



Universiteit  
Leiden  
The Netherlands

## **Initial properdin binding contributes to alternative pathway activation at the surface of viable and necrotic cells**

Essen, M.F. van; Schlagwein, N.; Hoven, E.M.P. van den; Gijlswijk-Janssen, D.J. van; Lubbers, R.; Bos, R.M. van den; ... ; COMBAT Consortium

### **Citation**

Essen, M. F. van, Schlagwein, N., Hoven, E. M. P. van den, Gijlswijk-Janssen, D. J. van, Lubbers, R., Bos, R. M. van den, ... Kooten, C. van. (2023). Initial properdin binding contributes to alternative pathway activation at the surface of viable and necrotic cells. *European Journal Of Immunology*, 52(4), 597-608. doi:10.1002/eji.202149259

Version: Publisher's Version

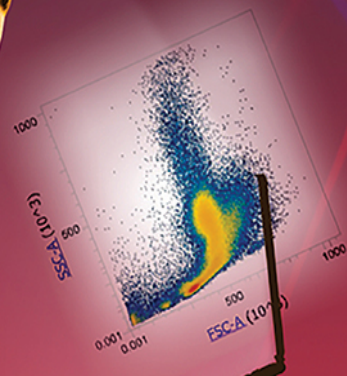
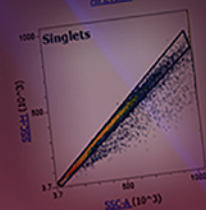
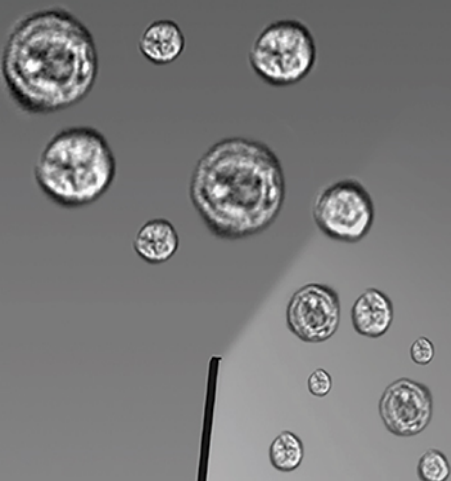
License: [Creative Commons CC BY-NC 4.0 license](https://creativecommons.org/licenses/by-nc/4.0/)

Downloaded from: <https://hdl.handle.net/1887/3515770>

**Note:** To cite this publication please use the final published version (if applicable).

invitrogen

Two data sets. One step. Zero doubt.



**Confidently confirm your cell profiles with a new flow cytometer that delivers flow cytometry and imaging data simultaneously.** Now, you can acquire dual data quickly and easily. The new Invitrogen™ Attune™ CytPix™ Flow Cytometer delivers both brightfield images and flow cytometry data sets simultaneously, so you can confirm cellular characteristics and sample quality confidently, without changing your protocols.



Enhance analysis and confidence at [thermofisher.com/cytpix](http://thermofisher.com/cytpix)

**ThermoFisher**  
SCIENTIFIC

For Research Use Only. Not for use in diagnostic procedures. © 2021 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. COL25211 0621

Initial properdin binding contributes to alternative pathway activation at the surface of viable and necrotic cells

Mieke F. van Essen<sup>\*</sup>, Nicole Schlagwein<sup>\*</sup>, Elisa M.P. van den Hoven<sup>\*</sup>, Daniëlle J. van Gijlswijk-Janssen<sup>\*</sup>, Rosalie Lubbers<sup>†</sup>, Ramon M. van den Bos<sup>‡</sup>, Jacob van den Born<sup>§</sup>, Jurjen M. Ruben<sup>\*1</sup>, Leendert A. Trouw<sup>†,¶</sup>, Cees van Kooten<sup>\*2</sup>, on behalf of the COMBAT consortium

<sup>\*</sup>Div. of Nephrology and Transplant Medicine, Dept. of Medicine, Leiden University Medical Center, Leiden, The Netherlands

<sup>†</sup>Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

<sup>‡</sup>Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, Utrecht, The Netherlands

<sup>§</sup>Department of Nephrology, University Medical Center Groningen, Groningen, The Netherlands

<sup>¶</sup>Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands.

Grant support: The COMBAT consortium is sponsored by the Dutch Kidney Foundation (grant 13OCA27).

<sup>1</sup>Present address: Amsterdam UMC, location VUmc, Cancer Center Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Received: 16/03/2021; Revised: 01/12/2021; Accepted: MONTH DD, YYY

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/eji.202149259](https://doi.org/10.1002/eji.202149259).

This article is protected by copyright. All rights reserved.

<sup>2</sup>Corresponding author at: Leiden University Medical Center, Div. of Nephrology and Transplant Medicine, Dept. of Medicine, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Tel: +31(0)71-5262148, email: kooten@lumc.nl

Email addresses: MvE: m.f.van\_essen@lumc.nl, NS: n.schlagwein@lumc.nl, EvdH: elisa.vandenhoven@gmail.com, DvG-J: D.J.van\_Gijlswijk-Janssen@lumc.nl, RL rosalie.lubbers@gmail.com, RvdB: r.m.vandenbos@uu.nl, JvdB: j.van.den.born@umcg.nl, JR: j.ruben@amsterdamumc.nl, LT: L.A.Trouw@lumc.nl, CvK: kooten@lumc.nl.

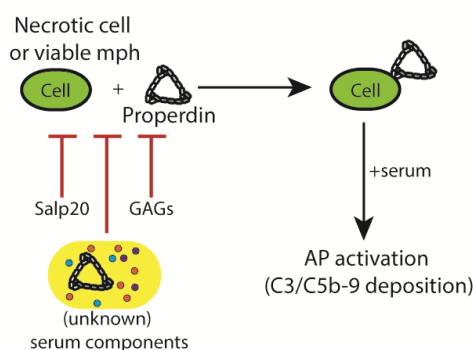
Keywords: Alternative pathway, Complement system, Dendritic cells, Macrophages, Properdin, Viable/death cells.

Abbreviations: AP, alternative pathway CP, classical pathway; LP, lectin pathway, NHS, normal human serum; P2, dimeric form of properdin; P3, trimeric form of properdin; P4, tetrameric form of properdin; PRM, pattern recognition molecule.

### Abstract

Properdin, the only known positive regulator of the complement system, stabilizes the C3 convertase, thereby increasing its half-life. In contrast to most other complement factors, properdin is mainly produced extrahepatically by myeloid cells. Recent data suggest a role for properdin as a pattern recognition molecule. Here we confirmed previous findings of properdin binding to different necrotic cells, including Jurkat T cells. Binding can occur independent of C3, as demonstrated by HAP-1 C3 knockout cells, excluding a role for endogenous C3. In view of the cellular source of properdin, interaction with myeloid cells was examined. Properdin bound to the surface of viable monocyte-derived pro- and anti-inflammatory macrophages, but not to dendritic cells. Binding was demonstrated for purified properdin as well as fractionated P2, P3 and P4 properdin oligomers. Binding contributed to local complement activation as determined by C3 and C5b-9 deposition on the cell surfaces and seems a prerequisite for alternative pathway activation. Interaction of properdin with cell surfaces could be inhibited with the tick protein Salp20 and by different polysaccharides, depending on sulfation and chain-length. These data identify properdin as a factor interacting with different cell surfaces, being either dead or alive, contributing to the local stimulation of complement activation.

Properdin binds to various necrotic cells as well as viable macrophages (mph), which seems a prerequisite for alternative pathway (AP) activation at the surface of these cells. Various factors, including the tick protein Salp20, (unknown) serum components and glycosaminoglycans (GAGs) are able to prevent properdin binding to these cell surfaces.



## Introduction

The complement system consists of three pathways, the classical (CP), lectin (LP) and alternative pathway (AP) and plays an important role in the defense against microorganisms and the removal of dead cells (reviewed in [1]). Properdin is the only known positive regulator of the AP, stabilizing the C3 convertase, increasing its half-life five to ten times [2]. Properdin appears in dimers, trimers and tetramers [3]. Recently, crystal structures of properdin provided new insights into the mechanism of C3 convertase stabilization [4, 5].

