TOWARDS A DETAILED UNDERSTANDING OF THE RED BLOOD CELL STORAGE LESION

and its consequences for in vivo survival following transfusion

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Till Simon och Elvira
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Abstract

Red blood cells (RBCs) are vital for oxygen delivery to tissues and constitute the vast majority of all cells in blood. After leaving the red bone marrow as mature cells, RBCs have a lifespan of approximately 120 days before they are removed from the circulation by macrophages, mainly in the spleen and liver. RBC transfusion is a common therapy in modern healthcare. Major surgery, numerous cancer treatments and other, often lifesaving, interventions would be unthinkable without available blood supply. For this reason, hospitals store donated RBCs in blood banks.

The metabolic and structural changes that occur during prolonged storage of RBCs (the storage lesion) have been studied in detail in vitro and include oxidative stress, a reduction in glycolysis, increased membrane rigidity and shedding of microparticles from the RBC membrane. Stored RBCs share several features of senescent RBCs, but also with RBCs undergoing an apoptotic-like process called eryptosis. A consequence of the storage lesion is the fact that as much as 25% of stored RBCs could be rapidly removed from the circulation within 24 hours after transfusion. The mechanisms behind this rapid macrophage-mediated recognition and removal of stored RBCs, and its immunological consequences, remain largely unknown. Therefore, the aims of this thesis were to investigate if cryopreserved human RBCs induced an inflammatory response following autologous transfusion into healthy volunteers, and to further understand the mechanisms behind macrophage recognition of stored RBCs in vitro and in vivo.

Autologous transfusion of two units of cryopreserved RBCs into healthy human recipients was found to be associated with an increased extravascular RBC elimination already at 2 hours after transfusion. However, there were no signs of an increased production of any of the investigated pro-inflammatory cytokines, indicating that an increase in the destruction of RBCs per se did not induce an inflammatory response.

Eryptosis is a form of induced RBC death associated with an increased cytoplasmic Ca$^{2+}$ uptake. We found that a subset of human RBCs increased their Ca$^{2+}$ permeability during prolonged storage at +4°C. Using a murine model, to further understand how RBCs with an increased Ca$^{2+}$ permeability were eliminated by phagocytic cells in the spleen, it was found that such RBCs were taken up by marginal zone macrophages and dendritic cells (DCs) in a manner distinct from that of naturally senescent RBCs. The DC population particularly efficient in this process expressed CD207 and are known for their ability to promote immunological tolerance. Eryptotic cell uptake was not regulated by the phagocytosis-inhibitory protein CD47 on the RBCs.
To investigate how RBCs damaged during liquid storage are recognized and taken up by macrophages, a model to store and transfuse murine RBCs was developed. This storage model generated murine RBCs with several characteristics similar to that of stored human RBCs (i.e. loss of ATP, formation of RBC microparticles and rapid clearance of up to 35% of the RBCs during the first 24 h after transfusion). In vitro phagocytosis of human as well as murine stored RBCs was serum dependent and could be inhibited by blocking class A scavenger receptors using fucoidan or dextran sulphate.

In conclusion, the findings of this thesis contribute to further understanding how changes inflicted to RBCs during storage direct the fate of these cells in their interaction with cells of the immune system after transfusion. The observation of an increased Ca\textsuperscript{2+} permeability of stored RBCs, and the possible recognition of such cells by tolerance-promoting DCs, in combination with the findings that class A scavenger receptors and serum factors may mediate recognition of stored RBCs, may result in novel new directions of research within the field of transfusion medicine.
### Abbreviations

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<td>2,3-DPG</td>
<td>2,3- Diphosphoglycerate</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>AS</td>
<td>Additive solution</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CPD</td>
<td>Citrate-Phosphate-Dextrose</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ERK-1/2</td>
<td>Extracellular signal-regulated kinase-1/2</td>
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<td>FCS</td>
<td>Follicular calf serum</td>
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<td>FcγR</td>
<td>Fc gamma receptor</td>
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<tr>
<td>FSC</td>
<td>Forward scatter</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>Hct</td>
<td>Hematocrit</td>
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<td>HS</td>
<td>Human serum</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MARCO</td>
<td>Macrophage receptor with collagenous structure</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MIP-1β</td>
<td>Macrophage inflammatory protein 1 beta</td>
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<td>MP</td>
<td>Microparticle</td>
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<td>MZ</td>
<td>Marginal zone</td>
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<td>MZM</td>
<td>Marginal zone macrophages</td>
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<td>NAbs</td>
<td>Naturally occurring antibodies</td>
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<td>NK-cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin bound iron</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>P38-MAPK</td>
<td>P38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PI3-Kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosinic polycytidylic acid</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<td>RBC</td>
<td>Red Blood Cell</td>
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<tr>
<td>SAG-M</td>
<td>Saline-Adenine-Glucose-Mannitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SIGLEC</td>
<td>Sialic acid-binding immunoglobulin-type lectins</td>
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<td>SR</td>
<td>Scavenger receptor</td>
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<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TH-cell</td>
<td>T-helper lymphocyte</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<td>TReg-cell</td>
<td>Regulatory T-lymphocyte</td>
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<tr>
<td>TSP</td>
<td>Thrombospondin</td>
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<tr>
<td>VO2max</td>
<td>Maximal aerobic capacity</td>
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Enkel sammanfattning på svenska

Mot en ökad förståelse för lagringsspecifika förändringar hos röda blodkroppar, vilka kan påverka dess överlevnad efter transfusion

Röda blodkroppar (RBK) är celler som saknar cellkärna och många andra cellulära komponenter och vars primära syfte är att transporterar syre från lungor till kroppens celler. De cirkulerar i c:a 120 dagar innan de har ådragit sig sådana åldersskador att de känns igen och bryts ner av immunförsvarets makrofager i lever och mjälte. Kunskapen om de åldersskador som leder till att cellerna tas upp och bryts ned är i dagsläge begränsad. Den normala livslängden hos RBK kan förkortas i situationer som skadar RBK, exempelvis sjukdom eller olika typer av läkemedel. Exakt hur makrofager känner igen skadade RBK är delvis oklart. Blodtransfusioner är en vanlig behandlingsform inom modern sjukvård, och direkt avgörande för att kunna utföra stora operationer, vid flertalet cancerbehandlingar samt för att rädda traumapatienter med stora blödningar. För att tillgodose behovet på blod vid dessa ingrepp tappas blodgivare på blod som separeras i olika komponenter (vanligtvis RBK, blodplättar och plasma) och lagras i +4°C vid blodcentraler i väntan på transfusion. Det är sedan länge känt att under lagringstiden, som i dagsläget är maximalt 42 dagar, ådrar sig RBK en rad skador. Detta leder till att en substantiell andel av lagrade RBK (upp till 25%) försvinner kort efter transfusion då de känns igen av mottagarens makrofager. De möjliga riskerna för mottagaren som kan vara kopplat till detta är i dagsläget ombatterat i den vetenskapliga litteraturen. Även om många lagringsspecifika skador har identifierats hos RBK så är mekanismerna varmed makrofager känner igen och bryter ner vissa RBK efter transfusion fortfarande mycket oklara.

Syftet med denna avhandling har därför varit att öka förståelsen för de igenkänningsmekanismer som immunförsvarets celler använder vid kontakt med RBK, samt hur immunförsvaret reagerar på denna igenkänning. Initialt studeras detta i transfusionsförsök utförda på frivilliga försökspersoner. Det finns dock inneboende etiska problem med att i tillräckligt stor detalj studera dessa förlopp i människokroppen, vilket gör djurmodeller och studier i cellkulturer till en mer framkomlig väg. Vi har därför satt upp en musmodell för lagring och transfusion av RBK.

I det första delarbetet undersöktes vilken effekt transfusion av två blodpåsar med fryslagrade RBK hade på inflammation i det akuta skedet (2 timmar) samt två dygn efter transfusion. Vi fann en ökad makrofagemedierad
nedbrytning av RBK i akutskedet efter transfusion, men att detta inte ledde
till mätbara nivåer av inflammatoriska substanser i blodet.

Skadade (även kallade eryptotiska) RBK kännetecknas av ett ökat upptag av
calcium, vilket följs av att dessa celler snabbt tas upp av makrofager. Vi
observerade att en liten andel humana RBK vid lagring får förhöjda
calciumnivåer, vilket var relaterat till en lång lagringstid. Delarbete två
utforskade med hjälp av en musmodell hur sådana skadade RBK känns igen
av immunförsvarets celler. Det visade sig att eryptotiska RBK togs upp av
marginalzonsmakrofager och dendritiska celler (dessa celler är viktiga för
reglering av immunförsvaret) i mjälten. Detta upptag liknar mycket hur
uttjänta kroppsegna celler (apoptotiska celler) tas upp, men skiljer sig från
upptag av normalt åldrade RBK. Upptag av apoptotiska celler motverkar
som regel inflammation och hjälper snarare till att förhindra
immunförsvaret från att attackera sina egna celler och vävnader. Då en liten
del av lagrade RBK påvisade eryptotiska drag, öppnas möjligheten att dessa
skadade celler faktiskt kan ha en positiv inverkan på mottagaren av en
blodtransfusion. Att eryptos kan utgöra en del av lagringsskadans stärks av
vårt fynd att upptag av lagrade RBK i mjälten delar likheter med både det
som ses hos normalt åldrade och eryptotiska RBK. Även om CD47 är ett
cellyteprotein som kan förhindra upptag av normala värdceller, så fann vi att
varken eryptotiska eller lagrade cellers upptag påverkades av mängden CD47
på dessa cellers yta.

Det tredje delarbetet visade att det finns anmärkningsvärdiga likheter varmed
lagrade RBK från mus och människa kan tas upp av makrofager, något som
stärker relevansen för upptäckter i vår musmodell. När färskas RBK från mus
och människa blandades med musmakrofager skedde inget upptag. Däremot
så togs lagrade RBK upp oberoende om de kom från mus eller människa.
Makrofagernas upptag visade sig vara serumberoende, vilket indikerar att
det finns någon komponent i serum som krävs för makrofagernas
igenkänning av lagrade celler. Vidare kunde vi förhindra upptaget genom att
blockera en viss typ av receptorer (klass-A scavenger receptorer) på
makrofagernas yta. Detta tyder på att lagrade RBK känns igen med hjälp av
dessa receptorer hos makrofagerna, samt att upptaget involverar någon
serumkomponent.

Sammanfattningsvis så har detta avhandlingsarbete påbörjat en kartläggning
av interaktionen mellan lagrade RBK och kroppens immunceller, vilket
medfört en utökad förståelse för de skador som uppkommer hos RBK under
lagring och konsekvenserna av detta efter återinförsel av dessa celler i
blodet. Vi har funnit att ökad nedbrytning av RBK efter transfusion i sig inte
leder till inflammation. Vidare har avhandlingsarbetet kunnat koppla en del
av lagringskadorna som uppstår till så kallad eryptos, vilket skulle kunna innebära att vissa lagringsskadade RBK kan ha en dämpande effekt på kroppens immunförsvar. Avslutningsvis tyder arbetet på att makrofagers upptag av lagrade RBK är beroende av komponenter i serum och class-A scavengerreceptorer på makrofagernas yta.

I en förlängning hoppas vi att kunskap om vilka förändringar hos lagrade RBK som är relevanta för deras igenkännning av makrofager kan leda till framtagandet av en mer optimerad lagringsmiljö för RBK. Sådana strategier kan leda till att minska mängden RBK som behöver överföras till patienter i samband med transfusioner om fler fungerande RBK kan behållas i cirkulationen.
List of original papers

1. **Transfusion of cryopreserved human red blood cells into healthy humans is associated with rapid extravascular hemolysis without a proinflammatory cytokine response**
   
   Hult A., Malm C. and Oldenborg P.A.
   

