Innate immune functions in kidney transplantation

Berger, S.P.

Chapter 6

Properdin binds to late apoptotic and necrotic cells independently of C3b, and regulates alternative pathway complement activation

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Summary

Cells that undergo apoptosis or necrosis are promptly removed by phagocytes. Soluble opsonins such as complement can opsonize dying cells, thereby promoting their removal by phagocytes and modulating the immune response. The pivotal role of the complement system in the handling of dying cell has been demonstrated for the classical pathway (via C1q) and lectin pathway (via MBL and ficolin). Here 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 independent of C3b, which is additional to the standard model wherein properdin binds to pre-existing 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 late stage of dying cells. Our data indicate that direct recognition of dying cells by properdin is essential to drive alternative pathway complement activation.

Introduction

Under steady-state conditions, cells that undergo apoptosis and necrosis can be safely and silently eliminated by professional phagocytes, i.e. immature dendritic cells (DCs) and macrophages (Mφ) [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 MBL and ficolin) [10-14]. It was 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 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 independent 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.

Material 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 ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2, followed by 3 or 30 hours culture 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 fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI, VPS Diagnostics, Hoeven, the Netherlands) according to established methods [22]. In addition, light microscopy and fluorescent microscopy (Leica DC300F, Leica, Rijswijk, the Netherlands) were performed to detect the morphology and Hoechst nuclear staining of these cells, respectively. Alternatively, human umbilical cord endothelial cells (HUVEC), U937 cell (monocytic cell lines), and Epstein-Barr virus-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 was 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 (Mφ2) were generated from $CD14^+$ monocytes in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 µg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) in the presence of 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands) for 6 days. Jurkat cells were used as the target for phagocytosis. Prior to the induction of apoptosis or necrosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands). Labeled early, late apoptotic or necrotic cells (1×10^5) were investigated with or without opsonization using normal human serum (NHS). Dying cells were co-cultured with Mφ2 in 1:1 ratio at 37°C for 0.5 h in 100 µl RPMI culture medium in round-bottom glass tubes. As a control, co-culture was performed at 4°C to detect the binding of dying cells to phagocytes. *M*φ2 were stained with a PE-conjugated mAb against CD11b (BD Biosciences, San Jose, CA) and uptake was analyzed by a two-color flow cytometry.

The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of Mφ2 that ingested and/or bound apoptotic cells.

Isolation of properdin, C1q and MBL, 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 (2×VBS, 1.8 mM Na-5,5-diethylbarbital, 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 Sulphopropyl 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, Uppsala, Sweden). Fractions containing properdin were dialyzed against PBS, 2 mM EDTA and further purified using human IgG coupled to a Biogel A5 (Bio-Rad, Hercules, CA) to remove contaminating C1q. Purity of the properdin preparation was determined by analysis on 10% SDS-PAGE gel. A single 220-kD 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 seconds) 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 following: Blood was obtained by venapuncture, allowed to clot at room temperature for 1 hour and then centrifuged. The serum was brought to a NaCl concentration of 0.3M by addition of NaCl and then mixed with an immunoabsorbent of rabbit IgG anti-human C4. Coupling of rabbit IgG anti-C4 to Sepharose was performed according to manufacturers' instructions (Amarsham Biosciences, Roosendaal, the Netherlands). Following absorption by gentle mixing for 30 minutes at 4ºC, the mixture was centrifuged at 1000g and the supernatant aliquotted and frozen at -80ºC. 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 C-activity at a dilution of 1/240. Additionally, C4 hemolytic activity could be restored in 1/25 diluted C4-deficient serum with 10 ug/ml purified C4.

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

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

Normal human sera from 9 healthy donors were used as C3 full sera as confirmed by C3 ELISA. The properdin concentration in those C3 full sera was ranging from 10.4 to 25.1 ug/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 37ºC 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. Then cells were extensively washed and incubated with a rabbit-anti-human properdin polyclonal Ab (generated by immunizing rabbit with purified properdin), and detected with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-rabbit Ig (Southern Biotechnology Associates, Birmingham, US). The cells were analyzed by flow cytometry. Data from 10^4 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 as 40%. In some experiments, purified C3 or C3b was used to detect its binding to properdin which has been pre-bound on necrotic cells.

Binding of C1q (30 µg/ml) and MBL (10 µg/ml) were performed in the same way as properdin binding and detected with a monoclonal antibody (mAb) directed against C1q (mAb 2204) or MBL (clone 3E7), respectively. Binding was visualized with phycoerythrin (PE)-conjugated goat $F(ab)_2$ anti-mouse Ig (DAKO, Glossstrup, Denmark).

In some experiments, cells were pre-incubated with C1q or7 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, Maarssen, the Netherlands) or human albumin (Sigma-Aldrich) were coated in PBS on microtiter plates overnight, and then blocked with 2%BSA before adding purified properdin.

