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C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend

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Abbreviations: aHUS, atypical hemolytic uremic syndrome; C3b, activated complement factor 3; C4b, activated complement factor 4; C4BP, C4b-binding protein; CCP, complement control protein (domain); CRP, C-reactive protein; Gla, γ-carboxyglutamic acid; FH, factor H; FI, factor I; PS, protein S;

Abstract

C4b-binding protein (C4BP) is best known as a potent soluble inhibitor of the classical and lectin pathways of the complement system. This large 500 kDa multimeric plasma glycoprotein is expressed mainly in the liver but also in lung and pancreas. It consists of several identical 75 kDa α-chains and often also one 40 kDa β-chain, both of which are mainly composed of complement control protein (CCP) domains. Structure-function studies revealed that one crucial binding site responsible for inhibition of complement is located to CCP1-3 of the α-chain. Binding of anticoagulant protein S to the CCP1 of the β-chain provides C4BP with the ability to strongly bind apoptotic and necrotic cells in order to prevent inflammation arising from activation of complement by these cells. Further, C4BP interacts strongly with various types of amyloid and enhances fibrillation of islet amyloid polypeptide secreted from pancreatic beta cells, which may attenuate pro-inflammatory and cytotoxic effects of this amyloid. Full deficiency of C4BP has not been identified but non-synonymous alterations in its sequence have been found in haemolytic uremic syndrome and recurrent pregnancy loss. Furthermore, C4BP is bound by several bacterial pathogens, notably *Streptococcus pyogenes*, which due to inhibition of complement and enhancement of bacterial adhesion to endothelial cells provides these bacteria with a survival advantage in the host. Thus, depending on the context, C4BP has a protective or detrimental role in the organism.

Key words: complement, C4b-binding protein, protein S, apoptosis, group A streptococcus

1. Introduction

Complement is a very powerful tool of the innate immune system used to eliminate invading pathogens, since it not only marks pathogens for removal and activates cellular immune responses but also directly lyses target cells [1,2]. Furthermore, complement is also important for opsonisation of apoptotic and necrotic cells, which, after being marked by complement, are removed by professional phagocytes (reviewed in [3]). Failure in the complement system leads therefore to an increased risk for infections and autoimmune diseases [4]. Complement is also important in therapies using monoclonal antibodies, such as rituximab [5] where cytotoxic effects by complement contribute significantly to the the therapeutic effect [6].

The complement cascade can be activated by three distinct pathways, which are defined by their initial recognition molecules [7]. The classical pathway is initiated by the C1 complex , a multimer consisting of C1q, C1s and C1r, which recognises surface bound antibodies, IgG and IgM. The lectin pathway recognises sugar moieties on the target using ficolins and mannan binding lectin (MBL) as a recognition molecule. Finally, the alternative pathway does not have a distinct recognition molecule but is triggered by spontaneous tick-over of C3 molecules and also serves as a strong amplification loop to the other initiating pathways.

All complement pathways converge at the level of the C3 convertase, initiating and amplifying C3b deposition on the target surface. At the same time as C3b is deposited, C3a, an anaphylatoxin, is released. As the complement cascade proceeds, C5 convertases are formed, which facilitate the cleavage of C5 into surface deposited C5b and the second anaphylatoxin, C5a. C5b deposition triggers the formation of the membrane attack complex (MAC), which forms a lytic pore and can directly lyse eukaryotic cellls as well as gram negative bacteria. To avoid killing the host's own cells, the complement cascade has to be tightly regulated by surface bound and soluble complement inhibitors. One of the main soluble complement inhibitors is C4b-binding protein (C4BP). In this review we will highlight three facets of C4BP:

1. **The good**: the indispensable functions to maintain proper homeostasis in the body
2. **The bad**: consequences of mutated or missing C4BP
3. **The deadly**: pathogens recruiting C4BP and thus evading complement and immune recognition

2. C4BP: The good

At 500 kDa, C4BP is one of the largest plasma glycoproteins and can be found in circulation at an estimated concentration of 200 mg/L [8]. The major isoform of C4BP in plasma [9] is composed of seven identical α-chains and one β-chain [10] linked together by interactions between amphiphatic alpha-helical regions and disulphide bridges in their C-terminal domains (Fig. 1A) [11,12]. Upon

inflammation, expression of a form of C4BP composed of exclusively α-chains is increased (Fig. 1A) [9,13,14]. All β-chain containing C4BP molecules circulate in plasma in a high affinity, calcium-dependent complex with vitamin K-dependent, anticoagulant protein S (PS) [15,16]. Only free PS can act an inhibitor of the coagulation system, due to its ability to act as protein C cofactor in degradation of activated coagulation factors Va and VIIIa. In comparison, C4BP with bound PS retains its full ability to inhibit complement [17], but the complex does not interfere with coagulation in a PS-mediated manner. On the other hand C4BP itself, particularly the form lacking PS, binds plasminogen and auguments its activation via urokinase-type plasminogen activator [18]. The high affinity complexes between C4BP and PS are formed already intracellularly in the endoplasmatic reticulum and β-chain secretion from cells increases dramatically in the presence of PS. In turn, β-chain secretion is clearly decreased in PS-deficient patients [19]. The reason for this tight regulation may lie in the thrombotic risk associated with decreased levels of free PS, which obviously has to be prevented. As with most circulating plasma proteins, C4BP is efficiently synthesized in the liver [20] but there are secondary sites of synthesis such as lung and pancreatic islets (http://www.gtexportal.org). In addition, mRNA for the β-chain, but not for the α-chain, is found in human ovary, the functional significance of which is still unknown [21].

C4BP is a major soluble inhibitor of both the classical and the lectin pathways of complement [22]. It exerts its inhibitory action by binding to and limiting the function of activated complement component C4b. Importantly, C4BP acts as a cofactor to a serine protease factor I (FI) in the proteolytic inactivation of both soluble and cell-bound C4b, thus preventing the formation of the C4bC2a complex i.e. classical C3-convertase [23-25]. Furthermore, C4BP accelerates the natural decay of the C3-convertase [26,27]. Finally, C4BP acts as a cofactor to FI in the cleavage of C3b in the fluid phase, thereby affecting the alternative pathway of complement [28], which is otherwise mainly controlled by factor H (FH).

