

UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: Expression, purification, and refolding of
a 152 aa functional fragment of the human
endotoxin receptor CD14 in *Escherichia coli*

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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Date: 12/4/03

Comments (Optional):

Expression, purification, and refolding of a 152aa functional fragment of the human endotoxin receptor CD14 in *Escherichia coli*

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Abstract

Severe sepsis and septic shock are major causes of morbidity and mortality in intensive care units worldwide, accounting for 250,000 deaths each year in the United States alone. Sepsis is the clinical manifestation of a dysregulated innate immune response to microbial infection. Over-activation of inflammatory mediators may result in multiple organ failure, acute circulatory failure, and death. Recognition of non-self microbial products by host cell receptors is fundamental to the innate inflammatory response. CD14 is a general pattern recognition receptor that is responsible for sensitive detection of many unique pathogen-associated toxins such as LPS, PGN, and LTA. The structure and function of CD14 has extensive implications in understanding the innate immune response and progression of the potentially fatal syndrome sepsis. However, no structural data of CD14 has been acquired; the nature of its interactions with endotoxins has yet to be characterized. Production of milligram quantities of protein is necessary for NMR structural studies of CD14. This paper discusses the endotoxin-induced innate response, treatment strategies for sepsis, and gives a protocol for the expression and purification of a 152 amino acid fragment of soluble CD14.

Background

Sepsis

Sepsis is a condition involving an out of control host systematic inflammatory response caused by the infection of pathogenic micro-organisms in humans. As the condition progresses, it can lead to multiple organ dysfunction 'severe sepsis', and even to a state of acute circulatory failure characterized by arterial hypotension 'septic shock' and death (Levy et al., 2003). Sepsis is the tenth most common cause of death in the United States—affecting approximately 750,000 patients annually and accounting for over 210,000 deaths per year (Hoyert et al., 2001; Angus et al., 2001). With the incidence of sepsis rising at rates between 1.5—8% per year despite technical improvements made in intensive care units (ICUs), and costs of approximately \$16.7 billion per year burdening the US health care system, massive research efforts have been undertaken in search of improved treatments and therapy (Martin et al., 2003).

It is the innate immune response that is the first line of defense when a pathogen crosses the host's natural defense barriers, however, it is the over-activation of the innate immune system that is directly responsible for the deleterious symptoms of sepsis.

Leukocytes play an important role, recognizing the pathogens and eliciting inflammatory initiators. The release of proinflammatory mediators, such as chemokines and cytokines facilitates the migration of serum-soluble components out of the vasculature and into the specific area where the microbial invasion has been detected (Glauser et al., 1991; Mirlashari & Lyberg, 2003; Dentener et al., 1993). This response is essential for effective combat of host microbial infection.

In septic shock, the endotoxin-induced over-synthesis of pro-inflammatory cytokines and chemokines, IL-1, IL-6, IL-8, IL-12, and TNF- α , and over-activation of

macrophages results in the release of toxic molecules from polymorphonuclear cells. These processes trigger the further production of cytokines and endothelial necrosis, resulting in coagulatory responses and multiple complex proteolytic cascades (Riedemann et al., 2003). This self-amplifying and dysregulated inflammatory response often leads to organ failure and mortality, if not curtailed by counter-regulatory systems and anti-inflammatory cytokines (Baumgartner & Calandra, 1999).

Endotoxins LPS & PGN

There are several components associated with pathogens that the immune defense system engages for detection and initiation of inflammatory response. Of the bacterial components commonly exploited by the immune system are lipopolysaccharide (LPS), peptidoglycan (PGN), lipotechoic acid (LTA), lipoarabinomannan (LAM), lipoprotein and unmethylated DNA with CpG motif. LPS is present in large quantities on the surface of Gram-negative bacteria, while PGN and LTA are important components of Gram-positive bacteria, though all three reside at low levels on the outer surfaces of both types of cells.

Although other bacterial components are recognized by the innate immune response system, LPS and PGN form the two major bacterial endotoxin components recognized by innate defenses. The outer protectant on the membrane of Gram-negative bacteria is constituted, in large part, of the phosphoglycolipid LPS (Joiner, 1988). The lipid A moiety of LPS (Fig. 1) is highly hydrophobic and tethers one end of the endotoxin within the phospholipid bilayer allowing the O-region of LPS to form a perimeter of

defense on the extracellular surface, thwarting the actions of bile salts and hydrophobic antibiotics (Bäckhed, 2003).

