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LETTER TO THE EDITOR

M-ficolin is present in Aspergillus fumigatus infected lung and modulates epithelial cell immune responses elicited by fungal cell wall polysaccharides

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Introduction

A. fumigatus is the most common mold pathogen in the developed world and commonly causes disease in individuals with an immunodeficiency. During fungal growth, cell wall polysaccharides β-glucan and chitin are exposed, which enables immunological detection by pattern recognition molecules such as the β-glucan binding receptor dectin-1. Several immune-regulating chitin receptors have been found including epithelial fibrinogen C domain containing 1 and IgG-Fc-γ receptors. β-glucan comprises a mixed group of β-D-glucose polysaccharides while chitin is a linear homopolymer consisting of N-acetylglucosamine (GlcNAc) residues linked by β-1,4 glycosidic bonds.

The three human ficolins (M, L, and H) play essential roles in pathogen recognition and complement activation through the lectin pathway. It was recently demonstrated that A. fumigatus infection resulted in decreased fungal clearance and cytokine production in ficolin-A/B double deficient mice although these effects were complement independent. Ficolins A and B are mouse homologues of L- and M-ficolin, respectively, while there is no mouse H-ficolin homolog. Different ficolins bind A. fumigatus conidia and elicit complement activation, phagocyte activation and modulation of epithelial signaling and L- and H-ficolin are increased in bronchoalveolar fluid in invasive aspergillosis. However, no direct interaction has been reported between M-ficolin and A. fumigatus and the potential role of M-ficolin in immunity against A. fumigatus remains unknown. M-ficolin is primarily produced by peripheral blood leukocytes, bone marrow cells and type II alveolar cells. M-ficolin binding is selective for acetylated compounds, including GlcNAc, where recognition and binding occurs through a conserved calcium-dependent binding site, termed S1.

The aim of this study was to investigate the hypothesis that M-ficolin interacts with A. fumigatus through interaction with chitin and β-1,3 glucan and thereby mediates complement activation and potentiates IL-8 secretion of A549 cells, a cell line with characteristics of type II epithelial cells.

Materials and methods

Buffers - TBS: 140 mM NaCl, 10 mM Tris-HCl, and 0.02% (w/v) Na2S, pH 7.4; TBS/Tw: TBS and 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate) (Merck KGaA); TBS/Tw/Ca2+: TBS/Tw, 5 mM CaCl2; TBS/Tw/EDTA: TBS/Tw, 10 mM EDTA; PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4; ELISA coating buffer: 15 mM Na2CO3 and 34.9 mM NaHCO3, pH 9.6; Horseradish peroxidase (HRP) substrate buffer: 35 mM citric acid and 67 mM Na2HPO4, pH 5; B1 buffer: 4 mM barbital, 145 mM NaCl, 2 mM CaCl2, and 1 mM MgCl2; ROSA buffer: 20 mM Tris/Base, 1 M NaCl, 0.05% (v/v) Triton X-100 (Bie & Berntsen), 10 mM CaCl2, and 1 mg/ml human serum albumin (HSA) (10 96 97, CSL Behring); M-ficolin buffer: TBS/Tw, 5 mM EDTA, 100 μg/ml heat-inactivated normal human Ig (beriglobin, CSL Behring), 50 μg/ml bovine Ig (Lampire Biological laboratories), 850 mM NaCl, and 1 mg/ml HSA; Fixative

1These authors contributed equally to this work.

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solution (8% formaldehyde in 50 mM PIPES, 25 mM EGTA pH 7.0; 5 mM MgSO4; 5% (v/v) DMSO); Complete medium (CM): RPMI medium 1640, 10% foetal bovine serum, 2 mM L-glutamine, 50 U penicillin/ml, and 50 μg streptomycin/ml (Gibco | Thermo Fisher Scientific, for all cell culture reagents).

Immunohistochemistry - Immunohistochemistry was performed essentially as described previously\textsuperscript{13} using anti-M-ficolin mAb 036–05 (Bioporto Diagnostics A/S). Stained tissue sections were analyzed by a trained pathologist.

Human tissue samples - Human control tissues and tissues from 2 anonymous patients with chronic pulmonary aspergillosis with A. fumigatus pulmonary infection were obtained from the Diagnostic Biobank at the Department of Pathology, Odense University Hospital (Odense, Denmark). The Regional Scientific Ethical Committee for Southern Denmark approved the use of the healthy human tissue sections for research purposes (Ref. No VF20050070), and samples were obtained from patients with written informed consent.

