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Chapter 2

Consequences of dysregulated complement regulators on red blood cells

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ABSTRACT

The complement system represents the first line of defense that is involved in the clearance of pathogens, dying cells and immune complexes via opsonization, induction of an inflammatory response and the formation of a lytic pore. Red blood cells (RBCs) are very important for the delivery of oxygen to tissues and are continuously in contact with complement proteins in the blood plasma. To prevent complement activation on RBCs, various complement regulatory proteins can be found in plasma and on the cell membrane. RBCs are special cells without a nucleus and having a slightly different make-up of complement regulators than nucleated cells, as membrane cofactor protein (MCP) is not expressed and complement receptor 1 (CR1) is highly expressed. Decreased expression and/or function of complement regulatory proteins may result in unwanted complement activation and accelerated removal of RBCs. This review describes complement regulation on RBCs and the consequences when this regulation is out of balance.

1. INTRODUCTION

Red blood cells (RBCs) are the most common cells in our blood and are indispensable in the delivery of oxygen to tissues. RBCs have a biconcave form, do not have a nucleus, and under normal conditions RBCs have a life span of ~120 days. While circulating in the body, RBCs are continuously in contact with complement components in the blood plasma[1].

The complement system is part of our innate immune system and is very important in the clearance of pathogens, dying cells and immune complexes. The complement system consists of at least 30 circulating and membrane-associated proteins that are mainly synthesized by the liver. There are three distinct pathways of complement activation: the classical pathway (CP), which is activated by antibody-antigen complexes, pattern recognition molecules such as CRP or directly by structures on pathogens or apoptotic cells, the lectin pathway (LP) is activated by pathogen associated carbohydrate structures and the alternative pathway (AP) which is activated by the spontaneous hydrolysis of C3, that can form the AP-initiating C3 convertase (called tick-over mechanism), and only proceeds on unregulated surfaces. Both the CP and LP lead to the cleavage of complement proteins C4, C2 and the subsequent formation of the C3 convertase C4bC2a on the activating surface, while the AP leads to the formation of the C3 convertase C3bBb on the surface.[1-5] Both C3 convertases cleave more C3 molecules to opsonize foreign or damaged material for phagocytosis and eventually leading to clearance of cells by formation of the membrane attack complex (MAC) and the chemotaxis of leukocytes by anaphylatoxins contributing to the inflammatory response[1,2,6,7].

Since complement activation has potentially dangerous strong inflammatory effects and may lead to cell lysis, it is of utmost importance that complement activation is tightly regulated. Complement regulation must act specifically on healthy host cell surfaces, while leaving pathogen clearance intact. Next to complement regulation on host cell surfaces, soluble complement regulators also control complement activation in solution to prevent consumption of complement proteins in plasma. Dysregulation of the complement system, due to either inefficient regulation or overstimulation, can be detrimental for the host and contributes to the pathology of many diseases including autoimmune diseases, transfusion reactions, and complement mediated conditions like paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS) and age-related macular degeneration (AMD)[1,2,8,9]. This review focuses on complement regulation on RBCs and the consequences of complement regulation dysbalance on RBCs.

2. Complement regulators

Complement regulatory proteins can be found both in the fluid phase in plasma (such as factor H (FH) and its splice variant FH-like 1 (FHL-1), factor I (FI), C1 inhibitor (C1-INH) and C4b-binding protein (C4BP)) and on the cell surface of most human cells (complement receptor 1 (CR1/CD35), membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55), and CD59)[2,10]. Genes expressing several regulatory proteins are located in the same region on chromosome 1. Therefore, this region is also called the regulator of complement activation (RCA) gene cluster. The genes in this cluster show a particular structural organization as they consist of short consensus repeats (SCR) domains, also called sushi domains or complement control protein, with a length of ~60 amino acids. Genes of this cluster include CR1, MCP, DAF, FH and C4BP[11,12]. However, not all genes for complement regulator proteins are located in this RCA cluster, as C1-INH and CD59 are located on chromosome 11 and the gene encoding for FI is located on chromosome 4[13-15].

