *in vitro* but some drug activity is retained *in vivo* post-infusion. (a) The inhibitory activity of eritoran against LPS-induced TNF-α release in whole blood after pre-incubation with different lipoprotein fractions, *in vitro*. Black bars represent eritoran (final concentration 10nM) pre-incubated with lipoprotein fractions at the given concentrations and gray bars represent saline. After 18-hours at 37ºC, solutions were diluted with 1:5 into fresh human whole blood and LPS was added (final concentration 10ng/mL). After 3-hours at 37ºC, plasma was collected and assayed for TNF-α. (b) TNF-α release by 10ng/mL LPS in *ex vivo* blood samples taken from healthy volunteers infused with varying doses of eritoran (inset). The values shown are the mean of samples taken from 5 subjects for all time points, except for 108h (3 subjects) and 144h (4 subjects). HDL, *high-density lipoprotein*; LDL, *low-density lipoprotein*; TRL, *triglyceride-rich lipoprotein*. Figures taken from Rossignol *et al. Antimicrobial Agents and Chemotherapy*, 2004 [32]

1.4 Septic patients are hypocholesterolemic and have altered lipoprotein metabolism

It is has been well-established that critically ill patients, such as those with sepsis, have a profound and consistent hypocholesterolemia in which TC, HDL, LDL, and their associated major apolipoproteins (apo A1 in HDL and apo B in LDL) are markedly reduced [33-40]. The mechanism by which this dyslipidemia occurs is not well-understood, however it has been shown that several cytokines can decrease secretion of apo A1 and apo B from HepG2 cells and may also decrease circulating HDL concentrations by affecting the activity of enzymes involved in the process of reverse cholesterol transport [41]. It is not surprising there has been considerable interest in therapeutic solutions aimed at improving lipid levels in sepsis, with a particular emphasis on HDL.

HDL has been shown to have LPS binding and neutralizing capacity by its ability to sequester LPS and reduce the release of pro-inflammatory cytokines such as TNF-α and IL-6 from monocytes/macrophages [42-48]. The binding capacity of circulating lipoproteins for LPS in normal plasma has been found to be in excess of 200μg/mL. Since the average concentration of LPS normally found in septic patients is ≤1ng/mL, even in lieu of low HDL levels, the question still arises, "Why doesn't endogenous HDL help provide some protection against gram-negative infection?" [49, 50]. This conundrum can be explained by a study that has demonstrated that binding of LPS to lipoproteins is much slower than the binding of LPS to peripheral blood mononuclear cells. Therefore, in the presence of high LPS concentrations LPS-induced cellular stimulation will occur before lipoproteins have a chance to sequester and neutralize the endotoxin [51]. However, increases in HDL and LDL concentrations accelerate the binding of LPS to lipoproteins and cause a reduction in TNF-α release. As expected, the lack of HDL protection due to lowered plasma HDL levels is apparent in

patients who have just been diagnosed with severe sepsis and are in the late stages of infection. A diagnosis of severe sepsis occurs around the same time that cholesterol levels, including HDL and LDL, are lowest with clinical studies showing nadirs close to 16mg/dL for HDL and 34mg/dL for LDL [52]. Normal levels of HDL and LDL cholesterol are between 40 and 60 mg/dL and close to or below 100 mg/dL, respectively [53]. Interestingly, cholesterol levels in severe septic patients recover to near normal levels over two weeks to one month, indicating that although HDL may be beneficial to survival, its neutralizing effects may only be seen during early-stage of infection when HDL levels are still relatively high (**Figure 9**) [52, 54].

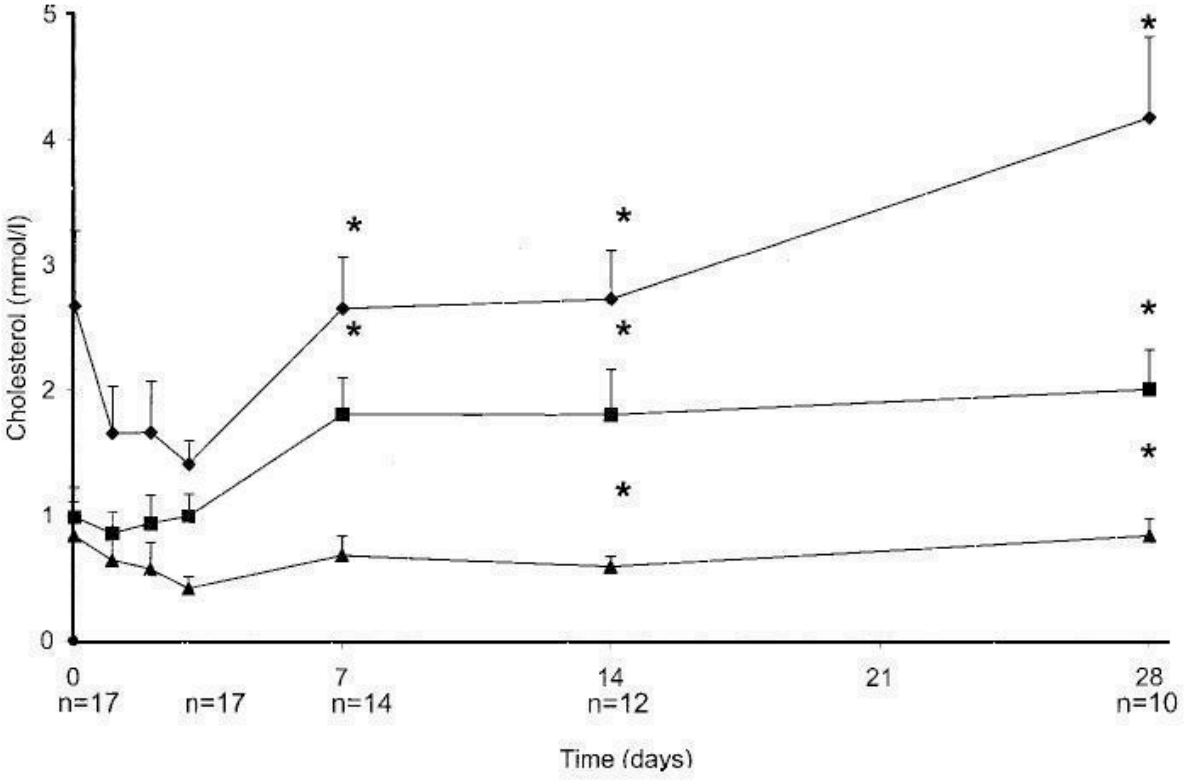


Figure 9. Total cholesterol levels in ICU patients are well below normal upon meeting the criteria for severe sepsis but improve over time in survivors. Blood samples from critically ill patients (n=17) admitted to ICU who developed severe sepsis were drawn on day 0 (study entry) and days 1, 2, 3, 7, 14 and 28 via an indwelling arterial catheter. Plasma was separated into lipoprotein fractions by density gradient ultracentrifugation and cholesterol was determined by enzymatic methods. Shown are the total plasma cholesterol (◆), LDL cholesterol (■), and HDL cholesterol (▲) from surviving patients during the 28-day period. Values are mean ± SEM. Figure taken from van Leeuwen *et al. Critical Care Medicine*, 2003 [52]

Therefore, endogenous HDL provides little protection against LPS during late-stage sepsis when HDL levels are low as their neutralizing ability is less effective. However, it is at this phase in gram-negative infection where eritoran is an appropriate therapy for treating sepsis since it acts as a competitive inhibitor at the TLR4 receptor complex, blocking the LPS-induced release of pro-inflammatory cytokines and allowing the host time to clear endotoxin and achieve some level of immunoregulation.

1.5 Apolipoproteins associated with HDL may play a role in eritoran binding and deactivation

HDL is distinguished from other lipoproteins by its role in reverse cholesterol transport, in which it removes excess cholesterol from peripheral tissues and transports it back to liver or steroidogenic tissues like the adrenal glands for excretion or the synthesis of steroid hormones, respectively. HDL particles are variable in size and density and can be divided into two main subclasses, HDL_2 and HDL_3, which respectively are larger and less dense and smaller and more dense [55]. In contrast to more lipid-rich lipoproteins like VLDL and LDL, HDL particles are 50% protein by composition, have very little triglycerides and contain mostly cholesterol esters in their core. The major distinguishing feature of HDL from other lipoprotein classes is their apolipoprotein composition.

Different from VLDL and LDL whose principal apolipoproteins are non-exchangeable, HDL contains exchangeable and soluble apolipoproteins that include apo A1 and A2 (its major apolipoproteins) and apo C1, C2, C3 and E (its minor apolipoproteins). All apolipoproteins are important in maintaining particle integrity, however each of them also has specific functions in cholesterol and lipoprotein metabolism outside its roles as structural adhesives (**Table 1**).

Table 1. Characteristics of apolipoproteins found in humans.* Figure modified from Wasan *et al. Nature Reviews: Drug Discovery*, 2008 [99]

Apolipo-protein	Plasma concentration (mg per dL)	Present on chylomicrons	Distribution into lipoproteins during fasted state (%)			Molecular weight (kDa)	Function
			VLDL	LDL	HDL		
ApoA1	130	Transiently	0	0	100	29.0	LCAT activator; substrate for SRB1
ApoA2	40	No	0	0	100	17.4	Structure; hepatic lipase inhibitor; substrate for SRB1
ApoA4	15	Yes	0	0	0	44.5	LCAT activator
ApoB48	Transient	Exclusively	0	0	0	241	Chylomicron structure
ApoB100	80–250	No	6–12	88–94	0	512	Structure; receptor ligand
ApoC1	3–6	No	3	0	97	6.6	LCAT activator
ApoC2	3–12	No	40	0	60	9.0	Lipoprotein lipase activator
ApoC3	12	No	30	10	60	9.0	Hepatic lipase inhibitor
ApoD	10–12	No	0	0	100	19.0	Many postulated
ApoE	5–7	Yes	40	10	50	34.0	Substrate for LDLr; chylomicron remnant receptor

* VLDL, *very low-density lipoprotein;* LDL, *low-density lipoprotein*; HDL, *high-density lipoprotein*; LCAT, *lecithin- cholesterol acyltransferase*; LDLr, *low-density lipoprotein receptor*; SRB1, *scavenger receptor class B1*

1.5.1 Do phospholipids exclusively facilitate LPS neutralization?

In LPS neutralization by HDL, it has been proposed that endotoxin likely anchors to the phospholipid surface of lipoproteins by insertion of the acyl chains of lipid A directly into this monolayer, conveniently concealing the bioactive component and neutralizing the toxicity of this molecule [44]. As previously mentioned, the LPS binding capacity of lipoproteins is quite large (> 200μg/mL) and it has been argued that increases in the concentration of HDL, for example, should therefore be without effect. If indeed LPS bound only to phospholipid, enough should be already available to incorporate LPS given the capacity for association, but this is not the case. In fact, transgenic mice that were high expressors of human apolipoprotein A1 had a greater survival and also lower TNF-α release after LPS challenge than low expressors, indicating that increased HDL levels were better at neutralizing LPS [44]. Additionally, another study using reconstituted HDL (rHDL) made with apolipoproteins, found that by increasing rHDL concentration there was an increased reduction in TNF-α production in human whole blood [43]. In spite of this though, the same study showed that there was a strong inverse correlation between TNF-α production and increased phospholipid content. Moreover, they showed that inclusion of other factors, such as protein, into a stepwise regression analysis did not improve this association. This implies that phospholipid concealment of LPS by membrane insertion still remains elusive.

In fact, intercalation of LPS into phospholipid membranes in a non-specific, hydrophobic manner has been disputed, as insertion of LPS into phospholipid membranes in protein-free buffer occurred only after long incubation periods or in the presence of high Ca^{2+} concentrations [56]. Likewise, co-administering LPS to rats in the presence of a protein-free

lipid emulsion did not reduce the release of pro-inflammatory cytokines, but later treatment with an apo E-enriched emulsion did cause a significant reduction [57]. Further clarity surrounding this matter has come with the recent failure of LIPOS, the Lipid Infusion and Patient Outcomes in Sepsis trial. This study, conducted to assess the efficacy of a phospholipid emulsion in increasing surface phospholipids on plasma lipoproteins, was completely ineffective at lowering 28-day all cause mortality compared to placebo and provided no benefit in preventing additional organ failure in the target group [58]. This was unexpected as the follow-up to earlier proof of concept studies that showed the promise of a phospholipid-rich emulsion in treating endotoxemia in healthy volunteers [59].

Ultimately, the aforementioned studies clearly outline an existing role for protein components in promoting LPS-lipoprotein association and demonstrate that although phospholipids may support these interactions they are not the sole facilitator. The negative results of the clinical study LIPOS illustrate the complex nature of these interactions and suggest that a simple insertion model may not be adequate to explain the mechanisms behind them.

1.5.2 Apolipoprotein A1, C1, and E can modulate the immune response to LPS

Conversely, much attention has been given to the study of apolipoproteins, of which several have been recently shown to undergo profound changes in expression and circulating plasma levels in patients with sepsis, in addition to acting as possible binding sites for LPS in HDL neutralization. Apolipoprotein E, whose plasma levels are raised during septic infections in response to LPS in both mice and humans, is also able to strongly inhibit the release of pro-inflammatory cytokines and prevent LPS-induced mortality in mice [57, 60-63].

Remarkably, free apo E and apo E-enriched emulsions have also been shown to redirect LPS within the liver resulting in an anti-inflammatory effect. In rats, specific uptake of LPS by Kupffer cells (cytokine-producing liver macrophages) decreased 8-fold after incubation with apo E or the apo E-emulsion, whilst parenchymal cell uptake (where LPS is typically secreted into the bile and deactivated) increased two-fold. Rensen *et al.* also explored the behaviour of endotoxin binding and found that chemical modification of arginine residues by cyclohexanedione (CHD) to form CHD-arginine, resulted in a decreased affinity of apo E for LPS, implying the participation of cationic residues in LPS binding [64]. In sepsis, 35% of circulating apo E is associated with HDL.

On the other hand, while normal apo C1 levels typically range from 6-10 mg/dL in a healthy person, in septic patients levels have been reported to be as low as 1.3mg/dL, a 5-fold decrease in plasma concentration [65]. What is interesting though is that apo C1 has been purported to behave as an immune system stimulant, improving presentation of LPS to macrophages and enhancing the host's immune response, as opposed to preventing it. Apo C1 was discovered to have a highly conserved sequence of residues also found in LPS-binding proteins, suggesting the association between endotoxin and apo C1 was likely to involve electrostatic interactions, as lysine residues accounted for 60% of the sequence and the lipid A moiety of LPS contains electronegative phosphates.[66].

Not surprisingly, apo A1 the major apolipoprotein on HDL, has also been shown to function as a regulator of the immune response to LPS. Several studies have shown that infusion of free Apo A1 or rHDL-containing A1 can prevent or diminish the release of inflammatory cytokines from both human and murine monocytes/macrophages in response to LPS [67, 68], attenuate LPS-induced lung and renal injury in mice [69, 70], increase the survival

rates of rats after LPS-induced endotoxemia [71], and inhibit leukocyte adhesion to endothelial cells [72]. Despite its beneficial anti-inflammatory effects, the plasma concentration of apo A1 becomes significantly lowered during the septic state. Hypocholesterolemia is characteristic of septic patients, and decreases in HDL levels are typically measured by decreases in the plasma concentration of apo A1. However, below normal levels of HDL, as measured by apo A1, are a product of both inherently lower cholesterol levels (there is lower total cholesterol) and apo A1 replacement by the acute-phase protein serum amyloid A (SAA).

1.6 Serum amyloid A (SAA): An acute phase reactant

Serum amyloid A is a 104 amino acid, highly conserved acute-phase protein with structural similarity to apolipoproteins that increases during injury, trauma and inflammation [73]. The liver is the major source of SAA production during the acute-phase response (APR) or chronic inflammation and it can be induced by stimulation with pro-inflammatory cytokines, including TNF-α following LPS administration. In humans, SAA plasma concentrations are elevated 500-2000 fold from basal levels of 20-50 μg/mL during an inflammatory state, and typically only 15-20% of total plasma SAA is free protein, most is lipid-bound [74, 75].

1.6.1 SAA-HDL formation can occur *de novo* or via displacement of apo A1

SAA has been shown to associate largely with the HDL fraction during the APR and several studies have sought to comprehend the process behind SAA-HDL formation. At present, it is evident that HDL containing SAA can be formed in one of two ways: (1) SAA displaces apo A1 in circulation, (2) SAA synthesized in the liver is secreted in a lipid-bound

form. Despite being able to displace apo A1, SAA-HDL can also be formed in its absence, as shown in apo A1 knockout mice stimulated with LPS [76].

Recently, however, SAA-HDL formation was shown to be ABCA1 dependent, similar to the production of alpha-HDL containing apo A1 [77, 78]. Nevertheless, in the absence of an acute phase response the exogenous expression of SAA does not alter the plasma concentration of HDL cholesterol or apo A1 [79]. Thus, decreases in apo A1 are not solely an effect of its displacement from HDL particles or the increased expression of SAA, indicating that changes in lipoprotein metabolism and composition during an APR could be caused by other factors affected during inflammation.

1.6.2 Composition and size of acute phase SAA-HDL differs from normal HDL

Regardless of whether there are clear functional differences in SAA-HDL formed via displacement of apo A1 or *de novo*, there are obvious differences in the composition and physicochemical properties of SAA-HDL versus native HDL enriched with apo A1. Consistently, acute phase HDL particles enriched with SAA are larger in size than normal HDL but with a density corresponding to the HDL_3 subclass. During trauma or inflammation, SAA can replace up to 80% of total HDL apolipoprotein and higher ratios of SAA protein-to-HDL_3 total mass have been shown to increase particle radii [80, 81]. *In vitro* formation of SAA-HDLs by HEK 293 cells expressing ABCA1, resulted in particles with an average diameter that was 2-3 nm larger than apo A1-generated HDL, more spherical and with differences in surface charge [78]. In accordance, formation of acute phase HDL in wild-type C57BL/6 mice injected with LPS, resulted in a larger surface-to-core ratio as estimated by increased protein content and a decrease in cholesterol ester from baseline [76]. An increase in protein content

may explain the discrepancy between the size and density of acute phase HDL. SAA is smaller than apo A1 (11.5 kDa vs. 28 kDa) and more SAA molecules by size could replace one apo A1, causing an expansion of the particle (increased diameter), but maintaining the density of the particle similar to that of HDL_3. However, this is speculative as it has yet to be reported.

1.6.3 The biological function of SAA is still ill-defined

Despite the breadth of information on SAA in the literature, to date much is still unknown about its *in vivo* functions, and its role as a pro-inflammatory or anti-inflammatory protein continues to remains elusive. Initially, SAA was determined to be a serum precursor for the subsequent formation of amyloid A protein (AA), which comprises the first 76-residues of SAA and can form amyloid fibrils that are deposited into various tissues [82, 83]. SAA (free and/or HDL-bound) has been purported to have a wide range of functions including: induction of extracellular matrix (ECM)-degrading enzymes important for tissue damage repair, chemoattractant properties for immune cells such as monocytes and T lymphocytes, alterations in reverse cholesterol transport (cholesterol removal from damaged tissue/delivery for tissue regeneration), and binding to vascular proteoglycans, implicated in the formation of macrophage foam cells in atherosclerosis (reviewed in [73, 84]). Despite the fact that its biological function is not well understood, its homology across vertebrate species and its predictable and consistent expression during an immune response highlights the importance of SAA and encourages further exploration within this field to better define its role as an immunomodulator.

