Original article

Aggregates in blood filter chambers used from the plasma donations of anti-D donors: evaluation for monoclonal antibody discovery using phage display

Eunike C. McGowan[1,2,3], Robert L. Flower[1,2], Martina L. Jones[2,3], David O. Irving[1,5], Ross T. Barnard[2,4], Catherine A. Hyland[1,2], Stephen M. Mahler[2,3], Xuan T. Bui[1,2,3]

[1]*Research and Development, Australian Red Cross Lifeblood, Brisbane, Australia;*
[2]*Australian Research Council Training Centre for Biopharmaceutical Innovation, The University of Queensland, Brisbane, Australia;*
[3]*Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia;*
[4]*School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia;*
[5]*University of Technology Sydney, Sydney, Australia*

Background - RhD-immunoglobulin (RhIg) prevents anti-D alloimmunisation in D-negative pregnant women when the fetus is D-positive, reducing the incidence of haemolytic disease of the fetus and newborn. Manufacturing RhIg is reliant on the limited supply of plasma donations with anti-D antibodies. Monoclonal antibody (mAb) development platforms such as phage display, require blood samples to be collected from anti-D donors, which may be a complicated process. The blood filter chamber (BFC) discarded after an anti-D donor's donation might provide a source of Ig-encoding RNA. This study aims to evaluate whether used BFCs are a suitable source of Ig-encoding RNA for phage display.

Material and methods - Haemonetics PCS2 BFCs were obtained from 10 anti-D donors for total RNA extraction, cDNA synthesis and amplification of VH and VL IgG sequences for assembly of single-chain variable fragments (scFvs). A scFv-phage display library was constructed and 3 rounds of biopanning were performed using D-positive and D-negative red blood cells (RBCs). Positive phage clones were isolated, Sanger sequenced and, where possible, reformatted into full-length human IgGs to define specificity. The BFC aggregates from 2 anti-D donors underwent a Wright-Giemsa stain and hematological cell count.

Results - Of 10 BFCs, a sufficient yield of total RNA for library construction was obtained from BFCs containing cellular aggregates (n=5). Aggregate analysis showed lymphocytes were the cellular source of Ig-encoding RNA. From the 5 samples with aggregates, scFvs were assembled from amplified IgG variable regions. The library constructed from 1 of these samples resulted in the isolation of clones binding to D-positive RBCs with *IGHV3* gene usage. Of the 4 reformatted IgG, 3 were anti-D and 1 had undefined specificity.

Discussion - BFC aggregates are a new and convenient source of Ig-encoding RNA which can be used to construct Ig gene libraries for mAb isolation and discovery via antibody phage display.

Keywords: *anti-D, blood filter, phage display, plasma donation, RhIg.*

INTRODUCTION

The administration of prophylactic polyclonal, RhD-immunoglobulin (RhIg), for RhD-negative pregnant women, remains a major success in reducing rates of maternal anti-D alloimmunisation, which when untreated, leads to haemolytic disease of the fetus and newborn[1]. RhIg is currently manufactured from a limited supply of polyclonal IgG

Arrived: 15 April 2020
Revision accepted: 17 July 2020
Correspondence: Xuan T. Bui
e-mail: x.bui@uq.edu.au

Blood Transfus 2021; 19: 64-72 DOI 10.2450/2020.0093-20

antibodies which have been fractionated from the plasma donations of volunteer donors who have antibodies recognizing the RhD antigen (anti-D donors)[1]. During shortages, RhD-negative male donors are preferentially recruited to produce anti-D antibodies by deliberately immunising them with RhD-positive red blood cells (RBCs). Over their donation career, these donors are "boosted" with injections of RhD-positive RBCs to produce or maintain high anti-D antibody levels. These immunisation programmes are not without risk. The development of monoclonal antibodies (mAbs) which parallel the activity and efficacy of RhIg provides the potential to eliminate these risks to RhD-negative donors and ensure a sufficient supply.

However, such developments are not possible yet as the mechanisms of action of RhIg remains to be fully understood[2]. Although anti-D mAbs with the ability to clear RhD-positive RBCs have been developed, the failure of prophylaxis in RhD-negative volunteers during clinical trials have suggested RhIg may have additional and/or other mechanisms of action/s involved in suppressing anti-D alloimmunisation[2,3]. A number of possible mechanism of actions have been hypothesised, including those related to the IgG-mediated inhibition of B-cell activation[4]. A valuable resource in the elucidation of these mechanisms includes antibody-producing lymphocytes from anti-D donors. This resource allows the properties of IgG antibodies in RhIg to be investigated and help facilitate development of its recombinant alternative.