PGN, a polymer of $\beta(1-4)$ -linked *N*-acetylmuramic acid cross-linked by short peptides (Fig. 1), is an essential component of the cell wall of Gram-positive bacteria (Schleifer & Kandler, 1972; Doyle & Dziarsky, 2001). The PGN polymers surround the membrane in bacteria and are responsible for maintaining the cellular shape and withstanding the force created by the osmotic gradient of the bacteria and the surrounding environment. LPS and PGN are necessary for bacterial vitality and are released during cell growth and death.

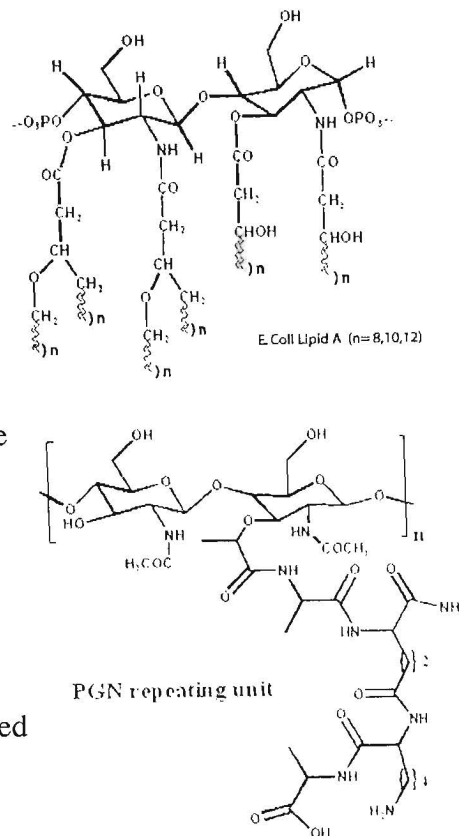


Figure 1. Molecular motifs recognized by CD14: Lipid A Moiety of LPS & PGN repeating units.

CD14 Human Endotoxin Receptor

The human endotoxin receptor CD14 is in the vanguard of the host innate immune defenses. Responsible for detection of endotoxins at subnanomolar concentrations, CD14 is a pattern recognition receptor that detects unique pathogen-associated molecular motifs (Wright et al., 1990). The 55 kDa glycosylphosphatidylinositol (GPI)-anchored protein is expressed on the membrane-surfaces of monocytes and macrophages and is critical for early mobilization of innate defenses and activation of anti-microbial pathways (Haziot et al. 1998; Lee et al., 1992; Weidemann et al., 1994). CD14 is also expressed as a soluble form (sCD14) lacking the

GPI anchor at concentrations of 2-6 $\mu\text{g/ml}$ in human serum. Soluble CD14 acts to reconnoiter and activate responses to endotoxin in endothelial, epithelial, and other cells devoid of the membrane-bound form (mCD14) (Grunwald et al., 1992; Pugin et al., 1993; Frey et al., 1992). Although microorganisms have tremendous variety, LPS and PGN are evolutionarily conserved and not expressed in higher eukaryotes, and as such, are ideal candidates for pattern recognition. Their distinct molecular-motifs provide a mechanism for highly-sensitive recognition by the pattern recognition receptor CD14. This allows the innate immune system to subdue and eliminate most infections quickly.

CD14 plays an important role in the initiation of sepsis. Previous studies have revealed attenuation of the systematic response during sepsis by blocking CD14, and it has been inferred that the primary role of CD14 is to enhance the sensitivity of the endotoxin-induced signal for cellular activation. A precise molecular-level understanding of the CD14-endotoxin interactions is necessary to understand the pathogenesis of sepsis.