After washing, bound properdin was detected with Dig-labelled rabbit-anti-human properdin. Bound antibody was developed with anti-Dig-HRP (Roche Diagnostics GmbH, Mannheim, Germany), and measured for absorbance at OD 451 nm.

To confirm that DNA is exposed on late apoptotic cells and necrotic cells, a monoclonal mouse anti-human dsDNA Ab (ImmunoTools, Friesoythe, Germany) was used to detect DNA and its binding assessed with a PE-conjugated goat $F(ab)_2$ anti-mouse Ig. For double staining, late apoptotic cells and necrotic cells were opsonized with properdin at 37ºC for 1 h in serum-free RPMI culture medium. Cells were then incubated 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 (BD Biosciences). For confocal microscopy analysis, primary Abs were visualized by Alexa 568 or Alexa 488-labelled secondary Abs. Above cells were followed by a Hoechst nuclear staining prior to fixation by 1% paraformaldyde, and then mounted onto the slides for analysis by a confocal laser scanning microscope LSM 510 (Carl Zeiss AG), as described previously [24]. Images were visualized using a 63 × /1.40 numeric aperture oil objective, and were processed using Zeiss LSM Image Examiner software.

Complement activation by dying cells

Activation of complement by dying cells was assessed as follows: early, late apoptotic or necrotic cells were pre-incubated with or without properdin (20 ug/ml) at 37ºC 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 37ºC. Deposition of C3, C4 and C5b-9 on the cell surfaces were 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. Mollnes, Nordland Central Hospital, Bodø, Norway), respectively.

Statistical analysis

Statistical analysis was performed by one sample *t* test using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05.

Results

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 (Figure 1A). The difference between late apoptotic cells and necrotic cells was confirmed by light microscopy and Hoechst nuclear staining (Figure 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 anti-inflammatory Mφ2 preferentially recognized and ingested early apoptotic cells, as compared to the ingestion of late apoptotic and necrotic cells [24] (Figure 1C). However, opsonization of early apoptotic cells with normal human serum (NHS) did not enhance their uptake by Mφ2 (Figure 1C, D), while opsonization of late apoptotic and necrotic cells with NHS significantly increased their uptake by Mφ2 ($p < 0.01$) (Figure 1C, 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 Mφ1 (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 (Figure 1E). Therefore we assessed the pathways involved in the activation of complement on these cells.

Properdin binds to late apoptotic and necrotic cells

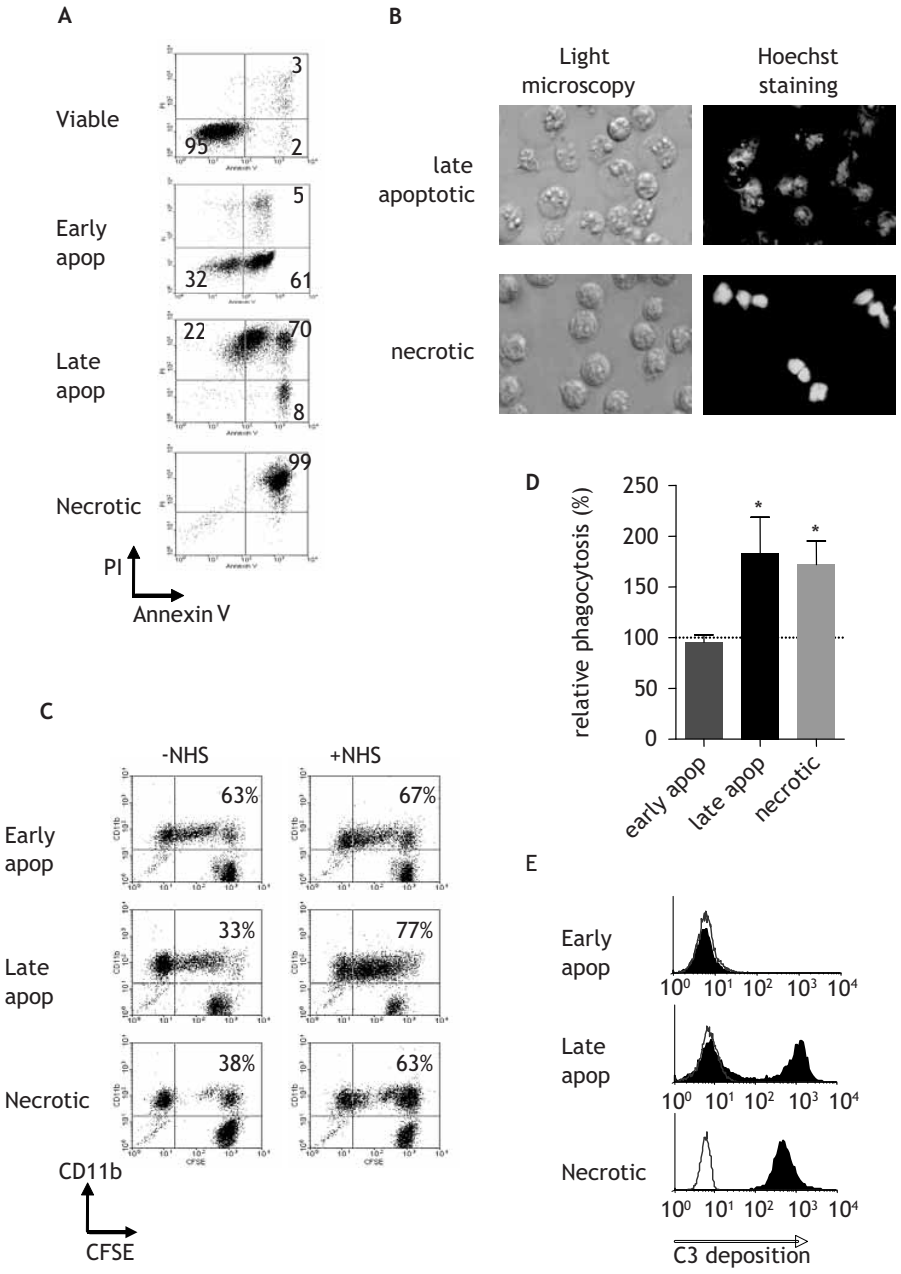
We investigated whether properdin, the only naturally occurring positive complement regulator, can bind directly to dying cells that are at different stages of cell death. Similar to C1q, and MBL, properdin showed a predominant interaction with late apoptotic cells and necrotic cells over early apoptotic or viable cells (Figure 2A, B). Properdin was shown to bind to both late apoptotic and necrotic cells in a dose-dependent manner (Figure 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 5 cycles of freeze-thawing (data not shown), suggesting that binding of properdin to necrotic cells is a universal phenomenon and irrespective of specific cell types.

