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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

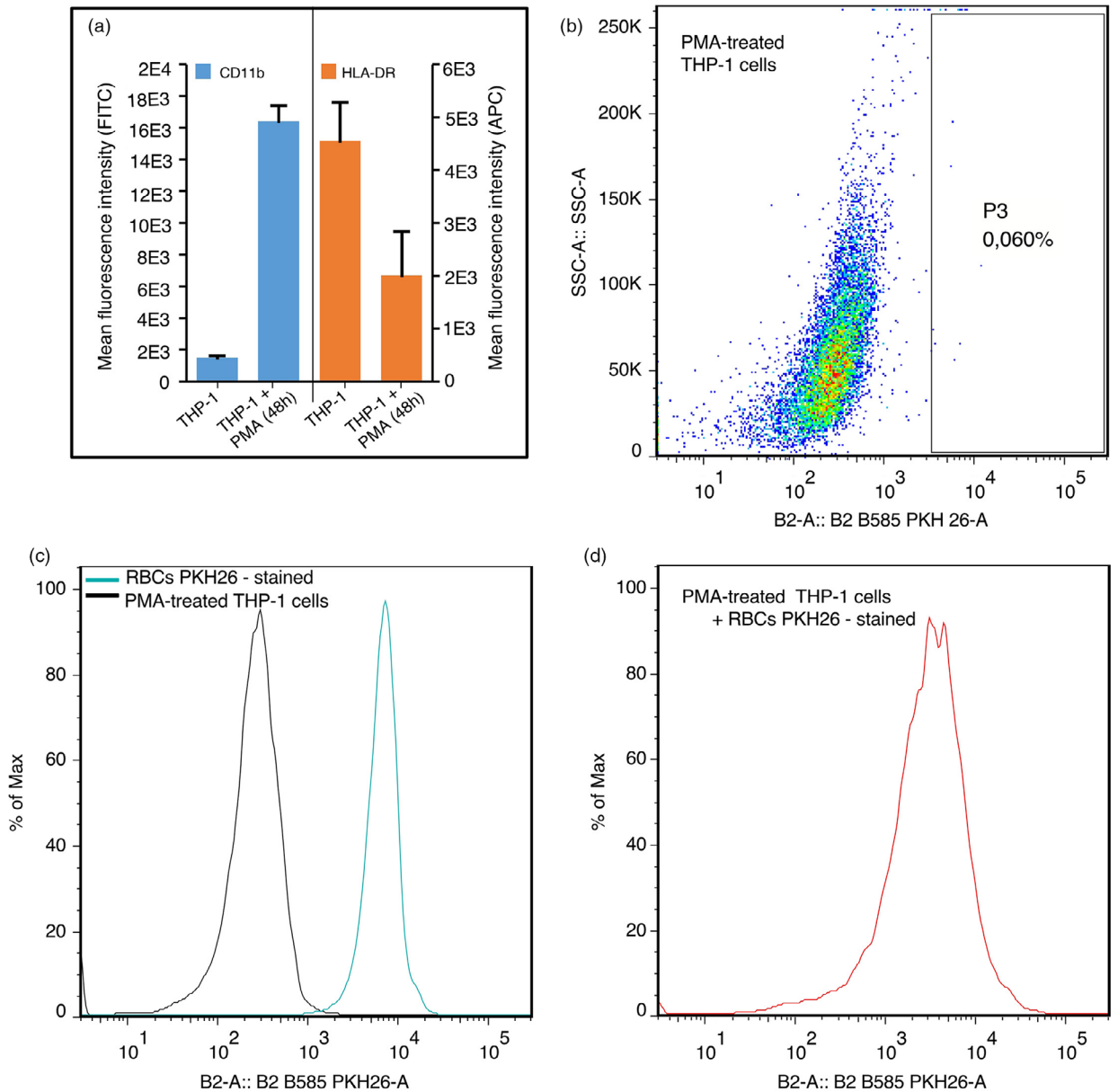


**Fig. 1** Characterization of PMA-treated THP-1 cells. (a) Effect of PMA differentiation on CD11b (left) and HLA-DR (right) expression in THP-1 cells at different times (0 and 48 h). (b) Scatter plot of PMA-treated THP-1 cells (blank control). Region P3 has been selected as contains ≤ 0·1% of events. (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). [Colour figure can be viewed at wileyonlinelibrary.com]