2. **Splenic uptake of RBCs with an elevated cytoplasmic Ca\(^{2+}\)-concentration primarily involves marginal zone macrophages and CD207+ dendritic cells**
   
   Larsson A., Hult A., Nilsson A., Olsson M. and Oldenborg, P.A.
   
   *Submitted*

3. **Phagocytosis of liquid-stored red blood cells in vitro requires serum and macrophage scavenger receptors**
   
   Hult A., Toss F., Malm C., and Oldenborg P.A.
   
   *Submitted*

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Introduction

The human body contains, depending on body size, between 4 and 6 liters of blood [4]. The main components of blood are the cell free plasma (50-60%) and erythrocytes (red blood cells/RBCs; 40-50%), while white blood cells (leukocytes) and platelets (thrombocytes) together accounts for only ~1% of total blood volume [5]. The cellular components of blood originate from pluripotent hematopoietic stem cells situated in the red bone marrow. Hematopoietic stem cells give rise to erythrocytes, megakaryocytes (which produce thrombocytes important for the coagulation process), and leukocytes of lymphoid or myeloid origin [6]. The formation of blood cells, or hematopoiesis, is strictly regulated by different hormones and cytokines in order to meet the demand of the ever shifting physiological milieu of the body, i.e. to produce more leukocytes upon infection or more erythrocytes when tissue oxygenation is low [5].

The leukocytes

The immune system is built up by a variety of lymphoid tissues, leukocytes and molecules, which are designed to protect us from infectious agents such as bacteria, viruses, fungi and parasites. In addition to protecting us from pathogens, the immune system also helps in maintaining body homeostasis by removing and restoring damaged endogenous cells and tissues. After differentiation and maturation, predominantly in the bone marrow, leukocytes migrate to the blood, peripheral tissues and the lymphatic system. In all these tissues, leukocytes carry out their various immunologic functions. The immune system can be divided into two branches; the innate and the adaptive system. The innate immune system is a rapidly responding system that recognizes and eliminates a wide range of pathogen-associated molecular patterns (PAMPs) found on various infectious agents. PAMPs are identified by their binding to different pattern recognition receptors (PRRs) expressed by the immune cells [6, 7]. The adaptive immune system is initially much slower, but instead more efficient in clearing the body from infectious agents. Furthermore, the adaptive system has a memory, and the ability to generate long lasting immunity against different pathogens. Via this memory function, pathogens will be swiftly recognized and eliminated if invading a second time [6].
**Innate immunity**

The cellular components of the innate immune system derive mainly from myeloid progenitors and include mast cells, monocytes, macrophages, dendritic cells (DCs), and neutrophil, eosinophil and basophil granulocytes. Despite their lymphoid origin, natural killer cells (NK-cells) are also considered as innate immune cells which identify and kill virus-infected or cancerous endogenous cells. Mast cells, macrophages and DCs are mainly found in various tissues, while monocytes and granulocytes are mainly found in the blood of a healthy individual. The neutrophil granulocyte is the most abundant leukocyte in blood, constituting 50-70% of all leukocytes. It is readily recruited to infected areas by inflammatory chemokines and cytokines and play a key role in the initial defense against pathogens. Blood monocytes eventually leave the circulation and migrate to organs and peripheral tissues where they mature into macrophages. As they mature, macrophages will display a unique receptor repertoire depending on their microenvironment, making for instance a liver macrophage somewhat phenotypically and functionally different from a marginal zone macrophage in the spleen [8]. Upon recognition of infectious agents, the innate immune system launches an inflammatory response by the release of histamine, pro-inflammatory cytokines and chemokines in order to recruit and activate other immune cells to the infected area for the participation in the fight of the infection [6]. The innate immune system also includes the ability of antigen presentation, whereby macrophages or DCs scavenge foreign or endogenous antigens in peripheral tissues by phagocytosis or macropinocytosis for later presentation to the adaptive immune system.

In addition to fighting infectious agents, the innate immune system (DCs and macrophages in particular) is also involved in maintaining normal homeostasis in its microenvironment by engulfment of senescent or apoptotic endogenous cells and cell fragments, a process mostly resulting in anti-inflammatory responses [9]. The discrimination between (modified) self and non-self is dependent on which receptor(s) that recognize(s) the cell or substance destined for phagocytosis (or endocytosis) by the phagocytic cells and ultimately determines if the cell will inhibit or induce an immune response [7]. Aside from the cellular components of the innate immune system, there is also the complement system which consists of several plasma proteins with the ability to bind to various pathogens and either initiate lysis of the pathogen (mainly bacteria) or elicit an inflammatory response by the release of pro-inflammatory molecules and the formation of opsonins on the surface of the pathogen. Opsonins target pathogens for elimination by phagocytic cells that carry complement receptors (CRs) [10].
Pattern recognition receptors (PRRs)

The cells of the innate immune system carry a panel of receptors, PRRs, that recognize evolutionary conserved patterns on pathogens - PAMPs as well as molecules from damaged endogenous cells and tissues (danger-associated molecular patterns - DAMPs) [11, 12]. These PRRs include the Toll-like receptors (TLRs), which recognize bacterial patterns like lipopolysaccharide (LPS) as well as viral RNA. Another member of the PRRs is the C-type lectin receptors (CLRs). These receptors recognize carbohydrate structures on pathogens. Endogenous carbohydrate structures, capable of binding to CLRs, are often hidden by terminal sialic acid, thus hindering auto-recognition [6]. Binding to TLRs and CLRs can elicit production of the pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 [11], (Figure 1).

Scavenger receptors (SRs) also belong to the PRRs and can be involved in recognition of PAMPs on pathogens as well as ligands on apoptotic cells [13]. The discrimination between self and non-self will ultimately determine if the

Figure 1. PRR mediated pathogen recognition by a phagocytic cell.

PAMP recognition by PRRs results in; 1, Phagocytosis and subsequent degradation of the pathogen. 2, Transcription, production and secretion of pro-inflammatory cytokines e.g. IL-1β, TNF-α and IL-6. The release of pro-inflammatory cytokines will initiate an inflammatory response that will recruit and activate other leukocytes to the site of infection.
phagocytic cell will generate a pro-, or anti-inflammatory response. SRs are a heterogeneous family of receptors with an extremely diverse ligand repertoire, where most ligands are polyanionic and include proteins, polyribonucleotides, polysaccharides and lipids [13, 14]. SRs are subdivided into classes (class A-I, each with several members) and are known to partner with various co-receptors for their function [13]. Macrophages express several SR classes, including class A-SRs. SR-A1 and MARCO are two receptors that belong to the class A-SRs found in the spleen. SR-A1 is expressed by most macrophages while the expression of MARCO is restricted to marginal zone macrophages (MZM) [13].

Adaptive immunity

The cells of the adaptive immune system derive from a common lymphoid progenitor cell and can be divided into B-lymphocytes (B-cells) and T-lymphocytes (T-cells). Lymphocytes are, in contrast to leukocytes of the innate immune system, highly specific in their ability to recognize antigen in that it will only bind to one specific motif. During development, the genes that are coding for the variable region of the B- or T-cell receptor undergoes rearrangements and hypermutations, resulting in a wide range of receptor variability and subsequent pathogen recognition within the lymphocyte population. Each individual lymphocyte, however, is still highly specific in its antigen recognition. Lymphocytes that recognize self-antigens undergo apoptosis (programmed cell death) during development in order to avoid autoimmune reactions. Those that survive this selection are allowed to migrate from the bone marrow (or thymus in the case of T-cells) into the blood and lymphatic system, including the spleen and lymph nodes [6].

When a B-cell has bound an antigen to its receptor (called B-cell receptor/BCR), the antigen is internalized and later presented on MHC class II to T-cells for T-cell dependent activation. Upon activation, the B-cell starts to proliferate and changes phenotype into either a memory cell or a plasma cell. The plasma cell starts to produce immunoglobulins (antibodies or ab:s) which are then distributed via the lymph system out into the body, with the ability to bind a specific antigen. Binding of ab:s to their antigens induces opsonization of the pathogen which can then be recognized by phagocytic cells having Fcγ-receptors (FcγR) that recognizes the constant region of the ab:s. The binding of an ab to its antigen can, in addition, initiate activation of the complement system which in turn increases the opsonization and leads to further promotion of phagocytosis [10]. The memory B-cells can swiftly be activated if presented with the antigen upon a second infection and hence establishes a long lasting immunity against its specific antigen [6].
T-cells recognize its antigen via the T-cell Receptor (TCR). Antigens are presented to T-cells by macrophages or DCs. When T-cells recognizes its antigen, it can be activated and will then differentiate into one of the following subsets; 1) Cytotoxic T-lymphocytes (CTL) whose main function is to kill infected host cells, 2) T-helper cells (T_H-cells) who are involved in co-activation of B-cells as well as other immune cells and, 3) Regulatory T-cells (TReg-cells) which can promote suppression of lymphocytes. [6] This is of course a simplified description of the extremely complex functions of the immune system in general and the adaptive immune system in particular.

**Tolerance vs. Immunity**

DCs are particularly efficient antigen presenting cells (APCs) that control the T-cell mediated immune response via paracrine and contact-dependent signaling mechanisms. When a DC has taken up and processed an antigen, it will migrate to the T-cell areas of secondary lymphoid organs and present the MHC class II-bound antigen to naïve T-cells. If the engulfed antigen was recognized as foreign by the DC, it will also express co-stimulatory receptors (e.g. CD80 and CD86) and secrete pro-inflammatory cytokines (i.e. interleukin 12 (IL-12) and IL-18) to promote activation of T_H-cells and CTLs [15] (Figure 2A). On the other hand, if the antigen was recognized as a self-antigen, the DC will display the antigen to T-cells together with lower levels of CD80 and CD86, which will not be accompanied by pro-inflammatory cytokine production. Instead, the anti-inflammatory cytokine IL-10 will be released. Presentation of self-antigens may result in either T-cell anergy or the formation of TReg-cells and promotion of tolerance against the self-antigen (Figure 2B) [16]. The activation of T-cells is hence a two-step process in which binding of the T-cell receptor to the MHC-bound antigen on DCs is the first step. The second step is either the ligation of CD80/CD86 on the DC to CD28 on the T-cell to promote an immune response, or binding of CD80/CD86 to CTLA-4 on the T-cell to promote tolerance.

**The Red blood cell**

**Function**

Erythrocytes (from now on referred to as RBCs), is the most abundant cell type in the human body, and play a key role in oxygen delivery from the lungs to tissues throughout the body. The transport of oxygen is mediated by the iron-containing metalloprotein hemoglobin (Hb), which is highly abundant in RBCs. The tertiary structure of adult Hb consists of four polypeptide chains (two α-globin and two β-globin) which all carry an iron-containing heme group to which oxygen is reversibly bound, forming
Figure 2. Recognition patterns on ingested material determine how antigens are co-presented to T-cells.

Ingestion of antigen by an APC is degraded and later presented on MHC class II on the cell surface. **A**, Recognition of foreign (e.g. pathogens) by APCs induce a pro-inflammatory response and subsequent T-cell mediated immunity. **B**, Recognition of modified self (e.g. apoptotic cells) by APCs induces T-cell mediated tolerance in an anti-inflammatory manner.

Antigen presentation on MHC class II

Pro-inflammatory cytokines
IL-1β, IL-6, IL-12, IL-18, TNF-α

Anti-inflammatory cytokines
IL-10
deoxyhemoglobin). The affinity of oxygen to hemoglobin is affected by the partial pressure of oxygen (pO$_2$) and carbon dioxide (pCO$_2$), temperature, pH and the intracellular concentration of 2,3-diphosphoglycerate (2,3-DPG). Thus, the affinity regulation of oxygen to Hb optimizes oxygen uptake in the lungs, and oxygen delivery to metabolically active tissues, dependent on the physiological milieu at the respective site [17, 18].