There is also evidence that properdin is able to interact with surfaces directly, thereby contributing to complement activation [6]. The exact mechanism of how properdin acts as a pattern recognition molecule (PRM) remains to be elucidated and is also still a matter of debate. Some investigations showed a need of initial C3b deposition prior to binding of properdin [7, 8]. Other studies have shown that properdin can interact directly with microbial surfaces, early and late apoptotic and necrotic cells, and proximal tubular epithelial cells (PTECs) [9–13]. Our group recently showed properdin deposition in the glomeruli of C3

knockout mice with induced acute anti-glomerular basement membrane disease [14], providing evidence of a C3b-independent binding of properdin. The binding of properdin to tubular epithelial cells was shown to be mediated by heparan sulfates expressed on the cell surface [15–17].

Properdin, in contrast to other complement factors which are produced by the liver, is mainly produced by myeloid cells. Neutrophils contain a pool of properdin, both dendritic cells and macrophages produce and secrete properdin [18–21]. In addition, myeloid cells are able to produce and secrete other complement factors, contributing to local complement activation [22]. In this study, we investigated the binding of properdin to different cell surfaces and not only confirmed binding to necrotic Jurkat T cells [11], but also observed binding to pro- and anti-inflammatory macrophages. Binding could be prevented by heparin, depending on the degree of sulfation, chain length and backbone composition of glycosaminoglycans (GAGs). Furthermore, Salp20, a tick protein from *Ixodes scapularis* [23, 24] blocked properdin binding. Altogether, our data suggests that the binding of properdin to the cell surfaces is a prerequisite for AP activation on the cell surface.

## Results

### *Properdin binds to the surface of necrotic but not viable Jurkat T cells*

To investigate the interaction of properdin with different cell surfaces, we incubated necrotic Jurkat T cells with properdin and used FACS analysis to show binding (Figure 1A and B, Suppl. Fig. 1A), confirming previous results [11]. Properdin binding was dose-dependent (Figure 1C), and was observed with properdin derived from various sources, including serum-purified and recombinant properdin (Suppl. Fig. 1B). To exclude the contribution of non-physiological aggregates, we fractionated properdin using size exclusion chromatography and peak-fractions were pooled as indicated (Figure 1D). Incubation of

viable Jurkat T cells with P2, P3 and P4 oligomers and unfractionated properdin did not result in binding, whereas binding was observed to necrotic Jurkat T cells (Figure 1E). The non-physiological P>4 form of properdin was able to bind to the surface of both viable and necrotic Jurkat T cells (Figure 1E). Binding capacity increased with increasing size, following a similar pattern as described before [25].

To exclude a contribution of endogenous C3 in facilitating properdin binding, we used C3 knockout HAP-1 cells, generated using CRISPR-Cas9. Properdin bound weakly to the surface of viable HAP-1 cells, and very strongly to necrotic HAP-1 cells. Binding of purified properdin to WT HAP-1, HAP-1 control and C3 knockout HAP-1 cells was comparable, indicating that properdin binding can occur independent from C3 (Figure 1F). Binding of properdin oligomers P2, P3 and P4 to C3 knockout HAP-1 cells was also observed, with higher oligomeric structures showing increased binding (Figure 1G).

#### *Properdin binding is a prerequisite for AP activation on necrotic cells*

To investigate complement activation at the surface of necrotic cells, we first verified complement activation in RPMI, the medium used in our cell cultures, in an ELISA system. In this system, all three pathways of complement could be efficiently activated in a dose-dependent manner (Suppl. Fig. 2). MgEGTA specifically inhibited the CP and LP, but AP activation was maintained. In contrast, 10mM EDTA completely prevented complement activation in all three pathways (Figure 2A and Suppl. Fig. 2).

Incubation of necrotic Jurkat T cells with 10% NHS resulted in complement activation, as measured by the deposition of C3 on the cell surface (Figure 2B). Addition of 10mM EDTA or MgEGTA completely prevented C3 deposition on necrotic cells, suggesting CP or LP activation in RPMI. Pre-incubation of necrotic Jurkat T cells with properdin, followed by NHS exposure, resulted in a strong increase of C3 deposition when compared to

NHS alone. In this case, C3 deposition was not inhibited by MgEGTA, thereby confirming complement activation through the AP (Figure 2B).

To determine subsequent steps of complement activation, we measured C5b-9 deposition on the surface of cells. Only minor C5b-9 deposition was found after exposure to NHS, but this was significantly increased upon pre-binding of properdin (Figure 2C). 10mM EDTA prevented C5b-9 deposition under all conditions. Again, in the presence of MgEGTA, pre-binding of properdin resulted in a strong C5b-9 deposition. Therefore, low level C3 and C5b-9 deposition on necrotic Jurkat T cells is mediated by the activation of the CP or LP. However, for AP activation the initial binding of properdin is a prerequisite, thereby resulting in high levels of C3 and C5b9. Also binding of purified oligomeric forms of properdin contributed to C3 deposition via the AP on the surface of necrotic Jurkat T cells, which was not the case for viable Jurkat T cells (Figure 2D). Similar results were observed for C3 deposition on the surface of C3 knockout HAP-1 cells (Figure 2E).

#### *Serum components and Salp20 prevent properdin binding, inhibiting AP activation*

Alternative pathway activation on the surface of necrotic Jurkat cells, as shown by C3 (Figure 2B) and C5b-9 (Figure 2C) deposition, was not observed when cells were exposed to NHS, despite properdin being present in this serum. Even with increasing amounts of NHS, up to 50% (containing >10  $\mu\text{g}/\text{mL}$  properdin), no binding was observed, whereas incubation with purified properdin did result in binding (Figure 3A). Even more, a dose-dependent inhibition of properdin binding to necrotic Jurkat T cells was observed when properdin was pre-incubated with different percentages of  $\Delta\text{NHS}$  (Figure 3B). Inhibition of properdin binding by these serum components also resulted in reduced complement activation on the necrotic cell surface, as determined by C3 deposition (Figure 3C). These results indicate that serum components are able to inhibit initial properdin binding to this cell surface.

This article is protected by copyright. All rights reserved.



To gain further insights into the mechanism of properdin binding to necrotic cells, we used the salivary tick protein Salp20. It has been shown that Salp20 interferes with properdin-C3b interaction and thereby inhibiting the activation of the AP [5, 23, 26], but it is unclear whether it interferes with the pattern-recognition function of properdin. Salp20 did not bind to the surface of necrotic Jurkat T cells directly, as demonstrated using fluorescently-labelled Salp20 (Figure 3D). Salp20 was able to dose-dependently inhibit properdin binding to the surface of necrotic Jurkat T cells when pre-incubated (Figure 3E and F). Furthermore, Salp20 prevented AP activation on necrotic Jurkat T cells, indicated by the absence of C3 deposition (Figure 3G).

#### *Properdin binds to viable pro- and anti-inflammatory macrophages*

Where most complement factors are produced by the liver, properdin is predominantly produced by myeloid cells like dendritic cells and macrophages [19, 20]. This raises the question whether locally produced properdin can contribute to local complement activation. Properdin was not able to interact with the myeloid cell lines MonoMac6 or U937, unless they were made necrotic (Figure 4A). Therefore, we generated monocyte-derived dendritic cells, pro-inflammatory macrophages and anti-inflammatory macrophages, and investigated the interaction of properdin with these subpopulations, distinguishing viable and dead cells (Suppl. Fig. 3 and Suppl. Fig. 4A and B). Properdin showed minimal binding to dendritic cells (Figure 4B, mean fold increase 1.3, varying from 0.9 to 1.7 fold). In contrast, a strong binding to the surface of viable pro-inflammatory macrophages (Figure 4C, mean fold increase 4.3, varying from 1.2 to 15.9 fold) was observed. In all donors tested, a more consistent binding of properdin to anti-inflammatory macrophages was observed (Figure 4D, mean fold increase 3.5, varying from 1.7 to 7.3 fold). In most cases, dendritic cells and pro- and anti-inflammatory macrophages were generated from the same monocyte-population, indicating that the difference in binding is not due to donor variation, but is a result from the differentiation process. The donor variation is also reflected by spread in basal gMFI levels in the absence of exogenous properdin. A

similar properdin binding was found upon gating on the small population of dead pro- and anti-inflammatory macrophages (Suppl. Fig. 4A-C).

