Regulation of complement activation on red blood cells

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Chapter 8

Summarizing Discussion

The complement system plays a pivotal role in human health and disease. On the one hand, the complement system is involved in the clearance of pathogens, dying cells and immune complexes. Consequently, deficiencies in the complement system may lead to increased risk of infections and autoimmunity. On the other hand, excessive complement activation due to inefficient regulation and/or overstimulation can contribute to inflammation and the pathogenesis of many (often immune-mediated) diseases[1-3]. Thus, it is of utmost importance that regulatory proteins of the complement system are present on human cell surfaces and in blood plasma that act specifically on healthy host cells to protect against unwanted complement activation, while leaving pathogen clearance intact[4-6]. In a healthy body, complement activation and regulation are well-balanced.

While circulating in the body, red blood cells (RBCs) are continuously in contact with complement components in the blood plasma[7], which makes proper complement regulation on these cells indispensable. This is illustrated by the fact that various diseases are associated with complement-mediated RBC destruction. This destruction may be antibody-mediated, such as in autoimmune hemolytic anemia (AIHA) and allo-immunization. Alternatively, RBC death may be the result of defective complement regulation, as occurring in paroxysmal nocturnal hemoglobinuria (PNH; CD55/CD59 deficiency) and atypical hemolytic uremic syndrome (aHUS; associated with mutations in complement regulation proteins). Complement-mediated destruction of RBCs can result in anemia, requiring treatment when severe. RBC transfusions are given to restore oxygen transporting capacity in patients suffering from sudden blood loss or to patients that have chronic breakdown of their RBCs[8-10]. Disease severity and response to existing (complement inhibiting) therapeutics is variable in patients. This may have to do with the natural genetic variation in complement components, that impacts the potency of complement activation. This genetic make-up has been coined the complotype. The complotype may have a major impact on susceptibility to inflammatory and infectious diseases and influences the severity of these conditions[11]. This illustrates the urgent need to better understand how the balance between complement activation and regulation is maintained on different cell surfaces. The studies in this thesis focus on various aspects of regulation of complement activation on RBCs, the results of which will be discussed in this chapter.

Complement regulation on RBCs

The importance of complement regulation on RBCs is emphasized by the fact that rabbit RBCs, which are completely devoid of any complement regulators that interact with human complement, are completely lysed within minutes upon incubation with human serum, while human RBCs have a normal life span of approximately 120 days in circulation[12,13]. Healthy human cells are equipped with several complement

regulating proteins, either expressed on the cell surface or acting in the fluid phase, for protection against unwanted detrimental effects of complement activation.
Interestingly, RBCs have a different composition of membrane expressed complement regulators than nucleated cells including hematopoietic stem and progenitor cells. Mature RBCs express the membrane bound complement regulators CD35, CD55 and CD59, but not CD46[4]. As hematopoietic progenitor cells do express CD46, this indicates that CD46 is lost during erythropoiesis. In **Chapter 3**, we investigated the dynamics of CD46 expression during erythropoiesis. We showed that CD46 follows a similar expression pattern as transferrin receptor CD71 during erythropoiesis and loss of expression occurred mainly before enucleation. CD46 may therefore be used as a surrogate erythroid differentiation progression marker next to CD71, thereby increasing the panel of markers that can be used to monitor progression of erythropoiesis. As CD46 can serve as an entry receptor for different pathogens, such as viruses (measles, human herpes virus 6, different serotypes of adenovirus) and bacteria (Streptococcus pyogenes and Neisseria species). However, RBCs do not have a nucleus, so they are incapable to support virus replication[14-16]. Therefore, it has been suggested that the lack of CD46 on mature RBCs prevents dissemination of pathogens. Most remarkable, we found that CD35 was already expressed early in erythropoiesis showing that proerythroblasts uniquely express both CD35 and CD46, a phenotype that possibly makes these cells extra resistant against complement activation.
Besides membrane bound complement regulators, fluid phase complement regulator factor H (FH) is important in the regulation of complement activation on RBCs. In **Chapter 5**, we demonstrate that FH plays a key role in protecting RBCs against spontaneous complement activation in homeostatic conditions by making use of 20 novel anti-FH monoclonal antibodies. These were generated and characterised for their binding site on the FH molecule and the functional consequences of this binding in several standard complement assays. Inhibition of FH resulted in C3 deposition on a subset of RBCs, despite the presence of membrane regulators CD35, CD55 and CD59. In addition, we showed that besides the well-established role of FH binding domains 19-20, binding domains 6-8 seem to be important for complement regulation on RBCs. So apparently, binding of FH via both binding domains is required for optimal protection of RBCs against complement regulation. It may be that SNPs or mutations in one of the two binding domains affect protection of the RBCs against complement-mediated lysis. aHUS is characterized by excessive complement activation in the kidney, due to SNPs or mutations in complement regulation proteins, such as FH. Destruction of RBCs in aHUS patients has been explained by mechanical rupture of RBCs in the small vasculature. It is, however, well possible that mutated FH can also result in more C3 deposition and C9 deposition on

