Doctoral Thesis

Naturally occurring anti-C3 antibodies and framework-specific anti-idiotypes prolong the life span of erythrocytes from healthy Coombs-positive blood donors

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Naturally occurring anti-C3 antibodies and framework-specific anti-idiotypes prolong the life span of erythrocytes from healthy Coombs-positive blood donors

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2 Summary

Over the years many blood donation and transfusion centers have found with a prevalence of one in 5000 to 10000 healthy individuals, who were tested positive in the Coombs test. These individuals, called “healthy Coombs-positive blood donors” have red blood cells (RBCs) carrying 2-5 times more IgG than normal senescent RBC. Despite the pathological number of RBC-bound IgG naturally occurring antibodies (NAbs), these Coombs-positive blood donors do not show signs of autoimmune hemolytic anemia. Nevertheless, they are excluded from blood donation. We studied why these RBCs continue to circulate, while normal senescent RBCs with fewer IgG per cell, are cleared from the circulation.

Senescent RBCs from healthy Coombs-positive blood donors carried extra amounts of IgG molecules as compared to old control RBCs. The extra amounts of IgG comprised anti-C3 NAbs and their corresponding framework-specific anti-idiotypes. The density distribution of RBCs from these rare blood donors differed from that of controls with some RBCs having even higher densities than that of the densest cells of controls. A measure of the absolute cell age revealed that these very dense RBCs from healthy Coombs-positive blood donors were even older than the oldest control RBCs, suggesting that they circulate as “overaged” cells. This anomalous behaviour is likely due to the fact that the plasma of healthy Coombs-positive blood donors contained high concentrations of anti-C3/C3b and even higher concentrations of anti-idiotypic NAbs, which associated with C3b or iC3b dimers or C3b2-IgG/i C3b2-IgG complexes on aged RBCs and prevented the interactions of RBC-bound C3b/iC3b with their receptors on phagocytic cells. Correspondingly, senescent RBCs from healthy Coombs-positive
donors were more readily phagocytosed in vitro than those from controls, because the
former RBC carried overall more IgG NAbs than aged control RBCs. On the contrary,
physiological concentrations of whole human IgG, which competitively inhibit Fc-
receptor mediated phagocytosis, lowered the ingestion of old Coombs-positive RBC to
the level or below the level of that observed for senescent control RBCs. Hence, anti-
C3 and corresponding anti-idiotypic NAbs down regulate the phagocytosis of RBC
from healthy Coombs-positive blood donors and thereby prolonge their life span.
Negli anni, diversi centri di donazione e trasfusione del sangue hanno trovato, con una prevalenza di uno su 5000-10000, individui sani, ma positivi al test di Coombs. Questi individui, definiti "sani donatori di sangue positivi al test di Coombs", hanno eritrociti sulla cui superficie portano da 2 a 5 più IgG rispetto a normali senescenti eritrociti. Nonostante il numero patologico di IgG legati agli eritrociti, questi sani donatori di sangue positivi al test di Coombs non presentano alcun segno di anemia emolitica autoimmune. Tuttavia, questi donatori vengono esclusi dal donare il proprio sangue. Noi abbiamo studiato il perché questi eritrociti continuano a circolare, mentre i normali vecchi eritrociti con pochi IgG per cellula scompaiono dalla circolazione sanguigna. I senescenti eritrociti dei sani donatori di sangue positivi al test di Coombs avevano legati una quantità extra di IgG rispetto a normali senescenti eritrociti. Questa quantità extra consisteva in anti-C3 e il suo corrispondente anti-idiotipo specifico della struttura. La distribuzione secondo la densità degli eritrociti provenienti dai sani donatori di sangue positivi al test di Coombs differiva da quella dei controlli avendo alcuni eritrociti con una densità maggiore rispetto alle cellule più dense dei controlli. La misura dell'età cellulare assoluta ha rivelato che questi eritrociti densi dei sani donatori di sangue positivi al test di Coombs erano anche più vecchi degli eritrociti più vecchi dei controlli. Questo aspetto suggerisce che essi circolano come cellule invecchiate in eccedenza. Questo comportamento anomalo è probabilmente legato al fatto che il plasma di questi sani donatori di sangue positivi al test di Coombs conteneva concentrazioni elevate di anti-C3/C3b e concentrazioni ancor più elevate di anti-idiotipi,
i quali associano con dimeri C3b o iC3b e con C3b2-IgG complessi sugli eritrociti vecchi e prevengono le interazioni di C3b/iC3b legati agli eritrociti con i loro recettori esposti sui fagociti. Contemporaneamente, gli eritrociti senescenti dei sani donatori di sangue positivi al test di Coombs erano maggiormente fagocitati di quelli dei controlli, poiché i primi avevano più IgG sulla loro superficie rispetto agli eritrociti senescenti dei controlli. Al contrario, concentrazioni fisiologiche di IgG, che inibiscono competitivamente la fagocitosi mediata dai recettori Fc, abbassano l’ingestione dei vecchi eritrociti dei sani donatori di sangue positivi al test di Coombs fino al livello o addirittura al di sotto del livello di quello osservato per i vecchi eritrociti dei controlli. Pertanto, anti C3 e il suo corrispettivo anti-idiotipo abbassano la fagocitosi di eritrociti dei sani donatori di sangue positivi al test di Coombs prolungandone la loro vita.
4 Introduction

This thesis deals with erythrocytes from rare healthy individuals carrying IgG complexes that inhibit complement-mediated erythrophagocytosis. The introductory part will focus first on erythrocytes and their membrane. The modifications occurring during aging will then be described with particular emphasis on band 3 alterations that are followed by the binding of naturally occurring autoantibodies and complement to old cells. The second part will review what is known about phagocytosis of senescent erythrocytes with regard to IgG and complement receptors and its modulation by CD47-SIRP interactions. Finally, some properties of the individuals studied in this project will be described in some details.

4.1 Erythrocyte

Erythrocytes are the most abundant blood cells, which are generated in the bone marrow from the pluripotent hematopoietic stem cells through several stages of maturation. During its development the immature erythrocyte extrudes the nucleus and then leaves the bone marrow as a reticulocyte. The circulating reticulocyte loses the mitochondria and ribosomes within two days and becomes a mature red blood cell (RBC). The major role of this highly specialized mature cell is to transport oxygen and carbon dioxide between the lungs and tissues (reviewed by Nikinmaa, 1997). Additionally, red cells play an important role in the immunologic defense by capturing C3b-opsonized immune complexes by their complement receptor 1 (CR1) and transferring them to the reticuloendothelial system. The number of circulating RBCs is tightly controlled by the
hormone erythropoietin, which is produced in response to low tissue oxygenation (reviewed by Koury, 2005). Mature erythrocytes have a finite life span of 120 days, in which they undergo an aging process characterized by many chemical and enzymatic modifications that occur at the membrane level (reviewed by Clark, 1988; Lutz, 2004). These changes are thought to culminate in a signal, which leads to erythrocyte elimination. In fact, at the end of their life span, red cells are selectively removed from the blood circulation by the reticuloendothelial system.

4.1.1

4.1.2 RBC membrane

The RBC membrane is a composite material of two coupled structures. The outer element is the lipid bilayer containing membrane-spanning proteins, predominantly glycophorins and band 3. Bridging proteins, such as ankyrin and protein 4.2, connect the outer structure to the inner portion, the skeleton. The latter consists of a two dimensional scaffold that comprises primarily spectrin, protein 4.1, and actin (Davis and Bennett, 1990; Van Dort et al., 2001).

Band 3 protein, also known as anion exchange protein 1 (AE1), exchanges \( \text{HCO}_3^- \) for \( \text{Cl}^- \). This protein, which exists as dimers, tetramers and oligomers, accounts for 30% of the total membrane proteins (10^6 copies per cell) and is the major integral membrane protein (reviewed by Tanner, 2002). Band 3 is a glycoprotein of 95 KDa comprised of two domains, a 55 KDa membrane-spanning domain and a cytoplasmic domain. The former traverses the lipid bilayer 12 times and forms the anion exchanger. The 55 KDa domain, furthermore, carries one N-glycosidically linked carbohydrate (Drickamer, 1978; Fukuda and Fukuda, 1981). The cytoplasmic domain is responsible
for the anchorage of the RBC membrane to the cytoskeleton. Indeed, band 3 has binding sites for ankyrin (Bennett and Stenbuck, 1980), protein 4.2 (Cohen et al., 1993; Rybicki et al., 1996), and protein 4.1 (An et al., 1996; Pasternack et al., 1985), which interact with the underlying skeleton. Additionally, band 3 protein regulates the glycolytic enzyme complex assembly on the erythrocyte membrane through tyrosine phosphorylation of the band 3 N-terminal region (Campanella et al., 2005). All these interactions turn band 3 into the organizing center of the RBC membrane. Furthermore, recent studies suggest that band 3 proteins form the central element of a macrocomplex of integral and peripheral proteins, called “metabolon”, in which band 3 may be linked to the Rh complex either directly or through the interaction of protein 4.2 with CD47 (Bruce et al., 2003; Bruce et al., 2002) (Fig. 1). This metabolon may function as an integrated gas exchanger, whereby the diffusion distance of all components is reduced.

The Rh complex is composed of a core of two Rh components, two RhAG subunits (Eyers et al., 1994) and the accessory proteins CD47, LW and GPB (Fig. 1). The Rh complex is supposed to facilitate the CH₃NH₂/NH₃ transport in RBCs (Ripoche et al., 2004). Since this complex associates with band 3 complex also through the CD47-protein 4.2 connection, band 3 deficiency results in a total absence of CD47 (Bruce et al., 2003). Furthermore, protein 4.2-deficient RBCs express less CD47 (Bruce et al., 2002). The highly glycosylated transmembrane protein CD47 is built by an immunoglobulin domain at the N-terminal region, a five transmembrane-spanning domain, and a short cytoplasmic domain (Lindberg et al., 1993). On the erythrocyte membrane, CD47 proteins are present both as an immobile fraction trapped with in the metabolon and as a mobile pool of CD47 in the lipid bilayer (Dahl et al., 2003).
Introduction

Figure 1. RBC membrane: protein interactions within the metabolon.
Band 3 tetramers are linked to spectrin through ankyrin. Glyceraldehyde-3-phosphate dehydrogenase, aldolase, deoxy-hemoglobin, and hemichromes bind to the N-terminal region of the band 3 cytoplasmic domain. Protein 4.2 connects band 3 and ankyrin to the Rh complex by interacting with CD47. Glycophorin A (GPA) dimers are close to the band 3 membrane domain. Rh tetramers are directly associated with band 3 (Bruce et al., 2003).

Glycophorins comprise five sialic acid-rich glycoproteins, which belong to the class of integral proteins. Glycophorin A (GPA) (10^6 copies per cell) is the most abundant sialylated glycoprotein of the human RBC membrane (reviewed by Fukuda, 1993). It contains 60% carbohydrate, of which 25% consist of sialic acid that conveys a strong negative charge to the molecule and prevents cell agglutination. This protein, like band 3, carries determinants of several blood group antigens (Laine and Rush, 1988).

The spectrin protein (25% of membrane proteins) accounts for 75% of the RBC membrane skeletal proteins. It consists of two subunits, α-spectrin and β-spectrin, which are aligned side by side in an anti-parallel conformation. Spectrin dimers further associate into tetramers (reviewed by Discher, 2000). Through the association with protein 4.1, spectrin binds to glycophorin C. Moreover, spectrin tetramers together with
actin form the skeletal polygonal network that interacts with the cytoplasmic domain of band 3 through the association of spectrin with ankyrin (Bennett and Lambert, 1991; Liu et al., 1987). Thus, spectrin is responsible for the cell shape, membrane deformability, and stability of AE1.

The RBC membrane is composed of 40% of phospholipids, cholesterol and glycolipids. Phospholipids (PL) are asymmetrically distributed across the membrane. This asymmetry is maintained by the aminophospholipid translocase activity (Morrot et al., 1989; Seigneuret and Devaux, 1984). Therefore, phosphatidylcholine and most of the sphingomyelins are in the outer leaflet, while most of the phosphatidylethanolamine and phosphatidylserine are in the inner leaflet. Phosphatidylserine (PS) interacts with spectrin in the inner component (Haest et al., 1978). Glycolipids are interspersed between the phospholipids in the outer leaflet, where their glycans protrude from the cell surface. Much like glycoproteins, glycolipids carry important RBC antigens, such as the ABO antigens.