Activation of the AP on the surface of pro-and anti-inflammatory macrophages, as measured by C3 and C5b-9 deposition, was only observed upon pre-binding of properdin and not with NHS alone (Figure 4E-F). Binding of properdin to both macrophage populations could also be demonstrated by the purified oligomers, again showing a more consistent binding to anti-inflammatory macrophages (Figure 4G). Similar results were observed in binding to dead pro-and anti-inflammatory macrophages (Suppl. Fig. 4D). In addition, binding of properdin oligomers contributed to some C3 deposition, especially onto the surface of anti-inflammatory macrophages (Figure 4H). These results show that AP activation can occur at the surface of pro- and anti-inflammatory macrophages, but this depends on initial properdin binding.

#### *Sulfation pattern and GAG-length are important for properdin binding to cell surfaces*

Binding of properdin to viable cells has also been demonstrated for renal proximal tubular epithelial cells, where heparan sulfate was identified as a ligand to which properdin binds [16, 17]. Therefore, we compared different polysaccharides to determine their inhibitory effect on the binding of properdin to different viable and dead cells. A strong and dose-dependent reduction of binding to necrotic Jurkat T cells as well as viable pro- and anti-inflammatory macrophages was observed when properdin was pre-incubated with unfractionated heparin (Figure 5A). The results of three independent experiments on necrotic Jurkat T cells, pro-inflammatory and anti-inflammatory macrophages, using 10  $\mu\text{g/mL}$  of both properdin and the specific polysaccharides, are indicated in a heatmap (Figure 5B, inhibition (blue), increased properdin binding (orange) compared to control (white)). Heparin, but not low-molecular weight (LMW) heparin, inhibited the binding of properdin to the surface of these cells (Figure 5B, no. 1 vs no. 2). This indicates the importance of the heparin chain length. Fucoidan (no. 8) and dextran sulfate (no. 9) inhibited properdin binding to all cells, whereas heparan sulfate from bovine kidney (no. 7), unsulfated dextran (no. 10), chondroitin sulfate C (no. 11) and chondroitin

This article is protected by copyright. All rights reserved.

sulfate B (no. 12) did not (Figure 5B). This indicates that high sulfation is more important than backbone structure for properdin binding to cell surfaces. This was confirmed using *O*-sulfated K5 (no. 5), showing a dose-dependent inhibition of properdin binding for all cell-types (Figure 5C). In contrast, unsulfated K5 (no. 6) did not affect properdin binding or in the case of necrotic Jurkat T cells even increased properdin binding (Figure 5C). These results show that binding of properdin to the surfaces of necrotic Jurkat T cells and both pro- and anti-inflammatory macrophages are dependent on GAG charge and length and less on backbone structure.

### Discussion

Properdin is the only known positive regulator of the complement system, able to interact with the AP C3 convertase, thereby stabilizing its half-life 5-10 times [2]. Recent investigations have shown a potential role for properdin as a pattern recognition molecule, thereby providing a platform for complement activation [9–13]. However, in other investigations, properdin was only indicated as a positive regulator of complement activation, completely depended on C3b deposition prior to properdin binding [7, 8]. Consistent with previous studies [11, 25], we demonstrated that properdin is able to bind to the surface of necrotic Jurkat T cells, but also to both viable and necrotic pro-and anti-inflammatory macrophages. It has been suggested that these interactions might be exclusive for non-physiological aggregates. However, we demonstrate that not only various forms of unfractionated properdin show this activity, but also that binding is observed with fractionated P2, P3 and P4 oligomeric properdin fractions. In addition, we show that properdin binding is a prerequisite for activation of the AP on the cell surfaces. Binding of properdin to the cell surfaces could be inhibited by the salivary tick protein Salp20 and by different polysaccharides, depending on the degree of sulfation and the length of the side chains.

The exact mechanism of how properdin acts as a PRM remains to be elucidated and is still a matter of debate. As discussed in the introduction, some investigations showed a need of initial C3b deposition prior to binding of properdin [7, 8]. In addition, it is known that, due to extensive freeze-thaw cycles, non-physiological forms (“Pn”/P>4) of properdin can be formed [27]. We confirmed the binding of P>4 to the surface of both viable and necrotic cells. In contrast, physiological forms were only observed to bind necrotic cells, as was also shown by [25]. We only observed binding of purified properdin to necrotic Jurkat T cells, but not to viable Jurkat T cells. In addition, binding of purified properdin was comparable to experiments in which a recombinant form of properdin was used. Fractionation of purified properdin by size exclusion chromatography resulted in a profile as described before [8]. These P2-P4 properdin oligomers were able to bind necrotic Jurkat T cells to a similar extent as described by [25], and binding was not observed to viable Jurkat T cells.

It has been proposed that initial C3b deposition is required, prior to binding of properdin [7, 8]. We have investigated the C3-dependence of properdin binding using C3 knockout HAP-1 cells. We have confirmed that properdin was able to bind to the surface of C3 deficient cells, to a similar extent as to WT and CRISPR-Cas9 control cells. Similar results were previously observed for properdin binding to necrotic C3<sup>-/-</sup> mice splenocytes [11] and recently, properdin binding to the bacterial surface of *Leptospira* was described to occur in the absence of C3b [28]. Furthermore, our group showed the deposition of properdin in the glomeruli of C3 knockout mice with induced acute anti-glomerular basement membrane disease, showing C3b-independent properdin binding [14]. In addition, it was recently described that P4 was able to bind to soluble collectin-12 opsonized bacteria *A. fumigatus* in a C3-independent manner [29]. Altogether, we conclude that properdin is able to interact with various cells or cell surfaces in the absence of C3.

Research in which properdin binding was shown to be C3b-dependent was performed in serum, and therefore properdin was surrounded by multiple serum components [7]. In contrast, direct properdin binding to zymosan or Chlamydia could be observed using purified properdin, but in both cases was reported to be inhibited by human serum [25, 30]. In line with previous results [25], we now confirm that human serum also dose-dependently inhibits the binding of purified properdin to necrotic cells and prevents subsequent alternative pathway activation (Figure 3 B-C). It has been suggested that pentraxin serum amyloid P (SAP) [31] and monomeric C-reactive protein (CRP) [32] might be partially responsible for this inhibition, but characterization of the inhibitory factor(s) needs further validation. Nevertheless, these results further underline the importance of myeloid cells as the source of properdin production. Local production of properdin in tissues and at sites of inflammation, in the absence of other serum components, will facilitate that properdin can act as a PRM.

An intriguing question is why such an important mechanism as the initiation of the alternative pathway of complement activation would depend on spontaneous hydrolysis of C3, combined with a random deposition. It has been demonstrated that properdin can interact with low affinity with the CTC domain present in intact C3/C3b (SPR experiments;  $K_D = 19 \pm 2 \mu\text{M}$ ) [5], which would be feasible in view of the high C3 concentrations in serum. Therefore, opsonization of different surfaces with properdin would provide a mechanism how the hydrolysis of C3 could preferentially occur in the close vicinity of these targeted surfaces. We recently confirmed that in a cell mixture, properdin selectively directs complement activation onto the cell surface to which properdin is bound. Upon exposure to NHS in the presence of MgEGTA, only allowing activation of the AP, C3 deposition was predominantly found on these properdin-opsonized cells (data not shown).

Properdin is able to bind to proximal tubular epithelial cells and it has been shown that heparan sulfates are involved in this interaction [12, 16, 17]. We also investigated the

properdin binding to viable dendritic cells and macrophages and observed a properdin binding to both pro- and anti-inflammatory macrophages (Figure 4), partially confirming data of properdin binding to anti-inflammatory macrophages [10]. Dendritic cells and macrophages express various proteo- and glycosaminoglycans at the cell surface [33, 34]. The less efficient properdin binding to dendritic cells, and the absence of interaction with the myeloid cell lines MonoMac6 and U937, might be explained by a distinct surface composition. However, further research is needed to identify the molecular differences. We investigated which proteoglycans were able to compete with the binding of properdin to the investigated cell surfaces. To reduce the risk of losing proteoglycan structures on these cell surfaces, cells were not harvested by trypsin digestion, instead a non-enzymatic solution was used. Reduced properdin binding to the cellular surface was observed with (unfractionated) heparin. The length of the heparin chain seems to be important, since low molecular weight heparin did not inhibit properdin binding to the cell surface. The degree of sulfate groups on GAGs seemed also important, since *O*-sulfated K5 was able to inhibit properdin binding, whereas unsulfated K5 was not. Furthermore, dextran sulfate is able to reduce properdin binding to the cell surface, whereas unsulfated dextran is not (Figure 5). These findings correspond with previous findings, highlighting the importance of certain GAGs in facilitating properdin binding [16]. The binding of properdin with GAGs is likely mediated through electrostatic interactions as properdin is highly positive and is known to interact with negatively charged ligands (e.g. GAGs and DNA) [11]. Factor H is also able to interact with the cell surface via proteoglycans, contributing to local regulation of complement activation [35, 36].