RBCs also facilitate CO$_2$ transport in two different forms; a small fraction of CO$_2$ is bound to the globin part of Hb, while the bulk is converted by the enzyme carbonic anhydrase into carbonic acid (H$_2$CO$_3$) which dissociates into H$^+$ and bicarbonate (HCO$_3^-$) in the cytosol of RBCs. The HCO$_3^-$ is then exchanged with chloride (Cl$^-$) across the plasma membrane by the anion transporter membrane protein Band 3 (an abundant membrane protein in RBCs) to further be transported in the blood plasma to the lungs where the reaction is reversed, allowing CO$_2$ to diffuse into the alveoli of the lungs and finally be exhaled. [5]

The delivery of O$_2$ to, and the removal of CO$_2$ from, the tissues enables adenosine triphosphate (ATP) production from oxidative phosphorylation to occur in the mitochondria of the target cells and thus permitting an efficient energy utilization throughout the body. Because mature RBCs do not contain any organelles, including mitochondria, it will not consume any of the oxygen it carries and are hence solely dependent on anaerobic glycolysis for ATP production, leaving lactate as an end product. Lactate is finally expelled from the RBC via symport of hydrogen ions in a pH dependent manner [5, 19].

**Erythropoiesis**

The maturation of RBCs from hematopoietic stem cells involves several intermediates as the precursor cells divide and proliferate, starting to produce Hb and extrudes organelles (including their nucleus), to finally reach the state of reticulocytes. Still carrying ribosomes and mRNA for protein synthesis, the reticulocytes are then released from the red bone marrow into the blood stream. During the first couple of days in circulation, the reticulocytes mature into RBCs which have lost their capability to produce proteins. At any given time, 1-2 % of all circulating RBCs are reticulocytes. The reticulocyte concentration can hence be used to assess the rate of the RBC production, since a higher percentage of reticulocytes is indicative of increased erythropoiesis [20].

The regulation of erythropoiesis under normal conditions is highly dependent on the peptide hormone erythropoietin (EPO), which is mainly
synthesized in the kidneys. EPO promotes committed erythroblasts in the red bone marrow to proliferate and differentiate to RBCs [21]. The EPO production is in turn regulated by the oxygen availability in the EPO producing cells, thus creating an efficient feedback loop in where more EPO, and consequently more RBCs, are produced when tissue oxygenation is low [20, 22]. However, in cases of increased hemolysis and chronic anemia, EPO regulation of erythropoiesis is not sufficient to compensate for the vast reduction of RBCs. Instead, glucocorticoids and stem cell factor have been suggested to facilitate the recovery from severe anemia by stimulating extended proliferation of early erythroid progenitor cells [23].

The life and death of RBCs

The mature human RBC has a lifespan of approximately 120 days before it is targeted for destruction by macrophages, mainly in the spleen and liver. Although not fully understood, the senescence of RBCs is likely to involve several structural changes on the cell surface, which ultimately leads to macrophage recognition and phagocytosis [24]. Following ingestion of RBCs, the macrophages recycle iron from Hb which is then transported back to the bone marrow by the transporter protein transferrin in order to facilitate continued erythropoiesis. In situations with increased RBC breakdown, transferrin can become saturated. The highly reactive iron ions, capable of forming reactive oxygen species and tissue damage in its unbound state, is then found as non-transferrin bound iron (NTBI) in the plasma [25]. The remaining heme-group of Hb (without iron) is converted to bilirubin in macrophages and later secreted in the blood plasma [26]. Changes in the plasma bilirubin concentration and transferrin saturation can hence be used as indicators to assess the rate of RBC phagocytosis.

Senescence markers of RBCs include the clustering and/or degradation of the Band 3 membrane protein which has been shown to be formed after various oxidation processes. This may be attributed to the binding of oxidized Hb to the cytoplasmic domain of Band 3 [24, 27, 28]. Alterations in Band 3 can also be induced through the activation of calcium-dependent proteases like calpain, at least in the elderly [29]. RBCs shrink in size and increase their density as they age, which can be seen by lower forward scatter (FSC) as assessed by flow cytometry and by density centrifugation fractionation [30]. The decrease in cell size is contributed to by the shedding of microparticles (MPs) throughout the lifespan of the RBC [31]. MPs contain various amounts of Hb and their formation seems to be, at least partly, facilitated by the spleen [31, 32]. The MPs display the phospholipid phosphatidylserine (PS) on their outer membrane leaflet and are quickly removed from the circulation by macrophages [33]. If human RBCs
themselves externalize PS as a natural part of their senescence is questionable [30], but cannot be completely ruled out due to the fast recognition of PS+ cells by macrophages, leaving them little time to be detected in circulation. Reductions in terminal sialic acids [34, 35] and in the expression of the cell surface glycoprotein CD47 [36] have also been observed in the membrane of senescent RBCs. Increased non-enzymatic glycation (forming advanced glycation end products (AGES)) of Hb as well as membrane proteins is also found to occur as RBCs age [37, 38]. Finally, the metabolic rate of RBCs decrease over time as seen by reduced activity of glycolytic enzymes and a consequent reduction in ATP and lactate production [39].

**Eryptosis**

Besides normal senescence, RBCs can also enter a programmed cell death (apoptotic)-like process called eryptosis, which can be induced by an number of endogenous and xenobiotic substances, as well as by oxidative stress, osmotic shock and energy depletion [40]. Eryptosis is not a pure analogue of apoptosis in nucleated cells, as RBCs lack mitochondria and nucleus. Eryptosis and apoptosis yet share many common features like cell shrinkage, MP formation and PS externalization, which in RBCs is induced by increased cytosolic Ca²⁺ levels [41, 42]. The actions of increased cytosolic Ca²⁺ involves the activation of K⁺ ion channels and subsequent reduction in cell size, cytoskeleton degradation (and membrane blebbing) by activated calpain, and an increased scrambling activity of the phospholipids in the lipid bilayer resulting in PS-externalization [42]. Eryptosis has also been reported to occur in a number of diseases, including type-2 diabetes [43, 44], thalassemia [45] and heart failure [46], often related to oxidative events.

**Recognition and phagocytosis of RBCs**

The mechanisms behind macrophage removal of normally senescent RBCs, reaching the 120 day limit of their life-span, are still not completely understood [2]. This lack of detailed knowledge is one important reason why there is an even greater deficit in the understanding of the mechanisms behind the rapid post-transfusion clearance of stored RBCs, which will be discussed further in a later section. In addition to the biochemical changes that occur during RBC senescence, clearance of senescent RBCs requires molecular changes to the cell surface which can then be recognized by macrophages (Figure 3). Most likely, senescent RBCs gradually accumulate these cell surface changes, suggested to include loss of terminal sialic acids and the subsequent exposure of underlying carbohydrate structures [34], a reduction of the anti-phagocytic protein CD47 on the cell surface [36],
binding of naturally occurring antibodies (NAbs) [30, 47] and oxidative damage to Hb and phospholipids [48]. Many of these changes may well be recognized by NAbs, but the most convincing target for such antibodies so far is the conformational changes that take place in the Band 3 protein as a result of oxidative damage to hemoglobin [27, 28, 49]. Although NAbs are of low affinity and at low concentrations in circulation, binding of NAbs could result in FcγR-mediated phagocytosis of senescent RBCs but also complement deposition and CR participation [50]. Finally, while being questioned [30], senescent RBCs may also, like nucleated apoptotic cells, eventually expose PS on the surface to promote their phagocytosis by macrophages [51]. PS can be recognized by several receptors on phagocytic cells, either directly by TIM1, TIM4 or stabilin-2, or via bridging molecules like Gas-6, thrombospondin (TSP) and lactadherin, following interaction with integrins on the macrophage [2].

**Figure 3. Pro-, and anti-phagocytic receptors on macrophages.**

Abbreviations: SIGLEC - Sialic acid binding immunoglobulin lectins, SIRP-α - Signal-regulatory protein-α, PS – Phosphatidylserine, TIM-4 - T-cell immunoglobulin mucin protein 4, TSP - Thrombospondin, NAbs - Naturally occurring antibodies, FcγR – Fc gamma receptor, CR – complement receptor, C3b – complement protein 3b.

*Modified from de Back et al. [2].*
In addition to the pro-phagocytic receptors mentioned above, macrophages also carry anti-phagocytic receptors (figure 3). Signal-regulatory protein α (SIRPα) is an inhibitory receptor expressed on phagocytic cells that recognizes the widely expressed “self-antigen” CD47 [52]. Mouse RBCs lacking CD47 was shown to be rapidly phagocytized by red pulp macrophages in the spleen, indicating the anti-phagocytic properties of the CD47-SIRPα interaction. Another family of receptors that mediates recognition of self and inhibits phagocytosis is the sialic acid binding immunoglobulin lectins (SIGLECs) that recognizes terminal sialic acids on host cells [53]. A reduction in sialic acid on RBCs (by neuraminidase treatment) leads to the rapid clearance of these cells by macrophages [54]. A reduction in CD47 and terminal sialic acid on aging RBCs have been observed [34-36], which may well contribute to macrophage recognition of senescent RBCs.

**Immune cells of the spleen**

Principally, the spleen has two separate compartments; the immunological and lymphocyte-rich white pulp, and the RBC-rich red pulp having more of a filtering function [1, 55, 56]. The splenic white pulp is divided into separate T-cell and B-cell areas. Macrophages can be found in both the red and white pulp, as well as in the area separating these two domains – the marginal zone (MZ). Separate DC subsets are also distributed within the red pulp, MZ and white pulp (T-cell) areas. In the murine spleen, three major macrophage populations can be identified; the F4/80+ red pulp macrophages, the MARCO+ MZ macrophages and the MOMA-1+ marginal metallophilic macrophages (MMM) [1, 3] (Figure 4). F4/80+ macrophages are mainly involved in the clearance of cells trapped in the red pulp, whereas the other two macrophage populations are more specialized in trapping microbes and PS+ apoptotic cells. Due to their close localization to the splenic white pulp, MARCO+ and MOMA-1+ macrophages (together with DCs) are more actively interacting with lymphocytes to regulate the adaptive immune system. When investigating macrophage phagocytosis of RBCs, it is important to remember that macrophages in vivo are very heterogeneous as a group, with many specific functions depending on their phenotype and anatomical location [8]. Adding on to this complexity, activated macrophages may also promote as well as dampen inflammation, making analysis of specific macrophage subsets even more important [8].
Figure 4. Anatomical structure of the spleen.
Large arteriole (lower part to the left) is branching and delivers blood to the marginal zone (MZ) and sinusoids of the spleen. Blood is then filtered through the MZ or arteriole sinusoids in to the red pulp and finally collected in sinusoids connected to venules. Upper part represent a magnification of the MZ. Marginal metallophilic macrophages (MMM) is lining the white pulp side of the MZ. Adjacent to MMM are dendritic cells (DCs) and MZ macrophages (MZM). Blood is filtered through the MZ an in to the red pulp where red pulp macrophages are situated. (Modified from Junqueira et al., [1] and Cyster [3]).
**RBC transfusion**

**History**

The first successful blood transfusion in humans was carried out by obstetrician Dr. James Blundell in 1818 when he transfused approximately 400 ml of whole blood, which was collected from multiple donors and administrated ten times over a 40 min period of time [57]. The patient, suffering from gastric carcinoma (described at the time as “scirrhosity of the pylorus”), showed clear symptoms of anemia and was barely conscious at the time of transfusion, but displayed marked improvement in the following 24 h post transfusion. His symptoms then reemerged and were followed by death a little over two days after the transfusion. Dr. Blundell continued his pioneering work in blood transfusion with a total of 10 documented transfusions of which 5 were successful [58].