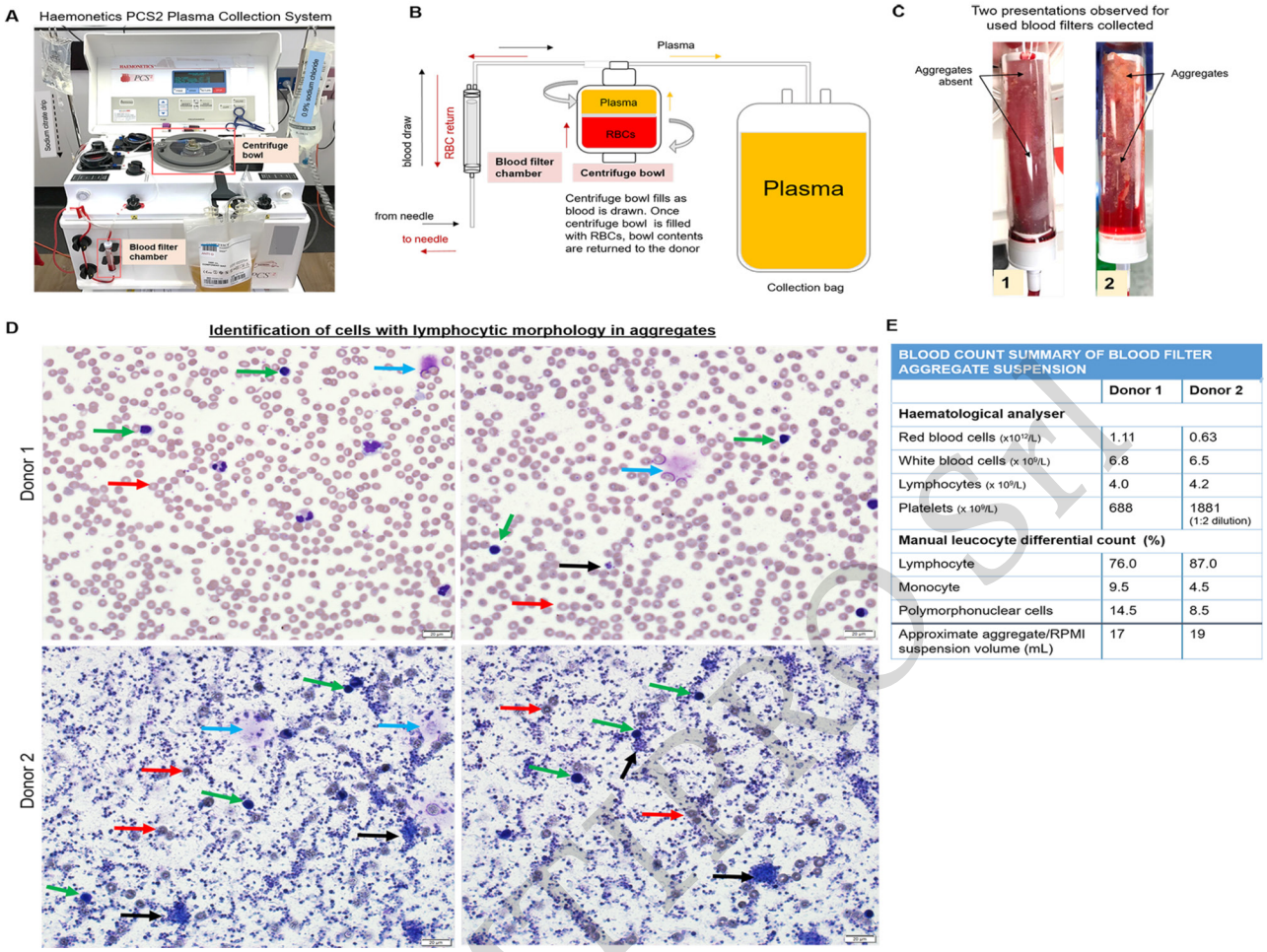
A proposed direction in developing an RhIg recombinant alternative may be to mimic the complexity of polyclonal IgG with the use of two or more mAbs. Human anti-D mAbs have been successfully developed using various antibody discovery technology platforms. One platform involves isolating human lymphoblastoid cell lines (LCLs) with the desired specificity, and then immortalizing the cell line using Epstein-Barr virus (EBV)-transformation[5]. Another is phage display technology which creates a library of cloned human antibody fragments, such as Fab or single-chain variable fragments (scFvs), derived from the immunoglobulin (Ig) RNA from an individual[6-10]. The reformatting and expression of these anti-D Ig variable regions to whole IgG molecules requires a mammalian cell production system such as Chinese Hamster Ovary (CHO) cells, for example[7,11,12].

Compared to EBV-transformed LCLs, phage display is advantageous in that it can: 1) shuffle VH-VL pairings 2) isolate several clones against a target and reveal their preferential VDJ usage 3) link the specificity of the variable region (phenotype) with the encoding nucleotide sequence (genotype) and 4) allow engineering of the Fc region[13]. Additionally, CHO cells have a favourable safety profile over human-derived cells for the manufacture of therapeutic antibodies, since they are less likely to propagate human viruses during the manufacturing process which may carry over to the final product[2,14,15]. These advantages highlight the value of phage display for mAb discovery.

Immune human phage display libraries from hyperimmune anti-D donors have been used in RBC biopanning to discover antibody fragments against various Rh blood group antigens, including RhD[7-10]. Nucleotide analysis of these anti-D variable regions showed preferential usage of the kappa light chain and *VH3-30* and *VH3-33* alleles, which has been referred to as the *"VH3-33 superspecies"*[7,16,17]. The key to construction of Ig gene libraries to facilitate antibody discovery by phage display, however, is to first source Ig-encoding RNA from an anti-D donor.

