Aus dem Institut für Transfusionsmedizin
der Medizinischen Fakultät Charité–Universitätsmedizin Berlin

# DISSERTATION

## Pathophysiologie des Erythrozytenabbaus bei Patienten mit Autoimmunhämolytischen Anämien (AIHA)

Pathophysiology of Erythrocyte Destruction in Patients with
Autoimmune Hemolytic Anemia (AIHA)

zur Erlangung des Akademischen Grades
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von

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**Abkürzungsverzeichnis**

| | |
|---|---|
| Aab | Autoantikörper |
| AIHA | Autoimmunhämolytische Anämie |
| cAIHA | Autoimmunhämolytische Anämie vom Kältetyp |
| C1-C9 | Komplement-Komponente 1-9 |
| $Ca^{2+}$ | Calcium |
| CD | Cluster of differentiation 95 |
| Ch | Chido, auf den Erythrozytenmembranen lokalisiertes Antigen |
| $CO_2$ | Kohlendioxid |
| CR | Komplementrezeptor |
| DAT | Direkter Coombstest |
| EPO | Erythropoetin |
| EDTA | Ethylendiamintetraessigsäure |
| FACS | Fluorescence Activated Cell Sorting *(*Durchflusszytometrie*)* |
| FCS | Fetales Kälberserum |
| FITC | Fluoreszenzthiozyanat |
| FSC | Forward Scatter |
| GPDH | Glucose-6-Phosphat-Dehydrogenase |
| HEPES | N-2- Hydroxyethyl-piperazin-N'-2-Ethansulfonsäure |
| Hb | Hämoglobin |
| Ig | Immunoglobulin |
| K | Kell, auf den Erythrozytenmembranen lokalisiertes Antigen |
| KAK | Kälteautoantikörper |
| LDH | Laktatdehydrogenase |
| MAC | Membrane-Attack-Complex |
| MMA | Monozyten-Monolayer-Assay |
| mM | millimolar |
| PBS | Phosphate-Buffered Saline |
| PKH26 | KIT zur Zellmembranfärbung |

| | |
|---|---|
| PS | Phosphatidylserin |
| PE | Phycoerythrin |
| PMA | Phorbol 12-Myristat 13-Acetat |
| RETR | Retikulozyten |
| RBC | Erythrozyten, rote Blutkörperchen |
| Rh | Rhesus „auf den Erythrozytenmembranen lokalisiertes Antigen“ |
| RPMI | Zellkulturmedium |
| wAIHA | Autoimmunhämolytische Anämie vom Wärmetyp |
| WAK | Wärmeautoantikörper |
| Yt | Antigensystem (bekannt als Cartwright), auf den Erythrozytenmembranen lokalisiertes Antigen |
| U | Units |

# 1 Zusammenfassung der Publikationen

## 1.1 Abstrakt (Deutsch)

Die Autoimmunhämolytische Anämie (AIHA) ist eine Autoimmunerkrankung mit verkürzter Überlebenszeit der roten Blutkörperchen (Erythrozyten). Die Erkrankung wird durch spezifische Autoantikörper gegen Erythrozyten verursacht. Die verkürzte Überlebenszeit der Erythrozyten erfolgt bekannter Weise durch Fc- und / oder C3b-vermittelte Phagozytose oder in seltenen Fällen durch direkte Zelllyse bei Aktivierung der terminalen Komplementkomponenten. Die Bildung von Antikörpern gegen Erythrozyten und ihre klinische Relevanz sind variabel. Die Reaktivität der Autoantikörper ergibt oft keinen sicheren Hinweis auf die Stärke der Autoimmunhämolyse (Zellzerstörung) und lässt daher keinen eindeutigen Rückschluss auf die klinische Relevanz zu.

In der vorliegenden Arbeit wurden drei Aspekte zur weiteren Charakterisierung der AIHA untersucht: 1. die klinische Relevanz nachweisbarer Autoantikörper; 2. eine mögliche Rolle der Eryptose, dem suizidalen Tod von Erythrozyten, bei AIHA. Die Eryptose ist durch die Exposition von Phosphatidylserin (PS) an der Zelloberfläche charakterisiert und kann durch eine Vielzahl von Faktoren wie oxidativen Stress, osmotischen Schock oder Energiemangel katalysiert werden. Aus dem Vorkommen der Eryptose bei AIHA ergab sich als 3. Aspekt, ob die Komplementaktivierung bei Eryptose eine Rolle spielt.

Um die klinische Relevanz der Antikörper gegen Erythrozyten zu bestimmen, wurde ein Durchflusszytometrie-basierter Erythrophagozytose-Assay unter Verwendung der monozytären THP-1-Zelllinie und PKH26 red-gefärbten Erythrozyten etabliert. In diesem Test korrelierte die Erythrophagozytose bei Patienten mit AIHA vom Wärmetyp mit der Hämolyse *in vivo*. Dagegen ließ sich bei Patienten mit einer signifikanten AIHA vom Kältetyp keine erhöhte Erythrophagozytose feststellen. Dies deutet darauf hin, dass die Erythrozyten-Zerstörung nicht immer durch Fc- bzw. C3b vermittelt wird. Die Frage, ob Autoantikörper gegen Erythrozyten eine Eryptose, den suizidalen Tod von Erythrozyten, auslösen können, war bisher nicht bekannt. In der vorliegenden Arbeit wurde festgestellt, dass Patienten mit Autoimmunhämolyse aufgrund von IgM- oder IgA-Autoantikörpern eine Eryptose zeigten, nicht jedoch Patienten mit IgG-Autoantikörpern. Da die klinisch relevanten Kälteautoantikörper vom IgM-Typ komplementaktivierend sind, stellte sich daraus die Frage, ob Komplementkomponenten auch bei der Eryptose eine Rolle spielen.

Dabei wurde in dieser Arbeit zum ersten Mal festgestellt, dass die Eryptose durch IgM-Autoantikörper von der Komplementaktivierung abhängt. Sie beginnt bereits bei der Formation des ersten Komplexes (C5b6) und nimmt mit weiterer Aktivierung der terminalen Komplexe bis zur Bildung des Komplexes C5b-8 zu. Im Vergleich dazu wird die IgA-induzierte Eryptose offensichtlich mechanisch und ohne Komplementaktivierung verursacht.

## 1.2 Abstract (English)

Autoimmune haemolytic anaemia (AIHA) is an autoimmune disease characterized by accelerated red blood cells (RBC) destruction. Autoantibodies to RBC are usually of IgG-, IgM- and rarely of IgA classes. It is known that the destruction of erythrocytes in patients with autoimmune hemolytic anemia is mediated by Fc- and / or C3b-phagocytosis, and in rare cases via direct cell-lysis by activating the terminal complement components. The clinical relevance of autoantibodies to erythrocytes is variable, and cannot usually be determined by standard serological testing.

In the present work, three aspects related to autoimmune haemolytic anaemia were studied: 1) the clinical relevance of detectable autoantibodies; 2) whether eryptosis occurs in autoimmune haemolytic anaemia. Eryptosis, the suicidal death of erythrocytes is characterized by exposure of phosphatidylserine (PS) on the erythrocyte surface, and can be catalyzed by a variety of factors such as oxidative stress, osmotic shock and lack of energy. The second aspect led to the question, 3) whether complement activation plays a role in eryptosis.

To assess the clinical relevance of the antibodies to erythrocytes, we established a flow-cytometry-based erythrophagocytosis assay using PMA-treated THP-1 cells and PKH26 red-labeled RBC. In this test, erythrophagocytosis in patients with AIHA of warm-type correlates with the haemolysis *in vivo*. In contrast, no increased erythrophagocytosis was observed in patients with significant AIHA of cold type. This suggests that RBC destruction is not always mediated by Fc or C3b. The question whether antibodies to RBC can also cause an eryptosis has not yet been addressed. In the present work, we demonstrated that RBC of patients with autoimmune haemolysis due to IgM- or IgA-autoantibodies may undergo eryptosis, but not those of patients with autoimmune haemolytic anaemia related to IgG autoantibodies. Hence, cold autoantibodies of the IgM class are complement-activating, the question raised whether complement components may play a role in eryptosis. We demonstrated for the first time that eryptosis due IgM autoantibodies is dependent on complement activation, which begins with the formation of the first terminal complex (C5b6), and becomes most significantly by the formation of C5b-8 complex.

# 2 Einleitung

## 2.1 Autoimmunhämolytische Anämie (AIHA)

Die AIHA ist eine Autoimmunerkrankung, die durch einen beschleunigten Erythrozytenabbau gekennzeichnet ist. Dieser wird durch die Bildung von Autoantikörpern gegen Erythrozyten verursacht (Barcellini, 2015; Packman, 2015; Salama, 2015). Erythrozytäre Autoantikörper sind gegen eigene Antigene gerichtet und gehören zur IgG-, IgM- und selten zur IgA-Klasse. Sie reagieren optimal bei 37°C bzw. bei 0-4°C und werden dementsprechend als Wärme- bzw. Kälteautoantikörper bezeichnet. Das zugehörige Krankheitsbild wird danach als autoimmunhämolytische Anämie vom Wärme- oder Kältetyp klassifiziert (Barcellini, 2015; Pachman, 2015; Wouters und Zeerleder, 2015).

## 2.2 Mechanismen der Erythrozyten-Zerstörung

Die Mechanismen der Erythrozyten-Zerstörung bei Patienten mit autoimmunhämolytischer Anämie folgen bekannterweise zwei unterschiedlichen Wegen:

1) Erythrozytäre Autoantikörper der IgG-Klasse können zur Zerstörung von Erythrozyten durch Fc-vermittelte Phagozytose und bei Aktivierung des Komplements durch C3b-vermittelte Phagozytose führen (Abbildung 1). IgM-Autoantikörper sind komplementaktivierend und können ebenso zur Zerstörung der Erythrozyten durch C3b-vermittelte Phagozytose führen oder

2) in seltenen Fällen durch direkte Zelllyse mittels Aktivierung der terminalen Komplementkomponenten C5b-9 (Membran Attack Complex, MAC), welche Poren in der Erythrozyten-Membran bilden (Barcellini, 2015; Packman, 2015; Petz und Garratty, 2004; Salama, 2002; Wouters und Zeerleder, 2015). Der erste Abbauweg, die Phagozytose, wird als extravasale Hämolyse bezeichnet und der zweite, die Zelllyse, als intravasale Hämolyse. Beide Mechanismen sind in Abbildung 2 schematisch dargestellt.

Die Lebensdauer der zirkulierenden menschlichen roten Blutkörperchen (Erythrozyten) beträgt ca. 120 Tage (Veale et al., 2014). Die beschädigten bzw. gealterten Erythrozyten werden von Makrophagen in der Milz oder in der Leber abgebaut (Healy et al., 2007). Der Abbaumechanismus dieser Zellen ist bisher nicht eindeutig geklärt. Möglicherweise spielt hierbei die Eryptose, der programmierte Tod von Erythrozyten, eine Rolle (Ghashghaeinia et al., 2012; Salama et al., 2015).

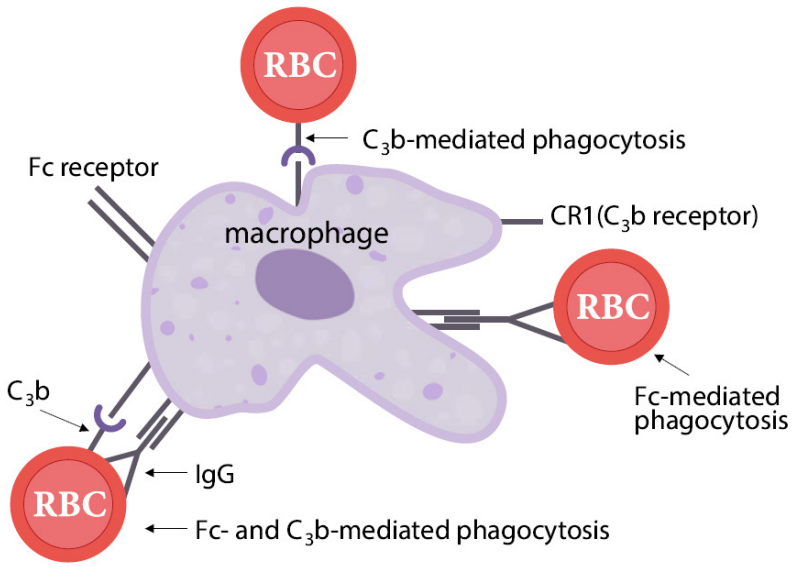


**Abbildung 1: Übersicht über die Fc- / oder C3-vemittelte Phagozytose**
Ery: Erythrozyten; C: Komplementkomponente; Fc: Fragment crystallisable (nach Salama et al., 2002).

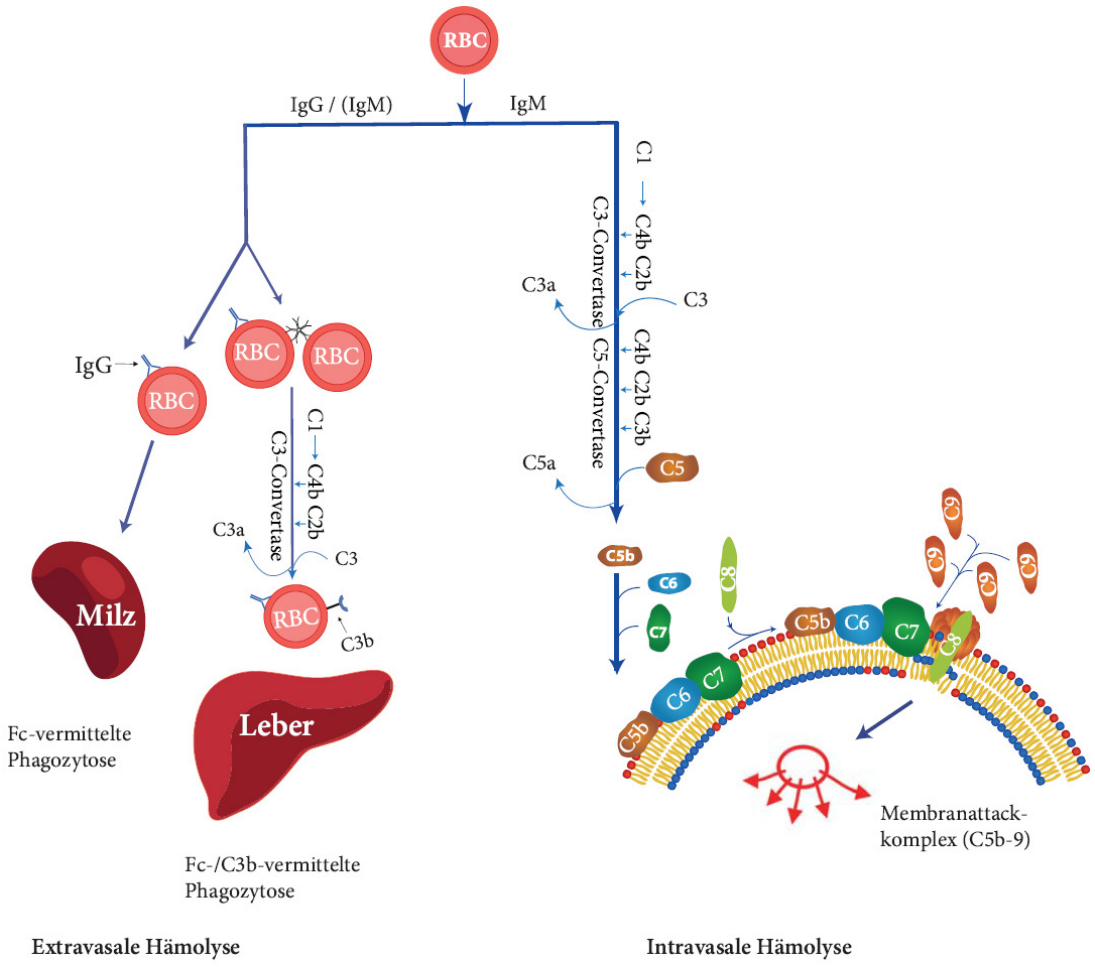


**Abbildung 2: Schematische Darstellung der Mechanismen von extravasaler und intravasaler autoimmunhämolytischer Anämie (selbst erstellt).**

## 2.3 Antiglobulintest (Coombstest)

Der Coombstest wird zum Nachweis der Antikörper und / oder Komplementfaktoren C3 verwendet. Der Test umfasst die an Erythrozyten gebundenen Antikörper bzw. Komplemente (Direkter Antiglobulintest, DAT) sowie freie erythrozytäre Antikörper im Serum bzw. Plasma (Indirekter Antiglobulintest, IAT). Der Nachweis beruht auf einer Agglutination von Testerythrozyten bzw. Patienten-Erythrozyten. Beim direkten Antiglobulintest (DAT) werden die Patientenerythrozyten mit PBS Lösung gewaschen und durch Zugabe von AHG (Antihuman-Globulinserum) untersucht.

Beim indirekten Antiglobulintest werden Testerythrozyten mit antikörperhaltigem Plasma bzw. Serum des Patienten inkubiert und unter Zugabe von AHG untersucht.

Als AHG wird üblicherweise ein polyspezifisches Antiserum gegen IgG und C3d verwendet (Salama und Heymann, 2010). Eine Zellagglutination nach Zugabe von AHG spricht für das Vorliegen spezifischer AK gegen Erythrozyten.

## 2.4 Monozyten-Monolayer-Assay

Vor mehr als 40 Jahren wurde der Monozyten-Monolayer-Assay entwickelt, um die klinische Relevanz der erythrozytären Alloantikörper zu ermitteln (Tong et al., 2019). Alloantikörper bilden sich gegen Fremdantigene anderer Individuen und können nach Transfusion oder Schwangerschaft auftreten (Reid und Mohanads, 2004). Die häufigsten klinisch signifikanten Alloantikörper sind Anti-Rh (Rhesus) und Kell-Antikörper (Arndt und Garratty, 2004). Der Assay basiert auf der Verwendung von Monozyten gesunder Blutspender, die in einer Monoschicht auf einer Platte unter optimalen Zellkultur-Bedingungen hergestellt werden und Erythrozyten, die vorher mit Patientenserum inkubiert wurden. Die phagozytierten Erythrozyten werden manuell durch ein Mikroskop quantifiziert (Tong et al., 2019).

Viele technische Variablen können die Ergebnisse der Monozyten-Monolayer-Assays beeinflussen. Dazu gehört die Quelle der Monozyten von Blutspendern. Dies kann zu unterschiedlicher Makrophagenaktivität bei Blutspendern führen. Die manuelle Quantifizierung ist ein weiterer Hauptkritikpunkt am klassischen Assay.

## 2.5 THP-1-Zellen

THP-1-Zellen wurden von einer monozytären Leukämiezelllinie aus dem peripheren Blut eines einjährigen Jungen abgeleitet (Tsuchiya et al., 1980). Sie haben einige Vorteile gegenüber den humanen Monozyten und sie sind ein häufig verwendetes Modell zur Untersuchung der Funktion von Makrophagen. Sie sind leicht zu kultivieren, fähig in Suspension zu wachsen, reproduzierbar und daher sind sie langfristig verfügbar. THP-1-Zellen können durch Behandlung mit einem Phorbolester, z.B. Phorbol 12-Myristat 13-Acetat (PMA) zu Makrophagen-ähnlichen Zellen differenziert und adhärent werden (Tsuchiya et al., 1982; Qin, 2012). Die Makrophagen besitzen auf ihrer Oberfläche Fc-Rezeptoren für IgG-Moleküle und C3b-Komplementrezeptoren (CR1), welche die Phagozytose vermitteln (Salama et al, 2002)**.** In einer früheren Studie zeigte der fluorometrische quantitative Erythrophagozytosetest unter Verwendung von humanen THP-1-Zellen und PKH26-markierten Erythrozyten eine hohe Empfindlichkeit bezüglich der Phagozytose in Abhängigkeit vom Erythrozytenalter (Healey et al., 2007; Veale et al., 2014).

