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Development of novel approaches to overcome daratumumab interference with pretransfusion testing using gel card and solid phase assay

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List of abbreviations

Ab	Antibody
ADCC	Ab-dependent cellular cytotoxicity
ADCP	Ab-dependent phagocytosis
AHG	Anti-Human Globulin
BC	Buffy Coat
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
cADPR	Cyclic ADP-ribose
CDC	Complement-dependent cytotoxicity
DARA	Daratumumab
FITC	Fluorescein Isothiocyanate
Hb	Hemoglobin
HRP	Horseradish Peroxidase
IAT	Indirect Antiglobulin Test
IgG	Immunoglobulin G
kDa	Kilodalton
LISS	Low-Ionic Saline Solution
mAb	Monoclonal Antibody
MAC	Membrane attack complex
MM	Multiple Myeloma
NAADP	Nicotinic acid adenine dinucleotide phosphate
NEG	Negative
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PLT	Platelet
RBC	Red Blood Cell
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SSP+	Storage Solution for Platelets

RT	Room Temperature
TBS	Tris-Buffered Saline
TRIS-HCl	Tris(hydroxymethyl)-aminomethane hydrochloride
UMG	Universitätsmedizin Greifswald
UV	Ultraviolet

1. Introduction

More than three decades have passed since the first therapeutic monoclonal antibody was approved by the US Food and Drug Administration (US FDA) in 1986, and during this time antibody engineering has undergone significant evolution.

Currently, a total of at least 570 therapeutic monoclonal antibodies (mAbs) have been tested in clinical trials by commercial companies, and 79 therapeutic mAbs have been approved by the US FDA and are currently on the market, including 30 mAbs for the treatment of cancer [1]. In recent years therapeutic antibodies have thus become a dominant class of new drugs for the treatment of diseases, and particularly for tumor therapy.

Therapeutic antibodies are selected for their ability to bind with high specificity and affinity to particular antigens. Unfortunately, some of these antigens are not restricted to the target cells, and hence may cause adverse effect. An example of this is seen with the monoclonal antibody to CD38 - daratumumab (DARA). This antibody was developed for the treatment of multiple myeloma, but in clinical trials was shown to interfere with laboratory tests that are performed prior to blood transfusion [2].

Patients suffering from multiple myeloma (MM) are frequently anaemic and require blood transfusion. In the SIRIUS trial about 40% of MM patients undergoing treatment with DARA required blood transfusions what was not related to the decrease of hemoglobin caused by the treatment but were due to the underlying hematologic malignancy or completely unrelated condition [3, 4]. Therefore, accurate detection of red cell specific alloantibody in pre-transfusion blood compatibility testing is imperative for preventing hemolytic transfusion reactions. Chapuy and colleagues first described that CD38 antibodies interfered with pre-transfusion antibody screening by giving rise to false positive results, so that specific red blood cell (RBC) alloantibody cannot be reliably identified [5]. Because of this, accurate detection of transfusion incompatibility due to the presence of red cell specific alloantibody in the patient's serum may be missed and may result in hemolytic transfusion reactions.

1.1 Daratumumab – CD38 antibody for treatment of MM

Daratumumab is an immunoglobulin IgG1k human mAb. It was developed by the immunization of human immunoglobulin loci transgenic mice with recombinant human CD38

protein [6, 7]. Daratumumab targets the CD38 protein, which is a 46 kDa type II transmembrane glycoprotein that is expressed on the surface of many immune cell populations. CD38 contains a relatively long C-terminal extracellular domain (258 aa), a transmembrane region (21 aa) and a short N-terminal cytoplasmic tail (21 aa) [8]. CD38 is a multifunctional protein involved in diverse functions. As an enzyme, it is responsible for the metabolism of two Ca^{2+} messengers, cADPR (cyclic ADP-ribose) and NAADP (nicotinic acid adenine dinucleotide phosphate); as a cell surface antigen, it is involved in regulating cell adhesion, differentiation, and proliferation [9].

CD38 is over-expressed in a large number of hematological malignancies e.g. multiple myeloma (MM), Waldenström's macroglobulinemia, primary systemic amyloidosis, mantle cell lymphoma, acute lymphoblastic leukemia, acute myeloid leukemia, NK cell leukemia, NK/T-cell lymphoma, and plasma cell leukemia [6]. Furthermore, expression of CD38 has been associated with a number of diseases including autoimmune diseases [e.g. systemic lupus erythematosus), type II diabetes mellitus and osteoporosis. It is a marker of progression in HIV-1 infection, and a negative prognostic marker of B-CLL [9-12].

Daratumumab represent a novel class of therapeutic antibody. It demonstrated anti-MM cytotoxicity through Fc dependent effector mechanisms including complement-dependent cytotoxicity (CDC), Ab-dependent cellular cytotoxicity (ADCC), Ab-dependent phagocytosis (ADCP), tumor cell apoptosis and modulation of the enzymatic activity of CD38 (Fig. 1) [6, 13]. The ADCC action is achieved through activation of Fc receptors on myeloid and NK effector cells by tumor cell-attached immunoglobins. Subsequent cytotoxicity is mediated either by a mechanism involving the release of perforin and granzymes from effector cells and/or by one involving the death ligands FasL and tumor necrosis factor related apoptosis-inducing ligand. In ADCP, phagocytosis of tumor cells is mediated by macrophages. CDC is dependent on the interaction of the antibody Fc domains with the classic complement-activating protein C1q leading to activation of downstream complement proteins, which results in the assembly of the membrane attack complex (MAC), that punches holes in the tumor cells [14, 15].

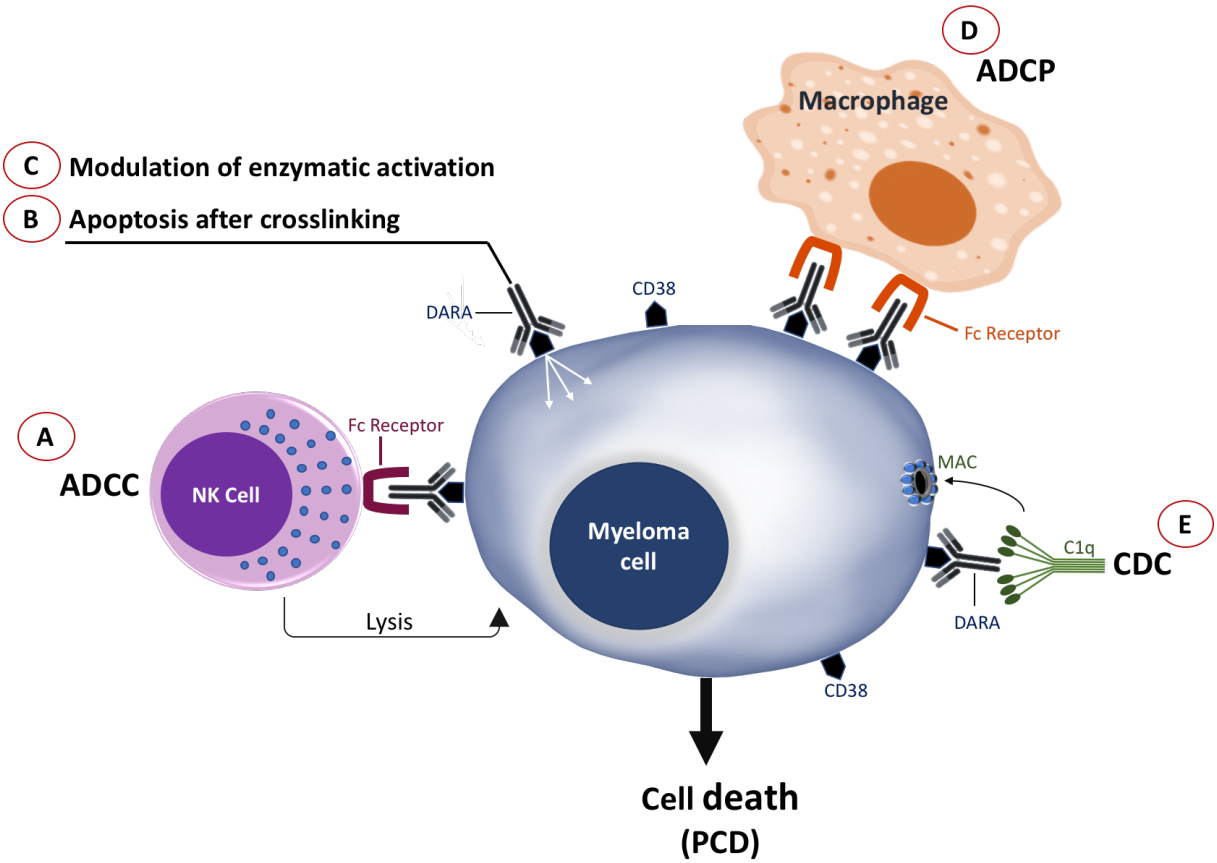


Figure 1. Mechanisms of action of daratumumab targeting surface antigens on MM cells [6]. (A) ADCC – daratumumab binds CD38, and its Fc fragment is then bound by an FcR-bearing effector cell, such as a natural killer cell, leading to destruction of the MM cells (B, C) enzymatic modulation - daratumumab modulates the enzymatic activities of CD38. Apoptosis – FcR-mediated crosslinking of daratumumab induces programmed cell death (PCD) [6, 7, 16, 17]. (39). (D) ADCP - daratumumab binds CD38, and its Fc fragment is then bound by an FcR-bearing macrophage, inducing phagocytosis. (E) CDC – daratumumab binds CD38, and its Fc fragment is bound by C1q, initiating the complement cascade and resulting in a MAC which leads to cell lysis and death.

ADCC - Ab-dependent cellular cytotoxicity; PCD - Programmed cell death; ADCP - Ab-dependent phagocytosis; CDC - complement-dependent cytotoxicity; MAC - membrane attack complex

Daratumumab showed anti-MM efficacy in combination with novel agents in heavily pretreated, relapsed, and refractory MM patients. This resulted in FDA approval in 2015. Its high efficacy and favorable safety profile in recent trials led to an expansion of indications for use in relapsed or refractory multiple myeloma. Current small molecule drugs for the treatment of MM include bortezomib, melphalan, and prednisone, and Daratumumab now joins them as the first front line monoclonal antibody approved for the treatment of transplant-ineligible MM, patients [18, 19]. It is therefore expected that an increasing number of patients will be treated with DARA.

1.3 Pretransfusion compatibility testing

Antibodies against circulating red blood cells (RBCs) are capable of causing clinically significant hemolytic transfusion reactions leading to accelerated destruction of a significant proportion of transfused RBCs [20]. Because of this, screening for antibodies directed against RBCs is a part of all pre-transfusion testing.

In 1908, Carlo Moreschi described the principle of the anti-globulin technique, and in 1945, Coombs et al. introduced the use of anti-human globulin (AHG) for the detection of red blood cells coated with non-agglutinating antibodies. This test was then rapidly applied in regular clinical laboratory practice [21].

The Direct Antiglobulin Test (DAT) allows the detection of red blood cells coated in vivo by immunoglobulin. The indirect antiglobulin test (IAT), in contrast, allows the detection of serum antibodies to allogeneic red blood cells, by incubating the serum with RBCs *in vitro*. Using this procedure, the target antigens on the red blood cells can be identified. IAT is an immunology laboratory procedure in general use as an essential part of transfusion compatibility screening tests.

In screening for antibodies directed against RBCs, the autocontrol will indicate whether the reaction is due to the presence of an autoantibody, an alloantibody or both. In the anti-globulin crossmatch test the donor's red blood cells combined with the recipient's serum or plasma will show the presence or absence of antibodies in the recipient's blood that will bind to antigens on the donor's red blood cells.

The principle of IAT is that patient serum or plasma is incubated with RBCs with individual antigen phenotypes. Antibodies present in the patient serum or plasma will bind to the RBCs with the corresponding antigen (Fig. 2). The IgG molecules are incapable of producing macroscopic visible agglutination of red cells, and therefore the indirect antiglobulin test utilizes a secondary antibody, anti-human globulin (AHG). This AHG binds the Fc portion of the immunoglobulin molecule and act as a bridge to crosslink coated RBCs, and hence to induce agglutination [22].

There are many different techniques of indirect antiglobulin test such as: tube tests, micro column, gel card or solid phase assays. Many laboratories work with fully automated systems for pre-transfusion diagnostics, and these mostly use gel card or solid phase IAT platforms. New treatment approaches for particular groups of patients, such as DARA treated patients,

should ensure that they are compatible with analysis on fully automated platforms. For these reasons, this study focused on using DARA F(ab')^2 fragments in IAT in the context of automation, rather than tube testing.

In the gel card technique, the gel column acts as a filter that traps agglutinated red blood cells as they pass through during centrifugation of the card. A positive result of IAT appears as red cell agglutinations trapped on the upper surface of the gel matrix, while non-agglutinated red cells pass through the gel and form a layer on the bottom of the column (Table 2). The strength of agglutination is a marker for either the antigen expression density or for the concentration of the antibody in the patient plasma.

In the solid phase technique, a positive result appears as a "carpet" of cells covering the bottom of the well, whereas a negative result shows centralized cells on the bottom of the well (see section 2.4.2). The strength of agglutination in gel card and solid phase assays is given from negative (no binding) to 4+ (the strongest reaction).

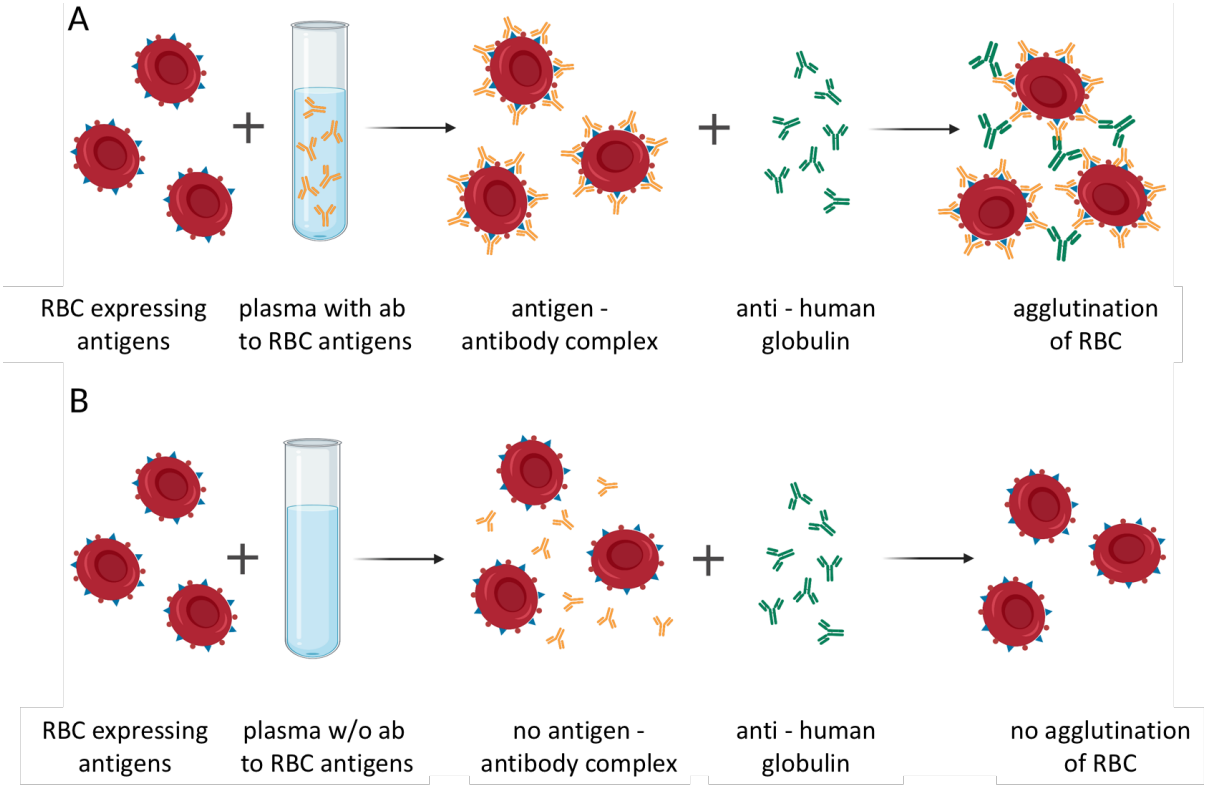


Figure 2. Principle of indirect antiglobulin test. (A) Negative IAT – plasma sample without RBC alloantibody, (B) Positive IAT due to present RBC alloantibody present in plasma [23].

1.4 Interference of daratumumab with pretransfusion testing

An important advantage of therapy using monoclonal antibodies like daratumumab lies in their specific targeting. However, many laboratory tests, including IAT, are also based on specific antibody-antigen interactions, and these laboratory diagnostic assays may be interfered with by the presence of the therapeutic monoclonal reagents.

Chapuy and colleagues described several lines of evidence indicating that CD38 antibodies interfere with blood compatibility testing [5]. First, by flow cytometry, fluorescently labeled antibodies to CD38 bind directly to RBCs. Second, plasma samples from healthy individuals spiked with DARA and incubated with RBCs gave false positive results in IAT, as did samples from DARA-treated patients. Third, an eluate prepared from the RBCs of a DARA-treated patient bound only to $CD38^{+}$ cells and not to $CD38^{-}$ control cells [5].

In a clinical trial with daratumumab those patients requiring transfusion who were receiving the DARA antibody, showed negative IAT before therapy but positive IAT after receiving the first dose of daratumumab [6]. Moreover, it was shown that after treatment of MM patients with daratumumab, false positive IAT results were obtained for 2-6 months after receiving the last dose [15, 24].

These false positive test results were caused by RBCs, which express CD38 and enable free daratumumab to bind *in vitro* during pretransfusion compatibility testing (Fig. 3). Agglutination due to daratumumab causes a weak, usually 1+ result, which can occur in all tests using AHG [23]. Furthermore, interference by daratumumab leads to pan-reactive agglutination in the IAT resulting in erroneous results in antibody detection (screening) tests, antibody identification panels, and antihuman globulin (AHG) crossmatches [25]. As a result, clinically relevant alloantibody may not be recognized in patients treated with daratumumab.

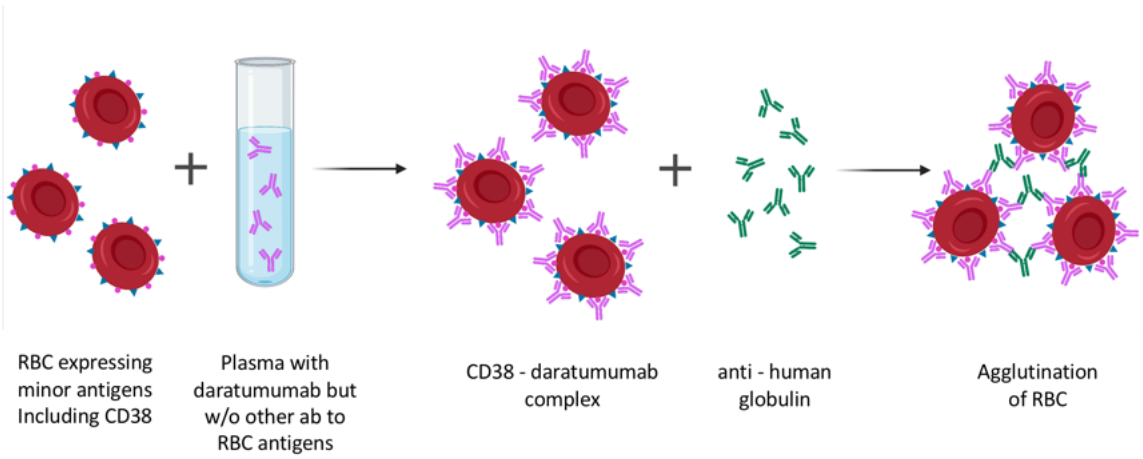


Figure 3. Positive IAT due to interference of daratumumab, which binds to CD38 on RBCs and causes agglutination after addition of antihuman globulin (AHG).

1.5 Proposed solutions to overcome DARA interference

As the interference caused by CD38 antibody therapy has created a unique situation in immunohematology labs, several methods to negate the DARA effect on RBCs have been investigated.