Obtaining samples from anti-D donors as a source of the starting Ig RNA for phage display library construction has some constraints. Firstly, there are ethical considerations when additional blood samples are requested from anti-D donors who have already donated large volumes of plasma. Secondly, a whole blood donation request, in place of the anti-D donor's routine plasma donation, may interrupt donation schedules and routine procedures. Although whole blood, buffy coat and leukapheresis donations have been collected from anti-D donors, these previous studies were undertaken more than 10 years ago[7,8]. Now, with an increasing demand for RhIg and an ageing anti-D donor population[18], the arrangement of a whole blood or leukapheresis donation request is likely to be cumbersome or impractical.

Discarded leucocyte reduction filters used from blood donations have been demonstrated to contain viable white blood cells (WBCs). These have been used as an alternative WBC source in a variety of applications[19,20]. A commercially-available instrument for collecting plasma donations, known as the Haemonetics Plasma Collection System 2 (PCS2) (**Figure 1A**), utilises a disposable collection



BLOOD COUNT SUMMARY OF BLOOD FILTER AGGREGATE SUSPENSION

	Donor 1	Donor 2
Haematological analyser		
Red blood cells ($\times 10^{12}$/L)	1.11	0.63
White blood cells ($\times 10^{9}$/L)	6.8	6.5
Lymphocytes ($\times 10^{9}$/L)	4.0	4.2
Platelets ($\times 10^{9}$/L)	688	1881 (1:2 dilution)
Manual leucocyte differential count (%)		
Lymphocyte	76.0	87.0
Monocyte	9.5	4.5
Polymorphonuclear cells	14.5	8.5
Approximate aggregate/RPMI suspension volume (mL)	17	19

Figure 1 – Blood filter aggregate analysis
(A) Photograph of the disposable donor harness installed in the Haemonetics PCS2 Plasma collection system after a plasma donation. (B) Diagram representing where the blood filter chamber is positioned for the blood draw (black arrow) and red cell return (red arrow) during the plasma donation (not to scale). (C) Photograph of the two different blood filter chamber presentations observed after the plasma donation of 10 anti-D donors. From each blood filter chamber of 10 anti-D donors, aggregates were absent in 5 blood filters (C1) and aggregates were present in 5 blood filter chambers (C2) (D) Wright-Giemsa smears of blood filter aggregates for donor 1 and donor 2 (×40 objective). Lymphocytes (green arrows) were identified in the blood filter aggregates despite differing amounts of platelet aggregates (black arrows) and RBCs (red arrows). Fibrin (blue arrows) were also observed. (E) Blood count summary from a haemotological analyser and manual differential count. Significant differences in RBCs and platelet counts were observed between the 2 anti-D donors.

tubing system for each plasma donation. The disposable tubing, called the donor harness, contains a blood filter chamber (BFC) (**Figure 1B**). At the end of the donation, the BFC is discarded as part of routine procedure.

This study aims to evaluate whether BFCs used from the plasma donation of anti-D donors are a convenient source of Ig-encoding RNA for mAb discovery using phage display.

MATERIALS AND METHODS

Donors

Ten volunteer anti-D donors who participate in the Rh programme at Australian Red Cross Lifeblood consented to participate in the study (Lifeblood Human Research Ethics Committee 2017#08). Additionally, anti-C and/or anti-E antibodies were present in 7 of 10 donors.

Recovery of cells from used BFC

After the 10 anti-D donors had completed their 450-800 mL of plasma donation, the used donor harness was removed from the PCS2 (Haemonetics, Braintree, MA, USA) by the standard procedure. The BFC was separated from the donor harness and the opposite ends of the BFC wall were snipped with scissors for removal. BFC aggregates were immersed, washed and/or scraped with a 21G needle into RPMI medium 1640 (ThermoFisher Scientific, Waltham, MA, USA).

Blood Transfus 2021; 19: 64-72 DOI 10.2450/2020.0093-20

Blood filter aggregate analysis

Resuspended aggregates from two anti-D donors were analysed on a haematological analyser and a Wright-Giemsa blood stain using the Aerospray® Hematology Pro Autostainer (ELITechGroup Inc, Puteaux, France). An estimation of leucocyte numbers was manually counted and images were obtained using an Olympus IX73 inverted microscope with a camera (Olympus, Tokyo, Japan).

Library construction

The material recovered from the BFCs, including RBCs, underwent total RNA extraction using a TRIzol-chloroform (Invitrogen, Carlsbad, CA, USA) and spin-column (RNeasy Mini Kit [QIAGEN, Hilden, Germany]) method. Total RNA yield was quantified using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific) and the quality was analysed using an Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Subsequently, cDNA was synthesised from the samples containing aggregates in the BFCs using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). A single-chain variable fragment (scFv) phagemid library was constructed, and phage particles were prepared, as previously described[21] for generation of a human IgG repertoire.

Biopanning

Prior to biopanning, 1 mL of phage particles (~10^{10}) and 1 mL of bromelain-treated packed RBCs (RhD-positive or RhD-negative) were blocked with 4 volumes of 2% bovine serum albumin in PBS (BSA/PBS; 1 hr, 4 °C). The blocked phage suspension was added to 10 mL of a 10% bromelain-treated RhD-negative RBC suspension (r"r for Round 1 and r'r for Round 2 and 3. The r'r" phenotype was not used given the frequency of r'r" RBCs in Australia is <0.1%[22]) for depletion (1 hr, 4 °C). The supernatant containing unbound phage was added to RhD-positive RBCs (R2R2; 1 hr, 4 °C) for panning. Unbound phage particles were removed with one cold 0.9% saline (pH 5.8) wash and three washes with cold PBS. Bound phage particles were eluted with 200 mM glycine (pH 2.8) and neutralised with Tris-HCl (pH7.4) before infection of *Escherichia coli* XL1-Blue (Agilent Technologies) at log phase of growth (OD_{600}=0.5-0.7).