The next big step towards modern transfusion medicine was taken by Karl Landsteiner in the early 20th century. He discovered that sera from different donors sometimes, but not always, agglutinated blood cells of other donors. He proposed the ABO blood group system (although Dr. Landsteiner at the time classified blood into groups A, B and C) [59] as a method to conduct compatible blood transfusions, a discovery that ultimately rendered him the Nobel prize in physiology or medicine in 1930. The identification of the Rhesus blood group system (the second most important blood group system after the ABO system) in the late 1930’s [60] is also to be considered an important step on the way to safer and more compatible blood transfusions.

Today, more than 300 antigens belonging to over 30 blood group systems have been described [61]. Blood group antigens are proteins or carbohydrate structures present on the cell membrane of RBCs. Alloimmunization against blood group antigens can occur after RBC transfusion, owing to blood group incompatibilities between donor and recipient [61]. Alloimmunization results in ab-mediated immunity against the foreign antigen. In most cases, the production of abs against blood group antigens is an induced response which follows after contact with the respective antigen. In contrast, abs against A and B antigens of the ABO blood group system starts to be produced during the first year after birth [62], irrespective of contact with the antigens. Enzymatic removal of the B-antigen from RBCs of blood group B has been described, making them transfusible to donors of all blood groups [63]. The combined removal of both the A and B antigens have later been reported [64], opening the door for universal RBCs that can be transfused to everyone, regardless of the ABO-blood group of the donor or recipient.
The use of citrate as an anticoagulant in donated blood made transfusions easier to manage as the risk for coagulation was vastly reduced [65]. The addition of glucose (or dextrose) in combination with citrate enabled prolonged storage of blood possible [66]. The introduction of first Acid-Citrate-Dextrose (ACD) and later Citrate-Phosphate-Dextrose (CPD) as a combined anticoagulant and energy source to the collected blood (stored in plastic bags) enabled donation and transfusion to be separated in time by as much as 28 days when stored at +4°C [67], thereby making storage of blood in blood banks a routine in many hospitals.

**Demographics**

According to the World Health Organization (WHO), 108 million units of blood were donated worldwide in 2012, with approximately half of these donations occurring in high-income countries where only 18% of the world’s population lives. In low-income countries, a majority of the collected blood is stored and later on transfused as whole blood, as compared to high income countries where 95% of the donated blood is further processed into RBC-, plasma-, and thrombocyte concentrates [68].

**Effects of RBC transfusion**

Blood transfusions are used both in the setting of acute and chronic bleeding, as well as in patients with bone marrow suppression, e.g. cytostatic treatment, and in patients with hemoglobinopathies (e.g. thalassemia and sickle cell disease) [69, 70]. Symptoms of anemia (i.e. fatigue, tachycardia and dyspnea) usually only arises at Hb concentrations below 90-100 g/L due to compensatory mechanisms that increase cardiac output and replenish blood plasma volume [71]. However, symptom onset is largely dependent on patients’ related factors such as age, cardiorespiratory fitness, rate of anemia development and functional requirements. The recommendations to transfuse hemodynamically stable patients is generally recommended at Hb 70-80 g/L [71]. The restoration of blood volume and viscosity after profound hemorrhagic shock and hemodilution is also important in order to restore a functional microcirculation by increasing capillary blood perfusion [72]. Moreover, the oxygen carrying capacity following transfusion has been documented to increase in non-anemic men as assessed by increased arterial oxygen content and greater resistance to hypoxic conditions during exercise [73], an observation that has opened up for the misuse of blood transfusions as a doping agent in endurance sports [74].
**RBC storage conditions**

To allow for a more efficient use of donated blood, blood donation and transfusion have to be optimally separated in place and time. This can be achieved by avoiding coagulation and assuring a sufficient *ex vivo* storage milieu with respect to nutrients and buffered pH conditions [66]. The first step involves collecting donated blood in CPD. As mentioned above, donated blood is today often separated into its different components. In this process, RBCs are isolated from the other cellular components of the blood, as well as from the majority of the plasma, by centrifugation and filtration techniques carried out inside a closed system containing the necessary components. The last step in this process involves the addition of an additive solution (AS) to the packed RBCs in order to optimize storage conditions. In Europe, a solution of Saline-Adenine-Glucose-Mannitol (SAG-M) is widely used, while closely related solutions (abbreviated AS-1, AS-3 and AS-5) are used in America. Blood stored at +4°C in these solutions can be kept for up to 42 days and typically display an *in vivo* recovery of 78-82% 24 h after transfusion into healthy individuals [75]. This may, however, not reflect the 24 h recovery in a clinical setting, since there are reports involving transfusions to patients with various underlying diseases showing 24 h recoveries after transfusion of around 75% already after 25-35 days of storage [76, 77]. Recently, a new additive solution (AS-7) has been approved for prolonged storage of RBCs for up to 8 weeks [78]. The AS-7 differs from the previous storage solutions by containing NaHCO₃ and NaPO₄ but no saline, having a higher pH and lower osmolality and displaying an *in vivo* 24-h recovery of 88% at day 42 and 82% at day 56 of storage [78].

**The storage lesion**

RBCs in liquid storage undergo time-dependent metabolic and structural changes, collectively referred to as the storage lesion. The storage lesion has been well characterized, based on *in vitro* measurements on RBC concentrates over time. Metabolic alterations are manifested by the reduction in ATP and 2,3-diphosphoglycerate (2,3-DPG) content. As RBCs are purely glycolytic, a time-dependent increase in levels of lactate and a corresponding decrease in pH is also noticed in the storage media. Structural changes, partly due to oxidative damage, include conformational changes in the Band 3 anion transport protein, IgG binding as well as increased rigidity and osmotic fragility. Shape shifting and a reduction in size is also noted in a subset of stored RBCs, attributed to the shedding of MPs and by reduced levels of cytoplasmic K⁺ [76, 79-82]. The amount of CD47 may also be reduced during storage [83, 84], although as to which extent is not clear. Thus, stored RBCs show several similarities to what has been described for
normally senescent RBCs in circulation (Figure 5). ATP and 2,3-DPG levels have been shown to recover shortly after transfusion, indicating that at least part of the storage lesion is reversible in vivo following transfusion [85]. However, it is presently unclear if the storage lesion simply reflects an accelerated aging of the RBCs, if it is merely a stress-induced eryptosis, a combination of both, or something else. Perhaps more important, the physiological consequences in vivo (after transfusion) remains largely unknown [86]. It is also unclear if the storage lesion affects all age-fractions of the RBCs in a random way, or if it primarily affects the older cells within the RBC population. This distinction may be of importance to understand the storage lesion and in the long run to develop strategies to prevent it.

In addition to liquid storage, RBCs can be frozen (cryopreserved) for extended periods of time, using glycerol as a cryoprotectant. Cryopreserved RBCs are more fragile and display increased hemolysis upon thawing and washing, as compared to liquid stored RBCs, but instead maintain their ATP and 2,3-DPG content better [87, 88]. In addition, RBC cryopreservation does not result in MP formation, PS exposure or reduced levels of CD47 [89]. The 24 h in vivo recovery of cryopreserved RBCs has been reported to be ~85%, making cryopreservation of RBCs a valuable alternative to liquid storage, especially for patients with rare blood groups or when there is a shortage of liquid stored RBCs [88].

<table>
<thead>
<tr>
<th>Microparticle formation</th>
<th>Senescent RBC</th>
<th>Stored RBC</th>
<th>Eryptotic RBC</th>
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<tr>
<td>Glycolysis</td>
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<tr>
<td>ATP</td>
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<td>↓</td>
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<tr>
<td>2,3-DPG</td>
<td>?</td>
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<tr>
<td>Lactate</td>
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<td>Oxidative damage</td>
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<tr>
<td>Ca^{2+} influx</td>
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<td>?</td>
<td>+</td>
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<tr>
<td>Band 3 aggregation/degradation</td>
<td>+</td>
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<td>Phosphatidylserine exposure</td>
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<td>IgG binding</td>
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<tr>
<td>Sialic acid</td>
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<td>CD47</td>
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**Figure 5. A comparison between senescent, stored or eryptotic RBCs.**
Presented is a selection of metabolic and structural changes reported in the literature.
Risks associated with transfusion

Transfusion-related acute lung injury (TRALI) is the leading cause of fatalities directly related to transfusion, followed by hemolytic transfusion reactions due to blood group incompatibility [71]. In a systemic review of 45 cohort studies, evaluating the efficacy of RBC transfusions in high-risk patients, the risks associated with transfusion were found to outweigh the benefits in all but three studies [90]. Firm conclusions of the relation of risks and benefits of transfusions are however difficult to draw because of the inherent bias in cohort studies as sicker patients tend to receive more transfusions [91]. There has also been an ongoing debate as to whether RBCs that have been stored for longer periods of time will have adverse effects following transfusion in various clinical settings. These effects include increased risks of infection, multiple organ failure, length of hospital stay and mortality [92-94]. A compilation of 32 studies on the topic by Aubron et al. [95] found that 18 studies reported clinically harmful effects and 14 did not. The discrepancy is likely due to various confounders not taken in to account, as well as problems with underpowered studies. It is also possible that transfusion of older blood can be particularly harmful to certain patient groups [92].

Recently, a large randomized controlled trial (Age of blood evaluation, ABLE) was published in which over 2400 ICU-patients had been enrolled and randomly selected to receive either fresh RBCs (stored for 6.1±4.9 days) or standard issued RBCs (stored for 22.0±8.4 days) [96]. The authors found neither an increased risk of 90 day mortality, nor any other risks that could be associated with transfusion of stored RBCs. Similar findings have been reported from randomized controlled trials conducted on cardiac surgery patients (the RECESS trial) [97] and in premature infants (the ARIPI trial) [98], indicating that there are no major risks associated with transfusion of older blood, at least not for the patient groups that have been investigated. Still, because the maximum storage of RBCs in AS ranges from 42 to 56 days, it could be argued that the average storage times of 22 days (ABLE), 28 days (RECESS) or 14.6 days (ARIPI) may not be relevant to asses “older” RBCs. This argument becomes particularly important in case the possible adverse effects related to prolonged RBC storage would be manifested closer to the end of the maximum storage time [99]. In addition, despite the relatively large number of participants, the above mentioned studies have been challenged as underpowered, thus failing to detect possible small but clinically relevant differences [99, 100].

Several animal studies have been conducted in the search for the mechanistic explanation to the supposed risks with transfusion of stored
RBCs. Hod et al. reported that mice receiving the equivalent of 2 units of stored (but not fresh) RBCs manifested with high levels of pro-inflammatory cytokines and NTBI in the plasma 2 h after transfusion [101]. In addition, they found that the pro-inflammatory response to lipopolysaccharide (LPS) stimulation was enhanced by the transfusion of stored, but not fresh RBCs [101]. Comparable inflammatory responses were obtained in a similar study in canines [102]. “The iron hypothesis” have been suggested to explain the inflammatory response seen in animal studies. This states that inflammation is mediated by an increased erythrophagocytosis and iron overload in the macrophages, resulting in release of reactive iron ions in a quantity that exceeds transferrin binding capacity [103]. However, in a human study where 1 unit of RBCs stored for 42 days was transfused, there were indications of NTBI in the plasma but no signs of a pro-inflammatory response [104].

How to study RBCs

Human studies

As mentioned earlier, the current (poor) understanding of the storage lesion and its effects upon transfusion is largely based on in vitro studies of stored RBC concentrates, which may or may not be relevant to explain the swift macrophage recognition of these cells after transfusion. In vivo studies are usually limited to non-invasive monitoring of 24 h survival assessments using $^{51}$Cr or biotin in vitro-labeled RBCs [105, 106]. As for the efficacy of transfusion, there are indications that stored RBCs actually decrease tissue oxygenation upon transfusion [107, 108]. This is possibly attributed to a reduced vasodilation activity as a consequence of a decreased ATP content and nitric oxide (NO) availability [109]. However, there are limitations in the studies conducted to measure tissue oxygenation in humans, which complicate the interpretation of these findings [110].

