Role of the CD47/SIRPα-interaction in regulation of macrophage phagocytosis

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Abbreviations

AIHA  Autoimmune hemolytic anemia
BSGC  Buffered saline glucose citrate
CMFDA  5-chloromethylfluorescein diacetate
CR  Complement receptor
DMEM  Dulbecco modified Eagle’s medium
DC  Dendritic cell
EDTA  ethylenediaminetetraacetic acid
FACS  Fluorescence-activated cell sorter
FCγR  Fc-gamma receptor
FCS  Fetal calf serum
HBSS  Hanks balanced salt solution
Hct  Hematocrit
HSA  Human serum albumin
IAP  Integrin-associated protein
ITAM  Immunoreceptor tyrosine-based activating motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
ITP  Idiopathic thrombocytic purpura
LPC  Lysophosphatidylcholine
mAb  Monoclonal antibody
pAb  Polyclonal antibody
PBS  Phosphate Buffered Saline
PI3K  Phosphoinositide 3-kinase
PRP  Platelet-rich plasma
RBC  Red blood cell
TSP  Trombospondin
Abstract

Role of the CD47/SIRPα-interaction in regulation of macrophage phagocytosis
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CD47 is a cell surface glycoprotein that is expressed by virtually all cells in the body. Binding of CD47 to the macrophage receptor Signal Regulatory Protein alpha (SIRPα) yields an inhibitory signal that counteracts phagocytosis. Red blood cells (RBCs) that lack CD47 are rapidly cleared from the circulation, whereas CD47 expressing cells have a normal turnover rate. CD47 has therefore been proposed to function as a marker of self, enabling the immune system to discriminate between self and foreign. Thus, the studies of the present thesis aimed at further investigating the role of CD47 as a marker of self in regulating phagocytosis of platelets, phagocytosis of viable or senescent RBCs, and the mechanisms involved.

CD47 on platelets was found to regulate their turnover in vivo, since platelets from CD47−/− mice transfused into wild type recipients were cleared more rapidly from the circulation than wild type platelets. In addition, CD47−/− mice were found to suffer from a mild spontaneous thrombocytopenia, without any signs of accelerated platelet apoptosis or increased platelet activation. CD47−/− mice were more sensitive to experimental immune thrombocytopenia (ITP), as compared with wild type mice. In vitro phagocytosis experiments proved that platelet CD47 was responsible for this effect, since blocking antibodies to macrophage SIRPα increased phagocytosis of wild type platelets to the levels seen for CD47−/− platelets. When unopsonized platelets or RBCs from CD47−/− mice (expressing about 50% less CD47 than wild type cells) were transfused into wild type recipients, they were cleared from the circulation at virtually the same rate as wild type cells. However, CD47−/− cells were cleared more rapidly than wild type cells when transfused animals were challenged with an antibody directed against the transfused cell type. In vitro, IgG-opsonized CD47−/− platelets and RBCs were ingested to a higher extent than wild type cells, but less than CD47+/− cells, suggesting that CD47 dose-dependently regulates phagocytosis in macrophages. It was also investigated if inhibitory SIRPα signaling is localized to the site of contact with the cell that is to be ingested, or whether the inhibition of phagocytosis is more general in the whole macrophage. Experiments with a mix of IgG-opsonized wild type and CD47−/− RBCs showed that the effect of inhibitory CD47-SIRPα signaling was local in the macrophage and limited to the site of contact with a specific target cell. Thus, contact with one or several wild type RBCs did not affect the increased phagocytosis of CD47−/− RBCs by the same macrophage.

RBC senescence involves oxidation of membrane lipids and proteins, as well as exposure of phosphatidylserine (PS) on the cell surface, and clearance of senescent RBCs is believed to be regulated by several different factors. To investigate the role of CD47 in uptake of experimentally senescent RBCs, RBCs were oxidized with CuSO4/ascorbic acid (Ox-RBCs). Phagocytosis of Ox-RBCs required recognition of PS on the RBCs, recognition by scavenger receptors on the macrophages, and was strongly dependent on serum. CD47 did not inhibit serum-dependent phagocytosis of experimentally senescent unopsonized RBCs, since phagocytosis of senescent wild type or CD47−/− RBCs was virtually similar. The ability of CD47 to cluster in the plasma membrane upon cross-linking with antibodies was reduced in senescent RBCs. Despite this, CD47 inhibited phagocytosis of IgG-opsonized viable or senescent RBCs to the same extent.

In summary, CD47 can function as a marker of self on both RBCs and platelets. The phagocytosis-inhibitory effect is dependent on the CD47 expression level, and CD47-SIRPα signaling acts locally in the macrophage at the contact with a target cell. In experimentally senescent RBCs, CD47 does not inhibit serum-dependent phagocytosis in the absence of opsonization, but still inhibits FcγR-mediated phagocytosis.

Key words: CD47, SIRPα, platelets, red blood cells, macrophages, phagocytosis, Fcγ receptor, senescence
**List of original papers**

This thesis is based on the following articles and manuscripts, which will be referred to in the text by their roman numerals (I-IV):

I. **Platelet homeostasis is regulated by platelet expression of CD47 under normal conditions and in passive immune thrombocytopenia**
   Olsson M., Bruhns P., Frazier WA., Ravetch JV., and Oldenborg PA.
   *Blood, 2005; 105 (9): 3577-3582*

II. **Dose-dependent inhibitory effect of CD47 in macrophage uptake of IgG-opsonized murine erythrocytes**
    Olsson M., Nilsson A., and Oldenborg PA.
    *Biochemical and Biophysical Research Communications, 2007; 352: 193-197*

III. **CD47 on experimentally senescent RBCs inhibits phagocytosis following Fcγ receptor-mediated but not scavenger receptor-mediated recognition by macrophages**
     Olsson M., and Oldenborg PA.
     *Manuscript*

IV. **Spatial regulation of Fcγ receptor-mediated phagocytosis by the inhibitory CD47/SIRPα interaction**
    Olsson M., and Oldenborg PA.
    *Manuscript*
Background

The innate immune system – overview

The innate immune system preexists and is ready to resist microbial intruders at any given time. This is the first line of host defense that reacts immediately in a non-specific manner. Pathogenic microorganisms may enter the body by many different routes, for example the skin, respiratory tract, or the gastrointestinal mucosa. These structures are covered by epithelia that act like a physical and chemical barrier. The normal flora of commensal bacteria also helps in fending off pathogenic bacteria by competing for nutrients. However, these barriers are sometime breached and pathogenic microorganisms start to duplicate in the host tissue. Inflammation occurs when bacteria, viruses, or parasites overcome these barriers. Often they immediately encounter macrophages that identify them as a threat. Macrophages are soon reinforced by neutrophils. Both macrophages and neutrophils rely on a repertoire of receptors that can discriminate between self and non-self. The innate immune system uses relatively few variations in their receptor repertoire compared to the adaptive immune system. The receptors used by the innate immune system recognize structures that are common to many different pathogens, but absent in the host.

When an infectious organism breaches the innate immune system, the adaptive immune system is activated. Unlike the innate immune system, the adaptive response is specific, and depends on activation of T- and B-lymphocytes, and generation of memory cells, which can protect against future infections. Dendritic cells (DC) and macrophages ingest infectious agents and present these antigens together with MHC. This is called antigen presentation and is typically an important link between the innate and the adaptive immune system. Importantly, macrophages are far less potent as antigen-presenting cells (APC) than the DC. Normally, expression of peptides on MHC is not enough to induce an adaptive immune response. A second signal to the lymphocytes is also required, which is delivered by co-stimulatory molecules expressed by activated APC.

Self vs. non-self discrimination in the innate immune system

It is pivotal for the immune system to be able to distinguish self from foreign. When this process fails, development of autoimmunity may occur. There are several
mechanisms involved in preventing the immune system from reacting to own tissues, i.e. selection in the development stage of activator cells.

Recognition of microorganisms is achieved by either recognition of non-self structures or missing self. Non-self structures are defined as structures unique for the microorganisms that the host is missing. Missing self relies on structures that are expressed by host cells identifying them as self. Microbes lacking these structures are therefore recognized as foreign.

NK cells rely, at least in part, on mechanisms enabling them to separate self from foreign. The missing self-hypothesis was postulated by Kärre, to describe how NK cells are activated when target cells lack or have a reduced expression of MHC class I on their surface. Thus, NK cells have inhibitory receptors, which can inhibit activation when they recognize normal levels of self-MHC class I on a target cell.

The alternative complement pathway is another example how cells in the innate immune system may recognize missing self. Complement factor 3 (C3) is a serum protein that can attach to both host and microbial cells. C3 is spontaneously cleaved and starts a chain of events that forms an active protease complex. However, these events are inhibited on host cells by several proteins, including CD46 and CD55. Therefore the alternative pathway is only activated when cells do not express certain self structures.

Surfactant proteins A and D (SP-A and SP-D) are found in the lungs and consist of a globular head and a collagenous tail. When pathogen-associated molecular patterns (PAMPs) are recognized by the head domains of SP-A or SP-D, the tail region can interact with phagocytes to initiate phagocytosis and/or trigger an inflammatory response in the lung. However there are data supporting the contrary effect of SP-A and SP-D, namely that they seem to suppress inflammatory response in the lung. Another study propose that SP-A and SP-D have dual functions, both proinflammatory and anti-inflammatory. By binding PAMPs with the globular domain, it acts proinflammatory. The surfactants may also bind the inhibitory receptor signal regulatory protein alpha (SIRPα – described in detail below) with the globular part, which results in an anti-inflammatory response.

CD47

CD47, or integrin associated protein (IAP), is a ubiquitously expressed transmembrane cell surface glycoprotein that is a member of the immunoglobulin superfamily. CD47 consists of a highly glycosylated extracellular immunoglobulin variable (IgV) domain,
a hydrophobic five transmembrane domain, and an intracellular domain with four different splicing alternatives\(^7^8\) (Fig. 1). CD47 was first recognized to associate with the \(\alpha\nu\beta3\) integrin on leukocytes and in placenta\(^9\), but has since then also been associated with \(\alpha1\beta3\) and \(\alpha\nu\beta3\) on platelets\(^10\), with \(\alpha\nu\beta3\) on melanoma cells\(^11\) and ovarian carcinoma cells, and with \(\alpha2\beta1\) on platelets and smooth muscle cells\(^12^13\). The cancer antigen OV-3 is highly up regulated on ovarian carcinoma cells, and was found by molecular cloning to be identical to CD47\(^14\).