The bacteria were then plated onto agar plates containing yeast, tryptone, ampicillin (100 µg/mL) and 2% glucose (w/v) (2YT-AmpGlu; overnight, 30 °C). The harvested cells were then grown and infected with M13K07 helper phage. Phage particles were then produced by growth (overnight, 30 °C) of the infected cells in 2YT-media containing ampicillin (100 µg/mL) and kanamycin (30 µg/mL) (2YT-AmpKana). The phage particles were then purified using polyethylene glycol-NaCl for subsequent panning rounds.

Phage screening

Individual *E.coli* XL1-Blue colonies containing phagemid from the third round of panning were randomly picked from 2YT-AmpGlu plates and infected with M13K07 helper phage for phage production in a 96-well format plate. Phage supernatant from each colony and 1 mL RBCs (R2R2 or r'r) were blocked with 100 µL and 10 mL of 2% BSA, respectively, for 1hr at 4 °C with agitation. Then, 100 µL of blocked 10% RBC suspension (R2R2 or r'r) was added to 100 µL blocked phage for 1 hr, at 4 °C with agitation then centrifuged at 1,000 *g* × 1 min for washing. After three washes with cold PBS, 50 µL of 5 µg/mL mouse anti-M13 phage antibody (clone MM05, Sino Biologicals; Beijing, China) was added to detect bound phage (30 min, room temperature [RT]) then washed three times with cold PBS. Subsequently, 50 µL of 10 µg/mL Alexa Fluor 488nm goat anti-mouse IgG (H+L) (Invitrogen) was added (30 min, RT) for flow cytometry analysis (CyFlow Cube 8 Robby [Sysmex, Kobe, Hyogo, Japan]).

A human monoclonal anti-D antibody (clone# R593)[23] and Alexa Fluor 488nm mouse anti-human IgG1 (Invitrogen) were used as the positive control. FCS Express V6 software (De Novo; Pasadena, CA, USA) was used to identify phage clones with reactivity to R2R2 but not to r'r RBCs.

RhD specificity confirmation and sequencing

Purified phage clones with reactivity with R2R2 RBCs but not to r'r RBCs were tested as described above to obtain their reactivity profiles with an Rh RBC panel (R1R1, R2R2, Ror, r'r, r"r and rr). Bacterial colonies identified as producing anti-D scFv phage clones were cultured overnight in 2 mL of 2YT-AmpGlu for phagemid DNA extraction using the Miniprep Kit (QIAGEN). Phagemid DNA containing scFv inserts were Sanger sequenced with phagemid-derived forward and reverse primers and the scFv nucleotide sequence was aligned with ImMunoGeneTics/V-QUEST (IMGT) to show V(D)J usage[24].

Reformatting of scFvs into full-length human IgG1

The isolated scFvs were reformatted into full-length human IgG1, expressed in ExpiCHO-S cells (ThermoFisher Scientific) and affinity-purified on Protein A using the AKTA explorer (GE Healthcare, Chicago, IL, USA) as previously described[26]. Flow cytometry was performed to confirm the specificity of reformatted human IgG1 mAbs. The staining was with 1 ug mAb and 100 µL of commercial screening cells at 0.8% RBC suspension (Bio-Rad) in a 30 min, room temperature (RT) incubation. Then, Alexa Fluor 488nm mouse anti-human IgG1 (Invitrogen) was added for 30 min, RT, in the dark for flow cytometric analysis using the Cytoflex (Beckman Coulter, Brea, CA, USA).

Additionally, an 11-cell reagent red cell panel with 0.8% RBC suspension (Bio-Rad, Hercules, CA, USA) was used to define the specificity of the monoclonal antibodies in a Coombs anti-human IgG card as per manufacturer's instructions (Bio-Rad).

RESULTS

Blood filter presentation correlated with total RNA yield

For the 10 donors, two different BFC presentations were noted (**Figure 1C**) and these correlated with total RNA yield (µg): 1) aggregates present (n=5; 80.4-172.7 µg) and 2) aggregates absent (n=5; 1.9-12.4 µg) (**Table I**). A trend was observed for the 5 blood filters lacking aggregates, as 4 out of 5 of these donors reported the use of aspirin (**Table I**).

Blood filter aggregate analysis

The Wright-Giemsa stained BFC samples from donor 1 and 2 revealed WBCs, RBCs, and platelets were captured by the filter at the end of the plasma donation (**Figure 1D**). Although the microscopic analysis and blood counts revealed a major difference in the number of RBCs and platelets captured in the BFC, the total numbers of WBCs (1.2×10^8) and lymphocytes (6.8×10^7 for donor 1 and 8.0×10^7 for donor 2) were similar between the two donor samples (**Figure 1E**). A manual leucocyte count showed lymphocytes constituted the highest proportion of WBCs (up to 87%) compared to monocytes and polymorphonuclear cells (**Figure 1E**).