Chapuy et al. investigated a method using 0.2 M DTT, which reduces disulfide bonds of RBC-bound CD38, changing the molecule's antigenic structure so that CD38 antibodies are no longer able to bind it. The study included 25 worldwide blood bank laboratories in North America, South America, Europe, Australia and the Asia Pacific region. Regardless of the method of testing, all sites observed daratumumab interference using patients' sample in an initial antibody screen using untreated cells, but after treatment of RBC with DTT all sites correctly identified an unknown alloantibody in samples spiked with daratumumab [26, 27]. The treatment of RBCs with DTT has been further internationally validated and became the most widely used method in routine laboratories [26, 28]

The major drawback of this method is that disulfide bonds of other RBC antigens e.g. Kell, Cartwright (Yt^a), John Milton Hagen (JMH), $Knops^a$ (Kn^a, McC^a, and Yk^a), Landsteiner-$Wiener^a$ (LW^a), Lutheran, Dombrock, and Cromer are also disrupted [26, 29, 30]. Matched transfusions for Kell antigens are already recommended for DARA treated patients, if the antibody screening test is performed with DTT treated test-RBCs. However, this does not solve the problem of the destruction of Yt^a, JMH, $Knops^a$, LW^a, Lutheran, Dombrock, and Cromer antigens by the DTT method. Hence, antibodies against antigens altered by DTT cannot be detected by this method [23, 30, 31]. Furthermore, incomplete inhibition of DARA binding or hemolysis of RBCs is often observed, causing time consuming laboratory work-up with a potentially critical delay of RBC transfusion [32].

To mitigate the destructive effects of 0.2 M DTT on the red cell membrane, Hosokawa et al. introduced the Osaka method using 0.01 M DTT in pH 7.3 phosphate-buffered saline (PBS), followed by washing and centrifugation to negate daratumumab interference while preserving K antigenicity. The authors noted that anti-K reactivity was detected and daratumumab interference was negated, but the method failed to detect low-titer (<4) anti-

K [33]. In 2020 Izaquirre and colleques also demonstrated a modification of the original DTT method based on lowering the DTT concentration to 0.04 M (final DTT concentration 0.01 M). They observed that complete elimination of anti-CD38 interference can be accomplished while preserving Kell and other blood group antigens. They noted, however, two limitation of their study: they were unable to test the integrity of other red blood cell antigens, because of scarcity of sera with anti-LW, -Dombrock, -Knops, -Indian and -Raph specificities; secondly, incubation of patient's sera with this concentration of DTT may diminish the ability to detect IgM alloantibody [34].

In an alternative approach, Chapuy et al. presented method using CD38+ human HL60 cells to absorb the free anti CD38 from patient sera. Dara-spiked plasma was incubated with HL60 CD38+ (1×10^6 and 5×10^6) cells and then assayed for the presence of residual DARA by flow cytometry. This resulted in detectable removal of DARA from spiked plasma only when a high number of adsorbing cells was used, and this approach to overcoming DARA interference was not further pursued [5].

Trembley at al. suggested a method using Daudi cell stroma which are well characterized B lymphoblast cell line employed extensively in studies of mechanisms of leukemogenesis. What is important Daudi cells express high level of CD38. Adsorbing a sample of plasma with Daudi cell stroma led to removal of daratumumab interference while allowing detection of alloantibody such as anti-K, anti-Yt^a, and anti-Gy^a. According to authors, Daudi cell stroma is relatively inexpensive and large quantities can be stored and frozen for convenience. The main limitation is the standardization of the method and the requirement for specialized and expensive equipment [27, 35].

Proteolytic enzymes like trypsin and papain were also investigated as a potential method to reduce daratumumab binding to CD38. Both enzymes cleave the CD38 antigen on RBCs, and neither of them degrades the Kell antigens. Unfortunately, trypsin destroys a number of other clinically significant antigens including M, N and the less immunogenic Ge2, Ge3, Ge4, Ch/Rg, and Lutheran antigens. Papain degrades antigens from the Duffy and MNS blood group systems, as well as several minor antigens including Ch/Rg, Ge2, and Ge4 [23, 29]. Due to that, both methods could be used just as a complementary method, however this would increase the time of laboratory procedures and may not be practical in a routine lab.

Schmidt et al. described testing plasma from patients treated with daratumumab not against RBCs, but rather against cord blood cells, which do not express the CD38 antigen. The drawback to this approach is that cord RBCs are not typically available in a routine transfusion lab, and they may have lack, or only weakly express, several antigens including P1, Lewis and Lutheran antigens, thus limiting the effectiveness of the antibody screen [23, 27, 36].

One further method to overcome DARA inference is by neutralization of CD38 antibody with the sCD38 extracellular domain, or by the use of anti-idiotype antibody. Both of these methods were shown by Oostendorp et al., to permit the identification of known irregular antibodies in DARA spiked plasma [24]. However, sCD38 is too expensive for routine testing, and anti-idiotype antibodies against DARA are not comercially available.

Genotyping has also been successfully used to provide blood product for transfusion to daratumumab treated patients. Antigen typing is a simple procedure that prevents mismatching for the most common blood groups and is commonly performed in blood banks where commercial kits that allow for rapid and accurate genotyping are available [4]. Prior to the first dose of an anti-CD38 monoclonal antibody, all patients should get an extended phenotype or/and genotype, depending on the resources available. Importantly, phenotyping may be inaccurate if the patient has received a blood transfusion in the preceding 3 months [4]. Unfortunately, also this method has some disadvantages. For extend genotyping only a limited number of matching donors will be available, and the presence of other irregular antibodies still cannot be excluded because of positive cross matching caused by CD38 antibody. These methods are however currently prohibitively expensive and therefore are used just in transfusion facilities with high numbers of blood donations and in immunohematology laboratories of big hospitals. But not in small hospitals [4, 23, 26].

In a recent publication, Werle et al. showed that the use of DARA-Fab fragments is also a potentially promising method to prevent DARA interference in IAT. According to these authors, incubation of the screening RBCs with DARA-Fab prevented pan-agglutination in pre-transfusion testing without affecting the detectability of all alloantibody tested. So far, this report has been a single experiment and requires further validation [25].

Finally, a commercial reagent (DaraEx, IMUSYN) has been introduced in Europe for research purposes, which is also able to inhibit interference of anti-CD38 antibodies during pre-

transfusion compatibility testing. The content of this reagent has not been disclosed by the company [37].

1.6 Future perspective of CD38 antibody

The treatment options in MM have changed dramatically over the past decade with the emergence of novel agents, including targeted immunotherapies. CD38 monoclonal antibodies are highly efficient therapies for MM, and their use is likely to increase as the range of therapeutic indications continues to grow [38]. Tests of Daratumumab are in various stages of development across a wide variety of cancers, as well as in a subcutaneous formulation.

Other CD38 monoclonal antibodies are also under investigation for the treatment of MM. These include Isatuximab, for which several ongoing phase 3 trials are in progress, as well as early clinical trials of MOR202 and TAK079 [23, 38]. Furthermore, these novel therapies are expected to be potentially useful in the treatment options available for patients who are ineligible for autologous stem cell transplantation followed by high-dose chemotherapy [38]. There may therefore soon be approval of other CD38 monoclonal antibodies for treatment of multiple myeloma.

Therapeutic CD38-targeting antibodies were shown to interfere with routine pre-transfusion laboratory tests due to the weak expression of CD38 on human red blood cells [24]. This interference with blood group serologic testing is an example of the type of interactions that may occur between biological therapeutic agents present in a patient's serum, and specific tests in the clinical laboratory. Such interference is not restricted to daratumumab, as it has also been observed with Isatuximab and MOR202 [14, 23]. The potential interference of therapeutic CD38 monoclonal antibodies with laboratory tests will become an increasing problem as its clinical use expands.

Therapies based on the use of monoclonal antibodies are becoming ever more common. Recently, the IgG4 monoclonal anti-CD47 agent (Hu5F9-G4) has gained attention as a treatment option for hematologic malignancies and solid tumors. Several therapeutic CD47 monoclonal antibodies have already entered clinical trials [31]. Valliquette and colleagues reported that anti-CD47 causes interference in both AB0 and antibody screening tests. Moreover, CD47 is not cleaved from test RBCs by treatment with papain, ficin, trypsin, alpha-chymotrypsin, 0.2 M DTT or warm autoantibody removal medium (W.A.R.M.) reagent, so

interference cannot be mitigated using these methods [31]. This makes it clear that there is a growing need for the improvement of methods to overcome interference produced by immunotherapies.

The main objective of the work presented here was to develop novel strategies to improve diagnostics of patients treated with daratumumab. Two approaches to overcome the interference of DARA with pre-transfusion antibody screening and red cell compatibility testing were tested.

In the first part of this study a method for blocking the binding of free daratumumab to CD38 expressed on red blood cells is presented. This approach was based on adsorption of DARA from patient plasma using CD38 expressing cells prior to antibody screening and crossmatching. Since not only red blood cells but also platelets and leucocytes express CD38, the feasibility of using all of these cells as an adsorbing matrix was examined.

A second approach to quench DARA interference was also tested. This relies on blocking the binding site of DARA on RBCs without influencing other RBC alloantibody binding sites. The hypothesis was that $F(ab')_2$ fragments bind to the same epitope on CD38 as intact daratumumab does, and thus block binding of free daratumumab in patient plasma to red blood cells in the diagnostic assay. For this purpose, $F(ab')_2$ fragments of daratumumab were prepared by pepsin digestion and tested for their ability to function as a blocking reagent.

2. Materials and Methods

2.1 Biological study material

CHEMICALS	PROVIDER
Pancoll Human, Separating Solution, (Density: 1.077 g/mL)	Panbiotech, Aidenbach, DE
PBS (w/o $Ca^{2+,}$ Mg^{2+})	Panbiotech, Aidenbach, DE

ANTIBODIES	PROVIDER
Daratumumab (Anti-CD38 mab) DARZALEX®	Johnson & Johnson, New Jersey, US/ UMG pharmacy

HUMAN CELLS	PROVIDER
ID I-II-III 0.8% RBC suspension in diluent 2	BioRad Medical Diagnostic, Hercules, US
Human Platelet Concentrates (Human Thrombozytenkonzentrat Gepoolt Leukozytendepletiert In Additivlösung/HGW)	Universitätsmedizin Greifswald, Institut für Immunologie und Transfusionsmedizin, Greifswald, DE
Human Buffy Coat from Whole Blood Donations of Healthy Volunteers	Universitätsmedizin Greifswald, Institut für Immunologie und Transfusionsmedizin, Greifswald, DE
Human Fresh Frozen Plasma (Gefrorenes Human-Frischplasma CPD/HGW)	Universitätsmedizin Greifswald, Institut für Immunologie und Transfusionsmedizin, Greifswald, DE
Human Red Blood Cell Concentrates (Human-Erythrozytenkonzentrat PAGGSM Leukozytendepleitert/HGW)	Universitätsmedizin Greifswald, Institut für Immunologie und Transfusionsmedizin, Greifswald, DE
Human Red Blood Cell Concentrates (Human-Erythrozytenkonzentrat PAGGSM Leukozytendepleitert/HGW)	Universitätsmedizin Greifswald, Institut für Immunologie und Transfusionsmedizin, Greifswald, DE

DEVICES AND SOFTWARE	PROVIDER
Centrifuge Rotanta 460 R	Hettich, Tuttlingen, DE
SYSMEX-POCH-100I Cellcounter	Hettich, Tuttlingen, DE

2.1.1 Red blood cells

Test-red blood cells (RBCs) were taken from commercially available screening cell panels BioRad ID I-II-III 0.8% RBC suspension in a modified low-ionic saline solution (diluent 2) or prepared from RBC concentrates (Human-Erythrozytenkonzentrat PAGGSM leukozytendepleitert//HGW) of voluntary blood donors. RBC concentrates containing 0.5 - 0.7 L/L of RBC (hematocrit), with addition of 0.01-0.02 L/L of CPD anticoagulant, 0.28 -0.38 L/L of PAGGS-M additive solution and 0.01-0.10 L/L of human plasma. Afterwards cells were prepared in various manners, depending on performed test (see paragraphs 2.2.1, 2.3.2 and 2.4).

2.1.2 Platelets

Platelets (PLT) were collected from platelet concentrate (Human Thrombozytenkonzentrat gepoolt leukozytendepletiert in Additivlösung/HGW).

Platelet concentrate containing 2-4 × 10^{11} platelets per unit with a total volume of 300-370 mL, 0.04-0.09 mL of CDP anticoagulant, 0.20-0.31 mL of human plasma and 0.6-0.7 mL of additive solution SSP+/mL of platelets was produced by pooling buffy coats of four healthy donors of the same blood type. Cells were then diluted to a concentration of 1 × 10^{7}/mL with SSP+ additive solution.

2.1.3 Peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were prepared from standard whole blood donations from healthy donors. After separation of whole blood (500 mL) most leukocytes are present in the buffy coat (BC) fraction. For isolation of PBMCs the BC was diluted with the same volume of phosphate-buffered saline (PBS, pH 7.4) and PBMCs were obtained by centrifugation on a Pancoll density gradient medium (1.077 g/mL; 400 × g, 40 min, RT) (Fig. 4). The cells were collected and washed three times with PBS (300 × g, 10 min, 4°C). The count of pooled PBMC was determined using an automated cell counter (Sysmex-pocH-100i Cell counter). Afterwards cells were prepared in various manners, depending on performed test (see paragraph 2.2).

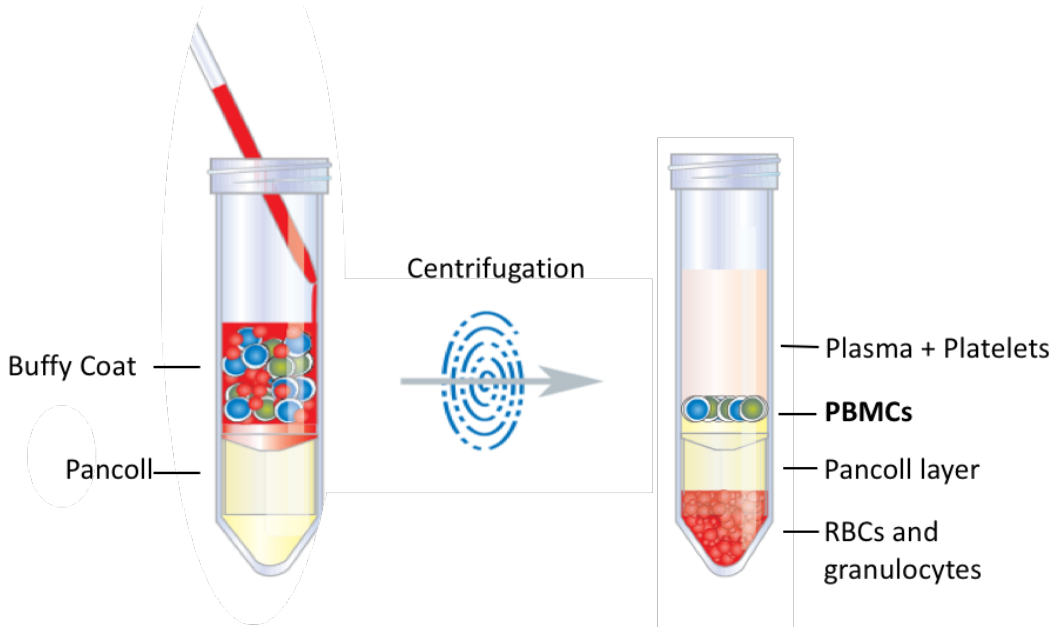


Figure 4. Principle of the density gradient centrifugation method for the separation of PBMCs [39]. The separation of blood cells is based on their density; centrifugation leads to differential migration of cells resulting in the formation of multiple layers. The top layer is a platelet rich plasma, a thin layer in between contains PBMCs, and a bottom layer contains most of the red blood cells and granulocytes.

2.1.4 Human plasma

RBC alloantibody negative plasma was collected from healthy blood donors. Plasma of patients containing specific RBC alloantibody (Table 1) were collected from the routine diagnostic laboratory and used as neat plasma or titrated by dilution with antibody negative plasma.

RBC alloantibody negative and positive plasmas were used with and without spiking with DARA (0.01, 0.05, 0.1, 0.25 and 0.5 mg/mL) to evaluate the performance of IAT after adsorption of DARA by PBMCs. This was then repeated in the presence of DARA-F(ab')$_2$ fragments. The final concentration of DARA for adsorption experiment was 0.05 mg/mL (see paragraph 2.2.2) and for IAT experiments with addition of DARA-F(ab')$_2$ fragments 0.5 mg/mL (see paragraph 2.4).

Plasma from patients with multiple myeloma before and after DARA infusion were also tested.

Table 1. 35 different patient plasma samples containing RBC alloantibody of single or multiple RBC antigen specificity were tested in IAT. Antibody specificities are listed according to the experimental protocol for which they were used.

PATIENT PLASMA SAMPLES TESTED IN IAT			
Antibody specificity	**Numbers of tested plasma containing single ab or multiple ab**	**Addition of DARA-F(ab')$_2$ fragments (Y/N)**	
		Gel card technique	**Solid phase technigue ***
Anti-D	6	Y	Y
Anti-E	4	Y	Y
Anti-C	4	Y	Y
Anti-C^w	1	Y	N
Anti-c	1	Y	Y
Anti-e	2	Y	Y
Anti-K	5	Y	Y
Anti-Kp^a	1	Y	N
Anti-M	3	Y	Y
Anti-S	2	Y	Y
Anti-Fy^a	4	Y	Y
Anti-Jk^a	1	Y	Y
Anti-Lu^a	1	Y	Y

*Because of the limited volume of the plasma samples not all could be tested in solid phase technique.

All patient samples were leftovers from routine patient diagnostics, had been anonymized before transfer from the routine laboratory to the research laboratory, and were stored at -20°C until use. The use of anonymized residual patient plasma from routine laboratory testing and residual blood from whole blood donations was approved by the ethic committee of the Universitätsmedizin Greifswald.

2.2 Experiments for DARA adsorption from patient plasma

CHEMICALS	PROVIDER
Bovine Serum Albumin (BSA) 22%	Ortho-Clinical Diagnostics, Pencoed, UK
PBS (w/o $Ca^{2+,}$ Mg^{2+})	Panbiotech, Aidenbach, DE

ANTIBODIES	PROVIDER
Daratumumab (Anti-CD38 mab) DARZALEX®	Johnson & Johnson, New Jersey, US/ UMG pharmacy
Human IgG	Invitrogen, Carlsbad, California, US
Polyclonal Rabbit Anti-Human IgG/FITC	DAKO, Glostrup, DK

DEVICES AND SOFTWARE	PROVIDER
Centrifuge Rotanta 460 R	Hettich, Tuttlingen, DE
Flow Cytometer: Cytomix FC500	Beckman Coulter, Brea, CA, US
CXP – Software (V 1.2)	Beckman Coulter, Brea, CA, US

BUFFER PREPARATION:

PBS/0.2% BSA-Buffer	BSA 0.2 % in PBS (w/o Ca^{2+}, Mg^{2+})

2.2.1 CD38 expression on different cell type

Flow cytometry experiment was performed to identify which blood cell type expresses the highest amount of CD38, and would therefore be suitable for DARA adsorption from patient plasma. For this, binding of DARA to RBC (n=9), PLT (n=9) and PBMCs (n=11) was analyzed.

For experiments, 100 µL of PBMCs, PLTs or RBCs suspension were first prepared at a concentration of 1×10^7 cells/mL in PBS/0.2% BSA (PBS containing 0.2% BSA). BSA was used to saturate nonspecific binding sites. To this cell suspension, 5 µL of DARA (20 µg/mL) or negative control (human IgG 20 µg/mL) was added, and incubated for 20 min at RT. To remove unbound antibodies, a washing step with 1.5 mL of PBS/0.2% BSA (650 × g for 7 min) was performed. Next, cells were stained with polyclonal rabbit anti-human IgG/FITC [1:75 dilution]

in 100 µL of PBS/0.2% BSA and incubated for 20 min at RT. Samples were then washed once (650 × g for 7 min), resuspended in 500 µl PBS/0.2% BSA and measured in an FC500 flow cytometer.

Because bi-modal cell distribution was expected, the results of flow cytometry analysis are presented as median MFI (mean fluorescence intensity) of positive labeled cells. Median is less influenced by skew or outliers, and is therefore considered a more robust statistic.