1.7 Do apolipoproteins interact with eritoran to cause HDL-mediated deactivation of the drug?

Previous research has postulated that distinctive components of high-density lipoproteins, namely shell-forming phospholipids and core cholesterols, should serve as regions of LPS binding and neutralization. However, contradictory evidence has demonstrated the complexity of LPS binding and indicates that alternative/auxiliary elements are being overlooked. Indeed, there is an extensive amount of research that has shown that apolipoproteins are affected by changes in lipid metabolism that occur during sepsis, as described above. Additionally, several of these apolipoproteins are now considered to be involved in modulating the immune response, being that they can bind LPS, prevent LPS-induced cytokine release and improve survival rates after endotoxemia in experimental models**.**

Given that eritoran is a structural analogue of the bioactive lipid A moiety found on LPS, it is plausible that HDL-mediated deactivation of the drug over time during sepsis is a consequence of its association with apolipoproteins found on HDL. There is still much uncertainty surrounding the site of eritoran association with HDL, but hitherto, there has been no literature to better describe the details of this event. Curiously though, eritoran preferentially associates with HDL_3, which has a higher protein content and a smaller lipid core than HDL_2. Also, considering its small hydrophobic volume and the amphipathic nature of the drug, it is highly unlikely that the drug would be able to reside within the apolar core of HDL_3 [31, 85]. When comparing total cholesterol, circulating LDL outnumbers HDL in a ratio of 6:1, suggesting that eritoran's preferential and rapid association with HDL must be

mediated by some factor specific to HDL and not LDL, especially at the subnormal levels of lipoproteins seen in sepsis patients [86].

Therefore, it's possible that one or more of the apolipoprotein components of HDL play a critical role in binding and sequestering eritoran and are responsible for the deactivation of drug observed over time in clinical studies. The ultimate goal of this research is to elucidate whether and which apolipoproteins play a role in facilitating deactivation by HDL in the hope that this will lend insight into how drug deactivation can be avoided, and thus improve the use of eritoran as a therapeutic for patients with sepsis.

2. OBJECTIVES AND HYPOTHESES

The ultimate goal of this research is to determine which, if any, HDL-associated apolipoproteins affect eritoran's ability to block LPS-induced TNF-α release from peripheral blood mononuclear cells in whole blood.

2.1 Hypotheses

(1) A2, C1, C2, C3, or E in the free form will cause a decrease in the activity of eritoran such that, in the presence of LPS, the release of TNF-α will be higher in groups treated with apolipoproteins than in the LPS/eritoran control group, in which TNF-α release should be abolished or close to zero.

(2) All apolipoproteins will cause a greater decrease in drug activity in a lipid-bound form, as part of reconstituted HDL (rHDL), than in their free form.

(3) The level of drug deactivation elicited by a combination of apolipoproteins in rHDL, as would be found on an HDL in the healthy state, will cause a greater decrease in drug activity when compared to rHDL containing individual apolipoproteins.

(4) The level of drug deactivation elicited by all apolipoproteins in combination, as would be found on an HDL during sepsis, will be not be significantly different from that observed when eritoran was tested in the presence of a native HDL.

2.2 Objectives

2.2.1 Objective 1: Free apolipoproteins

To evaluate the effect on eritoran antagonistic activity against LPS-induced TNF-α release in the presence of free apolipoproteins A2, C1, C2, C3, and E at two different concentrations reflecting the circulating levels of these apolipoproteins on HDL in septic (low) and healthy (high) individuals. To reveal whether or not the deactivation is concentration dependent, a third intermediate concentration was included to show a range.

Aim 1: Identify apolipoproteins that may be factors affecting the LPS antagonistic activity of the drug when associated with HDL. These apolipoproteins will be selected for further investigation in objective 2.

2.2.2 Objective 2: Reconstituted high-density lipoprotein (rHDL) containing apolipoproteins

To evaluate the effect on eritoran antagonistic activity against LPS-induced TNF-α release in the presence of individual, lipid-bound apolipoproteins A1, A2, C1, and SAA as part of a reconstituted HDL. Test a sole concentration based on a concentration-response curve of native HDL. The concentration chosen must be within a range in which at least 25% deactivation is observed so as to see an effect of the rHDL if indeed one exists.

Aim 2: Assess the effects of apolipoproteins on drug activity when they are in a physiologically relevant form (lipid-bound in an rHDL construct) as opposed to free.

2.2.3 Objective 3: Normal rHDL (NrHDL)

To evaluate the effect on eritoran antagonistic activity against LPS-induced TNF-α release in the presence of "normal" rHDL that contain all apolipoproteins (A1, A2, C1, C2, C3, E) in the quantities that would be found on native HDL particles. The concentration chosen for this objective will be selected based on the lowest concentration of native HDL shown to fully deactivate the drug in an HDL concentration-response curve.

Aim 3: Determine if there is a difference (or synergistic effect) in drug activity when all apolipoproteins are present together on rHDL, as opposed to individual apolipoproteins (objective 2).

2.2.4 Objective 4: Septic rHDL (SrHDL)

To evaluate the effect on eritoran antagonistic activity against LPS-induced TNF-α release in the presence of "septic" rHDL which contain all apolipoproteins found associated with HDL in the septic state (SAA, A2, C1, C2, C3, E) in their respective quantities. The concentration chosen for this objective will be selected as described for objective 3.

Aim 4: Determine if changes in apolipoprotein composition that occur in sepsis, as modeled in septic rHDL (SrHDL), affect drug activity when compared to the apolipoprotein composition in normal HDL (NrHDL).

2.3 Significance of research

Severe sepsis has become more frequent among patients in hospital ICUs, typically as a result of nosocomial infections contracted by the critically ill, necessitating longer patient stays, increased surveillance from healthcare personnel, and a burden on hospital resources. Unfortunately, current treatment of sepsis lies in managing physiological changes manifested by LPS rather than eliminating their source by inhibiting endotoxin activity and preventing activation of immune cells. Eritoran, an effective LPS antagonist, is a seemingly strong prospect for sepsis treatment that would enhance the existing approach to management and improve patient outcomes, the primary outcome being survival.

This research, which is trying to establish a clearer understanding for the time-dependent deactivation of eritoran by HDL *in vivo*, has obvious clinical implications. The results of this study will hopefully provide insight as to the site of eritoran association with HDL such that new strategies (e.g. drug delivery, dosing regimens, etc.) can be developed to prevent losses in eritoran pharmacologic action and prolong its therapeutic benefits.

However, from a purely scientific standpoint, the study of inflammation and inflammatory diseases has become a fascinating area of research and one that seems to be closely tied to the metabolism of lipids and cholesterol. Apolipoproteins, some of which have been observed to bind and mediate physiological responses to LPS, have never been investigated with regard to eritoran in sepsis. Any advances that could be made from information gathered from this research would help to better understand the physiological properties of apolipoproteins and how interactions occur between lipoproteins and certain types of xenobiotics.

3. EXPERIMENTAL DESIGN, PARTICLE CHARACTERIZATION AND METHODS

3.1 Reagents

Eritoran (E5564) was a kind gift of the Eisai Research Institute (Andover, MA). Initially, 10mg of lyophilized drug was re-suspended in 10mL of dH_20 to make a 1mg/mL solution. 50µL aliquots of the 1mg/mL solution were created for storage at -20ºC and fresh drug was used when required. Prior to use, the drug was further diluted to achieve a working concentration of 500nM. LPS from O55:B5 *Escherichia coli* (Sigma-Aldrich®) was reconstituted in dH_20 to achieve a concentration of 1mg/mL and was stored in 100µL aliquots at -20ºC. Before use, 100µL of this solution was diluted in 100mL dH_20 to achieve a working concentration of LPS at 1µg/mL. LPS is stable up to two weeks at 4ºC. Both drug and LPS were sonicated prior to use in a water bath sonicator for 1-2 minutes to break up existing aggregates.

Lyophilized apolipoproteins A2, C1, C2, C3, and E (Meridian Life Sciences®, Inc.) were spun for 15 seconds in a centrifuge prior to reconstituting in dH_20. As lyophilized powders do not contain preservatives, reconstituted vials were used in full for each free apolipoprotein assay. Apolipoprotein A1 used in making rHDL was generously provided by Dr. Gordon Francis. Crosslinking reagent, bis[sulfosuccinimidyl] suberate, also known as BS^3 was purchased from Pierce (Thermo Fisher Scientific, Inc.). Reagents for SDS-PAGE include β-mercaptoethanol (Sigma-Aldrich®), Laemmli sample buffer (Bio-Rad®), molecular weight marker (Fermentas) and 4-20% pre-cast gradient gels (Bio-Rad®). For native gels, a high molecular weight marker kit (GE Healthcare) containing proteins with defined diameters were used to estimate particle size and native sample buffer (Bio-Rad®), which

contained no SDS, was used to load protein. All 1X tris-glycine running buffers were diluted down from 10X stock solutions made in house with the exception of those used for native gels in which a 10X stock was purchased (Bio-Rad®) without SDS.

3.2 Plasma lipoprotein separation by ultracentrifugation

Human normolipidemic plasma (Bioreclamation) was separated into lipoprotein fractions by density gradient ultracentrifugation for the collection of HDL. Briefly, 1.02g of sodium bromide (Sigma-Aldrich®) was added to 3mL plasma in clear ultracentrifuge tubes (Beckman Coulter) and were cooled on ice for 2-3 hours at 4ºC alongside sodium bromide density solutions (1.006g/mL, 1.063g/mL, 1.21g/mL) before setting the gradient . Density solutions, 2.8mL each, were layered on top of cooled plasma in the order 1.21g/mL, 1.063 g/mL and 1.006g/mL (top layer). The tubes were placed into individual titanium buckets and capped. Buckets were loaded on a SW 41 Ti swing rotor and centrifuged at 202 x 1000 g, 15ºC for 18 hours.

Upon completion, the lipoprotein fractions were removed from the centrifuge tubes using glass Pasteur pipettes. The HDL fraction from each tube was pooled into a 50mL BD Falcon™ tube and stored at 4ºC while other lipoprotein fractions were discarded. Subsequently, HDL was desalted using desalting columns (Econo-Pac desalting pre-packed gravity flow columns, Bio-Rad®) as per manufacturer's instructions. Eluted fractions were measured for protein content using the DC-protein assay (Bio-Rad®) and bovine serum albumin as a standard (Thermo Pierce). Total cholesterol (TC) was measured using the Wako Cholesterol E kit (Wako Chemicals USA). Fractions with the highest concentration of protein and TC were pooled together and adjusted to a 0.9% NaCl (Sigma-Aldrich®) solution for

isotonicity. Total cholesterol and protein was measured again to determine the stock concentration of HDL to be used in subsequent assays.

3.3 Creating reconstituted HDL (rHDL) with apolipoproteins

rHDLs were produced in collaboration with the laboratory of Dr. Gordon Francis and with the generous help of Ted Chan. To create spherical particles that best mimic the physical structure of human HDL_3, the whole lipid fraction from delipidated human HDL was combined with protein in a molar ratio of 80:1 phospholipid:protein when using individual apolipoproteins**.** When creating rHDL with a combination of apolipoproteins, as with the 'normal' rHDL model, molar ratios of apolipoproteins used were based on the circulating plasma concentration of each protein and its association with HDL.

Initially, the whole lipid fraction was added to a glass vial and evaporated completely under nitrogen gas after which 1mL of reconstitution buffer (NaCl 150mM, Tris 10mM, DTPA 0.1mM, pH 8.0**)** is added to the tube and sonicated until all lipids are dissolved back into the buffer solution. Sodium cholate (cholate:phospholipid molar ratio, 1:1) is then added to the tube and mixed vigorously. Subsequently, the desired apolipoprotein(s) is added to the tube and mixed again. If the volume of the protein solution added is less than 1mL, more buffer can be added to make a final volume of 2mL. The mixture is stirred under argon gas at room temperature overnight and then centrifuged the following day in a 3 mL tube at 99,000 rpm at 4ºC for 4 hours to remove any free lipids. Once the upper lipid layer is removed, the density of the mixture is adjusted to 1.21 g/mL using KBr and the centrifugation is repeated for an additional 4 hours. The upper fraction of the mixture containing the rHDL is collected and dialyzed against 2L of reconstitution buffer for approximately one day with at least 2

changes of the buffer. Lastly, protein content is measured as described above. At least 1.5-2.5mg of protein was used for starting material and final concentrations ranged from 2.5 – 4 mg/mL. All rHDL and HDL were stored under nitrogen gas to prevent oxidation.

3.4 Crosslinking and particle size analysis

Characterizing the rHDL consisted of determining protein number per particle and its size. Protein content can be estimated using a water-soluble crosslinking reagent, BS^3, which links proteins together on the particle surface by reacting with primary amino groups ($-NH_2$) in the side chain of lysine residues and/or the N-terminus of the polypeptide. After crosslinking, linked products (i.e. dimers, trimers, etc.) can be visualized by differences in molecular weight from the monomer form by SDS-PAGE.

A crosslinking solution of 100nM is prepared immediately before use by dissolving 2mg of BS^3 in 35μL of water. This solution was added to rHDL samples in a 50-fold molar excess based on protein. The reaction mixture was incubated for 3 hours on ice and was quenched using a final concentration of 50mM Tris-HCL, pH 7.5 for 15 minutes at room temperature. 10 μg of protein from each reaction mixture was mixed with sample loading buffer and reducing agent and boiled for 5 minutes before being loaded on pre-cast gradient gels. Gels were run for 1 hour at 100V before being stained in Biosafe Coomassie stain (Bio-Rad®) and destained overnight in water. Protein number (i.e. dimers, trimers, tetramers, etc.) were estimated from the molecular weight of different bands resolved from crosslinked rHDL and confirmed by comparison to crosslinked free apolipoprotein. Particle size of rHDL was estimated by running samples on a non-denaturing gel in the presence of protein standards with a known Stokes radius (radius of a sphere or globular particle).

The relative mobility or R_f of smears were evaluated in LabWorks imaging software (UVP, Inc., Upland, CA). Briefly, smears were divided into three regions ('bands') – top, middle and bottom – in which an R_f value was assigned to each as a proportion of 1. Using Excel, particle diameter was approximated by comparing the distance migrated by the 'bands' of the sample of interest to distance migrated by standards. Human HDL was also included for comparison.

3.5 TNF-α assay in human whole blood: Testing eritoran inhibitory activity against LPS

Testing the inhibitory activity of E5564 using a whole blood assay was chosen as blood best simulates the environment that the drug will encounter upon infusion into a patient. Since cellular and/or soluble components of the blood system may adversely affect drug activity, as shown with HDL, testing the activity of eritoran in whole blood will provide an appropriate measure of its preference to bind the LPS receptor in the presence of potential deactivators [23]. Moreover, TNF-α was chosen as a marker for cellular activation by LPS because it is consistently released by macrophages during an immune response and plays a critical role in mediating deleterious effects on the host [87].

To start, apolipoproteins and rHDL-containing apolipoproteins were incubated in sterile 48-well plates in the presence of 50nM eritoran or Ca^{2+} and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) (Invitrogen™) for a total volume of 100 µL for 18 hours at 37°C with shaking. If apolipoproteins /rHDL do associate with eritoran this should provide ample time for binding. A range of concentrations for each free apolipoprotein were tested and a single concentration of rHDL-containing apolipoprotein. With regard to free protein,

low concentrations chosen are relevant to physiological levels present in association with HDL in a sepsis patient whereas high concentrations represent these levels in healthy persons [52, 88]. The concentration chosen for rHDL was based on the lowest concentration that could maximally inhibit drug activity in a concentration-response curve based on native HDL. Controls during the 18-hour incubation include HBSS with HDL alone and HBSS with HDL and 50nM eritoran. The desired final concentration of HDL added (as estimated by total protein) was 0.8mg/mL based on a dose response in which deactivation was optimal.

After the incubation additional controls are added to the plate as follows: 5.05µL of 1µg/mL LPS is added to all wells and treatment and control groups are diluted 5-fold by the addition 400µL EDTA-treated freshly drawn human blood from healthy volunteers to a final volume of 500µL, as previously described with some modifications [89]. Final concentrations of eritoran and LPS per well are 14ng/mL (10nM) and 10ng/mL, respectively. Plates are incubated for additional 3 hours (at which TNF-α response is maximal) with gentle shaking at 37ºC and then centrifuged at 1,000 x g for 10 minutes at 4ºC after which plasma samples are collected and frozen at -80ºC. The final concentrations of eritoran were chosen based on a previous study that demonstrates 10nM (14ng/mL) of the drug can fully inhibit the release of TNF-α after 10ng/mL LPS stimulation in human whole blood [28]. Thus, 10ng/mL LPS was also chosen for this study; however, it should be noted that clinically relevant levels of LPS in sepsis patients are typically below 1ng/mL, so using 10ng/mL is considered an extremely high concentration of endotoxin [30, 50]. All plasma samples were diluted $^{1}/_{10}$ in dH_20 and vortexed thoroughly before being analyzed for TNF-α using an enzyme-linked immunosorbent assay (ELISA) for human TNF-α (R&D Systems®).

3.6 Statistics

Treatment and control groups on each plate were assayed in triplicate. Moreover, plates for each apolipoprotein were repeated three times using blood from three separate subjects. Raw data were processed in Excel 97 (Microsoft, Redmond, WA.). Paired t-tests were performed using SigmaStat 3.5 (Systat®) to determine the effects of free apolipoproteins and rHDL-containing apolipoproteins on eritoran inhibitory activity against LPS-induced TNF-α release. A one-way analysis of variance (ANOVA) was used to determine the effects of increasing concentrations of native HDL on eritoran inhibitory activity against LPS. Error was reported as standard error of the mean (SEM). A p-value of < 0.05 is considered statistically significant.

3.7 Description of methods: Measuring protein, cholesterol, and TNF-α

3.7.1 Detergent-compatible (DC) protein assay by Bio-Rad®

The DC-protein assay by Bio-Rad® is based on the Lowry method of protein quantification which takes place in two steps [90]. First, an alkaline copper tartrate solution is added to samples in a microplate. The peptide bonds in the sample protein will complex to cupric ions (Cu^{2+}) reducing the copper to the monovalent, cuprous form (Cu^{+}) in the alkaline environment. Second, a dilute folin reagent is added to the wells. When the folin reagent reacts with the copper-protein complex it becomes reduced producing a characteristic blue colour that intensifies over time and reaches 90% of its maximal colour development after a 15-minute incubation at room temperature. At this point, absorbance is determined on a

microplate spectrophotometer in the 650-750nm range, in which colour development (increases in absorbance) are proportional to protein content (**Figure 10**).

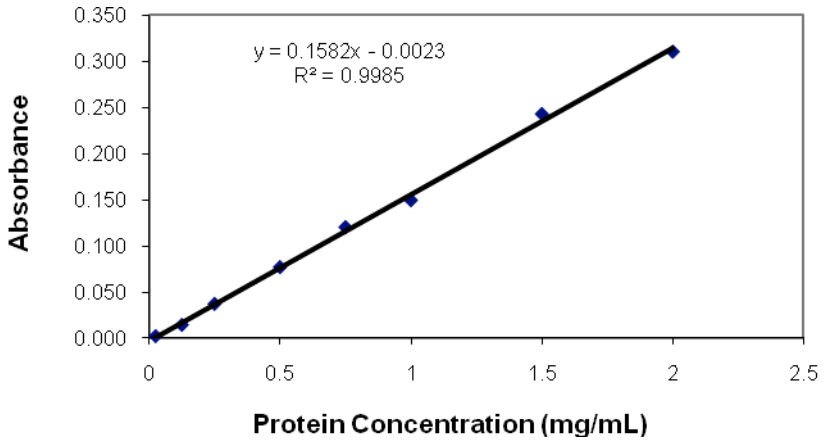


Figure 10. Example of a typical standard curve generated to measure protein using the DC protein assay. Nine standards are used ranging from 0 mg/mL to 2 mg/mL of bovine serum albumin (BSA). The line of best fit would be used to calculate protein concentrations in HDL or rHDL samples from absorbance.

3.7.2 The cholesterol E kit from Wako Diagnostics

The cholesterol E kit from Wako determines total cholesterol in plasma using a colorimetric method based on changes in absorbance. Briefly, cholesterol esters are cleaved back into free cholesterol by cholesterol ester hydrolase, at which point free cholesterol produced from esters and that already present in the sample are oxidized in a subsequent reaction by cholesterol oxidase. This reaction produces hydrogen peroxide which will contribute to the quantitative oxidative condensation between reagents 3,5-Dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline sodium salt (DAOS) and 4-aminoantipyrine in the

presence of peroxidase. The product of this reaction is a blue colour, the measurement of which is optimally read at 600nm. Simply, 2µL of sample are aliquoted in triplicate onto a 96-well plate after which 200µL of colour regent is added. Plates are given a quick shake and incubated at 37°C for 5 minutes before being read for absorbance on a spectrophotometer. The absorbance measured is directly proportional to the amount of total cholesterol present in the sample, expressed in mg/dL (**Figure 11**).