Animal models

Human physiology or pathophysiology is, of course, best studied directly in humans. However, that is not always practical or possible to do, in part due to ethical considerations. Therefore, the use of the mouse as a model system is common in order to enable studies not easily performed in humans. The mouse offers advantages by its small size, short generation time and both homologous and modifiable genetic backgrounds. Although mice are not a man, they are still a close relative when comparing the genomes with ~99% of the human genes having mouse homologues [111]. Even though the immune system of humans is quite similar to that of mice, it is nonetheless
important to mention that there are differences between species which needs to be taken in to account before making generalizations of findings in mice and its potential impact for humans. For instance; the main leukocyte cell type in human blood is neutrophil granulocytes which constitute 50-70% of total leukocytes. In mice, neutrophils only account for 10-25% and lymphocytes are instead the most abundant among blood leukocytes. Immunological differences between species also includes the occurrence and distribution of various kinds of Toll-like receptors (TLRs), CD4 and CD33, Fc receptors and immunoglobulin subclasses, as well as differences in cytokines secreted by the cells of the immune system. This can be seen as a reflection of different strategies used by the two species to cope with the environmental demands that they have been subjected to during the 65 to 75 million years of evolution that separates us [112].

Mouse RBCs have a shorter lifespan than human RBCs, typically about 50 days [113]. Senescent mouse RBCs are recognized by macrophages in spleen and liver [114]. The senescence mechanisms seem to be similar in mice and humans with respect to rheological properties and cell shrinkage [115]. Although at an accelerated rate, human and mouse RBCs also display similar storage characteristics [116]. Taken together, this indicates that a mouse model of storage and transfusion of RBCs could prove useful in getting a deeper understanding of implications of the human red blood cell storage lesion after transfusion.
Aims

Conflicting results have been reported regarding inflammation as a potential side effect of transfusion of stored RBCs.

- The aim was to investigate if cryopreserved human RBCs induce an increased erythrophagocytosis and subsequent inflammatory response following autologous transfusion into healthy volunteers.

The storage lesion of RBCs may resemble senescence, but could potentially also include eryptosis.

- We aimed at explore the role of eryptosis in the storage lesion and then investigate the splenic uptake of eryptotic cells after transfusion.

Although the biochemical changes described in stored RBCs may have an impact on their survival after transfusion, it seems logical that there is another level of the problem that needs to be understood in order to explain the rapid loss of RBCs after transfusion, namely; what molecular changes to the cell surface of the stored RBCs are recognized by macrophages and eventually results in RBC phagocytosis?

- The final aim was to study the receptor-ligand interaction between liquid stored RBCs and macrophages in order to better understand the consequences of the storage lesion after transfusion.
Materials and methods

Human studies (paper 1)

Ten healthy male participants, age 22 to 44 years, with Hb in the range of 140-155 g/L, were included in the study. The subjects, most of them committed to recreational training, were not allowed to compete in any events associated with the Swedish sports confederation during the entire time period of the study, including 4 weeks after reinfusion, due to the fact that they would potentially have a competitive advantage over any opponent related to the increase in oxygen carrying capacity after reinfusion. In addition, 7 control participants, matching the inclusion criteria, were also included for the evaluation of maximal aerobic capacity (VO₂max). Control participants, which did not donate or reinfuse RBCs, conducted the VO₂max tests at the same time points as the transfusion group.

Donation and reinfusion

Study participants donated one unit of blood (450 ml) on two occasions separated by one week. Blood was collected in CPD, followed by leukocyte and thrombocyte reduction, after which the majority of the plasma was removed by centrifugation. One hundred ml of SAG-M was then added to the remaining cells after which they were stored for 48 to 72 h before cryopreservation. Glycerolization of RBCs was carried out using the ACP 215 automated system (Hemonetics, Inc., Tamarac, FL) by the addition of a 57% glycerolyte solution (Fenwal, Lake Zurich, IL), after which the cells were kept frozen (-80°C) for 15 to 16 weeks. RBCs were then thawed, deglycerolized and washed, resulting in a post thaw recovery of ~80%. Reinfusion of RBC units was carried out by transfusing each unit under approximately 30 min. Venous whole blood samples from study participants were collected in EDTA, gel or Li-Heparin containing tubes (BD Vacutainer, Franklin Lake, NJ) at 2 h before, and at 2 and 48 h post transfusion for whole blood, serum and plasma analysis, respectively.

Blood analysis

Whole blood samples were used to analyze blood cell counts and Hb concentrations (Sysmex 2100, Sysmex, Kobe, Japan), while serum samples were used in the analysis of bilirubin, transferrin and serum iron (Vitros 5.1, Ortho Clinical Diagnostics, Inc., Raritan, NJ). Results from the two latter were combined to calculate transferrin saturation [117]. Serum samples were also used for the analysis of pro-, and anti-inflammatory cytokines, using a
cytokine bead array, custom designed to detect and quantify IL-1β, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1β (MIP-1β) and TNF-α (7-Plex kit, Bio-Rad, Hercules, CA). Analyses were made according to the manufactures instructions, with the addition of an extra concentration for the generation of the standard curve in order to increase detection of cytokines of minute concentrations. Plasma samples were used for the quantification of total haptoglobin by an immunoturbidimetric assay (Cobas 6000, Roche Diagnostics Scandinavia AB, Bromma, Sweden).

Maximal aerobic capacity (VO₂max) assessment

Study participants conducted treadmill running to exhaustion on a constant speed (individually set to be maintainable for 10 km) with a fixed stepwise incline increase (0.5-1°) each minute. Oxygen consumption (VO₂) was measured on a Jaeger Oxycon Pro (Erich Jaeger GmbH, Hoechberg, Germany). VO₂max tests were performed before and after blood donation, as well as before and (48 h) after reinfusion.

Animal studies (paper 2 and 3)

In the subsequent experiments, we used adult (8-25 weeks of age) male and female CD47−/−, CD47+/−, or wild type (wt) Balb/c mice, or wild type C57BL/6J mice. Mice were kept under low pathogen conditions and bred in our local facility with free access to water and food in accordance with local guidelines. All the experiments were performed in compliance with relevant Swedish and institutional laws and guidelines and approved by the Umeå research animal ethics committee (A14-12).

Labeling of RBCs

Labeling of RBCs before transfusion enabled the tracing of these cells after transfusion. We have used two different methods to label mouse RBCs. First, in vitro labeling with the fluorescent lipophilic dye PKH26 (according to the manufacturer’s instructions, Sigma Aldrich, St. Louis, MO). The PKH26 dye readily incorporates in the lipids of the RBC cell membrane [118]. Second, in vivo biotinylation with biotin (N-hydroxysuccinimido-biotin, EZ-Link NHS-Biotin, Thermo Scientific, Rockford, IL) which forms irreversible amine bonds to membrane proteins with exposed amine containing side chains (commonly lysine) and the N-terminal of proteins and polypeptides [106].

PKH26 labeling of RBCs was preferably used to identify splenic uptake of transfused cells. Tissue sections of spleens from transfused animals were
incubated with fluorescently labeled antibodies against surface markers of phagocytic cells in order to visualize specific cell populations, i.e. F4/80 on red pulp macrophages, MARCO on MZM, MOMA-1 on MMM and CD11c on dendritic cells. Sections were then analyzed by laser scanning confocal microscopy (Leica TSP-2, Heidelberg, Germany).

We used biotin-labeled cells in survival experiments, where blood samples could be drawn from mice at different time points after transfusion. Blood samples were then incubated with fluorochrome-conjugated streptavidin, which forms a strong bond to biotin. In this way, the fraction of the labeled cells, i.e. the transfused cells, could be monitored over time by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA). In vivo biotinylation was carried out by tail vein injection of 0.4 to 1.5 mg of biotin, dissolved in 150 µl of sterile PBS. This efficiently labeled all circulating cells. The mice were sacrificed the following day, and blood (with >98% biotin-labeled cells) collected by heart puncture. This blood was used for storage and transfusion. The double in vivo biotinylation technique enabled discrimination between different age fractions within the RBC population [119]. This was made possible by the injection of 1.1 mg of biotin per day for three days and then by waiting until half of the RBC population had been regenerated (~25 days), after which a lower dose of biotin (0.4 mg) was injected. This resulted in half of the RBCs binding higher levels of biotin (biotinhi; the older fraction) and the other half binding intermediate levels of biotin (biotinint; the young fraction). The two fractions of biotinylated cells can easily be discriminated individually from biotin negative RBCs of the recipient mice after transfusion.

We also labeled human RBCs with FLUO-3 (Molecular Probes) in order to quantify intracellular Ca²⁺ uptake. This was done by incubating RBCs with FLUO-3 in Ca²⁺-free media for 1 h in 37°C. Cells were then washed and later suspended in Ca²⁺-containing media, followed by incubation for 1 h in 37°C. Fluorescence intensity was then determined by flow-cytometric analysis (FACSCalibur flow cytometer, BD Biosciences).

The mouse model for RBC storage and transfusion

Whole blood was collected in CPD (blood to CPD-ratio - 7:1) by heart puncture of euthanized mice and spun at +4°C and 500 x g for 5 min. Plasma was then transferred to a separate tube, while the buffy coat and the top layer of the RBCs were discarded. Plasma was spun at +4°C and 1300 x g for 10 min, in order to remove any residual cells, after which the supernatant was transferred into a new tube. The RBCs were washed 3 times in PBS and finally mixed with SAG-M and plasma (ratio 3:1) and either used for direct
transfusion or stored for 10 days at +4°C in 0.5 ml test tubes. For Phthalate experiments, test tubes were coated in plastic film containing DEPH-phthalates.

In eryptosis experiments, the RBCs were incubated with the calcium ionophore ionomycin. For this, RBCs were suspended in Krebs-Ringer Hepes (KRH) medium at a hematocrit of 0.5% with 1µM ionomycin for 1 h at 37°C, followed by 3 washes in PBS. Ionophore-treated RBCs were finally resuspended in sterile PBS at a hematocrit 0.5% for phagocytosis experiments, or at 20% hematocrit for transfusion experiments.

Mice anesthetized with isofluorane (Baxter, Deerfield, IL) were transfused with 150 µl of fresh or 10 day stored RBCs, administered by tail vein injection. Blood samples from the opposite tail vein were taken at multiple time points after transfusion and analyzed by flow cytometry for the presence of transfused cells as described above.

**In vitro characteristics of stored RBCs**

To elucidate if the mouse model for RBC storage shared characteristics with that of human stored RBCs, *in vitro* measurements on intracellular ATP and the formation of MPs were conducted. For ATP measurements, 5 x 10^7 murine RBCs, stored for 0, 4, 7, or 10 days, were lysed in hypotonic buffer. An ATP standard curve was established by diluting ATP samples of known concentrations. Samples were mixed with ATP-reagent, containing luciferin and luciferase, followed by analysis of peak luminescence using a Triathler luminometer (Hidex, Turku, Finland). For MP quantification, fresh and 10 day stored murine RBC concentrates were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ter-119 (ImmunoTools, Freising, Germany) and analyzed for MPs by logarithmic scaling for FSC and SSC on a FACSCalibur (BD Biosciences) flow cytometer.

