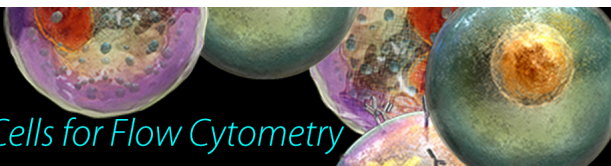


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Secreted M-Ficolin Anchors onto Monocyte Transmembrane G Protein-Coupled Receptor 43 and Cross Talks with Plasma C-Reactive Protein to Mediate Immune Signaling and Regulate Host Defense

Jing Zhang,^{*,†} Lifeng Yang,^{*,‡} Zhiwei Ang,^{*} Sia Lee Yoong,^{*} Thi Thu Thuy Tran,^{*} Ganesh Srinivasan Anand,^{*} Nguan Soon Tan,[§] Bow Ho,^{¶,1} and Jeak Ling Ding^{*,‡,1}

Although transmembrane C-type lectins (CLs) are known to initiate immune signaling, the participation and mechanism of action of soluble CLs have remained enigmatic. In this study, we found that M-ficolin, a conserved soluble CL of monocyte origin, overcomes its lack of membrane-anchor domain by docking constitutively onto a monocyte transmembrane receptor, G protein-coupled receptor 43 (GPCR43), to form a pathogen sensor-cum-signal transducer. On encountering microbial invaders, the M-ficolin–GPCR43 complex activates the NF- κ B cascade to upregulate IL-8 production. We showed that mild acidosis at the local site of infection induces conformational changes in the M-ficolin molecule, which provokes a strong interaction between the C-reactive protein (CRP) and the M-ficolin–GPCR43 complex. The collaboration among CRP–M-ficolin–GPCR43 under acidosis curtails IL-8 production thus preventing immune overactivation. Therefore, we propose that a soluble CL may become membrane-associated through interaction with a transmembrane protein, whereupon infection collaborates with other plasma protein to transduce the infection signal and regulate host defense. Our finding implies a possible mechanism whereby the host might expand its repertoire of immune recognition-cum-regulation tactics by promiscuous protein networking. Furthermore, our identification of the pH-sensitive interfaces of M-ficolin–CRP provides a powerful template for future design of potential immunomodulators. *The Journal of Immunology*, 2010, 185: 6899–6910.

Human leukocytes play critical roles in the early detection and efficient removal of virulent pathogens during bacteremia via various surface receptors/receptor complexes (1). Similar to TLRs (2), C-type lectins (CLs) are essential in recognizing pathogens and alerting the host to elicit immune responses against bacterial (3), fungal (4), and viral infections (5). Being structurally and functionally conserved (6), CLs bind

to the sugar moieties of pathogen-associated molecular patterns (PAMPs) displayed on microbes (7), activate complements (8, 9), promote phagocytosis (10), and induce cytokines (11). Whereas transmembrane CLs including dectins (12) and DC-SIGN (13) have been reported to induce signaling pathways through their cytoplasmic domains, little is known about soluble CLs, which encompass a large family of proteins and alert the host cells to an infection, although mannose binding lectin, which is also a soluble CL, was observed to enhance the proinflammatory response to *Staphylococcus aureus* independent of complement activation (14).

Ficolins, first documented as TGF- β 1-binding proteins on pig uterus membranes (15), constitute a family of secretory CLs that play critical roles as pattern-recognition receptors (PRRs) in host defense. Immune diseases such as rheumatoid arthritis (16) and necrotizing enterocolitis (17) are associated with defective M-ficolin. M-ficolin is the only ficolin isoform secreted by immune cells such as monocytes (18), neutrophils (19), and alveolar epithelial cells (20). Whereas the mouse homologues of L- or H-ficolins are evolutionarily divergent (21), the M-ficolin gene is well conserved in invertebrates (22), mouse (21), and human (9), suggesting the crucial and fundamental roles of M-ficolin. However, their varied locations in assorted tissues and species are suggestive of their differential functions. Notably, the tissue distribution and function of the human M-ficolin and its homologue (ficolin β) in pigs (23) and mice (24) are substantially different (25). Hitherto, no consensus has been reached on the subcellular localization and fate of M-ficolin in human monocytes, whether intracellular in secretory granules (19, 20), on the cell surface (18), or secreted into the serum (26), although the localization of M-ficolin in the neutrophils was recently reported (19). Similar to other CLs, M-ficolin binds to the acetylated sugar moieties

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CL, C-type lectin; CLD, collagen-like domain; CRP, C-reactive protein; FBG, fibrinogen-like domain; GlcNAc, N-acetylglucosamine; GPCR43, G protein-coupled receptor 43; HDMS, hydrogen–deuterium exchange mass spectrometry; LA, lipid A; LTA, lipoteichoic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MFL, M-ficolin; n.s., not significant; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; PRR, pattern-recognition receptors; QDO, SD-Trp-Leu-His-Ade; ReLPS, rough strain truncated LPS; shRNA, short hairpin RNA; siRNA, small interfering RNA; SPR, surface plasmon resonance.

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like *N*-acetylglucosamine (GlcNAc) in PAMPs displayed on microbes (20). Thus, M-ficolin recognizes invading microbes, and it was proposed to be a phagocytic receptor on immune cells (18). Complement activation was reported to be a consequence of specific carbohydrate recognition by M-ficolin. However, the expression of M-ficolin by immune responsive cells implies that it might play an additional role in cellular signal transduction.

At the initial phase of infection, the physiological condition of pH 7.4 exists (9). Subsequently, mild acidosis (pH 6.5) can occur at the local site of infection or in a severe systemic infection, due to the massive infiltration of immune cells to the site of infection, which activates the respiratory burst (27, 28). Several CLs containing a C-terminal Ca^{2+} -dependent C-type carbohydrate recognition domain have been reported to be sensitive to pH shift, which causes conformational changes (29, 30). This might affect their interaction with other partners and regulate their immune functions. Therefore, it was of interest to study the potential effects of pH change induced by infection on the functions of M-ficolin. Recently, we found that C-reactive protein (CRP), an acute-phase pentraxin, interacts with circulating L-ficolin at low pH condition (9), thus boosting the immune response during an infection. With 80% homology to L-ficolin, we envisage that M-ficolin and CRP might also interact at the later phase of infection where local acidosis occurs, with potential implications for the regulation of the immune response.

In this study, we demonstrate that secreted soluble M-ficolin docked onto the monocyte surface through a constitutive association with G protein-coupled receptor 43 (GPCR43). The M-ficolin-GPCR43 complex recognizes the pathogen, mediates signal transduction, and upregulates IL-8 through NF- κ B activation. During this process, under conditions mimicking the initial or later stage of infection, CRP associates with the M-ficolin-GPCR43 complex, which regulates IL-8 secretion. This was attributable to infection-induced local acidosis where perturbations of the micro-environment occurred, causing conformational changes in M-ficolin, which consequently enhanced its extracellular interaction with CRP. Therefore, M-ficolin collaborates with its interacting partners (GPCR43 and CRP) to initiate, mediate, and regulate the immune response.

Materials and Methods

Bacterial strains, reagents, Abs, human primary monocytes, and cell cultures

The *Pseudomonas aeruginosa* strain PAO1 was kindly provided by Prof. B.H. Iglewski (University of Rochester, Rochester, NY). GlcNAc, *Escherichia coli* 055:B5 LPS, *E. coli* F583 diphosphoryl lipid A (LA), and *Staphylococcus aureus* lipoteichoic acid (LTA) were purchased from Sigma-Aldrich (St. Louis, MO). Rough strain truncated LPS (ReLPS) of *Salmonella minnesota* was purchased from List Biological Laboratories (Campbell, CA). *Staphylococcus aureus* peptidoglycan (PGN) was purified from the *Staphylococcus aureus* culture as described previously (31). Pertussis toxin was purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-M-ficolin was from Hycult (Uden, The Netherlands). Mouse monoclonal anti-V5, anti-His, and TRIzol reagent were from Invitrogen. The NF- κ B inhibitors PDTC and MG-132 were from Calbiochem (San Diego, CA).

Primary human monocytes were purified from the buffy coat by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation (32) followed by magnetic cell sorting using the Monocyte Isolation Kit II (Miltenyi Biotech, Bergisch Gladbach, Germany). The primary monocytes and U937 cells were cultured at 37°C in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen) and 1% (v/v) penicillin and streptomycin (Invitrogen). COS-1 and HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin and streptomycin. All experiments were performed according to national and institutional guidelines on ethics and biosafety (National University of Singapore Institutional Review Board, Reference Code NUS-IRB 08-296).

Creating stable M-ficolin knockdown (M-ficolin⁻) clones and RT-PCR

Stable silencing of M-ficolin was performed with HuSHTM short hairpin RNA (shRNA) plasmid panel for M-ficolin (Origene, Rockville, MD). Four shRNA plasmids targeting different fragments of M-ficolin were separately transfected into U937 cells by the Nucleofector II machine (Amaza, Gaithersburg, MD) using the default program according to the manufacturer's instructions. Nucleofected cells were transferred to 1 ml medium and renewed after 24 h. Knockdown of M-ficolin was ascertained by RT-PCR 24 h after nucleofection, and a selection pressure with 400 ng/ml puromycin was applied for 2 wk to obtain a stable cell line, during which single colonies were selected. The successful stable M-ficolin knockdown clones were reconfirmed by RT-PCR. RNA was extracted by TRIzol reagent (Invitrogen). cDNA was first synthesized with the SuperScript RT-PCR System (Invitrogen) using random hexamer primers according to the manufacturer's instructions.

