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Anal Bioanal Chem (2013) 405:775–784
DOI 10.1007/s00216-012-6204-3

ORIGINAL PAPER

Innate immunity probed by lipopolysaccharides affinity strategy and proteomics

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Received: 17 April 2012 / Revised: 3 June 2012 / Accepted: 15 June 2012 / Published online: 1 July 2012
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Abstract Lipopolysaccharides (LPSs) are ubiquitous and vital components of the cell surface of Gram-negative bacteria that have been shown to play a relevant role in the induction of the immune-system response. In animal and plant cells, innate immune defenses toward microorganisms are triggered by the perception of pathogen associated molecular patterns. These are conserved and generally indispensable microbial structures such as LPSs that are fundamental in the Gram-negative immunity recognition. This paper reports the development of an integrated strategy based on lipopolysaccharide affinity methodology that represents a new starting point to elucidate the molecular mechanisms elicited by bacterial LPS and involved in the different steps of innate immunity response. Biotin-tagged LPS was immobilized on streptavidin column and used as a bait in an affinity capture procedure to identify protein partners from human serum specifically interacting with this effector. The complex proteins/lipopolysaccharide was isolated and the protein partners were fractionated by gel electrophoresis and identified by mass spectrometry. This procedure proved to be very effective in specifically binding proteins functionally correlated with the biological role of LPS. Proteins specifically bound to LPS essentially gathered within two functional groups, regulation of the complement system (factor H, C4b, C4BP, and alpha 2 macroglobulin) and inhibition of LPS-induced inflammation (HRG and Apolipoproteins). The reported strategy might have important applications in the elucidation of biological mechanisms involved in the LPSs-mediated molecular recognition and anti-infection responses.

Keywords Innate immunity · Lipopolysaccharide · Proteomics · Affinity

Published in the special issue *Analytical Science in Italy* with guest editor Aldo Roda.

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Introduction

In a biological context, the term immunity refers to the set of events and biological cascades that protect organisms against infectious diseases. This protection system is based on complex interconnections between cells and molecules whose synergic action determines immune response. The main physiological function of the immune system is the protection against infectious agents or simply against exogenous elements. The ability to distinguish “self” from “non-self” is critical to prepare an effective immune response [1].

Immune system response can be classified into two categories: adaptive immunity and innate immunity. The adaptive immune system is based on specialized processes whose function is to eliminate external threats during the late phase of infection with the generation of immunological memory. Specificity is developed by clonal gene rearrangements from a range of antigen-specific receptors on lymphocytes [2]. Only vertebrates are provided with this type of immunity, allowing the organism to recognize and remember the “non-self.” Innate immune system is necessary to activate adaptive immune system. It acts in a non-specific manner and does not confer any memory of the challenge. It is widespread among all animals and even in plants, and its main function is to provide immediate protection against pathogens. Innate immunity relies on specific cells as primary mediators, namely phagocytic cells and antigen presenting cells, such as granulocytes, macrophages, and dendritic cells [3]. Innate immunity mechanisms do not have specific pathogen targets, the principal

targets are evolutionarily conserved molecular structures, termed "pathogen associated molecular patterns" (PAMPs) whose expression is common to different pathogens. These structures are generally recognized by receptors on the membrane of the host cells that are responsible for the immune response. Such structures are called "pattern recognition receptors" (PRR). PRRs are constitutively expressed in the host on all cells of a given type [4]. Lipopolysaccharide (LPS) is a glycolipid, generally referred to as endotoxin in Gram negative bacteria, belonging to PAMP family. It is a potent inducer of the innate immune system and the main cause of septic shock [5].

The recognition of this endotoxin is achieved by the interplay of a variety of proteins, either receptors or serum secreted proteins. The cascades of events following LPS infection has been investigated in numerous studies. However, most of them focused on the interaction of single specific proteins with LPS, such as the LPS-binding protein (LBP), whose mechanism of action was clarified in many works [6, 7]. Further studies showed that other proteins possess LPS-binding domains [8–10]. Intracellular pathways elicited by LPS infection was investigated by in vitro and in vivo analyses on different cell types, ranging from immune cells to epithelial cells [11, 12].

