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### Regulation of complement activation on red blood cells

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# **Chapter 1**

**Short introduction and scope of this thesis**



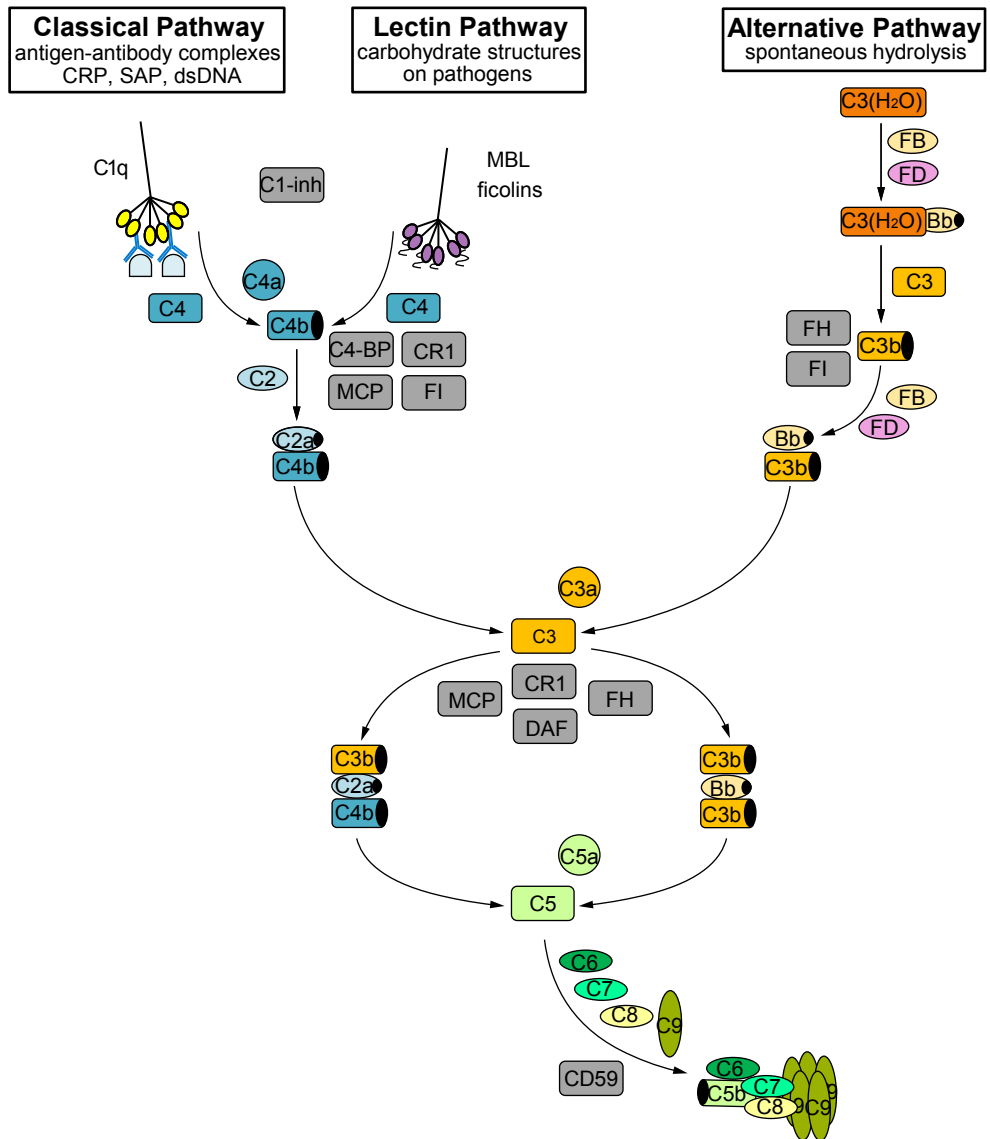
## Complement system

The complement system plays a central role in our innate immune system and consists of more than 30 plasma and membrane-associated proteins. It constitutes a first line of defense against many pathogens due to the facilitation of phagocytosis by opsonization via C3b and C4b, causing osmolytic lysis of bacteria via the formation of the membrane attack complex (MAC/C5b-9) and induction of an inflammatory response by the release of anaphylatoxins (C5a, C3a). Next to pathogen clearance, complement also plays an important role in removing damaged or dying cells from the body<sup>1-4</sup>.