The binding of properdin to necrotic Jurkat T cells might affect phagocytosis. Binding of properdin enhances complement activation, thereby increasing local C3b levels. C3b opsonized cells can be detected by complement receptors expressed on phagocytes, resulting

in removal of the dead cells [37, 38]. We observed binding of properdin to the surface of both necrotic cells and macrophages. Properdin-bound necrotic cells could therefore interact with macrophages, suggesting a role in phagocytosis. Such a potential role of properdin in facilitating the bridging of cells was discussed recently, and would likely require higher oligomeric properdin structures like P3 and P4, allowing binding to both surfaces via unoccupied binding sites [39]. Alternatively, specific properdin receptors, like NKp46, could play a role in these processes. NKp46 is expressed on innate lymphoid cells like NK cells and is able to bind properdin [40]. It was shown that this interaction was required for the survival of mice infected with *Neisseria meningitidis* [40]. However, in the context of the current research its contribution would be less likely, since NKp46 receptor expression is not describe on macrophages.

Altogether, our findings show that purified properdin and P2, P3 and P4 oligomers are able to bind to the surface of necrotic Jurkat T cells and pro- and anti-inflammatory macrophages, whereas only the non-physiological form of properdin (P>4) is able to bind to the surface of viable Jurkat T cells. The binding of properdin contributes to local complement activation. This work provides a foundation for further research about the role of locally produced properdin and its binding to the surface of these cells.

## Materials and methods

### *Cell culture*

Necrotic Jurkat T cells were generated as described previously (Suppl. Fig. 1) [11]. Monocytes were isolated and dendritic cells were generated as described before [41]. Pro- and anti-inflammatory macrophages were generated by supplementing monocyte-cultures with 10 ng/mL granulocyte-macrophages colony-stimulating factor (GM-CSF; premium grade, 130-093-868, Miltenyi Biotec) or 100 ng/mL macrophage colony-stimulating factor

(M-CSF; premium grade, 130-096-493, Miltenyi Biotec), respectively. Analysis of differentiation is shown in the supporting information (Suppl. Fig. 3).

#### *Protein production*

Recombinant properdin and Salp20 were generated as described previously [5, 26].

#### *Fractionation of properdin*

Purified properdin (~400 $\mu$ g, concentration 1 mg/ml, A412, lot 144323 and 181138, Quidel), or a 3x concentrated sample, was loaded on a Superdex 200 Increase 10/300 (GE Healthcare) pre-equilibrated using PBS. Fractions were eluted with PBS at 0.5 or 0.25 mL/min and collected in 250 $\mu$ L fractions. Fractions were pooled and properdin concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated (mouse monoclonal anti-human properdin, 0.3  $\mu$ g/mL, A233, Quidel, San Diego, CA, USA), blocked (PBS-1% bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA)) and pooled fractions were measured in several dilutions (diluted in PBS containing 0.05% Tween 20 (Sigma-Aldrich)-1%BSA). Next, plates were washed with wash buffer (PBS containing 0.05% Tween 20) and incubated with rabbit anti human-properdin-digoxigenin (DIG; in-house, 1:2500) for 1hr at 37°C. After washing, plates were incubated with peroxidase-conjugated IgG fraction monoclonal mouse anti-DIG (0.1  $\mu$ g/mL, 200-032-156, Jackson ImmunoResearch, Cambridgeshire, UK) for 1h at 37°C and developed using 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich). The reaction was stopped using 1M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 450nm (iMark Microplate Reader, Bio-Rad, Hercules, CA, USA).

#### *Properdin binding to cells*



Purified properdin, either from Quidel (A412, San Diego, CA, USA) or CompTech (A139, Tyler, TX, USA) was aliquoted and kept frozen (-80°C). Recombinant produced properdin was stored at 4°C. 10 µg/mL properdin (Quidel; or otherwise indicated at the figure legends), was diluted in RPMI-1640 medium (no phenol red), followed by incubation with the cells for 1h at 4°C. Cells were washed and incubated with mouse-monoclonal anti-human properdin (2 µg/mL, A233, Quidel) for 30 minutes at 4°C, followed by incubation with goat anti-mouse immunoglobulins/RPE, goat F(ab')<sub>2</sub>, RPE (5 µg/mL, R0480, DAKO, Santa Clara, CA, USA) for 30 minutes at 4°C. Binding of oligomeric properdin structures P2, P3 and P4 was examined following a similar protocol. Binding was assessed using flow cytometry (LSR-II, BD).

#### *Activation of classical (CP), lectin (LP) and alternative pathway (AP)*

CP, LP and AP activity in normal human serum (NHS) was determined by ELISA (Suppl. Fig. 2).

#### *C3 deposition on cell surfaces*

Properdin binding, using either purified or oligomeric forms of properdin (Quidel), was performed as described above. Cells were washed and incubated (30 minutes, 37°C) with 10% NHS either diluted in RPMI, RPMI-MgEGTA (RPMI-1640 containing 10mM EGTA and 5mM MgCl<sub>2</sub>) or in RPMI-EDTA (RPMI-1640 containing 10mM EDTA). Cells were washed and incubated (30 minutes, 4°C) with mouse-anti human C3 (1/600, RFK22, in-house generated), followed by incubation with goat anti mouse – PE(F(ab')<sub>2</sub> goat anti-mouse-IgG-RPE) (5 µg/mL, R0480, DAKO) diluted in RPMI (no phenol red, 30 minutes at 4°C). C3 deposition was determined by flow cytometry (LSR-II, BD).

### *C5b-9 deposition on cell surfaces*

Cells were blocked with pooled heat-inactivated NHS ( $\Delta$ NHS, 10%) for 15 minutes at room temperature. Next, cells were washed and incubated with properdin (Quidel). Cells were washed and incubated with 10% NHS diluted in the described complement buffers (30 minutes, 37°C). Cells were incubated with mouse monoclonal anti human C5b-9 (1  $\mu$ g/mL, AE11, Hycult Biotech; 30 minutes, 4°C) followed by incubation with goat anti mouse – PE(F(ab')<sub>2</sub> goat anti-mouse-IgG-RPE) (5  $\mu$ g/mL, R0480, DAKO), diluted in RPMI (no phenol red, 30 minutes at 4°C). Deposition was determined by flow cytometry.

### *Serum as inhibitor*

Properdin binding from NHS was examined by incubating necrotic Jurkat T cells with different percentages of NHS. Binding was examined as described above. In addition, properdin (10  $\mu$ g/mL, Quidel) was incubated with various percentages of  $\Delta$ NHS (2-fold dilutions starting from 50%) for 30 minutes at 4°C. Next, the mixture was added to necrotic Jurkat T cells and incubated for 1hr at 4°C. Cells were washed and properdin binding was determined as described above. For C3 deposition, cells were washed with RPMI-MgEGTA and incubated with 10% properdin-depleted serum (A339, Comptech) for 30 minutes at 4°C. Cells were washed and C3 deposition was determined by flow cytometry as described above.

### *Salp20 and properdin*

10  $\mu$ g/mL properdin (Quidel) was pre-incubated with various concentrations of Salp20 (30 minutes, 4°C). Next, cells were incubated with the mixture (1hr, 4°C). Properdin binding was analysed by flow cytometry as described above. A FITC labelled form of Salp20 was used to investigate the direct interaction of Salp20 with the cell surface.

The effect of Salp20 on properdin binding and AP activation was examined. Properdin and Salp20 (1000 nM) were pre-incubated and the mixture was added to necrotic Jurkat T cells. Cells were washed and incubated with 10% NHS diluted in RPMI-MgEGTA (30 minutes, 37°C). C3 deposition was determined as described above.

