Molecular basis and biological effects of endotoxin interactions with human H-ficolin and mannn-binding lectin

SUMMARY

Complement system is one of the most important elements of innate immunity and it is responsible for pathogen clearance from the organism. Among three pathways of complement activation, lectin pathway (LP) plays main role in the interaction with polysaccharides of Gram-negative bacteria including lipopolysaccharides (LPS, endotoxin). Molecules responsible for LP activation are: mannose-binding lectin (MBL), collectin-11 (CL-11), ficolin-2, and ficolin-3. There are analogues of some of these molecules in mice (*e.i.* MBL-A, MBL-C, ficolin B).

MBL and ficolin-3 in complex with serine proteases MASP interacts with ligands presented on the surface of the pathogens followed by LP activation and inactivation of the microorganisms. Contrary to other immunological mechanisms, LP activation initiated by described complexes, has a relatively low ligand specificity (broad spectrum of pathogen structures with characteristic structural features is recognized) and short time of reaction. Both ficolin-3 and MBL are present in relatively high concentration in comparison to other molecules activating LP. These proteins have many similar features in terms of the general structure. The main differences concerns the type and number of recognized ligands and knowledge about its significance in immunity and pathogenesis of infectious diseases, including nosocomial infections and sepsis, especially caused by Gram-negative bacteria, where one of the major virulence factor is LPS.

Studies presented herein focus on MBL and ficolin-3 – molecules that differ significantly in terms of the number of identified ligands and molecular basics of interactions. Many ligands were identified for MBL, including LPS, and the model of interactions with sugar residues and Ca^{2+} ions was presented. However the knowledge concerning molecular basis of interactions between MBL and LPS and biological effects thereof are limited to mannose homopolymer (O-PS) containing LPS and its ability to induce an early-phase endotoxic shock (anaphylactoid reaction). Unlike MBL, to date only three natural ligands for ficolin-3 where identified, two of which (O-PS of *H. alvei* LPS) were discovered by members of the Laboratory of Microbial Immunochemistry and Vaccines. Thus it was interesting to continue searching for

new ligands, conduct pioneering research on the molecular bases of ficolin-3 interaction with identified LPS and biological effects of their activity.

LPS is an integral part of outer membrane of Gram-negative bacteria and it is composed of lipid A which is anchored within the membrane, core oligosaccharide (core OS) and the most distal and variable region – O-specific polysaccharide (O-PS), built of oligosaccharide repeating units (RU). In the course of nosocomial infections and sepsis caused by Gram-negative bacteria the development of endotoxic shock (late phase shock) can occur and is characterised by high mortality. The mechanism of this phenomenon is generally based on TLR4:MD2:CD14 complex interactions with lipid A of LPS. On the other hand, in murine model after administration of LPS leading to LP activation in MBL-dependent manner, early-phase endotoxic shock (anaphylactoid reaction) is developed. It was suggested that observed activity could have implications for understanding the role of complement in the pathogenesis of sepsis. In both cases LPS structure plays major role due to its impact on host immunological system and risks of complications during nosocomial infections.

The purpose of the present study was therefore to investigate the molecular basis and biological effects of endotoxin interactions with two LP activating proteins – ficolin-3 and MBL.

It was demonstrated for MBL that LPS are common ligands among LPS from bacteria of *Enterobacteriaceae* family. Among 182 tested LPS originating mostly from smooth Gram-negative bacteria, such as *H. alvei*, *Proteus* spp., *K. pneumoniae* and *P. shigelloides*, 113 new ligands were identified. In most cases MBL interacted with core OS region that is relatively conservative in comparison with O-PS. Using the LPS of *H. alvei* as a model system it was demonstrated that core OS regions of of *H. alvei* LPS responsible for interactions with human MBL belonged to Ra and OS1 (typical for *H. alvei* oligosaccharide in hexasaccharide form) (Ryc. 6.33) types, including OS1 substituted with disaccharide L-α-D-Hep*p*3OAc-(1→4)-α-Kdo*p*. Moreover, the interaction depends on the presence of characteristic sugar residues recognized by MBL (e.i. GlcNAc, Glc, Hep) in terminal positions or well exposed in the LPS structure, mainly in the outer core OS region. Additionally, characteristic constituents of enterobacterial LPS such as Kdo and lipid A were excluded as MBL ligands, what was suggested previously in the literature. In addition, presence of mannose homopolymer in O-PS was not the only condition necessary for MBL-binding,

since all tested LPS of *H. alvei* recognised by this protein contain O-PS as a heteropolysaccharide.

