Synergy between Ficolin-2 and Pentraxin 3 Boosts Innate Immune Recognition and Complement Deposition*

Ying Jie Ma1, Andrea Doni2, Tina Hummelsøj3, Christian Honoré4, Antonio Bastone5, Alberto Mantovani5, Nicole M. Thielens6, and Peter Garred1

From the 1Laboratory of Molecular Medicine, Department of Clinical Immunology, Section 7631, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen, Copenhagen 2100, Denmark, the 2Istituto Clinico Humanitas, Rozzano 20089, Italy, the 3Department of Biochemistry and Molecular Pharmacology, Mario Negri Institute for Pharmacological Research, Milan 20157, Italy, and the 4Laboratoire d’Enzymologie Moléculaire, Institut de Biologie Structurale Jean-Pierre Ébel, UMR 5075, CNRS-CEA-Université Joseph Fourier, Grenoble 5075, France

The long pentraxin 3 (PTX3) is a multifunctional soluble pattern recognition molecule that is crucial in innate immune protection against opportunistic fungal pathogens such as Aspergillus fumigatus. The mechanisms that mediate downstream effects of PTX3 are largely unknown. However, PTX3 interacts with C1q from the classical pathway of the complement. The ficolins are recognition molecules of the lectin complement pathway sharing structural and functional characteristics with C1q. Thus, we investigated whether the ficolins (Ficolin-1, -2, and -3) interact with PTX3 and whether the complexes are able to modulate complement activation on A. fumigatus. Ficolin-2 could be affinity-isolated from human plasma on immobilized PTX3. In binding studies, Ficolin-1 and particularly Ficolin-2 interacted with PTX3 in a calcium-independent manner. Ficolin-2, but not Ficolin-1 and Ficolin-3, bound A. fumigatus directly, but this binding was enhanced by PTX3 and vice versa. Ficolin-2-dependent complement deposition on the surface of A. fumigatus was enhanced by PTX3. A polymorphism in the FCN2 gene causing a T236M amino acid change in the fibrinogen-like binding domain of Ficolin-2, which affects the binding to GlcNAc, reduced Ficolin-2 binding to PTX3 and A. fumigatus significantly. These results demonstrate that PTX3 and Ficolin-2 may recruit each other on pathogens. The effect was alleviated by a common amino acid change in the fibrinogen-like domain of Ficolin-2. Thus, components of the humoral innate immune system, which activate different complement pathways, cooperate and amplify microbial recognition and effector functions.

The ficolins are multimeric collagen-like proteins consisting of an N-terminal domain, a collagen-like domain (CD), and a C-terminal fibrinogen-like (FBG) domain involved in innate immune defense. In humans, three types of ficolins have been identified as follows: Ficolin-1 (M-ficolin), Ficolin-2 (L-ficolin), and Ficolin-3 (H-ficolin/Hakata antigen). They function as recognition molecules in the lectin complement pathway along with mannose-binding lectin but with differentiated complement activating capacity. Ficolin-2 and Ficolin-3 circulate in the blood with a median concentration of 5 and 25 µg/ml, respectively. Ficolin-2 is mainly produced in the liver, whereas Ficolin-3 is synthesized in both the liver and lungs, with the highest expression in the lungs. Ficolin-1 is primarily expressed by bone marrow-derived cells and lung epithelial cells. It has been shown to be present in the blood with a median plasma concentration of 60 ng/ml (9). The ficolin genes (FCN1, -2, and -3) are polymorphic, and particularly polymorphisms in FCN2 regulate both the level and function of Ficolin-2. In this respect, a base substitution in exon 8 at position 6359 (C→T) causing a threonine to be replaced by a methionine (T236M) in the FBG domain of Ficolin-2 has been shown to cause decreased binding activity toward GlcNAc.

Ficolin-1 has been reported to bind to GlcNAc, GalNAc, and sialic acid (8, 12). It may opsonize Staphylococcus aureus via GlcNAc and interact with a smooth-type strain of Salmonella typhimurium through an unknown ligand, the binding of which is not diminished by GlcNAc. Ficolin-2 has shown to recognize specific pathogen-associated molecular patterns, which are typically located in pathogen cell membranes, such as lipoteichoic acid and peptidoglycan in Gram-positive bacteria cell walls, lipopolysaccharide in Gram-negative bacteria cell walls, and 1,3-ß-d-glucan in yeast and fungal cell walls. The ligand specificity of Ficolin-2 may also be defined as acetyl groups, including those of N-acetylmannosamine, GlcNAc, GalNAc as well as acetyl groups on cysteine, glycine, and choline (15). Ficolin-2 recognizes clinically important pathogens, like S. typhimurium, S. aureus, and Streptococcus pneumoniae (13, 16, 17). Ficolin-3 shows affinity for GlcNAc, GalNAc, and ß-fucose and may interact with S. typhimurium, Salmonella minnesota, and Aerococcus viridans (17, 18).

* This work was supported by the grants from The Benzon Foundation, The Lundbeck Foundation, The Carlsberg Foundation, The Capital Region of Denmark, The European Commission (Project Tolerage), and the Italian Ministry of Health.
1 To whom correspondence should be addressed. Tel.: 45-35457631; Fax: 45-35398766; E-mail: garred@post5.tele.dk.
2 The abbreviations used are: CD, collagen-like domain; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; FBG, fibrinogen-like domain; G418, geneticin; LPS, lipopolysaccharide; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; MTX, methotrexate; PBS, phosphate-buffered saline; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MOPS, 4-morpholinepropanesulfonic acid; Bistris, 2-[bis(2-hydroxyethyl)amin]-2-(hydroxyethyl)propane-1,3-diol; PTX3, pentraxin 3; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorter.
The long pentraxin 3 (PTX3) is a soluble pattern recognition molecule mediating innate immune recognition (19). PTX3 is a glycoprotein of 45 kDa, which assembles into an octameric structure through protomer linkage by disulfide bonds (20). PTX3 shares C-terminal structural similarity with the classic short pentraxins, C-reactive protein (CRP), and serum amyloid P component, whereas the N-terminal sequence differs from the other proteins (21). Myeloid cells are a major source of PTX3, but PTX3 has also been shown in vitro to be produced by a variety of cells in response to inflammatory signals (21). During inflammation PTX3 is rapidly up-regulated and released into the surrounding tissue and into the bloodstream. PTX3 interacts with C1q and participates in activation of the classical complement pathway (22, 23). Moreover, it has also been shown that PTX3 binds the complement regulatory factor H and that this interaction regulates the alternative pathway of complement (24).