Expression of human rM-ficolin - Human wild-type rM-ficolin was expressed as described previously.\textsuperscript{14} For the expression of rM-ficolin applied in the complement activation assays, heat-inactivated FBS was used in the cultures.

rM-ficolin ELISA - The rM-ficolin ELISA was a standard sandwich ELISA using 0.5 μg/ml monoclonal anti-M-ficolin antibody (7G1 mAb) as catching antibody and 0.5 μg/ml biotinylated 7G1 antibody as detection antibody.

Purification of rM-ficolin from cell culture supernatant (CS) - A total of 40 ml of 50% (v/v) chitin bead slurry (New England Biolabs) was packed in a column and washed with TBS/Tw/Ca\textsuperscript{2+}, 0.5 M NaCl. The rM-ficolin-enriched and serum free CHO cell CS was added to the column connected to an ÄKTA-FPLC (GE Healthcare) and washed. rM-ficolin was eluted with acetate (TBS and 250 mM Na-Acetate, pH 7.4) and EDTA (TBS and 520 mM EDTA).

Fluorescence imaging - A. fumigatus conidia CBS 101355 (Centraalbureau van Schimmelcultures, Utrecht, Netherlands) (5·10\textsuperscript{7}/ml) were grown for 14–16 hours in Sabouraud dextrose broth (Difco\textsuperscript{TM}, BD Biosciences) to generate hyphae. Grown hyphae were washed twice in 50 mM PIPES, pH 6.7, and fixed in 2 ml fixative solution for 30 min. Purified rM-ficolin was diluted in TBS/Tw/Ca\textsuperscript{2+} and incubated with the hyphae for 2 hours at room temperature (RT). The hyphae were then washed in TBS/Tw/Ca\textsuperscript{2+} and incubated with monoclonal 7G1 anti-M-ficolin for 1 hour at 4°C, washed and incubated with FITC-labeled IgG goat-anti-mouse (Dako) for 30 min at 4°C. Then, hyphae were washed, incubated with Alexa Fluor 633-labeled wheat germ agglutinin (WGA) (5 μg/ml) (Life Technologies, Invitrogen, Thermo Scientific) for 30 min at 4°C and then washed again. Images were acquired using an Olympus IX71 fluorescence microscope equipped with 4-laser optics and an F-view fluorescence CCD camera. All images were acquired and processed using Olympus Cell\textsuperscript{\textregistered} soft imaging software.

Binding of M-ficolin to different A. fumigatus strains - Four A. fumigatus isolates derived from human patients having keratitis were included in this study. They were isolated at the Aravind Eye Hospital and Postgraduate Institute of Ophthalmology (Coimbatore, Tamilnadu, India) and deposited in the Szeged Microbiological Collection (SZMC, Szeged, Hungary, www.szmc.hu) under the following strain numbers: SZMC 2419, SZMC 2421, SZMC 2422, and SZMC 2430. One A. fumigatus isolate (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA; NRRL 174) from an unknown source was also included in this study. The fungal isolates were maintained on malt extract slants (0.5% (w/v) malt extract, 0.5% (w/v) yeast extract, 0.5% (w/v) glucose, 1.0% (w/v) KH\textsubscript{2}PO\textsubscript{4}, and 1.5% (w/v) agar) at 4°C. Conidia were incubated for 0 (conidia) or 8 hours (germ-lings) at 30°C in CM with shaking (150 rev/min), centrifuged (10,000 × g, 10 min at 25°C), and washed in TBS/Ca\textsuperscript{2+} or TBS/EDTA. The suspensions were mixed 1:1 with serum containing Ca\textsuperscript{2+} or EDTA and pre-diluted 1:10. The final concentration was 10\textsuperscript{7} conidia or germ-lings/ml. Positive controls with 10% (v/v) GlcNAc-coated Sepharose beads (CL\textsuperscript{4B} GE Healthcare) (50% v/v slurry) were added to serum pre-diluted 1:20 in TBS/ Ca\textsuperscript{2+} or TBS/EDTA. Suspensions were incubated at RT for 1 hour (150 rev/min), centrifuged (10,000 × g, 10 min at 25°C), and supernatants were stored at -80°C until analysis for M-ficolin content by time-resolved fluorometry (TRIFMA) as described previously.\textsuperscript{15}

