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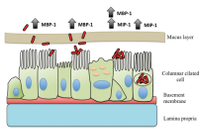

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LETTER TO THE EDITOR

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

Kasper Jensen[a,†], Kit P. Lund[a,†], Kimmie B. Christensen[a], Anne T. Holm[a], Lalit Kumar Dubey[a,b], Jesper B. Moeller[a,c], Christine S. Jepsen[a], Anders Schlosser[a], László Galgóczy[d,e], Steffen Thiel[f], Uffe Holmskov[a], and Grith L. Sorensen[a]

[a]Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark; [b]Global Health Institute, School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; [c]Jill Roberts Institute for Research in Inflammatory Bowel Disease, Weill Cornell Medicine, Cornell University, New York, NY, USA; [d]Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary; [e]Division of Molecular Biology, Biocenter, Medical University of Innsbruck, Innsbruck, Austria; [f]Department of Biomedicine, Aarhus University, Aarhus, Denmark

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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.[1] Several immune-regulating chitin receptors have been found including epithelial fibrinogen C domain containing 1 and IgG-Fc-γ receptors.[2,3] β-glucan comprises a mixed group of β-D-glucose polysaccharides while chitin is a linear homopolymer consisting of N-acetylglucoseamine (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.[4] 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.[5] Ficolins A and B are mouse homologues of L- and M-ficolin, respectively, while there is no mouse H-ficolin homolog. Different ficolins[6-9] 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.[7,9] However, no direct interaction has been reported between M-ficolin and *A. fumigatus*[10] 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.[4] M-ficolin binding is selective for acetylated compounds, including GlcNAc, where recognition and binding occurs through a conserved calcium-dependent binding site, termed S1.[11,12]

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) NaN_3, pH 7.4; TBS/Tw: TBS and 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate) (Merck KGaA); TBS/Tw/Ca^{2+}: TBS/Tw, 5 mM $CaCl_2$; TBS/Tw/EDTA: TBS/Tw, 10 mM EDTA; PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4; ELISA coating buffer: 15 mM Na_2CO_3 and 34.9 mM $NaHCO_3$, pH 9.6; Horseradish peroxidase (HRP) substrate buffer: 35 mM citric acid and 67 mM Na_2HPO_4, pH 5; B1 buffer: 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$; ROSA buffer: 20 mM Tris/Base, 1 M NaCl, 0.05% (v/v) Triton X-100 (Bie & Berntsen), 10 mM $CaCl_2$, 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

CONTACT Grith L. Sorensen glsorensen@health.sdu.dk University of Southern Denmark, J.B. Winsloews Vej 25.3, DK-5000 Odense, Denmark.
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[†]These authors contributed equally to this work.

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[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.[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^{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 von Schimmelcultures, Utrecht, Netherlands) ($5{\cdot}10^5$/ml) were grown for 14–16 hours in Sabouraud dextrose broth (Difco™, 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^{2+} and incubated with the hyphae for 2 hours at room temperature (RT). The hyphae were then washed in TBS/Tw/Ca^{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^F 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_2PO_4, and 1.5% (w/v) agar) at 4 °C. Conidia were incubated for 0 (conidia) or 8 hours (germlings) at 30°C in CM with shaking (150 rev/min), centrifuged (10,000×g, 10 min at 25°C), and washed in TBS/Ca^{2+} or TBS/EDTA. The suspensions were mixed 1:1 with serum containing Ca^{2+} or EDTA and prediluted 1:10. The final concentration was 10^7 conidia or germlings/ml. Positive controls with 10% (v/v) GlcNAc-coated Sepharose beads (CL$^-$4B GE Healthcare) (50% v/v slurry) were added to serum prediluted 1:20 in TBS/Ca^{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.[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.[14]

