Effect of red blood cell storage on *in vitro* erythrophagcytosis

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Áhrif geymslu rauðra blóðkorna á *in vitro* rauðkornaát

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Abstract

Blood transfusion is an essential part of modern healthcare. It is a life-saving intervention, which unfortunately, can have adverse clinical effects. Red blood cell transfusion is e.g. used in foetal medicine, in cases of trauma, in surgeries treating heart diseases and as a consequence of cancer therapy. Red blood cell concentrates are stored at 2-6 °C for up to 42 days. Storage of RBC concentrates makes it possible to have an inventory of relatively safe units available at most times. However longer storage results in damage to the RBCs, referred to as storage lesion. Storage lesions of RBCs are all the changes, both physical and metabolic, that occur to the RBC during the storage period. The longer the RBCs are stored, the more storage lesions they acquire, and a smaller proportion survives when it is transfused into the patient. Erythrophagocytosis is the process by which old or damaged RBCs are cleared in the body by phagocytic cells. The clinical implications of transfusing RBCs that have been stored for longer times are not well understood but are currently being studied vigorously. A recent meta-analysis of 21 studies concluded that a significantly increased mortality is associated with the transfusion of old blood compared to new blood. However, recent and larger studies found no links between older transfused blood and increased mortality. Whether old blood carries increased risk has been debated for quite some time and recent studies need to be reviewed thoroughly. The question remains whether results from numerous studies warrant changes in current clinical practices of blood transfusion.

The objective of this study was to analyse how the age of stored RBCs affects erythrophagocytosis using the human monocytic cell line THP-1. To achieve this, THP-1 cells were co-cultured with PKH26-labelled RBCs and analysed with flow cytometry and fluorescence microscopy.

Negligible phagocytosis was seen when undifferentiated THP-1 cells were used. However, differentiated THP-1 cells readily phagocytosed PKH26-labelled RBCs. Comparison of RBC units stored for 7, 19 and 40 days revealed minor differences.

The cell line THP-1 is sensitive to overgrowth and changes in culture conditions and as a result it readily loses its ability to phagocytose. This is avoided by using differentiated THP-1 cells. However, this introduces considerable variability to the assay. The red fluorescence seen in the flow cytometric analysis of the erythrophagocytosis assay was the result of phagocytosis of PKH26-labelled RBCs. This was confirmed with photomicrographs and the inhibition of phagocytosis with the use of cytochalasin D.

From our results it is not possible to conclude that longer storage affects erythrophagocytosis. Further development of the erythrophagocytosis assay is necessary.

Ágrip

Blóðgjöf er mikilvægur hlekkur í nútíma læknisfræðilegri meðferð. Þrátt fyrir að blóðgjöf geti svo sannarlega oft verið lífgjöf, geta afleiðingar blóðgjafar einnig valdið vandamálum. Rauðkornaþykkni er gefið við ýmsar aðstæður, s.s. vegna áverka, við fósturlækningar og aðgerðir m.a. vegna hjartasjúkdóma og sem stuðningsmeðferð vegna krabbameinslækninga. Rauðkornaþykkni er geymt við 2-6 °C í allt að 42 daga. Geymsla rauðkornaþykknis í svo langan tíma gerir það mögulegt að hafa blóð tiltækt hverju sinni. Hins vegar hefur geymsla til lengri tíma þau áhrif að rauðkornin verða fyrir skaða sem kallaður hefur verið geymsluáverki (storage lesion). Geymsluáverkar eru allar þær skemmdir sem rauðkorn verða fyrir yfir geymslutímabilið, bæði við efnaskipti og vegna áþreifanlegs tjóns. Þeim mun lengur sem rauðkornin eru geymd, því meiri skemmdir eiga sér stað og minna hlutfall rauðkorna lifir af inngjöf í sjúkling. Rauðkornaát (erythrophagocytosis) er það ferli sem sér um að hreinsa gömul og skemmd rauðkorn í líkamanum. Klínískar afleiðingar þess að gefa rauðkorn, sem geymd hafa verið til lengri tíma, eru ekki vel þekktar, en um þessar mundir er verið að rannsaka þær af krafti. Nýleg rannsókn sýndi fram á að að dánartíðni eykst marktækt við gjöf eldra blóðs samanborið við nýlegra blóð. Hins vegar greindi önnur rannsókn frá því að ekki væru tengsl þar á milli. Hvort gjöf eldra blóðs ber með sér aukna áhættu hefur verið til umræðu lengi og nauðsynlegt er að fara vel yfir nýlegar rannsóknir. Spurning er hvort niðurstöður fjölmargra rannsókna feli í sér nægileg rök til þess að rétt sé að breyta klínískum leiðbeiningum við blóðgjöf.

Markmið rannsóknarinnar var að rannsaka áhrif aldurs rauðkornaeininga á *in vitro* rauðkornaát með því að nota mennsku monocyta frumulínuna THP-1. THP-1 frumum var blandað saman við flúrmerkt rauðkorn og niðurstöðurnar greindar með frumuflæðissjá og flúrsmásjá.

Frumuát var hverfandi þegar ósérhæfðar THP-1 frumur voru notaðar. Hins vegar, átu THP-1 frumur, sem höfðu verið sérhæfðar, rauðkornin mjög auðveldlega. Samanburður á rauðkornaeiningum, sem höfðu verið geymdar mislengi, sýndi smávægilegan mun á áhrifum geymslu rauðkorna.

THP-1 frumulínan er viðkvæm fyrir ofvexti og breytingum á vaxtarskilyrðum og þar af leiðandi tapar hún getunni til frumuáts mjög auðveldlega. Þessu er hægt að komast hjá með því að nota THP-1 frumur sérhæfðar með PMA. Hins vegar, fjölgar það breytunum í rannsókninni. Rauða flúrljómunin sem sást í greiningu frumuflæðissjárinnar á rauðkornaátinu var afleiðing frumuáts THP-1 frumna á flúrmerktum rauðkornum. Þetta var staðfest með smásjármyndum og hindrun frumuáts með cytochalasin D.

Niðurstöðurnar benda ekki til þess að lengri geymsla hafi áhrif á rauðkornaát. Þróa þarf þessa aðferð frekar.

Acknowledgements

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Abbreviations

2,3-DPG	2,3-diphosphoglycerate
ACD	Acid-citrate-dextrose
ATP	Adenosine triphosphate
BC	Buffy coat
BFU$_E$	Burst-forming unit erythroid
CFU$_E$	Colony-forming unit erythroid
CPD	Citrate-phosphate-dextrose
DMSO	Dimethyl sulfoxide
Emp	Erythroblast macrophage protein
FBS	Foetal bovine serum
FDA	Food and Drug Administration
HIV	Human immunodeficiency virus
HTLV	Human T-lymphotropic virus
Nabs	Naturally occurring antibodies
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotineamide adenine dinucleotide phosphate (reduced)
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen recognition receptor
RBC	Red blood cell
SAGM	Saline-adenine-glucose-mannitol
SIRPα	Signal-regulatory protein alpha
VCAM-1	Vascular cell adhesion molecule 1
WBC	White blood cell
WHO	World Health Organization

1 Introduction

1.1 Blood transfusion

1.1.1 History

It seems that human beings have always been fascinated with blood. However, its medical benefits remained elusive for the longest of time. The first recorded attempt to transfuse blood, from human to human, was made in 1492 when Pope Innocent VII was transfused with blood from three other men. All four died and many failed blood transfusions followed. In 1818, James Blundell performed the first successful human blood transfusion. At that point, adverse transfusion reactions and blood clotting still remained as problematic factors. It was not until 1869, that Braxton Hicks introduced an anticoagulant, sodium phosphate, which was not toxic to cells. In 1901, the Austrian Karl Landsteiner made a huge contribution to modern transfusion medicine when he discovered the ABO system. This explained many of the adverse reactions seen when blood was transfused into patients. A further quest for improved storage solutions followed his important discovery (Harmening, 2012).

Citrate, which is still used in modern storage solutions as an anticoagulant, was introduced by Lee and Vincent in 1913. In 1916 Rous and Turner reported that adding glucose to citrate made it possible to store rabbit red blood cells (RBCs) for four weeks. During World War I, Oswald Hope Robertson used the Rous-Turner solution to store human blood. He developed ways to store the blood for up to 26 days on ice and used it to transfuse injured soldiers. Robertson has been called the world's first blood banker (Hess, 2010b; Hess & Schmidt, 2000). A major breakthrough came in 1943, when Loutit and Mollison presented acid-citrate-dextrose (ACD) as a storage solution. The acid concentration in the solution made it possible to autoclave the solution without damaging the dextrose (Hess, 2010b). Citrate-phosphate-dextrose (CPD) storage solution, developed by Gibson in 1957, later replaced ACD as a storage solution (Harmening, 2012). Since then important advances have been made to form today's modern practice of blood transfusion and storage.

1.1.2 Modern practice

Blood transfusion is an essential part of modern healthcare. According to the World Health Organization (WHO), around 92 million blood units are collected each year in 164 countries, covering 92% of the world population (World Health Organization, 2011), with red blood cell transfusion of around 85 million units every year, thereof 15 million in the US alone (Carson et al., 2012). Blood transfusion is a life-saving intervention, which unfortunately, can have adverse clinical effects (Green, Allard, & Cardigan, 2015). Safety is one of the most important aspect of the blood transfusion practice. The quality needs to be constantly monitored to ensure the health and safety of both the donor and recipient. Standard protocols need to be in place so this can be achieved. In recent decades, the safety of blood has increased significantly (Green et al., 2015). The WHO and the US Food and Drug Administration (FDA) have devoted a lot of time and effort in order to standardise procedures concerning the collection and processing of blood and the storage of individual blood components (Greening et al., 2010).

To be eligible to donate blood, one needs to be a healthy adult and fulfil the donor selection criteria. This includes a questionnaire and an interview to exclude high-risk individuals. There are both permanent deferrals, such as intravenous drug abusers, and temporary deferrals, e.g. when the person in question has just gotten a tattoo. Donated blood is tested for the presence of viruses such as hepatitis B and C viruses, human T-lymphotropic virus (HTLV) and human immunodeficiency virus (HIV). Bacterial contamination is limited with the cleaning of the donor's arm and most bacteria do not survive the refrigerated storage. ABO and Rh antigens are determined along with serum antibodies (Green et al., 2015; Hess, 2010a). A total of 62 countries reported that more than 99% are voluntary unpaid blood donors. This is associated with lower risk of infections and a more stable blood supply. In more developed countries, this is usually the case (World Health Organization, 2011).

Blood component transfusion is an integral part of health care, with transfusion of RBCs being the most common (Hess, 2010a). Transfusion of RBCs is performed when there is anaemia or severe blood loss caused by various conditions such as trauma or surgeries (Sparrow, 2010).

