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Soluble Collectin-12 (CL-12) Is a Pattern Recognition Molecule Initiating Complement Activation via the Alternative Pathway

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Soluble defense collagens including the collectins play important roles in innate immunity. Recently, a new member of the collectin family named collectin-12 (CL-12 or CL-P1) has been identified. CL-12 is highly expressed in umbilical cord vascular endothelial cells as a transmembrane receptor and may recognize certain bacteria and fungi, leading to opsonophagocytosis. However, based on its structural and functional similarities with soluble collectins, we hypothesized the existence of a fluid-phase analog of CL-12 released from cells, which may function as a soluble pattern-recognition molecule. Using recombinant CL-12 full length or CL-12 extracellular domain, we determined the occurrence of soluble CL-12 shed from in vitro cultured cells. Western blot showed that soluble recombinant CL-12 migrated with a band corresponding to ~120 kDa under reducing conditions, whereas under non-reducing conditions it presented multimeric assembly forms. Immunoprecipitation and Western blot analysis of human umbilical cord plasma enabled identification of a natural soluble form of CL-12 having an electrophoretic mobility pattern close to that of shed soluble recombinant CL-12. Soluble CL-12 could recognize *Aspergillus fumigatus* partially through the carbohydrate-recognition domain in a Ca^{2+}-independent manner. This led to activation of the alternative pathway of complement exclusively via association with properdin on *A. fumigatus* as validated by detection of C3b deposition and formation of the terminal complement complex. These results demonstrate the existence of CL-12 in a soluble form and indicate a novel mechanism by which the alternative pathway of complement may be triggered directly by a soluble pattern-recognition molecule. *The Journal of Immunology*, 2015, 195: 3365–3373.

T he complement system is an important humoral and cellular innate immune defense system that functions as a rapid and efficient primary host defense barrier against invading pathogens and altered host cells. Its initiation and amplification is mediated by three defined pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (1). The CP is mainly activated in an Ab-dependent fashion by IgM or IgG clusters via interaction with C1q, but it is also initiated by C1q directly through recognition of distinct struc-

tural moieties on microbial and apoptotic cells or through versatile soluble pattern-recognition molecules (sPRMs), such as PTX3 and C-reactive protein (2–4). However, LP activation is primarily induced by collagen-like sPRMs, such as mannose-binding lectin (MBL) and the ficolins (5). It has also been shown that the CP and LP could be cross-activated through formation of heterocomplexes among PTX3, C1q, and the ficolins, as well as MBL (6, 7). In contrast, the AP does not require Abs or sPRMs for its initiation, and it is often referred to as spontaneous and nondiscriminatory because it does not differentiate between self and nonself. Rather, its initiation occurs by spontaneous low-rate hydrolysis of the thioester in C3, which is quickly amplified on nearby surfaces (8).

Recently, three novel human defense collagens: collectin-10 (collectin liver 1, CL-L1, or CL-10), collectin-11 (collectin kidney 1, CL-K1, or CL-11), and collectin-12 (collectin placenta 1, CL-P1, or CL-12) derived from the *COLEC10*, *COLEC11*, and *COLEC12* genes, respectively, were identified and characterized (9–11). CL-10 was initially demonstrated as a cytoplasmic protein, whereas recent work has shown its potential presence in human blood circulation (12). CL-11 is a serum protein with a low concentration of ~0.3 μg/ml (13, 14), and it has been shown to bind certain microorganisms and apoptotic cells through its carbohydrate-recognition domains (CRDs) mainly to L-fucose and D-mannose (11, 15). Furthermore, CL-11 has recently been shown to be an LP-activating molecule associated with the LP-associated serine proteases (MASPs) in addition to MBL and the ficolins (16). Different from the other novel collectins, CL-12 was originally defined as scavenger receptor C-type lectin because it shares structural and functional similarities with type A scavenger receptor and collectins (10, 17). As a transmembrane scavenger receptor, CL-12 is mainly localized in vascular endothelial cells in general, but in particular

in umbilical cord endothelial cells (18). CL-12 revealed pattern-recognition characteristics toward yeast, as well as *Escherichia coli* and *Staphylococcus aureus*, by mediating phagocytosis by CL-12 transfected cells (17, 18). Furthermore, it has been shown that CL-12 selectively bind glycans bearing both terminal galactose and fucose moieties and presents a striking high-affinity selectivity for Lewis^X trisaccharide, which is commonly displayed on adhesion molecules of various leukocytes, suggesting that CL-12 could modulate leukocyte recruitment (19, 20).

CL-12 has also been shown to bind fibrillar β-amyloid protein, thus indicating a potential role in scavenging of amyloid (21). Intriguingly, recent findings in zebrafish embryo models have shown that knockdown of zebrafish *COLEC12* expression causes severe defects in vasculogenesis and development during the early embryogenic stages (22). These results suggest a pivotal role for CL-12 not only in innate immune defense, but also in fundamental developmental processes. Based on its structure and functional similarities with soluble collectins, we hypothesized the existence of a fluid-phase analog of CL-12 released from cells, which may function as a sPRM. Based on our data, we report a novel mechanism of complement activation where the AP may be directly initiated by sPRMs.

Materials and Methods

Expression of recombinant CL-12

Recombinant CL-12 full length (rCL-12FL) was produced using the Flp-In System (Invitrogen) according to the manufacturer's instructions. In brief, cDNA encoding CL-12 full length (Met^1~Leu^{742}) was designed into the Flp-In expression vector (pcDNA5/FRT) containing the Hygromycin B resistance gene and ordered from Invitrogen. Stably growing Flp-In-CHO cells containing the *lacZ*-Zeocin fusion gene were cotransfected with a mixture of Lipofectamine, the Flp recombinase expression plasmid pOG44, and the pcDNA5/FRT vector containing *COLEC12* gene. As negative control, the pcDNA5/FRT vector lacking the *COLEC12* gene was applied in parallel. The cells were cultivated in Ham's F-12 medium (Sigma) for 2 d. Stable transfectants were then selected by cultivation in the medium containing hygromycin (500 μg/ml; Invitrogen). In addition, recombinant CL-12 extracellular domain (rCL-12ED) (Lys^{60}~Leu^{742}) tagged with N-terminal 6× histidine was also produced as described earlier.

SDS-PAGE and Western blot

Proteins were separated by 4–12% SDS-PAGE gel under reducing or nonreducing conditions according to the method of Laemmli (23) and visualized by Coomassie Brilliant blue or Western blot, as described previously (6, 7).

Determination of CL-12 from rCL-12FL–expressing CHO cells

To determine soluble CL-12, we cultivated rCL-12FL–expressing CHO cells (CHO/CL-12FL) in EGM-2 BulletKit medium (Lonza). Expression level of rCL-12FL on CHO/CL-12FL was determined with goat anti-human CL-12 polyclonal Ab (CL-12 pAb; R&D Systems) and Alexa Fluor 488–conjugated donkey anti-goat IgG (Life Technologies), and finally analyzed with a BD FACSCalibur (Becton Dickinson) as previously described (6). When the culture flasks were 80–90% confluent, the supernatants were harvested and analyzed for the presence of CL-12 by Western blot as described earlier. In brief, the supernatants were subjected to SDS-PAGE, and CL-12 was detected with rat anti-human CL-12 mAb (R&D Systems) and HRP-conjugated rabbit anti-rat IgG (Dako). As a negative control, CHO cells that were transfected with the blank vectors lacking the *COLEC12* gene (CHO/Control) were applied in parallel. Alternatively, transmembrane CL-12 was isolated from CHO/CL-12FL using Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. The presence of CL-12 was then determined by Western blot as described earlier. As a negative control, CHO/Control was applied in parallel.