Table 1. Biological functions of CD47

<table>
<thead>
<tr>
<th>Function</th>
<th>Ligand or associated protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker of self, inhibits phagocytosis</td>
<td>SIRP(\alpha)</td>
<td>15-17</td>
</tr>
<tr>
<td>Regulation of integrin-mediated activation</td>
<td>(\beta_1, \beta_2, \beta_3, \beta_5) integrin-association</td>
<td>7</td>
</tr>
<tr>
<td>Induction of apoptosis</td>
<td>(\alpha)-CD47 mAbs</td>
<td>18-22</td>
</tr>
<tr>
<td></td>
<td>SIRP(\alpha/\gamma)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FAS/CD95-association</td>
<td></td>
</tr>
<tr>
<td>T cell activation/inhibition T(_{reg}) formation</td>
<td>SIRP(\alpha)</td>
<td>23-25</td>
</tr>
<tr>
<td>Regulation of cell migration</td>
<td>SIRP(\alpha)</td>
<td>26</td>
</tr>
<tr>
<td>Inhibits NO-dependent production of cGMP</td>
<td>TSP-1</td>
<td>27</td>
</tr>
<tr>
<td>Macrophage fusion and osteoclast formation</td>
<td>SIRP(\alpha)</td>
<td>28</td>
</tr>
</tbody>
</table>

Furthermore, CD47 also bind trombospondin (TSP). There are five types of TSP and CD47 has been confirmed to bind TSP-1\(^29\). The binding segment has been identified in all five forms of TSP. Therefore, CD47 is believed to bind all five TSP forms. In contrast to integrin binding, which occurs within the membrane of the same cell, CD47 can also bind to SIRP\(\alpha\) via cell to cell contact. CD47 has been associated with a number of different biological responses, which are summarized in table 1.

CD47 is highly expressed by RBCs, which do not express integrins. Individuals with the Rh\(_{null}\) phenotype show reduced expression levels of CD47, about 25% of normal CD47 levels\(^30^31\). The Rh complex is associated with several other proteins in the plasma membrane like, glycophorin B, and Rh associated glycoprotein (RhAG). Furthermore, studies have proposed that Band 3 is associated with the Rh complex\(^32\). Band 3 forms a complex with proteins like glycophorin A, protein 4.2 and ankyrin,
which are believed to be anchoring points to spectrin\textsuperscript{33,34}. Humans deficient in protein 4.2 and band 3, exhibit a strong reduction in CD47 expression\textsuperscript{32,35}. Based on these findings, there is reason to believe that CD47 in the Rh-complex in fact forms a link to the Band 3-complex, by binding to protein 4.2 (Fig. 2). The situation in murine RBCs is somewhat different from that in human cells. RBCs from protein 4.2-deficient mice show normal expression levels of CD47\textsuperscript{36}. The same is true also for Band 3-deficient RBCs, which have little or no expression of Rh polypeptides\textsuperscript{32}. In addition, in human RBCs, it was suggested that about 50\% of the CD47 molecules are anchored to the cytoskeleton, and the rest is more freely mobile in the plasma membrane\textsuperscript{37}. Murine RBCs, on the other hand, have almost no attachment of CD47 to the cytoskeleton\textsuperscript{38}. The mobility of CD47 and its ability to cluster at sites of contact with macrophages was proposed to be necessary for effective induction of phagocytosis-inhibitory signals (described below).

Figure 1. Structure of CD47. The extracellular part is highly glycosylated and contains two disulfide bonds, where one connects the extracellular domain with the five times membrane spanning domain. The intracellular part is numbered according to the different splice versions. Reproduced with permission from ref 8.
SIRPα

The signal regulatory protein (SIRP) family is a group of transmembrane proteins that are associated with regulation of leukocyte function. SIRP proteins have relatively similar extracellular domains, which belong to the immunoglobulin superfamily domains, but differ in their cytoplasmic region. The extracellular domain is the ligand-binding region, and typically contains a single Ig V-domain, and one or two constant Ig-like domains (Ig C). Today, there are three known members in the SIRP family, SIRPα (also known as SHPS-1, BIT, P84, MFR, or CD172a), SIRPβ, and SIRPγ. These three proteins mainly differ in their cytoplasmic region. SIRPα has an intracellular region containing two immunoreceptor tyrosine-based inhibitory motifs (ITIM). CD47 can function as a ligand for SIRPα. SIRPβ has a short sequence, only containing 6 amino acids, lacking signaling structures. However, SIRPβ signals via the adaptor protein DAP12, containing an immunoreceptor tyrosine-based activating motif (ITAM), which can generate an activating signal. Unlike SIRPα, SIRPβ does not bind CD47. SIRPγ, also called SIRPβ2, contains a short cytoplasmic region of only four amino acids, lacking signaling motifs and the ability to signal via DAP12. Like SIRPα, SIRPγ was found to bind CD47. However, SIRPγ is only expressed in T-cells, but not in myeloid cells. It has been suggested that

Figure 2. In human RBCs, CD47 is associated with RBC membrane proteins and the cytoskeleton. Reproduced with permission from ref 8.
SIRPγ expressed by T-cells binds CD47 on APCs, and by doing so it can support T-cell activation\textsuperscript{47}.

SIRPα was first identified in neurons\textsuperscript{42}, but was later also found to be expressed in myeloid cells, where high expression was noticed in granulocytes, monocytes, macrophages, and DC\textsuperscript{48}. As mentioned above, the extracellular part contains three or one Ig like domains (alternative splicing, at least in mice), where the outer part is an IgV-domain and the two inner parts are IgC-domains\textsuperscript{41}. When activated, the ITIMs are tyrosine phosphorylated and can bind the Src-homology 2 (SH2)-containing tyrosine phosphatases SHP-1 and SHP-2\textsuperscript{41}. SHP-1 and SHP-2 seem to have opposite functions, where SHP-1 primarily is associated with inhibitory functions, and SHP-2 is associated with activation of cellular responses (Fig. 3). In macrophages, inhibitory CD47/SIRPα-signaling is only dependent on SHP-1\textsuperscript{16,49}. Despite its interaction with CD47, SIRPα may also be regulated by other factors. These include, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, neutrophins, lysophosphatic acid (LPA), growth hormone (GH), and colony-stimulating factor (CSF)\textsuperscript{40}. In addition, integrin binding to extracellular matrix proteins can induce

![Figure 3. Ligation of SIRPα will result in phosphorylation of the two ITIMS, followed by recruitment of the tyrosine phosphatases SHP-1 and SHP-2. Reproduced with permission from ref 8.](image-url)
tyrosine phosphorylation of the SIRPα ITIMs\textsuperscript{50}. Interestingly, it was recently suggested that CD47 on the same cell is involved in regulating integrin/adhesion-dependent SIRPα phosphorylation in macrophages\textsuperscript{51}.

In myeloid cells, SIRPα has so far mostly been shown to act as an inhibitory receptor\textsuperscript{15;16;52}. However, ligation of SIRPα was also found to be associated with an enhanced phosphoinositide 3-kinase (PI3K) activity\textsuperscript{53}, and SIRPα-stimulated NO production was in part dependent on PI3K activity\textsuperscript{54}. Also, the SIRPα/CD47 interaction was found to be important for efficient formation of multinucleated giant cells from macrophages\textsuperscript{55;56}, and osteoclast formation both \textit{in vivo} and \textit{in vitro}\textsuperscript{28}. Thus, SIRPα may be involved in a complex array of intracellular signaling systems and biological functions. Still, the most investigated function of SIRPα is inhibition of phagocytosis when interacting with CD47 on a target cell (described below).

**Red blood cells**

RBCs are formed in the bone marrow. Before leaving the bone marrow and entering the circulation, the RBCs lose their nucleus and assume a biconcave shape. The main functions of RBCs are to deliver O\textsubscript{2} to the tissues, and to remove CO\textsubscript{2} from tissues and release it in the lungs. RBCs are highly viscous and easily change shape to squeeze through thin capillaries. The cytoskeleton and cell membrane contain several different proteins like spectrin, ankyrin, actin, glycophorin and Band 3, just to mention a few. Some of these proteins are important for the RBCs to maintain the biconcave shape, i.e. reduced amounts of spectrin results in spherocytosis.

Autoimmune hemolytic anemia (AIHA) is an autoimmune disease where RBCs are destroyed due to the presence of autoantibodies directed at RBCs. These antibodies may include both IgG and IgM, and may activate complement. Thus, macrophages may recognize both complement and antibody-opsonized RBCs via their complement receptors (CR) and/or Fc receptors (FcR)\textsuperscript{57}.

**RBC turnover**

The exact mechanism behind uptake and destruction of senescent RBCs is still open for debate. Understanding normal clearance of RBCs from the circulation is important to predict the fate of RBCs under pathological conditions, as well as the survival of transfused RBCs. Aged RBCs are mainly engulfed by Kupffer cells in the liver and by macrophages in the spleen\textsuperscript{58}. RBC senescence resembles apoptosis in general from
some points of view, however, since RBCs lack nuclei and mitochondria, the term eryptosis has been suggested as a synonym to apoptosis, to describe RBC senescence. The reasons for clearance of senescent RBCs are probably dependent on several mechanisms working together, like alterations in carbohydrate structure, PS exposure, naturally occurring antibodies, or oxidative damage. The hallmark of apoptosis is exposure of PS. Receptors involved in uptake of PS positive cells are scavenger receptor B, PS receptor, the vitronectin receptor and the LPS receptor CD14. Regarding PS-recognition, within a short period of time, three separate PS-receptors were recently described, referred to as TIM-1, TIM-4, and BAII.

Blood plasma contains several different anti-RBC antibodies. These antibodies have different affinities and specificity. Some bind intracellular proteins such as actin or spectrin, others bind extracellular proteins like Band 3, some antibodies have the capability to activate complement, and others do not. The fact that low concentrations of Abs are found on RBCs in vivo indicates that something more must happen to the senescent RBCs to enable their phagocytosis by macrophages.

Band 3 is the most expressed protein on the RBC surface where it is an anion transporter, that transports one HCO3− out from the RBC and at the same time takes one Cl− in. IgG in plasma often bind to Band 3 at the end of the RBC life cycle. Therefore the conclusion must be, that some type of change occurs in Band 3 during senescence, enabling binding of IgG present in plasma. There are two theories regarding this fact: 1) aggregation of Band 3 in senescent RBCs forms a new epitope for Abs, and 2) breakdown of Band 3 that creates new epitopes. In both theories, oxidation may be the source of newly formed epitopes. During aging of RBCs, the total cell volume is reduced and their density increases. This is associated with loss of cholesterol and phospholipids. Plasma membrane vesicles released from older RBCs have been shown to bind IgG and express PS. The hemoglobin of these vesicles also resembles that of old RBCs.