1.1.3 Collection and processing of blood components

Blood donation is either a whole blood collection or apheresis collection. Apheresis is performed when only a specific blood component is to be donated, usually plasma or platelets but also red blood cells. A specific machine is used to separate the required blood components from whole blood and the rest is then returned to the donor. When whole blood is donated, 450 ml are collected into a plastic blood collection bag containing around 63 ml of anticoagulant. The anticoagulant solution commonly used is the CPD solution. Small alterations to this solution include the addition of adenine in the CPDA-1 solution or more dextrose in the CP2D solution. Citrate prevents coagulation by binding calcium (Ca^{2+}) in the blood and dextrose serves as nutrition for the cells (Greening et al., 2010).
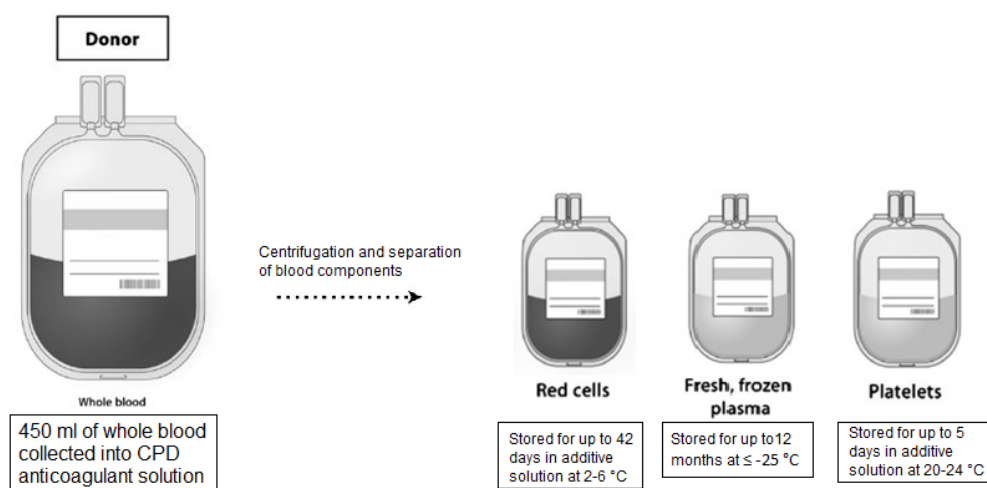
Figure 1 Blood component processing

Whole blood is collected into a plastic collection bag commonly containing citrate-phosphate-dextrose (CPD) anticoagulant solution. Blood components are separated by centrifugation into red blood cell concentrates, platelet concentrates and plasma. Modified from (Greening et al., 2010).

The whole blood is processed into blood components, including RBC concentrate, platelet concentrate and plasma (figure 1). To maintain adequate quality, this process needs to be done within 24 hours of collection (Green et al., 2015; Greening et al., 2010). The processing into blood components is done by centrifugation, which separates the blood into three layers. The RBCs form the bottom layer, the white blood cells (WBCs) form a buffy coat (BC) layer on top of them and the plasma remains at the top. Depending on the force of centrifugation, the platelets are either suspended in the plasma or become part of the BC with the WBCs (Greening et al., 2010).

1.1.3.1 *Red blood cell concentrates*

The blood components are sorted into separate plastic bags with the RBCs being transferred via sterile connected tubes into another plastic bag containing additive solution. The separated RBC concentrates are usually leukocyte-filtered, which reduces haemolysis significantly and increases storage time (Hess, 2010c). Also, if the WBCs are not filtered there is an increased risk that the donor WBCs develop immunity against the recipients' own cells (Greening et al., 2010). In Europe, the RBCs are usually suspended into the saline-adenine-glucose-mannitol (SAGM) additive solution, which is modified from the previous SAG solution, with mannitol added to maintain membrane stability and thus minimising haemolysis (Sparrow, Sran, Healey, Veale, & Norris, 2014). In the US, however, slightly different additive solutions are used; the AS-1, AS-3 and AS-5. AS-1 and AS-5 are very similar to SAGM but AS-3 is considerably different (Table 1). The RBC concentrates are stored at 2-6°C for up to 42 days (Greening et al., 2010).

Table 1 The composition of red blood cell additive solutions

[Adapted from (Greening, Glenister, Sparrow, & Simpson, 2010)]

	SAG-M (saline-adenine-glucose-mannitol)	AS-1 (additive solution 1)	AS-3 (additive solution 3)	AS-5 (additive solution 5)
Dextrose (mM)	45.4	111	55.5	45.4
Adenine (mM)	1.26	2	2.22	2.22
Monobasic sodium phosphate (mM)	0	0	23	0
Mannitol (mM)	29	41.2	0	45.4
Sodium chloride (mM)	150	154	70	150
Citric acid (mM)	N/A	N/A	2	N/A
Sodium citrate (mM)	N/A	N/A	23	N/A
Manufacturer(s)	Baxter, Fresenius, MacoPharma, Pall.	Baxter	Haemonetics	Terumo
Trade or alternate names	N/A	Adsol	Nutricel	Optisol

1.2 Red blood cells

1.2.1 Erythropoiesis

The formation of blood cells in adults occurs in the bone marrow and is called haematopoiesis. All blood cells are derived from a multipotent haematopoietic stem cell that has the ability to self-renew. It gives rise to the common myeloid and lymphoid progenitors, which are committed to a specific lineage and do not have the ability for self-renewal. The common myeloid progenitor gives rise to granulocytes, mast cells, platelets and red blood cells. The hormone erythropoietin is essential in inducing erythropoiesis, the formation of new RBCs, in the bone marrow. It is produced in the kidneys in response to decreased oxygen saturation or when RBC count is low (Alberts, 2015).
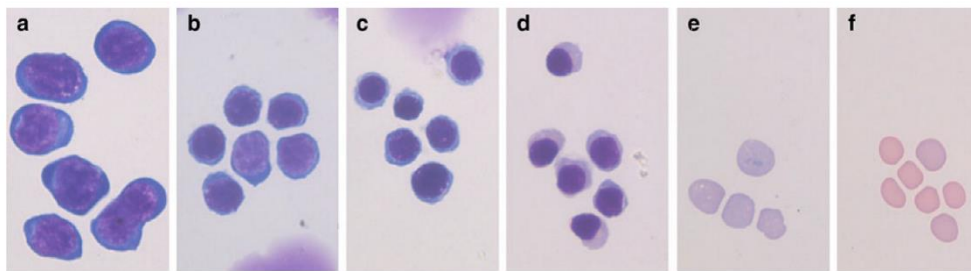
Figure 2 Erythropoiesis

The process of erythropoiesis includes several different stages of differentiation. These figures show the late stages of erythropoiesis from the earliest morphologically recognizable red blood cell precursor to the mature red blood cell: **a** proerythroblast, **b** basophilic erythroblast, **c** polychromatophilic erythroblast, **d** orthochromatophilic erythroblast, **e** reticulocyte and **f** mature red blood cells (An & Mohandas, 2011).

In the process of erythropoiesis, the common myeloid progenitor cell differentiates from the haematopoietic stem cell and then gives rise to the burst-forming unit (BFU$_E$) and colony forming unit (CFU$_E$) erythroid progenitors. These differentiate to the earliest morphologically recognizable RBC precursor, the proerythroblast, which divides progressively and gives rise to around 14-16 RBCs. The following stages of differentiation are basophilic erythroblast, polychromatophilic erythroblast, orthochromatophilic erythroblast, reticulocyte and finally the mature RBC (figure 2). During this differentiation, there is a progressive condensation of chromatin, decrease in RNA, decrease in mitochondria, and an increased synthesis of haemoglobin. The orthochromatophilic erythroblast is the last stage where a nucleus is still present. The condensed nucleus is expelled, which results in an immature red blood cell called a reticulocyte. The reticulocyte circulates the blood stream for 1-2 days before maturing into an RBC (Harmening, 2009; Hoffbrand & Moss, 2011).

Erythropoiesis occurs in distinct units in the bone marrow called erythroblastic islands. A macrophage is at the centre of the island and is surrounded by erythroblasts. The macrophage is in close contact with the developing erythroblasts. The communication occurring between the cells enhances the proliferation of erythroid progenitors. Other important functions of the macrophages in these erythroid niches is to provide the erythroblasts with iron for haemoglobin production and to phagocytose the expelled nucleus of the orthochromatophilic erythroblast (An & Mohandas, 2011). Developing erythroblasts express various adhesion molecules to attach to the central macrophage (figure 3). Erythroblast macrophage protein (Emp) is one such molecule found on both
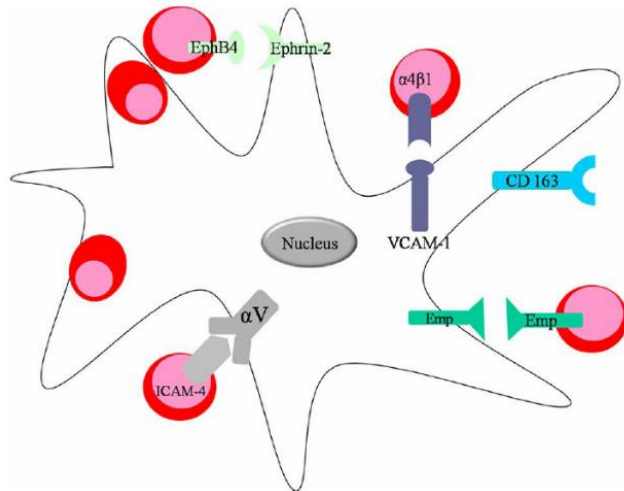
Figure 3 Adhesion molecules in erythroblastic islands

A macrophage is at the center of an erythroblastic island in the bone marrow, surrounded by developing erythroblasts. These cells communicate through several adhesion molecules (de Back, Kostova, van Kraaij, van den Berg, & van Bruggen, 2014).

erythroblasts and macrophages. Another important communication is through α4β1 integrin on erythroblasts and vascular cell adhesion molecule 1 (VCAM-1) on the central macrophage. Other surface adhesion molecules have been identified but the mechanism by which they promote erythropoiesis is mostly unknown and further research is needed (de Back et al., 2014).

1.2.2 Structure and function

1.2.2.1 Membrane

A normal red blood cell is 6-8 μm in diameter and 1.5-2.5 μm thick. The mature RBC is shaped like a biconcave disc and does not have a nucleus or other organelles. The RBC membrane consists of a lipid bilayer containing phospholipids, proteins, cholesterol, glycoproteins and glycolipids. It is an extremely complex and flexible structure with more than 300 membrane proteins identified (Harmening, 2009). The major component of the cytoskeleton is the protein spectrin, which forms complexes with other cytoskeletal proteins, such as ankyrin, actin and protein 4.1, which then associate with the lipid bilayer, strengthening and supporting it (figure 4). These spectrin complexes rely on an adenosine triphosphate (ATP)-dependent phosphorylation to function properly. Membrane integrity and stability is thus compromised when there is a decrease in ATP or when oxidative damage occurs to any of these proteins, leading to loss of membrane deformability (Hoffman, 2013). Deformability is one of the most important characteristic of the RBC membrane. Without this ability, it would not be able to pass through the small capillaries and the sinusoidal slits of the spleen, which are only 3-5 μm in diameter (Triulzi & Yazer, 2010).
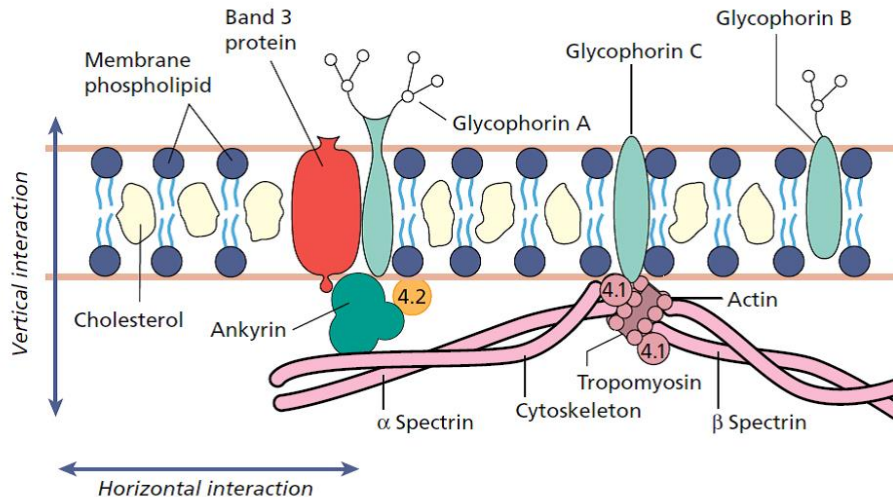
Figure 4 Red blood cell membrane structure

The membrane of red blood cells is a lipid bilayer containing many different proteins such as band 3 and glycophorins. Spectrin is the major component of the cytoskeleton and in association with other proteins, such as protein 4.1 and ankyrin, it stabilises and strengthens the lipid bilayer (Hoffbrand & Moss, 2011).

