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Regulation of complement activation on red blood cells

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Chapter 4

Complement deposition and IgG binding on stored red blood cells are independent of storage time

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ABSTRACT

Background: In the Netherlands, red blood cells (RBCs) are allowed to be stored up to 35 days at 2-6°C in saline-adenine-glucose-mannitol (SAGM). During storage, RBCs undergo several changes. Collectively known as storage lesion. We investigated to what extent complement deposition and antibody binding occurred during RBC storage and investigated phagocytic uptake in vitro.

Materials and methods: RBCs were stored for different lengths of time at 2-6°C in SAGM. Complement deposition and antibody binding were assessed upon storage and after incubation with serum. M1- and M2-type macrophages were generated from blood monocytes to investigate RBC phagocytosis.

Results: No complement deposition was directly observed on stored RBCs, while incubation of RBCs with serum resulted in variable donor dependent C3 deposition and IgG binding, both independent of storage time. Only 1-4% phagocytosis of stored RBCS was observed by macrophages.

Conclusion: RBCs are susceptible to complement deposition and antibody binding independent of storage time. Limited phagocytic uptake by macrophages was observed in vitro.

INTRODUCTION

Worldwide about 85 million units of red blood cells (RBCs) are transfused per year. Transfusion remains an essential therapy in the treatment of critically ill patients. However, 10-25% of donor RBCs are cleared from the circulation of transfused recipients within 24 hours¹. In the Netherlands, RBC concentrates (RCCs) may be stored up to 35 days at 2-6°C in saline-adenine-guanine-mannitol (SAGM) before transfusion, which is more conservative than in most countries². During storage, RBCs undergo several biochemical and structural changes, collectively known as “storage lesion”^{3,4}. Mostly retrospective and observational studies indicate that longer stored RBCs potentiate adverse clinical outcomes in transfusion recipients^{5,6}. However, more recent multicenter randomized prospective studies did not confirm adverse outcomes such as in-hospital mortality⁷⁻¹⁰. To potentially improve *in vivo* survival of transfused RBCs, it is important to understand the mechanisms and consequences of the rapid clearance of transfused RBCs.

In vivo RBCs have a life span of ~120 days. At the end of their lifespan, RBCs become senescent, have decreased deformability, and are eventually phagocytosed by macrophages of the reticuloendothelial system¹¹. Phagocytosis of senescent RBCs may be mediated by naturally occurring antibodies against band 3 and subsequent complement activation, exposure of phosphatidylserine (PS) and/or the molecular switch of CD47 that controls RBC phagocytosis¹².

During storage, the membrane of RBCs is altered which may result in complement activation e.g. due to PS exposure; decreased expression of complement regulators such as decay-accelerating factor (DAF/CD55), CD59 and complement receptor 1 (CR1/CD35)^{13,14}. Alternatively, complement deposition may be mediated by bystander deposition via contact with the filter used for leukoreduction and/or plastic from the bag¹⁵. Szymanski and colleagues showed that C3 fragments accumulate on the RBC membrane during storage of whole blood and that RBC survival was negatively correlated with both duration of storage and the amount of C3 deposition¹⁶. When whole blood is separated followed by leukocyte depletion by filtration and storage of RBCs in SAGM, C3, C4 and C5 are undetectable in the RCC¹⁷. However, it has been reported that despite leukoreduction, C3 is found on RBC membranes and soluble MAC levels are increased during RBC storage^{13,18}.

Here, we investigated to what extent antibody binding and complement activation occurred during RBC storage. Furthermore, by incubating stored RBCs with normal human serum (NHS), we studied whether opsonization with complement and/or antibodies may contribute to *in vitro* phagocytic uptake of RBCs by M1- and M2-type macrophages.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody (mAb) anti-C3-19, anti-C4-10 and anti-DAF-1 are in-house generated mAbs¹⁹. Anti-C3-19 was DyLight 488- or DyLight 647-conjugated (Thermo Scientific, Rockland, IL). Anti-C4-10 and anti-DAF-1 were DyLight 647-conjugated (Thermo Scientific). Polyclonal antibody anti-IgG-FITC was from Sanquin Reagents (Amsterdam, The Netherlands). Anti-CR1-PE (To5) was from Santa Cruz (Dallas, TX) and anti-CD59-CF405M (VJ1/12.2) was from Abcam (Cambridge, UK).

