Possible Therapeutic and Diagnostic Applications of the Endotoxin Binding Soluble CD14

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Head of the **Examination Committee**:   András Berta, DSc
Members of the Examination Committee: Tímea Berki, DSc
                                                                        Sándor Szántó, PhD

The Examination takes place at the library of the Ophtalmology Clinic, Medical and Health Science Center, University of Debrecen
 Debrecen, 15.01.2013, 11:00 am

Head of the **Defense Committee**:   András Berta, DSc
Reviewers: Emese Kiss, PhD
                    István Csípő, PhD
Members of the Defense Committee: Tímea Berki, DSc
                                                            Sándor Szántó, PhD

The Ph.D. Defense takes place at the Lecture Hall of the Department of Medicine, Medical and Health Science Center, University of Debrecen
 Debrecen, 15.01.2013, 1:00 pm

1. INTRODUCTION

1.1 The CD14 Molecule

The CD14 molecule is a pattern recognition receptor of the innate immune system. The gene encoding the protein is located at chromosome 5q23-31, and consists of two exons and transcribes into a 1.4 kb mRNA transcript. The mature protein is a 356 amino acid glycoprotein which is mainly expressed by myeloid cells. It has both a membrane bound (mCD14, 55 kDa) and soluble forms (sCD14, 48 kDa, 56 kDa). The mCD14 has no transmembrane region, it is anchored into the cell membrane via a glycosylphosphatidylinositol (GPI) tail. The mCD14 is highly expressed by monocytes (~100,000/cell) and to a much lesser extent by neutrophil granulocytes (~4,000/cell) while the serum concentration of sCD14 is in the range of few µg/mL.

1.2 The Function of CD14 and the LBP/TLR4/MD2 System

The main function of CD14 is the recognition of lipopolysaccharide (LPS) liberated from the membrane of Gram-negative bacteria, and the initialisation of cell activation. Recognition of LPS and initialisation of cell activation is mediated by the Lipopolysaccharide Binding Protein/Toll-like Receptor 4/ Myeloid differentiation protein 2 receptor system (LBP/TLR4/MD2). Free LPS monomerized by LBP is shuttled by CD14 to the TLR4/MD2 complex located in the cell membrane, where the LPS molecule reaches the TLR4 transmembrane receptor via MD2 which leads to the initialisation of the intracellular signalling cascade.

1.3 Potential Ligands of CD14

The CD14 molecule is a pattern recognition receptor with the main function of recognizing LPS originating from the membrane of Gram-negative bacteria. It has more ligands beside of LPS ranging from bacteria derived molecules, fungi

and apoptotic bodies to microbial cell wall components, and even to whole bacteria.

1.3.1 Soluble Molecules

The most known and most studied ligand of CD14 is LPS, an essential component of the outer membrane of Gram-negative bacteria. The LPS structure of different bacterium families is greatly similar. The wild type LPS (S-type) of the family *Enterobacteriaceae* consists of the O-antigen, an R-core and the lipid-A. In certain cases the O-antigen might be missing (R-type) which makes the lipid-A component more accessible for the immune system. Besides LPS many other ligands of CD14 have been described., such as lipoarabinomannan of *Mycobacteria*, soluble peptidoglycan, WI-1 antigen of *Blastomyces dermatitidis*, cell wall of *Staphylococcus aureus*, polyuronic acids of *Pseudomonas,* muramilpeptide and outer membrane lipoproteins of *Borrelia burgdorferi*. These molecules are all essential and conservative components of microbes which could make them an excellent therapeutic target.

1.3.2 Blebs

Not only free LPS is liberalized from the outer membrane of Gram-negative bacteria but outer membrane vesicles as well, also called blebs. Bleb production is very pronounced in case of *Neisseria meningitidis* where bleb production was also confirmed *in vivo* both in serum and in cerebrospinal fluid. Since bleb structure is basically identical to the structure of the outer membrane, blebs activate the immune system via the classic CD14-LBP and subsequently TLR4/MD2 mediated pathway.

1.3.3 Bacteria

In addition to microbial ligands multiple reports describe CD14 dependent phagocytosis mechanisms of different pathogens like *Mycobacterium*

tubercolosis, Salmonella typhimurium, Escherichia coli and *Listeria monocytogenes*. Although the role of CD14 is a well established fact, only one workgroup described the binding of sCD14 and mCD14 directly to bacteria.

1.4. CD14 and Diseases

1.4.1. Bacterial Infections

As a pattern recognition receptor sCD14 plays a crucial role during the initial phase of infections by promoting cytokine production which in turn leads to initialization of different immune responses. The serum concentration of sCD14 correlates with the severity of infections, and shows an elevated level both in local infections and sepsis. The exact mechanisms and reason of CD14 production during infections is not well-known, although it is very likely that beside a de novo production the shedding of mCD14 is also responsible for the elevated sCD14 levels. However de novo synthesis of sCD14 seems also important as its production is inducible by IL-6 and its serum concentration correlates with the concentration of C-reactive Protein (CRP), a well known acute phase protein (APP). Serum level of sCD14 also correlates with mortality, however it is not entirely clear whether elevated sCD14 concentrations are the reason behind the elevated mortality rate or that they are result of more severe infections.

1.4.2. Other Diseases

The change of sCD14 levels is characteristic not only for infections, but there are examples of sCD14 levels correlating with the severity of other diseases. There are reports of elevated sCD14 concentrations in the bronchoalveolar lavage fluid of sarcoidosis patients, and in the serum of psoriasis, systemic lupus erythematosus and inflammatory bowel disease patients.