The membrane has an asymmetrical distribution of phospholipids and is rich in glycoproteins called glycophorins. The major transmembrane protein of RBCs is the anion exchange-channel protein, commonly known as band 3, which catalyses chloride-bicarbonate exchange, contains binding sites for cytoskeletal proteins and glycolytic enzymes and plays a role in the removal of senescent RBCs (Harmening, 2009).

Water and anions, such as chloride (Cl^-) and bicarbonate (HCO^{3-}), flow freely across the RBC membrane. This is thought to occur through anion-exchange channels. Cations, however, such as sodium (Na^+) and potassium (K^+), need to be actively transported across the membrane. Sodium is primarily extracellular and potassium intracellular. The RBC maintains water homeostasis by controlling the transport of these cations with ATP-dependent Na^+/K^+ pumps. The concentration of calcium (Ca^{2+}) needs to be controlled as well, since excess intracellular concentration can have negative effect on RBC shape and flexibility. This is performed by ATP-dependent Ca^{2+} pumps. The shape and volume of the RBC is therefore dependent on ATP, with its depletion resulting in a rigid and dehydrated RBC (Harmening, 2009).

In summary, alterations in the proteins or lipids associated with the RBC membrane can result in decreased deformability and permeability and eventually the sequestration of the RBC in the spleen.

1.2.2.2 *Haemoglobin*

Haemoglobin is 95% of the dry weight and 35% of the weight by volume of the RBC. The majority, or 95-97% of haemoglobin in adults, is haemoglobin A (Hb A), which is made up by two alpha (α) and two beta (β) globin chains, each containing a haem group. Haem consists of a protoporphyrin ring and a ferrous iron (Fe^{2+}). Ferric iron (Fe^{3+}) is transported across the RBC membrane bound to transferrin. Most

of it is delivered to the mitochondria where it is reduced to ferrous iron (Fe^{2+}) before being inserted into the protoporphyrin ring. Haemoglobin contains approximately two-thirds of the body's iron supply (Harmening, 2009).

Haemoglobin binds oxygen (O_2) in the lungs and delivers it to the tissues, where it binds carbon dioxide (CO_2), which is released from the body through the lungs. When oxygen is released in the tissues, 2,3-diphosphoglycerate (2,3-DPG) produced in the RBC, binds to haemoglobin. This causes a conformational change in the haemoglobin and a decreased affinity for oxygen. On the contrary, when oxygen binds haemoglobin in the lungs, 2,3-DPG is released, causing an increase in oxygen affinity (Hoffman, 2013).

1.2.2.3   *Function and metabolism*

RBCs transport oxygen (O_2) and carbon dioxide (CO_2) via haemoglobin binding. Active production of ATP is essential for the function and survival of RBCs. The mature RBC does not have a nucleus or other organelles restricting the possibilities for ATP production. Additionally, since RBCs transport oxygen and do not consume it, this is mostly performed through the anaerobic breakdown of glucose (Hoffbrand & Moss, 2011).

Around 90% of the ATP is produced by the Embden-Meyerhof glycolytic pathway (figure 5), which generates ATP and reduced nicotinamide adenine dinucleotide (NADH). Additionally, 10% is produced via the hexose monophosphate shunt pathway, which also produces reduced nicotinamide adenine dinucleotide phosphate (NADPH). Reduced glutathione, along with NADPH, protects the RBC from oxidative injury (Hoffbrand & Moss, 2011).

Figure 5 Embden-Meyerhof pathway

Red blood cells produce adenosine triphosphate (ATP) through anaerobic glycolysis (Hoffbrand & Moss, 2011).

Methaemoglobin is an abnormal form of haemoglobin where ferrous iron (Fe^{2+}) is oxidised to ferric iron (Fe^{3+}). Methaemoglobin, which is incapable of binding and transporting oxygen, is reduced via the methaemoglobin reductase pathway. NADH and the enzyme methaemoglobin reductase are key factors of this pathway. The last pathway, important for RBC function, is the Luebering-Rapaport shunt. It results in the production and accumulation of 2,3-DPG (Hoffbrand & Moss, 2011).

1.2.3 The clearance of red blood cells *in vivo*

1.2.3.1 *Monocytes and macrophages*

Monocytes and macrophages are a heterogeneous group of immune cells that are professional phagocytes of pathogens and apoptotic cells. Together they belong to the mononuclear phagocyte system (MPS) and are derived from the granulocyte/macrophage progenitor cell. Monocytes circulate the bloodstream before migrating into tissues, where they mature into macrophages (Parihar, Eubank, & Doseff, 2010). Resident macrophages are found in all tissues of the body usually with specific immunophenotypes depending on their location. In the spleen, there are splenic macrophages, Kupffer cells are macrophages of the liver, and osteoclasts and microglia reside in the bone and the central nervous system respectively (Hoffman, 2013).

These cells produce inflammatory cytokines and are equipped with pathogen recognition receptors (PRRs) to recognize and phagocytose foreign microbes and molecules. Macrophages are especially rich in PRRs, which makes them highly effective phagocytes (Geissmann et al., 2010). They also have receptors for recognizing apoptotic or damaged cells. These so-called "eat me" signals promote binding and phagocytosis by macrophages (Alberts, 2015).

1.2.3.2 *Phagocytosis*

Phagocytosis is a form of endocytosis in which professional phagocytes, mainly macrophages and neutrophils, ingest large particles, such as microorganisms and senescent or dead cells. This process is dependent on binding and activation of cell-surface receptors triggering a signalling pathway leading to phagocytosis. Macrophages have several such receptors, including Fc receptors and complement receptors that recognise antibodies and opsonins, respectively, on the surface of a particle. Furthermore, in apoptotic cells phosphatidylserine, normally on the cytoplasmic side of the membrane, is exposed on the surface and serves as a signal for phagocytes. The process of phagocytosis involves the formation of a large endocytic vesicle called a phagosome. The phagosome fuses with a lysosome forming a phagolysosome where the ingested particle is broken down by enzymes, such as lysozyme and acid hydrolases. A person's own living cell is protected by so-called "don't eat me" signals that bind cell-surface receptors on phagocytes and inhibit phagocytosis. Healthy cells expose some phosphatidylserine on the cell surface but are not phagocytosed. Therefore, the activation of phagocytosis seems to require the loss of "don't eat me" signals and the presence of "eat me" signals (Alberts, 2015).

1.2.3.3 *Erythrophagocytosis*

Erythrophagocytosis is the process by which old or damaged RBCs are cleared in the body. Every day, macrophages phagocytose more than 10^{11} old RBCs (Alberts, 2015). As the RBC circulates the body during its 120-day life span, it goes through metabolic and physical changes referred to as senescence. Each day around 0.8% of RBCs are removed by macrophages of the MPS, mostly in the spleen but also in the liver (de Back et al., 2014). The exact mechanism is unknown but several signals have been suggested in tagging old RBCs for clearance. These include phosphatidylserine exposure and damage

to band 3 membrane protein, creating a neoantigen, which is bound by naturally occurring antibodies (Nabs). Studying the senescence signals that induce *in vivo* erythrophagocytosis is difficult because the cells that carry these signals will inevitably be phagocytosed before they can be analysed (Bosman, Willekens, & Werre, 2005; de Back et al., 2014; Lee, Park, Jung, Bae, & Kim, 2011). Furthermore, it is well known that the membrane protein CD47, expressed by RBCs, inhibits phagocytosis by macrophages by binding signal-regulatory protein alpha (SIRPα) on macrophages. When CD47 is absent on the surface of RBCs, they are rapidly phagocytosed by macrophages. However, it has recently been suggested that during aging CD47 goes through conformational changes turning it into a signal for clearance by SIRPα on macrophages. In other words, CD47 seems to play a part in both inhibiting and inducing erythrophagocytosis (Burger, Hilarius-Stokman, de Korte, van den Berg, & van Bruggen, 2012).

Around 80-90% of the removal of RBCs occurs as extravascular haemolysis. Old or damaged RBCs are phagocytosed by macrophages and broken down into their components. Iron is recycled and transported, via the protein carrier transferrin, to the bone marrow for the production of new haemoglobin but also stored in the macrophages as ferritin. Globins are broken down into amino acids and the protoporphyrin ring is converted to bilirubin, which is processed and excreted by the liver into the small intestines. The rest of the RBCs, around 10%, break down within the blood vessels as intravascular haemolysis and the RBC contents are released into the blood stream. Free haemoglobin is taken up by the protein carrier, haptoglobin, and carried to the liver for further breakdown (Ganz, 2012; Orlov & Karkouti, 2015).

1.3 Storage of RBCs

Red blood cell concentrates are stored in the SAGM additive solution at 2-6 °C for up to 42 days. As the storage time progresses, metabolic and physical changes affect the RBCs. These are referred to as storage lesions (Greening et al., 2010). By keeping RBCs refrigerated, these processes slow down. The rate of metabolism slows down by 10-15% for every Celsius degree. This means that the metabolic rate of the stored RBCs is ten times less at 4 °C than it is at 25 °C (Hess, 2014).

The golden standard for quality of stored red blood cells is the 24h *in vivo* recovery of radiolabelled RBCs. The mean recovery from 20 or more recovery assays, should be more than 75% (Dumont & AuBuchon, 2008). Additionally, haemolysis needs to be within 1% in the US and within 0.8% in Europe. Usually, there is only around 0.4% haemolysis at the end of storage (Hess et al., 2009). Other measurements are required but there is not a specific value for acceptance or rejection. Among these measurements are ATP concentration, 2,3-DPG, pH, glucose and lactate concentration (Dumont & AuBuchon, 2008). In the 24 hour *in vivo* recovery measurement, about 20 ml of sample is taken at the end of storage, and the RBCs are labelled with chromium 51. The labelled RBCs are then reinfused into the original donor and samples are taken at several time intervals to analyse the clearance. Finally, after 24 hours, a 75% recovery of the RBCs is the criteria for acceptance (Hess, 2012). For additive solutions used today, the recovery is usually around 84% (Hess, 2010b).

1.3.1 Storage lesions

Red blood cell transfusion is e.g. used in foetal medicine, in cases of trauma, in surgeries treating heart diseases and as a consequence of cancer therapy. Storage of RBC concentrates makes it possible to have an inventory of relatively safe units available at most times (Hess, 2010c). However, as previously stated, longer storage results in damage to the RBCs, referred to as storage lesion (Veale, Healey, & Sparrow, 2014).