RBCs in aHUS patients, thereby increasing the clearance of RBCs by opsonization or by MAC formation and thus lysis. Indeed, this has been reported to occur on platelets in aHUS patients[17]. In addition, also in PNH it has been shown that FH plays a role in the protection of RBCs against hemolysis[18,19]. Further research is required to investigate whether FH mutations indeed contribute to the destruction of RBCs in aHUS, for example by incubation of RBCs with serum of aHUS patients to investigate complement deposition. In addition, the set of anti-FH monoclonal antibodies may be helpful to narrow down the involved binding domain(s) of FH.

Complement and storage lesion

Worldwide about 85 million units of RBCs are transfused per year[9,20]. After collection, RBCs are stored at 2-6 ºC before use and during this storage RBCs undergo several biochemical and structural changes, collectively known as "storage lesion"[21-24]. Nowadays, it is still under debate whether storage of RBCs has harmful effects for transfusion recipients. It is clear though, that 10-25 percent of transfused donor RBCs are cleared within 24 hours from the circulation of the recipient[25]. Possibly, complement deposition and antibody binding, both facilitating phagocytosis, may contribute to the rapid clearance of donor RBCs. Previous research has demonstrated that upon storage of RBCs complement deposition increased significantly[26-29]. However, until recently it was unknown whether this occurs on RBCs that are collected and stored under current Dutch blood bank conditions and whether this indeed leads to the clearance of a substantial fraction of donor RBCs.

In **Chapter 4**, we report that under current Dutch blood bank conditions there is no complement deposition on stored RBCs as present in a red cell concentrate. However, we found that upon contact with complement in human serum *in vitro*, as also occurring during transfusion *in vivo*, RBCs are susceptible to both complement deposition and antibody binding, although independent of storage time. However, the extent of complement deposition and antibody binding on stored RBCs was not sufficient for the phagocytic uptake by monocyte-derived macrophages *in vitro*, suggesting that besides complement deposition and antibody binding on stored RBCs other factors such as decreased deformability of RBCs are involved in clearance of donor RBCs in recipients. As follow up study on the *in vitro* results, it would be interesting to verify *in vivo* whether complement deposition and IgG binding on transfused donor RBCs occurs in patients. Ideally, the cohort study will consist of patients who will experience multiple exposures to RBC transfusion (both short and long stored RBCs) and multiple measurements during follow-up (1 hour, 24 hours, 1 week and 2 weeks). The short time point measurement (1 hour and 24hour) will reflect the quality of the RBCs and the long time point measurements (1 week and 2 weeks) will provide insight on the inherent RBC turnover rate in the