4.1.3 Modifications during RBC aging

During their life span erythrocytes are confronted with many stressful events. In the kidney medulla red cells have to face osmotic stress, while in the lungs they are exposed to oxidative stress (Rice-Evans and Baysal, 1987). Moreover, red cells resist mechanical stress when traveling through capillaries with diameters smaller than their own dimensions. The inability to synthesize new proteins together with the decrease of enzymatic protection against oxidative and peroxidative damage leads to the accumulation of alterations in the membrane, which in turn cause the progressive RBC senescence process (reviewed by Clark, 1988; Lutz, 2004; Lutz et al., 1988).
4.1.3.1 Vesiculation

During aging the RBC surface area decreases by 20% accompanied by an increase in cell density. The membrane loss occurs by vesiculation that happens in vivo. A similar mechanism occurs during blood storage (reviewed by (Greenwalt, 2006), upon metabolic depletion (Lutz et al., 1977), or upon addition of ionophore A23187 and Ca\(^{2+}\) (Allan et al., 1976). Following metabolic depletion or during storage, younger cells shed more vesicles than old cells, since old RBCs are thought to have lost their ability to release “dangerous” material (Dumaswala and Greenwalt, 1984; Snyder et al., 1985). Vesicles released from ATP-depleted RBCs contain band 3 and hemoglobin, but are spectrin-free (Lutz et al., 1977). This is probably due to a partial detachment of the cytoskeleton from the lipid bilayer (Bobrowska-Hagerstrand et al., 1998). Moreover, ATP depletion induces the release of CR1 protein enriched vesicles (Pascual et al., 1993). In contrast, the increase in intracellular Ca\(^{2+}\) concentration in cells treated with ionophore leads to the production of diacylglycerol from negatively charged phospholipids on the inner membrane leaflet. The diffusion of the uncharged diacylglycerol into the outer membrane leaflet induces a morphological change from discocytes to echinocytes and the release of vesicles (Allan et al., 1976; Allan and Michell, 1975). The increase of intracellular Ca\(^{2+}\) concentration induces the budding of vesicles containing glycophorin, band 3, acetylcholinesterase, and some actin and little spectrin (Cole et al., 1978; Cole et al., 1979). In contrast to this, metabolically depleted RBCs release spectrin-free vesicles even in the absence of Ca\(^{2+}\) (Muller and Lutz, 1983). Vesicles are rapidly removed by the reticuloendothelial system (Bocci et al., 1980; Willekens et al., 2003). Based on animal experiments, Willekens and colleagues suggest that spectrin-free vesicles are cleared via recognition of externalized PS. However, spectrin-free vesicle obtained by ATP-depletions maintain their PL
asymmetry (de Jong et al., 1996). Even spectrin-free vesicles obtained by Ca\textsuperscript{2+} and ionophore maintained their normal distributions (Raval and Allan, 1984) and lost their asymmetry only after an incubation for 16 h at 37°C due to ATP depletion. By this time vesicles would have been removed upon interaction with NAbs and complement via their receptors by phagocytic cells. In fact, it is likely that vesicles are cleared upon recognition by autoantibodies that presumably bind to band 3 on vesicles (Müller and Lutz, 1983). Vesiculation appears to have a protective role, whereby it could prevent complement-mediated death by eliminating the membrane attack complex (Iida et al., 1991). Likewise, the release of opsonized band 3 containing vesicles prolongs the in vivo lifespan of erythrocytes with a spectrin/ankyrin deficiency as was shown for splenectomized patients with a hereditary spherocytosis (Reliene et al., 2002).

### 4.1.3.2 Phosphatidylserine exposure

Phosphatidylserine (PS) is located in the inner membrane leaflet of normal, mature erythrocytes and of all eukaryotic cell types (Kuypers, 1998). However, PS distribution is altered in the lipid bilayer of RBCs from patients with thalassemia (Kuypers et al., 1998; Srinivasan and Basu, 1996) and sickle cell anemia (Kuypers et al., 1996; Wood et al., 1996). PS is also exposed on apoptotic cells (Gregory, 2000; Hoffmann et al., 2001). Externalization of PS in vivo is the consequence of an increased intracellular Ca\textsuperscript{2+} concentration, which activates scramblase (Zhou et al., 1997). ATP depletion that leads to an impaired aminophospholipid translocase activity (Dekkers et al., 2002; Williamson et al., 1992; Zhou et al., 1997) can also result in PS externalization but does not occur in vivo (Kirkpatrick et al., 1979). Furthermore, PS exposed on apoptotic cells seems to be a recognition signal for cell removal (Fadok et al., 1998). Connor and his group showed that senescent normal RBCs have only 2 times more PS exposed on the
outer membrane leaflet than young cells (Connor et al., 1994). The phagocytosis rate of these cells was however 15 times higher than that of young cells. Thus, it is inconceivable that PS exposed on normal aged RBC could play the key role in RBC elimination. Even spectrin-free vesicles have an unperturbed PL asymmetry (de Jong et al., 1996) (Raval and Allan, 1984). Hence, their clearance may involve opsonization (Müller and Lutz, 1983; Reliene et al., 2002), instead of recognition of external PS (reviewed by Bosman et al., 2005).

4.1.3.3 Loss of CR1

Complement receptor 1 (CR1), which is located in clusters on erythrocytes (300-1000 copies per cell), functions as factor I cofactor in the cleavage of the activated complement protein C3b to iC3b and C3dg (Medof et al., 1982). Moreover, it binds immune complexes (ICs) containing C3b/C4b and facilitates their transport from the peripheral blood to the liver or spleen, where ICs are cleared without erythrocyte destruction (Cornacoff et al., 1983; Emlen et al., 1989; Reinagel and Taylor, 2000). CR1 is a transmembrane glycoprotein with a long extracellular portion composed of 30 short consensus repeats (SCRs), which contain binding sites for C3b and C4b (Klickstein et al., 1988). This receptor is embedded in the plasma membrane through a hydrophobic transmembrane region followed by a small cytoplasmic domain (Klickstein et al., 1987). In vivo studies on humans have demonstrated that intravenously injected ICs rapidly bind to red cells via CR1 and are cleared by macrophages during the passage of RBCs through the liver or the spleen (Schifferli and Taylor, 1989). The number of CR1 molecules decreases during RBC aging. In fact, young erythrocytes bear two to 16 times more CR1 than senescent cells (Fishelson and Marikovsky, 1993; Lutz et al., 1992; Ripoche and Sim, 1986). This data confirms the
fact that CR1 is lost from red cells in the IC transfer process (Reinagel and Taylor, 2000). Indeed, RBC complement receptor may be cleaved by membrane-associated proteases on the acceptor cell, when RBC-bound ICs interact with FcγRII and CR1 on macrophages (Ripoche and Sim, 1986). Alternatively, IC-containing CR1 could be lost intact from RBC surface by vesicle budding (Pascual et al., 1993).

### 4.1.3.4 Band 3 oligomerization

Oxidative events (reviewed by Arese et al., 2005) and ATP-depletion (Liu and Palek, 1979), lead to the oligomerization of band 3 proteins. While 40% band 3 molecules are confined to the macrocomplex, 60% of them diffuse within the lipid bilayer as dimers (Nigg et al., 1980). It is presumable that the mobile fraction aggregates either upon a tight association of hemoglobin degradation products (hemichromes) with the cytoplasmic domain of band 3 (Low et al., 1985; Schuck and Schubert, 1991) or upon SH group oxidation during aging or oxidative damage (Lutz et al., 1987). The cytoplasmic region of band 3 contains two highly oxidant-reactive cysteine residues that undergo cross-linking (Turrini et al., 1994). Oligomers, rather than dimers (Tanner, 1997) or tetramers (Hanspal et al., 1998) of intact band 3 proteins lead to the formation of binding sites for the low affinity anti-band 3 naturally occurring antibodies (anti-band 3 NAbs) (Lutz et al., 1987; Lutz et al., 1984; Turrini et al., 1991). It is conceivable that band 3 dimers are V shaped (Lutz, 2004), since a cross-linking study at 4°C showed that the exoplasmic regions of band 3 protein are further apart than 13 Angstrom, whereas the cytoplasmic tails are capable of SS-bond formation (Schweizer et al., 1982). Thus, anti-band 3 NAbs are unable to bridge the two exoplasmic regions of band 3, as long band 3 is dimeric.
Figure 2. Sequence of band 3 oligomerization on RBC.

A) Band 3 dimers are likely V shaped on younger erythrocytes and do not allow anti-band 3 NAb to bind bivalently. B) Upon oxidation the association of hemichromes with band 3 induces band 3 clustering C) Anti-band 3 NAbs bind bivalently to oligomerized band 3 protein and thereby gain 100-1000 fold in avidity.
4.1.4 Naturally occurring autoantibodies

In normal human serum a substantial portion of all immunoglobulins are naturally occurring autoantibodies (NAbs) (10-1000 ng/ml) of the IgG, IgM, and IgA classes (Avrameas et al., 1981; Cunningham, 1976; Guilbert et al., 1982). These NAbs are germline encoded and derive from B-1 cells (Solvason and Kearney, 1992) that develop in the fetal liver early in ontogeny and predominantly reside in the peritoneal cavity. Here, they persist as a self-replenishing population that carries CD5 receptors on their surface (Kasaian and Casali, 1993). Fetal B cell precursors do not express the enzyme terminal deoxyneucleotidyl transferase resulting in no addition of N regions in the heavy chain. Therefore, they exhibit a restriction in the diversity of the $V_H$ region repertoire (Gilfillan et al., 1993; Li et al., 1993). Furthermore, B-1 cells are positively selected by self-antigens (Carmack et al., 1990; Hayakawa et al., 1999; Pennell et al., 1989a; Pennell et al., 1989b). Indeed, the interaction of the B cell receptor (BCR) with self-antigens independently of T-cell help is the key for generation of mature B-1 cells (natural autoreactive B cells) in the periphery (Hayakawa et al., 1999; Hayakawa and Hardy, 2000). B-1 cell activation does neither lead to strong antibody responses nor to generation of B cell memory. In addition, CD5 seems to be a negative regulator of the BCR signaling preventing inappropriate activation of autoreactive B cells (Bikah et al., 1996).

NAbs, which are generated in the total absence of external antigenic stimulation (Avrameas, 1991; Coutinho et al., 1995), show low affinity binding to their antigens. Thus, naturally occurring autoantibodies need to bind bivalently to enhance their avidity (Lalor and Morahan, 1990; Lutz et al., 1984). These immunoglobulins exhibit a remarkably conserved and well-defined anti-self repertoire (Haury et al., 1997;
Mouthon et al., 1995). Moreover, NAbs initiate complement opsonization, whereby target cells are either phagocytosed or undergo cytotoxic lysis. Thus, they are thought to play an important role in tissue homeostasis. In fact, they recognize and induce the removal of oxidatively-stressed and senescent RBCs (Lutz et al., 1987; Lutz et al., 1988) and mediate clearance of apoptotic cells by binding to exposed lysophospholipids (Kim et al., 2002). Furthermore, NAbs play an important role in the first line of defense recognizing invading pathogens (Baumgarth et al., 1999). Naturally occurring autoantibodies are often described as polyreactive. However, it was shown that self and conserved non self-antigens recognized by NAbs are likely to share the same, or at least very similar, epitopes (Berland and Wortis, 2002). There may be other aspects for the polyreactivity (see idiotypic network).

4.1.4.1 NAbs against RBC components

Normal blood donors contain naturally occurring autoantibodies against RBC cytoskeletal elements, such as spectrin, actin, and band 6 protein (Lutz and Wipf, 1982). The existence of these NAbs directed against autoantigens, which are not normally expressed on the cell surface, suggests that they play an important role in the clearance of debris deriving from occasional RBC lysis. Among these NAbs there exist also NAbs directed to normally exposed antigens, like those against RBC band 3 (anti-band 3 NAbs), which represent $1.8 \times 10^5$ of the total IgG (Lutz et al., 1984). Anti-band 3 NAbs have a tissue homeostatic role in recognizing and inducing the removal of senescent RBCs (reviewed by Lutz et al., 1996a).
4.1.4.2 Anti-hinge NAbs

Terness and colleagues studied a special naturally occurring autoantibody, which recognizes epitopes within the hinge region of F(ab')\textsubscript{2} fragments of IgG molecules. This anti-F(ab')\textsubscript{2}, known as anti-hinge NAb, strongly binds to the exposed hinge region of F(ab')\textsubscript{2} fragment from IgG\textsubscript{i} molecules with high affinity (Kd= 3.6 x 10\textsuperscript{-8} M) (Welschof et al., 1997), but only weakly to intact IgG\textsubscript{i} molecules and Fab fragments (Terness et al., 1995; Welschof et al., 1999). In contrast to other NAbs, anti-hinge NAbs are apparently not generated by antigen-driven maturation. They are probably expressed in response to the hinge region exposure upon antibody-antigen interactions, which are shown to induce conformational changes in the antibody (Brown and Bekisz, 1984). Anti-hinge NAbs are thought to play a role in the suppression of autoreactive B cells and were able to suppress anti-erythrocyte autoantibody-producing B cells in vitro (Terness et al., 1993).