### 2.1 Fluid phase complement regulators

Fluid phase complement regulators are more pathway specific compared to membrane bound complement regulators, that have more common regulation mechanisms as described below. C1-INH is a plasma serine protease inhibitor (serpin) that inhibits the serine proteases in the C1-complex (C1r and C1s) of the CP and the mannan binding lectin-associated proteases (MASPs) of the LP, thereby inhibiting the cleavage of C4 and C2[2,10,16]. FI is a serine protease that can cleave both C3b and C4b into inactive forms iC3b and iC4b respectively, which do not contribute to further amplification of complement activation, and can further be degraded into C3d and C4d respectively. For efficient regulation, FI requires the activity of a co-factor[2,10]. Both fluid phase complement regulators (FH and C4BP) and membrane bound complement regulators (CR1 and MCP, described below) can act as co-factor for FI. FH consists of 20 SCR domains and besides acting as a cofactor for FI in the inactivation of deposited C3b, FH accelerates the decay of the AP C3 convertase (C3bBb)[2,10,17]. FHL-1 is a splice variant of FH and is identical to the first 7 SCR domains of FH, except for four additional amino acids at the C-terminus. FHL-1 has similar complement regulatory functions as FH, but is lacking the C-terminal surface binding domains of FH[2,10,18]. C4BP, which is often in complex with protein S, has an octopus-like structure, consisting of 7 arms with 8 SCR domains and 1 arm with 3 SCR domains. C4BP acts as a cofactor for FI in the inactivation of deposited C4b and accelerates the decay of the C3 convertase of the CP and LP (C4bC2a). C4BP can also act as co-factor for FI in the inactivation of deposited C3b, although FH can do this much more efficient[2,10,19].

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2.2 Membrane bound complement regulators

The membrane bound complement regulators regulate complement activation of all three pathways mainly by acting at the level of the C3 convertases by either promoting rapid dissociation via decay accelerating activity or by acting as cofactor for FI to enzymatically inactivate deposited C3b and C4b[2,10]. CR1 consists of 30 SCR domains and is attached to the cell membrane via a transmembrane domain. CR1 accelerates the decay of the C3 convertase of all three pathways and can act as a co-factor for FI in the inactivation of deposited C3b and C4b[2,10,20]. Besides acting as regulator of complement, CR1 on RBCs is involved in the clearance of immune complexes and pathogens that are opsonized with complement C3b, so called immune adherence clearance. RBCs transport CR1-bound immune complexes to the spleen and liver to be removed from the RBC membrane by phagocytic cells. MCP is, similar as CR1, attached to the cell membrane via a transmembrane domain and acts as cofactor for FI. MCP is much smaller compared to CR1, only consisting of 4 SCR domains[2,10,21]. Comparable to MCP, DAF consists of 4 SCR domains, being attached to the cell membrane via a glycophosphatidylinositol (GPI) anchor. DAF accelerates the decay of the C3 convertase of all three pathways. As CD59 is not present in the RCA cluster gene it has no significant similarities to any of the other complement regulator proteins[2,10,22]. CD59 is, just as DAF, linked to the cell membrane by a GPI anchor. CD59 prevents complement activation at a later stage compared to the other membrane bound complement regulators by binding to C8 and C9, thereby preventing C9 from polymerizing and forming the MAC[2,10,23].