#### *Heparinoids and properdin*

10 µg/mL purified properdin was pre-incubated with various concentrations of unfractionated heparin (LEO Pharma, Ballerup, Denmark), with either 0.1, 1 or 10 µg/mL *O*-sulfated and unsulfated K5 or with 10 µg/mL of polysaccharide derivatives: heparin, low molecular weight (LMW) heparin, *O*-sulfated and unsulfated K5, fucoidan, dextran sulfate, unsulfated dextran T40, N-desulfated/reacetylated heparin, periodate oxidized/reduced heparin, chondroitin sulfate C, chondroitin sulfate B and heparin sulfate from bovine kidney [17]. Mixtures were added to the cells (1hr, 4°C). Properdin binding was detected by flow cytometry as described above.

#### *Flow cytometric analysis*

Acquired flow cytometric data was analyzed using FlowJo Software version 10.6.1 (Tree Star, Ashland, OR, USA). Cells were gated based on FSC-A vs SSC-A, followed by selection of single cells using FSC-A vs FSC-H, and examined for properdin-binding (Suppl. Fig. 1, Suppl. Fig. 4A-B) or complement deposition, respecting the guidelines as described by [42].

#### *Statistical analysis*

Statistical analysis was performed by Wilcoxon matched-pairs signed rank test. Analysis between multiple groups was performed using a nonparametric one-way ANOVA (Friedman test with Dunn's multiple comparisons test). Significance was defined as  $P \leq 0.05$ . For

statistical analysis and graphical representations GraphPad Prism v.9.0.1 was used (San Diego, CA, USA).

#### Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### Conflict of interest disclosure

None.

#### Author contributions

MvE and CvK designed the study. MvE, NS, DvG-J and EvdH performed experiments, collected and analyzed data. RvdB and JvdB generated and provided critical reagents, RL and LT generated and provided CRISPR-Cas9 modified HAP-1 cells. JR and CvK supervised the project. MvE and CvK wrote the manuscript, which was reviewed and approved by all authors.

#### Acknowledgments

We thank Prof. R.C. Hoeben for his help in the generation of C3-deficient HAP-1 cells. The HAP-1 cell line used for the CRISPR-Cas9 experiments was a kind gift from R. Spaapen, Sanquin Research, Amsterdam, The Netherlands. The COMBAT consortium is sponsored by the Dutch Kidney Foundation (grant 13OCA27). The COMBAT consortium is an inter-university collaboration in the Netherlands that is formed to study basic mechanisms, assay development and therapeutic translation of complement-mediated renal diseases. Principal investigators are (in alphabetical order): S. Berger (Department of Internal Medicine-

Nephrology, University Medical Center Groningen, The Netherlands), J. van den Born (Department of Internal Medicine-Nephrology, University Medical Center Groningen, The Netherlands), P. Gros (Department of Chemistry, Utrecht University, The Netherlands), L. van den Heuvel (Department of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands), N. van de Kar (Department of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands), C. van Kooten (Department of Internal Medicine-Nephrology, Leiden University Medical Center, The Netherlands), M. Seelen (Department of Internal Medicine-Nephrology, University Medical Center Groningen, The Netherlands), A. de Vries (Department of Internal Medicine-Nephrology, Leiden University Medical Center, The Netherlands).

#### References:

1. **Walport, M. J.** Complement. First of two parts. *N. Engl. J. Med.* 2001. **344**: 1058–1066.
2. **Fearon, D. T., and K. F. Austen.** Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J. Exp. Med.* 1975. **142**: 856–863.
3. **Smith, C. A., M. K. Pangburn, C. W. Vogel, and H. J. Müller-Eberhard.** Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. *J. Biol. Chem.* 1984. **259**: 4582–4588.
4. **Pedersen, D. V., T. A. F. Gadeberg, C. Thomas, Y. Wang, N. Joram, R. K. Jensen, S. M. M. Mazarakis, et al.** Structural Basis for Properdin Oligomerization and Convertase Stimulation in the Human Complement System. *Front. Immunol.* 2019. **10**: 2007.
5. **van den Bos, R. M., N. M. Pearce, J. Granneman, T. H. C. Brondijk, and P. Gros.** Insights Into Enhanced Complement Activation by Structures of Properdin and Its Complex With the C-Terminal Domain of C3b. *Front. Immunol.* 2019. **10**: 2097.
6. **Kemper, C., J. P. Atkinson, and D. E. Hourcade.** Properdin: Emerging Roles of a Pattern-Recognition Molecule. *Annu. Rev. Immunol.* 2010. **28**: 131–155.
7. **Harboe, M., P. Garred, J. K. Lindstad, A. Pharo, F. Müller, G. L. Stahl, J. D. Lambris, and T. E. Mollnes.** The role of properdin in zymosan- and *Escherichia coli*-induced complement activation. *J Immunol.* 2012. **189**: 2606–2613.
8. **Harboe, M., C. Johnson, S. Nymo, K. Ekholm, C. Schjalm, J. K. Lindstad, A. Pharo, et al.** Properdin binding to complement activating surfaces depends on initial C3b deposition. *Proc.*

*Natl. Acad. Sci. USA* 2017. **114**: E534–E539.

9. **Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade.** Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J. Immunol.* 2007. **179**: 2600–2608.
10. **Kemper, C., L. M. Mitchell, L. Zhang, and D. E. Hourcade.** The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. *Proc. Natl. Acad. Sci. USA.* 2008. **105**: 9023–9028.
11. **Xu, W., S. P. Berger, L. A. Trouw, H. C. de Boer, N. Schlagwein, C. Mutsaers, M. R. Daha, and C. van Kooten.** Properdin Binds to Late Apoptotic and Necrotic Cells Independently of C3b and Regulates Alternative Pathway Complement Activation. *J. Immunol.* 2008. **180**: 7613–7621.
12. **Gaarkeuken, H., M. A. Siezenga, K. Zuidwijk, C. van Kooten, T. J. Rabelink, M. R. Daha, and S. P. Berger.** Complement activation by tubular cells is mediated by properdin binding. *Am. J. Physiol. Renal Physiol.* 2008. **295**: F1397-F1403.
13. **Kemper, C., and D. E. Hourcade.** Properdin: New roles in pattern recognition and target clearance. *Mol. Immunol.* 2008. **45**: 4048–4056.
14. **O’Flynn, J., J. Kotimaa, R. Faber-Krol, K. Koekkoek, N. Klar-Mohamad, A. Koudijs, W. J. Schwaeble, et al.** Properdin binds independent of complement activation in an in vivo model of anti-glomerular basement membrane disease. *Kidney Int.* 2018. **94**: 1141–1150.
15. **Zaferani, A., R. R. Vivès, P. van der Pol, G. J. Navis, M. R. Daha, C. van Kooten, H. Lortat-Jacob, et al.** Factor H and properdin recognize different epitopes on renal tubular epithelial heparan sulfate. *J. Biol. Chem.* 2012. **287**: 31471–31481.
16. **Zaferani, A., R. R. Vivès, P. van der Pol, J. J. Hakvoort, G. J. Navis, H. Van Goor, M. R. Daha, et al.** Identification of tubular heparan sulfate as a docking platform for the alternative complement component properdin in proteinuric renal disease. *J. Biol. Chem.* 2011. **286**: 5359–5367.
17. **Lammerts, R. G. M., D. T. Talsma, W. A. Dam, M. R. Daha, M. A. J. Seelen, S. P. Berger, and J. van den Born.** Properdin Pattern Recognition on Proximal Tubular Cells Is Heparan Sulfate/Syndecan-1 but Not C3b Dependent and Can Be Blocked by Tick Protein Salp20. *Front. Immunol.* 2020. **11**: 1643.
18. **Wirthmueller, U., B. Dewald, M. Thelen, M. K. Schäfer, C. Stover, K. Whaley, J. North, et al.** Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils. *J. Immunol.* 1997. **158**: 4444–4451.
19. **Dixon, K. O., J. O’Flynn, N. Klar-Mohamad, M. R. Daha, and C. van Kooten.** Properdin and factor H production by human dendritic cells modulates their T-cell stimulatory capacity and is regulated by IFN- $\gamma$ . *Eur. J. Immunol.* 2017. **47**: 470–480.
20. **Cortes, C., J. A. Ohtola, G. Saggi, and V. P. Ferreira.** Local release of properdin in the cellular microenvironment: Role in pattern recognition and amplification of the alternative pathway of