LPS & PGN Induced Signal Transduction

LPS and PGN induced intracellular signaling as part of the innate immune response is initiated by binding of CD14 to these endotoxins. CD14, however, lacks a transmembrane domain and is incapable of directly mediating the activation of signaling pathways within the cell. Instead, it associates with and presents the endotoxin to Toll-like receptor (TLR), activating the ensuing intracellular signaling cascade. The Toll-like receptor family has 17 known members and consists of an extracellular leucine rich repeat (LRR) domain and a cytoplasmic Toll/IL-1R (TIR) homology domain (Takeuchi & Akira, 2001). LRRs are short protein chains of 20 to 29 amino acids found in a diverse

group of proteins that includes CD14 and myeloid differentiation protein 88 (MyD88). Due to the evolutionary conservation of LRR's in major pattern recognition proteins and receptors, the LRR is putatively thought to be important in the interaction of complexed CD14, TLR-4, and MyD88 (Kobe & Kajava, 2001).

Upon endotoxin recognition by CD14, a legion of multiple signaling molecules are recruited and form receptor clusters on the cellular membrane (Fig 2)(Triantafilou, 2003). The particular signaling proteins that must be recruited differ depending on which endotoxin has been detected. Activation of innate LPS induced defense pathways are enhanced by LBP, a 60 kDa acute-phase protein that functions to transfer LPS to CD14 (Schumann et al., 1990; Knapp et

al., 2003). The LPS-CD14 complex primarily interacts with TLR-4 (Shimazu et al., 1999), though some studies suggest that TLR-2 may play a role in LPS induced signaling as well(Yang et al., 1998; Kirschning et al., 1998). Although studies have shown that TLR-2 alone can activate the PGN signaling cascade, heterodimers between TLR-2:TLR-1 or TLR-2:TLR-6 have also been suggested to associate with the PGN-CD14 complex (Ozinsky et al., 2000).

Though LPS and PGN are both recognized by CD14, they stimulate different TLRs, TLR-4 and TLR-2 respectively (Wang et al., 2001). However, both TLR-4 and TLR-2 have similar signaling paradigms upon CD14 mediated activation. Though only

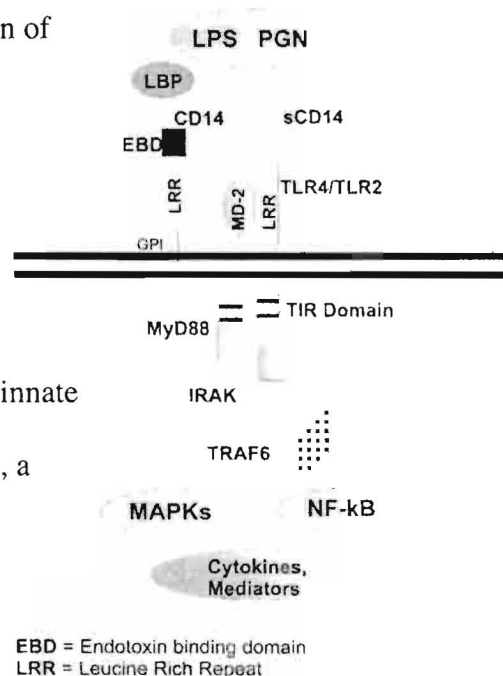


Figure 2. CD14 mediated intracellular signaling cascade in response to endotoxin detection.

two requisite proteins have been linked with PGN activation of TLR-2, at least four proteins are necessary for the optimal cellular response to LPS: CD14, LPS binding protein (LBP), Toll-like receptor 4 (TLR-4), and MD-2. The receptor cluster picture may not be so simple, however. Recently it was shown that TLR-4 when stimulated with LPS associates with a complex of receptors involving heat-shock proteins (hsp) 70 and 90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (GDF5) (Triantafilou et al., 2001; Triantafilou et al., 2002). Additionally, MyD88 associates with TLR-4 and contributes responsiveness to LPS in some cells (Andreaskos et al., 2003).

MD-2 is a secreted protein that interacts with the extracellular domain of TLR-4. The function of MD-2 has yet to be fully resolved. It has been suggested that MD-2 induces a conformational change in TLR-4 necessary for the transport of the nascent protein from the endoplasmic reticulum to the cellular membrane (Nagai et al., 2002). A more plausible explanation is that MD-2 functions to improve TLR-4 binding sensitivity to LPS (Akashi et al., 2000; da Silva Correia et al., 2001). Two domains responsible for TLR-4 binding and LPS responsiveness have been identified on MD-2 by site-directed mutagenesis (Re & Strominger, 2003; Mancek et al., 2002). The domain shown to increase LPS sensitivity is rich in aromatic and basic amino acids thought to interact with the negatively charged and hydrophobic regions of LPS (Re & Strominger, 2003).