## 2.6 Eryptose

Eryptose, der suizidale Tod der Erythrozyten, ist durch die Exposition von Phosphatidylserin (PS) an der Zelloberfläche gekennzeichnet. Ein wesentlicher Auslöser ist der Anstieg des zytosolischen Kalziums ($Ca^{2+}$), welcher die Scramblase stimuliert und damit zum Zusammenbruch der Phosphatidylserin-Asymmetrie (PS) führt (Lang et al., 2010; Lang und Qadri, 2012). Die PS-Exposition an der Erythrozytenoberfläche ist durch Annexinbindung nachweisbar. Faktoren wie oxidativer Stress, osmotischer Schock und Energiemangel wurden auch als Auslöser der Eryptose beschrieben (Ghashghaeinia et al., 2012; Lang et al., 2010). Die Eryptose wurde auch bei Patienten mit Leberversagen, Herzversagen, Nierenversagen, Sepsis, Malaria, Thalassämie, Glucose-6-Phosphat-Dehydrogenase-Mangel, Sphärozytose, metabolischem Syndrom, Diabetes, Mykoplasmeninfektion, Sichelzellenanämie und Eisenmangel beschrieben (Pretorius et al., 2016).

## 2.7 Zielsetzung der Doktorarbeit

Ziel dieser Doktorarbeit war es, einen neuen Durchflusszytometrie-basierten Erythrophagozytosetest unter Verwendung monozytärer THP-1-Zellen zu etablieren, um zu bestimmen, ob ein Makrophagenassay auf Basis von kultivierten Monozytenzellen die klinische Relevanz (Hämolyse) von Autoantikörpern abbilden kann.

Ein weiteres Ziel war die Beantwortung der Frage, ob die Eryptose bei der autoimmunhämolytischen Anämie (AIHA) eine Rolle spielt und wenn ja, welche Faktoren zur Eryptose führen.

## 3 Hintergrund, Methoden und Ergebnisse der Publikationen

Das Forschungsvorhaben wurde von der örtlichen Ethikkommission der Charité – Universitätsmedizin Berlin genehmigt (Nr. EA2 / 058/12).

In den folgenden drei Publikationen dieser Dissertation wurden drei Aspekte zur weiteren Charakterisierung der AIHA untersucht.

### 3.1 A fluorometric erythrophagocytosis assay using differentiated monocytic THP-1 cells to assess the clinical significance of antibodies to Red Blood Cells. (Vox Sanguinis 2021).

**Hintergrund**

Die klinische Relevanz von Antikörpern gegen Erythrozyten *in vivo* ist variabel und generell kann sie nicht allein durch den Coombstest vorhergesagt werden. In der vorliegenden Arbeit wurde ein neuer Durchflusszytometrie-basierter Erythrophagozytosetest unter Verwendung von Phorbol 12-Myristat 13-Acetat (PMA)-behandelten THP-1-Zellen und PKH26-red gefärbten Erythrozyten etabliert, um die Signifikanz von Allo- und Autoantikörpern gegen Erythrozyten zu bestimmen.

**Methoden**

In der vorliegenden Arbeit wurden die THP-1-Zellen durch Behandlung mit PMA in Makrophagen-ähnlichen Zellen differenziert. Erythrozyten von sechzehn Patienten mit klinisch signifikanter autoimmunhämolytischer Anämie, von fünf schwangeren Patientinnen mit nachweisbaren Wärmeautoantikörpern ohne Hämolyse; und die Erythrozyten, die mit bekannten klinisch signifikanten Alloantikörpern Anti-D und Anti-K beladen wurden, wurden mit dem lipophilen Fluoreszenzfarbstoff PKH26-red gefärbt und mit PMA-behandelten THP-1-Zellen inkubiert. Nach der Hämolyse von nichtphagozytierten Erythrozyten wurde die Erythrophagozytose mittels Durchflusszytometrie quantifiziert.

**Ergebnisse**

Die mit Anti-D und Anti-K beladene Erythrozyten wiesen eine verstärkte Erythrophagozytose auf, wohingegen mit Anti-Ch beladene Erythrozyten keine Erythrophagozytose verursachten. Die Alloantikörper Anti-Yt(a) und Anti-Lu(b) konnten eine Erythrophagozytose auslösen. Patienten mit klinisch relevanter autoimmunhämolytischer Anämie vom Wärmetyp wiesen eine erhöhte Erythrophagozytose auf. Die Erythrophagozytose bei allen fünf Patienten mit

nachweisbaren Wärmeautoantikörpern in der Schwangerschaft (ohne Hämolyse) war im Vergleich zur Negativkontrolle nicht erhöht.

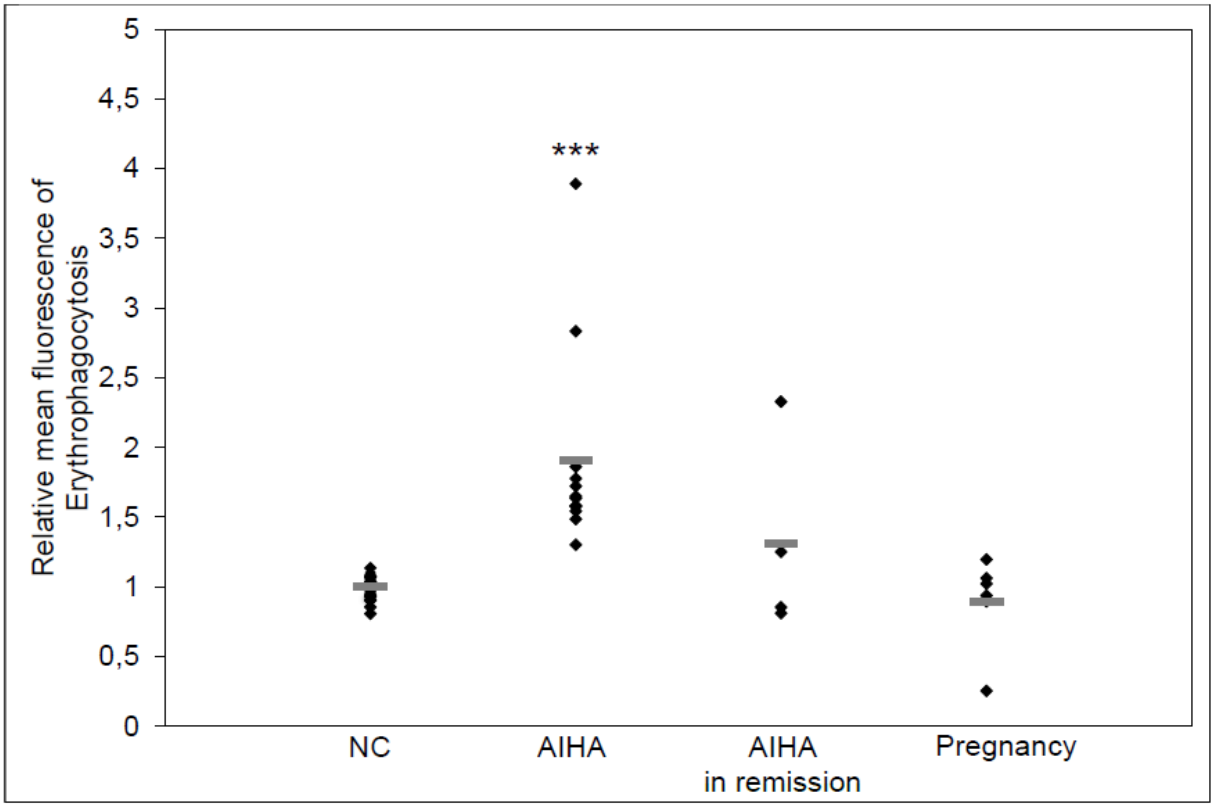


**Abbildung 3: Erythrophagozytose von Erythrozyten gesunder Blutspender im Vergleich zu Erythrozyten von Patienten mit AIHA vom Wärmetyp und schwangeren Patientinnen mit Wärmeautoantikörpern.** Dargestellt ist die relative mittlere Fluoreszenz (Kanal B2 B585 PKH26-red) von PMA-behandelten THP-1-Zellen nach Phagozytose von Erythrozyten aus gesunden Blutspendern (NC, erste Spalte), Patienten mit signifikanten wAIHA (zweite Spalte), Patienten mit wAIHA in Remission (dritte Spalte), schwangeren Patientinnen mit Wärmeautoantikörpern (vierte Spalte). Jeder Punkt gibt eine Erythrozyten-Probe an. Die grauen Linien repräsentieren den Mittelwert jeder Gruppe. Die mittlere Fluoreszenz wurde auf 1 normalisiert. (***) p <0,001 (Balola et al., 2021).

**3.2 Eryptosis in autoimmune haemolytic anaemia.** (Eur J Haematol 2018;100(1): 36-44).

**Hintergrund**

Im etablierten neuen Durchflusszytometrie-basierten Erythrophagozytosetest wurde bei Patienten mit einer signifikanten AIHA vom Kältetyp keine erhöhte Erythrophagozytose festgestellt, was darauf hinweist, dass die Zerstörung von Erythrozyten nicht immer durch Fc oder C3b vermittelt wird. Die Frage bleibt offen, ob die Autoantikörper die Erythrozyten von Patienten mit AIHA über einen anderen Mechanismus zerstören können. In dieser Arbeit wurde das Augenmerk darauf gelegt, ob die Autoantikörper gegen Erythrozyten möglicherweise eine Eryptose auslösen können.

**Methoden**

Es wurden Erythrozyten von vierundzwanzig Patienten mit AIHA vom Wärmetyp (darunter sieben Patienten mit signifikanter AIHA und elf Patienten in Remission), sieben Patienten mit AIHA vom Kältetyp und einem Patienten mit AIHA vom Mischtyp mit Phycoerythrin (PE)-Annexin-V (Phosphatidylserin (PS) Marker) gefärbt. Die Annexin-V Bindung an die PS-exponierten Erythrozyten wurde mittels Durchflusszytometrie untersucht.

**Ergebnisse**

Ein erhöhter Prozentsatz an PS-exponierenden Erythrozyten (PS+) war bei sieben Patienten mit klinisch signifikanter AIHA vom Wärmetyp nachweisbar. Bei diesen Patienten gehörten die Autoantikörper neben der IgG-Klasse auch zur IgM- und / oder IgA-Klasse. Im Gegensatz dazu war der Prozentsatz der PS-exponierenden Erythrozyten von sechs Patienten mit klinisch relevanter, ausschließlicher IgG-AIHA vom Wärmetyp und weiteren elf Patienten mit AIHA vom Wärmetyp in vollständiger Remission unverändert im Vergleich zu Erythrozyten von gesunden Blutspendern. Bei allen sieben Patienten mit AIHA vom Kältetyp war ein erhöhter Prozentsatz der PS-exponierenden Erythrozyten festzustellen. Die Hämolyse war bei diesen Patienten mit IgM- oder IgA-Autoantikörpern verbunden. Die Erythrozyten von Patienten mit AIHA vom Mischtyp zeigten ebenfalls eine erhöhte PS-Exposition. Zusammenfassend zeigt diese Arbeit, dass ein signifikanter Anstieg der PS-exponierenden Erythrozyten als Marker der Eryptose bei IgM-AIHA oder IgA-AIHA auftritt.

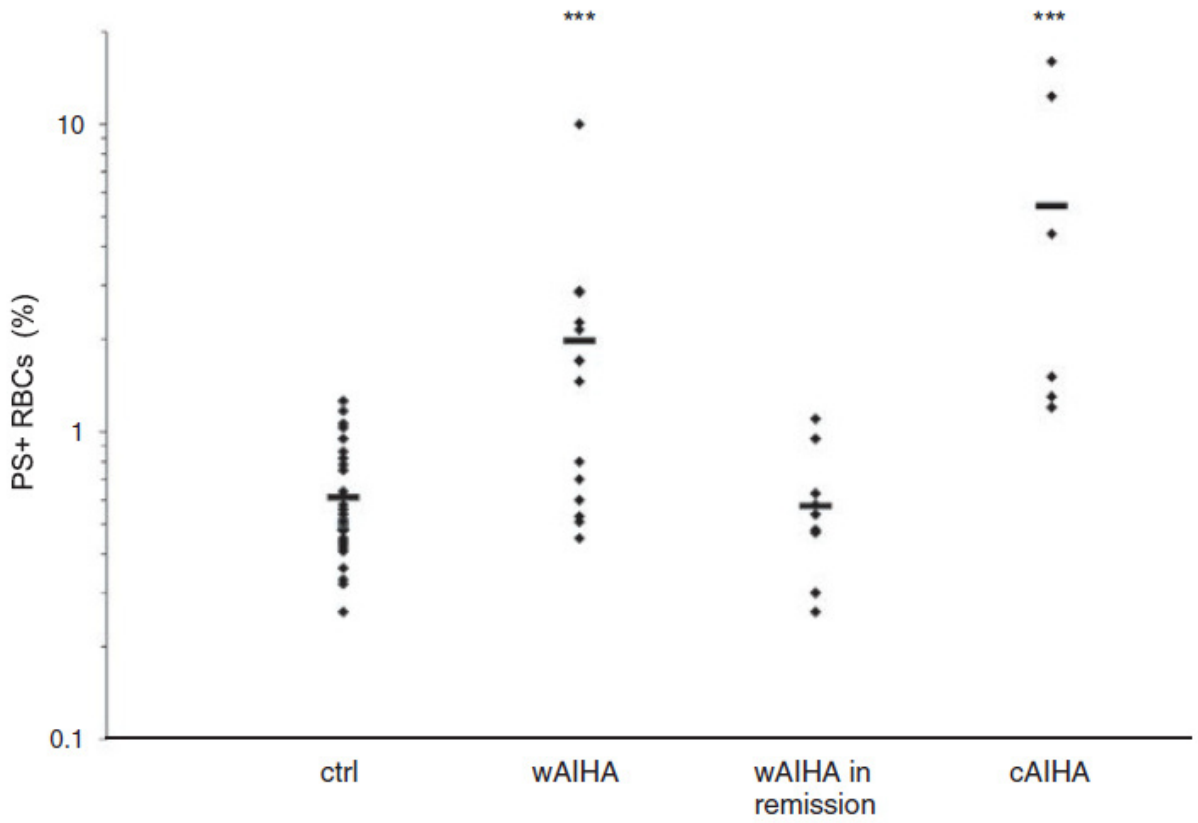


**Abbildung 3: Eryptose (PS+RBCs) von Erythrozyten gesunder Blutspender im Vergleich zu Erythrozyten von Patienten mit wAIHA, Patienten mit wAIHA in Remission und Patienten mit cAIHA.** Prozentsatz der PS+-Erythrozyten (logarithmische Skala) von gesunden Blutspendern (ctrl, erste Spalte), Patienten mit signifikanter wAIHA (zweite Spalte), Patienten mit wAIHA in Remission (dritte Spalte) und Patienten mit signifikanter cAIHA (vierte Spalte). Jeder Punkt stellt eine einzelne Erythrozyten-Probe dar. Die schwarzen Linien repräsentieren den Mittelwert jeder Gruppe. *** P<0,001 zeigt signifikante Unterschied zur Kontrolle gesunder Blutspender (Bartolmäs et al., 2018).

### 3.3 Sublytic terminal complement components induce eryptosis in autoimmune haemolytic anaemia related to IgM autoantibodies. Cell Physiol Biochem. 2019;53(3):453-464.

**Hintergrund**

Der zugrundeliegende Mechanismus der erhöhten PS-Exposition von Erythrozyten durch Autoantikörper in AIHA ist unbekannt, ebenso die Frage, welche Faktoren bei der Eryptose möglicherweise eine Rolle spielen könnten. Basierend auf den Kälteautoantikörpern vom IgM-Typ, die komplementaktivierend sind, wurde in der vorliegenden Arbeit die mögliche Rolle der Komplementkomponenten bei der Eryptose untersucht.

**Methoden**

In dieser Arbeit wurden Erythrozyten gesunder Blutspender der Blutgruppe 0 mit frischen Seren oder Plasmen von zwei Patienten mit klinisch relevanter AIHA vom Kältetyp inkubiert unter Verwendung von EDTA, hitze-inaktiviertem Serum, Anti C5-, C8- und C9-Antikörpern und C5-C9-depletierten Seren. Nach Färbung mit Phycoerythrin (PE)-Annexin-V wurden die Zellen mittels Durchflusszytometrie auf PS-Exposition untersucht.

**Ergebnisse**

Durch Inkubation mit frischen Seren von Patienten mit AIHA vom Kältetyp ließ sich in Spendererythrozyten eine erhöhte PS-Exposition induzieren. Durch Zugabe von EDTA oder bei Verwendung des Patienten-Plasmas oder von hitze-inaktivierten Patientenseren war keine erhöhte PS-Exposition nachweisbar. Durch Hitze-Inaktivierung wurden die Komplement-Komponenten zerstört, daher ist die Eryptose vermutlich Komplement-abhängig. Durch Zugabe von Anti-C5 wurde die erhöhte PS-Exposition vollständig inhibiert. Erstaunlicherweise inhibierte die Zugabe von Anti-C8 die PS-Exposition, während die Zugabe von Anti-C9 zu einem noch höheren Spiegel an PS-exponierenden Zellen führte. Um die Hypothese zu bestätigen, wurde hitze-inaktiviertes Serum von Patienten mit AIHA vom Kältetyp mit C5- bis C9-depletiertem Serum inkubiert. Die Inkubation mit dem Patientenserum und C5-depletiertem Serum führte nicht zu einer erhöhten PS-Exposition der Erythrozyten. Die Inkubation mit C6-, C7-, C8- oder C9-depletiertem Serum führte zu einem graduellen Anstieg der PS-Exposition. Dabei wurde in dieser Arbeit zum ersten Mal dargestellt, dass die Eryptose durch IgM Autoantikörper von der Komplementaktivierung abhängt. Der sublytische terminale

Komplementkomplex (C5-8) ist der effektivste Auslöser der Eryptose bei Patienten mit AIHA vom Kältetyp. Sie beginnt bei der Bildung des C5b/6-Komplexes und nimmt mit weiterer Aktivierung der terminalen Komplexe bis zur Bildung des C5b-8 Komplexes zu. Die IgA-induzierte Eryptose ist dagegen nicht Komplement-abhängig.

Unsere Ergebnisse zeigen einen neuen Abbauweg von Erythrozyten in AIHA anhand des Einflusses der terminalen Komplementkomplexe auf die Erythrozytenmembran.

