M-ficolin concentrations in cord blood are related to circulating phagocytes and to early-onset sepsis

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INTRODUCTION: The pattern-recognition molecule M-ficolin is synthesized by monocytes and neutrophils. M-ficolin activates the complement system in a manner similar to mannan-binding lectin (MBL), but little is known about its role in host defense. Neonates are highly vulnerable to bacterial sepsis, in particular, due to their decreased phagocytic function.

RESULTS: M-ficolin cord blood concentration was positively correlated with the absolute phagocyte count (ρ 0.51, P < 0.001) and with immature/total neutrophil ratio (ρ 0.34, P < 0.001). When comparing infants with sepsis and controls, a high M-ficolin cord blood concentration (>1,000 ng/ml) was associated with early-onset sepsis (EOS) (multivariate odds ratio 10.92, 95% confidence interval 2.21–54.02, P = 0.003). Experimental exposure of phagocytes isolated from adult donors to *Escherichia coli* resulted in a significant time- and dose-dependent release of M-ficolin.

DISCUSSION: In conclusion, M-ficolin concentrations were related to circulating phagocytes and EOS. Our results indicate that bacterial sepsis can trigger M-ficolin release by phagocytes. Future studies should investigate whether M-ficolin may be used as a marker of neutrophil activation during invasive infections.

METHODS: We investigated M-ficolin in 47 infants with culture-positive sepsis during the first 30 days of life (13 with EOS) and in 94 matched controls. M-ficolin was measured in cord blood using time-resolved immunofluorometric assay (TRIFMA). Multivariate logistic regression was performed.

INTRODUCTION

In spite of improved perinatal care, invasive bacterial infections still account for more than one million neonatal deaths worldwide annually (1). The extraordinary vulnerability of the human newborn to infection is generally attributed to its immature adaptive immunity on one hand, and to the immaturity of neonatal neutrophils on the other (2). Indeed, the pattern of infections in neonates closely parallels that seen in neutropenic oncologic patients. Neonatal phagocytes are characterized by a functional decrease in several defense mechanisms such as impaired chemotaxis and reduced respiratory burst, which severely limit the bactericidal capacity of the neonatal immune

system (3). These developmental immaturities are even more pronounced in premature neonates. Therefore, protection of the neonate by humoral innate defense mechanisms is crucial (4). Recently, deficiencies within the complement system have been proposed to explain a part of the neonatal susceptibility to infection (5).

The complement system eliminates microorganisms and enhances the adaptive immune response (6). Even in the absence of antibodies, the complement system can be activated by the evolutionarily conserved lectin pathway. The lectin pathway consists of four soluble pattern-recognition molecules, mannan-binding lectin (MBL) and M-, L-, and H-ficolins, and of the MBL-associated serine proteases, which are able to activate downstream complement compounds (7). MBL deficiency has been extensively investigated and is associated with an increased susceptibility to sepsis (5,8,9). In contrast, very little is known on the role of ficolins in health and disease (7).

M-ficolin (also termed ficolin-1) is unique among the lectin pathway proteins insofar it is synthesized not in the liver but mainly in bone marrow and in circulating leukocytes (10). M-ficolin is encoded by the *FCN1* gene localized on chromosome 9 and consists of subunits each containing a collagen-like strand and three C-terminal recognition domains. M-ficolin binds to microbial surfaces that express *N*-acetyl-D-glucosamine or *N*-acetylgalactosamine and to sialic acid residues (11,12). M-ficolin was detected first in monocytes (12,13) but Liu *et al.* later reported localization of M-ficolin to neutrophil granula (11), which has been confirmed by others (14). Only recently, M-ficolin has been shown to be a serum protein also (15). However, the role of M-ficolin in host defense against invasive infections remains unknown.

The aim of this study was to investigate the involvement of M-ficolin in neonatal sepsis.