4.1.4.3 Idiotypic network

Naturally occurring autoantibodies form a dense idiotypic network, in which they interact with other autoantibodies that either circulate in the blood or are exposed on cell membranes (Jerne, 1974). This is due to the fact that the antibody’s variable and hypervariable regions may act as antigenic determinants (idiotopes). NAbs bind to hypervariable region are known as paratope-specific anti-idiotypes. Thus, NAbs, like antigens, are able to induce generation of anti-idiotypes. These may trigger in turn the formation of anti-anti-idiotypes and so on (Jerne, 1974). The purpose of the idiotypic interaction is to bring into contact all classes of lymphocytes and regulate specific immune responses (Vakil et al., 1986; Varela and Coutinho, 1991). A defect in the idiotypic network may give rise to autoimmune diseases that are characterized by the
presence of uncovered autoantibodies, which are free to interact with self-antigens. Healthy individuals possess the same autoantibody repertoire as autoimmune disease patients (Stahl et al., 2000). In contrast to patients, these autoantibodies are blocked in healthy individuals by anti-idiotypes from binding to self-antigens (Pan et al., 1998; Zhang et al., 2001). Beside paratope specific anti-idiotypes, there are NAbs able to interact with idiotopes outside the antigenic determinants (framework-specific anti-idiotypes). Among the latter group, there are anti-idiotypes that when associated with idiotypes enhance the idiotypic binding to the antigens (Sawutz et al., 1987). Data from warm autoimmune hemolytic anemia (WAIHA) patients, in which IgM molecules have a strong affinity for IgG, showed that the increased IgG binding to RBC membranes was due to the interaction of IgM with anti-RBC specific IgG molecules (Stahl and Sibrowski, 2005). In whole pooled human IgG, Jelezarova and Lutz detected NAb complexes of anti-C3 NAbs and framework specific anti-F(\(\text{ab}'\))\(_2\) NAbs that could bind to the alternative C3 convertase precursor (C3b\(_2\)-IgG complexes) and modulate its activity in vitro (Jelezarova and Lutz, 2005). In fact, NAb complexes, which contained an excess of anti-C3 NAbs, promoted generation of the alternative C3 convertase. This was probably due to the fact that these NAb complexes blocked C3 convertase precursor interaction with complement regulatory proteins (Jelezarova and Lutz, 2005). Since framework-localized idiotopes may occur on NAbs having different paratopes, it is possible that framework-specific anti-idiotypes may form complexes of NAbs having different specificities. Hence, such complexes may in part be responsible for the polyreactivity of NAbs.
Figure 3. IgG structure and how paratope specific anti-idiotypes, framework-specific anti-idiotypes, and anti-hinge NAbs interact with IgG molecules.

4.1.4.4 NAbs associated with erythrocytes

The quantity of IgG molecules, which are associated with RBCs of different ages, differs. Indeed, senescent erythrocytes bear a higher amount of IgG molecules than young erythrocytes. Clark and Sorette measured RBC-associated IgG by binding of $^{125}$I-protein A. The data showed that young erythrocytes carried 50 IgG molecules per cell, whereas senescent RBC (0.5% of the total RBC population) bore 250 molecules per cell (Clark and Sorette, 1991). In contrast, Paleari and colleagues determined the number of RBC-bound IgG by an immunoenzymatic method. This study revealed that old erythrocytes (1% of the total RBC population) carried 400 copies of IgG per cell, whereas young cells bore 1.8 times less molecules than aged RBCs (Paleari et al.,
Most of the IgG molecules associated with old erythrocytes belong to the subclass 1 and 3 and only a small portion to the subclass 2. IgG₄ molecules have not been found being associated with old RBCs (Clark and Sorette, 1991). It is still unclear how many NAbs associate with erythrocytes of different ages. This is probably due to the difficulty in washing the cells sufficiently. Correspondingly, the specificity of bound NAbs has remained controversial. Kay found that NAbs, which were eluted from senescent RBCs, bound to band 3 and a breakdown product of band 3 on blots (Kay, 1984). On the contrary, Lutz and colleagues showed that pooled whole human IgG contained NAbs that specifically bound to band 3 monomers and preferentially to band 3 oligomers (Lutz et al., 1984). Turrini and his group confirmed the latter findings. In fact, erythrocytes, which were treated with protein clustering agents, bound NAbs when incubated with autologous serum (Turrini et al., 1991). Anti-band 3 NAbs are found to be bound to band 3 as NAbs (Lutz and Stringaro-Wipf, 1983) and as C3b-IgG complexes (Lutz et al., 1996b). Moreover, they bind exclusively to the N-terminal region of the 55 KD domain (Lutz et al., 1993a), rather than to the carbohydrate-rich fragment (Beppu et al., 1992). RBC-bound anti-band 3 NAbs amount to less than 20 IgG per oxidized RBCs (Lutz et al., 1987) indicating that these NAbs alone cannot efficiently stimulate phagocytosis of senescent erythrocytes. In fact, RBC clearance is induced when activated complement protein C3b associates with anti-band 3 covalently (Lutz et al., 1987).

### 4.1.5 Complement system

The complement system is part of the innate immunity. Its role is to recognize and kill pathogens and maintain tissue homeostasis by removing immune complexes, senescent, and apoptotic cells from the circulation. The complement system is activated by three
different pathways: the classical pathway, the alternative pathway and the lectin pathway. The classical route is activated by antigen-antibody complexes, while the lectine pathway is initiated by the association of mannose binding lectin or ficolin with specific carbohydrates. Both interactions activate C4 and C2 proteins, which generate the classical C3 convertase (C4b2b) that cleaves C3 into C3a and C3b. The binding of C3b to C4b2b leads to a sequence of reactions that culminate in the formation of the membrane attack complex and lysis of the target cell. The alternative pathway differs from the previous routes because it is capable of autoactivation, since C3 protein contains a thioester that undergoes hydrolysis at a slow rate, generating a functionally active C3 molecule, C3(H2O). Factor B can associate with this activated molecule and be cleaved by factor D. This initial C3 convertase cleave C3 into C3a and C3b, whereby a positive feedback is initiated, the amplification loop. Complement amplification can also be triggered by C3b generated by the classical or lectin pathway, by which new C3 protein is cleaved by the activity of a properdin stabilized C3bBb complex. Strong evidences suggest that the alternative complement pathway can occur not only on cell-bound C3b, but also in the fluid phase. Here nascent C3b reacts covalently with fluid phase IgG and generates C3b2-IgG complexes, which are the best C3 convertase precursor (reviewed by Lutz and Jelezarova, 2006). The complement system leads to the release of anaphylatoxins, C3a and C5a, which are important proinflammatory molecules. They are involved in stimulation and chemotaxis of mast cells and blood basophils. To prevent inappropriate host tissue damage, the complement system is under control of several regulatory proteins. The decay acceleration factor (DAF) facilitates the dissociation of C3 convertase. Moreover, the convertase assembly is suppressed by the proteolytic cleavage of C3b into iC3b, C3dg, and C3d by factor I in the presence of membrane cofactor protein (MCP, CD46), C4b-binding protein, CR1, or
factor H (Hourcade et al., 1989; Liszewski and Atkinson, 1998). Additionally, CR1 on erythrocytes binds ICs containing C3b and serves to remove them from the circulation (Reinagel and Taylor, 2000). CD59 protect cells from the membrane attack complex by binding to proteins of the last part of the complement cascade (Meri et al., 1990).

Figure 4. The Complement system activation and the amplification loop
4.1.5.1 Complement on senescent RBCs

Low numbers of cell-bound antibodies are insufficient to induce Fc receptor-mediated phagocytosis (Ehlenberger and Nussenzweig, 1977; Kurlander and Rosse, 1979). In association with C3b fragments, however, even low numbers of antibodies are able to strongly stimulate phagocytosis of RBCs (Ehlenberger and Nussenzweig, 1977; Kurlander and Rosse, 1979; Lutz et al., 1987). RBCs-bound anti-band 3 NAbs, like other NAbs, activate the complement system, whereby nascent C3b is deposited on the target cell. Furthermore, there exist some IgGs, like anti-band 3 NAbs, for which C3 protein has a higher affinity as compared to whole IgG (Kulics et al., 1983; Lutz et al., 1993b). Nascent C3b fragment reacts covalently with anti-band 3 heavy chain (Lutz et al., 1993c) and gives rise mainly to C3b$_2$-IgG complexes (Lutz et al., 1996b). The ester-bonded C3b dimers are covalently linked to the CH1 domain of a single IgG via ester bonds (Jelezarova et al., 2003; Vidarte et al., 2001). Unlike free C3b, C3b in C3b$_2$-IgG complexes exhibits increased resistance to factor H and I mediated inactivation (Lutz et al., 1996b). Therefore, when measured under 20% serum conditions, the half-life of C3b in C3b$_2$-IgG complexes is prolonged to 3-4 minutes as opposed to ≤1 second for free C3b. Furthermore, C3b$_2$-IgG complexes promote generation of the amplifying C3 convertase seven to ten fold better than C3b. In fact, the presence of dimeric C3b within C3b$_2$-IgG complexes facilitates the binding of properdin (Jelezarova et al., 2001; Jelezarova et al., 2000) leading to an increased association of factor B with C3b as is known for quite some time (DiScipio, 1981). Moreover, C3b trapped in ICs and presumably also C3b$_2$-IgG complexes tightly bind to CR1, because CR1 contains two to five C3b binding sites (Pascual and Schifferli, 1992; Schifferli et al., 1988). Hence,
fluid phase C3b-containing ICs and RBC-bound C3b$\_2$-IgG complexes interact efficiently with CR1 on erythrocytes and phagocytic cells (Fries et al., 1987; Malbran et al., 1987).
4.2 Phagocytosis of senescent erythrocytes

Numerous data strongly suggest that clearance of senescent erythrocytes requires the innate immune system (reviewed by Lutz, 2004), whereby aged RBCs are recognized by macrophages upon interaction of C3b₂-IgG/iC3b₂-IgG complexes with complement receptors and IgG receptors. Efficient phagocytosis of opsonized target cells is highly dependent on the recognition of cell-bound IgG and on covalently bound C3b (Ehlenberger and Nussenzweig, 1977). It has been shown that fluid phase C3bₙ-IgG complexes bind to phagocytic cells six times more than C3b monomers (Malbran et al., 1987). In vitro phagocytosis studies showed that RBCs opsonized with C3bₙ-IgG complexes are at least 10 times more efficiently engulfed by macrophages than RBCs carrying IgG alone (Fries et al., 1987). Furthermore, physiological concentrations of free plasma IgG fully inhibit phagocytosis of RBCs opsonized with IgGs but not substantially that of targets opsonized by C3bₙ-IgG complexes. Additionally, C3bₙ-IgG complexes induce phagocytosis twice as effectively as randomly deposited C3b and IgG molecules (Fries et al., 1987). Hence, initiation of erythrophagocytosis through low affinity antibodies is increased by complement components even though the number of cell-bound IgG is low.
Figure 5. C3b–IgG complexes interact with IgG- and complement- receptor

For a successful RBC internalization phagocytic cells need that IgG receptors and complement receptors on their surface interact with RBC-bound C3b/iC3b and IgG clusters or C3b–IgG complexes.