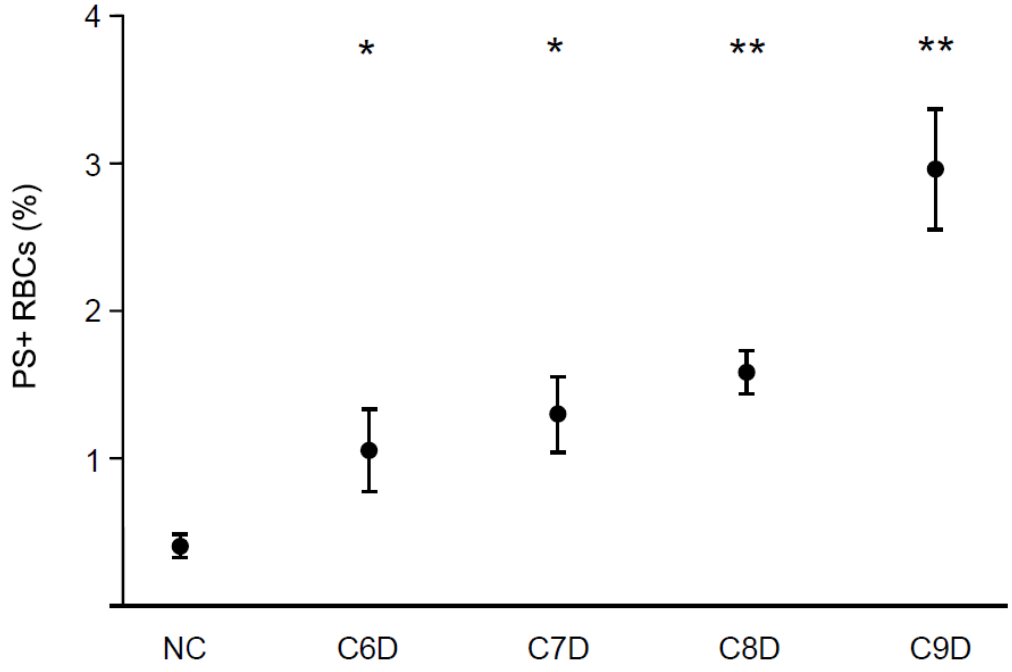


**Abbildung 5: Abhängigkeit der Eryptose von den Komplementfaktoren.** Prozentsatz der PS+-Erythrozyten (PS+RBCs) nach Inkubation von Erythrozyten gesunder Blutspender mit hitze-inaktiviertem Serum von Patienten mit AIHA vom Kältetyp und C6-, C7-, C8- oder C9-depletiertem Serum (C_D) im Vergleich zur PS-Exposition der Negativkontrolle (NC). Die Eryptose erhöht sich allmählich in Gegenwart von C6-, C7-, C8- und C9-depletiertem Serum. (*) p <0,05, (**) p <0,01 (Balola et al., 2019).

## 4 Allgemeine Diskussion

Die klassischen Abbauwege der Erythrozyten durch Fc- und / oder C3b-vermittelte Phagozytose oder Zelllyse durch Aktivierung der terminalen Komplementkomponenten C5-9 (Packman, 2015; Petz und Garratty, 2004; Barcellini, 2015; Wouters, 2015) erklären nicht eindeutig die Frage nach der Variabilität der Hämolyse und dem Ansprechen auf die Therapie bei AIHA.

Die Ergebnisse des Monozyten Monolayer Assays (MMA) von Tong et al. zeigten eine gute Korrelation mit klinisch signifikanten Alloantikörpern. In diesen Studien wurden Monozyten von gesunden Spendern verwendet (Tong et al., 2019). Viele technische Variablen können die erzielten Ergebnisse allerdings nachteilig beeinflussen. Dazu gehören die Auswahl von Monozyten aus Blutspendern, sowie Kulturbedingungen und Analysemethoden (Tong et al., 2019). Darüber hinaus sind wenige Informationen über die Verwendung der MMA zur Bestimmung der Signifikanz der Autoantikörper bekannt. In der vorliegenden Arbeit wurde ein neuer Durchflusszytometrie-basierter Erythrophagozytose-Assay unter Verwendung von PMA-behandelten THP-1 Zellen entwickelt. Dieser basiert auf der Messung von phagozytierten PKH26-red markierten Erythrozyten und ist zuverlässig und gut reproduzierbar, da die Phagozytoseaktivität unabhängig von Monozytenspendern ist und der Nachweis der phagozytierten Erythrozyten fluorozytometrisch erfolgt. Darüber hinaus sind die THP-1-Zellen leicht zugänglich und können durch Kultivierung reproduziert werden und daher jederzeit zur Verfügung stehen. In diesem Zusammenhang könnte der Assay sogar noch den weiteren Vorteil der geringen Kosten haben. Zuerst wurden gut charakterisierte Alloantikörper mit klinischer Relevanz untersucht. Die Ergebnisse zeigten, dass die Bindung der klinisch signifikanten Alloantikörper Anti-D und Anti-K an die Oberfläche von Erythrozyten zu einer verstärkten Erythrophagozytose führten, während der klinisch nicht signifikante Alloantikörper Anti-Ch keine gesteigerte Erythrophagozytose verursachte. Zwei weitere Alloantikörper Anti-Yt (a) und Anti-Lu (b) konnten eine Erythrophagozytose induzieren, die darauf hindeuten, dass sie möglicherweise klinisch signifikant sein können. In der Tat wurde in früheren Studien gezeigt, dass die klinische Signifikanz dieser Alloantikörper, Anti-Yt (a) und Anti-Lu (b), variabel ist (Cutbush and Mollison, 1958; AuBuchon et al., 1988; Eckrich et al., 1995). Die Ergebnisse der vorliegenden Arbeit korrelieren mit den Ergebnissen einer Studie, in der MMA-Daten für 46 Patienten über

20 Jahre analysiert wurden, um die klinische Signifikanz von Alloantikörpern zu bestimmen (Arndt und Garratty, 2004). Somit eignet sich der Test für die Auswahl kompatibler Erythrozyten bei alloimmunisierten Patienten mit positiver Kreuzprobe, die eine Bluttransfusion benötigen.

Bei Verwendung der Erythrozyten von Patienten mit AIHA vom Wärmetyp und von Schwangeren mit Wärmeautoantikörpern ohne Hämolyse wurde festgestellt, dass nur die Autoantikörper von Patienten mit AIHA vom Wärmetyp eine erhöhte Erythrophagozytose zeigen.

In ähnlicher Weise zeigten die Erythrozyten von drei Patienten ohne Hämolyse keine erhöhte Erythrophagozytose. Somit korrelierte die Erythrophagozytose bei Patienten mit AIHA vom Wärmetyp mit der Hämolyse *in vivo*.

Die Frage, warum die Wärmeautoantikörper bei Schwangeren (Hoppe et al., 2001; Surucu et al., 2015) und persistierende Autoantikörper bei AIHA nach vollständiger Remission nicht zu einer Phagozytose führen, ist noch nicht endgültig geklärt worden.

Basierend auf den Ergebnissen dieser Arbeit kann dieses Phänomen weder durch die Anzahl der an Erythrozyten gebundenen Antikörper noch durch die autologen Makrophagen erklärt werden. Eine mögliche Erklärung könnte sich auf die Struktur der IgG-Fc-Region beziehen, die durch eine spezifische Glykosylierung modifiziert und bestimmt wird, um Signalwege zu modifizieren, auf die die Makrophagen abzielen. Dieser Glykosylierungsprozess ist eine komplexe posttranslationale, enzymspezifische Modifikation, die mit der Bindung der Zuckerreste assoziiert (Arnold et al., 2007; Lux und Nimmerjahn, 2011; Biermann et al., 2016).

Bei Patienten mit einer signifikanten AIHA vom Kältetyp ließ sich keine erhöhte Erythrophagozytose feststellen. Dies weist darauf hin, dass die Erythrozyten-Zerstörung nicht immer durch Fc- bzw. C3b vermittelt wird.

Ein weiteres Ziel dieser Arbeit war es zu zeigen, ob diese Autoantikörper eine Eryptose induzieren können. Daher wurden Erythrozyten von Patienten mit AIHA vom Wärme-, Kälte- und Mischtyp mit Annexin-V behandelt und exponiertes PS auf Erythrozyten-Oberflächen mittels Durchflusszytometrie gemessen.

Es wurde festgestellt, dass bei Patienten mit signifikanter AIHA vom Wärme- und vom Kältetyp ein erhöhter Anstieg von PS-exponierenden Erythrozyten auftritt. Da die Hämolyse bei diesen Patienten mit IgM- oder IgA-Autoantikörpern verbunden war (Bartolmäs et al., 2018), konnte dadurch nachgewiesen werden, dass zusätzlich zu den klassischen Wegen der

Fc-, C3b-abhängigen Phagozytose und / oder der C5b-9-vermittelten -Lyse (Packman, 2015; Petz und Garratty, 2004) eine Eryptose als weiterer Abbauweg möglich ist. Es blieb jedoch unklar, wie diese Autoantikörper eine Eryptose verursachen und welche Faktoren möglicherweise hier die Eryptose beeinflussen können. Dies führte zur Hypothese, ob eine Komplementaktivierung die Eryptose beeinflussen könnte. In der vorliegenden Arbeit wurde die Eryptose durch Inkubation O-RBCs gesunder Blutspender mit frischem Serum von Patienten mit IgM-Kälteautoantikörpern induziert. Bei Verwendung von Patientenplasma oder hitze-inaktiviertem Serum fand keine Eryptose statt. Unter Verwendung von EDTA, hitze-inaktiviertem Serum, Antikörpern gegen C5, C8 und C9 und C5-C9-depletierten Seren wurde festgestellt, dass die Eryptose aufgrund der Autoantikörper der IgM-Klasse von der Aktivierung dieser Komplementkomponenten abhängt.

Nach Zugabe von Anti-C5 (Eculizumab, Soliris®) vor der Inkubation oder bei Verwendung von C5-depletiertem Serum als Komplementquelle trat keine Eryptose auf, was darauf deutet, dass C5 für IgM-induzierte Eryptose notwendig ist. Bei Verwendung von C6-, C7- und C8-depletierten Seren stieg die Eryptose schrittweise infolge der Bildung von C5b-7-Komplex weiter an und war am stärksten nach der Bildung von C5b-8-Komplex auf der RBC-Membran. Dies führt man auf den Anstieg des Kalzium-Einstroms zurück. Die C5b-7 heften sich an der Zellmembran an (DiScipio et al., 1988; Preissner et al., 1985), induzieren die Bildung von Ceramid und ermöglichen den Kalzium ($Ca^{+2}$)-Einstrom, der der wesentliche Auslöser der Eryptose ist (Lang et al., 2015; Qadri et al., 2017; Steffen et al., 2011; Preisner et al., 1985; Attanasio et al., 2007; Lang et al., 2010; Niculescus et al., 1993; Serna et al., 2016; Chung et al., 2007).

Die Bildung von C5b-8-Komplex führt zu einem weiteren Anstieg von Kalzium-Einstrom (Sharp et al., 2016; Fu et al., 2016; Morgan et al., 1986). Daher können sowohl sublytische C5b-7- als auch C5b-8-Komplexe die Eryptose induzieren. Die starke Zunahme der Eryptose nach der Hemmung von C9 durch Anti-C9-Antikörper oder bei Verwendung von C9-depletiertem Serum könnte durch die Akkumulation von sublytischen C5b-8-Komplexen auf der Zellmembran erklärt werden. Die Abnahme der Eryptose nach Zugabe von C9 bzw. der Bildung von C5b-9-Komplex (Membrane Attack Complex, MAC) könnte jedoch vor allem durch die im Vergleich zur Eryptose viel schneller ablaufende Zelllyse durch den MAC erklärt werden. Zusätzlich könnte die Bildung von löslichen C5b-9-Komplexen als Inhibitor der Komplementaktivierung und der Bildung des sublytischen C5b-8-Komplex wirken (Tegla et al., 2011). Im Gegensatz zu den IgM-Autoantikörpern scheinen die IgA-Autoantikörper das

Komplement nicht zu aktivieren und das Komplement zur Eryptose-Induktion nicht zu benötigen. Ihre eryptotische Wirkung blieb durch die Verwendung von EDTA-Plasma oder hitze-inaktiviertem Serum unverändert.

Zusammenfassend zeigt diese Arbeit, dass die IgM-induzierte Eryptose komplementabhängig ist und dass die sublytischen terminalen Komplementkomplexe C5b/6, C5b-7 und insbesondere C5b-8-Komplex zur Eryptose führen können. Damit stellt die Eryptose einen dritten Abbauweg von Erythrozyten bei Patienten mit AIHA dar (Abb. 6).

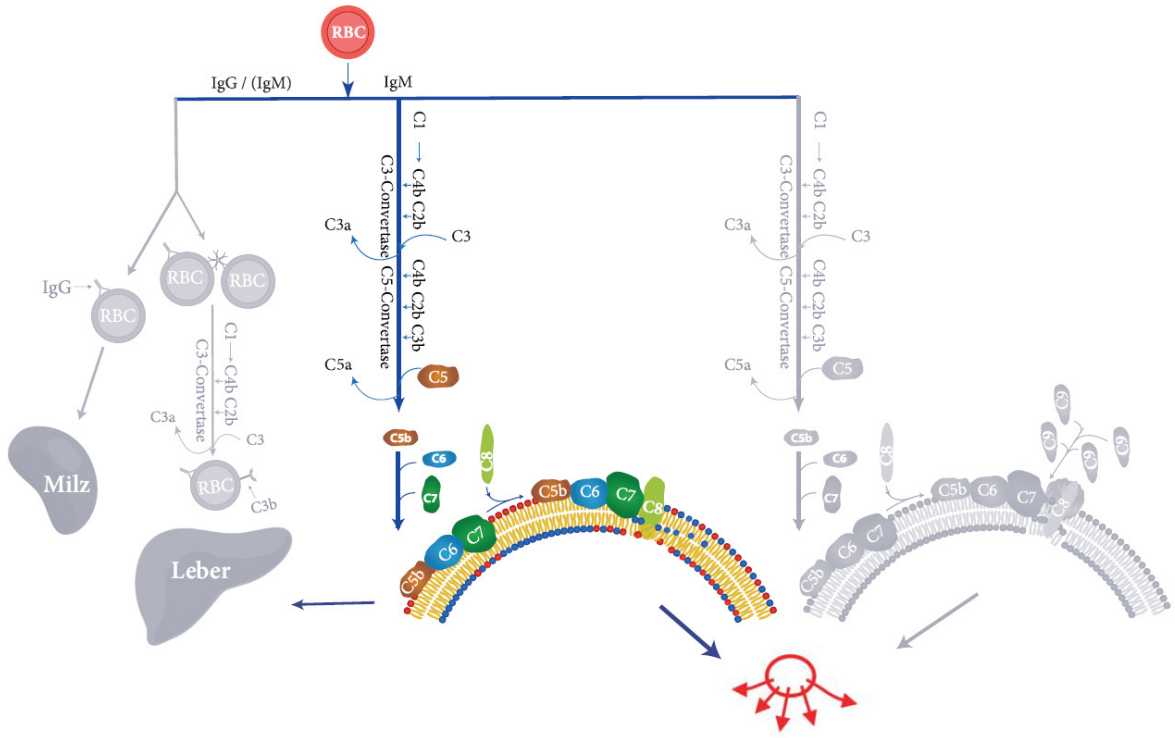


**Abbildung 6: Erythrozytäre Abbauwege.** Der Erythrozyten-Abbau bei Patienten mit autoimmunhämolytischer Anämie (AIHA) kann durch i) Phagozytose (links); ii) Eryptose (zentral) oder iii) intravasale Sofort-Lyse erfolgen (selbst erstellt).

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## 6 Eidesstattliche Versicherung

„Ich, Abdelwahab Hassan Ahmed Balola, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [Pathophysiologie des Erythrozytenabbaus bei Patienten mit Autoimmunhämolytischen Anämien (AIHA) / Pathophysiology of Erythrocyte Destruction in patients with Autoimmune Hemolytic Anemia (AIHA)] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.
Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

_____ _____

Datum Unterschrift

## 7 Anteilserklärung an der im Anhang ausgewählten Publikationen

**Publikation 1: Balola AHA**, Mayer B, Bartolmäs T, Salama A. A fluorometric erythrophagocytosis assay using differentiated monocytic THP-1 cells to assess the clinical significance of antibodies to red blood cells. Vox Sang 2021; **116**(10):1106-1116.

Impact factor: 2,347 (2019)

Experimentelles Design, experimentelle Durchführung wurden von mir unter Aufsicht von meinen Betreuern durchgeführt.

Ich habe die Daten-Auswertung unter Aufsicht der Betreuer analysiert und ausgewertet.

Alle Tabellen und Abbildungen wurden von mir nach Rücksprache mit Betreuern erstellt.

Literaturrecherche und Auswahl der relevanten Literatur wurden von mir durchgeführt.

Ich habe das Schreiben des ersten Publikationsentwurfs erstellt und mit den Betreuern endgültig überarbeitet.

**Publikation 2:** Bartolmäs T*, Mayer* B, **Balola AH**, Salama A.: Eryptosis in autoimmune haemolytic anaemia. Eur J Haematol, 2018.
Impact factor: 2,25

Experimentelle Durchführung wurde von mir unter Aufsicht von meinen Betreuern durchgeführt.

Die Daten-Auswertung habe ich teilweise gemeinsam mit den Betreuern durchgeführt.

Abbildungen (Abb. 1, 3 und 4) wurden von mir nach Rücksprache mit Betreuern erstellt.

Ich habe an der Literaturrecherche, der Erstellung und Überarbeitung des endgültigen Publikationsentwurfs mitgewirkt.

**Publikation 3: Balola AHA**, Mayer B, Bartolmäs T, Salama A. Sublytic Terminal Complement Components Induce Eryptosis in Autoimmune Haemolytic Anaemia Related to IgM Autoantibodies. Cell Physiol Biochem, 2019.

Impact factor: (Scopus Cite Score 2019): 8,0

Experimentelles Design, experimentelle Durchführung wurden von mir unter Aufsicht von meinen Betreuern durchgeführt.

Ich habe die Daten-Auswertung unter Aufsicht der Betreuer analysiert und ausgewertet.

Alle Abbildungen wurden von mir nach Rücksprache mit Betreuern erstellt.

Literaturrecherche und Auswahl der relevanten Literatur wurden von mir durchgeführt.

Ich habe das Schreiben des ersten Publikationsentwurfs erstellt und mit den Betreuern endgültig überarbeitet.