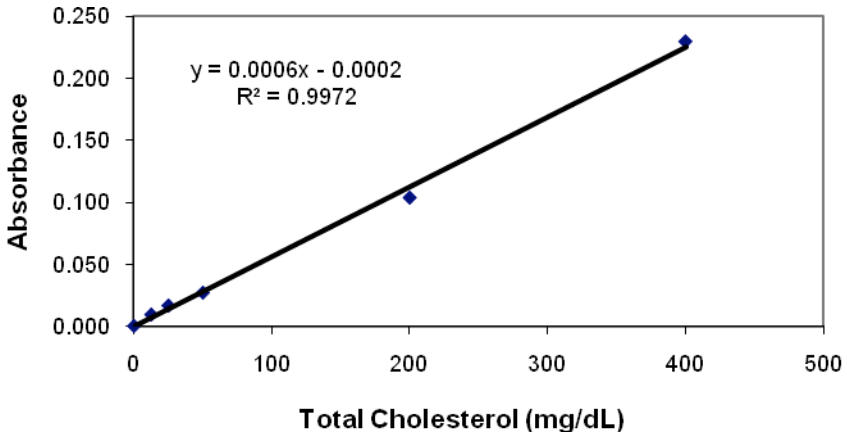


Figure 11. Example of a typical standard curve generated to measure total cholesterol using the Wako Cholesterol E kit. Six standards are used ranging from 0 mg/dL to 400 mg/dL of cholesterol. The line of best fit would be used to calculate cholesterol concentrations in HDL samples or fractions from absorbance.

3.7.3 Human TNF-α immunoassay by R&D Systems®

The human TNF-α immunoassay from R&D Systems® is a quantitative sandwich ELISA in which a mouse monoclonal antibody specific for TNF-α has been pre-coated onto a 96-well plate to which standards and samples are added. Any TNF-α present in these samples was immobilized by antibody. After several washes, any unbound material was

cleared and a polyclonal antibody against TNF-α conjugated to the enzyme horseradish peroxidase (HRP) will be added to wells. With both antibodies bound to TNF-α, a "sandwich" is formed around the antigen. Following subsequent washes, a substrate solution containing hydrogen peroxide and a chromogen (tetramethylbenzidine, TMB) was added to all wells. HRP will oxidize TMB using hydrogen peroxide at which point colour developed in the wells proportional to the amount of TNF-α initially bound in the first step. Stop solution (sulfuric acid) was added to stop the progression of colour development. The absorbances of plates were read on a microplate spectrophotometer at 450 nm (**Figure 12**).

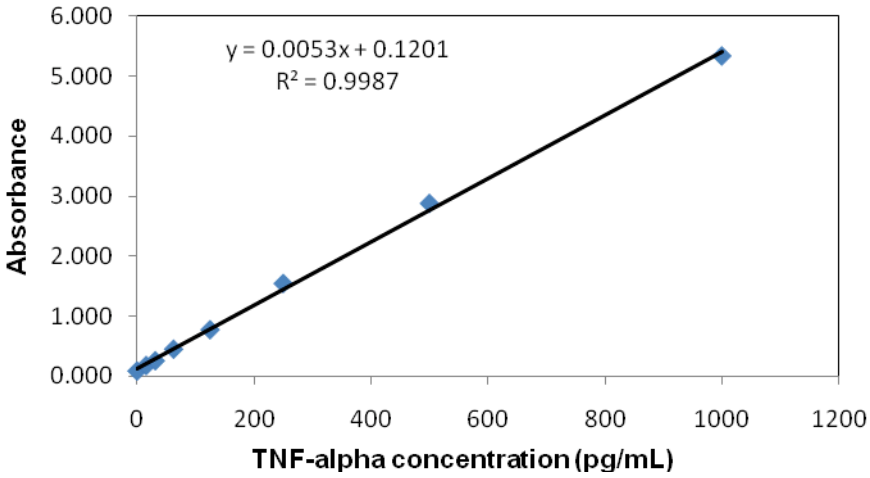


Figure 12. Example of a typical standard curve of TNF-α generated using ELISA. Eight standards are used ranging from 0 pg/mL to 1000 pg/mL of recombinant human TNF-α in animal serum. The line of best fit was used to calculate TNF-α concentration in plasma samples from absorbance.

4. RESULTS

4.1 The effect of free apolipoproteins on the antagonistic activity of eritoran against LPS

Apolipoproteins A2, C1, C2, C3, and E were incubated in the free form overnight with eritoran to allow for potential drug binding before being added to human whole blood and treatment with LPS to induce the release of cytokines. Each apolipoprotein was evaluated at three different concentrations to reflect circulating levels of the protein as associated with HDL in both the septic state (low concentration) and in a healthy state (high concentration). A third, intermediate concentration was chosen to complete a concentration range in order to observe any concentration-dependent effects on drug deactivation. Healthy concentrations were based on levels established in the literature and septic concentrations were approximated from HDL levels reported during sepsis and an estimation of the apolipoprotein content on HDL (**Tables 2 and 3**). Apolipoproteins A1 and SAA were not tested in the free form as the desired concentrations of these proteins were outside the volumetric limits of the whole blood assay.

Table 2. Apolipoprotein plasma levels found in healthy fasting humans and the percent associated with HDL [88].*

Apolipoprotein	Plasma Concentration in mg/dL	% found in HDL
Apo C1	6 (60µg/mL)	97
Apo C2	3 (30µg/mL)	60
Apo C3	12 (120µg/mL)	60
Apo A1	130 (1.3mg/mL)	100
Apo A2	40 (0.4mg/mL)	100
Apo E	5 (50µg/mL)	50

*Concentrations to use per well were based on these values.

Table 3. Final concentrations used for free apolipoproteins in whole blood *

	Final concentration of protein used (µg/mL)
A2	
Low	83
Intermediate	250
High	400
C1	
Low	5.1
Intermediate	25
High	58.2
C2	
Low	0.64
Intermediate	9
High	18
C3	
Low	9
Intermediate	36
High	72
E	
Low	0.51
Intermediate	12.5
High	25

* Low values calculated based on HDL cholesterol in septic blood. The estimation that the ratio of total protein: total cholesterol in HDL is 2:1. HDL cholesterol level in septic patients was found to be 32mg/dL, thus total protein associated with HDL would be ~ 64mg/dL. The estimated % of each apolipoprotein in HDL was calculated previously and concentrations in septic patients were derived by multiplying these composition percentages by the total protein concentration which was 64 mg/dL. Apolipoprotein Composition of HDL – Apo A1 (85%), Apo A2 (13%), Apo C1 (0.8%), Apo C2 (0.1%), Apo C3 (1.4%), Apo E (0.08%). [52, 55, 88]

In addition to the groups of interest, several quality controls were added to each plate to ensure no inherent release of TNF-α without LPS (blood only group) and that the whole blood assay was consistent and working (HDL controls) as HDL has already been shown to deactivate the drug (**Table 4**).

Table 4. Experimental and quality control groups included in TNF-α assay to test the inhibitory activity of eritoran (E5564) against LPS in the presence of different lipid-free apolipoproteins or as part of a reconstituted HDL particle (lipid-bound).

Experimental Groups
LPS Only LPS + E5564 Apo (low concentration) + LPS Apo (low concentration) + LPS + E5564 Apo (Inter. concentration) + LPS Apo (Inter. concentration) + LPS + E5564 Apo (high concentration) + LPS Apo (high concentration) + LPS + E5564 rHDL-apo + LPS rHDL-apo + LPS + E5564
Quality Control Groups
Blood LPS + HDL LPS + HDL + E5564

The release of TNF-α, a pro-inflammatory cytokine, was used as a marker for drug activity. Stimulation by LPS causes an increase in the release of TNF-α from peripheral blood mononuclear cells in the blood and drug activity is inferred by its ability to effectively reduce this response. Since TNF-α levels can vary widely from person to person after stimulation

with LPS, all values were normalized within subjects to their baseline TNF-α release after LPS stimulation in order to compare effects between subjects. Although absolute values of TNF-α may differ, overall trends should be the same from person to person. Quality controls were performed for every assay conducted (during the overall study) but have been omitted from graphs as they were not included in the statistical analyses as comparative groups. However, a representative figure of these controls is provided (**Figure 13**).

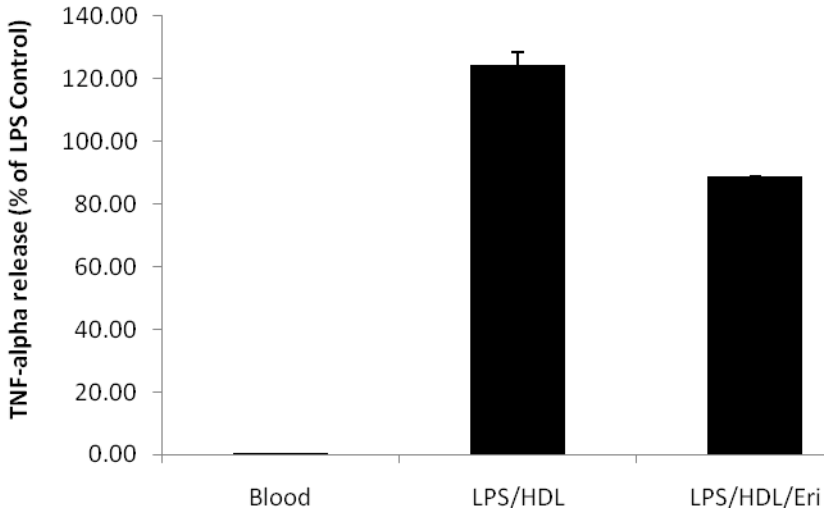


Figure 13. Example of the TNF-α release observed with quality controls. The above three controls were completed in the whole blood assay alongside comparative groups to ensure consistency in eritoran deactivation by HDL (already shown by Rossignol *et al. Antimicrobial agents and Chemotherapy,* 2004) and verify blood is endotoxin-free. Any unexpected deviations within these controls would warrant the experiment be repeated. This graph is a representation of the TNF-α levels expected. Similar results were observed with every assay. Error bars represent + SEM. N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*; HDL, *high-density lipoprotein*

In figure 14, apolipoprotein A2, the second most abundant apolipoprotein on healthy HDL, was shown to significantly deactivate eritoran at the low (mean=180.9%, p=0.041) and intermediate (69.6%, p=0.036) concentrations, but was less effective at the highest concentration (31.5%, p>0.05) (**Figure 14**). Furthermore, at the highest concentration, apo A2 reduced cytokine release without the presence of the drug (A2/LPS). This implies that at higher levels of the protein, a threshold concentration may be achieved where LPS response is diminished by the protein itself. However, it is not obvious if this effect is a result of LPS binding by the protein or secondary effects on the blood system (*i.e.* apo A2 affects other factors of inflammation which decrease cytokine release). Likewise, at the lowest concentration, apo A2 presence alone causes an unforeseen increase in TNF-α release in which levels exceed that of the LPS only group. This result is poorly understood at this time.

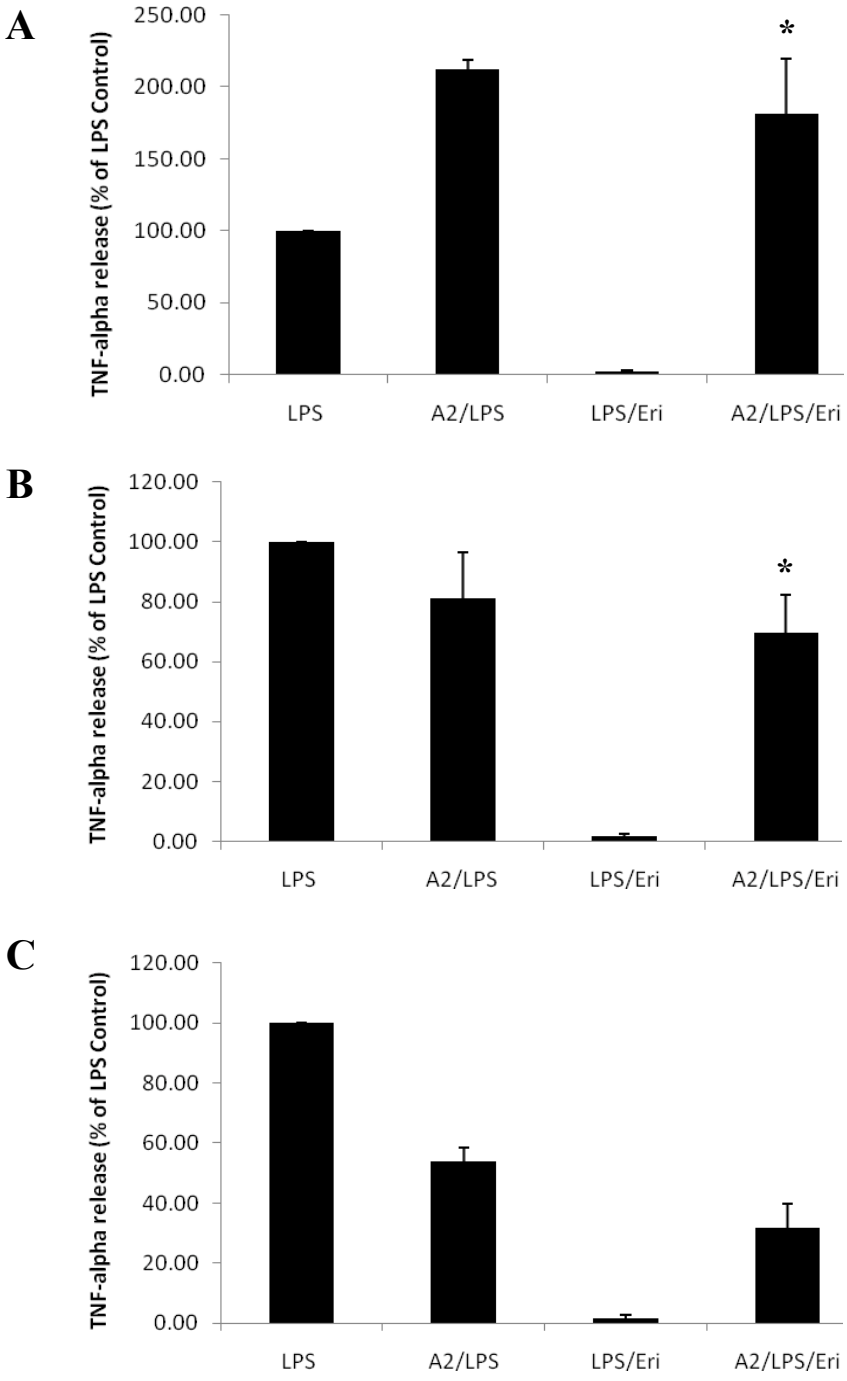


Figure 14. Eritoran deactivation by free apolipoprotein A2. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and apolipoprotein A2 at the *low (A)*, *intermediate (B)*, and *high (C)* concentrations. Final concentrations were 83µg/mL, 250µg/mL and 400µg/mL, respectively. Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using paired t-tests. Error bars represent + SEM. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor-α*; Eri, *eritoran*

In figure 15, apolipoprotein C1 fully inhibited drug activity at the lower, septic concentration, however was not statistically significant (101.2%, p=0.068) (**Figure 15**). This result must be interpreted cautiously however, since this particular t-test was underpowered and is probably a consequence of the large deviation from the mean observed in one subject (subject 3) at this particular concentration (**Appendix: Table 1**). Conversely, at the intermediate and high concentrations, apo C1 was shown to significantly deactivate the drug (87.3%, p=0.025; 45.4%, p=0.036).

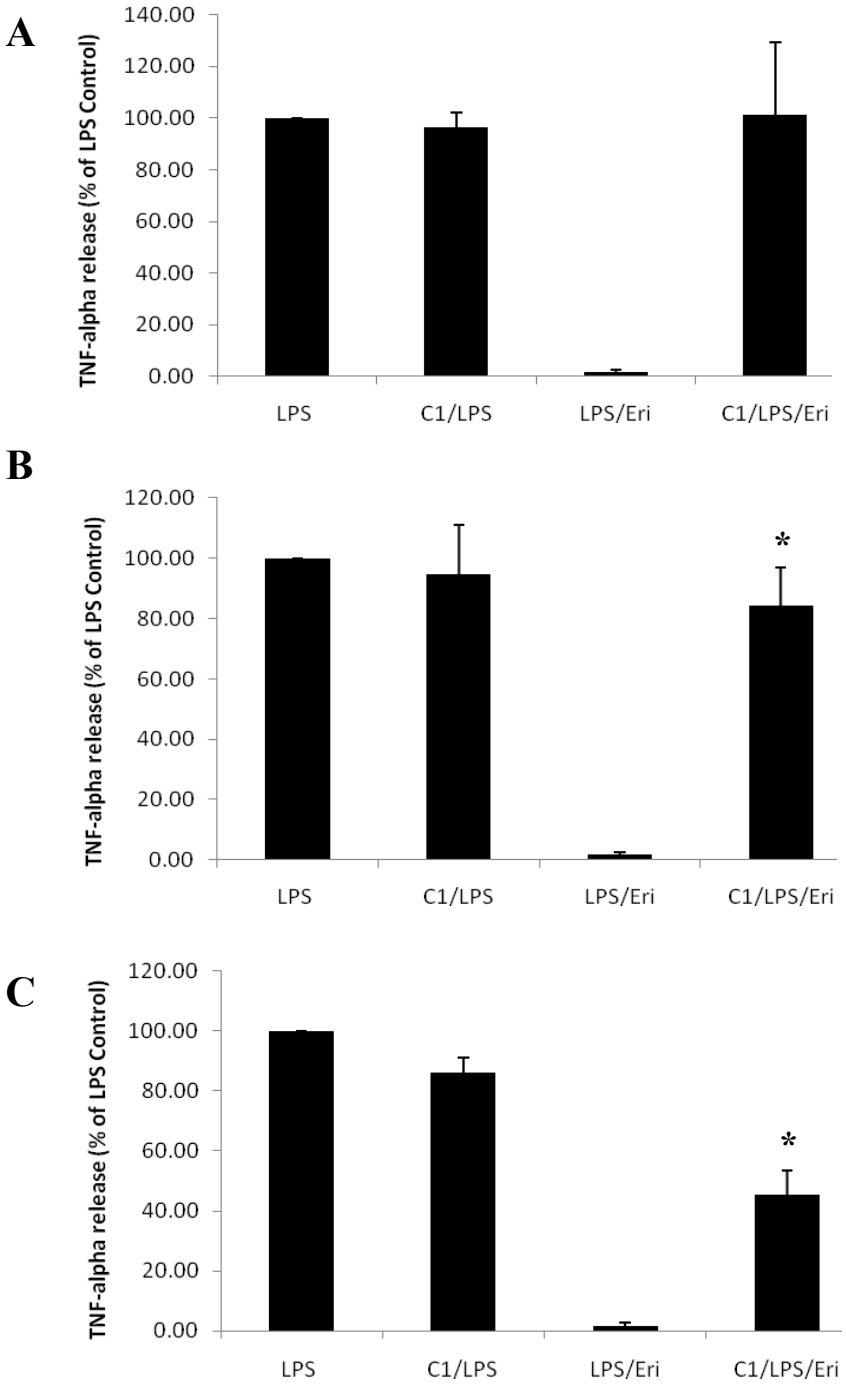


Figure 15. Eritoran deactivation by free apolipoprotein C1. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and apolipoprotein C1 at the *low (A)*, *intermediate (B)*, and *high (C)* concentrations. Final concentrations were 5.1µg/mL, 25µg/mL and 58.2µg/mL, respectively. Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using paired t-tests. Error bars represent + SEM. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor-α*; Eri, *eritoran*

In figure 16, apo C2 deactivated eritoran at the low (13.9%), intermediate (34.3), and high (29.0%) concentrations. However, the inhibitory effect on drug activity, as measured by increases in TNF-α, in the presence of apo C2 was not statistically different from the LPS/Eri group when tested at any of these concentrations. It must be noted that each t-test conducted for this protein was underpowered, again illustrating large variation between subjects (**Appendix: Tables 1, 2, 3**) and the reduced likelihood to detect differences when they actually exist.