Toll-like receptor 4 (TLR-4) is the signal transducing receptor of lipopolysaccharides; lipid A is the real effector required to activate TLR4 signaling pathway in conjunction with a soluble co-receptor protein myeloid differentiation protein 2 (MD2), which directly and physically binds to LPS [13, 14]. Entry of LPS or lipid A into the blood stream is responsible for the onset of septic shock [15]. Aggregates of LPS bind to certain plasma proteins, such as albumin, LBP and soluble CD14 to facilitate the interaction with host cells, via TLR4. The complex of protein bound to LPS, TLR4, and MD2, activates two intracellular pathways [16]: the MyD88-dependent pathway, responsible for early-phase NF-κB and MAPK activation, which controls the induction of proinflammatory cytokines TNF-a, IL-1, and IL-6 and the MyD88-independent, TRIF-dependent pathway that activates IRF3, which is necessary for the induction of IFN-β- and IFN-inducible genes [17]. The activation of the TLR4-MD2 complex triggers the induction of inflammatory cytokines acting as endogenous mediator of infection, as well as the superoxide anion (O_2^-), hydroxyl radicals (OH), nitric oxide, and antimicrobial peptides [18].

The low and balanced concentrations of these mediators as well as the presence of soluble immune response modulators lead to a resulting inflammation, which is one of the most important and ubiquitous aspect of the immune host defense against invading microorganisms. Beside these positive effects, an uncontrolled and massive immune response, due to the circulation of large amount of endotoxins, leads to the symptoms of the sepsis and of the septic shock. Therefore, identification of all the molecular actors involved in the recognition cascade is of pivotal importance with particular emphasis on the differences in elicitation and reaction by different LPSs.

Numerous studies have been performed following pro-inflammatory expression patterns via microarray analyses [19] and the phage display method was used to capture new LPS-binding peptides [20].

Recently, proteomics approaches were addressed to the study of innate immunity. Comparative analysis of the human plasma proteome prior and after LPS injection was performed [21] and the human innate immunity interactome was investigated using 58 genes involved in transcriptional regulation of type I IFN tagged with the FLAG epitope following cells stimulation with LPS [22].

The aim of this study was the setting up of a new methodology for the development of lipopolysaccharides analyses to be used to capture LPS-interacting proteins in order to better elucidate the molecular mechanism of the innate immunity. Here, a strategy based on chemical manipulations, biochemical procedure and mass spectrometry techniques integrated in a functional proteomic workflow is proposed. The procedure was developed using an LPS derived from *Salmonella typhimurium*, and the proteomic study was carried out by using human serum from a healthy donor as a model.

Materials and methods

LPS extraction

Dried cells of *S. typhimurium* were extracted by the phenol/water method [23]. The extracted phases were dialyzed three times against distilled water and subjected to enzymatic digestions in order to remove nucleic acids and protein contaminants. Both water and phenol fractions were analyzed through sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) 12.5 % and LPS bands were evidently present in the water phase (data not shown). The LPS was further purified and analyzed as described [24]. Briefly, extract was first both digested with DNase, RNase, and Proteinase K, dialyzed, lyophilized, and further purified by gel filtration chromatography on Sephacryl S-500 (yield, 4.3 % of the dry mass). The LPS was further analyzed for its carbohydrate composition which resulted to be the one expected in *S. typhimurium* LPS, with mannose galactose, glucose, rhamnose, and abequose.

LPS biotinylation

LPS biotinylation was achieved by a transesterification reaction with biotin-*p*-nitrophenylester (Sigma Aldrich).

Three milligrams of lyophilized LPS were solubilized in 200 μL pyridine (Romil), to which a 9-mg of biotin-*p*-nitrophenylester was added. LPS/biotin-p-nitrophenylester ratio was calculated as 1:3 (weight/weight). The reaction was carried out in the dark for 2 h at 80 °C. The reaction mixture was dried under nitrogen and then the sample was dissolved in MilliQ water. The excess of reagents was removed by molecular exclusion chromatography on PD-10 columns (GE-Healthcare). This is a fast and high recovery desalting procedure based on disposable columns. The sample was applied to the top of the column and eluted in water by gravity flow. LPS, being larger than the pores of the matrix was eluted first, whereas salts and the excess of reagents, penetrating the pores, were eluted after LPS, just before the total volume of the column. LPS containing fractions were pooled and lyophilized. Unlabeled and biotinylated LPS were dissolved in Laemmli buffer, supplemented with 100 mM dithiothreitol (DTT) and separated by SDS-PAGE in a 12.5 % polyacrylamide gel. LPS was detected by silver nitrate staining for carbohydrates (Tsai Frash, 1982) or electrotransferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 3 % non fat dry milk, 1 % bovine serum albumin (Sigma Aldrich), in PBS containing 0.05 % Tween 20 (Sigma Aldrich). Then it was incubated with 1:1,000 streptavidin/HRP conjugate (Sigma Aldrich). Biotinylated sample was detected using SuperSignal West FemtoChemiluminescent Kit from Pierce.