Storage lesions of RBCs are all the changes, both physical and metabolic, that occur to the RBC during the storage period. The longer the RBCs are stored, the more storage lesions they acquire, and a smaller proportion survives when transfused into the patient. These lesions include a drop in pH, decrease in ATP and 2,3-DPG concentrations, increased extracellular K^+ and haemoglobin, oxidative and enzymatic damage to proteins and lipids, and membrane loss through shedding of microvesicles (Figure 6). This leads to a change in the RBC membrane and shape and eventually sequestration by macrophages in the spleen and liver (Hess, 2010b).
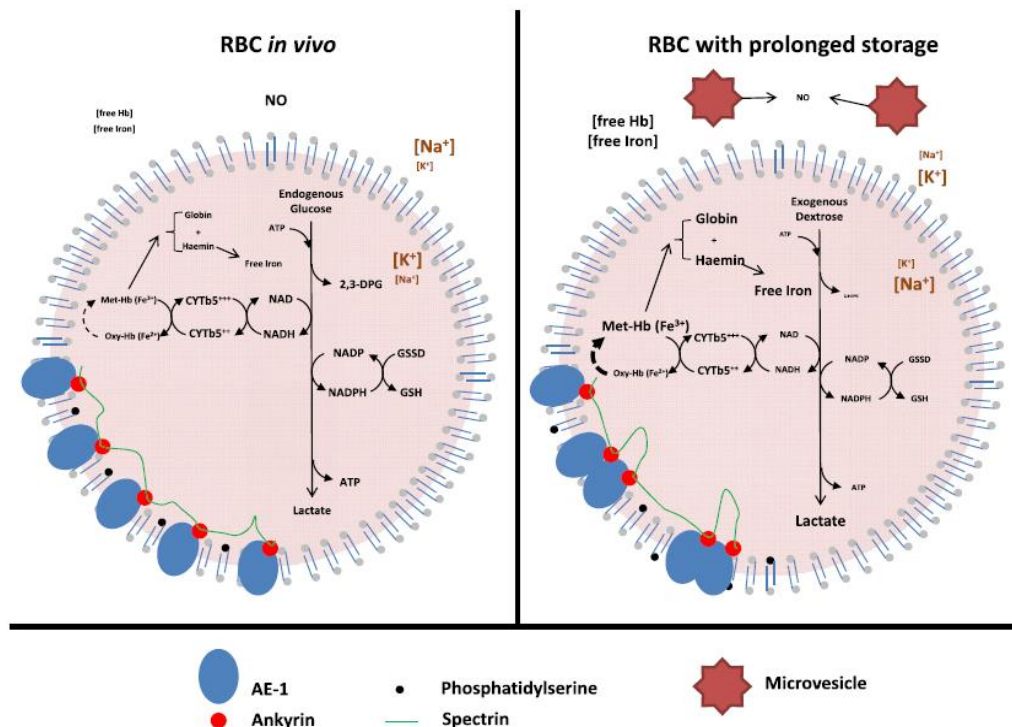
Figure 6 Red blood cell storage lesion

During storage, red blood cells accumulate storage lesions such as decreased ATP and 2.3 DPG, increased extracellular K^+ and haemoglobin, protein and lipid damage and membrane loss (Orlov & Karkouti, 2015).

As the RBCs break down glucose, the storage solution and the RBCs become more acidic because of the formation of lactic acid and protons in anaerobic glycolysis. This in turn slows down glycolysis so that less ATP and NADH is produced. After five to six weeks of storage, the pH usually drops from 7.0 to 6.5, and glucose is broken down at half the original rate. Since the RBC is dependent on ATP for its metabolic activity, a decrease in its concentration has widespread effects. The activity of ATP-dependent
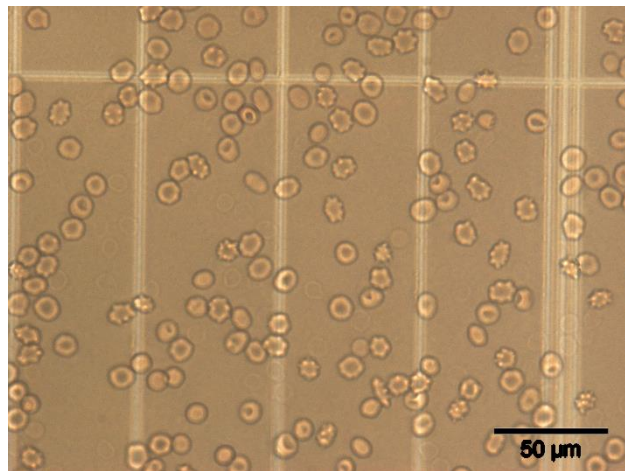
Na$^+$/K$^+$ and Ca^{2+} pumps in the RBC is reduced, causing K$^+$ to accumulate outside of the cell and Na$^+$ and Ca^{2+} inside (Hess, 2010b). Also, the ATP-dependent enzyme amino phospholipid translocase keeps phosphatidylserine from being exposed on the surface of the RBC, which is a sign of senescence recognised by macrophages. However, the contribution of the ATP concentration to the storage lesion might be less significant than previously thought (Orlov & Karkouti, 2015).

The CPD storage solution commonly used has a pH of 5 – 5.8, which protects the dextrose from damage when the solution is autoclaved. The pH of the resulting mixture of whole blood and these acidic solutions is about 7.1. This leads to increased breakdown of 2,3-DPG concentrations in the RBC (Hess, 2010a). The decreased 2.3-DPG concentration reduces the oxygen delivery of the RBCs but is quickly restored after transfusion (Hess, 2014).

A normal RBC has a biconcave disc shape, which is very important to its ability to squeeze through small capillaries and through the sinusoids of the spleen. During storage, the shape of the RBC changes dramatically. It becomes more round with rough edges and is called a spheroechinocyte (figure 7). At this stage, it loses membrane through vesiculation (Greenwalt, 2006).

Shedding of membrane microvesicles is a part of normal processes in the RBC in vivo. As the reticulocyte matures, it loses membrane until it reaches the appropriate size. Vesiculation of the membrane is also associated with apoptosis of RBCs (Hess, 2010b). During their lifetime, RBCs lose around 20% of haemoglobin by means of microvesicles, which are quickly

Figure 7 Spheroechinocytes during storage

During storage the red blood cells (RBCs) undergo a change in shape referred to as spheroechinocyte. The RBCs become more round and the membrane forms spikes, which eventually detach from the cell in the form of microvesicles.

removed by Kupffer cells in the liver (Willekens et al., 2005). It has been proposed that RBCs in storage shed these microvesicles to get rid of damaged oxidised proteins, such as haemoglobin and band 3 (Willekens et al., 2008). These microvesicles are a heterogeneous mix containing different compositions of lipids and proteins but their biological function is largely unknown (Parihar et al., 2010). The shape changes that occur during storage of RBCs probably affect its function in some way but are usually reversible by rejuvenation in the presence of nutrients at neutral pH (Hess, 2010c).

Oxidative damage is one of the main causes of the storage lesion. When blood is collected it has around 75% oxygen saturation. In this state oxygen is spontaneously lost from one ferrous (Fe2+) haemoglobin molecule and binds to another. As this is occurring, the oxygen sometimes removes an electron from the iron so that a ferric (Fe3+) methaemoglobin is formed. At the beginning of storage, there is enough NADH to rapidly remove it but as glycolysis is slowed down, the RBC capacity to form NADH in glycolysis is decreased. As the concentration of the unstable methaemoglobin rises, it becomes more likely that hydroxyl radicals will form. They are highly reactive and can damage proteins

and lipids (Hess, 2010c). Oxidative damage to proteins, such as haemoglobin and band 3, affects the function and deformability of the RBC and evidently decreases its ability to transport oxygen and survive in the circulation (Mohanty, Nagababu, & Rifkind, 2014).

1.3.2 Transfusion reactions related to storage lesions

The decision to transfuse blood must not be taken lightly. Despite its life-saving capacity, there are always certain risks involved. The clinical implications of transfusing RBCs that have been stored for longer times are not well understood but are currently being studied vigorously. When a unit of blood is transfused into a patient, up to 25% of the RBCs may need to be cleared by the MPS within the first hours of transfusion. This can be a burden for the monocytes and macrophages of the MPS and might overwhelm the system resulting in increased free haemoglobin, haem and iron. In addition, these substances will already have accumulated to some extent in the storage solution (Hod et al., 2010; Orlov & Karkouti, 2015). This can lead to increased risk of infection by affecting macrophage activation, in addition to providing iron to many bacteria (Ozment et al., 2013). Additionally, free haemoglobin and microvesicles bind nitric oxide (NO) in the circulation, thus inhibiting its activity. NO is a potent vasodilator in addition to inhibiting platelet aggregation and endothelial cell-surface expression of adhesion molecules, so the consequences can be various (Donadee et al., 2011). Furthermore, microvesicles have phosphatidylserine and other negatively charged phospholipids exposed on the surface and are considerably procoagulant (Rubin et al., 2013; Spinella, Sparrow, Hess, & Norris, 2011).

These are some of the possible consequences of transfusing older blood. However, the clinical effects have not been thoroughly concluded. Many studies have been performed in order to elucidate the possible association between transfusing older blood and adverse clinical outcomes. A recent meta-analysis of 21 studies concluded that there is a significantly increased mortality associated with the transfusion of old blood compared to new blood (Wang, Sun, Solomon, Klein, & Natanson, 2012). However, recent and larger studies report no link between older transfused blood and increased mortality (Heddle, Eikelboom, Liu, Barty, & Cook, 2015; Lacroix et al., 2015; Middelburg, van de Watering, Briet, & van der Bom, 2013). In addition, multiple organ dysfunction score (MODS) was not increased in patients undergoing cardiac surgery in another study (Steiner et al., 2015). Many of the studies that reported an increased mortality following transfusion were observational studies with certain limitations, such as the fact that severely ill patients received more units of older blood. Also, most patients received more than one unit, stored for different amounts of time, complicating the determination of unit age (Lacroix et al., 2015).

Whether old blood carries increased risk has been in the debate for some time. Recent studies need to be reviewed thoroughly. The question remains whether results from numerous studies warrant changes in current clinical practices of blood transfusion.

2 Objectives

The objective of this study was to analyse how the age of stored RBCs affects erythrophagocytosis, using the human monocytic cell line THP-1.

3 Materials and methods

3.1 Cell culture

### 3.1.1 General procedure for frozen cells

At the start of cell culture, cryovials, containing the human monocytic cell line THP-1 (TIB-202, American Type Culture Collection (ATCC), Manassas, VA, USA), were retrieved from storage in liquid nitrogen. A 37 °C water bath was used to gently thaw the cells. To reduce the risk of an external contamination, the cap of the vial was not allowed to touch the water and was sprayed with a 70% ethanol solution. As the cells were almost thawed, they were quickly but carefully transferred into a conical tube containing warmed RPMI 1640 GlutaMAX™ medium (Gibco, Grand Island, NY, USA), supplemented with 15% foetal bovine serum (FBS, Gibco) and penicillin/streptomycin antibiotics (Gibco), referred to as complete growth medium. To minimize the risk of a spontaneous monocyte differentiation, induced by the presence of the cryoprotectant dimethyl sulfoxide (DMSO), the cell suspension was centrifuged using Thermo Scientific Heraeus Multifuge X3 (Thermo Electron Led GmbH, Osterode Germany) at 280 g for 5 minutes. The supernatant was discarded. This centrifuge was used unless otherwise stated. Moreover, DMSO has confirmed cytotoxic properties. The cryoprotectant DMSO is commonly used for freezing cells, as it protects the cells from the formation of ice crystals during the freezing process. The cell pellet was resuspended with 5 ml of warmed medium to a concentration of $2x10^5$ cells/ml and transferred to a 25 cm^2 NUNC™ cell culture treated flasks with filter caps (Thermo Scientific, Roskilde, Denmark). The culture flask was placed in a Steri-Cult CO_2 incubator HEPA class 100 (Thermo Scientific, Marietta, OH, USA) at 37 °C, 5% CO_2 and 95% humidity, which are standard culture conditions.

