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Properdin Binds to Late Apoptotic and Necrotic Cells Independently of C3b and Regulates Alternative Pathway Complement Activation$^{1,2}$

Wei Xu,* Stefan P. Berger,* Leendert A. Trouw, † Hetty C. de Boer,**, Nicole Schlagwein,* Chantal Mutsaers,* Mohamed R. Daha,* and Cees van Kooten$^3$*

Cells that undergo apoptosis or necrosis are promptly removed by phagocytes. Soluble opsonins such as complement can opsonize dying cells, thereby prompting their removal by phagocytes and modulating the immune response. The pivotal role of the complement system in the handling of dying cells has been demonstrated for the classical pathway (via C1q and lectin pathway (via mannose-binding lectin and ficolin)). Herein we report that the only known naturally occurring positive regulator of complement, properdin, binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells. This binding occurs independently of C3b, which is additional to the standard model wherein properdin binds to preexisting clusters of C3b on targets and stabilizes the convertase C3bBb. By binding to late apoptotic or necrotic cells, properdin serves as a focal point for local amplification of alternative pathway complement activation. Furthermore, properdin exhibits a strong interaction with DNA that is exposed on the late stage of dying cells. Our data indicate that direct recognition of dying cells by properdin is essential to drive alternative pathway complement activation. *The Journal of Immunology, 2008, 180: 7613–7621.*

Under steady-state conditions, cells that undergo apoptosis or necrosis can be safely and silently eliminated by professional phagocytes, that is, immature dendritic cells and macrophages (Mφ)$^3$ (1–3). Apoptotic cells are a rich source of autoantigens, which are involved in the induction of self-tolerance and autoimmunity (4). Compelling evidence has emerged that abnormal clearance of apoptotic cells is associated with development of the autoimmune disease systemic lupus erythematosus (SLE) (5, 6).

Soluble factors from the innate immune system such as complement or pentraxins can opsonize apoptotic cells, thereby promoting their removal by phagocytes (6–8). In humans, homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE (9), suggesting that complement is involved in removal of dying cells and the immune regulation associated with this process. Complement-mediated clearance of apoptotic cells has been well documented both in vitro (10) and in vivo (11). Nevertheless, the role of the complement system in the handling of dying cells has been mostly linked to the classical pathway (via C1q and lectin pathway (via mannose-binding lectin (MBL) and ficolin)) (10–14). It has been suggested that the main product of complement activation, iC3b, facilitates the removal of dead material and mediates peripheral tolerance (10, 15, 16).

The alternative pathway of complement is thought to be activated following hydrolysis of C3, generation of C3b, and formation of a positive feedback loop to mount a rapid local response (17). The alternative pathway was initially recognized to amplify complement activation triggered by the classical pathway. Properdin, discovered in 1954 (18), is the only known naturally occurring positive regulator of complement activation (19). It was originally shown that properdin binds to C3b and increases the stability of the alternative pathway convertases at least 10-fold on target surfaces and immune complexes (20). It has been recently suggested that properdin could bind directly to microbial targets (21), which is consistent with a proposal made more than 50 years ago (18).

In the present study, we investigated whether properdin, like C1q and MBL, contributes to the recognition and opsonization of dying cells. We found that properdin binds predominantly to late apoptotic and necrotic cells independently of C3b, but not to early apoptotic cells, leading to alternative pathway-mediated complement activation. DNA was identified as one of the ligands on dying cells to which properdin binds. This accounts for a C3b-independent mechanism of properdin-initiated complement activation on dying cells.

Materials and Methods

Induction of apoptosis and necrosis

Jurkat cells were cultured in RPMI culture medium. Early or late apoptosis was induced in Jurkat cells by exposure to UV-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m², followed by 3 or 30 h culture...
FIGURE 1. Complement-mediated phagocytosis of dying cells. A, Early or late apoptosis was induced in Jurkat cells by exposure to UV-C light at a dose of 50 J/m², followed by 3 or 30 h culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h. Cells were stained with annexin V and PI by flow cytometry. B, Late apoptotic cells and necrotic cells are scored by light microscopy or fluorescent microscopy for Hoechst staining on cytospins of these cells. Magnification, ×200. C, CFSE-labeled early apoptotic, late apoptotic, or necrotic cells (1 × 10⁵) were first opsonized with or without NHS, then cocultured with Mδ2 in a 1:1 ratio at 37°C for 0.5 h. Mδ2 were stained with a PE-conjugated mAb against CD11b, and uptake was analyzed by two-color flow cytometry. CD11b⁺ CFSE⁺ cells were used as a measure for the percentage of Mδ2 that ingested apoptotic cells. D, Relative phagocytosis was calculated as uptake of NHS-opsonized dying cells vs nonopsonized cells. Data are mean ± SEM of three independent experiments. *p < 0.01, one-sample paired t test. E, C3 deposition (filled histogram) after NHS opsonization on early, late apoptotic, and necrotic cells. Open histograms are the matched isotype controls.