2.1 Species specific differences in C4BP

Complement inhibitors which resemble the C4BP α-chain in structure and function can be found already in the bony fish barred sand bass (*Parablax nebulifer*), evolving 300 million years before humans [29]. Notably, C4BP has been first discovered in mice [30], which then led to an identification of a similar protein in human serum [24]. The organisation of C4BP seems to be highly conserved. The α-chains from most species consist of 8 CCP domains and form a multimer of 6-8 α-chains, depending on the species. So far, all tested species do harbour a gene for a β-chain, which however is not always expressed *in vivo* (Table 1). Despite the general similarity in structure, on the amino acid level large variations do occur. That in turn would also explain why some pathogens only can bind C4BP from certain species, giving rise to their host-specificity, which will be discussed later.

Mouse C4BP is quite divergent from the human counterpart and consists of 7 identical α-chains, which in contrast to human C4BP are only noncovalently linked to one another [31]. Instead of 8 CCP domains as found in every human C4BP α-chain, mouse C4BP consists of only 6 CCP domains per α-chain [32]. Furthermore, mouse C4BP lacks the β-chain.

C4BP from monkeys and greater apes seem to be highly similar to human C4BP. Chimpanzee C4BP even displays a similar isoform pattern as detected in human serum using a western blot analysis [33]. Similar, although not identical proteins have been found in chicken (*Gallus gallus*) [34]; frogs (*Xenopus tropicalis*) [35] and rainbow trout (*Oncorhynchus mykiss*; genbank BT073759.1)

2.2 Structure-function relationships in C4BP molecule

The α- and β-chains contain eight and three CCP domains typical for complement inhibitors, respectively. So far, the 3D structure of whole C4BP has not been determined experimentally but the structure of the functionally crucial α-chain CCP1-2 domains was solved by NMR (Fig. 1B) [36]. Further, the structure of the central core responsible for polymerisation was solved by x-ray crystallography [12]. Interestingly, the short oligomerisation domain of C4BP provides extremely high oligomer stability, which may be necessary since the protein is large and may bind several ligands simultaneously, including cell surfaces, leading to a high degree of sheer stress. This property of C4BP is now being used to produce stable, polymeric scaffolds for recombinant proteins [37]. Interestingly, C4BP is a remarkably stable protein [38] able to withstand extreme temperature and pH conditions without losing activity. The overall 3D structures of CCP domains are generally highly conserved, allowing creation of useful homology-based 3D models, which has also been the case for all CCP domains from C4BP [39].

C4BP has a broad variety of binding partners including complement proteins, extracellular matrix components, amyloids as well as pathogens and cellular targets, which are all highlighted in Figure 2. The complement inhibitory activity of C4BP is localized to CCP1-3 where the C4b molecule binds (Fig. 1B) [40-43]. C4BP interacts with both C4d and C4c fragments of the C4b molecule [44]. The binding of C3b requires CCP1-4 for full interaction [28]. Both of these complexes are based on electrostatic interactions and involve a cluster of positively charged amino acids at the interface of CCP1 and CCP2 (R39, K63, R64, R66, H67; Fig. 1B amino acids marked in red) [45,46]. These amino acids are also involved in binding of DNA [47] and heparin. For most tested point mutations in the α-chain, the decrease in affinity to C4b and/or C3b was followed by the loss of all complement inhibitory activities of C4BP. However, for mutants K126Q/K128Q and F144S/F149S, clustered on CCP3, a selective loss of their ability to act as co-factors in the cleavage of both C4b and C3b was observed [48]. Both mutants show the same binding affinity for C4b/C3b and had the same inhibitory effect on formation and decay of the classical pathway C3-convertase as the wild type C4BP. It appears that C4b and C3b do not undergo the same conformational changes upon binding to the C4BP

mutants as during the interaction with the wild type C4BP, which then results in the observed loss of the cofactor activity.

The binding of PS resides in CCP1 of the β-chain and is mainly based on hydrophobic interactions [49]. The interaction involves mainly I16, V18, V31, and I33 from the CCP1, with secondary effects from L38 and V39. In addition, K41 and K42 contribute slightly to the interaction [50]. The role of CCP2 appears to be to orient and stabilize CCP1 rather than to be directly part of the binding site [51].

2.3 C4BP allows for silent phagocytosis of dying cells

It has been a mystery why C4BP forms such an extremely high affinity complex with PS and one answer to this question has been provided in recent years with the discovery of the binding of C4BP-PS complex to apoptotic [52] and necrotic cells [53]. PS alone also strongly binds to dying cells in a calcium dependent manner, which is due to an interaction between the γ-carboxyglutamic acid (Gla)-domain and phosphatidylserine. Binding of free PS to apoptotic cells enhances their phagocytosis [54]. Interestingly, the C4BP-PS complex has the opposite effect on phagocytosis, presumably due to steric hindrance exerted by this large protein complex [55]. Another ligand for C4BP-PS on dying cells is surface exposed DNA [53] and the interaction is enhanced by monomeric C-reactive protein (CRP) [56] and the long pentraxin 3 [57]. Interestingly, CRP is the so far only known ligand modulating C4BP's inhibitory function. Monomeric CRP bound to C4BP enhances the cofactor activity for degradation of C4b and C3b [56]. It appears that the main function of the C4BP-PS complex on dying cells is to protect these from complement over-activation, which could otherwise be elicited by binding and activation of C1 complex [58], mannose-binding lectin [59] and ficolins [60], which all recognize dying cells (Fig.3). The fact that almost all patients lacking C1q are affected by systemic lupus erythemathosus (SLE), an autoimmune disease characterized by impairment of dying cell clearance, supports the hypothesis of C1q/C1 complex being important for the removal of apoptotic cells [61]. Further, mice lacking C1q develop a similar autoimmune phenotype [62]. The binding of the initiators of the classical and lectin pathways leads to the opsonisation of dying cells with iC3b, which enhances phagocytosis (Fig. 3 right lane). However, full activation of complement would also lead to generation of the proinflammatory anaphylatoxin C5a and cell damage by MAC (Fig 3, left lane). This must be prevented by binding of C4BP as well as the inhibitor of the alternative pathway, FH [63]. Dying cells incubated with human serum lacking C4BP, presented larger amounts of C3b and MAC deposited on the surface. Furthermore, macrophages ingesting such cells showed an increased TNF-α production [53]. Binding of C4BP and FH appears to be of particular importance for dying cells as these cells lose significant amounts of membrane bound complement inhibitors that otherwise would protect their surface [63,64]. Apart from inhibiting complement and resulting inflammation, C4BP was recently shown to have an additional immunomodulatory function in inducing a semi-mature, tolerogenic and anti-inflammatory state in dendritic cells [65]. This effect seems to be depend on CCP6 and was observed only for the acute form of C4BP lacking the β-chain. Immature dendritic