3. Complement regulation on RBCs

Fluid phase and membrane bound complement regulators offer protection against ongoing spontaneous complement activation via the alternative pathway and hence prevent potential complement-mediated destruction of RBCs that are continuously in contact with complement proteins (Fig. 1A). On the membrane of RBCs complement regulators CR1, DAF and CD59 are present. Membrane regulator MCP is only expressed on nucleated cells. MCP is therefore lacking on mature RBCs while it is expressed on erythroblasts, the nucleated RBC precursors that are formed in the bone marrow (manuscript in preparation). During RBC development the expression of MCP is lost, which must have some evolutionary benefit. It has been described that MCP is a receptor for different pathogens such as viruses (measles, human herpes virus 6, different serotypes of adenovirus) and bacteria (*Streptococcus pyogenes* and *Neisseria species*)[24]. It has been suggested that the lack of MCP on mature RBCs rather prevents dissemination of pathogens than replication as they do not have a nucleus. Since CR1 is expressed on RBCs during all stages of maturation (manuscript in preparation), we speculate that CR1 may compensate
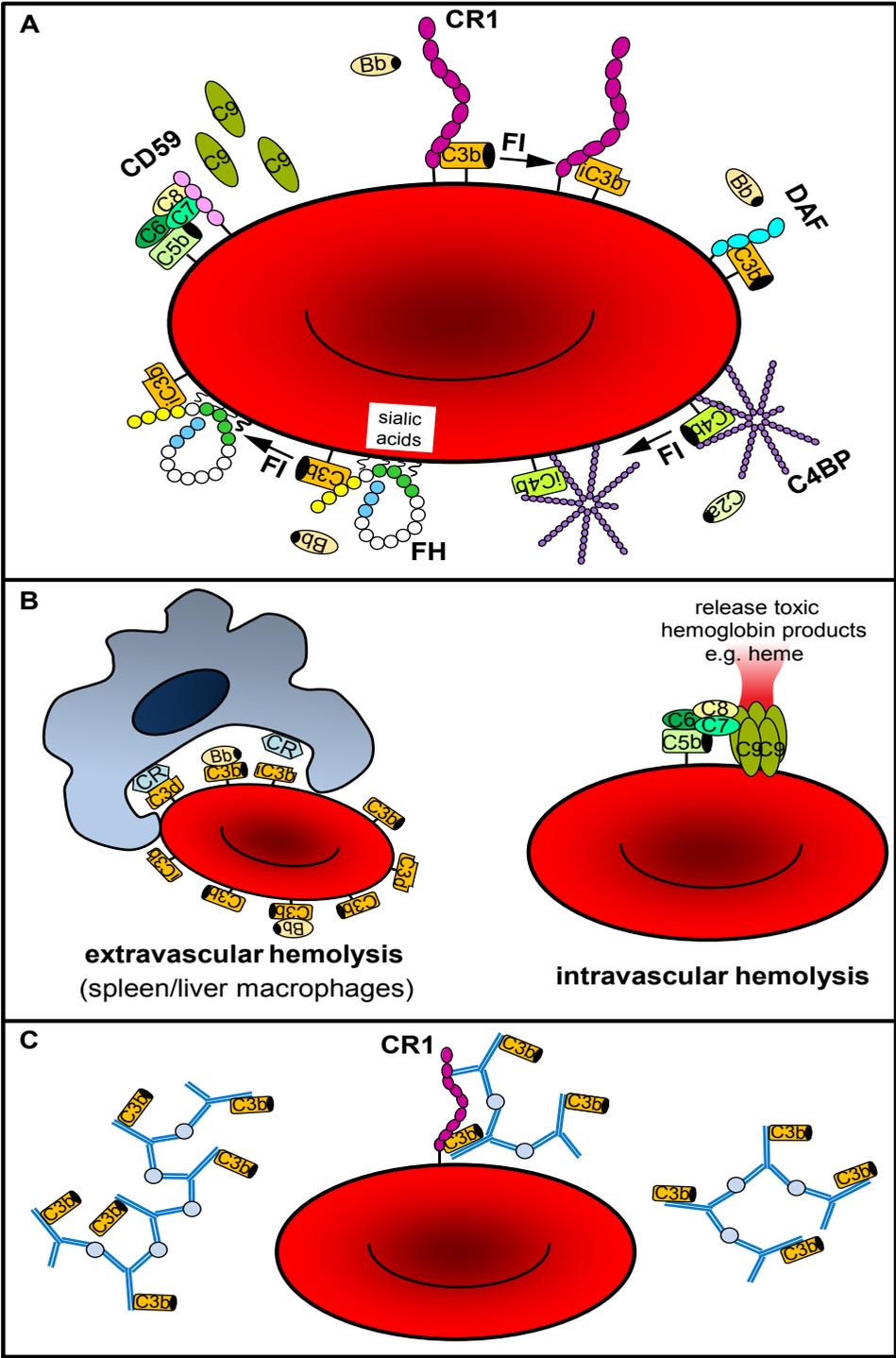
for the absence of MCP as both complement regulators have similar complement regulating activities. Moreover, CR1 bears the human RBC Knops/McCoy blood group antigen[25,26]. In addition, Chido and Rodgers blood groups are distinct epitopes on the C4 protein (C4A and C4B, respectively) that are absorbed passively onto the surface of RBCs[27]. Binding of AP regulator FH to RBCs mainly occurs via deposited C3b and sialic acids on the RBC membrane[28]. As the repertoire of complement regulating proteins on RBCs differs from nucleated cells and RBCs are in continuous contact with complement proteins, we discuss in this review the consequences of both primary genetic deficiencies and secondary downregulation of complement regulation on RBCs.

4. Primary genetic deficiencies of RBC complement regulation

Mutations in genes encoding the complement regulatory proteins can result in altered or missing expression and/or function of these complement regulatory proteins, and are associated with diseases such as PNH, aHUS and affect the severity of malaria[1,2,8,9].

4.1 Paroxysmal nocturnal hemoglobinuria

PNH is a rare hallmark disease of dysfunctional complement regulation, characterized by hemolytic anemia, strong thrombophilia and bone marrow failure. PNH results from the clonal expansion of hematopoietic stem cells that are mutated in the X-linked phosphatidylinositol glycan class A (*PIG-A*) gene, which encodes for an enzyme that is required in the GPI-anchorage of proteins to the cell membrane. Complement regulators DAF and CD59, both being GPI-linked proteins, are lacking