### 4.2.1 IgG Receptors

IgG molecules with their Fc fragment can bind to three major classes of receptors, known as FcyRI, FcyRII, and FcyRIII. All three classes of receptors are expressed on human phagocytic cells. They contain highly conserved extracellular Ig domains, but different cytoplasmic regions. IgG receptors that activate an immune response are characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM). This region can be intrinsic to the receptor, as in FcyRIIA, or an associated subunit, as in the case of FcyRI and FcyRIII A (Daeron, 1997). On the contrary, inhibitory Fc receptors, such as FcyRIIB, contain the immunoreceptor tyrosine-based inhibition motifs (ITIM) and negatively modulate the immune response. FcγRI show a
high affinity for free monomeric immunoglobulins, while the low affinity receptors FcγRII and FcγRIII bind to aggregated IgG molecules or to antibodies complexed with antigens (Ravetch and Kinet, 1991). Moreover, FcγRs differentiate between IgG subclasses. IgG1 and IgG3 interact with all FcγRs, whereas IgG2 is recognized by FcγRII and IgG4 by FcγRI. Furthermore, FcγRI has a stronger affinity for IgG1 and IgG3 than for IgG4. To initiate signal transduction, FcRs have to aggregate not only with other FcRs (Unkeless et al., 1995) but also with different receptors expressed on phagocytic cells. Malbran and his colleagues showed that ICs are internalized by phagocytic cells in the presence of increasing concentrations of fluid phase IgGs when FcRs interact with complement receptors (Malbran et al., 1987).

4.2.2 Complement Receptors

Complement proteins are recognized by CR1, CR3, and CR4 found on phagocytic cells. Since free C3b has a half-life of a second and iC3b of 45 minutes, the latter is the major C3 fragment that mediates recognition of opsonized target by macrophages (Cain et al., 1987; Rosenthal et al., 1996; Ross et al., 1987). CR1, a single chain type I transmembrane glycoprotein, binds C3b and has a slightly lower affinity for iC3b. In contrast, CR3 and CR4, members of the integrin family, bind primarily iC3b. Moreover, CR4 and CR3 are the most abundant C3 receptors on tissue macrophages (Hogg et al., 1986; Myones et al., 1988), but CR4 shows less affinity for iC3b than CR3. Moreover, CR3 cooperates with FcγRIII in transmitting signals from the IgG receptor to the inside of the cell independently of the interaction with complement proteins (Krauss et al., 1994). In addition, it seems that if CR1 mainly serves in the binding of the target cell,
CR3 and CR4 are responsible for the internalization of the opsonized cells (Yan et al., 2000).
4.3 Modulation of RBC phagocytosis

4.3.1 CD47 and SIRP

CD47 is exposed on virtually all cells including RBCs and mediates adhesion-related processes (Lindberg et al., 1994; McDonald et al., 2004; Reinhold et al., 1995). Data from a study in nonobese diabetic (NOD) mice suggested that CD47 is involved in the regulation of erythrocyte phagocytosis by splenic macrophages (Oldenborg et al., 2000). In fact, it was shown that erythrocytes from CD47 knock-out mice were rapidly eliminated once transfused into wild-type recipients (Oldenborg et al., 2002; Oldenborg et al., 2000). CD47 deficient RBCs were readily engulfed because of the absence of CD47 interaction with signal regulatory protein α (SIRPα) on the macrophage surface. SIRPα is a member of the immunoglobulin superfamily, highly expressed on phagocytic cells (Seiffert et al., 1999). It has three extracellular Ig domains and an intracellular domain with at least two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). When activated, phosphorylated motifs bind the src-homology 2 domain-containing phosphatases SHP-1 and SHP-2 (Veillette et al., 1998). Activation of these enzymes leads to the attenuation of the signaling initiated by the aggregation of FeγRs and CRs (Lienard et al., 1999; Oldenborg et al., 2001). In humans it is still unclear whether CD47-SIRPα interaction occurs. On human RBC membrane, CD47 trapped in the band 3 macrocomplex has less mobility than that in mice, where no association with band 3 and protein 4.2 is found (Bruce et al., 2003; Mouro-Chanteloup et al., 2002; Mouro-Chanteloup et al., 2003). Nevertheless, the extracellular domain of SIRPα interacts with CD47 on human RBC (Subramanian et al., 2006). Hence, it is possible that even in humans the loss of CD47 with RBC age may render phagocytosis of
weakly opsonized senescent RBCs more likely than if these weakly opsonized RBCs were young.
4.4 Healthy Coombs-positive blood donors

Over the years many blood donation and transfusion centers have found with a prevalence of one in 5000 to 10000 healthy individuals who were tested positive in the Coombs test (reviewed by Garratty, 1991). This immunologic test, also known as direct agglutination test (DAT), with anti-Ig antibodies on washed RBCs from whole blood detects RBC-bound Ig molecules if their number amounts to ≥200 IgG molecules per cell (Dupuy et al., 1964). A positive Coombs test and an increased reticulocyte number are the two major criteria to diagnose an autoimmune hemolytic anemia (AIHA) (reviewed by Gehrs and Friedberg, 2002). The healthy blood donors with a positive DAT value, called “healthy Coombs-positive blood donors”, have RBCs carrying two to five times more IgG molecules than normal senescent RBCs (reviewed by Garratty, 1991). These blood donors, however, do not show any sign of AIHA. Indeed, despite the high amount of cell-bound IgG molecules, these Coombs-positive RBCs do not undergo hemolysis, therefore the donors of these cells have normal numbers of reticulocytes and their concentration of hemoglobin breakdown products in blood is not increased. In the following, we will refer to the erythrocytes from healthy Coombs-positive blood donors as CRBCs. Due to an interference of the high anti-IgG reactivity with the assay for blood group detection and the fact that the positive DAT value may be a manifestation of an upcoming anemia or an unknown, covered illness, healthy Coombs-positive blood donors have so far been excluded from blood donation and were asked to visit a hematologist.

Gorst and colleagues studied 32 healthy Coombs-positive blood donors longitudinally, of which 26 individuals still had a positive DAT, when retested after a period of six
months to 14 years and only one donor developed AIHA (Gorst et al., 1980). Results from a similar analysis revealed that nine of 26 healthy Coombs-positive blood donors were again tested positive after a period of one to ten years (Bareford et al., 1985). Of these nine only one individual developed AIHA after ten years from the first Coombs test. Lutz and Frey-Wettstein performed another longitudinal study confirming this data: in seven blood donors, which were tested twice over a period of six years, six remained healthy and among these healthy individuals five retained a positive Coombs test (unpublished data). Moreover this group analyzed the RBC membrane profile and the IgG molecules bound to it. While the membrane protein pattern analysis did not reveal any abnormality, acid eluted IgG molecules from CRBCs showed binding to band 3, and surprisingly a strong binding to C3 and C3b, and IgG primarily to the light chain (LC) (unpublished data). Masouredis and colleagues analyzed the specificity of IgG molecules eluted from CRBCs (Masouredis et al., 1987). Immunoglobulins from CRBCs contained two different IgG populations. One population appeared to bind to RBC, while the other showed anti-idiotypic nature, since it associated with RBCs sensitized with anti-Rh(D) (Masouredis et al., 1987). However, these results are questionable, since eluates were obtained from blood units stored in their plasma for seven to 54 weeks that is longer than the RBC life span. Moreover, the eluted material was heated at 56°C for 1 hour. This procedure is known to aggregate IgG molecules via Fc portions and results in increased ELISA readings (Costa et al., 1984). Thus, this pretreatment may have induced the binding of unspecific IgG molecules. In a later study, the same group showed that IgG eluates, which were not heated as described before, contained autoantibodies that immunoprecipitated Rh antigens and band 3 (Pierce et al., 1990).
5 Aim of the study

As outlined in the introduction a large body of information suggests that senescent RBCs are cleared by phagocytes following their selective opsonization by NAbs and complement. These findings are in complete contrast to the existence of healthy Coombs-positive blood donors with RBCs that carry two to five times as many IgG NAbs and yet circulate. It was the aim of this study to clarify this apparent discrepancy.

This thesis addressed the following questions: Do IgG molecules bind to CRBCs of any age? What is the specificity of CRBC-bound IgG molecules? Why are CRBCs not readily phagocytosed? We could show that NAbs were primarily associated with aged CRBCs, as it occurs in controls. Eluted IgGs contained predominantly anti-band 3, anti-C3 and LC specific anti-IgG NAbs. To elucidate why CRBCs carry high numbers of IgG NAbs with the given specificity, we investigated whether plasma from healthy Coombs-positive blood donors contained higher titers than controls for anti-C3 and anti-idiotypic NAbs. The latter was analyzed in plasma depleted of anti-hinge region NAbs. We found significantly increased titers for both types of NAbs, suggesting that RBC-bound C3b/iC3b might be blocked on CRBC and could not interact with CR receptors on phagocytic cells. Such a blockade would result in decreased erythrophagocytosis in the presence of physiological concentrations of IgG. Therefore we studied in vitro phagocytosis of CRBCs in the presence and absence of fluid phase IgG.

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6 Materials and Methods

6.1 Blood samples

Blood from sixteen healthy adult donors and eleven healthy Coombs-positive blood donors were obtained from the Regional Blood Transfusion Service, SRK, Zurich, Switzerland. The blood was collected in CPDA-1 and freed of white cells by Leucoflex LST2 filter (MacoPharma, Quebec, Canada).

6.2 Isolation of F(\(ab\)'\(_2\)) fragments from whole pooled human IgG

Pooled whole human IgG (Sandoglobulin, ZLB, Bern, Switzerland) was dissolved in 25 mM NaAc (pH 4.5) and dialyzed against 25 mM NaAc. A dialyzed sample (100 mg) was digested with 1 mg pepsin (Sigma-Aldrich, St. Louis, MI, USA) on the rotating table for 24 h at 37°C. The digestion was stopped by the addition of 1 M Tris (pH 9) and 250 mM NaCl. To isolate F(\(ab\)'\(_2\)) fragments, the sample was applied to a Sephadex G75 (GE Healthcare, Uppsala, Sweden) in 20 mM Tris, 250 mM NaCl, and 0.08% Azide (pH 7.4) at a flow rate of 18 ml/h. Fractions were collected every 5 min. The void volume peak containing residual IgG and F(\(ab\)'\(_2\)) was pooled and dialyzed against 9.85 mM Na\(_2\)HPO\(_4\), 1.76 mM KH\(_2\)PO\(_4\), and 150 mM NaCl (PBS, pH 7.4). To remove IgG residual, the dialyzed material was absorbed overnight at 4°C on Protein G Sepharose 4 Fast flow (GE Healthcare) in a batch procedure. Purified F(\(ab\)'\(_2\)) fragments were concentrated to 1.5 mg/ml in Vivaspin 25000 molecular weight cut-off (MWCO) (Vivascience AG, Hannover, Germany) and stored frozen in aliquots.
6.3 Isolation of Fc fragments from whole pooled human IgG

Pooled whole human IgG (Sandoglobulin) was dissolved in PBS (pH 7.0) and dialyzed against the same buffer. A dialyzed sample (100 mg) was digested with 0.2 mg papain (Sigma-Aldrich) in 5 mM EDTA and 10 mM cysteine (Sigma-Aldrich) for 24 h at 37°C. The digestion was stopped by the addition of 250 mM N-ethylmaleimide (NEM). To isolate the Fc fragments, the sample was loaded onto a Sephadex G200 (GE Healthcare) equilibrated in PBS (pH 7.4) at a flow rate of 18 ml/h. Fractions were collected every 5 min. The Fc fragment peak was pooled, concentrated to 2 ml in Vivaspin 25000 MWCO (Vivascience). This Fc material was loaded to a Protein G Sepharose 4 Fast flow (GE Healthcare) column overnight at 12 ml/h in the cold. After washing the column, bound material was eluted with 0.1 M glycine, 1 M NaCl (pH 2.6), dialyzed against PBS (pH 7.4), concentrated to 1 mg/ml in Vivaspin 25000 MWCO (Vivascience), and stored frozen in aliquots.