in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h or 5 cycles of freeze-thaw from −80°C to 36°C. Both apoptosis and necrosis were confirmed by double staining with FITC-labeled annexin V and propidium iodide (PI, VPS Diagnostics) according to established methods (22). Additionally, light microscopy and fluorescent microscopy (Leica DC300F, Leica Microsystems) were performed to detect the morphology and Hoechst nuclear staining of these cells, respectively. Alternatively, HUVECs, U937 cells (monocytic cell lines), and EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) were used for the induction of necrosis.

In some experiments, splenocytes were obtained from C3 knockout (C3⁻/⁻) (23) or C57BL/6 wild-type (WT) mice (Harlan). Splenocytes were rendered necrotic by incubating them at 56°C, as described above.

Phagocytosis assay

Phagocytosis of early apoptotic, late apoptotic, and necrotic cells was assessed by using a protocol described previously (24). Briefly, a subset of macrophages (Mδ2) were generated from CD14⁺ monocytes in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 ng/ml penicillin, and 90 μg/ml streptomycin) (all from Invitrogen) in the presence of 5 ng/ml M-CSF (R&D Systems/ITK Diagnostics) for 6 days. Jurkat cells were used as the target for phagocytosis. Before the induction of apoptosis or necrosis, Jurkat cells were fluorescently labeled with CFSE (Molecular Probes, Leiden, the Netherlands). Labeled early, late apoptotic, or necrotic cells (1 × 10⁵) were investigated with or without opsonization using normal human serum (NHS). Dying cells were cocultured with Mδ2 in a 1:1 ratio at 37°C for 0.5 h in 100 μl RPMI culture medium in round-bottom glass tubes. As a control, coculture was performed at 4°C to detect the binding of dying cells to phagocytes. Mδ2 were stained with a PE-conjugated mAb against CD11b (BD Biosciences), and uptake was analyzed by two-color flow cytometry. The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of Mδ2 that ingested and/or bound apoptotic cells.

Isolation of properdin, C1q and MBL, and C3 and C3b

Properdin was isolated from pooled human serum from volunteer donors. Serum was first precipitated by dialysis against 5 mM EDTA (pH 6.0). The precipitate was dissolved in Veronal-buffered saline (2X VBS, 1.8 mM Na-5,5-diethylbarbitral, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) and then dialyzed against 0.01 M NaAc containing 2 mM EDTA (pH 6.0) and applied to a sulfopropyl C50 column. Properdin was eluted with a linear salt gradient. Properdin-containing fractions, as determined by ELISA, were pooled, concentrated, and subsequently applied to a Sephacryl S-300 gel filtration column (Pharmacia Biotech). Fractions containing properdin were dialyzed against PBS, 2 mM EDTA, and further purified using human IgG coupled to a Biogel A5 (Bio-Rad) to remove contaminating C1q. Purity of the properdin preparation was determined by analysis on 10% SDS-PAGE gel. A single 220-kDa band was observed. C1q and MBL were purified from pooled human plasma obtained from healthy donors as described previously (25, 26). C3 was purified from serum using different steps of chromatography, whereas C3b was generated by brief trypsin cleavage (60 s) of purified C3 followed by direct inactivation. The purity of C3 and C3b was determined by SDS-PAGE gel.
Serum

C4-depleted serum (C4ds) was used as a complement source lacking both classical and lectin pathway activity, and was prepared as follows: Blood was obtained by venapuncture, allowed to clot at room temperature for 1 h, and then centrifuged. The serum was brought to a NaCl concentration of 0.3 M by addition of NaCl and then mixed with an immunosorbent of rabbit IgG anti-human C4. Coupling of rabbit IgG anti-C4 to Sepharose was performed according to the manufacturer’s instructions (Amersham Biosciences). Following absorption by gentle mixing for 30 min at 4°C, the mixture was centrifuged at 1000 × g and the supernatant aliquotted and frozen at −80°C. The C4-depleted serum had no detectable complement activity at a dilution of 1/5 in a hemolytic test using sheep erythrocytes sensitized with rabbit anti-SRBC, while the starting serum induced 1 unit of C5a activity at a dilution of 1/240. Additionally, C4 hemolytic activity could be restored in 1/25 diluted C4-deficient serum with 10 µg/ml purified C4.