LPS immobilization onto avidin beads

Biotin-LPS immobilization was carried out using 3 mg biotin-LPS/mL of settled avidin agarose resin (settled gel, Pierce)

An aliquot of resin was extensively washed with binding buffer (0.1 M Na_3PO_4; 0.3 M NaCl; pH 7.2) and incubated with biotin-LPS at 4 °C overnight under shaking. Then the resin was washed three times with binding buffer.

Biotin-LPS and human serum interaction

The affinity experiment was preceded by a "precleaning step." A protease inhibitors cocktail (Sigma Aldrich) was added to 5 mg human serum (Servizio Analisi, Policlinico), to a final concentration of 1 mM. This sample was diluted to a final volume of 600 μL with binding buffer and incubated with 150 μL of settled resin overnight at 4 °C. The unbound fraction of precleaning step was therefore incubated with 150 μL of biotin-LPS immobilized resin overnight at 4 °C. Later on, both aliquots of resin, that we term "control" and "sample," were subjected to repeated washes with binding buffer. Elution was performed boiling the resin in Laemmli buffer and DTT.

SDS-PAGE was performed, loading on a 1.5 mm, 12.5 % gel all the samples deriving from the different steps of the affinity experiment. The gel was run at constant 25 mA for 1 h. The gel was stained with colloidal Coomassie (Pierce).

In gel trypsin digestion

The analysis was performed on the Coomassie blue-stained protein bands excised from the gels. Gel particles were washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced incubating the bands with 10 mM DTT for 45 min at 56 C. Cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The bands were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/μL) in 50 mM ammonium bicarbonate buffer, pH 8.5. Gel particles were incubated at 4 °C for 2 h, in order to allow the enzyme to enter the gel. The buffer solution was then removed and a new aliquot of buffer solution was added for 18 h at 37 C. A minimum reaction volume, enough for complete gel rehydration was used. At the end of the incubation the peptides were extracted by washing the gel particles 0.1 % formic acid in 50 % acetonitrile at room temperature and then lyophilised.

LC-MS/MS analyses

Peptides mixtures were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using a HPLC-Chip LC system (Agilent 1200) connected to a Q-TOF 6520 (Agilent Technologies). Samples were diluted in 10 μL of 0.1 % formic acid. After loading, the peptide mixtures were concentrated and washed at 4 μL/min in 40 nL enrichment column with 0.2 % formic acid in 2 % acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75 μm×43 mm) at a flow rate of 0.4 μL/min with a linear gradient of eluent B (95 % acetonitrile and 0.2 % formic acid) in A (2 % acetonitrile and 0.1 % formic acid) from 7 to 80 % in 51 min.

Mass spectrometry analyses were performed using data-dependent acquisition MS scans (mass range, 300–2,400 m/z), followed by MS/MS scans (mass range, 100–2,000 m/z) of the most intense ions of a chromatographic peak.

Raw data from LC-MS/MS were converted to mzData. The spectra were searched against the NCBI database (2006.10.17 version) using the licensed version of Mascot 2.1 (Matrix Science). The MASCOT search parameters were: taxonomy *Homo sapiens*; allowed number of missed cleavages 2; enzyme trypsin; variable post-translational modifications, methionine oxidation, and pyro-glu N-term Q; peptide tolerance 10 ppm and MS/MS tolerance 0.6 Da; and peptide charge from +2 to +3.

Results

An overview of the procedure, displaying the scheme of sample preparation till MS was provided in Fig. 1.

LPS were extracted by *S. typhimurium* cells as described in "Materials and methods" and analyzed by SDS-PAGE. The LPS fraction was found exclusively in the water phase as suggested by the presence of the typical ladder pattern in the gel analysis (data not shown).

LPS tagging

Immobilisation of the LPS represents the key step in the entire strategy to generate a labeled bait that could be linked to agarose beads. Biotinylation of LPS was chosen as the best suited derivatization process both for the simple modification reaction and to take advantage of the strong and specific interaction of biotin with avidin.

Biotinylated LPS was analyzed by SDS-PAGE following both silver staining and immunodetection with streptavidin/HRP conjugate. Figure 2 shows the corresponding gel. Staining procedures displayed the occurrence of two sample bands. The low molecular mass bands corresponds to the lipid A core component of LPS (R-type LPS) whereas the high molecular mass band represents the whole LPS molecule comprising the O-chain moiety (S-type LPS). Both components were responsive to western blot analysis demonstrating that they had been derivatized by biotin.