Sialic acid, which is negatively charged, is found on the RBC surface, and can bind to macrophage lectins (Siglecs) with inhibitory function, mediated via an ITIM. Sialic acid has therefore been proposed as a marker of self on RBCs, by binding Siglecs they generate an inhibitory signal. Loss of sialic acid is associated with RBC senescence, and may therefore be a signal for removal of senescent RBCs. CD47 can regulate RBC uptake depending on the amounts CD47 expressed by the RBC (paper II). It has been shown that CD47 expression is reduced on aged RBCs, and that CD47 is lost on RBCs stored for transfusion. Loss of phagocytosis inhibitory structures like CD47 or sialic acid may eventually reduce the prophagocytic signals needed for clearance.
Platelets

Platelets are small, disk shaped fragments, which are derived from megakaryocytes in the bone marrow. They do not carry a nucleus, but have several organelles such as mitochondria, RER, golgi apparatus, and some granules. Platelets normally circulate in the blood for 8-10 days in humans, and for about 4-5 days in mice. Platelets play a major role in hemostasis and have the ability to adhere to foreign or endogenously altered surfaces, as well as to other platelets. Upon activation platelets release several substances involved in coagulation. Reduced numbers of platelets in circulating blood is called thrombocytopenia, and results in increased risk of bleeding. Idiopathic thrombocytic purpura (ITP) is an autoimmune disease where autoantibodies directed against platelets are formed, which may lead to phagocytosis by macrophages in the liver and spleen.

Macrophages

Professional phagocytes, macrophages and neutrophils, are an important frontline of host defense, which must be overcome if microorganisms are to establish an infection. Macrophages are cells that participate in both the innate and the adaptive immune system. They are located throughout the body at several locations like connective tissue, the GI tract, and lungs. However, there are some places where they are highly concentrated like the red pulp of the spleen, and in the liver. Macrophages play an important role in protection against pathogens, like bacteria, but they are also central in removal of apoptotic cells. Only the macrophage precursor, monocyte, is found in circulating blood. From there the monocyte leaves the blood and differentiates to macrophages in the tissues.

Macrophages play an important role in the inflammatory response, and are often the first cell to encounter microorganisms in the tissues. By releasing cytokines and chemokines, they are soon reinforced by other cells like neutrophils. Due to the large heterogeneity among macrophages, and depending on the specific type of stimuli, there is a broad array of surface antigens, metabolic changes and secretory responses associated with macrophage activation.

Besides playing an important role in the innate immune response, macrophages are also involved in regulating the adaptive immune response. As mentioned above, together with DC, macrophages can act as APC. Resting macrophages express no or very few MHC class II receptors on the cell surface. However, when activated, MHC class II and co-stimulatory receptors are upregulated. This enables the macrophage to function as an APC.
Macrophage Subpopulations

Macrophages is a heterogeneous cell population, where the phenotype is affected by the local microenvironment. Examples of tissue specialized macrophages are macrophages in the spleen, Kupffer cells in the liver, alveolar macrophages in the lung, microglia in the nervous system, and peritoneal macrophages in the peritoneal cavity. Some of the above mentioned populations are further divided into subpopulations, such as splenic macrophages. Two types of macrophages are found in the marginal zone, metallophilic macrophages that seem to play a role in systemic infection, and phagocytic marginal zone macrophages which express several receptors, like scavenger receptors. Furthermore, the white pulp contains tingible body macrophages that are involved in phagocytosis of apoptotic B cells. The red pulp of the spleen holds yet another macrophage population, which in part may be involved in clearance of senescent RBCs. The receptor repertoire expressed by macrophages depends on their specialized function, which is related to their microenvironments.

Phagocytosis

Binding of a particle (pathogen or host cell) to macrophage receptors may result in phagocytosis, which is an evolutionary conserved process, where cells ingest particles or molecules from their surroundings. Phagocytosis is an active process where the particle is enclosed by the phagocyte cell membrane and then internalized. This process is specific and regulated through stimulation of receptors such as FcγRs, CRs, and scavenger receptors by ligands on the target particle. By definition, phagocytosis refers to uptake of particles, larger than 0.5 μm in diameter. Ingestion occurs when receptors on the phagocyte plasma-membrane recognize structures on target particles. Binding of particles to specific cell surface receptors is the first step preceding phagocytosis. Upon particle binding, phagocytic receptors form clusters in the plasma-membrane where the binding occurs. Clustering of receptors is believed to strengthen the binding and amplify the signal. As a result, intracellular signaling cascades induce polymerization of actin filaments, which are able to form fagocytic cups around the particle that eventually is internalized. When ingested, the particle is surrounded by the phagocyte cell membrane, forming what is called a phagosome. Macrophages have lysosomes in the cytoplasm, which contain enzymes, proteins and peptides that are toxic. Lysosomes fuse with the phagosome and form a phagolysosome, a process called phagosomal maturation. Upon fusing the lysosome releases its toxic contents. Macrophages also produce other substances, like H₂O₂, superoxide anion (O₂⁻) and nitric oxide (NO), which have a toxic effect on the phagocytosed microorganisms. These substances are formed by lysosomal NADPH.
oxidases and other enzymes that are a part of the respiratory burst\textsuperscript{92,93}. It was recently understood, that phagosomal maturation, and activation of gene-transcription associated with a proinflammatory response, are processes that are differentially regulated by spatial recognition of ligands on the target particle\textsuperscript{94}. Thus, macrophage recognition of microbial ligands via toll-like receptors can discriminate between microorganisms and apoptotic cells, affecting both internalization and phagosome maturation of each particle \textit{per se}, when both are internalized by the same macrophage\textsuperscript{94}.

\textbf{Pro-phagocytic receptors}

\textbf{Fc receptors}

The Fc receptors (FcR) bind the Fc portion of immunoglobulins. FcRs determined FcγR are the class that binds IgG. Subclasses of FcγR are defined by the affinity for IgG subclasses and the type of signaling pathway involved, inhibitory or activating\textsuperscript{95}. Today there are four known different classes of FcγRs in mice, FcγRI, FcγRIIB, FcγRIII and FcγRIV\textsuperscript{95}. Upon ligation by IgG, FcγI, FcγRIII, and FcγRIV generate activating signals via their ITAM motifs, whereas FcγRIIB acts as an inhibitory receptor\textsuperscript{96}. FcγRs are widely expressed on cells involved in the immune system. However, the relative amount of each FcγR differs depending on cell type. Monocytes, macrophages, DC, basophils and mast cells express both activating and inhibitory FcγRs. Neutrophils express FcγRIIB and FcγRIII and FcγRIV. NK cells only express the activating FcγRIII, and B-cells only express the inhibitory FcγRIIB\textsuperscript{97}. FcγRs recognize pathogens that are opsonized with antibodies. When activating FcγRs bind the Fc part of the antibodies, the cell is activated and engulfs the pathogen. Free antibodies may also bind FcγRs, however, to achieve activation, FcγRs are often dependant on cross linking of the FcγRs with an immune complex\textsuperscript{90}. Despite the fact that there are several activating FcγRs, the signaling after binding an immune complex is quite similar. Following receptor ligation, the ITAM is tyrosine phosphorylated by kinases of the Src family\textsuperscript{90}. This will result in accumulation of Syk, which can activate several downstream targets that in the end will lead to engulfment of the opsonized particle (Fig. 4). FcγRIIB signals via an ITIM in the cytoplasmic domain. FcγRIIB ITIM signaling starts with phosphorylation of the ITIM, followed by generation of SH2 domains that can bind SHP, which leads to abrogation of ITAM signaling from activating FcγRs\textsuperscript{98}.

FcγRIIB has an important role to regulate the strength of a response in cells participating in innate immunity. This is demonstrated in FcγRIIB-knockout mice, which have an enhanced macrophage immune response\textsuperscript{99}. The negative regulation of
the cell response differs dependent on the IgG isotype, which can be explained by the fact that IgG isotypes differ in their affinity for FcγRs. IgG2a and IgG2b are the most pro-inflammatory subclasses in mice. For example, antibodies of the IgG2b subclass were exclusively dependent on FcγRIV in vivo in an ITP model, but could also bind FcγRIII in vitro. IgG2a proved to behave in a similar manner, but under some conditions FcγRI and FcγRIII were involved in the activation by IgG2a. IgG1, on the other hand, proved to only bind FcγRIII and FcγRIIB. Both IgG2a and IgG2b bind the inhibitory FcγRIIB with low affinity, suggesting that these two isotypes are less sensitive to regulation by FcγRIIB than IgG1. A recent study has shown that FcγR binding of both IgG2a and IgG2b on opsonized RBCs is dependent on the opsonization level. IgG2b was found to mediate phagocytosis by binding to both FcγRIII and FcγRIV. However, at low opsonization levels of IgG2a, phagocytosis was exclusively mediated by FcγRIII. Higher IgG2a opsonization levels proved to be mediated by FcγRIII, but also with a contribution from both FcγRI and FcγRIV.

FcγRIIB has also been proposed to constitute a late peripheral checkpoint during B-lymphocyte development, and low levels of FcγRIIB are associated with development of autoimmune disease. Besides being important for phagocytosis and presentation of antigens in DC, FcγRs have shown to be important for setting a threshold in activation of DCs and by doing so regulate the adaptive immune response. Mice devoid of FcγRIIB generate a stronger and more long lasting immune response. Other experiments that used DC without FcγRIIB, or by blocking FcγRIIB, showed spontaneous DC maturation.

Complement receptors

The complement system is mainly a part of the innate immune system, but may also be activated by the adaptive immune system. Complement proteins are found in serum, and complement receptors (CR), which are expressed by several cell types, may trigger phagocytosis. There are three main pathways were complement proteins are involved: classical complement pathway (antibody dependent), the alternative complement pathway (not dependent on antibodies), and the mannose-binding lectin pathway (similar to the classical pathway but relies on mannose-binding lectin instead of antibodies). Complement proteins are numbered in the order of their discovery, hence C3 was the third complement protein found. C3 is a key protein of the complement system. Upon complement activation C3-convertase cleaves C3 to produce C3b that may function as an opsonin and aid in phagocytosis mediated by the complement receptors. The complement system acts in three distinct ways to prevent infection. First, it may opsonize cells (C3b and C3bi), second it recruits other immune cells (C3a and C5a), and third it can kill cells by creating pores in the
membrane (C5b, C6, C7, C8 and C9)\textsuperscript{106}. There are four complement receptors CR1-4, where CR1, CR3, and CR4 are phagocytic receptors. CR1 is expressed by RBCs, B cells, monocytes, neutrophils, eosinophils, and DC, and CR3 is found on monocytes, macrophages, neutrophils, DC, and NK cells\textsuperscript{107}. CR4 is less well characterized. CR2, however, has not been described as a phagocytic receptor\textsuperscript{107}. C3b and C3bi can engage CR1 or CR3/4, respectively, to stimulate phagocytosis\textsuperscript{107}.