Preparation of total protein extracts from human umbilical cord tissues and isolation of human umbilical cord plasma

Human umbilical cord tissue was obtained freshly from 10 healthy puerperal women with informed consent. After the umbilical cords had been cut from the newborn babies, the bloods that remained in the um-

bilical veins after parturition were collected in hirudin blood tubes (Roche Diagnostics). We also collected venous blood in hirudin tubes from 10 healthy staff members with informed consent. Preparation of total protein extracts from human umbilical cords was achieved by Chemicon's total protein extraction kit (Millipore A/S) according to the manufacturer's instructions. Furthermore, platelet-poor plasma was isolated by centrifugation (2000 × *g*, 10 min at room temperature) of human umbilical vein blood, and plasma samples were stored in small aliquots at −80˚C until analysis.

Determination of CL-12 in human umbilical cord and in plasma

To determine CL-12 in human umbilical cord, we subjected samples of the total protein extracts (50 μg) from human umbilical cords to SDS-PAGE, and the presence of CL-12 with biotinylated CL-12 mAb and streptavidin-conjugated HRP (Amersham Bioscience) was visualized in Western blot as described earlier. In some experiments, the samples were immunoprecipitated with Dynabead sheep anti-rat IgG (Invitrogen) in combination with CL-12 mAb or rat IgG1 (R&D Systems), and analyzed with CL-12 pAb by Western blot.

To determine the level of soluble CL-12 in human umbilical cord plasma, we coated ELISA plates with CL-12 pAb (1 μg/ml) in PBS buffer (10 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4). All reaction volumes were 100 μl, and plates were washed after each step in PBS containing 0.05% Tween 20. The plates were then incubated with purified recombinant CL-12 from R&D (rCL-12R&D; 100 ng/ml; N terminally 9× histidine tagged extracellular domain of human CL-12, Leu^{57}~Leu^{742}) or human umbilical cord plasma (20%) that was diluted with PBS containing 0.05% Tween 20 containing EDTA (10 mM) plus normal goat IgG (50 μg/ml; R&D system) at room temperature for 3 h with agitation in triplicate. Bound proteins were detected with CL-12 mAb or rat IgG1 as isotype control, followed by incubation of HRP-conjugated rabbit anti-rat IgG (Dako). Final peroxidase reaction was performed as previously described (24). CL-12 level in the plasma was calculated based on the standard curve fitted with rCL-12R&D. In some experiments, the plasma samples were incubated in ELISA plates coated with CL-12 pAb (1 μg/ml) or normal goat IgG (1 μg/ml). Bound proteins were stripped and visualized with biotinylated CL-12 mAb and streptavidin-conjugated HRP in Western blot as described earlier.

Binding of soluble CL-12 to *Aspergillus fumigatus* conidia

Binding of soluble CL-12 to *A. fumigatus* (clinical isolate 6871; University of Perugia, Perugia, Italy) was performed as described previously (6, 24). rCL-12R&D was applied as a soluble form of CL-12 in the following experiments. In brief, *A. fumigatus* were incubated with rCL-12R&D (~0.03–0.25 μg/ml) at 37˚C for 1 h. Bound proteins were detected with CL-12 pAb and Alexa Fluor 488–conjugated donkey anti-goat IgG and then finally analyzed with a BD FACSCalibur. In some experiments, *A. fumigates conidia* were incubated with rCL-12R&D in the presence of *N*-acetyl-D-glucosamine (GlcNAc; 0.3 M), curdlan (500 μg/ml), or a mixture of both (all from Sigma). Bound CL-12 was assessed as described earlier. Alternatively, rCL-12R&D binding to *A. fumigatus* was also determined in the presence of EDTA (10 mM) or EGTA (10 mM)-Mg^{2+} (5 mM) instead of Ca^{2+} (2.5 mM).

Human sera

Serum and plasma samples were obtained from healthy volunteer donors with informed consent. MBL-defect serum (serum^{MBL-}) and properdin-defect serum (serum^{fP-}) were obtained from an MBL-defect individual and a properdin-defect individual with normal complement activities, respectively. The sera were diluted with TBS/Ca^{2+} (10 mM Tris/150 mM NaCl/2.5 mM CaCl$_2$, pH 7.4) or TBS/EGTA-Mg^{2+} (10 mM Tris/150 mM NaCl/10 mM EGTA/5 mM Mg^{2+}, pH 7.4) before use based on experimental intentions.

Complement activation

Complement C4b deposition was determined as previously described (7). In brief, *A. fumigatus* were incubated with rCL-12R&D (5 μg/ml) before addition of serum^{MBL-} (10%) as a complement source in the presence of TBS/Ca^{2+}. After washing, C4b deposition was detected by FITC-linked rabbit anti-human C4c pAb (Dako) and analyzed by flow cytometry. Complement C3b and terminal complement complex (TCC) deposition were determined in the presence of TBS/EGTA-Mg^{2+} as follows: *A. fumigatus* were preincubated with rCL-12R&D (5 μg/ml) or elevated concentrations of rCL-12R&D (~0–4 μg/ml) before incubation of serum^{MBL-} (10%) in the presence of EGTA-Mg^{2+}. After washing, C3b and TCC deposition were detected by rabbit anti-human C3c pAb (Dako)/FITC-linked swine

anti-rabbit IgG (Dako) or mouse anti-human C5b-9 mAb (BioPorto)/
FITC-linked goat anti-mouse IgG (Dako), respectively, and analyzed as
described earlier.

In some experiments, complement C3b and TCC deposition were also
induced with serum[fP−] (10%) instead and assessed as described earlier.
Alternatively, serum[fP−] was spiked with exogenous properdin (5 μg/ml;
CompTech) under physiological concentration before induction of com-
plement activation. Properdin that was thawed once from initial prepara-
tion was analyzed by SDS-PAGE before use to determine formation of
properdin aggregates during storage. Preparations that formed aggregates
were excluded from testing. During complement activation, properdin that
bound *A. fumigatus* was also detected using mouse anti-human properdin
mAb (Hyb 39-6, characterized in our laboratory) and FITC-linked goat
anti-mouse IgG. Bound proteins were analyzed by flow cytometry as de-
scribed earlier. In some experiments, to determine whether properdin binds
the transmembrane form of CL-12, we incubated properdin (5 μg/ml) with
either CHO/CL-12FL or CHO/Control and analyzed it as described earlier.

Properdin binding to anchored soluble CL-12

A. fumigatus were preincubated with rCL-12R&D (5 μg/ml) and then
blocked residual exposed sites with serum[fP−] (10%). After washing, pro-
perdin binding was determined by incubation of exogenous properdin
(5 μg/ml) using goat anti-human properdin polyclonal Ab (CompTech) and
Alexa Fluor 488–conjugated donkey anti-goat IgG. Bound proteins were
analyzed by flow cytometry as described earlier.

ELISA

Microtiter plates were coated with rCL-12R&D or BSA in 2-fold serial
dilutions starting at 5 μg/ml in PBS. All reaction volumes were 100 μl, and
plates were washed after each step in TBS/Ca^{2+} containing 0.05% Tween 20.
After blocking with BSA (1%), the plates were incubated with properdin
(5 μg/ml) at room temperature for 3 h. Bound protein was detected using
goat anti-human properdin polyclonal Ab/HRP-conjugated donkey anti-goat
IgG (H&L; GenScript) and visualized by enzymatic reactions as described
earlier. In a reverse order, rCL-12R&D binding to the plates immobilized
with properdin (or BSA; 5 μg/ml) was analyzed by incubation in 2-fold se-
rial dilutions starting at 5 μg/ml, followed by detection with CL-12 mAb/
HRP-conjugated rabbit anti-rat IgG as described earlier.