PTX3 can interact with a number of different pathogens, bacteria as well as fungi and viruses. A specific binding has been observed for selected Gram-positive and Gram-negative bacteria, including S. aureus, Pseudomonas aeruginosa, S. typhi, murium, Klebsiella pneumoniae, S. pneumoniae, and Neisseria meningitidis (21). PTX3 also binds zymosan and conidia from Aspergillus fumigatus (25). Furthermore, it has been shown that ptx3 knock-out mice are extremely susceptible to invasive pulmonary aspergillosis. The phenotypic defect can be completely reversed by treatment with recombinant PTX3 (25, 26). These data indicate that PTX3 is important in protection against A. fumigatus, which has become a major cause of morbidity in medical institutions because of the increasing number of immunosuppressed patients (27).

Based on the knowledge of the structural and functional similarities between C1q and the ficolins, this study was designed to characterize a possible interaction between the ficolins and PTX3 using A. fumigatus as a model. Based on our data, we propose an important role for previously unlinked collaboration of PTX3 and Ficolin-2, but not Ficolin-1 and Ficolin-3, in the recognition of A. fumigatus and amplification of complement activation. Moreover, our results demonstrate functional consequences of the Ficolin-2 T236M substitution in the interaction between PTX3 and A. fumigatus.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Mouse monoclonal antibodies against human Ficolin-1 (clone FCN115), Ficolin-2 (clone FCN216, clone FCN219, and biotinylated FCN219), and Ficolin-3 (clone FCN334) were characterized in our laboratory (4, 5, 9). Mouse monoclonal antibody against MBL (HYB 131-11) was purchased from BioPorto (Gentofte, Denmark). Rabbit polyclonal anti-Ficolin-1 antibody and biotinylated rabbit polyclonal anti-Ficolin-2 and -3 antibody were obtained from Hycult Biotechnology (HP9039, Uden, The Netherlands) and R & D Systems, Inc. (BAF2367 and BAF2428, Minneapolis, MN), respectively. A rabbit polyclonal anti-C1q antibody and HRP-conjugated rabbit anti-mouse IgG were obtained from Dako (Glostrup, Denmark). HRP-conjugated donkey anti-rabbit IgG and streptavidin-HRP were purchased from Amersham Biosciences. Streptavidin-phycocerythrin was purchased from Pharmingen. Goat anti-mouse IgG-FITC conjugate, swine anti-rabbit IgG-FITC conjugate, and a rabbit polyclonal anti-C4 antibody were obtained from Dako (Glostrup, Denmark).

Restriction enzymes, Hybrid ECL nitrocellulose membrane, donkey horseradish peroxidase-conjugated anti-rabbit Ig (NAP340V), and streptavidin-horseradish peroxidase conjugate (RPN 1231) were from Amersham Biosciences. Liver and peripheral leukocyte cDNA was from Clontech. pSV2neo was from Clontech. KOD DNA polymerase was from Novagen (EMD Biosciences, San Diego). BigDye terminator cycle sequencing kit, the model 492 gas-phase sequencer, the model 140C HPLC system, and the model 610A data analysis software package (version 2.1) were from Applied Biosystems (Naerum, Denmark). Cell culture utensils were from CM-lab (Vordingborg, Denmark). Trypsin, genetin (G418), hyposxanthine/thymidine media supplement (HT-supplement), l-glutamine solution, penicillin/streptomycin solution, methotrexate (MTX), dialyzed fetal bovine serum, Iscove’s modified Dulbecco’s medium, GlcNac, mannose, n-galacto-D-mannan, Curdlan from Alcaligenes faecalis (β-1,3-glucan hydrate) (C7821), EDTA, EGTA, bovine serum albumin (BSA), and GlcNac-agarose were all from Sigma. PowerCHO-1 CD was from Lonza (Basel, Switzerland). EndoFree plasmid maxi kit was from Qia- gen (VWR International A/S, Albertlund, Denmark). Quantum Prep plasmid miniprep kit and the molecular weight standard, Precision prestained protein standard, were from Bio-Rad.

**Escherichia coli** One Shot TOP10F Competent Cells, Lipo- fectamine PLUS reagent kit, NuPAGE 3–8% Tris acetate gel, 10% Bistris gel, Tris acetate SDS running buffer, MOPS SDS running buffer, NuPAGE transfer buffer, and NuPAGE LDS Sample Buffer were all from Invitrogen. SuperSignal West Femto maximum sensitivity substrate was from Pierce, Bie and Berntsen A/S (Roedovre, Denmark). Microtiter plates (Maxi- Sorp) were from Nalge Nunc (Roskilde, Denmark). OPD tablets were from Dako (Glostrup, Denmark). Phosphate-buffered saline (PBS) buffer (10 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was obtained from Bie and Berntsen. HEPES buffer (25 mM HEPES, 155 mM NaCl, 5 mM CaCl2, pH 7.4) was obtained from the hospital pharmacy at Rigshospitalet, Copenhagen, Denmark. Recombinant PTX3, MBL, and MASP-2 were produced in CHO cells and immunopurified by ion-exchange chromatography. A. fumigatus conidia were obtained from a clinical isolate (26). Dynabeads® Pan mouse IgG was obtained from Invitrogen.