3.1.2 Subculturing procedure

The human monocytic cell line THP-1 was cultured in 25 cm^2 and 75 cm^2 NUNC™ culture flasks using warmed RPMI 1640 GlutaMAX™ medium, supplemented with 15% FBS in the beginning and then 10% when cells were growing in a steady logarithmic phase. The cells were maintained at 2-4 $x10^5$ cells/ml and split at around $8-9x10^5$ cells/ml. The cells were not allowed to exceed the density of $1x10^6$ cells/ml to avoid irreversible changes in their properties. The cell density was checked regularly and complete growth medium was added to maintain the proper cell density.

3.1.3 Cell counting

The THP-1 cells were counted using an improved Neubauer haemocytometer (figure 8) and Leica DM IRB inverted microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). The cell suspension was diluted 1:10 in phosphate buffered saline (PBS, Gibco), 900μl of PBS added to 100μl of the cells. Cells in the four large squares in the corners were counted in both chambers and divided by eight to get the mean number of cells. The following equation was used to calculate the concentration of cells in 1 ml. $\frac{Cells}{ml} = Cells_{mean} \times 10 \times 10^4$. The dilution factor is 10 and by multiplying with 10^4 the result is converted to ml.
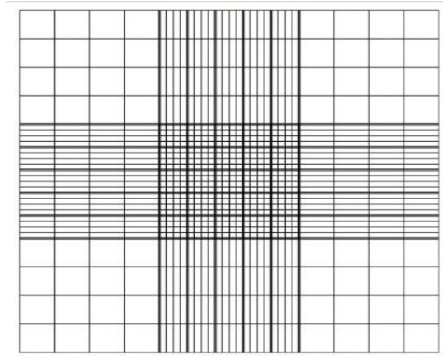
Figure 8 An improved Neubauer counting chamber

The cells were counted in the four large squares and concentration in 1 ml was calculated.

3.2 Preparation of RBC concentrates

Whole blood was collected and processed into RBC concentrates at the Blood Bank in Iceland, by using a 4-bag system. Whole blood was collected into bags containing 63 ml of CPD solution and the blood bags were rested on a cold plate to obtain the appropriate temperature of 22 ± 2°C. The blood bags were centrifuged in a Sorvall RC 12BP centrifuge (Thermo Scientific, Asheville, NC, USA) at 800 g for 11 minutes at 20 °C and then separated into blood components using Optipress II (Baxter Fenwal, Mont Saint Guibert, Belgium). The plasma was pressed into one bag and the RBCs into another containing 100 ml of SAGM solution. The separated RBCs were then leucocyte filtered into the final storage bag.

3.3 Fluorescence labelling of red blood cells

The stored RBCs (Blood Bank, Iceland) were labelled using the PKH26 red fluorescent cell linker kit (MINI26, Sigma-Aldrich, St. Louis, MO, USA). All reagents and RBCs were brought to room temperature before experiments. Samples of stored RBC concentrates were washed twice in 10 mM PBS. After washing in PBS, 100 μl of RBCs were diluted with 400 μl of diluent C and 5 μl of PKH26 dye diluted with 495 μl of diluent C. Prepared solutions were mixed with pipetting and incubated for four minutes at room temperature with periodic mixing. The insertion of the dye in the cell membrane is a very fast process, so longer incubation time is not necessary. The reaction was stopped with 1 ml of FBS for one minute and 8 ml of phenol red free RPMI 1640 medium (Gibco) supplemented with 10% FBS was added. The cell suspension was centrifuged at 1700 g for ten minutes. The supernatant was discarded and the cells were resuspended in phenol red free RPMI 1640 medium supplemented with 10% FBS to a total volume of 10 ml and transferred to a fresh tube. In addition, the cells were washed twice and resuspended in phenol red free RPMI 1640 medium supplemented with 10% FBS to a final volume of 10 ml. The cells were kept at 4 °C unless they were used within two hours. The efficiency of the labelling was estimated with an Olympus BX51 fluorescence microscope (Olympus Optical Co. GmbH, Hamburg, Germany). Photomicrographs were obtained with Cell A imaging software (version 2.5, Olympus Corporation, Tokyo, Japan).

3.4 Erythrophagocytosis assay
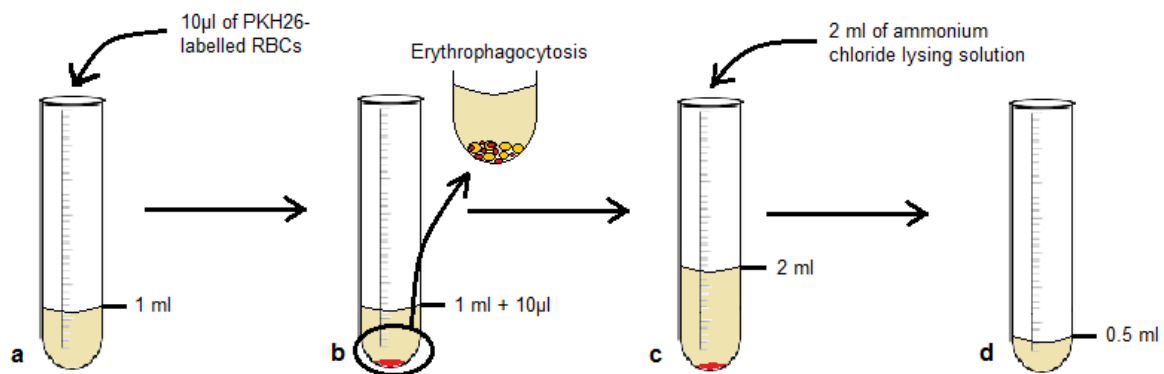
Figure 9 Erythrophagocytosis assay using undifferentiated THP-1 cells

a THP-1 cells at 1x10^6 cells/ml and 10 µl of PKH26-labelled red blood cells (RBCs) were mixed together. b The cells were centrifuged at 940 g for 20 s, to bring the cells closer together, and incubated for 45 minutes at 37 °C, 5% CO$_2$ and 95% humidity. c Unpagocytosed RBCs were lysed using ammonium chloride lysing solution. d Finally the cells were suspended in 0.5 ml phosphate-buffered saline (PBS) and kept on ice prior to flow cytometric analysis.

The erythrophagocytosis assay was performed as previously described (Healey, Veale, & Sparrow, 2007). In figure 9, the main steps of the experimental setup is shown. Prior to the assay, the THP-1 cells were centrifuged at 480 g for ten minutes and resuspended at the concentration of 1x10^6 cells/ml. Viability was determined using Trypan blue exclusion assay and required more than 95% to carry on experiments. Trypan blue (Gibco) at the concentration of 0.4% was mixed with the cell suspension at 1:10 dilution. The cell viability was calculated with the following equation: $\% \, viable \, cells = [1.00 - (Number \, of \, blue \, cells \div Number \, of \, total \, cells)] \, x \, 100$. The THP-1 cells (1x10^6 cells) were mixed with 10µl of the PKH26-labelled RBCs in 5 ml culture tubes (BD Biosciences, San Jose, CA, USA). THP-1 cells in complete medium alone were used as a control. The cells were sedimented at 940 g for 20 seconds using Universal 320 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen Germany) and incubated for 45 minutes at 37 °C, 5% CO$_2$ and 95% humidity. A 1:10 dilution of ammonium chloride lysing solution (BD Biosciences) was prepared with distilled water and kept on ice. After incubation, the culture tubes were vortexed and centrifuged at 940 g for two minutes and kept on ice to reduce further phagocytosis. Non-phagocytosed RBCs were lysed by adding 2 ml of the ice cold ammonium chloride lysing solution (BD Biosciences) for one minute and centrifuged at 940 g for two minutes. The lysis procedure was performed three times and then the THP-1 cells were resuspended in 0.5 ml PBS and kept on ice until analysis with flow cytometry.

3.5 Flow cytometry

The THP-1 cells were analysed using a FACSCalibur flow cytometer (BD Biosciences), equipped with a 488 nm argon laser and standard bandpass filters, and Cell Quest Pro software (version 6.0, BD Biosciences). The flow cytometer was calibrated weekly with CaliBRITE beads and FACS-Comp software (BD Biosciences). A total of 10,000 events were collected for each sample and the data was presented as dot plots of red fluorescence (FL2) and side scatter (SSC), and histograms of mean fluorescence intensity (MFI).

3.6 Morphological analysis

3.6.1 Fixation in 10% buffered formalin

Following the erythrophagocytosis assay, the THP-1 cells were centrifuged at 480 g for ten minutes. The supernatant was discarded and the cell pellet was suspended in the remaining liquid (around 50 µl). The cells were then fixed by adding 100 µl of 10% buffered formalin (Merck KGaA, Darmstadt, Germany) and kept refrigerated until the cytospin preparation.

3.6.2 Cytospin preparation

Cytospin 3 Shandon centrifuge (Thermo Shandon Limited, Cheshire, UK) was used to prepare cytospin slides. The sample chamber was assembled with a glass slide and a filter paper and placed in the centrifuge. Approximately 150 µl of each sample was loaded into the sample chambers and centrifuged at 113 g for three minutes. The specimens were air dried, stained with haematoxylin and eosin (H&E) at the pathology lab of Landspítali university hospital in Iceland, and examined with a microscope.

3.7 Differentiation of THP-1 monocytes to macrophages

3.7.1 Differentiation with phorbol 12-myristate 13-acetate (PMA)

Viability was determined as described above, using trypan blue. The THP-1 monocytes were seeded into 12 well plates (Becton Dickinson Labware Europe, Le Pont De Claix) at the final density of 6×10^5 cells/ml and differentiated into macrophages with 200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The cells were incubated for 72 hours at 37 °C, 5% CO_2 and 95% humidity. The RPMI 1640 GlutaMAX^{TM} medium supplemented with 10% FBS was used for culture.

3.7.2 Erythrophagocytosis assay
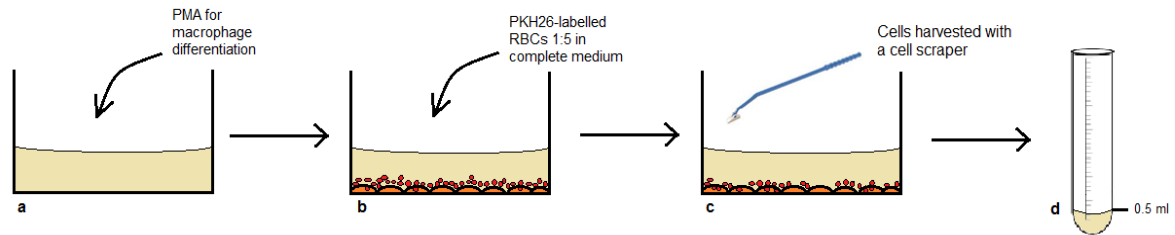
Figure 10 Erythrophagocytosis assay using PMA-differentiated THP-1 cells

a THP-1 cells at the concentration of 6x10^5 cells/ml (1.5 ml) were differentiated into macrophages with 200 ng/ml phorbol 12-myristate 13-acetate (PMA). b THP-1 macrophages were incubated with PKH26-labelled red blood cells (RBCs) for 45 minutes, 3 hours, 6 hours and 24 hours. c The cells were washed with phosphate-buffered saline (PBS) and harvested with a cell scraper. d Unphagocytosed RBCs were lysed with ammonium chloride lysing solution and the THP-1 cells were suspended in 0.5 ml of PBS and kept on ice prior to flow cytometric analysis.

