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The ficolin response to LPS challenge in mice

Ida Jarlhelt, Ninette Genster, Nikolaj Kirketerp-Møller, Mikkel-Ole Skjoedt, Peter Garred*

Laboratory of Molecular Medicine, Department of Clinical Immunology, Section 7631, Rigshospitalet, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT

The ficolins belong to an important family of pattern recognition molecules, which contributes to complement activation via the lectin pathway. How the ficolins respond to inflammatory stimuli remains only partly understood. In the present study, we investigated the ficolin A and ficolin B expression and protein distribution patterns in a mouse model of LPS-induced inflammation. The time- and tissue-specific expression of ficolin A and B was determined by real time PCR. Furthermore, ficolin protein levels in serum and bone marrow extracts from LPS challenged mice were determined by novel in-house developed sandwich ELISAs. Ficolin A was mainly expressed in liver and spleen. However, our data also suggested that ficolin A is expressed in bone marrow, which is the main site of ficolin B expression. The level of ficolin A and B expression was increased after stimulation with LPS in the investigated tissues. This was followed by a downregulation of expression, causing mRNA levels to return to baseline 24 h post LPS challenge. Protein levels appeared to follow the same pattern as the expression profiles, with an exception of ficolin B levels in serum, which kept increasing for 24 h. Ficolin A was likewise significantly increased in bronchoalveolar lavage fluid from mice infected with the fungi *A. fumigatus*, pointing towards a similar effect of the ficolins in non-sterile mouse models of inflammation. The results demonstrate that LPS-induced inflammation can induce a significant ficolin response, suggesting that the murine ficolins are acute phase reactants with increase in both mRNA expression and protein levels during systemic inflammation.

1. Introduction

The ficolins are an important part of innate immunity, capable of activating the complement system via the lectin pathway (Garred et al., 2016; Matsushita et al., 2000). To date three human ficolins have been identified, termed ficolin-1, ficolin-2 and ficolin-3 (Endo et al., 1996; Ichijo et al., 1993; Matsushita et al., 1996). Two murine ficolins have been identified - ficolin A and B. Detailed phylogenetic analysis has indicated that ficolin B is the mouse orthologue of human ficolin-1 and that the genes encoding the mouse ficolin A and human ficolin-2 have a shared evolutionary origin (Garred et al., 2010). The gene encoding the mouse variant of ficolin-3 exists as a pseudo-gene (Endo et al., 2004). Human and murine ficolins have been demonstrated to bind a variety of different pathogens suggesting a protective role of ficolins in host defense (Ali et al., 2012; Endo et al., 2012; Genster et al., 2014; Krarup et al., 2005; Pan et al., 2012). The ficolins recognize pathogen-associated molecular patterns on the surface of microorganisms where they form complexes with mannose-binding lectin (MBL), ficolin and collectin associated serine proteases termed the MASPs (Héja et al., 2012). Three MASP molecules have been identified, MASP-1, MASP-2 and MASP-3. MASP-2 catalyzes the cleavage of complement components C4 and C2, leading to the formation of the C3 convertase (Ricklin et al., 2010). The C3 convertase cleaves C3, which then leads to downstream formation of the C5 convertase. The cleavage of C5 initiates the formation of the terminal membrane attack complex, resulting in cell lysis (Muller-Eberhard, 1986). Also released during C3 and C5 cleavage are the soluble fragments C3a and C5a, which are important anaphylatoxins that attracts immune cells to the site of inflammation and mediate a range of biological effector functions (Peng et al., 2009). Collectively, the effector functions such as opsonization, lysis and generation of an inflammatory response result in elimination of the target pathogen. Ficolins and the complement system therefore generally have beneficial and host-protective effects. Exaggerated activation of the complement cascade can nevertheless have detrimental effects since uncontrolled production of pro-inflammatory cytokines can lead to tissue damage, multiple organ failure and ultimately, death (Cohen, 2002; Markiewski et al., 2008; Ward, 2004). Lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is

* Corresponding author at: Laboratory of Molecular Medicine, Department of Clinical Immunology, Section 7631, Rigshospitalet, Ole Maaloeesvej 26, 2200, Copenhagen N, Denmark.
E-mail address: peter.garred@regionh.dk (P. Garred).

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a potent inflammatory stimulator and has therefore been widely used as a sterile sepsis model (Doi et al., 2009). How the ficolins respond to inflammatory stimuli remains only partly understood and the aim was directed at gaining insight into the in vivo kinetics and biological role of murine ficolin A and B during systemic inflammation. This was achieved by investigating ficolin A and B expression and protein secretion in a mouse model of LPS-induced inflammation.

2. Methods

2.1. Reagents and buffers

LPS (*E. coli* 0111:B4, product no. L 2630) were obtained from Sigma-Aldrich. LPS was stored in concentrations of 5 mg/ml in aliquots at −20 °C and further diluted on the day of use. Bovine Serum Albumin, RPMI-1640 medium, Penicillin/streptomycin Solution, Cell Freeze Medium and Granulocyte-Macrophage Colony stimulating factor were obtained from Sigma-Adrich (Brøndby, Denmark). L-Glutamine, Fetal Bovine Serum and Hybridoma Serum Free Media were obtained from Gibco. RNA lysis buffer, β-Mercaptoethanol and Maxwell® 16 Tissue LEV Total RNA Purification kit were obtained from Promega. $CaCl_2$, NaCl, $MgCl_2$ and Tween-20 were obtained from Merc (Darmstadt, Germany). Phosphate buffered saline (PBS), Barbital buffer (VBS), Ethanol 96% and Protein A/G binding buffer were obtained the hospital pharmacy (Region H Apoteket at Rigshospitalet, Copenhagen, Denmark). HCL, Horseradish-peroxidase (HRP) conjugated Streptavidin, HiTrap Protein A and G HP antibody purification columns and CNBr-Activated Sepharose beads were obtained from GE healthcare life sciences. HRP-conjugated rabbit-anti-rat IgG were obtained from Dako and TMB ONE from Kem-En-Tec Diagnostics. The TaqMan gene expression assay 20x and TaqMan Universal PCR Master mix were obtained from Applied Biosystems. All TaqMan primers were obtained from Thermo Fisher.