LPS immobilization

Biotin-LPS was conjugated to avidin agarose beads by incubation in 0.1 M Na_3PO_4 overnight. Both unbound and bound fractions were analyzed by SDS-PAGE followed by blotting and incubation with streptavidin/HRP conjugate. Figure 3 shows the results of the immobilization procedure; two responsive bands corresponding to LPS and the lipid A moiety were clearly detected in the bound fraction while no responsive bands occurred in the unbound material.

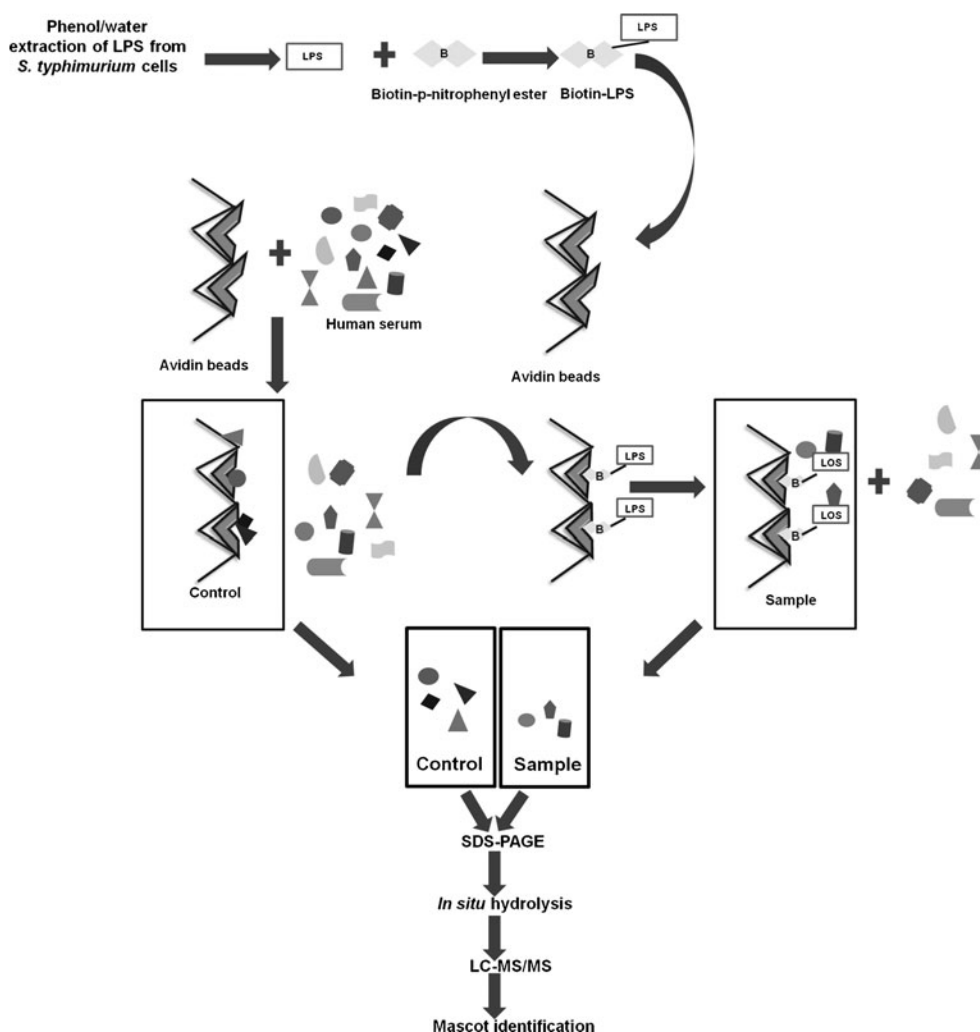

Fig. 1 Overview of the strategy. Lipopolysaccharide by *S. typhimurium* was extracted by phenol/water extraction and derivatized with biotin-*p*-nitrophenyl ester. Human serum was incubated with avidin agarose beads in order to detect a specific interaction between the resin and the beads (precleaning step giving rise to "Control"). Then, the unbound coming from the precleaning was incubated with avidin agarose beads after their incubation with biotin-LPS ("Sample"). "Control" and "Sample" were analyzed by SDS-PAGE, the bands were in situ digested, and proteins identified by LC-MS/MS