2.2.2 PBMC-based depletion of DARA from patient plasma

PBMCs were used for removal of DARA by adsorption from patient plasma. Antibody negative plasma was spiked with DARA (0.05 mg/mL) and incubated with a pellet of PBMCs (~6 × 10^8 cells) with end-over-end mixing for 15 min at 37°C. After centrifugation (4,400 × g, 10 min) the supernatant was again incubated with the same amount of PBMCs under the same conditions. A maximum of three consecutive adsorption steps were performed (Fig. 5). PBMCs adsorbed plasma was analyzed for residual DARA by IAT using ID I-II-III 0.8% RBC suspension, and by flow cytometry using fresh PBMCs (see paragraph 2.5).

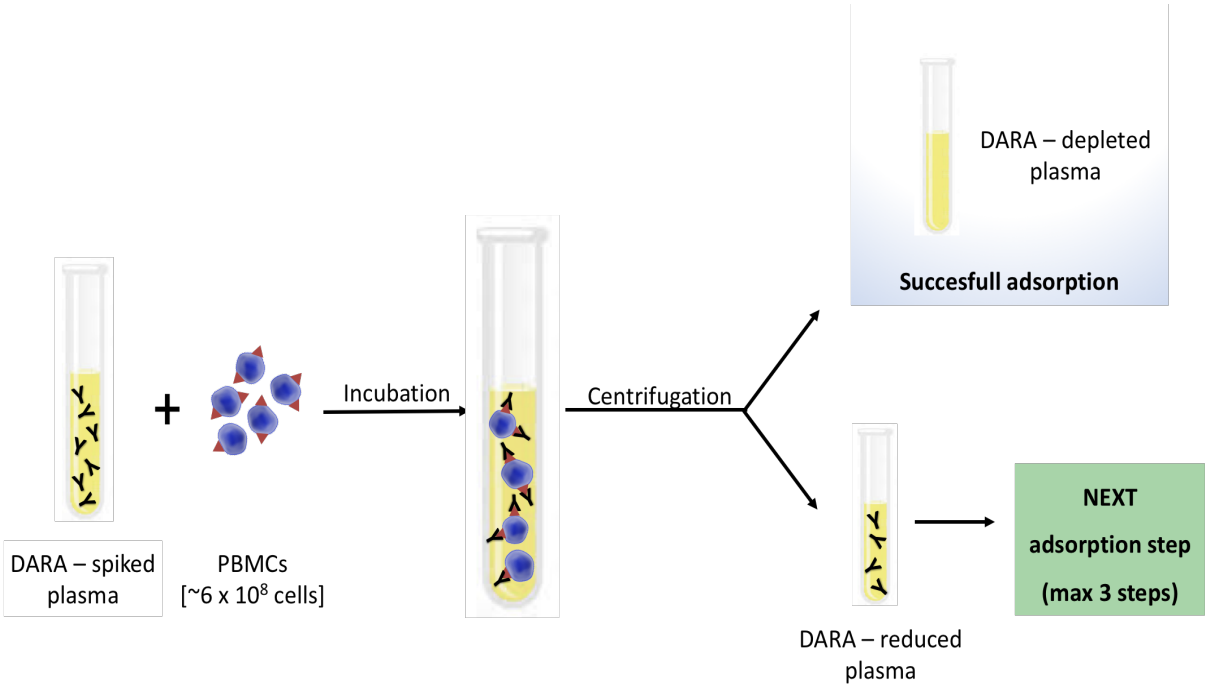


Figure 5. Adsorption of DARA from DARA spiked plasma by repeated incubation with PBMCs expressing CD38 protein.

2.3 Experiments for blocking DARA binding to CD38 on red blood cells

CHEMICALS	**PROVIDER**
Bovine Serum Albumin (BSA) 22%	Ortho-Clinical Diagnostics, Pencoed, UK
Digestion Buffer	GBioscience, St. Louis, US
Ethanol	Roth, Karlsruhe, DE
F(ab')$_2$ Fragmentation Kit	GBioscience, St. Louis, US
Imobilized Pepsin	GBioscience, St. Louis, US
Imperial Protein Stain (Coomassie R-250 Dye)	Thermo Scientific, Waltham, US
Laemmli Non-Red. Sample Buffer; 4 × Conc.	BioRad Medical Diagnostic, Hercules, US
Methanol	Roth, Karlsruhe, DE
PBS (w/o Ca^{2+}, Mg^{2+})	PanBiotech, Aidenbach, DE
Powdered Milk, Blotting Grade, Low Fat	Roth, Karlsruhe, DE
Protein G Resin	GBioscience, St. Louis, US
Quantipro BCA Assay Kit	Sigma, St. Louis, US
Roti-Load 1 Sample Buffer, Reducing, 4× Conc	Roth, Karlsruhe, DE
Sodium Dodecyl Sulfate (SDA)	Merck Millipore, Billerica, US
Supersignal West Femto	Thermo Scientific, Waltham, US
Tris-HCl	Sigma, St. Louis, US
Tween 20	BioRad Medical Diagnostic, Hercules, US

ANTIBODIES	**PROVIDER**
Daratumumab (Anti-CD38 mab) DARZALEX®	Johnson & Johnson, New Jersey, US/ UMG pharmacy
Human IgG	Invitrogen, Carlsbad, California, US
Goat Anti-Human IgG F(AB')$_2$ FITC	BIOZOL Diagnostica Vertrieb GmbH, Eching, DE
Goat Anti-Human IgG (Fab Specific)	Sigma-Aldrich, St. Louis, US
Donkey Anti-Goat IgG-HRP	Santa Cruz Biotechnology, Santa Cruz, US

EQUIPMENT	**PROVIDER**

Blotting Paper	Sarstedt AG &Co. KG, Sarstedt, DE
Mini-Protean TGX Stain-Free Gels 4-20%	BioRad Medical Diagnostic, Hercules, US
Protein G Spin Column, 1 mL	GBioscience, St. Louis, US
SpinOut^{tm} GT-600, 3 mL	GBioscience, St. Louis, US
Whatman® Protran® Nitrocellulose Membranes	Roth, Karlsruhe, DE
96well Plate Flatbottom	Sigma, St. Louis, US

DEVICES AND SOFTWARE	**PROVIDER**
Consort Electrophoresis Power Supply	CONSORT, Turnhout, BEL
Centrifue Rotanta 460 R	Hettich, Tuttlingen, DE
Incubator Binder BD 53	Binder, Bohemia, US
Flow Cytometer: Cytomix FC500	Beckman Coulter, Brea, CA, US
Fusion F×7	VILBER, FR
Ph-Meter Lab 850	Schott AG, Mainz, DE
Tecan Infinite F50	TECAN, Maennedorf, CH
Vortex – Genie 2	Scientific Industries, NY, US
CXP-Software (V1.2)	Beckman Coulter, Brea, CA, US
Fusion Capt Software	VILBER, FR
Magellan For F50	TECAN, Maennedorf, CH

BUFFERS PREPARATION:

BLOTTO Blocking Buffer	low-fat milk Tween20 in TBS	5 % 0.1 %
IgG Elution Buffer (pH 2.7)	Glycine in diH_2O	0.1 M
IgG Neutralization Buffer (pH 8.0)	TRIS-HCl in diH_2O	1 M
PBS/0.2% BSA	BSA solution in PBS (w/o Ca^{2+}, Mg^{2+})	0.2 %

SDS-PAGE Running Buffer (10 ×)	Tris base	0.25 M
	Glycine	1.924 M
	SDS	0.0347 M
	in diH_2O	
TBS (10 ×) (pH 7.6)	Tris base	0.5 M
	NaCl	1.5 M
	in diH_2O	
TBS – Tween (pH 7.6)	Tween20	0.05 %
	in TBS	
Transfer Buffer	Tris base	0.025 M
	Glycine	0.192 M
	Methanol	20 %
	in diH_2O	

2.3.1 Generation of DARA-F(ab')$_2$ fragments

F(ab')$_2$ fragments of daratumumab (DARA-F(ab')$_2$ fragments) were generated from whole daratumumab antibody by pepsin digestion using a F(ab')$_2$ fragmentation kit according to the manufacturer's protocol (Fig.6). In brief, samples containing 2.5 mg of DARA were applied to the SpinOUT GT-600 desalting column before digestion and centrifuged at 1000 × g for 2 min. The antibody containing flowthrough was adjusted to 0.5 mL with digestion buffer. The pepsin resin (2mg/mL) was suspended by gentle shaking and inverting. 0.25 mL of pepsin resin was transferred to a 1 mL column, placed into 2 mL collection tube and centrifuged at 5000 × g for 1 min to remove storage buffer. The resin was equilibrated by the addition of 0.5 mL of digestion buffer and centrifuged at 5000xg for 1 min. Then 0.5 mL of DARA sample was added and incubated at 37°C with end-over-end mixing for 7 h. The column was placed into a new 2 mL collection tube and centrifuged at 5000 × g for 1 min to collect the pepsin-digested antibody. The column was then washed twice by adding 0.5 mL of PBS. The flowthroughs were combined to a final volume of 1.5 mL (DARA-tube 1). Immobilization of pepsin on a slurry resin, instead of in solution, allows a quick removal of the pepsin from the solution. This stops the enzyme reaction and avoids enzyme contamination of the desired antibody fragments.

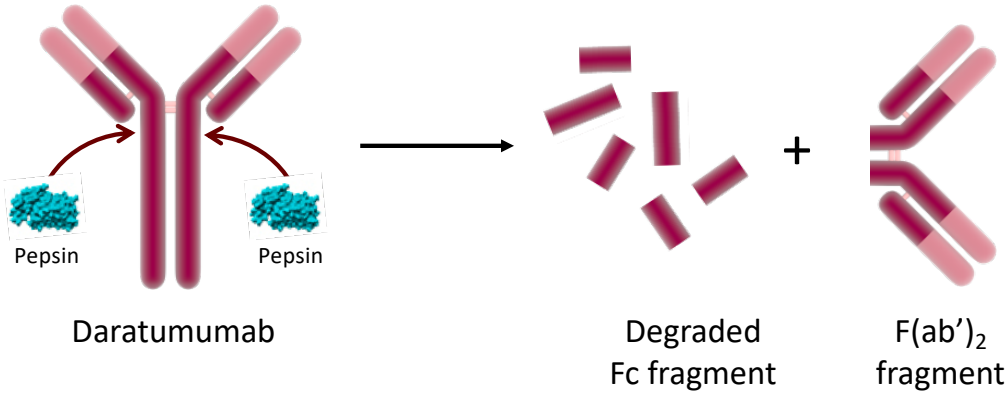


Figure 6. Principle of DARA $(Fab')_2$ fragment generation by pepsin digestion. Pepsin is a nonspecific endopeptidase, which is active only at acidic pH. Pepsin cleaves the heavy chain of antibody below the two disulfide bridges, resulting in divalent $F(ab')_2$ fragments and small peptides derived from the Fc fragment.

DARA-$F(ab')_2$ fragments were separated from undigested IgG and Fc fragments by affinity chromatography using immobilized Protein G (Fig. 7). A Protein G spin column (capacity: >20 mg human IgG/mL resin) was transferred to a 15 mL collection tube and centrifuged at 1000 × g for 1 min to remove storage buffer. The resin was equilibrated by addition of 2 mL PBS and centrifuged. Next, 1.5 mL sample (DARA-tube 1, Fig. 7) was applied to the protein G resin and incubated at RT with end-over-end mixing for 15 min. The column was placed into a 15 mL collection tube and centrifuged at 1000 × g for 1 min to collect the flowthrough containing unbound fragments of IgG. The column was then washed twice with 1 mL of PBS, and flow-throughs were combined (DARA-tube 2, Fig. 7). After washing, bound fragments of digested IgG were eluted with IgG elution buffer and neutralized by addition of Tris-HCl buffer (1M; pH 8). For maximum recovery, elution step was performed four times (DARA-tube 3.1 - 3.4). For next experiments two elution fraction 3.2 to 3.3 were combined (DARA-tube 3) and used.

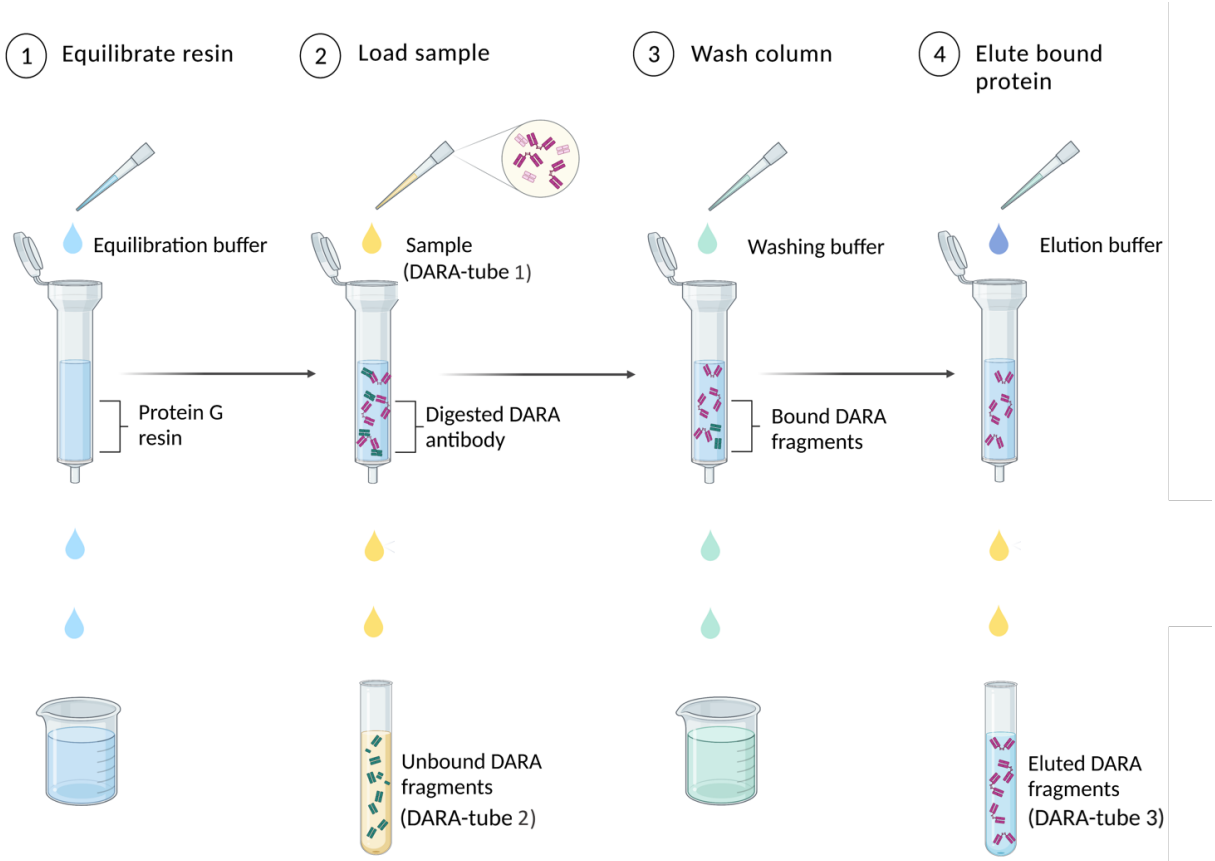


Figure 7. Affinity chromatography with Protein G ligand was used to separate DARA F(ab')$_2$ fragments from Fc molecules. (1) Protein G resin is equilibrated with binding buffer and packed in a column. (2) Sample containing digested daratumumab (DARA-tube 1) is loaded onto the column. DARA-F(ab')$_2$ was separated from Fc fragments by binding to the immobilized Protein-G. (3) The unbound material which has no affinity to the Protein G ligand pass through the column and are collected as DARA-tube 2. (4) Addition of elution buffer disrupts the interaction between the Protein-G and bound fragments and allows to purify the target molecule (DARA-tube 3) [40].

The concentration of DARA-F(ab')$_2$ fragments was determined using the QuantiPro BCA Assay Kit according to manufacturer's specification. In brief, a calibration curve was plotted between 0.5 µg/mL to 30 µg/mL diluted from 1 mg/mL bovine serum albumin (BSA) solution. BCA working reagent was prepared by mixing 25 parts of BCA Reagent A with 25 parts of BCA Reagent B and with 1 parts of Copper(II). 150 µL of combined flow through (DARA-tube 2) and combined elution fraction (DARA-tube 3) in several dilutions (1:2 to 1:50) and 150 µl of the BCA working reagent were mixed in flat bottom 96 well plate and incubated at 37°C for 2 h. The absorbance was measured at 560 nm using microplate reader (Infinite F50, TECAN) against blank (Tris-HCl in several dilutions 1:2 to 1:50). Sample concentration was calculated based on the standard BSA curve.

2.3.2 Detection of DARA-F(ab')$_2$ fragments

Purity and molecular mass of the pepsin digested samples were analyzed using SDS-PAGE and Western blot. SDS-PAGE analysis was performed under reducing and non-reducing conditions using Mini-PROTEAN TGX Stain-Free Gels (4-20%) according to the manufacturer's protocol. Samples (undigested DARA and DARA-tube 1, 2, 3.1 - 3.3) were each mix 1:4 with non-reducing Laemmli buffer or were reduced by addition of Roti-Load 1 sample buffer at 90°C for 5 min. Electrophoresis was performed at room temperature for approximately 60 min at 200V in running buffer. Stain-free gels were activated by exposure to UV light for 5 min.

After electrophoresis proteins were transferred to a nitrocellulose membrane (Amersham Protran® 0.2 µm NC, Roth) for 90 min in ice cold transfer buffer. The membrane was blocked with BLOTTO for 2 h to prevent nonspecific binding of the antibody to the membrane surface. The membrane was then incubated with the unconjugated primary goat anti-human IgG Fab specific antibody in BLOTTO under gentle agitation overnight at 4°C. Excess primary antibody was removed by washing the membrane with TBS/Tween20 three times for 5 min each. The membrane was then incubated with the HRP conjugated donkey anti-goat IgG-HRP secondary antibody in BLOTTO for one hour at room temperature. Excess secondary antibody was removed by washing the membrane with TBS/Tween20 three times for 5 min each. The membrane was incubated with chemiluminescence reagent SuperSignal West Femto for 5 min at room temperature and visualized using the Fusion F×7 gel documentation system. Detection of the protein bands was carried out using Fusion-CAPT software.

Flow cytometry was used to detect whether purified DARA-F(ab')$_2$ fragments bound to RBC similar to undigested DARA. 100 µL of RBCs (1×10^7 cells/mL) were incubated with 10 µL of DARA (0.01 mg/mL) or DARA-F(ab')$_2$ fragments (0.01 mg/mL of combined DARA elution fraction (DARA-tube 3) for 15 min at 37°C and washed once with PBS/0.2% BSA (650 × g, 7 min). Cells were then incubated with FITC-conjugated goat anti-human IgG-F(ab')$_2$-specific antibody (1:100 dilution) for 20 min and washed again. The samples were resuspended in 500 µL PBS/0.2% BSA and measured in an FC500 flow cytometer. The results of flow cytometry analysis are given as mean fluorescence intensity (MFI) multiplied by percentage of positive labeled cells.

2.4 Read out system to assess DARA interference in pretransfusional testing

CHEMICALS	PROVIDER
Capture LISS Buffer	Immucor Inc., GA, US
Capture-R Ready Indicator Cells	Immucor Inc., GA, US
Diluent 2	Immucor Inc., GA, US
PBS (w/o Ca^{2+}, Mg^{2+})	PanBiotech, Aidenbach, DE
Sodium Chloride NaCl (0.9% Sol.)	B.Braun Melsungen AG, Melsungen, DE

ANTIBODIES	PROVIDER
Daratumumab (Anti-CD38 mab) DARZALEX®	Johnson & Johnson, New Jersey, US/ UMG pharmacy

EQUIPMENT	PROVIDER
Capture-R Select Microtiter Strips	Immucor Inc., GA, US
ID-Card LISS/Coombs	BioRad Medical Diagnostic, Hercules, US

DEVICES AND SOFTWARE	PROVIDER
Gamma P2 Incubator	Immucor Inc., GA, US
ID-Centrifuge 12 SII	BioRad Medical Diagnostic, Hercules, US
ID-Incubator 37 SII	BioRad Medical Diagnostic, Hercules, US
Microplate Centrifuge Universal 320	Hettich,Tuttlingen, DE
Microplate Washer Gamma CSW 100	Immucor Inc., GA, US

IAT was performed using two different techniques: gel card (Fig. 8) and solid phase (Fig. 9). The strength of agglutination in gel card and solid phase IAT was given from negative (no agglutination) to 4+ (strong agglutination). If antigen-negative cells were agglutinated by the plasma this reactivity was defined as a false positive.