_____

Unterschrift, Datum und Stempel des erstbetreuenden Hochschullehrers

_____

Unterschrift des Doktoranden/der Doktorandin

## 8 Druckexemplare der ausgewählten Publikationen und entsprechender Auszug aus der Journal Sumary List

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI Selected Categories: **"HEMATOLOGY"** Selected Category Scheme: WoS **Gesamtanzahl: 63 Journale**

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 1 | BLOOD | 165,117 | 17.543 | 0.205320 |
| 2 | CIRCULATION RESEARCH | 51,539 | 14.467 | 0.071470 |
| 3 | Journal of Hematology & Oncology | 6,732 | 11.059 | 0.015550 |
| 4 | Lancet Haematology | 2,446 | 10.406 | 0.011070 |
| 5 | LEUKEMIA | 25,819 | 8.665 | 0.048640 |
| 6 | Blood Cancer Journal | 2,800 | 8.023 | 0.010400 |
| 7 | HAEMATOLOGICA | 17,314 | 7.116 | 0.034660 |
| 8 | AMERICAN JOURNAL OF HEMATOLOGY | 10,629 | 6.973 | 0.023080 |
| 9 | ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY | 32,385 | 6.604 | 0.032080 |
| 10 | STEM CELLS | 20,554 | 6.022 | 0.024110 |
| 11 | CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY | 8,477 | 5.833 | 0.014250 |
| 12 | BLOOD REVIEWS | 3,040 | 5.823 | 0.005280 |
| 13 | JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM | 19,492 | 5.681 | 0.024230 |
| 14 | BRITISH JOURNAL OF HAEMATOLOGY | 23,759 | 5.518 | 0.033130 |
| 15 | BONE MARROW TRANSPLANTATION | 13,085 | 4.725 | 0.015830 |
| 16 | Blood Advances | 3,312 | 4.584 | 0.015930 |
| 17 | SEMINARS IN HEMATOLOGY | 2,234 | 4.379 | 0.003270 |
| 17 | THROMBOSIS AND HAEMOSTASIS | 15,589 | 4.379 | 0.020570 |
| 19 | CYTOTHERAPY | 6,557 | 4.218 | 0.008100 |

| | | | | |
|---|---|---|---|---|
| 20 | JOURNAL OF THROMBOSIS AND HAEMOSTASIS | 17,598 | 4.157 | 0.025190 |
| 21 | BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION | 12,667 | 3.853 | 0.023760 |
| 22 | JOURNAL OF LEUKOCYTE BIOLOGY | 16,995 | 3.757 | 0.017010 |
| 23 | Blood Transfusion | 1,905 | 3.662 | 0.003730 |
| 24 | Experimental Hematology & Oncology | 633 | 3.492 | 0.001420 |
| 25 | Therapeutic Advances in Hematology | 679 | 3.456 | 0.001500 |
| 26 | PLATELETS | 3,276 | 3.378 | 0.005580 |
| 27 | TRANSFUSION MEDICINE REVIEWS | 1,409 | 3.328 | 0.002980 |
| 28 | HEMATOLOGY- ONCOLOGY CLINICS OF NORTH AMERICA | 2,581 | 3.107 | 0.004920 |
| 29 | CURRENT OPINION IN HEMATOLOGY | 2,701 | 3.097 | 0.004850 |
| 30 | STEM CELLS AND DEVELOPMENT | 7,874 | 3.082 | 0.010280 |
| 31 | Hematology-American Society of Hematology Education Program | 2,981 | 3.000 | 0.006680 |
| 32 | HAEMOPHILIA | 5,450 | 2.990 | 0.006390 |
| 33 | LEUKEMIA & LYMPHOMA | 8,640 | 2.969 | 0.013770 |
| 34 | SHOCK | 7,919 | 2.960 | 0.010370 |
| 35 | ANNALS OF HEMATOLOGY | 5,734 | 2.904 | 0.010750 |
| 36 | SEMINARS IN THROMBOSIS AND HEMOSTASIS | 4,074 | 2.892 | 0.005260 |
| 37 | THROMBOSIS RESEARCH | 10,616 | 2.869 | 0.017410 |
| 38 | HEMATOLOGICAL ONCOLOGY | 1,372 | 2.832 | 0.003500 |
| 39 | EXPERIMENTAL HEMATOLOGY | 5,153 | 2.820 | 0.005650 |
| 40 | TRANSFUSION | 13,708 | 2.800 | 0.020860 |
| 41 | BEST PRACTICE & RESEARCH CLINICAL | 1,342 | 2.792 | 0.001600 |

Selected JCR Year: 2019; Selected Categories: "HEMATOLOGY" 1-2

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 42 | Expert Review of Hematology | 1,459 | 2.573 | 0.004060 |
| 43 | Current Hematologic Malignancy Reports | 899 | 2.531 | 0.002580 |
| 44 | BLOOD CELLS MOLECULES AND DISEASES | 3,020 | 2.460 | 0.003180 |
| 45 | PEDIATRIC BLOOD & CANCER | 11,805 | 2.355 | 0.023440 |
| 46 | VOX SANGUINIS | 3,368 | 2.347 | 0.005020 |
| 47 | Clinical Lymphoma Myeloma & Leukemia | 2,374 | 2.298 | 0.005580 |
| 48 | Thrombosis Journal | 773 | 2.295 | 0.001890 |
| 49 | INTERNATIONAL JOURNAL OF HEMATOLOGY | 4,046 | 2.245 | 0.006200 |
| 50 | EUROPEAN JOURNAL OF HAEMATOLOGY | 4,515 | 2.220 | 0.006720 |
| 51 | LEUKEMIA RESEARCH | 5,918 | 2.214 | 0.006910 |
| 52 | TRANSFUSION MEDICINE | 1,433 | 2.159 | 0.002390 |
| 53 | International Journal of Laboratory Hematology | 1,844 | 2.141 | 0.003750 |
| 54 | BLOOD PURIFICATION | 2,062 | 2.116 | 0.003080 |
| 55 | MICROCIRCULATION | 2,462 | 2.110 | 0.002700 |
| 56 | JOURNAL OF THROMBOSIS AND THROMBOLYSIS | 2,794 | 2.054 | 0.005740 |
| 57 | TRANSFUSION MEDICINE AND HEMOTHERAPY | 1,223 | 1.937 | 0.002610 |
| 58 | CLINICAL HEMORHEOLOGY AND MICROCIRCULATION | 2,147 | 1.741 | 0.002180 |
| 59 | Turkish Journal of Hematology | 452 | 1.685 | 0.000690 |
| 60 | Hematology | 1,373 | 1.650 | 0.002050 |
| 61 | JOURNAL OF CLINICAL APHERESIS | 1,365 | 1.629 | 0.001670 |

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# A fluorometric erythrophagocytosis assay using differentiated monocytic THP-1 cells to assess the clinical significance of antibodies to red blood cells

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**Background and Objectives** The significance of antibodies to red blood cells (RBCs) is variable and cannot be predicted solely by serological testing. A flow cytometry-based erythrophagocytosis assay was established using phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells and RBCs labelled with PKH26 to assess allo- and autoantibodies to RBCs.

**Materials and Methods** THP-1 cells were differentiated into macrophage-like cells by treatment with PMA. RBC samples coated with alloantibodies or autoantibodies were obtained from 16 patients with autoimmune haemolytic anaemia of warm type (wAIHA) as well as from five pregnant women with warm autoantibodies. RBCs from healthy blood donors were used as controls. RBCs were labelled with the red lipophilic fluorescent dye PKH26 and incubated with PMA-treated THP-1 cells. After removal of nonadherent RBCs by washing and haemolysis of adherent RBCs, erythrophagocytosis was quantified by flow cytometry.

**Results** We observed significant phagocytosis of RBCs coated with clinically relevant alloantibodies (i.e. anti-D and anti-K) or autoantibodies from patients with active wAIHA, but not of those coated with alloantibodies (anti-Ch) or autoantibodies from patients and pregnant women without haemolysis.

**Conclusion** The flow cytometry-based erythrophagocytosis test described here is quantitative, highly reliable, and may be helpful for the assessment of the clinical significance of antibodies to RBCs.

**Key words:** AIHA, alloantibodies, autoantibodies, flow cytometry, macrophages, phagocytosis, THP-1 cells.

## Introduction

Antibodies to red blood cells (RBCs) may show various effects *in vivo* on circulating RBCs compared with their reactivity with RBCs in serological testing. In addition, their clinical significance often depends on the clinical condition of individual patients. Indeed, some patients may develop a severe haemolytic transfusion reaction (HTR) due to an incompatible RBC transfusion, whereas other patients may develop only mild or no reactions under identical serological findings [1, 2]. Therefore, the clinical significance of detectable antibodies cannot always be determined using standard serological testing.

During the last decades, numerous methods have been developed to measure or predict RBC survival. Currently, chromium-51 or biotin-labelled tests for survival measurement [3, 4] are most reliable; however, they cannot be used routinely. Alternative techniques, including the

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biological cross-match, antibody-dependent cellular cytotoxicity (ADCC) assay [5], chemiluminescence test and the monocyte monolayer assay (MMA) [6], are often predictive but do not invariably exclude HTRs. The MMA is the most widely used assay to evaluate the clinical significance of alloantibodies to RBCs. This test is based on the use of autologous or allogeneic monocytes and RBCs opsonized with recipient serum. It is yet not unanimously clear if the outcome of patients transfused with serologically incompatible RBCs demonstrates a sound correlation for all antibodies with results of the MMA. Since patients with alloantibodies potentially causing clinically relevant haemolysis are usually not transfused with incompatible blood, data correlating with results of the MMA with haemolysis parameters are difficult to obtain. A recent study [7] used a MMA to cross-match 61 RBC alloantibodies with RBC units. Thirty-one out of 61 patients with no or variable significant antibodies were transfused successfully with RBC units and negative MMA cross-match. Unlike macrophages, circulating monocytes are not known to exhibit erythrophagocytosis in alloantibody-mediated haemolytic anaemia [6]. Hence, it remained questionable whether the use of macrophages might more accurately reflect the *in vivo* outcome than the use of monocytes [6,8,9].

The human leukemic cell line THP-1 has several advantages over human peripheral monocytes and is commonly used for investigating the function and regulation of monocytes and macrophages. THP-1 cells express FcRI and FcRII receptors [10]. THP-1 monocytes can convert to macrophage-mimicking cells in the presence of phorbol 12-myristate 13-acetate (PMA) [11, 12]. With increasing cell adherence, FcRI and FcRII receptor expression is reduced [10]. Interestingly, phagocytosis of IgG-coated sheep RBCs increases much stronger than that of uncoated RBCs (64 % vs. 35 %) [12]. In a previous study that applied the fluorometric quantitative erythrophagocytosis assay using human THP-1 cells and PKH26-labelled RBCs, high sensitivity and good reproducibility were demonstrated [13]. We modified this assay by using PMA-treated THP-1 cells and PKH26-labelled RBCs in a shaking assay. Thus, we used adherent macrophage-like cells instead of monocytes, and gentle shaking may favour antibody-mediated interaction between macrophage-like cells and RBCs. Accordingly, a trypsinization step was needed. All other steps and analysis of the data were done as described by Healey *et al.* [13]. The test was initially used to assess the clinical relevance of several known alloantibodies. To demonstrate a correlation between the assay and antibody-dependent haemolysis, RBCs from three groups of patients were analysed as follows: patients with clinically significant AIHA, patients with AIHA in remission and pregnant women with detectable autoantibodies to RBCs.

## Materials and methods

### Patients

The research was approved by the local ethics committee (No EA2/058/12). Sixteen patients with AIHA of warm type were included. Routine haemolysis parameters (haemoglobin, LDH, haptoglobin, reticulocytes) were determined. In addition, five pregnant women with detectable autoantibodies without haemolysis were investigated. EDTA blood samples for the control group were obtained from healthy blood donors.

### Serological testing

Serological testing, including antibody screening and the monospecific direct antiglobulin test (DAT), was performed using standard gel technology as described elsewhere [14,15]. Acid eluates were performed according to the manufacturer's recommendation (BAG Health Care GmbH, Lich, Germany).

### THP-1 cells culture

THP-1 human monocytic leukaemia cells (TIB-202, American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured at a density of $1–5 \times 10^5$ cells/ml in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FCS; GIBCO, Invitrogen, Carlsbad, California, USA), and 1% penicillin 100 U/ml and 1% streptomycin 100 μg/ml (GIBCO Life Technologies, Carlsbad, California, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% $CO_2$. Cell suspensions were pelleted at 900 *g* for 2 min, resuspended and transferred at a density of $1 \times 10^5$ cells/ml in refreshed medium twice weekly.

### Differentiation of THP-1 cells to macrophage-mimicking cells

THP-1 cells were resuspended at a density of $1 \times 10^6$ cells/ml in fresh growth medium and PMA (Calbiochem, Darmstadt, Germany, hazardous substance, please acknowledge the safety data sheet) to a final concentration of $10^{-8}$ M [16]. Five hundred microlitres of the suspension was transferred into each well of 6-well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. Differentiation medium with $10^{-8}$ M PMA was

refreshed after 24 h. Following 48 h, the medium was replaced with fresh medium without PMA and incubated for a further 3 h. The adherent, macrophage-like THP-1 cells were almost confluent before use in the phagocytosis assay.

### Verification of THP-1 cell differentiation by flow cytometry

After a 10-min trypsinization period (GibcoTM TrypLE Express), PMA-treated THP-1 cells were resuspended in cell culture medium. PMA-treated and non-treated THP-1 cells ($0{\cdot}5 \times 10^6$) were diluted in 200 µl PBS and stained with 10 µl of the fluorescent labelled anti-human CD11b / MAC-1 (FITC) or anti-human HLA-DR (APC) antibodies (both from BD Biosciences Pharmingen TM). Cells were subsequently analysed by flow cytometry [16] with MACSQuant® Flow Cytometer (Miltenyi Biotech, Bergisch Gladbac, Germany).

### RBC preparation and labelling by PKH26 and the phagocytosis assay

RBCs from patients with AIHA, pregnant women with detectable autoantibodies and from healthy donors were washed three times with saline (0·9% NaCl) and pelleted at 900 *g* for 2 min before PKH26 staining. In contrast, patients' plasma containing alloantibodies (anti-D, anti-K, anti-Lu(b), anti-Yt(a), anti-Ch (200µl)) were incubated with 50 µl pelleted donor RBCs (expressing the correspondent antigen) for 2 h at 37°C and then washed three times with saline (0·9% NaCl). The alloantibody-coated RBCs were further tested in an identical way as patients' and healthy donors' RBC, respectively.

All RBCs were membrane-stained using PKH26 according to the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 10 µl of RBCs from pellet was diluted in 125 µl diluent C (supplied with the PKH26 kit), added to 0·5 µl PKH26 in 125 µl diluent C, followed by immediate mixing and incubation at room temperature for 4 min. The reaction was terminated by the addition of 100 µl of human AB plasma. After 1 min, 2 ml of cell culture medium was added. Cells were pelleted at 1700 *g* for 10 min, resuspended in 100 µl of cell culture medium and added to PMA-differentiated THP-1 cells in a 6-well plate. Subsequently, plates were incubated at 37°C for 45 min under soft continuous agitation.

### Flow cytometry

After incubation at 37°C for 45 min, non-phagocytic RBCs were removed by washing three times with 2 ml of ice-cold isotonic saline (0·9% NaCl). Residual RBCs were haemolysed with ice-cold hypotonic saline (0·2% NaCl) for 2 min, followed by the addition of 2 ml of ice-cold hypertonic saline (1·6% NaCl) for 2 min to restore isotonicity [13,17]. Subsequently, the supernatant was removed and the adherent macrophage cells were detached by trypsin. Before and after trypsinization, THP cells were checked microscopically for remaining RBCs or detritus THP cells were diluted with 500 µl PBS ($1 \times 10^6$ cells/ml) and kept on ice until analysis by flow cytometry (MACSQuant® Flow Cytometer, Miltenyi Biotech). At least 10 000 events were collected for each sample. Data were analysed using the FlowJo® software (FlowJo LLC, Ashland, OR, USA). The mean fluorescence and percentage of cells that were strongly positively stained for PKH26-red-labelled RBCs compared with control group were calculated. Controls using RBCs from two or three healthy blood donors with no selection of age, sex or blood group were included in each experiment.

### Analysis and statistics

Clinical relevance of allo- or autoantibodies was determined by comparing patients' results (MFI or % phagocytosis) with arithmetic means ± standard deviation (SD) of the same day control group. As a cut-off, antibodies generating results (MFI or % phagocytosis) higher than arithmetic means + 3 SD of controls were considered clinically relevant.

For statistical analysis of patient groups and control groups, data were calculated as arithmetic means ± standard error of the mean (SEM). Statistical analysis was performed using Microsoft Excel and SSPS. Significance between two groups was determined using Mann–Whitney U-test.

## Results

### Serological and clinical data

In total, RBCs from 16 patients with long-term AIHA were studied. All patients had a positive eluate (Table 1). Fourteen of 16 patients showed IgG-positive DAT and 11 patients C3d-positive DAT. One patient predominantly had an IgM-wAIHA (Pat. W7). Another patient (Pat. W12) had both warm and cold autoantibodies (mixed-type IgM-AIHA). Based on haemoglobin, reticulocyte, lactate dehydrogenase and haptoglobin levels, as well as the clinical picture, 12 patients showed clear signs of haemolysis. All five pregnant women had an IgG-positive DAT and detectable autoantibodies in the eluate (Table 1). There were no signs of haemolysis in the pregnant cohort [18].

**Table 1** Most relevant data of patients with AIHA

| Patient No. | Age (years) | DAT | | | | | Eluate | Hb (g/dl) | LDH (U/l) | Hp (mg/dl) | RETR 10E3/µl | Medication |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sex | IgG | C3d | IgM | IgA | | | | | | |
| W1 | 8 | M | 4+ | – | (+) | 1+ | P | 10•9 | 281 | nd | nd | Rituximab / Prednisolone |
| W2 | 64 | F | 4+ | 2+ | – | – | P | 13•4 | 583 | <5•8 | nd | Prednisolone 1 mg/d |
| W3 | 49 | F | 4+ | 4+ | – | – | P | 10 | 443 | <5•8 | 140 | Cyclosporin A 300 mg /d, Prednisolone 10 mg/d, Darbepoetin alfa |
| W4 | 82 | F | 3+ | 3+ | – | – | P | 11•4 | 348 | 122 | 50 | Prednisolone 5 mg/d, Darbepoetin alfa |
| W5 | 48 | F | 4+ | (+) | – | 2+ | P | 8•4 | 1326 | <5•8 | 454 | Prednisolone 30 mg/d, *Cyclophosphamide* 150 mg/d |
| W6 | 60 | F | 4+ | 4+ | – | – | P | 13•0 | 483 | <5•8 | nd | Prednisolone 5 mg/d |
| W7 | 36 | M | (+) | 4+ | 4+ | (+) | P | 12•4 | 281 | <5•8 | 169 | Prednisolone 75 mg/d |
| W8 | 43 | M | 4+ | 3+ | – | – | P | 10•4 | 460 | <5•8 | 112 | Prednisolone 10 mg/d, Azathioprine 200 mg/d |
| W9 | 68 | F | 4+ | 3+ | 2+ | – | P | 8•6 | 1214 | nd | 419 | Mycophenolic acid; 2000 mg/d, Prednisolone 10 mg |
| W10 | 50 | M | 4+ | – | – | – | P | 12•4 | 397 | <5•8 | nd | Prednisolone 7•5 mg/d, Cyclophosphamide 150 mg/d |
| W11 | 27 | M | 3+ | (+) | – | – | P | 12•9 | 252 | <5•8 | nd | – |
| W12 | 51 | F | – | 4+ | – | – | P | 12•5 | 314 | nd | nd | – |
| W13 | 55 | F | 4+ | 1+ | – | (+) | P | 11•5 | 227 | 116 | nd | Prednisolone 10 mg/d, Cyclophosphamide 150 mg/d |
| W14 | 73 | F | 1+ | – | – | – | P | 11•3 | 234 | 149 | 101 | Cyclophosphamide 25 mg/d |
| W15 | 27 | F | 4+ | – | – | – | P | 14•8 | 235 | 95•6 | 79 | Prednisolone 10 mg/d, Azathioprine 200 mg/d |
| W16 | 56 | M | – | – | – | – | P | 13•9 | 242 | 110 | 81 | Prednisolone 2•5 mg/d |
| G1 | 36 | F | 4+ | – | (+) | – | P | | | | | |
| G2 | 36 | F | 4+ | 2+ | – | – | P | | | | | |
| G3 | 29 | F | 3+ | – | – | – | P | | | | | |
| G4 | 32 | F | 1+ | – | – | – | P | | | | | |
| G5 | 40 | F | 1+ | – | – | – | P | | | | | |

DAT, direct antiglobulin test; G, pregnancy with autoantibodies of warm type; Hb, haemoglobin; Hp, haptoglobin; LDH, lactate dehydrogenase; nd, no data; P, positive; RETR, reticulocytes; W, patient with AIHA of warm type.