6.4 Isolation of complement C3

Complement C3 was isolated from plasma according to the method of Tack (Hammer et al., 1981; Tack and Prahl, 1976). Filtered 0 Rh+ blood was centrifuged in GSA bottles for 10 min at 1017 g. The supernatant fluid was collected and recentrifuged in the same bottles as before for 10 min at 16270 g. Polyethylenglycol 4000 (PEG, 5% final concentration) was slowly added to the supernatant fluid under stirring in the cold for 30 min. The pH was adjusted to 7.4. After centrifugation in GSA bottles for 15 min at 4923 g, the supernate was mixed with PEG 4000 (12% final concentration) as described above. After centrifugation in GSA bottles for 20 min at 6876 g, the supernate was discarded, while the pellet was resuspended in buffer I (100 mM Na2HPO4, 150 mM
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NaCl, 15 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4). The dissolved pellet was applied to a L-lys Sepharose column (38 ml packed in a 2.5 x 7.8 cm column) equilibrated with buffer I at a flow rate of 38 ml/h. The flow through was collected and diluted 7-8 times with 5 mM EDTA containing 200 μg/ml PMSF. Diluted material was mixed with EACA (50 mM final concentration), adjusted to pH 7.0 and loaded to the DEAE-Sepharcel (220 ml packed in a 2.5 x 45 cm column) equilibrated in DEAE buffer (25 mM K₂HPO₄, 5 mM EDTA, 0.08% Azide, 50 μg/ml PMSF, pH 7.0) at 68 ml/h. Prior to elute, the column was washed with DEAE buffer for 24 h. Bound material was eluted with a gradient of 500 ml buffer A (25 mM K₂HPO₄, 5 mM EDTA, 50 mM EACA, 50 μg/ml PMSF, pH 7.0) and 500 ml buffer B (25 mM K₂HPO₄, 5 mM EDTA, 50 mM EACA, 180 mM NaCl, 50 μg/ml PMSF, pH 7.0). Fractions were collected every 8 min. The C3/C5 peak was pooled and mixed with PEG 4000 (16% final concentration) as described above. The mixture was centrifuged in SS34 tubes for 15 min at 7649 g. The supernatant fluid was discarded, while the pellet was dissolved in 10 to 16 ml Sepharose CL 6B buffer (100 mM K₂HPO₄, 5 mM EDTA, 50 mM EACA, 150 mM NaCl, 50 μg/ml PMSF, pH 7.4). The resuspended pellet was applied to the equilibrated Sepharose CL 6B column (334 ml packed in 2.6 x 63 cm column) at 38 ml/h. Fractions were collected every 10 min. The C3/C5 peak was pooled and dialyzed against HA 5/70/10 buffer (5 mM K₂HPO₄, 70 mM KCl, 10 mM EACA, 50 μg/ml PMSF, pH 7.4). The dialyzed sample was loaded to the equilibrated Hydroxylapatite (HA) columns (70 ml packed in two columns each of 2.5 x 7 cm) at 18.0 ml/h. The columns were washed with HA 5/70/10 buffer until no protein was detected in the eluent. C3 was eluted with 125 mM K₂HPO₄, 70 mM KCl, 10 mM EACA, 50 μg/ml PMSF, pH 7.4. C3 was 100% pure by SDS-PAGE and could be fully activated by trypsin. The protein was concentrated to 2 mg/ml in Vivaspin 100000 MWCO.
(Vivascience), filtered in the sterile hood through a 0.2 μM filter, aliquotated and stored at 4°C.

6.5 **Coupling of proteins to Affigel**

Affigel 10 (BioRad Laboratories, Hercules, CA, USA) was washed three times with ice cold water and resuspended in ice cold PBS. Two to three mg protein/ml Affigel were incubated on a rotating wheel overnight at 4°C. The mixture was centrifuged for 5 min at 268 g in a Sorvall T6000 centrifuge. The gel was incubated with 1 volume of blocking solution (0.1 M ethanolamine in PBS) for 1 h at 4°C. The blocking buffer was removed by centrifugation at 268 g for 5 min. The gel was washed four times with cold PBS. Coupled Affigel was stored in PBS containing 0.08% azide (pH 7.4) at 4°C. In order to check the coupling efficiency, an aliquot from the supernatant fluid was saved after coupling and washing. These aliquots were subsequently analyzed by SDS-PAGE. Prior to use, the coupled Affigel was pre-eluted with 0.2 M Glycine pH 2.5.

6.6 **Iodination of protein G and isolated antibodies**

Recombinant protein G (Sigma-Aldrich), IgGs eluted from RBCs of controls and healthy Coombs-positive blood donors, anti-C3, and anti-F(ab')2 isolated from healthy Coombs-positive plasma were labeled with $^{125}$I-iodine (GE Healthcare). Protein G and isolated IgG molecules (50 μg) were iodinated with 1 mCi for 60 s using chloramine-T as an oxidant (Lutz et al., 1993b). The reaction was stopped by the addition of Na-metabisulfite. Free $^{125}$I was removed by passing the labeled material over a PD10 desalting column (GE Healthcare). Prior to use, the column was equilibrated with PBS (pH 7.4) containing 1 mM NaI, 1 mM EDTA, and 0.05% gelatine. Labeled proteins
were aliquoted and stored at -20°C. Before use, the thawed material was dialyzed against PBS (pH 7.4). Radioactivity was determined in a MR 480 gamma-counter (Kontron, Zürich, Switzerland). Based on earlier experiments, the specific activity of a particular protein was calculated from the recovered radioactivity and assuming a 50% loss of used protein.

6.7 SDS-PAGE and Western blot

Proteins were electrophoresed on SDS-PAGE containing 8 to 10% acrylamide and bisacrylamide at a ratio of 37.5:1 (Applichem GmbH, Darmstadt, Germany) for 45 min at 200 V. Before loading, proteins were prepared in sample buffer with or without 40 mM dithiothreitol (DTT). After heating for 3 min, reduced and unreduced proteins were alkylated with an excess of NEM. Reduced and not reduced polypeptides were pooled after alkylation and run in the same lane. The proteins in the gels were visualized by staining with Coomassie blue or silver stain (Swain and Ross, 1995) or they were blotted onto a membrane (Immobilon-P transfer membrane from Millipore Corporation, MA, USA) in the cold for 3 h at 70 V. One part of the membrane was stained with amidoblack. The other part of the membrane was incubated with 5x10⁵ to 1x10⁶ cpm/ml ¹²⁵I-iodinated proteins in Replica buffer (20 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 0.5% gelatine and 0.05% Tween 20 over night at room temperature on a shaker. The membrane was washed three times with Replica buffer containing 0.5% gelatine and 0.05% Tween 20 followed by three washes with Replica buffer only. Once dried, the membranes were exposed to a phosphor screen over night and scanned with a Phosphoimager Storm 820 (Molecular Dynamics).
6.8 Protein quantification by densitometry

Gels stained with Coomassie blue or Silver solution and immunoblots were scanned with the GS-800 calibrated densitometer (Biorad). The bands were quantified by using Quantity One software (Biorad).

6.9 RBC density separation on self-forming Percoll gradients

Filtered blood was centrifuged in GSA bottles for 10 min at 1017 g. The plasma was re-centrifuged in GSA bottles for 10 min at 16270 g, and stored frozen in aliquots. Pelleted erythocytes were resuspended with cold Percoll buffer (854g/L Percoll (GE Helthcare), 10 mM NaKHPO₄, 144 mM NaCl, 0.5 mM EDTA, 5g/L D-glucose, 30μg/mL PMSF, osmolality 300mOsm/Kg, pH 7.4) to a hematocrit of 10-15% (Lutz et al., 1992). The suspension was transferred to SS34 tubes with covers and immediately centrifuged for 25 min at 43140 g in a Sorvall RC-5B centrifuge. Density-separated RBCs were fractionated into 5 or 6 portions numbered from top to bottom of the tube. Fractionated RBCs were washed as indicated later.

6.10 Membrane preparation and determination of band 4.1 a/b ratio

Density-separated RBCs were washed three times with Gey's balanced salt (Sigma-Aldrich) in GSA bottles by centrifugation at 1017 g for 8 min. Membranes were prepared from RBC by lysis in 30 volumes of a hypotonic lysis buffer (5mM phosphate, 1mM EDTA pH 7.4.) Membranes were washed twice with the hypotonic lysis buffer. The first time the buffer was supplemented with 0.4 mM DFP. Pelleted membranes were resuspended in 1% SDS and 5 mM NEM. They were denaturated for 3 min at
100°C in electrophoresis sample buffer containing 40 mM DTT. Reduced proteins were alkylated with NEM. To quantify bands 4.1 a and 4.1 b for the calculation of band 4.1 a/b ratio, membrane proteins (7 µg) were electrophoresed at 195 V on 8% SDS-polyacrylamide gels that run for 45 min beyond the moment when the tracking dye had reached the front. The polypeptides were visualized by staining with Coomassie blue. Bands 4.1 a and 4.1 b were quantified with a GS-800 calibrated densitometer (Biorad).

6.11 125I-protein G binding assay for detection of RBC-bound IgG

125I-protein G binding to RBC was performed by a phthalate oil separation method described by Kelton and Denomme (Kelton and Denomme, 1982). Density-separated RBCs were washed three times with Gey’s balanced salt (Sigma-Aldrich) in GSA bottles by centrifugation at 1017 g for 8 min. Washed RBCs were adjusted to a cell number of 2 to 3 x 10⁹ cells/ml in PBS (pH 7.4) containing 1 g/l D-glucose (PBS-G). 125I-labeled protein G (8 x 10⁶ cpm/µg) in PBS (pH 7.4) supplemented with 5 mg/ml ovalbumin in a total volume of 25 µl was added to 25 µl RBC suspension (1:1). After 1 h incubation on ice, 150 µl PBS-G was added to the mixture. An aliquot of 150 µl of this suspension was immediately centrifuged on 200 µl of a pre-cooled phthalate oil mixture (70% dibutyl phthalate and 30% di-isonyl phthalate) in 400 µl polyethylene tubes for 4 min at 6500 g in a Beckman Microfuge. After freezing the tubes on dry ice, the tips containing pelleted RBCs were cut off. Cell-bound radioactivity was measured with a MR 480 gamma-counter (Kontron).
6.12 FACS analysis for detection of RBC-bound IgG

Density-separated RBCs were washed three times with Gey's balanced salt (Sigma-Aldrich) in GSA bottles by centrifugation at 1017 g for 8 min. Washed RBCs were adjusted to a number of 1x10^6 cells in 50 μl FACS buffer (PBS with 0.5% BSA, pH 7.4). To stain RBC-bound IgG, phycoerythrin (PE)-labeled F(ab')2 fragments from an anti-human IgG (1:24, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added to the RBCs. After 30 min at 4°C the cells were washed once with FACS buffer, centrifuged for 2 min at 2000 g in a 5415D Eppendorf centrifuge. RBCs were resuspended with 400 μl FACS buffer. The intensity of PE was analyzed in the FL2 channel of a FACS calibur (BD Biosciences, San Jose, CA, USA).

6.13 Elution of IgG from fractionated RBCs

Density-separated RBCs were washed twice with PBS supplemented with 1 mM EDTA and 50 μg/ml PMSF (pH 7.4) and once with 155 mM NaCl in GSA bottles by centrifugation at 1017 g for 8 min. To elute IgG from RBCs of different cell age, 40x10^10 cells of washed RBCs were resuspended in 200 ml elution buffer (0.2 M glycine, 10 mM NaCl, osmolality 270 mOsm/Kg, pH 2.5) and centrifuged in GSA bottles for 5 min at 5858 g. The supernatants were collected and the pH was measured. If the pH was not acid the elution was remade. Supernates from the first and the second elution were combined and neutralized with 1 M Tris (pH 9) containing 0.001% ovalbumin. The elutes were centrifuged for 5 min at 5858 g in new GSA bottles, to remove any residual cells. Solid urea (5M final concentration) was added to the eluates under stirring. After 1 hr urea treatment at room temperature, the mixtures was
supplemented with 0.05% Tween 20 and then dialyzed against PBS containing 0.08% Azide, 50μg/mL PMSF, and 0.05% Tween 20 (pH 7.4) (2 x changes) at 4°C.

6.14 Purification of acid-eluted IgG on Protein G Sepharose

Dialyzed eluates from density separated RBCs were passed through 1.5 ml Protein G Sepharose 4 fast flow (GE Healthcare) equilibrated in PBS (pH 7.4) at 7.5 ml/h at 4°C. RBC-eluted samples were applied to the Protein G beads in the cold. After washing the column until no protein was detected in the eluent, bound material was eluted with 0.2 M glycine (pH 2.7). Fractions were collected every 4 min. The IgG peak was pooled and neutralized with 1 M Tris (pH 9). Tween 20 (0.02% final concentration) was added to the purified IgGs. The sample was dialyzed against PBS containing 0.08% azide and 0.02% Tween 20 (pH 7.4) and, then, was concentrated up to 80 μl in Vivaspin 100000 MWCO (Vivascience). To test their purity, proteins were subjected to SDS-PAGE and visualized by staining with Silver stain. The protein concentration was determined by densitometric quantification. IgGs were iodinated as described in chapter 5.6.