Upon LPS-induced activation of TLR-4, the signal is transduced by phosphorylation of various adaptor proteins such as Myd88, IL-1 receptor-associated kinase (IRAK), and tumor necrosis factor-associated factor-6 (TRAF6) (Guillot et al., 2003). A cascade of intracellular signaling ensues that in turn activates mitogen-activated

protein kinases (MAPKs), NF- κ B-inducing kinase (NIK), and I κ B kinase (IKK) (Hoffmann, 2002). Upon stimulation of IKK, I κ B is phosphorylated and degraded.

NF- κ B is an inducible transcription factor that is constitutively expressed in most cells. However, in cells at rest it is sequestered to the cytoplasm because it is associated with I κ B. I κ B signals for active transport out of the nucleus (Woronicz et al., 1997). Only after I κ B has been phosphorylated, ubiquitinated, and degraded is NF- κ B active and able to effectively migrate to the nucleus (May et al., 2002). NF- κ B activation and migration into the nucleus results in the promotion of a cavalry of self-amplifying proinflammatory cytokines—IL-1, IL-4, IL-8, and TNF- α (Kopp & Medzhitov, 1999). Their release at the site of microbial invasion is the first step in the innate host response. This sophisticated innate armament allows for early detection and general defense against invading pathogens.

Strategies for Treatment of Sepsis Involving CD14

Previous treatment strategies in ICUs have involved the use of agents that block the activities of pro-inflammatory mediators, but have had limited success at decreasing the morbidity of patients (Glauser, 2000). In fact, clinical trials over the past 40 years including administration of polyclonal human antiserum against LPS, monoclonal antibodies against lipid A and TNF- α , and anti-coagulatory molecules have all failed to produce substantial improvements in patient mortality rates (Riedemann et al., 2003).

The new frontier of treatment strategies involves the design of antagonistic molecules that compete with microbial endotoxins by interacting with pattern recognition receptors, and the delineation of high-affinity compounds that bind endotoxins and

repress their ability to elicit an innate response. Potential targets include LBP, CD14, TLR-2, TLR-4, TLR-6, and MD-2. It is important, however, that complete inactivation of proteins pivotal to innate immunity be avoided.

No X-Ray or NMR structural studies on CD14 have been conducted so far and the tertiary structure, essential for understanding the binding of CD14 to LPS and PGN, is not yet known. Further, no structure of a protein homologous CD14 exists either, preventing homology modeling. Mutagenesis and limited proteolysis studies have identified two regions on CD14 thought to be responsible for binding LPS: residues 39-44 (Stelter et al., 1997) and residues 57-64

Amino acid sequence of CD14

Figure 3. CD14 sequence and motif diagram. The endotoxin binding domain was expressed and purified.

(McGinley et al., 1995). Efforts to crystallize CD14 have been largely unsuccessful. NMR spectroscopy is an alternative method for structure determination. However, milligram quantities of CD14 are needed for NMR experiments. An efficient expression and purification protocol is necessary to obtain these amounts of protein and that too in isotopically labeled forms required for NMR structural studies.

Materials & Methods

CD14 20-171 Vector & Transformation into BL21-Gold

The coding sequence for CD14 from amino acids 20-171 was codon optimized for expression in *E. coli* and synthesized (Bio ST). The resulting sequence was inserted into a pET30a vector (Novagen) between Xho I and Nde I restriction endonuclease sites such that only the C-terminal end would have the 6X-his tag. The N-terminal His6 tag was not included because of its proximity to the functionally active portion of the protein. The pET30a plasmid confers kanamycin resistance to transformed cells for which it is compatible and contains an upstream lac operator for controlled induction of protein synthesis with IPTG.

BL21-Gold(DE3) pLysS competent, chloramphenicol resistant cells from Stratagene were transformed after thawing on ice for 15 min. 3 µl, approximately 200 ng of plasmid DNA, was added to 35 µl of cells. After 10 minutes on ice, the solution was submitted to heat shock for 90 seconds at 42° C and then added to 35 µl of LB broth in a sterile Eppendorf tube. After incubation for 30 minutes at 37° C and 200 RPM, 35 µl were spread onto LB-Agar plates containing kanamycin and chloramphenicol to select transformed cells only, and incubated inverted at 37° C overnight. Single colonies were

picked for all growths. The BL21-Gold(DE3) pLysS strain of *E. coli* was selected because it encodes T7 lysozyme, a T7 RNA polymerase inhibitor.