**Construction of FCN Expression Plasmids**—Liver cDNA was used as a template for PCR amplification for FCN2 and FCN3 and peripheral leukocyte cDNA for FCN1. Oligonucleotide primer sets were designed (FCN-1, sense primer 5'-ctagtcagcagtaggtcaggtgacagca-3' and antisense primer 5'-acgg- gaattcctagggcggccaccttagat-3'; FCN-2, sense primer 5'-ctagtcagcagtaggtcaggtgacagca-3' and antisense primer 5'-acgggccggccacctgtagtct-3'; and FCN-3, sense primer 5'-ctagtcagcagtaggtcaggtgacagca-3' and antisense primer 5'-acgggcggccacctgtagtct-3') to obtain an XbaI site at the 5'-end and an EcoRI site at the 3'-end for FCN1 and a Sall site at the 5'-end and an Smal site at the...
performe with SuperSignal West Femto maximum sensitivity substrate on autoradiograph films. Precision prestained protein standard (Bio-Rad) was utilized as a molecular weight standard.

Affinity Chromatography—Human EDTA-plasma (25 ml) from healthy donors was diluted in PBS and applied to CNBr-Sepharose beads (Amersham Biosciences) derivatized with recombinant PTX3. CNBr-Sepharose beads derivatized with BSA (Sigma) and recombinant MBL were used as control. Thebound proteins were eluted with 0.1 M glycine-HCl, pH 2.8, immediately adjusted to pH 7.5 with 1.5 M Tris-HCl, pH 8.8, and collected directly into a 5-kDa cut-off Vivaspin® centrifuge filter (Sartorius, Epsom, UK). Fractions (5 µg of total proteins) were analyzed by SDS-PAGE and Western blot.

Binding of Ficolins to Solid Phase PTX3—To determine whether ficolins interact with PTX3, ELISA microtiter plates were coated overnight at 4 °C with various concentrations of PTX3 (0.3, 1, 3, and 10 µg/ml) and BSA (10 µg/ml) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6). All reaction volumes were 100 µl, and plates were washed after each step in TBS-Ca2+ (20 mM Tris, 150 mM NaCl, 5 mM CaCl2, pH 7.4) containing 0.05% (v/v) Tween 20. Plates were incubated for 1 h at room temperature with TBS-Ca2+ containing 1% (w/v) BSA to block-exposed wells. Ficolin-1, -2, or -3 dilution series in TBS-Ca2+ (6.65, 2, 0.6, and 0.2 µg/ml) were added to the wells and incubated for 5 h at 37 °C. Bound Ficolin-1, -2, or -3 was detected using polyclonal anti-Ficolin-1 antibody, biotinylated monoclonal anti-Ficolin-2 antibody (clone FCN219), and biotinylated monoclonal anti-Ficolin-3 antibody (clone FCN334) by overnight incubation at 4 °C, followed by 1 h of incubation at 37 °C with HRP-conjugated F(ab´)2 from anti-rabbit donkey IgG for Ficolin-1 and HRP-conjugated streptavidin for Ficolin-2 and -3, respectively. Finally, peroxidase reaction was performed by using ortho-phenylenediamine/hydrogen peroxide solution as substrate. The reactions were terminated using 1 M H2SO4, and absorbance was read at 490 nm.

In some experiments, plates were coated with PTX3, followed by incubation of Ficolin-1, -2, or -3 in the presence or absence of GlcNAc at concentrations ranging from 0 to 300 mM, and also with 5 mM of the calcium chelator EGTA. Bound ficolins were detected as depicted above.

To compare natural ficolins and recombinant ficolins, plates were coated with recombinant PTX3, followed by addition of natural and recombinant Ficolin-2 or -3, respectively. Bound ficolins were detected as described above. Alternatively, wells coated with natural PTX3 or recombinant PTX3 were incubated with recombinant Ficolin-1, -2, or -3, respectively.

Binding of Ficolin-2 to PTX3 Using Surface Plasmon Resonance Spectroscopy—Analyses were performed using a BIAcore 3000 instrument (GE Healthcare). Recombinant PTX3 was diluted to 100 µg/ml in 10 mM sodium formate, pH 3.5, and immobilized on the surface of a CM5 sensor chip (GE Healthcare) using the amine coupling chemistry. Binding of recombinant purified Ficolin-2 (29) to immobilized PTX3 (4000 resonance units) was measured at a flow rate of 20 µl/min in 150 mM NaCl, 2 mM CaCl2, 20 mM HEPES, pH 7.4, containing 0.005%
surfactant P20 (GE Healthcare). Regeneration of the surface was achieved by injection of 10 μl of 1 m sodium acetate, pH 7.2 followed by 10 μl of 10 mM NaOH. Equivalent volumes of each protein sample were injected over an activated-deactivated surface to serve as blank sensorgrams for subtraction of the bulk refractive index background.

Data were analyzed by global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for six concentrations simultaneously, using the BIAevaluation 3.2 software (GE Healthcare). The apparent equilibrium dissociation constants ($K_d$) were calculated from the ratio of the dissociation and association rate constants ($k_{on}/k_{off}$). The molar concentration of Ficolin-2 was estimated assuming a tetrameric structure of 414 kDa (12 polypeptide chains of 34.5 kDa each) (29).

**Binding of Wild Type Ficolin-2 and Its Mutant to GlcNAc Beads**—Three μg of wild type Ficolin-2 or Ficolin-2 T236M in eluates was measured by ELISA as described above.

**Binding of Ficolin-1, -2, and -3 to A. fumigatus** —A. fumigatus conidia were washed and resuspended in 100 μl of HEPES buffer containing 1% heat-inactivated FCS at 2.4 × 10^7 cells/ml and incubated with Ficolin-1, -2, or -3 at 37 °C for 1 h. All reaction volumes were 100 μl, and A. fumigatus conidia were washed after each step in HEPES buffer containing 1% heat-inactivated FCS. Bound proteins were eluted with 0.4 mM GlcNAc in binding buffer. The concentration of wild type Ficolin-2 and Ficolin-2 T236M in eluates was measured by ELISA as described above.

**Binding of Ficolin-2 and PTX3**—To verify PTX3 binding to A. fumigatus, PTX3 was incubated with A. fumigatus at a final concentration of 10 μg/ml in the absence or presence of 20 μg/ml galactomannan at 37 °C for 1 h, followed by incubation of biotinylated anti-PTX3 monoclonal antibody at 4 °C for 30 min. PTX3 binding was detected by incubation of streptavidin/phycocerythrin at 4 °C for 15 min and measured by FACS analysis.