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

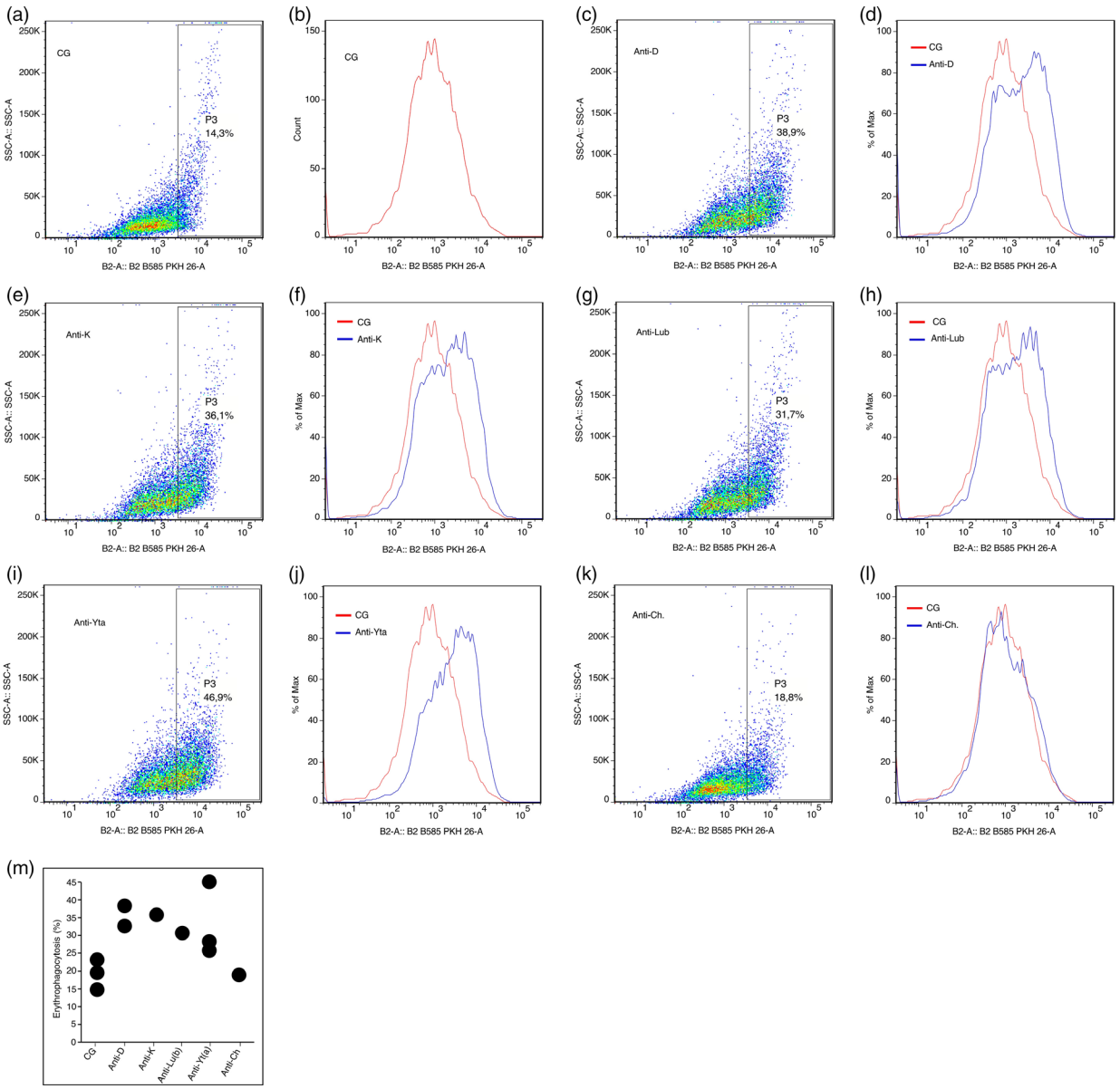


**Fig. 2** Erythrophagocytosis of RBCs coated with alloantibodies. (a-l) Scatter plot (a) and histogram (b) of PMA-treated THP-1 cells after phagocytosis of RBCs from a healthy blood donor (control group). Scatter plots (c, e, g, i, k) and overlay of histograms with a control group (d, f, h, j, l) of 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. [Colour figure can be viewed at wileyonlinelibrary.com]