**In vitro phagocytosis**

Mouse peritoneal macrophages were used for phagocytosis experiments, basically as described elsewhere [120]. Fresh or stored human and mouse RBCs, or eryptotic mouse RBCs, were then incubated together with adherent macrophages for 1 h at 37°C, 5% CO₂. Incubation was carried out with or without the addition of 10% FCS or human serum (HS). The effect of the class A-SR-inhibitors fucoidan and dextran sulphate, or the control substance chondroitin sulphate, was also evaluated. For signaling inhibitor experiments, macrophages were pre-incubated for 30 min in the presence or absence of the phosphatidylinositol 3-kinases (PI3-kinase)–inhibitor
LY294002 (50µM), the P38 mitogen-activated protein kinase (p38 MAPK)-inhibitor SB203580 (30 µM), or the extracellular signal-regulated kinase-1/2 (ERK1/2)-inhibitor PD98059 (30 µM). When phagocytosis was terminated, non-ingested RBCs were lysed with dH₂O for 30 sec, followed by washing, fixation and staining, using May-Grunewald/Giemsa. In every experiment, at least two technical replications per condition were made. Macrophages with ingested RBCs were then counted to generate a phagocytosis index by dividing the number of phagocytized RBCs with the total number of macrophages.

**Ethics**

The human transfusion study was conducted in accordance with the declaration of Helsinki and approved by the Swedish central ethics committee (Dnr Ö 1-2009). For *in vitro* experiments, human RBCs were acquired from blood bags (containing saline-adenine-glucose-mannitol (SAG-M), kept at a constant temperature of +4°C and stored in the normal deposit at the Blood Bank at Norrlands University Hospital (Umeå, Sweden). A part of a plastic hose, filled with ~1 ml RBCs, was welded off from blood bags stored for up to six weeks. The acquired samples were anonymized and carried only information of blood group and the date of donation. Since the results obtained in the present study could not be traced back to a specific blood donor, ethical approval was not needed according to Swedish legislation (41, SFS 2003:460). All animal experiments were approved by Umeå research animal ethics committee (A14-12).

**Statistical analysis**

Two tailed student’s paired and unpaired t-test was used for comparisons between samples and groups respectively, when normal distribution could be assumed. Wilcoxon’s related samples signed rank test were used of paired comparisons when data were not normally distributed (IBM SPSS statistics Base 19, IBM, Armonk, NY, USA and JMP 11.0, SAS Campus Drive, Cary, NC).
Results

Paper 1

Transfusion of liquid-stored RBCs has been associated with worsened outcomes after transfusion [92, 121]. In addition, an animal study has shown that transfusion of the equivalent of 2 units of liquid-stored RBCs resulted in a profound proinflammatory response, suggested to be the result of an increased RBC uptake by macrophages shortly after transfusion [101]. To investigate the response to transfusion of cryopreserved RBCs in humans, we transfused 2 units of cryopreserved RBCs into 10 healthy human volunteers. Indicators of erythrophagocytosis, inflammation and the number of leukocytes in blood were assessed at 2 h and 48 h after transfusion and compared with that at 2 h before transfusion.

Transfusion of cryopreserved RBCs leads to acute accelerated RBC breakdown

As expected, these RBC transfusions resulted in a significant increase in blood Hb. The number of circulating neutrophil granulocytes was significantly higher at 2 h and 48 h after transfusion. This was accompanied by an increase in circulating monocytes at 48 h after transfusion, thus indicating a transfusion-mediated effect on the immune system. We also found evidence of an acute clearance of transfused RBCs as the serum iron concentration, the transferrin saturation and the serum bilirubin concentration were all significantly increased at 2 h post transfusion, but had then returned toward baseline at 48 h. We further noticed a decrease in serum haptoglobin levels at 2 h after transfusion, which again returned towards baseline levels at 48 h, indicating that part of the hemolysis was in fact intravascular.

Absent pro-inflammatory response in the acute phase after transfusion

After concluding that an acute hemolysis occurred after transfusion, we next investigated if there were any signs of inflammation. For this, we screened for known pro-inflammatory cytokines; IL-1β, IL-6, IL-8, MCP-1, MIP-1β, TNF-α, and the anti-inflammatory cytokine IL-10. None of the investigated cytokines increased in serum samples, neither at 2 h nor at 48 h after transfusion.
**Additional results**

Paper 1 is based on data that originates from a larger study aimed at evaluating the enhanced aerobic capacity as a result of autologous transfusion and to identify biological markers of blood doping. Maximal aerobic capacity (VO₂max) was evaluated before and after blood donation as well as reinfusion. Donation of 900 ml of blood led to a significant reduction in VO₂max of 8±3%. Surprisingly, this reduction was not compensated for during the 15 weeks of recovery after donation even though Hb concentration was restored (Figure 6). In contrast, reinfusion of the cryopreserved RBCs led to a 13±8% increase in VO₂max, close to the starting value.

![Maximal oxygen uptake (VO₂max)](image1)

**Blood Hb concentration**

![Blood Hb concentration](image2)

**Figure 6. Restoration of [Hb] 15 weeks after donation does not result in restored maximal aerobic capacity (VO₂max).**

VO₂MAX and blood Hb concentration was measured before and after blood donation as well as reinfusion. Values for control group (dotted line, n=7) and transfusion group (continuous line, n=9), are given as means±SEM. Unpaired student’s t-test was used for group comparisons. Indicated is the time of blood donation (D) and time of RBC reinfusion (R).
Paper 2

Eryptosis, a Ca$^{2+}$ dependent stress-induced cell death in RBCs, results in a shorter than normal lifespan and could theoretically be a part of the RBC storage lesion. However, the understanding on how eryptotic RBCs are taken up by phagocytic cells in the spleen is virtually unknown. Thus, this study aimed at first investigating if there were any signs of eryptosis in liquid store RBCs, and second to investigate what phagocytic populations of the spleen that mediate uptake of eryptotic cells.

Stored human RBCs contain a subset of Ca$^{2+}$-high cells

It is currently unknown if eryptosis plays a role in the storage lesion. Because calcium uptake is a hallmark of eryptosis, we tested if liquid stored human RBCs display an increased Ca$^{2+}$ permeability. RBCs, stored in SAG-M for 0, 2, 4 and 6 weeks were labelled with the FLUO-3 calcium indicator followed by incubation in Ca$^{2+}$ containing medium for 1 h at 37°C. At the beginning of storage, human RBCs stored in SAG-M contained a low fraction of cells with an increased cytoplasmic Ca$^{2+}$-level (0.7±0.3%). However, we found a time-dependent increase in the fraction of cells with increased Ca$^{2+}$, reaching 3.6±0.8% after 6 weeks of storage. This suggests that part of the storage lesion could be associated with some RBCs having an eryptotic phenotype.

Characteristics of eryptotic murine RBCs

A mouse model was used to investigate the clearance of transfused eryptotic cells, as this is difficult to assess in humans. It was found that RBCs treated with 1 µM ionomycin increased their intracellular Ca$^{2+}$ concentration and that around 50% of the cells were PS+ as accessed with Annexin V binding. This was accompanied by a reduction in cell size as seen by lower FSC in flow cytometric analysis.

In vivo uptake of Ca$^{2+}$-RBCs is located to the splenic marginal zone in a CD47-independent manner

Transfusion of untreated RBCs, labeled with PKH26 or biotin, resulted in a rather linear clearance over the entire expected lifespan of 50 days, where the splenic uptake of these cells was located to F4/80+ macrophages in the red pulp area. The fact that the clearance kinetics of PKH26 or biotin labeled cells was virtually identical indicated that the labeling per se had no effect on the removal of the transfused cells. In contrast to that for freshly isolated RBCs, transfusion of PKH26-labeled Ca$^{2+}$-RBCs (ionophore treated) resulted in a swift clearance of the majority of the transfused cells within 30 minutes. The splenic uptake of Ca$^{2+}$-RBCs was located to the MZ, and appeared to be preferentially mediated by MARCO+ MZ macrophages (MZM). Uptake of Ca$^{2+}$-RBCs was also seen in CD11c+ DCs in the MZ at 1 h after transfusion. At
24 h after transfusion, we also found CD11c+ DCs that were positive for PKH26 in the T-cell area of the splenic white pulp. Flow cytometric analysis at 24 h after transfusion revealed that more than 20% of the total CD11c^hi DCs, and about half of the CD11c^hi/CD207^hi DC subset, were positive for PKH26^+ Ca^{2+}-RBCs. The uptake of Ca^{2+}-RBCs by macrophages or DCs was not affected by CD47 on the RBCs since CD47^−/− Ca^{2+}-RBCs were equally ingested by peritoneal macrophages in vitro, or by DCs in vivo, as compared with that for CD47^+/+ Ca^{2+}-RBCs.

Additional results
Murine RBCs, labeled with PKH26 and stored for 10 days were injected into recipient mice. Spleens were harvested after 24 h and cryosections were labeled with antibodies against of MARCO^+ MZM and F4/80^+ red pulp macrophages. The splenic uptake of stored RBCs was found to co-localize with both F4/80^+, as well as MARCO^+, splenic macrophages (Figure 7.)

Figure 7. Splenic uptake of liquid stored murine erythrocytes is located both in the marginal zone and the red pulp.
PKH26^+ stored erythrocytes were shown to be taken up by both F4/80^+ red pulp macrophages (top row), and MARCO^+ MZM (bottom row). Co-location of erythrocytes and macrophages is seen as yellow on merged pictures to the right.
Paper 3

Much is still unknown regarding the storage lesion and the mechanisms mediating rapid RBC elimination after transfusion. The lack of detailed knowledge can in part be explained by ethical limitations in human studies. To circumvent these limitations, we have adopted a murine model of RBC storage and transfusion. This model was designed to mimic that of the human setting as closely as possible, e.g. by using CPD and SAG-M in the collection and storage of the RBCs. In addition to the in vivo model of RBC transfusions in mice, we also investigated if scavenger receptors were involved in the phagocytosis of stored murine or human RBCs, using an in vitro mouse macrophage phagocytosis assay.

Characteristics of the murine model for storage and transfusion
Murine RBCs have a shorter lifespan and are generally considered as more fragile than human RBCs [116], which was also reflected in the shorter storage time needed to induce the storage lesion (10 days for mouse RBCs and 42 days for human RBCs). At this time point, stored murine RBCs displayed similar characteristics as previously described for stored human RBCs, including about 50% reduction in ATP content and formation of MP. In addition, about 35% of the stored murine RBCs were eliminated from the circulation within 24 h, while the RBCs surviving this time period showed normal long-term clearance kinetics after transfusion.

Phagocytosis of stored human and mouse RBCs is serum dependent and mediated by scavenger receptors
The initial rapid clearance kinetics of stored RBCs likely represents molecular changes caused to a fraction of the RBCs during storage, which are efficiently recognized by macrophages leading to phagocytosis after transfusion. We hypothesized that these changes may not be species-specific, but rather that the recognition mechanisms could be part of the evolutionary conserved systems that identify damaged cells. To test this hypothesis, both human and murine RBCs (fresh or stored) were incubated with murine peritoneal macrophages in an in vitro phagocytosis assay. When macrophages were incubated with fresh RBCs, neither murine nor human RBCs were phagocytized. In contrast, stored murine and human RBCs were readily phagocytized by macrophages in the presence of heat inactivated FCS (and heat inactivated HS for human RBCs). Interestingly, phagocytosis of stored murine or human RBCs was markedly reduced by 70-75% under serum-free conditions. To investigate if phagocytosis of stored RBCs was dependent on class A-SR, we studied phagocytosis in the presence of the class A-SR ligands fucoidan and dextran sulphate. The results showed that both fucoidan and dextran sulphate inhibited phagocytosis of stored murine
or human RBCs by more than 90%. Phagocytosis of stored RBCs was also significantly reduced in the presence of the PI3 kinase-inhibitor LY294002. In addition, the ERK1/2-inhibitor PD98059 and the p38MAPK-inhibitor SB203580 each reduced phagocytosis of stored RBCs by 30% and 36%, respectively.