recipient. Moreover, this would give more insight on the influence of storage period of RBCs *in vivo* and complement deposition and antibody binding to the transfused RBCs can be assessed. Tracing donor RBCs in recipient patients might be done for example by discriminating donor RBCs from recipient RBCs by staining for differences in minor antigens, i.e. minor blood groups that are not matched between donor and recipient or by labeling donor RBCs with biotin[30,31]. Both techniques have advantages and disadvantages that are mentioned in [32]. In short, the advantages of using minor antigen mismatch are that RBCs are not manipulated before transfusion and recovery of a whole concentrate can be determined, but disadvantages are that only compatible transfusion with at least one minor antigen difference can be studied. Advantages for biotin labeling are that also autologous RBC transfusions can be studied and the characteristics of the transfused RBCs can also be observed after transfusion, but disadvantages are that recipients can develop anti-biotin antibodies, RBCs are manipulated before transfusion and only a small volume can be transfused[32]. These disadvantages may not be counting anymore as recently a sterile closed system has been described for biotin labelling of a whole concentrate and the biological activity of these RBCs was not affected[33]. Apart from their capacity to detect donor RBCs in blood samples by flow cytometry, both techniques can also be used to isolate the donor RBCs from blood samples of the recipient. This allows the analysis of other properties of the donor RBCs such as their metabolic status, rheologic properties and susceptibility to *in vitro* phagocytosis. In addition, a pure population of donor RBCs can be used for proteomic analysis, which may lead to the identification of yet unknown changes occurring in RBCs after transfusion.

Complement and hemolytic disease

PNH is a hallmark disease of defective complement regulation, which is characterized by anemia, thrombophilia and bone marrow failure. PNH RBCs are deficient for membrane bound regulators CD55 and CD59, due to a mutation in the PIG-A gene that is responsible for GPI-anchorage of proteins including CD55 and CD59[34,35]. As mentioned in **Chapter 3**, RBCs lack CD46, but they do express CD35, which has a similar complement regulatory function as CD46. Due to the lack of complement regulators CD55 and CD59 on a clone of hematopoietic cells, these cells are dependent on CD35 and FH for protection and are therefore very sensitive for complement-mediated destruction. So far, it is not well defined via which mechanism C3 deposition on RBCs of PNH patients occurs and subsequently results into hemolysis. One postulated mechanism is that low levels of continuous complement activation via spontaneous hydrolysis of C3 result in initial C3 deposition, which is then propagated by persistent alternative pathway (AP)-activation due to lack of CD55 expression[34].

As access to patient cells may be limited and cells can be heterogeneous between different patients, we generated single and combined complement regulator human HAP1 knockout cells using CRISPR/Cas9 to provide us with a tool for future research on *in vitro* complement activation and dysregulation on human cells. The results are described in **Chapter 6**. We confirmed the reported function of complement regulation by CD46 and CD55 upon CP activation, namely deletion of CD46 resulted in increased C3 and C4 deposition, while deleting CD55 mainly resulted to increased C3 deposition. Remarkably, we observed no spontaneous AP-mediated C3 deposition on the HAP1 cells lacking CD46 and/or CD55. This result surprised us, as it has been described that C3 deposition occurs on RBCs of PNH patients that are deficient for CD55 and CD59. C3 deposition is however mainly observed on circulating RBCs in PNH patients that are treated with eculizumab (anti-C5), that prevents hemolysis upon complement deposition on the RBCs. The amount of the observed C3 deposition is very variable between PNH patients[34], which is as yet unexplained and deserves further research. When, in addition to knocking out CD46/CD55, FH in the serum was inhibited by a monoclonal antibody, we observed significant AP-mediated C3 deposition, again pointing out the importance of fluid phase FH for the protection of the cell surface against spontaneous C3 activation. It is however remarkable that we only observed an effect of FH inhibition on AP-mediated C3 deposition on HAP1 cells when both CD46 and CD55 are deleted, and not on the wildtype cells, considering that blocking of FH resulted in AP-mediated C3 deposition on healthy RBCs that express CD35, CD55 and CD59 (**Chapter 5**). As FH binds to RBCs mainly via deposited C3b and poly-anionic residues such as sialic acid[36], it is possible that RBCs express more sialic acids compared to HAP1 cells, thus rendering RBCs more dependent on FH for protection against complement.