RESULTS

Description of Patients

A total of 47 patients who developed blood culture-positive sepsis within the first 30 days of life were enrolled in the study. Their median gestational age was 31 weeks (range 24–41)

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with a median birth weight of 1,500 g (range 555–3,700 g). Demographic and perinatal characteristics did not differ significantly between the patients and the 94 matched controls ($P > 0.05$). Further details of this cohort have been published elsewhere (5). Patients developed sepsis at a median age of 7 days (range 0–27) with 13 (28%) episodes occurring within the first 72 h of life (early-onset sepsis (EOS)) and 34 (72%) episodes after 72 h of life (late-onset sepsis). Five infants (10%) died due to septic shock. Thirty-one episodes (66%) were due to gram-positive organisms, 15 (32%) due to gram-negative organisms, and 1 (2%) due to fungal infection. Sepsis was caused by *Staphylococcus aureus* in 16 cases, coagulase-negative staphylococci in 10, *Escherichia coli* in 9, group B streptococci in 3, *Enterobacter cloacae* in 3, and one each by α -hemolytic viridans streptococci, *Listeria monocytogenes*, *Haemophilus influenzae*, *Proteus mirabilis*, *Acinetobacter baumannii*, and *Candida albicans*.

M-ficolin Cord Blood Concentrations in the Entire Study Population

When analyzing M-ficolin cord blood concentrations in the entire study population (patients and controls, $n = 141$), concentrations ranged from 17 to 4,532 ng/ml with a median of 628 ng/ml (interquartile range 377–891). The distribution of M-ficolin concentrations was non-normal, whereas the distribution of the logarithmized concentrations was approximately normal (Kolmogorov–Smirnov statistic $Z = 0.06$, $P = 0.20$; Figure 1). M-ficolin cord blood concentrations were positively correlated with gestational age (Spearman's $\rho = 0.33$, $P < 0.001$) and birth weight ($\rho = 0.32$, $P < 0.001$). Infants born after maternal chorioamnionitis had higher M-ficolin levels as compared with those without chorioamnionitis (adjusted for gestational age, $P = 0.003$).

M-ficolin Concentration and Differential Leukocyte Count

The *FCN1* gene is expressed mainly in the bone marrow and peripheral blood cells (16). We, therefore, investigated the association of M-ficolin cord blood concentration with differential leukocyte counts obtained immediately after birth (Figure 2). M-ficolin cord blood concentration was highly significantly correlated with leukocytes, metamyelocytes, band neutrophils, segmented neutrophils, and monocytes ($P < 0.001$ for all), whereas myelocytes were less correlated ($P = 0.016$) and lymphocytes not associated ($P = 0.19$). The strongest correlations were found for absolute neutrophil count ($\rho = 0.53$, $P < 0.001$; Figure 2a) and absolute phagocyte count (i.e., absolute neutrophil count plus monocytes, $\rho = 0.51$, $P < 0.001$; Figure 2e). Left shift, calculated as immature/total (I/T) neutrophil ratio, was positively correlated with M-ficolin concentrations ($\rho = 0.34$, $P < 0.001$; Figure 2f).

To investigate M-ficolin expression at various neutrophil developmental stages, we labeled cord blood cells with combinations of anti-CD16, anti-CD45, anti-CD66, and anti-M-ficolin antibodies. Granulocytes were gated based on their side scatter and CD45 staining intensity. The gated granulocytes were >99% CD66-positive (data not shown), which indicates

no presence of monocytes or lymphocytes. The granulocytes were divided into three groups (I, II, and III) based on their surface CD16 expression. No CD16 expression was present on the cells in group I, whereas an intermediate and high expression was present on the cells in groups II and III, respectively (Figure 3a). Higher staining for anti-M-ficolin was correlated with an increase in CD16 expression (Figure 3b).

M-ficolin Cord Blood Concentration in Sepsis Patients vs. Controls

M-ficolin cord blood concentrations were higher in infants with EOS than in controls (median 837 vs. 628 ng/ml, Figure 4 and Table 1). Based on receiver-operating-characteristic curve analysis, high M-ficolin cord blood concentrations ($>1,000$ ng/ml) were defined in contrast to normal M-ficolin concentrations ($\leq 1,000$ ng/ml). Six of the 13 (46%) infants with EOS and 6 of 15 (40%) infants with gram-negative sepsis as compared with 17 of 94 (18%) controls had high M-ficolin cord blood concentrations ($P = 0.028$ for EOS, $P = 0.062$ for gram-negative sepsis). Multivariate analysis confirmed that high M-ficolin was associated with EOS with an odds ratio of 10.92 (95% confidence interval 2.21–54.02, $P = 0.003$). In addition, high

Figure 1. Distribution of M-ficolin. Distribution of M-ficolin concentrations in cord blood in the whole cohort (patients and controls, $n = 141$) is shown. The frequency of patients is stated on the y-axis, the concentration of M-ficolin on the x-axis. Shown are (a) linear and (b) logarithmized M-ficolin concentrations.