The following buffers were used: PBS (10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), VBS (4 mM $C_8H_{11}N_2NaO_3$, 145 mM NaCl, 2.6 mM $CaCl_2$, 2.1 mM $MgCl_2$, pH 7.4), VBS-T (VBS, 0.5% Tween-20), TBS + Ca_2 (10 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$), Krebs Ringer buffer (115 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.6 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM D-glucose, pH 7.4), low pH wash buffer (0.1 M $C_2H_3NaO_2$, 0.5 M NaCl, pH: 2.8)

2.2. Mice

Mice double deficient of ficolin A and ficolin B were generated as previously described (Endo et al., 2012; Genster et al., 2016). Heterozygous mice were crossed and the resulting offspring were used for homozygous breeding of WT and double KO mice; mice used in this study belong to the F4 generations. The female mice used were 10–12 weeks of age and housed in pathogen-free, temperature-controlled and air-conditioned facilities with a 12 h light/dark cycle. Breeding as well as all animal experiments were conducted at the Department of Experimental Medicine, University of Copenhagen, and Rigshospitalet University Hospital, Copenhagen, Denmark. All animal experiments were approved by the Danish Animal Experiments Inspectorate (permission No. 2016-15-0201- 00896) and conducted according to the national Animal Experimentation Act (LBK No. 475 from May 15, 2014). The review board at the Faculty of Health and Medical Sciences, University of Copenhagen, approved this study (P16-247).

2.3. LPS challenge

To induce a strong inflammatory response, we injected 100 µl of a sub-lethal dose (2.5 mg/kg) of LPS intraperitoneal in age and weight-matched mice. The animals were then sacrificed by cervical dislocation at different time points post LPS challenge (after 1, 6 and 24 h). Control mice were sacrificed without intervention. The organs collected from the animals included liver, lung, spleen, heart, kidney and bone marrow. Bone marrow was collected from femurs and tibiae bones of both legs of a mouse. Furthermore, blood samples from mice was collected during a previous in-house experiment (Genster et al., 2017). After the administration of a sub-lethal (2.5 mg/kg) dose of LPS, serum was obtained by submandibular blood collection before the animal was sacrificed. Blood was collected at 1 h, 6 h, 12 h or 24 h after stimulation with LPS. Blood was additionally collected from control littermates (0 h). Additionally, to generate WT and KO serum pools, blood was collected from 6 mice WT mice and 6 KO mice (15 weeks of age).

2.4. Aspergillus fumigatus challenge

Ficolin A and B protein levels were measured in bronchoalveolar lavage fluid (BALF) from *Aspergillus fumigatus* infected mice. Mice were inoculated with *A. fumigatus* and samples were collected as described previously in another paper (Genster et al., 2016). The assay included 6 WT mice infected with *Aspergillus*, 3 WT mice treated with PBS, 3 KO mice infected with *Aspergillus* and 3 KO mice treated with PBS. All samples were diluted 1:2 in VBS-T.

2.5. Real time PCR

Tissue samples were homogenized in RNA lysis buffer with a mechanical homogenizer (TissueRuptor, Qiagen) and RNA was extracted using the Maxwell 16 tissue LEV Total RNA Purification Kit (Promega) following the manufacturer's recommendations. Total RNA was quantified using a Qubit Fluorometer (Invitrogen) and 1 µg of total RNA was reverse transcribed with the MuLV Reverse transcriptase (Applied Biosystems #N808-0018). Ficolin A and B gene expression in organs was assessed by quantitative real-time PCR with TaqMan probes specific for mouse TBP (mM00446971_m1), Ficolin A (Mm00484287_m1) and Ficolin B (Mm01332438_m1), obtained from Applied Biosystems. Quantitative Real-time PCR was performed in triplicate loading 10 ng of cDNA per reaction and carried out on the Stratagene Mx3005 P Real-Time PCR system (Agilent Technologies). Reaction conditions included incubation at 50 °C for 2 min. and 95 °C for 10 min. followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. PCR runs included no-template controls and no-reverse transcriptase controls. The target genes were normalized to the housekeeping gene, TBP, and results were represented as the relative expression of ficolin A and B.

2.6. Production of monoclonal antibodies against mouse ficolins

Monoclonal antibodies against recombinant mouse ficolin A or B were raised in Sprague Dawley rats. The recombinant ficolin A and B had been synthesized as previously described for ficolin-2 (Hummelshoj et al., 2007). The fusion and selection of the antibodies was performed essentially as described previously for human ficolin-2 (Skjoedt et al., 2010). Five different hybridoma clones were cultured – two against ficolin A (clone 21 and 22) and three against ficolin B (clone 1, 2 and 3). The hybridomas were initially grown in RPMI 1640 medium containing 10% fetal calve serum, 1% penicillin/streptomycin and 1% L-glutamine (200 nM). The cells afterwards relocated to serum free medium containing 1% penicillin/streptomycin. The ficolin antibodies was afterwards purified by use of a HiTrap Protein A for the ficolin B antibodies or a HiTrap Protein G column for the ficolin A antibodies in an ÄKTA pure protein purification system (GE Healthcare, UK).

2.7. Purification of recombinant ficolin A and B

Recombinant ficolin A and B was purified by the virtue of the purified antibodies coupled to a CNBr column. 1 g of CNBr-activated sepharose beads were added to 20 ml cold 1 mM HCl and mixed for 15 min at RT. The suspension was transferred through a glass-filter and

the dry beads were washed with 3 × 10 ml 1 mM HCl followed by 10 ml PBS. The dried sepharose beads were transferred to a tube and blocked with PBS for 2.5 h at RT. The beads were incubated with the antibody solution (5 mg antibody in total in a concentration of 1 mg/ml in PBS) for 5 min. The absorbance of the supernatant was measured at 280 nm, and the coupling percent should be around 70–80%. The antibody coupled beads were blocked overnight at 4 °C using 1 M glycine. Finally, the beads were transferred to a column and were allowed to precipitate. The column was washed with RPMI medium containing 10% fetal bovine serum (FBS) and low pH wash buffer. The column was stored in 25% ethanol and sealed at 4 °C until further use.

A stock of supernatant from Chinese hamster ovary cells expressing recombinant ficolin A and B was produced in earlier studies (Hummelshoj et al., 2007). Upon purification, 5 mM EDTA was added to the supernatant before it was applied to the antibody column at 4 °C. The column was washed with the binding/washing buffer (5 mM EDTA in PBS) both before and after flow through of the supernatant. The bound recombinant ficolin A and B was eluted in 10 × 2 ml fractions by use of elution buffer (0.5% citric acid in distilled water, pH: 2.5). 50 µl neutralization buffer (1 M Tris) was added to each fraction. The protein-fractions were pooled and dialyzed in TBS + Ca_2 buffer for ficolin A or Krebs Ringer buffer for ficolin B overnight at 4 °C. The protein samples were afterwards aliquoted and stored at −80 °C.