In **Chapter 6** we described a potential novel role of CD59 in regulating the AP C3 convertase, next to its well-described function in inhibiting the MAC. As opposed to PNH RBCs that are deficient for both CD55 and CD59, isolated CD55 deficiency (Inab phenotype) is not associated with clinically evident hemolytic disease, although higher C3 deposition has been observed on RBCs from Inab individuals[37-39]. In line with our results on CD46/CD55/CD59 deficient HAP1 cells, *in vitro* inhibition of CD59 with a blocking antibody on both healthy RBCs and RBCs from the Inab phenotype, resulted in increased C3 deposition[39-41]. Our results on HAP1 KO cells and the reported results on Inab RBCs suggest a role for CD59 on C3 regulation. However, it still needs to be elucidated via which mechanism CD59 affects C3 regulation and whether this is physiologically relevant. Two obvious options for C3 regulation by CD59 are decay accelerating activity and cofactor activity for factor I, that are known mechanisms for CD55, CD35, CD46 and FH regulation. However, it can also be a yet unknown mechanism. The finding that CD59 can act as regulator for C3 activation,

in addition to its function as inhibitor of the MAC, adds to the understanding of the pathogenesis of PNH on which mechanism C3 deposition on RBCs of PNH patients occurs.

Instead of studying the role of specific complement regulators, the CRISPR/Cas9 approach can also be used to directly target the PIG-A gene in HAP1 cells or even more clinically relevant in erythroblasts before transforming them into reticulocytes to further investigate the pathogenesis of PNH in a constant cell line rather than in patient-derived RBCs. The PIG-A gene is located on the X-chromosome[35], thus a single mutation in erythroblasts derived from a male donor should be sufficient to lead to a complete knockdown of the gene. Moreover, as described in **Chapter 3** during *in vitro* culturing of erythroblasts towards reticulocytes, the cells loose CD46 expression during maturation which seems mainly to occur before enucleation of the cells. Therefore, we expect that the manipulated cells will be positive for CD35, but negative for CD55, CD59 and CD46 and thus resembling PNH RBCs. In addition, targeting the complete PIG-A gene may also shed light on why we did not observe AP-mediated C3 deposition on HAP1 cells that were targeted for CD46 and/or CD55, while C3 deposition does seem to occur on RBCs of PNH patients that are deficient for CD55/CD59 due to a mutation in the PIG-A gene. Moreover, in the above-mentioned chapters, we only investigated the role of the known membrane bound and fluid phase complement regulators. Genome wide screens can be performed to identify yet unknown complement regulatory proteins. Two examples of genome-wide screens that can be performed are based on haploid genetics or CRISPR/Cas9. For haploid genetic screens, HAP1 cells undergo randomly gene trap mutagenesis, while for CRISPR/Cas9 screens, specific genomic loci in human cells are targeted[42,43]. Both methods would lead to a heterogeneous population of cells each knockout for a different set of genes. These cells can be exposed to complement in normal human serum and subsequently be analyzed for C3 and C4 deposition. Cells that are screened with significantly lower or higher complement deposition compared to wild type cells are of interest, because this might be caused by hit genes of known complement regulators (that serve as control) or genes are hit that might shed light on yet unknown complement regulatory proteins. Haploid genetic screens seem to be more powerful compared to the CRISPR/Cas9 screens; the latter however can be used on diploid cells allowing to elucidate mechanisms that do not occur in haploid HAP1 cells.

Complement and therapeutic options

Besides PNH, AIHA is another disease that is associated with clinical anemic conditions. AIHA is characterized by the formation of autoantibodies against RBCs, with or without complement activation. RBC specific antibodies can activate the

complement system resulting in antibody and/or complement opsonized RBCs that may be cleared by phagocytes via Fc-gamma receptors and/or complement receptors causing extravascular lysis. Moreover, strong complement activation results in formation of the MAC, causing intravascular lysis[44-46].

As the AP amplification loop is considered to have a major contribution to total complement activation (80%) regardless which pathway originally initiated the response[47,48], the AP may be a potential therapeutic target for several complement-mediated diseases, including autoantibody-mediated diseases such as AIHA, by intervening in the amplification loop of complement activation. In **Chapter 7**, we demonstrated however that in the context of AIHA, there is only limited (if any) contribution of the amplification loop to total complement activation on human RBCS when inducing classical pathway (CP) activation via antibodies. This indicates that the CP can induce efficient complement activation by itself while bypassing the amplification loop. Therefore, for antibody-mediated diseases, such as AIHA, therapeutic options that target the upstream activation of the CP of complement, for example C1-INH or TNT009, may be the best option[44,49-51]. Besides using the described HAP1 knockout cell lines to investigate the pathogenesis of complement-mediated diseases, these cell lines may assist in the development and testing of novel therapeutic complement inhibitors for the treatment of complement-mediated diseases. This includes AIHA, as we demonstrated in **Chapter 6** that also on HAP1 cells complement deposition was most probably caused by CP activation via antibodies.