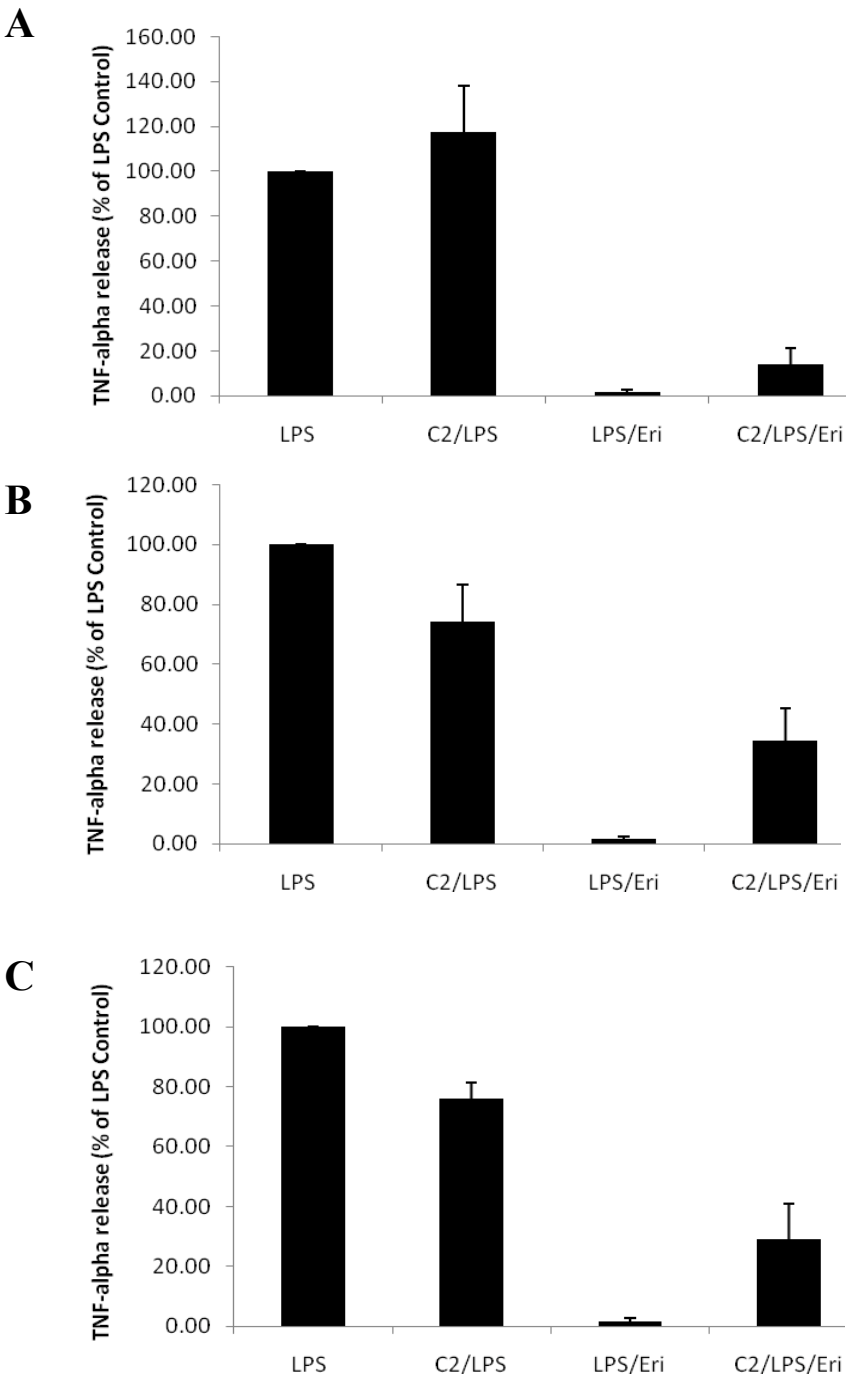


Figure 16. Eritoran deactivation by free apolipoprotein C2. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and apolipoprotein C2 at the *low (A)*, *intermediate (B)*, and *high (C)* concentrations. Final concentrations were 0.64µg/mL, 9µg/mL and 18µg/mL, respectively. Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using paired t-tests. Error bars represent + SEM. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*

In figure 17, apo C3 caused drug deactivation at the low (16.0%), intermediate (67.2%) and high (23.4%) concentrations, but was not statistically significant in any case as a consequence of the low power to detect differences (**Figure 17**). As with apo C2, there is no evidence from these data that apo C3 causes a concentration-dependent decrease in eritoran activity, however the failure to see any trend with apo C3 is likely due to inter-subject variability (**Appendix: Tables 1, 2, 3**).

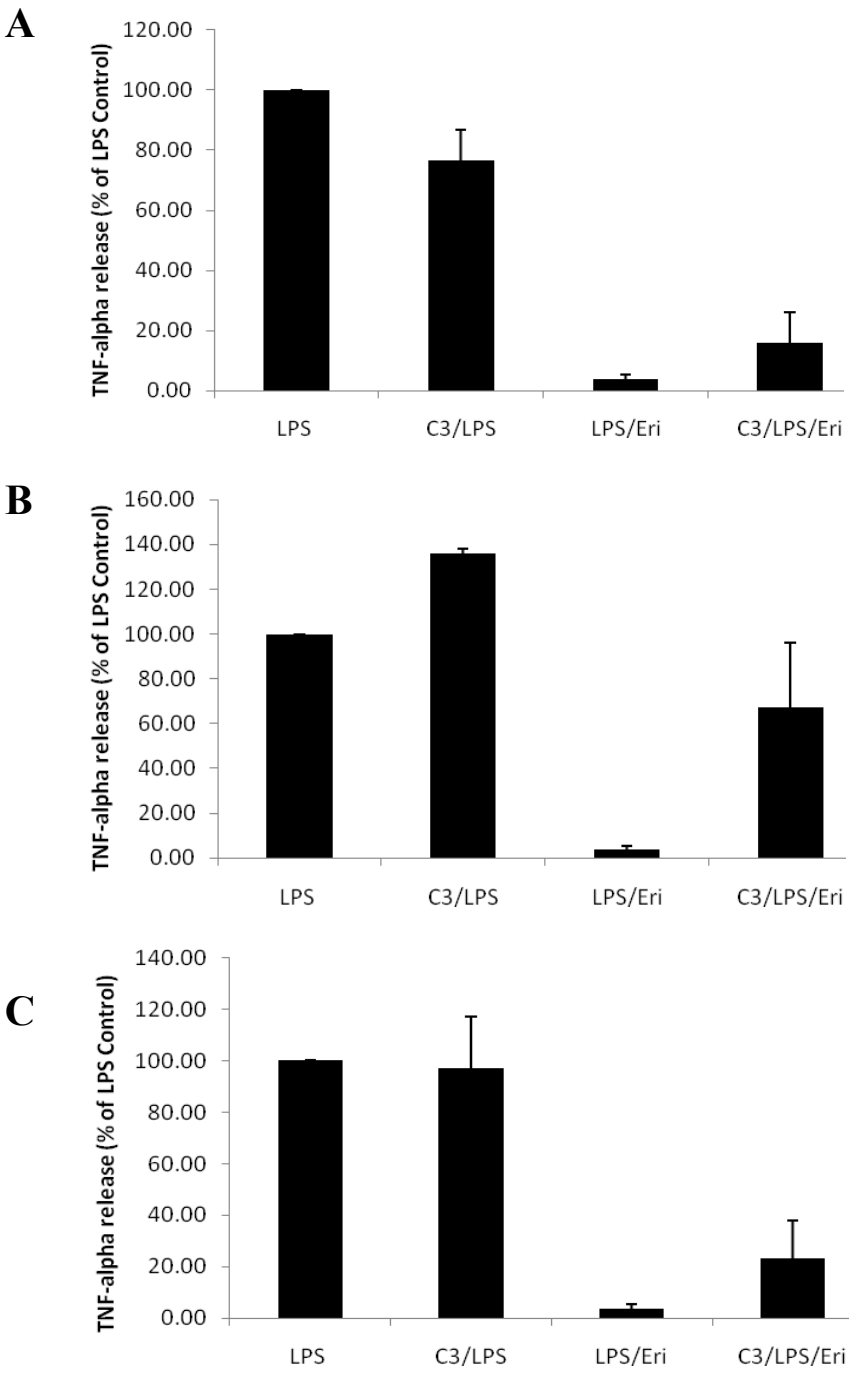


Figure 17. Eritoran deactivation by free apolipoprotein C3. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and apolipoprotein C3 at the *low (A)*, *intermediate (B)*, and *high (C)* concentrations. Final concentrations were 9µg/mL, 36µg/mL and 72µg/mL, respectively. Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using paired t-tests. Error bars represent + SEM. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*

In figure 18, there were differences observed in drug activity at the low (66.5%), intermediate (141.8%) and high (75.1%) concentrations of apolipoprotein E tested (**Figure 18**). However, again due to variability between subjects, statistical tests were underpowered and there was no significant difference in drug activity from the LPS/Eri group in groups treated with apo E at the low (septic) and intermediate concentrations. On the other hand, at the high (healthy) concentration apo E was shown to cause a significant reduction in drug activity (75.1%, $p=0.004$).

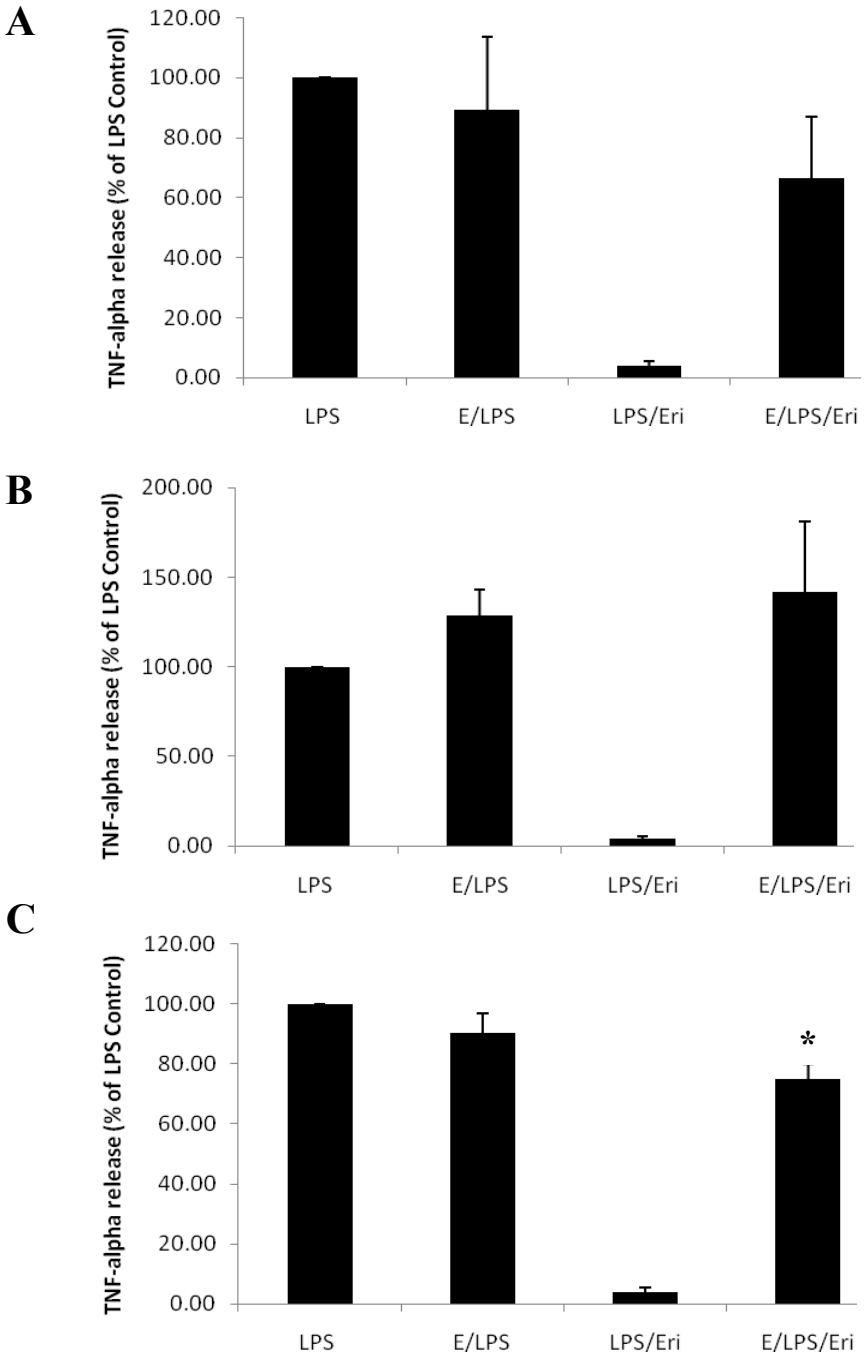


Figure 18. Eritoran deactivation by free apolipoprotein E. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and apolipoprotein E at the *low (A)*, *intermediate (B)*, and *high (C)* concentrations. Final concentrations were 0.51µg/mL, 12.5µg/mL and 25µg/mL, respectively. Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using paired t-tests. Error bars represent + SEM. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor-α*; Eri, *eritoran*

4.2 The effect of reconstituted HDL (rHDL) containing apolipoproteins on the antagonistic activity of eritoran against LPS

The results of the free apolipoprotein study prompted us to further investigate apolipoproteins A2 and C1 as potential deactivators in an rHDL construct. Moreover, it was imperative to test apo A1 and SAA to determine whether or not they affect eritoran activity, being the most abundant apolipoproteins on HDL in both the healthy and septic state. As rHDL constructs containing individual apolipoproteins have never been explored as a means of reducing eritoran activity, the first stage of this study required us to establish the concentration range we wanted to use. This was important, because, as the limiting factor we wanted rHDL to be "in excess" such that it would ensure we saw significant deactivation of the drug if indeed it existed and not fail to observe drug effects due to low concentrations.

Based on an earlier HDL concentration-response curve (data not shown) it was deemed that HDL at a concentration of 1.5 mg/mL, as measured by protein, would impart almost full deactivation. However, since our protein supply for most of the apos was limited we settled on a range of 0.4 - 0.75 mg/mL, which caused roughly 30-50% deactivation. Surprisingly, a preliminary whole blood assay performed using rHDL-A1 illustrated that rHDL at a protein concentration as high as 0.7 mg/mL greatly reduced TNF-α release by itself without the presence of the drug. As a result, this impaired the ability to detect possible apolipoprotein effects on the eritoran activity since the release was negligible to begin with. Thus, an HDL concentration-response curve was repeated at a range of protein concentrations below 0.7 mg/mL (**Figure 19**). It is evident that HDL deactivates eritoran completely at 0.8 mg/mL, by protein. Nevertheless, there is still 20-30% deactivation below

0.8 mg/mL, at which HDL levels are considered sub-normal. This demonstrates how little HDL is required, by protein, to affect eritoran activity.

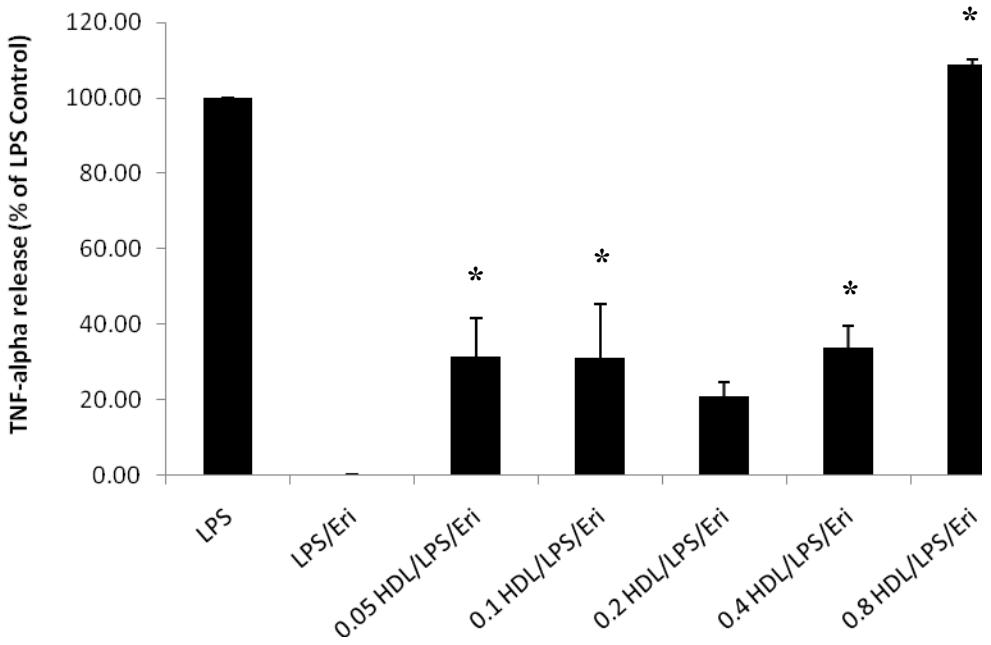


Figure 19. Concentration-dependent effects of high-density lipoprotein (HDL) on eritoran activity. TNF-α release from human whole blood after stimulation with 10ng/mL LPS in the presence of the LPS antagonist eritoran (10nM final) and increasing concentrations of high-density lipoprotein (HDL). HDL concentrations are expressed in mg/mL by protein and cytokine release in % of LPS control. All HDL controls (without eritoran) were conducted at the same time for each concentration and all were similar to LPS (not shown). Means were calculated from the TNF-α release observed in three independent subjects. Statistical analysis was performed using a one-way ANOVA ($p<0.001$). * Denotes significant differences between groups to the LPS/Eri group as assessed by Dunnett's test. A p-value of <0.05 is considered statistically significant. Error bars represent + SEM. N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor-α*; HDL, *high-density lipoprotein*; Eri, *eritoran*

Therefore, 0.1 mg/mL was chosen as the final protein concentration that was to be used for all rHDL studies. At this concentration, we could make rHDL using less protein and still be within the level of deactivation desired.

To estimate particle size, each set of rHDL were characterized for the number of protein molecules/particle using crosslinking with BS^3 and run on a native gel alongside a high molecular weight marker (HMW) (**Appendix: Figures 2-7**). A table providing the particles sizes (**Appendix: Table 4**) and an example of the particle analysis performed in excel is shown for rHDL-A1 and A2 (**Appendix: Figure 1**). Reconstituted HDL were used in the same week as their preparation, to limit oxidation and/or contamination, and tested for their ability to inhibit eritoran activity. TNF-α release from whole blood was analyzed in plasma collected as described in methods.

In Figure 20, illustrating deactivation of eritoran by rHDL-A1, lipid-bound A1 caused a significant loss in drug activity (mean=47.4%, p=0.02) as shown by the inability of eritoran to fully suppress TNF-α release (**Figure 20**).