Serum samples

Normal human serum (NHS) was drawn from thirteen donors, after informed consent. Eight sera were pooled (NHS pool), of which an aliquot was heat inactivated (HI-NHS) for 30 minutes at 56°C, five sera were individually aliquoted. All aliquots were stored at -80°C.

Isolation and storage of RBCs

RBCs from RCCs, collected in a quadruple citrate phosphate dextrose (CPD)-SAGM top-and-bottom bag system (Composelect, Fresenius HemoCare, Emmer-Compascuum, the Netherlands), were used from the Dutch Sanquin Blood Supply Foundation after informed consent from the donors. Alternatively, RBCs were isolated from whole blood collected in sodium citrate tubes by centrifugation at 1080g for 10 minutes. After removing plasma and peripheral blood mononuclear cells, RBCs were washed three times with PBS by centrifugation at 1080g for 10 minutes (Fresenius Kabi, Zeist, The Netherlands) and stored as a 50% cell suspension in SAGM (Fresenius Kabi).

Both RCCs and RBCs from tubes were stored for a short (2-6 days) or long (35-40 days) time period at 2-6°C and before use, stored RBCs were washed with PBS to investigate the intrinsic capacity of the RBCs to bind antibodies and activate the complement system.

RBC incubation with human serum for complement deposition and antibody binding

0.16% RBCs in a final volume of 100µL were incubated with 50% NHS or HI-NHS (v/v) diluted in veronal buffer (3mM barbital, 1.8mM sodium barbital, 145mM NaCl, pH 7.4) containing 0.05% gelatin (w/v), 10mM CaCl₂ and 2mM MgCl₂ at 37°C overnight. To detect antibody binding, RBCs were incubated 1 hour at RT. Allogenic NHS was used, unless indicated otherwise.

FACS analysis of complement deposition, antibody binding and markers on RBCs

NHS exposed RBCs were washed with Hepes buffer containing 1% human serum albumin (v/v), 10mM glucose and 2mM CaCl₂ and stained with antibodies in the dark for 30 minutes at RT. Anti-C3-19, anti-C4-10 and anti-DAF-1: 1µg/ml; anti-IgG 1:100 dilution; anti-CR1: 1:25 and anti-CD59: 1:12.5. RBCs were analyzed using fluorescence-activated cell sorting (FACS) analysis. Percentage positive RBCs was determined for complement deposition and antibody binding. Median fluorescence intensity (MFI) were used for the expression of DAF, CR1 and CD59.

Phagocytosis assay

A phagocytosis assay was performed as described before²⁰. In short, M1- and M2-type macrophages were generated from human monocytes by stimulation for 9 days with 10ng/ml GM-CSF (CellGenix, Freiburg, Germany) or 50ng/ml M-CSF (eBioscience, Vienna, Austria) respectively. Subsequently, M1- and M2-type macrophages were incubated with NHS opsonized or untreated carboxyfluorescein succinimidyl ester (CFSE)-labelled RBCs. As positive control, RBCs were opsonized with anti-RhD antibody. RBCs were added to M1- or M2-type macrophages for 2 hours and 1 hour respectively. After incubation, non-phagocytosed RBCs were removed, using an isotonic ammonium chloride lysis buffer. Uptake of CFSE-labelled RBCs by macrophages was analyzed by FACS.

In addition, the cells were stained with May-Grünwald eosin-methylene blue modified solution (Merck) for five minutes and subsequently with Giemsa solution (Merck) for 30 minutes. Light micrographs were taken using an Axiovert Scope.A1 microscope (Zeiss, Oberkochen, Germany).