cells treated with C4BP retained high endocytotic activity but upon LPS treatment they did not upregulate surface expression of CD83, CD80, and CD86. These cells also secreted less proinflammatory Th1 cytokines (IL-12, TNF-α, IFN-γ, IL-6, IL-8) but instead released more IL-10. Treatment with C4BP also prevented surface CCR7 overexpression, which resulted in reduced chemotaxis. Moreover, C4BP treated dendritic cells failed to enhance allogeneic T cell proliferation, impairing IFN-γ production in these cells and, conversely, promoting CD4+CD127low/neg CD25highFoxp3+ T cells. Our recent unpublished data show that apart from the C4BP-PS complex, also C4BP lacking β-chain binds strongly to dying cells. Ongoing studies will elucidate the nature of the involved molecules. Nonetheless it seems obvious that during inflammation large amounts of C4BP lacking the β-chain will be produced. These in turn will likely interact with dying cells, limiting not only complement related inflammation but also activation of dendritic cells, to prevent autoimmune reactions.

2.4 Role of C4BP in the pancreas

The expression of C4BP in pancreatic islets is intriguing. One potential function of C4BP relevant to this tissue could rely on its interaction with various types of amyloid, including that formed by islet amyloid polypeptide (IAPP). IAPP is a peptide co-secreted from pancreatic beta-cells together with insulin. During the initial stages of type 2 diabetes characterized by insulin resistance, insulin and IAPP secretion increase simultaneously [66,67]. Once IAPP reaches a critical concentration the protein starts to aggregate and forms amyloid deposits, which can be found in the majority of type 2 diabetes patients [68]. Human IAPP, but not the rat homologue which is unable to form amyloid fibrils, has cytotoxic effects on beta cells. Even though the exact nature of the cytotoxic form of IAPP (monomers, oligomers or fibrils) is still discussed, for Alzheimer's beta-amyloid (Aβ) there is a growing consensus that small oligomers rather than the mature fibrils of Aβ are responsible for the cytotoxic effect. We found that C4BP not only binds IAPP fibrils and monomers, but also increases the speed of fibril formation as assessed by thioflavin T assay in conjunction with electron microscopy [69]. Furthermore, there was a clear co-localisation of C4BP and amyloid detected by Congo red staining in human pancreatic tissue. A physiological function associated with the C4BP-IAPP interaction is the protection from overt complement activation, since amyloid deposits bind C1q, a strong activator of the classical complement pathway. Simultaneous binding of C1q allows for controlled opsonisation of the amyloid with C3b/iC3b, which allows for removal by phagocytosis, but does not proceed to inflammation mediated by C5a or formation of cytotoxic membrane attack complex. Interestingly, this phenomenon of C1q/C4BP binding was observed also for other types of amyloid such as Aβ [70] and prions [71]. Another possible function of the C4BP-IAPP interaction might be the protection of beta-cells from cytotoxicity elicited by IAPP oligomers, as these will in the presence of C4BP be faster assembled into safer amyloid fibrils. Apart from apoptotic cells and

amyloid there are other examples of molecules, which bind simultaneously C1q and C4BP. These are CRP [72] and components of extracellular matrix such as osteodherin, fibromodulin and chondroadherin [73]. Interestingly, related proteins decorin and biglycan, which do not bind and activate C1q but rather inhibit complement, do not bind C4BP. This implies that these interactions are not coincidental but serve some functional purpose. This phenomenon should allow phagocytosis of the material labeled with C1q, which functions as an opsonin. The low level of complement activation, which occurs as a consequence of these interactions, can also give rise to phagocytosis via receptors for fragments of C3. Deposited C3b together with C3a, which has been shown to be anti-inflammatory under certain conditions [74], could lead to anti-inflammatory clearance. Importantly, the whole process is kept under tight control by inhibitors of the later stages of the complement cascade such as C4BP, which prevents release of C5a and formation of the membrane attack complex and therefore down-regulate inflammation.

3. C4BP: the bad

In contrast to other complement proteins and inhibitors no full or even clear partial deficiency has been described for C4BP. This underlines the importance of C4BP for maintaining homeostasis in the body.

3.1 atypical hemolytic uremic syndrome (aHUS)

The first non-synonymous polymorphism in C4BP was described in aHUS [75], a disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure [76]. aHUS is in the majority of cases associated with loss of function mutations in complement inhibitors or gain of function mutations in complement factors, and thus resulting complement dysregulation. The rare non-synonymous polymorphism in the α-chain of C4BP (R240H) was weakly associated with aHUS in two independent cohorts and found also in some healthy volunteers. No statistically significant association was found in a third cohort [77]. The alteration had an effect on the ability of C4BP to bind C3b and to act as cofactor to FI in degradation of C3b, while the inhibitory effect of C4BP on C4b was not affected [75]. This observation supports the current hypothesis that dysregulation of the alternative pathway of complement is causative for aHUS [76].