To investigate the ability of daratumumab to induce *in vitro* RBC agglutination, plasma was spiked with 0.01, 0.05, 0.1, 0.25 and 0.5 mg/mL daratumumab and IAT gel card and solid phase assays were performed (see supplementary material).

IAT using DARA-spiked plasma (0.05 - 0.5 mg/mL), was carried out before and after adsorption of DARA by PBMCs, or addition of DARA-F(ab')$_2$ fragments (0.5 mg/mL). The final

concentration of DARA for IAT adsorption experiments and for IAT experiments after addition of DARA-F(ab')$_2$ fragments was 0.05 mg/mL and 0.5 mg/mL, respectively.

Several concentrations of DARA-F(ab')$_2$ fragments (0.01, 0.05, 0.10, 0.15, 0.25, 0.5 mg/mL) were tested using the IAT gel card technique to determine the lowest working concentration of DARA-F(ab')2 fragments.

RBC alloantibody (single or combined specificities, see Table 1) were recognized by the agglutination pattern of the antigen positive and negative cells. RBC alloantibody containing plasma samples were titrated to the lowest concentration detectable by the gel card technique. In this way whether the addition of DARA-F(ab')$_2$ fragments alone would interfere with the sensitivity of IAT was evaluated.

IAT was also performed using plasma from randomly selected patients with multiple myeloma, before and after DARA infusion therapy (n=8).

2.4.1 IAT gel card technique

IAT gel card technique was performed using commercially available gel cards (ID-Card LISS/Coombs) containing anti-IgG and anti-C3d (Fig. 8). The RBC were taken from commercially available screening cell panels BioRad ID I-II-III 0.8% RBC suspension in a diluent 2 or prepared from RBC concentrates as a 1% suspension in diluent 2. First, 50 µL of RBC suspension and 25 µl of human plasma were pipetted into the upper reaction chamber and incubated for 15 min at 37°C (Fig. 8A). The gel cards were then centrifuged for 10 min at 1030 rpm (ID-Centrifuge 12 SII) and agglutination of red cells was evaluated (Fig. 8B).

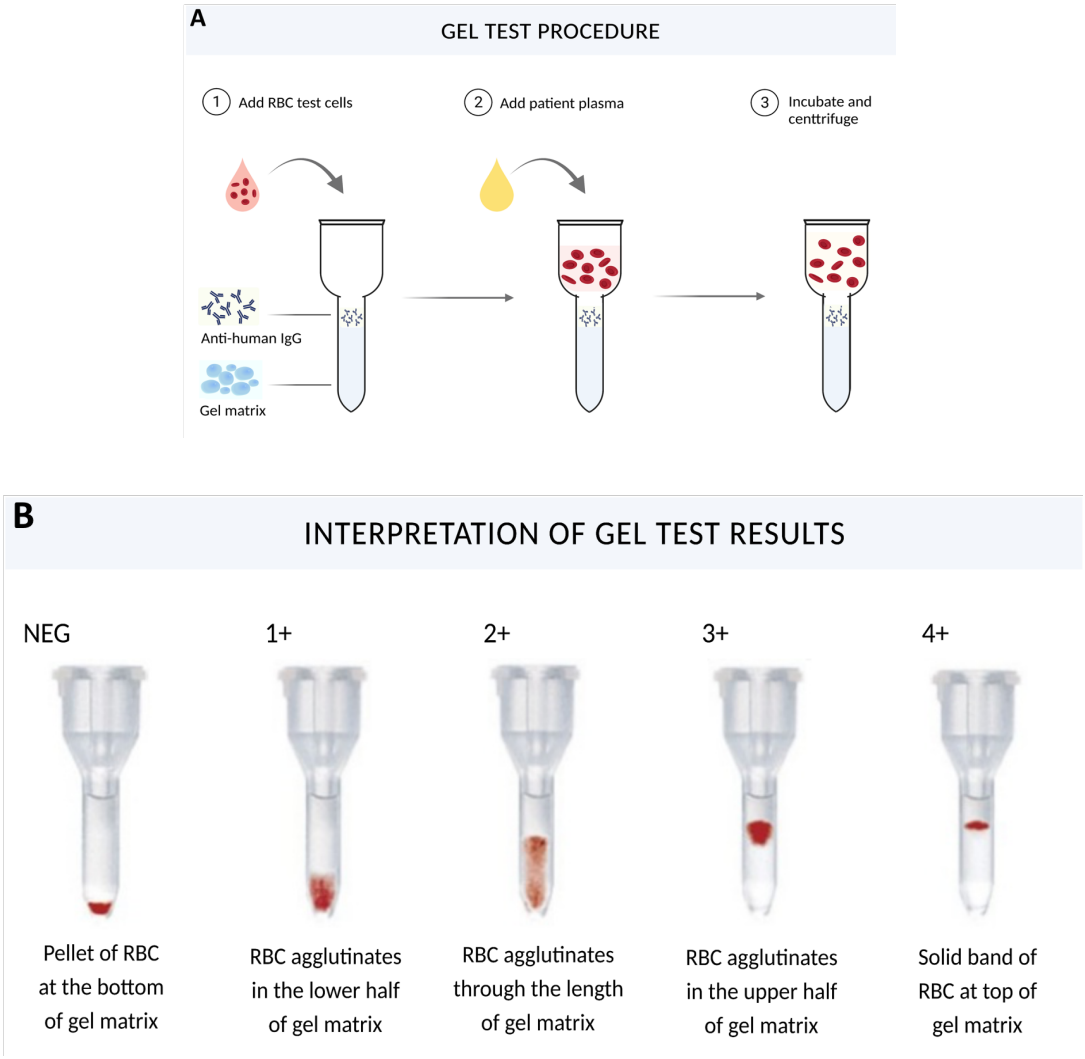


Figure 8. (A) Principle of indirect antiglobulin test gel card technique and (B) interpretation of agglutination of RBCs appearing in the gel matrix [41]. A solid red pellet at the bottom of the tube indicates a negative result, whereas suspended particles (red cell agglutinates) within the gel matrix indicate a positive test result (1+ to 4+).

2.4.2 IAT solid phase technique

For the solid phase IAT technique Capture-R Select microtiter strips, precoated with RBCs were used for antibody detection according to the manufacturer's instructions as a semi-manual procedure (Fig. 9A). Briefly, 100 µL of PBS and 50 µL of 2% RBCs suspension in a modified low-ionic saline solution buffer (diluent 2) were added to microtiter strips, agitated and centrifuged for 5 min at 350 × g. To remove unattached red blood cells, the plate was agitated vigorously and washed six times with saline buffer in an automatic microplate washer (CSW 100). Next, 100 µL of Capture LISS buffer and 50 µL of patient plasma were added to each well

and incubated for 40 min at 37°C. The purple blue colour of LISS buffer turned turquoise in the presence of plasma. After incubation, the plate was washed twice with saline buffer, 50 µL of Capture-R Ready Indicator-RBCs (RBCs coated with Fc specific AHG) were added to each well and centrifuged immediately for 2 min at 500 × g. The plate was placed on an illuminated surface and the binding of the indicator-RBCs to the test cell layer was graded from negative (no binding) to 4+ (complete homogenous distribution over the red cell layer, strongest reaction) (Fig.9B).

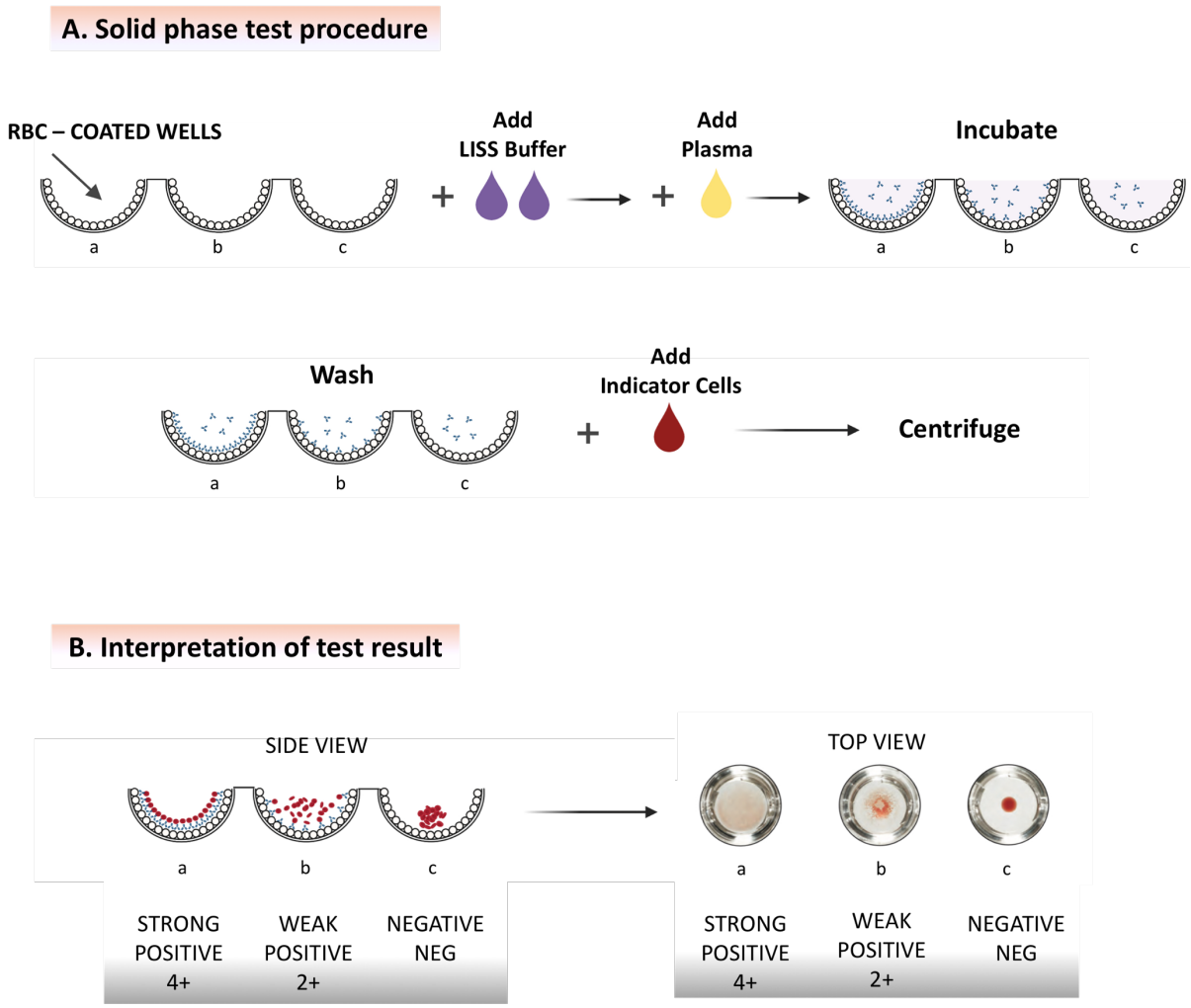


Figure 9. (A) Principle of the indirect antiglobulin test solid phase technique**:** a) high titer of RBC alloantibody, b) low titer of RBC alloantibody, c) no RBC alloantibody (Ref.) (B) Interpretation of solid phase results. A red spot in the middle of reaction well indicates a negative result (NEG), whereas particles spread over the surface of a reaction well indicate a positive test result (1+ to 4+).

2.5 Read out system to examine method for overcoming DARA interference in pretransfusion testing

To confirm the efficacy of both approaches, adsorption of DARA from patient plasma and pretreatment of RBCs with DARA F(ab')$_2$ fragments, series of IAT were performed. For experiments with DARA adsorbed plasma the IAT gel technique was used. Experiments with DARA F(ab')$_2$ fragments were performed using the IAT gel technique as well as the IAT solid phase technique.

2.5.1 Experiments with DARA adsorbing cells

To determine if the use of PBMCs as a DARA adsorbing cell is effective, a series of IAT gel card technique and flow cytometry experiments was performed.

For IAT experiments, 50 µL of RBC screening cell ID I-II-III (0.8% suspension) and 25 µL of PBMC adsorbed plasma were incubated in a reaction chamber of a gel card microtube. After 15 min of incubation at 37°C the gel card was centrifuged for 10 min at 1030 rpm (ID-Centrifuge 12 SII).

For flow cytometry analysis, 100 µL of PBMC cell suspension in concentration of 1×10^7 cells/mL was prepared. To this, 10 µL of DARA spiked plasma (0.05 mg/mL) or DARA spiked plasma after every adsorption step was added. After 20 min of incubation at RT, a washing step with 1.5 mL PBS/0.2% BSA (650 × g for 7 min) was performed. Cells were then resuspended in 100 µL of polyclonal rabbit anti-human IgG/FITC [1:75 dilution], incubated for 20 min and then washed once (650xg for 7 min). Finally, cells were resuspended in 0.5 mL of PBS/0.2% BSA and analysis of the samples was performed immediately afterwards.

2.5.2 Experiments with DARA-F(ab')$_2$ fragments

DARA-F(ab')$_2$ fragments were used for blocking the binding side of DARA on RBCs before and during the IAT procedure. For the gel card technique 25 µL DARA-F(ab')$_2$ (0.5 mg/mL) fragments were preincubated with RBCs in the reaction well of the gel card for 5 min at 37°C before adding patient plasma. For solid phase technique DARA-F(ab')$_2$ fragments were added into the wells coated with RBCs, incubated for 20 min at 37°C and washed. After incubation

of RBCs with plasma and washing, indicator-RBCs in parallel with and without DARA-F(ab')$_2$ fragments were added to each well, centrifuged and results were analyzed macroscopically.

2.6 Stability testing of DARA-F(ab')$_2$ fragments

DARA-F(ab')$_2$ fragments (0.5 mg/mL) were stored at 4°C, -20°C and -80°C and tested after 7, 14, 30, 45, 60 and 180 days by the IAT gel card technique. Two plasmas in absence of RBC alloantibody were used with and without spiking with DARA and with and without addition of DARA-F(ab')$_2$ fragments. DARA-F(ab')$_2$ fragments were stored as 1 mL aliquots to avoid repeated freezing and thawing, since this may result in degraded functionality caused by formation of ice crystals and loss of functionality due to agglutination. Furthermore, DARA-F(ab')$_2$ fragments were used to perform IAT by routine transfusion laboratory procedures (Greifswald, Transfusionmedizin) after storing at -80°C for up to 330 days.

3 Results

3.1 Adsorption of free DARA from plasma

3.1.1 Detection of DARA binding to different blood cell types by flow cytometry

Flow cytometry analysis was performed to detect CD38 expression levels on different cell surfaces, such as RBC (n=9), PLT (n=9), and PBMC (n=11), so as to select cell population suitable for testing as a DARA adsorption matrix. The protocol that was used is described in section 2.2.1. The number of detected cells (events) was plotted in a histogram against the fluorescence intensity for CD38. The results are given as median MFI multiplied by percentage of positive labeled cells. Expression of CD38 was detected on all cell types tested by a shift of the fluorescence peak compared to the corresponding negative control (Fig. 10A, B, C). The highest signal was detected on PBMCs (median MFI_{PBMC} of positive cells = 6.505; 4.43-7.54) (Fig. 10D). CD38 fluorescence signal on PLTs and RBCs was considerably lower (median MFI_{PLT} of positive cells = 0.997; 0.93-1.08; median MFI_{RBC} of positive cells = 1.380; 1.11-1.6) (Fig. 10D). Therefore, PBMCs were expected to have the highest adsorbent capacity for the removal of DARA from patient plasma, and were used for further experiments.

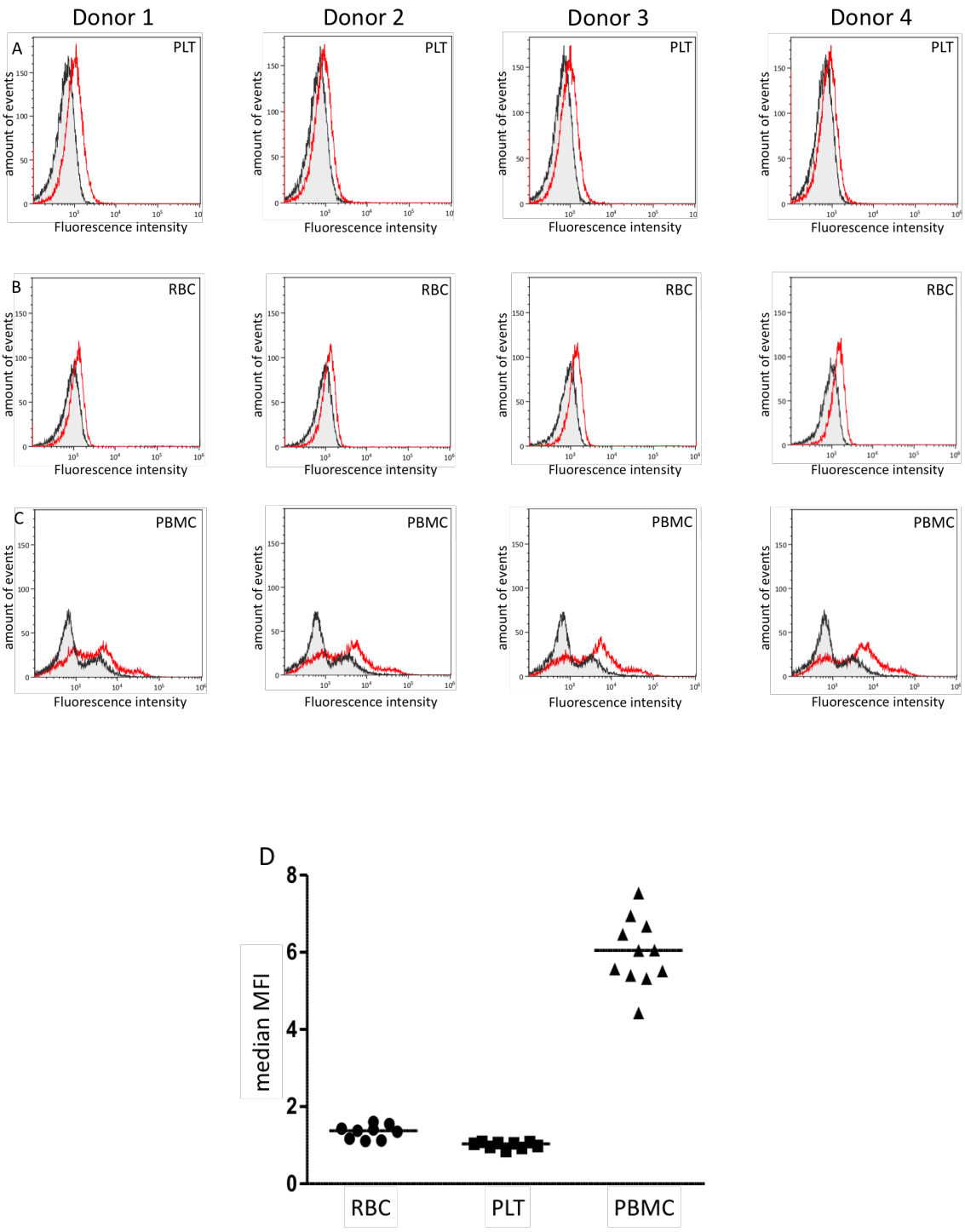


Figure 10. Representative examples of histograms of CD38+ cells (A) PLT, (B) RBC, (C) PBMC (red peaks) compared to negative control (grey peaks); (D) CD38 expression on different cell types (RBC n=9, PLT n=9, PBMC n=11).

3.1.2 Effect of DARA adsorption on IAT performance

DARA spiked plasma (50 µg/mL) induced 1+ to 3+ agglutination of red cells (Table 2B). After three consecutive adsorption steps with PBMC (~6 × 10^8 cells/step) agglutination strength became weaker but complete inhibition was not achieved (Table 2C). Further adsorption steps were not performed due to large reduction of sample volume after each adsorption step.