Activation of the complement system can occur via three pathways, which are activated via separate mechanisms, but all eventually converge to a common terminal pathway (Fig. 1). The classical pathway (CP) of complement system is activated by binding of C1q to antibody-antigen complexes or to structures like CRP, SAP and dsDNA. The lectin pathway (LP) of the complement system is activated by binding of mannan binding lectin (MBL) or ficolins to carbohydrate structures on pathogens. Finally, the alternative pathway (AP) of complement system is activated by the spontaneous hydrolysis of C3 followed by a tick-over to become completely activated, which only proceeds on unprotected surfaces. Upon activation of the CP and the LP, complement proteins C4 and C2 are cleaved and subsequently the C3 convertase C4bC2a is formed on the activating surface. Upon activation of the AP, complement components C3 and factor B (FB) are cleaved leading to the formation of the C3 convertase C3bBb on the activating surface. The three complement activation pathways merge at the level of C3, where both C3 convertases cleave more C3 molecules resulting in the formation of the C5 convertases C4bC2aC3b and C3bBbC3b. Both C5 convertases are able to cleave C5 into C5a and C5b, and subsequently C6, C7, C8 bind to C5b on the surface that mediates the polymerization of C9 molecules into a tube-like pore that is inserted in the membrane of pathogens, which is called the MAC<sup>1-7</sup>.

## Complement regulation

Since complement is always activated at low level and it may have strong pro-inflammatory effects, complement activation can potentially harm host cells. Therefore, a balance between activation on foreign or modified self-surfaces and inhibition on intact host cells is of utmost importance to avoid tissue damage and to sustain homeostasis. To maintain this balance of complement activation, complement regulatory proteins are present on the cell surface of most human cells and in the fluid phase in blood plasma to prevent damage through unwanted complement activation (Fig. 1 grey boxes)<sup>8-10</sup>.



**Figure 1:** The complement system is activated via three pathways: the classical pathway, the lectin pathway and the alternative pathway. Complement activation results in opsonization (C3b and C4b), formation of the membrane attack complex (MAC/C5b-9) and induction of an inflammatory response by the release of anaphylatoxins (C3a, C5a). The complement system is tightly regulated by membrane bound and fluid phase complement regulators, which are indicated in grey boxes.

There are two major mechanisms by which complement activation is regulated: decay accelerating activity (DAA) that promotes the dissociation of the C3 and C5 convertases and cofactor activity (CA) for factor I (FI) that enzymatically inactivates deposited C3b and C4b into inactive forms that are unable to proceed further into the complement activation cascade. DAA is exerted by the membrane regulators complement receptor 1 (CR1)/CD35, membrane cofactor protein (MCP)/CD46, decay-accelerating factor (DAF)/CD55 and the fluid phase regulator factor H (FH). CA is exerted by the membrane regulators CR1 and MCP, and the fluid phase regulators FH and C4b-binding protein (C4BP). CD59 prevents C9 from polymerizing and forming the MAC and C1-inhibitor (C1-INH) inhibits the upstream activation of the CP and the LP by forming covalent complexes with the CP and LP proteases<sup>8-10</sup>.

### **Red blood cells**

Red blood cells (RBCs), also called erythrocytes, are the most common type of blood cells in vertebrates including humans. RBCs are formed during a process called erythropoiesis, which occurs in the bone marrow and consists of several developmental stages and finally results in mature RBCs in the blood stream<sup>11</sup>. RBCs are classified by different blood groups based on the presence or absence of surface antigens. To date there are 36 blood group systems, of which the most well-known blood groups are the ABO blood group system and the Rhesus blood group system<sup>12</sup>. RBCs are crucial in the delivery of oxygen to tissues as RBCs contain hemoglobin, which is responsible for the transport of oxygen in our body. In order to do this, RBCs have a flexible membrane that allows them to pass through the small capillaries in the circulation. RBCs have a biconcave form, with a diameter  $\sim 7.5\mu\text{M}$ , and they do not contain a nucleus or organelles<sup>13,14</sup>. Adults have on average  $20\text{-}25 \times 10^{12}$  circulating RBCs. The life span of RBCs is  $\sim 120$  days in the blood stream and every day  $\sim 0.8\%$  of the total number of RBCs are cleared by macrophages of the reticuloendothelial system and replenished by production in the bone marrow<sup>13-15</sup>. The clinical importance of RBCs is best exemplified in patients suffering from a sudden decrease in the amount of RBCs, such as during trauma. Under these conditions, transfusion of RBCs remains an essential therapy to restore the oxygen transporting capacity.

### **Blood transfusion**

Allogeneic blood transfusion involves the blood donation by healthy volunteers, testing and processing of the blood, and storage under stringent conditions for preservation of the stored blood components. Different blood component can be used for transfusions, such as RBCs, platelets and plasma, to support different clinical problems<sup>16,17</sup>. RBC transfusions are given to restore oxygen transporting

capacity when there are clear signals of lack of oxygen due to anemia. Worldwide about 85 million units of red blood cells (RBCs) are transfused per year<sup>17,18</sup>. In the Netherlands, RBCs for transfusions, also called red cell concentrates, are obtained from whole blood that is collected in citrate solution that prevents clotting. The blood is centrifuged, and the different components, such as RBCs, plasma and buffy coat (containing leukocytes and platelets) are separately collected in plastic bags. Residual white blood cells and platelets are removed from the RBCs by a leukoreduction filter, so less than  $1 \times 10^6$  leukocytes remain present in the RBC bag. In the Netherlands, RBCs may be stored up to 35 days at 2-6 °C in storage solution containing saline-adenine-guanine-mannitol (SAGM), to meet the quality standards that 75% of the transfused RBCs must survive for 24 hours in the recipient and a maximum hemolysis of 0.8% in the bag<sup>19-21</sup>.