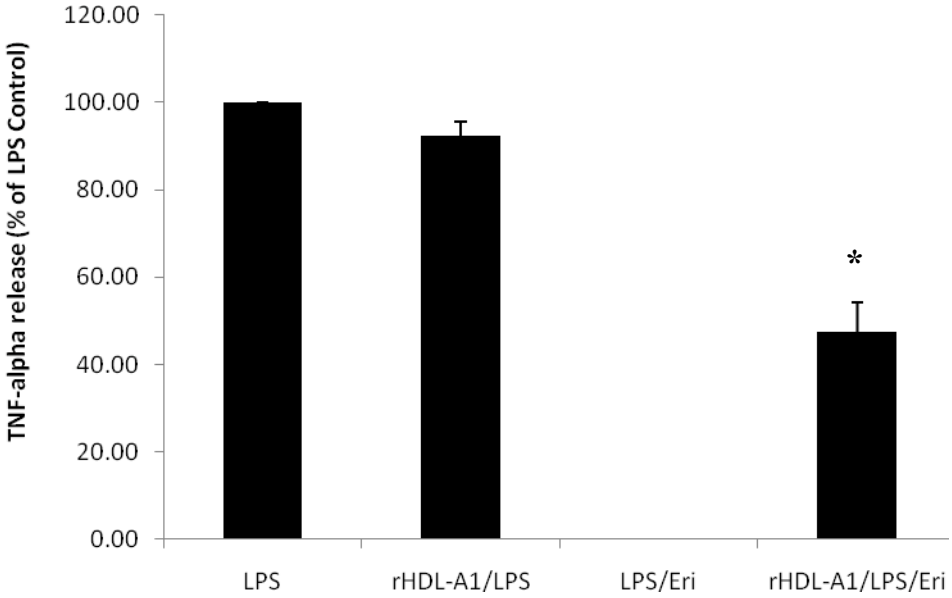


Figure 20. Eritoran activity as measured by TNF-α release in the presence of rHDL-A1. Values shown here are the means calculated from all three subjects. TNF-α release is shown as % of LPS. Final concentrations of rHDL-A1 are 0.1mg/mL by protein. Error bars represent + SEM. Statistical analysis was performed using a paired t-test. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*; rHDL, *reconstituted high-density lipoprotein*

Similarly, as shown in figure 21, eritoran antagonistic activity against LPS-induced TNF-α release in whole blood was considerably inhibited in the presence of rHDL-SAA. At 0.1 mg/mL, lipid-bound SAA decreased drug activity by 26.4% ($p<0.001$) (**Figure 21)**.

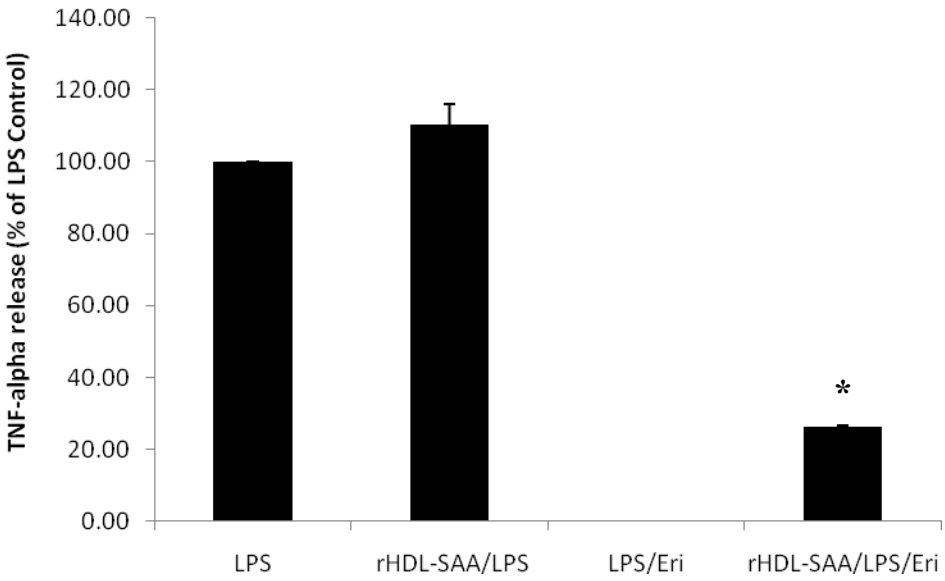


Figure 21. Eritoran activity as measured by TNF-α release in the presence of rHDL-SAA. Values shown here are the means calculated from all three subjects. TNF-α release is shown as % of LPS. Final concentrations of rHDL-SAA are 0.1mg/mL by protein. Error bars represent + SEM. Statistical analysis was performed using a paired t-test. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*; rHDL, *reconstituted high-density lipoprotein*

On the other hand, the sizeable reductions in drug effect seen with apo A1 were not observed when eritoran activity was tested after pre-incubation with rHDL-C1 (13.0%, $p>0.05$) (**Figure 22**). Although, there was some drug inhibition in the presence of rHDL-C1, it was not statistically significant from the LPS/Eri group, in which TNF-α release was zero.

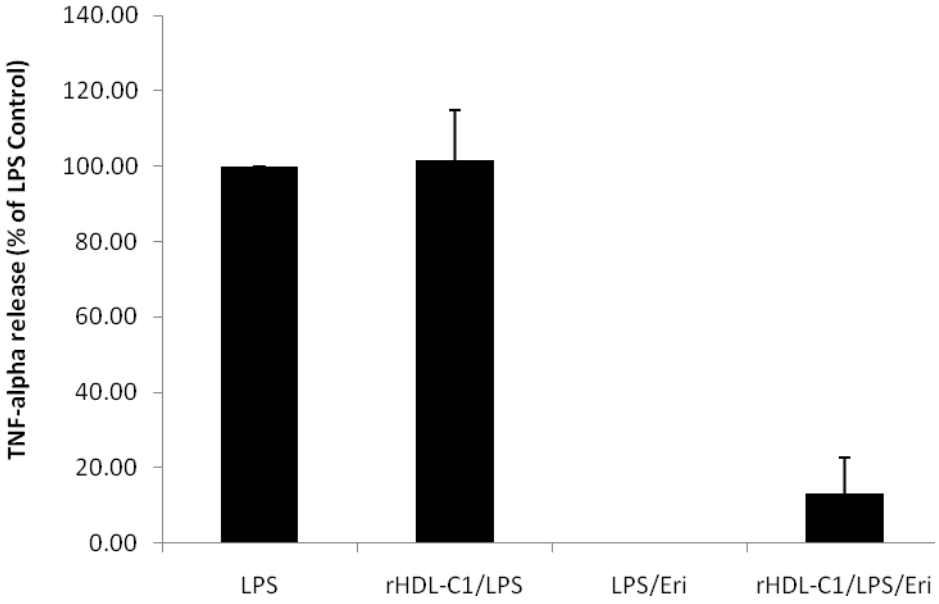


Figure 22. Eritoran activity as measured by TNF-α release in the presence of rHDL-C1. Values shown here are the means calculated from all three subjects. TNF-α release is shown as % of LPS. Final concentrations of rHDL-C1 are 0.1mg/mL by protein. Error bars represent + SEM. Statistical analysis was performed using a paired t-test. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*; rHDL, *reconstituted high-density lipoprotein*

Similarly, a less than 15% decrease in drug activity was observed when eritoran was tested for its ability to block TNF-α release in the presence of rHDL-A2 (13.1%, $p>0.05$) (**Figure 23**). Although apo A2 in the lipid-bound form still prevented the drug from fully inhibiting cytokine release as shown in the LPS/Eri group, this difference was not statistically significant and therefore, no effect can be inferred.

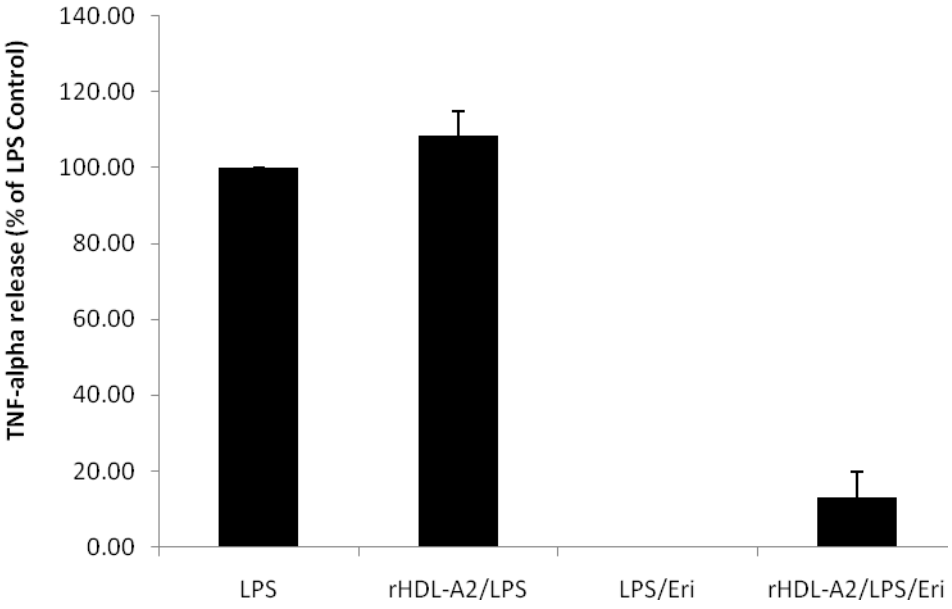


Figure 23. Eritoran activity as measured by TNF-α release in the presence of rHDL-A2. Values shown here are the mean calculated for all three subjects. TNF-α release is shown as % of LPS. Final concentrations of rHDL-A2 are 0.1mg/mL by protein. Error bars represent + SEM. Statistical analysis was performed using a paired t-test. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor*-α; Eri, *eritoran*; rHDL, *reconstituted high-density lipoprotein*

It is important to suggest why significant differences may not have been observed between rHDL-A2 and rHDL-C1 when compared to the LPS/Eritoran group. Briefly, one subject from each treatment group (A2 or C1) had zero release of TNF-α indicating no effect of the rHDL-apolipoprotein construct on the drug. However, the other two subjects did release low amounts of cytokine, in comparison, but the zero-release data point caused an increase in inter-subject variability. Ultimately, this lessened the power of these two tests making it less likely to detect differences even though graphically there is some loss of drug

activity in both instances. Regardless, in contrast to their free forms, apolipoprotein A2 and C1 were less effective at reducing eritoran activity when they were lipid-bound (in an rHDL). As most of these exchangeable apolipoproteins will typically be associated with an HDL particle rather than free the majority of the time, it is expected that changes in protein structure as a result of binding lipids may affect the degree to which each individual apolipoprotein dampens drug function.

4.3 Modelling HDL: The effect of "normal" reconstituted HDL (NrHDL) on the antagonistic activity of eritoran against LPS

Lipid-free apolipoproteins A1, SAA, A2 and C1 were all shown to have distinctive effects on eritoran ranging from strong deactivation for apo A1 to weak deactivation for apo C1. In all instances however, at 0.1 mg/mL by protein, all apolipoproteins did elicit some level of drug inhibition. When interpreting this data it becomes essential to account for the composition of these apolipoproteins as part of a native HDL particle. For example, apo A1 is far more abundant on HDL than apo C1 (70% of total protein versus 3.1%) and hence the net effects on drug deactivation observed with individual apolipoproteins in rHDL are either downplayed or embellished by true, physiological levels.

HDL will always contain an assortment of apolipoproteins in varied amounts. Being able to manipulate apolipoprotein composition is a powerful method by which to probe how changes in the apolipoprotein class and quantity affect drug deactivation by HDL. Accordingly, to better understand how the protein constituents on HDL cooperate to facilitate drug deactivation we created a model rHDL containing all apolipoproteins in the molar ratios they would be found associated with HDL in the healthy state. This rHDL was dubbed

normal rHDL (NrHDL). Later, this concept would be extended to making model rHDL mimicking the apo composition found in the septic state for comparison.

Molar ratios for normal rHDL were established from Havel and Kane *OMBID*, 2001 [88]. The ratio used for this objective was 63: 31: 12: 3: 10: 1 (A1 : A2 : C1 : C2 : C3 : E). Reconstituted HDL containing all apolipoproteins were made in a similar fashion to the process described in methods. NrHDL were characterized by SDS-PAGE to determine apolipoprotein content (**Appendix: Figure 8**). Moreover, a spherical rHDL structure was ascertained through imaging with transmission electronmicroscopy (TEM) (**Appendix: Figure 9**). To reproduce the degree of deactivation observed with native HDL shown in figure 19, these model constructs were used at 0.8mg/mL by protein where the loss in drug activity was maximal. When treated with 0.8 mg/mL NrHDL, there was a considerable loss of eritoran activity against LPS-induced TNF-α release (**Figure 24**). However, this change was not significantly different from the LPS/Eritoran group (mean=46.9%, $p>0.05$), perhaps as a result of variation around the mean, produced by the divergent cytokine release in one subject.

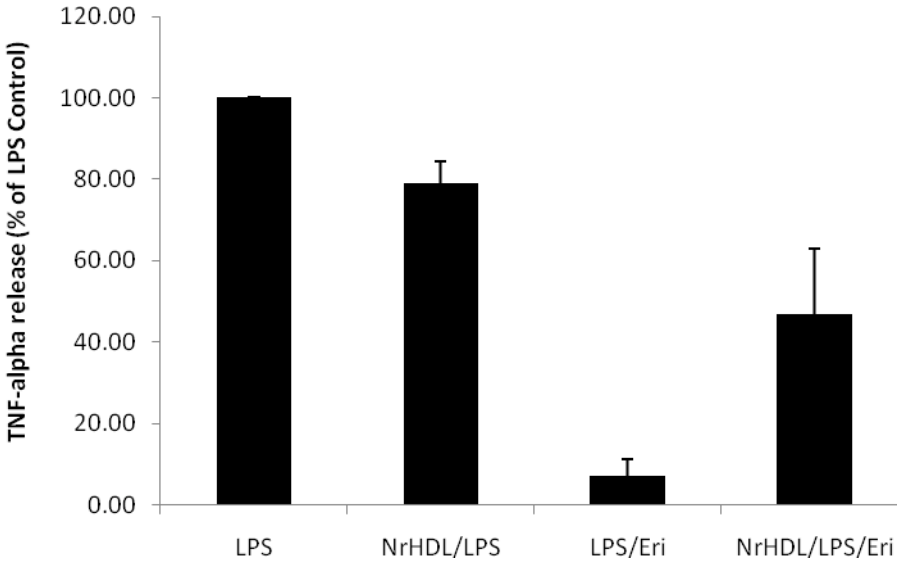


Figure 24. Loss of eritoran antagonistic activity as measured by TNF-α release in the presence of 'normal' rHDL (NrHDL). The "normal" rHDL model is composed of all native HDL apolipoproteins (A1, A2, C1, C2, C3, and E) in the molar ratio they would be found associated with HDL in a healthy person. Values shown here are the mean for all three subjects. TNF-α release is shown as % of LPS. Final concentrations of NrHDL are 0.8 mg/mL by protein. Error bars represent + SEM. Statistical analysis was performed using a paired t-test. *Denotes a significant difference from LPS/Eri ($p<0.05$). N=3 LPS, *lipopolysaccharide*; TNF-alpha, *tumour necrosis factor-α*; Eri, *eritoran*; NrHDL, '*normal' reconstituted high-density lipoprotein*

4.4 Modelling HDL: The effect of "septic" reconstituted HDL (SrHDL) on the antagonistic activity of eritoran against LPS

With the same rationale as proposed in the previous section, another set of model rHDL were created to simulate the apolipoprotein composition that would be found on HDL in septic patients. Since serum amyloid A almost entirely replaces apo A1 on the HDL particle during sepsis, SAA was the most abundant apolipoprotein on SrHDL relative to other apos based on the molar ratio used. The molar ratio was established by estimating apolipoprotein composition from confirmed plasma concentrations of apolipoproteins during

sepsis and the percentage of that concentration circulating as part of a HDL [52, 54, 60, 63, 65, 80, 81, 91, 92]. If there was no published literature on the changes in concentration for a particular apolipoprotein during sepsis, then the values used for normal HDL were retained for this study. The ratio employed for this objective was 1461: 17: 22: 2: 2: 8: 1 (SAA: A1: A2: C1: C2: C3: E).

Unfortunately, when this rHDL preparation was made the sample was laden with lipid and it appeared cloudy and white-gray. As a surface active agent, cholate should help drive the formation of lipid-protein particles forming a solution that is clearer and not as milky. This implied that rHDL particles did not form properly and moreover, interference from floating lipid prevented an accurate reading of the sample's protein concentration. Therefore, we were unable to test this preparation for its ability to deactivate eritoran. Further, absolutely no particle-like forms (spherical or discoidal) could be discerned from TEM images of this particular preparation, supporting the notion that particle formation was incomplete (**Appendix: Figure 10**).

5. DISCUSSION

5.1 Summary and major findings

Sepsis, clinically defined when the host experiences the symptoms of a systemic inflammatory response (SIRS) to infection, corresponds to a spectrum of pathophysiological states that fluctuate in their degree of severity. Severe sepsis, distinguished by multiple organ dysfunction and hypotension is frequently caused by LPS, an endotoxin found on gram-negative bacteria. The rising incidence rate of sepsis during the last 20 years has drawn much attention to lack of effective therapies available and has initiated the development of an assortment of compounds aimed at disrupting elements of the inflammatory response. In lieu of several failed clinical studies concerning pharmaceuticals aimed for the treatment of sepsis, eritoran tetrasodium, a second-generation lipid A analogue, has remained as a strong candidate. However, time-dependent deactivation of eritoran by HDL in clinical subjects has become a point of both interest and concern regarding drug efficacy and dosing regimens in critically ill patients. Septic patients are typically hypocholesterolemic, experiencing a sharp decline in the circulating levels of HDL, with a mutual shift in apolipoprotein content. Notable changes in the levels of HDL-associated apolipoproteins and their capacity to bind or associate with LPS during sepsis has led to the belief that the apolipoprotein components of HDL are important in sequestering eritoran during which drug activity is diminished.

Therefore, the overall aim of this project was to identify whether apolipoproteins were involved in reducing eritoran antagonistic activity against LPS, and if so, which proteins. Briefly, the four objectives established for this study were to test eritoran deactivation in the presence of: (1) free apolipoproteins, (2) rHDL-apolipoproteins (lipid-

bound), (3) normal rHDL (NrHDL) (combined apolipoproteins), and (4) septic rHDL (SrHDL) (combined apolipoproteins in the septic state).

All objectives were tested in the same fashion using an *ex vivo* whole blood assay in which eritoran pre-incubated with apolipoprotein or the rHDL-apolipoprotein of interest, was subsequently added to human whole blood with simultaneous stimulation using LPS. Drug activity was indirectly determined by measuring changes in the release of the pro-inflammatory cytokine TNF-α, which is consistently released from macrophages activated by LPS. In summary, the major findings of this study are: (1) differences exist in the effect on drug activity when apolipoproteins are free versus lipid-bound, (2) the major apolipoproteins in both the healthy and septic state, A1 and SAA, reduced eritoran antagonistic activity in rHDL, (3) A1 and SAA in rHDL were superior to minor apolipoproteins A2 and C1 in dampening drug activity and (3) normal rHDL (NrHDL) deactivates eritoran to a similar degree as rHDL-A1.

5.1.1 Differential effects of apolipoproteins in the free state or as part of reconstituted HDLs on eritoran antagonistic activity: The role of amphipathic helices

When eritoran activity was measured in the presence of free apolipoproteins, several proteins exhibited strong deactivation of the drug. In particular, apo A2, C1 and E caused fairly large reductions in drug activity when incubated at the low (septic) and high (healthy) concentrations. Particularly, at the septic-like concentration, both apo A2 and C1 completely abolished the antagonistic effect of the drug on TNF-α release. In comparison, at a slightly higher concentration of apo A2 (0.1 mg/mL vs. 0.08 mg/mL), rHDL-A2 reduced eritoran activity by less than 15%. Likewise, apo C1 within an rHDL construct, albeit at a much

higher concentration than when tested free (0.1mg/mL vs. 0.005mg/mL), caused only a minor reduction in drug activity, also below 15%. The inconsistency of drug deactivation observed between free apolipoproteins and those as part of a reconstituted HDL is important when considering whether apolipoproteins *in vivo* truly reduce drug antagonism of LPS.

In the body, apolipoproteins will be bound to lipids as part of an HDL the majority of the time, and thus, their ability to deactivate eritoran as part of an HDL is more relevant than when free. Apolipoproteins are part of a multigene family that has evolved through a series of complete and internal gene duplications from a single ancestral gene to form various proteins ranging in length, complexity, and function [93]. Structurally, they are highly similar and contain a signature feature that connects them, the presence of several 11-residue long (11-mer) amino acid repeats. The resulting physical feature of these 11-mer repeats is their ability to form amphipathic α-helices, bestowing them with the ability to avidly bind lipid and maintain the structural integrity of lipoproteins as carriers of hydrophobic molecules throughout the blood. When bound to phospholipids, the original structure of the apolipoprotein will be radically altered, exposing lipid-binding sites and/or concealing them. The effect on eritoran, which has an amphipathic nature owing to its negatively-charged phosphate head groups and hydrophobic acyl chains, will likely change depending on the availability of these sites and their capacity for interaction with the drug.