Preparation of acetylated human serum albumin (achSA) beads - Cyanogen bromide-activated Sepharose beads 4B (GE Healthcare) were coupled to HSA and subsequently acetylated as previously described.\textsuperscript{14}

Growth of A. fumigatus and preparation of AIF - A. fumigatus conidia (CBS 101355) were grown at 37°C on Sabouraud dextrose agar (Difco\textsuperscript{TM}) plates and harvested in PBS/Tw. A. fumigatus AIF was produced essentially as described previously\textsuperscript{16} and tested for endotoxin contamination using limulus amebocyte lysate assay (Lanza). The endotoxin level was <0.5 EU/ml. The amount of A. fumigatus AIF obtained was determined by weighing after vacuum centrifugation at 55°C.

Pull-down assays with rM-ficolin and polysaccharides - Pull-down experiments, which were analyzed by western
Pull-down experiments, which were analyzed by ELISA, were performed with 10 μg chitin from shrimp shell (Sigma-Aldrich), 10 μg A. fumigatus AIF, or 50 μl 50% (v/v) acHSA bead slurry. Beads and particles were washed with TBS/Tw/Ca²⁺ and mixed with 500 μl rM-ficolin CS diluted to 100 ng/ml in TBS/Tw/Ca²⁺ and in the presence of 50 mM glucose, glucosamine, GlcNAc, and acetate or 10 μM EDTA. After ON incubation (300 rev/min) at 4°C, the samples were centrifuged (10,000 g, 10 min at 25°C). Then, 100 μl of normal human serum containing MBL/MASP complexes, diluted 1:25 in ROSA-buffer, was added to each well and incubated for 2 hours at RT. Finally, the wells were washed 3 times before the sample material was loaded into duplicate into 96-well microtiter plates (Nunc™ FluoroNunc™, Thermo Scientific) and incubated for 1.5 hours at 37°C, enabling binding of the remaining C4 present in the supernatant. The plate was washed 3 times in TBS/Tw/Ca²⁺, added a freshly prepared mixture of 2 biotinylated anti-C4 mAbs (Hyb 161–1 and 161–2, BioPorto) in a concentration of 0.5 μg/ml and incubated ON at 4°C. The plate was washed 3 times and Europium³⁺-labeled streptavidin (Perkin Elmer) diluted 1:1000 in TBS/Tw/EDTA was added. The plate was incubated for 1 hour, washed and 200 μl enhancement buffer (Perkin Elmer) was added. The amount of europium was measured using TRIFMA as described previously.⁶

**rM-ficolin-mediated C4b generation -** C4b generation assays were conducted using β-1,3 glucan (curdlan) (Sigma-Aldrich), A. fumigatus AIF and acHSA beads. A total of 0.5 mg curdlan or A. fumigatus AIF or 10 μl 50% (v/v) acHSA bead slurry was washed with TBS/Tw/Ca²⁺ and incubated ON at 4°C with 500 μl rM-ficolin serially diluted in TBS/Tw/Ca²⁺. Samples were washed and incubated with 300 μl MASP-2 diluted to 200 ng/ml in TBS/Tw/Ca²⁺ and incubated end-over-end for 2 hours at RT. Samples were washed in TBS/Tw/Ca²⁺ and 300 μl C4 diluted to 80 ng/ml in B1 buffer was added. Then, the samples were incubated end-over-end for 1.5 hours at 37°C. After pelleting, 100 μl supernatant was added to 500 μl TBS/Tw/EDTA to stop the reaction and then loaded in duplicate into 96-well microtiter plates (Nunc™ FluoroNunc™, Thermo Scientific) and incubated ON at 4°C. The wells were previously coated with 1 μg/ml anti-C4–1 in 100 μl ELISA coating buffer ON at 4°C and blocked with 200 μl TBS containing 0.1% HSA (v/v) for 2 hours at RT. Polyclonal biotinylated rabbit anti-C4 was used as a detector antibody. The plates were developed using TRIFMA as described previously.⁷