Growth of A. fumigatus and preparation of AIF - *A. fumigatus* conidia (CBS 101355) were grown at 37°C on Sabouraud dextrose agar (Difco™) plates and harvested in PBS/Tw. *A. fumigatus* AIF was produced essentially as described previously[16] and tested for endotoxin contamination using limulus amebocyte lysate assay (Lonza). 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

blotting, were performed with 100 μl 50% (v/v) chitin bead slurry (New England Biolabs), 100 μl 50% (v/v) acHSA bead slurry, 2 mg β-1,3 glucan (curdlan) from *Alcaligenes faecalis* (Sigma-Aldrich), 2 mg *A. fumigatus* AIF or 100 μl 50% (v/v) acHSA bead slurry in incubation with 1 ml 7 μg/ml rM-ficolin CS ON (300 rev/min) at 4°C. Pull-down experiments were further performed in the presence of 50 mM glucose, glucosamine, GlcNAc, acetate and propionate or 10 mM EDTA (Sigma-Aldrich).

Pull-down experiments, which were analyzed by ELISA, were performed with 10 mg chitin from shrimp shell (Sigma-Aldrich), 10 mg β-1,3 glucan (curdlan), 10 mg *A. fumigatus* AIF, or 50 μl 50% (v/v) acHSA bead slurry. Beads and particles were washed with TBS/Tw/Ca^{2+} and mixed with 500 μl rM-ficolin CS diluted to 100 ng/ml in TBS/Tw/Ca^{2+} and in the presence of 50 mM glucose, glucosamine, GlcNAc, and acetate or 10 mM EDTA. After ON incubation (300 rev/min) at 4°C, the samples were centrifuged (10,000 × g), and 100 μl of the supernatant was analyzed by ELISA.

SDS-PAGE and western blotting - The pelleted particles and beads were washed 3 times in TBS/Tw/Ca^{2+}. Bound protein was eluted by boiling pelleted particles in SDS-PAGE buffer and resolved under non-reduced conditions by SDS-PAGE followed by western blotting using the 7G1 mAb for rM-ficolin detection.

Mannan-MBL-MASP coating of microtiter plates - A 96-well microtiter plate was coated with 10 μg/ml mannan (purified in house from *Saccharomyces cerevisiae*) in ELISA coating buffer and incubated ON. The wells were blocked with 1 mg/ml HSA in TBS and incubated for 1 hour, followed by washing 3 times in TBS/Tw/Ca^{2+}. 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 as described below.

rM-ficolin-mediated C4 consumption - C4 consumption assays were conducted using chitin beads (New England Biolabs). A total of 10 μl 50% (v/v) chitin bead slurry was washed with TBS/Tw/Ca^{2+} and incubated ON with 500 μl rM-ficolin CS in serial dilution at 4°C. The samples were washed and incubated end-over-end for 2 hours at RT with 300 μl recombinant MASP-2 prepared as described previously[14] and diluted to 100 ng/ml in TBS/Tw/Ca^{2+}. The samples were then washed, combined with 300 μl purified human C4[14] diluted to 80 ng/ml in B1 buffer, and incubated end-over-end for 1.5 hours at 37°C. After centrifugation (10,000 × g), the reaction was stopped by adding 100 μl supernatant to 500 μl TBS/Tw/EDTA, and then loaded onto mannan-MBL-MASP-2-coated 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/Ca^{2+}, 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 Europium^{3+}-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.[15]

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/Ca^{2+} and incubated ON at 4°C with 500 μl rM-ficolin serially diluted in TBS/Tw/Ca^{2+}. Samples were washed and incubated with 300 μl MASP-2 diluted to 200 ng/ml in TBS/Tw/Ca^{2+} and incubated end-over-end for 2 hours at RT. Samples were washed in TBS/Tw/Ca^{2+} 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.[15]

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 10^7 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^{TM}) 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 analyzes. 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 Holm-Sidak'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. 2J) and was inhibited by co-incubation with GlcNAc (data not shown). rM-ficolin also bound regions with low chitin content.