Since the eluates contained proteins other than IgG and the eluted IgGs themselves appeared to be complexed, the iodinated samples were further treated with 5 M urea for 1h at room temperature. The mixtures were applied to a Sephcryl 300 (50 ml matrix packed in a 1.5x30 cm column) equilibrated in PBS (pH 7.4) supplemented with 0.02% Tween 20 and 5 M urea. The flow rate was 2.6 ml/h and fractions were collected every 20 min. The peak corresponding to IgG was pooled and dialyzed against PBS (pH 7.4). Radioactivity was measured and the purity of the material was tested on SDS-PAGE and autoradiography of dried gels.
6.15 ELISA

Chemobond plates (Dr. E. Fischer, Dübendorf, Switzerland) were covalently coated by Schiff base chemistry with the desired proteins according to the methods described by Lutz (Lutz et al., 1990) and stored at 4°C. Prior to use, the plates were blocked (300 μl/well) with 0.5% gelatine in PBS (pH 7.4) for 1 hr at 37°C. To measure the total concentration of IgG NAbs in plasma, 50μl of plasma samples were treated with 50μl 10 M urea in PBS containing 10 mM EDTA for 1hr at room temperature. To analyze the accessible portion of IgG NAbs 50μl of plasma samples were incubated with 50μl PBS containing 10 mM EDTA. Afterwards, all samples were diluted with PBS containing 0,1% gelatine (GPBS pH 7.4) to 0.5% plasma and added to the plates (150μl/well) in triplicates. After a 90 min incubation at 37°C the plates were washed three times with GPBS containing 0.05% Tween 20 (300μl/well). Plates were incubated (150 μl/well) with anti-human γ-chain-specific IgG conjugated to alkaline phosphatase (1:9000) in GPBS for 60 min at 37°C. Prior to incubation with p-nitrophenyl phosphate diluted in substrate buffer to 1 mg/ml (Sigma-Aldrich, Fast Tablet Set), the plates were washed three times with GPBS containing 0,05% Tween 20, three times with PBS, and once with water. After 60 min incubation at 37°C, the optical density (OD) at 405 nm was determined with a Dynatech MR5000 microplate reader (Bioconcept, Allschwil, Switzerland).

Where indicated anti-hinge IgG NAbs were depleted by incubating 1 ml of plasma for 30 min with 0,4 mg/0,2 ml F(ab')2 –Affigel at 4°C in a batch procedure. The mixture was centrifuged for 5 min at 268 g in a Sorvall T6000 centrifuge. The supernatant fluid was analyzed by ELISA as described above.
6.16 Cells

Human monocytic leukemia cells, THP-1, were maintained in suspension at a density of 2.0 x 10^5/ml to 1.0 x 10^6/ml in RPMI 1640 medium containing 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (Sigma-Aldrich), and 1% penicillin-streptavidin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. For the phagocytosis assay 1x10^6 cells/ml (500μl/well) were transferred into 24-well plates containing glass coverslips. THP-1 cells were differentiated into macrophage-like cells by incubation of the cells with fresh medium supplemented with 10 nM phorbol myristic acid (PMA) for 48 h.

6.17 Phagocytosis assay

Density-separated RBCs were washed three times with Gey’s balanced salt (Sigma-Aldrich) in GSA bottles by centrifugation at 1017 g for 8 min. RBCs (0.5 ml/well, 1x10^7/ml) from fractions 1, 3, 5 and 6 were incubated with differentiated THP-1 cells. Assuming that all plated THP-1 cells adhere to the coverslips, the theoretical ratio of RBC versus THP-1 cells was estimated to be 10:1. After 1 hr incubation at 37°C in a humidified atmosphere containing 5% CO2, plates were washed 3 times with Gey’s balanced salt (Sigma-Aldrich). Non-ingested erythrocytes were lysed for 1 min with 0.1% NaCl (0.5 ml/well). The reaction was stopped by adding an equal volume of 1.7% NaCl. After three washes with PBS, cells were fixed with 3% paraformaldehyde and 5% sucrose for 30 min at room temperature. After three washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS (pH 7.4) for 5 min. After 3 washes with PBS supplemented with 0.01% Triton X-100, cells were blocked with 3% BSA in PBS (pH 7.4) for 1 hr. Erythrocytes were stained with goat anti-human hemoglobin FITC.
conjugated in PBS containing 3% BSA, and 0.05% Triton X-100 (1:2000, Bethyl, Montgomery, TX, USA) for 1 hr. Coverslips were washed once with PBS containing 1% BSA and 0.01% Triton X-100, and twice with PBS containing 0.05% Triton X-100. The coverslips were mounted on slides and examined using a Axiovert 100 M ZEISS fluorescent microscope (Carl Zeiss AG, Feldbach, ZH, Switzerland). Four equally sized quadrants of 100-200 THP-1 cells were defined for each coverslip. The number of phagocytosed fluorescently labeled RBCs was counted in each quadrant. The number of internalized RBCs was referred to the that of phagocytic cells per quadrant. The resulting values were averaged over the number of quadrants of a coverslip.
7 Results

7.1 Healthy Coombs-positive blood donors

Several healthy Coombs-positive blood donors could be studied longitudinally. These blood donors, whose characteristics are listed in Table 1, have RBCs that bear large amounts of IgG molecules, but not revealed pathological numbers of IgA, IgM, C3c and C3d. Furthermore, these healthy blood donors were not taking any medication and have not received blood transfusions at all. The DAT values of some individuals changed over the years (Table 1).

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Table 1. DAT values, measured with the DiaMed ID-system, over time from healthy Coombs-positive blood donors
7.2 Detection of IgG bound to erythrocytes of different cell age.

A positive DAT value signifies an increased number of cell-bound IgG molecules, but does not yield information on whether these IgG molecules are equally spread among RBCs of different age or not. To investigate whether the extra amount of IgG molecules was confined to a particular RBC subpopulation or not, we determined IgG molecules bound to erythrocytes of different densities. RBCs from controls and healthy Coombs-positive blood donors were separated with respect to their density that for normal RBCs yields a distribution according to their cell age with the oldest cells having the highest density (Clark et al., 1983; Lutz et al., 1992). The density distributions were similar for healthy Coombs-positive blood donors and age/sex matched controls, except that healthy Coombs-positive blood donors had significantly more RBCs banding at high densities and had RBCs with even higher densities than that of the oldest cells of controls (Fig. 6). The occurrence of very dense RBCs was best seen in Percoll gradients generated from well-packed RBCs, where the majority of the cells banded in the first third of the tube (Coombs 400953 Fig. 6). The densest RBCs of Coombs 253343 and control B were fractionated in two tiny fractions a and b (Fig 6). Fractions a and b of CRBCs contained 3.2%, and 2.2% of total CRBCs respectively, whereas fraction a of control RBCs contained 2% of total RBCs and fraction b did not contain any cells. Fractionated RBCs were studied for the absolute cell age by determining the ratio of the protein content in band 4.1 a to that in band 4.1 b (Inaba et al., 1992; Inaba and Maede, 1988). The results show that CRBCs in fractions a and b had band 4.1 a/b ratios of 10 and 12, respectively. RBCs in fraction a had a band 4.1 a/b ratio of 6.
For analysis of RBC-bound IgG, we collected six fractions numbered in growing order from the top to the bottom of the tubes, in which the sixth fraction contained ~ 5% of total RBCs. The absolute cell age of the fractionated RBCs was determined as described above (Fig. 7B). The results show that both types of cells had increased band 4.1 a/b ratios with density, but the absolute cell ages were lower than in Figure 6, because larger portions of the whole population were analyzed. Nevertheless, the lightest cells were young, and the densest cells were old erythrocytes. The densest CRBCs had even higher band 4.1 a/b ratios than the densest RBCs.

The amount of IgG associated with erythrocytes of different cell age was measured by $^{125}$I-Protein G binding assays (Fig. 7C). The number of RBC-bound IgG NAbs increased with cell age for both controls and healthy Coombs-positive blood donors. In controls, young red blood cells with band 4.1 a/b ratios of 1 to 3,0 carried 100 to 150 IgGs per cells, whereas, old cells with band 4.1 a/b ratios between 3,5 and 4,5 bore 150 to 210 IgGs per cell. On the contrary, in healthy Coombs-positive blood donors young RBCs with band 4.1 a/b ratios from 1,5 and 3,5 carried 140 to 250 IgGs per cell. Old cells were characterized by band 4.1 a/b ratios between 4 and 6,3 and carried 250 to 580 IgGs per cell. RBCs from healthy individuals with DAT 1+ had similar amounts of IgG molecules per cell as normal RBCs. However, healthy Coombs-positive blood donors with DAT 2+ and 3+ had RBCs that reached a higher absolute cell age than control cells and carried even more NAbs than the oldest control RBCs. In the healthy individual with DAT 3+ RBCs of any cell age carried more IgG molecules than cells from all other samples. However, the majority of IgG was again bound to aged cells with a band 4.1 a/b ratio that was even higher than that of aged RBCs from blood donors with a low DAT value.
Figure 6. Density distribution of CRBCs and RBCs.

RBCs from healthy Coombs-positive blood donors were density separated by Percoll gradients in two different experiments, along with RBCs from age and sex matched controls (for a and b see text).
Figure 7. IgG NAbs bound to erythrocytes of different cell age.

(A) RBCs from controls and healthy Coombs-positive blood donors were density separated into 6 fractions on self forming Percoll gradient. (B) Membrane proteins from fractionated RBCs were separated on SDS-PAGE and stained by Coomassie blue. Band 4.1 a/b ratios were calculated from values obtained by densitometric quantification of band 4.1 a and band 4.1 b. (C) IgG per cell measured by $^{125}$I-protein G binding versus band 4.1 a/b ratios.
7.3 Characterization of RBC-bound IgG NAbs

So far nobody analyzed the specificity of cell-bound IgG eluted from young and old RBCs, but several authors reported the difficulty to recover enough IgG molecules (reviewed by (Bosman et al., 2005). Erythrocytes from controls and healthy Coombs-positive blood donors were density separated. Since we knew from the $^{125\text{I}}$-Protein G binding assays (Fig. 7C) that one RBC carries $\geq 100$ IgG molecules, $10^{10}$ RBCs would only yield 0.25mg IgG NAbs. To elute enough IgG molecules for their characterization, we fractionated the density separated RBCs into five large portions to elute $40 \times 10^{10}$ RBCs (Fig. 8A). The lightest cells (fraction 1), middle dense (fraction 3), and the densest cells (fraction 5) were studied. The number of IgG molecules bound to the fractionated RBCs was quantified by $^{125\text{I}}$-Protein G binding assays and FACS analysis (Fig. 8B). The results revealed that cell-associated NAbs did not significantly increase in these large fractions, although the densest cells from each separation were indeed enriched in old RBC based on the band 4.1 a/b ratio. IgG molecules were acid eluted from the fractionated RBCs, purified on Protein G Sepharose and quantified. More IgG could be eluted from old than from young and middle-aged RBCs (Fig. 8B).

Isolated IgG molecules were iodinated and their binding specificity was analyzed on blots from electrophoresed C3, IgG, RBC membrane proteins, and F(ab')$_2$ fragments (Fig. 9). Since small amounts of IgG molecules were eluted from young and middle-aged RBCs of all samples, we pooled these IgGs from each subject and referred to it as "IgGs from younger cells". Surprisingly, IgG eluted from old erythrocytes of control and healthy Coombs-positive blood donors bound to all immobilized proteins, whereas IgG from younger cells from controls as well as from healthy Coombs-positive blood
Results

donors bound almost exclusively to intact IgG. The only exception was IgG eluted from younger RBCs of one healthy Coombs-positive blood donor (number 1831), which also bound to C3 proteins. Thus, IgG eluted from younger erythrocytes of controls and healthy individuals with a positive DAT value contained primarily anti-IgG molecules. These antibodies were Fc specific because they did not bind to F(\(\text{ab}'\))2 fragments. In contrast, IgG eluted from senescent red cells of all samples contained anti-band 3, anti-C3, anti-IgG, and anti- F(\(\text{ab}'\))2 IgG NAbs. Hence, IgG eluted from Coombs-positive RBCs and controls did not differ qualitatively. Knowing that acid eluted IgG may be comprised of protein complexes, labeled IgGs eluted from RBCs were gel filtrated in 5M urea and the IgG peak was collected. Interestingly, binding of IgGs from old control and CRBCs increased to all immobilized proteins after complex dissociation (Fig. 10). Thus, NAbs bound to RBCs and CRBCs were either complexed to begin with or formed complexes during acid elution. We determined the urea-mediated increase in the binding intensities for all individual polypeptides (Table 2). Urea increased IgG binding by 0.92 (binding to C3) up to 3 fold (binding to Fab) in controls. In contrast, the binding of IgG eluted from CRBCs increased significantly more by a urea treatment for C3, its peptides, for IgG LC, and band 3. Thus, senescent CRBCs carried more IgG complexes than control cells and these complexes presumably contained primarily anti-C3, LC-specific anti-F(\(\text{ab}'\))2, and anti-band 3 NAbs. In contrast to the results shown for NAbs eluted from old RBCs and CRBCs, IgG eluted from younger CRBCs showed upon dissociation and repurification binding to IgG and also some binding to C3 and F(\(\text{ab}'\))2 fragment, but hardly any binding to RBC membrane proteins, except for one healthy Coombs-positive blood donor (number 1831) with some binding to band 3 (Fig. 11). Thus, IgG NAb associated with younger cells differed significantly in their specificities from those eluted from old CRBCs and RBCs.
**Results**

**A**

![Image](image_url)

- **Collected fractions for characterization of RBC-bound IgG**

**B**

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<tr>
<th>Samples</th>
<th>DAT</th>
<th>RBC density fractionation</th>
<th>percentage of all RBCs</th>
<th>ratios of band 4.1 a/b</th>
<th>¹²⁵I-Protein G binding assay</th>
<th>FACS analysis</th>
<th>Tot of IgG µg eluted from fractionated RBCs</th>
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Figure 8. IgG NAbs bound to RBCs density separated and collected as large fractions.