← Fig. 1: Complement regulation on RBCs and consequences of dysregulation. (A) Membrane-bound complement regulators (DAF, CR1, CD59) and fluid phase complement regulators (C4BP, FH, FHL-1, FI) prevent further amplification of complement activation on RBCs. CR1 and DAF accelerates dissociation of the C3 convertase of all three complement activation pathways (C4bC2a and C3bBb). CR1 can also act as a co-factor for FI to inactivate both deposited C3b and C4b. FH and FHL-1 can bind to RBCs via sialic acids and accelerates the dissociation of the C3 convertase of the AP (C3bBb) and can inactivate deposited C3b together with FI, while C4BP accelerates the dissociation of the C3 convertase of the CP and LP (C4bC2a) and mainly inactivates deposited C4b together with FI. CD59 prevents the binding and polymerization of C9 and thus formation of the MAC. Lack of MCP expression on RBCs is the major difference as compared to complement regulation on nucleated cells. (B) Decreased expression and/or function of complement regulatory proteins results in opsonization of RBCs with the C3 convertase of all three complement activation pathways (C4bC2a and C3bBb) and degradation products (iC4b, C4d and iC3b, C3d) contributing to extravascular lysis due to clearance by spleen and liver macrophages and/or results in the formation of the MAC causing intravascular hemolysis of RBCs. Both ways of hemolysis contribute to the development of anemia. (C) Decreased expression and/or function of CR1 reduces immune adherence clearance that results in accumulation of immune complexes that contribute to a stronger inflammatory response and thus disease progression in patients.

on PNH cells, rendering these cells extremely vulnerable to complement mediated intravascular hemolysis. Hereby, cell-free hemoglobin and heme are released into the circulation resulting in oxidation and inactivation of plasma proteins and widespread cytotoxicity. In addition, cell-free heme may act as nitric oxide (NO) sink decreasing the bioavailability of NO, which finally results in endothelial cell dysfunction and platelet activation. Although being positive acute phase proteins, the natural scavengers for cell-free hemoglobin and heme, that are haptoglobin and hemopexin, are rapidly depleted due to consumption. Upregulation of inducible heme-oxygenase-1 degrading heme into carbon monoxide, ferric iron and biliverdin, the latter subsequently reduced to bilirubin, may in part compensate for the exhausted plasma scavengers[29-32]. Since complement is continuously spontaneously activated via the tick-over of the AP, hemolysis in PNH patients is mostly chronic, but may exacerbate during acute situations characterized by additional complement activation, such as infections[33-38]. The exact mechanisms leading to initial complement activation and C3 deposition on the RBCs of PNH patients and subsequent hemolysis are not well defined. It has been postulated that continuously low levels of complement activation via the spontaneous hydrolysis of C3 results in initial C3 deposition continued by persistent AP activation due to lack of DAF expression[37].

The only Food and Drug Administration (FDA)-approved drug for the treatment of PNH is eculizumab, a humanized monoclonal antibody that blocks C5 and thereby prevents the formation of the MAC. The drug can hereby compensate for the function of CD59 and abrogates intravascular hemolysis. Eculizumab is very effective as a treatment for PNH patients and it improves the quality of life of many PNH patients[39-41]. However, a subset of PNH patients treated with eculizumab, is still dependent on RBC transfusion which may be attributed to increased C3 deposition on the RBC membrane, which leads to clearance of RBCs by spleen and liver macrophages in a process called extravascular hemolysis. Large variation in C3 deposition on PNH RBCs is observed between patients which is not completely understood yet, although an association was found with a polymorphism in CR1. The L/L genotype results in lower expression of CR1 compared to the H/L genotype (intermediate expression) or H/H genotype (high expression)[42,43]. It has been reported that PNH patients with the CR1 L/L genotype have more C3 deposition on their RBC membranes and are less well responding to eculizumab[44]. Finally, it has been shown that, to a certain extent, FH protects PNH RBCs against hemolysis. Since polymorphisms and mutation for FH are described, it is suggested that PNH patients with lower levels of functional FH are more susceptible for hemolysis[45].

As opposed to PNH, where hematopoietic cells are deficient for both DAF and CD59, isolated DAF deficiency (Inab phenotype) is not associated with clinically evident hemolytic disease, although higher C3 deposition was observed on RBCs from

Inab individuals[46-48]. *In vitro* inhibition of CD59 on RBCs from the Inab phenotype using a blocking CD59 specific antibody resulted in increased C3 deposition and hemolysis[48]. This suggests that CD59 is superior to DAF in controlling the hemolysis of RBCs. This critical role of CD59 is supported by a case of a 7 month old patient presenting with bulbar syndrome. Acute febrile illness results in this child in a Coombs-negative hemolysis, acute kidney failure and neurological impairment[49]. In addition, Nevo et al.[50,51], reported several cases of a familial chronic Coombs-negative hemolytic anemia and relapsing polyneuropathy, which was finally caused by a missense mutation (p.Cys89Tyr) in CD59.