Statistical analysis

Comparisons between groups were made by one-way ANOVA and
GraphPad Prism, version 5.0 (GraphPad Software). The *p* values <0.01
were considered significant.

Results

Expression of soluble CL-12

To determine a soluble form of CL-12, we established stably trans-
fected Flp-In-CHO cells expressing rCL-12FL and rCL-12ED, re-
spectively. The supernatants derived from in vitro–cultured CHO/
CL-12FL were used to analyze soluble CL-12 expression. Under non-
reducing conditions, rCL-12FL presented multimeric ladder bands
with monomers, dimers, and trimers, whereas under reducing
conditions it migrated as a single band of ∼140 kDa. Consistently,
rCL-12 from R&D (rCL-12R&D) and rCL-12ED displayed elec-
trophoretic mobility similar to that of rCL-12FL, showing slight
difference in gel mobility presumably because both rCL-12R&D
and rCL-12ED lack in domain structure integrity compared with
rCL-12FL (Fig. 1A, 1B). When the supernatants from in vitro–
cultured CHO/CL-12FL were analyzed, as shown in Fig. 1A and 1B,
we could show distinct bands of soluble form of CL-12 in the culture
supernatants, but not in the supernatants harvested from mock-
transfected CHO cells (CHO/Control). No changes in the growth
and morphology of the cells were observed during the cultivation.
However, the soluble form of recombinant CL-12 in the culture
supernatants indicated three bands with gel mobility corresponding
to ∼150, ∼250, and ∼400 kDa under nonreducing conditions. The
∼150-kDa band represented bovine IgG that cross-reacted with the
detection Ab. Nevertheless, under reducing conditions it restored to
∼120 kDa (Fig. 1A, 1B).

To further substantiate the potential existence of a soluble form
of CL-12 under physiological conditions, we collected fresh hu-
man umbilical cord tissue and plasma from healthy puerperal
women and used them to analyze CL-12 expression. Western
blot showed that native CL-12 derived from human umbilical cord
tissue had an electrophoretic mobility equal to that of rCL-
12R&D (∼130 kDa) under reducing conditions, which was further
confirmed by the subsequent immunoprecipitation of the total
protein extract (Supplemental Fig. 1). ELISA for human um-
bilical cord plasma also revealed that the median concentration
of soluble CL-12 from 10 healthy subjects was measured to be
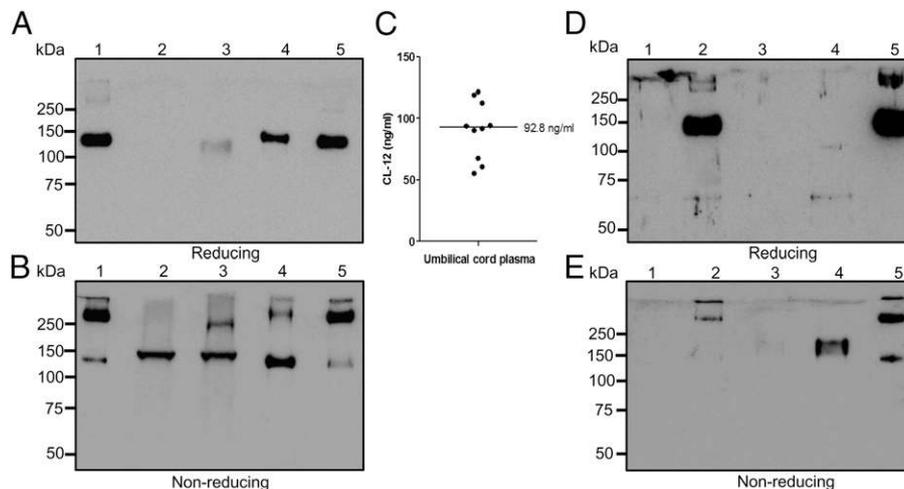
FIGURE 1. Characterization of soluble CL-12. (**A** and **B**) Western blot of recombinant CL-12. Samples were subjected to SDS-PAGE and analyzed
by Western blot. *Lane 1*, rCL-12R&D; *lane 2*, supernatant from cultivation of CHO/Control; *lane 3*, supernatant from cultivation of CHO/CL-12FL;
lane 4, membrane protein extracts from CHO/CL-12FL; *lane 5*, supernatant from cultivation of rCL-12ED–expressing CHO cells. (**C**) Expression of
soluble CL-12 in human umbilical cord plasma. The level of soluble CL-12 (*n* = 10) was determined in ELISA. Bar represents median concentration.
(**D** and **E**) Western blot of soluble CL-12 in human umbilical cord plasma. The sample of human umbilical cord plasma was incubated with ELISA plate
that was coated with either CL-12 pAb or normal goat IgG. Bound proteins were stripped and analyzed with biotinylated CL-12 mAb in Western blot.
Results are representative of five independent experiments with the plasma from five individuals that yields similar results. *Lane 1*, Proteins from control
ELISA wells incubated with rCL-12R&D; *lane 2*, proteins from CL-12 pAb–immobilized ELISA wells incubated with rCL-12R&D; *lane 3*, proteins
from control ELISA wells incubated with human umbilical cord plasma; *lane 4*, proteins from CL-12 pAb–immobilized ELISA wells incubated with
human umbilical cord plasma; *lane 5*, rCL-12R&D.

92.8 ng/ml (range ~55–121.4 ng/ml; Fig. 1C), whereas reactivity in venous plasma from healthy adults (*n* = 10) was shown to be negligible. Furthermore, Western blot analysis showed that soluble CL-12 in the plasma migrated mainly as a doublet with molecular mass of ~150 and ~230 kDa, whereas under reducing conditions it migrated as single band with a molecular mass of ~110 kDa (Fig. 1D, 1E). No apparent bands emerged with similar gel mobility in the negative control (Fig. 1D, 1E).

Binding specificity of soluble CL-12 on A. fumigatus

To characterize the potential physiological relevance of soluble CL-12 as recognition molecule in the complement activation, we used *A. fumigatus* as a model of infection and determined the binding of soluble CL-12 by purified rCL-12R&D. As shown in Fig. 2A and 2B, rCL-12R&D bound *A. fumigatus* in a dose-dependent manner and induced substantial agglutination of *A. fumigatus* conidia. However, addition of GlcNAc or curdlan inhibited the binding of rCL-12R&D, and when combined together, the inhibitory effects were even increased (Fig. 2C). Of interest, removal of Ca^{2+} by addition of EDTA or EGTA-Mg^{2+} did not reduce, but apparently enhanced CL-12 binding to the conidia (Fig. 2D).

Soluble CL-12 boosts activation of the AP of complement

To determine the potential effects of soluble CL-12 on complement activation, we used serum[MBL−] as a source of complement and determined the effect on *A. fumigatus* in TBS/Ca^{2+} or TBS/EGTA-Mg^{2+}. In TBS/Ca^{2+}, no clear effect of rCL-12R&D was detected on C4b deposition (Supplemental Fig. 2). In TBS/EGTA-Mg^{2+}, which inhibits activation of the Ca^{2+}-dependent CP and LP and permits selective activation of the Mg^{2+}-dependent AP, C4b deposition was minimal, whereas both C3b and TCC deposition were easily detected (Fig. 3). However, addition of rCL-12R&D enhanced both C3b and TCC deposition induced by serum[MBL−] alone in the presence of EGTA-Mg^{2+} (Fig. 3B, 3C). Furthermore, a shift in concentration of rCL-12R&D from 0 to 4 μg/ml was accompanied with a significant amplification in both C3b and TCC deposition on *A. fumigatus* (Fig. 4).