Properdin-depleted serum (Pds) was obtained by immune absorption using Biogel-coupled mAb against human properdin (a gift of Statens Serum Institut, Copenhagen, Denmark). Pds showed normal classical and lectin pathway activities in hemolytic assays.

C4-deficient serum was obtained from a patient who was deficient for C3 while containing normal properdin concentration (17.5 µg/ml). In C3-deficient serum, the C3 level was below detection limit as measured by ELISA (data not shown).

Normal human sera from nine healthy donors were used as C3 full sera as confirmed by C3 ELISA. The properdin concentration in those C3 full sera ranged from 10.4 to 25.1 µg/ml.

Binding assay for properdin, C1q, and MBL

Binding of properdin to viable, early apoptotic, late apoptotic, or necrotic cells was investigated by incubating cells with up to 40 µg/ml human purified properdin at 37°C for 1 h in serum-free RPMI culture medium. We used serum-free medium as a standard buffer to exclude a possible contribution of serum constituents, unless specifically indicated. Cells were then extensively washed and incubated with a rabbit anti-human properdin polyclonal Ab (generated by immunizing rabbit with purified properdin) and detected with PE-conjugated goat F(ab’2), anti-rabbit Ig (Southern Biotechnology Associates). The cells were analyzed by flow cytometry. Data from 10⁶ events were acquired. Alternatively, C3-deficient serum or C3 full serum was used as a source of properdin to detect binding of properdin. These sera were diluted in serum-free RPMI medium at 40%. In some experiments, purified C3 or C3b was used to detect its binding to properdin that had been prebound on necrotic cells.

Binding of C1q (30 µg/ml) and MBL (10 µg/ml) was performed in the same way as properdin binding and was detected with a mAb directed against C1q (mAb 2204) or MBL (clone 3E7), respectively. Binding was visualized with PE-conjugated goat F(ab’2), anti-mouse Ig (DakoCytomation).

In some experiments, cells were preincubated with C1q or MBL, followed by incubation of properdin, and vice versa. To detect the binding of properdin to DNA, double-stranded DNA (dsDNA) from calf thymus (Sigma-Aldrich), single-stranded DNA (ssDNA, Isogen Life Science), or human albumin (Sigma-Aldrich) was coated in PBS on microtiter plates overnight and then blocked with 2% BSA before adding purified properdin. After washing, bound properdin was detected with digoxigenin (Dig)-labeled rabbit anti-human properdin. Bound Ab was developed with anti-Dig-HRP (Roche Diagnostics) and was detected with digoxigenin (Dig)-labeled rabbit anti-human properdin.

Results

Complement activation by dying cells

Activation of complement by dying cells was assessed as follows: early, late apoptotic, or necrotic cells were preincubated with or without properdin (20 µg/ml) at 37°C for 1 h in serum-free RPMI culture medium, washed extensively and then exposed to different dilutions of Pds, C4ds, or NHS for 0.5 h at 37°C. Deposition of C3, C4, and C5b-9 on the cell surfaces was detected by flow cytometry using mAbs against C3 (RFK22 (27)), C4 (anti-C4–4 (28)) and C5b-9 (AE11, kindly provided by Dr. T. E. Molinres, Nordland Central Hospital, Bodø, Norway), respectively.

Statistical analysis

Statistical analysis was performed by one-sample t tests using GraphPad Prism software. Differences were considered statistically significant when p values were <0.05.

Complement-mediated phagocytosis of late apoptotic and necrotic cells

Relatively pure populations of viable (90–98%), early apoptotic (40–70%), late apoptotic (90–100%), and necrotic cells (100%) were obtained, based on the annexin V and PI staining (Fig. 1A). The difference between late apoptotic cells and necrotic cells was confirmed by light microscopy and Hoechst nuclear staining (Fig. 1B), demonstrating that late apoptotic cells showed blebbing on the
cell surfaces and nuclear segmentation, whereas necrotic cells showed condensed nuclei.