Binding of properdin to dying cells can occur independent of C3

It has been established for a long time that properdin can bind to pre-existing 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 independent 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 (Figure 3A). Properdin did not bind to viable splenocytes derived from either $C3^{-/-}$ or WT mice (Figure 3A).

In addition, to confirm C3 independence, we also used C3 deficient serum in EDTA containing medium (obtained from a C3 deficient patients 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 to C3 full serum (NHS) and purified properdin (Figure 3B), although the extent of the binding is low since the concentration of properdin in these sera was around 7ug/ml. In the absence of EDTA, C3 full sera showed much higher binding of properdin, indicating that complement activation amplifies the properdin binding. 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 monoclonal antibody recognizing both C3 and C3b. Under these conditions, C3 or C3b did not bind to non-opsonized 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 (Figure 3C). Although preparations were pure as based on SDS-PAGE analysis (data not shown), small contamination with C3b cannot be excluded. 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.


A
Viable
Early apop
Late apop
Necrotic
3
95
2
5
32
61
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PI
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Light microscopy
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late apoptotic
necrotic
C
-NHS
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Early apop
Late apop
Necrotic
63%
67%
33%
77%
38%
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CD11b
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250
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early apop
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E
Early apop
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10^0 10^1 10^2 10^3 10^4
C3 deposition

Figure 1. Complement-mediated phagocytosis of dying cells. (**A**) Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light at a dose of 50 J/m^2, followed by 3 or 30 hours 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 (1×10^5) were first opsonized with or without normal human serum (NHS), then co-cultured with Mφ2 in 1:1 ratio at 37°C for 0.5 h. Mφ2 were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. $CD11b^+CFSE^+$ cells were used as a measure for the percentage of Mφ2 that ingested apoptotic cells. (**D**) Relative phagocytosis was calculated as uptake of NHS-opsonized dying cells versus non-opsonized cells. Data are mean ±SEM of 3 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 was detected by flow cytometry. Open histograms are the matched isotype controls (see page 154 for color image B).