1.4.3. Acute Phase Protein? Diagnostic Marker?

The use of sCD14 as a diagnostic marker emerges primarily in inflammatory diseases. The sCD14 concentration shows a good correlation with the progression of different diseases, CRP concentration and the level of IL-6, the cytokine that regulates the liver's APP production. Furthermore, real-time PCR experiments confirm on mRNA level that sCD14 production is inducible by IL-6. Based on these findings the sCD14 acts as an APP and it is worthwhile to investigate its possible use as a diagnostic marker.

1.5. Clinical Significance of Cirrhosis and Accompanying Infections

Cirrhosis is the result of a chronic liver disease during which liver tissue is replaced by scar tissue while normal liver functions degrade. Main complications are ascites, varix bleeding, hepatic encephalopathy and elevated risk of different bacterial infections.

1.5.1. Etiology and Clinical Properties of Cirrhosis

Mostly alcoholism or Hepatitis B/C infections are the reasons behind cirrhosis but there may be several other associations like diabetes, obesity, autoimmune diseases, inheritable diseases concerning iron metabolism, cystic fibrosis and the use of hepatotoxic drugs.

1.5.2. Clinical Significance of Cirrhosis Related Infections

One of the most common complication of cirrhosis is the development of bacterial infections which may lead to further degradation of liver functions and ultimately to death of the affected patient. Furthermore, bacterial infections may cause other complications, like varix bleeding, hepatic encephalopathy, renal failure or coagulation disorders. Different infections may elevate mortality

significantly, bacterial infections are in the background in about 30-50% of cirrhosis related deaths.

1.5.3. Diagnostics of Cirrhosis Related Infections

About 50% of infections in cirrhosis are of atypical clinical appearance and some other properties of cirrhosis makes identification of infections even more difficult. Elevation of bacteraemia related serological markers are an early sign of infections which makes identification of infections possible. However, these APPs are mainly produced by the liver, which makes their use as a serological marker rather difficult in cirrhosis. On the other hand, infection unrelated factors like hepatocellular carcinoma, necrosis, local inflammation of liver tissue or bacterial translocation may induce synthesis of these markers, which makes their clinical use even less effective. Earlier studies conclude that CRP and procalcitonin (PCT) levels are the most reliable markers of diagnosing infections, however their diagnostic accuracy and cut-off values are not properly established.

1.6. Difficulties of Treating Infections. Antibiotic Resistance. Potential New Therapeutic Agents: Fc-fusion Proteins

Abuse of antibiotics lead to development of antibiotic resistant pathogens which are more and more difficult to combat especially in cases where the immune system of the infected patient is compromised. One possible approach to solve this problem is the combination of certain antimicrobial proteins with the Fc part of the immunoglobulin molecule. The resulting proteins act as artificial antibodies and may have bactericidal properties while involving the immune system in the neutralization of the pathogens.

With the advancement of biotechnology and molecular genetic techniques the door to creating various artificial proteins is opened widely. The common characteristics of receptor/Fc fusion proteins are that one or more receptor

molecules or receptor domains are coupled to the Fc part of human IgG1. The receptor/Fc fusion can be established by genetic engineering or chemical reactions. In these proteins the Fc part remains intact (CH2, CH3 domain and hinge region), and the receptor protein(s) potentially recognizing a broad spectrum of microbes or microbial ligands are coupled to the N-terminal part.

The Fc fusion has many advantageous traits regarding both the use of the "finished" protein, and during the expression and purification steps. The most important property of the Fc part regarding the antimicrobial activity of the fusion protein is the fact that the dimerization conferred by the Fc part may increase the receptor-ligand avidity of the receptor part, and that the Fc part retains its original functions like the ability of complement activation and being able to mediate FcR dependent phagocytosis.

There are two important examples of these types of artificial antibodies, one is the fusion of BPI_{23} to the Fc part of human IgG1, while the other example is also a cationic protein, CAP18 fused to the Fc part of human IgG1. In both cases increased survival rate was reported in mice treated with the fusion proteins in the used sepsis models.

2. Aims of the Study

The CD14 molecule, as a pattern recognition receptor, has several ligands that are of a microbial origin. This raises the possibility of a therapeutical use: the pattern recognition properties of CD14 combined with the advantages conferred by the Fc fusion may result in an opsonin with a broad spectrum providing therapical possibilities in the treatment of infections caused by antibiotic resistant bacteria. Furthermore the fusion protein may be useful in cases like *N. meningitidis* infections, where the pathogen's outer membrane vesicle production plays an important role in the escalation of inflammatory events.

- Our goal is to create a CD14/Fc protein, test its structural integrity and biological functions, and describe its binding ability to bacteria, and prove the LPS-CD14 specificity of this binding. Furthermore we investigate its effect on the phagocytosis of neutrophil granulocytes, macrophages and dendritic cells.

- We investigate the possible LPS and N. meningitidis bleb neutralizing capability of the fusion protein, and how these endotoxin preparates behave against other neutralizing agents.

The concentration of human sCD14 correlates with the severity of infectious diseases which raises the possibility that monitoring the sCD14 concentration may be of use in establishing diagnosis and severity of infections. In cases like cirrhosis where liver damage handicaps the use of classic APP markers the application of sCD14 might be even more useful.

- In the second part of our work we seek answer whether measurement of serum sCD14 concentration, either alone or in combination with other APP markers, is of any diagnostic value in identifying infections that accompany cirrhosis.

3. Patients, Materials and Methods

3.1. Patients (cirrhosis)

Three hundred and sixty-eight, well-characterized, consecutive patients with cirrhosis (male/female: 204/164, age: 56.4 ± 10.8 years, disease duration: 3.9 ± 4.2 years) were included from the Gastroenterology Division of the Department of Medicine (University of Debrecen). Diagnosis of cirrhosis was established based on clinical, biochemical, ultrasonographic and when available histological features. Presence of actual bacterial infection was sought actively on enrolment.