### PMA-treated THP-1 cells transform into macrophage-like cells

Phorbol 12-myristate 13-acetate-differentiated THP cells were adherent, demonstrating increasing CD11b expression, as well as a downregulation of HLA-DR (Fig. 1a). The strongest changes in CD11b/HLA-DR expression were observed from 48 to 72 h. Therefore, THP cells were used after 48 h of differentiation in further experiments. Scatter plots of THP-1 cells and differentiated THP-1 cells (macrophage-like cells; blank control) were similar and showed only background fluorescence in channel B2 B585 (Fig. 1b) and a very low number of cells in region P3 (<0·1 %). After the phagocytosis assay, trypsinated THP-1 cells were checked for remaining RBCs by the use of Hayem's solution and the improved Neubauer haemocytometer showing an almost complete haemolysis of non-phagocyted RBCs (data not shown). Figure 1c shows an overlay of the histograms of PMA-treated THP-1 cells and PKH26-stained RBCs from healthy donors. After incubation with PKH26-stained RBCs, PMA-treated THP-1 cells showed increased fluorescence from ingested PKH26-stained RBCs (control group; Fig. 1d). This reflects already published results showing that non-opsonized RBCs were also digested in larger amounts [13,17].

### RBCs coated with alloantibodies

RBCs loaded with significant (anti-D, anti-K) or potentially significant alloantibodies for haemolysis (anti-Lu(b), anti-Yt(a)) were observed to further enhance phagocytosis by PMA-treated THP-1 cells (Fig. 2a–j, m). However, two of three anti-Yt(a) did not enhance phagocytosis significantly by more than mean ± 3 SD of the control group, which corresponds to the clinical experience with this antibody. In contrast, pre-incubation of RBCs with the non-haemolytic alloantibody anti-Ch did not affect phagocytosis (Fig. 2k,l,m).

### Enhanced phagocytosis of RBCs from patients with active wAIHA

A typical result obtained from patients with significant wAIHA is depicted in Fig. 3a–c using the example of patient no. W9 (Table 2). Erythrophagocytosis was significantly increased (> average mean of control + 3 SD) in patients with active AIHA compared with healthy donors (control group). This significant increase in the mean fluorescence of all THP-1 cells and percentage of strongly positive stained cells in region P3 indicates a strong avidity of macrophages to patient RBCs. In comparison, erythrophagocytosis of RBCs from a pregnant woman (G4, Table 2) was not increased (Fig. 3d–f). Patients with clinically relevant AIHA of warm type showed a highly significant enhanced erythrophagocytosis as shown by mean fluorescence (Fig. 3g). In contrast, a similar erythrophagocytosis was observed in patients with AIHA of warm type in remission or patients with autoantibodies due to pregnancy and healthy blood donors, respectively (Fig. 3g). Further analysis did not demonstrate a correlation with the amount of bound IgG or C3d (Tables 1 and 2).

Positivity of haemolysis parameter (LDH/haptoglobin) was found to correlate strongly positive with erythrophagocytosis. Using our assay, RBCs of all patients with significant haemolysis showed increased phagocytosis by THP-1 cells. Increased phagocytosis was not observed of RBCs from three AIHA patients without haemolysis (W14-W16) and all pregnant women with warm autoantibodies (G1–G5; Table 2).

## Discussion

The MMA has been shown to correlate well with the significance of alloantibodies [6]. However, numerous technical variables may affect assay results. These include the selection of monocytes, RBCs, culture conditions and analytical method [6]. In addition, compared to abounding MMA results about the significance of alloantibodies there is little information available on the use of the MMA to determine the significance of autoantibodies to RBCs. In one study, it has been shown that the amount of IgG1 autoantibodies on $^{51}$Cr-labelled RBCs corresponds well with the phagocytosis by monocytes and the haemolysis *in vivo* [19]. In the work of Gallagher *et al.* [20], all of the 16 patients with AIHA and clinical evidence of haemolysis showed an enhanced phagocytic index (PRBC) in the MMA, whereas six non-haemolysing patients showed a normal PRBC.

In the present study, we focused on further optimization of the MMA and on assessing the relevance of autoantibodies rather than alloantibodies to RBCs. The assay described here is both quantitative and highly reliable. Furthermore, it has some advantages as compared to previously described assays. First, the macrophage-like cells are derived from the immortalized monoclonal cell line THP-1 after differentiation with PMA. The cells are easily accessible, and their phagocytic activity is highly reproducible compared with monocytes from healthy donors. As in most other MMA, only phagocytosed RBCs are measured since free or adherent erythrocytes are removed quantitatively by washing and RBC lysis. Second, measurement by flow cytometry is easier and allows for a more precise quantification through higher numbers of counted cells compared with microscopic analysis. One notable disadvantage lies in the necessity of staining the

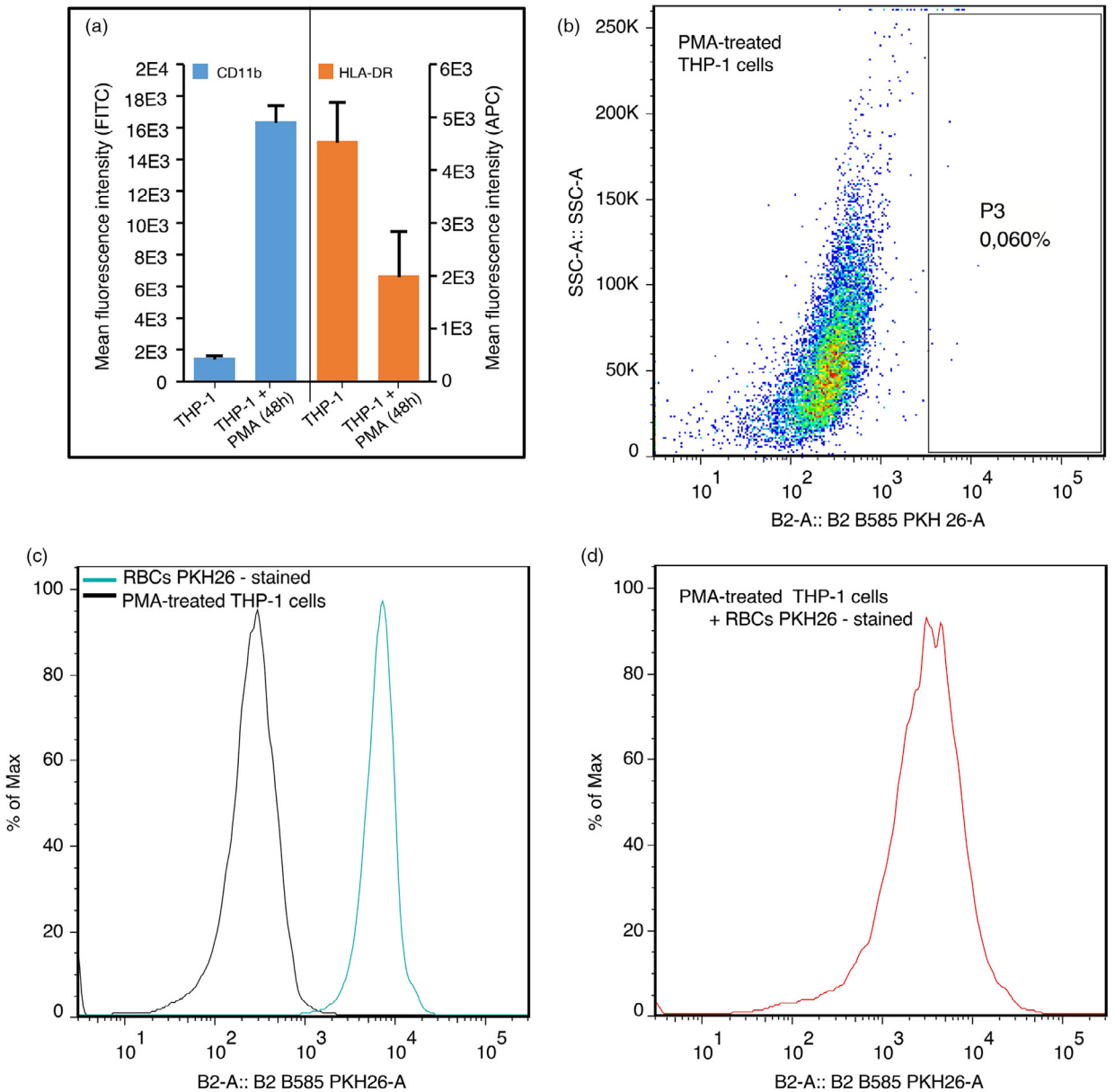


**Fig. 1** Characterization of PMA-treated THP-1 cells. (a) Effect of PMA differentiation on CD11b (left) and HLA-DR (right) expression in THP-1 cells at different times (0 and 48 h). (b) Scatter plot of PMA-treated THP-1 cells (blank control). Region P3 has been selected as contains ≤ 0·1% of events. (c) Histogram overlay of PMA-treated THP-1 cells (black) and PKH26-stained RBCs (blue). (d) Histogram of PMA-treated THP-1 cells after phagocytosis of PKH26 stained RBCs from healthy blood donors (control group).

RBCs; however, it is required for fluorocytometric analysis and is easy to perform. Another drawback may be the permanent culturing of the THP-1 cells and the time-consuming differentiation of 48 h.

Initially, we determined the reliability by testing well-characterized alloantibodies known to induce phagocytosis. Two such antibodies, anti-D and anti-K, led to significant phagocytosis, whereas there was no enhanced phagocytosis observed for the insignificant antibody anti-Ch. Two other alloantibodies, anti-Yt(a) and anti-Lu(b), were able to induce enhanced phagocytosis, albeit two of three anti-Yt(a) not significantly. The results indicate a potential clinical significance, which is, however, variable [21–23]. Our findings also correlate with the results of a retrospective study that analysed MMA data from 46 patients over 20 years to predict the clinical significance of alloantibodies [24]. Therefore, the assay could be used to select compatible RBCs in alloimmunized patients

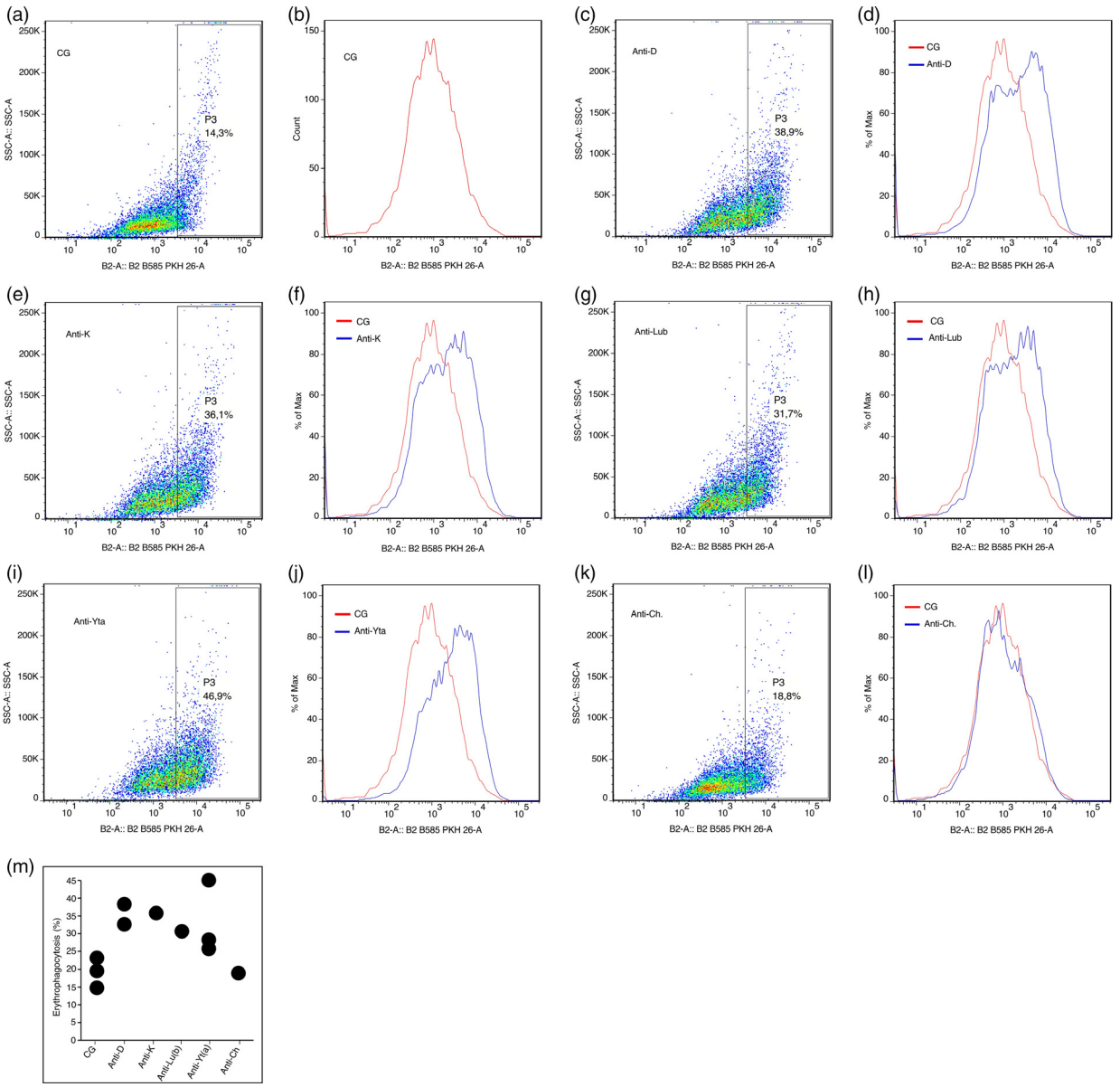


**Fig. 2** Erythrophagocytosis of RBCs coated with alloantibodies. (a-l) Scatter plot (a) and histogram (b) of PMA-treated THP-1 cells after phagocytosis of RBCs from a healthy blood donor (control group). Scatter plots (c, e, g, i, k) and overlay of histograms with a control group (d, f, h, j, l) of PMA-treated THP-1 cells after phagocytosis of alloantibody-coated RBCs [anti-D, anti-K, anti-Lu(b), anti-Yt(a) and anti-Ch (blue).]. (m) Results of THP-1 phagocytosis of RBCs pre-incubated with alloantibodies in comparison with control group RBCs.

requiring blood transfusion in cases where all cross-matched units are serologically incompatible. Noumsi et al. used the Monocyte index (MI) of <5 % in MMA for cross-matching sera with alloantibodies to select RBC units for patients [7]. The evaluation of Noumsi's test is difficult due to the fact that also clinically relevant antibodies (anti-s, anti-e, anti-hrS, anti-Fy3, anti-Jkb) showed no significant phagocytosis in MMA. Nevertheless, patient with these alloantibodies has not been transfused. Therefore, the reliability of this assay for assessing the clinical relevance of these antibodies remains unclear.

In some cases, the cause of anaemia cannot be completely attributed to the presence of autoantibodies, even when affected patients were previously or currently afflicted with AIHA. Though rarely observed, some patients may have diseases associated with mild haemolysis such as hereditary or acquired non-immune haemolytic anaemia [25]. For example, eryptosis, the programmed

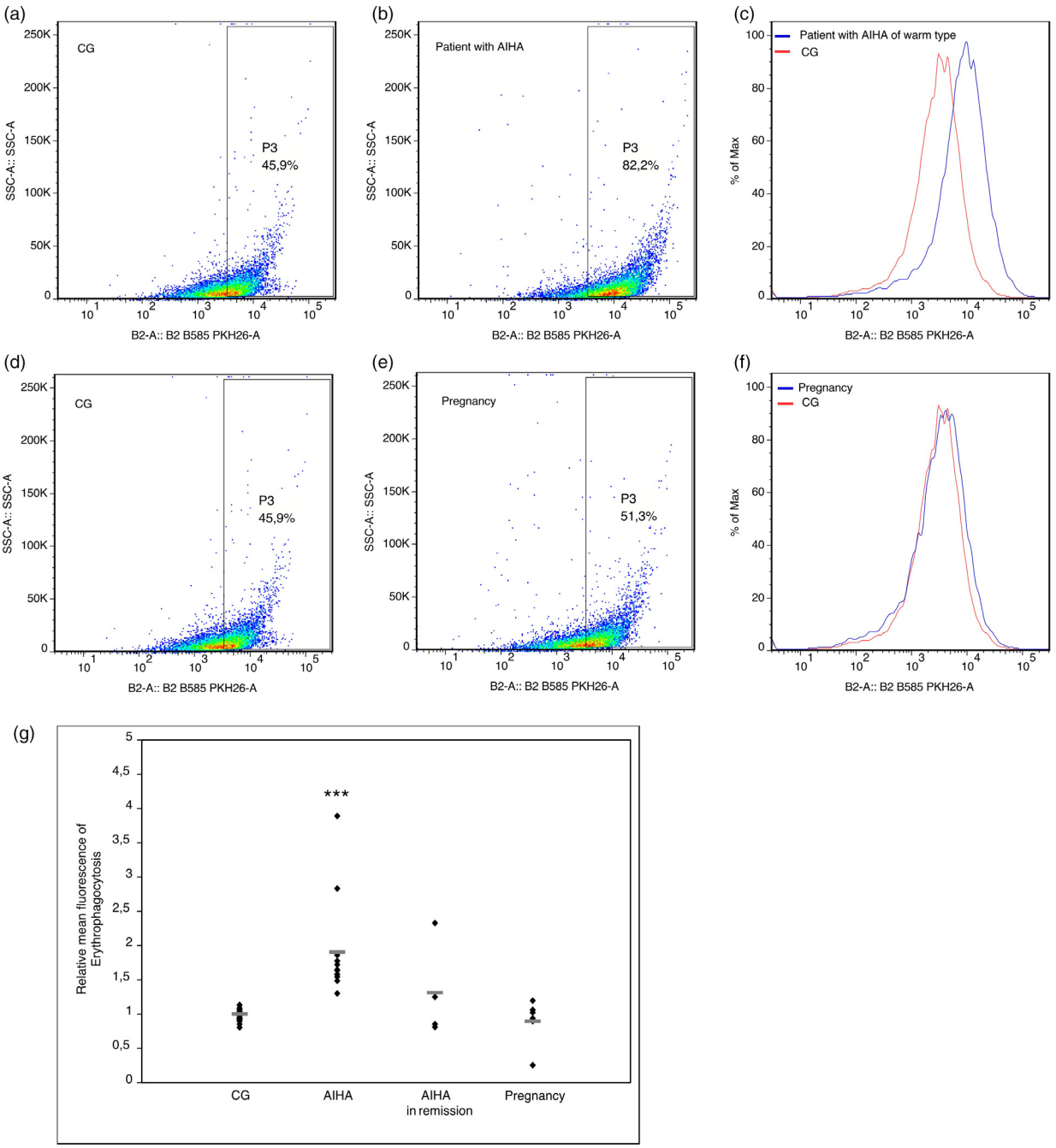


**Fig. 3** Phagocytosis of RBCs from a patient with AIHA and a pregnant woman with warm autoantibodies. Scatter plots and overlay histogram of PMA-treated THP-1 cells after phagocytosis of RBCs from a healthy blood donor (a, c: red line) and of RBCs from a patient with clinically relevant AIHA (b, c: blue line). Scatter plots and overlay histogram of PMA-treated THP-1 cells after phagocytosis of RBCs from a healthy blood donor (d, f: red line) and of RBCs from a pregnant woman with warm autoantibodies (e, f: blue line). (g) Comparison of THP-1 phagocytosis of RBCs from healthy blood donors (CG, first column), patients with significant wAIHA (second column), patients with wAIHA in complete remission (third column) and pregnant patients with autoantibodies (fourth column). Each point indicates one THP-1 sample after incubation with the respective RBCs. The grey lines represent the mean of each group. The mean fluorescence for the CG was normalized at 1. ***$P < 0{\cdot}001$ significant difference from control group.