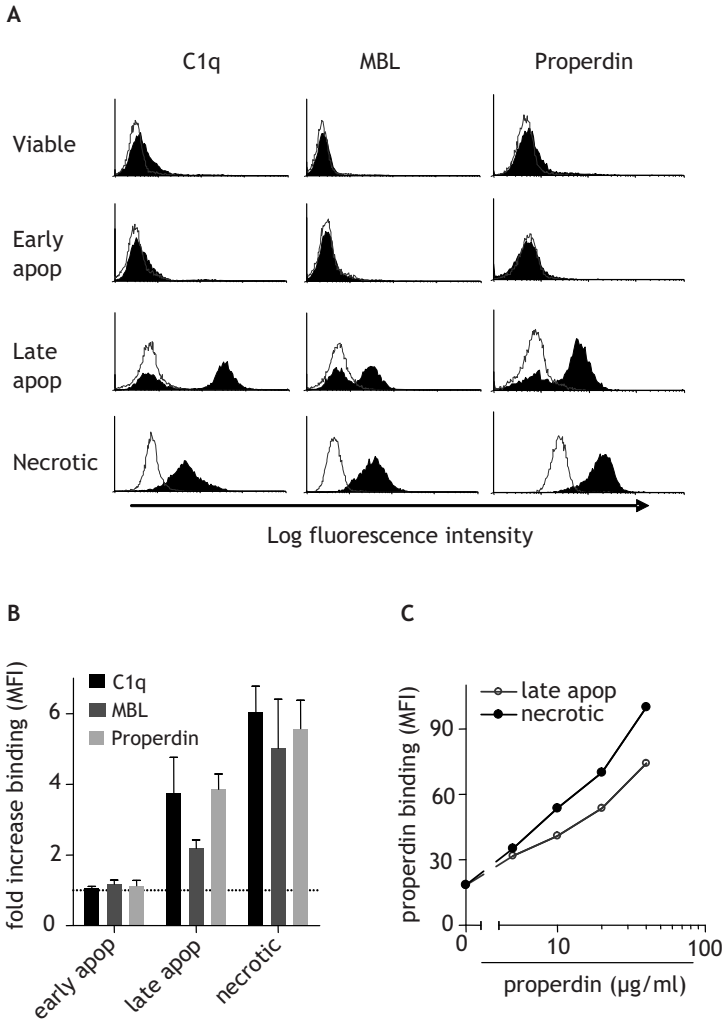

Figure 2. Properdin binds to late apoptotic and necrotic cells. (**A**) Viable, early apoptotic, late apoptotic and necrotic cells were collected and incubated with C1q, MBL or properdin. Specific binding (filled histogram) of C1q, MBL and properdin were detected by mAb against C1q (clone 2204), MBL (clone 3E7) and rabbit-anti-human properdin. Open histograms are the matched isotype controls. (**B**) Fold increase of binding was calculated as the mean fluorescence intensity (MFI) of C1q, MBL, or properdin divided by the MFI of matched isotype controls. Data shown are mean ±SEM of at least 4 independent experiments. (**C**) Late apoptotic and necrotic cells were incubated with increasing concentration of properdin (up to 40 µg/ml), and detected for properdin binding. Data shown are MFI.

To test the physiological relevance of properdin binding to dying cells, we used sera from nine healthy donors (properdin concentration ranges from 10.4 to 25.1 ug/ml). All sera tested showed predominant binding to late apoptotic cells and necrotic cells, but not to early apoptotic or viable Jurkat cells (Figure 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 properdin-depleted serum (Pds). Cells pre-incubated with purified properdin alone, as expected, did not show C3 and C5b-9 deposition (Figure 4A). In Pds, a reduced C3 deposition was observed, which is accompanied with a lack of deposition of the membrane attack complex C5b-9 (Figure 4A). Necrotic cells that had been pre-exposed to properdin, washed extensively, and subsequently incubated with Pds, displayed significantly increased C3 and C5b-9 deposition (Figure 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 C4-depleted serum (C4ds) as a source of complement, since C4 is a crucial factor for both pathways. Exposure of both late apoptotic cells (Figure 4B) and necrotic cells (Figure 4C) that had been pre-incubated 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 neither on late apoptotic (Figure 4B) nor on necrotic cells (Figure 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 (Figure 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.