Soluble CL-12 triggers activation of the AP of complement via properdin

Properdin is the only known positive regulator of complement activation so far. Therefore, to determine the involvement of properdin in CL-12–amplified AP activation, we used serum[fP−] as a complement source instead. To allow the AP to work at serum concentration with minimal functional activity, we treated serum[fP−] with TBS/EGTA-Mg^{2+} as a final concentration of 10% in the reaction mixtures. As shown in Fig. 5A and 5B, the AP activation was drastically reduced when EGTA-Mg^{2+}–treated serum[fP−] was applied and only minimal autoactivation of the AP was observed. However, reconstitution of exogenous rCL-12R&D or properdin alone under physiological concentration did not restore AP activation. However, when reconstituting both rCL-12R&D and properdin, we were able to restore AP activation validated by detection of C3b and TCC deposition (Fig. 5A, 5B). Furthermore, we found that properdin binding was substantially increased on the conidia when rCL-12R&D was present (Fig. 5C). In some experiments, the results were also confirmed by the rCL-12ED instead of purified rCL-12R&D as an independent example of soluble CL-12 (Supplemental Fig. 3). However, when incubating properdin with CHO/CL-12FL, we could not detect any apparent binding of properdin on the cell surfaces where rCL-12FL was abundantly expressed (data not shown).
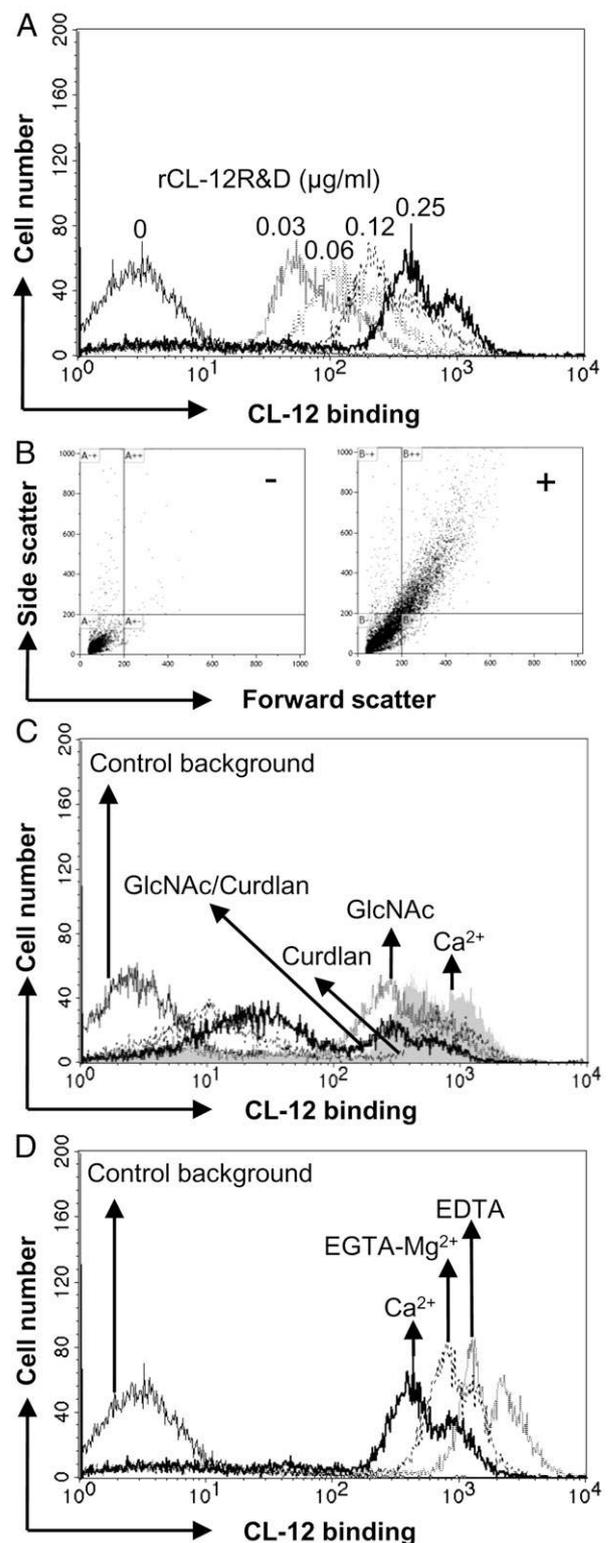
FIGURE 2. Binding of soluble CL-12 to *A. fumigatus*. (**A**) Dose-dependent binding of soluble CL-12 to *A. fumigatus*. (**B**) Soluble CL-12–induced agglutination of *A. fumigatus*. Agglutination was assessed by change in forward and side scatter morphology, and shown in the absence or presence of rCL-12R&D (10 μg/ml). (**C**) Binding specificity of soluble CL-12 on *A. fumigatus*. rCL-12R&D binding to *A. fumigatus* was determined in the presence of GlcNAc (0.3 M), curdlan (500 μg/ml), or a mixture of both. (**D**) Effect of Ca^{2+} chelator on soluble CL-12 binding to *A. fumigatus*. rCL-12R&D binding to *A. fumigatus* was determined in the presence of Ca^{2+} (2.5 mM), EDTA (10 mM), or EGTA (10 mM)-Mg^{2+} (5 mM). The mean fluorescence intensity (MFI) was used to assess protein binding. Results are representative of at least three independent experiments.

FIGURE 3. Effect of soluble CL-12 on the AP of complement activation. *A. fumigatus* were incubated with or without rCL-12R&D (5 μg/ml) prior to induce complement activation by serum^{MBL-} (10%) in the presence of EGTA-Mg^{2+}. C4b (**A**), C3b (**B**), or TCC (**C**) deposition was analyzed by flow cytometry. Mean fluorescence intensity (MFI) was used to assess protein binding. Results are representative of at least three independent experiments.

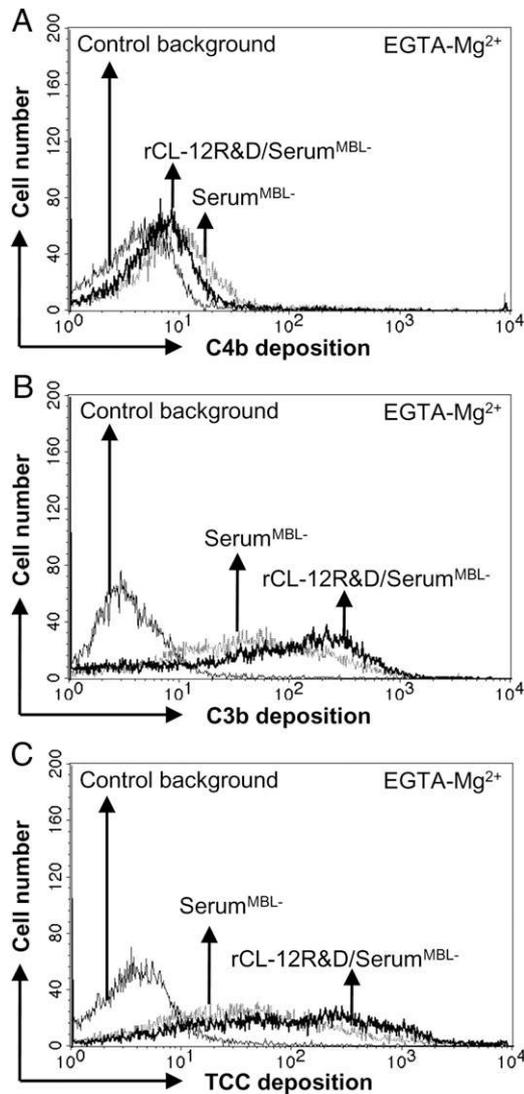*Binding of properdin to anchored soluble CL-12*

Properdin binding to immobilized CL-12 was determined in both cell-based context and microtiter plate. In FACS analysis, in contrast with the case without serum, properdin binding was drastically inhibited when *A. fumigatus* was preincubated with serum^{fP-} (Supplemental Fig. 4A), indicating that some serum factors block access of properdin to the conidia by competing the surface binding sites with properdin. Therefore, to assess solely CL-12–mediated properdin binding, we minimized direct properdin binding to the cell surface glycosaminoglycans by blocking with serum^{fP-} after preopsonization of CL-12. As shown in Fig. 6A, opsonization of rCL-12R&D apparently enhanced the binding of properdin in contrast with the controls where rCL-12R&D was not present. Moreover, the results were also substantiated by the rCL-12ED that was evaluated by quantitative ELISA instead of purified rCL-12R&D (Supplemental Fig. 4B). In some experiments, this was also verified in ELISA where rCL-12R&D–immobilized wells

bound properdin in a dose-dependent manner (Fig. 6B) and vice versa (Supplemental Fig. 4C).