**Growth of A. fumigatus isolates in the presence of rM-ficolin -** MBL-deficient human serum was incubated with fungal hyphae for 30 min on ice (150 rev/min), centrifuged (10,000 × g, 10 min at 25°C), and serum supernatant was used for the cultures described beneath. A total of 25 μl 10⁷ conidia was grown ON in RPMI-1640 medium (Sigma-Aldrich) at 4°C (150 rev/min). The resulting fungal hyphae were centrifuged (10,000 × g for 10 min at 25°C) and incubated at 4°C for one hour in 25 μl RPMI with a serial dilution of purified rM-ficolin. Then, 25 μl MBL-deficient serum supernatant was added to the test tubes resulting in final concentrations of rM-ficolin of 1500, 150, 15, 1.5, and 0.15 ng/ml. The pH was adjusted to 7.4 if necessary. The suspensions were incubated at 37°C for 0 and 8 hours (150 rev/min). Finally, pelleted (10,000 × g, 10 min at 25°C) fungal material was lyophilized and weighed.

**IL-8 secretion from A549 lung epithelial cells challenged with A. fumigatus AIF and rM-ficolin -** A total...
of 10^5 human A549 type II alveolar adenocarcinoma cells were seeded on 24-well plates (Nunc™) in 500 µl CM. The following solutions and suspensions were freshly prepared in serum-free medium and incubated for 1 hour at 37°C: rM-ficolin control, which was the supernatant produced by centrifugation of 2 mg/ml A. fumigatus AIF and 20 µg/ml purified rM-ficolin at 1,000 × g for 5 min; 2 mg/ml A. fumigatus AIF control; and 2 mg/ml A. fumigatus AIF + 5 µg/ml, 10 µg/ml M-ficolin or 20 µg/ml rM-ficolin. The ON cultures of A549 cells were washed twice in cold sterile PBS and then incubated with 200 µl of the appropriate challenge for 6 hours at 37°C. Next, the cell CSs were centrifuged at 1,000 × g for 5 min and stored at -20°C until analysis.

**IL-8 ELISA** – IL-8 measurements on cell CSs were performed with the Human CXCL8/IL-8 DuoSet ELISA, DY208 (R&D Systems) according to the manufacturer’s recommendations.

**Statistical analysis** – Prism (GraphPad Software, Inc.) version 6.0b was used for all graphs and statistical analyses. Bindings between rM-ficolin and different A. fumigatus strains or polysaccharides and growth of A. fumigatus isolates with different culture conditions were analyzed by ANOVA with Tukey’s multiple comparisons test. Secretion of IL-8 was analyzed by one-way ANOVA with Tukey’s multiple comparison test. P-value < 0.05 was considered statistically significant.

**Results**

**Localization of M-ficolin to the periphery of the aspergilloma** – Positive control immunostaining of M-ficolin in monocytes/granulocytes was observed in the spleen (Fig. 1A). Weak alveolar macrophage staining was observed in non-infected tissue (Fig. 1B). Strong M-ficolin immunoreactivity was detected in monocytes/granulocytes in the interface between fungal balls and the surrounding pulmonary scar tissue (Fig. 1C-G) and in all blood vessels (shown from infected lung) (Fig. 1H). M-ficolin immunoreactivity was undetectable in scar tissue and in central necrotic zones of fungal balls.

**Characterization of M-ficolin binding to A. fumigatus** – Conidia and germlings from 5 different A. fumigatus strains were incubated with purified rM-ficolin, and the residual rM-ficolin in the supernatant after centrifugation was measured (Fig. 2A-B). Conidia and germlings pulled out 40–70% of rM-ficolin in the presence of calcium and binding was significantly calcium-dependent for strains SZMC 2419 (p < 0.01), SZMC 2430 (p < 0.01) and NRRL 174 (p < 0.05).

Next, fluorescence microscopy showed that rM-ficolin bound to the surface of the A. fumigatus hyphae and mother-bud, while the tip of polarized growing buds were undetected (Fig. 2C-J). Chitin (WGA) was localized to sites of septum formation, the mother-bud, and to evolving hyphae with polarized growth (Fig. 2H). The binding of rM-ficolin to the hyphae was partially co-localized with chitin (WGA) in the mother-bud (Fig. 2I) and was inhibited by co-incubation with GlcNAc (data not shown). rM-ficolin also bound regions with low chitin content.

