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Author: O’Flynn, Joseph
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Myeloperoxidase directs properdin mediated complement activation

Joseph O’Flynn, Karen O. Dixon, Maria C. Faber Krol, Mohamed R. Daha, Cees van Kooten.
**Abstract**

Neutrophils and complement are key members of innate immunity. The alternative pathway (AP) of complement consists of C3, factor B, factor D and properdin which amplifies the AP activation. The AP has been implicated in many neutrophil mediated diseases such as ANCA vasculitis. The exact mechanism by which the AP and neutrophil interact remains largely unstudied. We investigated the ability of the AP to interact with neutrophil components which can be exposed and released upon activation. Our studies focused on neutrophil enzymes including myeloperoxidase (MPO), elastase, lysozyme and cathepsin G. All enzymes demonstrated an ability to bind properdin compared to azurocidin which did not. However, only MPO illustrated a capability to induce C3 activation. We demonstrated MPO-mediated AP complement activation as it occurred in the presence of MgEGTA compared to the EDTA control. This activation resulted in C3 deposition and required properdin to occur. Furthermore, we could show that MPO binds properdin directly which then serves as a focus for AP activation. In summary, we show that properdin can interact directly with neutrophil components. MPO demonstrates the ability to activate the AP which is dependent on properdin. Finally, MPO is capable of inducing properdin initiated C3 and C5b-9 deposition *in-vitro.*

**Introduction**

The complement system consists of three pathways, the classical, lectin and alternative pathway. The AP is composed of C3, factor B, factor D and properdin. The AP is unique in that it can become autoactivated, transforming C3 to C3H₂O (C3b like molecule). This activated C3 binds factor B which is then cleaved by factor D forming the C3bBb (AP C3 convertase) which generates more C3b. The C3bBb is stabilised by properdin, promoting further C3b generation (1). More recently, it was discovered that properdin has a pattern recognition capability allowing it to bind, and become initiator of the AP. Properdin is then capable of binding C3b followed by factor B and factor D generating the stabilised AP C3 convertase (2, 3). In short, properdin is the only known positive regulator in the complement system and can act as initiator of the AP. Properdin can recognise various foreign structures such as *Neisseria gonorrhoeae, Escherichia coli* as well as lipopolysaccharide (LPS)(4). Properdin can also bind altered self such as apoptotic and necrotic cells. Studies by Xu et al, demonstrated that properdin can bind necrotic splenocytes from both wild type and C3 deficient mice excluding any role of C3b in the binding of properdin (5). Furthermore, the pattern recognition ability of properdin can promote phagocytosis of apoptotic
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cells (4). This was illustrated by Kemper et al by demonstrating that properdin can bind apoptotic T lymphocytes which can then induce AP complement activation. Intriguingly, in this study the apoptotic T cells with properdin attached were phagocytosed in the absence of any complement activation (6). Properdin can also bind to non-apoptotic/necrotic cells such as the surface of viable stimulated neutrophils allowing for complement activation which demonstrates overlap between the AP and neutrophil mediated processes (7).

The AP has been implicated in many neutrophil mediated diseases such as rheumatoid arthritis, ischemia reperfusion injury and in particular anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis. With this in mind, exposure of various neutrophil constituents may allow for interaction with the alternative pathway of complement. Human neutrophils have demonstrated the capability to produce C3, while in mouse studies it has been demonstrated that neutrophils can produce factor B, both integral to the alternative pathway (8, 9). Moreover, neutrophils store the only positive regulator of complement, properdin in their secondary specific granules (10). In contrast, the majority of ANCA autoantigens including MPO are stored in the primary azurophilic granules of neutrophils. Activation of neutrophils followed by degranulation allows for an assortment of neutrophil enzymes to interact with complement factors. Various azurophil granule proteins and oxidants have demonstrated the capacity to activate complement (11, 12). MPO itself can activate human C5 to generate a haemolytic C5-9 (13). Interestingly, neutrophils activated with anti-MPO or anti-PR3 IgG released factors capable of activating the complement system (14). With this in mind, exposure of various neutrophil constituents may allow for interaction with the alternative pathway of complement. We investigated the ability of the AP including properdin to interact with these purified constituents in-vitro. We show that various neutrophil enzymes can interact with properdin. In addition, MPO demonstrates a clear ability to induce AP activation which is dependent upon properdin. Intriguingly, MPO illustrated an ability to anchor properdin providing a platform for AP C3 and C5b-9 deposition.