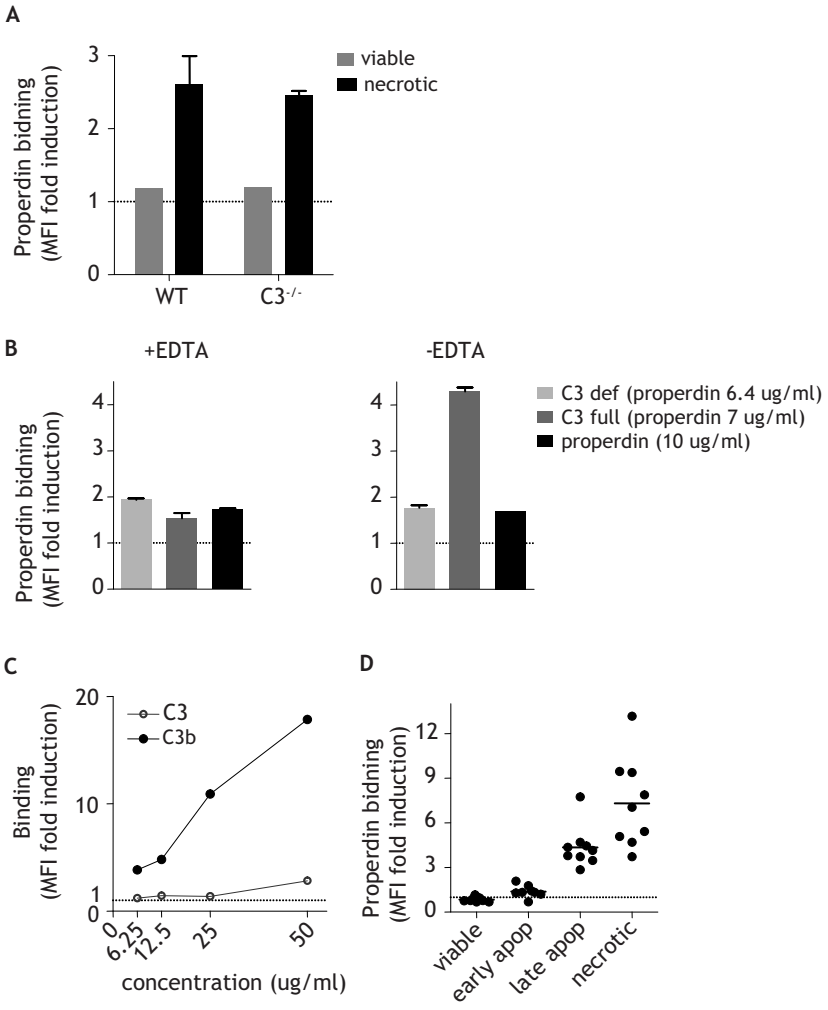

Figure 3. Binding of properdin to dying cells is independent of C3b. (**A**) Splenocytes from $C3^{-/-}$ or WT mice were first rendered necrotic by heating or kept viable, and measured for properdin binding. Fold increase of MFI was shown. Data shown are mean ±SEM of 4 independent experiments. (**B**) Necrotic cells were opsonized with C3 full serum (normal human serum from a healthy donor, properdin concentration was 16 ug/ml), or C3 deficient serum (obtained from a patient who was deficient for C3, properdin concentration was 17.5 ug/ml) for 1 hour at 37°C in the presence or absence of 10 mM EDTA. 40% of the serum (diluted in serum-free RPMI medium) was used, thus the final concentration of

properdin presented in the opsonization was 6.4 ug/ml (C3-full serum) and 7 ug/ml (C3-def serum), respectively. As a control, purified properdin (10 ug/ml) was used for the binding assay. Binding of properdin was detected with a polyclonal rabbit anti-human properdin antibody and developed by a goat anti-rabbit Ab. Fold induction of MFI in flow cytometry is shown. Data are mean ±SD of two experiments. (**C**) Necrotic cells were first opsonized with properdin (30ug/ml) for 1 hour at 37°C to allow sufficient binding of properdin on the surface of necrotic cells. Next increasing concentrations of C3 and C3b were added (up to 50 ug/ml), and detected with a monoclonal antibody (RFK22) recognizing both C3 and C3b. Fold induction of MFI in flow cytometry is shown. Data are representative of three independent experiments. (**D**) Sera from 9 healthy donors (properdin concentration ranges from 10.4 to 25.1 ug/ml), diluted as 40% in RPMI serum-free medium, were used to opsonize viable, early apoptotic, late apoptotic or necrotic cells. Properdin binding (MFI fold induction) is shown.

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]. Since properdin was shown to bind to late apoptotic and necrotic cells in a similar pattern as C1q and MBL (Figure 1A), we hypothesized that properdin may bind to a similar structure on dying cells. Necrotic cells were pre-incubated with properdin, followed by incubation with increasing concentrations of C1q. A dose-dependent binding of C1q was observed, but pre-incubation with properdin did not inhibit the binding of C1q to the cells (Figure 5A). In a reverse way, pre-incubation of necrotic cells with C1q did not decrease properdin binding either (Figure 5B). Similarly, pre-incubation of necrotic cells with properdin did not interfere with MBL binding and *vice versa* (Figure 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 (Figure 6A). Furthermore, pre-incubation of properdin with calf thymus dsDNA dose-dependently inhibited binding of properdin to necrotic cells (Figure 6B), suggesting a strong interaction between DNA and properdin.