Binding of rM-ficolin to chitin, β-1,3 glucan and A. fumigatus AIF – Pull-down assays showed binding of rM-ficolin to chitin beads, β-1,3 glucan, *A. fumigatus* AIF, and acHSA beads (Fig. 3A-D). These bindings were partially inhibited by EDTA, acetate, and GlcNAc, as shown by the disappearance of the lowest MW rM-ficolin bands. The presence of the non-acetylated compounds glucose, glucosamine, and propionate showed no or weak inhibition. In a separate set of similar pull-down experiments, quantitative ELISA showed that the concentration of rM-ficolin in the supernatant was significantly reduced by chitin, β-1,3 glucan, *A. fumigatus* AIF, and acHSA beads (Fig. 3E-H). The presence of inhibitors appeared to affect the interaction with assay detection antibodies. This resulted in apparent inhibition > 100% in some ELISA assays.

M-ficolin-mediated complement activation – Two different assays were used to demonstrate dose-dependent complement activation; a "C4 consumption" assay (Fig. 4A) and a "C4b deposition" assay (Fig. 4B-D), respectively. Dose-dependent rM-ficolin-mediated complement activation was observed in response to chitin beads, β-1,3 glucan and *A. fumigatus* AIF (Fig. 4A-C), while no detectable C4b deposition by the known M-ficolin ligand acHSA was observed using the same conditions (Fig. 4D).

No rM-ficolin modulation of A. fumigatus growth – The ability of rM-ficolin to mediate growth inhibition of *A. fumigatus* clinical isolates was tested in cultures containing 50% serum. No rM-ficolin-dependent growth inhibition was detected after 8 hours-old culture of *A. fumigatus* (Fig. 4E-F).

rM-ficolin-enhanced AIF stimulation of lung epithelial cell IL-8 secretion – rM-ficolin opsonization of *A. fumigatus* AIF induced a significant and dose-dependent increase in the A549 lung epithelial cell secretion of IL-8 compared with challenge with un-opsonized AIF and rM-ficolin alone after 6 hours of treatment (Fig. 4G).