(A) Control and Coombs-positive RBCs were density separated into 5 large fractions on self forming Percoll gradients. Fractions 1, 3, 5 were collected. (B) The cell age was determined by the band 4.1 a/b ratio. RBC-bound IgG NAbs were measured by ¹²⁵I-protein G binding and FACS. IgGs eluted from the factonated RBCs were quantified by densitometry of stained SDS-PAGE gels.
Figure 9. Binding specificity of $^{125}$I-IgG NAbs eluted from RBCs.

IgG NAbs acid-eluted from fractionated RBCs as described in Fig. 6 were purified on Protein G Sepharose. IgG NAbs eluted from RBC fractions 1 and 3 were pooled and referred to as “IgG from younger cells”. IgG molecules were $^{125}$I-iodinated and their binding specificity was studied on immunoblots. Reduced and not reduced (red= reduction) proteins (2 μg/lane) were blotted onto a membrane that was incubated with iodinated proteins (0,5 x $10^6$ cpm/ml).
Figure 10. Binding specificity of IgG NAbs eluted from old RBCs before and after dissociation and repurification of eluted IgG.

$^{125}$I-labeled IgG NAbs eluted from old RBCs of control and healthy Coombs-positive blood donors were gelfiltrated in 5 M urea on a Sephacryl S-200. The binding specificity of the dissociated IgG NAbs was analyzed on immunoblots and compared to that before dissociation (from Fig. 9).
Table 2. Coombs-positive RBCs carry more NAb complexes than control RBCs.

Binding of IgGs from old CRBCs and RBCs were quantified by densitometry of the autoradiography from the blots shown in Fig. 10 before and after dissociation of NAb complexes with 5 M urea. Total binding (after urea treatment), which is given as intensities x mm² as obtained by Quantity One, and the relative binding (after/before urea treatment) are shown in the above table.
### Results

<table>
<thead>
<tr>
<th>Before NAb complexes dissociation</th>
<th>After NAb complexes dissociation</th>
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<tr>
<td>Coombs 1831</td>
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<td>Coombs 400953</td>
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**Legend:**
- C3 / IgG
- α C3
- F(ab')<sub>2</sub>
- Band 3
- β C3
- HC
- LC

**Figure 11. Binding specificity of IgG NAbs eluted from younger RBCs upon dissociation and repurification.**

\(^{125}\)I-iodinated IgG NAbs eluted from younger Coombs-positive RBCs were gelfiltrated in 5M urea on a Sephacryl S-200 column. The binding specificity of the dissociated NAbs was studied on immunoblots and compared to that before dissociation (from Fig. 9).
7.4 Anti-C3 and anti-F(\text{ab}')\textsubscript{2} IgG NAbs in plasma

The fact that the DAT value of healthy Coombs-positive blood donors can fluctuate over the years and that the RBC membranes of these donors did not reveal any pathology suggested that the extra number of NAbs bound to the CRBCs may originate from higher NAb titers. Since the immunoblots from acid-eluted IgG strongly suggested that healthy Coombs-positive blood donors had more anti-C3 and presumably anti-idiotypic anti-IgG trapped in complexes, we investigated their plasma for anti-C3 and anti-F(\text{ab}')\textsubscript{2} NAbs by ELISA before and after dissociation of IgG complexes with 5M urea. Urea pre-treated and untreated plasma samples from healthy Coombs-positive blood donors bound significantly better to C3b and F(\text{ab}')\textsubscript{2} fragments than control plasma (Fig. 12). The binding of untreated plasma from healthy Coombs-positive blood donors to C3b was 1,1 to 5,8 times stronger than that of untreated control plasma. After urea treatment it was also 1,5 to 5,9 times higher than that of control plasma. The binding of IgG from plasma of healthy Coombs-positive blood donors to F(\text{ab}')\textsubscript{2} fragments was 0,6 to 3 times higher than that of controls before urea treatment, whereas after urea treatment it was 1,4 to 4,9 times stronger. Thus, plasma from healthy individuals with a positive DAT value contained more anti-C3 and anti-F(\text{ab}')\textsubscript{2} IgG than normal plasma.

Since normal human plasma contains not only anti-idiotypes, but also a substantial concentration of anti-hinge NAbs (Terness et al., 2002), we determined the binding to C3b and F(\text{ab}')\textsubscript{2} fragments before and after depletion of anti-hinge IgG NAbs (Fig.13). The conditions chosen for absorption of anti-hinge NAbs did not also deplete the plasma from anti-idiotypic NAbs (Jelezarova and Lutz, 2005). The untreated samples
showed the portion of accessible IgG NAbs in the plasma, while the urea pre-treated samples revealed the total concentration of the analyzed antibodies. Moreover, the binding of plasma IgG depleted of anti-hinge NAbs to F(ab')2 fragments yielded the concentration of anti-idiotypes. The total NAb concentrations exceeded the accessible concentrations by 2 to 9 fold in all samples (Fig. 13), indicating that the majority of these NAbs were complexed in the plasma of controls and healthy Coombs-positive blood donors. Nine out of ten Coombs-positive plasma had higher total concentrations of anti-C3 NAbs than controls before anti-hinge depletion (Fig. 13 A). Expectedly, the depletion of anti-hinge NAbs did not significantly alter anti-C3 NAb determination in controls and healthy Coombs-positive blood donors, except that only 6 out of 10 had higher anti-C3 NAbs than controls (Fig. 13 B). The total binding of plasma from all healthy Coombs-positive blood donors to F(ab')2 fragments was 1.3 to 2.5 times higher than that of controls before anti-hinge NAb depletion (Fig. 13C). Plasma from five healthy Coombs-positive blood donors contained high concentrations of accessible anti-F(ab')2 NAbs, which in three samples were anti-hinge NAbs, because the signal was lowered by absorbption of anti-hinge NAbs. In the other two samples the accessible portion of anti-F(ab')2 NAbs remained high, implying the presence of a 6 to 7 fold higher concentration of accessible anti-idiotypes (Fig. 13 D). In this experiment, two Coombs-positive samples (No. 4 and 5) shown in figure 11 were re-tested. Sample No. 5 showed that the high accessible portion of anti-F(ab')2 NAbs was due to anti-hinge NAbs. On the contrary, the other re-tested sample (No. 4) having a low accessible portion of anti-F(ab')2 NAbs did not reveal differences before and after anti-hinge NAb depletion (Fig. 13 C and D).
Antibody concentrations detected by ELISA

Figure 12. Anti-C3 and anti-F(ab')2 NAbs in plasma from healthy Coombs-positive blood donors and controls.

The binding of 9 control plasma samples and 5 Coombs-positive plasma samples to C3b and F(ab')2 plates was assayed by ELISA before and after 6 M urea treatment.
Figure 13. Total versus accessible plasma anti-C3 and anti-F(ab')2 NAbs before and after depletion of anti-hinge NAbs.

Binding of plasma IgG from controls and healthy Coombs-positive blood donors to C3b and F(ab')2 was analyzed by ELISA before and after anti-hinge NAbs depletion. Total anti-C3 and anti-F(ab')2 NAbs (urea-pretreated samples) are plotted versus accessible NAb concentrations (untreated samples). The open symbol (o) indicates sample No. 4 and the star symbol (*) sample No. 5 also analyzed in the experiment shown in Fig. 12.
7.5 **Phagocytosis of Coombs-positive RBCs.**

To clarify why RBCs from healthy Coombs-positive blood donors carried more NAbs than senescent control RBCs and evidently continued to circulate, we investigated their in vitro phagocytosis. RBCs from density fraction 1 (=young RBCs), fraction 3 (=middle-aged RBCs), fraction 5 (=old RBCs), and fraction 6 (=very old RBCs) were incubated with THP-1 macrophage-like cells in the presence and absence of fluid phase whole human IgGs. Ingested RBCs were fluorescently labeled and the extent of phagocytosis was determined. In the presence and absence of physiological concentrations of IgG, young and middle-aged RBCs from controls and healthy Coombs-positive blood donors did not show a significant phagocytosis (Fig. 14). On the contrary, ingestion of old and very old RBCs from both sources was significant. Moreover, phagocytosis of very old RBCs from healthy Coombs-positive blood donors was 1.5 to 2 times higher than that of control cells in the absence of fluid phase IgGs. This implies that very old Coombs-positive RBCs carried more opsonins than aged control RBCs. The uptake of old and very old cells from healthy individual with DAT 1+ was up to 7 ± 0.72% and 10.5 ± 0.9%, respectively. We could not detect phagocytosis of the very old cells from the healthy individual with DAT 3+, most likely because highly opsonized RBCs agglutinated as was evident from the slides. However, the ingestion of old cells from this individual was up to 9.9 ± 0.7%. In contrast, old and very old RBCs from controls were ingested up to 5.7 ± 1.2% and 6.3 ± 0.6%, respectively. Thus, very old erythrocytes from healthy Coombs-positive blood donors were phagocytosed to a higher extent than control cells in the absence of fluid phase IgG (Fig. 14). On the other hand, addition of physiological concentration of pooled...
whole human IgG during phagocytosis lowered the uptake of RBCs from both sources. Phagocytosis of very old CRBCs from one healthy Coombs-positive blood donor (No. 430097) was even lower than that of controls, with the results being significantly different (p ≤ 0.03).
Figure 14. Old and very old CRBCs are more readily phagocytosed than RBCs, but only in the absence of fluid phase IgGs.

Young, middle-aged, old and very old RBCs from controls and blood donors with DAT 1+ and DAT 3+ were incubated for 1h at 37° with THP-1 cells in the presence and absence of 12 mg/ml pooled whole human IgG. Not ingested RBCs were lysed with 0.1% NaCl and ingested RBCs were fluorescently labeled. The mean value of ingested RBCs per 100 THP-1 was determined.
8 Discussion

Erythrocytes of healthy Coombs-positive blood donors bear higher numbers of IgG molecules than control senescent RBCs. In contrast to aged red cells from controls, erythrocytes from healthy Coombs-positive blood donors are not immediately removed from the circulation, despite their high numbers of bound IgGs. During aging normal RBCs undergo several membrane modifications, whereby anti-band 3 NAbs associate with band 3 clusters and induce C3b deposition (Lutz et al., 1987; Lutz et al., 1984). Macrophages within the reticuloendothelial system recognize RBCs carrying C3b/iC3b and IgG NAb complexes and eliminate them. Here we show that senescent erythrocytes from healthy Coombs-positive blood donors carry significantly more NAbs than control old RBCs and some of these NAbs inhibited the erythrophagocytosis in vivo and in the presence of IgG in vitro. The extra number of RBC-bound immunoglobulins appear to comprise anti-C3 and framework-specific anti-idiotypic NAb complexes, which bind to complement proteins on aged RBCs and may block the interaction of C3b/iC3b with CRs on phagocytic cells. Hence, erythrocytes from healthy Coombs-positive blood donors circulate longer and reach an older age than control RBCs.