In figure 10, the main steps of the experimental setup is shown. After 72 hours of incubation, the cells were washed twice with warmed PBS before the erythrophagocytosis assay, where 100 µl of PKH26-labelled RBCs were mixed with 400 µl of phenol red free RPMI medium supplemented with 10% FBS. This was added to the culture wells with the PMA-differentiated THP-1 cells, and incubated for 45 minutes, 3 hours, 6 hours and 24 hours at 37 °C. At the end of each incubation time, the plates were put on ice and the cells were washed with 2 ml of ice cold PBS to enable their detachment from the surface of the well. Ice cold PBS (1 ml) was added and PMA-differentiated THP-1 cells were harvested with a rubber cell scraper (BD Biosciences Europe, Erembodegem, Belgium). The resulting cell suspension was centrifuged at 940 g for five minutes. The supernatant was discarded and unphagocytosed RBCs were lysed using 1:10 ice cold ammonium chloride lysing solution. The solution was vortexed and centrifuged at 940 g for two minutes. The washing procedure was performed twice, first with 4 ml and then with 2 ml of the lysing solution. Finally, the cells were suspended in 0.5 ml of ice cold PBS and kept on ice until flow cytometric analysis.

3.7.3 Comparing units of RBCs at different storage times

The THP-1 cells were harvested at passage 15 prior to differentiation with PMA. The PMA-differentiated THP-1 cells were used to analyse the effect of RBC storage on erythrophagocytosis. Samples were taken from RBC units stored for 7, 19 and 40 days. As described above, the RBCs were labelled with the fluorescent dye PKH26 and kept refrigerated or used within two hours. The erythrophagocytosis assay was performed as described above, with the addition of a one hour pre-incubation with cytochalasin D at a concentration of 10 µg/ml as a control. Cytochalasin D is a fungal toxin that is a powerful inhibitor of actin polymerisation. Adding cytochalasin D inhibits the phagocytosis of macrophages. Instead of mixing the PKH26-labelled RBCs with the medium before adding to the wells, as was done before, the medium was added to appropriate wells at the same time as cytochalasin D in medium was added. The RBCs were then transferred directly to the wells.

4   Results

4.1   Efficiency of RBC labelling with red fluorescent dye PKH26

To evaluate the efficiency of the labelling of RBCs with the fluorescent dye PKH26, the cells were viewed under a fluorescent microscope. The majority of the cells was labelled and the RBC membrane labelling appeared fairly bright and uniform, as seen in figure 11. Some cells appeared slightly fainter.
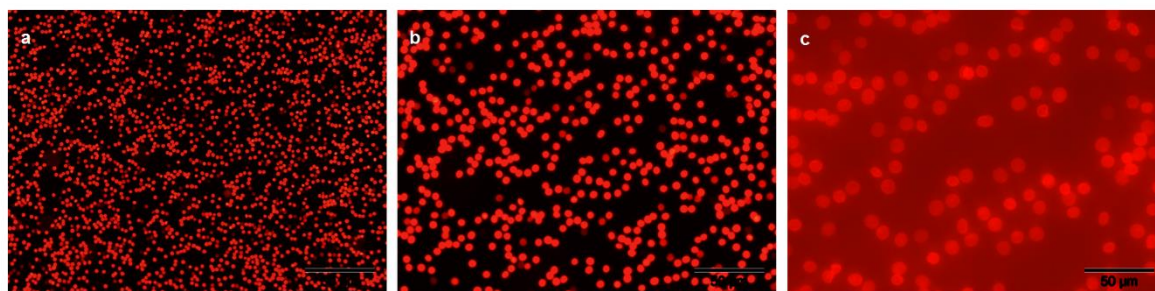
Figure 11 PKH26-labelled red blood cells

Photomicrographs of stored red blood cells labelled with the red fluorescent dye PKH26: **a** *10x magnification,* **b** *20x magnification,* **c** *40x magnification.*

4.2   Erythrophagocytosis using undifferentiated THP-1 cells

THP-1 cells were harvested at passage 8 and the RBCs were sampled and labelled at day 41. The viability of the THP-1 cells, according to trypan blue exclusion assay, was more than 99%. The ability of the cultured THP-1 monocytes to phagocytose the RBCs was analysed with flow cytometry. The flow cytometric data of the erythrophagocytosis, using passage 8 THP-1 monocytes, is shown in figure 12. The negative control, THP-1 cells in medium alone, showed a very small percentage of cells displaying autofluorescence or 0.1%. The sample, additionally containing PKH26-labelled RBCs, showed a small increase in fluorescence, or 1.3% of the total cells.
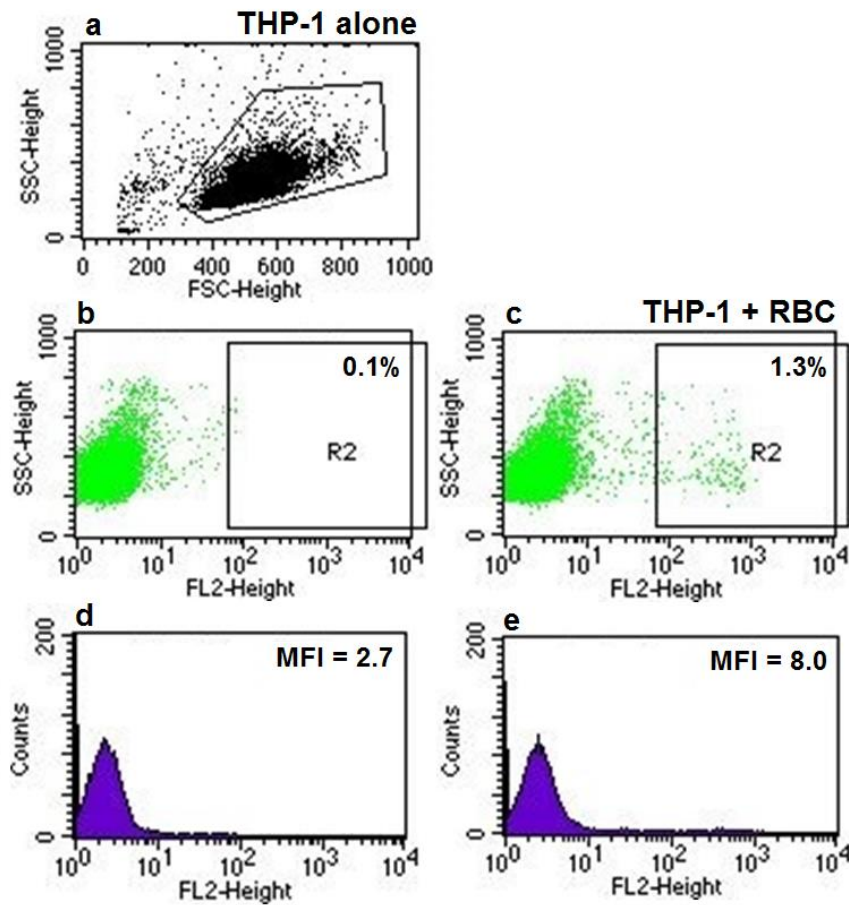
Figure 12 Flow cytometric data of erythrophagocytosis using undifferentiated THP-1 cells

a *The dot plot shows the 10.000 events collected from the sample containing only THP-1 cells. Forward scatter (FSC) versus side scatter (SSC) was used. FSC represents the size of the events counted and SSC the granularity or internal complexity. Considering the size and granularity of monocytes, the appropriate group was gated and analysed further. The dot plots below show undifferentiated THP-1 cells in* *b* *medium alone or* *c* *incubated with PKH26-labelled red blood cells. PKH26 (FL2) versus SSC was used. The percentage of THP-1 cells that were positive (R2 gate) is shown. The mean fluorescence intensity (MFI) is shown in the histograms for both* *d* *the control and* *e* *sample.*

The THP-1 cells were morphologically analysed after the erythrophagocytosis assay, using a fluorescence microscope. Figure 13 shows photomicrographs of PKH26-labelled RBCs within the undifferentiated THP-1 cells. There was a small proportion of THP-1 cells that had ingested RBCs during the 45 minute incubation
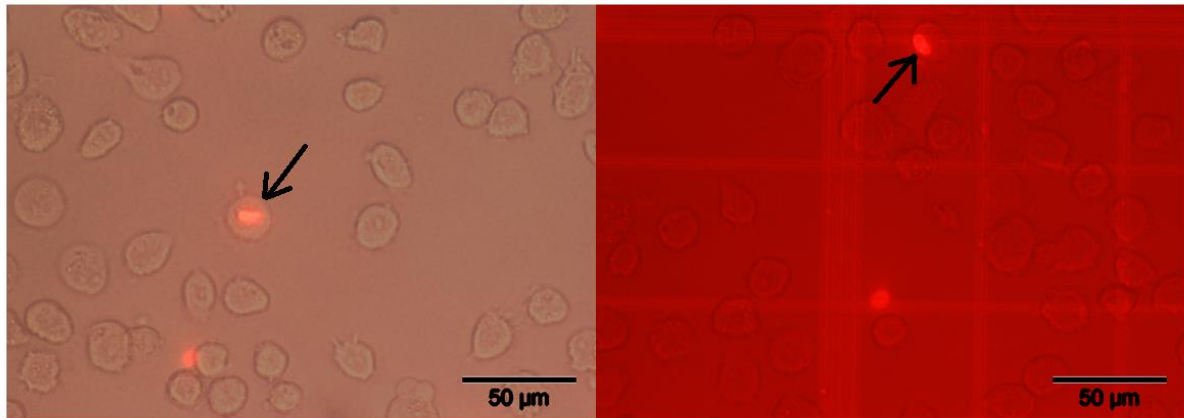
Figure 13 Erythrophagocytosis of undifferentiated THP-1 monocytes

Photomicrographs of PKH26-labelled RBCs that have been phagocytosed by the THP-1 cells.

4.3 Erythrophagocytosis using PMA-differentiated THP-1 cells

THP-1 cells were harvested at passage 10 and the RBCs were sampled and labelled at day 12 of storage. The viability of the THP-1 cells according to the trypan blue exclusion assay, prior to PMA differentiation, was more than 99%.

4.3.1 Comparison of different incubation times

Phorbol 13-myristate 12-acetate-differentiated THP-1 cells were incubated for different periods of time with PKH26-labelled RBCs and analysed using flow cytometry. In figure 14, the results from the flow cytometric analysis are shown. As storage time was longer, both the MFI and the proportion of THP-1 cells positive for red fluorescence were increased. In the negative control, THP-1 cells in medium alone, autofluorescence was small, or only 0.2% of the cells with 3.7 MFI. After 45 minutes, 32.4% of the THP-1 cells displayed red fluorescence and MFI had increased to 39.1. The percentage was very similar at three and six hour incubation times, or 83% with 182.2 MFI and 86.2% with 203.7 MFI respectively. After 24 hours of incubation, the majority of the THP-1 cells, or 96.1%, were positive for red fluorescence with MFI 397.8.
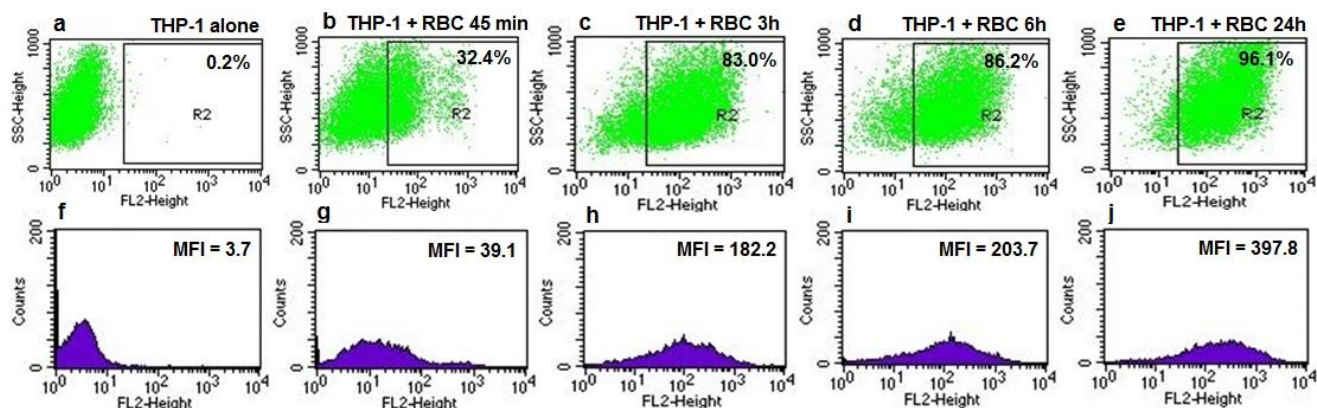
Figure 14 Flow cytometric data of erythrophagocytosis using PMA-differentiated THP-1 cells