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

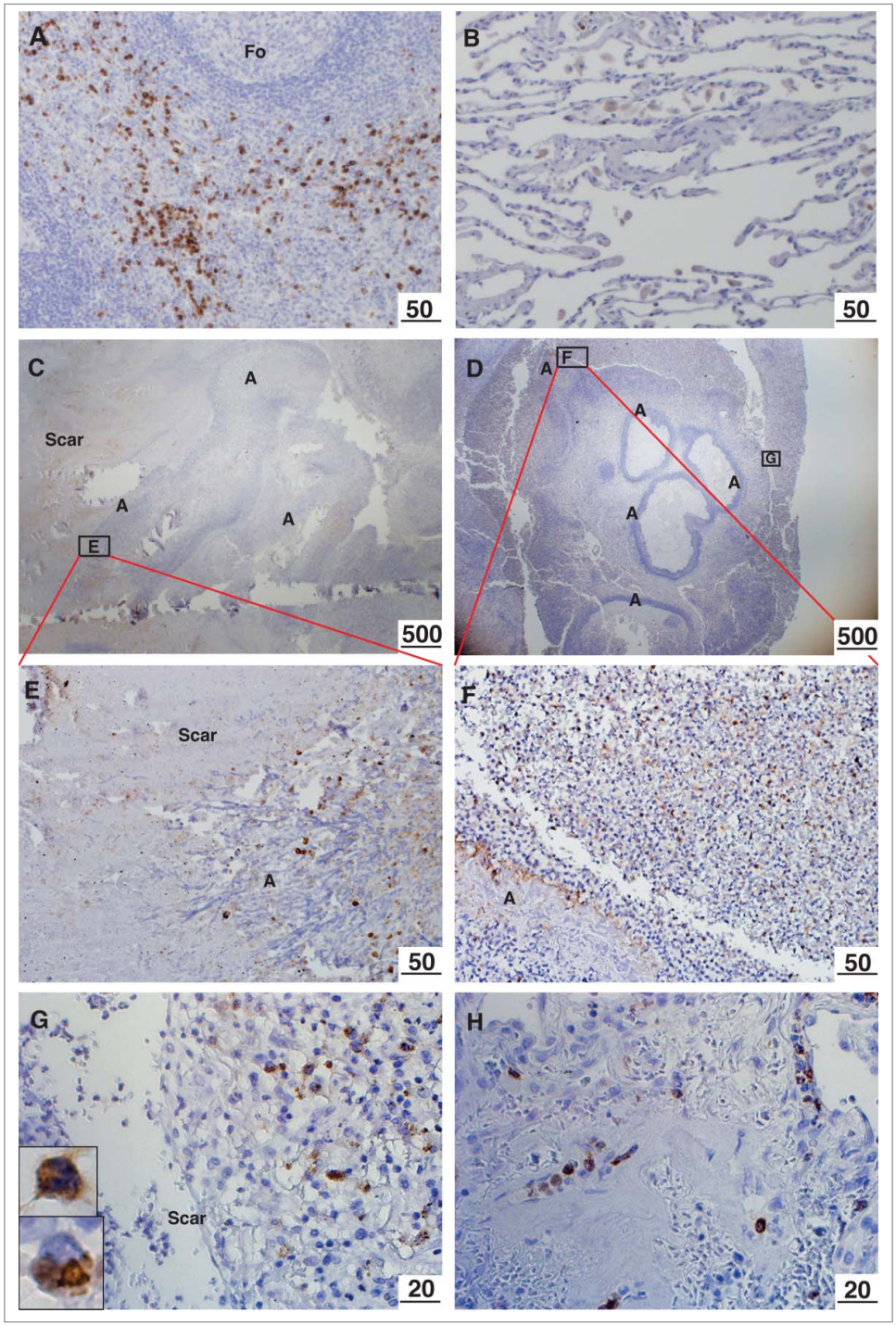

Figure 1. Immunohistochemical localization of M-ficolin to the aspergilloma. Brown staining indicates presence of M-ficolin. *(A)* Control immunostaining of monocytes/granulocytes in the spleen. *(B)* Control alveolar tissue. Overview of elongated *A. fumigatus* fungal balls surrounded by pulmonary scar tissue in patient 1 *(C)* and patient 2 *(D)*. The boxes indicate the location of images E-G. *(E)* Pulmonary scar tissue and *A. fumigatus* mycelial zone. *(F-G)* Peripheral zone of aspergilloma (Upper insert: granulocyte. Lower insert: monocyte). *(H)* Pulmonary blood vessels in scar tissue from a lung with *A. fumigatus* infection. Fo = follicle. A = *A. fumigatus*. Scar = scar tissue. The lengths of the bars are in micrometers.