In agreement with our earlier findings, M-CSF-driven antiinflammatory Mδ2 preferentially recognized and ingested early apoptotic cells, as compared with the ingestion of late apoptotic and necrotic cells (24) (Fig. 1C). However, opsonization of early apoptotic cells with NHS did not enhance their uptake by Mδ2 (Fig. 1, C and D), while opsonization of late apoptotic and necrotic cells with NHS significantly increased their uptake by Mδ2 (p < 0.01) (Fig. 1, C and D). Enhancement of phagocytosis by NHS was also restricted to late apoptotic and necrotic cells when using monocyte-derived dendritic cells and GM-CSF-driven Mδ1 (data not shown). We next questioned whether the observed enhanced phagocytosis is associated with complement deposition on the dying cells. Indeed, NHS-exposed late apoptotic and necrotic cells, but not early apoptotic cells, displayed strong deposition of C3 by flow cytometry (Fig. 1E). Therefore, we assessed the pathways involved in the activation of complement on these cells.

Properdin binds to late apoptotic and necrotic cells

We investigated whether properdin, the only naturally occurring positive complement regulator, can bind directly to dying cells that are at different stages of cell death. Similar to C1q and MBL, properdin showed a predominant interaction with late apoptotic cells and necrotic cells over early apoptotic or viable cells (Fig. 2, A and B). Properdin was shown to bind to both late apoptotic and necrotic cells in a dose-dependent manner (Fig. 2C). To rule out the possibility that the observed binding of properdin is cell type or method specific, different cell lines and methods for induction of necrosis were used. Properdin was shown to bind to necrotic HUVEC, U937, HK-2, and EBV-LCL cells, and also to Jurkat cells that were rendered necrotic by five cycles of freeze-thawing (data not shown), suggesting that binding of properdin to necrotic cells is a universal phenomenon irrespective of specific cell types.

Binding of properdin to dying cells can occur independently of C3

It has long been established that properdin can bind to preexisting clusters of surface-bound C3b (29), thereby stabilizing the C3b-dependent C3 convertase C3bBb (20). However, the experiments presented above were performed in the absence of serum, suggesting that binding of properdin to dying cells occurs independently of C3b. This seems to be consistent with a recent publication showing that properdin binds directly to bacterial targets (30). To exclude the possibility for endogenous generation of C3b by dying Jurkat cells, we investigated whether properdin could bind to necrotic splenocytes derived from C3 knockout (C3−/−) mice. Properdin was shown to bind to necrotic splenocytes of C3−/− mice to a similar extent as binding to necrotic cells from WT mice (Fig. 3A). Properdin did not bind to viable splenocytes derived from either C3−/− or WT mice (Fig. 3A).

Additionally, to confirm C3 independence, we also used C3-deficient serum in EDTA containing medium (obtained from a C3-deficient patient with a normal concentration of properdin) as a source of properdin to opsonize necrotic cells. We found similar binding of properdin to necrotic cells in C3-deficient serum as
compared with C3 full serum (NHS) and purified properdin (Fig. 3B), although the extent of the binding is low because the concentration of properdin in these sera was \( \sim 7 \mu g/ml \). In the absence of EDTA, C3 full sera showed much higher binding of properdin, indicating that complement activation amplifies the properdin binding. Taken together, these data suggest that binding of properdin to dying cells can occur independently of C3.

We next investigated whether purified C3 or C3b can bind to properdin bound on the surface of dying cells. We first opsonized necrotic cells with properdin to allow sufficient binding of properdin on the surface of necrotic cells. Next, increasing concentrations of C3 and C3b were added, and binding was detected with a mAb recognizing both C3 and C3b. Under these conditions, C3 or C3b did not bind to nonopsonized necrotic cells, showing specificity for the interaction with properdin (data not shown). Binding to properdin was almost exclusive for C3b, and only minor interaction with intact C3 was seen at the highest concentration (Fig. 3C). Although preparations were pure, as based on SDS-PAGE analysis (data not shown), small contamination with C3b cannot be excluded. Taken together, these data show that properdin binds C3b instead of intact C3 and suggest that local generation of C3b is a prerequisite for the focal properdin-driven complement activation/amplification.