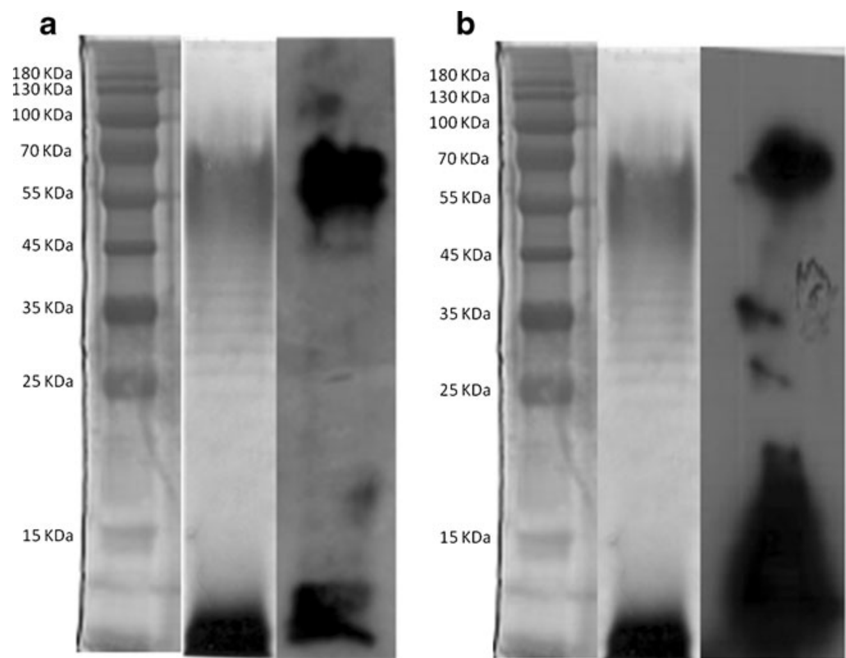

Fig. 2 SDS-PAGE of LPS after biotin-*p*-nitrophenylesterderivatition detected by silver staining (*on the left*) and blotting with streptavidin/HRP incubation (*on the right*). The figures represent an entire lane of LPS. Nevertheless, protein markers have been added in the figure in order to compare LPS electrophoretic mobility with protein markers. A biological replicate is provided in (**b**)

Identification of LPS-interacting proteins

The final step of the proposed strategy relies on the use of the immobilized LPS as bait in a functional proteomic experiment [25] aiming at capturing specific LPS interactors to investigate innate immunity molecular mechanisms in human serum. An aliquot of human serum proteins was incubated with underivatized avidin agarose beads overnight at 4 °C as a pre-cleaning step to remove all the proteins that non-specifically interact with agarose or avidin. The unbound fraction was then incubated with biotin-LPS avidin agarose beads and after extensively washing in binding buffer both bound fractions (control and sample) were individually eluted in Laemmli buffer and fractionated by SDS-PAGE gel. An aliquot of 15 μg of human serum was also loaded onto the gel (Fig. 4). Due to the complexity of the gel patterns and the low resolution of 1D electrophoresis, several proteins can occur in the same gel band. Therefore, protein bands specifically present in the sample lane and absent in the control lane cannot be identified by simply comparing the two gel profiles. Thus the entire sample lane of the gel was cut in 25 slices. Each slice was destained and in situ trypsin digested. To check for non specific proteins, the same procedure was applied to the control lane. The resulting peptide mixtures were extracted from the gel and submitted to nano-LC-MS/MS analysis generating sequence information on individual peptides.

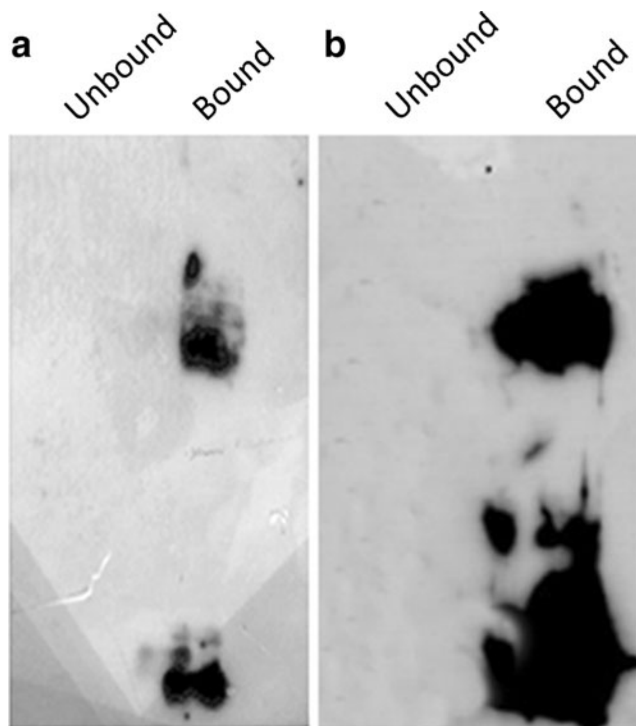

Fig. 3 Immobilization procedure probed by blotting with streptavidin/HRP incubation. Two bands corresponding to LPS and lipid A moiety were clearly detected in the bound fraction while no bands occurred in the unbound material. A biological replicate is provided in (**b**)