The majority of amphipathic helices on apolipoproteins are those belonging to class A, which are defined by a unique clustering of positively charged residues at the polar/non-polar interface and negatively charged proteins at the centre of the polar face [94]. As part of an extensive review of the helical structure of exchangeable apolipoproteins, Segrest *et al.* proposed the 'snorkel' hypothesis in which positively charged residues (e.g. Lysine) when

associated with phospholipid behave in an amphipathic manner and can actually extend into the polar face, exposing their charged moieties to the aqueous milieu [95]. In aforementioned studies, mutant apo E and apo C1 proteins, whose cationic residues were replaced by neutral residues in predicted LPS binding motifs, were less effective at modulating LPS response [64, 66].

Hence, the likelihood exists that the interaction between LPS and apolipoproteins may involve electrostatic interactions, presumably between positive residues in proposed binding regions and the negatively-charged phosphates bound to the lipid A moiety of LPS. As eritoran is a lipid A analogue, it is likely that drug deactivation by apolipoproteins occurs in a similar manner to that of LPS and that the diminished effect of A2 and C1 on the drug as part of an rHDL is a consequence of the re-arrangement/masking of these binding sites when they are part of a lipoprotein.

5.1.2 Apo A1 and SAA cause significant reductions in eritoran activity: Major apolipoproteins have a greater deactivating effect on the drug than minor apolipoproteins

It was not unexpected that apolipoprotein A1 and serum amyloid A had a notable impact on drug activity in the physiological, lipid-bound form. As the principal and most abundant apolipoproteins on HDL in both the healthy and septic state, apolipoprotein A1 and SAA account for up to 70 and 80% of HDL protein content during normal and inflammatory conditions, respectively. Thus, from a quantitative standpoint the availability of these proteins on HDL particles make them more likely to physically associate with the drug as opposed to other apolipoproteins. A recent study by Silva *et al.* describes the current 'trefoil' model of apolipoprotein A1 structure on spherical high density lipoproteins in which three

apo A1 molecules are linked by intermolecular salt bridges dividing the phospholipid sphere into three equal lunes or slices [96]. This arrangement allows for the incorporation of additional apo A1 molecules by changing the bend angle for existing molecules and illustrates the extensive degree of coverage attained by this protein on a single particle.

Similarly, SAA-HDL are typically larger in size than native HDL, but are characterized by the same density. The results of native gel particle sizing of rHDL-SAA indicates that the average diameter of our sample was 13.5nm, a 5nm increase from native HDL (~8.2nm). As a consequence of its smaller size, more SAA molecules (11.5 kDa) could replace one apo A1 (28 kDa), which would facilitate expansion of the particle (increased diameter) while maintaining its density similar to that of HDL_3. Although this concept has not yet been verified it would surely augment SAA presence on HDL, increasing the probability of the drug first associating with this protein upon encountering circulating HDL during sepsis.

Nevertheless, as opposed to HDL found *in vivo,* in this particular study these apolipoproteins were tested individually at a defined protein concentration, 0.1mg/mL, which suggests there is something specific or exclusive about apo A1 and SAA that confers a greater deactivating effect on drug activity than the minor apolipoproteins, A2 and C1. Although it is beyond the scope of this project, this possibility suggests that protein arrangement/structure within a phospholipid shell is fundamental to drug sequestration.

5.1.3 Normal rHDL (NrHDL) has a similar drug deactivation profile to rHDL-A1

To follow-up on the drug deactivation seen with individual proteins, all apolipoproteins were combined together to produce reconstituted particles that best mimicked the proportions found on native HDL. The purpose of this analysis was to determine if there were differences in the effect on drug activity when all proteins were present on an rHDL and whether or not a synergistic effect could be achieved. NrHDL caused an approximate 46% decrease in drug activity similar to the 47% reduction observed when the drug was pre-incubated with rHDL-A1. The observed effects for NrHDL might be slightly inflated, due to the divergent release of TNF-α in one subject, however the drug was deactivated by this construct at a minimum of 26% in all subjects. Whether or not this apparent loss of drug activity is due to the cooperative effect of all apolipoproteins is not clear. The reason for this lies in the fact that the molar ratio of apo A1 to other apolipoproteins was at least 2-fold higher than the second most abundant apolipoprotein, apo A2.

When making these 'combined' rHDL particles the assumption was that all apolipoproteins will come together in the devised molar ratio to "solubilize" floating lipids into particles. However, when run out on an SDS-PAGE gel and stained for protein, there was a definitive band for apo A1, but no obvious bands for other apolipoproteins (see Appendix: Figure 8). This implies that, although some particles may have formed with all apolipoproteins (as suggested by faint smears at the molecular weights of apos C, E and A2), it is likely that the majority of the particles contained mainly apo A1, thus producing a similar effect on drug activity as rHDL-containing only apo A1. However, this is speculative, without further particle characterization one cannot be certain that the particles formed in this

sample were homogenous with respect to protein composition. Therefore, additive or cooperative effects of apolipoproteins in deactivating eritoran could not be assessed from this study.

5.2 Limitations of the study

Investigation of the elements involved in eritoran deactivation by HDL is an uncharted area of research, the results of which contribute new insights to the lack of information available about this association and lend way to other scientific approaches by which to study it. The exploratory nature of this study employed a "shotgun" type approach, in which multiple apolipoproteins were tested for their deactivating potential in an effort to narrow the lot down to a few likely prospects. The data gathered offer valuable information about which apolipoproteins may cause a loss of drug activity when lipid-bound as part of an HDL particle, however, there are some limitations of this project that must be acknowledged.

5.2.1 Sample size and statistical power

First, graphical representation of drug deactivation by apolipoproteins in the free and lipid-bound form illustrate that there was some level of deactivation in the presence of all proteins tested, however the degree of deactivation varied dependent on the apo used. However, when analyzed statistically some differences were not observed to be significantly different when compared to the LPS/Eri group, who's TNF-α release was typically zero or almost zero. The major reason for this was the variability in TNF-α release that was frequently seen between subjects, which in turn caused large deviations around the sample mean. This was not completely surprising as TNF-α release after stimulation with 10ng/mL

LPS in blood from human subjects has been shown to range from roughly 700 to 3800 pg/mL [89]. Unfortunately, the consequence of the inconsistent release among only three subjects reduced the power of statistical tests, making them unable to discern differences when they may actually exist (i.e. the probability of type II errors). Also, it should be noted here for future studies that the apolipoprotein composition on HDL used in the first objective (shown in '*' table 3), where free apolipoproteins were tested for their effects on eritoran, was calculated slightly incorrectly. The actual composition should be A1 (70%), A2 (21%), C1 (~3%), C2 (~1%), C3 (~4%), and E (~1%) (calculated from [88]). These percentages are slightly different from the aforementioned composition used in the study, however the concentrations of apolipoprotein incubated with the drug were low to begin with that even when you correct for the revised composition it doesn't greatly change the concentrations tested.

Ultimately, one serious limitation of this study was not having an adequate sample size (great enough number of subjects) to detect substantial effects on drug activity while maintaining 80% power for all statistical analyses. Despite this, this pilot study still demonstrates a consistent drug deactivation when in the presence of several apolipoproteins, especially apo A1 and SAA. Therefore, these results are still relevant but necessitate additional authentication in more subjects.

5.2.2 Techniques used for particle characterization

Secondly, the ability with which to make inferences on drug deactivation in the presence of rHDL-containing apolipoproteins is contingent upon the proper formation of reconstituted particles. When making rHDLs, the assumption is that lipid-protein spheres will

be produced when apolipoproteins naturally combine with amphipathic (phospholipid) and hydrophobic (cholesterol, cholesterol esters) lipids in the presence of cholate. It is imperative that every sample be thoroughly characterized to ensure the existence of the apolipoprotein of interest and if possible, the shape and size of rHDLs.

In this study, in order to determine apolipoprotein content (molecules of protein/particle) and particle size both SDS-PAGE and native gel analysis were employed. To visualize protein, gels were stained with Coomassie blue. Qualitatively, bands could be discerned and crosslinking gels used for rHDL containing individual apolipoproteins indicated different populations of rHDL with varying numbers of proteins/particle. However, in the case of NrHDL it was difficult to detect all apolipoproteins in combination, owing to the differences in the mass of proteins added due to the molar ratio used. Perhaps, the application of stronger and more sensitive techniques such as western blotting (using specific antibodies for each protein) may have better defined the composition of these particles and ensured that the majority of particles formed with all apolipoproteins.

In addition, native gels provided an estimate of particle size however, it has become increasingly common to employ several different techniques to confirm particle size, such as gel-filtration chromatography (GPC) and transmission electronmicroscopy (TEM). We did use TEM to image NrHDL particles, which were spherical, however it would have been helpful to have shape information on all rHDL preparations.

5.2.3 Limited supply of commercially produced apolipoproteins

Last, in order to establish new protocols, as was the situation with crosslinking gels and native gel analysis of rHDL in our lab, there was a period of method development in which rHDL-A1 was characterized several times to determine the optimal conditions for future preparations. Ultimately, these conditions were applied to all other rHDL preparations due to the limited supply of other apos, as all apolipoproteins used to make rHDLs were bought commercially, except for apo A1, which was generously supplied by the Francis lab. However, in many instances it would have been helpful to have an in-house supply of apolipoproteins to better characterize reconstituted particles of all types before using them in the whole blood assay.

Additionally, commercial protein preparations can be finicky and at times interfere with specific assays. In particular, commercial SAA protein could not be accurately determined using SDS-PAGE, as this protein requires the presence of dilute acid to go into solution, and it tended to aggregate at some point during this process. An unlimited supply of all apolipoproteins would have been helpful, but the high costs and low quantities provided by commercial suppliers make this unreasonable. In consequence, producing and purifying recombinant proteins in-house would further assist developing techniques required for precise characterization of reconstituted particles and the establishment of new methodologies for testing eritoran activity in the presence of apolipoproteins.

5.3 Clinical implications

Eritoran, currently in phase III as part of the clinical ACCESS trial (A Controlled Comparison of Eritoran tetrasodium and placebo in patients with Severe Sepsis), has been shown to be highly effective *in vitro* against LPS-induced stimulation of immune cells and *ex vivo* from healthy volunteers administered a short-term infusion of the drug [28, 30]. However, observed deactivation of the drug shortly after the end of infusion, despite its relatively long elimination half-life (40-50 hours), demonstrated a loss of drug activity resultant of binding to and remaining associated with high-density lipoprotein. As was noted with rHDL-containing A1, SAA, A2 and C1, even at sub-normal levels akin to those reported during sepsis (0.1mg/mL by protein) all lipid-bound apolipoproteins can reduce eritoran activity, reaching up to ~ 50% as seen with A1. From a clinical standpoint this is highly pertinent since HDL is a major obstacle with regard to drug efficacy and must be taken into account when considering drug dosing in the clinic. As a result, it is necessary to ensure that sufficient levels of eritoran remain in circulation such that activity can be maintained against varying levels of LPS, ranging from low (~0.05 ng/mL) to extremely high (~10 ng/mL). In the initial stages of drug testing, healthy volunteers given a 72-hour continuous infusion of eritoran in a high dose group, 252 mg, experienced a C_{max} as high as 41μg/mL but also maintained long-term drug activity against 1ng/mL LPS up to 3 days post-infusion [32].

In contrast, a more recent study aimed at reducing infusion time, found that intermittent, twice daily dosing of 105 mg was sufficient to maintain steady-state plasma levels of ~10μg/mL in which eritoran pharmacodynamic activity was 100% against 10ng/mL LPS [97]. This latter study showed that lower doses of the drug were still effective against the highest endotoxin concentration reported without the need of a continuous infusion to

promote excessively high plasma concentrations. Granted, this adjusted dosing regimen is still only a temporary solution to thwart major drug inactivation by HDL. Since the drug is administered intravenously, twice daily infusions still confine patients to the hospital for long periods of time and may interfere with other ongoing treatments as severe sepsis occurring from infection is normally a by-product of a primary illness (e.g. bodily trauma, transplant recipient, HIV-related, etc.). Ultimately, in the absence of drug association with HDL, eritoran would be able to retain a more lengthy duration of action in patients without the need for supplemental maintenance doses.

The relevance of investigating eritoran deactivation by apolipoproteins is valuable in providing insight into the cause or mechanism of drug binding by HDL, in an effort to facilitate approaches to reduce or prevent this interaction. When eritoran was pre-incubated with increasing concentrations of the different lipoprotein fractions, VLDL, LDL and HDL, there were differences in their effects on drug action. In summary, VLDL and LDL produced minimal, non-significant reductions in activity as compared with HDL, which caused full deactivation [32]. In this particular study, Rossignol *et al.* reported measurable pharmacodynamic activity at 72-hours post infusion and indicated that extended drug activity may be a consequence of the accumulation of eritoran in combined VLDL and LDL fractions, where ~17 % of the drug is found after a high dose (252 mg) and drug action is basically unaffected. This information lends itself to the idea that driving the drug into the more lipid-rich lipoproteins would function to avoid deactivation by HDL and extend pharmacodynamic activity of eritoran. It is possible that within a VLDL or LDL the drug is able to dissociate freely from the particle, continuing to reach its therapeutic targets as opposed to association with HDL, which appears to render the drug inactive.

Thus, the growth in general knowledge gained from studying apolipoprotein effects on eritoran may be useful in better understanding how to target or shuttle the drug within the body (new formulations) or cause acute and temporary changes to patient lipid profiles to favour drug activity and effectiveness *in vivo* in patients with sepsis (changing diet, increasing lipid-rich lipoproteins). Ultimately, elucidation of the HDL components causing drug deactivation will improve strategies aimed at preventing HDL-drug interactions and enhance eritoran treatment of severe sepsis.

5.4 Conclusions and future Studies

In this study, it was shown that exchangeable apolipoproteins are potential factors in eritoran deactivation against LPS by HDL. Their ability to reduce drug action as part of reconstituted HDL has confirmed that changes made to the protein components or composition of rHDL will produce changes in drug activity (i.e. different apos cause varying degrees of deactivation) -- supporting the concept that apolipoproteins and not other lipid constituents (e.g. phospholipids or cholesterol) are the reason for eritoran association and deactivation by HDL. In light of these advances, the belief that apolipoproteins are the fundamental element facilitating drug-lipoprotein interactions has led to several other avenues of exploration.

First, to assess changes in apolipoprotein composition on HDL during the septic state (and its subsequent effect on eritoran activity) it would be useful to have a quantitative estimate of HDL protein content in septic patients. This would include evaluating which apolipoproteins are found on HDL and in what proportion during sepsis.

Likewise, as apolipoproteins have become a focal point of drug association, a more in-depth analysis of protein structure and potential binding regions on apolipoproteins would be an exciting an area of research. Investigating the presence of LPS-binding domains in apolipoproteins could be analyzed using *in silico* techniques such as sequence alignment against proteins already known to bind endotoxin. In turn, this would provide information about putative drug binding sites along the apoprotein as eritoran is analogue of the lipid A moiety found on LPS.

Furthermore, conclusive information on probable regions of LPS/drug association could lead to protein-deletion studies in which recombinant mutant proteins are produced with and without the presence of alleged binding regions and tested for their ability to deactivate eritoran in an rHDL. An alternative study in which peptides corresponding only to binding regions, as opposed to full length proteins, could be tested in a similar fashion to those above. Other follow-up studies may include: determining the binding kinetics of radiolabeled eritoran for various rHDL-apolipoproteins, investigating variations in the release of TNF-α between males and females and the differences in eritoran action, and the use of protein-degrading elements (e.g. Trypsin, heat, etc.) on rHDL-apolipoprotein drug deactivation.

REFERENCES

1. Goldsby, R. A., Kindt, T. J. & Osborne, B. A. in *Immunology, 4th Edition* (W.H. Freeman and Company, New York, NY, USA, 2001).

2. Dinarello, C. A. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest* **112**, 321 (1997).

3. Netea, M. G. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends in immunology* **24**, 254 (2003).

4. Bone, R. C. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* **101**, 1644 (1992).

5. Medzhitov, R. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394 (1997).

6. Dombrovskiy, V. Y. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. *Critical care medicine* **35**, 1244 (2007).

7. Angus, D. C. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical care medicine* **29**, 1303 (2001).

8. Freitas, F. G. R. The impact of duration of organ dysfunction on the outcome of patients with severe sepsis and septic shock. *Clinics* **63**, 483 (2008).

9. Martin, G. S., Mannino, D. M., Eaton, S. & Moss, M. The Epidemiology of Sepsis in the United States from 1979 through 2000. *N Engl J Med* **348**, 1546-1554 (2003).

10. Polderman, K. H. Drug intervention trials in sepsis: divergent results. *Lancet, The* **363**, 1721 (2004).

11. Girbes, A. R. J. Pharmacological treatment of sepsis. *Fundamental clinical pharmacology* **22**, 355 (2008).

12. Janeway, C. A. Innate immune recognition. *Annual review of immunology* **20**, 197 (2002).

13. Munford, R. S. Severe sepsis and septic shock: the role of gram-negative bacteremia. *Annual review of pathology* **1**, 467 (2006).

14. Raetz, C. R. Biochemistry of endotoxins. *Annual Review of Biochemistry* **59**, 129 (1990).

15. Galanos, C. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. *European journal of biochemistry* **148**, 1 (1985).

16. Gay, N. J. Structure and function of Toll receptors and their ligands. *Annual Review of Biochemistry* **76**, 141 (2007).

17. O'Neill, L. A. J. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature reviews. Immunology* **7**, 353 (2007).

18. Morisato, D. The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. *Cell* **76**, 677 (1994).

19. Lemaitre, B. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973 (1996).

20. Hultmark, D. Insect immunology. Ancient relationships. *Nature* **367**, 116 (1994).

21. Poltorak, A. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085 (1998).

22. Fujihara, M. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacology therapeutics* **100**, 171 (2003).

23. Hawkins, L. D., Christ, W. J. & Rossignol, D. P. Inhibition of endotoxin response by synthetic TLR4 antagonists. *Curr. Top. Med. Chem.* **4**, 1147-1171 (2004).

24. Leon, C. G. Discovery and development of toll-like receptor 4 (TLR4) antagonists: a new paradigm for treating sepsis and other diseases. *Pharmaceutical research* **25**, 1751 (2008).

25. Pålsson-McDermott, E. M. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **113**, 153 (2004).

26. Visintin, A., Halmen, K. A., Latz, E., Monks, B. G. & Golenbock, D. T. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. *J. Immunol.* **175**, 6465-6472 (2005).

27. Kim, H. M. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* **130**, 906 (2007).

28. Mullarkey, M. *et al*. Inhibition of endotoxin response by E5564, a novel toll-like receptor 4-directed endotoxin antagonist. *J. Pharmacol. Exp. Ther.* **304**, 1093-1102 (2003).

29. Lynn, M. *et al*. Blocking of responses to endotoxin by E5564 in healthy volunteers with experimental endotoxemia. *The journal of infectious diseases* **187**, 631 (2003).

30. Wong, Y. N. *et al*. Safety, pharmacokinetics, and pharmacodynamics of E5564, a lipid A antagonist, during an ascending single-dose clinical study. *J. Clin. Pharmacol.* **43**, 735-742 (2003).

31. Wasan, K. M. *et al*. Association of the endotoxin antagonist E5564 with high-density lipoproteins in vitro: dependence on low-density and triglyceride-rich lipoprotein concentrations. *Antimicrob. Agents Chemother.* **47**, 2796-2803 (2003).

32. Rossignol, D. P. *et al*. Safety, pharmacokinetics, pharmacodynamics, and plasma lipoprotein distribution of eritoran (E5564) during continuous intravenous infusion into healthy volunteers. *Antimicrob. Agents Chemother.* **48**, 3233-3240 (2004).

33. Alvarez, C. & Ramos, A. Lipids, lipoproteins, and apoproteins in serum during infection. *Clin. Chem.* **32**, 142-145 (1986).

