Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity

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Properdin regulates alternative pathway complement activation on late apoptotic and necrotic cells

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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. However, the pivotal role of the complement system in the handling of dying cell has been mainly linked to 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 is independent of C3b, and we show that properdin becomes a focus point for local amplification of alternative pathway complement activation. Properdin does not compete with C1q and MBL for binding to late apoptotic and necrotic cells, but exhibits a strong interaction with DNA which is released and exposed on these cells. Although purified properdin does not enhance phagocytosis, it limits the pro-inflammatory potential of necrotic cells by reducing TNF-α release by dendritic cells and macrophages, whereas IL-10 production remains unchanged. Our data indicate that recognition of dying cells by properdin is essential to drive alternative pathway complement activation and actively promotes an anti-inflammatory response.

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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 and lectin pathways. Properdin, discovered in 1954 [18], is the only known naturally occurring positive regulator of complement activation [19]. It was 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]. Moreover, it has been suggested that properdin amplifies complement activation on a target surface through an organized assembly process of C3 convertase C3bBbP, accounting for a direct complement activation capacity of properdin [21]. Properdin has long been recognized as one of the key players in the regulation of the complement system [18], nevertheless, the interaction of properdin with dying cells and the involvement of the alternative pathway in this process have not been investigated.

In present study, we investigated whether properdin, like C1q and MBL, contributes to the processing 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. Our data suggest that properdin is one of the important regulators involved in the handling of dying

cells via: 1.) activation of alternative pathway complement; 2.) modulation of the immune response by antigen presenting cells by dying cells.

Material and methods

Generation of phagocytes

Generation of dendritic cells (DCs), Mφ1 and Mφ2 from human peripheral blood monocytes was performed using the methods described previously [22]. Briefly, Mφ1 and 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 GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively, for 6 days. DCs were cultured with combination of GM-CSF and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ).

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 [23]. Alternatively, human umbilical cord endothelial cells (HUVEC), U937 cell (monocytic cell lines), HK-2 cell (Renal tubular epithelial cells) 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 ($C3^{-/-}$) [24] 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 [22]. Briefly, 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 either alone or following opsonization with normal human serum (NHS) or purified properdin. Dying cells were co-cultured with DCs, Mφ1 or 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. Mφ2 were stained with a PE-conjugated mAb against CD11b 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 DCs, Mφ1 or Mφ2 that ingested and/or bound apoptotic cells.

Isolation of properdin, C1q and MBL

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. C1q and MBL were purified from pooled human plasma obtained from healthy donors as described previously [25,26].

Properdin binding assay

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. Then cells were extensively washed and incubated with a rabbit-anti-human properdin polyclonal Ab, 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.

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 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) 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.

Serum

C4-depleted serum (C4ds) was used as a complement source that is lacking both classical and lectin pathway activity, and was prepared by affinity absorption using goat anti-human C4 IgG coupled to CNBr-activated Sepharose 4 fast flow (Amersham Bioscience Europe GmBH, Roosendaal, the Netherlands). C4ds was free of C4 antigen and hemolytic activity of the classical pathway could be resorted by 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.

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 at 37°C for 1 h, 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 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.

Detection of cytokine production by DCs, Mφ1 and Mφ2.

DCs, Mφ1 and Mφ2 (1×10^5) were incubated for 4 hours at 37°C with necrotic cells that were pre-opsonized with or without properdin, followed by activation with 200 ng/ml LPS for another 20 h in 48-well-plate and supernatants were harvested. The amounts of TNF-α and IL-10 in the supernatants were quantified by ELISA, as described previously [22].

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 (Fig. 1A). In agreement with our earlier findings, M-CSF-driven anti-inflammatory Mφ2 preferentially recognized and ingested early apoptotic cells, as compared to the ingestions of late apoptotic and necrotic cells [22] (Fig. 1B). However, opsonization of early apoptotic cells with normal human serum (NHS) did not enhance their uptake by Mφ2 (Fig. 1B, C), while opsonization of late apoptotic and necrotic cells with

NHS significantly increased their uptake by Mφ2 ($p<0.01$) (Fig. 1B, C). 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. 1D). Therefore we next assessed the pathways involved in the activation of complement on these cells.