In situ proximity ligation assay

To determine protein-protein interaction, in situ proximity ligation assay was performed using the Duolink detection 563 kit (Olink Biosciences, Uppsala, Sweden) following the manufacturer's instructions. Primary monocytes or transfected cells were plated onto 4-well chamber slides (Sterilin, London, UK) 24 h before the assay. Cells were washed once with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4) and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS. To select the compatible Abs for this particular experiment, M-ficolin-GPCR43 was detected by the primary Abs of rabbit anti-M-ficolin and mouse anti-GPCR43 (COSMO Bio Company, Tokyo, Japan), respectively. GPCR43-CRP was detected by the primary Abs of rabbit anti-GPCR43 (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-CRP (Sigma-Aldrich), respectively. M-ficolin-CRP was detected by the primary Abs of rabbit anti-M-ficolin and goat anti-CRP, respectively. The corresponding probes of anti-rabbit PLUS, anti-mouse MINUS, and anti-goat MINUS were from Olink Biosciences. Imaging of the cells was carried out using an LSM META 510 confocal microscope (Carl Zeiss, Jena, Germany) under a 100 \times oil objective. Samples without primary Abs served as negative controls. Optimal interval was used for Z sectioning.

Immunofluorescence microscopy

Cells at a density of 1×10^5 cells/ml were plated onto 4-well chamber slides (Sterilin) for 24 h, washed once with PBS, and fixed in 4% paraformaldehyde for 15 min following which primary and secondary Abs were added and washed three times with PBS containing 0.05% Tween 20. For colocalization analysis of M-ficolin and GPCR43, the M-ficolin-GFP and GPCR43-m-cherry plasmids were co-transfected into HEK293 cells, which had been plated onto a 4-well chamber slide. Twenty-four hours after transfection, cells were washed once with PBS and fixed in 4% paraformaldehyde for 15 min. To stain the nucleus, a drop of Prolong Gold antifade reagent with DAPI (Invitrogen) was added before overlaying with the coverslip. Imaging of the cells was performed using an LSM META 510 confocal microscope (Carl Zeiss) under a 100 \times oil objective. Optimal interval was used for Z sectioning.

Flow cytometry

Flow cytometry was carried out as described previously (18). Briefly, 2×10^6 cells were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde for 15 min. After two washes with PBS containing 0.05% Tween 20, cells were sequentially stained with primary rabbit anti-M-ficolin (1:200) or rabbit anti-GPCR43 (1:50) and PE-conjugated secondary Ab (goat anti-rabbit, 1:200; Invitrogen). After three washes, cells were diluted into a density of 1×10^5 cells/ml, and flow cytometry was performed using the Beckman Coulter Epics Altra (Beckman Coulter, Brea, CA).

Membrane protein extraction and ELISA

The total membrane proteins from primary monocytes were purified using a native membrane protein extraction kit (proteoExtract; Calbiochem, Gibbstown, NJ) according to the manufacturer's instructions. To test the interaction between M-ficolin and the extracted membrane protein, ELISA was performed as described previously (9) with 1 μ g membrane protein extracts immobilized on the Maxisorp plate (NUNC, Roskilde, Denmark). Similarly, the binding of M-ficolin to immobilized CRP under pH 7.4 and pH 6.5 was carried out with 0.8 μ g CRP immobilized on the Maxisorp plate. Increasing concentrations of M-ficolin in 25 mM Tris, 145 mM NaCl, pH 7.4, or 25 mM 2-(N-morpholino)ethanesulfonic acid (MES), 145 mM NaCl, pH 6.5, were added. OD_{405nm} was read. One microgram

M-ficolin directly immobilized onto the ELISA plates was the positive control serving as 100% binding. The percentage of the bound protein was calculated by comparison with the positive control. To test the effect of CRP on the interaction between the fibrinogen-like domain (FBG) and GlcNAc, 1 μ g GlcNAc-BSA was immobilized onto a Maxisorp plate. One microgram FBG and increasing amount of CRP were preincubated at room temperature for 2 h in 25 mM MES, 145 mM NaCl, 2 mM calcium, pH 6.5, before adding to the ELISA wells, which were precoated with GlcNAc-BSA. FBG was identified using anti-His fusion Ab. One microgram FBG directly immobilized onto the ELISA plates was the positive control serving as 100% binding. The percentage of the bound protein was calculated by comparison with the positive control.

Surface plasmon resonance

Surface plasmon resonance (SPR) analysis of real-time biointeraction between GPCR43 and M-ficolin was performed using the Biacore 2000 instrument (Biacore International AB, Uppsala, Sweden). GlcNAc-BSA was immobilized on the CM5 chip as described previously (33). M-ficolin at 2 μ M was injected over the Biacore chip in the running buffer (50 mM Tris, 145 mM NaCl with 2 mM calcium, pH 7.4). The bound M-ficolin was blocked by injecting 50 μ l 1 μ g/ml BSA. The native membrane extracts of i) wild-type RAW 264.7, not expressing any GPCR43, ii) RAW 264.7 transfected with GPCR43, and iii) primary monocytes were concentrated and buffer-exchanged to the same running buffer using Vivaspin columns (Sartorius stedim biotech, Aubagne, France). Fifty microliters of these membrane extracts, at 0.5 μ g/ml each, were then injected over the bound M-ficolin. The surface was again blocked by injecting 50 μ l 1 μ g/ml BSA before introducing 5 μ M polyclonal rabbit anti-GPCR43 (Santa Cruz Biotechnology), which recognizes GPCR43 (at amino acids 61–180). The dissociation was for 180 s at the same flow rate. Regeneration of the chip surface was achieved by injection of 20 μ l 0.1 M NaOH. The binding affinities were calculated using BIAevaluation software, version 3.2 (Biacore International AB). The resonance unit difference before and after the injection represents the protein–protein interaction.

Silencing of GPCR43 in primary monocytes

The silencing of GPCR43 was performed by transfecting primary monocytes with small interfering RNA (siRNA; Santa Cruz Biotechnology) using N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (Roche Applied Science, Indianapolis, IN). The ratio of RNA to transfection reagent was 16.5 pmol per μ l N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate. Transfections were performed within 2 h of cell plating. Control siRNA (Santa Cruz Biotechnology) was used for comparison. To check the transfection efficiency, control siRNA labeled with FITC (Santa Cruz Biotechnology) was subsequently transfected into the cells under the same condition. The fluorescent cells were enumerated under the microscope to ensure that the transfection efficiency was above 70%. The knockdown effect of siRNA was checked by flow cytometry.

Yeast two-hybrid and yeast three-hybrid screening system

The M-ficolin cDNA sequence was cloned without its secretion signal into yeast two-hybrid bait vector, pGBKT7, and was used to screen the human leukocyte cDNA library. The co-transformants of yeast, *Saccharomyces cerevisiae* (AH109), were plated onto the SD-Trp-Leu-His-Ade (QDO) plates for up to 5 d. The prey plasmids in the yeast were extracted, electroporated into the *E. coli*, then purified and sequenced. To confirm the protein–protein interaction, the isolated preys were individually co-transformed with the bait and replated onto the QDO plates. β -Galactosidase assay was performed to indicate the intensity of the protein–protein interaction. To confirm the interaction between M-ficolin and GPCR43, the bait and prey vectors were switched with M-ficolin in pACT2 and GPCR43 in pGBKT7, and the constructs were again co-transformed. The transformants were plated onto SD-Trp-Leu and restreaked on QDO plates for verification. To compare the binding strength, one colony of the transformants was diluted in 50 μ l water, and 2 μ l of the diluted transformants were dotted onto the SD-Trp-Leu and QDO plates. Serial 10-fold dilutions were performed five times for each colony to test the strength of the interaction between the different GPCR43 domains and M-ficolin.

Yeast three-hybrid system was used to test for potential protein–protein interaction among M-ficolin, GPCR43, and CRP. The third protein in this system can participate in the interaction in several ways: as a bridge or a stabilizer or an inhibitor. GPCR43 in pACT2 vector was used as prey. CRP and M-ficolin were cloned into MCSI and MCSII sites, respectively, of the pBridge vector, and reciprocally, as baits. The bait and prey were co-transformed into yeast and plated onto SD-Trp-Leu for 3 d. The

transformants were then restreaked onto the SD-Trp-Leu, QDO, and QDO-Met plates for 5 d. The growth of the yeast in the QDO-Met plates would indicate a ternary relationship among CRP–M-ficolin–GPCR43. All cloning vectors, human leukocyte cDNA library, and culture media used in yeast two- and three-hybrid assays were from Clontech (Palo Alto, CA).