34. Carpentier, Y. A. & Scruel, O. Changes in the concentration and composition of plasma lipoproteins during the acute phase response. *Curr. Opin. Clin. Nutr. Metab. Care* **5**, 153-158 (2002).

35. Gordon, B. R. Relationship of hypolipidemia to cytokine concentrations and outcomes in critically ill surgical patients. *Critical care medicine* **29**, 1563 (2001).

36. Gordon, B. R. Low lipid concentrations in critical illness: implications for preventing and treating endotoxemia. *Critical care medicine* **24**, 584 (1996).

37. Hudgins, L. C. *et al*. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. *J. Lipid Res.* **44**, 1489-1498 (2003).

38. Kitchens, R. L. & Thompson, P. A. Impact of sepsis-induced changes in plasma on LPS interactions with monocytes and plasma lipoproteins: roles of soluble CD14, LBP, and acute phase lipoproteins. *J. Endotoxin Res.* **9**, 113-118 (2003).

39. Levels, J. H. M. Alterations in lipoprotein homeostasis during human experimental endotoxemia and clinical sepsis. *Biochimica et biophysica acta* **1771**, 1429 (2007).

40. Marik, P. E. Dyslipidemia in the critically ill. *Crit. Care Clin.* **22**, 151-9, viii (2006).

41. Khovidhunkit, W. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *Journal of lipid research* **45**, 1169 (2004).

42. Ulevitch, R. J., Johnston, A. R. & Weinstein, D. B. New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. *J. Clin. Invest.* **64**, 1516-1524 (1979).

43. Parker, T. S. *et al*. Reconstituted high-density lipoprotein neutralizes gram-negative bacterial lipopolysaccharides in human whole blood. *Infect. Immun.* **63**, 253-258 (1995).

44. Levine, D. M., Parker, T. S., Donnelly, T. M., Walsh, A. & Rubin, A. L. In vivo protection against endotoxin by plasma high density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 12040-12044 (1993).

45. Harris, H. W., Grunfeld, C., Feingold, K. R. & Rapp, J. H. Human very low density lipoproteins and chylomicrons can protect against endotoxin-induced death in mice. *J. Clin. Invest.* **86**, 696-702 (1990).

46. Flegel, W. A., Wolpl, A., Mannel, D. N. & Northoff, H. Inhibition of endotoxin-induced activation of human monocytes by human lipoproteins. *Infect. Immun.* **57**, 2237-2245 (1989).

47. Murch, O., Collin, M., Hinds, C. J. & Thiemermann, C. Lipoproteins in inflammation and sepsis. I. Basic science. *Intensive Care Med.* **33**, 13-24 (2007).

48. Wu, A., Hinds, C. J. & Thiemermann, C. High-density lipoproteins in sepsis and septic shock: metabolism, actions, and therapeutic applications. *Shock* **21**, 210-221 (2004).

49. Levels, J. H. Distribution and kinetics of lipoprotein-bound endotoxin. *Infection and immunity* **69**, 2821 (2001).

50. Opal, Steven M., Scannon, Patrick J., Vincent, Jean-Louis , White, Mark, Carroll, Stephen F., Palardy, John E., Parejo,Nicolas A., Pribble, John P., Lemke, Jon H. Relationship between Plasma Levels of Lipopolysaccharide (LPS) and LPS-Binding Protein in Patients with Severe Sepsis and Septic Shock. *J. Infect. Dis.* **180**, 1584-1589 (1999).

51. Netea, M. G. Bacterial lipopolysaccharide binds and stimulates cytokine-producing cells before neutralization by endogenous lipoproteins can occur. *Cytokine* **10**, 766 (1998).

52. van Leeuwen, H. J. Lipoprotein metabolism in patients with severe sepsis. *Critical care medicine* **31**, 1359 (2003).

53. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* **285**, 2486-2497 (2001).

54. Chien, J. Low serum level of high-density lipoprotein cholesterol is a poor prognostic factor for severe sepsis. *Critical care medicine* **33**, 1688 (2005).

55. Jonas, Ana and Michael C. Phillips. in *Biochemistry of Lipids, Lipoproteins and Membranes* (ed Vance, D.E. and J. E. Vance) 485-506 (Elsevier B.V., Amsterdam, The Netherlands; Oxford, UK., 2008).

56. Schromm, A. B., Brandenburg, K., Rietschel, E. T. & Seydel, U. Do endotoxin aggregates intercalate into phospholipid membranes in a nonspecific, hydrophobic manner? *Innate Immun.* **2**, 313 (1995).

57. Van Oosten, M. *et al*. Apolipoprotein E protects against bacterial lipopolysaccharide-induced lethality. A new therapeutic approach to treat gram-negative sepsis. *J. Biol. Chem.* **276**, 8820-8824 (2001).

58. Dellinger, R. P. *et al*. Efficacy and safety of a phospholipid emulsion (GR270773) in Gram-negative severe sepsis: results of a phase II multicenter, randomized, placebo-controlled, dose-finding clinical trial. *Crit. Care Med.* **37**, 2929-2938 (2009).

59. Gordon, B. R. *et al*. Neutralization of endotoxin by a phospholipid emulsion in healthy volunteers. *J. Infect. Dis.* **191**, 1515-1522 (2005).

60. Barlage, S. *et al*. ApoE-containing high density lipoproteins and phospholipid transfer protein activity increase in patients with a systemic inflammatory response. *J. Lipid Res.* **42**, 281-290 (2001).

61. de Bont, N. *et al*. Apolipoprotein E knock-out mice are highly susceptible to endotoxemia and Klebsiella pneumoniae infection. *J. Lipid Res.* **40**, 680-685 (1999).

62. Kattan, O. M., Kasravi, F. B., Elford, E. L., Schell, M. T. & Harris, H. W. Apolipoprotein E-mediated immune regulation in sepsis. *J. Immunol.* **181**, 1399-1408 (2008).

63. Li, L. Infection induces a positive acute phase apolipoprotein E response from a negative acute phase gene: role of hepatic LDL receptors. *Journal of lipid research* **49**, 1782 (2008).

64. Rensen, P. C. *et al*. Human recombinant apolipoprotein E redirects lipopolysaccharide from Kupffer cells to liver parenchymal cells in rats In vivo. *J. Clin. Invest.* **99**, 2438-2445 (1997).

65. Berbee, J. F. *et al*. Plasma apolipoprotein CI correlates with increased survival in patients with severe sepsis. *Intensive Care Med.* **34**, 907-911 (2008).

66. Berbee, J. F. *et al*. Apolipoprotein CI stimulates the response to lipopolysaccharide and reduces mortality in gram-negative sepsis. *FASEB J.* **20**, 2162-2164 (2006).

67. Flegel, W. A., Baumstark, M. W., Weinstock, C., Berg, A. & Northoff, H. Prevention of endotoxin-induced monokine release by human low- and high-density lipoproteins and by apolipoprotein A-I. *Infect. Immun.* **61**, 5140-5146 (1993).

68. Wang, Y. Effect of lipid-bound apoA-I cysteine mutants on lipopolysaccharide-induced endotoxemia in mice. *Journal of lipid research* **49**, 1640 (2008).

69. Li, Y., Dong, J. B. & Wu, M. P. Human ApoA-I overexpression diminishes LPS-induced systemic inflammation and multiple organ damage in mice. *Eur. J. Pharmacol.* **590**, 417-422 (2008).

70. Yan, Y. J., Li, Y., Lou, B. & Wu, M. P. Beneficial effects of ApoA-I on LPS-induced acute lung injury and endotoxemia in mice. *Life Sci.* **79**, 210-215 (2006).

71. Imai, T., Fujita, T. & Yamazaki, Y. Beneficial effects of apolipoprotein A-I on endotoxemia. *Surg. Today* **33**, 684-687 (2003).

72. Thaveeratitham, P., Plengpanich, W., Naen-Udorn, W., Patumraj, S. & Khovidhunkit, W. Effects of human apolipoprotein A-I on endotoxin-induced leukocyte adhesion on endothelial cells in vivo and on the growth of Escherichia coli in vitro. *J. Endotoxin Res.* **13**, 58-64 (2007).

73. Uhlar, C. M. & Whitehead, A. S. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265**, 501-523 (1999).

74. Marsche, G. The lipidation status of acute-phase protein serum amyloid A determines cholesterol mobilization via scavenger receptor class B, type I. *The Biochemical journal* **402**, 117 (2007).

75. Cabana, V. G. Influence of apoA-I and apoE on the formation of serum amyloid A-containing lipoproteins in vivo and in vitro. *Journal of lipid research* **45**, 317 (2004).

76. Cabana, V. G., Reardon, C. A., Wei, B., Lukens, J. R. & Getz, G. S. SAA-only HDL formed during the acute phase response in apoA-I+/+ and apoA-I-/- mice. *J. Lipid Res.* **40**, 1090-1103 (1999).

77. Hu, W. *et al*. Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo. *J. Lipid Res.* **49**, 386-393 (2008).

78. Abe-Dohmae, S. *et al*. Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner. *J. Lipid Res.* **47**, 1542-1550 (2006).

79. Hosoai, H. *et al*. Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice. *J. Lipid Res.* **40**, 648-653 (1999).

80. Coetzee, G. A. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. *The Journal of biological chemistry* **261**, 9644 (1986).

81. Clifton, P. M., Mackinnon, A. M. & Barter, P. J. Effects of serum amyloid A protein (SAA) on composition, size, and density of high density lipoproteins in subjects with myocardial infarction. *J. Lipid Res.* **26**, 1389-1398 (1985).

82. Husebekk, A., Skogen, B., Husby, G. & Marhaug, G. Transformation of amyloid precursor SAA to protein AA and incorporation in amyloid fibrils in vivo. *Scand. J. Immunol.* **21**, 283-287 (1985).

83. Tape, C., Tan, R., Nesheim, M. & Kisilevsky, R. Direct evidence for circulating apoSAA as the precursor of tissue AA amyloid deposits. *Scand. J. Immunol.* **28**, 317-324 (1988).

84. Chait, A., Han, C. Y., Oram, J. F. & Heinecke, J. W. Thematic review series: The immune system and atherogenesis. Lipoprotein-associated inflammatory proteins: markers or mediators of cardiovascular disease? *J. Lipid Res.* **46**, 389-403 (2005).

85. Kennedy, A. L. Preferential distribution of amphotericin B lipid complex into human HDL3 is a consequence of high density lipoprotein coat lipid content. *Journal of pharmaceutical sciences* **88**, 1149 (1999).

86. Wasan, Kishor M. and Gabriel Lopez-Bernstein. Targeted liposomes in fungi: Modifying the therapeutic index of amphotericin b by its incorporation into negatively charged liposomes. *Journal of liposome research* **5**, 893-903 (1995).

87. Beutler, B. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* **229**, 869 (1985).

88. Havel, R.J. and J.P. Kane. in *The Online Metabolic and Molecular Basis of Inherited Disease* (eds Valle, D. et al.) 1-7 (The McGraw-Hill Companies, Inc., 2001).

89. Rose, J. R. *et al*. Consequences of interaction of a lipophilic endotoxin antagonist with plasma lipoproteins. *Antimicrob. Agents Chemother.* **44**, 504-510 (2000).

90. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951).

91. Fraunberger, P., Schaefer, S., Werdan, K., Walli, A. K. & Seidel, D. Reduction of circulating cholesterol and apolipoprotein levels during sepsis. *Clin. Chem. Lab. Med.* **37**, 357-362 (1999).

92. Berbee, J. F., Havekes, L. M. & Rensen, P. C. Apolipoproteins modulate the inflammatory response to lipopolysaccharide. *J. Endotoxin Res.* **11**, 97-103 (2005).

93. Luo, C. C., Li, W. H., Moore, M. N. & Chan, L. Structure and evolution of the apolipoprotein multigene family. *J. Mol. Biol.* **187**, 325-340 (1986).

94. Segrest, J. P., De Loof, H., Dohlman, J. G., Brouillette, C. G. & Anantharamaiah, G. M. Amphipathic helix motif: classes and properties. *Proteins* **8**, 103-117 (1990).

95. Segrest, J. P. *et al*. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J. Lipid Res.* **33**, 141-166 (1992).

96. Silva, R. A. *et al*. Structure of apolipoprotein A-I in spherical high density lipoproteins of different sizes. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12176-12181 (2008).

97. Rossignol, D. P., Wong, N., Noveck, R. & Lynn, M. Continuous pharmacodynamic activity of eritoran tetrasodium, a TLR4 antagonist, during intermittent intravenous infusion into normal volunteers. *Innate Immun.* **14**, 383-394 (2008).

98. Hennessey, E.J., Parker, A.E. & O'Neill, L.A. Targeting Toll-like receptors: emerging therapeutics? *Nat. Rev. Drug Discov.* **9**, 293-307 (2010)

99. Wasan, K.M., Brocks, D.R., Lee, S.D., Sachs-Barrable, K. & Thornton, S. J. Impact of lipoproteins on the biological activity and disposition of hydrophobic drugs: implications for drug discovery. *Nat. Rev. Drug Discov.* **7**, 84-99 (2008)

APPENDIX

Table A1. The effect of free apolipoproteins on eritoran activity between subjects at the low concentration. TNF-α release during the whole blood assay in the presence of free apolipoproteins at the low concentration. Values shown here were normalized to the LPS control within each of the three subjects (S1, S2, S3). LPS, *lipopolysaccharide*; Apo, *apolipoprotein*, Eri, *eritoran*; TNF-α, *tumour necrosis factor alpha*

		LPS	Apo/LPS	LPS/Eri	Apo/LPS/Eri
A2	S1	100.00	211.31	2.41	209.90
	S2	100.00	224.27	2.80	228.04
	S3	100.00	199.94	0.05	104.77
C1	S1	100.00	102.27	2.41	125.69
	S2	100.00	102.33	2.80	133.02
	S3	100.00	85.02	0.05	44.88
C2	S1	100.00	118.38	2.41	16.60
	S2	100.00	152.36	2.80	24.92
	S3	100.00	81.15	0.05	0.00
C3	S1	100.00	65.86	3.99	35.14
	S2	100.00	66.60	1.14	0.00
	S3	100.00	97.02	6.40	12.82
E	S1	100.00	56.78	3.99	41.82
	S2	100.00	73.80	1.14	50.82
	S3	100.00	136.99	6.40	106.73

Table A2. The effect of free apolipoproteins on eritoran activity between subjects at the intermediate concentration. TNF-α release during the whole blood assay in the presence of free apolipoproteins at the intermediate concentration. Values shown here were normalized to the LPS control within each of the three subjects (S1, S2, S3). LPS, *lipopolysaccharide*; Apo, *apolipoprotein*, Eri, *eritoran*; TNF-α, *tumour necrosis factor alpha*

		LPS	Apo/LPS	LPS/Eri	Apo/LPS/Eri
A2	S1	100.00	102.99	2.41	79.78
	S2	100.00	52.05	2.80	44.50
	S3	100.00	88.76	0.05	84.52
C1	S1	100.00	92.91	2.41	83.99
	S2	100.00	67.52	2.80	63.00
	S3	100.00	124.07	0.05	106.13
C2	S1	100.00	78.62	2.41	38.21
	S2	100.00	50.41	2.80	13.94
	S3	100.00	93.15	0.05	50.78
C3	S1	100.00	136.30	3.99	118.78
	S2	100.00	131.34	1.14	19.17
	S3	100.00	139.64	6.40	63.58
E	S1	100.00	108.06	3.99	102.20
	S2	100.00	120.40	1.14	103.46
	S3	100.00	157.22	6.40	219.81

Table A3. The effect of free apolipoproteins on eritoran activity between subjects at the high concentration. TNF-α release during the whole blood assay in the presence of free apolipoproteins at the high concentration. Values shown here were normalized to the LPS control within each of the three subjects (S1, S2, S3). LPS, *lipopolysaccharide*; Apo, *apolipoprotein*, Eri, *eritoran*; TNF-α, *tumour necrosis factor alpha*

		LPS	Apo/LPS	LPS/Eri	Apo/LPS/Eri
A2	S1	100.00	62.83	2.41	44.65
	S2	100.00	47.64	2.80	16.18
	S3	100.00	51.06	0.05	33.86
CI	S1	100.00	77.46	2.41	50.75
	S2	100.00	87.02	2.80	29.94
	S3	100.00	94.03	0.05	55.73
C2	S1	100.00	79.83	2.41	38.33
	S2	100.00	65.33	2.80	5.16
	S3	100.00	82.67	0.05	43.52
C3	S1	100.00	129.82	3.99	52.06
	S2	100.00	100.69	1.14	11.55
	S3	100.00	60.85	6.40	6.72
E	S1	100.00	102.79	3.99	84.19
	S2	100.00	86.22	1.14	68.93
	S3	100.00	81.76	6.40	72.05

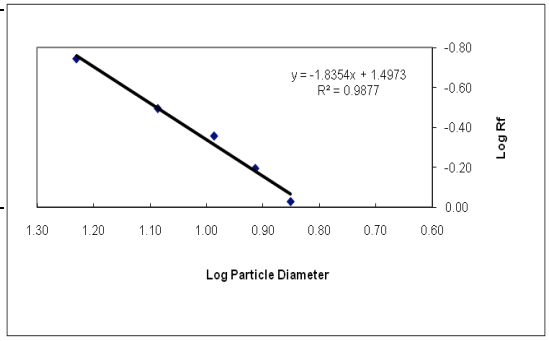
Table A4. Average particle diameters of apolipoprotein-containing rHDLs as estimated by native gel analysis.*

rHDL-Apo	Average estimated particle diameter (nm)
HDL	8.2
A1	7.5
A2	8.6
C1	13.5
SAA	13.6

*Molecular weights: A1 (28 kDA), A2 (8.7 kDA), C1 (6.6 kDa) and SAA (11.5 kDA). Please note that these are the averages of the potential population of rHDLs constructed. Some rHDL would fall on either side of this diameter.