Figure 2. M-ficolin correlates with peripheral phagocyte counts (patients and controls, $n = 141$). M-ficolin cord blood concentration and differential peripheral leukocyte counts are shown for (a) leukocytes, (b) lymphocytes, (c) monocytes, (d) absolute neutrophil count, (e) absolute phagocyte count, and (f) immature/total neutrophil ratio. The regression line is shown as dotted line.

M-ficolin was associated with a significantly increased risk for gram-negative sepsis (odds ratio 5.48, 95% confidence interval 1.30–23.05, $P = 0.020$). No association was found between M-ficolin and gram-positive sepsis or late-onset sepsis.

When limiting the analysis to infants exposed to chorioamnionitis ($n = 57$), patients developing EOS had higher M-ficolin cord blood concentrations as compared with those not developing EOS (median 844 vs. 571 ng/ml) and high M-ficolin ($>1,000$ ng/ml) was associated with EOS with an odds ratio of 5.33 (univariate 95% confidence interval 1.17–24.28, $P = 0.03$).

M-ficolin Release After Exposure to Gram-Negative Bacteria

To investigate whether sepsis may trigger the release of M-ficolin from phagocytes, we measured M-ficolin after experimental exposure to *E. coli*. In brief, granulocytes, peripheral blood mononuclear cells (PBMCs), and whole citrate blood from adult blood donors were incubated with different inocula of *E. coli* for 0.5 or 3 h at 37°C. Subsequently, concentrations of M-ficolin, MBL, L-ficolin, and H-ficolin were measured in the supernatants. **Figure 5** shows a time- and dose-dependent increase of M-ficolin after exposure of whole blood (**Figure 5a**), purified PBMCs (**Figure 5b**), or purified granulocytes (**Figure 5c**) to *E. coli*. No change was found in the other lectin pathway members studied (**Figure 5d**). Experiments using *Salmonella typhimurium* instead of *E. coli*, yielded similar results (data not shown).

DISCUSSION

We observed elevated M-ficolin cord blood concentrations in infants with EOS. In addition, M-ficolin concentrations correlated with the absolute phagocyte count and left shift. Experimental exposure of phagocytes to *E. coli* resulted in a time- and dose-dependent increase in M-ficolin. These findings therefore suggest that bacterial sepsis can trigger M-ficolin

release by phagocytes. To the best of our knowledge, this is the first study assessing M-ficolin in invasive infections.

The range and distribution of M-ficolin measured in our cohort are in agreement with the published data (17), but the overall concentrations were lower as compared with adults (15). M-ficolin cord blood concentration increased with gestational age, although less as compared with the hepatically synthesized lectin pathway components (5,18). We have previously described an increase of M-ficolin over the first year of life in a cohort including controls from this study (19).

M-ficolin cord blood concentrations were strongly correlated with absolute phagocyte counts and with both mature and immature neutrophils (16). We have confirmed this recently in an independent cohort of children with cancer, where M-ficolin serum concentration was correlated with phagocyte counts in peripheral blood and in bone marrow (20). In our current study, we found that granulocyte M-ficolin surface expression was positively correlated with increased CD16 surface expression. CD16 is differentially expressed during granulocyte development and both bands and segmented neutrophilic granulocytes show a high CD16 surface expression (21,22). Rorvig *et al.* (14) previously reported increasing M-ficolin mRNA expression in adult myelocytes/metamyelocytes as compared with myeloblasts/promyelocytes and band/segmented cells.