To test the physiological relevance of properdin binding to dying cells, we used sera from nine healthy donors (properdin concentration ranges of 10.4–25.1 \( \mu g/ml \)). All sera tested showed predominant binding to late apoptotic cells and necrotic cells, but not to early apoptotic or viable Jurkat cells (Fig. 3D).

Properdin is a focal point for amplification of alternative pathway complement on dying cells

To investigate whether binding of properdin to dying cells might act as a focal point for local amplification of the complement system, we analyzed complement activation on necrotic cells using Pds. Cells preincubated with purified properdin alone, as expected, did not show C3 and C5b-9 deposition (Fig. 4A). In Pds, a reduced C3 deposition was observed, which is accompanied by a lack of deposition of the membrane attack complex C5b-9 (Fig. 4A). Necrotic cells that had been preexposed to properdin, washed extensively, and subsequently incubated with Pds displayed significantly increased C3 and C5b-9 deposition (Fig. 4A), suggesting that properdin is essential for local amplification of the complement cascade on necrotic cells.

To prove that cell-bound properdin can activate complement independently of the classical and lectin pathways, we used C4ds as a source of complement, because C4 is a crucial factor for both pathways. Exposure of both late apoptotic cells (Fig. 4B) and necrotic cells (Fig. 4C) that had been preincubated with properdin to C4ds significantly induced the deposition of C5b-9 in a dose-dependent manner, as compared with those without properdin. There was no C4 deposition either on late apoptotic (Fig. 4B) or on necrotic cells (Fig. 4C) after opsonization of C4ds, confirming that C4 had been effectively depleted in our C4ds preparation. When a fixed amount (30%) of C4ds was used, the increase of C5b-9 on the cell surface was dose-dependently affected by the amount of properdin (Fig. 4D). Overall, this shows that properdin is a rate-limiting factor that mediates complement activation at the surface of late apoptotic and necrotic cells via alternative pathway activation.

Properdin does not compete with binding of C1q and MBL to necrotic cells

We showed previously that C1q and MBL share binding ligands on apoptotic cells (31). Because properdin was shown to bind to late apoptotic and necrotic cells in a similar pattern as C1q and MBL (Fig. 1A), we hypothesized that properdin may bind to a similar structure on dying cells. Necrotic cells were preincubated with properdin, followed by incubation with increasing concentrations of C1q. A dose-dependent binding of C1q was observed, but preincubation with properdin did not inhibit the binding of C1q to the cells (Fig. 5A). In a reverse way, preincubation of necrotic cells with C1q did not decrease properdin binding either (Fig. 5B). Similarly, preincubation of necrotic cells with properdin did not interfere with MBL binding, and vice versa (Fig. 5C). Therefore, our data suggest that properdin binds to a yet unknown ligand, which is different from the one to which C1q and MBL bind.
Properdin binds to DNA

One of the autoantigens exposed on apoptotic cells and necrotic cells is DNA (4, 32). Based on the finding that properdin specifically binds to late apoptotic and necrotic cells, we hypothesized that properdin might bind to DNA exposed on the surface of dying cells. Properdin showed a strong binding to both dsDNA and ssDNA at concentrations of 1 µg/ml and higher on microtiter plates (Fig. 6A). Furthermore, preincubation of properdin with calf thymus dsDNA dose-dependently inhibited binding of properdin to necrotic cells (Fig. 6B), suggesting a strong interaction between DNA and properdin.

We confirmed that DNA is indeed exposed on late apoptotic cells and necrotic cells using a monoclonal anti-dsDNA Ab as detected by flow cytometry (Figs. 6C and 7A). Necrotic cells that were preincubated with properdin showed double positivity for both properdin binding and anti-DNA (Fig. 6C). The binding of properdin to DNA on necrotic cells was further confirmed by confocal microscopy showing that properdin and DNA are colocalized on necrotic cells (Fig. 6D). As a control, properdin-opsonized viable cells were negative for either properdin binding or DNA (data not shown).