HMW	Particle Diameter	Rf
Thyroglobulin	17.0	0.18
Ferritin	12.2	0.32
Catalase	9.7	0.44
Lactate Dehydrogenase	8.2	0.64
Albumin	7.1	0.94

	Log Particle Diam.	Log Rf
Thyroglobulin	1.23	-0.74
Ferritin	1.09	-0.49
Catalase	0.99	-0.36
Lactate Dehydrogenase	0.91	-0.19
Albumin	0.85	-0.03



Rf (distance migrated)

HDL Rf Values	Log Rf Values	Est. Log Diameter	Actual Particle Diameter (nm)		
0.46	-0.337	1.00	10.0	Band 1	
0.64	-0.194	0.92	8.3	Band 2	**Avg**
0.75	-0.125	0.88	7.7	Band 3	
0.89	-0.051	0.84	7.0	Albumin	

rHDL-A1 Rf Values	Log Rf Values	Est. Log Diameter	Actual Particle Diameter (nm)		
0.68	-0.167	0.91	8.1	Band 1	
0.78	-0.108	0.87	7.5	Band 2	**Avg**
0.84	-0.076	0.86	7.2	Band 3	

rHDL-A2 Rf Values	Log Rf Values	Est. Log Diameter	Actual Particle Diameter (nm)		
0.50	-0.301	0.98	9.5	Band 1	
0.60	-0.222	0.94	8.6	Band 2	**Avg**
0.71	-0.149	0.90	7.9	Band 3	

Figure A1. Particle size analysis of rHDL-A1 and A2. The average diameter of rHDLs were calculated by comparing the distance migrated (R_f) of protein standards with a known diameter to the distance migrated of the sample of interest. Typically, there is a range of particle sizes formed and banding will diffuse or "smeared" looking. All smears were split into three imaginary bands (top, middle and bottom of the smear corresponding to Band 1, 2, 3) and size was estimated from a log-transformed standard curve. Avg, *average*; HMW, *high molecular weight*, HDL, *high density lipoprotein*; rHDL, *reconstituted high density lipoprotein*

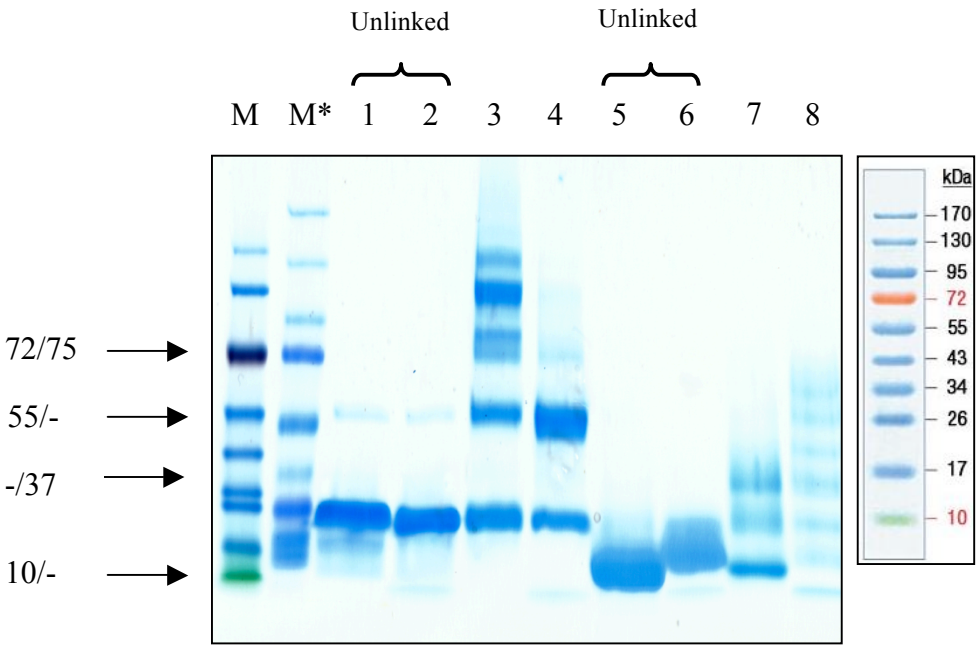


Figure A2. Crosslinking gel of rHDL-A1 and rHDL-A2. Crosslinker BS^3 was used in 50-fold excess to protein added in the crosslink reaction. 10 µg of protein were loaded from each group. Gels were stained with Coomassie Biosafe and destained in water. M is the MW marker from Fermentas (box). M* is the molecular weight marker from Biorad. Lanes 1-8: (1) Free A1, (2) rHDL-A1, (3) Free A1, (4) rHDL-A1, (5) Free A2, (6) rHDL-A2, (7) Free A2, (8) rHDL-A2. Numbers to the left-hand side of gel denote the molecular weight of bands. (Fermentas/Biorad). *NB: Two different protein standards were used to confirm the molecular weights of crosslinking products since certain bands in the marker from Fermentas seemed to overlap during electrophoresis (i.e. 95 and 72 kDa).*

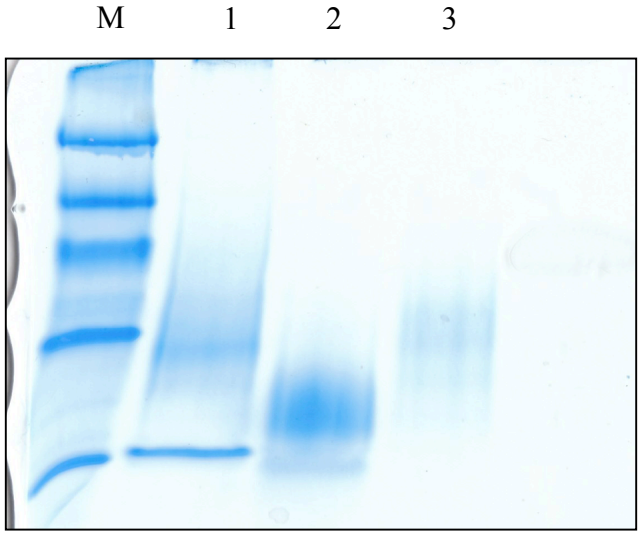


Figure A3. Native gel of rHDL-A1 and A2. 5ug of each sample were loaded. Gels were stained with Coomassie Biosafe and destained in water. Standards in the high molecular weight marker (HMW) include: Thyroglobulin (669kDA; 17nm diameter), Ferritin (440kDa; 12.2 nm), Catalase (232kDa, 9.7nm), Lactate Dehydrogenase (140kDa; 8.2nm) and Albumin (66kDa; 7.1nm). M is the HMW. Lanes 1-3: (1) HDL, (2) rHDL-A1, (3) rHDL-A2.

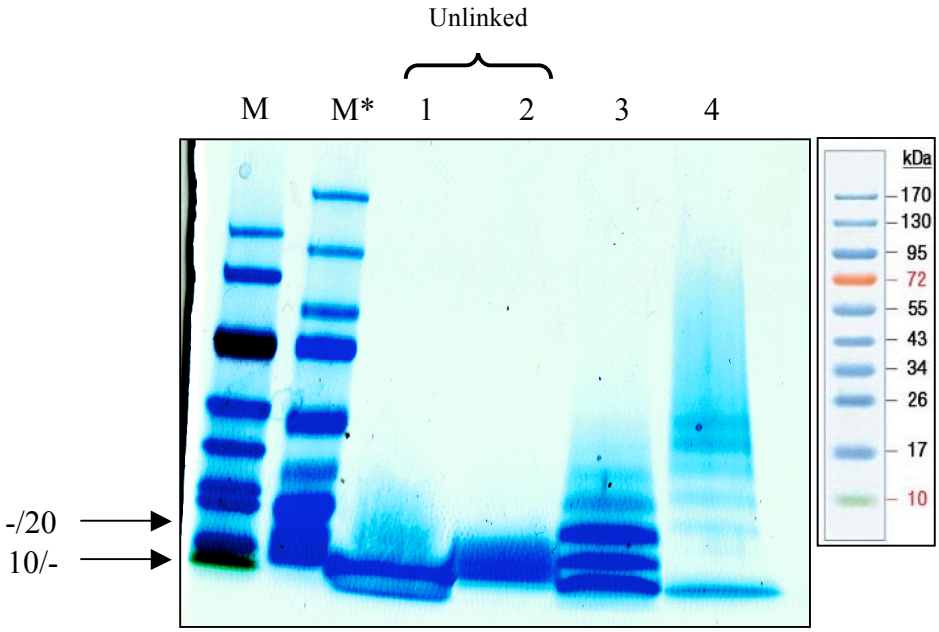


Figure A4. Crosslinking gel of rHDL-C1. Crosslinker BS^3 was used in 50-fold excess to protein added in crosslink reaction. 10 µg of protein were loaded for each group. Gels were stained with Coomassie Biosafe and destained in water. M is the MW marker from Fermentas. M* is the molecular weight marker from Biorad. Lanes 1-4: (1) Free C1, (2) rHDL-C1, (3) Free C1, (4) rHDL-C1. *NB: Gel was overconstrasted to show x-linking in the rHDL-C1 lane as it was too faint to see after staining with the naked eye since less protein was used in the crosslinking reaction. Numbers to the left-hand side of gel denote the molecular weight of bands. (Fermentas/Biorad).*

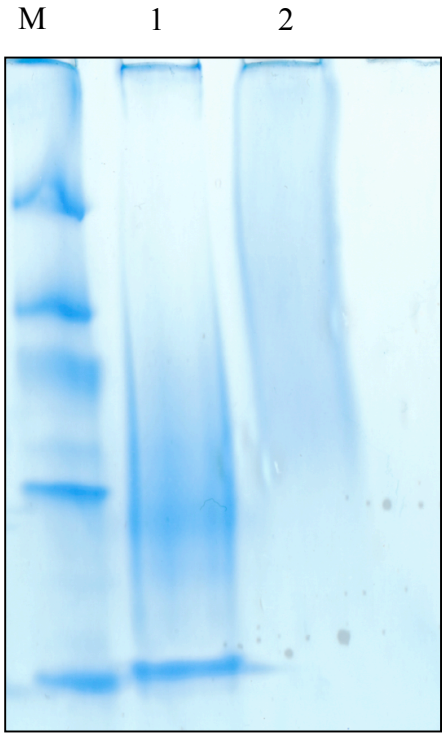


Figure A5. Native gel of rHDL-C1. 10 μg of each sample were loaded. Gels were stained with Coomassie Biosafe and destained in water. Standards in the high molecular weight marker (HMW) include: Thyroglobulin (669kDA; 17nm diameter), Ferritin (440kDa; 12.2 nm), Catalase (232kDa, 9.7nm), Lactate Dehydrogenase (140kDa; 8.2nm) and Albumin (66kDa; 7.1nm). M is the HMW. Lanes 1-2: (1) HDL, (2) rHDL-C1.

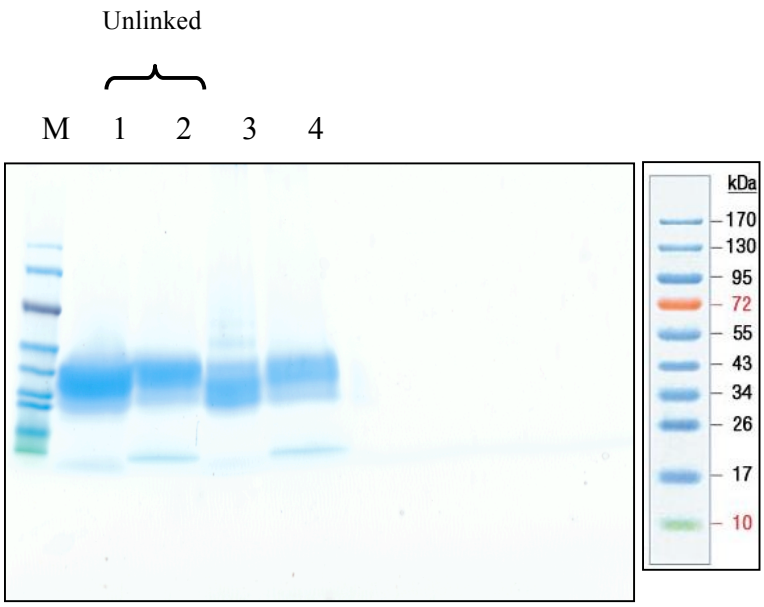


Figure A6. Crosslinking gel of rHDL-SAA. Crosslinker BS^3 was used in 50-fold excess to protein added in crosslink reaction. 10 µg of protein were loaded for each group. Gels were stained with Coomassie Biosafe and destained in water. M is the MW marker (Fermentas). Lanes 1-4: (1) Free SAA, (2) rHDL-SAA, (3) Free SAA, (4) rHDL-SAA. *NB: It appears that SAA aggregates were formed during some stage in the preparation for SDS-PAGE, forming the thick, higher molecular weight banding observed. SAA is an 11.5 kDa protein, some of which can be seen by the faint lower molecular weight bands running near the 10 kDa standard (in green). Therefore, protein number/particle could not be discerned for this sample.*

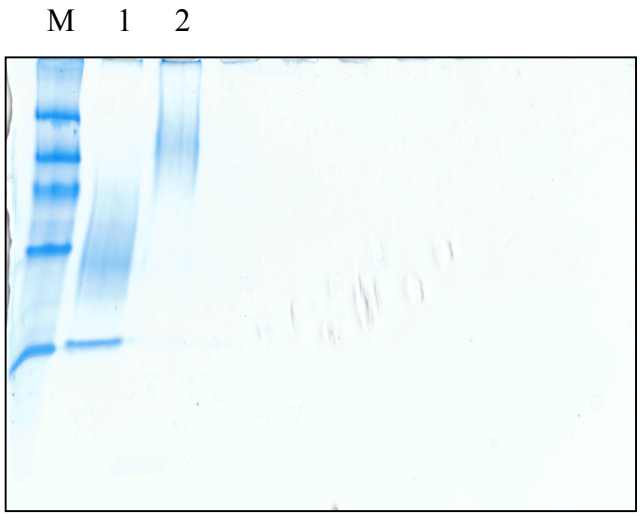


Figure A7. Native gel of rHDL-SAA. 5 μg of each sample was loaded. Gels were stained with Coomassie Biosafe and destained in water. Standards in the high molecular weight marker (HMW) include: Thyroglobulin (669kDA; 17nm diameter), Ferritin (440kDa; 12.2 nm), Catalase (232kDa, 9.7nm), Lactate Dehydrogenase (140kDa; 8.2nm) and Albumin (66kDa; 7.1nm). M is the HMW. Lanes 1 and 2: (1) HDL, (2) rHDL-SAA.

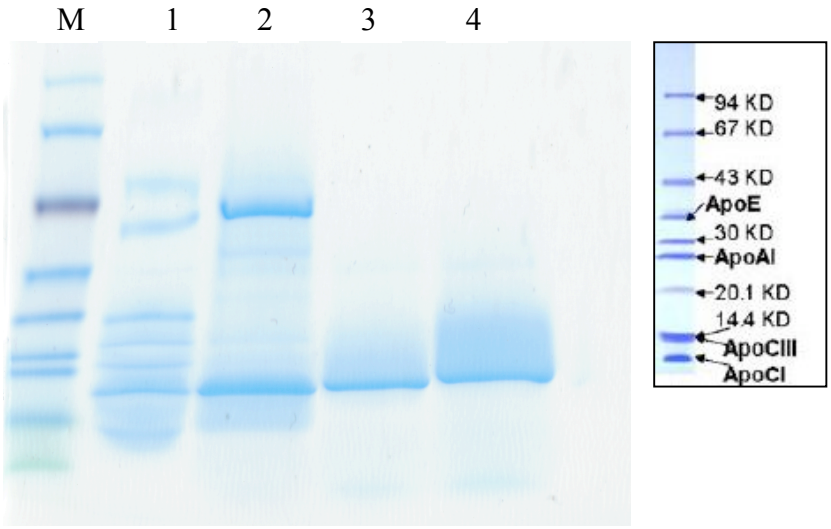


Figure A8. SDS-PAGE gel of NrHDL. NrHDL were characterized for protein composition and the inclusion of all apolipoproteins after rHDL preparation using SDS-PAGE. 10µg and 20µg of protein were loaded. Gels were stained with Coomassie Biosafe and destained in water. M is the MW marker (Fermentas). Lanes 1-4: (1) Human apolipoprotein marker (box), (2) HDL (3) 10µg NrHDL, (4) 20µg NrHDL. The apolipoprotein marker (Academy Bio-medical Co.) contained a mixture of Apo C1 (6.6kDa), C3 (8.7kDa), A1 (28kDa), and E (~37kDa) and other standards at 94, 67, 43, 30, 20.1 and 14.4 kDa.

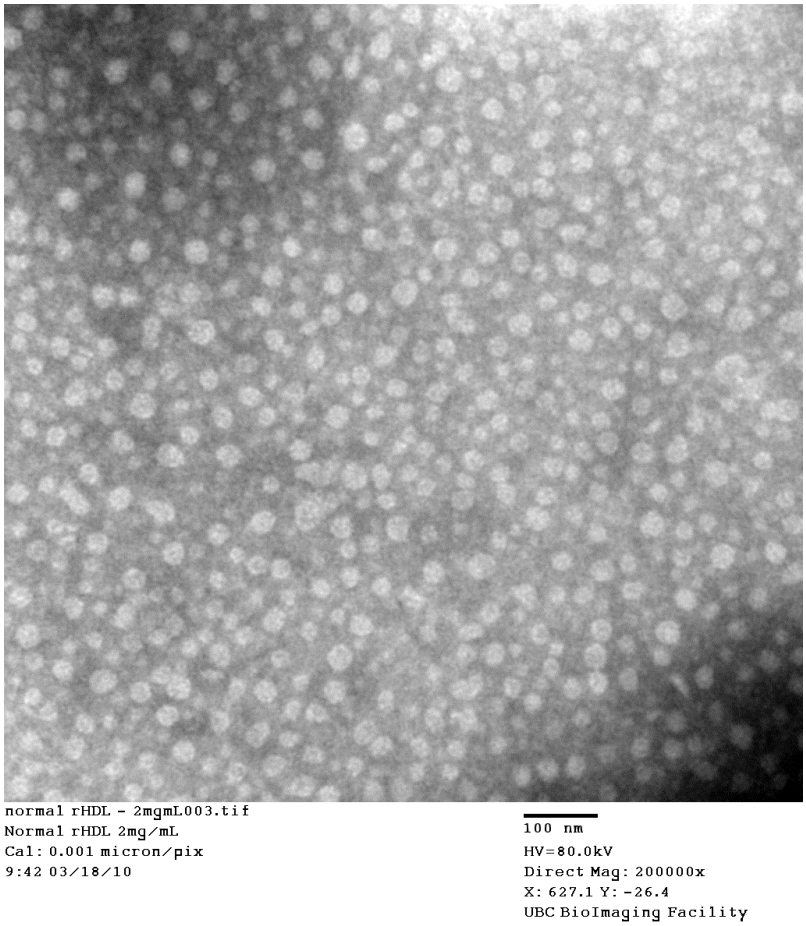


Figure A9. TEM image of spherical NrHDL. NrHDL were negatively stained with 1% phosphotungstic acid (PTA) imaged using a Hitachi H7600 transmission electromicroscope. The concentration of this sample was 2mg/mL by protein and was mounted on formvar-coated grids. The magnification shown here is 200,000x. The black bar at the bottom of the image represents a scale of 100nm. Negatively stained regions (distinguished as lighter staining) illustrate a range of particle sizes all with a spherical shape.

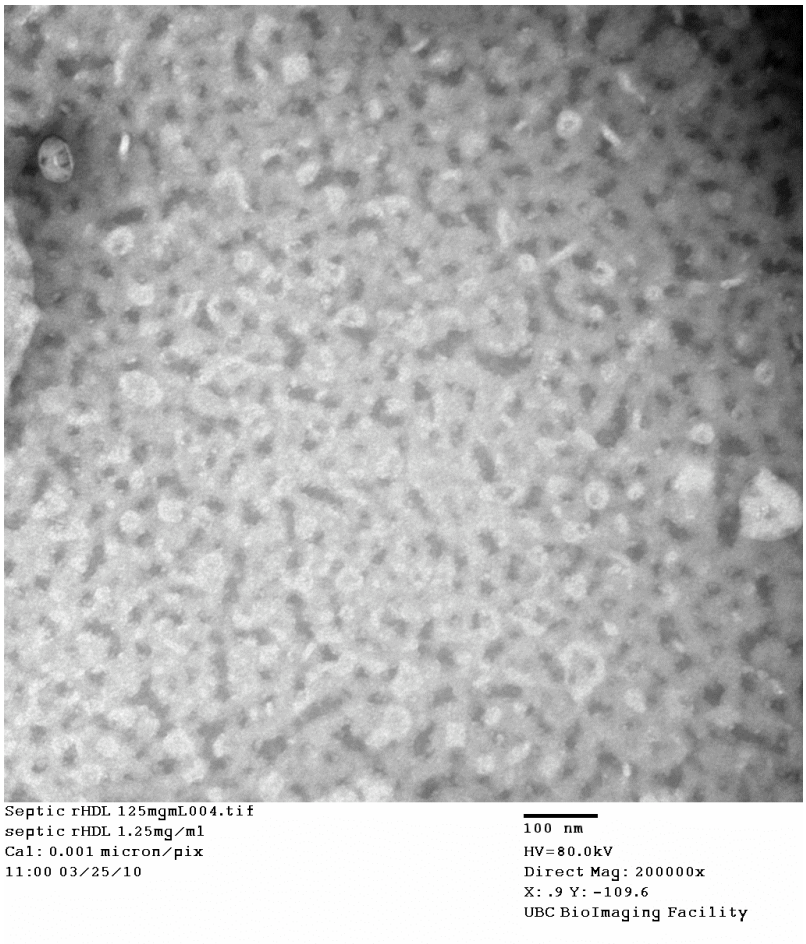


Figure A10. TEM image of SrHDL preparation. SrHDL were negatively stained with 1% phosphotungstic acid (PTA) imaged using a Hitachi H7600 transmission electromicroscope. The concentration of this sample could not be determined as the sample was very cloudy (floating lipid) and interfered with the assay. To verify if particles even formed properly it was still imaged. The magnification shown here is 200,000x. The black bar at the bottom of the image represents a scale of 100nm. No rHDL particles could be easily detected. Negatively stained regions (lighter) may or may not represent lipid droplets/aggregates or unstained open space on the grid.