4.2 Atypical hemolytic uremic syndrome

aHUS is a rare disease that is characterized by thrombocytopenia, microangiopathic hemolytic anemia and renal failure. About half of the aHUS cases can be explained by mutations in complement regulation genes, including FH, FI and MCP. Mutations in FH are the most abundant mutations in aHUS patients and account for 20-30% of all reported mutations and in addition 5-10% aHUS patients have autoantibodies against FH[52-57]. Most of the aHUS associated mutations are clustered in the C-terminal domain of FH (especially in domain 19 and 20), an important surface binding region which is also a hotspot for autoantibodies against FH[56-62]. As a result, the binding of FH to C3b and glycosaminoglycans on host cell surfaces is impaired, leading to unwarranted alternative pathway activation on these cells, resulting in the formation of inflammatory mediators (such as C5a) and introduction of the MAC on endothelial cells and platelets. Subsequent platelet activation and endothelial damage result in thrombosis in the microvasculature and microangiopathic hemolysis due to mechanical rupture of RBCs[28,62-68]. Hemolysis can result in locally high levels of heme. It has been described that heme *in vitro* can activate complement via the AP in fluid phase and on endothelial cells. This indicates that heme may act as a secondary hit in amplifying damage in aHUS due to complement activation[69].

Little is known about the contribution of complement activation to the destruction of RBCs in aHUS. However, considering the contribution of FH in protecting PNH cells from lysis[45], it is conceivable that dysfunctional complement regulation may contribute to complement activation on the RBC membrane. C3 deposition on platelets of aHUS patients with FH mutations has been demonstrated by others[67]. We observed increased C3 deposition on RBCs when incubated with normal human serum in which FH was inhibited by monoclonal antibodies, indicating that intact FH function is mandatory to protect RBCs from spontaneous AP activation (unpublished data). However, in aHUS the Coombs test for complement is usually negative. This may indicate that either complement decorated RBCs are cleared rapidly, or complement deposition does not substantially occur in aHUS. Further research should establish

the consequence of compromised FH function and C3 deposition on RBC clearance in aHUS.

4.3 Dual role of genetic polymorphisms of CR1 in the pathogenesis of malaria

Malaria is an infectious disease that is caused by the transfer of the parasite *Plasmodium* by mosquitos. In humans *P. falciparum* is responsible for the most severe infections, accounting for most malaria related deaths. Severe anemia is a possibly lethal complication of malaria infection. *P. falciparum* infects and multiplies in RBCs causing rupture of the RBCs and invasion of even more RBCs. However, the total degree of RBC destruction cannot solely be explained by direct destruction by the parasite, as uninfected RBCs also undergo accelerated clearance by macrophages in the liver and spleen. It has been postulated that membrane destabilization on uninfected RBCs occurs due to antibody and heme binding both promoting C3 deposition on RBCs and subsequent clearance. In addition, it has been described that patients with malaria have dysregulated erythropoiesis, also contributing to anemia in malaria infected patients[70-73].

In malaria, the formation of RBC clusters due to the binding of infected to uninfected RBCs, called rosetting, can obstruct blood vessels and lead to impaired perfusion. Rosetting is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) that binds to CR1[74]. The level of CR1 expression on RBCs is associated with formation of rosettes and occurrence of severe malaria[75,76]. CR1 can also be used by P. falciparum as an entry receptor to invade RBCs, mediated by *P. falciparum* erythrocyte receptor 4 (PfRh4), which is important for *P. falciparum* for survival and reproduction. RBCs with the L/L genotype, so with lower expression of CR1, were shown to be less infected compared to RBCs with the H/H genotype, and soluble CR1 can block PfRh4-mediated *P. falciparum* growth. Interfering with the CR1-parasite interaction may therefore be a therapeutic approach for malaria[77,78]. However, the downside of lower CR1 expression is increased complement deposition on the membrane of RBCs and accelerated clearance via macrophages. In addition, the clearance of immune complexes via CR1 on RBCs will be reduced which may result in more tissue damage[79-82]. These results indicate a dual role of genetic polymorphisms of CR1 in the pathogenesis of malaria.

5. Secondary downregulation of complement regulation on RBCs

Besides primary genetic disorders leading to impaired complement regulation on RBCs, there are several clinical conditions associated with decreased complement regulation that is secondary to the condition, which will be reviewed below.