Table 2. Representative results of IAT using 3 different RBCs for testing the DARA adsorbent efficacy of PBMCs. (A) plasma without DARA (negative control), (B) DARA spiked plasma (50 µg/mL) (positive control) and (C) DARA spiked plasma (50 µg/mL) after adsorption with PBMC (~6 × 10^8 cells/step). A solid red pellet at the bottom of the tube indicates a negative result (NEG), red cell agglutinates within the gel matrix indicate a positive test result (1+ to 2+).

	A	B	C		
	Negative control	Positive control	Adsorption steps:		
			1st	2nd	3rd
Normal plasma	+	+	+	+	+
DARA 0.5 mg/mL	-	+	+	+	+
Plasma preadsorped with PBMC	-	-	+	+	+
Test-RBC I					
Result	**NEG**	**3+**	**2+**	**2+**	**1+**
Test-RBC II					
Result	**NEG**	**3+**	**2+**	**2+**	**1+**
Test-RBC III					
Result	**NEG**	**2+**	**1+**	**1+**	**1+**

Patient plasma adsorbed on PBMCs was analyzed for residual DARA by flow cytometry using fresh PBMCs (Fig. 11). Samples were collected after each adsorption step. Percentage of measured CD38 positive cells was similar for both, plasma before (34.24 %) and after DARA adsorption (1st step: 33.80 %; 2nd step: 33.48 %; 3rd step: 30.41 %) compared to the negative control (4.50 %). This showed that the expression of CD38 on PBMCs is too low to adsorb DARA completely from patient plasma. This confirms the result of the IAT analysis shown in Table 2.

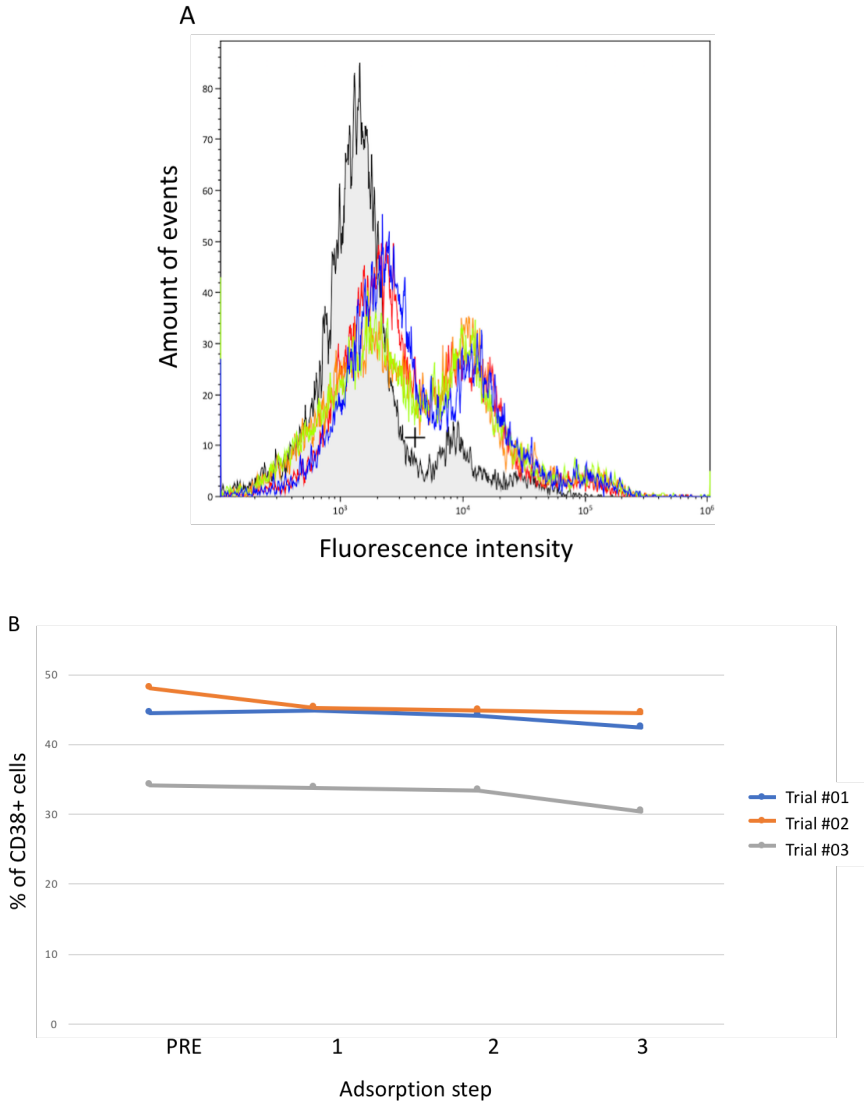


Figure 11. A representative result of flow cytometry analysis of PBMCs adsorbed patient plasma. (A) Histogram displaying fluorescence intensity results of a binding of DARA spiked plasma to PBMCs: 1. DARA spiked plasma before adsorption step (red peak), 2. after three consecutive adsorption steps (1st step - blue peak; 2nd step - orange peak; 3rd step - green peak) in comparison to corresponding negative control (grey peak). (B) Percentage of CD38+ cells analyzed to control residual DARA in patient plasma.

3.2 Inhibition of DARA induced RBCs agglutination in IAT by DARA-F(ab')$_2$ fragments

DARA-(Fab')$_2$ fragments were prepared by pepsin digestion of DARA antibody. Purification of DARA-(Fab')$_2$ fragments was performed by protein G affinity chromatography. The BCA method was employed to determine the concentration of purified DARA-(Fab')$_2$ fragments. SDS-PAGE and Western blot experiments were performed to determine the purity and molecular weight of the DARA-(Fab')$_2$ fragments generated.

3.2.1 Determination of DARA-F(ab')$_2$ fragments concentration

The BCA assay used for protein quantitation showed that the highest DARA-(Fab')$_2$ fragment concentrations were observed in elution fraction 2 and 3 within a range of 0.540- 0.701 and 0.312 - 0.476 mg/mL respectively (Fig. 12). The lowest concentration was observed in fraction 1 and 4 and was within the range of 0.148 - 0.362 and 0.012 - 0.067 mg/mL respectively (Fig. 12). Therefore, elution fractions 2 (DARA-tube 3.2) and 3 (DARA-tube 3.3) were combined (DARA-tube 3) and used for further experiments.

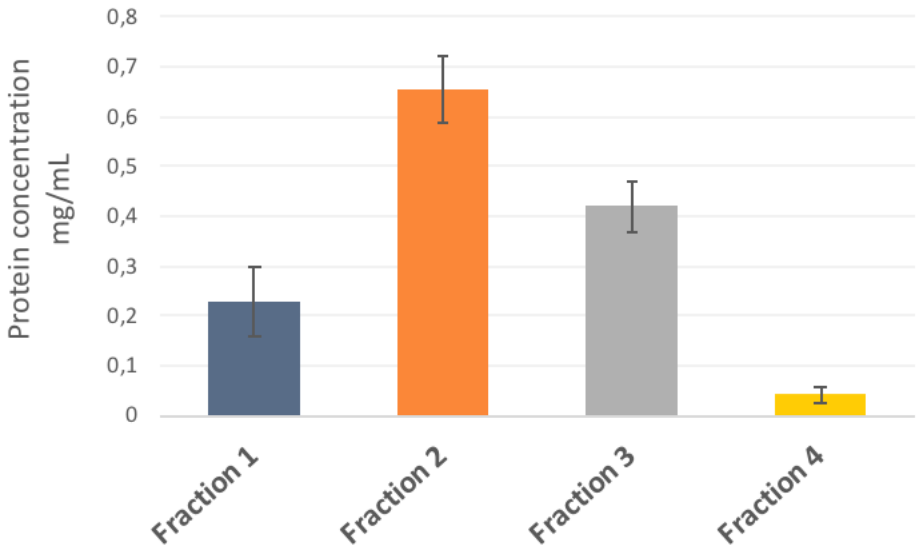
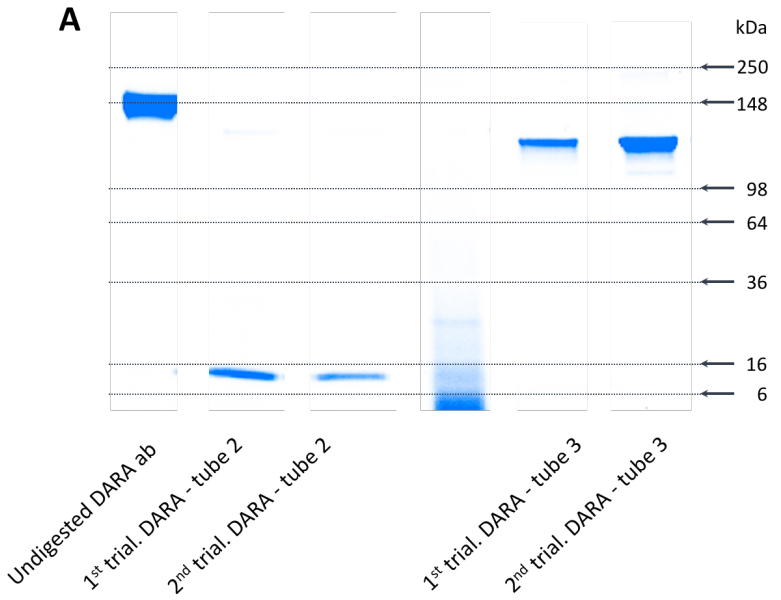
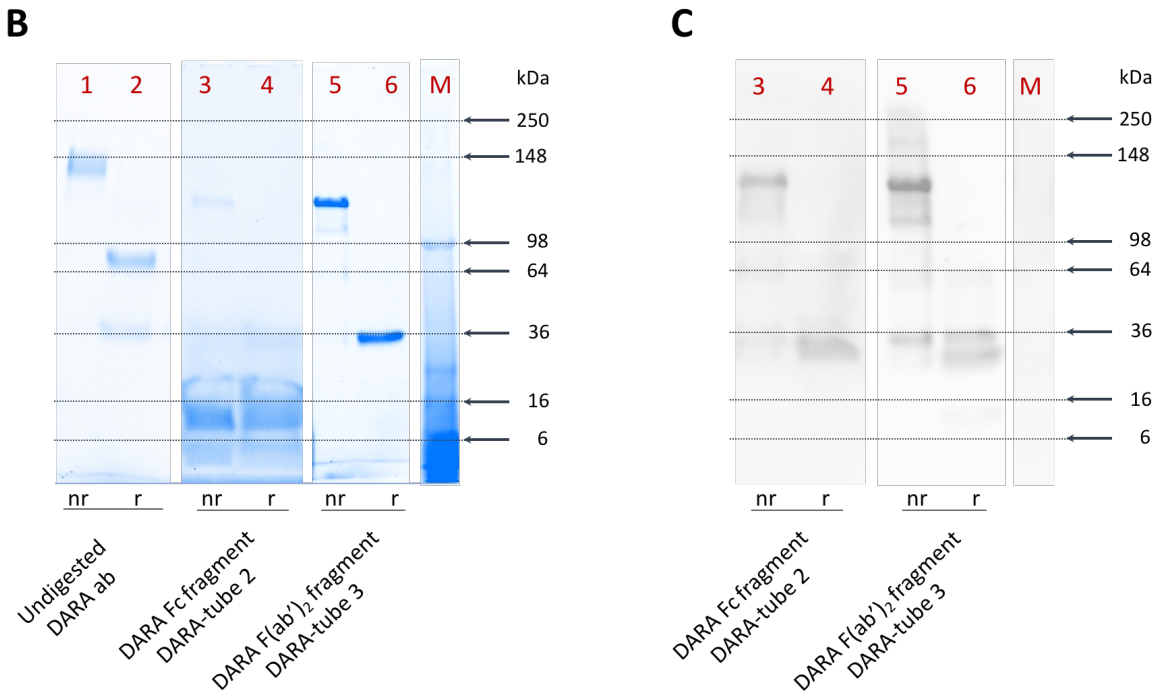


Figure 12. DARA-F(ab')$_2$ fragment concentrations of each elution step of 8 separate purification trials presented in mg/mL, calculated on the basis of a standard protein calibration curve.

3.2.2 Detection of DARA-F(ab')$_2$ fragments by SDS-PAGE and Western Blot

SDS-PAGE and Western blots were performed to confirm the presence of DARA-(Fab')$_2$ fragments. Samples were subjected to electrophoresis under non-reducing and reducing conditions. The relative molecular mass of non-reduced whole DARA antibody was approximately 148 kDa. The flowthrough fraction (DARA-tube 2) and eluted fraction (DARA-tube 3) showed bands at approximately 10 and 110 kDa respectively (Fig. 13A). The band at 110 kDa corresponds to (Fab')$_2$ fragments. Separation of reduced complete DARA antibody showed two bands at approximately 64 and 36 kDa (Figure 13B-2). Analysis of reduced flowthrough fraction (DARA-tube 2) and eluted fraction (DARA-tube 3) showed bands at approximately 10 and 36 kDa respectively (Fig. 13B-4; 13B-6). This indicates that the DARA-(Fab')$_2$ fragments appeared in the elution fraction (DARA-tube 3) instead of in flowthough fraction (Fig.13). Furthermore, DARA-(Fab')$_2$ fragments in elution fraction (DARA-tube 3) were detected by goat anti-human IgG Fab specific antibody at around 110 kDa (Fig. 13C). This showed that DARA-(Fab')$_2$ bounded to Protein G.





nr – non-reducing conditions; r – reducing conditions

Figure 13. Pepsin digested DARA antibody analysis using SDS-PAGE (A; B) and Western blot (C). SDS-PAGE was carried out under non-reducing (A; B Lanes: 1, 3, 5) and reducing (B Lanes 2, 4, 6) conditions. Panel A shows two trials of digestion of DARA antibody and separation of $(Fab')_2$ fragments from digested Fc fragments. Bands from DARA-tube 2 and DARA-tube 3 appeared around 10 and 110 kDa respectively. This corresponds to presence of digested Fc fragments in DARA-tube 2 and $(Fab')_2$ fragments in DARA-tube 3. Lanes 1 and 2 in B show non-reduced and reduced whole DARA antibody. Lanes 3 and 4 show non-reduced and reduced Fc fragments of DARA and lane 5 and 6 show DARA-$F(ab')_2$ fragments. Panel C shows Western blot analysis performed using unconjugated primary goat anti-human IgG Fab specific and HRP conjugated secondary antibody donkey anti-goat IgG-HRP. Molecular mass marker (M) is indicated in 10^3 Daltons (kDa).

3.2.3 Detection of DARA-$F(ab')_2$ fragments binding to RBCs

To confirm that DARA-$(Fab')_2$ fragments bind to RBCs in the same way as the nondigested DARA antibody, both were analyzed by flow cytometry. CD38 fluorescence signal was similar for both nondigested antibody and DARA-$(Fab')_2$ fragments (MFI 2.26 for and MFI 2.20 for DARA and DARA-$(Fab')_2$ fragments respectively). Both species showed 1.8-fold increase in fluorescence signal compared to the negative control (Fig. 14). Moreover, the signal was similar to MFI of CD38 expression on RBCs (Fig. 10B).

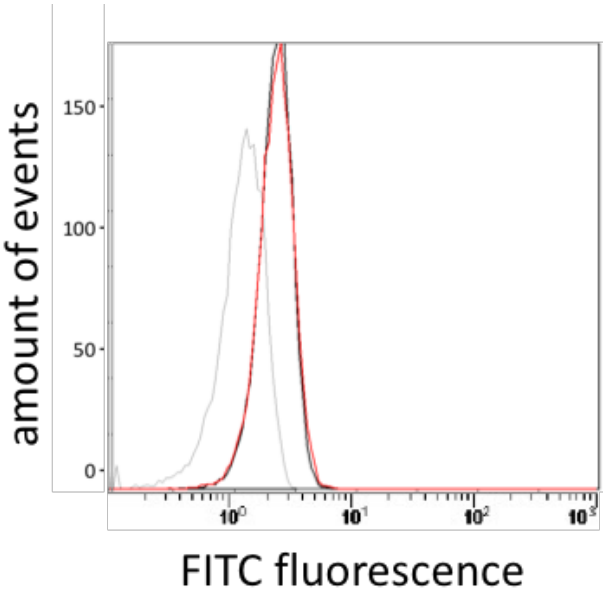


Figure 14. Representative fluorescence histogram of DARA (red peak) and DARA-F(ab')$_2$ fragments (DARA-tube 3) (black peak) binding to RBCs in comparison to human IgG negative control (grey peak) n=10.

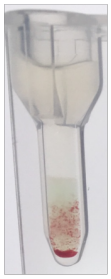
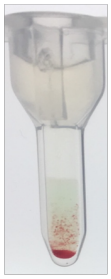
3.2.4 IAT results after blocking DARA binding to RBCs by DARA-F(ab')$_2$ fragments

A series of IAT experiments were carried out using DARA-spiked plasma (max. 0.5 mg/mL) containing previously identified specific RBC alloantibody (single or combined specificities as presented in Table 1).

3.2.4.1 Gel card technique

Plasma samples (n=35) spiked with daratumumab (0.5 mg/mL) were investigated with the gel card technique as a crossmatch experiment using antigen positive and negative RBCs [42]. Experiments with daratumumab containing plasma showed that addition of DARA-F(ab')$_2$ fragments in concentration below 0.25 mg/mL reduced the extent of agglutination induced by 0.5 mg/mL of daratumumab, but complete inhibition was not observed. Therefore, DARA-F(ab')$_2$ fragments at a concentration of 0.5 mg/mL were used for IAT to overcome daratumumab interference (Table 3).

Table 3 Representative results of IAT in the presence of daratumumab (0.5 mg/mL) and in presence or absence of DARA-F(ab')$_2$ fragments using commercially available antibody screening RBCs and plasma without RBC antibodies.

	Plasma w/o RBC antibodies						
Daratumumab [0.5 mg/mL]	+	+	+	+	+	+	+
DARA-F(ab`)$_2$ fragments [mg/mL]	0	0.01	0.05	0.10	0.15	0.25	0.5
Results:	 **3+**	 **3+**	 **2+**	 **0.5+**	 **0.5+**	 **NEG**	 **NEG**

IAT gel card technique performed using RBC antibodies containing plasma, which were titrated to the lowest detectable concentration, showed that incubation of DARA-F(ab')$_2$ fragments (0.5 mg/mL) with RBCs did not reduce the agglutination induced by the low titer RBC alloantibody reaction. All tested RBC alloantibody were further detectable with the same sensitivity in IAT gel card technique (Table 4).

In contrast, preincubation of RBCs with DARA-F(ab')$_2$ fragments completely blocked DARA induced RBC agglutination with all tested concentrations up to 0.5 g/mL in plasma (Table 5).

Table 4. Representative results of IAT for testing sensitivity of antibody detection. RBC antibodies containing plasma samples were titrated to the lowest concentration detectable by gel card technique. Tests were performed in the presence (+) and absence (-) of DARA-F(ab´)$_2$ fragments 0.5 mg/mL.

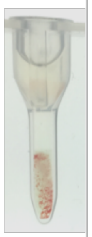
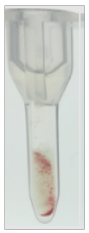
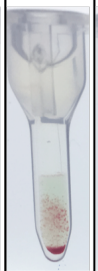
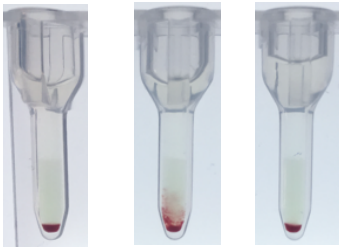
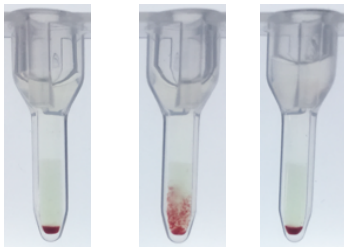
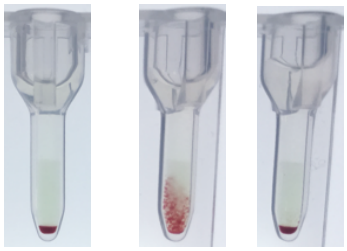
RBC ab present in plasma	**Anti - D**								**Anti - C**							
RBC phenotype	0+ CcD.Ee K+								0+ CcD.Ee K+							
Dilution factor	1:4	1:4	1:16	1:16	1:32	1:32	1:40	1:40	1:2	1:2	1:5	1:5	1:8	1:8	1:10	1:10
DARA-F(ab`)$_2$ fragments	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Results:																
	2+	2+	1+	1+	0.5+	0.5+	NEG	NEG	2+	2+	1+	1+	0.5+	0.5+	NEG	NEG

Table 5. Results of IAT gel card technique using RBC alloantibody containing plasmas in the presence (+) and absence (-) of DARA and DARA-F(ab´)$_2$ fragments 0.5 mg/mL. Plasmas were spiked with DARA (0.5 mg/mL) and tested. As representative examples, four different plasmas containing anti-D, anti-K, anti-E, or anti-Fya are shown. A solid red pellet at the bottom of the tube indicates a negative result (NEG), whereas suspended particles (red cell agglutinates) within the gel matrix indicate a positive test result (1+, 2+) [42].