Materials and Method

Reagents
Myeloperoxidase was isolated from human neutrophils (15). Commercially available myeloperoxidase from Athens research (Georgia, USA) and Cedarlane (Ontario, Canada) was also tested. Azurocidin, lysozyme and cathepsin G isolated from human neutrophils were from Athens research. Native neutrophil elastase tested from AbD serotec (Oxford, UK). Human properdin was isolated from serum by anion exchange and size exclusion chromatography (5), or was

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obtained from commercial source (Quidel, California, USA). The molecular characteristics of properdin were investigated by SDS-PAGE (Fig 1). Properdin deficient serum was generated in house using zymosan depletion (16). C3 was isolated from human serum and treated with trypsin for the generation of C3b (17). The rabbit anti-human properdin DIG, mouse anti-human C3 DIG (RFK-22) are in house reagents, and the mouse anti human C5b-9 DIG (a kind gift from Dr. T.E. Mollnes, Bodo, Norway) (18). Mouse anti human factor P #2 was supplied by Quidel while the goat anti human factor P was from Complement Technology (Texas, USA). Goat anti-mouse immunoglobulins Horseradish peroxidase (HRP) and rabbit anti-goat immunoglobulins HRP were purchased from Dako (Heverlee, Belgium). Sheep Anti Digoxigenin (DIG) peroxidase was from Roche (Mannheim, Germany). For use in ELISA, all antibodies were diluted in PBS BSA (1%) Tween-20 (0.05%) (PTB).

Properdin binding to neutrophil components.
The neutrophil enzymes MPO, elastase, azurocidin, lysozyme, cathepsin G or bovine serum albumin (BSA) were diluted in phosphate buffered saline (PBS) and coated in a NUNC 96-well plate. The plate was blocked using 1% BSA/ PBS for a minimum of 30 minutes incubated at 37°C. Properdin was diluted in PTB and added to the wells for 1 hr. Followed by detection of properdin using rabbit anti-human properdin-DIG for 1 hr and sheep anti-DIG HRP for 1 hr with development using 3-3', 5, 5-tetramethylbenzidine (TMB). The plate is washed in PBS Tween-20 (0.05%) between each step. Data are expressed as optical density values at 450nm (OD 450nm). Furthermore properdin binding was demonstrated using mouse anti-human factor P #2 and goat anti-human factor P respectively. The primary antibodies were developed using corresponding goat anti-mouse HRP and rabbit anti-goat HRP followed by TMB.

Complement activation assays.
For the C3 activation assay, a variety of neutrophil constituents or BSA was coated on a 96-well plate using PBS. After coating, the plate was blocked using 1% BSA/PBS and incubated at 37°C for at least 30 minutes. The wells were exposed to normal human serum diluted in GVB++ MgEGTA (33.3mM) or EDTA (20mM) for 1 hr at 37°C. The plate was washed in PBS Tween-20 (0.05%) with mouse anti human C3-DIG (RFK-22) added after for 1 hr. Detection was completed using sheep anti-DIG HRP for 1 hr and TMB. The plate was washed in PBS Tween-20 (0.05%) between each step. The properdin deposition assays involving NHS were performed similar to above however detection was performed using rabbit anti-human properdin DIG and sheep anti-DIG HRP using TMB as chromogen. All TMB readings were
performed at 450nm.
To show that the alternative pathway activation is dependent upon properdin we performed the following experiment. MPO was coated at 2.5 µg/ml using PBS on a 96 well plate. A blocking step of 1% BSA/PBS was added for a minimum of 30 minutes at 37°C following coating. The wells were exposed to a fixed concentration of properdin deficient serum with or without properdin or just NHS in GVB++ MgEGTA (33.3mM) or EDTA (20mM) for 1 hr. The plate was washed in PBS Tween-20 (0.05%). Mouse anti-human C3 DIG was diluted in PTB and added to all wells for 1 hr followed by addition of sheep anti-DIG HRP in PTB for 1 hr. The enzymatic reaction was developed using TMB with OD values measured at 450nm. Studies testing the ability of properdin to initiate complement activation were performed on a 96 well plate coated with MPO and BSA respectively. All MPO and BSA coated wells were blocked using 1% BSA/PBS for at least 30 minutes at 37°C. Increasing concentrations of properdin diluted in PTB were added to the individual wells. The wells were washed using PBS Tween-20 (0.05%). A fixed concentration of properdin deficient human serum diluted in GVB−/- MgEGTA (33.3mM) or GVB−/- EDTA (20mM) was then added for 1 hr. The plate was washed using PBS Tween-20 (0.05%) followed by addition of mouse anti-human C3 DIG or mouse anti-human C5b-9 DIG, both were then detected using sheep anti-DIG HRP as mentioned earlier. The assay was developed using TMB and read at 450nm.