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

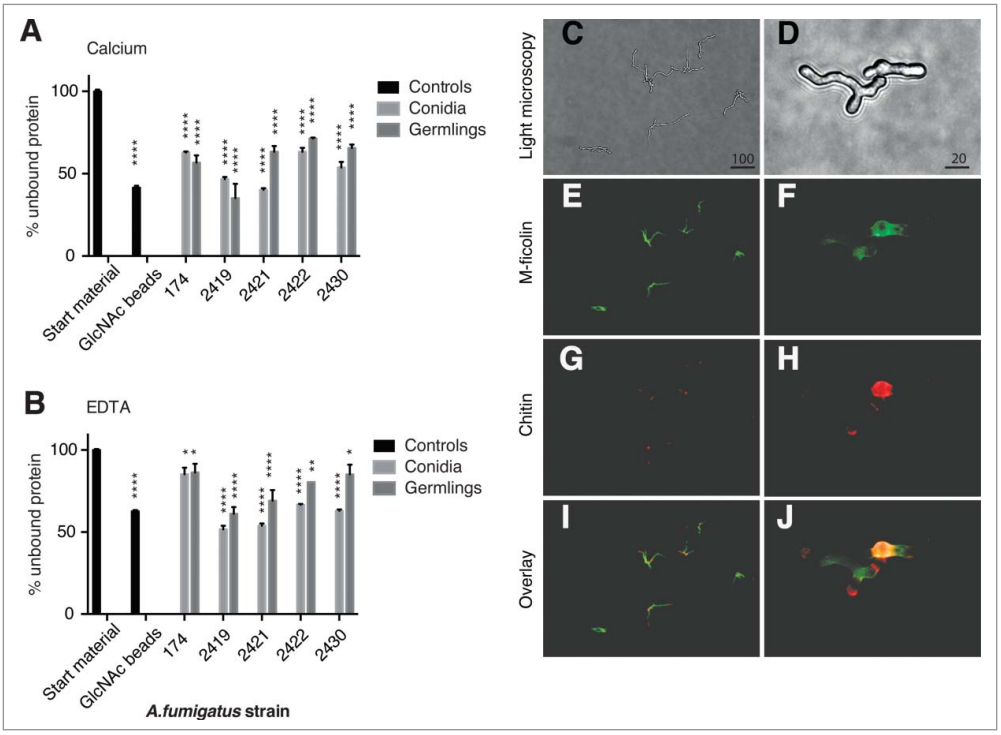

Figure 2. Characterization of M-ficolin binding to *A. fumigatus*. The binding between M-ficolin and *A. fumigatus* strains NRRL 174 (174), SZMC 2419 (2419), SZMC 2421 (2421), SZMC 2422 (2422) and SZMC 2430 (2430) was on conidia (0 hours) and germlings (8 hours) using pull-down assays in the presence of *(A)* 5 mM Ca^{2+} 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.

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,[10] 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.[17] 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.[16] 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.[12] 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