2.8. Sandwich ELISA

Nunc plate MaxiSorp plates were coated with antibodies for ficolin A or B – clone 21 and 1 respectively. 1 µg/ml antibody in PBS incubated overnight at 4 °C. The plates were blocked for 15 min in VBS-T. The sample consisting of mouse serum or tissue homogenates was applied in appropriate dilutions for 3 h at 37 °C. Detection antibodies included biotinylated antibody clone 22 for ficolin A and biotinylated antibody clone 2 for ficolin B. These were applied in a total concentration of 1 µg/ml for 1 h shaking at RT. Streptavidin HRP (RPN1231V, GE Healthcare) was added in a concentration of 1:2000 in VBS-T for 1 h shaking at RT. The substrate TMB ONE was applied and allowed to react for 10–20 min, H_2SO_4 0,2 M stopped the reaction and the OD of the samples was measured at 450 nm. Plates were washed 3 times with VBS-T by the use of a Nunc Immuno Washer between steps unless otherwise stated.

2.9. Assay validation

The calibrator consisted of in-house produced recombinant ficolin A and B, stored at −20 °C in single-use aliquots. The concentration of calibrators was determined by spectrophotometry (Eppendorf BioSpectrometer® fluorescence) and the given concentrations was divided by the extinction coefficients of the murine ficolins, determined by the ExPASy ProtParam tool. Two-fold serial dilutions of the recombinant ficolin A or B were used to generate a twelve-point calibrator curve ranging from 10 to 0 µg/ml. Recombinant ficolin A and B, mouse serum and bone marrow were serially diluted across the dynamic range of the assay and OD values were logistically transformed to evaluate the parallelism between the best-fit hill slopes. Linear regression was fitted to at least eight data points and multiple comparisons were used to determine differences among the slopes. The limit of detection of the assay was expressed as the background absorbance plus two times the standard deviation (SD). Intra-assay variation was calculated as the coefficient of variation (CV) of a serum pool in 40 wells of a single microtiter plate. To calculate the inter-assay variation, four serum samples were run in triplicates on four different days. Furthermore, the stability of recombinant ficolins and biological samples was tested by storing sampled at 4 °C, −20 °C and −80 °C and measuring ficolin levels initially and once again after 1 week of storage. The binding of all antibodies to recombinant ficolin A and B was tested right after purification and again after one month of storage at 4 °C. Additionally, the concentration of recombinant ficolin A and B was measured in aliquots before and after storage at either −20 °C or −80 °C for one month. The recombinant ficolin aliquots were also subjected to three freeze-thaw cycles (−80 °C and RT, respectively) and protein levels were measured after the first and third freeze-thaw cycle.

2.10. Statistics

All data was analyzed using GraphPad Prism Software, version 7. Measurements of samples and the calibrator were performed in duplicates unless otherwise stated. The concentration of ficolin A and B in serum was interpolated by regression analysis using a four-parameter logistic curve fitting. Comparisons of ficolin expression and protein levels were analyzed using two-way ANOVA with Bonferroni's multiple comparisons tests (BF's correction), $p < 0.05$ was considered statistically significant. Data are represented as mean + or ± SD. Significance levels: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3. Results

3.1. Tissue-specific expression of murine ficolins during LPS challenge

We investigated the expression of the murine ficolins in various organs and detected expression of ficolin A in liver, spleen and bone marrow. Expression of ficolin B was detected in bone marrow only. The ficolin mRNA levels were determined in control littermates and compared to mice sacrificed 1, 6 and 24 h post LPS stimulation. Ficolin expression was also measured in KO mice. The resulting data is presented as relative expression in Figs. 1 and 2. We observed similar expression patterns in all investigated tissues. The expression of ficolin A and B peaked within one hour after LPS-induced inflammation. This was followed by a decrease in expression, with levels returning close to or below baseline after 24 h. No ficolin A or B was detected in either tissue in KO mice at any time point (data not shown).

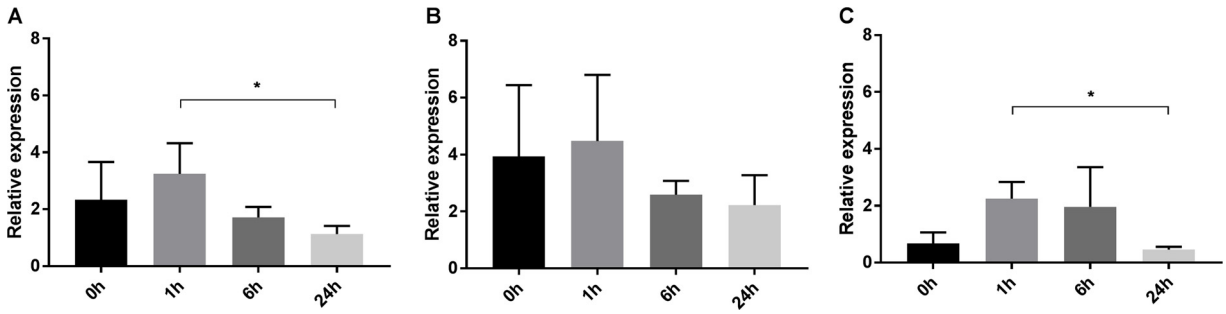

Fig. 1. Expression of ficolin A in tissues after stimulation with LPS. Mice were injected with a sub-lethal dose (2.5 mg/kg) of LPS and euthanized at the indicated time points post treatment. Relative mRNA expression of murine ficolin A was detected in A) liver, B) spleen and C) bone marrow. Groups are compared with a one-way ANOVA with BF's correction (n = 4). Data is represented as mean + SD. Significance levels: * = $p \leq 0.05$.

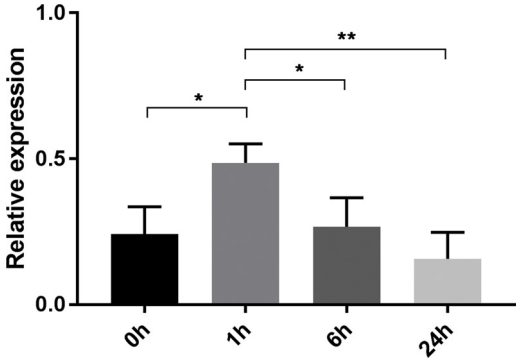

Fig. 2. Expression of ficolin B in bone marrow after stimulation with LPS. Mice were injected with a sub-lethal dose (2.5 mg/kg) of LPS and euthanized at the indicated time points. Relative mRNA expression of murine ficolin B was detected in bone marrow. Groups are compared with a one-way ANOVA with BF's correction (n = 4). Data is represented as mean + SD. Significance levels: * = $p \leq 0.05$, ** = $p \leq 0.01$.