Additional results

**Discrimination of RBC age fractions by in vivo biotinylation**

Double *in vivo* biotinylation was carried out with 25 days in between biotin injections. This enabled the RBCs to be separated into age fractions. Biotin\textsuperscript{hi} cells represented RBCs >25 days old and biotin\textsuperscript{int} cells represented the remaining RBCs which were <25 days old (figure 8A). On day 26, the mice were bled and RBCs were isolated before transfusion into recipient mice. Blood samples from transfused mice showed a close to linear clearance of >25 days old biotin\textsuperscript{hi} cells during the first 25 days after transfusion. The <25 days old biotin\textsuperscript{int} RBCs were, as expected, initially cleared slower and could be detected up to 55 days after transfusion (figure 8B).

![Figure 8. The use of biotin to label circulating erythrocytes in donor mice and subsequent analysis of their clearance following transfusion into recipient mice.](image)
Role of the RBC age at the start of storage for their 24 h survival post transfusion

RBCs from double in vivo biotinylated mice, freshly isolated or stored for 10 days, were transfused into recipient mice in order to investigate if RBCs >25 days old at the start of storage would be more severely affected by storage and therefore eliminated to a larger extent during the first 24 h after transfusion. The 24 h recovery of freshly transfused RBCs was high among both younger and older fractions (figure 9 A and B). Interestingly, when 10 days stored RBCs were transfused, we found that virtually all cells disappearing rapidly within the first 24 h belonged to the fraction of RBCs >25 days old at the start of storage (figure 9 B). When comparing the survival kinetics of the fraction of >25 days old freshly transfused or stored RBCs that remained in circulation at 24 h after the transfusion, we found that old fresh and stored RBCs had virtually similar recovery and expected life span (figure 9 C).

Figure 9. Virtually all of the stored murine RBCs cleared within 24 h belong to the fraction of older RBCs.

Murine RBCs were labeled twice with biotin in vivo to enable separation of RBCs with an age of <25 days (young) or >25 days (old). Isolated RBCs were either freshly transfused (Fresh) or stored in SAG-M and autologous plasma at +4°C for 10 days before transfusion. (A) Recovery of fresh or stored younger RBCs, or (B) fresh or stored older RBCs, at 24 h after transfusion. (C) Recovery kinetics of older fresh or older stored RBCs, where the amount of each RBC fraction remaining in circulation at 24 h after transfusion was set to 100 per cent. Data are means±SD of 4 separate recipient mice.

Phthalates during storage

Plastic softeners or phthalates are known to be released from blood bags in to the membranes of the stored RBCs [122-124]. Trying to investigate a potential effect of phthalates in our murine storage model, RBCs were stored in test tubes coated with or without phthalate containing PVC-plastic film.
Transfusion of phthalate stored RBCs demonstrated near identical 24 h survival characteristics as RBCs stored without phthalates (Figure 10A.) This finding could indicate that plastic softeners may not affect murine RBCs during storage with respect to their chances of surviving after transfusion.

**CD47 on stored murine RBCs does not influence the rapid elimination that occurs during the first 24 h**

The cell surface protein CD47 has been shown to serve as an anti-phagocytic ligand, protecting RBCs from being phagocytized by macrophages [52]. Furthermore, the amount of CD47 on the cell surface has been implicated to decrease during storage of human RBCs [83]. To test if a reduction in CD47 on stored RBCs could affect the rate of rapid 24 h elimination after transfusion, stored RBCs from Wt (CD47+/+) or CD47+/- mice (in which the CD47 expression is 50 % of that in CD47 Wt) were transfused into Wt mice. The 24 h survival was nearly identical when comparing Wt and CD47+/- RBCs, indicating that CD47 may not be involved in regulating the acute phagocytosis of stored erythrocytes (figure 10B).

![Figure 10](image_url)

**Figure 10. Acute clearance of stored murine RBCs is not affected by phthalates of RBC CD47.**

(A) RBCs were stored in the presence or absence of phthalates before transfusion to syngenic mice. Data are means±SD of 2 separate recipient mice. (B) Transfusion of stored Wt (CD47+/+) or CD47+/- RBCs displays near identical RBC recovery during the first 24 h after transfusion. Data are means±SD of 4 separate recipient mice.
Discussion

Frozen vs. liquid stored RBCs

At present, the vast majority of RBC concentrates are stored under liquid conditions [125]. Cryopreservation of RBCs are mainly restricted to donors with rare blood groups and for autologous transfusions [126]. Cryopreserved RBCs have been shown to be spared from many of the hallmarks of the liquid storage lesion including a reduction in ATP and 2,3-DPG and the formation of MPs [87-89]. Since little is known about the clinical consequences of the storage lesion, it is problematic to evaluate the two storing methods based on in vitro characteristics, but the phenotype of cryopreserved RBCs suggest that the method may have advantages over liquid storage. On the other hand, the time consuming procedure (3-4 hours) of thawing RBCs limits its use to planned procedures. Our data indicated that transfusion of two units of cryopreserved RBCs had a modest effect on the Hb concentration, which was only significantly elevated at 2 h after transfusion (from 145.4±4.9 g/L to 151.1±4.6 g/L). After 48 h, it had dropped 3 g/L and was no longer significantly different from the Hb concentration before transfusion. On the other hand, aerobic capacity assessment at 48 h after transfusion, revealed a 13% increase in VO₂max as compared to values obtained the week before transfusion (Figure 6). Thus, differences in the venous Hb concentration may not be the best assessment of neither RBC volume nor aerobic capacity after RBC transfusion to non-anemic men, possibly explained by the capacity of the spleen to store RBCs [127, 128].

Inflammation after transfusion?

There is at present a controversy regarding risks with transfusion of RBCs stored for prolonged periods of time [92, 93]. Alarming results from animal studies have also emerged, in were Hod et al. [101] could show a profound pro-inflammatory cytokine storm and the presence of NTBI 2 h after transfusion of the equivalent of two units of stored RBCs into mice. The cytokine release was suggested to originate from the large amount of iron delivered to macrophages upon the rapid phagocytosis of a fraction of the transfused RBCs [103]. However, no clear evidence has been presented for such a response in humans, with the exception of a report in preterm infants [129]. To test if increased erythrophagocytosis alone could provoke such a response in healthy humans, we conducted experiments where human subjects received two units of cryopreserved RBCs. Although the storage conditions was not the same (frozen vs. liquid stored), it has been shown that approximately 15 % of the previously frozen, transfused RBCs are rapidly
cleared from circulation upon transfusion [88, 130]. Indeed, our results also showed a marked increase in serum bilirubin, iron and transferrin saturation at 2 h post transfusion, which all indicate increased erythrophagocytosis. However, the rise in transferrin saturation (from 28.1 ±1.3 % to 43.2 ±1.5 %) did not indicate that non-transferrin bound iron were present in the plasma since most of the transferrin were still not saturated with iron. It can however not be ruled out that the peak of serum iron came about after the measurement at 2 h post transfusion and that NTBI might have been present at a later time point. A human study on transfusion of one unit of liquid stored RBCs, stored for 42 days, to healthy recipients showed a larger increase in transferrin saturation than observed in our study [104]. That study also detected NTBI in plasma after transfusion, indicating that there could be a difference as to how frozen and liquid stored RBCs are received after transfusion [104]. This discrepancy is probably related to the storage lesion which primarily is restricted to liquid stored RBCs.

To elucidate if the hemolysis was primarily intra- or extra-vascular (i.e. if the red blood cells burst in circulation or were phagocytized in a controlled way by macrophages), we measured the levels of haptoglobin in serum. Haptoglobin binds free Hb and a diminished plasma level of haptoglobin is an indirect marker for intravascular hemolysis, although profound extravascular hemolysis could also lead to reduced haptoglobin levels [131]. We found that haptoglobin was reduced by 0.18 g/L at 2 h after transfusion, which corresponds to the binding of approximately 0.8 g of Hb [132, 133]. Since the total amount of Hb transfused was about 80 g, intravascular hemolysis only accounted for 1% of the transfused RBCs, strongly indicating that the majority of the hemolysis was in fact extravascular. After concluding that we had an acute, extravascular hemolysis, we investigated the presence of pro-inflammatory cytokines in the plasma of transfused individuals, in search of similarities with findings in the above mentioned animal study. None of the investigated pro-inflammatory cytokines IL-1β, IL-6, IL-8, MCP-1, MIP-1β or TNF-α increased in plasma at 2 h or 48 h after transfusion. Similar findings were also reported after transfusion of one unit of liquid stored RBCs in healthy recipients [104]. The discrepancy in the data concerning signs of inflammation between animal and human studies could be due to differences in the storage and/or administration procedure, but also due to species differences [112].

**The mouse model for storage and transfusion**

In order to expand our understanding on post transfusion events related to the storage lesion, we turned to an animal model. There are always drawbacks when using model systems, and one must be cautious when
transferring findings in animal models to human physiology [112]. Nevertheless, due to ethical considerations, an animal model can present opportunities to study mechanisms that are not easily assessed in human subjects. The use of inbred mice is an attractive alternative due to practical considerations and the genetic kinship between man and mouse [111]. Together with the increasing availability of genetic manipulations, the mouse model offers unique possibilities compared to humans investigations.

The use of a mouse model in RBC storage and transfusion has been shown to resemble human settings [134], albeit with shorter storage time of the RBCs prior to transfusion. As expected, we observed that 10 days of storage resulted in an ATP reduction, MP formation and 24 h survival kinetics resembling that found in human RBCs after prolonged storage. Human RBCs are stored in PVC-plastic bags that contain plasticizers (phthalates), known to leak into the membranes of the RBCs and thus improve their storage characteristics as they reduce the hemolysis incidence [122-124]. In an attempt to evaluate the effect of phthalates on mouse RBC storage, we coated the storage test tube with PVC-plastic film prior to the addition of the cells. RBCs stored in phthalate plastic showed similar 24 h clearance kinetics as control RBCs stored without phthalates (Figure 10A), indicating that plasticizers may not have a major influence on the 24 h survival of stored mouse RBCs after transfusion in our model.

The role of calcium uptake in the storage lesion

Increased intracellular Ca²⁺ is a hallmark of eryptotic cells, which leads to PS externalization and the subsequent uptake by phagocytic cells [40]. The observation that a small fraction of RBCs had an increased Ca²⁺ permeability after 6 weeks of storage suggests that eryptosis could be a part of the RBC storage lesion. It is not known if the fraction of Ca²⁺ RBCs could further increase after transfusion, but the observation that human stored RBCs starts to externalize PS after overnight incubation at 37°C suggests the possibility that transfused cells may acquire additional removing signals after transfusion [135].

The spleen consists of distinct populations of phagocytic cells with different functions and locations [56]. By studying the splenic uptake of experimentally induced eryptotic cells after transfusion, we found that the vast majority of RBCs with increased Ca²⁺ were taken up in the marginal zone (MZ), preferentially by MARCO⁺ MZM. This is in contrast to the uptake of normally senescent RBCs, which generally are phagocytized by F4/80⁺ red pulp macrophages. One explanation to the different micro-anatomical locations of phagocytosis could be that a substantial amount of the arterial
blood in the murine (but also human) spleen flows through the MZ before entering the red pulp, placing phagocytic cells of the MZ first in line to recognize the cryptotic RBCs. The uptake of stored murine RBCs was found to be located both in the marginal zone and in the red pulp (Figure 7) and as such, agrees with the hypothesis that a part of the storage lesion could in fact be explained by cryptosis. However, it also supports the view that the storage lesion is multifactorial, and that other RBC changes lead to their recognition by the red pulp macrophages.