3.2. LPS, Bleb Preparates, Bacterium and Fungi Strains

For functional testing of CD14/Fc and CD14/His molecules *Salmonella Minnesota* Re (#595) LPS (Sigma-Aldrich) was used. Bleb preparates were isolated from *N. meningitidis* and characterized at the Department of Medical Microbiology, University of Utrecht, the Netherlands. For binding and phagocytosis studies the following bacteria and fungi were tested: *Escherichia coli* (ATCC 25922)*, Salmonella hartford* (Hungarian National Center of Epidemiology 100063)*, Lysteria monocytogenes* (Hungarian National Center of Epidemiology 130001)*, Streptococcus pneumoniae* (ATCC 49619), *Salmonella typhimurium* wild type (#657), *S. typhimurium* Ra (#656), *S. typhimurium* Re (#658), *S. minnesota* Re (#595), *Candida albicans* (#10231), *C. tropicalis* (#555) (clinical isolates). Bacteria and fungi were heat inactivated (80°C, 2 h, and 65°C, 2 h), and labelled by FITC (40 μg/mL, 45 minutes at 4°C; 0,2 μg/mL, 45 minutes at 4°C respectively). The fluorescently labelled microbes were aliquoted after washing and kept at -20°C until further use.

3.3. Isolation and Culturing of PMN, Monocytes, Macrophages, Dendritic and SKBr-3 cells

Polimorphonuclear cells: Polymorphonuclear cells were isolated from peripheral blood on a discontinuous Ficoll gradient (1077/1119 g/L, Sigma-Aldrich). After

aspiration of the PMN layer red blood cells were lysed with distilled water for 30 seconds, and subsequently isotonic osmolarity was restored by adding 1/10 volume of 10x PBS. After washing twice with PBS cells were resuspended at 5×10^6 cells/mL.

Monocyte-derived macrophages and dendritic cells: PBMCs were isolated by centrifugation on a Ficoll-Paque (1077 g/l; GE Healthcare) density gradient, and monocytes were separated from PBMCs using magnetic anti-CD14 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. For monocyte-derived macrophages and dendritic cells monocytes were cultured in complete RPMI medium (Sigma-Aldrich) supplemented with 50 ng/mL M-CSF (Peprotech EC) in case of macrophages, and 80 ng/ML GM-CSF (Gentaur Molecular Products), 100 ng/mL IL-4 (Peprotech EC) and 50 ng/mL M-CSF (Peprotech EC) in case of dendritic cells. Cells were used for the experiments on the 5th day. Phenotype of cells was verified by flow cytometry.

SKBr-3 cells: The SKBr-3 human breast carcinoma cell-line (ATCC) was cultured in complete DMEM (Sigma-Aldrich) medium.

3.4 Cloning and Expression of CD14/Fc and CD14/His

The cDNA of CD14 obtained from the cDNA library of Medical Microbiology, University Medical Center, Utrecht was amplified with the following primers, which introduced *Bam*HI and *Not*I restriction sites to the 5' and 3' ends of the PCR product, respectively: P1-5'TC<u>GGATCC</u>ACCACGCCAGAACCTTG3', P2-5'CAGCGGCCGCCAG-CACCA<u>GGGTTCCC</u>GA3'. The amplicon was ligated into an intermediate vector (pCR4-TOPO, Invitrogen) according to the manufacturers description. One shot TOP10 bacteria (Invitrogen) were transformed with the CD14 insert containing vector, and after selection cultured in liquid LB medium containing 50 μg/mL carbenicillin. Subsequently plasmids were isolated using QIAprep Miniprep Kit (Qiagen), according to the manufacturers' instructions. Presence of the CD14 insert was verified by double

restriction digestion (*BamH*I/*Not*I, New England Biolabs) and DNA sequencing (BigDye 3.1 sequencing kit, Applied Biosystems). Subcloning of the CD14 insert into the expression vectors, and the expression of CD14/Fc and CD14/His proteins in HEK293 cells was performed by U-Protein Express B.V. (Utrecht, the Netherlands). The expression vectors contained the Fc and His tags in the appropriate reading frames. Purification of the CD14/Fc and CD14/His proteins was performed with a Protein G and Nickel column (GE Healthcare) according to manufacturers description. Purity of both protein preparates was verified by SDS-PAGE. Biological function of the expressed molecules was verified by their LBP dependent LPS binding ability using ELISA.

3.5 Binding of CD14/Fc to Bacteria and Fungi

Fluorescently labelled bacteria and fungi (5 x 10^7/mL) were incubated in the presence or absence of LBP (100 ng/mL) and CD14/Fc or CD14/His (5 µg/mL). After incubation microbes were washed twice with PBS containing 0.1% BSA. Bound CD14/Fc and CD14/His was detected by a Cy5-goat-anti-human-IgG (Light + Heavy Chain, Jackson Laboratory; 1:100 dilution) antibody and a PE-anti-His antibody (Miltenyi Biotec, 1:100) respectively. After washing bound proteins were evaluated by flow cytometry based on their Cy5 (CD14/Fc) and PE (CD14/His) positivity.

For LPS competition and blocking experiments CD14/Fc was pre-incubated with LPS (two fold dilution sequence: 20 – 0.2 µg/mL) or anti-CD14 blocking antibody (60bca ATCC; two fold dilution sequence: 40 – 0.6 µg/mL), and subsequently the binding experiments were performed as described above. For CD14/Fc and CD14/His competition experiments 1 µg/mL CD14/Fc fusion protein was incubated with different concentrations of CD14/His protein (two fold dilution sequence: 350 – 0.7 µg/mL) together with *S. typhimurium* Re suspension. Bound CD14/Fc was detected by flow cytometry as described

above, level of blocking was determined by comparing samples to unblocked samples and result was given as percentage.