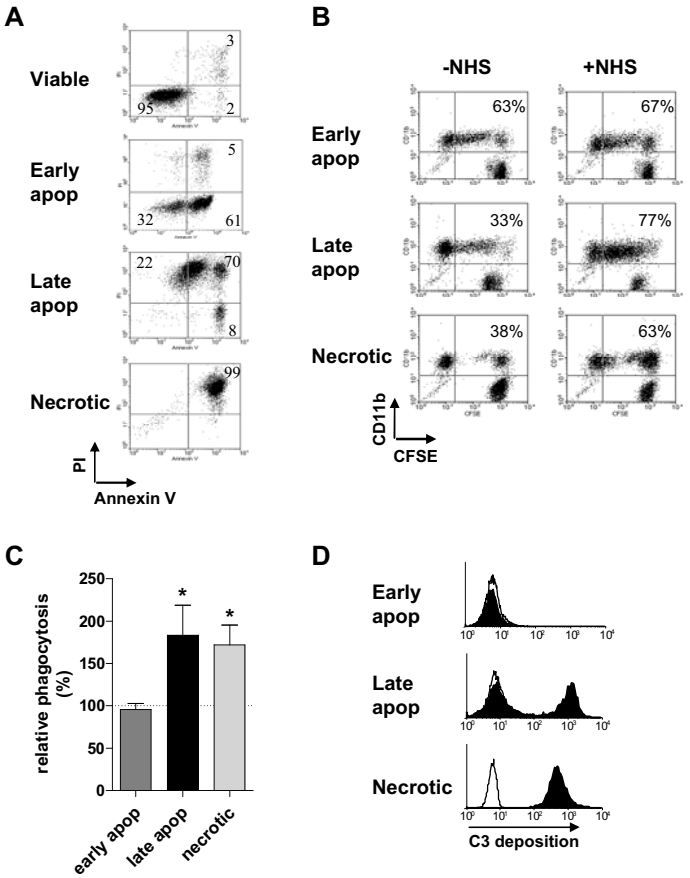

Figure 1. Complement-mediated phagocytosis of dying cells. (A) Early or late apoptosis was induced in Jurkat cells by exposure to ultra violet (UV)-C light at a dose of 50 J/m^2, followed by 3 or 30 hours culture in RPMI serum-free medium, respectively. Necrosis was induced by treating cells at 56°C for 0.5 h. Cells were stained with annexin V and PI by flow cytometry. (B) 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. (C) 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. (D) 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.

Properdin binds to late apoptotic and necrotic cells.

It has been shown that several serum factors bind to apoptotic cells (see review [8]). C1q, MBL and properdin are complement factors which activate complement via the respective 3 pathways. We here 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, and no significant binding to early apoptotic or viable cells (Fig 2A, 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 strongly 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.

It is accepted for a long time that properdin binds to a pre-existing cluster of surface-bound C3b [29], thereby stabilizing the C3b-dependent C3 convertase C3bBb [20]. However, the data presented above propose a new model for the binding of properdin to dying cells independent of C3b. To confirm our hypothesis, we investigated whether properdin could bind to necrotic splenocytes derived from C3 knockout ($C3^{-/-}$) mice. Properdin was shown to bind strongly to necrotic splenocytes of $C3^{-/-}$mice and the extent of binding was similar to the binding of properdin to necrotic cells from WT mice (Fig. 2D). Importantly, properdin did not bind to viable splenocytes derived from either $C3^{-/-}$ or WT mice (Fig. 2D).

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

Since properdin is central in alternative pathway activation, we investigated whether binding of properdin to these dying cells might act as a focus point for local amplification of the complement system. For this purpose, we analyzed complement activation on necrotic cells using properdin-depleted serum (Pds). Cells pre-incubated with purified properdin alone, as expected, do not show C3 and C5b-9 deposition. (Fig. 3A). Also, in Pds, no properdin binding to necrotic cells was seen (not shown), however a reduced C3 deposition was observed, which is accompanied with a lack of deposition of the membrane attack complex C5b-9 (Fig. 3A). Necrotic cells that had been pre-exposed to properdin, washed extensively, and subsequently incubated with Pds, displayed significantly increased C3 and C5b-9 deposition, suggesting that properdin is essential for local amplification of the complement cascade on necrotic cells.