Treatment of monocytes and measurement of cytokines

Monocytes were plated at 2×10^5 cells/ml into 6- or 24-well plates. The cell culture medium was replaced with fresh medium before different amounts of PAMPs or GlcNAc were added to replicate wells for 24 h, and the medium and cells were collected and separated by centrifugation. The inhibitory effect of M-ficolin Ab was tested by adding 500 ng/ml Ab and incubating for 2 h at 37°C before the addition of GlcNAc. To confirm the role of NF- κ B in the expression of IL-8, NF- κ B inhibitors (PDTC or MG-132) were added to monocytes 2 h before treatment with GlcNAc. DMSO used to dissolve the NF- κ B inhibitors was controlled below 0.1% (v/v) at which it had no significant effect on the cells. To test for possible involvement of G protein pathways, pertussis toxin, which is known to inhibit the association of G_i , G_o , and G_i with GPCRs (34), was used to pretreat the primary monocytes for 12 h before GlcNAc stimulation. To test the functional significance of the interplay of CRP, M-ficolin, and GPCR43 in the GlcNAc-induced IL-8 secretion, 500 ng/ml polyclonal rabbit anti-GPCR43 Ab targeting the amino acids 61–180 of GPCR43 was added to M-ficolin[−] cells and incubated for 1 h at 37°C before the addition of purified M-ficolin for 1 h. CRP was added subsequently and incubated for another hour following which 100 mM GlcNAc was added. The cell supernatant was collected 24 h after stimulation. The secreted IL-8 or RANTES was quantified with OptEIA human IL-8 ELISA (BD Biosciences) or Quantikine human CCL5/RANTES Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The cell culture supernatant was diluted 50 times, and 100 μ l diluted supernatant was added to each well. OD_{405nm} was read.

EMSA

Nuclear extract was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the product manual. The mammalian κ B DNA sequence (5'-AGTTGAGGGGACTT-TCCCAGGC-3') was synthesized in both the forward and reverse orientations. The complementary oligonucleotides were labeled with biotin using the biotin 3' end DNA labeling kit (Pierce). Biotin end-labeled DNA (20 fmol) was incubated with 2 μ l nuclear extract, and EMSA was performed using the lightshift chemiluminescent kit (Pierce).

Dual luciferase reporter assay

HEK293 cells were plated onto 24-well plates. Twenty-four hours later, cells were co-transfected with the indicated amounts of GPCR43 and M-ficolin plasmids together with 20 μ g pRL-CMV plasmid (Promega, Madison, WI) and 200 μ g NF- κ B reporter plasmid (Promega), using Lipofectamine 2000 (Invitrogen). pcDNA 3.1 plasmid (Invitrogen) was used to make up the total amount of DNA to 800 μ g. Subsequently, cells were stimulated with 100 mM GlcNAc. The reporter gene activity for NF- κ B was measured 24 h after transfection using dual luciferase assay (Promega). Readings from the untransfected cells stimulated with GlcNAc served as the negative control. The results were calculated from three independent experiments.

Expression and purification of the FBG of M-ficolin

The functional fragment of M-ficolin, the FBG, was cloned into the pSectag 2C vector (Invitrogen). The protein was expressed and purified as described previously (9).

Hydrogen–deuterium exchange mass spectrometry

To determine the interaction interface between M-ficolin and CRP at pH 7.4 and 6.5, hydrogen–deuterium exchange mass spectrometry (HDMS) was performed by combining 2 μ l each of the protein solutions at concentrations >2.5 μ g/ml with 18 μ l of a deuterated buffer (pH_{read} = 7.4/6.5) at the corresponding pH. This changed the composition of the aqueous buffer to 90% deuterium oxide buffer. After 0, 0.5, 1, 2, 5, or 10 min, the hydrogen–deuterium exchange reactions were quenched by the addition of 180 μ l of ice-cold, 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich) to lower the pH_{read} of the reaction to 2.5. An aliquot of 100 μ l of the quenched reaction was then mixed with 50 μ l pepsin bead slurry (Pierce), previously activated by washing three times in 500 μ l 0.1% trifluoroacetic acid, pH 2.5, at 4°C. After mixing with pepsin, the mixture was vortexed

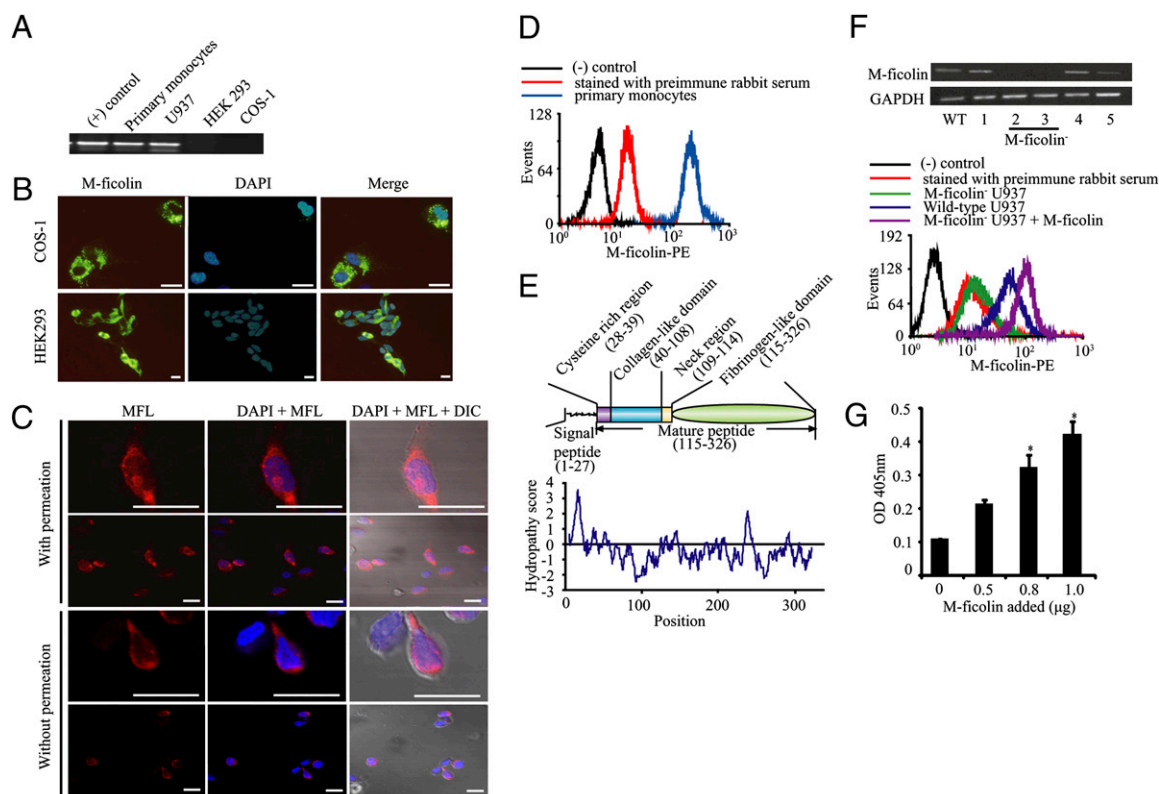


FIGURE 1. Localization of M-ficolin. *A*, Detection of the expression of M-ficolin in primary monocytes, U937, HEK293, and COS-1 cells by RT-PCR. Plasmids containing M-ficolin fragment was used as positive control [(+) control]. *B*, Detection of M-ficolin localization in HEK293 and COS-1 cells after transfection with M-ficolin fused with GFP tag (green). Scale bar, 10 μ m. *C*, The localization of M-ficolin (MFL, red) in primary monocytes was detected by anti-M-ficolin with or without permeation with Triton X-100. The cell nucleus was stained with DAPI (blue). Scale bar, 20 μ m. *D*, The expression of M-ficolin on the surfaces of primary monocytes was detected by FACS and compared with the unstained wild-type U937 cells [indicated as (–) control] and cells stained with preimmune rabbit serum as the negative controls. Original magnification $\times 100$. *E*, Hydropathy plot to analyze the hydrophobic region of M-ficolin. The full-length M-ficolin sequence was obtained from the National Center for Biotechnology Information Web site (accession number NM_002003; <http://www.ncbi.nlm.nih.gov/>), and the hydropathy plot based on the values given in the original Kyte–Doolittle paper was made using the ProtScale online service (<http://au.expasy.org/tools/protscale.html>). *F*, M-ficolin of U937 cells was knocked down by shRNA to create M-ficolin[–] cells. Complete knockdown of M-ficolin in clones 2 and 3 was detected by RT-PCR with GAPDH as the positive control (upper panel). The M-ficolin on the surfaces of wild-type U937 cells and M-ficolin[–] cells with and without preincubation with purified M-ficolin protein was detected by FACS and compared with the unstained wild-type U937 cells [indicated as (–) control] and cells stained with preimmune rabbit serum as the negative controls (lower panel). *G*, Dose-dependent binding of M-ficolin to the membrane extract of primary monocytes immobilized on the plates. * $p < 0.05$.

for 30 s followed by 30-s incubation on ice. This alternating cycle was repeated for 5 min. The exchanged mixture was then centrifuged for 1 min at $7000 \times g$ at 4°C , divided into three aliquots, flash-frozen in liquid N_2 , and stored at -80°C until analyzed. The pepsin-digested protein was analyzed by mass spectrometry with MALDI using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). Deuterium back-exchange that occurred during the analysis was determined by carrying out control experiments where the FBG and CRP were individually deuterated for 24 h at 25°C . The spectra were viewed and calibrated by Data Explorer version 4.9 (Applied Biosystems).