FcγRs can initiate phagocytosis without further stimuli than binding to antibody opsonized particles\textsuperscript{87}. CRs differ from FcγRs, in the way that CRs often need additional stimuli to ingest complement opsonized particles. Such a stimuli may be PMA, TNF-α, or GM-CSF\textsuperscript{87}. Both FcγRs and CRs are dependent on polymerization of actin, the process in which the particle is ingested differs\textsuperscript{108;109}. In FCγR-mediated phagocytosis pseudopodia protrude from the cell membrane and surround the IgG opsonized particle in a zipper like process before it is ingested. CR-mediated phagocytosis seems to be a more passive process, where the complement-opsonized particles seem to sink into the cell, without formation of any distinct pseudopodia\textsuperscript{108;109}.

**Scavenger receptors**

Apoptosis is a form of programmed cell death that continuously occurs in our body. When cells undergo apoptosis they are removed by phagocytes without triggering the inflammatory response that is seen in removal of bacteria\textsuperscript{110}. Apoptotic cells acquire different prophagocytic cell surface ligands, which can be recognized by phagocytic cells\textsuperscript{111}. Scavenger receptors are part of this recognition and participate in removal of apoptotic cells by recognizing structures that are not normally present on healthy cells. In addition, scavenger receptors also participate in the defense against microorganisms\textsuperscript{87}. Suggested ligands for apoptotic cells are PS, changed patterns of glycosylation of cell surface proteins, and surface charge\textsuperscript{89}. Known receptors on the macrophage that perform these duties are class A scavenger receptors, class B scavenger receptors, class E scavenger receptors, and CD14 (Table 2.). It should be noted that SR-B on murine macrophages does not mediate uptake of apoptotic cells\textsuperscript{112}.

**Cytoskeleton and signaling in phagocytosis**

Thyrosine phosphorylation of the ITAM moiety on the activating FcγR’s is crucial for FcγR-mediated phagocytosis. Phosphorylation of ITAMs is believed to be mediated by tyrosine kinases belonging to the src family\textsuperscript{113}. Syk kinase is then recruited to the
Table 2. Scavenger receptors expressed by macrophages, which have been implicated in uptake of senescent cells.⁸⁷⁻¹¹¹.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Functions</th>
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<tbody>
<tr>
<td><strong>Scavenger receptor class A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- SR-AI-III</td>
<td>Acetylated or oxidized LDL</td>
<td>Phagocytosis of apoptotic cells and bacteria</td>
</tr>
<tr>
<td>- MARCO</td>
<td>PS</td>
<td></td>
</tr>
<tr>
<td>- Polyanionic compounds (SR-A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scavenger receptor class B</strong></td>
<td>Native LDL, VLDL and HDL</td>
<td>Phagocytosis of apoptotic cells</td>
</tr>
<tr>
<td>- SR-B</td>
<td>Oxidized LDL</td>
<td></td>
</tr>
<tr>
<td>- CD36</td>
<td>Oxidized PS</td>
<td></td>
</tr>
<tr>
<td>- Trombospondin</td>
<td>Collagen type I</td>
<td></td>
</tr>
<tr>
<td><strong>Scavenger receptor class E</strong></td>
<td>Oxidized LDL</td>
<td>Phagocytosis of apoptotic cells</td>
</tr>
<tr>
<td>- LOX-1</td>
<td>PS</td>
<td></td>
</tr>
<tr>
<td>- Hsp70</td>
<td>Fibronectin</td>
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</tr>
<tr>
<td>CD14</td>
<td>LPS</td>
<td>Phagocytosis of apoptotic cells</td>
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Figure 4. Simplified schematic overview of some main initial signaling events during FcγR-mediated phagocytosis. The hypothetical inhibitory actions of SIRPα-signaling are also depicted.
phosphorylated ITAM, which results in activation of Syk (Fig. 4). This then leads to activation of several different downstream intracellular signaling pathways. Eventually, actin polymerizes and forms a phagocytic cup/pseudopodia surrounding the antibody-opsonized particle. The Rho family GTPases rac and Cdc42 are mandatory for regulation of actin polymerization during FcγR-mediated phagocytosis, but are not required for CR-mediated phagocytosis. PI3K is important in the phagocytosis process, and pharmacological inhibitors of PI3K blocks both FcγR-mediated and CR-mediated phagocytosis. PI3K does not seem to be required for particle binding or initial F-actin polymerization. However, the formation of a phagocytic cup and internalization process is PI3K-dependent. Phagocytosis of smaller particles (<3 μm) is less dependent on PI3K, which may be explained by the proposed role of this kinase in mediating fusion of cytoplasmic vesicles with the plasma membrane, supplying additional membrane to facilitate phagocytosis.

**Inhibitory regulation through CD47/SIRPα-interaction**

CD47 can function as a marker of self on RBCs, and CD47−/− RBCs are rapidly cleared from the circulation of CD47+/+, but not CD47−/− mice. This clearance mechanism is virtually entirely based in the spleen, and the cleared cells are specifically ingested by splenic F4/80+ red pulp macrophages. Elimination of CD47−/− RBCs is independent of complement and antibody. Furthermore, when SIRPα on isolated splenic macrophages was blocked (mAb P84) it increased the level of phagocytosis of CD47+/+ RBCs to that seen with CD47−/− RBCs, whereas phagocytosis of the CD47−/− targets was unaffected. Since inhibition of SIRPα prevents the CD47 effect, and CD47 is a known ligand for SIRPα, it is highly likely that the effect of CD47 is due to ligation of macrophage SIRPα by CD47 on the RBCs. In addition, IgG-opsonized CD47+/+ and CD47−/− RBCs are taken up equally well by macrophages from SIRPα/SHPS-1-mutant mice, which have a truncated non-signaling SIRPα cytoplasmic domain. CD47-deficient mice are more sensitive to AIHA. Furthermore, both IgG-opsonized CD47−/− and CD47+/+ RBCs are more rapidly cleared *in vivo* and more rapidly phagocytosed *in vitro* as the level of IgG-opsonization is increased. This demonstrates that the inhibitory CD47-SIRPα signal is integrated with the Fcγ receptor signal proximal to the decision to phagocytose, and that neither the SIRPα nor the Fcγ receptor signal is dominant. Rather the outcome is determined by the relative strength of the two signals (Fig. 5). The same results were obtained with C3bi-opsonized CD47−/− or CD47+/+ RBCs. Thus, it seems that the inhibitory signals generated by macrophage SIRPα upon ligation of CD47 affects prophagocytic signaling.
mechanisms that are important for phagocytosis stimulated via Fcγ receptors, as well as via CR.

During RBC ageing in vivo, changes to the RBCs take place, which are involved in regulating their final elimination by macrophages\textsuperscript{58,116,117}. As mentioned above, although the exact mechanisms involved are still not completely understood, it is likely that a combination of changes in the RBC plasma membrane and its components, biomechanical changes in the RBC, and the presence of natural antibodies together create enough prophagocytic stimuli\textsuperscript{116}. In addition, CD47 may be lost or redistributed on senescent RBCs. The prophagocytic stimuli may thus overcome the mechanisms that prevent phagocytosis (e.g. CD47/SIRPα interaction) during RBC interaction with macrophages.

The exact mechanisms mediating the phagocytosis-inhibitory effects of SIRPα are not yet fully understood. When the receptor is ligated by CD47, its ITIMs become tyrosine phosphorylated\textsuperscript{15,114}. The tyrosine phosphorylated ITIMs then recruit both SHP-1 and SHP-2\textsuperscript{114}. However, for inhibition of FcγR-mediated phagocytosis, only SHP-1 is important. This was clearly shown in experiments using mogenous viable mice\textsuperscript{16}, which express normal levels of SHP-2, but due to a point mutation have only about 20% of normal levels of SHP-1\textsuperscript{118}. Studies in macrophages from SIRPα/SHPS-1-
mutant mice have suggested that the inhibition mediated by SIRPα might be at the level of Syk-activation, or PI3K-activation\(^{114}\) (Fig. 4).

Despite the fact that the exact mechanisms behind inhibitory SIRPα-signaling are not completely clear, based on the findings described above, the following model can be proposed: to macrophages all particles are “foreign” and targeted for elimination unless they carry the correct “passport”: CD47.
Materials and methods

Animals

Generation of CD47\(-/-\) mice has previously been described\(^{119}\). Balb/c and C57BL/6J mice were backcrossed to Balb/c or C57BL/6J for 16 (Jackson Laboratory, Bar Harbor, ME) or more generations. Mice were kept in accordance with local guidelines and maintained in a specific pathogen-free barrier facility. All animal experiments were approved by the Institutional Review Committee.

Preparation of bone marrow derived macrophages

Preparation of bone marrow macrophages (BMM) has previously been described\(^{120}\). In short, mice were euthanized and femoral bones were removed. Bone marrow was flushed out with PBS/HSA 1\% and red blood cells were lysed in dH\(_2\)O. Bone marrow cells were resuspended in Dulbecco modified Eagle’s medium (DMEM) containing FCS 10\%, plated on tissue-culture dishes, and incubated for 2 h at 37\(^\circ\)C and 5\% CO\(_2\). Non adherent cells were collected and resuspended in DMEM/FCS 10\% supplemented with 15\% L929 cell supernatant (as a source of macrophage colony-stimulating factor), penicillin 100 U/ml and streptomycin. Cells were then plated on bacterial plastic dishes and allowed to mature for 6 days before use.

Measurement of platelet and red blood cell concentration

Platelet count, and RBC Hct or count, were determined using an automated blood analyser (Sysmex KX-21; sysmex Europe GMBH, Norderstedt, Germany). Blood was obtained from the lateral tail vein and immediately diluted in 95\(\mu\)l PBS containing 5 mM EDTA (PBS/EDTA 5 mM). The blood was further diluted in cellpack to obtain values that was within the normal range of the machine. Collected blood was kept on ice, and was always analyzed within 1 h from sampling.