3.6 Phagocytosis of Opsonised Bacteria, *Ex Vivo* Killing Assay

FITC labelled bacteria opsonised by CD14/Fc were incubated in the presence of 15 μL PBS or 10% serum at 37°C for 15 minutes. Subsequently 35 μL PMN, macrophage, dendritic cell or SKBr-3 suspension (1.7 x 10^5 cell) was added to the samples and then incubated for another 30 minutes at 37°C. After washing samples were evaluated by flow cytometry and phagocyting cells were identified by their FITC positivity. Intracellular localisation of cell associated bacteria was confirmed by confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss AG).

For phagocytosis blocking experiments cells were incubated for 45 minutes at 4°C with anti-CD16 (3G8, Abcam) and/or anti-CD32 (ATCC: "IV-3" myeloma cell line) blocking antibodies (20 μg/mL).

For killing assay experiments 3 x 10^3 CFU of *S. typhimurium* wt and Re bacteria were added to 1 mL heparinised whole blood in the presence or absence of 5 μg/mL CD14/Fc. After gently shaking for 60 minutes at 37°C, 100 μL of blood was spread on pre-warmed blood agar plates. After 24 hours of culturing, colonies were counted and compared to the appropriate controls.

3.7. Activation of Neutrophil Granulocytes by LPS and blebs

Isolated neutrophil granulocytes were activated for 30 minutes at 37°C with LPS or blebs in the presence of 1% serum or 100 ng/mL LBP. 1 x 10^5 neutrophil cells were stimulated with 10^{-6} M formyl-MET-LEU-PHE (fMLP, Sigma-Aldrich) in the presence of 150 μM luminol (Sigma-Aldrich), and the chemiluminescence response was measured with a luminometer (Autolumat LB 953, Berthold) for

10 minutes and the chemiluminescence response was expressed as the area under the curve.

3.8. Neutralization of LPS and Blebs by BPI, Plasma and CD14/Fc Fusion Protein – Neutrophil Chemiluminescence Assay, LAL Assay, L-selectin Expression

500 µL heparinised blood was fractioned into plasma and cell fractions by centrifugation (3,000g, 2 minutes); subsequently plasma was centrifuged again at 10,000g for 1 minute. For neutralization LPS or bleb was incubated with plasma for 60 minutes at 37°C, or BPI (10 µg/mL) or CD14/Fc (5 µg/mL) for 30 minutes in the presence of 3% serum or buffer. Subsequently LPS activity was detected either by LAL assay (Lonza) or isolated neutrophils were added to the samples and chemiluminescence response was measured as described above.

Effect of CD14/Fc was also evaluated in whole blood samples by measuring L-selectin expression levels of monocytes and granulocytes. Heparinised blood samples (50 µL/sample) were incubated with different concentrations of CD14/Fc (two fold dilution sequence: 10 µg/mL – 0.16 µg/mL) fusion protein in the presence of LPS, blebs or buffer for 30 minutes at 37°C. After incubation samples were labelled with anti-human-CD62L-APC (BD Pharmingen) antibody. Subsequently red blood cells were lysed (BD FACS Lysing Solution) and samples were washed and measured by flow cytometry (BD FACS Calibur). CD62L positivity was expressed as mean FL4 fluorescence intensity.

3.9. Serum sCD14, LBP, PCT and CRP Concentration Measurement

Analyses were performed on serum samples stored at -70°C. CRP levels were measured with an Integra 700 automated analyser (Roche). PCT was determined by a two-site immunoluminometric assay (Liaison) with a Liaison analyser (DiaSorin). LBP concentrations were determined by solid phase ELISA (Hycult

Biotechnology), and sCD14 concentrations were measured by ELISA (Quantikine, R&D Systems).

3.10. Statistical analysis

Binding of CD14/Fc and CD14/His to bacteria, phagocytosis-, *ex vivo* killing-, and blocking experiments were statistically evaluated by matched Wilcoxon analysis. LAL assay and chemiluminescence assay results regarding bleb and LPS neutralization were evaluated by Mann-Whitney U test. For cirrhosis diagnostic markers variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as means [standard deviation (SD)] or as medians [interquartile range (IQR)] according to their homogeneity. Categorical variables were compared with the Chi square-test or Chi square-test with Yates correction as appropriate. Continuous variables were compared with the Mann–Whitney U test or Student's t test. Spearman SRO tests were used to analyse the association between continuous variables. To test the accuracy and cut-off values of different APPs, receiver operating characteristics (ROC) curves were plotted. Statistical calculations were performed with softwares Statistica for Windows, GraphPadPrism 5 and SPSS 15.0; differences were considered significant at $p < 0.05$.

4. Results

4.1. Expression and Characterisation of CD14/Fc and CD14/His Proteins

The CD14/Fc fusion protein and the CD14/His protein were expressed in HEK293 cells and purified on Protein G and nickel columns respectively. Size and purity was verified by reducing and non-reducing SDS-PAGE. The CD14/Fc molecule forms dimers similarly to human IgG and has a molecular weight of 160 kDa under non-reducing conditions, and a molecular weight of 80 kDa under reducing conditions. Being a monomer the CD14/His has a molecular weight of 50 kDa under both reducing and non-reducing conditions. Biological activity of both expressed proteins was verified by their LBP dependent LPS binding capabilities in ELISA. The OD values for 1 µg/mL CD14/Fc and CD14/His were 0.75±0.16 and 0.38±0.03 while backgrounds were 0.03±0.02 and 0.04±0.02 respectively.