**Interaction between Ficolin-2 and PTX3 on A. fumigatus**—The procedure of Ficolin-2 binding to A. fumigatus was the same as mentioned above. A. fumigatus was incubated with Ficolin-2 (7.0 μg/ml) and incubated with PTX3 (10 μg/ml) at 37 °C for 1 h. Bound PTX3 was detected as indicated above. Alternatively, PTX3 was incubated with A. fumigatus at 37 °C for 1 h prior to incubation with Ficolin-2. Binding of Ficolin-2 in the presence of PTX3 was also measured using the same method.

In some experiments, A. fumigatus conidia were incubated with Ficolin-2 T236M instead of wild type Ficolin-2. Ficolin-2 T236M binding was detected with monoclonal anti-Ficolin-2 antibody FCN219 by incubation at 4 °C for 30 min and measured by FACS analysis.

**C4 Deposition on A. fumigatus**—C4 deposition on A. fumigatus was assessed by FACS analysis. As source of complement component, C1q-deficient serum was used in subsequent experiments. C1q-deficient serum was obtained from a C1q-deficient individual with normal levels of Ficolin-2 and MBL (31). Subsequently, MBL was depleted by agitating the C1q-deficient serum with mannan-agarose beads at 4 °C for 2 h. To deplete serum Ficolin-2, magnetic pan-mouse IgG Dynabeads was applied according to the manufacturer’s instructions. In brief, Ficolin-2 was depleted by incubation of the MBL-depleted and C1q-deficient serum with FCN219 (10 μg/ml) and Dynabeads at 4 °C with rotation. 20% C1q-deficient serum depleted of MBL (C1q /MBL depleted serum) and Ficolin-2 (C1q /MBL, Ficolin-2 depleted serum) was prepared by dilution of the C1q /MBL depleted serum in HEPES buffer.

For C4 deposition, A. fumigatus conidia were incubated with 20% C1q /MBL depleted serum with supplementary C4 (10 μg/ml) at 37 °C for 1 h. Alternatively, 20% C1q /MBL, Ficolin-2 depleted serum reconstituted by Ficolin-2 (7 μg/ml) was incubated with A. fumigatus conidia with supplementary C4 (10 μg/ml) at 37 °C for 1 h. All reaction volumes were 100 μl, and A. fumigatus was washed after each step in HEPES buffer containing 1% heat-inactivated FCS. Bound proteins were eluted with monoclonal anti-Ficolin-1 antibody (clone FCN115), anti-Ficolin-2 antibody (clone FCN219), or anti-Ficolin-3 antibody (clone FCN334) by incubation at 4 °C for 30 min, respectively, followed by 15 min of incubation at 4 °C with FITC-conjugated anti-mouse IgG from goat. Finally, the A. fumigatus conidia were washed and resuspended in 200 μl of HEPES buffer containing 1% heat-inactivated FCS. Flow cytometry was carried out on a FACS Calibur (BD Biosciences), and data were analyzed by CellQuest Pro software (BD Biosciences).

In some experiments, A. fumigatus conidia were incubated with Ficolin-2 in the presence of GlcNAc (0.3 mM), mannose (0.3 mM), EDTA (20 mM), Curdlan (β-1,3-glucan hydrate) (250 μg/ml), or a combination of GlcNAc (0.3 mM) and Curdlan (250 μg/ml). Ficolin-2 binding was detected as described above.

**PTX3 Binding to A. fumigatus**—To verify PTX3 binding to A. fumigatus, PTX3 was incubated with A. fumigatus at a final concentration of 10 μg/ml in the absence or presence of 20 μg/ml galactomannan at 37 °C for 1 h, followed by incubation of biotinylated anti-PTX3 monoclonal antibody at 4 °C for 30 min. PTX3 binding was detected by incubation of streptavidin/phycocerythrin at 4 °C for 15 min and measured by FACS analysis.

**Interaction between Ficolin-2 and PTX3 on A. fumigatus**—The procedure of Ficolin-2 binding to A. fumigatus was the same as mentioned above. A. fumigatus was incubated with Ficolin-2 (7.0 μg/ml), followed by incubation with PTX3 (10 μg/ml) at 37 °C for 1 h. Bound PTX3 was detected as indicated above. Alternatively, PTX3 was incubated with A. fumigatus at 37 °C for 1 h prior to incubation with Ficolin-2. Binding of Ficolin-2 in the presence of PTX3 was also measured using the same method.

In some experiments, A. fumigatus conidia were incubated with Ficolin-2 T236M instead of wild type Ficolin-2. Ficolin-2 T236M binding was detected with monoclonal anti-Ficolin-2 antibody FCN219 by incubation at 4 °C for 30 min and measured by FACS analysis.
S.D. were calculated by Excel software (Microsoft). Student’s t test was used to calculate significance, and p < 0.05 was considered to represent a statistical significant difference between two sample means.

RESULTS

Ficolin-2 Isolation from Human Plasma by Sepharose-PTX3 Beads—In ligand fishing experiments of human plasma involving PTX3 affinity chromatography coupled to mass spectrometry, several serum proteins such as C1q, factor H, properdin, MASPz, and Ficolin-2 were found to potentially interact to immobilized PTX3. Analysis with SDS-PAGE and subsequent Western blot was conducted on the fraction eluted from Sepharose-PTX3 beads incubated with human plasma. In Fig. 1, representative experiments with Ficolin-2 and C1q are shown. Sepharose-BSA and MBL beads were used as controls.