Significantly higher M-ficolin cord blood concentrations were found in infants with EOS as compared with controls. Multivariate analysis confirmed that M-ficolin concentrations $>1,000$ ng/ml were associated with a 10-fold increased risk of EOS. Although exposure to maternal chorioamnionitis may affect both M-ficolin concentrations and phagocyte counts at birth, the results of the multivariate analyses indicate that culture-proven EOS contributes to higher M-ficolin levels independently of chorioamnionitis and phagocyte counts. We observed a similar association between high M-ficolin and

Figure 3. Flow cytometry of granulocytes from human cord blood. Granulocytes were isolated from human cord blood, and gated from a side-scatter vs. CD45 plot (data not shown). The gate contained >99% CD66-positive cells (data not shown). (a) Based on their CD16 intensity (black line), the granulocytes were separated into three groups (group I: no CD16 expression; group II: intermediate CD16 expression; group III: high CD16 expression). (b) The cells in these three groups were subsequently assessed for surface M-ficolin. Cells in group I, II, and III showed a low, intermediate, and high M-ficolin surface expression, respectively. Isotype controls are indicated as shaded histograms.

gram-negative sepsis, probably because the majority of EOS infections (8 of 13) were caused by gram-negative organisms. The fact that most episodes of gram-positive sepsis had late onset (26 of 31) probably accounts for the lack of association between gram-positive sepsis and M-ficolin cord blood levels.

This clinical observation is supported in an *in vitro* bacteremia model assessing M-ficolin release after bacterial challenge. We were able to show a time- and dose-dependent release of M-ficolin in whole blood after exposure to *E. coli* and to *S. typhimurium*. This was also observed when isolated cells

Figure 4. Cord blood concentration of M-ficolin in neonates with EOS and LOS and controls. EOS, early-onset sepsis; LOS, late-onset sepsis. The dotted line is set at 1,000 ng/ml. Medians are shown. **P* value <0.05.

were studied: a high M-ficolin release was seen from isolated granulocytes, while PBMCs released less M-ficolin. The stimulation experiment was performed using adult blood cells as the amount of blood needed was considered too large for neonates. Future studies should quantitatively compare preterm, term, and adult phagocytes in their ability to express and secrete M-ficolin during sepsis.

Notably, M-ficolin concentrations were correlated with left shift, i.e., *I/T* ratio, which is an established laboratory marker of neonatal infection (23). In contrast to commonly used acute-phase markers, M-ficolin may potentially be of interest as a direct marker of neutrophil activity. Prospective studies with sequential determinations of M-ficolin in patients with neonatal sepsis are needed to assess the sensitivity and specificity of elevated M-ficolin.

In spite of the close structural and functional similarities between M-ficolin and MBL, little is known about the physiological role of M-ficolin. M-ficolin serves as a pattern-recognition molecule and binds to surface structures containing acetyl groups, such as *N*-acetyl-D-glucosamine and *N*-acetylgalactosamine, which are presented by a large variety of pathogens. Recently, M-ficolin has been shown to bind to group B streptococcus, an important pathogen causing EOS (7,11,12,24). Furthermore, M-ficolin can bind via its fibrinogen-like domain to C-reactive protein, which enhances the binding of C-reactive protein to bacteria and activates the lectin pathway of complement (25,26). This pattern-recognition receptor crosstalk is evolutionarily highly conserved, suggesting a crucial function for the initiation of the inflammatory response. Given that neutrophils represent the first and most numerous immune cells to encounter invading pathogens (27), M-ficolin release by neutrophils may permit the rapid increase of local M-ficolin concentrations in an infectious focus, thereby enhancing opsonization and bactericidal capacity.