3.2 Implications in recurrent pregnancy loss

Although partly elusive, C4BP and especially mutations in C4BP seem to be involved in recurrent, spontaneous pregnancy loss [78]. Several alterations were found in the α-chain in women with recurrent pregnancy loss, of which the R120H, I126T, and the G423T mutations affected the expression level and/or the ability of recombinant C4BP to serve as cofactor for FI. The only variant

identified in β-chain was located in the C-terminal part but did not impair the polymerization of the molecule. Miscarriage is a common complication of pregnancy with approximately 40% of cases remaining idiopathic. Complement complexes are involved and seem to be important in healthy pregnancies, while the role is yet unclear [79]. Unexpectedly, transgenic mice lacking C4BP appear to have an overall normal phenotype [80]. However, we observed that homozygous C4BP knockout mice often die during second or third pregnancy (unpublished observation). This would imply a pivotal role of this protein in maintenance of successful pregnancy, although the mechanism is not known.

4. C4BP: the deadly

Surface bound inhibitors of complement are indispensable to protect the body's own cells from complement over-activation and subsequent cell lysis. However not only host cells benefit from protection from complement, so do invading pathogens. To achieve that necessary protection, microorganisms employ a variety of strategies: alteration and inhibition of the complement cascade through direct interaction with virulence factors, enzymatic inactivation of complement and recruitment of complement inhibitors, surface bound as well as soluble [81]. C4BP and other soluble complement inhibitors are abundantly available, not only in serum but also on mucosal surfaces, wound- and interstitial fluids; typical points of entry for pathogens. The easy availability renders the host regulators to a prime target to be recruited to the surface of the invading microbe. Since C4BP retains its functionality on the pathogen's surface, C4BP protects pathogens and thus is detrimental for the body in that particular aspect.

4.1 Many pathogens recruit C4BP to inhibit complement

So far a broad variety of pathogens have been identified to bind C4BP (Table 2). It is known that gram positive [36,82-88] and negative bacteria [33,89-104] bind C4BP. However, not only bacteria benefit from complement protection, but viruses [105,106] and fungi [107-109] do so as well. The reasons why microbes recruit C4BP are similar: preventing MAC formation and subsequent lysis (in the case of gram negative bacteria and viruses) as well as preventing opsonisation and the activation of the immune system through the anaphylatoxins C3a/C5a. For example, C4BP inactivates C4b on the surface and accelerates the decay of the C3-convertase [88]. As a result, C4BP protects the pathogens from opsonisation [86]. In turn, reduced opsonisation directly diminishes phagocytosis. However, not only opsonisation, representing the early step of complement activation, is disturbed, but also, the formation of MAC and eventually lysis of the target cell is prevented (Fig.4). Accompanying that, release of anaphylatoxins, which activate the innate and adaptive immune system, is suppressed. Interestingly, some pathogens such as *Streptococcus pyogenes* can only bind human, but not mouse C4BP [85,86]. This might be one reason for the distinct host specificity of *S. pyogenes* (see also 4.3).

Taken together, these facts underline, how advantageous and important complement inhibition is for pathogens, especially in preventing early steps of activation.

4.1.1 *Streptococcus pyogenes* recruits C4BP using a hyper-variable domain on a surface bound virulence factor

Streptococcus pyogenes (Group A streptococcus) binds C4BP via its M-protein, a major surface bound virulence factor [84]. For a specific M-protein, M4, NMR analysis of a C4BP-M protein complex revealed the binding site for the bacterial M protein on C4BP to be 28 residues on CCP1-2 of the C4BP α-chain [110]. Compared to the natural ligand C4b, the M protein exploits an overlapping, but not identical binding site on the α-chain of C4BP [85,111]. Due to its multimeric structure with 7 identical α-chains, C4BP can bind to several ligands simultaneously even if these share the same binding site on C4BP. That in turn ensures a tight binding to dying cells or microbes but still allows interaction with other ligands, such as C3b or C4b. For example, while binding sites on C4BP α-chain for protein M and C4b/C3b are overlapping, C4BP bound to these bacterial proteins or the intact bacteria clearly retains its ability to bind C4b/C3b and thereby inhibit complement. The majority of all C4BP related publications describe that in fact C4BP retains its cofactor activity to degrade C3b and C4b although it is bound to a certain ligand or surface.

For the majority of M-proteins (90%) it has been shown that they can bind C4BP [112]. Interestingly, the C4BP binding site is located within the hyper-variable region of the M protein [113]. This region is located at the N-terminal part of the M protein outside the cell. Due to the hypervariable regions within the M protein, no immunological cross reactivity between the strains could be found [114], which could serve as a protection against the hosts immune defense. Interestingly, C4BP binding is conserved despite a significant sequence divergence in this region [112]. In most *S. pyogenes* strains, the M-protein is exclusively responsible for C4BP binding; therefore it would be a most suitable target for neutralizing antibodies and vaccinations. Despite various attempts, a functional vaccine could not be found so far, most likely due to the very unique properties of the M-protein including the hyper-variable region.

Beside M proteins, in some strains other members of the M protein family are able to bind C4BP [84]. Recently, we mapped the binding site of C4BP on *S. pyogenes* protein H [88] (not to be confused with complement factor H), a surface bound virulence factor expressed exclusively in highly virulent M1 strains [115]. Similar to M proteins, the C4BP binding site on protein H is also located in the very N-terminal part of the protein, corresponding to the hyper-variable region of the M protein.

Although *S. pyogenes* is a gram positive bacterium and cannot be directly lysed by complement, it seems to be pivotal for the bacteria to bind C4BP in order to decrease complement-mediated opsonisation and phagocytosis [87].

4.1.2 C4BP CCP1-3 are the primary binding site for the majority of pathogens

Interestingly, 2/3 of all pathogens for which the binding site for C4BP is defined, exploit the CCP1-3 (see table 2). Only two pathogens do not bind the N-terminal part of C4BP but rather in the core region encompassing CCP7-8. Interestingly, exactly the same CCP1-3 harbor the binding site for C4b and C3b and are also required for cofactor activity to FI [41]. For pathogens it must be important to bind C4BP and at the same time maintain the complement regulatory function of C4BP. Obviously, the microbes found ways to utilize a highly conserved and similar, but not identical binding site to C3b/C4b, still allowing inhibitory functions of C4BP when bound to the pathogens surface. Of note, the polymeric nature of C4BP with its seven arms would allow a tight binding to any surface and still having free arms to bind C3b/C4b and accelerate C3 convertase decay.