Interestingly, different from necrotic cells, cells made late apoptotic were not all recognized by properdin (Fig. 2A), which prompted us to further dissect these cell populations in detail. To better analyze the data, we divided these cells into two populations based on the forward and side scatter characteristics, namely R1 and R2 (Fig. 7A). Cells in R2 are annexin V+/PI+, characteristics of early apoptotic cells (Fig. 7A), and as expected these cells did not bind properdin and did not expose DNA (Fig. 7B). Cells in R1 are all annexin V−PI−, indicative of late apoptotic cells (Fig. 7A). Within the R1 population, part of the cells were both negative for properdin binding and DNA, and binding of properdin is related to

FIGURE 5. Properdin does not compete for binding with C1q and MBL. A, Necrotic cells were preincubated with properdin (40 µg/ml) and followed by incubation with increasing concentrations of C1q (up to 60 µg/ml). C1q binding was measured. Data shown are mean ± SEM of two independent experiments. B, Necrotic cells were preincubated with properdin (40 µg/ml) or C1q (30 µg/ml), then followed by incubation with C1q (10 µg/ml) or properdin (20 µg/ml), respectively. C1q and properdin binding were measured by flow cytometry. C, Competition between properdin and MBL (10 µg/ml) was investigated as described in B.

FIGURE 6. Properdin binds to DNA. A, Different concentrations of dsDNA and ssDNA or human albumin were coated on microtiter plates overnight and then blocked with 2% BSA before adding properdin. After washing, plates were incubated with digoxigenin (Dig)-labeled rabbit anti-human properdin. Signal was developed by anti-Dig-HRP and measured for absorbance at OD 451 nm. B, Properdin was preincubated with increasing concentrations of calf thymus dsDNA and then incubated with necrotic cells. Data shown are properdin binding to the cells (MFI). C, Necrotic cells were incubated with a mouse anti-human dsDNA Ab and developed by PE-conjugated goat F(ab')2 anti-mouse Ig. For double staining, cells were first opsonized with properdin (40 µg/ml) at 37°C for 1 h in serum-free RPMI culture medium, followed by incubation with a rabbit anti-human properdin Ab and a mouse anti-human dsDNA Ab, and developed by PE-conjugated goat F(ab')2 anti-rabbit Ig and FITC-conjugated goat F(ab')2 anti-mouse Ig. D, Confocal laser scanning microscopy was performed on properdin-opsonized necrotic cells that were stained for properdin, DNA, and Hoechst. Green, DNA; red, properdin; blue, Hoechst; yellow, DNA colocalizes with properdin; DIC, differential interference contrast. Magnification, ×400.
the degree that DNA is exposed on these dying cells (Fig. 7B). This suggests that during reorganization of dying cells, including blebbing, ligands for properdin are not equally distributed over the cellular fragments. Such unequal distribution of late apoptotic cells was not only applied to binding of properdin, but also to complement activation after NHS opsonization as measured for C3 and C5b-9 deposition on these cells (Fig. 7C). Confocal microscopy confirmed that properdin was colocalized exclusively with fragmented DNA exposed on these cells (Fig. 7D).

Discussion
We describe herein that properdin specifically binds to late apoptotic or necrotic cells, but not to early apoptotic cells. Furthermore, DNA exposed on dying cells is one of the ligands to which properdin binds. We provide evidence that binding of properdin to late apoptotic cells and necrotic cells can occur independently of C3b, and that it serves as a focal point for the local amplification of the alternative pathway of complement.

In the past, studies on complement-mediated clearance of dying cells have mainly focused on the classical pathway (10–12). Properdin is a positive regulator of the alternative pathway, which has been shown to bind to C3b and to stabilize the labile C3b-dependent C3 convertase C3bBb (19, 20). Two models have been proposed for the role of properdin in alternative pathway activation of complement. The first model suggests that properdin binds to a preformed C3bBb, resulting in stabilization of the alternative pathway of C3 convertase (20). The other model suggests that properdin first binds to a surface ligand via one of its subunits and then promotes the assembly C3bBb at the ligand-binding sites of its adjoining subunits (33). Very recently, it has been shown that properdin can bind directly to bacterial surfaces (34). Our data showed that properdin binds to late apoptotic and necrotic cells before C3 deposition on the cell surface. Evidence that binding of properdin to dying cells can occur independently of C3b was further supported by experiments showing that properdin binds strongly to necrotic splenocytes derived from C3−/− mice (Fig. 3A) and by experiments using C3-deficient serum (Fig. 3B). We further showed that properdin prebound on necrotic cells binds to purified C3b instead of intact C3. Thus, we suggest that properdin binds to dying cells first in the absence of C3, and following the generation of C3b it serves as a focal point for local amplification and boost of the properdin-driven complement activation cascade.