Library construction

Only the 5 BFCs presenting with aggregates yielded sufficient total RNA for cDNA synthesis. From all 5 of these cDNA samples, human IgG VH and VL gene fragments were amplified in all PCR reactions. This was demonstrated by the visualisation of PCR products of the expected size (*data not shown*). Using the human scFv PCR products from donor 2, which had been cloned into a phagemid vector, a primary phagemid library was constructed with a size of 3.2×10^7 colony forming units (CFU) per µg of vector DNA.

Biopanning and specificity analysis of phage clones with anti-D scFvs

Three rounds of biopanning were performed to isolate phage clones expressing anti-D scFvs. Out of 84 random colonies, 14 were found to produce phage clones which were reactive R2R2 RBCs but not r'r RBCs. Purified phage clones from all 14 colonies had a reactivity profile consistent with anti-D antibodies: reactive with RhD-positive (R1R1, R2R2 and Ror) and nonreactive with RhD-negative (r'r, r"r and rr) RBCs (*data not shown*). Sequencing of these 14 phage clones showed 7 unique scFv amino acid sequences.

IMGT analysis of these isolated anti-D scFv nucleotide sequences revealed usage of the VH3-30*03 allele, and kappa light chains of various alleles (**Table II**). This is consistent with previous reports of preferential use of VH3-30 alleles amongst RhD-specific antibodies[7,8,16].

Table I - *Summary of used, discarded blood filter chamber presentations from 10 anti-D donors*

Anti-D donors	Blood filter chamber presentation									
	Aggregates present					Aggregates absent				
Donor number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Rh antibodies recorded	Anti-D, -C	Anti-D, -C	Anti-D	Anti-D, -C, -E	Anti-D, -C, -E	Anti-D	Anti-D	Anti-D, -C	Anti-D, -C, -E	Anti-D, -C
Plasma donation volume (mL)	633	750	600	800	800	602	450	800	675	800
Total RNA yield (µg)	96.5	134.4	80.4	111.7	172.7	2.3	1.9	11.4	12.4	8.6
Aspirin medication	No	No	No	No	No	Yes	Yes	No	Yes	Yes

Blood Transfus 2021; 19: 64-72 DOI 10.2450/2020.0093-20

Table II - *IMGT® analysis of 7 unique anti-D scFv nucleotide sequences*

Clone	VH					VL			
	IGHV		*IGHD*	*IGHJ*		*IGKV*		*IGKJ*	
	Allele name	%	Allele name	Allele name	%	Allele name	%	Allele name	%
1	3-30*03	93.4	3-22*01	3*01	84.0	1-27*01	94.3	3*01	91.4
2	3-30*03	92.0	3-22*01	3*01	84.0	1-27*01	98.2	1*01	89.5
3	3-30*03	93.8	3-22*01	3*01	84.0	1-39*01	93.2	4*01	94.3
4,6-8, 10-13	3-30*03	92.0	3-22*01	3*01	84.0	1-27*01	98.2	1*01	94.7
5	3-30*03	93.4	3-22*01	3*01	84.0	1-27*01	98.2	1*01	94.7
9	3-30*03	93.0	3-22*01	3*01	84.0	1-27*01	94.6	4*01	91.4
14	3-30*03	91.7	3-22*01	3*01	78.0	1-27*01	97.9	1*01	91.9

The V-REGION allele with the most similarity to the 7 unique anti-D scFv nucleotide sequence was identified using ImMunoGeneTics/V-QUEST (IMGT). When available, the similarity was provided as a percentage. When the percentage was identical with two or more alleles, the closest germline allele identified from the IMGT "V-REGION translation" and "V-REGION protein display" was selected.
(Allele name/% similarity). VH: variable heavy chain; VL: variable light chain.