Statistics

Data were analyzed using GraphPad Prism (version 6; GraphPad Software, San Diego, CA). To compare C3 deposition and IgG binding between short and long stored RBCs a Wilcoxon test was performed. For the phagocytosis assay a two-way Anova test and as post-test a Tukey multiple comparisons test was performed.

RESULTS

Incubation of RBCs with normal human serum leads to C3 deposition

To determine whether complement activation occurs on RBCs during isolation and storage, C3 and C4 deposition on RBC membranes were analyzed by flow cytometry. No C3 or C4 deposition was observed on the membranes of RBCs obtained from either RCCs or tubes that were stored for a short period or a long period (Fig. 1A).

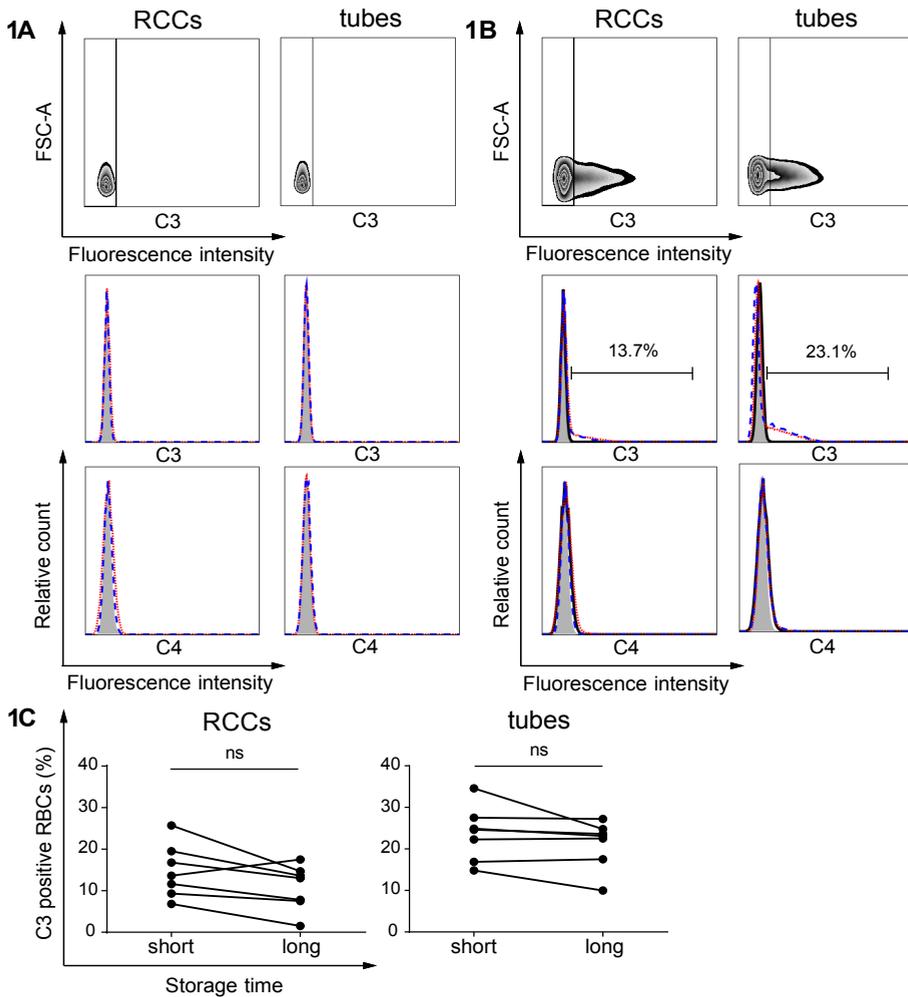


Figure 1. C3 deposition on RBCs upon incubation with normal human serum.