	Serum without anti-RBC abs			**Anti-D**			**Anti-K**			**Anti-E**			**Anti-Fy^a**		
RBC phenotype				D.	D.	dd	Kk	Kk	kk	Ee	Ee	ee	Fy^a+	Fy^a+	Fy^a-
DARA (0.5 mg/mL)	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
DARA-F(ab')$_2$ fragments (0.5 mg/mL)	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+
Result of IAT															
	NEG	2+	NEG	2+	2+	NEG	1+	1+	NEG	1+	2+	NEG	1+	1+	NEG

Plasma of all randomly selected patients (n=8) with multiple myeloma before DARA infusion showed negative results in the antibody screening test (Table 6A). After DARA infusion positive results (1+ to 3+ agglutination strength) were observed (Table 6B). DARA induced agglutination in the antibody screening test was completely inhibited by a 5 min preincubation of the RBCs with DARA-F(ab')$_2$ fragments (0.5 mg/mL) (Table 6C).

Table 6. Representative results of IAT in the presence (+) and absence (-) of DARA-F(ab')$_2$ fragments (0.5 mg/mL) using commercially available antibody screening RBCs and plasma from a patient with multiple myeloma: (A) patient plasma before the first DARA infusion, (B) plasma tested after DARA infusion, (C) plasma after DARA infusion tested after 5 min pre-incubation of RBCs with DARA-F(ab')$_2$ fragments.

Plasma of a multiple myeloma patient	**A**	**B**	**C**
	before DARA infusion	after DARA infusion	
F(ab')$_2$ fragment:	**-**	**-**	**+**
Test RBC I			
Result	**NEG**	**1+**	**NEG**
Test RBC II			
Result	**NEG**	**2+**	**NEG**
Test RBC III			
Result	**NEG**	**2+**	**NEG**

3.2.4.2 Solid phase technique

22 plasma samples (specificities shown in Table 1) were spiked with DARA and were investigated by the solid phase technique as a crossmatch experiment using antigen positive and negative RBCs (Table 7, Table 8). After pre-incubation of the RBC monolayer with DARA-F(ab')$_2$ fragments (DARA-tube 3), DARA spiked RBC-antibody negative plasma further induced a weak positive reaction (Table 7C). A second dose of DARA-F(ab')$_2$ fragments was therefore added in parallel to the indicator-RBCs. This blocked the DARA interference, and resulted in a negative crossmatch (Table 7D). Contact time of indicator-RBCs with DARA-F(ab')$_2$ fragments affected the IAT result. Starting centrifugation more than 30 s after the parallel addition of DARA-F(ab')$_2$ fragments and indicator-RBCs resulted in false negative results (Table 8). Using this modification, specific RBC alloantibody could be detected with the same sensitivity as when using the native strips (Table 9).

Table 7. Representative result of IAT solid phase technique in presence (+) and absence (-) of DARA and DARA-F(ab')$_2$ fragments (DARA-tube 3). The incubation of DARA-F(ab')$_2$ fragments only with RBC layer or only with the indicator-RBCs did not block completely the interference of DARA. DARA-F(ab')$_2$ fragments have to be added to RBCs layer as well as to the indicator-RBCs.

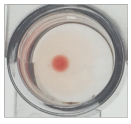
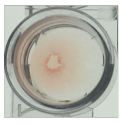
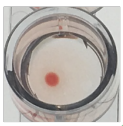
	A Negative Control	**B** Positive Control	**C**	**D**	**E**
Plasma spiked with DARA [0.5 mg/mL]	-	+	+	+	+
RBC monolayer + DARA-F(ab')$_2$ preincubated 20 min	-	-	+	+	-
Indicator-RBC + DARA-F(ab')$_2$ (10 sec)	-	-	-	+	+
Results:	 NEG	 4+	 1+	 NEG ✓	 4+

Table 8. Representative result of IAT the solid phase assay using RBC alloantibody (anti-D) testing several incubation times (0 to 5 min) of indicator-RBCs with DARA-F(ab')$_2$ fragments. (A) negative control in the absence of anti-D, (B) positive control in presence of anti-D. The delay of starting centrifugation after addition of DARA-F(ab')$_2$ fragments and indicator-RBCs (C, D, E) leads to false negative results.

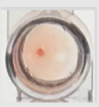
	CONTROLS		INCUBATION TIME of Indicator RBCs			
			5 min	2.5 min	1 min	NON
	A	B	C	D	E	F
Plasma with anti-D	-	+	+	+	+	+
RBC monolayer + DARA-F(ab')$_2$ preincubated 20 min	-	-	+	+	+	+
Indicator-RBC + DARA-F(ab')$_2$	-	-	+	+	+	+
Results:	 NEG	 4+	 NEG	 1+	 2+	 4+ ✓

Table 9. Results of IAT solid phase technique using RBC alloantibody containing plasmas in the presence (+) and absence (-) of DARA and DARA-F(ab')$_2$ fragments. For illustration, four different sera containing ant-Fy^a, anti-e, or anti-Jk^a, are shown. A red spot in the middle of reaction well indicates a negative result (NEG), whereas particles spread across the surface of a reaction well indicate a positive test result (1+ to 4+)

	Plasma without anti-RBC abs			Anti-Fy^a			Anti-e			Anti-Jk^a		
RBC phenotype				Fy^a+	Fy^a+	Fy^a-	ee	ee	EE	Jk^a +	Jk^a +	Jk^a -
DARA [0.5 mg/mL]	-	+	+	-	+	+	-	+	+	-	+	+
RBC monolayer + DARA-F(ab')$_2$ fr.	-	-	+	-	+	+	-	+	+	-	+	+
Indicator-RBCs + DARA-F(ab')$_2$ fr.	-	-	+	-	+	+	-	+	+	-	+	+
Results:	 NEG	 1+	 NEG	 4+	 4+	 NEG	 4+	 4+	 NEG	 4+	 4+	 NEG

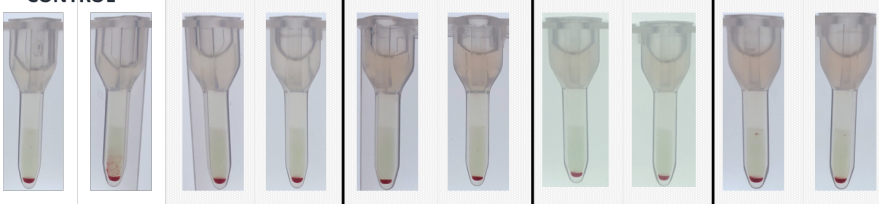
3.3 Stability testing of DARA-F(ab')$_2$ fragments

Results of IAT gel card assays showed that DARA-F(ab')$_2$ fragments can be stored at 4°C, -20°C and -80°C for 180 days without losing their functionality. DARA-F(ab')$_2$ fragments maintain their function of preventing DARA induced RBC agglutinations (up to 0.5 mg/mL in plasma) (Table 10). Freezing and thawing did not influence the IAT results.

DARA-F(ab')$_2$ fragments were stored for up to 360 days at -80°C, and used by the transfusion diagnostic laboratory (Greifswald, Transfusionmedizin). IAT results generated in the transfusion laboratory showed that long storage of DARA-F(ab')$_2$ fragments did not reduce their effectiveness or sensitivity in detecting RBC alloantibody

(tested for anti-D, anti-Fy^a).

Table 10. Representative result of IAT using DARA-F(ab')$_2$ fragments stored under different conditions. IAT was performed with and without spiking with DARA and in the presence (+) and absence (-) of DARA-F(ab')$_2$ fragments. For illustration if DARA-F(ab')$_2$ fragments is able to overcome DARA interference after storage at - 20°C and - 80°C for up to 180 days, IAT were performed using plasma without RBC alloantibody.

	THAWING AFTER:									
Storage time of of DARA F(ab`)$_2$ fragments	**Day 1**				**Day 30**		**Day 90**		**Day 180**	
Storage temp. of DARA F(ab`)$_2$ fragments [°C]			**- 20**	**- 80**	**- 20**	**- 80**	**- 20**	**- 80**	**- 20**	**- 80**
Plasma spiked with DARA [0.5 mg/mL]	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
DARA-F(ab`)$_2$ fragments	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Results:	**CONTROL*** 									
	NEG	2+	**NEG**	**NEG**	**NEG**	**NEG**	**NEG**	**NEG**	**NEG**	**NEG**

* Controls performed in each experiment series

4. Discussion

This work describes a robust and easy to apply method for overcoming the interference of daratumumab in pre-transfusion antibody screens and in red cell compatibility testing in daratumumab treated patients.

DARA is the first FDA-approved CD38 monoclonal antibody used to treat relapsed and treatment-refractory multiple myeloma, and it is currently approved as part of a front-line therapy [6, 7]. The anti-CD38 monoclonal Isatuximab (Sarclisa) has also been approved for the treatment of multiple myeloma in different combinations with other agents since May 2020 and April 2021. Other CD38 antibodies such as MOR202 are currently being investigated in clinical trials [20, 43]. As a result, the number of patients treated with these agents will increase in the near future. The target antigen CD38 is strongly expressed on multiple myeloma cells, but it is also present on several other human cell types, such as RBC [5, 44]. Thus, the plasma of DARA-treated patients yields false positive results in antibody screens, and pan-reactivity on blood compatibility testing. The DARA-induced agglutination occurs in all IAT tests, independent of the method, including column agglutination technology, tube or solid phase assays [5]. In addition, DARA interference may persist for up to 6 months after DARA treatment is discontinued [45]. This finding is clinically relevant as multiple myeloma patients undergoing treatment with DARA may require blood transfusions (38%, n=40, in the SIRIUS trial). It is important to note that the requirement for these transfusions is not directly related to the small Hb decrease caused by DARA-induced hemolysis, but rather to the underlying hematologic malignancy or to an unrelated condition [3, 4]. Therefore, accurate detection of red cell specific alloantibody in pre-transfusional blood compatibility testing is imperative to prevent hemolytic transfusion reactions.

In search of a method to quench the DARA interference in IAT this study investigated two approaches: adsorbing free DARA by immobilized CD38, and blocking the binding site of DARA on RBCs.

Plasma samples spiked with daratumumab at a concentration of up to 0.5 mg/mL were used to mimic the concentration of daratumumab present in the plasma of patients treated with this antibody. From pharmacokinetic data a dose regimen of daratumumab (16 mg/kg weekly for 8 weeks, every 2 weeks for 16 weeks, and every 4 weeks thereafter) is recommended, so as to rapidly saturate target-mediated clearance and maintain a therapeutic concentration.

Daratumumab serum concentrations in groups being administered 16 mg of daratumumab/kg of body weight was 0.575 mg/mL at the end of the weekly treatment applications [46].

Adsorbing free DARA from patient plasma by CD38 immobilized on blood cells was looked at because RBC, PLT as well as PMBC are easily available in blood donation facilities. Furthermore, PBMCs are spin-off products of whole blood donation. Therefore, the feasibility of using these cells as an adsorbing matrix was examined.

To analyze the capacity of cell surfaces to adsorb free DARA from patient plasma flow cytometry experiments were performed with RBCs, PBMCs and PLT. On all cell types CD38 expression could be detected by a slight shift of the fluorescence peak compared to the corresponding negative control, and the highest signal was detected on PBMCs. These results showed that PBMCs could be taken into consideration for removal of DARA by adsorption from patient plasma. Importantly in this consideration, PBMCs do not express the RBC specific antigens. Therefore, red cell specific antibodies will not be adsorbed by PBMC and can be further identified by IAT.

DARA spiked plasma (0.05 mg/mL) was therefore incubated with PBMCs to remove free daratumumab from patient plasma (three consecutive adsorption steps with ~6 x 10^8 PBMCs /step were performed). Agglutination strength did become weaker after each step, but it could not be completely inhibited. According to flow cytometry results, surface expression of CD38 on PBMCs appeared to be relatively low, and this could therefore explain the result. In addition, each adsorption step notably reduced the plasma volume. In consequence, performing more than three steps seems not to be feasible with a patient plasma sample in routine diagnostics because of the limited sample volume available. In vivo the 0.5 mg/ml concentration in patient plasma is not depleted by adsorption to the patient's PBMC, suggesting that peripheral mononuclear cells may not be a useful approach for adsorption of DARA in the therapeutic range.

This is comparable with the results of Chapuy et al. who tried to neutralize free DARA in plasma by adding soluble CD38 or mouse anti-DARA idiotype. This method was effective in DARA neutralization, however large quantities of soluble CD38 or anti-DARA idiotype were needed to treat clinical samples from DARA-treated patients. In the meanwhile, researchers from Grifols company have presented successful results of a neutralization method using highly concentrated recombinant CD38 [47]. Nevertheless, their novel sCD38 is still not commercially

available. Furthermore, experiments with transduced CD38+ HL60 cells as adsorbing cells demonstrated detectable removal of daratumumab from spiked plasma only when high numbers of adsorbing cells were used [26]. This is what we saw when neutralizing free DARA with PBMCs. These large quantities of neutralizing agents are needed in a small sample volume, which is difficult to achieve, and large volumes added into the patient sample can reduce the sensitivity and can therefore thwart detection of RBC alloantibody.

Recently, Ehrend E. et al. presented nonhuman cells "Darasorb cells" which express 5000fold more human CD38 than RBC. Using these cells one adsorption step with a very low plasma volume was sufficient to deplete free Dara from the patient plasma, whereas other antibodies remained unaffected [48]. Nevertheless, this product is not yet commercially available.

The second approach presented in this work was to block the binding side of DARA on RBC. This has been performed by incubation of RBC with DARA F(ab')$_2$ fragments which were generated by pepsin proteolysis of DARA antibodies. After digestion of DARA, the DARA F(ab')$_2$ fragments were to be separated from undigested DARA and the Fc fragments by affinity chromatography using immobilized Protein G. SDS-PAGE and Western blot analysis showed that the DARA F(ab')$_2$ fragments were in fact retained on the column, rather than being in the flowthrough. This was somewhat unexpected, though protein G is known to be able to bind to both the Fab and the Fc portions of the antibody molecule [49]. The constant domains of Fab and Fc are structurally related but are recognized by protein G in quite different ways [49, 50]. Full Fc fragments of IgG (~50 kDa) are also expected to bind to protein G, but pepsin digests Fc fragments to small fragments (~10 kDa), which probably cannot bind any longer. The most important result of this analysis was that after pepsin digestion there was no detectable intact DARA left. Flow cytometry analysis confirmed that DARA F(ab')$_2$ fragments bound to RBCs as well as the whole DARA antibody.

In parallel other groups worked with the same idea. Thus, a patent [51] was approved shortly after our results were published [42] and a product named DaraEx (Invitrogen) [37] is now commercially available. Furthermore, Werle et al. also showed that Fab fragments of DARA are sufficient to overcome the interference in the IAT [25]. The authors suggested that two Fab fragments mask more CD38 antigens than one F(ab')$_2$ fragment. If this is true, the preparation of Fab fragments from the same amount of DARA should be usable for more tests than the preparation of F(ab´)$_2$ fragment, which would probably be more cost effective.

The blocking capacity of DARA induced RBCs agglutination by DARA F(ab')$_2$ fragments was tested in IAT assays using gel card and solid phase assay. Both methods are available for fully automated laboratory diagnostics. We therefore tested the conditions and limits for an introduction of our principle into automation. 35 plasma samples containing RBC alloantibody spiked with DARA in increasing concentrations up to 0.5 mg/mL were investigated including a broad spectrum of RBC alloantibody specificities (Anti-D, -E, -e, -K, -Fy^a, -Lu^a, -Jk^a, -C, -c, -S and -M). Addition of DARA F(ab')$_2$ fragments directly into the gel card, without preincubation of test RBCs separately - as is necessary for DaraEx [52] - did not decrease the agglutination strength of low titer RBC alloantibody reaction. The sensitivity of detection of RBC alloantibody remained unchanged and DARA F(ab')$_2$ fragments completely blocked DARA induced RBC agglutination up to the clinically relevant concentration of 0.5 mg/ml in the IAT gel card assay. Moreover, in a recent publication F.F. Wagner stated that when using DaraEx under real-world conditions, the effect of overcoming DARA interference was sometimes incomplete [53]. This may indicate that the concentration of Dara F(ab´)$_2$ fragments as well as the DARA concentration in the patient plasma play a role for blocking efficacy.

22 plasma samples (Table 1) spiked with DARA were investigated by solid phase assay. When freshly prepared RBC monolayer wells were pre-incubated with DARA F(ab')$_2$ fragments, DARA spiked RBC antibody negative plasma still induced a weak positive reaction. Based on this observation and the fact that the reaction wells coated with RBC had been washed after incubation with DARA-F(ab')$_2$ fragments I hypothesized that the indicator RBC which also express CD38 were linked to the red cell layer by free F(ab)$_2$ fragment binding sites.

Therefore, together with the indicator RBCs a new dose of DARA F(ab')$_2$ fragments was added to block CD38 expressed on the indicator cells. Thereafter alloantibody could again be specifically detected with the same sensitivity as before (Table 4, Table 5). An important observation was that a delay of more than 30s in centrifugation after addition of DARA F(ab')$_2$ fragments to the indicator RBCs in the reaction well could lead to false negative results. This shows that standardized conditions, such as incubation times and simultaneous pipetting, are absolutely necessary to maintain reproducible performance and valid results. This may be achieved by using only standardized automation processes for the solid phase IAT with DARA F(ab')$_2$ fragments.

Because of the limitation with the solid phase IAT, the DARA F(ab')$_2$ gel card method was established for the routine laboratory. Plasma samples from randomly selected patients with multiple myeloma were investigated, both before and after DARA infusion therapy (n=8). Before DARA infusion, plasma of all patients showed negative IAT results. After DARA infusion positive results (1+ to 2+ agglutination strength) were observed. DARA induced agglutination in the antibody screening test was completely inhibited by a 5 min pre-incubation of the RBC with DARA F(ab')$_2$ fragments. Prevention of RBC agglutination by DARA F(ab')$_2$ fragments shows that DARA F(ab')$_2$ fragments do not lead to RBC agglutination by interaction with AHG in the gel card method. Unfortunately, there were no RBC allo-immunized patients undergoing DARA treatment.

Stability testing of DARA-F(ab')$_2$ fragments is also an important issue, because up to now DARA treated patients requiring transfusion are relatively rare. Aliquots of DARA-F(ab')$_2$ fragments were successfully stored at -20°C for up to 180 days and at -80°C for up to 360 days without losing their functionality. Freezing and thawing did not adversely affect the IAT results and allowed for further detection of RBC alloantibody.

As the interference of CD38 antibody therapy has created a unique situation in immunohematology labs, several other laboratory methods to negate the DARA effect on RBCs have been investigated, as already described in this work.

The first useful method to overcome IAT interference of DARA was the treatment of RBCs with dithiothreitol (DTT). This has been internationally validated and became the most widely used method in routine laboratories [26, 28] DTT reduces the disulfide bonds of CD38 so that CD38 antibodies are unable to bind this antigen on RBCs. The major drawback of this method is that disulfide bonds of other clinically relevant RBC antigens e.g. KEL, Cartwright (Yt^a), John Milton Hagen (JMH), Knops^a (Kn^a, McC^a, and Yk^a), Landsteiner-Wiener^a (LW^a), Lutheran, Dombrock, and Cromer are also disrupted [26, 29, 30]. Matched transfusions for Kell antigens are already recommended for DARA treated patients, if the antibody screening test is performed with DTT treated test-RBCs. However, this does not provide a complete solution of the problem of the destruction of Yt^a, JMH, Knops^a, LW^a, Lutheran, Dombrock, and Cromer antigens by the DTT method [23, 30, 31]. Hence, antibodies against antigens altered by DTT cannot be detected by this method. Moreover, Selleng et al. demonstrated unspecific agglutination after DTT treatment which did not reflect the presence of specific anti red cell antigen antibodies [54].