The HDMS spectra were analyzed using Data Explorer version 4.9 based on the theoretical mass of two prominent peptides (theoretical $m/z = 803.34$ and 1637.82). The average mass of a peptide was calculated by determining the centroid of its isotopic envelope using Decapp software (University of California San Diego, La Jolla, CA). Differences between the centroid value of the deuterated and nondeuterated peptides enabled determination of the average number of deuterons incorporated. Exchange at side chains was determined to be 4.5% of fast-exchanging side-chain hydrogen atoms based on dilution factors. Data analysis corrected for the side-chain deuteration was carried out prior to back-exchange correction. Finally, a correction factor was applied to account for the amount of back exchange. Kinetic plots of deuteration best fit were made to a single exponential model accounting for deuterons that were exchanging at a rapid rate (mainly solvent-accessible amides). The best fit was implemented in GraphPad Prism version 5 (GraphPad Software, San Diego, CA). Changes in deuterium incorporation of $>\pm 10\%$ were considered significant (35).

Molecular dynamics simulations

The crystal structures of the CRP monomers (Research Collaboratory for Structural Bioinformatics Protein Data Bank code 1B09) and M-ficolin (Research Collaboratory for Structural Bioinformatics Protein Data Bank code 2JHM) were used for molecular dynamics simulations at constant pH. The structure simulations were conducted with a 30 Å cutoff and a 2-fs time step based on the generalized Born implicit solvation model (36). A constant value for the dielectric constant was used during the simulations in water. A 2-ns simulation was conducted at a constant temperature of 300 K. AMBER simulations were conducted with the sander module in the AMBER 9.0 MD package (University of California, San Francisco, CA) with all simulation parameters. The simulated structure was used for Zdock and Rdock.

Computer-aided molecular modeling and predictions of interaction between M-ficolin and CRP: ZDOCK and RDOCK

Computer-assisted docking of CRP and M-ficolin was based on ZDOCK, a rigid-body protein–protein docking algorithm, followed by RDOCK, an interface refinement minimization algorithm. It was used to explore the rotational and translational space of a protein–protein interaction system. The averaged structure was extracted at equal intervals from the last 1-ns molecular dynamics simulation and used as the starting structures for ZDOCK simulations. An angular step of 6° was used, which resulted in 54,000 poses. In the refinement stage of RDOCK, the 2000 best poses of near native structures obtained in the initial stage were refined and re-

ranked using a more detailed energy function that took into account conformational changes as well as a solvation term. Based on the random docking result, further optimization to fit the experimental results was carried out using the constitutive binding region as the constraint.

Statistical analysis

Data represent means \pm SEM of three independent experiments conducted in triplicate. The p values <0.05 were considered significant by two-tailed Student t test.

Results

Secreted M-ficolin associates with the cell membrane

First, we sought to clarify the cellular localization of M-ficolin, which has hitherto been discordant (18, 20, 26). To select the appropriate cells for our experiments, the expression of M-ficolin in different cells or cell lines was identified. It was found that M-ficolin was endogenously expressed in primary monocytes and U937 cells but not HEK293 or COS-1 cells (Fig. 1A). By expressing M-ficolin–GFP fusion construct in HEK293 and COS-1 cells, we showed that the majority of the M-ficolin protein is

located in the cytoplasm with a probable presence on the plasma membrane (Fig. 1B). By immunodetection with or without permeation, we further localized M-ficolin in and on primary monocytes (Fig. 1C). The membrane localization of M-ficolin in primary monocytes was further confirmed by FACS analysis (Fig. 1D). As the human M-ficolin is encoded by one gene without any splice variants (37), we do not anticipate isoforms. However, the hydrophilicity and lack of a membrane-anchor domain in the M-ficolin molecule (Fig. 1E) necessitated our investigation on how it might be membrane localized. In this study, we hypothesized that M-ficolin, which contains a secretory peptide, is synthesized and secreted from the monocytes and then associates with the cell surface to exert its immunological functions while the major reserve of M-ficolin remains cytoplasmic. To prove this, M-ficolin was completely knocked down in the monocytic U937 cells. The M-ficolin[−] clone was confirmed by RT-PCR (Fig. 1F, upper panel) and FACS analysis (Fig. 1F, lower panel), and we observed that when supplemented with purified M-ficolin, the FACS signal on M-ficolin[−] cells was rescued to the level of wild-type cells (Fig. 1F, lower panel) confirming the association of

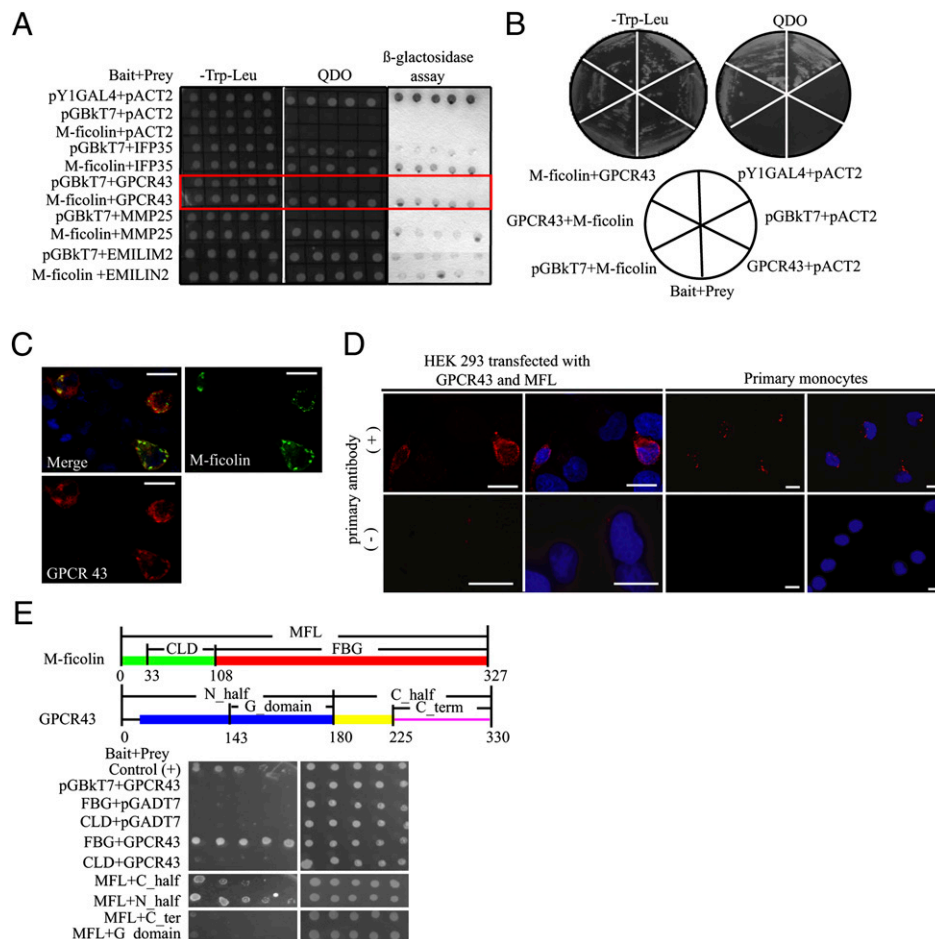


FIGURE 2. Identification of the potential interacting partners of M-ficolin. **A**, Yeast two-hybrid screening: M-ficolin in pGBKT7 was the bait to screen a human leukocyte cDNA library. Four positive transformants were further confirmed by β -galactosidase assay. **B**, M-ficolin in pGBKT7 and GPCR43 in pACT2 vectors or M-ficolin in pACT2 and GPCR43 in pGBKT7, were co-transformed into the yeast. pY1GAL4 co-transformed with pACT2 was the positive control. **C**, Colocalization of M-ficolin–GFP (green) and GPCR43–m-cherry (red) in HEK293 cells by fluorescence microscopy. Cell nucleus was stained with DAPI (blue). **D**, In situ proximity ligation assay to identify the in situ interaction between GPCR43 and M-ficolin in co-transfected HEK293 cells and purified primary monocytes. For **C** and **D**, images were taken after Z-stack with optimal interval. Original magnification $\times 100$. **E**, Delineation of the interaction domains of M-ficolin on GPCR43 by yeast two-hybrid. The FBG and collagen-like domain (CLD) in M-ficolin and N-terminal half (N_half), C-terminal half (C_half), C-terminal intracellular region (C_term), and one single region covering two glycosylation sites (G_domain) in GPCR43 were individually subcloned. To compare the interaction strength, yeast colonies were serially diluted and plated. All scale bars, 10 μ m. Data are representative of three independent experiments.

M-ficolin with the monocyte surface. By ELISA, we found that M-ficolin was bound to the membrane protein extracts of primary monocytes in a dose-dependent manner (Fig. 1G), implying that in vivo, it might associate with membrane protein(s).