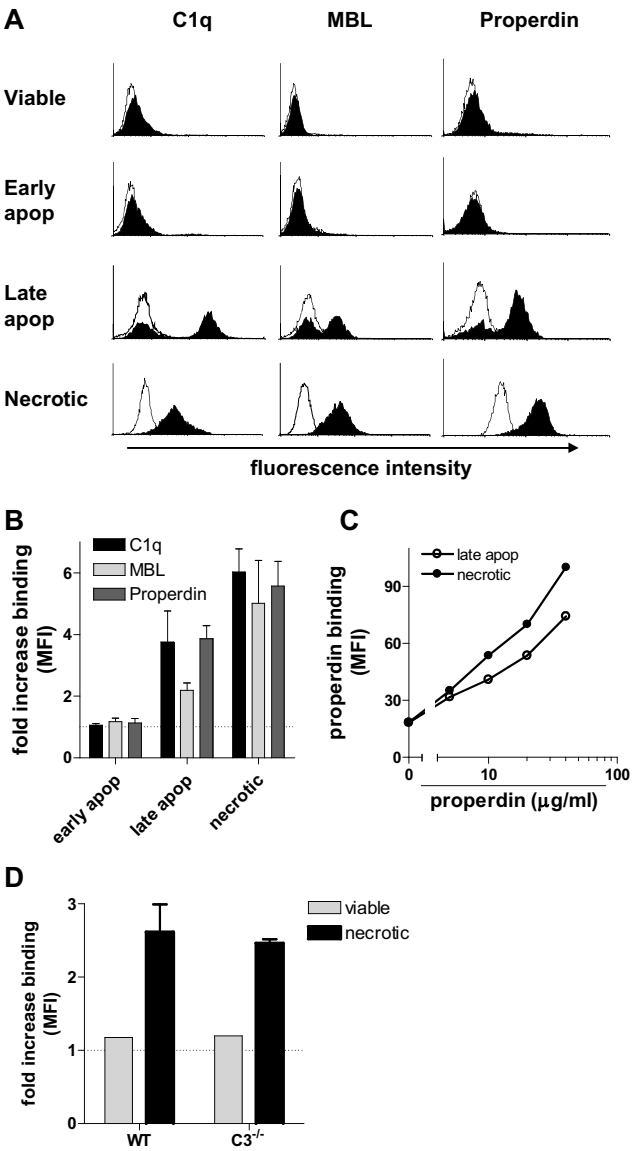

Figure 2. Properdin binds to late apoptotic and necrotic cells. (A) Viable, early apoptotic, late apoptotic and necrotic cells were collected and incubated with C1q, MBL or properdin. Specific binding (filled histogram) of C1q, MBL and properdin were detected by mAb against C1q (clone 2204), MBL (clone 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. (D) 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.

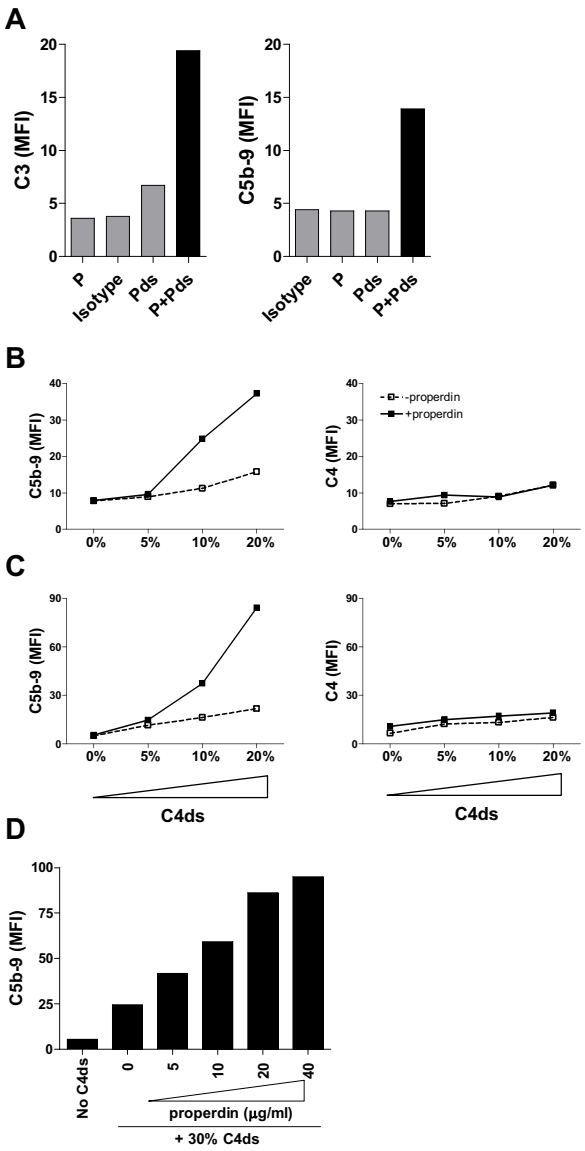

Figure 3. Properdin activates complement via alternative pathway

(A) Necrotic cells were pre-incubated with or without properdin, 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.