**Properdin-C3b assay**

Based on previous studies demonstrating MPO ability to activate complement we set out to determine the exact mechanism involved in the AP activation. We coated MPO or BSA using PBS onto a 96 well plate. The coated plate was incubated with 1% BSA/PBS as a blocking step for a minimum of 30 minutes at 37°C. Increasing concentrations of properdin was added to the individual wells for 1 hr. The wells were washed using PBS Tween-20 (0.05%) and a fixed concentration of human C3b (30 µg/ml) was added to all wells for 1 hr. The C3b binding was detected using mouse anti-human C3 DIG (RFK-22) and sheep anti-DIG HRP each incubated for 1 hr respectively. TMB was used as chromogen and the plate was read at 450nm.

**Results**

**Properdin interacts with various components of neutrophil granules**

It has been described that properdin may play a role in neutrophil mediated diseases such as ANCA vasculitis (4, 19). Properdin is present in secondary granules of neutrophils, and after degranulation can act as a pattern recognition
molecule (4, 19). Therefore we investigated the ability of properdin to interact with a selection of neutrophil components.

**Figure 1. Molecular characteristics of properdin.** Preparations of properdin were analyzed under non reducing conditions in a 10% SDS PAGE gel. Following electrophoresis, the gel was stained with coomassie brilliant blue and destained using methanol. The commercial preparation (lane 1) showed dimeric properdin, but no presence of aggregates, even when high concentrations are loaded (25µg). While in another preparation (lane 2, 1µg loaded), aggregates were formed following storage and this preparation was excluded from testing.

We used an ELISA based system and immobilised the following purified neutrophil factors: myeloperoxidase (MPO), elastase, azurocidin, lysozyme, proteinase 3 (PR3) and cathepsin-G (Fig 2). Incubation with a fixed concentration of purified human properdin (5µg/ml), resulted in a strong binding to MPO, elastase, azurocidin, lysozyme, proteinase 3 (PR3) and cathepsin-G (Fig 2). Incubation with a fixed concentration of purified human properdin (5µg/ml), resulted in a strong binding to MPO,

**Figure 2. The interaction between neutrophil components and the AP.** In panel (a), ELISA wells were coated with MPO, azurocidin, lysozyme, proteinase 3, cathepsin G all at 2 µg/ml respectively. Furthermore, elastase was coated at 8 µg/ml with 1% BSA coated as negative control. The coated plate was washed and 1% BSA/PBS was added as a blocking step for at least 30 minutes at 37°C. The wells were washed and exposed to 5µg/ml of properdin, followed by detection using rabbit anti human properdin. In panel (b) MPO, elastase, cathepsin G, proteinase 3 and lysozyme were coated at 2 µg/ml with 1% BSA coated as a control. Following coating, the plate was blocked using 1% BSA/PBS for a minimum of 30 minutes at 37°C. The wells were washed, with 5% NHS diluted in GVB ++ MgEGTA then added followed by detection using mouse anti human C3. The values in panel (b) were measured in triplicate and plotted as mean with standard deviation (SD).
intermediate binding lysozyme, cathepsin G, proteinase 3, elastase and a weak binding to azurocidin or BSA as a control protein (Fig 2a).