Regarding biological activity, it was demonstrated that complexes of MBL and *H. alvei* LPS are able to activate LP through MASP proteases, what was demonstrated by C4b and C4c deposition tests. For LPS which interacted with MBL through O-PS and core OS region, the complex activity was similar to *K. pneumoniae* O3 LPS (O-PS in mannose homopolymer form) and slightly lower for LPS which interacted with MBL exclusively by core OS (LPS 1209). Moreover, human MBL was able to interact with LPS on the surface of bacterial cell in growth phase-dependent manner. It is interesting taking into account natural course of nosocomial infection and the role of complement in its elimination and pathogenesis. The highest percentage of labeled cells was observed for *H. alvei* bacteria in lag phase of growth, where rough LPS form were dominant.

Presented studies allowed to explain the ability of a selected *H. alvei* LPS to induce early-phase endotoxic shock *in vivo* in murine model. Specificity of human MBL and mice MBL-C is similar and mainly concerns core OS regions of *H. alvei* LPS and core OS and O-PS of *K. pneumoniae* O3 LPS. Murine MBL-A, in addition to the core OS of *H. alvei* LPS, recognize also O-PS region of the *H. alvei* PCM 1200 LPS, which also interacts with human ficolin-3. Reactivities of murine ficolin-A and -B with selected *H. alvei* LPS were very weak and limited to O-PS regions of two LPS only. It seems that mice need both murine lectins to recognize the same spectrum of target structures, that are recognized by human MBL. Additionally, murine MBL-A partially plays the role of human ficolin-3, since it strongly interacts with LPS PCM 1200. It was demonstrated that early-phase endotoxic shock, which is the consequence of interaction between murine MBL and identified *H. alvei* LPS, may be developed not only after injection of mannose homopolymer-containing LPS (LPS *K. pneumoniae* O3), but also by heteropolysaccharide O-PS-containing LPS (*H. alvei* 1190 LPS). Moreover, rough population of LPS molecules (without O-PS) having core OS recognized by MBL (LPS *H. alvei* 1209) is also able to induce anaphilactoidal reaction. These observations may be important in order to explain the pathogenesis of sepsis and associated complications with regard to the structure of the LPS, a major surface antigen of Gram-negative bacteria. Assuming that MBL or LP may be important in pathogenesis of sepsis caused by Gram-negative bacteria, a key aspect of interpretation the clinical results should be a reference to serotype O of a pathogen, what is missing in most publications concerning the role of the complement in the pathogenesis of sepsis.

For ficolin-3 there are few identified ligands. Among 143 tested LPS of smooth Gram-negative bacteria, such as *Proteus* spp., *K. pneumoniae* and *P. shigelloides*, only four new ligands have been identified: O-PS of LPS of *P. vulgaris* O4 (strain 9/57), *P. penneri* O58 (strain 12), *P. shigelloides* O41 (strain 48/89) and O50 (strain 102/89). New ligands has joined the group containing LPS of *H. alvei* 23, PCM 1200, 1203, 1205.

To study molecular basis and biological effects of interactions between ficolin-3 and LPS, the following preliminary steps were conducted: (i) structural analysis of *H. alvei* LPS 1200 and *Proteus* spp. degradation products, (ii) isolation of oligosaccharides suitable for SPR and STD NMR analyses, with the use of hydrophilic interaction chromatography on a ZIC® HILIC® column and (iii) isolation of ficolin-3 from human plasma. Developed method of ficolin-3 separation allows to obtain c.a. 1,3 mg of ficolin-3 from 500 ml plasma. The method comprises the following steps: (i) precipitation of proteins according to the Zacho and co-workers [Zacho et al., 2012]; (ii) incubation with *Sacharomyces cerevisiae* and L-Fuc to get rid of MBL and ficolin-2; (iii) affinity chromatography on a O-PS 1200-Sepharose CL-4B resins based on the interaction between ficolin-3 and O-PS of *H. alvei* 1200 LPS [Swierzko et al., 2012]; and (iv) size exclusion-high-performance liquid chromatography (SEC-HPLC).