**Discussion**

In the present study, we investigated the possible role of M-ficolin in the recognition of fungal cell wall polysaccharides, which are exposed during fungal growth. We found that M-ficolin is present in human lung with aspergilloma and binds...
A. *fumigatus* calcium-dependently. M-ficolin further binds cell wall components chitin, β-1,3 glucan and *A. fumigatus* AIF and mediates complement activation, but provides no initial growth disadvantage of *A. fumigatus*. Finally, we found that rM-ficolin opsonization of *A. fumigatus* AIF increases IL-8 secretion in A549 lung epithelial cells.

M-ficolin immunoreactivity was located to monocytes/granulocytes in the vicinity of the pulmonary...
aspergilloma in accordance with a role in limiting the growth in a surface reaction. This was an intriguing observation, but it did not reveal whether soluble M-ficolin could react with *A. fumigatus*. Previous studies have reported that M-ficolin does not bind *A. fumigatus* conidia, however, we detected binding to conidia of 5 different *A. fumigatus* strains. This discrepancy between observations may be due to the high variability in the sialic acid ligand density on conidia of *A. fumigatus* strains. However, the focus of this study was related to recognition of polysaccharides in the cell wall of growing fungus and we initially envisioned chitin as the main M-ficolin ligand because chitin is a polymer of the known ligand GlcNAc. Purified rM-ficolin bound directly to the growing *A. fumigatus* hyphal cell wall but was only partially co-localized to chitin-rich zones, which suggests that M-ficolin recognizes alternative *A. fumigatus* ligands as well. Following, we studied the most abundant polysaccharide of the fungal cell wall, β-1,3 glucan, after determining that M-ficolin interactions with the growing fungal cell wall were observed for various different *A. fumigatus* strains. Our demonstration of binding of rM-ficolin to β-1,3 glucan is a novel observation, as no other non-acetylated compound has been demonstrated as a ligand for M-ficolin.

We further demonstrated functional interaction with *A. fumigatus* AIF, which mainly consists of a branched β-1,3/1,6 glucan backbone, but also comprises linear β-1,3/1,4 glucan and chitin. The binding profiles of rM-ficolin to chitin, β-1,3 glucan, and *A. fumigatus* AIF were highly similar to each other and to the binding profile for the positive control acHSA with inhibition by acetate, GlcNAc, and EDTA. The acetylated small molecule propionate was moreover included as inhibitor in some experiments. Thus, the M-ficolin binding profiles showed specificity and indicated involvement of the conserved ficolin S1 binding site, which mediates interaction with GlcNAc, N-acetylgalactose and N-acetylneuramic acid. However, due to the unexpected interaction with β-1,3 glucan we cannot rule out that additional binding sites may exist and support S1-mediated binding of this polysaccharide. Such additional interactions may further be suggested based on the non-significant inhibition by EDTA seen in some of *A. fumigatus* strains in the performed pull-down assays. Following, successful

Figure 2. Characterization of M-ficolin binding to *A. fumigatus*. The binding between M-ficolin and *A. fumigatus* strains NRRL 174 (174), SZMC 2419 (2419), SZMC 2421 (2421), SZMC 2422 (2422) and SZMC 2430 (2430) was on conidia (0 hours) and germlings (8 hours) using pull-down assays in the presence of (A) 5 mM Ca2+ or (B) 10 mM EDTA. The data are triplicates from 2 independently performed experiments. Data shown are mean ± SEM. Significance was determined using 2-way ANOVA with Holm-Sidak’s multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001. (C) Light microscopy of growing hyphae, original magnification 100×. (D) Light microscopy of growing hyphae, original magnification 400×. (E-F) Localization of regions recognized by M-ficolin (green) and (G-H) localization of chitin (WGA, red). (I-J) Overlay images. The lengths of the bars are in micrometers.
complement activation by the polysaccharides was performed using physiologically relevant concentrations of rM-ficolin that, however, were insufficient to mediate complement activation by the control ligand aHSA. We observed no apparent growth inhibition of *A. fumigatus* with increasing concentrations of M-ficolin. The assay was performed in the presence of 50% MBL-deficient serum, which was preincubated with fungal hyphae to remove potential anti-fungal antibodies. The negative outcome suggests that M-ficolin-mediated complement activation either may not result in a functional complement membrane attack complex or predominantly may occur with free polysaccharide particles liberated from dying cells. However, this observation does not exclude potential complement-mediated effects on inflammation and opsonization.