3.2. Assay validation

Cysteine bridge mediated oligomerization of the recombinant and native ficolins were verified by SDS-PAGE followed by InstantBlue™ staining of proteins. The oligomerization of recombinant ficolin A and B was similar to the native forms found in mouse serum (data not shown). The oligomerization patterns was also comparable to that of human ficolins (Hummelshoj et al., 2007) and rat ficolin A and B (Girija et al., 2011, 2007). We used recombinant ficolin A and B as calibrators in our sandwich ELISAs. Parallelism was observed between the slopes of the calibrators and biological material in the form of serum and bone marrow samples, indicating that the calibrators can be used to accurately measure sample concentrations in mouse serum and tissue homogenates. Following logistic transformation, the data from the ficolin A assay fitted a linear regression with $R^2 > 0.97$ for both curves with slopes between 0.54 and 0.64. The data from the ficolin B assay exhibited equal slopes between 0.81 and 0.88, but the linear regression model did not fit the bone marrow data well ($R^2 = 0.78$) (Fig. 3). Comparison tests nevertheless revealed that there was no significant difference of the slopes of the serial dilution in any of the assays ($p > 0.05$). The limit of detection, defined as the mean background absorbance plus two times the SD, was 0.010 µg/ml in serum for the ficolin A assay and 0.005 µg/ml in serum for the ficolin B assay. Intra- and inter-assay variations were calculated to estimate the variation of the assay. The intra-assay CV, calculated by measuring one sample forty times on the same plate, was 8.44% for ficolin A and 7.09% for ficolin B (Table 1). The average inter-assay CV was 7.07% for ficolin A and 16.04% for ficolin B, each determined from 4 serum samples measured in duplicates on four different occasions.

Neither antibodies nor biological samples were affected by storage conditions. All antibodies appeared to precipitate slightly when stored at 4 °C but were easily resuspended upon shaking and concentrations were not affected during one month of storage. Recombinant ficolin A was likewise not affected by either storage conditions or three freeze-thaw cycles. In general, the recombinant ficolin B appear unstable and thawing caused a significant drop in ficolin B concentration (data not shown). Aliquots of recombinant ficolin B were therefore stored at −80 °C and the concentration should ideally be determined before assayed.

Table 1
Intra- and inter-assay variation in the ficolin A and B sandwich ELISAs. The concentration of ficolin A and B was measured in murine WT serum 40 times on a plate within a single ELISA run to determine the intra-assay variation. The concentration of ficolin A and B in serum from four WT samples were tested at four different occasions to determine the inter-assay variation. The CVs were calculated for each assay by determining the ratio of the standard deviation to the mean.

	Sample	n	Mean	SD	CV%
Intra-Assay Variation					
Ficolin A assay	Serum	40	8.78	0.74	8.44
Ficolin B assay	Serum	40	0.009	1.12	7.09

	Sample	n	Occasions	Average CV%
Inter-Assay Variation				
Ficolin A assay	Serum	4	4	7.07
Ficolin B assay	Serum	4	4	16.04

3.3. Ficolin protein levels during LPS challenge

In accordance with previous findings, the average ficolin A concentration in serum was determined to be 4.8 µg/ml in control littermates injected with PBS. The amount of ficolin A in serum increased after one hour and this increase continued and became significant 12 h post LPS challenge (Fig. 4). The average concentration reached close to 10 µg/ml after 12 h, before returning to baseline or values below baseline after 24 h. The ficolin B protein levels in control littermates were close to undetectable, but an increase in concentration was detected during one-hour post LPS injection. The increase became significant after 12 h and the average ficolin B concentration reached a maximum above 0.1 µg/ml after 24 h. No ficolin A or B was detected in serum from KO mice at any time point (data not shown).

The concentration of ficolin B in serum was measurable, but low. Since ficolin B is expressed mainly in bone marrow, we reasoned that a possibility to achieve more quantitative protein measurements was to measure the protein levels in bone marrow extracts. Ficolin B protein levels were therefore determined by our ficolin sandwich ELISA in the bone marrow homogenates. In agreement with the mRNA expression patterns, the ficolin B protein levels in bone marrow increased significantly during the first hour post LPS challenge, followed by a decrease that reached baseline after 24 h (Fig. 5). The average concentration of ficolin B in bone marrow homogenate from control

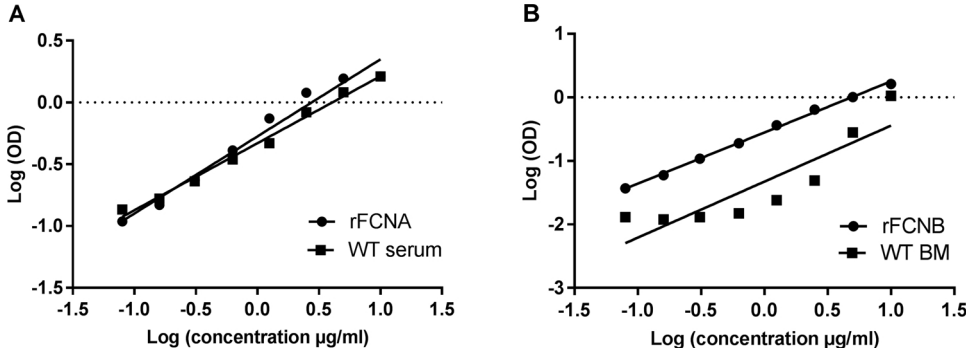

Fig. 3. Assay validation of the ficolin A and B sandwich ELISAs. Parallelism between calibrators and samples was determined over an 8-point serial dilution series using a log(agonist) vs. response equation. A) Parallelism between serial dilutions of serum and a recombinant ficolin A. B) Parallelism between serial dilutions of bone marrow homogenate and a recombinant ficolin B.