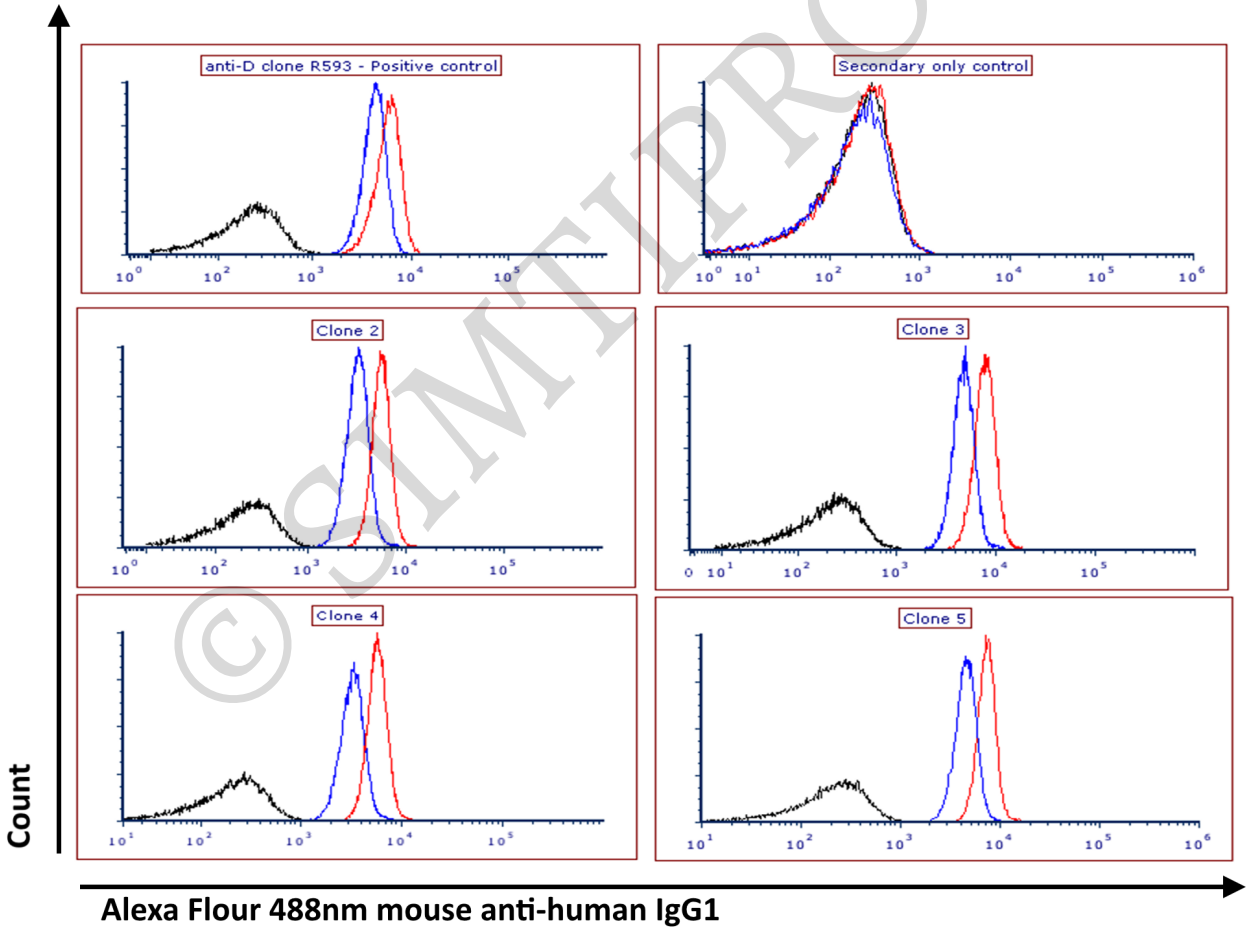


Figure 2 – Reactivity profile of reformatted monoclonal antibodies against screening cells
Flow histograms showing the reactivity of an anti-D monoclonal antibody clone R593, a secondary only control and 4 reformatted mAbs (clone 2 to 5) against a commercial screening cell panel comprising of red cells with three different phenotypes: R1R1 (blue), R2R2 (red) and rr (black).

Table III - *Reactivity profile of reformatted monoclonal antibodies against 11-cell reagent red blood cell panel*

Clone number	Rh phenotype of panel cell											
	1	2	3	4	5	6	7	8	9	10	11	Monoclonal antibody identity
	R1wR1	R1R1	R2R2	r'r	r"r	rr	rr	Ror	rr	rr	rr	
2	+	+	+	–	–	–	–	+	–	–	–	Anti-D
3	+	+	+	w	w	w	w	+	w	w	–	unresolved
4, 6-8, 10-13	+	+	+	–	–	–	–	+	–	–	–	Anti-D
5	+	+	+	–	–	–	–	+	–	–	–	Anti-D

The VH and VL sequences of phage-scFv clone 4, 6-8 and 10-13 were identical and are reported collectively.
w: weak; +: positive; –: negative; VH: variable heavy chain; VL: variable light chain.

From IMGT comparison in the 3 framework regions (FR) and 3 complementarity determining regions (CDR), all clones shared similar VH-, D- and J-region usages. For the variable heavy chain, the majority of differences existed in the CDR-2 and FR-3 regions. For the variable light chain, there were two Vκ alleles, *IGKV1-27*01* and *IGKV1-39*01*, with differences in the FR-1 and CDR-3 for the former, and FR-1 and CDR-1 for the latter.

After 4 out of 7 unique scFvs were reformatted into full-length human IgG1 mAbs, flow cytometry of the mAbs with screening cells showed reactivity with D-positive and no reactivity with D-negative cells (**Figure 2**). Furthermore, Coombs card testing against an 11-cell reagent red cell panel revealed 3 out of 4 mAbs were anti-D mAbs and the remaining 1 mAb had unresolved specificity (**Table III**).

DISCUSSION

Obtaining Ig-encoding RNA from an anti-D donor is a requirement in the construction of an immune human phage display library for mAb discovery related to RhIg. This study aimed to evaluate used BFCs from anti-D donors as a novel and convenient source of Ig-encoding RNA for phage display. This would help to overcome the constraints associated with the collection of blood samples from anti-D donors.

This study has found that when aggregates were present, routinely discarded BFCs were a suitable source of WBCs containing Ig-encoding RNA. Aggregates were present in 5 out of 10 BFCs. The remaining 5 BFCs without aggregates had total RNA yields insufficient for the human antibody phage display library construction, which showed that it was necessary to inspect the BFCs for aggregates prior to processing. With 4 out of 5 of these donors reported to have used aspirin, we hypothesise that the effect of aspirin on platelets[25] may have resulted in reduction in the number of lymphocytes captured by the blood filter, although a larger sample size would be required to confirm this hypothesis.