4.2. Binding of CD14/Fc to Bacteria and Fungi

Flow cytometry was used to determine binding of CD14/Fc to bacteria. The CD14/Fc fusion protein bound to all wild type and short LPS (Ra & Re) mutant Gram-negative bacteria. Binding to bacteria was LBP, time and CD14/Fc concentration dependent. In case of wild type bacteria maximal binding was achieved after 4 hours of incubation at 5-10 µg/mL CD14/Fc concentrations. We experienced exceptionally strong binding to Re mutants where maximal binding could be reached after 2 hours of incubation at 1-5 µg/mL CD14/Fc concentrations. On the other hand CD14/His showed significantly weaker binding to bacteria than the CD14/Fc dimer. It associated only with short LPS (Re) mutants, and even in those cases we experienced only a slight elevation in fluorescence levels. Neither of the recombinant proteins bound to tested fungi. Binding of CD14/Fc to bacteria (*S. typhimurium* wt és *S. typhimurium* Re) could be dose dependently inhibited by pre-incubating CD14/Fc with LPS and an anti-CD14 blocking antibody.

We also compared the avidity of CD14/Fc and CD14/His to bacteria. Both proteins were mixed at different ratios and added to *S. typhimurium* Re and binding of CD14/Fc was evaluated. We found that CD14/His at approximately 50 fold molar excess was able to inhibit binding of CD14/Fc to bacteria by 50% (1 µg/ml CD14/Fc vs. 10-20 µg/ml CD14/His, 160 kDa vs. 50 kDa).

4.3 CD14/Fc Enhances Phagocytosis of Gram-negative Bacteria

Fluorescently labelled bacteria (*S. typhimurium* Ra and Re, S. *minnesota* Re) were opsonised with 5 µg/mL CD14/Fc fusion protein and after washing added to PMN cells, macrophages, dendritic cells, or to FcR negative SKBr-3 cells used as a control. In case of PMN cells CD14/Fc enhanced phagocytosis both in the presence and absence of human serum. The phagocytosis enhancing effect was remarkably strong in the absence of serum (percent of positive cells with and without CD14/Fc: *S. minnesota* Re – 10.65 ± 0.97 vs. 3.72 ± 0.45. $p<0.05$; *S.typhimurium* Re – 12.60 ± 3.03 vs. 1.57 ± 0.37. $p<0.05$; *S. typhimurium* Ra – 13.85 ± 1.64 vs. 5.47 ± 0.42. $p<0.05$), while there was a significantly lesser effect in the presence of human serum.

In case of macrophages phagocytosis increased by 17% as compared to the control, giving the CD14/Fc the same opsonising property as human serum (percentage of positive cells with and without CD14/Fc: 69.01 ± 2.99 vs. 52.54 ± 1.33, $p<0.01$). Experiments with dendritic cells showed a less pronounced phagocytosis enhancing effect than human serum, but even in this case there was a significant raise in phagocytosis when compared to the control samples (percentage of positive cells with and without CD14/Fc: 20.46 ± 0.88 vs. 15.36 ± 4.40, $p<0.01$). SKBr-3 cells used as negative control did not associate with bacteria.

Trypan blue quenching showed that the majority (50-70%) of cell associated bacteria were internalized. The exact localisation of bacteria was confirmed by confocal microscopy. The membrane of PMN cells was labelled by an

AlexaFluor647 conjugated anti MHC-I antibody (blue), the nucleus was stained with propidium iodide (red), while bacteria were FITC labelled (green). Microscopy images confirmed that both serum and CD14/Fc opsonised bacteria were localised intracellularly.

4.4. CD14/Fc Opsonised Bacteria are Internalized via Receptors CD16 and CD32

To prove that uptake of bacteria is a result of the interaction of the Fc part of CD14/Fc and Fc receptors of phagocytes, Fc receptors (CD16 and CD32) of PMN cells were blocked with specific monoclonal antibodies. Our results showed, that internalisation of *S. typhimurium* Re could be partially inhibited by blocking receptors CD16 (61.64±24.16% inhibition. $p<0.05$), or CD32 (42.76±20.52% inhibition. $p=0.07$). When both receptors were blocked simultaneously phagocytosis was completely inhibited (91.65±3.54% inhibition. $p<0.01$). We experienced similar results in the case of the other tested short LPS mutant, *S. Minnesota* Re.

4.5 CD14/Fc Enhances *Ex Vivo* Killing of Gram-negative Bacteria

S. typhimurium wt and *S. typhimurium* Re bacteria were added to whole blood containing 5 μg/mL CD14/Fc or buffer, and subsequently incubated for 60 minutes at 37°C. After incubation 100 μL sample was spread on blood agar plates and bacteria were cultured for 24 hours. Colonies were counted and compared to buffer-only controls. We found no difference in case of wild type bacteria. Untreated blood decreased CFU count from 3×10^3/mL to 853±199/mL. Similarly, CD14/Fc treated blood decreased CFU count to 830±72/mL. However, in case of *S. typhimurium* Re significantly less bacteria survived if the blood contained 5 μg/mL CD14/Fc. CFU count decreased to 550±40/mL in case of untreated blood, while in case of CD14/Fc treated blood CFU count was 337±84/mL ($p<0.05$, n=3).

4.6. Effect of CD14/Fc and Other Neutralizing Agents On the Biological Activity of Different LPS Preparates and *N. meningitidis* Derived Blebs

In addition to killing pathogens it is crucial to neutralize biologically active molecules liberated from bacteria, especially in the therapy of septic patients. These molecules may emerge in free form, but they are also common in the form of outer membrane vesicles, also called "blebs".

4.6.1. Effect of CD14/Fc on the Biological Activity of LPS and Bleb Preparates

To evaluate the neutralizing effect of CD14/Fc LPS and bleb samples were incubated with or without CD14/Fc and their priming effect on isolated neutrophil granulocytes was measured in a chemiluminescence assay. CD14/Fc did not alter the effect of LPS and blebs, furthermore, it stimulated PMN cells dose dependently on its own. We found similar results in another assay, where L-selectin expression was measured on granulocytes by flow cytometry. The CD14/Fc molecule was able to down-regulate CD62L expression on its own similarly to LPS and bleb preparates. We tested our CD14/Fc samples with LAL assay for a possible LPS contamination but no endotoxin could be detected. As the LAL assay was the only experimental setting where CD14/Fc had no activity on its own, we evaluated its LPS and bleb neutralizing capabilities utilizing this assay but found no neutralizing effect.