**Impact of storage on RBCs - Senescence, cryptosis, or both?**

To further understand the effect of the storage lesion, we applied the double *in vivo* biotinylation technique [119], in which RBCs in circulation can be separated with regard to their age (Figure 8). This grants the ability to follow younger and older RBCs separately, in order to evaluate the effect of the storage lesion in separate age fractions. Interestingly, we found that almost the entire loss of transfused stored RBCs during the first 24 h involved the fraction of older RBCs (>25 days of age prior to storage), which strongly indicates that the storage lesion has a more profound effect on aged RBCs (Figure 9A, B). Markedly, the older cells that remained in circulation after 24 h showed similar clearance kinetics as freshly transfused RBCs of the same age (Figure 9C). This therefore suggests that the storage lesion could have a more profound effect on older RBCs, and that cells surviving the first 24h after transfusion seem to be rather unaffected by the storage lesion.

It is known that some of the RBCs having reduced levels of ATP and 2,3-DPG after storage may restore these levels within 24 h after transfusion [85]. Such recovery could theoretically also be connected with restoring some of the RBC cell surface changes that are involved in macrophage recognition and clearance. Furthermore, it has been suggested that the shedding of PS+ MPs from stored RBCs could reduce the amount of the externalized PS on the RBCs and thus reduce the risk of phagocytosis of these cells [135]. To investigate the possibility that some of the stored RBCs, which are normally rapidly eliminated after transfusion, may have the capacity to recover in the absence of macrophages, one could deplete recipient mice of macrophages in the spleen and liver before transfusion. The efficient depletion of macrophages in contact with the bloodstream by i.v. injection of clodronate liposomes has been demonstrated [136]. An improvement in 24 h recovery of stored RBCs in clodronate depleted macrophages have also been shown [137]. By transfusing biotin-labeled stored RBCs, we could investigate how different age fractions among the transfused cells remain in circulation over time until macrophages start to return around day 6, and elucidate if all of the rapidly cleared transfused RBCs are taken up by macrophages.
Transfused RBCs remaining in circulation can also be recovered by FACS-sorting for further biochemical analysis.

**How are stored RBCs presented to the immune system upon transfusion?**

DCs are professional APCs that trap foreign or endogenous antigens by phagocytosis or macropinocytosis. The antigens are then intracellularly processed, attached to MHC, and finally presented on the cell surface together with costimulatory molecules (Figure 2). Depending on the phenotype of the DC, and the recognition pattern of the antigen, the DC will present the antigen in either an immunogenic or tolerogenic way [15, 138]. The balance between tolerance and immunity can further be affected by the inflammatory state of the organism. Alloimmunization has been shown to be propagated after RBC transfusion of transgenic hen egg lysosome (HEL)-RBCs in mice, but only after co-stimulation with the immunostimulant poly(I:C) [139]. In contrast, the administration of LPS together with HEL-RBC transfusion not only failed to increase alloimmunization, but was also found to inhibit the same process [140]. Thus, inflammation as such may be a too broad of a label to use when assessing risks of transfusions since (mimicked) viral or bacterial inflammatory stimuli seem to have quite different effects on immunomodulation in relation to alloimmunization, at least in mice. This also highlights the importance of finding the receptor(s) involved in recognition of stored RBCs by the immune cells.

A subset of DCs which express the cell surface proteins CD103 (integrin αE) and CD207 (langerin) (CD207+ DCs) has been identified as responsible for the uptake of apoptotic blood borne cells in the marginal zone of the (mouse) spleen [141]. Their function is to maintain self-tolerance by migrating to the T-cell area of the spleen and present self-antigens to naïve T-cells. Depletion of MZ phagocytes has been shown to result in loss of immunosuppression against apoptotic cells [142]. After stimulation with either poly(I:C) or LPS, the number of CD207+ DCs was found to be drastically reduced in murine spleens [141]. The finding in paper 2 of the present thesis, that stored human RBCs may contain a population of eryptotic cells and that experimentally eryptotic RBCs were recognized and taken up by CD207+ DCs in the spleen, therefore opens the door for a somewhat unexpected possibility: that part of the storage lesion may in fact have beneficial effects in terms of propagating tolerance rather than immunity. Inflammatory stimuli are likely to interfere with maintenance of this tolerance, again bringing the role of inflammation after transfusion of stored RBCs to life. In a next step, it would be intriguing to transfuse PHK26+ stored RBCs, collect spleens and (by flow cytometry) determine to which degree different macrophage and DC subsets ingest
stored RBCs. Different activation markers on PKH26 + phagocytic cells can then be assessed in order to understand the immunologic impact of transfusion of stored RBCs.

**CD47 in senescence, eryptosis and the storage lesion**

The presence or absence of CD47 on experimentally eryptotic RBCs affected neither macrophage phagocytosis in vitro nor DC uptake of these cells in vivo. There have been indications in the literature that senescent, and possibly also stored RBCs, may have reduced amounts of CD47 on their surface [36, 83]. We found that CD47 +/- RBCs (expressing half of normal CD47 levels) had virtually identical clearance kinetics as normal CD47+/+ RBCs after storage and transfusion (Figure 10B). The long-term survival of CD47 +/- RBCs was on the other hand slightly reduced as compared to CD47+/+ controls (data not shown). This could support the hypothesis that senescent RBCs are recognized by phagocytic cells after opsonization by NAbs, since IgG-opsonized CD47 +/- RBCs were found to be cleared more rapidly after transfusion than equally IgG-opsonized CD47+/+ RBCs in vivo [143]. We have not specifically investigated the effect of CD47 in our phagocytosis assay of stored RBCs. However, since macrophage phagocytosis of stored RBCs could be efficiently blocked by addition of SR inhibitors, and SR-mediated phagocytosis of oxidatively damaged RBCs has been shown to be CD47 independent [120], there are no strong indications from our studies that CD47 (or lack thereof) is influencing macrophage recognition of stored RBCs.

Furthermore, the CD47-SIRPα interaction has been shown to be dependent on RBC membrane flexibility, which could imply that the ability of CD47 to inhibit phagocytosis could be further limited in senescent or stored RBCs [144]. In addition, an alternative role of the CD47-SIRPα interaction has been presented by Burger et al. [145], who showed that the serum protein thrombospondin-1 (TSP-1) could bind to CD47 on senescent or stored RBCs to promote their phagocytosis. A conformational change in CD47 enabled binding of TSP-1 which resulted in SIRPα-dependent phagocytosis. This indicates that the CD47-SIRPα interaction can have both pro-, and anti-phagocytic properties, thus making quantification of total CD47 per se an unsuitable indicator to predict the uptake of senescent and/or stored RBCs.

**Scavenger receptor mediated phagocytosis of stored RBCs**

Scavenger receptors are evolutionary conserved receptors that can mediate endocytosis and phagocytosis of molecules or cells carrying certain molecular signatures [13]. Fucoidan and dextran sulphate are ligands for
class A-SRs and can efficiently block binding of other ligands to these receptors [146]. Interestingly, we found that both fucoidan and dextran sulphate strongly inhibited phagocytosis of stored human as well as murine RBCs in vitro. Thus, class A-SRs may be a previously unappreciated class of receptors involved in macrophage phagocytosis of RBCs damaged by storage. It has previously been shown that fucoidan and dextran sulphate can inhibit phagocytosis of oxidatively damaged murine RBCs [120]. Inhibition of the signal transduction proteins PI3-kinase, p38-MAPK and ERK1/2 was shown to affect phagocytosis of IgG opsonized or oxidized RBCs [120]. Since we also found that the same inhibitors reduced the uptake of stored RBC, this could suggest that oxidative stress may in fact be one important contribution to the storage lesion with respect to the 24 h recovery of transfused cells. PI3-kinase is essential for phagocytosis of endogenous material as well as pathogens [147], and has been suggested to also regulate phagocytosis of stored RBCs [137]. Scavenger receptor class A-1 (SRA-1 or CD204) lacks a signaling cytoplasmic domain and may only mediate binding of damaged RBCs to the macrophage, while the intracellular signaling resulting in phagocytosis could be contributed by other receptors binding to the same RBC [148]. Ligation of fucoidan to SRA-1 has been shown to enhance TLR-4 mediated activation of nuclear factor-κβ (NF-κβ), resulting in TNF-α and IL-1β production by LPS stimulated murine peritoneal macrophages [149]. Ligation of fucoidan alone did not, however, mediate an inflammatory response. Importantly, the impact of this recognition mechanism in vivo, as well as in the macrophage subpopulations normally involved in elimination of stored RBCs in spleen or liver, has to be further investigated in both the murine and human system.

**The role of serum in phagocytosis of stored cells**

Both human and murine stored RBCs required heat-stable serum factors for efficient phagocytosis by macrophages in vitro, since removal of heat-inactivated FCS strongly reduced RBC uptake. Phagocytosis of stored human RBCs could also be restored by heat-inactivated human AB serum. Thus, heat-stable serum factors seem to be required for macrophage phagocytosis of stored RBCs. The fact that serum was required for phagocytosis of both human and murine stored RBCs, and that stored RBCs of both species were similarly recognized by murine macrophages, suggest that macrophages can use evolutionarily conserved species-independent mechanisms for uptake of stored RBCs. Complement is not a likely candidate, since it is destroyed by heat-inactivation [150]. However, several serum proteins have been described to function as bridge molecules in macrophage recognition of damaged or apoptotic cells [2, 151]. Thus, further investigations will be
required to narrow down on the serum component(s) and corresponding receptor(s) that promote phagocytosis of RBCs affected by storage.

**Concluding remarks**

The findings in paper 3 of this thesis indicate that the recognition of liquid stored RBCs is mediated by class A scavenger receptors. SR-A1 is a possible candidate which is expressed on many different macrophage populations, including murine peritoneal macrophages [149], which were used in our experiments. Inhibition of phagocytosis in our *in vitro* model, both at receptor and signaling transduction level, were shown to resemble that found for oxidatively damaged RBCs [120]. Together with our observation that the oldest RBCs are more prone to be rapidly removed after transfusion, this indicates that oxidative damage may very well lead to important phenotypic changes of stored RBCs that ultimately leads to their swift recognition after transfusion.

The phagocytosis of stored murine or human RBCs was found to be serum dependent. In addition, while SR-A1 lacks a signaling domain, PI3 kinase, p38MAPK and ERK1/2 were found to be important for phagocytosis. This suggests that (an)other receptor(s), possibly requiring serum proteins, could provide signaling and partner with SR-A1 for phagocytosis of stored RBCs (Figure 11). In contrast to phagocytosis of opsonized RBCs, CD47 does not seem to inhibit SR mediated RBC phagocytosis [120]. Our observation that stored CD47⁺/⁻ RBCs (expressing half of normal CD47 levels) were cleared at the same rate as normal (CD47⁺/+⁷) RBCs, strengthen the hypothesis for the involvement of SR in uptake of stored RBCs.

![Figure 11. Working hypothesis for a two-receptor model mediating phagocytosis of stored RBCs.](image_url)
Conclusions

This thesis is based on human and murine in vivo and in vitro studies. From human studies it is concluded:

- Increased erythrophagocytosis did not provoke a pro-inflammatory response in healthy men, thus questioning the iron hypothesis that have been suggested to occur after transfusion.

- A subset of liquid stored RBCs display a time dependent increase in eryptotic-like phenotype.

From studies conducted in the murine model system it is concluded:

- Eryptotic cells are phagocytized in the splenic marginal zone by macrophages and DCs in ways resembling uptake of apoptotic cells.

- Liquid-stored RBCs is phagocytized by macrophages in the marginal zone and red pulp of the spleen.

- The oldest fraction of liquid-stored murine RBCs is responsible for the reduced 24 h survival seen after transfusion. A reduction of CD47 on the cell surface did not affect the acute clearance of stored RBCs after transfusion.

- In vitro phagocytosis of stored human and mouse RBCs is serum dependent and can be blocked by class A scavenger receptor inhibition.
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References