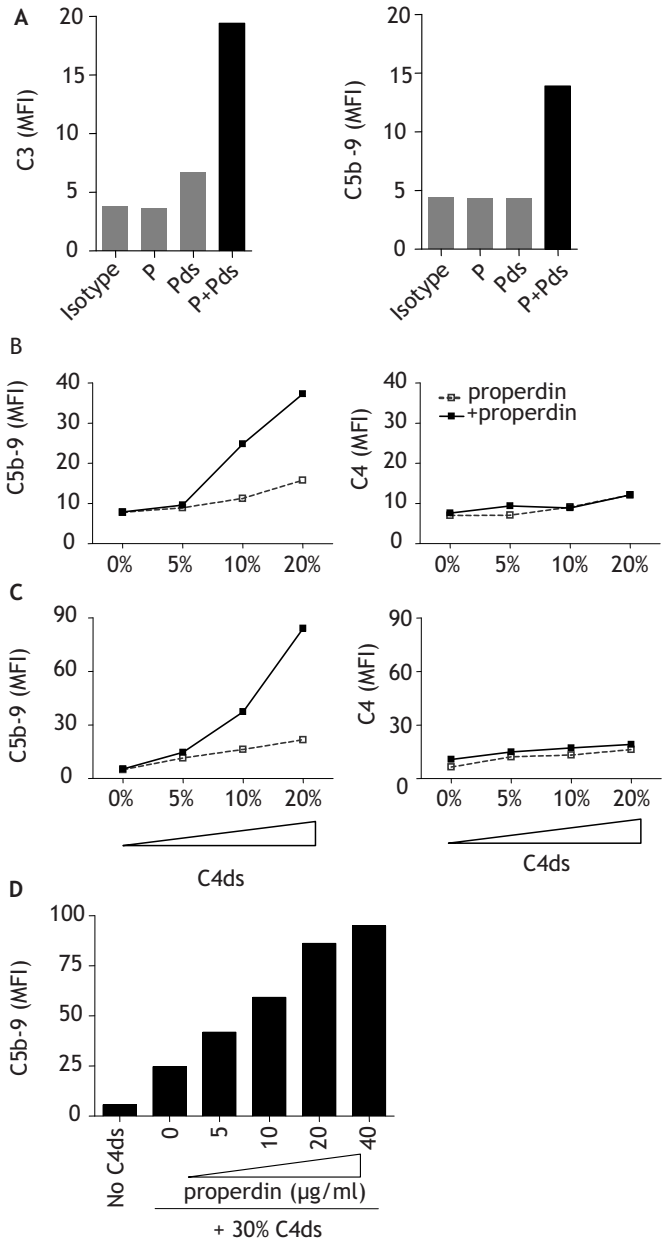

Figure 4. Properdin activates complement via alternative pathway. (**A**) Necrotic Jurkat cells were pre-incubated with or without properdin (20 µg/ml), and then washed extensively before adding 30% properdin-depleted serum (Pds). Data shown are C3 and C5b-9 deposition

on the cells. (**B**) Late apoptotic and (**C**) necrotic cells were pre-incubated with or without properdin, and then washed extensively before adding increasing concentration of C4-depleted serum (C4ds). C5b-9 and C4 deposition on the cells were measured. (**D**) Necrotic cells were pre-incubated with increasing concentration of properdin, and then washed extensively before adding 30% C4ds. Cells were measured for C5b-9 formation. Data are representative of 2 independent experiments.

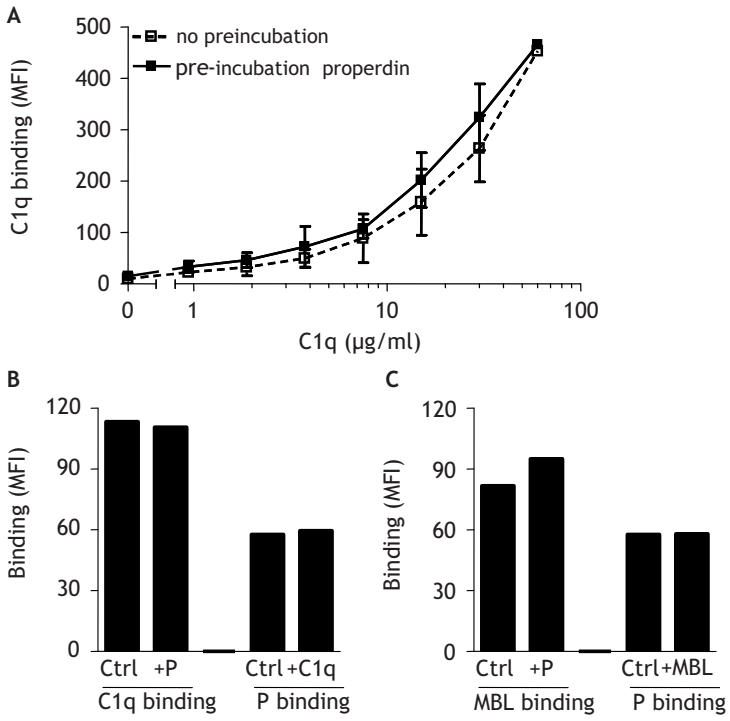

Figure 5. Properdin does not compete for binding with C1q and MBL. (**A**) Necrotic cells were pre-incubated with properdin (40 µg/ml) and followed by incubation with increasing concentrations of C1q (up to 60 µg/ml). C1q binding was measured. Data shown are mean ±SEM of 2 independent experiments. (**B**) Necrotic cells were pre-incubated 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).