In addition, as the complement activation pathways merge at the level of C3 and C5, these are logical targets for the development of therapeutic complement inhibitors. Blocking at the level of C3 shuts down the formation of anaphylatoxins, and prevents opsonization with C3b and thus rapidly reduces the inflammatory reaction as well as clearance via opsonophagocytosis. Blocking at the level of C5 prevents the formation of the MAC and the production of the anaphylatoxin C5a, while leaving opsonophagocytosis via C3 deposition intact. As already mentioned in **Chapter 2**, targeting complement at the level of C3 will probably be most successful to prevent anemia in conditions where there is extravascular clearance, while blocking at the level of C5 will be most successful to prevent anemia in conditions where there is intravascular clearance. Indeed the first *in vitro* results for C3 inhibiting peptide Compstatin are promising to reduce C3 deposition on RBCs in malaria[52] and eculizumab is successfully being used in PNH patients to completely abrogate MAC formation and thus inhibit intravascular hemolysis[53-55]. The use of eculizumab is also successfully used for the treatment of aHUS[56,57]. Therapeutic blocking of complement activation at the level of C3 or C5 however, results in the shutdown of the final effector cascades of the complement system. Furthermore, deficiencies in

the early complement components, such as C1q, C2, C3 and C4 are associated with recurrent bacterial infections and auto-immune disease, while deficiencies in the terminal complement components, such as C5, C6, C7, C8 and C9 are associated with increased risk of infections with encapsulated bacteria and in particular Neisseria infections[58,59]. This indicates that therapeutic blocking of complement may result in increased risk for infections. However, as shown in **Chapter 7**, in the presence of antibodies the CP is sufficient for *in vitro* killing of bacteria while bypassing the amplification loop. Thus, as already mentioned in **Chapter 2**, the benefits and risks of complement inhibition should be carefully considered. As such, mild anemia might not require complement inhibition as therapy, while complement inhibition may be a good therapeutic option in severe anemia with complement-mediated destruction of RBCs.

Future recommendations

Figure 1 shows an overview of how complement activation is regulated on RBCs as described in this thesis. Our results demonstrated that both membrane bound complement regulators CD35, CD55 and CD59 as well as fluid phase complement regulator FH are important in protecting RBCs against unwanted complement activation. To be able to restore oxygen transporting capacity in anemic patients, RBCs are collected from healthy donors and in The Netherlands may be stored up to 35 days at 2-6 ºC before transfusion. During storage, RBCs undergo several biochemical and structural changes that might underlie the fast clearance of part of the donor RBCs. We demonstrated *in vitro* that complement deposition and IgG binding occur only on stored RBCs upon contact with normal human serum independent of the storage time, while not occurring in the red cell concentrate itself. We however recommend to perform *in vivo* studies to verify whether complement deposition and antibody binding occur on transfused donor RBCs in patients, and whether this affects clearance of donor RBCs. RBC transfusions are given in clinical anemic conditions, such as PNH, in which RBC death results from defective complement regulation and AIHA, that is characterized by antibody-mediated RBC destruction. By creating HAP1 KO cell lines, we resembled PNH cells and obtained new insights about the function of complement regulators. We think it is very useful to also directly target the PIG-A gene in erythroblasts before transforming them into reticulocytes to further obtain knowledge about the pathogenesis of PNH. The HAP1 KO cell lines are also a promising tool for testing novel therapeutic complement inhibitors for the treatment of antibody-mediated diseases such as AIHA and may shed light on possible novel roles of complement regulatory proteins. Furthermore, for therapeutic treatments, we have to bear in mind that *in vitro* results cannot always be extrapolated to the situation on human cells or bacteria *in vivo*. We demonstrated

that in presence of antibodies, CP initiated complement activation can result in total complement activation while bypassing the amplification loop, indicating that intervention in the amplification loop may not be an effective therapy for antibody-mediated diseases.