5.1 Autoimmune disorders

Systemic lupus erythematosus (SLE) is an autoimmune disease in which patients have autoantibodies against nuclear antigens such as DNA and histones released from apoptotic cells, resulting in the formation of immune complexes. These immune complexes can deposit in tissues, including skin, joints and kidneys resulting in local complement activation, inflammation and progression of the disease[83,84]. SLE is characterized by chronic complement activation, and consumption of complement proteins C3 and C4 levels are used as diagnostic marker for disease severity. In addition, it has been shown that SLE patients have increased levels of C4d on their RBCs and C4d-positive RBCs are suggested as a biomarker for SLE disease activity[85-88]. Highest C4 deposition on RBCs was observed in SLE patients with hemolytic anemia, although levels of C4d deposition on RBCs did not correlate with disease activity due to persistent high levels on C4d on RBCs[86]. Moreover, C4d deposition on RBCs has also been observed for patients with primary antiphospholipid syndrome[88]. This indicates that C4d deposition on RBCs to serve as biomarker for disease activity has limitations and is not completely specific for SLE. Although C4d deposition on RBCs may not be disease specific, it has been shown in SLE patients that C4d deposition on RBCs results in production of NO and decreased cell deformability[89] rendering the RBCs more vulnerable for lysis while passing microvessels, thereby contributing to the pathogenesis of SLE. Many SLE patients suffer from secondary anemia caused by chronic inflammation, iron deficiency, renal insufficiencies or drug-induced bone marrow suppression[90,91]. Next to producing autoantibodies against intracellular material, SLE patients may also generate autoantibodies against their own RBCs. These autoantibodies can activate the complement system and cause hemolysis of RBCs, a phenomenon called autoimmune hemolytic anemia (AIHA), which has been reported as secondary condition in approximately 10% of SLE patients[90,91]. Moreover, AIHA is not only associated with SLE, but also lymphoproliferative disorders, immunodeficiency disorders, infections and drugs can trigger AIHA[92,93].

As mentioned above, CR1 on RBCs is very important in clearing complement opsonized particles via so called immune adherence clearance, including cell debris and immune complexes covered with complement. In SLE reduced expression of CR1 on RBCs was described compared to healthy individuals. Therefore, immune adherence clearance may be impaired and these immune complexes can deposit in tissues resulting in the progression of disease in SLE patients[94-97]. Decreased CR1 expression may be explained by proteolytic cleavage, during removal of immune complexes by phagocytic cells containing proteases. This was shown *in vitro* by mild trypsin treatment, plasmin or thrombin digestion[98]. Of note, other autoimmune diseases, such as Sjögren's syndrome and rheumatoid arthritis, are also associated

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with decreased expression of CR1 on RBCs, but no hemolysis or anemia were reported[99-101].

In addition to decreased CR1 expression, it has been reported that the expression of DAF and/or CD59 is decreased on the RBCs of SLE patients, suggesting that RBCs from SLE patients are more vulnerable to complement mediated hemolysis[97,102,103]. It has been shown in one study that SLE patients have decreased expression of both CR1 and CD59[97]. However, in the other two mentioned studies, CR1 was not measured simultaneously with DAF and CD59 expression[102,103]. Moreover, reduced DAF expression was not always associated with decreased CD59 expression and vice versa[97,103]. So, it is not clear whether decreased complement regulator expression in SLE is linked or heterogeneous. Moreover, it is still under debate whether this decreased expression of DAF and CD59 occurs only in SLE patients with secondary AIHA, as conflicting data were published[97,102,103].

5.2 Infectious diseases

Besides that genetic polymorphisms of CR1 affecting malaria have been reported (described above in section 4.3 Dual role of genetic polymorphisms of CR1 in the pathogenesis of malaria), it has been described that severe malarial anemia is associated with decreased levels of CR1 and DAF on RBCs. These deficiencies corrected upon treatment of malaria indicating that this reduction in complement regulatory proteins is most likely acquired and thus secondary to the disease. It is thought that CR1 is lost from the RBC membranes during removal of the immune complexes by macrophages of the reticuloendothelial system (immune adherence clearance) whereby CR1 is proteolytically cleaved off. The mechanism by which DAF is lost from the membrane is unknown. Decreased levels of RBC CR1 and DAF result in reduced immune complex binding and increased complement deposition on RBCs during malarial infection, leading to accelerated clearance of these RBCs[79-82,104]. However, it has been reported that decreased expression of DAF may also be beneficial as DAF is important for P. falciparum to invade RBCs, but via which mechanism is still unknown[105]. One study reported that the expression of CD59 was increased during *P. falciparum* infection[79], while another study showed that the expression of CD59 decreased, although not significantly[104]. For *P. falciparum* infected RBCs it has been shown that CD59 protects against hemolysis[106]. Altogether, complement regulatory proteins CR1 and DAF seem to play a dual role in malaria infection. On the one hand, both membrane proteins have been described to play a role in parasite entry into RBCs. On the other hand, secondary downregulation of CR1 and DAF are associated with accelerated destruction of RBCs and are found in patients with severe malaria anemia. Noteworthy, also patients with human immunodeficiency virus (HIV) infections have decreased expression of DAF and

CD59 on their RBCs, but this was not associated with hemolysis and anemia[107].