Recombinant Ficolin-1, -2, and -3 Characterization—Recombinant ficolins without His tag were expressed by using a CHO cell expressing system. The cDNA of FCN1, -2, and -3 was cloned and inserted into the pEDdC expression vector containing a dihydrofolate reductase expression site for MTX-induced amplification. CHO DG44 cell lines were transfected with the expression vectors containing the FCN1, -2, and -3 insert along with pSV2neo containing a neomycin resistance site and selected with G418. Clones expressing ficolins from G418 selection were generated through multicycle of amplification with elevated concentration of MTX. Using ELISA, the concentrations of Ficolin-1, -2, and -3 in the culture supernatants were quantified as 12, 7, and 10 μg/ml, respectively. To determine the oligomerization state of ficolins, recombinant and natural Ficolin-1, -2, and -3 were analyzed by 3–8% Tris acetate SDS-PAGE under nonreducing or reducing conditions, followed by Western blot (Fig. 2). Under nonreducing conditions, Ficolin-1, -2, and -3 presented multimeric assembly laddered with >10 bands (lane 1 in Fig. 2, A–C) but ran as a single band of ~34, 35, and 34 kDa under reducing conditions on Western blot, respectively (lane 2 in Fig. 2, A–C). The multimeric pattern is in correspondence with the pattern seen in a serum pool (Fig. 2, D–F). This result showed that CHO cells secreted recombinant Ficolin-1, -2, and -3 were oligomerized in subunits stabilized via
disulfide bonds, ranging from two to more than four subunits in a similar fashion to natural proteins.

**Binding of Ficolin-1, -2, and -3 to PTX3**—To determine whether Ficolin-1, -2, or -3 binds to PTX3, ELISA wells coated with various concentrations of PTX3 were incubated with the indicated concentrations of Ficolin-1 (A), Ficolin-2 (B) or Ficolin-3 (C) by two-dimensional serial dilutions. Results are represented as mean ± S.E. from at least six independent experiments.

Ficolin-1 exhibited better binding to PTX3 than Ficolin-3. To further determine the binding specificity of Ficolin-1, -2, and -3, we next examined their binding to immobilized PTX3 in the presence of GlcNAc (Fig. 4, A–C). Inclusion of GlcNAc in the binding buffer attenuated the binding of Ficolin-1 and -2 to immobilized PTX3, although no difference in Ficolin-3 binding was detected in the presence or absence of GlcNAc. These results implied that Ficolin-2, in particular, but also Ficolin-1 bind to immobilized PTX3 via the FBG domain but not Fico-
lin-3. We then removed calcium by adding a buffer containing 5 mM EGTA in experiments, including Ficolin-1 and Ficolin-2 (Fig. 5, A and B). These experiments resulted in a substantial increase in binding of both Ficolin-1 and Ficolin-2 to PTX3, but GlcNAc could still inhibit the interactions.

The ability of recombinant Ficolin-2 to interact with PTX3 was also investigated by surface plasmon resonance spectroscopy. As shown in Fig. 6, Ficolin-2 bound to immobilized PTX3 in the presence of 2 mM CaCl₂. The kinetic parameters of the interaction were determined by recording sensorgrams at varying ficolin concentrations (Fig. 6A) and evaluating the data by global fitting as described under “Experimental Procedures.” A $k_{on}$ value of $6.0 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and a $k_{off}$ value of $3.9 \times 10^{-3} \text{s}^{-1}$ were determined, yielding a resulting apparent $K_D$ value of 6.5 nM, indicative of a high affinity interaction. Binding was maintained when EDTA was substituted for Ca²⁺ in the running buffer, and the complex appeared even more stable, as reflected by the slower dissociation phase (Fig. 6B).

Relative Binding of Natural and Recombinant Ficolins to PTX3—To determine binding capacity of recombinant ficolins to PTX3 compared with ficolins from natural material, ELISA wells coated with recombinant PTX3 were incubated with each natural or recombinant ficolin. Both natural and recombinant Ficolin-2 showed vigorous binding to recombinant PTX3 in a similar manner (Fig. 7A). However, natural Ficolin-3 presented no binding compared with slight binding of recombinant Ficolin-3 (Fig. 7A). Subsequently, we also evaluated binding of recombinant ficolins to natural and recombinant PTX3. ELISA wells coated with natural or recombinant PTX3 were incubated with each recombinant ficolin. Recombinant Ficolin-2 bound both natural and recombinant PTX3; however, the binding to natural PTX3 is dominant, approximately three times higher than to recombinant PTX3 (Fig. 7B). Furthermore, recombinant Ficolin-1 and -3 presented very low binding to both natural and recombinant PTX3 (Fig. 7B). No signal was detected in BSA-coated wells (Fig. 7).

Binding of Ficolin-2 to A. fumigatus—To further provide evidence that the observed interaction between ficolins and PTX3 may be physiologically relevant, we focused our attention on A. fumigatus as an anchored matrix. To assess interaction of the ficolins with PTX3 on A. fumigatus, we first examined whether ficolins by themselves were able to bind to the surface of A. fumigatus by FACS analysis.

As shown in Fig. 8, A and B, we found that Ficolin-2 did bind to A. fumigatus directly in a dose-dependent manner, but neither Ficolin-1 nor -3 did. As a control, binding of recombinant MBL was also assessed concurrently. The binding of Ficolin-2
to *A. fumigatus* was significantly inhibited in the presence of 0.3 mM GlcNAc (Fig. 8C). Excess mannose or EDTA had no significant effect on Ficolin-2 binding to *A. fumigatus* (Fig. 8C). In contrast, binding of MBL to *A. fumigatus* was drastically decreased in the presence of GlcNAc or mannose or EDTA (data not shown).

**Recognition of β-1,3-Glucan on A. fumigatus**—Ficolin-2 can recognize β-1,3-glucan, a major cell wall component of yeast and fungi (14), and cell wall analysis of *A. fumigatus* has shown that β-1,3-glucan is extensively distributed on its surface (32). To further clarify whether the binding of Ficolin-2 is brought about by recognition of β-1,3-glucan on *A. fumigatus*, we attempted to inhibit the binding of Ficolin-2 using Curdlan (β-1,3-glucan hydrate). As shown in Fig. 8C, the binding of Ficolin-2 was impaired in the presence of Curdlan (250 μg/ml). Moreover, the combination of GlcNAc and Curdlan drastically inhibited the binding of Ficolin-2 (Fig. 8C), suggesting that Ficolin-2 bound to *A. fumigatus* in a GlcNAc- and Curdlan-dependent manner.