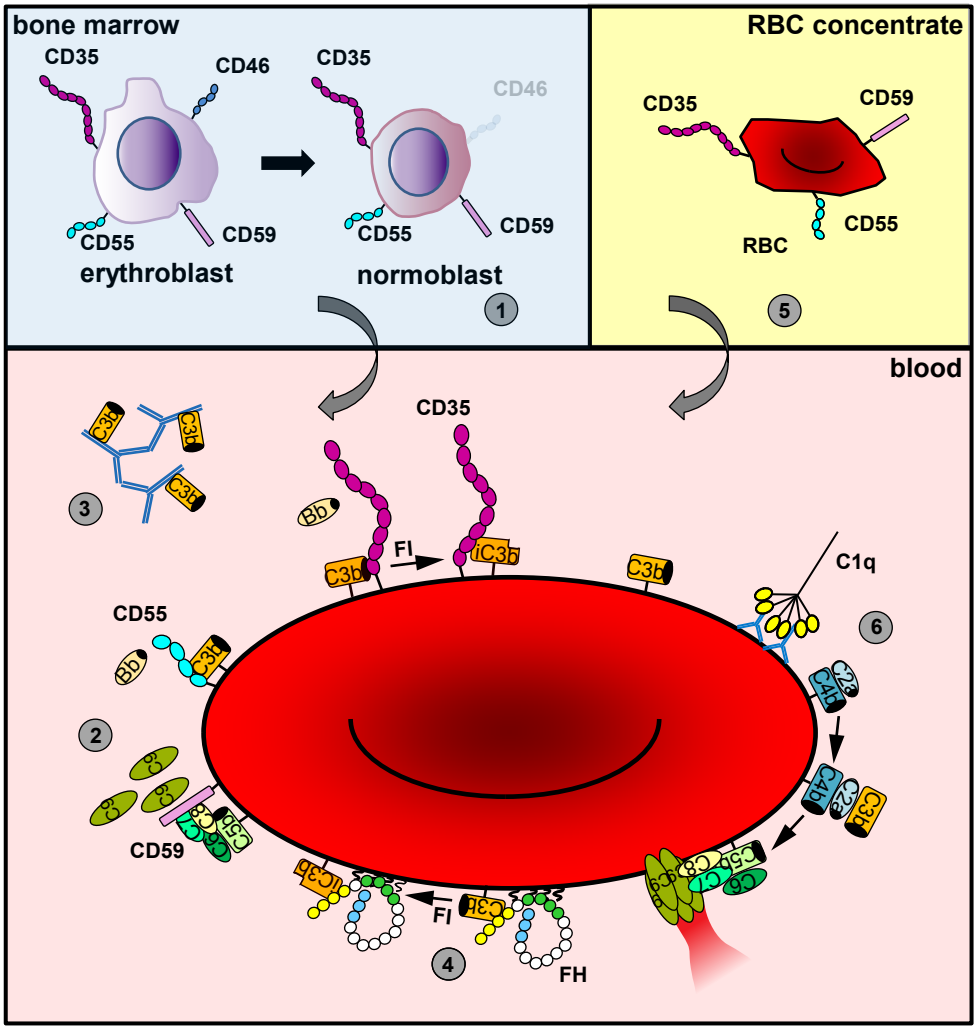

Figure 1: Overview of complement regulation on red blood cells (RBCs). (1) During erythropoiesis the expression of CD46 on precursors cells decreases (faded symbol) and is completely lost on mature RBCs. **(2)** Mature RBCs express complement regulators CD35, CD55 and CD59 on the membrane. (3) CD35 is important for immune complex adherence clearance. **(4) F**luid phase complement regulator FH plays a key role in protecting RBCs against spontaneous complement activation. **(5) I**n a RBC concentrate (storage lesion indicated in figure by deformed RBC) no complement deposition occurs, but upon *in vitro* contact with complement in human serum RBCs are susceptible for complement deposition and antibody binding. **(6)** Antibody binding can induce efficient CP complement activation resulting in total complement activation while bypassing the amplification loop.

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