M-ficolin anchors on the monocyte membrane via GPCR43

Because secreted M-ficolin is devoid of a membrane-anchor domain and was found to be localized on the monocyte surface possibly by interacting with cell surface proteins, it was imperative for us to elucidate the potential membrane receptor partner(s) of M-ficolin. Using yeast two-hybrid screening of a human leukocyte cDNA library, we short-listed 41 preys (Supplemental Table I), of which four candidates with highest scores were further validated. These plasmids were individually co-transformed with M-ficolin into yeast to exclude self-activating genes. β -Galactosidase assay indicated GPCR43 to be the most probable interacting partner of M-ficolin (Fig. 2A). Switching vectors for the bait and prey still yielded consistent interaction, confirming the association between M-ficolin and GPCR43 (Fig. 2B). Coexpression of M-ficolin-GFP and GPCR43-m-cherry in HEK293 cells demonstrated their colocalization (Fig. 2C and Supplemental Fig. 1A). Proximity ligation assay on both HEK293 (co-transfected with M-ficolin and GPCR43) and primary monocytes confirmed their in situ interaction (Fig. 2D and Supplemental Fig. 1B). These findings corroborate the likelihood that secreted M-ficolin interacts with transmembrane GPCR43 to become cell-surface localized. Next, we used yeast two-hybrid library screening method to delineate the binding regions between M-ficolin and GPCR43 (Fig. 2E). We found that GPCR43 binds to the FBG region of M-ficolin, which is known to harbor various ligand binding sites (38, 39). We showed that the M-ficolin was bound to both the extracellular domains of the N- and C-terminal halves of GPCR43 but not to the intracellular region (225–330) or the predicted glycosylation region (143–180), implying that the M-ficolin FBG might be stabilized in the cavity of GPCR43 formed by the extracellular loops of its seven-transmembrane domains. This implies that upon ligand binding, the conformational change of the FBG might distort the seven-transmembrane domains, which is the common mode of activation of GPCRs (40). To support further the interaction between GPCR43 and M-ficolin, we transfected the mouse macrophage RAW 264.7 cells with an expression vector harboring GPCR43 cDNA, following which membrane fractions were prepared under native conditions from both the transfected and control untransfected cells. Using SPR, we showed that an increase in resonance units occurred after injection of anti-GPCR43 over the immobilized GPCR43 (contained in the membrane extract from GPCR43-transfected RAW264.7 cells) compared with that in untransfected RAW264.7 (Supplemental Fig. 1C, 1D). In the control experiment, we noted that GPCR43-containing membrane extract did not bind to GlcNAc directly when in the absence of M-ficolin (Supplemental Fig. 1E). This indicates the specificity and direct interaction between GPCR43 and M-ficolin. To strengthen our finding, the membrane extract of primary human monocytes was also used for similar SPR analysis. Results again indicated the interaction between M-ficolin and native GPCR43 (Supplemental Fig. 1F).

PAMPs containing GlcNAc induce M-ficolin, which upregulates IL-8 expression

Because M-ficolin recognizes bacterial ligands and is tethered to a transmembrane receptor, it was imperative for us to investigate whether upon infection, the M-ficolin will transduce an infection signal intracellularly. Therefore, we challenged primary monocytes

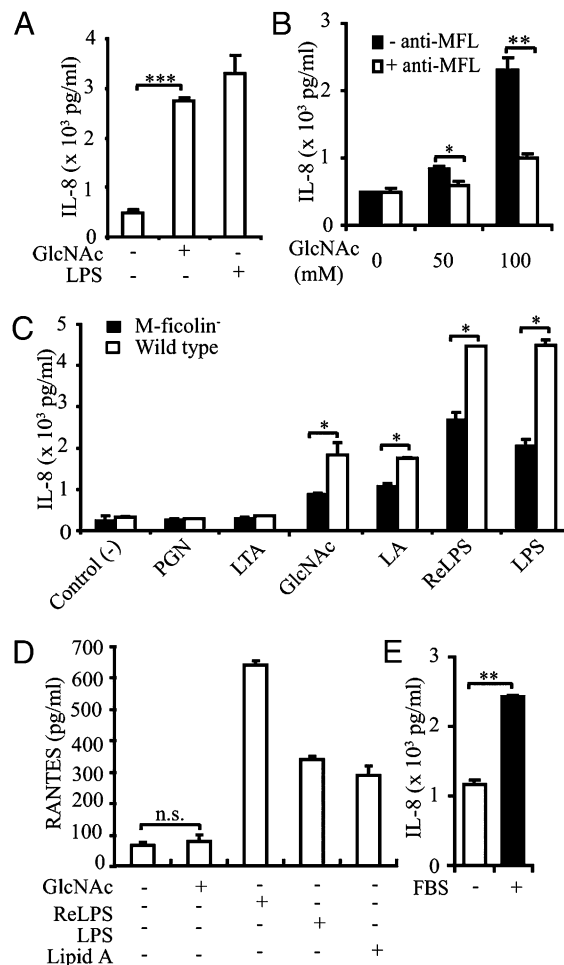


FIGURE 3. M-ficolin mediates IL-8 secretion upon PAMP/sugar stimulation. **A**, Same level of IL-8 secretion was observed by stimulating primary monocytes with 100 mM GlcNAc or 4 ng/ml LPS. **B**, M-ficolin Ab at 0.5 ng/ μ l blocked the GlcNAc-induced IL-8 upregulation in primary monocytes. GlcNAc at 50 mM and 100 mM was used as stimulant. **C**, IL-8 expression in wild-type and M-ficolin⁻ U937 cells when stimulated with 100 mM GlcNAc and 10 ng/ml PGN, LTA, LA, ReLPS and LPS. **D**, RANTES secretion by monocytes stimulated with 100 mM GlcNAc, 4 ng/ml ReLPS, LPS, and LA. **E**, Effect of FBS on the expression of IL-8. Freshly purified monocytes were changed to RPMI 1640 medium with or without FBS before stimulation with 100 mM GlcNAc. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant ($p > 0.05$).

with an M-ficolin-specific ligand, GlcNAc, representing the acetylated sugars commonly found in PAMPs, and to which ficolins bind (8). It was observed that similar to LPS, GlcNAc dramatically induced IL-8 (Fig. 3A), although a higher concentration of GlcNAc was required, probably due to the lack of a clustered pattern that would normally be present if it were displayed in situ on PAMPs and/or on pathogens. This was concordant with previous reports that up to 500 mM GlcNAc was required for co-crystallization with M-ficolin (41, 42), suggesting that only at such high concentrations is GlcNAc sufficiently clustered/patterned for the M-ficolin to bind. In fact, it is known that during infection, sugar groups like sialic acid, *N*-acetyl-D-glactosamine, or GlcNAc can reach very high levels (43, 44) attributable to either their release from the invading pathogens or the exposure of host sugar residues from injured cells. The possibility that contaminating LPS might have upregulated IL-8 via TLR4 was precluded by using LPS-free reagents (GlcNAc, FBS,

and culture media). PyroGene assay ascertained these materials to be LPS-free (Supplemental Fig. 2A, 2B). Polymyxin B, which is well known to bind and inhibit LPS, was used to confirm the LPS-free status of these materials. Up to 50 $\mu\text{g/ml}$ polymyxin B was unable to inhibit the GlcNAc-induced production of IL-8, thus corroborating the LPS-free status of the GlcNAc solution and the cell and medium preparations. This also ascertained that the IL-8 production was attributable to GlcNAc rather than to contaminating LPS (Supplemental Fig. 2C). To examine the potential role of M-ficolin in GlcNAc-induced IL-8 upregulation, we pre-treated the primary monocytes with anti-M-ficolin Ab before stimulation with GlcNAc. The anti-M-ficolin Ab significantly inhibited the GlcNAc-induced IL-8 production (Fig. 3B), thus revealing the overriding role of M-ficolin in mediating GlcNAc-induced signal transduction. We further authenticated the involvement of M-ficolin by challenging the wild-type and M-ficolin⁻ U937 cells with different PAMPs containing GlcNAc moiety. We observed that IL-8 was significantly upregulated when M-ficolin was induced by GlcNAc and other GlcNAc-containing PAMPs such as LA, ReLPS, and LPS, but not by PGN and LTA (Fig. 3C), implying the importance of the orientation of GlcNAc in the PAMPs and clustering/"patterning" of glycans/PAMPs to make them recognizable by M-ficolin (43). However, different from LPS, ReLPS, and LA, which might activate various other cell surface receptors, GlcNAc was unable to induce the upregulation of RANTES indicating the specificity of GlcNAc-induced cytokine production (Fig. 3D). In addition, the presence of serum enhanced the secretion of IL-8, implying that other components in the blood may regulate this process (Fig. 3E).

Overall, we have shown that by sensing PAMPs/sugars, M-ficolin transduces the infection signal intracellularly to upregulate IL-8 secretion.