(A) Representative FACS histograms are shown of C3 and C4 deposition measured directly on short (2-6 days) and long stored (35-40 days) RBCs. (B) Representative FACS histograms are shown of C3 and C4 deposition measured on short (2-6 days) and long stored (35-40 days) RBCs upon incubation with NHS. (C) Percentages of C3 positive RBCs upon incubation with NHS are shown that were short (2-6 days) or long (35-40 days) stored (n=7 RBC donors). The Wilcoxon test showed no significance. Grey: RBCs without fluorescently labelled antibody, black solid: HI-NHS, blue dashed: short storage, and red dotted: long storage.

Next, we incubated RBCs with allogeneic NHS as complement source. Using ABO mismatch as model system, C3 and C4 deposition was observed on the RBC membrane (Fig. 2A). Matched RBCs and NHS were used in all further experiments, to rule out complement activation due to ABO mismatches. Incubation of stored RBCs from either RCCs or tubes with NHS resulted in C3 deposition on the membrane

of a subpopulation of RBCs. No C4 deposition was observed (Fig. 1B), indicating alternative complement pathway activation. When analyzing RBCs from seven individual donors that were incubated with the same NHS pool, the percentage of C3 positive RBCs varied between 2-27% for RCCs and 10-35% for tubes (Fig. 1C). However, C3 deposition was not significantly different between RBCs that were stored for a short or a long period, indicating that C3 activation is RBC donor-dependent and irrespective of storage time. As no major differences were observed between RBCs from RCCs and tubes and to study the intrinsic capacity of the RBCs themselves, all further experiments were done with RBCs isolated from citrate tubes. The total population of RBCs expressed DAF, CR1 or CD59 equally well on RBCs that were stored for a short or long period of time (Table 1) and no correlation was found between expression of complement regulators and C3 deposition, excluding this as explanation for the observed C3 positive subset of RBCs.

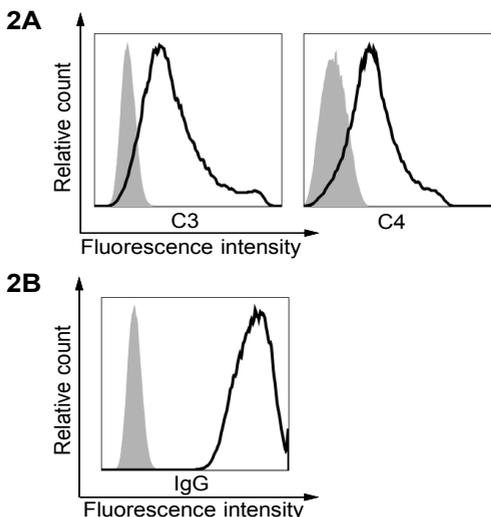
Table 1. DAF, CR1 and CD59 expression on short and long stored RBCs.

	Short	Long
DAF	780.6 ± 125.7	812.4 ± 109.6
CR1	506.8 ± 151.9	502.8 ± 151.0
CD59	9012.8 ± 1257.4	8925.8 ± 903.4

Data shown as mean MFI ± SD, n=5. No significant difference in Wilcoxon test between short (2-6 days) and long (35-40 days) stored RBCs.

IgG binds to stored RBCs and partly coincides with complement activation

Using ABO mismatch as model system, IgG binding was observed on the RBC membrane (Fig. 2B). Matched RBCs and NHS were used in all further experiments,



to rule out antibody binding due to ABO mismatches. As shown in Fig. 3A, IgG binding was detected on the RBCs after incubation with NHS. When analyzing RBCs from five individual donors incubated with the same NHS pool, the

Figure 2. Complement deposition and IgG binding in ABO mismatch model. Representative FACS histograms are shown of (A) C3 and C4 deposition and (B) IgG binding measured on blood group AB RBCs upon incubation with O-typed NHS. Grey: no NHS, black solid: NHS (representative graph of 3 individual experiments).

percentage of cells positive for IgG binding varied between 10-21% and no significant difference was observed between RBCs that were stored for a short or a long period of time (Fig. 3B).