For RBC count or Hct measurement, 7\(\mu\)l blood was obtained from the tail vein and diluted in 95\(\mu\)l PBS/EDTA 5 mM followed by 200\(\mu\)l cellpack to give a final dilution of 2.3/100. To confirm accuracy of the Sysmex-KX21, experiments were initially preformed in parallel with traditional Hct measurement, were blood is collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) from the retroorbital vein plexa of anesthetized mice. The capillary tubes were then spun and the Hct was
determined as the relative height of the RBC column expressed in percent. No differences in results from the two methods were observed.

For platelet counts, 5 μl blood was obtained from the tailvein and diluted in 95 μl PBS/EDTA 5 mM followed by addition of 400 μl cellpack yielding a final dilution of 1/100. Initially cell counts were compared to a traditional method were platelets were stained with stromatol (according to manufacture instruction) and counted in a bürker chamber. The two methods gave virtually the same results.

**Platelet and RBC staining**

**PKH26 and PKH67 staining**

RBCs were washed in PBS followed by resuspension in Dilluent C to a concentration of 1x10^9 RBC/ml. The cells were then incubated with 4 μM of red fluorescent dye PKH26, or green fluorescent dye PKH67, for 5 min at room temperature, followed by addition of an equivalent volume of PBS/HSA 2 % for 1 min. The cells were finally washed two times with PBS.

**CMFDA staining**

Isolation of platelet from whole-blood and subsequent 5-chloromethylfluorescein diacetate (CMFDA)-labeling was done as previously described\textsuperscript{121}, with some modifications. Anticoagulation buffer contained trisodium citrate 130 mM, EDTA 10 mM and theophylline 10 mM and carbacyclin 2 μg/ml. Buffered saline glucose citrate (BSGC) contained NaCl 116 mM, trisodium citrate 13.6 mM, Na₂HPO₄·7H₂O 8.6 mM, KH₂PO₄ 1.6 mM, EDTA 0.9 mM, glucose 11.1 mM and carbacyclin 1 μg/ml. Blood was obtained by cardiac puncture, using a syringe containing 200 μl of a 1:1 solution of anticoagulant buffer and BSGC. The blood cells were then centrifuged at 300 g for 3 min to obtain platelet-rich plasma (PRP). The pellet was resuspended in in BSG and the procedure was repeated. The two PRP fractions were then combined and centrifuged for 10 min at 1200 g, and resuspended in BSGC to a concentration of 5x10^8 platelets/ml. CMFDA was added to a concentration of 2.5 μM and the cells were incubated in dark for 45 min at room temperature. After washing, the platelets were resuspended in appropriate medium to a desired concentration.
**RBC in vitro phagocytosis**

RBCs were obtained by cardiac puncture and washed in PBS, resuspended to a cell concentration of $5 \times 10^8$/ml, followed by opsonization with pAb rabbit anti-murine RBC (200 μg/ml) at 37°C for 20 min. RBCs were then washed in PBS and samples from the different phenotypes were analyzed for the level of opsonization. This was done using a secondary antibody, Alexa488-cojugated goat anti-rabbit IgG, followed by flow cytometric analysis. The remaining RBCs were resuspended in DMEM/FCS 10 % to a concentration of $1 \times 10^8$/ml. $1 \times 10^7$ RBCs were added to each well with macrophages adherent to 11 mm glass coverslips. The cells were incubated for 30 min at 37°C in an atmosphere with 5 % CO₂. Phagocytosis was stopped by putting the cells on ice, followed by lysis of non-ingested RBCs with dH₂O for 40 s. The cells were then fixed and stained with May-Grünewald and Giemsa followed by mounting in DPX or PERTEX. In some experiments RBC were stained with PKH26 or PKH67, as described above, before antibody opsonization. After opsonization, RBCs were washed and resuspended in DMEM/FCS 10%, followed by addition of $1 \times 10^7$ RBCs to each well with macrophages. In some experiments CD47⁺/⁻ and CD47⁻/⁻ RBC, labeled with PKH26 and PKH67 respectively, were mixed 1:1 before addition to macrophages. The cells were left on ice for 20 min to ensure RBC and macrophage contact, followed by incubation for 30 min at 37°C in an atmosphere with 5 % CO₂. In experiments of phagocytic cup-formation, the cells were incubated for 5 min in a 37°C water bath. Cover slips were then washed in PBS and fixed in PFA for 20 min, followed by mounting in Vectashield.

**May-Grünewald and Giemsa staining**

After lysis of RBC and washing, the cover slips were incubated with May-Grünewald working solution (2:1 May-Grünewald stock solution:methanol) for 5 min. The cells were then carefully washed two times with dH₂O. Next, the cells were incubated with Giemsa working solution (1:5 Giemsa stock solution:dH₂O) for 5 min, followed by two washes with dH₂O. The cover slips were then allowed to air dry for at least 20 min before a few drops of xylene were added to dehydrate the cells. The cells were then mounted in DPX or PERTEX.

**Staining of F-actin with phalloidin probes**

Macrophages adherent to glass cover slips were fixed in 2% formaldehyde for 20 min followed by washing in PBS. The cover slips were then incubated with an F-actin-staining solution (10 μM phalloidin probe and 10μg/ml LPC in PBS) for 30 min. The
phalloidin was either conjugated to Alexa350, FITC or Alexa633. The cover slips were then carefully rinsed with PBS before they were mounted in Vectashield.

**Flow cytometric analysis of platelets**

Five μl blood was obtained from the lateral tail vein and was immediately diluted in PBS/EDTA 5 mM, followed by washing in PBS. Samples were then incubated with primary antibody in 96-well plates in dark at -4°C for 20 min. Cells were then washed with PBS, and when necessary incubated with a secondary antibody, followed by another wash in PBS. Cells were then analyzed using flow cytometry. Platelets give a separate forward scatter/side scatter-population and are easy to separate from RBCs when using logarithmic scales on forward and side scatter.

**Platelet survival**

Platelets were isolated and labeled with CMFDA as previously described. In the final step of CMFDA labeling, platelets were resuspended in a sterile saline solution to a cell-concentration of 3x10⁸/ml. Of this solution, 350 μl was intravenously injected in the lateral tail vein of recipient mice. To analyze clearance, 5 μl blood was obtained from the tail vein and diluted in PBS/EDTA 5 mM and directly analyzed by flow cytometry. Baseline values were determined at 10 min or 2 h after platelet injection. The relative amount of CMFDA positive platelets at later time points were correlated to the baseline values and expressed in percent.

**Thrombin induced CD62P expression**

Blood was collected in heparinized capillary tubes from the retro-orbital vein plexa of anesthetized mice. The cells were added to a solution of PBS/EDTA 5 mM and PRP was obtained as previously described. Combined PRP fractions were centrifuged at 1200 g for 10 min and the platelets were washed in wash solution (PBS/NaN₃ 0.1 %/HSA 1 %). Platelets were then incubated with thrombin at different concentrations ranging from 0-1 U/L for 10 min, followed by fixation with 2% formaldehyde for 30 min at room temperature. After fixation, the platelets were washed in wash solution and incubated with an anti-CD62P-FITC or FITC-conjugated isotype control antibody for 30 min on ice. CD62P expression was then analyzed with flow cytometry.
Induction of platelet phosphatidylserine exposure

Phosphatidylserine (PS) exposure was induced and analyzed as previously described\textsuperscript{122}, with a few modifications. Blood was obtained by cardiac puncture and cells were transferred to BSGC without carbacyclin. Platelets were isolated by centrifugation as previously described, and resuspended in BSGC without carbacyclin to a concentration of $3 \times 10^8$/ml. The samples were then incubated at 37°C, and at relevant time points, 5 μl platelets were transferred to 500 μl annexin V binding buffer. To this was added 1 μl annexin V-FITC and the platelets were incubated for 10 min at room temperature before analysis by flow cytometry.

In Vitro platelet phagocytosis assay

\textit{In vitro} phagocytosis of platelets was preformed as previously described\textsuperscript{123;124}, with some modifications. Platelets were isolated and labeled with CMFDA as previously described. The cells were resuspended in BSGC to a concentration of $5 \times 10^8$/ml and incubated in dark for 30 min to remove excess CMFDA dye. Platelets were then centrifuged at 1200 g for 10 min and resuspended in Hanks balanced salt solution (HBSS) with carbacyclin 1 μg/ml. Opsonization was performed at room temperature for 20 min with mAb 6A6 at a concentration of 0.65 μg/ml. Platelets were then washed with HBSS and resuspended in DMEM/FCS 10 % to a concentration of $3 \times 10^8$/ml. Macrophages were incubated for 15 min with anti-SIRPα mAb P84 or isotype control mAb at a concentration of 75 μg/ml before platelets were added. Platelets were added at a concentration of $3 \times 10^7$/well and plates were centrifuged at 200 g for 1 min to make sure that the macrophages were in close contact with the platelets. The cells were then incubated for 30 min at 37°C in an atmosphere with 5 % CO\textsubscript{2}. The wells were then washed with HBSS to remove uningested platelets, followed by incubation for 5 min with 0.5 mM EDTA and 0.05 % trypsin at 37°C. Detachment of macrophages was done using PBS/EDTA 5 mM on ice. The cells were transferred to eppendorf tubes and centrifuged at 200 g for 5 min, followed by incubation with FcγR blocking mAb 2.4G2 (20 μg/ml) for 15 min on ice. Thereafter the cells were incubated for 20 min on ice with anti-CD61 mAb conjugated to PE. Phagocytosis was then assessed using flow cytometry.

Experimental ITP

Thrombocytopenia was induced by injection of mAb 6A6. Mice were anesthetized using isoflurane (Baxter) and intravenously injected in the lateral tailvein with 6A6 or
saline as a control. Progression was followed by blood sampling and cell count on Sysmex-KX21.

**Red blood cell survival**

Red blood cells were labeled with PKH26 or PKH67 as previously described. RBCs (350μl) were then intravenously injected in the lateral tailvein at a concentration of 2x10⁹/ml in experiments were only one phenotype was injected in respective recipient. This volume and concentration gives about 3 % PKH positive RBCs relative to total RBCs in circulation. Experiments were two phenotypes were injected in the same recipient, the RBCs from each phenotype was labeled with either PKH26 or PKH67 respectively. The cells were then mixed 1:1 and intravenously injected (350 μl) at a concentration of 3x10⁹/ml, yielding about 2 % positive RBCs from respective phenotype relative to total circulating RBCs. Clearance of RBCs was measured by flow cytometry on whole blood diluted in PBS.