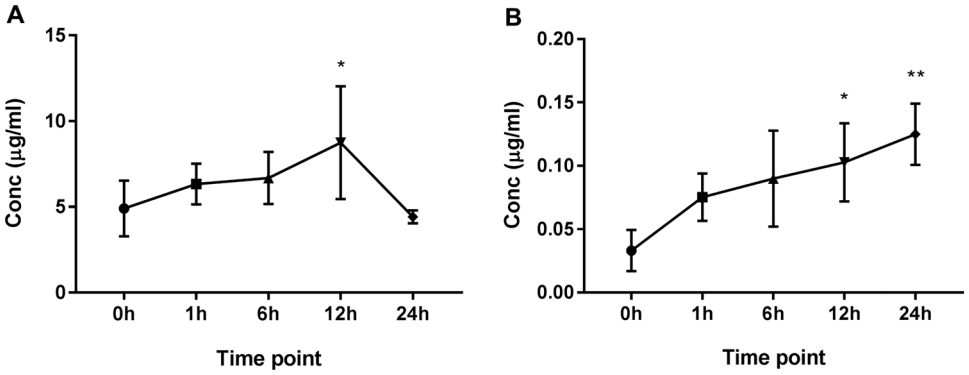

Fig. 4. Ficolin A and B in serum during LPS-induced inflammation. Mice were injected with a sublethal dose (2.5 mg/kg) of LPS and blood samples were collected at the indicated time points. Concentration of murine ficolin A and B was measured by two novel sandwich ELISAs. A) Ficolin A in serum. B) Ficolin B in serum. Each group was held against control littermates (0 h) with a one-way ANOVA with BF's correction (n = 7). One statistical outlier was excluded from the ficolin B dataset, confirmed by Grubbs' test (α = 001). Data is represented as mean ± SD. Significance levels: * = $p \leq 0.05$, ** = $p \leq 0.01$.

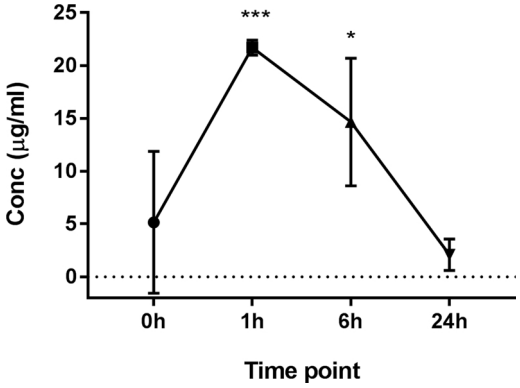

Fig. 5. Ficolin B in bone marrow during LPS-induced inflammation. Mice were injected with a sublethal dose (2.5 mg/kg) of LPS and euthanized at the indicated time points. The ficolin B protein concentrations in bone marrow homogenates were measured by a novel sandwich ELISA. Each group was held against control littermates (0 h) with a one-way ANOVA with BF's correction (n = 4). Data is represented mean ± SD. Significance levels: * = $p \leq 0.05$, *** = $p \leq 0.001$.

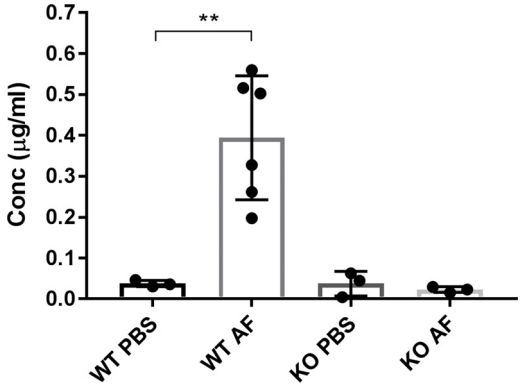

Fig. 6. The effect of *A. fumigatus* infection on ficolin A levels in BALF. WT or double KO mice was inoculated with *A. fumigatus* or PBS, and BALF was collected after 24 h. An ELISA was used to determine concentrations of ficolin A in BALF from infected mice and control animals. A one-way ANOVA with BF's correction was performed. Data is represented as mean ± SD. Significance levels: ** = $p \leq 0.01$.

littermates were 5 µg/ml and the average concentration reached above 20 µg/ml one-hour post LPS injection. No ficolin B was detected in bone marrow of KO mice.

3.4. Mouse model of A. fumigatus infection

An additional setup was prepared to assess the behavior of the ficolins in another mouse model of inflammation. The *A. fumigatus* model is a non-sterile model of infection, which can provide information about the ficolins under more biologically relevant conditions. Furthermore, it was of interest to verify the application of the developed sandwich ELISA assays in another experimental setup. Ficolin A and B protein levels was quantified in BALF from mice infected with the fungi *Aspergillus fumigatus* versus mice treated with PBS (Fig. 6). The resulting data indicated that no or very low amounts of ficolin A was present in BALF of WT mice in general, but that protein levels increased significantly upon infection with *A. fumigatus*. The average concentration of ficolin A in BALF from mice infected with *A. fumigatus* was close to 0.4 µg/ml. No ficolin B was detected in BALF from neither WT or *A. fumigatus* infected mice (data not shown).

4. Discussion

It is commonly accepted that LPS extracted from gram-negative bacteria can induce systemic inflammation in both mice and humans, mainly through activation of the TLR4/CD14 sensing complex (Pålsson-McDermott and O'Neill, 2004). Challenge with LPS mimics many of the early clinical features of sepsis including an increase in pro-inflammatory cytokines but without bacteremia (Doi et al., 2009; Lewis et al., 2016). Previously, it was acknowledged that complement activation after LPS administration mainly was associated with activation of the classical and alternative pathway (Morrison and Kline, 1977). Data is nevertheless emerging, indicating that the lectin pathway might as well be involved in an LPS-induced inflammatory response. Mouse MBL and ficolin A has been demonstrated to bind to LPS in vitro (Genster et al., 2017; Zhao et al., 2002). We present data that further indicates an involvement of ficolins in LPS mediated inflammation. We have studied the pattern of expression and protein secretion of murine ficolin A and B in tissue homogenates during LPS-induced inflammation. We found that treatment with LPS induce both ficolin A and B gene expression in various tissues (Figs. 1 and 2). Other studies have likewise suggested that the expression of ficolins are inducible since murine ficolin B expression has been shown to be upregulated upon macrophage activation (Runza et al., 2006). Additionally, the human analog ficolin-1, has been demonstrated to increase in expression in monocyte-derived macrophages after treatment with the TLR2 and TLR4 ligands (Frankenberger et al., 2008). Interestingly, the murine ficolin expression levels in our study additionally exert negative feedback behavior. In the just mentioned study by Frankenberg et al. they likewise experienced a decrease in ficolin expression four days after cells were treated with either LPS or Pam3Cys (Frankenberger et al., 2008). In a previous study conducted by our group (Genster et al., 2017), we compared the expression of murine ficolin A and B in control littermates and mice sacrificed 6 h post treatment with a lethal dose of LPS (10 mg/kg). We found no significant difference in ficolin mRNA levels in either liver, spleen or bone marrow. It is a possibility that the short time frame (6 h) caused us to "miss out" on the increase before the expression was downregulated. This could also be the reasoning for a human study conducted in 2016, in which it was investigated whether

LPS-induced lung and systemic inflammation could induce recruitment of lectins in healthy individuals (Plovsing et al., 2016). *FCN1* and *FCN3* mRNA expression was measured in BALF cells and blood monocytes before and 24 h after challenge with LPS and no significant effect was found of either. It is important to keep in mind that many factors differ between the two species. The human and mouse ficolins have unique distribution and binding capacities – indicating that they may participate differently during an immune response in humans and mice, respectively (Garred et al., 2010; Genster et al., 2014). Additionally, the structural features of the lectin pathway and the host response to LPS in general differs between human and mouse (Endo et al., 2015).