This information, together with the peptide mass values, was then used to search protein databases using an in house version of the Mascot software, leading to the identification of the protein components. The experiments were performed in two biological replicates and each protein mixture was run through LC-MS/MS three times. All proteins identified in both the sample and control lanes were discarded, while only those solely occurring in the sample lane and absent in the control lane were selected as putative LPS interactions. All the proteins identified in this study have a score greater

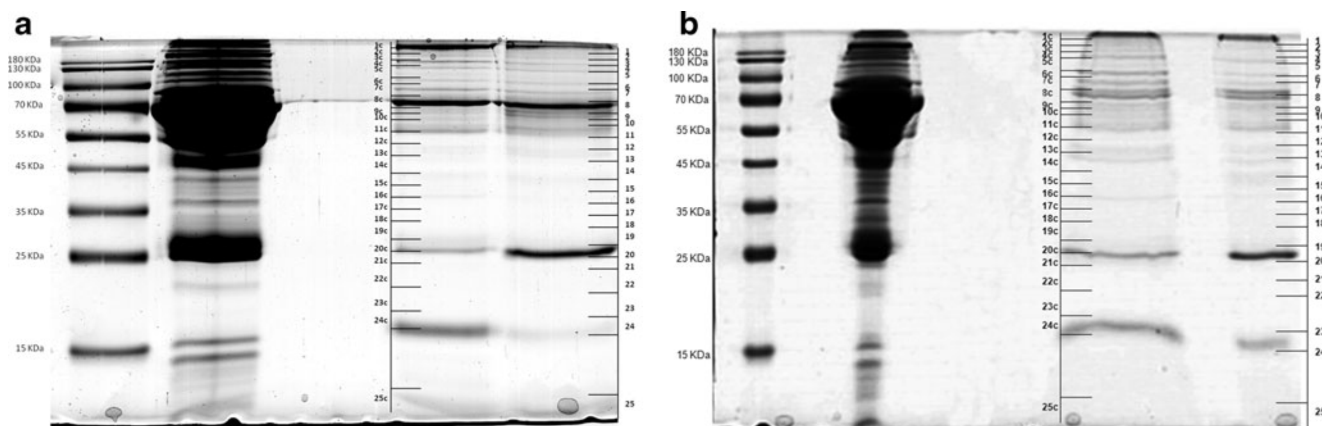

Fig. 4 12.5 % SDS-PAGE gel containing 15 μg of human serum together with control and sample bound fractions after elution in Laemmli buffer. The term "Control" refers to the proteins bound to the avidin agarose system during the precleaning step. The term "Sample" refers to the proteins specifically binding to the biotin-LPS adsorbed to the avidin agarose. The entire control and sample lanes of the gel were cut in 25 slices, as reported in the figure. Each slice was destained and trypsin digested, in situ. A biological replicate is provided in (**b**)

than the significance threshold, whose value was set at 0.05. This represents the probability that the match is a random event and is correlated to the score by the rule $S=-10*\text{Log}\ P$, where S is the Mascot score and P is the probability of the random event, implying that if a protein fall in the region of probability of ≤0.05, the hit could be a random match. All the identified proteins had a score greater than the threshold indicating identity or extensive homology. In addition to this, BLAST search engine has been used to assess if a peptide sequence is unique for one particular protein or for one particular organism. The research has been run using SwissProt as database. As further selection criteria, only proteins found in both biological replicates and identified with at least two significant peptides in MASCOT search were considered, providing a full list of putative LPS interactors. The results are summarized in Table 1, where even the peptide sequences are also reported.

Discussion

Lipopolysaccharides are major constituents of the outer membrane of Gram-negative bacteria and important molecules in the induction of the immune system response. The innate immune system constitutes the first line of defense against microorganisms and plays a primordial role in the activation and regulation of adaptive immunity. In humans, components of the innate immune system include members of the complement cascade and soluble pattern recognition molecules which act as functional ancestors of antibodies [26]. In particular, the complement system plays a key role in the elimination of micro-organisms after entrance in the human host.