To confirm that cell-bound properdin activates complement via the alternative pathway, we used C4-depleted serum (C4ds) as a source of complement, since C4 is a crucial factor for both the classical and lectin pathways. Late apoptotic and necrotic cells were pre-incubated with properdin and washed extensively before the addition of increasing concentrations of C4ds. Exposure of both late apoptotic cells (Fig. 3B) and necrotic cells (Fig. 3C) 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, suggesting that properdin is the limiting factor that mediates complement activation via alternative pathway. As a control, we measured deposition of C4 on both late apoptotic and necrotic cells that had been exposed to C4ds. There was no C4 deposition neither on late apoptotic (Fig. 3B) nor on necrotic cells (Fig. 3C) 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. 3D).

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 [30]. Since 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 was pre-incubated with properdin and followed by increasing concentrations of C1q. A dose-dependent binding of C1q was observed, but properdin did not inhibit the binding of C1q to the cells (Fig. 4A). In a reverse way, pre-incubation of necrotic cells with C1q did not decrease properdin binding either (Fig. 4B). Similarly, pre-incubation of necrotic cells with properdin did not interfere with MBL binding and *vice versa* (Fig. 4C). 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] and it has been shown to be released from these cells [31]. Several serum components have been suggested to interact with DNA, including C1q [32], MBL [33], serum amyloid-P component (SAP) [34], and C4b-binding protein (C4BP) [35]. 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. Pre-incubation of properdin with calf thymus dsDNA dose-dependently inhibited binding of properdin to necrotic cells (Fig. 4D), suggesting a strong interaction between DNA and properdin. To further confirm whether properdin binds to DNA, various concentrations of dsDNA and ssDNA were coated on microtiter plates prior to addition of properdin. Human albumin was used as a negative control. The binding was detected by a Dig-labelled rabbit-anti-human-properdin Ab. Properdin showed a strong binding to both dsDNA and ssDNA at concentrations of 1 μg/ml and higher (Fig. 4E).