- complement. *Front. Immunol.* 2013. **3**: 412.
21. **Li, K., H. Fazekasova, N. Wang, P. Sagoo, Q. Peng, W. Khamri, C. Gomes, et al.** Expression of complement components, receptors and regulators by human dendritic cells. *Mol. Immunol.* 2011. **48**: 1121–1127.
  22. **Lubbers, R., M. F. van Essen, C. van Kooten, and L. A. Trouw.** Production of complement components by cells of the immune system. *Clin. Exp. Immunol.* 2017. **188**: 183–194.
  23. **Tyson, K. R., C. Elkins, and A. M. de Silva.** A Novel Mechanism of Complement Inhibition Unmasked by a Tick Salivary Protein That Binds to Properdin. *J Immunol* 2008. **180**: 3964–3968.
  24. **Hourcade, D. E., A. M. Akk, L. M. Mitchell, H.-F. Zhou, R. Hauhart, and C. T. N. Pham.** Anti-complement activity of the Ixodes scapularis salivary protein Salp20. *Mol. Immunol.* 2016. **69**: 62–69.
  25. **Ferreira, V. P., C. Cortes, and M. K. Pangburn.** Native polymeric forms of properdin selectively bind to targets and promote activation of the alternative pathway of complement. *Immunobiology* 2010. **215**: 932–940.
  26. **Michels, M. A. H. M., N. C. A. J. van de Kar, R. M. van den Bos, T. J. A. M. van der Velden, S. A. W. van Kraaij, S. A. Sarlea, V. Gracchi, et al.** Novel Assays to Distinguish Between Properdin-Dependent and Properdin-Independent C3 Nephritic Factors Provide Insight Into Properdin-Inhibiting Therapy. *Front. Immunol.* 2019. **10**: 1350.
  27. **Farries, T. C., J. T. Finch, P. J. Lachmann, and R. A. Harrison.** Resolution and analysis of “native” and “activated” properdin. *Biochem. J.* 1987. **243**: 507–517.
  28. **Martinez, A. P. G., P. A. E. Abreu, S. de Arruda Vasconcellos, P. L. Ho, V. P. Ferreira, G. Saggi, A. S. Barbosa, and L. Isaac.** The Role of Properdin in Killing of Non-Pathogenic *Leptospira biflexa*. *Front. Immunol.* 2020. **11**: 572562.
  29. **Zhang, J., L. Song, D. V. Pedersen, A. Li, J. D. Lambris, G. R. Andersen, T. E. Mollnes, et al.** Soluble collectin-12 mediates C3-independent docking of properdin that activates the alternative pathway of complement. *Elife* 2020. **9**: e60908.
  30. **Cortes, C., V. P. Ferreira, and M. K. Pangburn.** Native properdin binds to *Chlamydia pneumoniae* and promotes complement activation. *Infect. Immun.* 2011. **79**: 724–731.
  31. **Mitchell, L., and D. Hourcade.** Inhibition of properdin-directed complement activation by serum amyloid P component. *Mol. Immunol.* 2008. **45**: 4103–4104.
  32. **O Flynn, J., P. van der Pol, K. O. Dixon, Z. Prohászka, M. R. Daha, and C. van Kooten.** Monomeric C-reactive protein inhibits renal cell-directed complement activation mediated by properdin. *Am. J. Physiol. Renal Physiol.* 2016. **310**: F1308–F1316.
  33. **Wegrowski, Y., A.-L. Milard, G. Kotlarz, E. Toulmonde, F.-X. Maquart, and J. Bernard.** Cell surface proteoglycan expression during maturation of human monocytes-derived dendritic cells and macrophages. *Clin. Exp. Immunol.* 2006. **144**: 485–493.

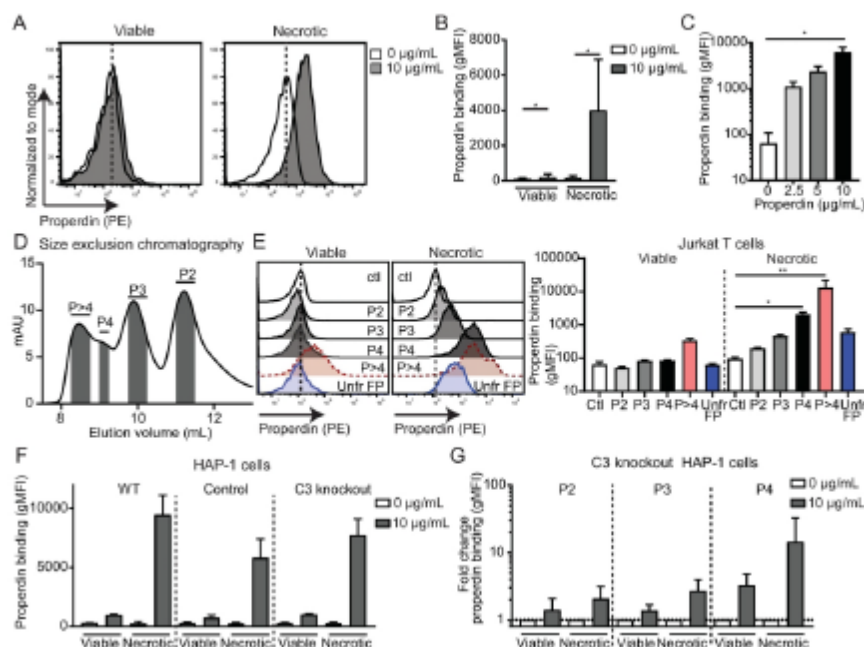
34. **Hatano, S., and H. Watanabe.** Regulation of Macrophage and Dendritic Cell Function by Chondroitin Sulfate in Innate to Antigen-Specific Adaptive Immunity. *Front. Immunol.* 2020. **11**: 232.
35. **Józsi, M., and P. F. Zipfel.** Factor H family proteins and human diseases. *Trends Immunol.* 2008. **29**: 380–387.
36. **Langford-Smith, A., A. J. Day, P. N. Bishop, and S. J. Clark.** Complementing the sugar code: Role of GAGs and sialic acid in complement regulation. *Front. Immunol.* 2015. **6**: 25.
37. **Martin, M., and A. M. Blom.** Complement in removal of the dead – balancing inflammation. *Immunol. Rev.* 2016. **274**: 218–232.
38. **Westman, J., S. Grinstein, and P. E. Marques.** Phagocytosis of Necrotic Debris at Sites of Injury and Inflammation. *Front. Immunol.* 2020. **10**: 3030.
39. **Pedersen, D. V., M. N. Pedersen, S. M. M. Mazarakis, Y. Wang, K. Lindorff-Larsen, L. Arleth, and G. R. Andersen.** Properdin oligomers adopt rigid extended conformations supporting function. *Elife* 2021. **10**: e63356.
40. **Narni-Mancinelli, E., L. Gauthier, M. Baratin, S. Guia, A. Fenis, A. E. Deghmane, B. Rossi, et al.** Complement factor P is a ligand for the natural killer cell-activating receptor NKp46. *Sci. Immunol.* 2017. **2**: eaam9628.
41. **van Essen, M. F., N. Schlagwein, D. J. van Gijlswijk-Janssen, J. D. H. Anholts, M. Eikmans, J. M. Ruben, and C. van Kooten.** Culture medium used during small interfering RNA (siRNA) transfection determines the maturation status of dendritic cells. *J. Immunol. Methods* 2020. **479**: 112748.
42. **Cossarizza, A., H. D. Chang, A. Radbruch, A. Acs, D. Adam, S. Adam-Klages, W. W. Agace, et al.** Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur. J. Immunol.* 2021. **51**: 1457–1973.