As properdin is an instrumental protein in the alternative pathway (AP) of complement, we addressed whether under similar conditions immobilised neutrophil enzymes are capable of activating the AP of complement. After addition of a fixed concentration of 5% normal human serum (NHS) in MgEGTA, to specifically allow only AP activation, complement activation was measured by the deposition of C3. MPO, and to a lesser extent also cathepsin G was capable of activating the AP of complement (Fig 2b). Cathepsin G-induced complement activation has been demonstrated previously (20).

**Properdin binding to MPO**

We further concentrated on the novel interaction between properdin and MPO. Although initial interaction of properdin with MPO was demonstrated with an in-house purified preparation (15), a similar binding was observed with two commercial preparations (Fig 3a).

![Figure 3. Properdin binding to MPO.](image)

Figure 3. Properdin binding to MPO. In panel (a) the various MPO preparations were coated to the ELISA wells at 0.5 µg/ml with 1% BSA as negative control. The coated plate was incubated at 37°C with 1% BSA/PBS for at least 30 minutes as a blocking step. Increasing concentrations of properdin were added to the wells followed by detection using rabbit anti human properdin. Panel (b) shows MPO coated at various concentrations or 1% BSA coated to the wells. All MPO or BSA coated wells had 1% BSA/PBS added

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Binding of purified human properdin was demonstrated using a polyclonal antibody against properdin. Increasing the concentration of properdin, showed dose-dependent binding to MPO, which was already clearly observed with 2.5 µg/ml of properdin, which is within the physiological range (Fig 3a). The binding of properdin was also dependent on the concentration of MPO immobilised on the plate when a fixed concentration of properdin was added (Fig 3b). Similar to panel 3a, a dose-dependent binding of properdin could be demonstrated with either a mouse monoclonal directed against properdin (Fig 3c), or with a goat anti-human properdin polyclonal (Fig 3d). In all cases only limited interaction was observed with the control protein BSA.

**MPO activates the alternative pathway of complement inducing C3 deposition.**

It has previously been reported that MPO can interact directly with the human complement component C5 allowing for the generation of a haemolytic C5b-9 complex (13). Therefore, we investigated the conditions required for MPO-mediated complement activation. MPO or BSA were exposed to 5% NHS diluted in either MgEGTA or EDTA conditions, followed by detection of bound C3. MPO incubated with NHS in MgEGTA demonstrated a strong C3 deposition compared to its respective EDTA and BSA controls (Fig 4a). Furthermore testing of a titration of NHS diluted in MgEGTA showed that the MPO-mediated C3 deposition is dose-dependent compared to the control protein (Fig 4b). This suggests that MPO-mediated complement C3 deposition is supported under AP conditions.
MPO mediated AP activation is dependent on properdin.

From earlier studies it has been reported that properdin is integral in promoting AP complement activation (2). To investigate whether properdin had a role in the MPO mediated C3 activation, we added 10% NHS in MgEGTA or EDTA to immobilised MPO and detected properdin binding. The findings demonstrate significant properdin deposition on MPO in the presence of MgEGTA, but with diminished signals observed with the EDTA conditions. These findings were reaffirmed by comparing NHS and properdin deficient serum under AP conditions (Fig 5a). The results illustrate a clear properdin binding in the NHS wells as compared to the properdin deficient serum, demonstrating the need for properdin for activation in this setting (Fig 5a). Similar results were obtained with recombinant mouse MPO using normal mouse serum and properdin deficient serum (data not shown).