Next, individual differences in IgG binding and C3 deposition were investigated by cross-incubating RBCs of five donors with serum of the same five donors (Fig. 4A). Without serum incubation no IgG binding or C3 deposition were observed on the RBCs (Q4). However, incubation of RBCs with allogeneic serum resulted in subsets of RBCs that were double-positive for both IgG and C3 (Q2) or single positive for either C3 (Q1) or IgG only (Q3). The percentage of C3 and/or IgG positive RBCs varied depending on the combination of RBCs and sera. Remarkably, even RBCs incubated with autologous serum showed C3 deposition and IgG binding (Fig. 4B).

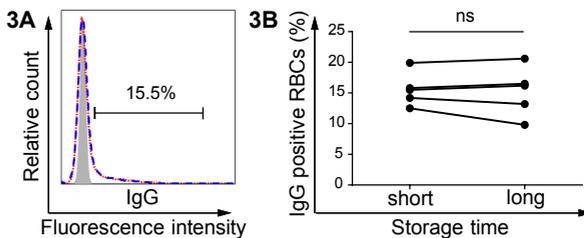


Figure 3. IgG binding on RBCs upon incubation with normal human serum. (A) Representative FACS histograms are shown of IgG binding on short (2-6 days) and long (35-40 days) stored RBCs upon incubation with NHS. (B) Percentages of IgG positive RBCs upon incubation with NHS are shown that were short (2-6 days) or long (35-40 days) stored (n=5 RBC donors). The Wilcoxon test showed no significance. Grey: RBCs without fluorescently labelled antibody, blue dashed: short storage, and red dotted: long storage.

Phagocytic uptake of RBCs by macrophages was independent of storage time

Finally, we studied the phagocytic uptake of stored RBCs either or not opsonized with NHS. RBCs opsonized with anti-RhD antibody (positive control) were readily taken up, indicating that both in vitro generated allogeneic M1- and M2-type macrophages were capable of phagocytosing opsonized RBCs. M2-type macrophages phagocytosed the RhD-opsonized RBCs significantly more (64-80%) than M1-type macrophages (21-26%), irrespective of storage time (Fig. 5A). The extent of phagocytosis of both untreated RBCs and NHS-opsonized RBCs was low compared to RhD-opsonized RBCs and no significant difference was observed between short and long stored RBCs. The percentage phagocytosis of both untreated RBCs and NHS-opsonized RBCs was lower for the M1-type macrophages (average 1%) compared to the M2-type macrophages (average 4%) (Fig. 5B).