**Red blood cell survival in combination with a pathogenic Ab**

RBCs were labeled with PKH26 or PKH67 and transfused to recipient mice as described above. Twenty four hours after the transfusion, recipient mice were intraperitoneally (i.p) injected with the anti-RBC mAb 34-3C at 1 or 3 μg/g bodyweight. Clearance of labeled RBCs was followed by flow cytometry and Hct was measured on Sysmex-KX21.

**In vitro oxidation of RBCs**

The procedure of creating *in vitro* oxidized RBCs has previously been described¹²⁵;¹²⁶. In brief, RBCs were washed with PBS and incubated at 4 % hct in PBS with 0.2 mM CuSO₄ and 5 mM ascorbic acid for 60 min at 37°C. After incubation, RBCs were washed and resuspended in the desired medium. In some experiments RBCs were opsonized with antibodies before addition to macrophages.

**Liposomes**

Preparation of unilamellar phospholipid liposomes has previously been described¹²⁷. In short, stock-solutions of cholesterol, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (PC), and 1-palmitoyl-2-oleyl-sn-glycero-3-phospho-L-serine (PS) in chloroform and
methanol (90:10), were mixed in disposable glass test tubes and evaporated under nitrogen. Lipids were then resuspended in DMEM and sonicated for 20 min. PC liposomes were mixed with cholesterol at an 80:20 molar ratio, and PS liposomes were mixed with PC and cholesterol at a 30:50:20 molar ratio. In experiments, 100 nmoles of lipids were used for each sample.

**Macrophage phagocytosis of oxidized RBCs in vitro**

Macrophages were either BMM or resident peritoneal macrophages. BMM were prepared as described above, and peritoneal macrophages were obtained by flushing the peritoneal cavity of euthanized mice with cold DMEM/10% FCS. Peritoneal cells were then sedimented by centrifugation at 1000 rpm for 5 min, and resuspended to 1-1.5x10⁶/ml in DMEM/10% FCS. Cells were plated in 4-well plates with 11 mm coverslips and incubated for 4 h at 37°C. The coverslips were then rinsed in PBS to remove non-adherent cells, followed by addition of 1x10⁷ RBCs/well. Macrophages and RBCs were incubated for 30 or 60 min, followed by washing with PBS and lysis of noningested RBCs with dH₂O. Cells were then fixed and stained with May-Grünewald and Gimsa.

**Microscopy of CD47 clustering on RBCs**

Distribution of CD47 on viable or oxidized RBCs was studied using laser scanning confocal microscopy. RBCs were incubated with an anti-CD47 antibody (mAb miap430) at room temperature for 30 min, followed by washing in PBS, and labeling with Cy-3 goat anti mouse IgG secondary antibody at 4°C for 30 min. This was followed by fixation in 3 % paraformaldehyde with 0.01 % glutaraldehyde for 30 min at room temperature. RBCs were then adhered to coverslips coated with poly-L-lysine and mounted in Vectashield.
Aims

➢ To investigate if CD47 is involved in regulating platelet turnover.

➢ To investigate if CD47/SIRPα-signaling can negatively regulate FcγR-mediated phagocytosis of platelets.

➢ To investigate if the amount of cell surface CD47 affects CD47/SIRPα-signaling.

➢ To study the effect of CD47 on phagocytosis of experimentally senescent RBCs.

➢ To find out whether inhibitory CD47/SIRPα-signaling has a local or more general distribution in the macrophage.
Results

(In the following section, figure references refer to the figures in the respective paper (I-IV))

Paper I

CD47⁻/⁻ mice are thrombocytopenic

Initial experiments to determine platelet counts in our mice revealed that CD47⁻/⁻ mice were to some extent thrombocytopenic. It is known that male mice normally have slightly higher platelet counts than female mice. Experiments confirmed, that male and female CD47⁻/⁻ mice had statistically significant lower platelet counts, as compared with their wild-type or heterozygous littermates of the same gender (Fig. 2). These data were consistent in CD47⁻/⁻ mice on both the Balb/c and the C57BL/6J background.

Platelet clearance

When mice were transfused with platelets from their own respective genotype, all mice exhibited the same rate of clearance, and nearly all platelets were cleared within 96 h (Fig. 3A). However, when CD47⁻/⁻ platelets were transfused into CD47⁺/+ mice, they were cleared within 4 h (Fig. 3B). Transfusion of CD47⁺/+ platelets into CD47⁻/⁻ or CD47⁺/+ recipients, or CD47⁺/+ platelets into CD47⁻/⁻ recipients showed no significant difference from the normal rate of clearance.

In Vitro aging or activation of platelets

Because CD47⁻/⁻ platelets were more rapidly cleared from the circulation of wild-type recipients, one may argue that CD47⁺/+ platelets could more readily become activated, or that they more easily become senescent. To address these questions, we activated platelets using thrombin at different concentrations, followed by analysis of cell surface P-selectin (CD62P) upregulation. We also aged platelets in vitro by incubating PRP at 37°C followed by measurement of PS-exposure at different time points. There were no differences in CD62P-expression or PS-exposure on freshly isolated platelets, or at any time points following in vitro treatment, when platelets from CD47⁻/⁻ and CD47⁺/+ mice were compared (Fig. 4A-C).
Experimental ITP and FcγR-mediated phagocytosis

To investigate if CD47/SIRPα-signaling is important in regulating platelet uptake in ITP, we injected a pathogenic anti-platelet antibody (mAb 6A6) at different concentrations in CD47+/+ or CD47−/− mice. Mice lacking CD47−/− were significantly more sensitive to ITP, as compared with CD47+/+ mice injected with the same amount of antibody. The experiments were performed in both Balb/c and C57BL/6J mice, and the relative difference between CD47+/+ and CD47−/− mice were the same for the two mouse strains (Fig. 5 B-C).

To confirm the role of CD47/SIRPα-signaling in regulating FcγR-mediated phagocytosis of platelets, we performed in vitro platelet phagocytosis experiments, where the functional blocking anti-SIRPα mAb P84 was used to block the CD47/SIRPα-interaction. In the absence of blocking antibody, or in the presence of an isotype control antibody, we found that CD47−/− platelets were phagocytosed to a significantly higher extent than CD47+/+ platelets. However, when SIRPα was blocked, phagocytosis of CD47+/+ platelets was increased to the level seen with CD47−/− platelets. The blocking antibody against SIRPα did not affect phagocytosis of CD47−/− platelets (Fig. 6A).

Gene-dose effect of CD47/SIRPα-signaling

CD47 expression was found to be reduced by 45% on platelets from CD47+/− mice, as compared with wild-type mice (Fig. 1). Interestingly, the in vitro experiments showed that CD47+/− platelets were phagocytosed to a lesser extent than CD47−/− platelets, but considerably more than CD47+/+ platelets (Fig. 6A). In vivo experiments showed that CD47+/+ and CD47+/− platelets injected in CD47+/+ recipients had the same half-life in circulation. However, when mice in the same experimental setup were also injected with mAb 6A6, CD47+/− platelets were much more rapidly cleared, as compared with CD47+/+ platelets (Fig. 6B).

Paper II

CD47 expression on RBCs

RBC expression of CD47 was measured using flow cytometry. Experiments, using RBCs from a cohort of CD47+/+ and CD47+/− mice, revealed that CD47+/− mice expressed 46.5±1.0% of the CD47-levels found on CD47+/+ RBCs (Fig. 1).
In Vitro phagocytosis

Opsonized RBCs were added to bone marrow derived macrophages and incubated for 30 min at 37°C. The phagocytosis index was significantly higher for CD47−/− RBCs, as compared with equally opsonized CD47+/+ RBCs. Furthermore, CD47+/− RBCs were ingested to a higher extent than CD47−/− RBCs, but to a lower extent than CD47+/+ RBCs, which indicates that the amount of CD47 expressed by cells can regulate phagocytosis in a gene-dose-dependent manner (Fig. 4).

RBC survival

Previous studies showed that CD47−/− RBCs are rapidly cleared when injected in CD47+/+ recipients. Here we investigated if CD47+/− RBCs circulated normally in CD47+/+ recipients. A 50/50 mixture of CD47+/+ and CD47+/− RBCs were labeled green or red, respectively, and intravenously injected into CD47+/+ recipients. Clearance was followed for 7 days, showing no difference between CD47+/+ and CD47+/− RBCs in CD47+/+ recipients (Fig. 2). To investigate the effects of an increased prophagocytic signaling on clearance of CD47+/− RBCs in CD47+/+ recipients, we injected transfused mice with the pathogenic antibody 34-3C at two different concentrations. Hematocrit (hct) and clearance rate were followed daily for 7 days. Mice injected with either a high (3 μg/g body weight) or low concentration (1 μg/g) of antibody all showed an accelerated clearance of CD47+/− RBCs compared to CD47+/+ RBCs (Fig. 3B and D). The hct dropped 22 % in mice injected with the low concentration, and 44 % in mice injected with the high dose of 34-3C (Fig. 3A and C).

Paper III

Phagocytosis of ox-RBCs is dependent on serum, scavenger receptors and PS expression

Oxidation of RBC cell membrane lipids occurs in vivo and has been proposed as one of many pro-phagocytic stimuli for senescent RBCs58. Several studies have used in vitro oxidized RBCs to study phagocytosis of ox-RBCs125,129,130. To test the role of CD47-SIRPα signaling in uptake of ox-RBCs, we used in vitro oxidized RBCs from CD47+/+ or CD47−/− mice. After oxidation, both CD47+/+ and CD47−/− RBCs expressed intermediate and equal amounts of PS (Fig. 3B, C). When ox-RBCs were added to macrophages, we found that ox-RBCs were readily phagocytosed (Fig. 1B-C). Previous studies showed that uptake of ox-RBCs required serum proteins131, which we confirmed (Fig. 1D).
Dextran sulfate, fucoidan, and OxLDL are known ligands for certain scavenger receptors that have been proposed to be involved in uptake of ox-RBCs. In our experiments we found that both dextran sulfate and fucoidan effectively inhibited phagocytosis in a dose-dependent manner (Fig. 1E-F). To study the effect of PS in phagocytosis of ox-RBCs, we used PS-liposomes. Treatment with PS-liposomes resulted in almost complete inhibition of ox-RBC uptake, whereas treatment with phosphatidylcholine liposomes did not have an effect (Fig. 1G). Thus, uptake of ox-RBCs is dependent on recognition by scavenger receptors, and requires both serum and recognition of PS.