Discussion

CL-12 was originally identified and characterized from placenta and liver as a membrane-anchored molecule functioning as an endocytic receptor present on vascular endothelial cells (10, 17). It has previously been shown that macrophage-specific scavenger receptor/collagen-like domain 163, which mediates the internalization of hemoglobin–haptoglobin complexes by macrophages, can be shed as a soluble form from the cell surface by activation of TLR, cross-linking of the Fcγ receptor or oxidative stress, and so on during inflammation and macrophage activation (25). The level of soluble collagen-like domain 163 has been found to be elevated in patients with different types of infections and inflammatory conditions (26), implying potential generation of soluble scavenger receptor under certain pathophysiological conditions. Interestingly, scavenger receptor CL-12 also shows homology with soluble collectins like MBL, CL-10, and CL-11, all of which activate the LP of complement and are characterized by structural features of collagen-like domain and CRD (27). Thus, we hypothesized the potential generation of a soluble CL-12 from the cell membrane–anchored receptor and a putative role in the complement system.

Determining the content of CL-12 in the culture supernatant of CHO/CL-12FL, we found that CL-12 was shed from the cell surfaces during cultivation. However, the resulting soluble CL-12 (~120 kDa) displayed a slight difference in electrophoretic mobility from that of rCL-12FL (~140 kDa) and rCL-12ED (~130 kDa) under reducing conditions, whereas under nonreducing conditions it represented dimer and trimer, suggesting that membrane-anchored CL-12 might be cleaved in close vicinity of the plasma membrane at its N terminus by a hitherto unknown mechanism. This is a subject of our future investigation. Because CL-12 has previously been shown to be highly expressed in HUVEC and placenta, we reasoned that membrane CL-12 might be shed constitutively into human umbilical cord blood circulation. Analysis of human umbilical cord tissues and plasma further identified the expression of native soluble CL-12. Using our ELISA setup, we demonstrated that CL-12 circulates in normal human umbilical cord plasma as a soluble protein at ~55–121.4 ng/ml, but we were not able to clearly detect CL-12 in normal adult venous plasma. Taken together, shedding of a soluble form of CL-12 from in vitro–cultured CHO/CL-12FL and detection of CL-12 immunoreactivity in human umbilical cord plasma show the existence of a soluble form of CL-12. Western blot analysis demonstrated that like soluble recombinant CL-12, the soluble plasma CL-12 was able to assemble into multimeric structures under nonreducing conditions. However, under reducing conditions it had electrophoretic mobility corresponding to ~110 kDa, which differs from that of soluble recombinant CL-12 (~120 kDa). Nevertheless, immunoprecipitation and Western blot analysis of human umbilical cord revealed that CL-12 migrated with gel mobility corresponding to ~130 kDa. CL-12 possesses 13 putative *N*-glycosylation sites in the entire extracellular domain, thus making it possible to be modified posttranslationally via *N*-glycosylation. Previous studies have shown that HUVEC (or placenta) cell lysates revealed two major bands of CL-12 corresponding to 140 and 110 kDa under reducing conditions in immunoblotting due to differential glycosylation status in vivo, which could be depleted by transfection of small interfering RNAs specific to the *COLEC12* gene (17, 18). These results suggest that there is considerable heterogeneity in posttranslational modification. Therefore, the remaining size difference between the two resultant soluble CL-12 forms from in vitro–cultured
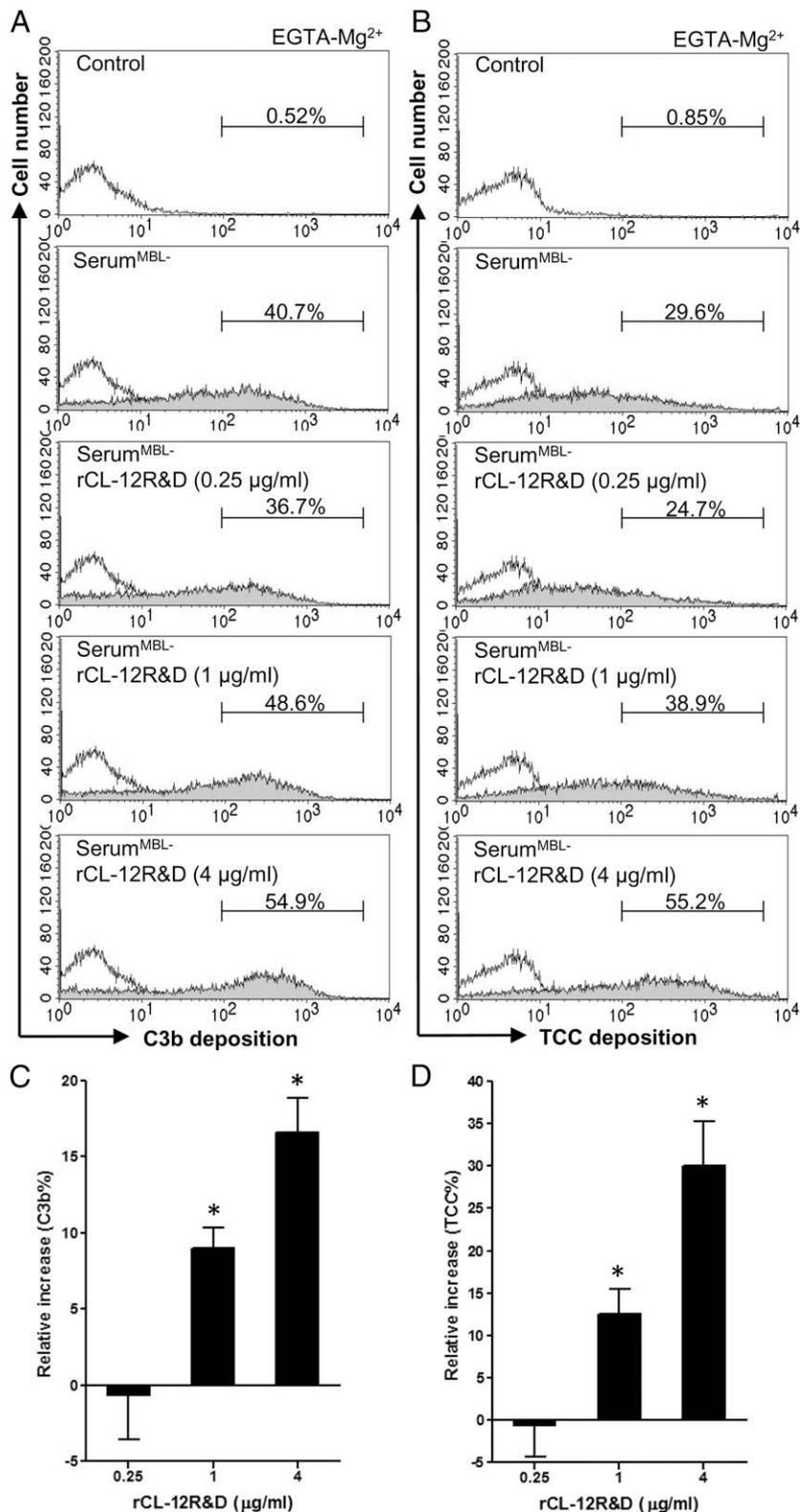
FIGURE 4. Dose-dependent amplification of the AP of complement activation by soluble CL-12. *A. fumigatus* were incubated with elevated concentration of rCL-12R&D (∼0–4 μg/ml) prior to induce complement activation; then C3b (**A**) or TCC (**B**) deposition was analyzed as described earlier. Mean fluorescence intensity (MFI) was used to assess protein binding. Results are representative of at least three independent experiments. (**C** and **D**) Quantification of the complement deposition by flow cytometry under different conditions. Results are expressed as percentage of relative C3b (C) or TCC (D) increase by subtracting the background reading from the percentage of complement deposition and plotted together. The value from the fungus incubated with serum^{MBL-} in the absence of rCL-12R&D was regarded as the background reading. *$p < 0.001$.