Structural analysis of two newly discovered ligands for ficolin-3, LPS *P. vulgaris* 9/57 and *P. penneri* 12, confirmed previously published O-PS structures and demonstrated that some of observed fragments did not correspond to literature data. In SPR analyses of intermolecular interactions fractions I (O-PS) and II of identified *Proteus* LPS and *H. alvei* 1200 LPS were used. Fraction II of *P. penneri* 12 contained $[RU]_3$. Fraction II of *P. vulgaris* 9/57 was composed of $[RU]_2$ or $[RU]_3$ substituting incomplete core OS fragments. Recombinant ficolin-3 interacted with all tested fractions (in concentration-dependent manner), showing greater specificity toward fraction I which in all cases is characterized by the highest degree of polymerization. Moreover, ficolin-3 interacted stronger with *P. vulgaris* 9/57 fractions than with *P. penneri* 12 fractions. In both cases fractions suitable for analyses of interactions by STD-NMR were not isolated. Results of SPR analyses indicate very strong interaction between ficolin-3 and O-PS of *H. alvei* 1200 LPS. K_D constant for interaction with oligosaccharide $[RU]_2$-[Hep]-Kdo was $3{,}2 \cdot 10^{-9}$ M.

Interaction specificity of recombinant and plasma-derived ficolin-3 towards monosaccharides and O-PS was similar. In SPR analyses monosaccharides and O-PS of *H. alvei* 1200, *P. vulgaris* 9/57 and *P. penneri* 12 LPS were used. Recombinant ficolin-3 interacted

with D-Glc*p*A, D-Gal*p*A, D-Qui*p*NAc, D-Man*p*NAc, L-Ara*p*, D-Fuc*p*. Both proteins showed higher specificity towards *P. vulgaris* 9/57 O-PS.

Studies on epitope mapping of ficolin-3/O-PS *H. alvei* 1200 LPS (oligosaccharide RU-[Hep]-Kdo) using STD-NMR method allowed to identify H2-H6 protons and N-acetamide group of →3)-β-D-Qui*p*4NAc-(1→ and H1 and H2 protons of →1)-Gro-(3-P→ as residues involved in interaction with recombinant and plasma isolated ficolin-3.

Complexes of ficolin-3/MBL:MASP and O-PS of *H. alvei* 1200, *P. vulgaris* 9/57 (O4), *P. penneri* 12 (O58), *P. shigelloides* 48/89, *P. shigelloides* 102/89 LPS are biologically active. They are able *in vitro* to activate LP (activation of C4). The strongest binding of ficolin-3 was observed for LPS *P. vulgaris* 9/57, *P. shigelloides* 48/89 and *H. alvei* 1200. The results obtained for *P. vulgaris* 9/57 and *P. penneri* 12 LPS corresponds to the data obtained by SPR analysis, where both ficolin-3 forms exhibited higher affinity for O-PS of *P. vulgaris* 9/57 LPS. Similar pattern of reactivity was observed in tests for C4 activation via LP (dependent mainly on activity of MASP-2 protease) of selected LPS – the highest deposition of C4 was observed for *P. vulgaris* 9/57, *P. shigelloides* 48/89 and *H. alvei* 1200 LPS. Unexpected results were obtained for MASP-1 activity assay. While almost all of selected LPS activated MASP-1 at similar, very low level, the ability of *P. vulgaris* 9/57 LPS was significantly higher. It means that O-PS region of *P. vulgaris* 9/57 LPS is recognized not only by serum ficolin-3/MASP-2 complexes (as it takes place in most of the selected LPS), but also strongly by MASP-1-containing complexes.

In terms of biological activity, interesting results were obtained during development and optimization of method for human serum ficolin-3 isolation. Contrary to other published protocols, optimisation procedures demonstrated how difficult is purification of this kind of proteins. It was shown that in assessing the quality and purity of such preparations, impurities such as MBL, ficolin-1, ficolin-2 and interactions of ficolin-3 with immunoglobulins (Ig) A, M and G must be taken into account. Previously published methods did not take under consideration monitoring of MBL and immunoglobulin as well as the level of total proteins. Obtained ficolin-3 preparation is complexed with MASP and immunoglobulins and is able to activate complement *in vitro*. Ficolin-3:MASP complexes were able to cleave C4 and C3 and selectively activate LP. Isolation of ficolin-3:MASP:Ig complex as instead of ficolin-3:MASP indicates another proof of cross-talk between molecules involved in innate and adaptive immunity. Moreover, ficolin-3 was able to simultaneously bind human IgG and O-PS of *H. alvei* 1200 LPS suggesting the presence of different binding sites for these ligands. These results

correspond with the discovery by Panda et al. [2013, 2014], who have shown that the ficolin-3 additional binding site for IgG may be activated under inflammatory conditions (different levels of Ca^{2+} and pH). These results represent the third report on this phenomenon and a new, interesting direction for further research.