It is important to note that no previous data have demonstrated that murine ficolin A is expressed in bone marrow. We performed an additional qPCR assay, to ensure that the ficolin A primer was not in fact recognizing ficolin B. In the assay, three different ficolin A TaqMan primers were used for determining the levels of ficolin A mRNA in WT, B KO and AB double KO mice. A one-way ANOVA test with multiple comparisons revealed significant differences in the average relative expression between AB double KOs and the two remaining groups. Therefore, the resulting data verified that ficolin A is expressed in bone marrow homogenate (data not shown). A comparative study of the human ficolins have likewise detected minor expression of ficolin-2 in bone marrow (Hummelshoj et al., 2008), suggesting that human ficolin-2 and mouse ficolin A is not exclusively expressed in the liver as previously anticipated. Interestingly, when looking at the relative expression of the murine ficolins, it appears that there is a greater expression of ficolin A than ficolin B in bone marrow (Figs. 1 and 2).

We succeeded in establishing two reliable novel sandwich ELISAs for measuring ficolin A and B protein levels. The assays were thoroughly validated and demonstrated satisfactory variability and parallelism between calibrators and samples. Our protein measurements indicate that the ficolin levels in serum and bone marrow increases during LPS-induced inflammation. Ficolin A and B protein levels in serum both demonstrate a significant increase after 12 h (Fig. 4). The level of ficolin B in bone marrow seems to follow the same pattern as mRNA expression peaking one-hour post LPS challenge (Fig. 5). The induced protein levels of bone marrow ficolin B corresponds to an increase of 300% from 5 μg/ml to 20 μg/ml within one-hour post LPS challenge. This is in agreement with another study, where they demonstrated that the expression of ficolin B increase in fresh granulocytes isolated from bone marrow after LPS treatment in vitro (Webersteffens et al., 2013). The significant increase of ficolin A and B levels upon stimulation with LPS indicates an acute-phase behavior of the ficolins. Several acute-phase proteins are produced in the liver and serve different physiological functions for the immune system. MBL and ficolin-2 has likewise been described as acute phase reactants (Chen et al., 2015; Suankratay et al., 1998). The protein secretion pattern of ficolin A and B in serum nevertheless seem to differ from each other 24 h post LPS challenge. The levels of ficolin A in serum is clearly reduced after 24 h, while the average concentration of ficolin B keeps increasing (Fig. 4). The different pattern of downregulation (or perhaps turnover) of the two murine ficolins could indicate that they have distinct physiological functions. This could be further reinforced by the data presented in another study where they found that the circulating ficolin A protects against *S. pneumoniae* infection via activation of the lectin pathway, whereas no ficolin B induced complement activation could be detected in ficolin A KOs (Endo et al., 2012). Another study investigated intracellular expression of human ficolin-1 co-localization and found that the protein is localized to secretory vesicles (Liu et al., 2005). This collectively indicates that ficolin B may partially execute its function without activation of complement, most likely at local sites rather than in the circulation. Although the measured concentrations of murine ficolin B in serum are low, our study is among the first to confirm that ficolin B can be detected in mouse serum – especially during LPS-induced inflammation. Our results are in agreement with the study by Endo et al. where they measured the average ficolin B serum concentration in WT mice to be around 0.1 μg/ml (Endo et al., 2012).

A recent study from our group investigated the cytokine response upon LPS stimulation with a lethal or a sub-lethal dose and found no significant difference between ficolin WT and KO mice (Genster et al., 2017). Furthermore, we performed whole genome transcriptome analysis on ficolin WT and KO mice and concluded that murine ficolins are not implicated in LPS-induced inflammation in mice. This is an interesting parallel to draw, indicating that the main inflammatory pathway is through TLR4 signaling. It appears that the ficolins will not directly disturb the interaction between LPS and TLR4, nor will ficolin-LPS induced complement activation modify the cell response to LPS. In the present paper we nevertheless demonstrate that LPS stimulation may cause (direct or indirect) increases in ficolin expression. Future studies will hopefully reveal additional information about the biological function of the ficolins during inflammation. It would also be of great interest to study the contribution of the ficolins in other infectious mouse models, in order to investigate whether protein levels increase during other inflammatory conditions. Especially because LPS-induced inflammation is a sterile inflammation and we know that the ficolins bind to many different pathogens. The significance of the ficolins in clinical situations involving polymicrobial infections have been demonstrated before (Hansen et al., 2016; Luo et al., 2016, 2013). Importantly, data from the BALF assay in the present study demonstrate that ficolin A levels increase significantly in lung fluid post infection with *A. fumigatus*. This implies that at least ficolin A exhibits acute phase behavior or is found in the exudate after a local response also in a different model of inflammation (Fig. 6). The mice were sacrificed and the BALF were collected 24 h post inoculation with *A. fumigatus*, so within a day, the concentration of ficolin A exhibited a five-fold increase. In line with these results, a previous in-house study additionally found a significant difference in the load of *A. fumigatus* in the lungs of ficolin WT compared to KO mice (Genster et al., 2016). Furthermore, the cytokine levels in BALF differed between WT and KO mice. The results from our study thereby supports the literature suggesting that murine ficolins plays a role in the defense against *A. fumigatus*. Human ficolin-2, the homologue of mouse ficolin A, has also been observed to recognize *A. fumigatus* (Bidula et al., 2015; Ma et al., 2009), which indicate that the protein is likely to participate in fungal defense in humans as well.

The data presented here demonstrate that murine ficolins are upregulated in response to LPS and *A. fumigatus* stimuli. However, it is important to keep in mind that the healthy mouse model of LPS challenge hardly replicate the clinical conditions of severe infection in a patient. The insight gained here may nevertheless provide the basis for further studying the ficolins in infectious diseases. We anticipate that the provided knowledge and the newly developed sandwich ELISAs can contribute to further assessment of the functional role and underlying mechanisms of ficolins during inflammation.