M-ficolin-GPCR43 cross talk mediates NF- κ B activation to upregulate IL-8 expression

Because NF- κ B activity is canonical to signaling the upregulation of IL-8, we investigated the GlcNAc-induced NF- κ B activity by EMSA to understand the mechanism underlying the M-ficolin-mediated IL-8 secretion. Indeed, a dose-dependent increase in the DNA-binding activity of NF- κ B p50 and p65 occurred, thus accounting for the transcription and expression of IL-8 (Fig. 4A). This was supported by the complete inhibition of IL-8 secretion by inhibitors of NF- κ B (5 μM PDTC and 20 μM MG-132) (Fig. 4B). Therefore, we used the NF- κ B reporter construct to assess the effect of the coexpression of GPCR43 and M-ficolin on the IL-8 secretion. By dual luciferase assay, we found that low level of GPCR43, which showed no self-activation of NF- κ B, enabled M-ficolin to respond to GlcNAc in HEK293 cells via NF- κ B (Fig. 4C). To test the functional significance of GPCR43, it was knocked down in primary monocytes by siRNA. The transfection efficiency was viewed by fluorescence microscopy, and the knocked down efficiency was quantified by flow cytometry to be around 50% (Supplemental Fig. 3). In comparison with the untransfected and control siRNA transfected cells, we observed that the GPCR43 knocked-down cells were much less responsive to GlcNAc (Fig. 4D), thus confirming that docking of M-ficolin to GPCR43 contributed significantly to the M-ficolin-mediated NF- κ B activation. We observed that pertussis toxin, a known in-

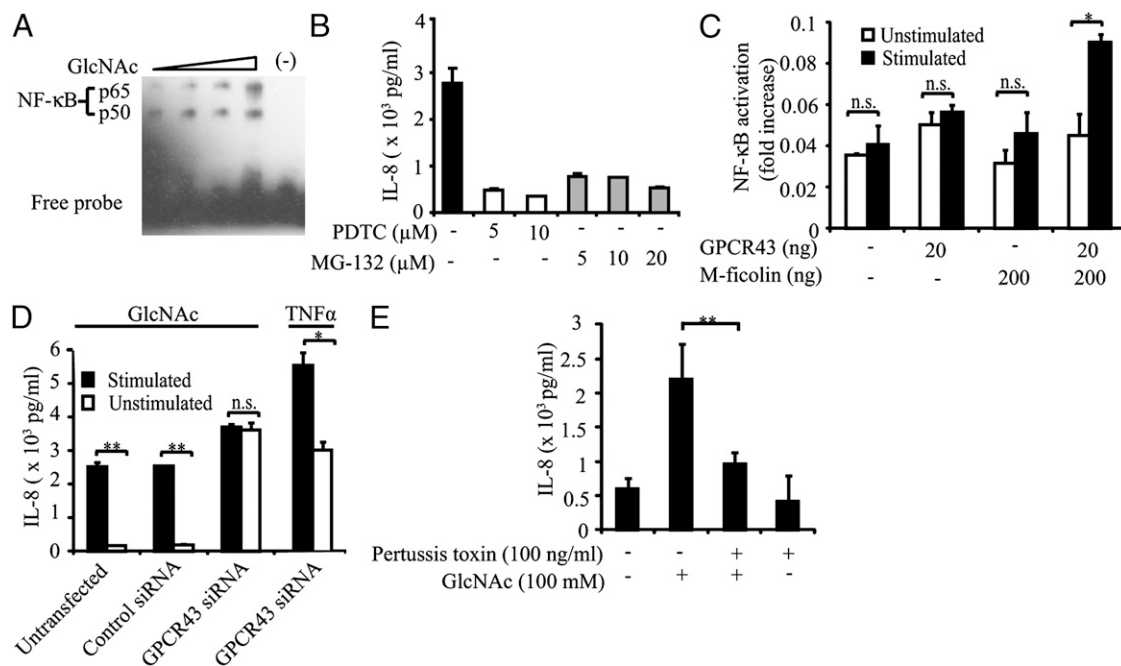


FIGURE 4. M-ficolin-GPCR43 interaction mediates NF- κ B activation to upregulate IL-8 expression. *A*, EMSA of monocytes stimulated with 10, 50, 100, and 200 mM GlcNAc showed enhanced DNA binding activity of p65 and p50 [(-) annotates cells without stimulation]. *B*, The NF- κ B activation was inhibited by the indicated amount of NF- κ B inhibitors PDTC and MG-132. Primary monocytes were incubated with the inhibitors for 2 h prior to challenging with 100 mM GlcNAc. *C*, The collaborative effect of M-ficolin and GPCR43 on the NF- κ B activation in HEK293 cells upon GlcNAc stimulation was quantified by dual luciferase assay. The untransfected cells stimulated with the same amount of GlcNAc served as the negative control. The fold increase was calculated by comparison with the negative control. *D*, Cells with GPCR43 knocked down by siRNA were much less responsive to GlcNAc stimulation. Cells transfected with control siRNA were used as a negative control, whereas cells stimulated with TNF- α were used as a positive control to indicate that the IL-8 level of GPCR43 knocked-down cells can be further activated by other ligands. *E*, Pertussis toxin 100 ng/ml significantly inhibits the GlcNAc-induced IL-8 secretion in primary monocytes. Data are representative of three independent experiments presented as means \pm SD. * $p < 0.05$; ** $p < 0.01$; n.s., not significant ($p > 0.05$).

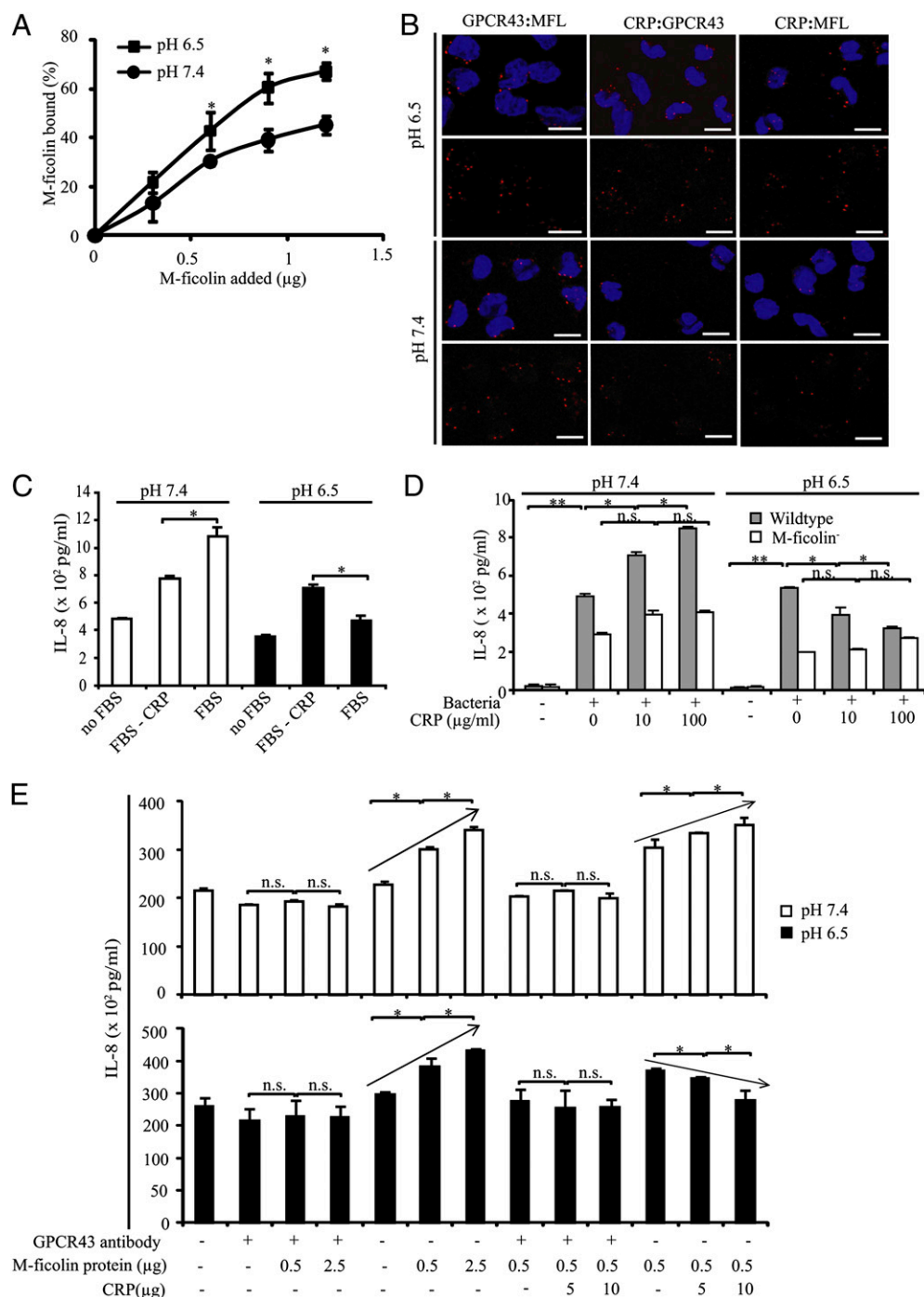


FIGURE 5. M-ficolin-GPCR43 interaction mediates NF- κ B activation to upregulate IL-8 expression. **A**, M-ficolin (MFL) shows dose-dependent binding to CRP immobilized on the plate under both pH 7.4 and pH 6.5 with stronger binding at pH 6.5. CRP, at 0.8 μ g/well, was immobilized onto each well of the ELISA plate. **B**, Proximity ligation assay to identify the interactions of MFL-GPCR43, GPCR43-CRP, and MFL-CRP in human primary monocytes at pH 7.4 and pH 6.5. All scale bars, 10 μ m. Original magnification \times 100. **C**, The effects of FBS depleted or undepleted of CRP on GlcNAc-induced IL-8 secretion at pH 7.4 and 6.5. FBS depleted or undepleted of CRP was supplemented into RPMI 1640 medium before stimulation with GlcNAc. **D**, The effect of CRP on regulating the expression of IL-8 in wild-type or M-ficolin⁻ U937 cells under normal condition (pH 7.4) or acidosis (pH 6.5) was analyzed by supplementing the cells with 0, 10, or 100 μ g/ml of CRP before challenge with *P. aeruginosa*. **E**, The effect of anti-GPCR43 Ab on the function of M-ficolin and CRP under pH 7.4 and pH 6.5. Increasing amounts of purified M-ficolin protein was added to the M-ficolin⁻ cells with or without pretreatment with GPCR43 Ab. Increasing doses of CRP were subsequently added to the culture medium. The IL-8 was measured 24 h after GlcNAc stimulation. Data are representative of three independent experiments. * p < 0.05; ** p < 0.01; n.s., not significant (p > 0.05).

hibitor of the association of G_i , G_o , and G_t with GPCRs (34), significantly inhibited the GlcNAc-induced IL-8 secretion (Fig. 4E). This implies that GlcNAc-induced GPCR43 activation might be acting via these protein pathways (34), although further studies

are needed to confirm this observation. At this juncture, our results have evidently shown that M-ficolin anchors to GPCR43, through which the M-ficolin transduces the infection signal intracellularly to activate NF- κ B and upregulate IL-8 secretion.