**Table 2** In vitro erythrophagocytosis by PMA-treated THP-1 cells

| Patient no | Haemolysis[a] Yes/No | MFI | | % Phagocytosis | |
|---|---|---|---|---|---|
| | | Patient | CG (mean ± SD) | Patient | CG (mean ± SD) |
| W1 | Yes | **3271** | 2119 ± 195 | **37** | 19·5 ± 3·5 |
| W2 | Yes | **3943** | 2119 ± 195 | **42** | 19·5 ± 3·5 |
| W3 | Yes | **2745** | 1738 ± 51 | **28** | 14·3 ± 0·6 |
| W4 | Yes | **3540** | 2148 ± 188 | **33** | 16·3 ± 1·5 |
| W5 | Yes | **5908** | 3431 ± 169 | **60** | 33·7 ± 5·9 |
| W6 | Yes | **4301** | 2723 ± 44 | **34** | 20·5 ± 1·2 |
| W7 | Yes | **6774** | 4140 ± 585 | **63** | 42·0 ± 4·2 |
| W8 | Yes | **5096** | 3431 ± 169 | **56** | 33·7 ± 5·9 |
| W9 | Yes | **12032** | 4247 ± 596 | **82** | 45·7 ± 7·5 |
| W10 | Yes | **3513** | 2700 ± 186 | **41** | 27·3 ± 3·2 |
| W11 | Yes | **1912** | 1076 ± 192 | **41** | 20·3 ± 6·7 |
| W12 | Yes | **4189** | 1076 ± 192 | **70** | 20·3 ± 6·7 |
| W13 | No | **2507** | 1076 ± 192 | **55** | 20·3 ± 6·7 |
| W14 | No | 5176 | 4140 ± 585 | 54 | 42·0 ± 4·2 |
| W15 | No | 1822 | 2248 ± 141 | 12 | 19·7 ± 2·5 |
| W16 | No | 2324 | 2723 ± 448 | 15 | 20·5 ± 2·1 |
| G1 | No | 3218 | 3431 ± 169 | 36 | 33·7 ± 5·9 |
| G2 | No | 3644 | 3431 ± 169 | 40 | 33·7 ± 5·9 |
| G3 | No | 5084 | 4247 ± 596 | 51 | 45·7 ± 7·5 |
| G4 | No | 4467 | 4981 ± 557 | 47 | 58·5 ± 6·4 |
| G5 | No | 5091 | 4981 ± 557 | 55 | 58·5 ± 6·4 |

**Bold MFI** or % **Phagocytosis** indication of a significant result, higher than mean ± 3 SD of the control group (CG).
[a]Haemolysis from laboratory findings and as judged by the treating physician; MFI, mean fluorescence intensity of the THP-1 cells after phagocytosis; % phagocytosis, proportion of THP-1 cells that contained fluorescence signal in p3 area; CG, control group; SD, standard deviation.

death of RBCs, has been described to occur in a numerous clinical conditions including sickle cell anaemia, thalassemia, glucose-6-phosphate dehydrogenase deficiency, hereditary spherocytosis, paroxysmal haemoglobinuria, myelodysplastic syndrome, phosphate depletion, iron deficiency, sepsis, haemolytic–uremic syndrome, renal insufficiency, diabetes mellitus, malaria, mycoplasma infection and Wilson disease [26, 27]. Importantly, eryptosis has been described recently in AIHA related to IgA or IgM autoantibodies [28]. The clinical significance of autoantibodies in AIHA is reflected usually by the rate of ongoing haemolysis. Nevertheless, confusion may arise in a number of cases due to co-morbidities associated with anaemia and/or non-immune haemolytic anaemia [25]. However, the results obtained by the DAT, as the main marker for the presence of AIHA, are of little value without sufficient clinical information [25]. The phenomenon related to the long-term persistence of detectable autoantibodies in patients whose AIHA had entered into complete remission is intriguing. It is unclear whether the causative autoantibodies and/or macrophages in these patients have acquired a new character following treatment. In fact, the finding in patient no. 13 reflects that these patients' autoantibodies appear to be clinically relevant as has been demonstrated by our test. This may indicate that the macrophages of affected patients were incapable to phagocyte the opsonized RBCs, probably, due to treatment with immunosuppressive drugs. This observation has been shown before [19, 20]. Similarly, it remains unknown why pregnancy-induced autoantibodies to RBCs do not appear to cause significant haemolysis [18, 29].

The present study analysed heterogeneous autoantibodies to RBCs. The results obtained largely reflect conditions *in vivo*. RBCs from patients with positive DAT and recognizable haemolysis were preferentially digested from macrophage-like cells in contrast to RBCs from patients without signs of haemolysis and pregnant women.

Therefore, a positive result in the described assay strongly indicates haemolysis *in vivo*. This finding may be helpful in the management of patients who had been successfully treated or are still under treatment for AIHA but cannot compensate for their anaemia. While *in vitro* phagocytosis of patient RBCs supports the clinical significance of the detectable autoantibodies, a negative result may indicate that the anaemia is related to another disease such as an infection, tumour or renal or cardiac failure. In addition, autoantibodies are frequently associated

with HTRs due to alloantibodies [30, 31]. It remains unknown whether these autoantibodies are also involved in RBC destruction and our assay may be useful to clarify that question.

Finally, the question of whether pregnancy-induced autoantibodies [18, 29] and persisting autoantibodies in AIHA following complete remission do not lead to phagocytosis has not yet been completely resolved. The results obtained in this study support our hypothesis that these autoantibodies are incapable of causing haemolysis. Based on our findings, this phenomenon cannot be either explained by IgG subclasses, the number of antibodies attached to the RBCs or autologous macrophages. A possible explanation might be related to the IgG Fc region structure, which is determined by a specific glycosylation to modify signal pathways targeted by the macrophages [32–34]. Further studies are required to clarify this phenomenon.

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## Conflict of interests

The authors declare no conflict of interests.

## Author contributions

AS, TB and AB designed the experiments. BM and AS treated the patients. AB and TB performed the experiments. TB and AB analysed the data. AS wrote the paper with input from all other authors. All authors reviewed the manuscript and approved the final version.

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Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI Selected Categories: **"HEMATOLOGY"** Selected Category Scheme: WoS **Gesamtanzahl: 71 Journale**

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 1 | CIRCULATION RESEARCH | 52,753 | 15.211 | 0.08282 |
| 2 | BLOOD | 1 | 15.132 | 0.27804 |
| 3 | Lancet Haematology | 1,307 | 10.698 | 0.00735 |
| 4 | LEUKEMIA | 25,265 | 10.023 | 0.05958 |
| 5 | HAEMATOLOGICA | 16,138 | 9.090 | 0.03893 |
| 6 | Journal of Hematology & Oncology | 4,098 | 7.333 | 0.00975 |
| 7 | BLOOD REVIEWS | 2,810 | 6.600 | 0.00601 |
| 8 | ARTERIOSCLEROSIS THROMBOSIS AND VAS-CULAR BIOLOGY | 34,074 | 6.086 | 0.04482 |
| 9 | JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM | 19,450 | 6.045 | 0.02828 |
| 10 | STEM CELLS | 21,694 | 5.587 | 0.03568 |
| 11 | AMERICAN JOURNAL OF HEMATOLOGY | 9,458 | 5.303 | 0.02262 |
| 12 | BRITISH JOURNAL OF HAEMATOLOGY | 23,861 | 5.128 | 0.03901 |
| 13 | THROMBOSIS AND HAEMOSTASIS | 16,701 | 4.952 | 0.02577 |
| 14 | JOURNAL OF THROMBOSIS AND HAE-MOSTASIS | 17,663 | 4.899 | 0.03438 |
| 15 | BONE MARROW TRANSPLANTATION | 12,506 | 4.497 | 0.02081 |
| 16 | CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY | 6,956 | 4.495 | 0.01219 |
| 17 | BIOLOGY OF BLOOD AND MARROW TRANS-PLANTATION | 10,583 | 4.484 | 0.02694 |
| 18 | JOURNAL OF LEUKOCYTE BIOLOGY | 17,244 | 4.224 | 0.02120 |
| 19 | TRANSFUSION MEDICINE REVIEWS | 1,292 | 4.111 | 0.00235 |
| 20 | CYTOTHERAPY | 5,589 | 3.993 | 0.00902 |
| 21 | SEMINARS IN HEMATOLOGY | 2,305 | 3.926 | 0.00378 |
| 22 | TRANSFUSION | 13,045 | 3.423 | 0.02205 |
| 23 | SEMINARS IN THROMBOSIS AND HEMOSTASIS | 3,876 | 3.345 | 0.00627 |
| 24 | STEM CELLS AND DEVELOPMENT | 7,589 | 3.315 | 0.01644 |

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 25 | HEMATOLOGICAL ONCOLOGY | 1,007 | 3.193 | 0.002060 |
| 26 | HEMATOLOGY-ONCOLOGY CLINICS OF NORTH AMERICA | 2,277 | 3.098 | 0.004500 |
| 27 | SHOCK | 7,437 | 3.005 | 0.011650 |
| 28 | ANNALS OF HEMATOLOGY | 5,137 | 2.845 | 0.011130 |
| 29 | CURRENT OPINION IN HEMATOLOGY | 2,587 | 2.821 | 0.005830 |
| 30 | MICROCIRCULATION | 2,444 | 2.797 | 0.003610 |
| 31 | THROMBOSIS RESEARCH | 10,486 | 2.779 | 0.021660 |
| 32 | HAEMOPHILIA | 5,776 | 2.768 | 0.009000 |
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| 34 | LEUKEMIA & LYMPHOMA | 8,243 | 2.644 | 0.016110 |
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| 39 | Current Hematologic Malignancy Reports | 658 | 2.388 | 0.002310 |
| 40 | PLATELETS | 2,438 | 2.356 | 0.004180 |
| 41 | LEUKEMIA RESEARCH | 6,335 | 2.319 | 0.009870 |
| 42 | Clinical Lymphoma Myeloma & Leukemia | 1,944 | 2.308 | 0.005070 |
| 43 | Hematology-American Society of Hematology Education Program | 2,797 | 2.238 | 0.007710 |
| 44 | TRANSFUSION MEDICINE AND HEMOTHERAPY | 897 | 2.152 | 0.002490 |
| 45 | Blood Transfusion | 1,620 | 2.138 | 0.003910 |
| 46 | VOX SANGUINIS | 3,204 | 2.107 | 0.005200 |
| 47 | INTERNATIONAL JOURNAL OF HEMATOLOGY | 3,716 | 1.942 | 0.006130 |
| 48 | Expert Review of Hematology | 1,042 | 1.937 | 0.003300 |
| 49 | BLOOD PURIFICATION | 1,864 | 1.919 | 0.002970 |

**Publikation 2:** Bartolmäs T*, Mayer* **B, Balola AH**, Salama A. Eryptosis in auto-immune haemolytic anaemia. Eur J Haematol 2018;**100**(1): 36-44.

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Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI Selected Categories: **"CELL BIOLOGY"** Selected Category Scheme: WoS **Gesamtanzahl: 190 Journale**

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 1 | NATURE REVIEWS MOLECULAR CELL BIOLOGY | 43,667 | 35.612 | 0.095540 |
| 2 | NATURE MEDICINE | 75,461 | 32.621 | 0.171980 |
| 3 | CELL | 230,625 | 31.398 | 0.583260 |
| 4 | Cell Stem Cell | 23,493 | 23.290 | 0.096030 |
| 5 | CANCER CELL | 35,217 | 22.844 | 0.096910 |
| 6 | Cell Metabolism | 29,834 | 20.565 | 0.101740 |
| 7 | NATURE CELL BIOLOGY | 39,896 | 19.064 | 0.092960 |
| 8 | TRENDS IN CELL BIOLOGY | 13,708 | 18.564 | 0.037630 |
| 9 | Science Translational Medicine | 26,691 | 16.710 | 0.126450 |
| 10 | CELL RESEARCH | 13,728 | 15.393 | 0.037450 |
| 11 | MOLECULAR CELL | 61,604 | 14.248 | 0.181170 |
| 12 | NATURE STRUCTURAL & MOLECULAR BIOLOGY | 27,547 | 13.333 | 0.081820 |
| 13 | Autophagy | 14,923 | 11.100 | 0.035510 |
| 14 | TRENDS IN MOLECULAR MEDICINE | 9,213 | 11.021 | 0.019720 |
| 15 | EMBO JOURNAL | 67,036 | 10.557 | 0.079780 |
| 16 | CURRENT OPINION IN CELL BIOLOGY | 13,339 | 10.015 | 0.027790 |
| 17 | DEVELOPMENTAL CELL | 26,896 | 9.616 | 0.074980 |
| 18 | GENES & DEVELOPMENT | 57,469 | 9.462 | 0.092720 |
| 19 | CURRENT BIOLOGY | 56,595 | 9.251 | 0.137200 |
| 20 | Cold Spring Harbor Perspectives in Biology | 13,275 | 9.247 | 0.049360 |
| 21 | Annual Review of Cell and Developmental Biology | 9,812 | 9.032 | 0.016870 |
| 22 | Cell Systems | 1,129 | 8.982 | 0.009600 |
| 23 | AGEING RESEARCH REVIEWS | 5,297 | 8.973 | 0.012030 |
| 24 | JOURNAL OF CELL BIOLOGY | 68,915 | 8.784 | 0.085170 |
| 25 | EMBO REPORTS | 13,293 | 8.749 | 0.031350 |
| 26 | PLANT CELL | 48,393 | 8.228 | 0.063640 |
| 27 | MATRIX BIOLOGY | 4,803 | 8.136 | 0.008500 |
| 28 | Cell Reports | 29,789 | 8.032 | 0.210690 |

| Rank | Full Journal Title | Total Cites | Journal Impact Factor | Eigenfactor Score |
|---|---|---|---|---|
| 29 | CELL DEATH AND DIFFERENTIATION | 18,865 | 8.000 | 0.031540 |
| 30 | AGING CELL | 8,067 | 7.627 | 0.018910 |
| 31 | CURRENT OPINION IN STRUCTURAL BIOLOGY | 10,619 | 7.179 | 0.024320 |
| 32 | ONCOGENE | 66,411 | 6.854 | 0.075960 |
| 33 | CELLULAR AND MOLECULAR LIFE SCIENCES | 23,341 | 6.721 | 0.041340 |
| 34 | Stem Cell Reports | 4,525 | 6.537 | 0.026290 |
| 35 | CYTOKINE & GROWTH FACTOR REVIEWS | 5,668 | 6.395 | 0.008050 |
| 36 | Science Signaling | 10,316 | 6.378 | 0.037220 |
| 37 | Protein & Cell | 2,363 | 6.228 | 0.008060 |
| 38 | SEMINARS IN CELL & DEVELOPMENTAL BIOLOGY | 9,024 | 6.138 | 0.024200 |
| 39 | Pigment Cell & Melanoma Research | 4,430 | 6.115 | 0.007840 |
| 40 | Wiley Interdisciplinary Reviews-RNA | 2,142 | 5.844 | 0.009350 |
| 41 | Cell Death & Disease | 14,475 | 5.638 | 0.046010 |
| 42 | FASEB JOURNAL | 41,572 | 5.595 | 0.051640 |
| 42 | Journal of Molecular Cell Biology | 1,877 | 5.595 | 0.005920 |
| 44 | STEM CELLS | 21,694 | 5.587 | 0.035680 |
| 45 | CELLULAR PHYSIOLOGY AND BIOCHEMISTRY | 11,234 | 5.500 | 0.017450 |
| 46 | Cell Communication and Signaling | 2,034 | 5.324 | 0.005510 |
| 47 | JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY | 14,186 | 5.296 | 0.025030 |
| 48 | Aging-US | 4,410 | 5.179 | 0.010910 |
| 49 | CURRENT OPINION IN GENETICS & DEVELOPMENT | 7,791 | 4.995 | 0.018550 |
| 51 | Stem Cell Research & Therapy | 4,578 | 4.963 | 0.012630 |
| 52 | CELL PROLIFERATION | 2,663 | 4.936 | 0.003440 |
| 52 | Oxidative Medicine and Cellular Longevity | 9,180 | 4.936 | 0.022930 |
| 54 | STRUCTURE | 14,417 | 4.907 | 0.036760 |
| 55 | Cells | 1,005 | 4.829 | 0.004100 |
| 56 | CELLULAR ONCOLOGY | 1,322 | 4.761 | 0.002020 |

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**Original Paper**

# Sublytic Terminal Complement Components Induce Eryptosis in Autoimmune Haemolytic Anaemia Related to IgM Autoantibodies

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**Key Words**
Phosphatidylserine • C5b6 • C5b-7 • C5b-8 • Annexin • Haemolysis • IgM autoantibodies

**Abstract**
***Background/Aims:*** Eryptosis, the suicidal death of red blood cells (RBCs), is characterized by phosphatidylserine (PS) exposure at the cell surface. It can be catalysed by a variety of abnormal conditions and diseases. Until now, the many questions surrounding the physiology and pathophysiology of eryptosis have not been sufficiently answered. Recently, we demonstrated IgM and IgA autoantibodies (aab) to induce PS exposure on circulating RBCs of patients with autoimmune haemolytic anaemia (AIHA). However, it remained unclear how these aab lead to eryptosis. ***Methods:*** Serum and plasma samples from patients with clinically relevant AIHA of cold type were used to induce eryptosis in O RBCs. Serum containing fresh complement from healthy donors, antibodies to complement component, and complement factor depleted sera were added to examine the influence of the complement on PS-exposure. RBC bound annexin V PE were analysed by flow cytometry. ***Results:*** Eryptosis related to IgM aab was found to be dependent on complement activation and could be effectively inhibited by EDTA, serum heat inactivation and anti-C5. PS exposure increased with sequential activation of the sublytic terminal complement components C5b6, C5b-7 and was most significant at the C5b-8 stage. A decrease was observed following the formation of the lytic membrane attack complex C5b-9, either because of lysis of eryptotic RBCs or because of inhibition of eryptosis by C9. ***Conclusion:*** Our findings reflect new aspects on RBC destruction in AIHA as well the impact of the terminal complement complexes on the RBC membrane. The striking differences to nucleated cell apoptosis may even have physiological meaning of RBC acting as a buffer of the complement system.