Figure legends:

**Figure 1: C3-independent properdin binding to necrotic cell surfaces.** (A) Representative histograms of the analysis of properdin binding to viable and necrotic Jurkat T cells by flow cytometry after incubation with 10  $\mu\text{g}/\text{mL}$  properdin (Quidel). (B) Quantification of properdin (10  $\mu\text{g}/\text{mL}$ , Quidel) binding to both viable and necrotic Jurkat T cells. Data are presented as mean $\pm$ SD of 6 independent experiments. (C) Quantification of properdin binding (0, 2.5, 5 or 10  $\mu\text{g}/\text{mL}$ ; Quidel) to necrotic Jurkat T cells. Data are presented as mean $\pm$ SD of 3 independent experiments. (D) Separation of purified properdin by size exclusion chromatography (SEC) examined in three independent experiments. Fractions were pooled as indicated by the shading in the SEC-profile. Properdin concentration of the pooled

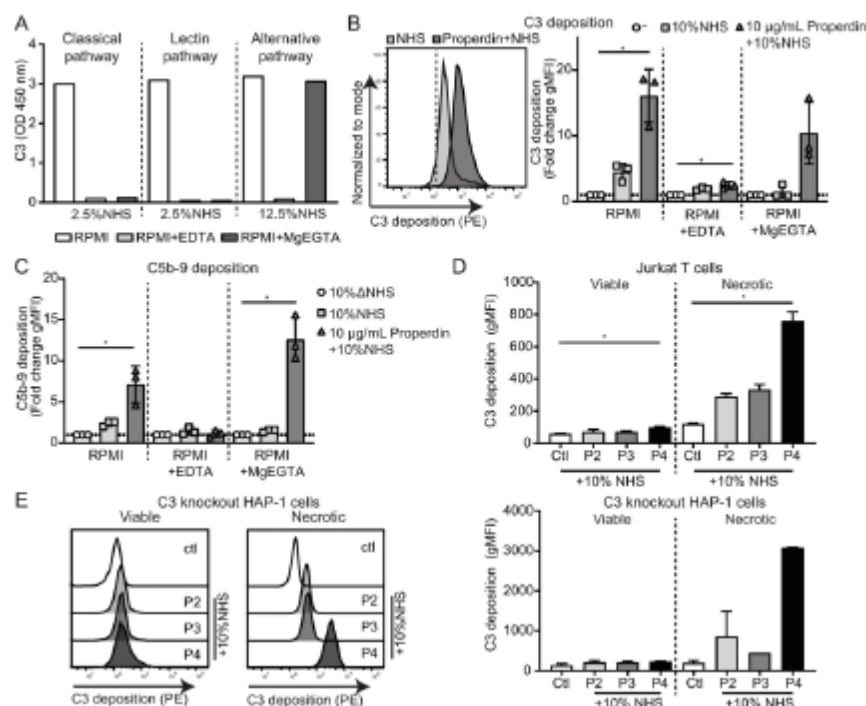


fractions were determined by ELISA (P2: 39.3  $\mu\text{g/mL}$ , P3: 41.9  $\mu\text{g/mL}$ , P4: 25.8  $\mu\text{g/mL}$ , P>4: 14.4  $\mu\text{g/mL}$ ). (E) Representative histograms of the binding of 10  $\mu\text{g/mL}$  P2, P3, P4, P>4 and unfractionated properdin (unfr FP) to viable and necrotic Jurkat T cells. Quantification of the data are presented as mean $\pm$ SD of the fractions tested on 3 different viable and necrotic Jurkat T cells batches. The fractions used are a representative for four individual experiments, all showing the interaction of P2, P3 and P4 with necrotic Jurkat T cells. Ctl represent the control conditions of cells incubated with primary and secondary antibodies in the absence of properdin. (F) Binding of properdin (10  $\mu\text{g/mL}$ ; Quidel) to the surface of viable and necrotic wild type HAP-1 cells (WT), CRISPR-Cas9 control cells (control) and a CRISPR-Cas9 derived C3 knockout (C3 knockout). Data are presented as mean $\pm$ SD of 3 independent experiments (WT necrotic: mean $\pm$ SD of 2 independent experiments). (G) Binding of 10  $\mu\text{g/mL}$  P2, P3 and P4 properdin oligomers, obtained from 3 independent SEC experiments, to viable and necrotic C3 knockout HAP-1 cells. Data are presented as mean  $\pm$ SD of 3 independent experiments (for necrotic C3 knockout HAP-1 cells) or 4 independent experiments (for viable C3 knockout HAP-1 cells). One way ANOVA-Friedman test and Dunn's multiple comparison test, \* $P\leq.05$ , \*\* $P\leq.01$ . Wilcoxon matched-pairs signed rank test, paired, \* $P\leq.05$ .



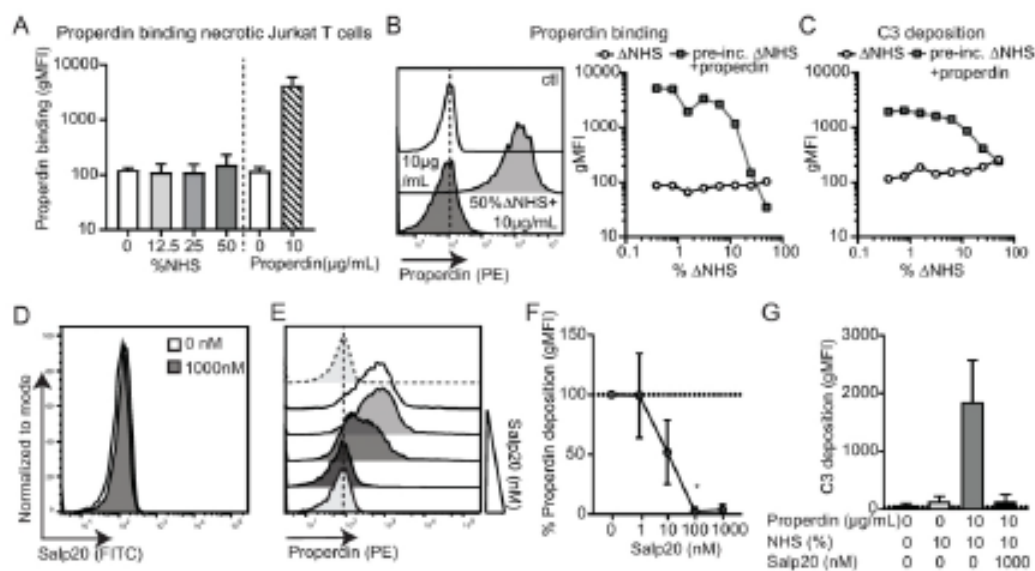
**Figure 2: Binding of properdin increases C3 and C5b-9 deposition on necrotic Jurkat T cells.** (A) Activation of the complement pathways in RPMI-1640 medium was determined by ELISA. Representative of 2 independent experiments. (B) C3 deposition on necrotic Jurkat T cells. This article is protected by copyright. All rights reserved. 23

cells in the absence (light grey histograms and bars) or presence (dark grey histograms and bars) of properdin (10  $\mu\text{g}/\text{mL}$ , Quidel). Dotted lines in histogram represent the incubation of cells with primary and secondary antibody only. (C) C5b-9 deposition on the surface of necrotic Jurkat T cells after exposure to 10% NHS in the absence (light grey bars) or presence of pre-binding of properdin (10  $\mu\text{g}/\text{mL}$ , Quidel; dark grey bars). gMFI values were used to calculate the fold change compared to non-exposed (for C3) or 10%  $\Delta\text{NHS}$  exposed (for C5b-9) necrotic Jurkat T cells. Data are presented as the mean  $\pm\text{SD}$  of 3 independent experiments. (D) AP activation on viable and necrotic Jurkat T cells in the absence or presence of 10  $\mu\text{g}/\text{mL}$  P2, P3 or P4, determined by C3 deposition. Data are presented as mean  $\pm\text{SD}$  of 3 independent experiments using P2-P4 fractions from one SEC fractionation. (E) AP activation on viable and necrotic C3 knockout HAP-1 cells in the absence or presence of 10  $\mu\text{g}/\text{mL}$  P2, P3 or P4, determined by C3 deposition. Data are presented as mean  $\pm\text{SD}$  of 2 independent experiments (P3 necrotic C3 knockout HAP-1: n=1) using P2-P4 fractions from two different SEC fractionations. One-Way ANOVA-Friedman test and Dunn's multiple comparison test, \* $P\leq 0.05$ .