We believe that this study has several strengths. M-ficolin concentrations were determined using time-resolved immunofluorometric assay (TRIFMA), an assay that is far superior to enzyme-linked immunosorbent assay in terms of sensitivity,

Table 1. Associations of M-ficolin cord blood concentration with sepsis, early-onset sepsis, late-onset sepsis, gram-negative sepsis, and gram-positive sepsis

	Median and frequency (%)		Univariate analysis ^a		Multivariate analysis ^{a,b}	
	Cases	Controls (n = 94)	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Sepsis (n = 47)						
M-ficolin	636 ng/ml	628 ng/ml	2.18 (0.70–6.76)	0.177	5.85 (1.24–27.78)	0.026*
M-ficolin >1,000 ng/ml	11 (23%)	17 (18%)	1.38 (0.59–3.26)	0.457	2.34 (0.80–6.89)	0.122
Early-onset sepsis (n = 13)						
M-ficolin	837 ng/ml	628 ng/ml	5.03 (0.79–31.90)	0.087	15.54 (1.55–156.19)	0.020*
M-ficolin >1,000 ng/ml	6 (46%)	17 (18%)	3.88 (1.16–13.02)	0.028*	10.92 (2.21–54.02)	0.003*
Late-onset sepsis (n = 34)						
M-ficolin	599 ng/ml	628 ng/ml	1.52 (0.43–5.42)	0.519	3.71 (0.66–20.90)	0.138
M-ficolin >1,000 ng/ml	5 (15%)	17 (18%)	0.78 (0.26–2.31)	0.655	0.91 (0.21–3.82)	0.891
Gram-negative sepsis (n = 15)						
M-ficolin	809 ng/ml	628 ng/ml	6.38 (1.01–40.46)	0.049*	15.37 (1.44–164.25)	0.024*
M-ficolin >1,000 ng/ml	6 (40%)	17 (18%)	3.02 (0.95–9.62)	0.062	5.48 (1.30–23.05)	0.020*
Gram-positive sepsis (n = 31)						
M-ficolin	585 ng/ml	628 ng/ml	1.32 (0.37–4.66)	0.668	2.20 (0.36–13.62)	0.395
M-ficolin >1,000 ng/ml	5 (16%)	17 (18%)	0.87 (0.29–2.60)	0.804	0.89 (0.19–4.15)	0.880

CI, confidence interval.

^aResults of binary logistic regression. ^bIncluding gestational age, chorioamnionitis, mode of delivery, mechanical ventilation, and absolute phagocyte count as covariates.

*P < 0.05.

dynamic range, and reproducibility (15). In our study, the definition of sepsis required the presence of both positive blood cultures and clinical signs of infection, avoiding false diagnosis due to potential contaminations. Furthermore, patients were carefully matched with controls for gestational age, sex, and chorioamnionitis, which strongly influence the risk of sepsis.

However, possible limitations need to be addressed. Only infants in whom cord blood was available were included. A selection bias, however, seems unlikely, as cord blood was routinely taken for determining *Toxoplasma gondii* serostatus in infants with unknown or negative maternal serologies. Second, similar to many studies assessing acute-phase markers in neonates (23), the small sample size reduces power and as a consequence the results need to be considered preliminary, warranting confirmation by larger prospective studies.

In conclusion, we report that M-ficolin cord blood concentrations were increased in infants with EOS. M-ficolin was secreted upon exposure of phagocytes to bacteria and M-ficolin serum concentration was related to circulating phagocytes. These preliminary results suggest that M-ficolin is involved in the early innate immune response against bacterial infections and may provide a basis for an improved understanding of the biological role of this pattern-recognition molecule. Future studies should investigate whether M-ficolin may be used as a direct marker of neutrophil activation in the course of infection.

METHODS

Patients

Infants born between November 2002 and November 2007 at the Department of Obstetrics, University of Bern, Switzerland, were eligible for this study if cord blood serum had been retrieved and

stored. Sepsis cases were defined as infants fulfilling all of the following criteria: (i) clinical signs of sepsis (temperature instability, irritability, apatheia, feeding difficulties, prolonged capillary refill, apnea, tachycardia, tachypnea); (ii) abnormal infectious parameters (C-reactive protein >20 mg/l, leukocytes <5 × 10⁹/l, immature/total neutrophil ratio >0.2); (iii) recovery of pathogens in blood culture within the first 30 days of life; and (iv) treatment for at least 7 days with intravenous antibiotics (28). Blood cultures yielding either coagulase-negative staphylococci or *S. aureus* were considered to be contaminants if the infant did not fulfill all the aforementioned criteria, or if the attending physician had considered the bacterium to be a contaminant. The study was approved by the institutional review board (Kantonale Ethikkommission Bern, Bern, Switzerland). The study of cord blood sera was approved by the same review board based on the institutional policy on use of stored biological material. Informed consent was obtained for flow cytometric and stimulation assays.