CHO/CL-12FL and human umbilical cord plasma could be because of the difference between the glycan produced by human and animal cells and/or the type of posttranslational modification.

Previous studies have pointed to scavenger receptor CL-12 as the phagocytic mediator responsible for recognition and phagocytosis of yeast, as well as *E. coli* and *S. aureus* (17, 18), indicating that CL-12 could recognize some constituent of cell-wall component on fungi and bacteria and may play a role in innate immunity. Intrigu-

ingly, we found that soluble CL-12 could bind *A. fumigatus* and subsequently induce massive agglutination via recognition of specific sugar moieties on the conidia, for instance, *N*-acetylglucosamine and β-1,3-glucan, which comprises the core of the cell wall through complex structure with repeating sugar arrays (28). Previously, Coombs et al. (20) have shown that recombinant CL-12 ED has affinities with some monosaccharides, such as galactose, fucose, *N*-acetylgalactosamine, and α-methyl galactoside, and selectively
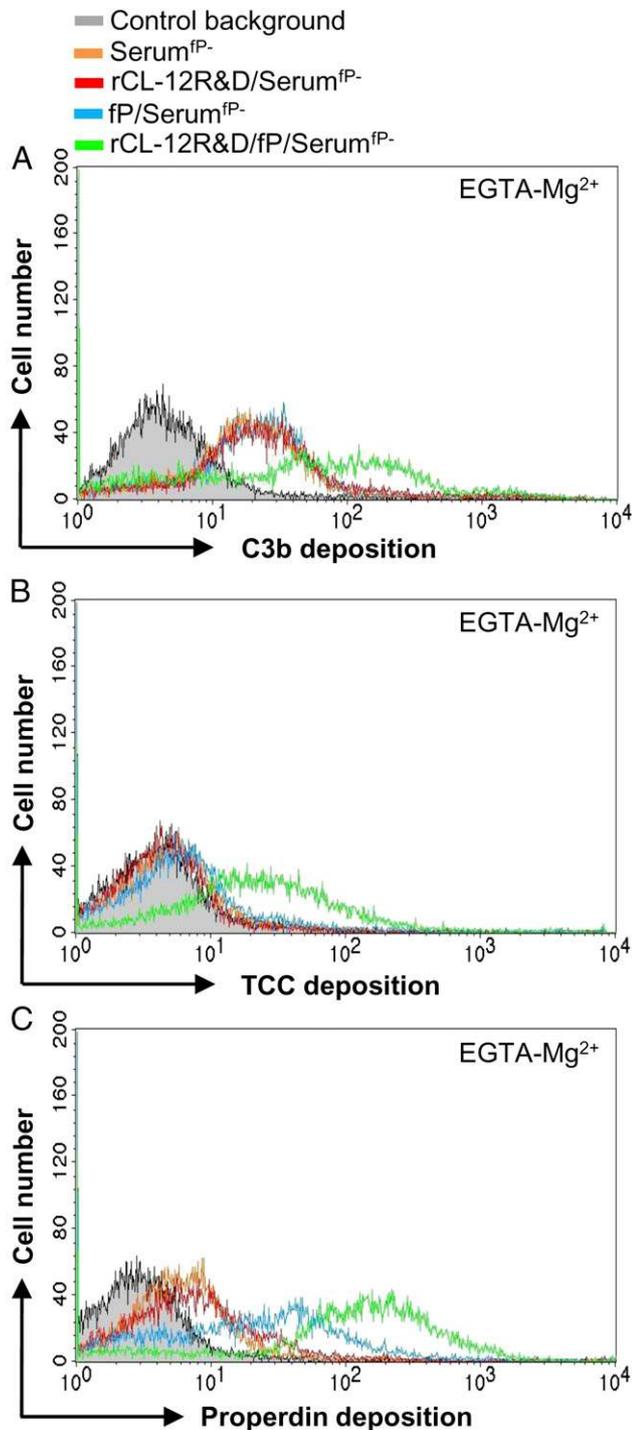
FIGURE 5. Soluble CL-12–induced complement amplification is properdin dependent. Complement activation was induced on *A. fumigatus* by serum[fP−] (10%) and analyzed as described earlier. The effect of rCL-12R&D (5 µg/ml), properdin (5 µg/ml), or a combination of both was determined by preincubation of *A. fumigatus* before induction of complement C3b (**A**) or TCC (**B**) deposition. During the AP activation, properdin that bound to *A. fumigatus* was also analyzed (**C**). Mean fluorescence intensity (MFI) was used to assess protein binding. Results are representative of at least three independent experiments.

FIGURE 6. Binding of properdin to anchored soluble CL-12. (**A**) Binding of properdin to rCL-12R&D in cell-based context. Properdin (5 µg/ml) binding to *A. fumigatus* was analyzed with or without preopsonization of rCL-12R&D (5 µg/ml) as described in *Materials and Methods*. Mean fluorescence intensity (MFI) was used to assess protein binding. Results are representative of at least three independent experiments. (**B**) Binding of properdin to rCL-12R&D in ELISA. Microtiter wells were coated with rCL-12R&D or BSA in elevated concentrations and incubated with properdin (5 µg/ml). Bound properdin was analyzed as described in *Materials and Methods*. The measured background value was subtracted from the presented results. Results are expressed as mean ± SD from triplicate experiments.

recognizes glycans containing Lewis[X] trisaccharide Galβ (1-4) [Fucα (1-3)]GlcNAc or the closely related Lewis[a] trisaccharide Galβ (1-3)[Fucα (1-3)]GlcNAc. Different from these reports, we revealed that soluble CL-12 might have affinity for glycans bearing *N*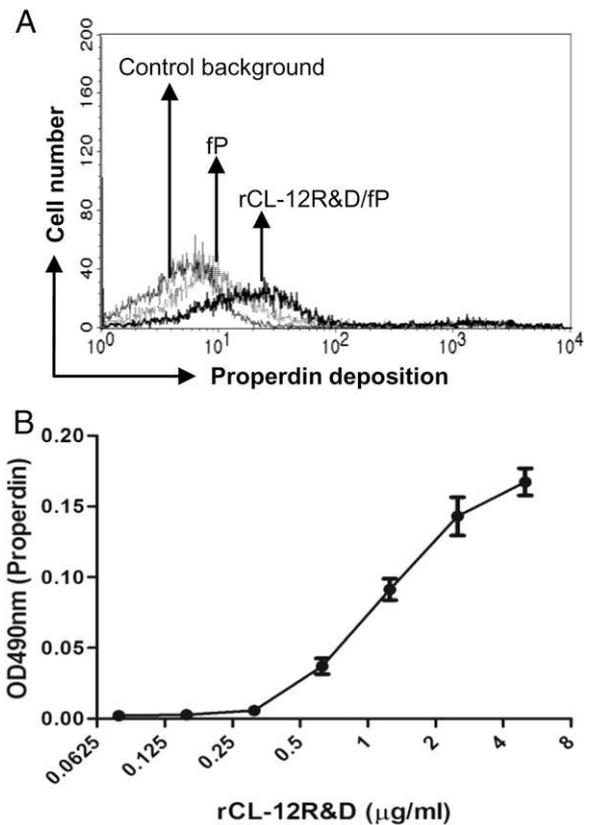-acetylglucosamine and β-1,3-glucan, suggesting that soluble CL-12 could bind to certain fungal pathogens by interaction with repeating carbohydrate arrays on the cell wall.