Although transfusions generally are life-saving in order to restore oxygen transporting capacity, transfusion of RBCs can also have serious adverse side effects, such as alloantibody formation with subsequent occasional hemolytic transfusion reaction, transfusion-related acute lung injury and iron overload. During storage at 2-6 °C, RBCs undergo several biochemical and structural changes such as consumption of metabolic species, accumulation of reactive oxygen species, altered lipid and membranes collectively known as “storage lesion”<sup>22-25</sup>. The exact nature of these changes remains unclear, but it seems that oxidative stress and defective adenosine triphosphate metabolism have some driving forces. Moreover, it has not been established yet how these changes lead to the rapid clearance of donor RBCs after transfusion. Furthermore, it is still under debate whether increased storage time results in changes of the RBCs that potentiate adverse clinical outcomes in transfusion recipients<sup>16,26-31</sup>. Therefore, it is of utmost importance to identify and understand the mechanisms that underlie the clearance of donor RBCs as well as the adverse outcomes for transfusion recipients that may be associated with the so-called storage lesion.

### **Complement regulation on RBCs**

Different cell types express different combinations of complement regulating proteins, and in addition, fluid phase complement regulators in plasma are involved in protecting the cell surface. Since RBCs are constantly exposed to complement proteins in blood plasma, regulation is very important to protect them from unwanted complement activation. RBCs express complement regulators CR1 (CD35), DAF (CD55) and CD59 on their cell membrane, and also fluid phase regulator FH is involved in complement regulation on RBCs. In contrast to nucleated cells, RBCs do not express MCP (CD46)<sup>8,32</sup>. Inefficient regulation or overstimulation of the complement system may cause complement deposition on RBCs. This complement

deposition can result into accelerated removal of RBCs intravascularly via the formation of MAC or extravascularly via complement receptor-mediated phagocytosis by macrophages, which mainly occurs in the liver<sup>4,33</sup>.

Complement-mediated clearance of RBCs is associated with clinical anemic conditions such as autoimmune hemolytic anemia (AIHA) and paroxysmal nocturnal hemoglobinuria (PNH). In AIHA, RBCs are cleared due to the formation of autoantibodies against RBCs, either or not followed by complement activation<sup>33-35</sup>. PNH is caused by a mutation in the PIG-A gene that is responsible for GPI-anchorage of membrane proteins, including membrane bound complement regulators DAF and CD59. Absence of DAF and CD59 renders RBCs from PNH patients vulnerable for complement activation<sup>36,37</sup>. So, anemia in AIHA is caused by autoantibodies, while in PNH this is caused by defective complement regulation. In both clinical anemic conditions, RBCs transfusion may be given to restore oxygen transporting capacity.

### Scope of this thesis

The scope of this thesis is to investigate how complement activation is regulated on RBCs. Within that context, we examined if and how complement is activated upon RBC storage and may have influence on RBC survival. In addition, the differential role of complement regulatory proteins, both membrane bound and fluid phase was investigated.

In **Chapter 2**, we reviewed the literature on complement regulation on RBCs and the (clinical) consequences when this regulation is out of balance. Healthy host cells are equipped with several membrane bound complement regulators, including CD35, CD46, CD55 and CD59. As in contrast to hematopoietic stem and progenitor cells, mature RBCs do not express CD46 but express CD35 as a complement regulator, we investigated in **Chapter 3** the dynamics of CD46 expression during erythropoiesis.

**Chapter 4** demonstrates the effect of RBC storage on complement deposition and antibody binding, both directly in the storage bag as well as upon contact with complement in serum. Besides membrane bound complement regulators, fluid phase regulator FH is important in the regulation of complement activation on RBCs. By using blocking anti-FH monoclonal antibodies we examined which binding domains of FH are important in protecting RBCs against complement mediated clearance. The results are described in **Chapter 5**. Dysregulation of complement activation can result in damage to host cells and contributes to the pathology of many diseases. We generated human cell lines knocked out for membrane bound complement regulators CD46, CD55 and/or CD59, to have unlimited availability of complement regulator deficient cells as model *in vitro* system. The results are described in **Chapter 6**. It is assumed that the alternative pathway is important for efficient complement activation by amplifying the response regardless which pathway originally initiated



## Chapter 1

the complement activation. In **Chapter 7** we describe the contribution of the amplification loop on results of classical pathway-initiated complement activation on various physiologically relevant cell surfaces such as RBCs in the context of autoimmune hemolytic anemia. Finally, **Chapter 8** summarizes and discusses the work described in this thesis.

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