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

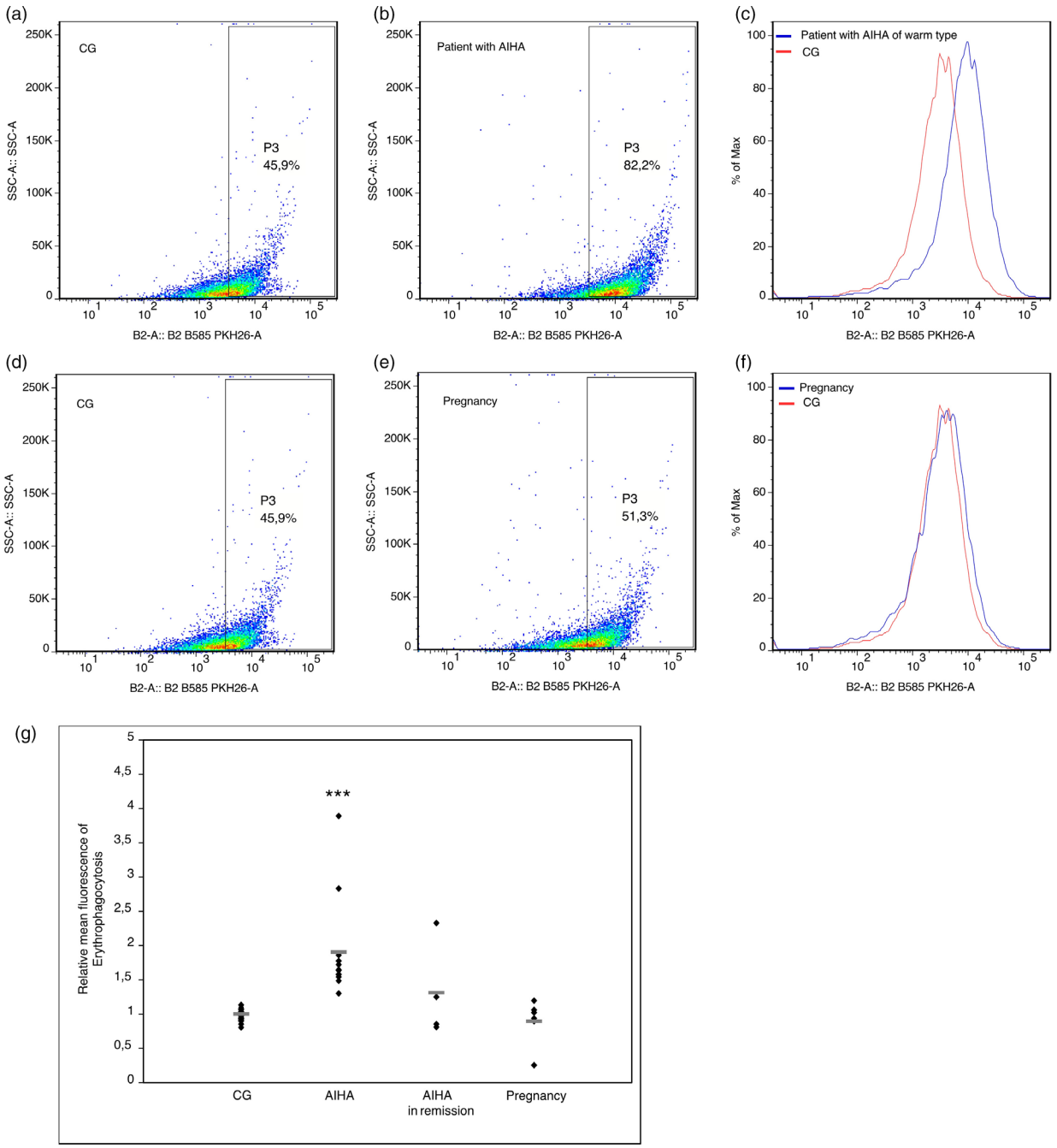


**Fig. 3** Phagocytosis of RBCs from a patient with AIHA and a pregnant woman with warm autoantibodies. Scatter plots and overlay histogram of PMA-treated THP-1 cells after phagocytosis of RBCs from a healthy blood donor (a, c: red line) and of RBCs from a patient with clinically relevant AIHA (b, c: 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. [Colour figure can be viewed at wileyonlinelibrary.com]


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**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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