Contributions

I.J., N.G. and P.G. designed the experiments. I.J., N.G. and N.K.M performed and analyzed the experiments. MO.S provided antibodies and general guidance. N.G. and P.G. supervised the study. I.J. wrote the manuscript. P.G. revised the manuscript and all authors critically reviewed and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

Ali, Y.M., Lynch, N.J., Haleem, K.S., Fujita, T., Endo, Y., Hansen, S., Holmskov, U., Takahashi, K., Stahl, G.L., Dudler, T., Girija, U.V., Wallis, R., Kadioglu, A., Stover, C.M., Andrew, P.W., Schwaeble, W.J., 2012. The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. PLoS Pathog. 8, 46. https://doi.org/10.1371/journal.ppat.1002793.

Bidula, S., Sexton, D.W., Abdolrasouli, A., Shah, A., Reed, A., Armstrong-James, D., Schelenz, S., 2015. The serum opsonin L-ficolin is detected in lungs of human transplant recipients following fungal infections and modulates inflammation and killing of Aspergillus fumigatus. J. Infect. Dis. 212, 234–246. https://doi.org/10.1093/infdis/jiv027.

Chen, T., Hu, Y., Ding, Q., Yu, J., Wang, F., Luo, F., 2015. Serum ficolin-2 concentrations are significantly changed in patients with hepatitis B virus infection and liver diseases. Virol. Sin. 30, 1–12. https://doi.org/10.1007/s12250-015-3605-4.

Cohen, J., 2002. The immunopathogenesis of sepsis. Nature 420, 885.

Doi, K., Yuen, P.S.T., Star, R.A., Doi, K., Leelahavanichkul, A., Yuen, P.S.T., Star, R.A., 2009. Animal models of sepsis and sepsis-induced kidney injury. J. Clin. Invest. 119, 2868–2878. https://doi.org/10.1172/JCI39421.2868.

Endo, Y., Sato, Y., Matsushita, M., Fujita, T., 1996. Cloning and characterization of the human lectin P35 gene and its related gene. Genomics 36, 515–521. https://doi.org/10.1006/geno.1996.0497.

Endo, Y., Liu, Y., Kanno, K., Takahashi, M., Matsushita, M., Fujita, T., 2004. Identification of the mouse H-ficolin gene as a pseudogene and orthology between mouse ficolins A/B and human L-/M-ficolins. Genomics 84, 737–744. https://doi.org/10.1016/j.ygeno.2004.07.006.

Endo, Y., Takahashi, M., Iwaki, D., Ishida, Y., Nakazawa, N., Kodama, T., Matsuzaka, T., Kanno, K., Liu, Y., Tsuchiya, K., Kawamura, I., Ikawa, M., Waguri, S., Wada, I., Matsushita, M., Schwaeble, W.J., Fujita, T., 2012. Mice deficient in ficolin, a lectin complement pathway recognition molecule, are susceptible to Streptococcus pneumoniae infection. J. Immunol. 189, 5860–5866. https://doi.org/10.4049/jimmunol.1200836.

Endo, Y., Matsushita, M., Fujita, T., 2015. New Insights into the Role of Ficolins in the Lectin Pathway of Innate Immunity, International Review of Cell and Molecular Biology. Elsevier Ltd.https://doi.org/10.1016/bs.ircmb.2015.01.003.

Frankenberger, M., Schwaeble, W., Ziegler-Heitbrock, L., 2008. Expression of M-ficolin in human monocytes and macrophages. Mol. Immunol. 45, 1424–1430. https://doi.org/10.1016/J.MOLIMM.2007.08.014.

Garred, P., Honore, C., Ma, Y.J., Rorvig, S., Cowland, J., Borregaard, N., Hummelshoj, T., 2010. The genetics of ficolins. J. Innate Immun. 2, 3–16. https://doi.org/10.1159/000242419.

Garred, P., Genster, N., Pilely, K., Bayarri-Olmos, R., Rosbjerg, A., Ma, Y.J., Skjoedt, M.O., 2016. A journey through the lectin pathway of complement – MBL and beyond. Immunol. Rev. 274, 74–97. https://doi.org/10.1111/imr.12468.

Genster, N., Takahashi, M., Sekine, H., Endo, Y., Garred, P., Fujita, T., 2014. Lessons learned from mice deficient in lectin complement pathway molecules. Mol. Immunol. 61, 59–68. https://doi.org/10.1016/j.molimm.2014.07.007.

Genster, N., Prætekjær Cramer, E., Rosbjerg, A., Pilely, K., Cowland, J.B., Garred, P., 2016. Ficolins promote fungal clearance in vivo and modulate the inflammatory cytokine response in host defense against Aspergillus fumigatus. J. Innate Immun. 8, 579–588. https://doi.org/10.1159/000447714.

Genster, N., Østrup, O., Schjalm, C., Eirik Mollnes, T., Cowland, J.B., Garred, P., 2017. Ficolins do not alter host immune responses to lipopolysaccharide-induced inflammation in vivo. Sci. Rep. 7, 1–12. https://doi.org/10.1038/s41598-017-04121-w.

Girija, U.V., Dodds, A.W., Roscher, S., Reid, K.B.M., Wallis, R., 2007. Localization and characterization of the mannose-binding lectin (MBL)-associated-serine protease-2 binding site in rat ficolin-a: equivalent binding sites within the collagenous domains of MBLs and ficolins. J. Immunol. 179, 455–462. https://doi.org/10.4049/jimmunol.179.1.455.

Girija, U.V., Mitchell, D.A., Roscher, S., Wallis, R., 2011. Carbohydrate recognition and complement activation by rat ficolin-B. Eur. J. Immunol. 41, 214–223. https://doi.org/10.1002/eji.201040612.

Hansen, M.B., Rasmussen, L.S., Pilely, K., Hellemann, D., Hein, E., Madsen, M.B., Hyldegaard, O., Garred, P., 2016. The lectin complement pathway in patients with necrotizing soft tissue infection. J. Innate Immun. 8, 507–516. https://doi.org/10.1159/000447327.

Héja, D., Harmats, V., Fodor, K., Wilmanns, M., Dobó, J., Kékesi, K.A., Závodszky, P., Gál, P., Pál, G., 2012. Monospecific inhibitors show that both mannan-binding lectin-associated serine protease-1 (MASP-1) and -2 are essential for lectin pathway activation and reveal structural plasticity of MASP-2*. J. Biol. Chem. 287, 20290–20300. https://doi.org/10.1074/jbc.M112.354332.

Hummelshoj, T., Thielens, N.M., Madsen, H.O., Arlaud, G.J., Sim, R.B., Garred, P., 2007. Molecular organization of human ficolin-2. Mol. Immunol. 44, 401–411. https://doi.org/10.1016/j.molimm.2006.02.023.

Hummelshoj, T., Fog, L.M., Madsen, H.O., Sim, R.B., Garred, P., 2008. Comparative study of the human ficolins reveals unique features of ficolin-3 (Hakata antigen). Mol. Immunol. 45, 1623–1632. https://doi.org/10.1016/j.molimm.2007.10.006.