**Collaboration of Ficolin-2 and PTX3 on A. fumigatus**—To address whether an interaction between Ficolin-2 and PTX3 may occur on the surface of *A. fumigatus*, we assessed binding by FACS analysis and compared the reactions in the presence of either PTX3 or Ficolin-2 alone or together (Fig. 9, A–C). As shown in Fig. 9, B and C, Ficolin-2 bound to *A. fumigatus* much better than PTX3, but most interestingly preincubation of PTX3 increased Ficolin-2 binding to *A. fumigatus* significantly (*p < 0.05*) (Fig. 9B) and vice versa (*p < 0.01*) (Fig. 9C).

**Enhancement of Ficolin-2-induced C4 Deposition on A. fumigatus by PTX3**—To further characterize the physiological relevance of the Ficolin-2-PTX3 interaction on *A. fumigatus*, we investigated its effect on Ficolin-2-dependent complement pathway activation as assessed by C4 deposition using C1q−/MBL-depleted serum. MBL depletion was assessed by analysis of MBL binding to *A. fumigatus*, compared with control reaction without MBL (Fig. 10A). Furthermore, binding of serum Ficolin-2 to *A. fumigatus* was also confirmed using C1q−/MBL-depleted serum (Fig. 10B) before testing C4 deposition, showing that serum-derived and recombinant Ficolin-2 have the same binding pattern to *A. fumigatus*. Next, we focused our attention on C4 deposition using the C1q−/MBL-depleted serum verified above. In C1q−/MBL-depleted serum we could observe deposition of C4 in the absence of PTX3 (Fig. 10C). However, this deposition was significantly increased in the presence of PTX3 (*p < 0.05*), compared with control experiment without PTX3 (Fig. 10C).

To verify the above implication regarding the effect of PTX3/Ficolin-2 interaction on C4 deposition, Ficolin-2 was depleted from C1q−/MBL-depleted serum and assessed by Western blot as described under “Experimental Procedures” (Fig. 10D, inset), followed by assessment of C4 deposition on *A. fumigatus*. As shown in Fig. 10D, C4 deposition was reinforced when C1q−/MBL, Ficolin-2-depleted serum was reconstituted by addition of Ficolin-2. Consistently, this deposition was significantly increased in the presence of PTX3 (*p < 0.05*), compared with control experiment without PTX3 (Fig. 10D).

To further provide evidence for the preceding results, C1q−/MBL-depleted serum was replaced with a more simple system, recombinant Ficolin-2 and MASP-2 as substitute complement initiators in the presence of exogenous C4. As shown in Fig. 10E, additional Ficolin-2-MASP-2 complex leads to C4 deposition on *A. fumigatus*. Consistent with the above results, PTX3 significantly enhanced Ficolin-2-MASP-2 complex-induced C4 deposition on *A. fumigatus* (*p < 0.05*), compared with control experiment without PTX3 (Fig. 10E).

**Reduced Ficolin-2 T236M Binding to GlcNAc, PTX3, and A. fumigatus**—Previously we found an allelic variant in the FCN2 gene that replaced a threonine with a methionine at amino acid position 236 (Ficolin-2 T236M), which when purified from serum exhibited reduced binding capacity to GlcNAc compared with wild type Ficolin-2 (10). To clarify whether recombinant Ficolin-2 T236M also exhibited altered or decreased ligand-binding capacity, an expression vector coding for the T236M gene that replaced a threonine with a methionine at amino acid 236 (Ficolin-2 T236M) was generated by site-directed mutagenesis, and the mutated protein was expressed in CHO cells. SDS-PAGE followed by Western blot analysis showed that the protein had a multimeric pattern comparable with that of the wild type protein (Fig. 11A). Moreover, binding of Ficolin-2 T236M to previously substantiated ligands GlcNAc, PTX3, and *A. fumigatus* was assessed by ELISA and FACS analysis, respectively, as described under “Experimental Procedures.” Differential binding capacity was detected between wild type Ficolin-2 and its T236M mutant. In accordance with our previous data, recombinant Ficolin-2 T236M showed signif-
significantly lower binding capacity toward GlcNAc-agarose beads \((p < 0.05)\) (Fig. 11B) compared with wild type, as was the binding toward immobilized PTX3 and \(A. \ fumigatus\) \((p < 0.05)\) (Fig. 11, C and D).

**DISCUSSION**

It is well described that C1q interacts with the short pentraxin family members CRP and serum amyloid P component and that this complex formation may mediate classical pathway complement activation (33, 34). The same has been shown for solid phase bound PTX3 (22, 23). This complex triggers activation of the classical pathway of complement via the globular heads of C1q.

Whether the lectin pathway initiators may have similar functions is largely unknown. However, it has recently been shown that both Ficolin-1 and Ficolin-2 interact with CRP and that this complex may mediate complement killing of \(P. \ aeruginosa\) (35, 36). We have demonstrated that circulating plasma-derived Ficolin-2 interacted with immobilized PTX3 in affinity chromatography experiments. This is similar to what was previously shown in the case of C1q in binding experiments (22). Moreover, Ficolin-2 and Ficolin-1 were able to bind to PTX3 adsorbed to microtiter wells in a dose-dependent fashion. These interactions were partially inhibited by GlcNAc. We also observed some binding with Ficolin-3, but we were not able to inhibit the binding with GlcNAc and interpret this result as a nonspecific interaction. Zhang et al. (36) showed that at pH 7.4 an increased calcium concentration dramatically inhibited the CRP/Ficolin-2 interaction, indicating that calcium under normal conditions prevents the CRP/Ficolin-2 interaction. Our
experiments were usually performed at pH 7.4 at a calcium concentration of 5 mM showing that the interaction between PTX3 and Ficolin-1 and in particular Ficolin-2 could be operating in the presence of calcium. However, calcium chelation with 5 mM EGTA led to a substantial increase in the binding of Ficolin-2, and especially of Ficolin-1. Accordingly, surface plasmon resonance spectroscopy experiments showed an increased stability of the PTX3-Ficolin-2 interaction in the presence of EDTA. The increased binding observed by ELISA could still be inhibited with GlcNAc indicating that it was not because of nonspecific trapping in the absence of calcium. It is difficult to relate these findings directly to a physiological situation, but it indicates that the interaction between Ficolin-1, Ficolin-2, and PTX3 is calcium-independent. However, use of a dilution buffer without calcium added yielded the same interaction pattern as in the presence of 5 mM calcium (data not shown). Thus, we choose to continue the experiments using calcium-containing buffers, which are closer to physiological conditions. However, according to our results using natural and recombinant proteins, it should be noted that natural PTX3 and recombinant Ficolin-2 behave in a similar fashion and that natural PTX3 had a higher binding capacity to recombinant Ficolin-2, compared with recombinant PTX3. On the contrary, natural Ficolin-3 showed negligible binding to both natural and recombinant PTX3 compared with slight binding of recombinant Ficolin-3. These results indicated that the interaction between native PTX3 and native Ficolin-2 in vivo is highly significant and suggest that natural PTX3 and Ficolin-2 might cause a vigorous interaction in vivo. Nevertheless, because of very low levels of Ficolin-1 in the serum (9), we could not perform the corresponding experiments concerning serum versus recombinant Ficolin-1. Furthermore, in our previous reports (20, 37), we have described the folding and sugar composition of recombinant and natural PTX3, indicating no significant differences between PTX3 from natural material or CHO expression system.