Taking into account the ability of MPO to activate the AP and inducing both C3 and properdin deposition, we wanted to elucidate the role of properdin in this C3 activation. With this in mind, NHS or properdin deficient serum with/without properdin was added to MPO. Properdin deficient serum exhibited significantly less C3 bound as compared to its NHS control; however the C3 deposition can be restored with the addition of properdin to the deficient serum (Fig 5b). To investigate the role of properdin further, we added increasing concentrations of properdin to 5% properdin deficient serum (MgEGTA) which was then added to
MPO. The C3 deposited on the well is increased with the dose dependent addition of properdin to the deficient serum (Fig 5c). In short, these results indicate a significant role for properdin in MPO-mediated complement activation.

Figure 5. MPO induces AP properdin consumption. In panel (a) MPO was coated at 2.5 µg/ml to ELISA wells. Following coating, all MPO coated wells had 1% BSA/PBS added as a blocking step which incubated at 37°C for a minimum of 30 minutes. The wells were washed and exposed to increasing concentrations of NHS or properdin deficient serum diluted in GVB++ MgEGTA or EDTA followed by detection using the rabbit anti human properdin antibody. In panel (b) ELISA wells were coated with MPO at a concentration of 2.5 µg/ml. The wells were washed and incubated with 1% BSA/PBS as a blocking agent and incubated at 37°C for at least 30 minutes. A fixed concentration of 5% properdin deficient serum was added alone or with 5µg/ml of properdin diluted in GVB++ MgEGTA or EDTA. Also, 5% NHS was diluted in GVB++ MgEGTA or EDTA. Detection was performed using the monoclonal mouse anti human C3. In panel (c) MPO was coated at 2.5 µg/ml to ELISA wells. The wells were blocked using 1% BSA/PBS for a minimum of 30 minutes at 37°C. A fixed concentration of 5% properdin deficient serum was added alone or with increasing concentrations of properdin diluted in GVB++ MgEGTA. The C3 deposited was detected using mouse anti human C3. P = properdin; P- = properdin deficient.

Properdin initiates C3 deposition on MPO.
Recently published data have suggested that properdin may act as an initiator of the alternative pathway inducing C3 activation (3). Therefore, we coated MPO or BSA exposed it to increasing concentrations of properdin or buffer alone. Next, the plate was washed and properdin deficient human serum diluted in MgEGTA or EDTA was added to all wells, followed by C3 detection. The results showed
only background levels of C3 bound in BSA wells, with a slight increase in the MPO wells which did not receive properdin. MPO wells supplemented with properdin prior to the addition of properdin deficient serum, demonstrated a clear dose-dependent increase in C3 deposition as compared to MPO wells unexposed to properdin (Fig 6). Similar results were obtained with a different properdin batch (data not shown). In summary, the results indicate that properdin may act as an initiator of alternative pathway C3 deposition involving MPO.

Properdin mediated AP generation of C5b-9
Results described above demonstrate a role for properdin in generating AP activation up to the C3 level. Therefore, we investigated whether this AP activation would lead to further complement activation and generation of C5b-9. This was performed similar to our assay for properdin initiated C3 deposition on MPO, with detection performed using a mouse monoclonal antibody specific to human C5b-9.

These results show a clear C5b-9 deposition on MPO compared to BSA coated wells which had received 5 ug/ml of properdin followed by serum diluted in MgEGTA. The C5b-9 was generated in a complement dependent manner as illustrated by its inhibition using EDTA. Furthermore, the AP C5b-9 is generated in a dose dependent manner when increasing concentrations of properdin were added before addition of properdin deficient serum (Fig 7).
Properdin directed C3b deposition on MPO

Our results indicated a role for MPO in activating the AP of complement, with properdin remaining key for this activation. With a previous study demonstrating that MPO can induce complement activation (13), we examined the exact mechanism involved in our studies using purified components. MPO and BSA were coated followed by the addition of increasing concentrations of properdin. All wells were exposed to 30µg/ml of C3b followed by detection of C3 deposition. The results demonstrate a clear binding of C3b which is dependent on the presence of properdin for binding to MPO (Fig 8).

Properdin mediated AP generation of C5b-9.