CRP collaborates with M-ficolin to regulate IL-8 secretion

As circulating L-ficolin (with 80% homology to M-ficolin) binds to plasma CRP in a pH-dependent manner, we reasoned that M-ficolin might also interact with CRP at different phases of infection where shifts in pH occur. This was confirmed by ELISA, which demonstrated that M-ficolin dose-dependently binds to CRP, with stronger binding at pH 6.5 (Fig. 5A). Because we have demonstrated that i) M-ficolin associates with GPCR43 and ii) plasma CRP interacts with M-ficolin, it was imperative for us to examine whether these interactions coexist. We performed proximity ligation assay of CRP–M-ficolin and M-ficolin–GPCR43 from the same batch of primary monocytes. In agreement with the results of biochemical and biophysical assays, we observed that M-ficolin–GPCR43 interaction was consistently prominent regardless of pH shift, whereas M-ficolin–CRP interaction was only strong under pH 6.5 (Fig. 5B and Supplemental Fig. 4A). This indicates that the interactions between CRP and M-ficolin and between M-ficolin and GPCR43 probably coexist under local acidosis. Proximity ligation assay of CRP–GPCR43 under pH 6.5 and pH 7.4 showed that M-ficolin might connect CRP to GPCR43 under local acidosis, which was further supported by yeast three-hybrid results (Supplemental Fig. 4B).

Because we observed that serum components might contribute to IL-8 secretion (Fig. 3E), it was pertinent for us to analyze the effect of CRP on the functions of M-ficolin. Therefore, cells were cultured in medium with FBS depleted or undepleted of CRP before stimulation with GlcNAc. We found that the presence of CRP boosted IL-8 secretion at physiological pH (pH 7.4) prevailing at the initial phase of infection (Fig. 5C), whereas under acidosis, CRP inhibited IL-8 secretion (Fig. 5C). This indicates a pH-dependent dual-opposing regulatory effect of CRP on M-ficolin function. To corroborate this observation, the cells were challenged with heat-inactivated *P. aeruginosa*, and increasing amounts of exogenous CRP was supplemented to the culture medium, which had been depleted of CRP. Consistently, we ob-

served that at the initial phase of infection (pH 7.4), CRP dose-dependently increased the secretion of IL-8 (Fig. 5D). Conversely, at the later phase of infection (pH 6.5), CRP also dose-dependently inhibited the secretion of IL-8 (Fig. 5D). This reaffirms the pH-dependent dual-opposing regulatory effect of CRP on IL-8 secretion and also implies that both the pathogen- and GlcNAc-induced IL-8 secretion share the same pathway, supporting the feasibility of using GlcNAc to represent the PAMPs that bind M-ficolin. Importantly, this regulatory effect of CRP was markedly reduced in M-ficolin[−] cells (Fig. 5D), supporting that CRP functions through M-ficolin.

To test the functional significance of the interactions between CRP and M-ficolin–GPCR43, we pretreated the M-ficolin[−] U937 cells with the GPCR43 Ab, following which the purified M-ficolin and CRP were added to the cells. Consistent with our previous results, we showed that without GPCR43 Ab, the addition of M-ficolin dose-dependently increased the IL-8 secretion under both pH 7.4 and pH 6.5. However, the addition of CRP dose-dependently suppressed the IL-8 secretion at pH 6.5 but not at pH 7.4 (Fig. 5E). Importantly, we found that GPCR43 Ab, which might have inhibited the GPCR43–M-ficolin, attenuated the effect of both M-ficolin and CRP under pH 7.4 and pH 6.5, indicating that both M-ficolin and CRP possibly function through GPCR43 (Fig. 5E). Overall, we have demonstrated that CRP, associating with M-ficolin–GPCR43, serves as a regulator under different stages of the infection in a pH-sensitive manner. Therefore, it is conceivable that in vivo, M-ficolin recruits CRP to balance the level of IL-8 and thus achieves homeostasis.

Conformational change in M-ficolin complexed with CRP modulates IL-8 secretion

The liaison between GPCR43 and M-ficolin is consistently strong under pH 6.5 or 7.4 when in the presence of CRP (Fig. 5B and Supplemental Fig. 4), suggesting that the plasma CRP might not inhibit the binding of M-ficolin to GPCR43. It has recently been

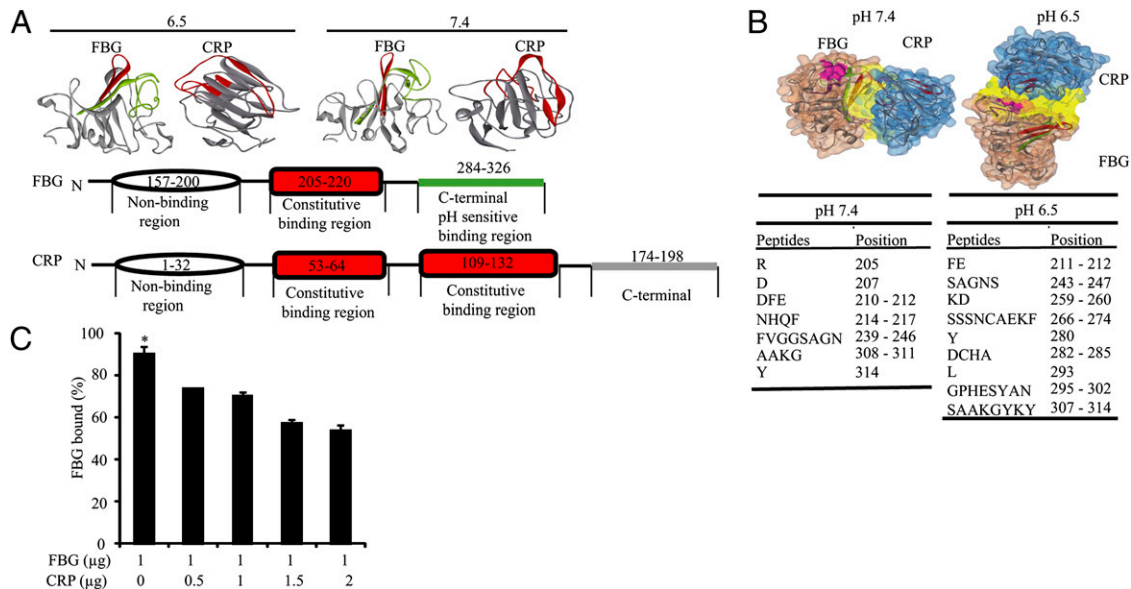


FIGURE 6. Conformational change in M-ficolin complexed with CRP modulates IL-8 secretion. **A**, CRP and the FBG binding interfaces were identified by HDMS and highlighted in their corresponding simulated structures under different pHs (upper panel) or schematic diagrams (lower panel): constitutive binding sites (red) and the pH-sensitive binding region (green). **B**, Computational docking model of the FBG (brown) and CRP (blue) at pH 7.4 and 6.5 optimized according to HDMS results. The binding interface is in yellow. The conserved PAMP/sugar binding pocket (pink) is exposed at pH 7.4 and blocked by CRP at pH 6.5. The predicted CRP binding sites on the FBG are tabulated below the models. **C**, Increasing amount of CRP inhibits the binding of the FBG to GlcNAc–BSA immobilized on the ELISA plate. The FBG and CRP were preincubated at room temperature for 2 h before adding to the ELISA wells, which were precoated with GlcNAc–BSA. The FBG was identified using anti-His fusion Ab. **p* < 0.05.

reported that CRP interacts with the FBG of M-ficolin (45). To understand the mechanisms underlying the influence of infection-mediated local acidosis on CRP-M-ficolin liaison, which suppresses IL-8 secretion, we performed HDMS to elucidate the binding interfaces between CRP and the FBG and the consequential conformational change under i) pH 7.4 and ii) pH 6.5. The differential incorporation of deuterium for each peptide was calculated across all time points of interaction (Supplemental Figs. 5, 6). Under both pH 7.4 and 6.5, three peptides of M-ficolin in the FBG (205–213, 209–216, 209–220) and three peptides of CRP (53–62, 54–64, 109–132) showed significant decrease in deuterium exchange, suggesting that these regions are probably constitutively interactive with each other (Fig. 6A, red ribbon). Notably, the binding interface located in M-ficolin_{284–326} (Fig. 6A, green ribbon) was more accessible to CRP at pH 6.5 than at pH 7.4, which might explain the enhanced binding of M-ficolin to CRP under local acidosis. In contrast, the CRP structure, which does not harbor such a pH-sensitive region, is invariant to pH change (Supplemental Fig. 6). Overall, these results indicate that relative to CRP, the M-ficolin structure is more adaptable to pH shift implying that it plays a dominant role in controlling the activity of the CRP-M-ficolin complex.