*Dot plots of erythrophagocytosis **a** THP-1 in medium only and THP-1 cells incubated with PKH26-labelled red blood cells at **b** 45 minutes, **c** 3 hours, **d** 6 hours and **e** 24 hours. The percentages represent the mean from 3 experiments of the proportion of THP-1 cells positive for red fluorescence. The mean fluorescence intensity (MFI) is shown in the histograms (**f-j**).*

Figure 15 shows photomicrographs of THP-1 cells incubated with PKH26-labelled RBCs for 3 or 24 hours and negative controls for each containing THP-1 cells in medium alone. Red blood cells are clearly seen within the THP-1 cells. The flow cytometric data from the erythrophagocytosis is summarised in figure 16.
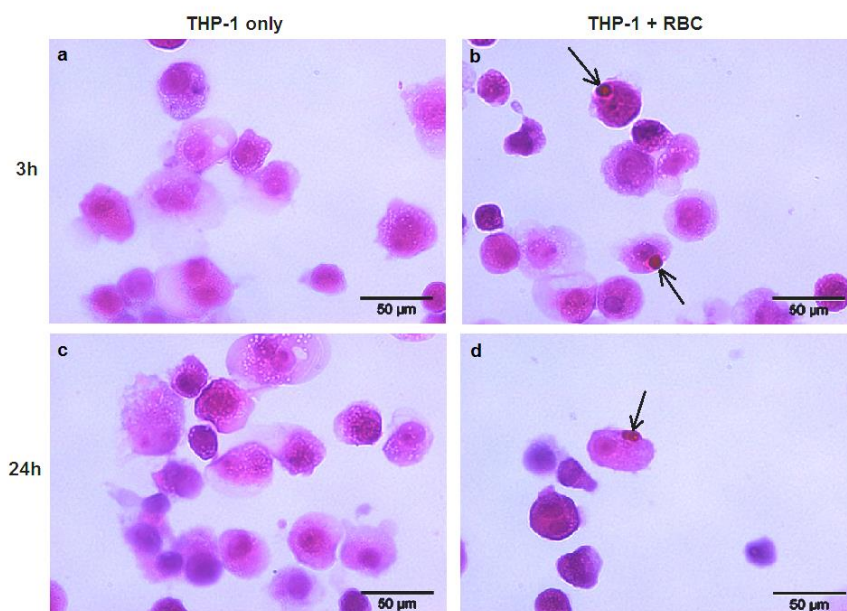
Figure 15 Cytospin of PMA-differentiated THP-1 cells

*Photomicrographs of cytospin performed on THP-1 cells after incubation for 3 hours with **a** medium alone and **b** PKH26-labelled RBCs or 24 hours with **c** medium alone and **d** PKH26-labelled RBCs.*
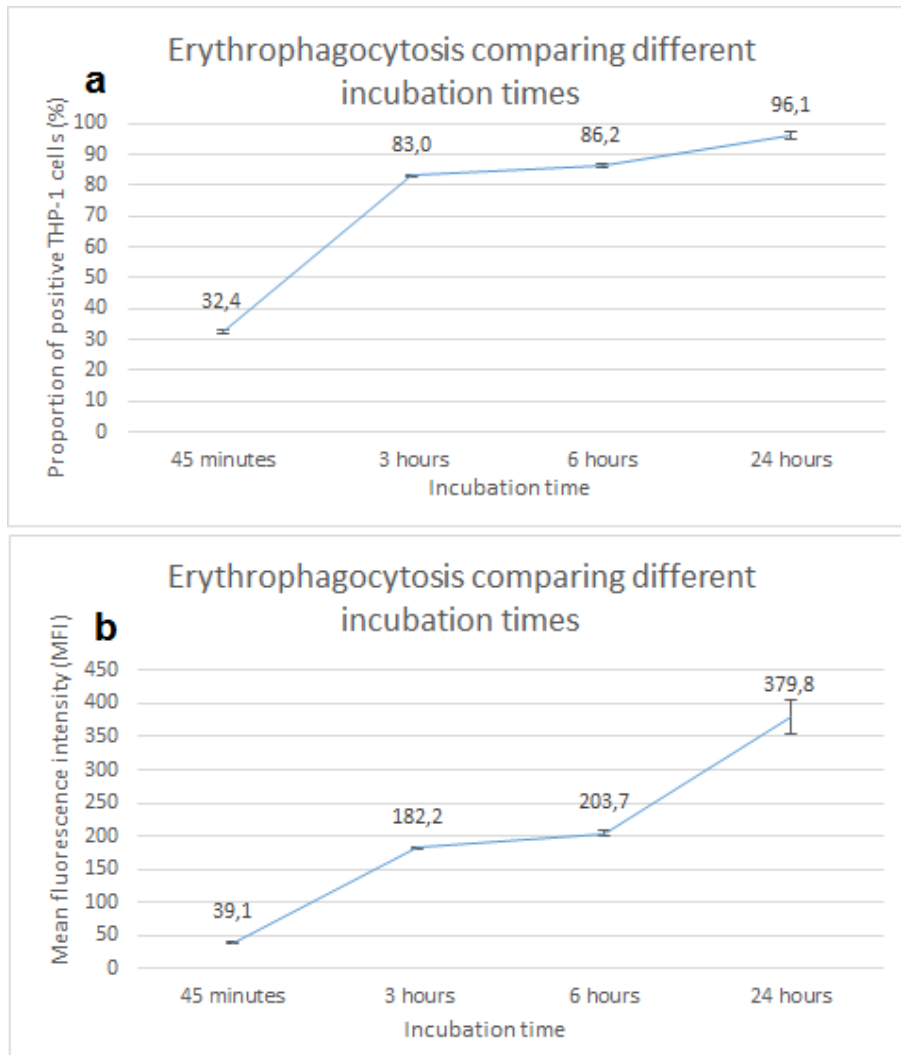
Figure 16 Erythrophagocytosis comparing different incubation times

*a The average proportion of THP-1 cells positive for red fluorescence is shown at different incubation times of the PMA-differentiated THP-1 cells and PKH26-labelled RBCs. The proportion of positive cells is increased with longer incubation time. **b** The average mean fluorescence intensity (MFI) at different incubation times of the PMA-differentiated THP-1 cells and PKH26-labelled RBCs. The MFI is increased with longer incubation time. The data comes from three experiments and error bars are presented as standard error.*

4.3.2 Comparison of different storage times

Erythrophagocytosis of RBC concentrates, stored for 7, 19 and 40 days, was compared using flow cytometry (figure 17). The negative control showed small autofluorescence, or 0.2 % of the cells with 6.4 MFI. Sample containing day 7 RBCs showed increased red fluorescence in 7.4% of the cells with 15.1 MFI. The fluorescence was similar for samples containing day 19 and 40 RBCs, or 11.8% with 18.1 MFI and 10.5% with 17.2 MF respectively. Samples containing cytochalasin D showed considerably less fluorescence compared to the samples without cytochalasin D. The flow cytometric data from the erythrophagocytosis is summarised in figure 18.
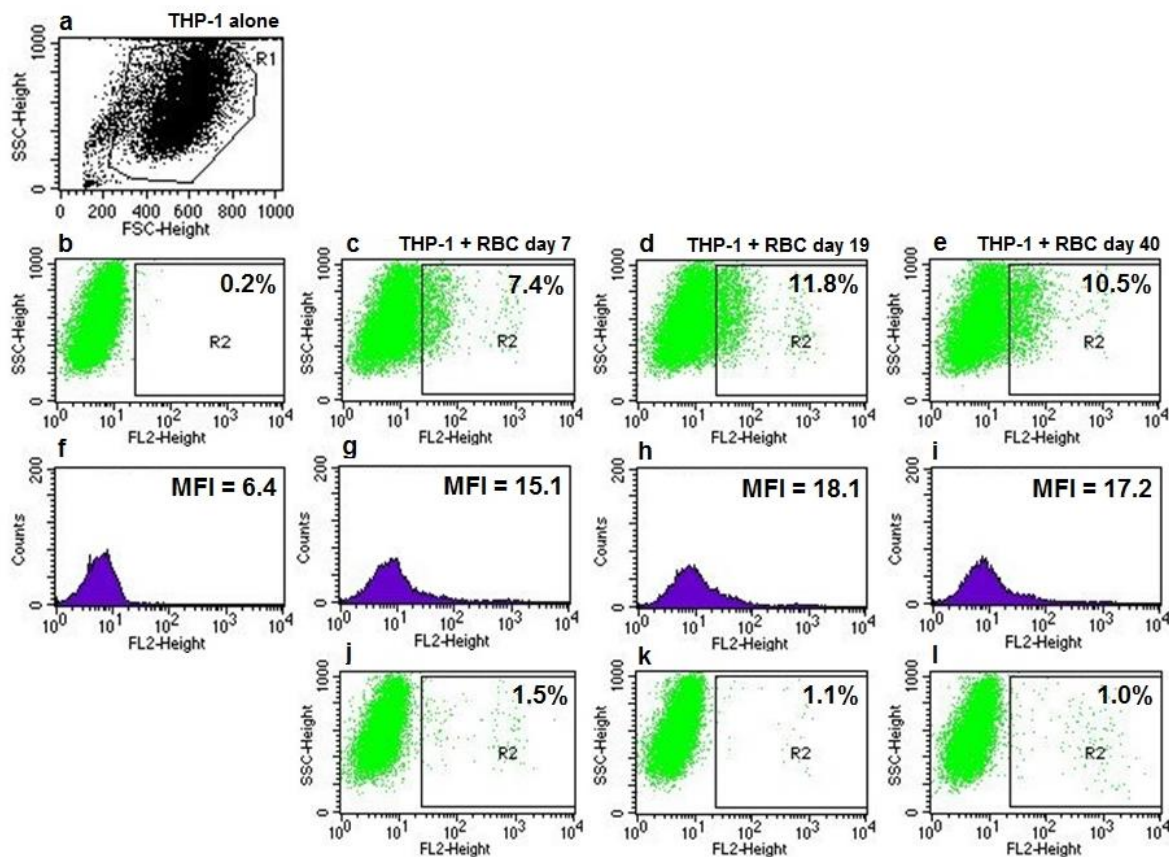
Figure 17 Flow cytometric data of erythrophagocytosis of red blood cell concentrates