Infection with *A. fumigatus* has become an increasing health problem in immunocompromised patients (27). PTX3 has been shown to be critical in the protection against *A. fumigatus* (25, 26), and Ficolin-2 has been shown to bind to β-1,3-glucan (14), which is a major constituent of many fungi. This prompted us to investigate whether the ficolins and PTX3 could cooperate at the surface of *A. fumigatus*. First, we established by flow cytometry that conidia of *A. fumigatus* served as a very good ligand for Ficolin-2. However, no binding could be observed for either Ficolin-1 or Ficolin-3. Consistent with previous reports, we confirmed specific binding of MBL to *A. fumigatus* (38) that could be inhibited by GlcNAc, mannose, or EDTA (data not shown). Unlike MBL, the binding of Ficolin-2 to *A. fumigatus* was not decreased in the presence of mannose or EDTA, but it was reduced to ~11% in the presence of GlcNAc. The latter finding suggests that more than one binding site in the FBG domain of Ficolin-2 is involved in the interaction with *A. fumigatus*. Recently, the crystal structure of the FBG domain of ficolins has been solved and has revealed the location of the major ligand-binding sites of Ficolin-2 (39). MBL has only one specific binding site in the CRD domain to recognize C3-OH and C4-OH of terminal carbohydrate residues by a typical hydrogen bonding interaction (40). In addition to a common external S1-binding site that it shares with Ficolin-1 and Ficolin-3, Ficolin-2 has three additional binding sites (S2–S4) in the

![FIGURE 9. Ficolin-2/PTX3 cross-talk on *A. fumigatus*. A, binding of PTX3 to *A. fumigatus* assessed by flow cytometry. Fluorescence intensity (FL2-H) was plotted against cell number. B, interaction of Ficolin-2 with PTX3. *A. fumigatus* was incubated with Ficolin-2 alone or preincubated with PTX3 before addition of Ficolin-2. C, interaction of PTX3 with Ficolin-2. *A. fumigatus* was incubated with PTX3 alone or preincubated with Ficolin-2 before addition of PTX3. The mean fluorescence intensity (MFI) was used as assessment of PTX3 or Ficolin-2 binding. Asterisks refer to the statistical significance versus controls: *, p < 0.05; **, p < 0.01. Results shown are mean ± S.D. and are representative of at least three independent experiments.](image-url)
These findings explain why Ficolin-2 harbors the capacity of versatile recognition of a variety of acetylated and carbohydrate-containing compounds. Most of the acetylated ligands bind to sites S2 and S3 of the FBG domain of Ficolin-2, whereas the binding of \( \alpha \)-1,3-glucan involves the edge of site S3 and S4 via water-mediated hydrogen bonding interactions. However, the S3-binding site is shared by both the acetyl group of GlcNAc and the first glucose residue of an elongated \( \alpha \)-1,3-glucan molecule (39). Because the cell membrane of \( A. fumigatus \) is composed mainly of chitin (a polymer of \( \alpha \)-1,4-N-acetyl-glucosamine), \( \alpha \)-1,3-glucan, and galactomannan (41), it is reasonable to hypothesize that two kinds of recognition sites are involved in the binding of Ficolin-2 to \( A. fumigatus \), which may both include a GlcNAc and a \( \alpha \)-1,3-glucan moiety.

Our competition experiments using Curdlan (a \( \alpha \)-1,3-Glucan hydrate from \( A. faecalis \)) suggest that the FBG domain of Ficolin-2 preferentially recognizes the \( \alpha \)-1,3-glucan moiety in addition to the GlcNAc moiety on \( A. fumigatus \), which accounts for the strong combined inhibiting effect of the two compounds. In agreement with the fact that the ligand-binding sites are distant from the \( \text{Ca}^{2+} \)-binding site in the Ficolin-2 structure, \( A. fumigatus \) recognition is not sensitive to EDTA.

PTX3 clearly bound \( A. fumigatus \) as has been shown before (25, 26), but to a lesser degree than Ficolin-2. Of particular interest was the observation that PTX3 could significantly augment the deposition of Ficolin-2 on the surface of \( A. fumigatus \). However, a possibly more intriguing finding was the observation that Ficolin-2 by itself could enhance the binding of PTX3 to \( A. fumigatus \). This indicates that PTX3 also interacts with Ficolin-2 at sites that are not involved in Ficolin-2 interaction with \( A. fumigatus \) per se. This may also explain the partial inhibition of the interaction between Ficolin-2 and PTX3 that we observed with GlcNAc on the solid phase polystyrene matrix. To further elucidate whether the Ficolin-2/PTX3 interactions with \( A. fumigatus \) may be of physiological relevance, we investigated their influence on complement activation induced by Ficolin-2. We could show that Ficolin-2 and PTX3 collaborate to boost Ficolin-2-mediated complement deposition. Thus, it is tempting to speculate that this interaction may play a significant physiological role.