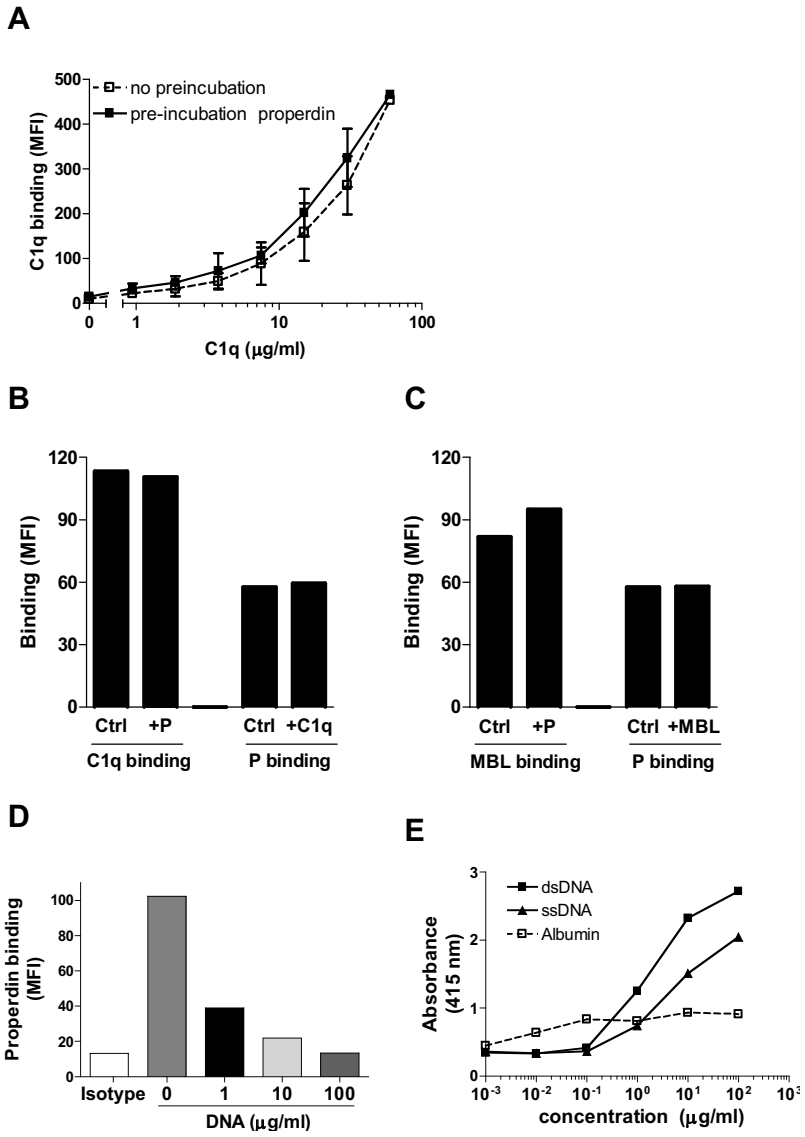

Figure 4. Properdin does not compete for binding with C1q and MBL, but binds to DNA. (A) Necrotic cells were pre-incubated with properdin (40 μg/ml) and followed by incubation with increasing concentrations of C1q (up to 60 μg/ml). C1q binding was measured. Data shown are mean ±SEM of 2 independent experiments. (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 (10μg/ml) was investigated similar as described in (B). (D) 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). (E) 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.

Purified properdin does not enhance phagocytosis of necrotic cells, but limits their pro-inflammatory potential.

It has been shown earlier that both C1q and MBL facilitate phagocytosis of apoptotic cells [12,13] prior to complement activation, accounting for a direct recognition of C1q and MBL by receptors on phagocytes. We here questioned whether properdin can enhance the removal of late apoptotic and necrotic cells by phagocytes. DCs, Mφ1 and Mφ2 generated in parallel from the same donor efficiently phagocytose late apoptotic cells (not shown) and necrotic cells. While opsonisation of necrotic cells with NHS clearly enhanced phagcytosis by Mφ1, opsonisation of necrotic cells with purified properdin did not show any enhancing activity (Fig. 5A). This was found to be true for all three types of phagocytes, namely DCs, Mφ1 and Mφ2 (Fig. 5B), suggesting that there are probably no high affinity properdin receptors on these phagocytes.

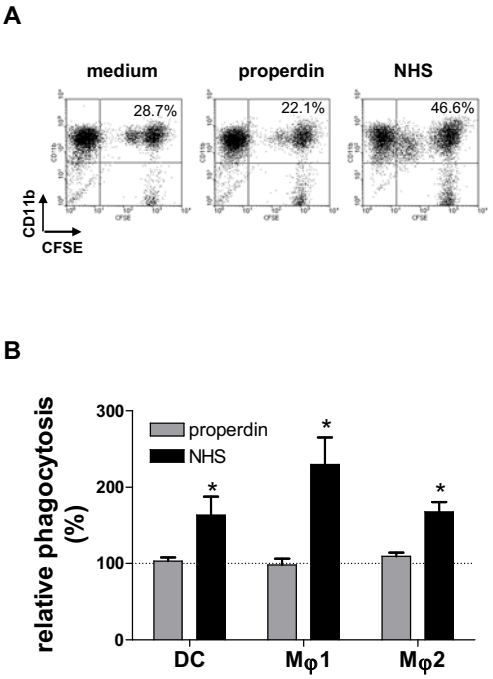

Figure 5. Properdin alone does not enhance phagocytosis

CFSE-labeled necrotic cells (1 × 10^5) were opsonized with or without properdin or 30% NHS, then co-cultured with DC, Mφ1 and Mφ2 in 1:1 ratio at 37°C for 0.5 h. Phagocytes were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. (A) A representative experiment of phagocytosis by Mφ1. (B) Relative phagocytosis was calculated as uptake of properdin- or NHS-opsonized necrotic cells versus non-opsonized cells. Data are mean ±SEM of 4 independent experiments. *, p<0.01, one sample *t* test.