T. Bartolmäs and A. Salama contributed equally to this work.

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## Introduction

It has been generally accepted for long time that red blood cells (RBCs) in patients with autoimmune haemolytic anaemia (AIHA) are destroyed by phagocytosis due to their coating with IgG autoantibodies (aab) and / or C3b complement component or by cell lysis due to IgM-mediated activation of the terminal complement components C5b-9 (membrane attack complexes), which form pores on cell membranes [1-3]. A single pore in the RBC membrane has been demonstrated to lyse the affected cell [4]. IgA aab alone may also cause *in vivo* haemolysis [5, 6]. It remains speculative how IgA aab lead to RBC destruction in patients with AIHA [3].

We recently demonstrated for the first time that eryptosis, the suicidal death of RBCs resembling the apoptosis of nucleated cells [7, 8], is also involved in AIHA related to IgA and IgM but not IgG aab [9]. Eryptosis has also been described to occur in oldest erythrocytes triggered by oxidative stress [10], and in tumor suppressor protein p53 deficiency [11]. Also it has been related to patients with a variety of diseases, including metabolic syndrome, diabetes, malignancy, hepatic failure, heart failure, renal failure, sepsis, malaria, mycoplasma infection, iron deficiency, sickle cell anaemia, spherocytosis, thalassaemia, glucose-6-phosphate dehydrogenase deficiency, and Wilson's disease [7, 12, 13]. Upon $Ca^{2+}$ influx, RBCs become eryptotic. This process is characterized by cell shrinkage and cell membrane scrambling, leading to the breakdown of the cell membrane's phospholipid asymmetry and exposition of phospholipid phosphatidylserine (PS) from the inner to the exterior leaflet [14-18]. These cells are usually recognized by macrophages and rapidly removed from the circulation [12].

The mechanisms by which IgM and IgA aab cause eryptosis remain unclear. The present study investigated the potential role of complement activation in these processes. The results provide insight into various aspects related to apoptosis, complement activation, and the interaction of lytic and sublytic terminal complement components with the RBC membrane.

## Materials and Methods

*Samples and reagents*

Serum and EDTA samples were from two patients with clinically relevant AIHA of cold type [9] and from one patient who had severe AIHA due to IgA aab [19]. Fresh RBCs and serum from healthy blood donors were used as controls and source of complement, respectively. Anti-C8b and anti-C9 polyclonal antibodies were from Thermo Fisher Scientific, Rockford, Il, USA, and anti-C5 (Eculizumab, Soliris®) from Alexion Europe (Rueil-Malmaison, France). C5-, C6-, C7- and C8-deficient serum and complement C5 were obtained from Sigma-Aldrich (Saint Louis, MO, USA) and C9-depleted serum from EMD Millipore (Merck KgaA, Darmstadt, Germany).

*In vitro eryptosis experiments*

RBCs from EDTA samples of healthy blood donors were washed two times in NaCl and once in HEPES-buffered Ringer's solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 $CaCl_2$ (p*H* 7.4). In general, ten µl of packed RBCs were used and 100 µl patient serum and where necessary 10 µl complement antibodies added as described. The mixture was incubated at 4 °C for 15 min, afterwards at 37 °C for 2 min, and then stored at 4 °C for 24–48 h. In the two-step experiments, 10 µl of RBCs were incubated with 50 µl heat-inactivated patient serum at 4 °C for 15 min, washed in 4 °C HEPES-buffered Ringer's solution, centrifuged, followed by the addition of 100 µl AB serum or complement factor-depleted sera. The mixture was incubated at 37 °C for 2 min and stored at 4 °C for 24–48 h. RBCs were handled with care and not pipetted vigorously to avoid physical damage. After incubation, RBCs were pre-warmed and washed three times at 37 °C to remove IgM aab. RBCs were diluted in 400 µl of HEPES-buffered Ringer's solution. Experiments were performed at least in triplicate.

*Flow cytometry*

Eryptosis was measured as previously described [9]. Annexin binding to extracellular exposed PS was used as a marker of eryptosis. A positive control was generated by incubating 5 µl of RBCs in 200 µl of a HEPES-buffered Ringer's solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 $CaCl_2$ (p*H* 7.4) with the $Ca^{2+}$ ionophore ionomycin (1 µM) as described elsewhere [20].

Five µl of RBCs were diluted in 200 µl HEPES-buffered Ringer's solution. Therefrom five µl of RBCs were added to 5 µl of PE (phycoerythrin) annexin V in 45 µl of annexin V binding buffer (BD Biosciences, Heidelberg, Germany). After incubation at room temperature for 15 min, the mixture was diluted with 450 µl of annexin V binding buffer and analysed by flow cytometry (MACSQuant® Flow Cytometer, Miltenyi Biotech, Germany). At least 20 000 events were collected for each sample. Data were analysed using the FlowJo® software (FlowJo, LLC; USA) and the percentage of PE annexin V positive cells compared to the negative controls was calculated.

*Statistics*

Data are expressed as arithmetic means ± standard error of the mean (SEM) and statistical analysis was performed using IBM SSPS statistics software v24. Significance between two groups was determined using student's T test with probabilities of $P < 0.05$ considered statistically significant.

## Results

*IgM aab induce eryptosis in patients with autoimmune haemolytic anaemia*

In our previous study, we could show that RBCs from patients with clinically relevant AIHA of cold type had significant increase of PS exposure [9]. Similarly, in the present study fresh serum samples from these patients induced significant PS exposure on O RBCs of healthy blood donors (Fig. 1A–E). The supernatant of the mixtures of RBCs with patient serum samples was haemolytic after incubation, indicating mild RBC lysis.

*Effect of EDTA and serum heat-inactivation on eryptosis*

In contrast, patient plasma as well as patient serum pre-treated with EDTA failed to provoke significant externalization of PS in RBCs (Fig. 1F–I, C). This can be explained by the fact that EDTA sequesters $Ca^{2+}$ by forming a metal complex, thereby inhibiting $Ca^{2+}$ influx into the cells as a trigger of eryptosis. However, the capability of patients' sera to induce eryptosis was also abolished after 2 days (Fig. 2A) but restored in the presence of fresh donor serum (Fig. 2B). Furthermore, heat-inactivation of fresh patient serum before its addition to RBCs did not lead to increased levels of exposed PS (Fig. 2C). The latter two findings indicate a further trigger of eryptosis in AIHA patients apart from $Ca^{2+}$.

*IgM aab and fresh serum proteins are necessary for inducing eryptosis*

To further subdivide the components necessary for inducing eryptosis in donor RBCs, heat-inactivated (i.a.) serum from a patient with IgM cold aab and fresh donor serum were added separately in a two-step experiment (Fig. 2D). As a result, RBCs were coated with IgM cold aab before adding fresh donor serum. The percentage of PS+ RBC was similar to that of the one-step addition of patient serum and fresh AB serum sera (Fig. 2E). The control, addition of i.a. patient serum and i.a. donor serum in two-steps showed no eryptosis (Fig. 2F). Taken together, the results indicate that fresh serum is necessary to induce significant eryptosis in patients with IgM aab (Fig. 2G).

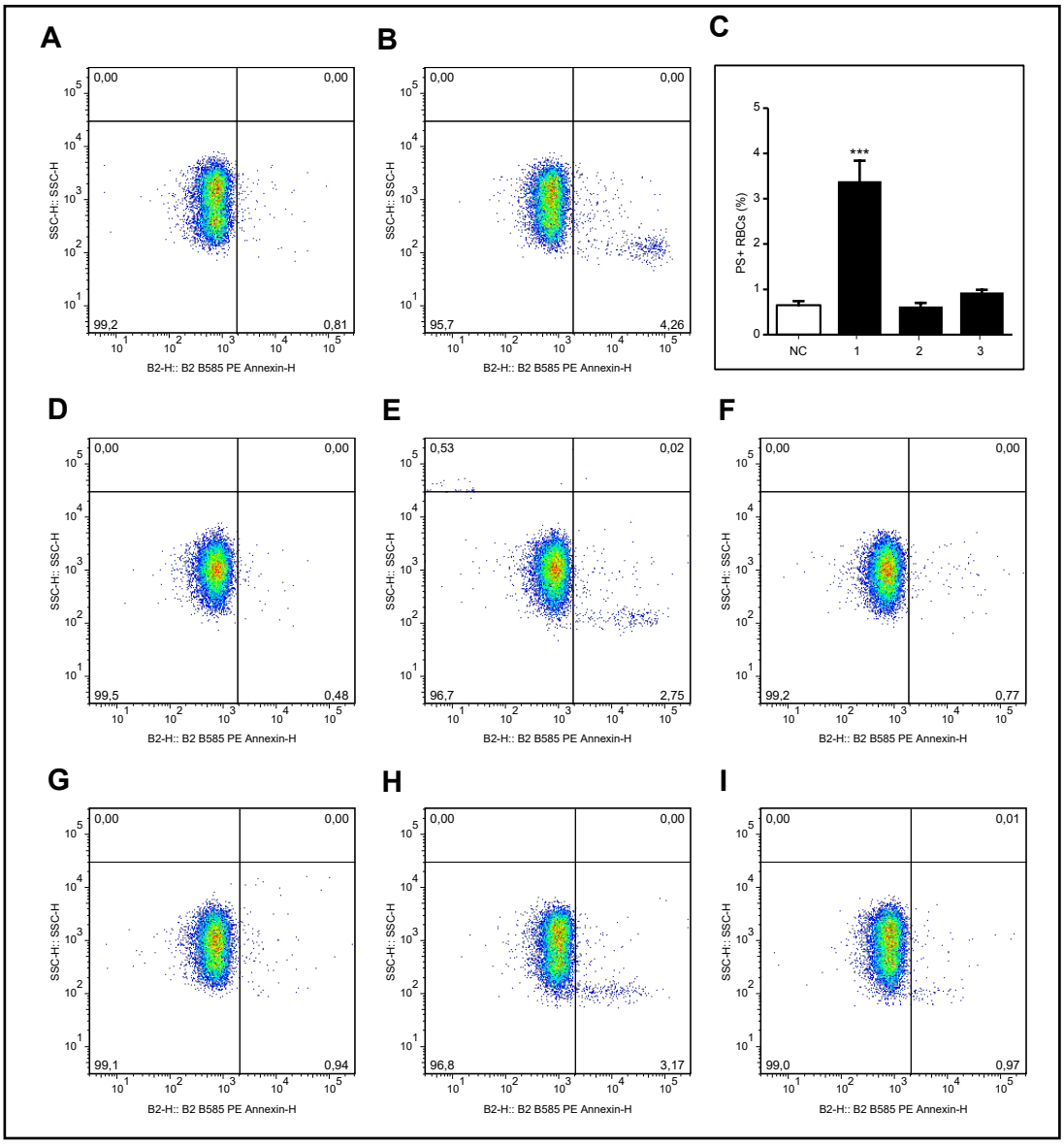


**Fig. 1.** Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs after incubation with serum/plasma from patients with cold AIHA. Compared to negative controls (A, D, G, and C bar "NC"), phosphatidylserin (PS) exposure was significantly ($p < 0.001$) increased in the presence of fresh serum (containing complement) from patients with cold AIHA (B, E, H, and C bar "1"). Incubation with patient EDTA plasma (F, and C bar "2") or addition of EDTA to patient serum (I, C bar "3") did not result in an increase of PS exposure. (***) $p < 0.001$.

*IgA-induced eryptosis is independent of fresh serum*

In a complementary experiment, serum and plasma from a patient with strongly agglutinating fatal IgA aab [19] were incubated with donor O RBCs. Although strong eryptosis was induced, the strength of PS exposure was not related to fresh serum but was equal after incubation with plasma or heat-inactivated serum (data not shown).

*Inhibition of IgM-related eryptosis by complement C5 antibody and C5-depleted serum*

Our results indicate that $Ca^{2+}$, IgM cold aab, and a number of heat-sensitive compounds in fresh serum are necessary to induce eryptosis in IgM AIHA patients. Since IgM cold aab were recently shown to activate complement even at core temperatures [21], we hypothesized

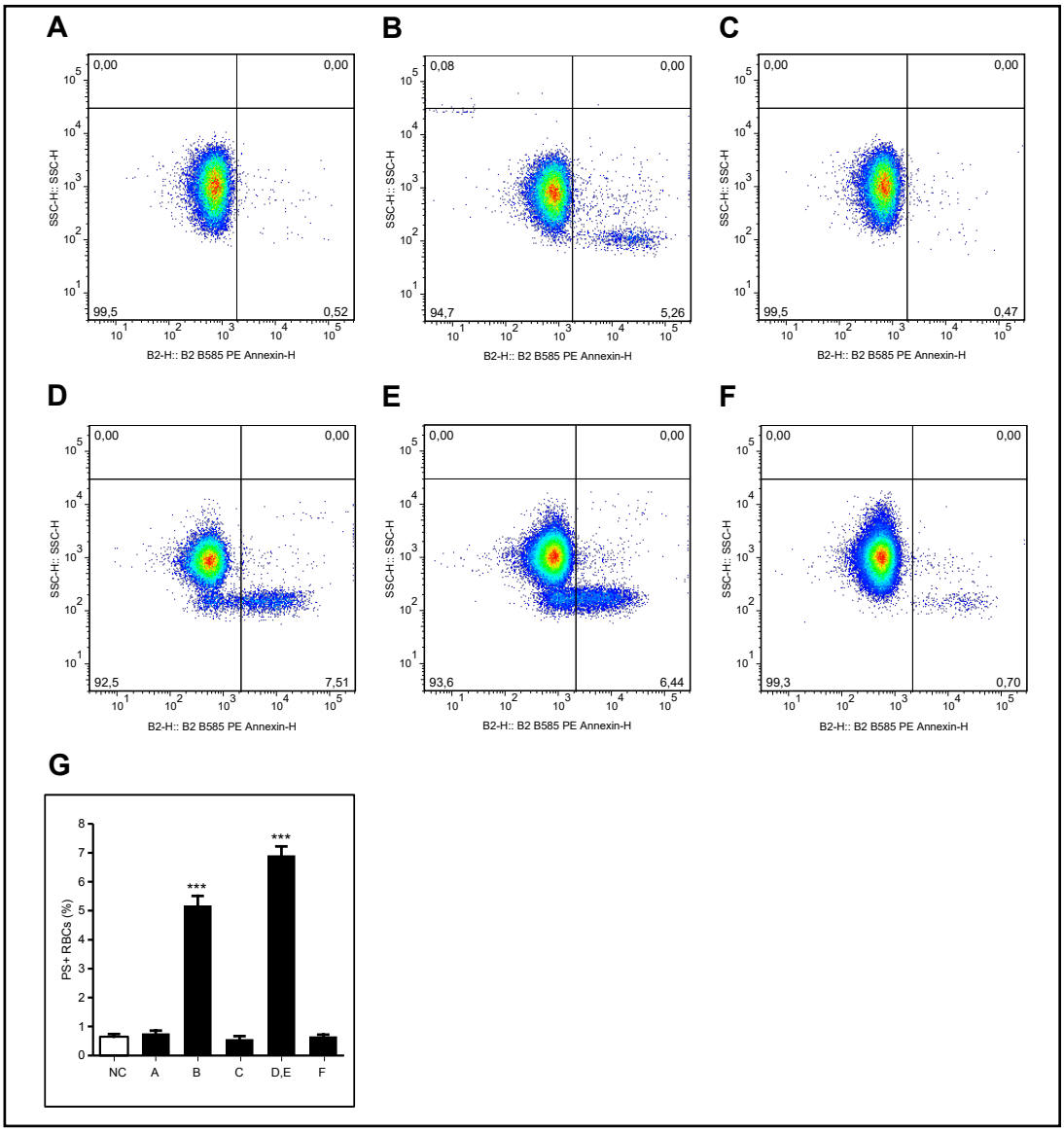


**Fig. 2.** Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and its dependence on serum complement activity. O RBCs incubated with serum (stored at 4° C for 48 h) from a patient with cold AIHA did not show increased PS exposure (A). Whereas supplementary addition of fresh AB serum as a source of complement induced significantly ($p<0.001$) PS exposure (B, G), no effect could be seen after addition of heat-inactivated (i.a.) AB serum (C, G). Similarly, i.a. serum from a patient with cold AIHA did induce significantly ($p<0.001$) PS exposure in O RBCs after subsequent addition of fresh AB serum (D, G) or simultaneous addition of fresh AB serum (E, G), but not after subsequent addition of i.a. AB serum (F). (***) $p<0.001$.

that the complement system is most likely involved in eryptosis. To test this hypothesis, fresh donor serum (Fig. 3A, negative control), a mixture of patient serum and fresh donor serum (Fig. 3B), or patient serum and fresh donor serum incubated prior to its addition to RBCs with anti-C5 antibody (Eculizumab, Soliris®) was added to RBCs (Fig. 3C). Whereas patient serum and fresh donor serum together induced strong eryptosis, PS externalization was almost completely prevented by pre-incubation with anti-C5 (Fig. 3G). Next, we used C5-depleted serum for independent evidence of the involvement of complement factor C5 in IgM aab-induced eryptosis. As expected, incubation with patient serum and C5-depleted

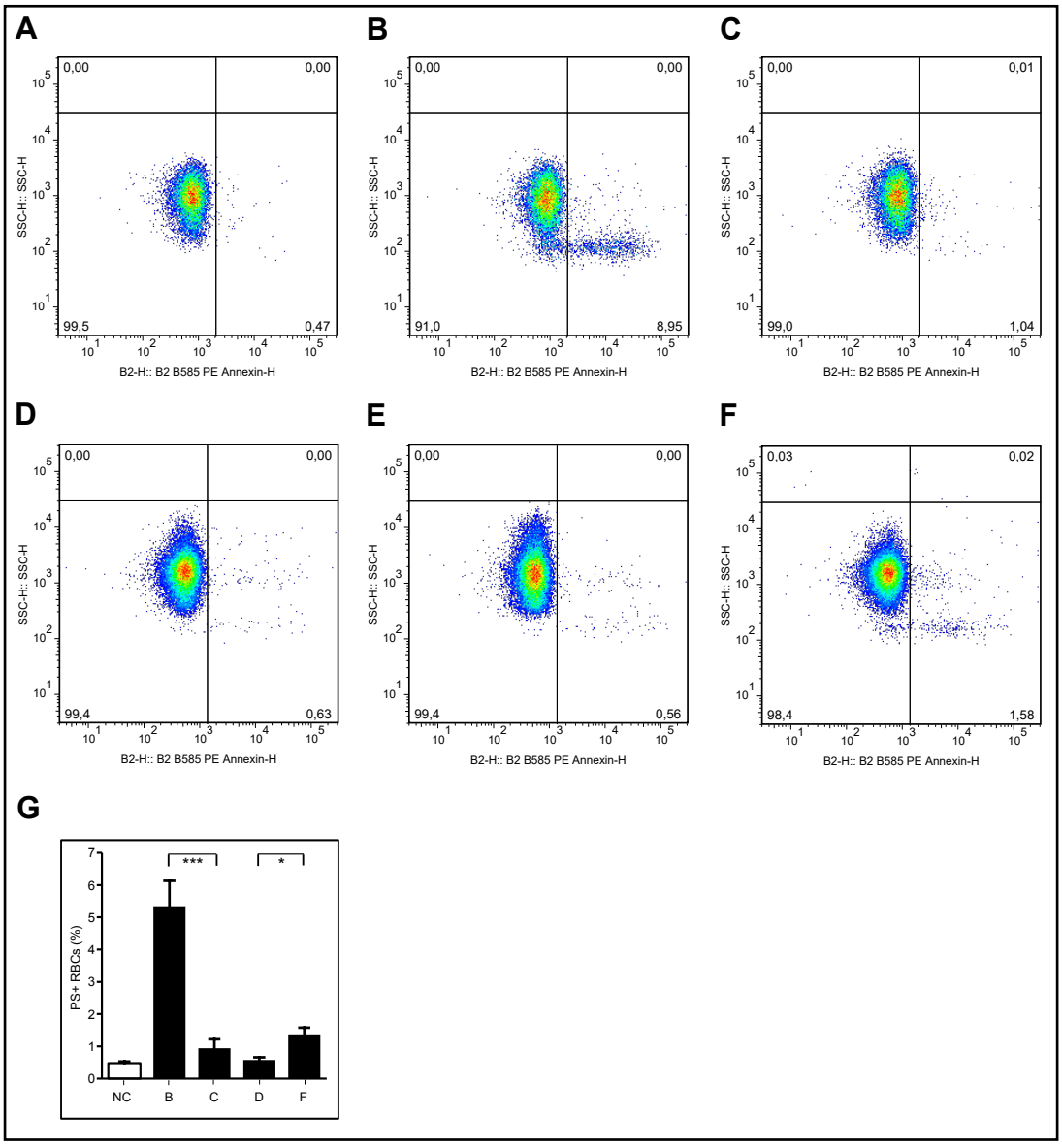


**Fig. 3.** Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and effect of C5 complement component. Compared to negative control (A), phosphatidylserin (PS) exposure was significantly ($p<0.001$) increased after incubation with fresh serum from patients with cold AIHA (B, G). Pre-treatment of patient serum with anti-C5 (Eculizumab, Soliris®) significantly ($p<0.001$) reduced PS exposure (C, G). Heat-inactivated patient serum and C5-depleted serum were incapable of inducing PS exposure (D) as compared to negative control (E, G) but supplementation with the missing C5 complement component resulted in a significant ($p<0.05$) increase of PS exposure (F, G). (***) $p<0.001$, (*) $p<0.05$ between the bracketed bars.

serum (Fig. 3D) did not result in PS externalization above the negative control level (Fig. 3E). However, the addition of C5 to this mixture restored significantly the eryptosis-inducing capacity (Fig. 3F, 3G), providing strong evidence of the specific role of the complement system in the eryptosis of RBCs from patients with IgM AIHA.