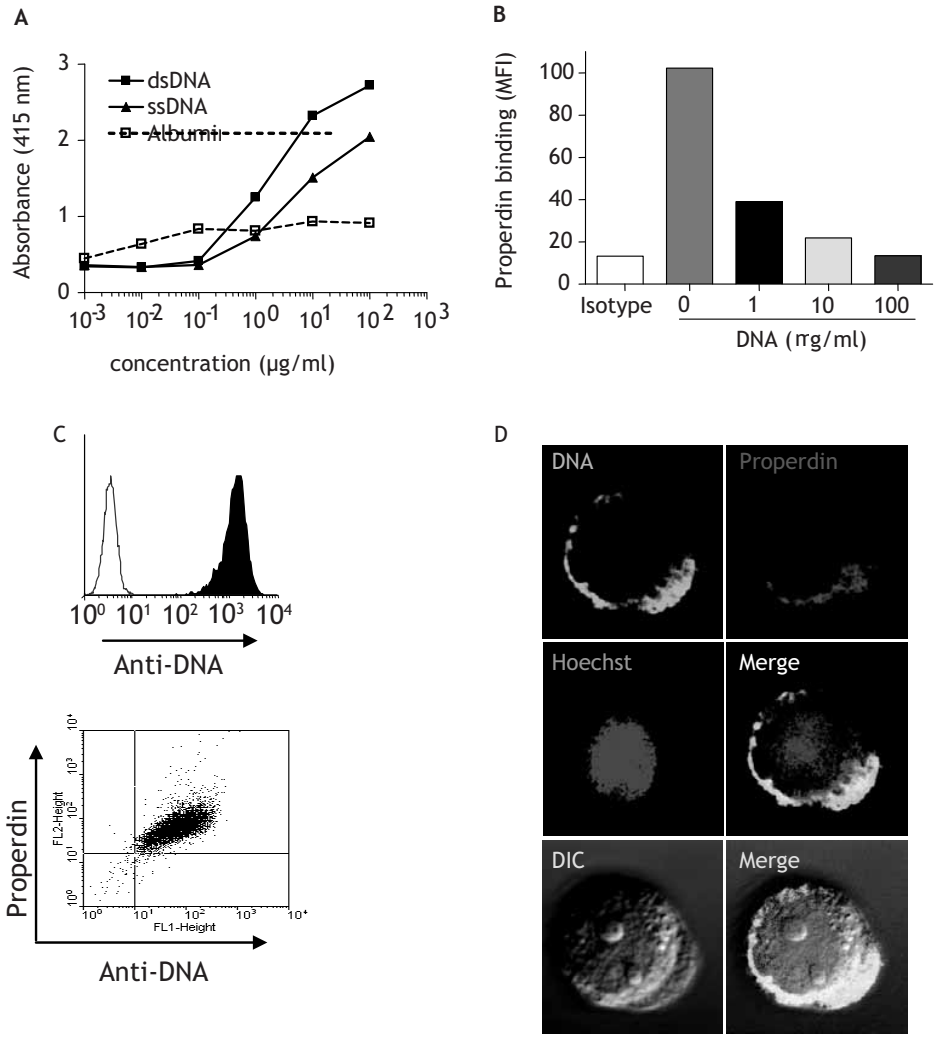

Figure 6. Properdin binds to DNA. (**A**) Different concentration of dsDNA and single strand DNA (ssDNA) or human albumin were coated on microtiter plates overnight, and then blocked with 2%BSA before adding properdin. After washing, plates were incubated with Dig-labelled rabbit-anti-human properdin. Signal was developed by anti-Dig-HRP, and measured for absorbance at OD 451 nm. (**B**) Properdin was pre-incubated with increasing concentrations of calf thymus double strand DNA (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 ug/ml) at 37ºC 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 (BD Biosciences). (**D**) Confocal laser scanning microscopy (LSM 510, Carl Zeiss AG), was performed on properdin-opsonized necrotic cells that were stained for properdin, DNA and Hoechst. Green: DNA; Red: properdin; Blue: Hoechst; Yellow: DNA co-localizes with properdin; DIC: differential interference contrast. Magnification, 400× (see page 155 for color image D).

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

Interestingly, different from necrotic cells, cells made late apoptotic were not all recognized by properdin (Figure 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 (Figure 7A). Cells in R2 are Annexin V^+PI^-, characteristics of early apoptotic cells (Figure 7A)., and as expected these cells did not bind properdin and did not expose DNA (Figure 7B). Cells in R1 are all Annexin V^+PI^+, indicative for late apoptotic cells (Figure 7A). Within the R1 population, part of the cells were both negative for properdin binding and DNA, and binding of properdin is related to the degree that DNA is exposed on these dying cells (Figure 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 (Figure 7C). Confocal microscopy confirmed that properdin was co-localized exclusively with fragmented DNA exposed on these cells (Figure 7D).