In contrast, aggregates from the BFCs of 5 anti-D donors resulted in a total RNA yield which was sufficient for cDNA synthesis, the next stage of library construction. Lymphocytes, among other peripheral blood cells, detected in the BFC aggregates of 2 anti-D donors provided evidence as to the cellular source from which the human Ig fragments from all 5 BFCs aggregates were amplified. Although human scFv PCR products were amplified in all aggregate-presenting samples, for this study, only the scFvs from 1 anti-D donor was reported as an example for immune human phage display library construction.

The library constructed in this study was found to be a similar size (3.2×10^7 CFU per μg of vector DNA) compared to that of the immune human antibody phage display libraries previously constructed from the whole blood of anti-D donors (10^6 to 10^7 CFU per ug)[7,9,10]. The germline analysis of anti-D scFv clones isolated from this library showed *VH3-30* usage, which is similar to previously isolated human monoclonal anti-D antibody fragments[7,9,10]. These showed that the aggregates captured in the BFCs can be used as a source of human Ig-encoding RNA for human mAb discovery using phage display.

An advantage of phage display is that the VH and VL sequences can then be reformatted into an IgG subclass of choice[26] for further characterisation, including defining specificity. The reformatted human IgG1 were shown to be suitable for use in a Coombs test and 3 mAbs were found to have a serological profile consistent with an

Blood Transfus 2021; 19: 64-72 DOI 10.2450/2020.0093-20

anti-D mAb. These novel anti-D mAbs now require further characterisation, which may include: 1) the D-epitope/s recognised 2) its mechanism of action on anti-D mAb coated RBCs (e.g triggering of monocyte phagocytic function) and 3) the pharmacokinetics profile (e.g absorption rate constant [Ka]).

The reformatting of the anti-D mAbs into various IgG subclasses may be useful in understanding the possible mechanisms of action, including the crosslinking of Fcγ receptors on B-cells in antibody-mediated B-cell inhibition and interactions with Fc receptors on macrophages and natural killer cells in the clearance of sensitised D+ RBCs[27]. Further elucidation of these mechanisms combined with the capability of engineering the Fc region of the anti-D IgG molecules may lead to improved anti-D mAb pharmacodynamic profiles[28]. The remaining mAb may be recognizing an epitope generated by the interaction of the D antigen with other RBC surface structures, but this requires further serological investigation.

The Haemonetics PCS2 is used to collect plasma donations in Australia and other countries[29,30]. The single-use donor harness, which includes the BFC, is routinely discarded after each donor has completed their donation on the Haemonetics PCS2. This provided the opportunity to conveniently retrieve the aggregates from the BFC each time the anti-D donor finishes their plasma donation. With this convenience, we also suggest the potential for these aggregates to be used in other applications apart from phage display, such as the isolation of immune cells for flow cytometry, as previously described for leucocyte reduction filters[19,20].

CONCLUSIONS

In conclusion, we have demonstrated that aggregates from used BFCs are a suitable source of Ig-encoding RNA required for mAb discovery using phage display and, potentially, a variety of other applications. This collection of used BFCs is convenient and accessible after the completion of the anti-D donor's plasma donation.

ACKNOWLEDGEMENTS

We would like to thank the anti-D donors for their participation in this study. We also thank Louise Taylor, Hannah Redding, Xiaoming Gibb, Zoe Horne and Rochelle Jackson for their help in participant enrolment and collection of blood filters at the donor centre; Scientific and Technical Services team who obtained the samples we used for biopanning; Dr Yew-Wah Liew and Dr Elizna Schoeman for their advice. EM is a recipient of an Australian Government Research Training Program Scholarship and gratefully acknowledges this support.

FUNDING AND RESOURCES

Australian governments fund the Australian Red Cross Lifeblood for the provision of blood, blood products, and services to the Australian community.

Elements of this research utilised the facilities, and the scientific and technical assistance of the National Biologics Facility (NBF) at The University of Queensland. NBF is supported by Therapeutic Innovation Australia (TIA).

TIA is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS) Program.

Funding support by the Australian Research Council through the Industrial Transformation Research Program (ITRP) scheme.

AUTHORSHIP CONTRIBUTIONS

EM and XB developed the aggregate recovery method, performed the experiments and wrote this paper. EM, XB and RF developed the biopanning strategy. XB, RF, MJ, SM, DI, RB, CH supervised this study and reviewed the manuscript.

The Authors declare no conflict of interest.

REFERENCES

1. Thyer J, Wong J, Thomson A, et al. Fifty years of RhD immunoglobulin (anti-D) therapy in Australia: celebrating a public health success story. Med J Aust 2018; **209**: 336-9.
2. Kumpel BM. Efficacy of RhD monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. Vox Sang 2007; **93**: 99-111.
3. Beliard R. Monoclonal anti-D antibodies to prevent alloimmunization: lessons from clinical trials. Transfus Clin Biol 2006; **13**: 58-64.
4. Brinc D, Denomme GA, Lazarus AH. Mechanisms of anti-D action in the prevention of hemolytic disease of the fetus and newborn: what can we learn from rodent models? Curr Opin Hematol 2009; **16**: 488-96.
5. Kumpel BM, Goodrick MJ, Pamphilon D, et al. Human Rh D monoclonal antibodies (BRAD-3 and BRAD-5) cause accelerated clearance of Rh D+ red blood cells and suppression of Rh D immunization in Rh D-volunteers. Blood 1995; **85**: 1701-9.
6. Jones ML. Use of bacteriophage for discovery of therapeutically relevant antibodies against infectious diseases. Microbiology Australia 2019; **40**: 33-6.
7. Andersen PS, Haahr-Hansen M, Coljee VW, et al. Extensive restrictions in the VH sequence usage of the human antibody response against the Rhesus D antigen. Mol Immunol 2007; **44**: 412-22.