The integrated strategy based on functional proteomic approach described in this paper represents a new starting point to elucidate the molecular mechanisms elicited by bacterial LPS and involved in the different steps of innate immunity response. Identification of the specific LPS-binding proteins in human serum was rarely addressed since most investigations focused on the LPS-mediated effects on cellular pathways and transcriptional responses. Only recently, a similar approach using covalently immobilized LPS [27] or lipotechoic acid [28] to screen for serum binding proteins was reported. In this procedure, following coupling to the bait, a further defunctionalization step was needed to chemically block any residual reactive group. Moreover, depletion of the most abundant serum proteins might have also caused the removal of the putative LPS or LTA-binding proteins. The article by Kim et al. describes a very similar approach, based on LPS-immobilized solid support to screen for LPS-binding proteins in human serum. Yet, the use of a depletion kit, allowing the removal of the most abundant proteins, could lead to the elimination of potential LPS-binding proteins from the system. In addition to this, most of the proteins, though already reported as innate immunity elicitors, are identified with only one peptide.

The strategy based on affinity capture procedure using biotin LPS as a bait was optimized and non-depleted human serum from a healthy donor was used as model. A number of proteins were specifically retained by the LPS bait and subsequently identified by mass spectrometric methodologies. The identity of these proteins represented per se a validation of the developed affinity-capture procedure. These proteins, in fact, essentially gathered within two functional groups, regulation of the complement system (factor H, C4b, C4BP, and alpha 2 macroglobulin) and inhibition of LPS induced inflammation (HRG and Apolipoproteins). Both groups are functionally correlated with the biological

Table 1 List of putative LPS-interacting proteins after in situ digestion of the bands from control and sample bound fractions

Sample	Protein	ID	Score	Peptides
3	Alpha-2-macroglobulin	P01023	170	QTVSWAVTPK
				QGIPFFGQVR
				AIGYLNTGYQR
				VTAAPQSVCALR
				MVSGFIPLKPTVK
				ALLAYAFALAGNQDK
				VDLSFSPSQSLPASHAHLR
				QQNAQGGFSSTQDTVVALHALSK
	Factor H	P08603	44	RPYFPVAVGK
				SSNLIILEEHLK
4	Ceruloplasmin	P00450	136	GAYPLSIEPIGVR
				QSEDSTFYLGER
				RQSEDSTFYLGER
				LISVDTEHSNIYLQNGPDR
				KLISVDTEHSNIYLQNGPDR
				KAEEEHLGILGPQLHADVGDKVK
	Phospholipase D	P80108	114	FGSSLITVR
				ILEGFQPSGR
				LSGALHVYSLGSD
				AQYVLISPEASSR
6	Coagulation factor II (Thrombin)	P00734	56	ETAASLLQAGYK
				VTGWGNLKETWTANVGK
				GQPSVLQVVNLPIVERPVCK
				KPVAFSDYIHPVCLPDRETAASLLQAGYK
	S plasma protein	P07225	140	YLVCLR
				VYFAGFPR
				QLAVLDKAMK
				QSTNAYPDLR
				SFQTGLFTAAR
				QGASGIKEIIQEK
				KVESELIKPINPR
7	Histidine rich glycoprotein precursor	P04196	54	QIGSVYR
				RPSEIVIGQCK
	C4 binding protein C4bp	P04003	266	QSTLDKEL
				YTCLPGYVR
				EDVYVVGTVLR
				LMQCLPNPEDVK
				LSLEIEQLELQR
				FSAICQGDGTWSPR
				GVGWSHPLPQCEIVK
				TGTTLKYTCLPGYVR
				KPDVSHGEMVSGFGPIYNYK
				KPDVSHGEMVSGFGPIYNYKDTIVFK
				KSRPANHCVYFYGDEISFSCHETSR
10	Beta 2-glycoprotein I	P02749	91	VSFFCK
				VCPFAGILENGAVR
				TCPKPDDLPFSTVVPLK
				GRTCPKPDDLPFSTVVPLK
				TFYEPGEEITYSCKPGYVSR

Table 1 (continued)

Sample	Protein	ID	Score	Peptides
	Proapolipoprotein A1	P02647	145	ATEHLSTLSEK
				VQPYLDDFQKK
				LLDNWDSVTSTFSK
				QKVEPLRAELQEGAR
				DSGRDYVSQFEGSALGK
13	Complement C4B	P0C0L5	92	QGSFQGGFR
				GSSTWLTAFVLK
				VLSLAQEQVGGSPEK
18	Apolipoprotein D	P05090	56	QAFHLGK
				VLNQELR
19	Apolipoprotein A-IV precursor	P06727	74	LLPHANEVSQK
				ISASAEELRQR
				IDQNVEELKGR
				TQVNTQAEQLRR
24	Prolactin-inducible precursor	P12273	64	NFDIPK
				FYTIEILKVE