It has been shown that a variant form of CL-12 lacking the CRD still shows binding activity for bacteria (10). Recently, studies using CL-12 domain deletion mutants have shown that the collagen-like domain in CL-12 mainly recognizes microbial and fungal ligands and the CRD specifically binds some sugar substances, whereas the coiled-coil domain additionally interacts with modified low density lipoprotein (29). These findings suggest that CL-12 differs from the classical collectins both in the arrangement and the functions of these domains. Combining with these reports, our observations that soluble CL-12 bound *A. fumigatus* in a divalent cation-independent fashion also imply that other functional domains could be involved in the opsonization in addition to the CRD. Previously, we have shown that recombinant CL-11 could bind *A. fumigatus* in a Ca^{2+}-independent fashion, which could not be inhibited by its defined sugar ligands, such as L-fucose or D-mannose, and this did not lead to the subsequent serum-induced complement activation (16). However, binding of CL-11 to *C. albicans* occurring via its CRD led to MASP binding and complement activation (16). This suggests that if the binding site involves the collagen-like domain, it might hamper complex formation with MASPs and thus preclude LP activation. In analogy with this finding, soluble CL-12 did not

show a clear influence in complement C4b deposition upon support of only Ca^{2+}. These results suggest that soluble CL-12 might be incapable of activating the complement system via the CP or LP. Because of this, as soluble CL-12 binding to *A. fumigatus* was Ca^{2+} independent, we investigated whether soluble CL-12 could activate complement in the presence of EGTA-Mg^{2+}, which selectively allows activation of the AP. Our results demonstrated that soluble CL-12 drastically augmented the AP-mediated C3b and TCC deposition in a dose-dependent fashion. However, it is noteworthy that properdin is capable of binding only to the soluble form of CL-12, but not to the CHO cell membrane-anchored form, emphasizing that appropriate orientation of CL-12 may be critical during interaction with properdin. These findings suggest that the CL-12/properdin–directed AP complement amplification might be highly restricted to the soluble form of CL-12 perhaps to prevent constitutive AP complement activation.

Properdin is a plasma glycoprotein of the complement system and plays a crucial role in host defenses against bacteria and fungi (30). However, isolated properdin and properdin in serum have shown striking differences in target-binding activities (31, 32). In agreement with another previous recent report, we observed that isolated properdin binding was drastically interfered with in the context of serum on the microbial surface as on *A. fumigatus* (33). However, our results revealed that soluble CL-12 might also provoke and direct target-binding activity of properdin from serum under certain physiological and pathophysiological conditions.

Decades of research have solidified the role of properdin as a stabilizer of the AP C3 convertase C3bBb, but the properdin protein itself was viewed upon having a relatively nonspecific role (34). Previously, properdin has been shown to bind AP activator surfaces strictly in a C3-dependent manner (33, 35). However, several lines of recent evidence suggest that properdin by itself may provide a platform for the in situ assembly of C3bBb complex on target surfaces, therefore providing new insights into properdin function (31, 36). Nevertheless, it must be emphasized that this is a disputed area of research (33). In this study, we demonstrated that soluble CL-12 recruits properdin to target surfaces, thereby allowing AP activation. However, our results also indicated that unlike the soluble form of CL-12, the membrane-anchored CL-12 is incapable of mediating properdin-directed complement activation, implying that nonspecific and uncontrolled self-activation may not occur under normal conditions. Taken together, our results revealed that given a platform via a cooperative immune response integrating signals from another "sensory" input, for instance, sPRM as CL-12, properdin is able to mediate de novo C3 convertase assembly. Therefore, properdin may initiate complement activation on certain pathogens more selectively via recognition of specific target surfaces by its "sensory" input, compared with standard AP of complement activation via a highly reactive but nondiscriminating nascent C3b, which binds covalently to a target. Thus, the evidence presented in this article supports the idea that properdin as a PRM may direct AP of complement activation (30, 31). Nevertheless, we envision that properdin plays a role in AP of complement activation not only by stabilizing preformed C3bBb, but also by providing a platform for de novo C3 convertase assembly once surface-bound through either surface glycosaminoglycans or cooperative recognition by the humoral innate immune system. However, both mechanisms require tick-over–generated nascent C3b.

In summary, like the CP and LP of complement relying on sPRMs for initiation, the AP of complement may also be initiated by specific recognition molecules via collaboration with properdin. This soluble CL-12/properdin collaboration–directed AP of complement activation could be particularly effective against *A. fumigatus*,

an opportunistic fungal pathogen, which causes mortality in critically ill and immunocompromised patients. Therefore, we provide insight into understanding a novel mechanism of the complement amplification via the AP, which might contribute adjunct to antibiotic therapy in the treatment of certain fungal infections or possibly other pathogens.

Disclosures

The authors have no financial conflicts of interest.