**FIGURE 10.** C4 deposition depends on Ficolin-2 and Ficolin-2/PTX3 collaboration. A, MBL binding to \( A. fumigatus \) in C1q-deficient serum and in MBL-depleted C1q-deficient serum. B, binding of Ficolin-2 in MBL-depleted C1q-deficient serum and of recombinant Ficolin-2 (5.5 \( \mu \)g/ml) to \( A. fumigatus \). Fluorescence intensity (FL1-H) was plotted against cell number in A and B and is representative of at least three independent experiments. C, C4 deposition induced by interaction of Ficolin-2 and PTX3 in MBL-depleted and C1q-deficient serum on \( A. fumigatus \). D, Ficolin-2, MBL-depleted and C1q-deficient serum was reconstituted by addition of recombinant Ficolin-2 and induced C4 deposition in the presence of PTX3 on \( A. fumigatus \). Depletion of Ficolin-2 from MBL-depleted and C1q-deficient serum analyzed by Western blot is shown in the inset. Lane 1, before depletion; lane 2, after depletion. E, reconstruction of PTX3-Ficolin-2-MASP-2 complex-induced C4 activation on \( A. fumigatus \) using recombinant MASP-2. The mean fluorescence intensity (MFI) was used to assess C4 deposition. Asterisk indicates the statistical significance versus controls: *, \( p < 0.05 \). Results represent mean ± S.D. of four independent experiments.
Ficolins and PTX3

FIGURE 11. Ligand binding disability of the Ficolin-2 T236M variant. A, multimerization of Ficolin-2 T236M. Wild type (WT) Ficolin-2 (lane 1) and Ficolin-2 T236M variant (lane 2) were analyzed by 10% SDS-PAGE followed by Western blot under nonreducing (left) or reducing (right) conditions. B, binding of wild type and Ficolin-2 T236M to GlcNAC-agarose beads. Ficolin-2 was incubated with GlcNAC-agarose beads and recovered by elution with GlcNAC, respectively. The concentration of Ficolin-2 in the eluates was assessed by ELISA. The amount of bound protein (left y axis) and the recovery yield (right y axis) were calculated from ELISA measurements. Results represent mean ± S.D. of three independent experiments. C, binding of wild type and Ficolin-2 T236M to PTX3 in ELISA. Microtiter wells coated with PTX3 or BSA as negative control were incubated with wild type (WT) Ficolin-2 and binding was detected with an anti-Ficolin-2 antibody. The mean fluorescence intensity (MFI) was used to assess Ficolin-2 binding. Asterisks refer to the statistical significance versus controls: *p < 0.05. Results represent mean ± S.D. of three independent experiments.

No deficiency state of Ficolin-2 in humans has been described so far, but promoter variants in the Ficolin-2 gene (FCN2) are associated with differences in the serum concentrations (4, 10). However, the GlcNAC binding ability of Ficolin-2 is hampered by a polymorphism situated in exon 8 in the FCN2 gene causing a threonine to be replaced with methionine (T236M) (10). This observation was based on binding studies performed with Ficolin-2 in whole serum. To bring the understanding of this observation a step further, we produced the variant T236M. This Ficolin-2 variant folded correctly into high order oligomeric structures comparable with those obtained for wild type Ficolin-2, as judged from SDS-PAGE and Western blot analysis. As we have shown for serum Ficolin-2 T236M variant (10), the GlcNAC binding capacity of the recombinant variant was dramatically decreased when compared with the wild type protein as was the interaction with PTX3 and A. fumigatus. Thus, this finding demonstrates the existence of a functional Ficolin-2 deficiency that may be of pathophysiological relevance not only in the direct interaction between Ficolin-2 and microorganisms but also between Ficolin-2 and PTX3. Based on the crystal structure of Ficolin-2 (39), the T236M polymorphism does not directly influence the four putative binding sites in the FBG domain. Thus, it is more likely that the resulting T236M change in the amino acid composition may affect the tertiary structure of the FBG domain of Ficolin-2, which may have an indirect effect of the binding and interaction properties of the protein. Alternatively, additional binding pockets may exist in the FBG domain of Ficolin-2 not resolved by the present crystallographic observations.

In summary, plasma Ficolin-2 could be purified with immobilized PTX3. Ficolin-1 and in particular Ficolin-2 but not Ficolin-3 interacted with PTX3 in a calcium-independent manner. Only Ficolin-2 bound A. fumigatus directly, but this binding was enhanced by PTX3 and vice versa. Ficolin-2-mediated complement deposition on the surface of A. fumigatus was enhanced by PTX3. These results demonstrate that PTX3 and Ficolin-2 may recruit each other on pathogens and enhance complement activation. This effect was dramatically reduced by a common amino acid change in the FBG domain of Ficolin-2. Thus, components of the humoral innate immune system, which activate different complement pathways, cooperate and amplify microbial recognition and effector functions.

Acknowledgment—We thank Vibeke Weirup for excellent technical assistance.

REFERENCES
1. Endo, Y., Matsushita, M., and Fujita, T. (2007) Immunobiology 212, 371–379
2. Runza, V. L., Schwaeble, W., and Männel, D. N. (2008) Immunobiology 213, 297–306
3. Hummelshøj, T., Fog, L. M., Madsen, H. O., Sim, R. B., and Garred, P. (2008) Mol. Immunol. 45, 1623–1632
4. Munthe-Fog, L., Hummelshøj, T., Hansen, B. E., Koch, C., Madsen, H. O., Skjædt, K., and Garred, P. (2007) Scand. J. Immunol. 65, 383–392
5. Munthe-Fog, L., Hummelshøj, T., Ma, Y. J., Hansen, B. E., Koch, C., Madsen, H. O., Skjædt, K., and Garred, P. (2008) Mol. Immunol. 45, 2660–2666
6. Lu, J., Tay, P. N., Kon, O. L., and Reid, K. B. (1996) Biochem. J. 313, 473–478
7. Teh, C., Le, Y., Lee, S. H., and Lu, J. (2000) Immunology 101, 225–232
8. Liu, Y., Endo, Y., Iwaki, D., Nakata, M., Matsushita, M., Wada, I., Inoue, K.,...