4.2 C4BP mediates invasion and promotes adhesion of pathogens

Beside its role as a complement inhibitor, C4BP seems to also influence adhesion and invasion of *S. pyogenes* to endothelial cells, which is related to its polymeric form and ability to bind several different ligands on bacterial and cell surfaces simultaneously. C4BP elevated bacterial adhesion to endothelial cells by more than four fold. Using a gentamicin protection assay, invasion of *S. pyogenes* to HUVEC cells was tested. Interestingly, and even more pronounced than adhesion, in the presence of C4BP, about 1 $\log_{10}$ more bacteria invaded HUVECs cells than in the absence of C4BP [88]. Accordingly, a *S. pyogenes* strain unable to bind C4BP did not show any significant differences either in invasion nor adhesion. It is not exactly clear, how C4BP increases adhesion and invasion, but it has been speculated that C4BP might serve directly as a bridging molecule, bringing bacteria and cells into close proximity. Cellular ligands for C4BP are poorly characterized but it is known to bind cell surface proteoglycans and CD91/LDL receptor related protein [116].

C4BP interacts with plasminogen and while both molecules are able to bind independently to some bacteria such as *Streptococcus pneumoniae*, these interactions are enhanced when C4BP and plasminogen are present simultaneously [18]. Since C4BP enhances plasminogen activation this may result in generation of larger amounts of plasmin on bacterial surface, leading to further attenuation of deposition of C3, which can be degraded by plasmin [117]. During infection, activation of the coagulation system leads to entrapment of bacteria in a fibrin clot, and the presence of plasmin, degrading the fibrin network, leads to liberation of the bacteria providing yet another advantage [118].

Similarly, adenoviruses use C4BP to dock to liver cells and induce their own uptake [105]. Although adenoviruses can also infect liver cells in a C4BP independent, coxsackie-adenovirus receptor dependent way, this report was the first one to show a so far unknown function of C4BP.

Adenoviruses are often used for gene therapy approaches and seem to be promising especially in cancer and cardiovascular diseases. However, a systemic administration of viruses unintentionally and quite specifically targets the liver. The interaction of adenovirus-bound C4BP and hepatocytes might be an explanation for these off-target effects.

These unexpected observations have to be analysed further to elucidate, which pathogens can use C4BP to enhance their invasiveness. It seems highly likely, that especially intracellular pathogens able to bind C4BP such as *Salmonella*, *Neisseria* or *Yersinia* could utilize C4BP not only to prevent opsonisation, but also increase uptake and invasion into their target cells.

4.3 Animal models expressing human C4BP

Although human C4BP differs significantly from mouse C4BP, it has been shown that the human variant can regulate mouse complement in different setups of experimental arthritis [119]. As expected, C4BP injected intraperitoneally into mice was able regulate the classical pathway, but not the alternative pathway of complement activation. Despite its very large size, C4BP was cleared rapidly, with a half-life of just 10h. However even small amounts of C4BP showed clear signs of regulatory activity and a tremendous effect on arthritis onset and severity. Manual administration of human complement components into mice however has to face obvious problems: a) fast clearance, b) constant supply of purified proteins to maintain serum levels, c) permanent manipulation of the experimental animal to supply the protein and d) possibly eliciting an immune reaction to a foreign protein. Those problems underline the necessity for a different approach, for example transgenic animals expressing human complement proteins in addition to or instead of the endogenous.

The importance of human complement inhibitors for infections has been difficult to prove. *In vivo* infection experiments are only possible, for obvious reasons, in non-human mammals. Some pathogens, such as *S. pyogenes* however exhibit extraordinary host specificity and do bind exclusively complement inhibitors from higher primates such as humans, chimpanzees and gorillas [85]. To overcome the above-mentioned problems with an external supply of human complement inhibitors, we generated transgenic mice. Recently we described the first transgenic mouse model expressing human C4BP [86]. Hu-C4BP was expressed in those animals additionally to their endogenous C4BP and at levels similar to human serum. Despite the large amounts of C4BP in serum, we could not detect any excessive inhibition of complement. Opsonization of zymosan was identical compared to wild animals. However we could demonstrate that C4BP indeed has the predicted detrimental impact on *S. pyogenes* infections in mice. The bacteria were covered in C4BP and prevent C3b deposition on their surface. As expected, decreased opsonisation led to decreased phagocytosis, elevated bacterial burden in multiple organs and subsequently significantly increased morbidity of the animals. It is highly likely, that these findings could be translated to different pathogens, which protect themselves by binding human complement inhibitors. Similar results have been achieved by using transgenic mice, which express human FH [86,120]. Taken together, it has been shown that pathogens recruit complement inhibitors to benefit from diminishing complement activation and deposition. In turn, the pathogens are enabled to replicate and manifest a severe infection.

5. Concluding remarks

C4BP is indispensable for the human body and fullfills important tasks in regulating the complement cascade. The significance is underpinned by the fact that there are no patients known lacking C4BP or having a significant loss of function mutation. Interestingly, mutations found so far to cause only a small functional dysfunction, already seem to influence the organism negatively, either causing aHUS or leading to spontaneous pregnancy loss. Similarly, in an animal model, C4BP seems to have fundamental role in reproduction as well.

Even though C4BP is essential for the human body, pathogens also benefit from recruiting it to their surface. Thus, C4BP does regulate complement activation regardless of the localisation becoming: friend or foe. For example, in wild type mice *S. pyogenes* is avirulent and does not cause disease. The presence of human C4BP in the animals however renders the bacteria virulent causing 100% mortality in less than four days. Taken together, having C4BP is always good, it just depends how you look at it: from the point of view of humans or of pathogens.