8.1 Coombs-positive RBCs carrying anti-C3 and framework-specific anti-idiotypic NAbs have a prolonged lifespan.

We could show that IgG molecules eluted from old and very old erythrocytes of all samples contained anti-band 3, anti-C3, LC-specific anti-IgG NAbs (Fig. 9 and 10). Since these RBCs-bound IgG NAbs induced in vitro erythrophagocytosis (Fig. 14), it is conceivable that anti-band 3 NAbs associated with band 3 clusters and induced C3b
deposition to the cell and bound anti-band 3 as C3b$_2$-IgG complexes in controls as well as in healthy Coombs-positive blood donors (Fig. 15) (Low et al., 1985; Lutz and Stringaro-Wipf, 1983; Turrini et al., 1991) (Lutz et al., 1987; Lutz et al., 1984) (Lutz et al., 1993b; Lutz et al., 1993c). Anti-C3 NAbs that were higher in plasma of healthy Coombs-positive blood donors (Fig. 12 and 13) presumably bound to these C3b/iC3b dimers or C3b$_2$-IgG/iC3b$_2$-IgG complexes. The majority of RBC-bound anti-IgG NAbs were presumably anti-idiotypes rather than anti-hinge NAbs (Fig. 9 and 10), as they were predominantly light chain-specific. Furthermore, they appeared to be framework-specific anti-idiotypes, since they bound to antibodies, which were associated with their antigens. Additionally, these framework-specific anti-idiotypes might even be specific for anti-C3 NAbs, knowing that anti-C3 NAbs are associated with framework-specific anti-idiotypes in pooled whole human IgG (Jelezarova and Lutz, 2005). Thus, anti-C3 and anti-C3- and framework-specific anti-idiotypic NAb complexes (IgG complexes) may have been associated with C3b/iC3b dimers and C3b$_2$-anti-band 3 complexes on senescent erythrocytes from Coombs-positive blood donors and less to those from controls (Fig. 15).

Our data show that senescent RBCs from healthy Coombs-positive blood donors bore more IgG molecules than aged control RBCs. In fact, the total binding of IgGs eluted from aged CRBCs to all immobilized proteins was 1.2 to 1.7 times that of IgGs from old RBCs (Fig. 10 and Table 2). Additionally, dissociation of NAb complexes from IgG eluates and their purification yielded IgG molecules that bound considerably better to C3 and LC, when obtained from senescent RBCs of healthy Coombs-positive blood donors as compared to those from controls (Table 2). For example, the binding of IgGs eluted from aged CRBCs to C3 and its subunits increased by 1.3 to 4.9 fold after urea treatment and the binding to the light chain of IgG increased by 7 to 31.5 fold. This
suggests that senescent CRBCs carried aside of anti-band 3 NAbs IgG complexes that contained anti-C3 and framework-specific anti-idiotypic NAbs. Thus, the extra amounts of IgG molecules, which were bound to senescent CRBCs contained anti-C3 and anti-C3-specific- and framework anti-idiotypic NAbs, which may have blocked CRBC-bound C3b/iC3b molecules and thereby prolonged their life span.

We indeed showed that erythrocytes from healthy Coombs-positive blood donors circulate longer in vivo than control cells, because band 4.1 a/b ratio, which is a measure of the absolute cell age (Inaba and Maede, 1988), revealed that the densest CRBCs were significant older than RBCs (Figs. 6, 7B and 7C). Correspondingly, these CRBCs had higher densities than the densest RBCs of age matched controls (Fig. 6A). We further showed that it was primarily this fraction of the whole CRBC population, which carried high numbers of IgG molecules (Fig. 7C). It appears that the prolonged RBC survival increased with increased DAT values. Indeed, erythrocytes from healthy individuals with a high DAT value carried even more IgG molecules than senescent cells from individuals with a low DAT value (Fig. 7C).

8.2 Younger RBCs from controls and healthy Coombs-positive blood donors carry C3b-containing ICs associated with CR1

We show for the first time that the small number of IgG molecules, which associate with young and middle-aged RBCs differ in their specificity from those bound to old RBCs. Moreover, control and Coombs-positive erythrocytes did not differ in this respect (Fig. 9). Due to minimal amounts, NAbs eluted from young and middle-aged RBCs were pooled and referred to as “NAbs from younger RBCs”. These IgG molecules liberated from protein complexes contained primarily Fc-specific anti-IgG and little anti-C3 and anti-F(ab')2 NAbs. Since these RBC-associated IgG molecules
promoted hardly any erythrophagocytosis (Fig. 12) and did not contain anti-band 3 NAbs (Fig. 9 and 11), it is conceivable that they were bound to CR1 by interacting with C3b/iC3b-containing ICs, which themselves bound to CR1 (Fig. 15) (Reinagel et al., 1997). We could isolate only minimal amounts of these NAbs as compared to what we measured by \(^{125}\text{I}-\text{Protein G binding assays and FACS analysis (Fig. 8B). Several reasons can account for this discrepancy. The washing procedure for isolation of bound NAbs differed from that to detect cell-bound IgG. Another explanation might be that IgGs trapped in ICs could only partly interact with Protein G Sepharose during antibody purifications.}

NAbs from younger RBCs of one Coombs-positive blood donor (No. 1831), who was the only elderly donor (Table 1), also contained some anti-band 3 NAbs (Fig. 11). Several studies showed that erythrocytes from elderly rodents had a shortened half-life as compared to cells from young rodents (Glass et al., 1983; Magnani et al., 1988). The shortened RBC life span in elderly mice appeared to be the result of a premature exposure of a surface antigen recognized by the immune system (Glass et al., 1983). In analogy to these findings, younger RBCs from the elderly Coombs-positive blood donor (No. 1831) may have carried some anti-band 3 NAbs, presumably because these CRBCs exposed band 3 clusters earlier than RBCs from young donors.
Figure 15. Hypothesis on how IgG NAbs may bind to young and old RBCs.
A) Younger RBCs from controls and healthy Coombs-positive blood donors may carry C3b-containing ICs bound to CR1 clusters. B) Old RBCs and CRBCs carry anti-band 3 NAbs, of which some represent C3b$_2$-anti-band 3 complexes. Old RBCs from healthy Coombs-positive blood donors carry in addition more anti-C3 and framework-specific anti-idiotypes bound to C3b$_2$-IgG complexes.
8.3 High plasma anti-C3 and corresponding anti-idiotypic NAb concentrations lead to a positive DAT value

Healthy Coombs-positive blood donors can maintain a positive DAT value over years, during which in some donors the DAT value fluctuated, while in others it became negative (Table 1). The occurrence of a positive DAT value was accompanied by increased concentrations of anti-C3 and anti-idiotypic NAbss in plasma of these blood donors (Fig. 13). The finding of increased NAb titters as such was unexpected, because the concentration of several other NAbss appears to be constant throughout life for healthy adults (Lacroix-Desmazes et al., 1999). Our data show that the occurrence of a positive DAT value was independent of the blood donors’ age, except for healthy Coombs-positive blood donor No. 1831 (Table 1). This is in contrast to the results reported by Gorst and colleagues, who analyzed three times as many cases as we did. They found a strong correlation between a positive DAT value and the age of the individuals (Gorst et al., 1980). Furthermore, we did neither detect a correlation between the DAT value and the gender nor did we detect a correlation between the DAT value and the blood group of the donors (Table 1).

The mechanism that leads to a positive DAT value is still elusive. We can, however, conclude that healthy Coombs-positive blood donors had higher anti-C3 and anti-idiotypic NAb concentrations in their plasma than controls (Fig. 12 and 13). Nevertheless, the magnitude of the DAT value appeared to be independent of the total plasma concentration of anti-C3 and anti-F(ab')2, and anti-idiotypic NAbss (Fig. 16). We were not able to show any relation between the DAT value and the total plasma concentration of anti-C3- and framework-specific anti-idiotypic NAbss, presumably because we measured their binding to F(ab')2 fragments of pooled whole human IgG,
Discussion

rather than to F(\text{ab}')_2 fragments from anti-C3 N Abs. Furthermore, ELISA data showed that these N Abs existed as complexes in the plasma of Coombs-positive donors (Figs 12 and 13). The ratios of total anti-C3/anti-idiotypic N Abs (1.1 ± 0.2) in plasma of healthy Coombs-positive blood donors were in the same range as those in plasma of controls, indicating that CRBC-bound anti-C3 and framework-specific anti-idiotypic N Abs did not promote C3 convertase generation as observed with ratios higher ~ 1.8 (Jelezarova and Lutz, 2005). Interestingly, the ratio of CRBC-bound anti-C3/anti-idiotypic N Abs was considerably lower than that of controls based on the relative binding of eluted, dissociated, and purified IgG to IgG LC and C3 (Table 2). If this finding could be established for unlabeled material, it would imply that bound N Abs had an inhibitory effect on C3 convertase generation. However, the analysis of anti-C3 N Ab and its corresponding framework-specific anti-idiotypes is still incomplete. To clarify the role played by this N Ab pair, we would have to isolate pure anti-C3 and framework-specific anti-idiotypic N Abs. The isolation of these individual IgG N Abs is extremely difficult, because they tend to form complexes as shown here and when obtained from IVIG (Jelezarova and Lutz, 2005).
Discussion

Figure 16. Relations between DAT values and concentrations of anti-C3, anti-F(ab')2 and anti-idiotype NAbs in plasma from healthy Coombs-positive blood donors (from Fig. 14).
8.4 Complement-mediated phagocytosis of aged Coombs-positive RBCs is inhibited by anti-C3 and framework-specific anti-idiotypic NAbs.

We could demonstrate that senescent CRBCs are more efficiently phagocytosed than senescent RBCs in the absence of plasma proteins (Fig. 14). In fact, the extent of erythrophagocytosis was clearly dependent on the number of cell-bound IgG molecules and therefore it was higher for senescent CRBCs than old RBCs and even higher for CRBCs from a blood donor with DAT 3+ as compared to CRBCs from a blood donor with DAT 1+ (Fig. 14). On the contrary, the extent of phagocytosis of CRBCs in the presence of physiological concentrations of IgG was less or the same as for senescent RBCs. These findings provide in vitro evidence for why CRBCs are not readily eliminated in vivo despite their increased number of cell-bound IgG. Anti-C3 NAbs and framework-specific anti-idiotypes, which were associated with complement proteins on aged CRBCs, evidently inhibited the interaction of C3b/iC3b with their receptors on phagocytic cells in vivo as well as in vitro when FcR dependent phagocytosis was inhibited by physiological concentrations of IgG. The lack of accessible C3b/iC3b may have reduced the extent of erythrophagocytosis, while fluid phase IgG competitively inhibited cell-bound IgG interactions with FcRs on phagocytic cells, as shown earlier by Fries and coworkers for RBCs opsonized by IgG or C3b2-IgG complexes (Fries et al., 1987; Malbran et al., 1987). Thus, in vivo uptake of CRBCs may be delayed because the highly opsonic C3b/iC3b molecules are prevented from stimulating phagocytosis via CRs. This possibility is supported by the long known effect of intravenously applied pooled whole human IgG in idiopathic thrombocytopenic purpura (ITP) patients, where ingestion of IgG-opsonized targets is reduced (Fehr et al., 1982; Kimberly et al., 1984).
Taken together the findings presented in this thesis suggest that plasma of healthy Coombs-positive blood donors contain high concentrations of anti-C3 and framework-specific anti-idiotypic NAbs that form complexes and may associate with C3b/iC3b dimers or C3b$_2$-IgG/iC3b$_2$-IgG complexes on CRBCs, whereby they prevent the interaction of C3b/iC3b with CRs on phagocytes and decrease the extent of phagocytosis of these erythrocytes (Fig. 17).

**Figure 17. Hypothesis on how anti-C3 and their framework-specific anti-idiotypic NAbs may prevent efficient phagocytosis in the presence of fluid phase IgG.**

Anti-C3 NAbs together with anti-idiotypic NAbs bind to C3b/iC3b dimers or C3b$_2$-IgG/iC3b$_2$-IgG complexes on aged Coombs-positive RBCs. This association hinders the interaction of RBC-bound C3b$_2$/iC3b$_2$ with their receptor on macrophages in the presence of physiological concentrations of fluid phase IgG.
9 Bibliography


Bibliography


## Abbreviations

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<tr>
<td>AE1</td>
<td>Anion exchanger protein 1</td>
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<tr>
<td>AHIA</td>
<td>Autoimmune hemolytic anemia</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<td>SIRP</td>
<td>Signal regulatory protein</td>
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<td>WAHIA</td>
<td>Warm autoimmune hemolytic anemia</td>
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