Some opsonins from the innate immune system like C1q [13] and C4bP [36] have been suggested to modulate the inflammatory response. We therefore investigated whether properdin might regulate the inflammatory response by phagocytes. Necrotic cells were opsonized with or without properdin and then co-cultured with DCs, Mφ1 and Mφ2 in the presence of LPS. In three independent experiments, properdin significantly inhibited necrotic cells-induced TNF-α production by DCs (mean ±SEM of fold induction: 0.76 ±0.14, p<0.05) and Mφ1 (mean ±SEM: 0.59 ±0.29, p<0.05) (Fig. 6A). Since the anti-inflammatory Mφ2 did not produce TNF-α

[22], properdin did not show an effect (Fig. 6A). In contrast to the production of TNF-α, properdin opsonization of necrotic cells did not inhibit the anti-inflammatory cytokine IL-10 by both DCs (mean ±SEM: 1.16 ±0.28) and Mφ1 (mean ±SEM: 1.0 ±0.29). Interestingly, a significant increase of IL-10 (mean ±SEM: 2.99 ±1.2, $p<0.05$) by properdin-opsonized necrotic cells on Mφ2 was observed (Fig. 6B). Our data therefore suggest that properdin is a regulatory factor which limits the pro-inflammatory potential of necrotic cells.

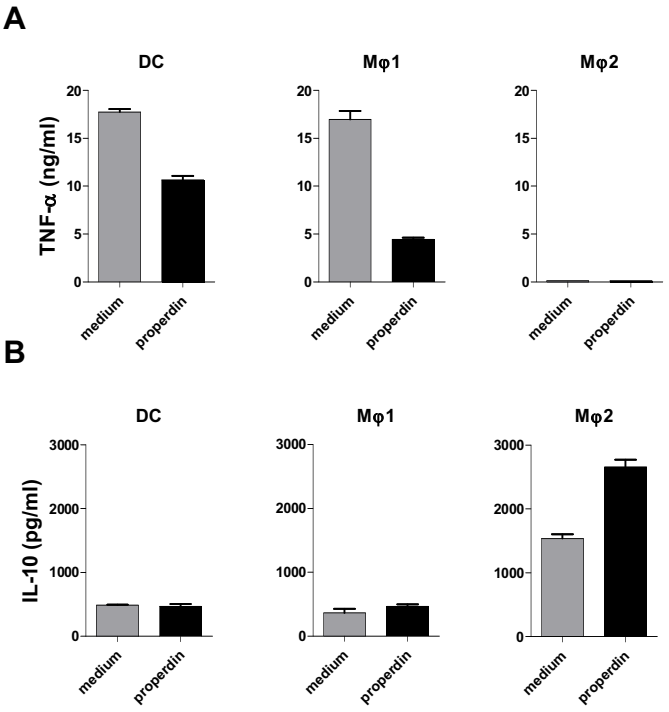

Figure 6. Properdin limits the pro-inflammatory potential of necrotic cells. Necrotic cells were opsonized with or without properdin, then co-cultured with DCs, Mφ1 and Mφ2 for 4 h followed by the addition of LPS (200 ng/ml) for another 20 h. Supernatants were collected for the measurement of TNF-α (A) and IL-10 (B) by DCs, Mφ1, and Mφ2. Data are mean ±SD of duplicate cultures and represent 3 independent experiments.

Discussion

We describe here that properdin, the only known naturally occurring positive regulator of complement, specifically binds to late apoptotic or necrotic cells, but not to early apoptotic cells. Furthermore, properdin binds to DNA and down-modulates the pro-inflammatory response induced by necrotic cells. We provide evidence that binding of properdin to late apoptotic cells and necrotic cells is independent of C3b, and serves as a focus point for the local amplification of the alternative pathway complement activation.

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-existing clusters of surface-bound C3b [20]. The other model suggests that properdin first binds to a surface ligand (C3b, C3bB or C3bBb) via one of its subunits and then promotes the assembly of further C3bBb at the ligand-binding sites of its adjoining subunits [21]. Our data showed that properdin binds to late apoptotic and necrotic cells prior to C3 deposition on the cell surface, therefore we exclude the possibility of initial binding of properdin to the cells is via C3b. Evidence that binding of properdin to dying cells is independent of C3b was further supported by experiments showing that properdin binds strongly to necrotic splenocytes derived from $C3^{-/-}$ mice (Fig. 2D). Thus, we suggest that properdin binds to the cell surface of late apoptotic or necrotic cells first in the absence of C3 to ligands like DNA, and serves as a focus point for the local amplification of complement activation as soon as a C3 source is available. Properdin is indispensable for the alternative pathway activation of complement on these dying cells.