Wells were coated with MPO at a concentration of 2.5 µg/ml or 1% BSA diluted in PBS. The coated plate had 1% BSA/PBS added as a blocking step which was incubated at 37°C for a minimum of 30 minutes. A dose response of properdin (5µg/ml) diluted in PTB was added to the wells followed by addition of 5% properdin deficient serum diluted in GVB/-/MgEGTA (33.3mM) or EDTA (20 mM). C5b-9 was detected using a monoclonal antibody (AE-11) specific to the neoepitope of the MAC complex.

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Discussion

Several studies have demonstrated a role for the alternative pathway in neutrophil-mediated diseases. Activated neutrophils and the release of soluble mediators have increasingly become the focus of research in this field. In particular in ANCA, TNF-stimulated neutrophils, as well as neutrophil extracellular traps (NETS), have been postulated as sources of immune activation in the disease (21, 22). Enzymes stored within the granules of neutrophils were shown to activate complement C5 with oxidants also illustrating similar capabilities (11-13). Intriguingly, ANCA activated neutrophils themselves release factors capable of activating the complement system (14). This led us to address the question whether neutrophil components may be involved in the alternative pathway activation, and by what possible mechanism it may become activated.

Initially, we focused on properdin and its interplay with neutrophil components, as it promotes AP activation. We found that properdin was capable of binding to MPO, elastase, cathepsin G and lysozyme but not azurocidin. With these results in mind, we wanted to assess the ability of these enzymes which bound properdin to activate alternative pathway C3 deposition. Interestingly, only MPO appeared capable of inducing significant C3 deposition. It is intriguing to see that not all properdin binding enzymes such as proteinase 3, elastase and lysozyme could activate complement, suggesting a mode of possibly regulating properdin. To ensure our results were reproducible we altered the MPO, properdin and antibody detection systems and attained similar results.

After demonstrating that MPO can mediate AP C3 deposition, we focused on the exact conditions and whether the process is complement dependent. Our results showed clear complement activation in MgEGTA but not in EDTA conditions indicating that activation is dependent on the AP of complement. In addition, we illustrated that during AP activation on MPO, properdin becomes deposited on the antigen in a dose dependent manner. To address the importance of properdin in this AP induced C3 deposition we tested the ability of properdin deficient serum to induce activation as compared to NHS. The results show that properdin-deficient serum lacked the ability to induce AP activation as compared to NHS. However, this activation could be restored by the addition of properdin to the properdin-deficient serum, and restoration of activation occurred in a dose-dependent manner. Properdin has two known distinct roles in the complement field. It can stabilise the AP C3 convertase and act as a pattern recognition molecule inducing AP activation (2, 4). We evaluated the ability of properdin to interact with MPO initially, followed by exposure to properdin deficient serum as a source of complement factors. Our results demonstrated that MPO was capable of inducing properdin mediated C3 and C5b-9 deposition, as compared to the
control. These results indicate a new role for MPO in activating the complement system. In addition, this data demonstrates that MPO and the other neutrophil granule components demonstrate a pleiotropic range of capabilities in regulating complement. It would be interesting to take into account the role of autoantibodies to these autoantigens, such as to whether it would enhance AP activation. Furthermore, MPO and other enzymes such as elastase are instrumental in the process of NETS which may possess capabilities to control AP activation. A recent study by Camous et al reinforced the link between neutrophils and the AP. Their interesting findings showed that normal activated neutrophils could bind properdin released during activation, and subsequently induced AP C3 convertase generation, indicating another link between the AP and the neutrophil (7). The diverse mechanism of AP activation involving the activated neutrophil, acting as a source of properdin and a platform for AP activation using enzymes such as MPO demonstrates the complex and varied interactions at work. Recent publications in the field of ANCA vasculitis, demonstrated the presence of AP components at the site of damage, as well as AP activation in the circulation of patients with active ANCA vasculitis (23, 24). These findings were reinforced by a mouse model of MPO ANCA vasculitis using various complement deficient mice, which demonstrated that the AP is involved in the pathogenesis of the condition (14). Our findings are all in-vitro but are comparable with data in-vivo (human and mice in ANCA vasculitis); however it will be important to test the principles in-vivo in the setting of neutrophil immune and autoimmune mediated diseases.

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References