**Oxidized RBCs have reduce mobility of CD47 in the plasma membrane**

CD47 on mouse RBCs is freely mobile in the plasma membrane. Labeling CD47 with the anti-CD47 antibody miap430 resulted in clustering of CD47 on viable RBCs. Clustering of CD47 has been proposed to be important in mediating CD47/SIRPα-signaling. When labeling CD47 on ox-RBCs, CD47 did not cluster at the same high level, as compared with viable cells. This indicates that CD47 has a reduced mobility in the plasma-membrane of in vitro oxidized RBCs (Fig. 2).

**Effects of CD47 on phagocytosis of ox-RBCs**

The CD47/SIRPα-interaction can negatively regulate FcγR and complement receptor-mediated phagocytosis. Here we investigated the role of CD47-SIRPα signaling in uptake of unopsonized in vitro oxidized RBCs. We found that ox-RBCs from CD47^+/− and CD47^-/- mice were equally phagocytosed in the absence of opsonization (Fig. 3D). Similar results were obtained when inducing RBC PS-exposure using the Ca^{2+} ionophore A23187 (Fig. 3E).

When oxidizing RBCs, there is a possibility that changes occur in the protein structure of CD47, and thereby preventing effective binding to SIRPα. To investigate if CD47 was still able to bind and signal via SIRPα, we opsonized ox-RBCs with the antibody 34-3C, before adding them to the macrophages. These experiments showed, that CD47^-/- ox-RBCs were ingested to a much higher extent than equally opsonized CD47^+/− ox-RBCs (Fig. 4). In paper I and II we showed that CD47 dose-dependently negatively regulate FcR-mediated phagocytosis of platelets and RBCs. We found that cells from CD47^-/- mice were taken up to a lesser extent than CD47^-/- cells but more
than CD47\(^{+/+}\) cells. In similar experiments with opsonized ox-RBCs we obtained virtually the same results (Fig. 5).

**Paper IV**

**Discrimination between wild-type and CD47\(^{-/-}\) RBCs during Fc\(\gamma\)R-mediated phagocytosis in macrophages**

To investigate to what extent the macrophage is spatially inhibited by CD47/SIRP\(\alpha\)-signaling, we used equally opsonized CD47\(^{+/+}\) and CD47\(^{-/-}\) RBCs labeled with PKH26 and PKH67, respectively. CD47\(^{+/+}\) and CD47\(^{-/-}\) RBCs were mixed 1:1, and were allowed to bind to macrophages. Macrophages were then predicted to be in contact with CD47\(^{+/+}\) and CD47\(^{-/-}\) RBCs to the same extent. CD47\(^{-/-}\) RBCs were found to be ingested to a much higher extent, as compared with CD47\(^{+/+}\) RBCs (Fig. 3). The levels of phagocytosis of RBCs of the respective genotype were comparable to that when they were given separately to macrophages (Fig. 2). These results show that CD47/SIRP\(\alpha\)-inhibition of FC\(\gamma\)R-mediated phagocytosis occurs locally at the phagocytic synapse with a high degree of spatial resolution. Experiments with phagocytic cup formation showed that F-actin polymerization and phagocytic cups were almost exclusively formed at binding sites of CD47\(^{-/-}\) RBCs, but only rarely where CD47\(^{+/+}\) RBCs were bound to the macrophages (Fig. 4). Furthermore, specific analysis of F-actin polymerization at contact sites with CD47\(^{-/-}\) RBCs having CD47\(^{+/+}\) RBCs in close proximity, showed that adjacent CD47\(^{+/+}\) RBCs did not affect the polymerization of F-actin at CD47\(^{-/-}\) RBC contacts (Fig. 4).
Discussion

Paper I: Experimental ITP and macrophage phagocytosis of platelets

In this paper, we show that the CD47/SIRPα-interaction can regulate normal platelet turn-over \textit{in vivo} and that it also negatively regulates FcγR-mediated phagocytosis of antibody opsonized platelets both \textit{in vivo} and \textit{in vitro}.

Previous studies have shown that CD47 on RBCs is important in regulating RBC clearance. This is due to the fact that CD47 on RBCs bind SIRPα on macrophages, which generates a phagocytosis-inhibitory signal. RBCs missing CD47 are rapidly cleared from the circulation by splenic red pulp macrophages\textsuperscript{15}. In paper I, we extended those studies to platelets, and investigated if platelet clearance and phagocytosis was also regulated by CD47 on the platelets. We performed transfusion experiments, where platelets from CD47\textsuperscript{+/+} or CD47\textsuperscript{-/-} mice were injected into CD47\textsuperscript{+/+} mice. Platelets that lacked CD47 were cleared from the circulation within four hours, whereas CD47\textsuperscript{+/+} platelets had a normal half-life of about 34 hours. CD47\textsuperscript{-/-} or CD47\textsuperscript{+/+} platelets injected in CD47\textsuperscript{-/-} recipient mice showed normal clearance from the circulation. These results are therefore strengthening the hypothesis that the CD47/SIRPα-interaction is important to regulate clearance of all circulating blood cells, not only RBCs.

Mice that lack CD47 have the same amount of circulating RBC as CD47\textsuperscript{+/+} mice\textsuperscript{115}. In contrast, we found that platelet levels were slightly lower in CD47\textsuperscript{-/-} mice, as compared with CD47\textsuperscript{+/+} mice. In agreement with our findings, it was reported that SIRPα mutant mice (in which the extracellular domain of SIRPα is expressed, but the intracellular domain is truncated and non-signaling) also had reduced numbers of circulating platelets\textsuperscript{134}. This reduction was explained by an increased consumption of these cells. However, we could not explain the reduced numbers of platelets in our CD47\textsuperscript{-/-} mice with an increased uptake. P-selectin and PS have been suggested as candidates involved in regulating platelet clearance, but only PS has been shown to decrease survival of platelets\textsuperscript{135}. We could neither detect higher baseline levels of PS on circulating CD47\textsuperscript{-/-} platelets, nor any difference in the increased amounts of PS when platelets were aged \textit{in vitro}, as compared to CD47\textsuperscript{+/+} platelets. Furthermore, stimulation of platelets with thrombin to induce P-selectin expression did not reveal any difference between CD47\textsuperscript{+/+} and CD47\textsuperscript{-/-} platelets. Thus, the discrepancy between SIRPα-mutant mice and CD47\textsuperscript{-/-} mice, in terms of reduced platelet counts in relation to half-life of transfused platelets, may be due to different methods for measuring
clearance. We used platelet activation inhibitors (carbacyclin and theophylline) when platelets were labeled before injection. This may result in an long lasting effect that still persist in transfused platelets, and thereby making them less prone to activation and/or clearance. This possibility could explain why we were unable to detect an increased platelet turn-over despite lower numbers of circulating platelets in CD47−/− mice. On the other hand, despite the use of these inhibitors for preparation of platelets, CD47−/− platelets were rapidly cleared in CD47+/+ recipients. Thus, the exact mechanisms behind the mild thrombocytopenia in CD47−/− mice needs to be further investigated.

Experimental AIHA can be induced by injection of anti-mouse-RBC antibodies of both IgG1 and IgG2 isotypes100. In that model, mice lacking CD47 proved to be much more sensitive to AIHA, as compared with CD47+/+ mice, regardless of Ab isotype115. Those results showed that CD47/SIRPα-signaling can regulate FcγR mediated phagocytosis both in vivo and in vitro. We hypothesized that this would also be true for platelets. Indeed, by using an established model for experimental ITP, we found that CD47−/− mice were more sensitive to experimental ITP, as compared with their CD47+/+ counterparts. To verify that CD47 on platelets protects against FcγR-mediated phagocytosis by binding to SIRPα on macrophages, we performed in vitro phagocytosis experiments were SIRPα on macrophages was blocked with the functional blocking mAb P84. When SIRPα was blocked, CD47+/+ platelets were ingested to the same levels as CD47−/− platelets, which confirmed that CD47α/SIRPα-signaling is able to regulate FcγR-mediated platelet phagocytosis. Block of SIRPα did not affect phagocytosis of CD47−/− platelets.

In early studies, it was suggested that the IgG2a mAb 6A6, used for experimental ITP in vivo, and to opsonize platelets for in vitro phagocytosis experiments, mediates phagocytosis by binding to macrophage FcγRIII136. However, more recent studies have shown that IgG2a stimulates phagocytosis through the FcγRIV receptor137. This FcγRIV receptor proved to bind IgG2a with a 40-fold higher affinity than FcγRIII. It was therefore likely that RBCs opsonized with IgG2a antibodies also triggers a prophagocytic signal by binding FcγRIV. However, an even more recent study showed that involvement of different FcγRs in AIHA is depended on the level of IgG2a opsonization. Low opsonization levels of IgG2a proved to be dependent on only FcγRIII, while high doses of IgG2a involved FcγRIII with contribution of FcγRI and FcγRIV100.

Interestingly, we found that IgG-opsonized CD47+/− platelets, which express about 55% of normal CD47-levels, were ingested significantly more than equally opsonized CD47+/+ platelets, but significantly less than CD47−/− platelets. These results show that CD47 can regulate FcγR mediated phagocytosis in a gene-dose-dependent manner.
However, we did not detect accelerated clearance of CD47\(^{++/--}\) platelets \textit{in vivo}, as compared to CD47\(^{++/+}\) platelets, when platelets were transfused into CD47\(^{++/+}\) mice. In contrast, when CD47\(^{++/+}\) mice were transfused with both CD47\(^{++/--}\) and CD47\(^{++/+}\) platelets, followed by an injection of the anti-platelet mAb 6A6, CD47\(^{++/--}\) platelets were cleared much faster than CD47\(^{++/+}\) platelets. The reason for normal half-life of CD47\(^{++/--}\) in circulation may be due to phagocytosis-inhibiting structures on the cell surface, like CD47. These inhibiting structures could possibly create a threshold that must be overcome by prophagocytic signals for phagocytosis to take place. The mechanisms involved in clearance of circulating platelets from the circulation are mostly unknown, but the sum of these prophagocytic signals may be weak enough for CD47 on CD47\(^{++/--}\) cells to suffice in balancing the phagocytosis signal. However, when an anti-platelet antibody is injected, prophagocytic signaling is stronger, and the balance is shifted in favor of phagocytosis. Thus, it is reasonable to assume that CD47\(^{++/--}\) platelets expressing lower levels of CD47 can no longer present an inhibitory signal strong enough to prevent phagocytosis when the prophagocytic signals reaches a certain threshold.

**Paper II: Gene-dose effect of CD47 in regulation of Fc\(\gamma\)R-mediated phagocytosis**

In this paper, we show that a 55% reduction of the CD47-level on RBCs does not affect normal half-life of circulating RBCs, but becomes important in the presence of only low levels of anti-RBC antibodies \textit{in vivo} and \textit{in vitro}.