In order to ensure the purity of the vector DNA and reduce the frequency of nicked copies for optimal expression, the CD14 vector transformed BL21 cells were grown in 50 ml of LB broth for approximately 4 ½ hours, OD₆₀₀ of .1-.2, and plasmid DNA was purified using a QIAprep Miniprep kit from Qiagen. To measure purity of the miniprep vector DNA, the absorbance was measured at 260 and 280 nm, and 260/280 ratios between 1.6 and 1.8 were accepted as pure.

Expression of soluble CD14 20-171

For growth of transformed cells, 25g of LB, Miller powder from Fisher was dissolved per liter of distilled/deionized water. The solution was autoclaved for 25 minutes, and cooled to room temperature before inoculation. Colonies from plates prepared as described above were transferred using sterile loops to 50 ml of flasks containing LB broth with kanamycin and chloramphenicol at concentrations of 50 and 35 µg/ml, respectively. The cells were grown at 37° C with agitation of 200 rpm. Once the cells entered the log phase of growth, OD₆₀₀ of .3, they were transferred to larger shaker flasks. The cells were then grown to an OD₆₀₀ of .6 before induction with 5 mM IPTG. After 3-4 hours, the cells were harvested by centrifugation at 6000 rpm for 15 minutes, and stored at -80° C until purification. Typical yields were 3-5 grams per liter of cells containing CD14 20-171 in the form of inclusion bodies which required resolubilization.

Optimization of Resolubilization, Refolding & Purification of CD14 20-171

The frozen cells were resuspended in 50 mM Tris buffer pH 8.0 and stirred at 4°C for one hour. A cocktail protease inhibitor tablet (Roche) was added to prevent protein cleavage. In order to lyse the cells, the solution was sonicated for five 30 second bursts using a Branson Sonifier 450.

PBS containing 6 M urea was originally used to resolubilize the CD14 20-171 inclusion bodies. This refolding buffer when added to the lysate formed a highly viscous solution that hindered purification. Dilution of the solution 8 fold after the addition of urea did not resolve the problem. The solution remained viscous and clogged the Ni-NTA Superflow column (Qiagen) used for the purification of His6 tagged proteins. As a means of reducing the viscosity, a preliminary purification step was added to the protocol. Gravity columns were packed with anion-exchanging Sepharose 6B (Sigma-Aldrich). Although, flow-rates were initially higher, these columns eventually became congested as well. The loaded solution was then eluted with stepwise increments of increasing KCl concentration. The fractions of elutant containing CD14 were then successfully loaded onto the Ni-NTA column (Qiagen), however only small quantities of CD14 were purified in this fashion.

As an alternative, the viscous 6M urea lysate solution was subjected to ultracentrifugation at 50,000g. This cleared the lysate considerably and was followed by passing the supernatant through .44 µm Stericup filters (Millipore). After ultracentrifugation and filtration, the cleared lysate passed easily over Ni-NTA columns. This purification method effectively produces CD14 20-171, however it is taxing and

requires the use of several Stericup filters. An effective alternative in which resolubilation by addition of 6 M GuHCl is described below.

For resolubilization of inclusion bodies, guanidine hydrochloride was added to the lysed cell suspension to make it 60% by weight, and the solution was stirred at 4° C for ½ hour. Afterwards, the solution was centrifuged for 30 minutes at 12,000 rpm. The supernatant was then diluted 6 fold in refolding buffer (50mM Tris pH 8, 2M glycine, 5mM cysteine) and stirred over-night. The solution containing the solubilized and refolded CD14 20-171 was then centrifuged for 30 minutes at 12,000 rpm to remove any precipitated debris. Ammonium sulfate precipitation was then performed at 60% by weight to further purify the protein, and the resulting pellet was dissolved in phosphate buffer saline (PBS), pH 8.