H-ficolin is reported to increase *A. fumigatus* induced IL-8 secretion from A549 cells. Whether this could also be achieved following M-ficolin opsonization was previously unknown. We observed potentiation of IL-8 secretion following A549 cell challenge with rM-ficolin-opsonized *A. fumigatus* AIF. Thus, our data support that M-ficolin mediates the initiation of inflammation and enhancement of neutrophil recruitment.

**Figure 3.** Pull-down assays with rM-ficolin binding the polysaccharides chitin, β-glucan and *A. fumigatus* AIF. Western blotting assays using the monoclonal anti-M-ficolin 7G1 antibody to detect rM-ficolin in the pellet resulting from incubation with (A) chitin beads, (B) β-1,3 glucan, (C) *A. fumigatus* AIF and (D) aHSA beads (control). The binding was performed in the presence of 10 mM EDTA, 50 mM acetate, propionate, glucose, glucosamine, or GlcNAc. The results are representative of 3 independent experiments. rM-ficolin was further measured by ELISA in the supernatant resulting from incubation with (E) chitin, (F) β-1,3 glucan and (G) *A. fumigatus* AIF and (H) aHSA beads. The data are from 3 independent experiments. The data shown are mean ± SEM. The data were analyzed by one-way ANOVA with Holm-Sidak’s multiple comparisons test, *p* < 0.05, **p** < 0.01, ***p*** < 0.001.
Figure 4. Functional interactions between rM-ficolin and fungal polysaccharides. (A) Concentration-dependent rM-ficolin-mediated chitin complement C4 consumption assay and complement C4b generation assays for (B) β-1,3 glucan, (C) A. fumigatus AIF and (D) acHSA (control). Dry weight (mg) of A. fumigatus (E) NRRL 174 and (F) SZMC 2430 cultures before and after an 8-hours incubation in 50% MBL-deficient serum and in the presence of various concentrations (0–1500 ng/ml) of rM-ficolin. (G) IL-8 secretion in A549 cell CS collected 6 hours after challenge with rM-ficolin alone or after incubation with A. fumigatus AIF or increasing concentrations of rM-ficolin opsonized A. fumigatus AIF. Blank control = serum free medium. The data shown are mean ± SEM of quadruplicate measurements representative of 2 (A) and duplicates from 3 (B-G) independent experiments, *p < 0.5, **p < 0.01, ***p < 0.001. #relative to background, relative to A. fumigatus AIF control, 1relative to rM-ficolin control.
Effects of M-ficolin modulation of phagocyte activity may further be anticipated, but were not explored. A model of the M-ficolin-mediated effects observed in this study is provided in Fig. 5.

In summary, the data are in support of recent in vivo data showing reduced fungal clearance in ficolin-deficiency.5 These first observations of binding of rM-ficolin to fungal polysaccharides, including the novel M-ficolin ligands chitin and β-1,3 glucan and resulting modulation of human epithelial cells, may be essential for efficient immune activation during fungal infection of the human lung.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| acBSA        | acetylated BSA |
| A. fumigatus | Aspergillus fumigatus |
| AIF          | alkali-insoluble fraction |
| BSA          | bovine serum albumin |
| CHO cells    | Chinese hamster ovary cells |
| CS           | culture supernatant |
| CM           | complete medium |
| FReDs        | fibrinogen-related domains |
| GlcNAc       | N-acetylglucosamine |
| IL-8         | interleukin 8 |
| MASP-2       | MBL-associated serine protease 2 |
| MBL          | mannan-binding lectin |
| ON           | overnight |
| rM-ficolin    | recombinant M-ficolin |
| RT           | room temperature. |
| S. typhimurium | Salmonella typhimurium |
| TRIFMA       | time-resolved fluorometry |
| WGA          | wheat germ agglutinin |

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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Jensen-K, Lund-KP, Christensen-KB, Holm-AT, Jepsen-CS, Dubey-LK and Galgóczy-L performed experiments; Jensen-K, Lund-KP and Sorensen-GL wrote the manuscript; Moeller-JB, Schlosser-A, Thiel-S, Holmskov-U and Sorensen-GL designed experiments; Thiel-S and Sorensen-GL conceived study; all co-authors approved final version of the manuscript.

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