This was observed in 40% of plasma by gel card analysis and in 7% using the solid phase technique. Furthermore, DTT treatment of red cells in the solid phase assay reduced or even abolished the sensitivity for anti-Fy^a antibodies [54]. Incomplete inhibition of DARA binding, or hemolysis of DTT treated red cells after a few days of storage, is also often observed and causes time consuming laboratory work with a potentially critical delay of RBC transfusion [32].

Modification of the DTT method by reducing the DTT concentration to 0.04M is a potential solution for the disadvantages observed with 0.2M DTT [33, 34]. However, the disadvantages of the DTT method could be easily overcome by using $F(ab)_2$ fragments. DTT is easy to prepare and cost effective. It could be a useful strategy to start diagnostics with DTT treated red cells. If residual agglutinations were to be observed, more cost intensive $F(ab´)_2$ fragments could be used as a confirming test.

In conclusion, work presented here was developed to solve a clinically relevant risk in transfusion therapy with red blood cells caused by therapeutic monoclonal antibodies which interfere with pretransfusion compatibility testing. For this, different approaches were tested. The interference could be resolved by addition of DARA $F(ab')_2$ fragments to gel card and – with some considerations – to solid phase IAT. This again allows the detection and identification of specific RBC alloantibody by IAT and re-establishes transfusion safety for DARA treated patients.

The use of monoclonal antibodies directed to molecules expressed on tumor cells is an expanding area of drug development. The transfusion challenges that arise with daratumumab can be expected to occur also with other similar agents e.g. Hu5F9-G4 anti CD47 agent [55]. Indeed, a recent published report showed that anti-CD47 causes interference in both AB0, Rhesus D and antibody screening tests [31]. Moreover, the CD47 is not cleaved from test RBCs by treatment with papain, ficin, trypsin, alfa-chymotrypsin, 0.2 M DTT or W.A.R.M. reagent so that interference cannot be mitigated using these methods [31, 56].

A statement by transfusion specialists has highlighted the transfusion safety problem associated with the potential interference of recently introduced therapeutic monoclonal antibodies with in vitro pre-transfusion compatibility testing. It may well be that the use of $F(ab')_2$ fragments of any monoclonal antibody can prevent interference caused by therapeutic

monoclonal antibodies that recognize antigens expressed on RBCs and will turn out to be an important and novel general approach.

5. Summary

Antibodies directed against the CD38 antigen like daratumumab (DARA), were developed for the treatment of multiple myeloma. Unfortunately, these antibodies interfere with pretransfusional laboratory testing.

Red blood cells (RBCs) as well as multiple myeloma cells express CD38, which leads to panreactive results in the indirect antiglobulin test (IAT) if serum or plasma from DARA-treated patients is screened for alloantibody against RBCs. As a result, specific RBC alloantibody cannot reliably be identified before red cell transfusion, and this increases the risk for hemolytic transfusion reactions. This important safety issue for RBC transfusions led us to search for a method for patients´ diagnostics, which is easy to perform and does not cause transfusion delay. To overcome interference of DARA with pretransfusion compatibility testing I have investigated two approaches: i) DARA adsorption from patient plasma using CD38 expressing cells, ii) blocking the binding site of DARA on RBCs without influencing the binding of transfusion relevant RBC alloantibody.

For the first approach, I have studied peripheral blood cells, which are easily available from a blood donation center, for their CD38 surface expression. PBMCs were shown to have the highest expression level and were tested as adsorption matrix to deplete free DARA from DARA-spiked plasma. After three consecutive adsorption steps agglutination strength in the IAT became weaker but could not be completely inhibited. Considering the conditions of a routine immunohematology laboratory this approach was not feasible for patients´ diagnostics because of limited patient plasma volume and because of the delay to transfusion caused by the time-consuming adsorption steps.

For the second approach I generated $F(ab')_2$ fragments of DARA using pepsin digestion. Experiments using DARA-spiked plasma with and without red cell alloantibody and plasma from randomly selected patients with multiple myeloma before and after DARA infusion were performed in presence and absence of DARA $F(ab')_2$ fragments in the IAT. Preincubation of RBCs with DARA $F(ab')_2$ fragments prevented DARA induced RBC agglutinations up to DARA concentrations found in patients´ plasma. I tested the method for two IAT methods widely used in routine immunohematology laboratories (microcolumn gel card and Capture®). I showed that the sensitivity to detect RBC alloantibody using the gel card and Capture® method was not influenced by addition of DARA $F(ab')_2$ fragments.

The use of $F(ab')_2$ fragments is a promising tool to resolve the interference of daratumumab with blood compatibility testing in the IAT. This approach may be more generally applicable to other therapeutic antibodies interfering with blood compatibility testing.

Zusammenfassung

Therapeutische Antikörper können unerwartete Wirkungen verursachen, wenn das Zielantigen nicht nur auf den Zielzellen exprimiert wird. Ein gegen das CD38-Antigen gerichteter Antikörper, Daratumumab (DARA), wurde für die Behandlung des multiplen Myeloms entwickelt. Allerdings beeinträchtigt dieser Antikörper erheblich die Verträglichkeitsuntersuchungen zwischen Blutkonserve und Patientenplasma vor der Transfusion von Erythrozytenkonzentraten.

CD38 wird auch auf Erythrozyten (RBCs) exprimiert. Durch die Bindung von DARA an die Spendererythrozyten wird im indirekten Antihumanglobulintest (IAT) eine in vitro Unverträglichkeit mit allen Testerythrozyten angezeigt. Dies wird dadurch verursacht, dass das erforderliche Antihumanglubulin (AHG) humanes IgG bindet, unabhängig davon, welches Zielantigen dieser Antikörper hat. Infolgedessen können Agglutinationen durch transfusionsrelevante Antikörper im Patientenplasma von DARA-induziereten Agglutinationen nicht unterschieden werden, wodurch das Risiko für akute hämolytische Transfusionsreaktionen steigt.

Daraus ergab sich die Fragestellung für meine Arbeit – eine Modifikation für den IAT zu finden, der diese Interferenz auflöst. Ich habe zwei neue Strategien verfolgt: i) die Adsorption von DARA aus dem Patientenplasma mit CD38-exprimierenden peripheren Blutzellen, ii) die Blockung der DARA-Bindungsstelle auf Erythrozyten, ohne dass die Bindung von transfusionsrelevanten erythrozytären Antikörpern behindert wird.

Für den ersten Ansatz konnte ich PBMCs als die Zellen identifizieren, die die höchste CD38 Expression zeigten. Leider konnte die Inkubation von DARA-gespiktem Plasma selbst nach mehreren Adsorptionsschritten die Interferenz im IAT nicht vollständig beseitigen. Auch die Durchführung der Methode erwies sich als nicht praktikabel für ein Routine-Diagnostiklabor.

Für den zweiten Ansatz habe ich mit Hilfe von Pepsin $F(ab')_2$ Fragmente von DARA hergestellt um damit die DARA-Bindungsstelle auf den Erythrozyten zu blockieren, damit das AHG nur an gebundene transfusionsrelevante Antikörper bindet. Die Zugabe von DARA $F(ab')_2$ Fragmenten zu den Testerythrozyten konnte die DARA-induzierten Agglutinationen im IAT verhindern und im Plasma vorhandene erythrozytäre Alloantikörper sichtbar und differenzierbar machen. Weiterhin konnte ich nachweisen, dass die Zugabe von DARA $(Fab')_2$ Fragmenten nicht die Sensitivität der Teste im Gelzentrifugationstest und im Capture® beeinträchtigt. Experimente mit Plasma von Myelom-Patienten vor und nach der DARA-Infusion bestätigten die Ergebnisse. Die Verwendung von $F(ab')_2$ Fragmenten ist ein vielversprechendes Verfahren, um Interferenzen von therapeutischen Antikörpern im IAT der prätransfusionellen Diagnostik aufzulösen - nicht nur für Daratumumab.

6. References

1. Kaplon, H. and J.M. Reichert, *Antibodies to watch in 2019.* MAbs, 2019. **11**(2): p. 219-238.
2. Murphy, M.F., et al., *Interference of New Drugs with Compatibility Testing for Blood Transfusion.* N Engl J Med, 2016. **375**(3): p. 295-6.
3. Lonial, S., et al., *Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial.* Lancet, 2016.
4. Chari, A., et al., *Blood Transfusion Management and Transfusion-Related Outcomes in Daratumumab-Treated Patients With Relapsed or Refractory Multiple Myeloma.* Clin Lymphoma Myeloma Leuk, 2018. **18**(1): p. 44-51.
5. Chapuy, C.I., et al., *Resolving the daratumumab interference with blood compatibility testing.* Transfusion, 2015. **55**(6 Pt 2): p. 1545-54.
6. van de Donk, N.W., et al., *Monoclonal antibodies targeting CD38 in hematological malignancies and beyond.* Immunol Rev, 2016. **270**(1): p. 95-112.
7. Sanchez, L., et al., *Daratumumab: a first-in-class CD38 monoclonal antibody for the treatment of multiple myeloma.* J Hematol Oncol, 2016. **9**(1): p. 51.
8. Morandi, F., et al., *CD38, a Receptor with Multifunctional Activities: From Modulatory Functions on Regulatory Cell Subsets and Extracellular Vesicles, to a Target for Therapeutic Strategies.* Cells, 2019. **8**(12).
9. Liu, Q., et al., *Crystal structure of human CD38 extracellular domain.* Structure, 2005. **13**(9): p. 1331-9.
10. Henriques, A., et al., *CD38, CD81 and BAFFR combined expression by transitional B cells distinguishes active from inactive systemic lupus erythematosus.* Clin Exp Med, 2016. **16**(2): p. 227-32.
11. Matsuoka, T., et al., *Expression of CD38 gene, but not of mitochondrial glycerol-3-phosphate dehydrogenase gene, is impaired in pancreatic islets of GK rats.* Biochem Biophys Res Commun, 1995. **214**(1): p. 239-46.
12. Sun, L., et al., *CD38/ADP-ribosyl cyclase: A new role in the regulation of osteoclastic bone resorption.* J Cell Biol, 1999. **146**(5): p. 1161-72.
13. Overdijk, M.B., et al., *The Therapeutic CD38 Monoclonal Antibody Daratumumab Induces Programmed Cell Death via Fcgamma Receptor-Mediated Cross-Linking.* J Immunol, 2016. **197**(3): p. 807-13.
14. van de Donk, N.W., et al., *Clinical efficacy and management of monoclonal antibodies targeting CD38 and SLAMF7 in multiple myeloma.* Blood, 2016. **127**(6): p. 681-95.
15. De Vooght, K.M., M. Oostendorp, and W.W. van Solinge, *New mAb therapies in multiple myeloma: interference with blood transfusion compatibility testing.* Curr Opin Hematol, 2016. **23**(6): p. 557-562.
16. Nijhof, I.S., et al., *Upregulation of CD38 expression on multiple myeloma cells by all-trans retinoic acid improves the efficacy of daratumumab.* Leukemia, 2015. **29**(10): p. 2039-49.
17. Overdijk, M.B., et al., *Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma.* MAbs, 2015. **7**(2): p. 311-21.

18. Dimopoulos, M.A., et al., *Daratumumab plus lenalidomide and dexamethasone versus lenalidomide and dexamethasone in relapsed or refractory multiple myeloma: updated analysis of POLLUX.* Haematologica, 2018. **103**(12): p. 2088-2096.
19. Palumbo, A., et al., *Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma.* N Engl J Med, 2016. **375**(8): p. 754-66.
20. Strobel, E., *Hemolytic Transfusion Reactions.* Transfus Med Hemother, 2008. **35**(5): p. 346-353.
21. Diagnostic Grifols, S.A., *ANTI-HUMAN GLOBULIN, Instructions for Use*, S.A. Diagnostic Grifols, Editor. 2019.
22. Theis, S.R. and M.F. Hashmi, *Coombs Test*, in *StatPearls*. 2019: Treasure Island (FL).
23. Lancman, G., et al., *Blood Transfusion Management for Patients Treated With Anti-CD38 Monoclonal Antibodies.* Front Immunol, 2018. **9**: p. 2616.
24. Oostendorp, M., et al., *When blood transfusion medicine becomes complicated due to interference by monoclonal antibody therapy.* Transfusion, 2015. **55**(6 Pt 2): p. 1555-62.
25. Werle, E., *Daratumumab Interference in Pretransfusion Testing Is Overcome by Addition of Daratumumab Fab Fragments to Patients Plasma.* 2019.
26. Chapuy, C.I., et al., *International validation of a dithiothreitol (DTT)-based method to resolve the daratumumab interference with blood compatibility testing.* Transfusion, 2016. **56**(12): p. 2964-2972.
27. Nedumcheril, M.T., et al., *Overcoming Drug Interference in Transfusion Testing: A Spotlight on Daratumumab.* J Blood Med, 2021. **12**: p. 327-336.
28. de Vooght, K.M.K., et al., *Vox Sanguinis International Forum on typing and matching strategies in patients on anti-CD38 monoclonal therapy: summary.* Vox Sang, 2018.
29. Reid ME, L.-F.C., Olsson ML., *The Blood Group Antigen FactsBook*. 2021.
30. Dizon, M.F., *The Challenges of Daratumumab in Transfusion Medicine.* Lab Med, 2016.
31. Velliquette, R.W., et al., *Monoclonal anti-CD47 interference in red cell and platelet testing.* Transfusion, 2019. **59**(2): p. 730-737.
32. Lorenze H., A.N.L., Nielsen M, Svendsen L., Andersen P., *Thirty-three-day storage of dithiothreitol-treated red blood cells used to eliminate daratumumab interference in serological testing.* VoxSanguinis, 2018. **12699**.
33. Hosokawa, M., et al., *Distinct effects of daratumumab on indirect and direct antiglobulin tests: a new method employing 0.01 mol/L dithiothreitol for negating the daratumumab interference with preserving K antigenicity (Osaka method).* Transfusion, 2018. **58**(12): p. 3003-3013.
34. Izaguirre, E.C., et al., *New method for overcoming the interference produced by anti-CD38 monoclonal antibodies in compatibility testing.* Blood Transfus, 2020. **18**(4): p. 290-294.
35. Tremblay, T., D.R. Branch, and L. Loubaki, *Daudi cell stroma: An alternative to dithiothreitol to resolve daratumumab interference in pretransfusion testing.* Transfusion, 2020. **60**(9): p. 2090-2096.
36. Schmidt, A.E., et al., *An alternative method to dithiothreitol treatment for antibody screening in patients receiving daratumumab.* Transfusion, 2015. **55**(9): p. 2292-3.
37. IMUSYN. *https://www.imusyn.de/products/daraex/*.
38. Nishida, H. and T. Yamada, *Monoclonal Antibody Therapies in Multiple Myeloma: A Challenge to Develop Novel Targets.* J Oncol, 2019. **2019**: p. 6084012.

39. Dagur, P.K. and J.P. McCoy, Jr., *Collection, Storage, and Preparation of Human Blood Cells.* Curr Protoc Cytom, 2015. **73**: p. 5 1 1-16.
40. György Hegyi, J.K., Mihály Kovács, *Introduction to Practical Biochemistry*, ed. T.-. A1. 2013.
41. Delaflor-Weiss E., C.V., *Implementation of Gel Testing for Antibody Screening and Identification in a Community Hospital, a 3-Year Experience.* Lab Med, 2005.
42. Selleng, K., P.D. Gebicka, and T. Thiele, *F(ab')2 Fragments to Overcome Daratumumab Interference in Transfusion Tests.* N Engl J Med, 2018. **379**(1): p. 90-91.
43. Mateos, M.V., et al., *Daratumumab plus Bortezomib, Melphalan, and Prednisone for Untreated Myeloma.* N Engl J Med, 2018. **378**(6): p. 518-528.
44. Malavasi, F., et al., *Human CD38: a glycoprotein in search of a function.* Immunol Today, 1994. **15**(3): p. 95-7.
45. Janssen, *Understanding Daratumumab Interference With Blood Compatibility Testing*. 2020.
46. Clemens, P.L., et al., *Pharmacokinetics of Daratumumab Following Intravenous Infusion in Relapsed or Refractory Multiple Myeloma After Prior Proteasome Inhibitor and Immunomodulatory Drug Treatment.* Clin Pharmacokinet, 2017. **56**(8): p. 915-924.
47. Binda M., F.V., Piel N., Berry J. D., Schwind P., *Detection of masked irregular antibodies in Anti-CD38 containing plasma after neutralization with a novel recombinant CD38.*, in *Transfusion Medicine and Hemotherapy*. 2018: Luebeck.
48. Ehrend, E., et al., *Preanalytic depletion of medicinal anti-CD38 antibody from patient plasma for immunohematology testing.* Blood, 2021. **138**(9): p. 814-817.
49. G-Biosciences, *F(ab)2 Fragmentation Kit_human_mouse_rabbit*. 2010.
50. Kato, K., et al., *Model for the complex between protein G and an antibody Fc fragment in solution.* Structure, 1995. **3**(1): p. 79-85.
51. Gruger D., S.C., *ANALYSIS OF ANTI-ERYTHROCYTE ANTIBODY IN THE PRESENCE OF ANTIBODY DIRECTED AGAINST A SURFACE-BOUND ERYTHROCYTE ANTIGEN*, in *USPTO*. 2019.
52. Schneeweiß C, G.g.D., Heuft H-G, *DaraEx verhindert Daratumumab-Interferenz im indirekten Antihumanglobulintest.* Transfusionsmedizin, 2017. **7**: p. 229-232.
53. F.F., W., *Antibody testing in patients treated with anti-CD38- there is still room for improvement.* Blood Transfusion, 2020.
54. Selleng, K., et al., *Performance qualification of the pretransfusional antibody screening test of plasma containing Daratumumab (anti-CD 38 antibody) after DTT-treatment of the test cells using gel card and Capture®.* Transfus Med Hemother, 2017. **44 (suppl. 1)**: p. 57.
55. Brierley, C.K., et al., *The effects of monoclonal anti-CD47 on RBCs, compatibility testing, and transfusion requirements in refractory acute myeloid leukemia.* Transfusion, 2019. **59**(7): p. 2248-2254.
56. Perram J., B.B., Ackerman L., Beig A., Khoo L., *Solid phase antibody screening in the presence of anti-CD38 monoclonal antibodies: a potential alternative to avoid interference.* Pathology, 2020.

7. Appendix

Supplementary material

Table S1. Results of IAT gel card method using plasma with increasing DARA concentration.

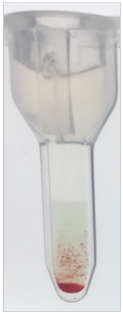
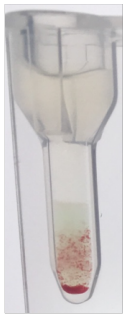
Plasma without anti-RBC abs	+	+	+	+	+	+
DARA concentration [mg/mL]	0	0.01	0.05	0.1	0.25	0.5
Results:	 NEG	 0.5+	 0.5+	 2+	 3+	 3+

Table S2. Results of IAT solid phase method using plasma with increasing DARA concentration.

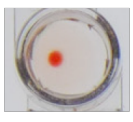
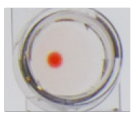
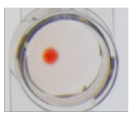
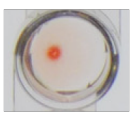
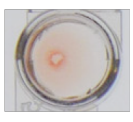
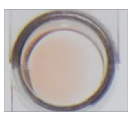
Plasma without anti-RBC abs	+	+	+	+	+	+
DARA concentration [mg/mL]	0	0.01	0.05	0.1	0.25	0.5
Results:	 NEG	 NEG	 NEG	 1+	 2+	 4+

Table S3. Results of IAT gel card method using plasma with increasing DARA-F(ab')$_2$ concentration.