Gravity flow columns packed with Ni-NTA Superflow from Qiagen were then loaded with the CD14 solution and washed with 100 ml PBS followed by 50 ml of PBS, 25 mM imidazole. The bound CD14 was eluted with PBS containing 250 mM imidazole, and the elutant was concentrated in amicon Centripreps (MWCO 10,000) from Millipore to a volume less than 1ml.

Gels and Western Blotting

PhastGel Homogenous 20% gels from Amersham Biosciences were used to run purified samples of CD14 20-171. The gels were silver-stained and developed with formaldehyde. Prior to running the samples, they were boiled for 10 minutes in SDS and β -ME. The BioRad Precision Plus Protein Dual Color ladder was used to consistently mark the CD14 peak.

Western blots were performed after boiling the samples in a denaturing, non-reducing buffer containing SDS. Novex pre-cast gels, 18% Tris-Glycine from Invitrogen were used. 15µl of SDS-boiled sample solution were loaded per well, and gels were run at 125V for 1½ hours in an Xcell SureLock Mini-Cell electrophoresis unit from Invitrogen. After separation, an Xcell II Blot Module was used to transfer the protein to a PVDF membrane at 25V for 1½ hours. A Tris based solution containing 5% milk, .1% Tween was used to block the membrane. Membranes were blocked overnight at 4° with slow agitation. Primary and secondary antibodies were added and incubated for 1 hour at room temperature each. Mouse monoclonal human anti-CD14 (ab8103) from Novus was diluted 1:400 and used as the primary antibody for CD14 detection with an HRP conjugated anti-mouse polyclonal secondary. Additionally, HRP conjugated mouse monoclonal His6 (ab7857) from Novus was diluted 1:4,000 and used to determine that the entire protein and His6 tag had been expressed. CN/DAB precipitating substrates from Pierce were used to develop the bound HRP conjugated antibodies.

Results & Discussion

Analysis of the primary structure of CD14 shows that the carboxy terminal end of CD14 consists of 10 leucine rich repeats (Fig 3), of which 7 leucine rich repeats are predicted to be involved in interactions with TLRs and other signaling molecules. Additionally, the first 19 amino terminal residues are a signal peptide that is cleaved before the protein leaves the endoplasmic reticulum. The functional region of the protein that recognizes and binds endotoxins is located within the first 152 N-terminal amino acids after the signal peptide region. Therefore, for ligand binding and structural studies

of CD14, residues 20-171 would be appropriate for expression. Shorter fragments of N-terminal CD14 would be subject to protease degradation and were therefore not considered. *E. coli* was chosen as an expression system for CD14 since the use of mammalian cells is unlikely to generate sufficient quantities of CD14 for NMR based structural studies. Expression of CD14 in yeast has been characterized, however, protein glycosylation occurs which presents a problem for NMR studies due to the presence of multiple conformations as a result of glycosylation.

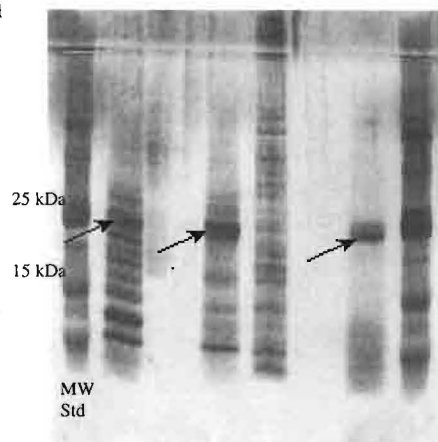


Figure 4. SDS-Page analysis of partially purified CD14. Arrows show the CD14 band

CD14 20-171 expression in *E. coli* using the pET30a expression system was successful. Yields of CD14 were improved when cultures were induced at an OD₆₀₀ of .6. When grown to higher concentrations before induction, the yield of cells was greater, but less total protein was produced. Figure 4 shows that CD14 20-171 migrated with the 20 kDa marker in the PhastGels confirming expression of protein with correct molecular weight. Expression using the protocol described here results in production of about 1-2 mg of protein/liter of medium. Western blotting results show that anti-human CD14 monoclonal antibody (Novus-ab8103) does bind to N-terminal residues 20-171 when transferred to PVDF membrane from SDS, non-denaturing gels (Fig 5). It has been reported that some CD14 antibodies do not recognize CD14 when ran in SDS-PAGE conditions (Majerle et al., 1999).