4.6.2. Effect of Other Neutralizing Agents on the Biological Activity of LPS and Bleb Preparates

LAL assay was used to evaluate the neutralisation of LPS and blebs by plasma. We found that plasma inhibited the LAL-cascade inducing ability of both LPS and blebs, confirming that human plasma plays an important role in neutralizing free LPS and outer membrane vesicles. BPI is an important antibacterial protein

of neutrophils which is known for its LPS neutralizing effect both *in vivo* and *in vitro*. After incubating free LPS and blebs with 10 μg/mL BPI we were not able to detect endotoxin activity in the LAL assay.

4.7. sCD14 as a Biomarker – Investigating Cirrhotic Patients

4.7.1. Patients

Three hundred and sixty-eight, well characterized, consecutive patients were included from the Gastroenterology Division of the Department of Medicine, University of Debrecen. Of the 368 patients with cirrhosis, 139 had clinically proven bacterial infection, whereas 229 had no infection at the time of the blood sampling. The infected and the non-infected patient groups did not differ in gender, co-morbidities and HCC. However, patients with infections were significantly older, with more severe cirrhosis and had frequent disease specific complications (ascites, variceal bleeding).

4.7.2 APP Levels in the Infected and Non-infected Patient Groups

When comparing infected and non-infected patients we found that CRP, PCT and LBP levels were significantly higher in the infected group. There was no difference in sCD14 levels, however. Investigating the relation of different markers, we found positive correlation between PCT, sCD14, WBC count, absolute PMN count and CRP levels in the infected group.

4.7.3 Discriminative Power of Different Markers in Identification of Cirrhosis Associated Infections

We utilized ROC analysis to determine whether the tested APP markers are able to differentiate between the infected and non-infected group, and if they possess any diagnostic value. The largest area under the curve was found in case of CRP [AUC, (95%CI): 0.93 (0.90–0.95)], which was lower in case of PCT [0.84 (0.79–0.88)], while in case of the other APP parameters the discriminating

power was found rather low [LBP: 0.67 (0.61–0.73); sCD14: 0.57 (0.51–0.63); WBC count: 0.73 (0.67–0.78); absolute PMN count: 0.77 (0.72–0.82)]. According to our results, serum sCD14 has low sensitivity/specificity and low positive/negative predictive values and therefore it is not suitable to predict infections in cirrhosis patients. On the other hand CRP seems as a sensitive marker of bacterial infections, especially when combined with PCT.

5. Discussion

5.1. CD14/Fc Fusion Protein – a Potential Antimicrobial Agent?

5.1.1. Interaction of CD14/Fc with Bacteria

CD14 together with LBP plays an important role in LPS signalisation interacting via the TLR4/MD2 receptor complex, but still there is not much information about the interaction of CD14 and whole bacteria. Although several authors mention CD14 dependent phagocytosis mechanisms, these results do not provide direct evidence of CD14-bacteria binding.

Properties of an antimicrobial protein fused to the Fc part of human IgG1 may change vastly as the result of the fusion. For example the dimerization may enhance the avidity of the fusion protein to its original ligand. As we hoped for a similar effect, we created a CD14/Fc fusion protein and a CD14/His as a control, and evaluated the bacteria binding properties of both proteins. In addition to avidity differences such a molecule may act as an opsonin via its Fc part which may confer other effector functions like enhancing phagocytosis by binding to different Fc receptors. Our experiments proved that the CD14/Fc fusion protein interacted in an LBP dependent manner with free LPS and whole bacteria.

The CD14/Fc protein bound to all tested Gram-negative bacteria, and this binding was especially strong in case of R-type mutants. This phenomenon is most likely due to the fact that these mutants lack the O-antigen from their LPS which makes the lipid-A part of the LPS molecule more accessible for the CD14/Fc protein. In contrast, the CD14/His molecule bound only to R-type mutants, and showed only a weak binding even in these cases. CD14/Fc showed an approximately 50 fold higher avidity to bacteria than CD14/His, supporting the anticipation that dimerization enhances the interaction potential with bacteria remarkably.

In spite of the many ligands, the pattern recognition property and thus the suspected broad species specificity CD14/Fc bound only to Gram-negative bacteria but not fungi. Amongst the tested Gram-positive bacteria we found that

CD14/Fc interacted with *L. monocytgenes* which is in accordance with the findings of Janot *et al.* who described an important role of CD14 in overcoming *L. monocytogenes* infections, suggesting at least one CD14 ligand in this pathogen.

Phagocytosis tests confirmed that in case of short LPS (Ra and Re) mutants CD14/Fc did not only bind to bacteria, but also enhanced the uptake by isolated neutrophil granulocytes, monocyte derived macrophages and dendritic cells. In addition of the phagocytosis enhancing effect CD14/Fc did also enhance the bacteria killing potential of whole blood. Unfortunately we were not able to observe either effect in case of wild type bacteria. It is very likely, that the lesser amount of CD14/Fc binding to wild type bacteria is not enough to crosslink Fc receptors which is necessary to initiate FcR mediated phagocytosis.

FcR blocking experiments confirm, that the phagocytosis enhancing effect of CD14/Fc is the result of the Fc part interacting with the PMN's FcRγIII/FcRγII (CD16/CD32) receptors. The fact that simultaneous blocking of both receptors reduces phagocytosis to background suggests that there are no other mechanisms involved beside the Fc-FcR interactions in the CD14/Fc mediated bacterium uptake.