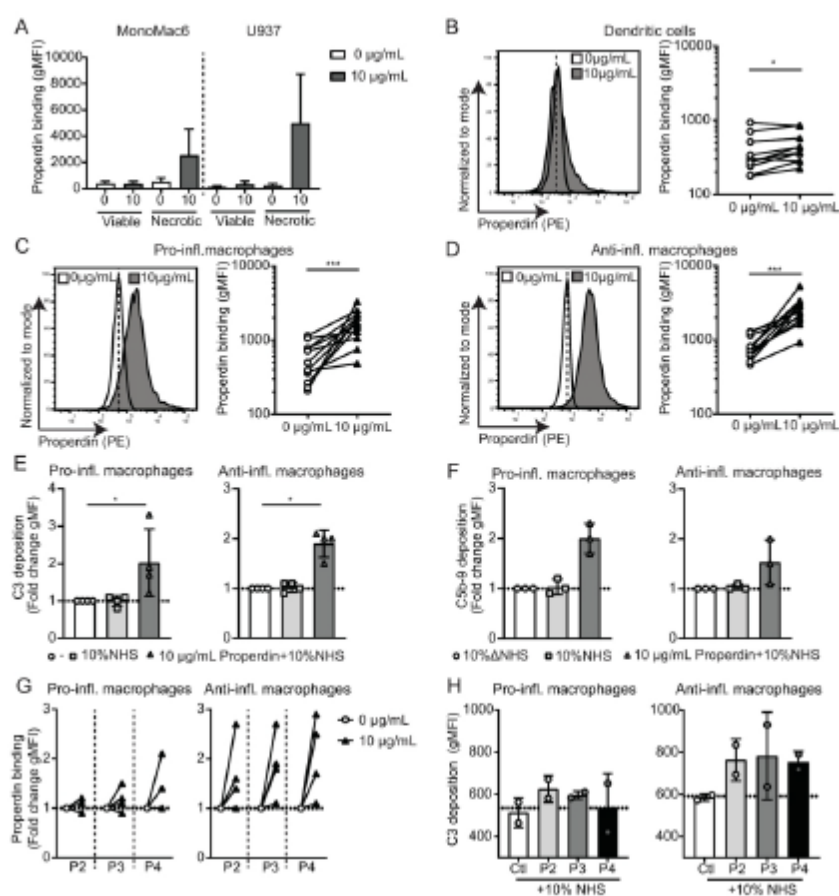
**Figure 3: Serum components and Salp20 interfere with properdin binding, disrupting alternative pathway activation.** (A) Properdin binding to necrotic Jurkat T cells when incubated with various percentages of NHS or with purified properdin (10  $\mu\text{g}/\text{mL}$ , Quidel).

Data are presented as the mean $\pm$ SD of 2 independent experiments. (B) Properdin (10  $\mu$ g/mL, Quidel) was pre-incubated with various percentages of  $\Delta$ NHS. Effect on properdin binding to necrotic Jurkat T cells was determined using flow cytometry. Ctl in the histogram represents the incubation of cells with primary and secondary antibody only. Representative of 2 independent experiments. (C) Properdin (10  $\mu$ g/mL, Quidel) was pre-incubated with various percentages of  $\Delta$ NHS, followed by incubation with 10% properdin-depleted serum in RPMI-MgEGTA, allowing AP activation. C3 deposition was determined using flow cytometry. Representative of 2 independent experiments. (D) Incubation of necrotic Jurkat T cells with a FITC-labelled Salp20. Representative of 2 independent experiments. (E) Representative histogram of the effect of various concentrations of Salp20 (1,10,100,1000 nM) on properdin (10  $\mu$ g/mL; Quidel) binding to necrotic Jurkat T cells, analysed by flow cytometry. (F) Quantification of the effect of various Salp20 concentrations on properdin (10  $\mu$ g/mL, Quidel) binding to necrotic Jurkat T cells. % properdin binding was calculated compared to properdin binding in the absence of Salp20 after background correction. Data are presented as mean $\pm$ SD of 4 independent experiments (1000nM Salp20: 3 independent experiments). (G) Effect of Salp20 (1000 nM) on AP activation, detected at the level of C3, on necrotic Jurkat T cells. Data are presented as mean $\pm$ SD of 3 independent experiments. One-Way ANOVA-Friedman test and Dunn's multiple comparison test, \* $P\leq.05$ .



**Figure 4: Properdin interacts with the surface of both pro and anti-inflammatory macrophages, contributing to complement activation.** (A) Properdin binding to viable and

necrotic monocyte-like cell lines MonoMac6 and U937 analysed using flow cytometry. Data are presented as mean $\pm$ SD of 3 independent experiments. (B-D) Representative histogram and quantification of properdin binding (10  $\mu$ g/mL; Quidel) to (B) dendritic cells (data are obtained from independent experiments performed on dendritic cells generated from 10 different monocyte donors), (C) pro-inflammatory macrophages (data are obtained from independent experiments performed on pro-inflammatory macrophages generated from 12 different monocyte donors) and (D) anti-inflammatory macrophages (data are obtained from independent experiments performed on pro-inflammatory macrophages generated from 12 different monocyte donors). (E) Effect of properdin binding on AP activation on pro- and anti-inflammatory macrophages, determined by C3 deposition. Data are presented as mean $\pm$ SD of independent experiments performed on pro- and anti-inflammatory macrophages generated from 4 different monocyte-donors. (F) Effect of properdin binding to pro- and anti-inflammatory macrophages on AP activation measured by C5b-9 deposition. Data are presented as mean $\pm$ SD of independent experiments performed on pro- and anti-inflammatory macrophages generated from 3 different monocyte-donors. (G) Pro-inflammatory macrophages and anti-inflammatory macrophages were incubated with 10  $\mu$ g/mL P2, P3 and P4 and binding was determined by flow cytometry. Data shown obtained on pro- and anti-inflammatory macrophages generated from 4 different monocyte donors (of which 2 were tested with oligomers from the first SEC, and 2 with the oligomers from the second SEC). (H) Effect of properdin oligomer binding on AP activation on pro- and anti-inflammatory macrophages, determined by C3 deposition. Data are presented as mean $\pm$ SD of independent experiments performed on pro- and anti-inflammatory macrophages generated from 2 different monocyte-donors (incubated with oligomers from the second SEC). Dotted line represents the control condition of cells incubated with primary and secondary antibodies in the absence of properdin and 10%NHS. Wilcoxon matched-pairs signed rank test, paired, \* $P\leq.05$ , \*\*\* $P\leq.001$ . One-Way ANOVA-Friedman test and Dunn's multiple comparison test, \* $P\leq.05$ .



**Figure 5: Interaction of properdin with the cell surface of necrotic Jurkat T cells and pro- and anti-inflammatory macrophages depends on glycosaminoglycan charge and length.** (A) Quantification of the effect of various concentrations of unfractionated heparin on properdin (10  $\mu\text{g}/\text{mL}$ , Quidel) binding to the surface of necrotic Jurkat T cells (left graph), pro-inflammatory macrophages (middle graph) and anti-inflammatory macrophages (right graph). % properdin binding was calculated compared to properdin binding in the absence of unfractionated heparin (3 independent experiments; except for 0.1 and 1000 U/mL, 2 independent experiments) after background correction. (B) Effect of pre-incubation of properdin (10  $\mu\text{g}/\text{mL}$ , Quidel) with various heparins, K5-derived polysaccharides, heparan sulfate and glycosaminoglycans (10  $\mu\text{g}/\text{mL}$ ) on properdin binding to the surface of necrotic Jurkat T cells, pro-inflammatory macrophages, and anti-inflammatory macrophages. Data are shown for 3 independent experiments (pro- and anti-inflammatory macrophages generated from 3 different monocyte-donors). White squares represent no change in properdin binding, blue squares indicate a lower properdin binding, orange squares indicate an increased properdin binding, all compared to properdin binding without pre-incubation with heparin-

like structures. (C) Effect of various concentrations *O*-sulfated K5 and unsulfated K5 (0.1, 1, 10  $\mu\text{g}/\text{mL}$ ) on properdin (10  $\mu\text{g}/\text{mL}$ , Quidel) binding to the surface on necrotic Jurkat T cells (left graph), pro-inflammatory macrophages (middle graph) and anti-inflammatory macrophages (right graph). Data are presented as mean $\pm$ SD of 2 independent experiments (pro- and anti-inflammatory macrophages were generated from 2 different monocyte donors). Data are presented as the mean  $\pm$ SD. One-Way ANOVA-Friedman test and Dunn's multiple comparison test, \* $P\leq 0.05$ .