We have tried to identify the ligands on late apoptotic and necrotic cells to which properdin binds. Our data suggest that DNA is one of the targets for properdin on dying cells. Indeed, DNA is massively released by apoptotic and necrotic cells [31,37], and has been shown to be one of the major autoantigens exposed on apoptotic cell surfaces [4]. In pathological situations, DNA is considered as one of the immunologically active autoantigens [38], that can stimulate immune cells via Toll-like receptors [39,40]. In autoimmune lupus, DNA is one of the major immunogens to trigger autoantibody production [41]. Our finding that properdin binds strongly to DNA suggests a role for properdin in preventing unwanted immune activation when DNA is exposed on dying cells during a large scale cell death.

Next to DNA, we also showed that properdin does not compete with the binding of C1q and MBL to dying cells, suggesting that properdin binds to other as yet unknown ligands which are different from those for C1q and MBL. It remains a challenging task to identify the exact additional ligands for properdin on dying cells. We have excluded the possibility that C3b is the ligand on dying cells, which initially facilitates binding of properdin. It is also not likely that phosphatidylserine (PS) exposed on the surface of dying cells is the ligand since properdin does not bind to early apoptotic cells although they do express PS. Recent data suggest that properdin binds to sulfatide (sulfated glycosphingolipids) [42]. Whether sulfatide or other phospholipids are one of the key ligands on dying cells for properdin recognition is currently under investigation.

Interestingly, although purified properdin binds strongly to late apoptotic and necrotic cells, it does not lead to an enhanced phagocytosis by DCs or Mφ. This might suggest that there are probably no high affinity properdin receptors on these phagocytes. In agreement with the earlier observations that complement-mediated

phagocytosis is mainly dependent on bound iC3b [10,11], we propose that a role of properdin in the augmentation of phagocytosis is associated with the triggering of alternative pathway activation of complement.

It has been suggested that some serum factors such as C4BP [35] are able to dampen the pro-inflammatory potential induced by necrotic cells. The current paper shows that properdin prevents the pro-inflammatory response of the pro-inflammatory Mφ1 and DCs induced by necrotic cells. Importantly, the anti-inflammatory cytokine IL-10 is not inhibited, or even increased after opsonization of dying cells with properdin. This indicates that although necrotic cells are generally thought to release danger signals and usually uncontrolled in their dying process, the host is able to minimize the danger. Since we did not find any evidence of the presence of a potential properdin receptor on these phagocytes which could directly interact with properdin, it is tempting to hypothesize that down-regulation of pro-inflammatory potential of dying cells by properdin might be via an indirect way where properdin inhibits the pro-inflammatory signals exposed on dying cells. Therefore, we propose that properdin plays a regulatory role in the resolution of inflammation and maintenance of peripheral tolerance towards self antigen.

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 [43]. Here we demonstrate that properdin binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells, which is consistent with the binding of other complement opsonins like C1q, MBL (review in [8]) and Ficolin [14]. Indeed, not only complement factors, but also natural IgM [44] and pentraxin family members such as SAP [45] and PTX3 [46], bind to late apoptotic and necrotic cells, but hardly to early apoptotic cells. Therefore, it seems that most, if not all, of the serum opsonins engage with late apoptotic or necrotic cells. It has been accepted that removal of dying cells during their early stage of cell death ensures a silent process, whereas late apoptotic or necrotic cells might provide danger signals that activate phagocytes and thereby break peripheral tolerance [47-49]. Therefore, soluble opsonins are critical to promote 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 [22], we suggest that a hierarchy exists in the clearance mechanism of dying cells [9], where 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, most likely ensuring a safe clearance when an overload of apoptosis or defects in phagocytic capacity occur.

In conclusion, we provide evidence that properdin binds specifically to late apoptotic and necrotic cells independent of C3b, and acts as a focus point for the local amplification of alternative pathway complement activation. Furthermore, properdin binds to DNA and limits the pro-inflammatory response by necrotic cells.

We propose here that properdin plays a dual role in the handling of dying cells. Firstly, it binds to DNA and probably other structures on late apoptotic and necrotic cells, potentially preventing the immune activation by the “danger” signals. Secondly, it is the limiting factor and focus point for local alternative pathway complement activation on these dying cells, thereby generating the vital opsonin iC3b to ensure a safe clearance.

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