Next, we predicted the binding pattern of CRP-M-ficolin by computational docking using the simulated structures of CRP and the FBG under pH 7.4 and 6.5 (Supplemental Fig. 7). Consistent with the binding and nonbinding regions identified by HDMS, the computational docking showed that at pH 6.5, more regions in the C terminus of the FBG were translocated to the binding interface with CRP (Fig. 6B). Importantly, we observed that under pH 7.4, the CRP binding site and the PAMP/sugar binding site (42) on the M-ficolin are separate. This is consistent with a recent speculation

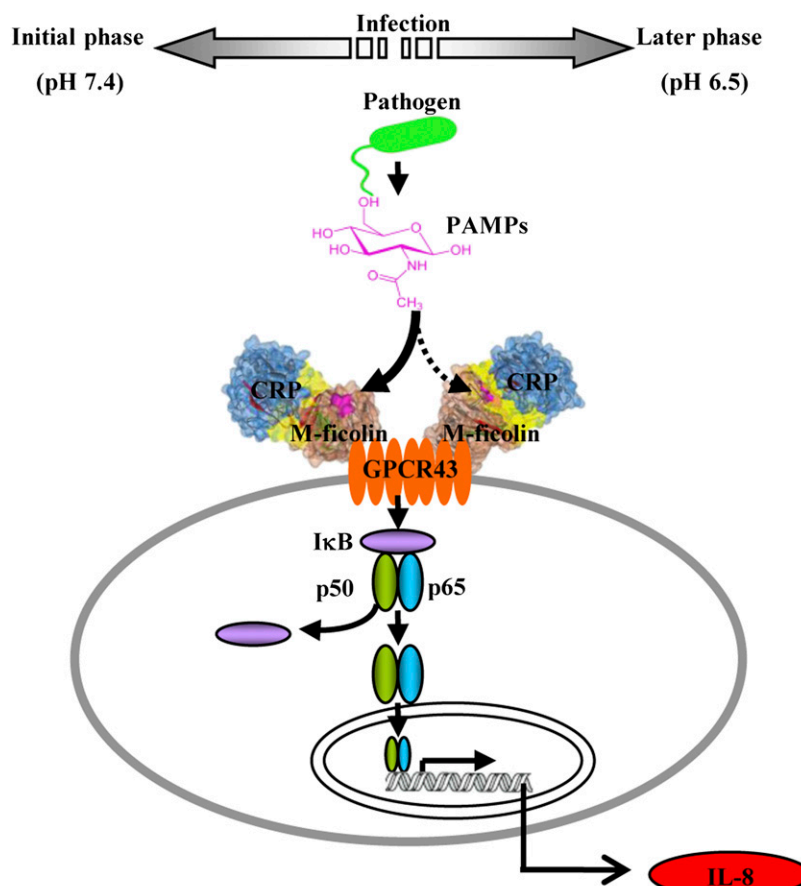
that under physiological condition, CRP is not anticipated to compete with the PAMP/sugar binding site on M-ficolin (46). In addition, we found that upon CRP binding, the FBG forms an extended conformation (Supplemental Fig. 4) making it more accessible to sugar ligands. This structural change in M-ficolin is facilitated by its interaction with CRP, which upregulates IL-8. In contrast, under pH 6.5, CRP was bound to the M-ficolin at motif 282–285, which is the evolutionarily conserved binding pocket for the *N*-acetyl group (42). The conformational maneuver of the M-ficolin motif 282–285, due to CRP binding, might block the ability of M-ficolin to recognize GlcNAc/pathogens, thus resulting in a reduction in IL-8 production, as was shown by the ELISA result. This indicates the role of CRP in restoring the homeostasis of IL-8 secretion (Fig. 6C).

Discussion

Innate immunity plays a central role in front-line defense against pathogens. However, severe inflammation response syndrome, which occurs during an overwhelming infection, tends to overactivate the immune system in an attempt to clear the pathogens. Unfortunately, this defense action can backfire, damaging the host's own tissues. Thus, therapeutic intervention of the immune pathways, particularly targeting the custodian cell-surface protein(s), is clinically valuable. Toward this goal, a detailed understanding of the molecular mechanisms underlying protein-protein interaction on the cell surface and the subsequent signal transduction inside the cells would provide a powerful rationale for future drug design and development.

Transmembrane CLs are well known to transduce signals into the cells independently of or collaboratively with TLRs. However, the functions and underlying mechanisms of action of the soluble

FIGURE 7. A model to illustrate the mechanism of M-ficolin-mediated signal transduction. Upon sensing the GlcNAc moiety in bacterial PAMPs, interaction between M-ficolin (brown) and GPCR43 (orange) activates the NF- κ B pathway leading to the upregulation of IL-8. Infection at the initial or later phases perturbs the microenvironmental pH, which induces conformational changes in the M-ficolin molecule and in turn regulates IL-8 secretion. At the initial phase, where the pH is still physiological, CRP binds weakly with M-ficolin. This protein-protein interplay exposes the PAMP/sugar binding region (pink) of M-ficolin (thick arrow), which upregulates IL-8. At the later phase, local acidosis strongly enhances the CRP-M-ficolin interaction, thus blocking the PAMP/sugar binding site on the M-ficolin (dashed arrow). This process downregulates IL-8 secretion and restores homeostasis.



CLs in signal transduction remain unclear, although galectin (47) and MBL (14) were reported to sense danger signals and boost the proinflammatory immune response. We demonstrated that M-ficolin, a representative soluble CL, mediates NF- κ B activation and IL-8 secretion upon challenge by pathogens/PAMPs/GlcNAc, which was confirmed by abrogation of the effect in M-ficolin⁻ cells, as well as cells pretreated with M-ficolin Ab. We showed that M-ficolin performs this key role by being localized on the monocyte surface through interaction with its cognate transmembrane receptor partner, GPCR43, which probably alerts the host cell of microbial invaders through transducing an infection signal via the activation of NF- κ B (Fig. 7). Although M-ficolin expression in macrophages was significantly lower, it can be up-regulated in monocyte/macrophage lineage upon LPS stimulation (48), indicating its critical function in immune cells during infection. Notably, such interplay of proteins leading to signal transduction was also observed in other membrane-associated molecules such as angiopoietins, which coincidentally also contain an FBG (49), implying the fundamental significance of such a phenomenon. As the three isoforms of ficolin share sequence homology with each other, albeit to different extents, it was of interest to examine whether the L- and H-ficolins also interacted with GPCR43. We found that L- and M-ficolins but not H-ficolin interacted with GPCR43 (Supplemental Fig. 8), which is probably due to the lower amino acid sequence homology (40%) of H-ficolin with L- and M-ficolins. As both M-ficolin and GPCR43 are secreted by monocytes and M-ficolin is proposed to be released upon stimulation of the monocytes during local infection (20), its concentration at the local area is probably high thus implying its greater likelihood to interact with GPCR43. Nevertheless, the potential participation of both the L- and M-ficolins in collaboration with GPCR43 could possibly broaden the spectrum of pathogen recognition and immune response.

The conformational change of CLs upon pH or ligand/protein binding is universal (6, 50), although the biological consequence remains unclear. We found that infection-mediated local acidosis induces conformational changes to the M-ficolin, altering its interaction with CRP. Under acidosis, the flexible C terminus in the FBG of M-ficolin exposes more binding sites for CRP, which completely blocked the PAMP/sugar binding sites of the FBG and inhibited the consequential IL-8 secretion. In contrast, at the initial phase of infection (pH 7.4), CRP associates weakly with the FBG and exposes the PAMP/sugar binding site of the FBG to bacteria, thus promoting IL-8 production. Therefore, CRP plays a dual reciprocal role. These results clearly support a refined regulatory mechanism where environmental perturbation at the later phase of infection induces the flexible structure of M-ficolin to change its conformation to accommodate CRP at a different site on the M-ficolin molecule, which regulates its pathogen recognition and IL-8 production. Our delineation of the binding sites of these proteins provides important clues for future drug design to target the CRP–M-ficolin interface for immunomodulation.

Overall, we have shown how the host exercises plasticity in its immune recognition-cum-regulation via monocyte-secreted M-ficolin, which exploits its extracellular interacting partners to form a trio-complex to act bidirectionally: i) transduce infection signal via GPCR43 into the host cell and b) regulate the immune response to restore homeostasis through the reciprocal role of CRP, which modulates the M-ficolin structure–activity relationship to downregulate IL-8 secretion. We envisage that the host can expand its repertoire of immune recognition-cum-regulation mechanisms by promiscuous protein networking among members of the families of soluble CLs, pentraxins, and transmembrane proteins, where cues from the infection-mediated environmental perturbation

induce the protein complex to immunomodulate and attain homeostasis. Furthermore, our elucidation of the binding interface and the infection and inflammation sensitive regulatory region of M-ficolin provides insights into the bioactive center of the M-ficolin molecule, which might be useful for future drug development.

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Disclosures

The authors have no financial conflicts of interest.

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