References

1. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11: 785–797.
2. Bottazzi, B., V. Vouret-Craviari, A. Bastone, L. De Gioia, C. Matteucci, G. Peri, F. Spreafico, M. Pausa, C. D'Ettorre, E. Gianazza, et al. 1997. Multimer formation and ligand recognition by the long pentraxin PTX3. Similarities and differences with the short pentraxins C-reactive protein and serum amyloid P component. *J. Biol. Chem.* 272: 32817–32823.
3. Nauta, A. J., B. Bottazzi, A. Mantovani, G. Salvatori, U. Kishore, W. J. Schwaeble, A. R. Gingras, S. Tzima, F. Vivanco, J. Egido, et al. 2003. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur. J. Immunol.* 33: 465–473.
4. Baruah, P., I. E. Dumitriu, G. Peri, V. Russo, A. Mantovani, A. A. Manfredi, and P. Rovere-Querini. 2006. The tissue pentraxin PTX3 limits C1q-mediated complement activation and phagocytosis of apoptotic cells by dendritic cells. *J. Leukoc. Biol.* 80: 87–95.
5. Garred, P., C. Honoré, Y. J. Ma, L. Munthe-Fog, and T. Hummelshøj. 2009. MBL2, FCN1, FCN2 and FCN3-The genes behind the initiation of the lectin pathway of complement. *Mol. Immunol.* 46: 2737–2744.
6. Ma, Y. J., A. Doni, T. Hummelshøj, C. Honoré, A. Bastone, A. Mantovani, N. M. Thielens, and P. Garred. 2009. Synergy between ficolin-2 and pentraxin 3 boosts innate immune recognition and complement deposition. *J. Biol. Chem.* 284: 28263–28275.
7. Ma, Y. J., A. Doni, M. O. Skjoedt, C. Honoré, M. Arendrup, A. Mantovani, and P. Garred. 2011. Heterocomplexes of mannose-binding lectin and the pentraxins PTX3 or serum amyloid P component trigger cross-activation of the complement system. *J. Biol. Chem.* 286: 3405–3417.
8. Alcorlo, M., A. Tortajada, S. Rodríguez de Córdoba, and O. Llorca. 2013. Structural basis for the stabilization of the complement alternative pathway C3 convertase by properdin. *Proc. Natl. Acad. Sci. USA* 110: 13504–13509.
9. Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, H. Yamazaki, T. Shimada, H. Keshi, Y. Sakai, A. Fukuoh, et al. 1999. Molecular cloning of a novel human collectin from liver (CL-L1). *J. Biol. Chem.* 274: 13681–13689.
10. Nakamura, K., H. Funakoshi, K. Miyamoto, F. Tokunaga, and T. Nakamura. 2001. Molecular cloning and functional characterization of a human scavenger receptor with C-type lectin (SRCL), a novel member of a scavenger receptor family. *Biochem. Biophys. Res. Commun.* 280: 1028–1035.
11. Keshi, H., T. Sakamoto, T. Kawai, K. Ohtani, T. Katoh, S. J. Jang, W. Motomura, T. Yoshizaki, M. Fukuda, S. Koyama, et al. 2006. Identification and characterization of a novel human collectin CL-K1. *Microbiol. Immunol.* 50: 1001–1013.
12. Axelgaard, E., L. Jensen, T. F. Dyrlund, H. J. Nielsen, J. J. Enghild, S. Thiel, and J. C. Jensenius. 2013. Investigations on collectin liver 1. *J. Biol. Chem.* 288: 23407–23420.
13. Yoshizaki, T., K. Ohtani, W. Motomura, S. J. Jang, K. Mori, N. Kitamoto, I. Yoshida, Y. Suzuki, and N. Wakamiya. 2012. Comparison of human blood concentrations of collectin kidney 1 and mannan-binding lectin. *J. Biochem.* 151: 57–64.
14. Selman, L., M. L. Henriksen, J. Brandt, Y. Palarasah, A. Waters, P. L. Beales, U. Holmskov, T. J. Jørgensen, C. Nielsen, K. Skjodt, and S. Hansen. 2012. An enzyme-linked immunosorbent assay (ELISA) for quantification of human collectin 11 (CL-11, CL-K1). *J. Immunol. Methods* 375: 182–188.
15. Hansen, S., L. Selman, N. Palaniyar, K. Ziegler, J. Brandt, A. Kliem, M. Jonasson, M. O. Skjoedt, O. Nielsen, K. Hartshorn, et al. 2010. Collectin 11 (CL-11, CL-K1) is a MASP-1/3-associated plasma collectin with microbial-binding activity. *J. Immunol.* 185: 6096–6104.
16. Ma, Y. J., M. O. Skjoedt, and P. Garred. 2013. Collectin-11/MASP complex formation triggers activation of the lectin complement pathway–the fifth lectin pathway initiation complex. *J. Innate Immun.* 5: 242–250.
17. Ohtani, K., Y. Suzuki, S. Eda, T. Kawai, T. Kase, H. Keshi, Y. Sakai, A. Fukuoh, T. Sakamoto, H. Itabe, et al. 2001. The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J. Biol. Chem.* 276: 44222–44228.
18. Jang, S., K. Ohtani, A. Fukuoh, T. Yoshizaki, M. Fukuda, W. Motomura, K. Mori, J. Fukuzawa, N. Kitamoto, I. Yoshida, et al. 2009. Scavenger receptor collectin placenta 1 (CL-P1) predominantly mediates zymosan phagocytosis by human vascular endothelial cells. *J. Biol. Chem.* 284: 3956–3965.
19. Elola, M. T., M. I. Capurro, M. M. Barrio, P. J. Coombs, M. E. Taylor, K. Drickamer, and J. Mordoh. 2007. Lewis x antigen mediates adhesion of human breast carcinoma cells to activated endothelium. Possible involvement of

the endothelial scavenger receptor C-type lectin. *Breast Cancer Res. Treat.* 101: 161–174.

20. Coombs, P. J., S. A. Graham, K. Drickamer, and M. E. Taylor. 2005. Selective binding of the scavenger receptor C-type lectin to Lewisx trisaccharide and related glycan ligands. *J. Biol. Chem.* 280: 22993–22999.

21. Nakamura, K., W. Ohya, H. Funakoshi, G. Sakaguchi, A. Kato, M. Takeda, T. Kudo, and T. Nakamura. 2006. Possible role of scavenger receptor SRCL in the clearance of amyloid-beta in Alzheimer's disease. *J. Neurosci. Res.* 84: 874–890.

22. Fukuda, M., K. Ohtani, S. J. Jang, T. Yoshizaki, K. Mori, W. Motomura, I. Yoshida, Y. Suzuki, Y. Kohgo, and N. Wakamiya. 2011. Molecular cloning and functional analysis of scavenger receptor zebrafish CL-P1. *Biochim. Biophys. Acta* 1810: 1150–1159.

23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

24. Ma, Y. J., A. Doni, L. Romani, H. J. Jürgensen, N. Behrendt, A. Mantovani, and P. Garred. 2013. Ficolin-1-PTX3 complex formation promotes clearance of altered self-cells and modulates IL-8 production. *J. Immunol.* 191: 1324–1333.

25. Etzerodt, A., and S. K. Moestrup. 2013. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. *Antioxid. Redox Signal.* 18: 2352–2363.

26. Møller, H. J. 2012. Soluble CD163. *Scand. J. Clin. Lab. Invest.* 72: 1–13.

27. Ohtani, K., Y. Suzuki, and N. Wakamiya. 2012. Biological functions of the novel collectins CL-L1, CL-K1, and CL-P1. *J. Biomed. Biotechnol.* 2012: 493945.

28. Beauvais, A., and J. P. Latgé. 2001. Membrane and cell wall targets in *Aspergillus fumigatus. Drug Resist. Updat.* 4: 38–49.

29. Mori, K., K. Ohtani, S. Jang, Y. Kim, I. Hwang, N. Roy, Y. Matsuda, Y. Suzuki, and N. Wakamiya. 2014. Scavenger receptor CL-P1 mainly utilizes a collagen-like domain to uptake microbes and modified LDL. *Biochim. Biophys. Acta* 1840: 3345–3356.

30. Lesher, A. M., B. Nilsson, and W. C. Song. 2013. Properdin in complement activation and tissue injury. *Mol. Immunol.* 56: 191–198.

31. Kemper, C., J. P. Atkinson, and D. E. Hourcade. 2010. Properdin: emerging roles of a pattern-recognition molecule. *Annu. Rev. Immunol.* 28: 131–155.

32. Kemper, C., L. M. Mitchell, L. Zhang, and D. E. Hourcade. 2008. The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. *Proc. Natl. Acad. Sci. USA* 105: 9023–9028.

33. Harboe, M., P. Garred, J. K. Lindstad, A. Pharo, F. Müller, G. L. Stahl, J. D. Lambris, and T. E. Mollnes. 2012. The role of properdin in zymosan-and *Escherichia coli*-induced complement activation. *J. Immunol.* 189: 2606–2613.

34. Fearon, D. T., and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J. Exp. Med.* 142: 856–863.

35. Agarwal, S., C. A. Specht, H. Haibin, G. R. Ostroff, S. Ram, P. A. Rice, and S. M. Levitz. 2011. Linkage specificity and role of properdin in activation of the alternative complement pathway by fungal glycans. *MBio* 2: 2.

36. Kemper, C., and D. E. Hourcade. 2008. Properdin: New roles in pattern recognition and target clearance. *Mol. Immunol.* 45: 4048–4056.