Ichijo, H., Hellman, U., Wernstedt, C., Gonez, L.J., Claesson-Welsh, L., Heldin, C.H., Miyazono, K., 1993. Molecular cloning and characterization of ficolin, a multimeric protein with fibrinogen- and collagen-like domains. J. Biol. Chem. 268, 14505–14513.

Krarup, A., Sørensen, U.B.S., Matsushita, M., Jensenius, J.C., Thiel, S., 2005. Effect of capsulation of opportunistic pathogenic bacteria on binding of the pattern recognition molecules mannan-binding lectin. Infect. Immun. 73, 1052–1060. https://doi.org/10.1128/IAI.73.2.1052.

Lewis, A.J., Seymour, C.W., Rosengart, M.R., 2016. Current murine models of sepsis. Surg. Infect. (Larchmt) 17, 385–393. https://doi.org/10.1089/sur.2016.021.

Liu, Y., Endo, Y., Iwaki, D., Nakata, M., Matsushita, M., Wada, I., Inoue, K., Munakata, M., Fujita, T., 2005. Human M-ficolin is a secretory protein that activates the lectin complement pathway. J. Immunol. 175, 3150–3156. https://doi.org/10.4049/jimmunol.175.5.3150.

Luo, F., Sun, X., Wang, Y., Wang, Q., Wu, Y., Pan, Q., Fang, C., Zhang, X.L., 2013. Ficolin-2 defends against virulent mycobacteria tuberculosis infection in vivo, and its insufficiency is associated with infection in humans. PLoS One 8, 1–15. https://doi.org/10.1371/journal.pone.0073859.

Luo, F., Chen, T., Liu, J., Shen, X., Zhao, Y., Yang, R., Zhang, X., 2016. Ficolin-2 binds to HIV-1 gp120 and blocks viral infection. Virol. Sin. 31, 406–414. https://doi.org/10.1007/s12250-016-3808-3.

Ma, Y.J., Doni, A., Hummelshøj, T., Honoré, C., Bastone, A., Mantovani, A., Thielens, N.M., Garred, P., 2009. Synergy between ficolin-2 and pentraxin 3 boosts innate immune recognition and complement deposition. J. Biol. Chem. 284, 28263–28275. https://doi.org/10.1074/jbc.M109.009225.

Markiewski, M.M., DeAngelis, R.A., Lambris, J.D., 2008. Complexity of complement activation in sepsis. J. Cell. Mol. Med. 12, 2245–2254. https://doi.org/10.1111/j.1582-4934.2008.00504.x.

Matsushita, M., Endo, Y., Sato, Y., Fujita, T., Chem, J.B., 1996. Protein chemistry and structure: a novel human serum lectin with collagen- and fibrinogen-like domains that functions as an opsonin. J. Biol. Chem. 271, 2448–2454. https://doi.org/10.1074/jbc.271.5.2448.

Matsushita, M., Endo, Y., Fujita, T., 2000. Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. J. Immunol. 164, 2281–2284. https://doi.org/10.4049/jimmunol.164.5.2281.

Morrison, D.C., Kline, L.F., 1977. Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). J. Immunol. 118 362LP-368.

Muller-Eberhard, H.J., 1986. The membrane attack complex of complement. Annu. Rev. Immunol. 4, 503–528. https://doi.org/10.1146/annurev.iy.04.040186.002443.

Pålsson-McDermott, E.M., O'Neill, L.A.J., 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113, 153–162. https://doi.org/10.1111/j.1365-2567.2004.01976.x.

Pan, Q., Chen, H., Wang, F., Jeza, V.T., Hou, W., Zhao, Y., Xiang, T., Zhu, Y., Endo, Y., Fujita, T., Zhang, X.L., 2012. L-Ficolin binds to the glycoproteins hemagglutinin and neuraminidase and inhibits influenza a virus infection both in vitro and in vivo. J. Innate Immun. 4, 312–324. https://doi.org/10.1159/000335670.

Peng, Q., Li, K., Sacks, S.H., Zhou, W., 2009. The role of anaphylatoxins C3a and C5a in regulating innate and adaptive immune responses. Inflamm. Allergy Drug Targets 8, 236–246.

Plovsing, R.R., Berg, R.M.G., Munthe-fog, L., Konge, L., Iversen, M., Møller, K., Garred, P., 2016. Alveolar recruitment of ficolin-3 in response to acute pulmonary inflammation in humans. Immunobiology 221, 690–697. https://doi.org/10.1016/j.imbio.2015.11.015.

Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11, 785–797. https://doi.org/10.1038/ni.1923.

Runza, V.L., Hehlgans, T., Echtenacher, B., Zähringer, U., Schwaeble, W.J., Männel, D.N., 2006. Localization of the mouse defense lectin ficolin B in lysosomes of activated macrophages. J. Endotoxin Res. 12, 120–126. https://doi.org/10.1179/096805106X102147.

Skjoedt, M.O., Palarasah, Y., Rasmussen, K., Vitved, L., Salomonsen, J., Kliem, A., Hansen, S., Koch, C., Skjodt, K., 2010. Two mannose-binding lectin homologues and an MBL-associated serine protease are expressed in the gut epithelia of the urochordate species Ciona intestinalis. Dev. Comp. Immunol. 34, 59–68. https://doi.org/10.1016/j.dci.2009.08.004.

Suankratay, C., Mold, C., Zhang, Y., Potempa, L.A., Lint, T.F., 1998. Complement regulation in innate immunity and the acute-phase response: inhibition of mannan-binding lectin-initiated complement cytolysis by C-reactive protein (CRP). Clin. Exp. Immunol. 113, 353–359.

Ward, P.A., 2004. The dark side of C5a in sepsis. Nat. Rev. Immunol. 4, 133.

Weber-steffens, D., Hunold, K., Kürschner, J., Giraldez, S., Elumalai, P., Schmidt, D., Trevani, A., Runza, V.L., Männel, D.N., 2013. Immature mouse granulocytic myeloid cells are characterized by production of ficolin-B. Mol. Immunol. 56, 488–496. https://doi.org/10.1016/j.molimm.2013.06.015.

Zhao, L., Ohtaki, Y., Yamaguchi, K., Matsushita, M., Fujita, T., Yokochi, T., Takada, H., Endo, Y., 2002. LPS-induced platelet response and rapid shock in mice: contribution of O-antigen region of LPS and involvement of the lectin pathway of the complement system. Blood 100, 3233–3239. https://doi.org/10.1182/blood-2002-01-0252. Supported.