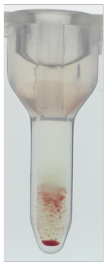
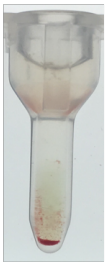
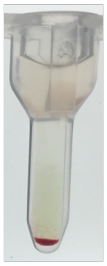
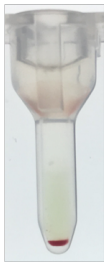
Plasma without anti-RBC abs	+	+	+	+	+	+
DARA [0.5 mg/mL]	+	+	+	+	+	+
DARA-F(ab')$_2$ concentration [mg/mL]	0	0.01	0.05	0.1	0.25	0.5
Results:	 2+	 2+	 2+	 1+	 0.5+	 NEG

Table S4. Results of IAT solid phase method using plasma with increasing DARA-F(ab')$_2$ concentration.

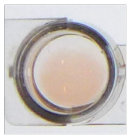
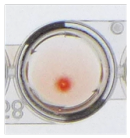
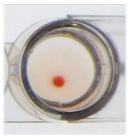
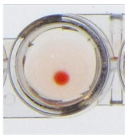
Plasma without anti-RBC abs	+	+	+	+	+	+
DARA [0.5 mg/mL]	+	+	+	+	+	+
DARA-F(ab')$_2$ concentration [mg/mL]	0	0.01	0.05	0.1	0.25	0.5
Results:	 4+	 2+	 2+	 1+	 NEG	 NEG

Table S5 - S14. Results of IAT for testing sensitivity of antibody detection. Different RBC antibodies containing plasma samples were titrated to the lowest concentration detectable by gel card technique. Tests were performed in the presence (+) and absence (-) of DARA-F(ab´)$_2$ fragments 0.5 mg/mL.

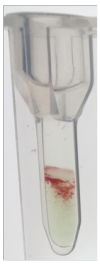
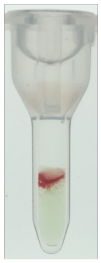
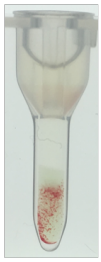
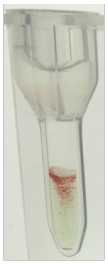
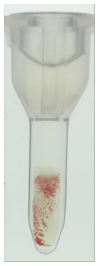
RBC phenotype	0 K+k+/Cc.D.Ee				
Plasma with anti-D abs	+	+	+	+	+
Plasma dilution factor	**0**	**1:8**	**1:16**	**1:24**	**1:32**
DARA [0.5 mg/mL]	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
Results of IAT	 4+	 3+	 2+	 1+	 NEG
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
	 4+	 3+	 3+	 3+	 3+
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+
	 4+	 3+	 2+	 1+	 NEG

Table S6.

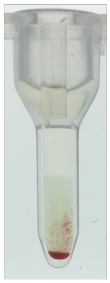
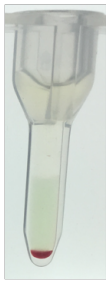
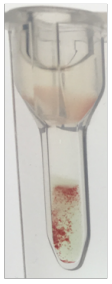
RBC phenotype	0 K+k+/Cc.D.Ee				
Plasma with anti-C abs	+	+	+	+	+
Plasma dilution factor	**0**	**1:4**	**1:6**	**1:8**	**1:12**
DARA [0.5 mg/mL]	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
Results of IAT	 2+	 2+	 1+	 0.5+	 NEG
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
	 3+	 3+	 2+	 2+	 2+
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+
	 2+	 2+	 1+	 0.5+	 NEG

Table S7.

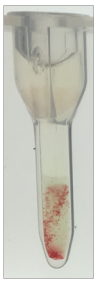
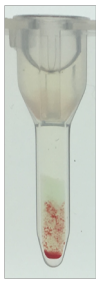
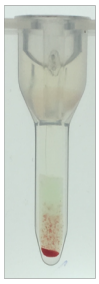
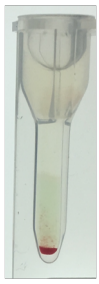
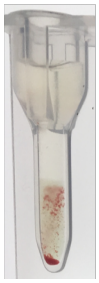
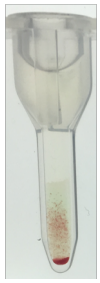
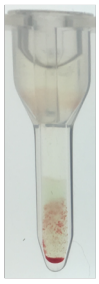
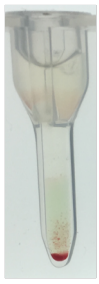
RBC phenotype	0 K+k+/Cc.D.Ee							
Plasma with anti-K abs	+	+	+	+	+			
Plasma dilution factor	**0**	**1:4**	**1:16**	**1:24**	**1:32**	**1:40**	**1:50**	**1:80**
DARA [0.5 mg/mL]	-	-	-	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-	-	-	-
Results of IAT	 3+	 3+	 2+	 2+	 2+	 1+	 1+	 NEG
DARA [0.5 mg/mL]			+	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]			-	-	-	-	-	-
			 2+	 2+	 2+	 2+	 2+	 NEG
DARA [0.5 mg/mL]			+	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]			+	+	+	+	+	+
			 2+	 2+	 2+	 1+	 1+	 NEG

Table S8.

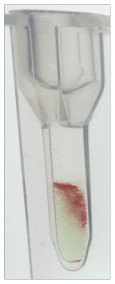
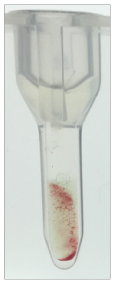
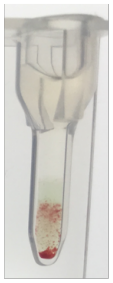
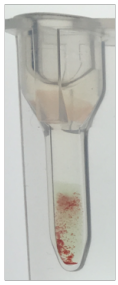
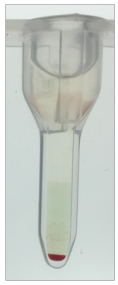
RBC phenotype	RBC Cc.D.Ee				
Plasma with anti-c abs	+	+	+	+	+
Plasma dilution factor	**0**	**1:2**	**1:4**	**1:8**	**1:16**
DARA [0.5 mg/mL]	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
Results of IAT	 3+	 2+	 2+	 0.5+	 NEG
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
	 3+	 3+	 2+	 2+	 2+
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+
	 3+	 2+	 1+	 0.5+	 NEG

Table S9.

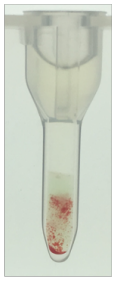
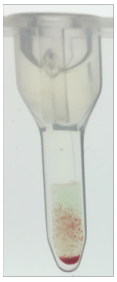
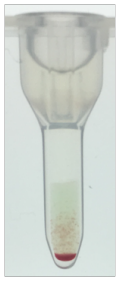
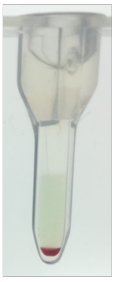
RBC phenotype	0 K+k+/Cc.D.Ee			
Plasma with anti-E abs	+	+	+	+
Plasma dilution factor	**0**	**1:2**	**1:2,5**	**1:3**
DARA [0.5 mg/mL]	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-
Results of IAT	 **3+**	 **1+**	 **1+**	 **NEG**
DARA [0.5 mg/mL]	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-
	 **3+**	 **2+**	 **2+**	 **2+**
DARA [0.5 mg/mL]	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+
	 **3+**	 **2+**	 **1+**	 **NEG**

Table S10.

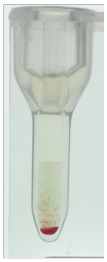
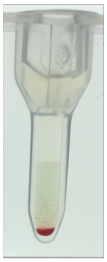
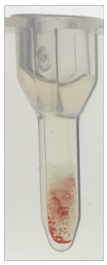
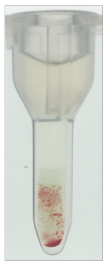
RBC phenotype	0 K-/cc.dd.ee						
Plasma with anti-e abs	+	+	+	+	+		
Plasma dilution factor	**1:4**	**1:8**	**1:16**	**1:24**	**1:32**	**1:40**	**1:50**
DARA [0.5 mg/mL]	-	-	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-	-	-
Results of IAT	 3+	 2+	 2+	 1+	 1+	 0.5+	 NEG
DARA [0.5 mg/mL]		+	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]		-	-	-	-	-	-
		 3+	 2+	 2+	 2+	 2+	 2+
DARA [0.5 mg/mL]	+	+	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+	+	+
	 3+	 2+	 2+	 1+	 1+	 0.5+	 NEG

Table S11.

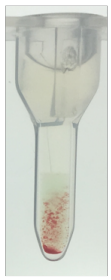
RBC phenotype	0 K-/cc.dd.ee Fy^a+			
Plasma with anti-Fy^a abs	+	+	+	+
Plasma dilution factor	**1:2**	**1:4**	**1:8**	**1:16**
DARA [0.5 mg/mL]	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-
Results of IAT	 2+	 1+	 0.5+	 NEG
DARA [0.5 mg/mL]	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-
	 2+	 2+	 2+	 2+
DARA [0.5 mg/mL]	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+
	 2+	 1+	 0.5+	 NEG

Table S12.

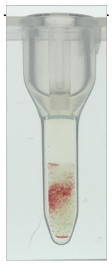
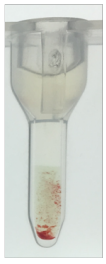
RBC phenotype	0 K-/cc.dd.ee Lu^a+						
Plasma with anti-Lu^a abs	+	+	+	+	+		
Plasma dilution factor	**1:4**	**1:16**	**1:24**	**1:40**	**1:50**	**1:60**	**1:80**
DARA [0.5 mg/mL]	-	-	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-	-	-
Results of IAT	 4+	 3+	 2+	 2+	 1+	 0.5+	 NEG
DARA [0.5 mg/mL]			+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]			-	-	-	-	-
			 2+	 2+	 2+	 2+	 2+
DARA [0.5 mg/mL]			+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]			+	+	+	+	+
			 2+	 1+	 1+	 0.5+	 NEG

Table S13.

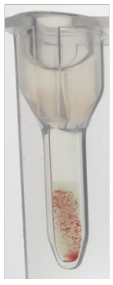
RBC phenotype	0 K-/cc.dd.ee M+				
Plasma with anti-M abs	+	+	+	+	+
Plasma dilution factor	**0**	**1:2**	**1:3**	**1:4**	**1:8**
DARA [0.5 mg/mL]	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
Results of IAT	 2+	 2+	 1+	 0.5+	 NEG
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
	 3+	 3+	 2+	 2+	 2+
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+
	 2+	 2+	 1+	 0.5+	 NEG

Table S14.

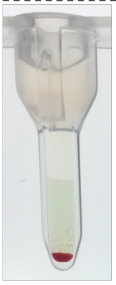
RBC phenotype	0 K-/cc.dd.ee S+s+				
Plasma with anti-S abs	+	+	+	+	+
Plasma dilution factor	**0**	**1:2**	**1:3**	**1:8**	**1:12**
DARA [0.5 mg/mL]	-	-	-	-	-
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
Results of IAT	 **2+**	 **2+**	 **1+**	 **0.5+**	 **NEG**
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	-	-	-	-	-
	 **3+**	 **3+**	 **3+**	 **2+**	 **2+**
DARA [0.5 mg/mL]	+	+	+	+	+
DARA-F(ab')$_2$ fragments [0.5 mg/mL]	+	+	+	+	+
	 **2+**	 **2+**	 **1+**	 **0.5+**	 **NEG**

Table S15 S18. Results of IAT in the presence (+) and absence (-) of DARA-F(ab')$_2$ fragments (0.5 mg/mL) using commercially available antibody screening RBCs and plasma from a patient with multiple myeloma: (A) patient plasma before the first DARA infusion, (B) plasma tested after DARA infusion, (C) plasma after DARA infusion tested after 5 min pre-incubation of RBCs with DARA-F(ab')$_2$ fragments.

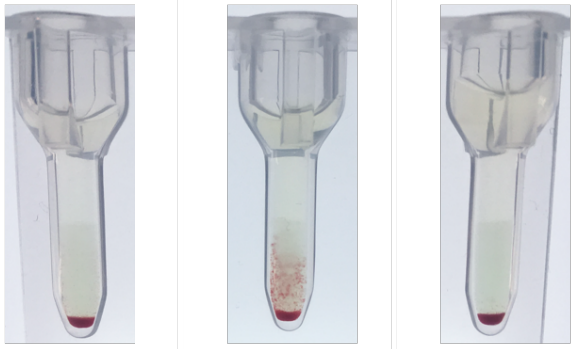
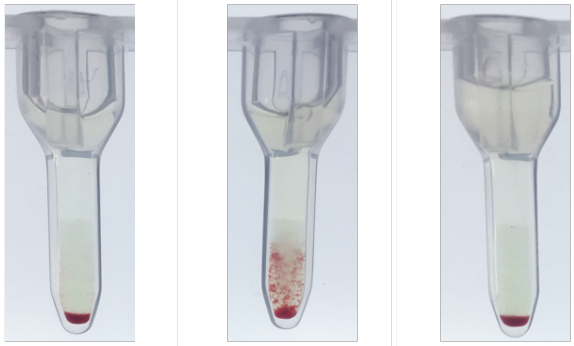
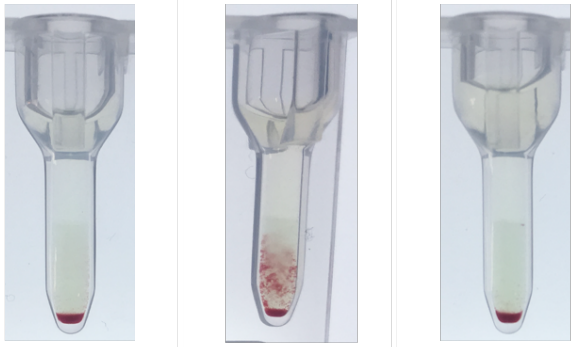
Plasma of a multi myloma patient	A	B	C
	before DARA infusion	after DARA infusion	
F(ab')$_2$ fragment:	-	-	+
Test RBC I			
Result	NEG	2+	NEG
Test RBC II			
Result	NEG	2+	NEG
Test RBC III			
Result	NEG	2+	NEG

Table S16.

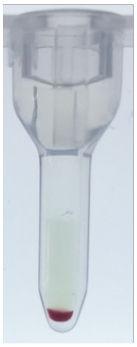
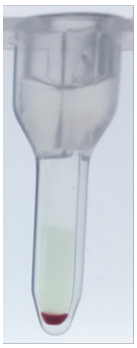
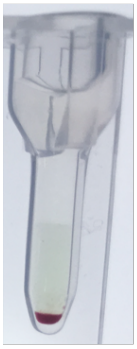
Plasma of a multi myloma patient	A	B	C
	before DARA infusion	after DARA infusion	
$F(ab')_2$ fragment:	-	-	+
Test RBC I			
Result	NEG	2+	NEG
Test RBC II			
Result	NEG	2+	NEG
Test RBC III			
Result	NEG	2+	NEG

Table S17.

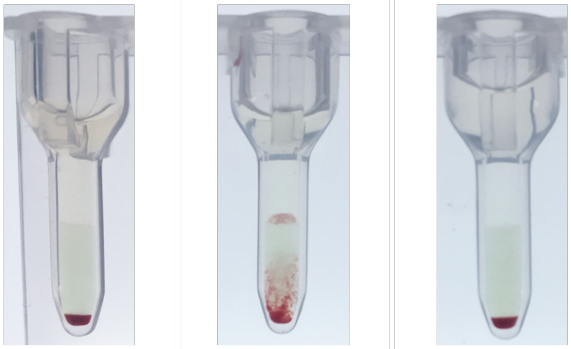
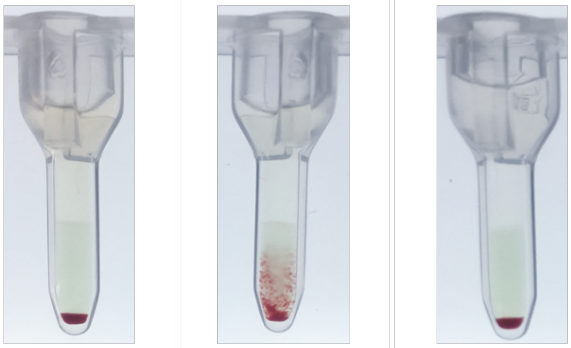
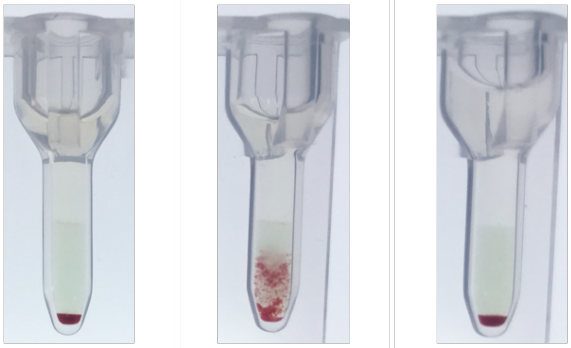
Plasma of a multi myloma patient	A	B	C
	before DARA infusion	after DARA infusion	
$F(ab')_2$ fragment:	-	-	+
Test RBC I			
Result	NEG	2+	NEG
Test RBC II			
Result	NEG	2+	NEG
Test RBC III			
Result	NEG	2+	NEG

Table S18.

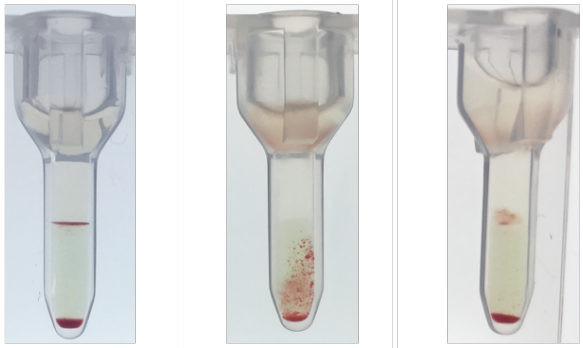
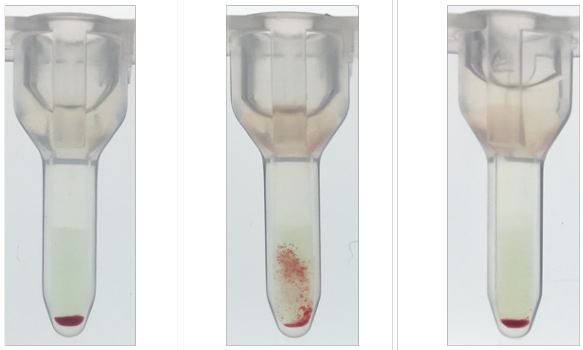
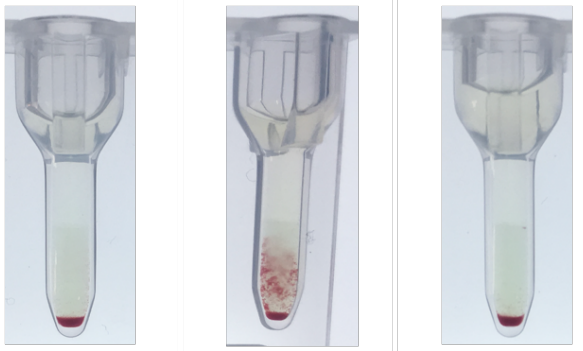
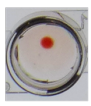
Plasma of a multi myloma patient	A	B	C
	before DARA infusion	after DARA infusion	
$F(ab')_2$ fragment:	-	-	+
Test RBC I			
Result	NEG	2+	NEG
Test RBC II			
Result	NEG	2+	NEG
Test RBC III			
Result	NEG	2+	NEG

Table S19. Results of IAT solid phase technique using RBC alloantibody containing plasmas in the presence (+) and absence (-) of DARA and DARA-F(ab')$_2$ fragments. A red spot in the middle of reaction well indicates a negative result (NEG), whereas particles spread across the surface of a reaction well indicate a positive test result (1+ to 4+).

		Plasma without anti-RBC abs			Anti-E			Anti-D			Anti-c		
RBC phenotype					Ee	Ee	ee	DD	DD	dd	cc	cc	CC
DARA (0.5 mg/mL)		-	+	+	-	+	+	-	+	+	-	+	+
RBC monolayer + DARA-F(ab')$_2$ fr.		-	-	+	-	+	+	-	+	+	-	+	+
Indicator-RBCs + DARA-F(ab')$_2$ fr.		-	-	+	-	+	+	-	+	+	-	+	+
Results:		NEG	2+	NEG	3+	3+	NEG	4+	4+	NEG	1+	1+	NEG