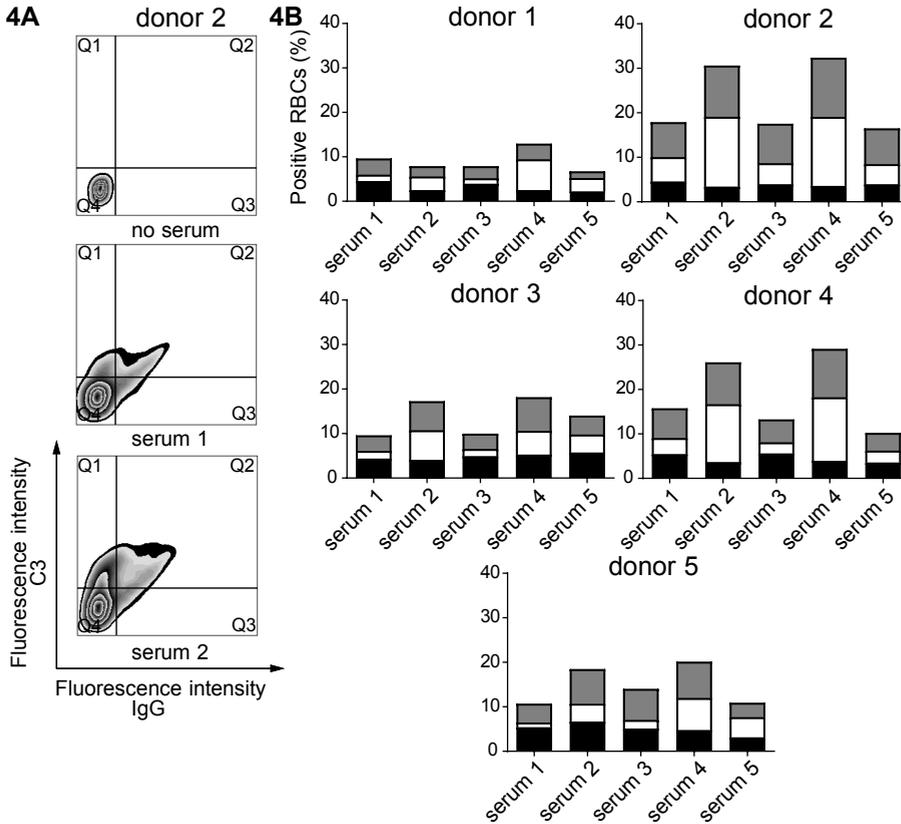


Figure 4. C3 deposition and IgG binding partly coincides on RBCs upon incubation with normal human serum. **(A)** Representative FACS dot plots are shown of RBCs cross-incubated with (autologous and allogeneic) serum of the same donors. Q1: C3 deposition, Q2: both C3 deposition and IgG binding, Q3: IgG binding and Q4: no C3 deposition and no IgG binding. **(B)** Percentages of IgG positive RBCs (black), C3 positive RBCs (white) and C3 and IgG positive RBCs (grey) are shown (n=5 RBC donors and serum).

DISCUSSION

Complement deposition and/or antibody binding may contribute to rapid clearance of donor RBCs after blood transfusion. Here, we investigated whether complement activation and antibody binding occurred during RBC storage and whether this may contribute to phagocytic uptake *in vitro*.

No complement deposition or IgG binding were detected on stored RBCs from both RCCs and tubes stored at 2-6°C in the standard storage solution SAGM. However, by incubating stored RBCs with allogeneic NHS, we observed C3 deposition on a

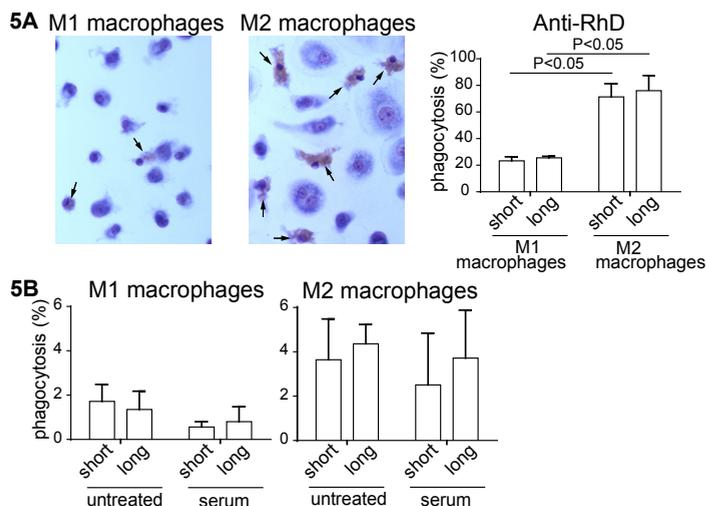


Figure 5. IgG and C3 opsonization on stored RBCs is not sufficient for phagocytic uptake by macrophages. (A) Wide field images and percentages phagocytosis are shown of the anti-RhD antibody (positive control) opsonized short (2-6 days) and long stored (35-40 days) RBCs (n=2) by M1- and M2-type macrophages. Two-way Anova test and as post-test a Tukey multiple comparisons test was performed and showed significance. **(B)** Percentages phagocytosis by M1- and M2-type macrophages are shown of the untreated short (2-6 days) and long (35-40 days) stored RBCs (n=3) and NHS-opsonized short (2-6 days) and long (35-40 days) stored RBCs (n=3). Bars show mean \pm standard deviation.

subset of RBCs from both RCC and tubes. RCC storage bags contain plasticizers, which are not present in the tubes, that can activate the complement system^{21,22}. However, the percentage C3 deposition was somewhat lower on RBCs from the RCCs compared to the tubes, suggesting that under the investigated conditions plasticizers did not have a major effect on complement activation.