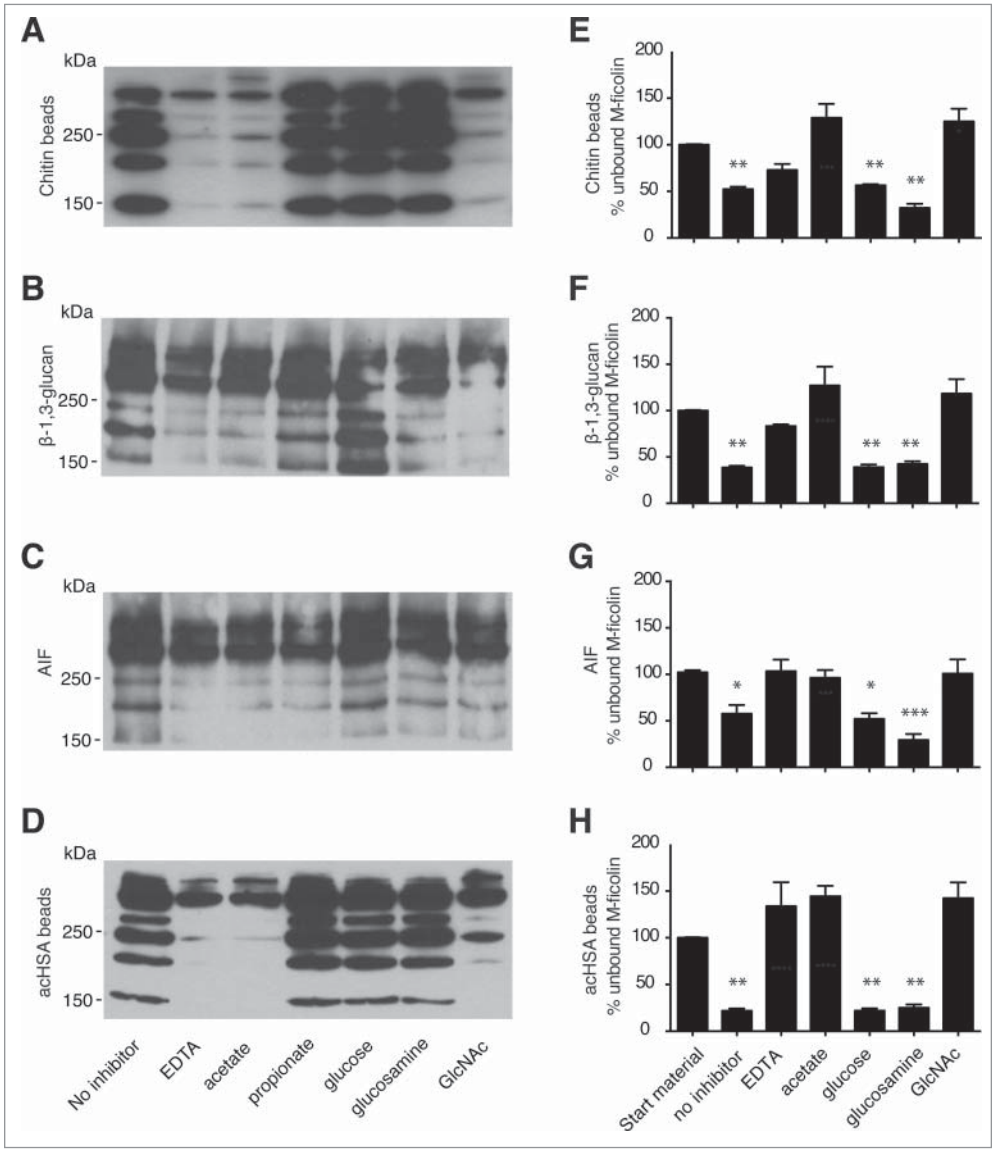

Figure 3. Pull-down assays with rM-ficolin binding the polysaccharides chitin, β-glucan and *A. fumigatus* AIF. Western blotting assays using the monoclonal anti-M-ficolin 7G1 antibody to detect rM-ficolin in the pellet resulting from incubation with (*A)* chitin beads, (*B)* β-1,3 glucan, (*C) A. fumigatus* AIF and *(D)* acHSA 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)* acHSA 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, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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 acHSA.

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.[9] 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.