To better predict the outcome of blood transfusions, it is important to identify structures on RBCs that can inhibit RBC clearance. A low molecular weight phagocytosis-inhibitory factor (PIF) has been described, which can inhibit uptake of RBCs in an unspecific manner. PIF inhibits both Fc\(\gamma\)R and complement receptor mediated phagocytosis\(^{138}\). CD47 has been shown to act as a marker of self, and be able to regulate Fc\(\gamma\)R mediated phagocytosis of opsonized RBCs\(^{15;16}\). There are two studies showing that the levels of CD47 is continuously reduced over time on RBCs stored for transfusion\(^{81;82}\). Annis \textit{et al}. showed a CD47 reduction of ~10%\(^{81}\), whereas Stewart \textit{et al}. showed a CD47 loss of ~50%, during storage for more than 28 days\(^{82}\). These differences may be due to different storage methods or different assays to measure CD47. Despite the discrepancy between these two studies, with regard to what actual amount of CD47 that is lost during storage, both studies show that it is likely that CD47-levels might be reduced on stored RBCs.

The fact that RBCs from CD47\(^{++/--}\) mice express about 55% less CD47, as compared to RBCs from CD47\(^{++/+}\) mice, makes cells from CD47\(^{++/--}\) mice a good model to study what
effects reduced levels of CD47 have on transfused RBCs. A 50% reduction of CD47 did not result in a different half-life, as compared to CD47+/+ RBCs, when CD47+/− RBCs were transfused into CD47+/+ recipients. However, when the mice in the same experiment were injected with a pathogenic antibody against RBCs, CD47+/− RBCs were cleared more rapidly than CD47+/+ RBCs. The effects were most pronounced in experiments were the mice were given a low dose of antibody, resulting in only 22% drop in hct. In vitro phagocytosis experiments yielded similar results, showing that RBCs from CD47+/− mice were ingested to a higher extent than CD47+/+ RBCs, but less than CD47+/− RBCs.

Storage, or natural aging of RBCs, result in several changes to the cells, which will finally lead to their uptake by macrophages58;75;139. Such changes may take place in the plasma membrane, the biochemical environment inside the cell, and may result in opsonization by naturally occurring antibodies. When all these changes add up, they present strong prophagocytic stimuli for the macrophages75, that may override any inhibitory effects of molecules such as CD47 or PIF. Even though we could not detect a reduced circulation time for CD47+/− RBCs transfused into CD47+/+ mice over a seven day period, it is still possible that the amount of CD47 may be important when prophagocytic stimuli, like binding of auto antibodies, increases. More CD47 on the cell surface may then keep the blood cells in circulation for a longer time.

**Paper III: Role of CD47 in phagocytosis of oxidized RBCs**

In this paper, we studied phagocytosis of in vitro oxidized RBCs, and found that CD47 did not affect the serum-dependent phagocytosis of oxidized RBCs in the absence of IgG-opsonization, but could still potently inhibit FcγR-mediated phagocytosis.

As mentioned above, senescence of RBCs has been associated with several biochemical changes to the cells, such as increased cell density, intracellular changes, membrane oxidation, PS exposure, or formation of neoantigens able to bind naturally occurring antibodies72. The exact mechanisms for uptake of senescent RBCs are not yet fully understood, but probably rely on accumulation of several prophagocytic stimuli that finally triggers phagocytosis. As long as phagocytes detect self markers (like sialic acid or CD47) on RBCs, the cells are spared. However, when prophagocytic signals accumulate over time, or if inhibitory structures on RBCs are lost, it will eventually result in phagocytosis72. Some of the changes that occur in the RBC plasma-membrane may be directly recognized by macrophages, others may depend on indirect binding bridged by plasma proteins125;126;130;131;140;141. Oxidation of RBCs is believed to induce prophagocytic changes on the RBCs62;63. Lipid oxidation increases during RBC aging58;142, and RBCs oxidized in vitro (ox-RBC) are readily
taken up by macrophages\textsuperscript{58}. Recognition of ox-RBC is believed to be mediated, at least in part, by scavenger receptors. When blocking these receptors with OxLDL, fucoidan or dextran sulfate, phagocytosis is reduced. In our study, we found that RBCs oxidized \textit{in vitro} were efficiently phagocytosed by macrophages without further opsonization. However, this uptake was strongly dependent on serum, required recognition of PS on the RBCs, and involved recognition by macrophage scavenger receptors, since dextran sulfate or fucoidan could block phagocytosis. Interestingly, despite the fact that CD47 on viable RBCs can strongly inhibit RBC phagocytosis by splenic red pulp macrophages\textsuperscript{15}, and complement receptor or Fc\textgamma R-mediated phagocytosis\textsuperscript{16}, we found that CD47\textsuperscript{+/+} and CD47\textsuperscript{−/−} ox-RBC were taken up to the same extent by the macrophages.

The phagocytosis-inhibitory effect of CD47 has been suggested to be dependent on the ability of CD47 to stay mobile in the RBC plasma membrane, to allow for efficient clustering at the phagocytic synapse\textsuperscript{38,133}. We could show that clusters of CD47 were readily formed on viable RBCs following cross linking with antibodies, but that this was less evident on ox-RBCs. This could explain why the inhibitory effects of CD47 was absent in uptake of ox-RBCs. However, when oxidized CD47\textsuperscript{+/+} or CD47\textsuperscript{−/−} were IgG-opsonized, CD47\textsuperscript{−/−} RBCs were phagocytosed significantly more than CD47\textsuperscript{+/+} RBCs, suggesting that inhibitory CD47/SIRP\textalpha-signaling is still intact, despite the fact that CD47 is less mobile in the cell membrane of ox-RBCs.

Our data, showing that in \textit{in vitro} uptake of ox-RBCs is dependent on serum is in agreement with previous studies\textsuperscript{131}. Serum may contain both complement and antibodies, which can induce prophagocytic signals and increase the uptake of ox-RBCs. However, in our experiments we used heat-inactivated serum, so complement effects could not be involved. Furthermore, our experiments showed that CD47\textsuperscript{+/+} ox-RBCs and CD47\textsuperscript{−/−} ox-RBCs were ingested equally in the absence of opsonization. Since CD47 on the RBCs can strongly negatively regulate Fc\textgamma R-mediated phagocytosis, but was without effect on serum-dependent phagocytosis of ox-RBCs, it is reasonable to assume that antibodies in serum were not involved.

Studies in mice showed that circulating RBCs lose CD47 with increasing age\textsuperscript{79,80}. Other reports showed that CD47 is lost on RBCs stored to be used in transfusions\textsuperscript{81,82}. Thus, a reduced level of CD47 in those conditions was suggested to facilitate clearance of aged or stored RBCs. However, the present study shows that the inhibitory effect of CD47 is dependent on what kind of prophagocytic recognition that is established between the RBC and the macrophage. Never the less, it cannot be ruled out that CD47 play a role in clearance of senescent RBCs. Naturally occurring antibodies have been shown to bind to aged RBCs. These antibodies would induce a prophagocytic signals via Fc\textgamma Rs, which we know that the CD47/SIRP\textalpha-interaction can inhibit.
Furthermore, these naturally occurring antibodies have been associated with increased deposition of C3bi on the aged RBC cell surface. C3bi triggers phagocytosis by binding complement receptors, which are also known to be negatively regulated by CD47/SIRPα-signaling. In conclusion, it is likely that removal of senescent RBCs is dependent on several stimuli that add up over time. The CD47 contribution in clearance of aged RBCs depends on to the exact interaction with the phagocytes, which will then be more or less affected by inhibitory signals from the CD47/SIRPα-interaction.

**Paper IV: Spatial resolution of the inhibitory CD47/SIRPα-interaction**

In this paper, we asked whether ligation of macrophage SIRPα by CD47 on a target cell induced a local inhibition of phagocytosis, or a more general inhibition of phagocytosis in the whole macrophage, and found that the inhibitory response was spatially limited to the phagocytic synapse.

It is a well known fact that CD47 can bind SIRPα on macrophages and thereby inhibit the macrophage. However, little is known to what extent the macrophage is inhibited. The macrophage could be generally inactivated, or it could be locally inhibited at the phagocytic synapse. A general inactivation would result in inhibition of the whole cell, regardless of where on the macrophage the SIRPα signal is initiated. On the other hand, if it is a local inhibition, the effects will only occur adjacent to the binding site were CD47 binds SIRPα. To test this, a 1:1 mixture of IgG-opsonized CD47+/+ and CD47−/− RBCs was allowed to bind to the macrophages, followed by incubation at 37°C to initiate phagocytosis. This resulted in a much higher phagocytosis of CD47−/− RBCs, as compared to CD47+/+ RBCs. In addition, the enhanced uptake of CD47−/− RBCs correlated well with experiments where CD47+/+ or CD47−/− RBCs were given to macrophages separately. This suggests that the inhibitory signal is indeed located to a very close proximity of the binding site between the macrophage and the target cell.

Formation of a phagocytic cup, when the macrophage cell membrane starts to surround the cell that is to be ingested, is one of the first events proceeding phagocytosis of an IgG-opsonized particle bound to a phagocyte. The movement of the plasma membrane is achieved by polymerization of F-actin. By terminating phagocytosis after just a few minutes, we identified phagocytic cups and found that the cups were almost exclusively formed were the macrophages were in contact with CD47−/− RBCs, but rarely upon contact with CD47+/+ RBCs. In addition, the presence of CD47+/+ RBCs in close proximity of a CD47−/− RBC did not affect the formation of a
phagocytic cup just beneath that cell. This strongly suggests that CD47/SIRPα-signaling does not spread throughout the whole macrophage, but is confined to the phagocytic synapse.
Conclusions

- Platelet turnover is regulated by CD47. Furthermore, inhibitory CD47/SIRPα-signaling can regulate Fcγ-mediated uptake of platelets in a dose dependent manner both in vivo and in vitro.

- CD47 on RBCs can dose-dependently inhibit FcγR-mediated phagocytosis.

- Oxidized RBCs are readily engulfed by macrophages. Inhibitory signals from the CD47/SIRPα-interaction may or may not have an effect on the macrophage receptors engaged in phagocytosis of oxidized RBCs. The FcγR activating signals are inhibited, while receptors involved in mediating serum-dependent uptake of oxidized RBCs are not inhibited.

- Macrophage inhibition by CD47/SIRPα signaling is spatially localized to the binding site of the target cell that is recognized by FcγR, without affecting the phagocytosis capacity of target particles lacking CD47 in close proximity at the surface of the same macrophage.
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