In contrast to a previously published study¹³, we observed that storage time of RBCs had no influence on C3 deposition and IgG binding. In addition, we observed no difference in expression of complement regulators DAF (Cromer blood group system), CR1 (Knops blood group system) and CD59 over time, while Kamhieh-Milz et al.¹³ suggested that increased complement deposition during storage may be caused by diminished expression of complement regulators on RBCs. Kamhieh-Milz et al.¹³ used phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGS-M) as storage solution, while we used SAGM. It has been described that PAGGS-M prevents initial RBC swelling, spontaneous hemolysis and osmotic fragility of the RBCs was lower compared to RBCs stored in SAGM²³. However, it has not been investigated whether PAGGS-M, influences complement activation, antibody binding and the expression of complement regulators.

Interestingly, we observed large donor variation for both C3 deposition and IgG binding on RBCs. For other factors such as hemolysis, osmotic and mechanical fragility also large donor variations are reported^{4,24}. It has been described that genetic background and environmental factors may influence the properties of RBCs of individual blood donors, which make RBCs from some donors more prone to storage lesion than others^{4,24}. The amount of C3 and IgG opsonization may be an indication of the degree of storage lesion and therefore may act in the future as a biomarker for the storage lesion. However, more studies are needed to investigate whether C3 and IgG opsonization on the subset of RBCs correlate with other known biomarkers of the storage lesion.

Most remarkably, even incubation with autologous serum resulted in both C3 deposition and IgG binding on RBCs. Changes are apparently, induced in RBC membrane structures during blood collection and processing, which may lead to complement activation and antibody binding after transfusion.

Phagocytosis assays were performed to investigate whether the observed C3 deposition and IgG binding may contribute to clearance of stored RBCs. We observed that phagocytosis of RhD-opsonized RBCs by M1-type macrophages was significantly lower compared to M2-type macrophages. This is in agreement with other studies showing that monocytes activated with GM-CSF or interferon- γ (M1-type macrophages), have a decreased phagocytic capacity compared to monocytes activated with M-CSF or IL-10 (M2-type macrophages)^{20,25}. Phagocytosis of untreated and NHS-opsonized RBCs was comparable, but low compared to RhD-opsonized RBCs. Others have shown before that in case of allo- or autoantibodies, as occurring in a transfusion reaction or autoimmune hemolytic anemia, RBCs are cleared from the circulation by macrophages via Fc-receptors and/or complement receptors^{26,27}. It seems that the extent of C3 deposition and/or IgG binding on stored RBCs observed in our study was not sufficient for phagocytic uptake by macrophages.

In conclusion, no C3 deposition was observed on RBCs as present in a RCC, even upon prolonged storage. However, upon incubation of stored RBCs with serum, C3 deposition and IgG binding occur on a subset of RBCs. So likely, after transfusion a fraction of donor RBCs will be opsonized with IgG and/or C3. This needs to be verified in vivo by tracing transfused RBCs in patients receiving a blood transfusion and measuring complement and IgG binding on the donor RBCs, which is subject of current research. Only minimal phagocytosis of RBCs was observed in vitro by allogeneic M1-and M2-type macrophages, which seemed to be independent of NHS opsonization. Apparently, phagocytosis by these cells was not significantly increased by C3 and IgG opsonization on the RBCs, although these cells do express complement receptors and Fc receptors.

Chapter 4

Acknowledgments

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Disclosure Statement

The authors declare that they have no competing interests.

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