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species	α-chain		β-chain			references
	Number of α-chains	Number of CCPs	Number of β-chains	Number of CCPs	PS binding	
Human	6/7 (P04003)	8	1	3 (P20851)	Yes	[13,121]
Mouse	7 (P08607)	6	Pseudo	---	No	[122]
Rat	(Q63514)	8	1	3 (Q63515)	Yes	[123]
Rabbit		8	Pseudo	---	No	[124]
Cattle	(Q28065)	8	1	2 (Q28066)	No	[125]
Guinea pig	7-8 ? (Q99N98)	7 [BAB39739.1]	?	?	No	[126]
Chimpanzee	6-7 ?	8 [XP_009439671.1]	1?	3 [XP_009439664.1]	unknown	[33]
Pig		8 [XP_005667608.1]	1?	3 [XP_003130503.1]	unknown	
Dog		8 [XP_003434998.1]	1?	3 [XP_851611.1]	unknown	

Table 1: Structure of C4BP from different species. In mouse and rabbit, the β-chain has evolved to a pseudogene and is not expressed *in vivo*. Numbers indicate () UniProt or [] genebank acession or numbers.

Pathogen	Disease	Surface ligand	CCP domains bound	references
Bordetella pertussis	whooping cough	hemagglutinin	CCP1-2	[89]
Borrelia burgdorferi	Lyme disease	?	?	[90]
Borrelia recurrentis and *duttonii*	relapsing fever	?	?	[127]
Escherichia coli K1	neonatal meningitis	OmpA : Outer membrane protein A (N-terminus)	CCP3, CCP8	[91,92,128]
Haemophilus influenzae	respiratory tract infections	?	CCP2, CCP7	[93]
Leptospira spp	leptospirosis	LigA, LigB LcpA Lsa30	CCP4, 7-8 CCP 7-8	[129,130] [131] [132]
Moraxella catarrhalis	otitis media, sinusitis	Ubiquitous surface protein A1 and A2	CCP2, CCP7	[94]
Neisseria gonorrhoeae	gonorrhea, disseminated gonococcal infection	porin 1A (loop 1) porin 1B (loops 5, 6) type IV pili (pilC)	CCP1 CCP1 CCP1-2	[95] [96] [97]
Neisseria meningitides	meningitis	porin	CCP2-3	[98]
Porphyromonas gingivalis	periodontitis	Gingipain A	CCP1, CCP6-7	[99]
Prevotella intermedia	periodontitis	?	?	[100]
Salmonella enterica	salmonella poisoning	Rck	CCP7-8	[101]
Staphylococcus aureus	Skin and systemic infections	SdrE, Bbp	?	[133]
Streptococcus pneumoniae	pneumonia	PspC enolase	CCP1-2, CCP8	[83] [82]
Streptococcus pyogenes	strep throat, necrotizing fasciitis, rheumatic fever	M and H proteins (hypervariable region)	CCP1-2	[36,84-87]
Yersinia enterocolitica	food-borne enteric pathogen	YadA and Ail	?	[103]
Yersinia pestis	plague	Ail	CCP6, CCP8	[33,104]
Yersinia pseudotuberculosis		Ail	CCP6-8	[102]
Aspergillus spp	systemic infections in immunocompromised	?	?	[109]
Candida albicans	candidiasis in immuno-compromised	pH-regulated antigen 1	CCP1-2, CCP7-8	[107] [108]
Adenovirus	Used for gene therapy	Fiber knob domain	CCP1-3	[105]
Flavivirus		NS1	CCP2-5, CCP8	[106]

Table 2: Pathogens, which bind C4BP.

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7. Vitae

Anna M. Blom

Anna Blom is a professor of medical protein chemistry at Lund University, Sweden. She obtained a PhD in medical and physiological chemistry from Uppsala University, Sweden followed by postdoctoral training at Lund University. Her research group focuses on regulation of the complement system in health and autoimmune and inflammatory diseases as well as infections.

David Ermert

David Ermert is a research scientist working at Lund University, Sweden. He started as a graduate student at the Max Planck Institute for Infection Biology, Berlin, Germany and obtained his PhD in immunology. He did a postdoctoral training at Lund University, Sweden and worked at the University of Massachusetts Medical School in Worcester, MA, USA. His research is focusing on Group A Streptococcus and the interplay with the human host with special emphasis on the complement system.