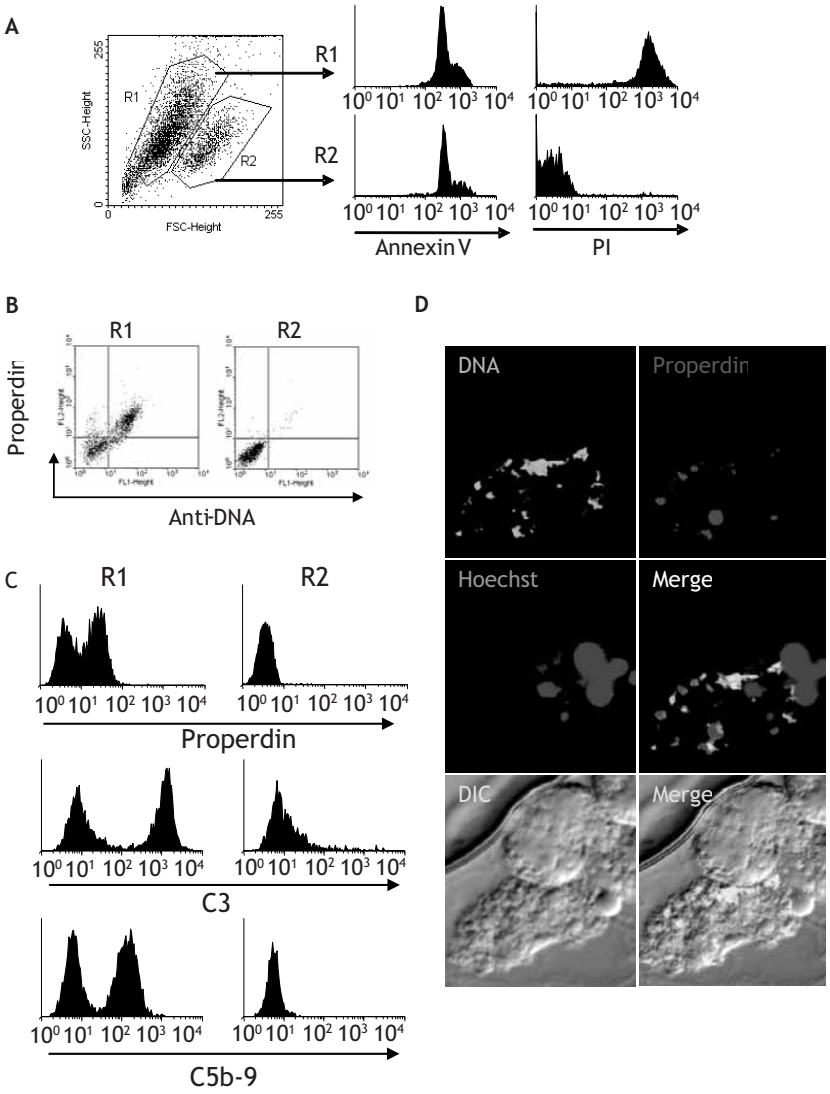

Figure 7. Properdin binds to a subpopulation of late apoptotic cells. (**A**) Jurkat cells made late apoptotic by culturing cells for 30 h after UV irradiation were stained with Annexin V and PI by flow cytometry. Cells were divided into R1 (late apoptotic cells) and R2 (early apoptotic cells). (**B**) Double staining of properdin and DNA to late apoptotic cells that were pre-opsonized with properdin was shown based on different gate on R1 or R2 region. (**C**) Histogram of properdin binding to late apoptotic cells was shown based on gate R1 and R2. C3 and C5b-9 depositions are shown on these cells after opsonization with NHS. (**D**) Confocal microscopy was performed on properdin-opsonized late apoptotic cells

that were stained for properdin, DNA and Hoechst. Green: DNA; Red: properdin; Blue: Hoechst; Yellow: DNA co-localizes with properdin; DIC: differential interference contrast. Magnification, 400× (see page 158 for color image D).

Discussion

We describe here 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 independent of C3b, and 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 pre-formed 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 prior to C3 deposition on the cell surface. Evidence that binding of properdin to dying cells can occur independent of C3b was further supported by experiments showing that properdin binds strongly to necrotic splenocytes derived from $C3^{-/-}$ mice (Figure 3A), and by experiments using C3 deficient serum (Figure 3B). We further showed that properdin pre-bound 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 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 antibody, we demonstrate that both late apoptotic cells and necrotic cells expose DNA. Confocal images indicate that properdin and DNA are

co-localized 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 Toll-like receptors [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 (SAP)[43], and C4b-binding protein (C4BP) [44]. We showed that properdin does not compete with the binding of C1q and MBL to dying cells, suggesting that C1q and MBL interacts with DNA structures different from the ones that properdin recognizes. It has been suggested that properdin binds to sulfatide (sulfated glycosphingolipids) and weakly to phosphatidylserine (PS) [45]. However, it is not likely that PS exposed on the surface of dying cells is a major ligand, since properdin does not bind to early apoptotic cells although these cells do express PS. 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*. reporting that properdin binds to early and late apoptotic cells [46]. Here 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 [8]), Ficolin [14], natural IgM [47] and pentraxin family members SAP [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 anti-inflammatory 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 pyonenic (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 of other opsonins such as MBL, CRP and PTX3 also bind to dying cells and help their clearance, deficiency of these opsonins do 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 those 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 attempting to speculate that properdin might also have a dual function: 1.) amplifying complement activation on dying cells to promote complement-mediated clearance; 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 acts as a focal point for the local amplification of alternative pathway complement activation. This process occurs independently of C3b. We propose here 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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