Our data suggest that DNA is one of the targets for properdin on dying cells. During apoptosis, DNase cleaves DNA into nucleosomal units (35). Indeed, DNA has been shown to be one of the major autoantigens exposed on apoptotic cell surfaces (4, 36). Using a monoclonal anti-DNA Ab, we demonstrate that both late apoptotic cells and necrotic cells expose DNA. Confocal images indicate that properdin and DNA are colocalized on late apoptotic cells or necrotic cells. Interestingly, it seems that only the DNA recognized by anti-DNA Abs was accessible for properdin. It is likely that during apoptosis, DNase digested small fragments of DNA, which are targeted by properdin, while properdin does not bind to the complete nucleosomal units of DNA. In pathological situations, DNA is considered as one of the immunologically active autoantigens (37) that can stimulate immune cells via TLRs (38, 39). In autoimmune lupus, DNA is one of the major immunogens to trigger autoantibody production (40). Our finding that properdin binds to DNA opens the possibility that properdin may
interfere with unwanted immune activation when DNA is exposed on dying cells during a large-scale cell death.

Several other serum components have been suggested to interact with DNA, including C1q (41), MBL (42), serum amyloid-P component (43), and C4b-binding protein (44). We showed that properdin does not compete with the binding of C1q and MBL to dying cells, suggesting that C1q and MBL interact with DNA structures different from the ones that properdin recognizes. It has been suggested that properdin binds to sulfatide (sulfated glyco-sphingolipids) and weakly to phosphatidylinerine (45). However, it is not likely that phosphatidylinerine exposed on the surface of dying cells is a major ligand, because properdin does not bind to early apoptotic cells although these cells do express phosphatidylinerine. Whether sulfatide or other phospholipids are one of the additional ligands on dying cells for properdin recognition is currently under investigation.

Involvement of properdin in the handling of dying cells was initially suggested by Kemper et al., who reported that properdin binds to early and late apoptotic cells (46). Herein we demonstrate that properdin binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells. A similar restriction has been demonstrated for the binding of C1q, MBL (reviewed in Ref. 8), ficolin (14), natural IgM (47), and pentraxin family members serum amyloid-P component (48) and PTX3 (49). Therefore, soluble opsonins especially seem to contribute to a safe clearance of late apoptotic and necrotic material. Together with our previous findings that early apoptotic cells are preferentially cleared by anti-inflammatory macrophages (24), we suggest that a hierarchy exists in the clearance mechanism of dying cells. Uptake of early apoptotic cells by local macrophages with antiinflammatory properties is an initial step, whereas complement-mediated processes via all three pathways are a rather late event (9), most likely ensuring a safe clearance when an overload of apoptosis or defects in phagocytic capacity occur, thereby preventing a break of tolerance (50–52).

In humans, individuals deficient for properdin are prone to lethal pyogenic (particular neisserial) infections (53, 54). Mice deficient for properdin provided evidence that properdin is essential in driving LPS-mediated alternative complement activation (55). No reports have shown that deficiency for properdin predisposes to the development of SLE, whereas in humans homozygous deficiency of any of the early components of the classical pathway of complement activation (C1q, C1r, C1s, C4, and C2) predisposes to the development of SLE (9). Although many other opsonins such as MBL, CRP, and PTX3 also bind to dying cells and help their clearance, deficiency of these opsonins does not lead to the development of autoimmunity (8). This might indicate a differential role for opsonins in the handling of dying cells, including augmentation of phagocytosis and/or a role in immune regulation. Among such opsonins, C1q is the strongest genetic factor that is linked to the development of SLE. Next to promoting clearance (13), C1q has been suggested to modulate dendritic cell function by imprinting these cells with tolerogenic properties (56). Based on this, it is tempting to speculate that properdin might also have a dual function: 1) amplifying complement activation on dying cells to promote complement-mediated clearance, and 2) immunomodulating properties, which deserve to be studied in detail.

In conclusion, we provide evidence that properdin binds specifically to late apoptotic and necrotic cells via ligands such as DNA, and that it acts as a focal point for the local amplification of alternative pathway complement activation. This process occurs independently of C3b. We propose herein that properdin is a rate-limiting factor and focal point for local alternative pathway complement activation on late stages of dying cells, thereby supporting a safe clearance.

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Disclosures
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