As selection criteria only proteins solely present in the sample lane and completely absent in the control lane were considered putative interactors. Analyses were carried out in replicates and only proteins proteins present in both replicates and identified with at least two peptides in MASCOT search were considered. The term "Sample" refers to the number of the gel slice where the protein was identified. In some slices, more than one protein were identified. Peptides sequences were included in the table

role of LPS. Interestingly, some of the LPS interactors identified in this study were coincident with those reported in other systems [28].

The complement system is an essential component of the innate immune system that participates in elimination of pathogens. Three different pathways synergistically contribute to the protection mechanism elicited by the complement proteins, the classical pathway, the alternative pathway and the lectin pathway. Despite the specific pathway they belong to, complement proteins provide host defense towards bacterial infections by binding to cell surface components of exogenous microorganisms. In contrast, pathogenic microorganisms have evolved several strategies to escape these defence mechanisms. The most common procedure consists in the recruitment of complement inhibitory proteins to the bacterial surface thus impairing the immune system.

In this respect, the identification of well characterized protein inhibitors of the complement system among the LPS interactors has a clear biological significance. C4BP, factor H, and alpha-2-macroglobulin are all complement inhibitors able to downregulate activation of the classical, alternative, and lectin pathways, respectively. *Streptococcus pyrogenes*, the ethiologic agent of important human infections, was shown to bind factor H, C4BP, and other complement proteins as a crucial step in the pathogenesis of these infections [29]. *Bordetella pertussis* was able to escape the classical pathway of complement by binding the classical pathway inhibitor C4BP. In addition, very recently, it was shown that this pathogen can also evade the alternative complement pathway by recruitment of factor H on the cell surface [30].

On the other hand, alpha-2-macroglobulin exerts a specific inhibitory activity towards the lectin pathway by binding the mannose-binding lectin (MBL) and the MBL-associated serine proteases MASP-1 and MASP-2 [31]. Adding of alpha-2-macroglobulin to human serum totally reversed killing of *Neisseria gonorrhoeae*, preventing MBL-mediated activation of the complement system [32].

However, the most effective complement inhibitor is factor H, a soluble protein regulator controlling the alternative immune pathway [33]. Recognition and the binding to factor H constitute the infection mechanism adopted by several pathogens as a common immune evasion strategy. In addition several recent reports pointed out that besides its role as an alternative pathway downregulator, factor H has an additional complement regulatory role in inhibiting activation of the classical pathway [34, 35].

A totally different biological role can be ascribed to the second group of proteins identified by the LPS affinity capture strategy. HRG and Apolipoproteins, in fact, are known to exert protective effects against LPS induced systemic inflammation preventing inhibition of the complement immune system. HRG binds strongly to several complement protein inhibitors including factor H and C4BP thus assisting in maintenance of normal immune function and enhancing complement activation [36]. *S. pyrogenes* was shown to

grow more efficiently in HRG-deficient plasma while the presence of overexpressed HRG greatly increased clots formation, bacterial entrapment and killing [37].

Apolipoproteins AI, A-IV, and D are all components of the high-density lipoproteins that have long been reported to bind bacterial LPS neutralizing its toxicity and preventing initiation of innate immunity. More recently, evidences for an Apo AI-LPS specific interaction have been obtained [38]. Moreover, the adenovirus mediated overexpression of this protein led to protection of mice against LPS-mediated systemic inflammation [39].

Conclusions

In conclusion, the novel LPS-mediated affinity capture strategy developed in this paper proved to be very effective in specifically binding proteins involved either in inhibiting the activation of the complement system or in preventing bacterial infection by sequestering the LPS moiety. Both processes are of relevant biological significance and the results obtained make sense with the bait used. Moreover, although a proper sensitivity test was not performed, the LPS affinity procedure led to the identification of low abundant serum proteins even without any depletion step, like phospholipase D, whose concentration in serum is considerably low. The sensitivity of the LC-MS/MS analyses allowed us to identify specific proteins even when the protein band showed only a very faint staining. A further advantage of this procedure concerns the possibility of determining both the proteins directly bound to LPS bait and other components involved in the functional complex but not linking the LPS moiety. It will now be possible to foresee applications of this strategy to investigate biological mechanisms exerted by pathogenic bacteria at the molecular level and to possibly define new mechanisms of infection.

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