*Dot plots of erythrophagocytosis using PMA-differentiated THP-1 cells incubated for 45 minutes with **b** medium alone, PKH26-labelled RBCs stored for **c** 7 days, **d** 19 days and **e** 40 days. Phagocytosis was inhibited with 1 hour pre-incubation with cytochalasin D and incubated with PKH26-labelled RBCs stored for **j** 7 days, **k** 19 days and **l** 40 days. The percentages represent the mean from 3 experiments of the proportion of THP-1 cells positive for red fluorescence. The mean fluorescence intensity (MFI) is shown in the histograms (**f-i**).*
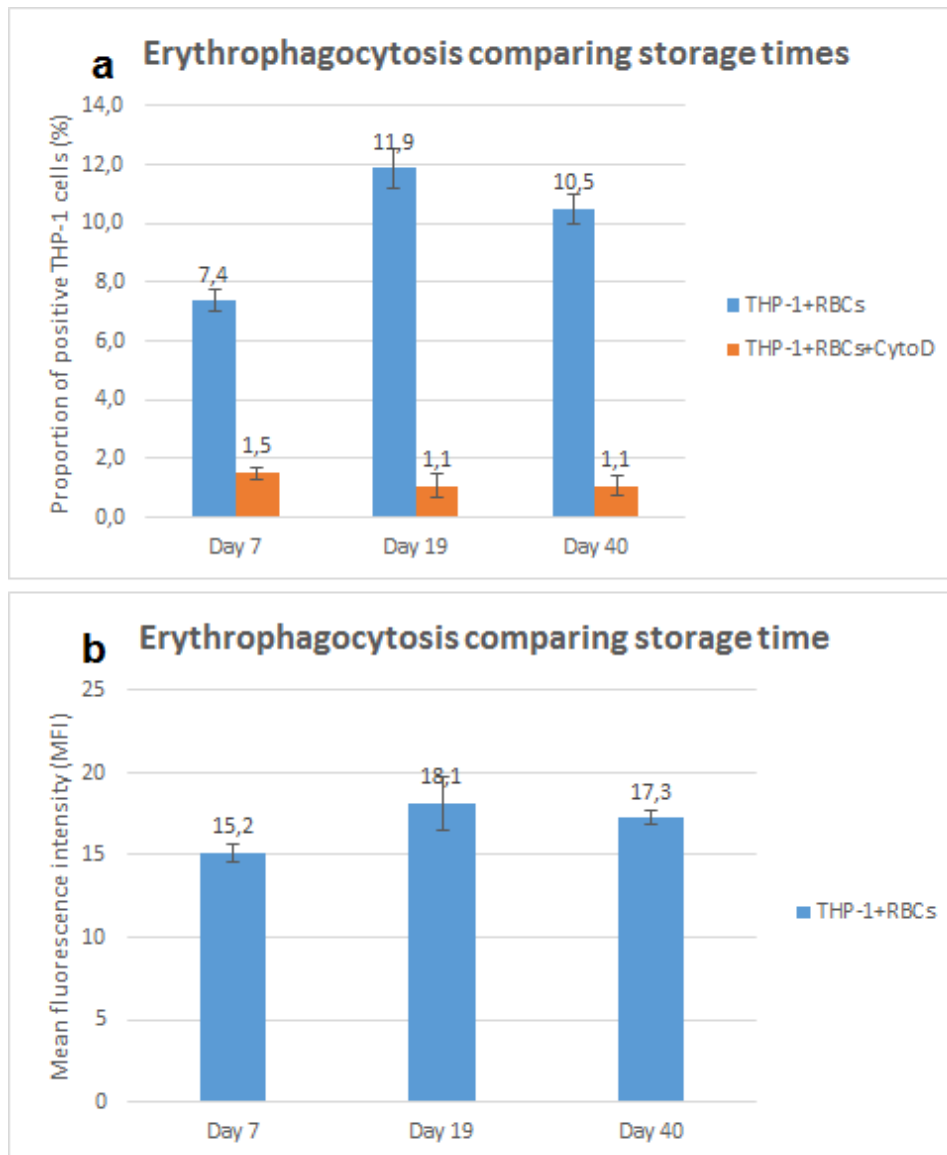
Figure 18 Erythrophagocytosis comparing different storage time

a The average proportion of THP-1 cells positive for red fluorescence for RBC units stored for different amount of time (7, 19 or 40 days). There was a slight increase from day 7 RBCs to day 19 and the results were very similar for day 19 and 40 RBCs. Erythrophagocytosis was inhibited with cytochalasin D. b The average mean fluorescence intensity (MFI) for the RBC units stored for different amount of time (7, 19 or 40 days). The MFI is very similar for all units. The data comes from three experiments and error bars are presented as standard error.

5   Discussion

In this study, the objective was to analyse how the age of RBC units affects erythrophagocytosis of RBCs during storage under blood bank conditions. To achieve this, we established two different methods to analyse erythrophagocytosis of RBC during storage. Both methods included co-cultures, flow cytometry and fluorescence microscopy.

5.1   Efficiency of erythrophagocytosis using undifferentiated THP-1 cells

The results of the erythrophagocytosis using passage 8 THP-1 cells were not as expected, with only 1.3% of the cells phagocytosing RBCs. It was confirmed with fluorescent photomicrographs that the THP-1 were in fact ingesting the RBCs (figure 13) but it was not enough to perform an assay to compare different storage times on erythrophagocytosis. The same erythrophagocytosis assay has been previously performed with much better results; around 40% of THP-1 cells phagocytosed when incubated with end of storage RBCs (Veale et al., 2014).

Several things need to be considered as possible variables that might have affected the assay outcome. These are overgrowth, which irreversibly changes the THP-1 characteristics and ability to phagocytose, culture conditions, such as the medium quality and temperature, and incubator conditions, such as temperature, CO_2 concentration and humidity. At one point, there was a problem with the incubator and as a consequence, the CO_2 levels dropped considerably and remained that way for at least an hour or more. It is possible that enough stress was introduced to the cells during this period to change their characteristics. There is also the possibility that an overgrowth was left unnoticed because of cell counting inaccuracy inherent in haemocytometers, which is augmented by the use of an inexperienced user.

If overgrowth and the general culture conditions were not the issue, the problem might lie in the fact that during prolonged subculturing, the THP-1 cells also have the tendency to lose their abilities to phagocytose. This means that the cells would need to be used at very early passages (R. Sparrow, personal communication, February 10, 2015); (Healey et al., 2007).

To confirm that the THP-1 cells were the problem, the THP-1 cells could have been incubated with sheep RBCs (positive control), since they are readily phagocytosed by THP-1 cells. However, obtaining sheep blood was not an option at the time of this experiment.

It is difficult to know precisely what caused the THP-1 cells to lose their phagocytic characteristics. However, these cells are very sensitive to culturing, and therefore it is likely that the issue originated in changes in their culture conditions.

5.2   Efficiency of erythrophagocytosis using PMA-differentiated THP-1 cells

Since the THP-1 cells did not phagocytose the RBCs when in an undifferentiated state, it was decided to stimulate the cells using PMA. The PMA-differentiated THP-1 cells phagocytosed the RBCs vigorously; after only 45 minutes of incubation more than 30% of the macrophages had ingested RBCs

(figure 14 **b**). The phagocytosis dramatically increased for three hours of incubation when more than 80% of macrophages were phagocytosing (figure 14 **c**). Also, MFI increased considerably, suggesting that each cell continued to ingest more RBCs and thus emitted a stronger fluorescence signal (figure 14 **g**, **h**). After six hours of incubation, the proportion and MFI were very similar, which suggests that the phagocytosis had slowed down (figure 14 **d, i**). After 24 hours, majority of the cells had phagocytosed RBCs, indicating that the PMA-differentiated THP-1 macrophages were highly effective at phagocytosing the RBCs (figure 14 **e**). Incubation for 45 minutes displayed considerable phagocytosis, as well as being the incubation time used for the erythrophagocytosis assay of undifferentiated THP-1 cells. It was thus decided that this time point would be used in further experiments.

When the units of blood, stored for different periods of time, were compared, considerably less phagocytosis was seen. This might be explained by the fact that the RBCs were added to the wells in a different manner. In the first experiment, RBCs were mixed with medium and then added into the wells, but in the second, medium had been added beforehand and the RBCs were added afterwards. In the second experiment, it is plausible that it took the RBCs a longer time to fall down to the bottom of the wells, where they came in contact with the PMA-differentiated THP-1 cells. Overall, the phagocytosis was similar for all three units of blood. The percentage of THP-1 cells that had ingested PKH26-labelled RBCs was lower for the unit stored for seven days compared to 19 or 40 days. However, the difference was minor, thus making it impossible to draw any conclusions from it. The proportion of positive THP-1 cells in the presence of cytochalasin D was only around 1% (figure 17 **j**, **k**, **l**). This was similar to the negative control, indicating that the inhibition of polymerisation and thus phagocytosis was successful. This confirmed that the red fluorescence displayed in the erythrophagocytosis assays was the result of phagocytosis of PKH26-labelled RBCs.

The drawback of PMA-differentiation of THP-1 monocytes to macrophages is that it presents considerable assay variability, making it more troublesome to get reproducible results. The concentration of PMA and incubation time and the culture conditions prior to differentiation can have considerable effects on the phenotype (Aldo, Craveiro, Guller, & Mor, 2013; Park et al., 2007). In addition, the THP-1 cells become adherent when differentiated, thus becoming more difficult to handle. On the other hand, if the experimental conditions are well controlled, this assay is relatively simple and easy to perform.

5.3 THP-1 cell line and erythrophagocytosis assays

The question remains whether THP-1 cells can adequately represent normal phagocytes. They have been widely used for this purpose, usually after PMA differentiation, to mimic the behaviour of normal tissue macrophages. The THP-1 cell line was derived from an immortal human leukemic cell and thus will never behave in exactly the same way as normal human cells. However, there are advantages to using a cell line instead of primary monocytes derived from peripheral blood mononuclear cells (PBMC), e.g. the homogeneous genetic background of the THP-1 cells compared to considerable donor variability in primary monocytes. THP-1 cells grow faster and can be stored indefinitely in liquid nitrogen. In addition, monocytes are only around 2-10% of PBMC. It is therefore difficult to retrieve enough uncontaminated samples from other components, such as platelets (Chanput, Mes, & Wichers, 2014).

The erythrophagocytosis assays using both undifferentiated and PMA-differentiated THP-1 cells are fairly simple and easy to perform. The problem with using undifferentiated THP-1 cells is that they are very sensitive to culture conditions and readily lose their phagocytic abilities. This is not a problem with the PMA-differentiated THP-1 cells but more variability is introduced to the assay when they are used. Also, it makes the work more tedious, since for each well, the adherent THP-1 cells need to be scraped from the bottom. It would be more convenient to use the one with undifferentiated THP-1 cells. To achieve this, the problems with culturing the THP-1 cells need to be solved, so that they retain their ability to phagocytose.

5.4 Limitations

One major limitation to this study was that a positive control, such as sheep RBCs, was not used. Also, the experiments were only performed a few times, which was not enough to acquire statistical significance. Units from different donors were used when comparing the effect of storage time on RBC units. The results would be more valid if the same unit had been sampled throughout the storage period. That would have limited the variability introduced by using RBCs from different donors. For future experiments, this would be the ideal setup.

6 Conclusion

This study demonstrates an erythrophagocytosis assay that requires impeccable culturing of THP-1 cells. If the cells experience sub-optimal culture conditions at any time, they can lose their ability to phagocytose. This makes it difficult to reproduce the assay, so that it is perhaps not the most suitable assay for repeated experiments. Differentiation of the THP-1 cells with PMA is an option to avoid problems with the passaging status of the THP-1 cells. From our results, it is not possible to conclude that longer storage affects erythrophagocytosis. Further development of the erythrophagocytosis assay is necessary. The first steps would be to use the undifferentiated THP-1 cells at earlier passages and to use a positive control. Also, further optimisation of the concentrations and incubation times is needed for the erythrophagocytosis assay using PMA-differentiated THP-1 cells.

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