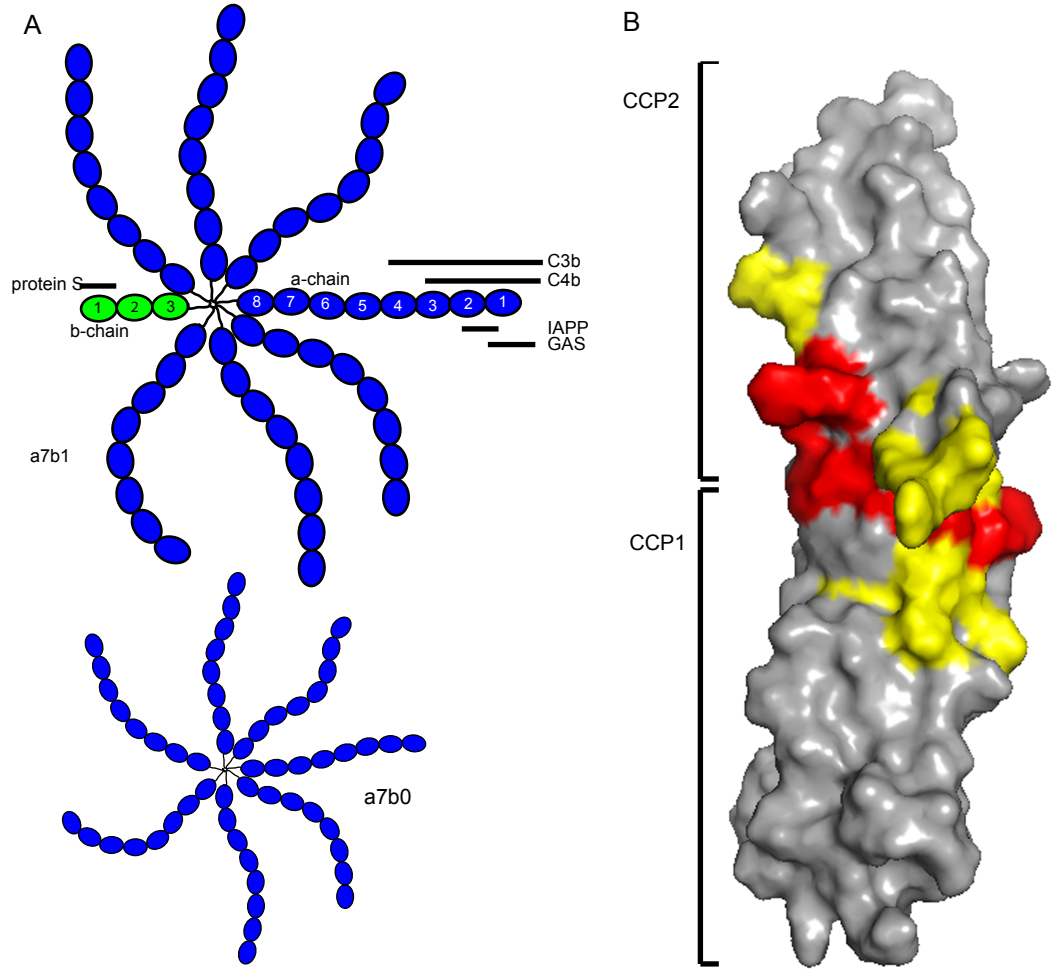
Figure legends

Figure 1: Structure of C4BP and binding sites to its ligands. C4BP is a multimeric protein consisting of 7 α-chains and a β-chain (α7β1)(A). During infections, C4BP can be expressed without the β-chain in the so called α7β0 configuration. Each α-chain and β-chain consists of 8 and 3 similar CCP domains, respectively. Protein S binds to CCP1 of the β-chain, while the complement regulatory activity is located on the α-chains. Binding sites for C3b, C4b, IAPP and *S. pyogenes* are indicated. In the solution structure of CCP1-2 (B) the responsible amino acids are highlighted for binding to C4b (red) and protein H (red and yellow). Compared to C4b, protein H utilizes a similar but not identical binding site on C4BP.

Figure 2: Known binding partners of C4BP. C4BP can bind to a variety of different proteins localized on cells, pathogens as well as in extracellular matrix, plasma and amyloids. For a more detailed descriptions of pathogens binding C4BP see table 2.

Figure 3: C4BP enables silent removal of apoptotic and necrotic cells by macrophages. During the natural turnover of cells or in case of external damage, cells have to be removed from the system. Apoptotic and necrotic cells are recognised by C1q, MBL and ficolins, which initiate opsonization of the cells. In healthy individuals, C4BP binds to those cells and together with FI cleaves C3b to iC3b (right column). This allows macrophages to encounter the so marked cells and remove them from the system in a silent way, without initiating inflammation. In fact, macrophages with phagocytosed C4BP-labeled apoptotic/necrotic cells rather release antiinflammatory cytokines. However, if C4BP is not present or binding fails, the complement cascade is not inhibited and the membrane attack complex, a lytic pore, forms and releases the intracellular content, including DNA (left column). Professional phagocytes, which encounter the opsonized and lysed cells attack them, release significant amounts of TNF-α and thus create a proinflammatory environment.