Controls and Matching Criteria

For each patient, two controls who did not suffer from infections during the neonatal period were matched for the following criteria: (i) gestational age (±1 week); (ii) sex; (iii) chorioamnionitis, defined as maternal fever, elevated maternal C-reactive protein, fetal tachycardia, prolonged rupture of membranes, and/or placental histology indicative of chorioamnionitis (29). Infants were not eligible as controls if they had developed proven or probable neonatal infection or if they had received antibiotic treatment for suspected neonatal infection for >72 h.

Differential leukocyte counts were routinely obtained at admission to the neonatal intensive care unit. The first differential leukocyte count after birth, if taken within a maximum of 12 h after birth, was included in the analysis.

M-ficolin Measurements

Cord blood is routinely collected and stored at our institution to determine *T. gondii* serology in the infants of mothers with unknown or negative serostatus. After centrifugation, cord blood sera were frozen in sterile tubes at –80 °C.

Figure 5. Release of M-ficolin after experimental bacteremia. *Escherichia coli* was added in increasing inocula to (a) 1 ml citrated whole blood, (b) 2×10^6 isolated peripheral blood mononuclear cells (PBMCs), or (c) 4×10^6 granulocytes. Controls with no *E. coli* exposure are represented on the left (three bars at the left end of the panels). M-ficolin was measured in the supernatant before adding *E. coli*, and after 30 min and 3 h of incubation with *E. coli*. (d) Control experiment showing no release of MBL (gray bars), H-ficolin (white bars), or L-ficolin (black bars) after experimental bacteremia. *E. coli* (10^8) was added to citrated whole blood. Controls with no *E. coli* exposure are represented on the left for each protein (start and 3 h). MBL, H-ficolin, and L-ficolin were measured in the supernatant after 30 min and 3 h of incubation with *E. coli*. MBL, mannan-binding lectin.

The concentrations of M-ficolin were measured by TRIFMA as previously described (15). TRIFMA is a sandwich assay taking advantage of the long-lasting fluorescence of the europium ion, which can be measured with great sensitivity by photon counting without interference from the rapidly fading background fluorescence. The assay uses a monoclonal antibody specific for M-ficolin (7G1) for coating and biotinylated 7G1 (followed by europium-labeled streptavidin) for development.

Flow Cytometric Analysis

Whole cord blood (stabilized with citrate; 100 μ l) was added to 5-ml fluorescence-activated cell-sorting tubes (55.476; Sarsted, Nümbrecht, Germany) and Fc receptors were blocked by adding 10 μ g heat-aggregated normal mouse IgG. This was prepared by incubating normal mouse IgG at 63°C for 30 min. Specific antibodies were then added and incubated for 30 min at room temperature in the dark. Red blood cells were lysed by adding 2 ml BD FACS lysis solution (349202; BD Biosciences, San Jose, CA) and incubating for 10 min at room temperature in the dark. After centrifugation and wash in Tris-buffered saline/azide/0.5% human serum albumin (10 mmol/l Tris, 140 mmol/l NaCl, 15 mmol/l Na₂HPO₄, pH 7.4 (Tris-buffered saline/azide) with 0.5% (w/v) human serum albumin), the cells were fixed in 0.9% formaldehyde (v/v) in phosphate-buffered saline (137 mmol/l NaCl, 8.1 mmol/l Na₂HPO₄, 2.7 mmol/l KCl, 1.5 mmol/l KH₂PO₄, pH 7.4). The cells were analyzed on a flow cytometer (Cytomics FC500; Beckman Coulter, Brea, CA) and the data were analyzed using FlowJo (Treestar, Ashland, OR), and compensated via the fluorescence-minus-one method. For each sample, 30,000 cells were analyzed.