5.3 Hemoglobin disorders

Thalassemias are blood disorders resulting from abnormalities in the synthesis of subunits of hemoglobin. β-thalassemia major is the most severe form of thalassemia where the β-globin subunit is affected. β-thalassemia major patients suffer from severe anemia and are critically dependent on blood transfusions[108-110]. Reduced expression of DAF on RBCs has been described in β-thalassemia major, which correlated with disease severity, while CD59 expression was normal. This suggests downregulation of DAF via an unknown mechanism that is secondary to the disease[109,110]. Evidently, DAF deficiency in β-thalassemia major patients is not the major cause of hemolysis, but it may contribute to the severity of the disease by increased complement deposition on affected RBCs. Worth mentioning, it has been reported that patients with sickle cell disease, another disease of dysfunctional hemoglobin and hemolytic anemia, show decreased expression of both DAF and CD59 on their RBCs[111,112]. The potential contribution to RBCs destruction in both diseases still needs to be established.

5.4 Pregnancy and pre-eclampsia

Pre-eclampsia is a condition of pregnancy, in which the mother suffers from high blood pressure and proteinuria, possibly being lethal for both mother and fetus. In some women, pre-eclampsia is accompanied by RBC hemolysis. Reduced expression of CR1 and DAF on RBCs has been reported in pregnant women, with the lowest expression reached in the third trimester of the pregnancy. The expression of CD59 on RBCs was not affected[113]. The expression of CR1 was lowest in women with pre-eclampsia[114]. However, the observed decreased expression of DAF and CR1 was not associated with increased C3d binding on the RBCs[113], suggesting that this is not the cause of hemolysis in pregnant women.

5.5 Storage lesion

Red cell concentrates (RCCs) are used for transfusion during conditions associated with tissue hypoxia, which is most often for the treatment of critically ill patients and patients undergoing surgery. Worldwide about 85 million units of RCCs are transfused per year[115]. These RCCs are stored at 4ºC before they are given to patients. During storage, RBCs undergo several biochemical and structural changes, that are collectively known as the "storage lesion"[116-119].

After a RCC transfusion, 10-25 percent of donor RBCs are cleared from the circulation of transfused recipients within 24 hours[120]. It has been shown that C3 fragments and MAC components accumulate on the membrane of RBCs during

storage[121-124]. Moreover, survival of RBCs was negatively correlated with both the duration of storage and the amount of C3 deposition[121,122], suggesting that complement activation contributes to the accelerated clearance of transfused RBCs. It has been shown that the expression of membrane regulators CR1, DAF and CD59 is reduced on stored RBCs[124-126]. However, we did not observe decreased expression of membrane bound complement regulators CR1, DAF and CD59 on RBCs that were stored under contemporary storage conditions. Moreover, in our hands, both complement deposition and phagocytosis by macrophages of stored RBCs was very low (manuscript in submission). Altogether, it may be questioned whether decreased expression of complement regulatory proteins on RBCs is responsible for the rapid loss of transfused RBCs.

6. CONCLUSION & DISCUSSION

Membrane-bound complement regulators CR1, DAF and CD59, and fluid phase complement regulator FH and FI are important to prevent complement activation on RBCs (Fig. 1A) during their constant contact with complement in blood plasma. Complement regulation on RBCs differs from that on nucleated cells in a sense that expression of MCP is lost during erythropoiesis, as erythroblasts do express MCP and mature RBCs are lacking MCP. Under normal conditions, complement regulatory proteins seem to be redundant. However, decreased expression and/or function of complement regulatory proteins, due to either primary genetic mutations in genes encoding for complement regulators or secondary downregulation, may result in detrimental complement activation on RBCs depending on which regulator is affected and the underlying disease (Fig. 1B). Complement deposition on RBCs, as result of defective regulation, may lead to extravascular hemolysis by macrophages. This is caused by the opsonization of RBCs with C3b, iC3b and/or C3d that interact with complement receptors present on the membrane of spleen and liver macrophages[127-130]. Strong complement activation on RBCs may lead to intravascular hemolysis via the formation of MAC, resulting in the release of toxic hemoglobin products in the circulation such as free heme. Both extravascular and/or intravascular hemolysis can contribute to the development of anemia. Moreover, extravascular and/or intravascular hemolysis occurs not only in conditions were decreased expression and/or function of complement regulatory proteins are described, but also in conditions in which antibodies against RBCs are present such as AIHA. In this disease, the CP is activated which therefore might be a good target for complement inhibition[127,128,131]. In addition, decreased CR1 expression and/or function may lead to impaired immune adherence clearance. This does not have a direct effect on RBCs, but contributes to the progression of disease by the deposition