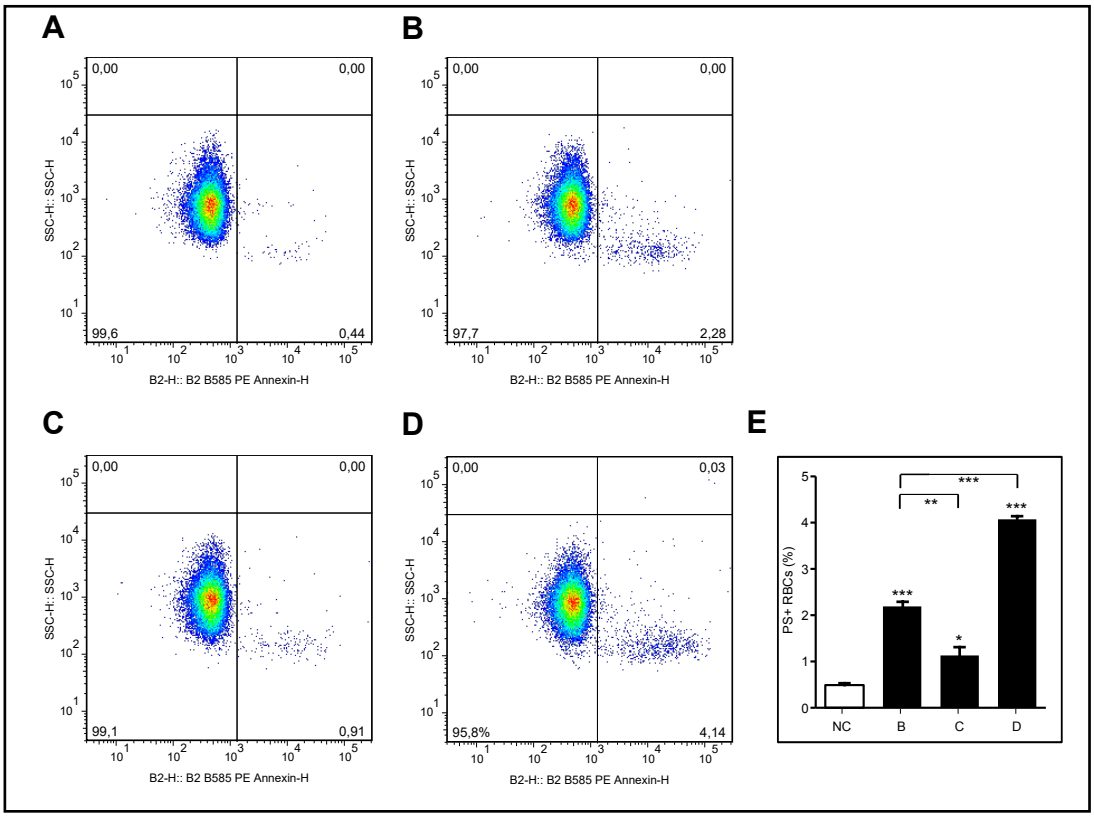


**Fig. 4.** Scatter plots and bar graph with arithmetic means ± SEM showing annexin-PE binding to O RBCs and effect of anti-C8 and anti-C9. Compared to negative control (A), phosphatidylserin (PS) exposure was significantly ($p<0.001$) increased after incubation with fresh serum from patients with cold AIHA (B). Pre-treatment of patient serum with anti-C8 significantly ($p<0.01$) reduced PS exposure (C) but RBCs still had a significantly ($p<0.05$) higher level of eryptotic cells compared to negative control. Pre-treatment with anti-C9 further significantly ($p<0.001$) increased PS exposure (D) compared to untreated patient serum. (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ compared to negative control or between the bracketed bars, respectively.

*Effect of anti-C8 and anti-C9 on eryptosis*

To further elucidate the role of complement in eryptosis, particularly regarding the potential involvement of the MAC, anti-C8 and anti-C9 antibodies were used in an analogous experiment (Fig. 4A–E). The addition of anti-C8 inhibited significantly the formation of eryptotic cells; however, incompletely (Fig. 4C, 4E). In contrast, the addition of anti-C9 resulted in a significant increase of eryptotic cells (Fig. 4D, 4E). One potential explanation for this unexpected result lies in the presumption that C9 may not be required for inducing eryptosis. However, since C9 is responsible for forming the lytic pore in the final step of the MAC, part of the eryptotic cells may be haemolysed completely in presence of C9 and escape detection with Annexin-PE. Therefore, C9 inhibition by anti-C9 may prevent eryptotic cells from lysis *in vitro*. Altogether, our results indicate that C5 is necessary for inducing eryptosis in IgM AIHA and C8 may support the formation of eryptotic cells; however, C9 may not be involved.

*Induction of eryptosis by the terminal complement complexes C5b/6, C5b-7, and C5b-8*

To further confirm our hypothesis, heat-inactivated serum from patients with cold IgM AIHA were used with commercially available serum depleted of the single complement proteins C6, C7, C8, or C9. Compared to the negative control (i.a. serum from patient + i.a. AB serum; Fig. 5A), the use of C6-, C7-, C8-. or C9-depleted serum led to a steady rise in the

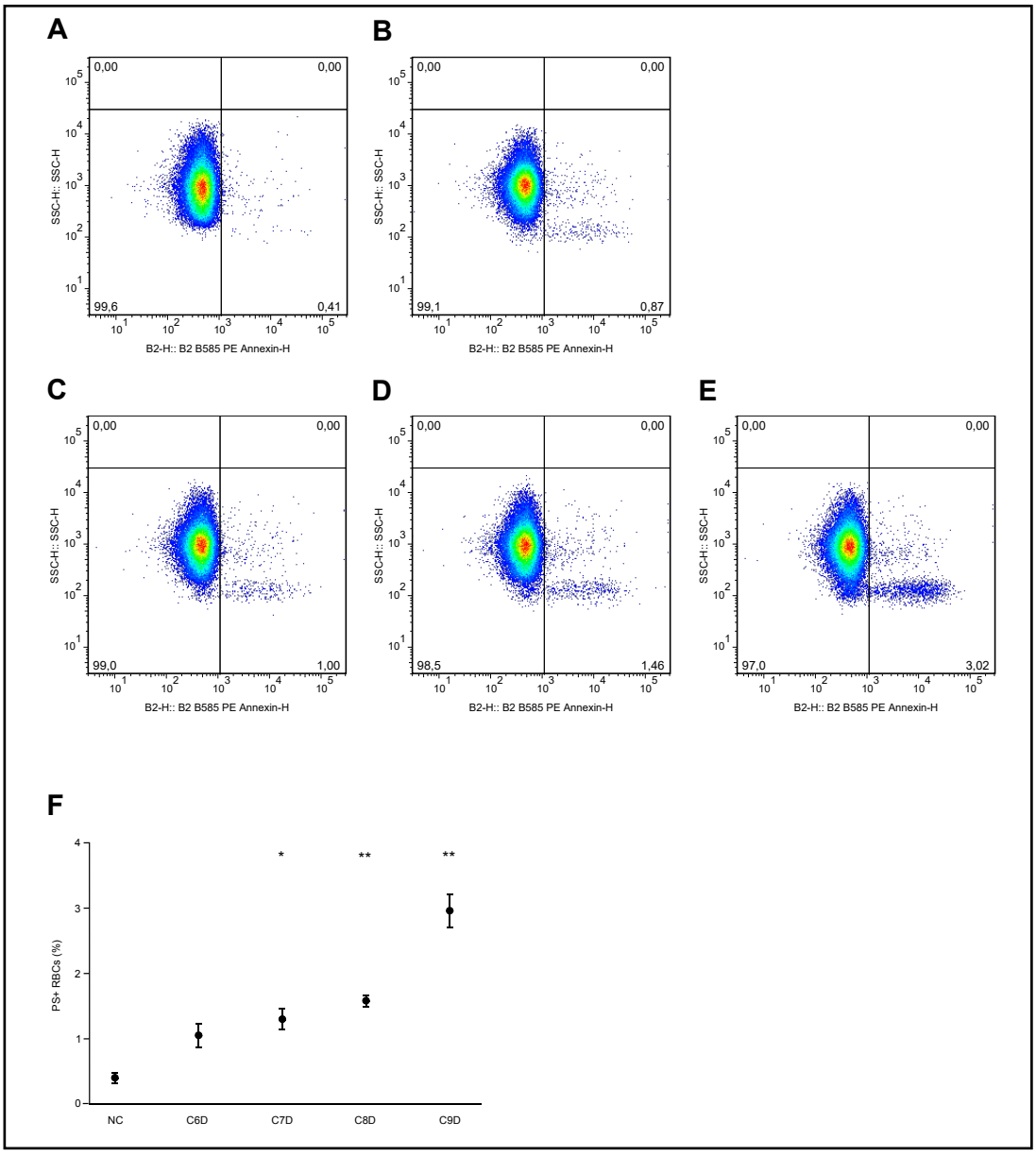


**Fig. 5.** Scatter plots showing annexin-PE binding to O RBCs and effect of the terminal complement components C5b/6, C5-7, and C5-8. Compared to negative control (A) PS exposure of RBCs incubated with i.a. patient serum gradually increased in the presence of C6-depleted serum (B), C7-depleted serum (C), C8-depleted serum (D), and C9-depleted serum (E). Graph showing increase of PS exposure in the presence of sera depleted of complement component C6, C7, C8 and C9, respectively (F). (*) $p<0.05$, (**) $p<0.01$.

number of PS+ cells (Fig. 5B–E) showing that the intermediate formation of distinct phases, collectively referred to as terminal complement complexes (TCCs) [22], increasingly induce eryptosis. Of notice is the strong increase in PS+ RBCs when C9-depleted serum was used and the TCC C5b-C8 complex is present (Fig. 5F).

## Discussion

The obtained results are intriguing in many respects and may shed light on the diversity and complexity of RBC destruction in patients with AIHA and diseases associated with anaemia. In addition to the classical pathways Fc-, C3b-, and/or C5b-9-mediated RBC phagocytosis and/or lysis, respectively [2, 5], we recently demonstrated eryptosis to be involved in AIHA due to IgM and IgA aab [9]. However, how these aab cause PS exposure on RBCs and consequently eryptosis remained unknown. In the aforementioned study, three possible mechanisms by which these aab may lead to PS translocations were discussed. These include physical stress as a result of strong agglutination, the release of inflammatory signals, and $Ca^{2+}$ influx. In the present study, we hypothesized whether complement activation may be involved in this process. Using EDTA, heat-inactivated serum, C5–C9 depleted sera, and antibodies to C5, C8, and C9, eryptosis due to IgM aab was found to depend on the activation of these components.

Eryptosis can be induced in donor O RBCs by incubation with fresh serum of patients with cold IgM aab. In contrast, an increase of PS exposure could not be induced in donor O RBCs by using patient plasma or heat-inactivated serum. Furthermore, there was no eryptosis following the addition of anti-C5 (Eculizumab, Soliris®) prior to incubation or when using C5-depleted serum as a source of complement. These data indicate that C5 may be mandatory for IgM-induced eryptosis. However, C5 activation does not suffice in significantly increasing PS exposure as demonstrated using C6-depleted serum. In contrast, the use of C7-depleted serum resulted in a further increase. This could be explained by the formation of labile C5b6 complexes. Though these complexes have not been reported to cause cellular changes [23, 24], they have the potential ability to interact with the hydrophobic domains of the lipid bilayer [22, 25, 26]. Whether this interaction could be enforced by the cell agglutination induced by the aab remains speculative. This assumption is supported by the observation that PS exposure can be stimulated by cell-cell adhesion of human RBCs [27-29], which is reflected in our experiment by the strong agglutination in the presence of the causative aab. In addition, C5b as a "collateral effect" of C5 activation has also been described to be involved in some apoptotic cell conditions [30]. Ultimately, it remains questionable whether all cellular responses induced by the sublytic MAC complexes including C5b6, C5b-7, and C5b-8 can be reproduced and/or measured *in vitro*.

PS exposure further increased as a result of the formation of C5b-7 and was most significant following C5b-8 complexes formation after the incubation of RBCs with IgM aab and C8- or C9-depleted serum, respectively. C5b-7 complexes adhere to the cell membrane [31, 32] and induce the generation of ceramide as well as $Ca^{2+}$ influx, which are principal mediators of eryptosis [7, 8, 32-36]. Hence, PS exposure at this stage may be due to $Ca^{2+}$ influx. This is not surprising since $Ca^{2+}$ alone is sufficient to induce PS exposure in human RBCs [29, 37]. C5b-8 formation leads to a further increase of $Ca^{2+}$ influx, membrane deformation, and may cause cell lysis [38-40]. Therefore, both sublytic C5b-7 and C5b-8 complexes are capable of inducing eryptosis. Future studies should focus on determining whether complement activation through the classical pathway, i.e., due to agglutinating IgM antibodies, might be more effective in causing eryptosis than the alternative pathway, i.e., in the absence of antibodies. The strong increase of PS exposure following inhibition at the last stage by anti-C9 antibody or the use of C9-depleted serum could be explained by the accumulation of C5b-8 sublytic complexes on the cell membrane. However, the decrease of PS on the cell membrane following C9 assembly and C5b-9 channel (MAC) formation could be explained by cell lysis or the formation of soluble sC5b-9 complexes, which are inhibitors of complement activation and sublytic C5b-8 complex formation [22].

In contrast to IgM aab, IgA aab do not appear to activate complement or require complement for inducing PS exposure and eryptosis, respectively. Their eryptotic effect remained unaltered through the use of EDTA plasma or heat-inactivated serum. This is supported by previous studies including ours [41] and from our group [19]. The question of whether IgA coated RBCs in patients with AIHA is eliminated by phagocytosis due

to PS exposure and, at least, in part due to autoagglutination remains unanswered. Haemagglutination due to IgM or IgA aab may result in RBC sequestration independent of Fc receptor and complement activation. This was previously demonstrated using a mouse model and polymeric forms of IgM and IgA aab [42]. In addition, $Ca^{2+}$ influx, the principal stimulator of eryptosis, may occur as a result of mechanical RBC deformation [43]. Therefore, agglutinating IgA and IgM antibodies may simultaneously lead to RBC sequestration by eryptosis and or agglutination.

In conclusion, the present study provides evidence that IgM-induced eryptosis is complement-dependent and that the sublytic terminal complement complexes C5b/6, C5b-7, and especially C5b-8 are sufficient for eryptosis, potentially representing a third degradation pathway in patients with AIHA. Eryptotic cells are usually removed rapidly from the circulation by macrophages, suffering the same fate as RBCs that undergo extravascular hemolysis. However, if phagocytotic activity is diminished, eryptotic cells may undergo intravascular haemolysis. This may explain why a number of patients with AIHA cannot be treated successfully with drugs that intervene in the monocyte/macrophage system. Further studies are required to elucidate the significance of this third RBC degradation pathway and its use in drug therapy.

Unlike eryptosis, the apoptosis of nuclear cells does not seem to be complement-dependent and complement-induced cell death is rather necrotic [44]. Sublytic MAC doses failed to produce DNA-ladder formation as a typical indicator of apoptosis in nuclear cells [44] and may even protect cells from apoptosis [45]. Furthermore, apoptotic cells have been shown to activate complement [46] and on the other hand complement facilitates the clearance of apoptotic cells [47]. In that context, the exciting difference between apoptosis of nucleated cells and complement-induced eryptosis could even have a further significance; RBCs may act as a buffer of the complement system, preventing inflammation and necrosis in living tissue by self-sacrifice and fast macrophage clearance.

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All authors reviewed the manuscript and approved the final version.

## Disclosure Statement

All authors declare that they have no competing interests.

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## 9 LEBENSLAUF

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

## 10 Publikationsliste

**Publikation 1: Balola AHA**, Mayer B, Bartolmäs T, Salama A. A fluorometric erythrophagocytosis assay using differentiated monocytic THP-1 cells to assess the clinical significance of antibodies to red blood cells. Vox Sang 2021; **116**(10):1106-1116.

Impact factor: 2,347 (2019)

https://doi.org/10.1111/vox.13105

**Publikation 2:** Bartolmäs T*, Mayer* **B, Balola AH**, Salama A. Eryptosis in auto-immune haemolytic anaemia. Eur J Haematol 2018;**100**(1): 36-44.

Impact factor: 2,595

https://doi.org/10.1111/ejh.12976

**Publikation 3: Balola AHA,** Mayer B, Bartolmäs T, Salama A. Sublytic Terminal Complement Components Induce Eryptosis in Autoimmune Haemolytic Anaemia Related to IgM Autoantibodies. Cell Physiol Biochem. 2019; **53**(3):453-464.

Impact factor: (Scopus Cite Score 2019): 8,0

https://doi.org/10.33594/000000150

**Publikation 4:** Bartolmäs T, Yürek S, **Balola AHA**, Mayer B, Salama A. Evidence Suggesting Complement Activation and Haemolysis at Core Temperature in Patients with Cold Autoimmune Haemolytic Anaemia. Transfus Med Hemother 2015; **42**(5):328–332.

Impact factor: 2,2

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