8. Dohmen SE, Verhagen OJ, de Groot SM, et al. The analysis and quantification of a clonal B cell response in a hyperimmunized anti-D donor. Clin Exp Immunol 2006; **144**: 223-32.
9. Dohmen SE, Verhagen OJ, Muit J, et al. The restricted use of IGHV3 superspecies genes in anti-Rh is not limited to hyperimmunized anti-D donors. Transfusion 2006; **46**: 2162-8.
10. Miescher S, Vogel M, Biaggi C, et al. Sequence and specificity analysis of recombinant human Fab anti-Rh D isolated by phage display. Vox Sang 1998; **75**: 278-87.
11. Robak T, Windyga J, Trelinski J, et al. Rozrolimupab, a mixture of 25 recombinant human monoclonal RhD antibodies, in the treatment of primary immune thrombocytopenia. Blood 2012; **120**: 3670-6.
12. Frandsen TP, Næsted H, Rasmussen SK, et al. Consistent manufacturing and quality control of a highly complex recombinant polyclonal antibody product for human therapeutic use. Biotechnology and Bioengineering 2011; **108**: 2171-81.
13. Siegel D. Recombinant monoclonal antibody technology. Transfusion clinique et biologique 2002; **9**: 15-22.
14. Council of the European Union. *Production and quality control of monoclonal antibodies [monograph on the internet]*. 1995. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/production-quality-control-monoclonal-antibodies_en.pdf. Accessed on 06/07/2020.
15. European Medicines Agency. *Quality of biotechnological products: Viral safety evaluation of biotechnology products derived from cell lines of human or animal orgin [monograph on the internet]*. 1997. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-r1-viral-safety-evaluation-biotechnology-products-derived-cell-lines-human-animal-origin_en.pdf. Accessed on 06/07/2020.
16. Chang TY, Siegel DL. Genetic and immunological properties of phage-displayed human anti-Rh(D) antibodies: implications for Rh(D) epitope topology. Blood 1998; **91**: 3066-78.
17. Siegel D, Czerwinski M, Spitalnik S. Section 5: Structural/genetic analysis of mAbs to blood group antigens. Coordinator's report. Transfusion clinique et biologique 2002; **9**: 83-97.
18. van Hoeven LR, Berkowska MA, Verhagen OJHM, *et al.* Prediction of the anti-RhD donor population size for managerial decision-making. Vox Sanguinis 2016; **111**: 171-7.
19. Dietz AB, Bulur PA, Emery RL, et al. A novel source of viable peripheral blood mononuclear cells from leukoreduction system chambers. Transfusion 2006; **46**: 2083-9.
20. Patel KR, Roberts JT, Subedi GP, et al. Restricted processing of CD16a/Fc gamma receptor IIIa N-glycans from primary human NK cells impacts structure and function. J Biol Chem 2018; **293**: 3477-89.
21. Jones ML, Alfaleh MA, Kumble S, et al. Targeting membrane proteins for antibody discovery using phage display. Scientific Reports 2016; **6**: 26240.
22. Australian Red Cross Lifeblood. *Red Cell Phenotype Calculator: RH - r"r' [monograph on the internet]*. 2020. Available at: https://transfusion.com.au/blood_basics/blood_groups/red_cell_phenotype_calculator. Accessed on 06/07/2020.
23. Gaucher C, Jorieux S, de Romeuf C. Anti-Rhesus D monoclonal antibody. Patent Application Pubblication US20110311556A1. Available at: https://patentimages.storage.googleapis.com/22/63/33/1046cb969deee9/US20110311556A1.pdf. Accessed on: 13/04/2020.
24. Brochet X, Lefranc M-P, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Research 2008; **36**: W503-W8.
25. Taylor ML, Misso NL, Stewart GA, et al. The effects of varying doses of aspirin on human platelet activation induced by PAF, collagen and arachidonic acid. Br J Clin Pharmacol 1992; **33**: 25-31.
26. Jones ML, Seldon T, Smede M, et al. A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies. J Immunol Methods 2010; **354**: 85-90.
27. Kumpel BM, Elson CJ. Mechanism of anti-D-mediated immune suppression--a paradox awaiting resolution? Trends Immunol 2001; **22**: 26-31.
28. Siegel DL. Research and clinical applications of antibody phage display in transfusion medicine. Transfus Med Rev 2001; **15**: 35-52.
29. Burgstaler EA. Blood component collection by apheresis. J Clin Apher 2006; **21**: 142-51.
30. Vrielink H, Van Der Meer PF. Collection of white blood cell-reduced plasma by apheresis. Transfusion 2004; **44**: 917-23.