of these immune complexes in tissues and the induction of an inflammatory response (Fig. 1C). Secondary downregulation of complement regulatory proteins on RBCs is not due to gene regulation since RBCs do not contain a nucleus anymore. It has been shown though, that RBCs can release vesicles containing CR1, DAF or CD59[132,133]. Increased vesiculation of RBCS during disease may therefore be a mechanism of the observed secondary downregulation of complement regulatory proteins in several diseases. In addition, it has been postulated that CR1 is lost from the RBC membrane by proteolytic cleavage during transfer of immune complexes to macrophages in immune adherence clearance98. The primary hematological consequences of all conditions mentioned in this review are summarized in Table 1. As complement activation can lead to accelerated RBC clearance via extravascular and/or intravascular lysis, inhibition of complement activation may be a potential therapeutic strategy to prevent anemia. As blocking complement may infer risk for infections, the benefits of complement inhibition should be balanced with the possible risks. Mild anemia due to reduced complement regulation probably does not require complement inhibition as therapy. However, in diseases with strong complement-mediated destruction of RBCs, therapeutic complement inhibition may be a good option. The best example of this is the successful use of eculizumab (anti-C5) in PNH patients, that completely abrogates intravascular hemolysis. Severe malarial anemia may be a target for complement inhibition as well, as anemia is one of the most life threatening complications of malaria. First *in vitro* results with C3 inhibiting peptide Compstatin Cp40 in malaria induced C3 deposition on RBCs were promising[134]. Targeting complement at the level of C3 may be preferred to prevent anemia as most conditions with reduced complement regulation lead to increased C3 deposition and extravascular RBC clearance, rather than intravascular hemolysis. Destruction of RBCs by antibodies, as occurring in AIHA (either or not secondary to SLE), may be best targeted by upstream inhibition of the classical pathway of complement, for example by C1-INH or TNT009, a therapeutic monoclonal antibody that inhibits the classical pathway protease C1s. Both plasma-purified and recombinant C1-INH are already used in the clinic and the therapeutic antibody TNT009 is currently under investigation in clinical trials[127,128,135-141].

In summary, complement regulation on RBCs may be affected by primary genetic mutations or secondary downregulation of complement regulators from the membrane. Reduced complement regulation may lead to complement deposition on RBCs which is most obvious on RBCs with primary genetic deficiency of complement regulators as in PNH. Targeting complement activation in hemolytic conditions with reduced complement regulation may be a therapeutic option to recue severe anemia. For some conditions, such as severe malaria anemia, this is already under investigation and first results seem to be promising.

Table 1

Conditions with primary genetic deficiencies or secondary downregulation of complement regulator proteins on RBCs

Condition	Affected regulators	Primarily Hematological Consequence	Refs
Primary genetic deficiencies of complement regulators			
Paroxysmal nocturnal hemoglobinuria (PNH)	DAF, CD59	Anemia	[34,37,38]
Inab phenotype	DAF	x	[46-48]
Inherited CD59 deficiency	CD59	Anemia	[49-51]
Atypical hemolytic uremic syndrome (aHUS)	FH, FI	unknown	[28,62,66]
Malaria	CR1	Anemia More immune complexes	[76,78,79]
Secondary downregulation of complement regulators			
Systemic lupus erythematosus (SLE)	CR1, DAF, CD59	Anemia/AIHA More immune complexes	[87,97,102]
Sjögren's syndrome	CR1	More immune complexes	[99]
Rheumatoid arthritis	CR1	More immune complexes	[100,101]
Malaria	CR1, DAF, CD59	Anemia More immune complexes	[79,82,104]
Human immunodeficiency virus (HIV)	DAF, CD59	x	[107]
β-thalassemia major	DAF	x	[109,110]
Sickle cell disease	DAF, CD59	x	[111,112]
Pregnancy and pre-eclampsia	CR1, DAF	More immune complexes	[113,114]
Storage lesion	CR1, DAF, CD59	x	[124-126]

7. Practice Points

- RBCs do not have a nucleus, do not express MCP and CR1 is highly expressed compared to nucleated cells.
- Decreased expression and/or function of complement regulatory proteins may result in detrimental complement activation on RBCs depending on which regulator is affected and the underlying disease.
- Both extravascular and/or intravascular hemolysis can contribute to the development of anemia
- Decreased CR1 expression and/or function may lead to impaired immune adherence clearance.

8. Research Agenda

Options of therapeutic complement inhibition in disease with very strong complement-mediated destruction of RBCs need further investigation.

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