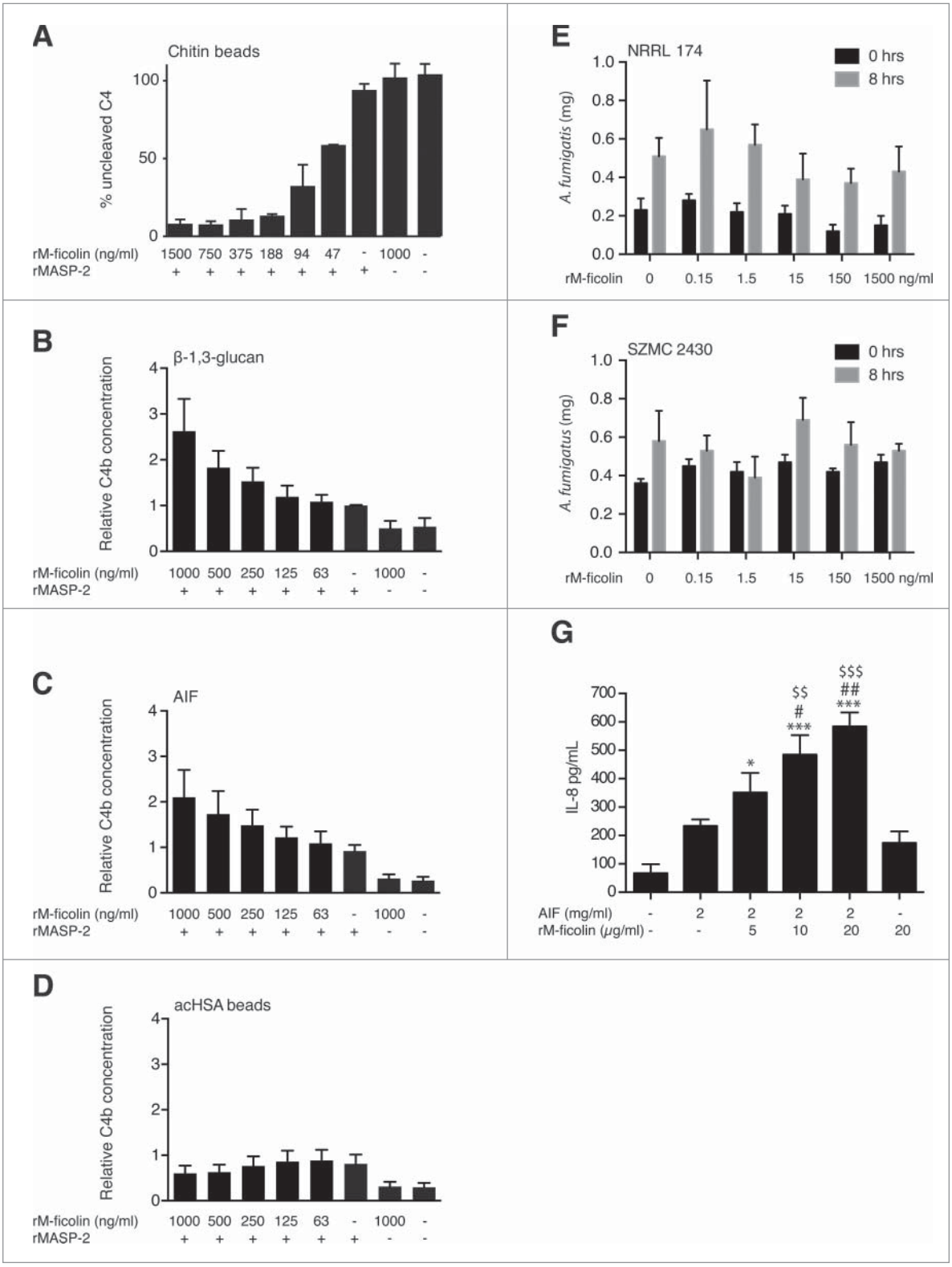

Figure 4. Functional interactions between rM-ficolin and fungal polysaccharides. *(A)* Concentration-dependent rM-ficolin-mediated chitin complement C4 consumption assay and complement C4b generation assays for *(B)* β-1,3 glucan, *(C) A. fumigatus* AIF and *(D)* acHSA (control). Dry weight (mg) of *A. 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, $^{\$}$relative to rM-ficolin control.

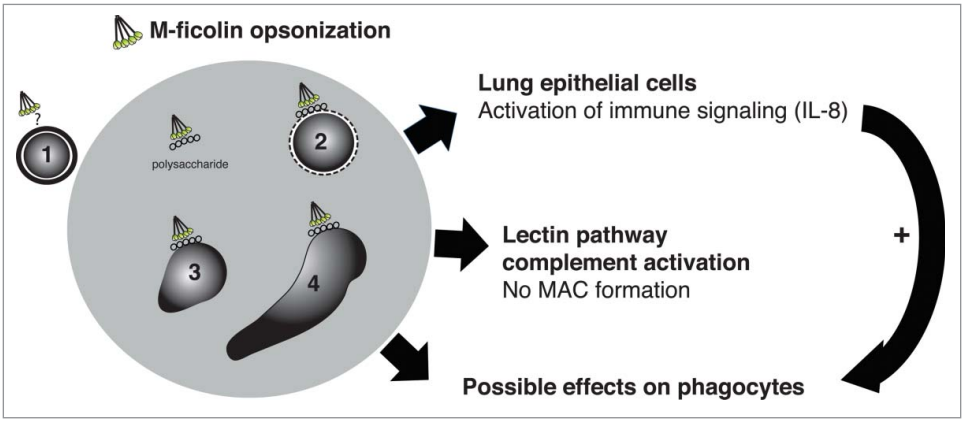

Figure 5. Schematic depiction of the interaction between M-ficolin-opsonized *A. fumigatus* and the innate immune system of the host. M-ficolin interacts with different life stages of *A. fumigatus*. The M-ficolin ligand on the surface of resting conidia (1) is unknown, however the effects of opsonization may be overlapping with other *A. fumigatus* life stages. Swollen conidia (2), germlings (3) and hyphae forming stages (4) expose cell wall polysaccharides β-glucan and chitin, which mediates M-ficolin interaction. M-ficolin enhances fungal polysaccharide-mediated pulmonary epithelial IL-8 secretion involved in phagocyte recruitment and enhances complement activation. The complement activation does not result in the formation of membrane attack complexes (MAC). Possible effects of M-ficolin-enhanced activation of phagocyte functions are unknown.

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

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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