Figure 4: C4BP protects pathogens from complement attack and eventual elimination. Once they have invaded the body, pathogens face the first line of defense, the complement system. Different pathogens, such as gram positive and gram negative bacteria, viruses and fungi are able to recruit the complement inhibitor C4BP to their surface (left column). So protected, the microbes evade complement attack, opsonization and do not activate further immune defense. Finally the pathogens are able to spread, replicate and establish a fulminant infection. Pathogens, which cannot bind C4BP fail to circumvent complement activation (right column). Complement deposits C3b on their surface, releasing the anaphylatoxin C5a and eventually forming MAC. Gram negative bacteria can be directly lysed by MAC. Other pathogens are not directly killed by complement, but all are marked for removal. Subsequently, professional phagocytes are recruited to the site and finally clear the infection.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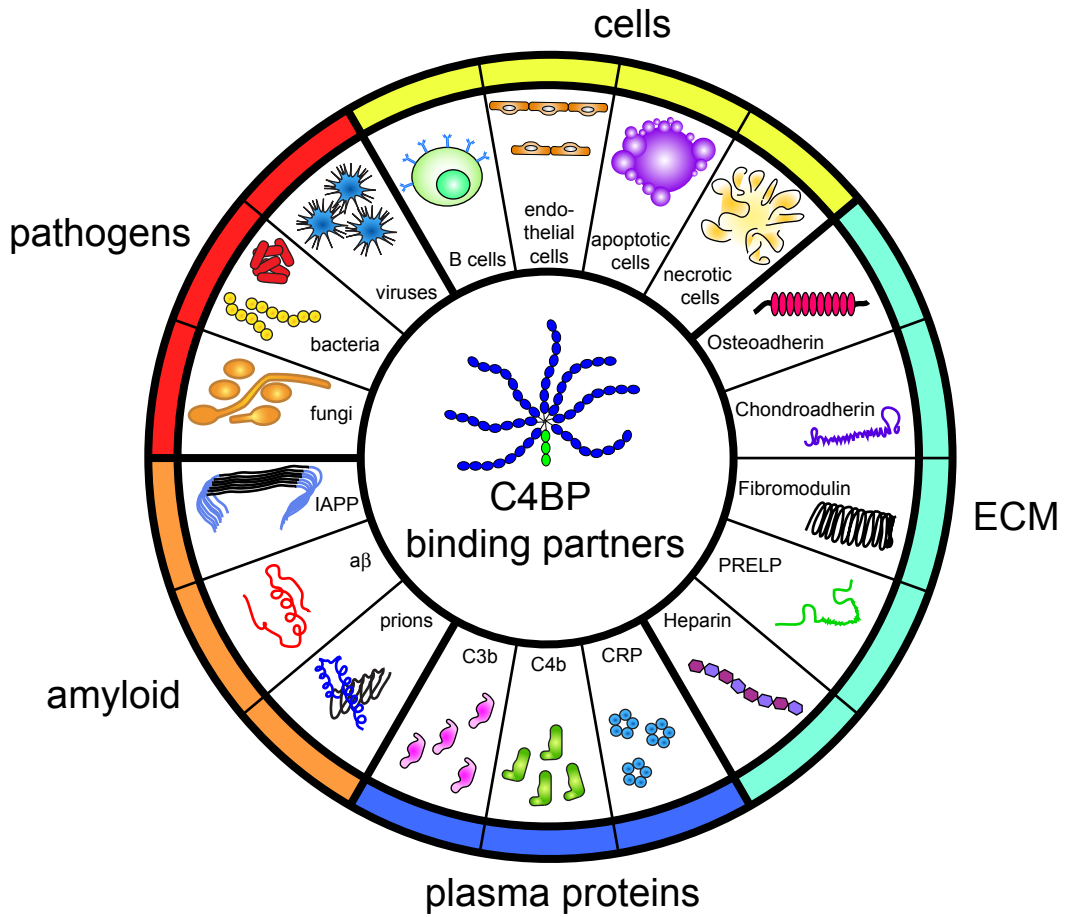
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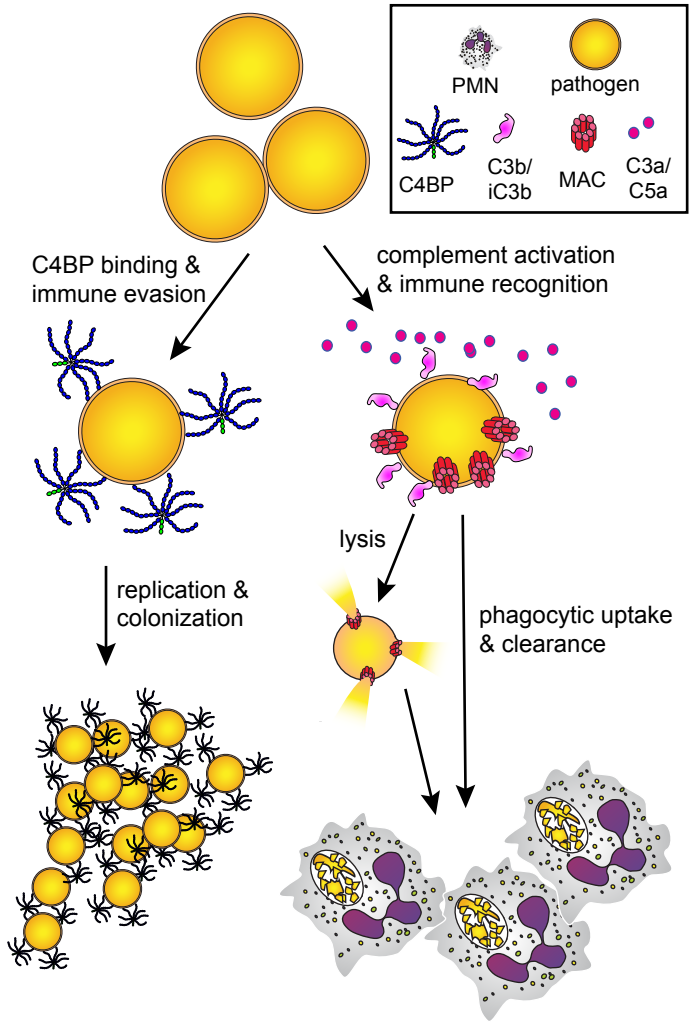
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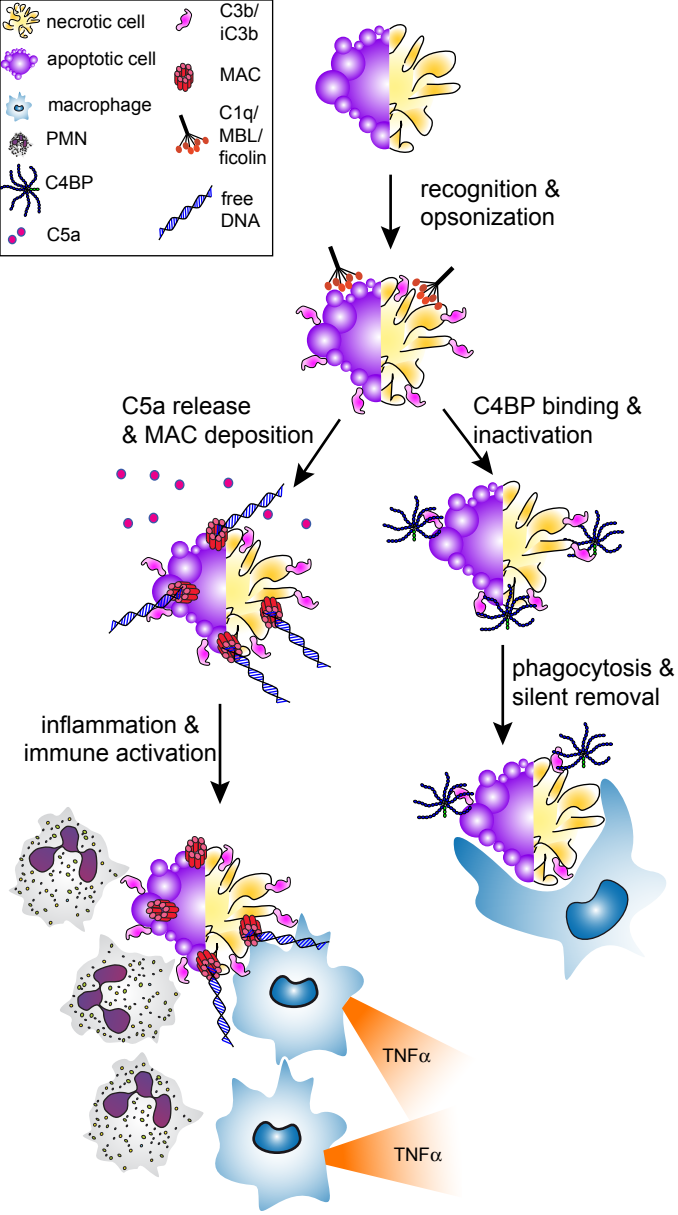
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