Antibodies against the following antigens were used (clones in parentheses): CD16-PE (B73.1), CD45-PerCP (2b1; BD Bioscience); CD66abce-FITC (Kat4c; Dako, Glostrup, Denmark). The following isotype controls were used (clones in parentheses): IgG1-PE (DAK-GO1; Dako); IgG1-PerCP (MOPC-31C; BD Bioscience). Monoclonal anti-M-ficolin antibody (7G1) and its corresponding isotype control (mouse IgG1_{kappa}, M-7894; Sigma-Aldrich, St Louis, MO) purified on protein G from ascites) were fluorescein-labeled with fluorescein-5-EX N-hydroxysuccinimide ester (F9551; Sigma-Aldrich) following the manufacturers' protocol.

Granulocyte Isolation

Granulocytes were isolated from freshly drawn blood. Blood was collected by venipuncture from healthy adults into citrate-venoject tubes. The blood was diluted with an equal amount of 3% dextran—3% (w/v) dextran 500 (31392-50G; Sigma-Aldrich) in 0.9% NaCl—and mixed. The mixture was incubated for 45 min at room temperature, and the supernatant was collected and the cells sedimented (10 min at 200g, 4°C). The pellet was resuspended in cold H₂O for 30 s to

lyse the red blood cells. Tonicity was restored by adding 1.8% NaCl. The cells were sedimented (10 min at 200g, 4°C), resuspended in cold phosphate-buffered saline and layered on top of Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI), followed by centrifugation for 30 min at 500g, 4°C. The pelleted granulocytes were washed once and resuspended in binding buffer—10 mmol/l Tris, 140 mmol/l NaCl, pH 7.4 (Tris-buffered saline) with 0.5% (w/v) human serum albumin and 2 mmol/l CaCl₂.

PBMC Isolation

PBMCs were isolated from freshly drawn blood collected by venipuncture from healthy adults into citrate-venoject tubes. The blood was diluted with an equal amount of 0.9% NaCl, layered on top of Ficoll-Paque PLUS, and centrifuged for 25 min at 900g, 20°C. The PBMCs were harvested, washed once, and resuspended in binding buffer.

Stimulation With *E. coli*

E. coli (Y1090) was grown on blood agar plates at 37°C. A single colony was transferred to 10 ml of Luria-Bertani medium and incubated for 24 h at 37°C. This bacteria stock was kept at 4°C. Before the bacteria were to be used in the stimulation experiment, 250 μ l of the stock was resuspended in 10 ml Luria-Bertani medium and incubated for 24 h at 37°C. This was repeated once. To estimate the concentration of the bacteria, colony-forming units on blood agar plates were counted.

Granulocytes (4×10^6) in 1 ml binding buffer, PBMCs (2×10^6) in 1 ml binding buffer, or 1 ml whole blood (stabilized with citrate; all isolated from adult blood donors) were incubated with different concentrations of *E. coli* (1.1 ml total volume). The tubes were incubated for 0.5 or 3 h at 37°C, with gentle mixing every 15 min. The supernatant was collected by centrifugation (10 min at 400g). To ensure the removal of residual bacteria, the supernatant was further centrifuged (15 min at 6,000g). The supernatants were analyzed by TRIFMA.

Statistical Analysis

Association of M-ficolin concentration with sepsis, with EOS vs. late-onset sepsis (EOS, within 72 h of life; late-onset sepsis, >72 h of life), and with gram-positive vs. gram-negative sepsis was investigated using uni- and multivariate logistic regression. First, association with numeric M-ficolin concentration was assessed. Second, by means of receiver-operating-characteristic curve analysis, the optimal cutoff was defined. Gestational age, chorioamnionitis, mode of delivery, mechanical ventilation, and absolute phagocyte count were included as covariates in multivariate analyses. Sensitivity analyses using mixed logistic regression with random-effects per patient-control triplets confirmed the results (R 2.10.1; R Foundation for Statistical

Computing, Vienna, Austria; data not shown). Spearman's rank correlation and multivariate linear regression were used to assess the association of logarithmized M-ficolin concentrations with differential leukocyte counts. Two-sided tests were used throughout, and *P* values <0.05 were considered significant. SPSS 18.0 software was used (SPSS, Chicago, IL).

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