5.1.2. Interaction of CD14/Fc with LPS and Outer Membrane Vesicles

Virulent factors (e.g.: bleb production) and other molecular components released by Gram negative bacteria play a huge role in the development of infection related complications, like sepsis, septic shock and multiorgan failure. Bleb production is exceptionally pronounced in *N. meningitidis* infections resulting in extraordinary high LPS concentrations. Uncontrolled effector functions caused by LPS, blebs and other bacterial components are usually irreversible and lead ultimately to the loss of patients. Thus it is very important to develop agents that are able to neutralize these microbial virulent factors beside killing pathogens.

We tested the CD14/Fc fusion protein to characterize its possible LPS and outer membrane vesicle neutralizing properties.

The CD14/Fc protein did not neutralize the LPS and bleb activity in our two, cell-based experimental systems. In addition, it was able to dose dependently activate PMN cells on its own. This phenomenon can't be explained by LPS contamination as the LAL assay turned out to be negative. It is very likely that the Fc part interacts with the Fc receptors which results in cell activation. Although the production of oxygen intermediers might be useful in killing bacteria, activation of cellular components might lead to the escalation of general inflammatory reactions.

As the LAL assay was the only experimental setting where CD14/Fc had no effect on its own we set out to test its LPS and bleb neutralizing ability utilizing this assay as well. The CD14/Fc fusion protein did not neutralize the different LPS and bleb praparates. It is possible, that the cascade inducing component(s) of the LAL assay bind(s) LPS/bleb stronger than CD14 which counters the neutralizing effect of the fusion protein. The main function of the CD14/LBP system is to shuttle LPS from aggregated LPS micelles to the appropriate donor molecules like for example the CD14/TLR4/MD2 complex located in the cell membrane (cell activation), or the high density lipoprotein (HDL) molecule (neutralization). This function makes it possible that the CD14/Fc molecule behaves in a similar fashion in the LAL assay: although it binds LPS monomers it shuttles endotoxin to the LAL assay components resulting in the lack of inhibitory effects.

Since the CD14/Fc fusion protein showed no LPS/Bleb neutralizing effect, we sought to find other possible neutralizing options. We showed that human plasma completely neutralized both LPS and blebs according to the LAL assay results. The background of this effect is very likely that LBP transports free LPS and LPS liberalized from blebs to HDL where LPS is not accessible anymore for the LAL assay components. Other experiments confirmed that pre-incubation

with BPI resulted in almost complete blocking of the LAL assay inducing capability of LPS and blebs.

Bleb vesicles contain huge amounts of LPS but are particles and might thus behave differently than free LPS. However, our results suggest that there are no differences in neutralizing LPS and bleb activity, neither by CD14/Fc nor by plasma or BPI, which means that blebs are similar to free LPS regarding their biological activity.

5.2. sCD14 – A Useful Biomarker in Confirming and/or Predicting Cirrhosis Associated Infections?

Several inflammatory and non-inflammatory conditions are accompanied by elevated mCD14 expression levels or the elevation of local or systemic sCD14 concentrations, and there are several applications of sCD14 measurement which provide aid in detecting infections related to different diseases. Since accompanying infections impact the condition, related complications and ultimately the life prospects of cirrhosis patients greatly, identification of infections are extraordinarily important in cirrhosis. Several studies confirm elevated LBP and sCD14 concentrations especially in decompensated cirrhosis, but only two studies show correlation between infections and LBP and sCD14 levels. Unfortunately the analytical properties of the tested markers were not evaluated in these cases. Thus we decided to perform additional investigations in this field and evaluated serum levels and analytical parameters of sCD14 and other 3 acute phase proteins from cirrhosis patients' samples. Strength of our work is that to our best knowledge ours is the largest study, involving 368 patients, that simultaneously investigates the clinical significance of different APP markers (CRP, PCT, LBP, sCD14) in identification of cirrhosis related infections.

Our results indicate that sCD14 measurement is not a sensitive method in detecting cirrhosis associated infections; we found no significant differences in

serum sCD14 levels between the infected and non-infected groups. According to this the analytical parameters (sensitivity, specificity, negative- and positive predictive values) were rather low. Investigating other APP markers we determined, that CRP level at 10 mg/L cut-off is alone a sensitive marker of infections. Combining CRP with PCT measurement raised the diagnostic accuracy slightly, but resulted in significant cost elevation. CRP, as a marker of bacterial infection is known for a long time, even in cirrhosis. However there are several open questions of CRP's optimal usage in this patient group. Earlier clinical studies that worked with 20-127 cases reported that CRP and PCT are the best markers for identifying bacterial infections, but their diagnostic accuracy and cut-off values varied greatly across different studies. In case of CRP the sensitivity was between 62-80%, and specificity was between 68-96%, while the ROC area under the curve was between 0.72-0.91. In the present study sensitivity was 84%, specificity was 91%, and ROC area under the curve was 0.93. Our defined optimal cut-off value (9.2 mg/mL) was lower than those in other studies (20 mg/L, 24.7 mg/L, 55.9 mg/L and 80.0 mg/L). One possible explanation of these differences is the heterogeneity of the patient groups. Previous studies used smaller patient groups for their investigations which may alter the results. Furthermore, infections in previous studies were often accompanied by systemic inflammatory response syndrome (SIRS), sepsis, or in some cases septic shock. Our entire infected group represents the everyday clinical case more faithfully. About 25% of the infections were mild, and localized to the urinary tracts. Since the immune system of cirrhosis patients does not operate properly, they are more prone to infection spreading which may lead to further degradation of liver functions and other severe complications; thus early diagnosis and active treatment of infections is recommended even in less severe cases.