Our recombinant CD14 contains 4 Cysteine residues, and their redox state must be controlled for proper refolding. This is effectively accomplished by the addition of 5

mM cysteine to the refolding buffer. Resolubilization of inclusion bodies by addition of urea is cumbersome and presents many problems associated with the high viscosity of the solution. Guanidine hydrochloride is an effective alternative for resolubilizing the protein. The ammonium sulfate precipitation is an essential step that contributes greatly to the purification protocol. After dissolving the precipitated protein, the CD14 solution is clear and roughly 50% pure. The CD14 sample purity is increased to approximately 70% following elution from the Ni-NTA column. To achieve the greater than 95% purity necessary for acquiring NMR spectra with a high signal to noise ratio, the sample will be run on a molecular size exclusion column.

Although studies indicate that endotoxin binding takes place between residues 39-64 (Stelter et al., 1997; McGinley et al., 1995; Juan et al., 1995), attempts at expressing fragments of CD14 much shorter than 152 residues have been unsuccessful (Majerle et al., 2000). Shorter fragments may not form a compact domain and be rapidly degraded upon translation. Therefore, for ligand binding studies of CD14, residues 20-171 were chosen. Bacterial

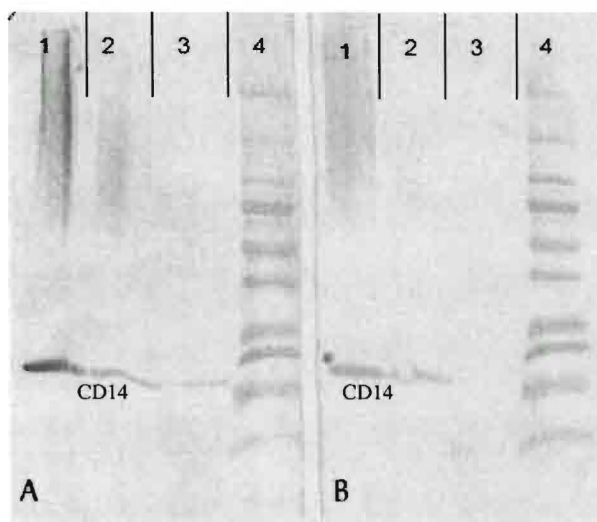


Figure 5. Western blots confirming CD14 expression by using α -CD14 1° (A) and translation of entire protein and His6-tag by using α -His6 1° (B). Lanes 1 & 2 in each blot are from preps induced at OD of .6; Lane 3 was induced at OD 1.2.

expression of CD14 is quick and scaleable, however, one caveat is that many strains of bacteria possess LPS, a natural ligand of CD14, and care must be taken not to

contaminate the sample of purified protein. This problem is avoided in part by expressing the CD14 in inclusion bodies.

Not only does bacterial expression of CD14 produce milligram quantities of protein required for structural studies in NMR, but the protocols used for unlabeled expression are easily applied to isotopic labeling of the protein. *E. coli* BL21 cells grow and express well in minimal media which allows for unproblematic ^{13}C and ^{15}N incorporation into the protein backbone and sidechains, which is necessary for high resolution of NMR spectra.

As the progression of sepsis is intimately linked with the induction of several endotoxin-activated signaling cascades, describing its pathogenesis is exceedingly complex. CD14 is an ideal candidate for therapy designed to suppress the progression of sepsis because it confers sensitivity to multiple transmembrane signaling molecules that are responsible for detection of an array of endotoxins. Although several eloquent mutagenesis studies have identified epitopes of CD14 responsible for endotoxin binding and monoclonal antibodies that competitively bind CD14 developed, no sophisticated structural studies have been conducted on the protein. The shortcomings of current treatments of sepsis can be overcome once the structure-function relationships are determined by NMR spectroscopy. Rational drug design can be accomplished by identifying high-affinity ligands of CD14 that antagonize the effects of endotoxins by performing NMR binding studies.

NMR binding studies will resolve the nature of the binding, identifying the precise residues interacting on CD14 and the binding portions on the endotoxin ligands.

However, for this to be possible, expression of sufficient quantity of protein is required, which has been accomplished during the course of this work.

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