PCT turned out to be the second best marker, but its performance was behind of CRP's in every aspect. Only few studies investigate the diagnostic properties of

PCT in liver diseases, although their results concur highly. In these cases PCT level was elevated in the infected group compared to the control, although median values varied somewhat: 0.74 µg/L, 2.8 µg/L, 3.2 µg/L, 9.8 µg/L, 10.1 µg/L. We also found significantly elevated PCT levels in infected patients, but the median value was lower: 0.24 µg/mL. Discriminative power of PCT was also lower (sensitivity: 72%, specificity: 84%, AUC-ROC: 0.84) with a cut-off value of 0.15 µg/L than in previous two studies (sensitivity: 85 and 95%, specificity: 96 and 98%, AUC-ROC: 0.97 and 0.98). About one-fourth of our infected patient group suffered only from mild infections, which leads only to slight elevation of PCT, if at all. These discrepancies can also be explained by technical differences. In spite of the fact that classic (non sensitive) PCT tests were used in all previous studies, differences in detection techniques may have lead to different results.

6. Summary

Antibiotic resistant microbes are more and more frequent during the past decades in the clinical practice while the rate of discovery of new antibiotics decreased. With the current limited treatment possibilities there is a growing need for antimicrobials with a new approach. One type of these novel antimicrobials are PRR/Fc fusion proteins. These molecules bind microbial components with their pattern recognition receptor part while the human IgG1 Fc parts activates immunological functions and promotes pathogen neutralization. The CD14 molecule as the most important LPS receptor seemed as a worthy candidate in such an artificial antibody as the pattern recognition component, thus we expressed a CD14/Fc fusion protein using recombinant DNA techniques. The resulting dimer molecule bound Gram-negative bacteria in a time and dose dependent manner and this interaction could be specifically inhibited. The dimer form showed an approximately 50-fold higher affinity to bacteria than the monomer sCD14. The CD14/Fc fusion molecule increased the FcR mediated phagocytosis of bacteria by PMN cells, macrophages and dendritic cells, and we also proved internalization of the opsonised bacteria by these cells. In an *ex vivo* whole blood experimental setting the CD14/Fc molecule enhanced killing of bacteria. However these effects were only significant for R-type LPS mutant bacteria, which decreases this protein's practical value. The CD14/Fc protein was able to activate PMN cells and monocytes on its own, but was unable to neutralize the effect of LPS neither in its free form nor in the form of outer membrane vesicles (blebs). We determined that these vesicles act as free LPS rather than particles as they can be neutralized similarly by plasma and BPI in the LAL assay.

The serum concentration of the soluble form of CD14 (sCD14) correlates well with the severity of infections and with the activity of autoimmune diseases. We evaluated a possible new diagnostic use of sCD14 in cirrhosis patients and tried to evaluate if changes in serum sCD14 concentrations can predict or confirm

cirrhosis associated infections. Our results show that serum sCD14 concentration does not differ significantly in infected and non-infected patient samples. However, we found that CRP is a sensitive and specific marker of cirrhosis associated infections at a cut-off value of 10 mg/L.

7. New findings, results and clinical relevances

1., Using recombinant DNA techniques we created a CD14/Fc fusion protein and characterized its biological properties. The CD14/Fc fusion protein binds to Gram-negative bacteria and to the Gram-positive *L. monocytogenes*. It enhances FcR dependent phagocytosis/internalization of Gram-negative bacteria by PMN cells, and monocyte derived macrophages and dendritic cells. CD14/Fc also promotes killing of Gram-negative bacteria in whole blood. Since these effects were only significant on R-type bacteria, the use of this molecule as a therapeutic agent is out of question. We think, however, that similar fusion proteins with higher affinity and broader antimicrobial spectrum might be effective agents against antibiotic resistant pathogens.

2., We investigated the neutralizing effect of CD14/Fc on LPS/bleb preparates and found that it is unable to neutralize endotoxin, furthermore, it has cell activating effect on its own. According to this finding its use as a therapeutic agent in sepsis is not an option.

3., We investigated whether LPS and bleb vesicles are effectively neutralized by human plasma and BPI in the LAL assay, and found complete neutralization in both cases. According to this finding we conclude that as long as biological activity is concerned, blebs behave like free LPS molecules.

4. We determined that serum sCD14 measurement is not suitable for detecting infections in cirrhosis patients, while CRP measurement (especially when combined with PCT measurement) is a sensitive marker of bacterial infections.

8. Publication List

Register Number: DEENKÉTK/285/2012.
Item Number:

Subject: Ph.D. List of Publications

Candidate: András Vida

Neptun ID: ALAJGD

Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. Papp, M., Vitális, Z., Altorjay, I., Tornai, I., Udvardy, M., Hársfalvi, J., **Vida, A.**, Kappelmayer, J., Lakatos, P.L., Antal-Szalmás, P.: Acute phase proteins in the diagnosis and prediction of cirrhosis associated bacterial infections.
   Liver Int. 32 (4), 603-611, 2012.
   DOI: http://dx.doi.org/10.1111/j.1478-3231.2011.02689.x
   IF:3.824 (2011)

2. **Vida, A.**, Bardoel, B., Milder, F., Majoros, L., Sümegi, A., Bácsi, A., jr. Vereb, G., van Kessel, K.P.M., van Strijp, J.A.G., Antal-Szalmás, P.: Fusion of the Fc part of human IgG1 to CD14 enhances its binding to gram-negative bacteria and mediates phagocytosis by Fc receptors of neutrophils.
   Immunol. Lett. 146 (1-2), 31-39, 2012.
   DOI: http://dx.doi.org/10.1016/j.imlet.2012.04.008
   IF:2.526 (2011)

3. **Vida, A.**, Troelstra, A., Antal-Szalmás, P., van Bommel, T.J.P., Verheul, A.F.M., Verhoef, J., van Kessel, K.P.M., van Strijp, J.A.G.: Neutralization of Neisseria meningitidis outer membrane vesicles.
   Inflamm. Res. 60 (9), 801-805, 2011.
   DOI: http://dx.doi.org/10.1007/s00011-011-0344-3
   IF:2.109

Total IF: 8.459

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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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9. Supports