Received: 4 December 2024 | Revised: 21 March 2025 | Accepted: 26 March 2025

DOI: 10.1111/trf.18245

ORIGINAL RESEARCH

Transfusion Medicine

TRANSFUSION

A novel acridine flow cytometry marker to track post-transfusion amustaline/glutathione pathogen-reduced red blood cell survival in sickle cell disease patients

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Funding information
Biomedical Advanced Research and Development Authority, Grant/Award Number: HHS010020160009c; NIH/NHLBI, Grant/Award Number: K23HL146904 PI: Yee

Abstract

Background: Measurement of transfused red blood cell (RBC) survival is relevant to the effective management of sickle cell disease (SCD). Following amustaline/glutathione pathogen-reduced (PR) RBC transfusion, small quantities of PR-RBC surface-bound acridine are detectable by flow cytometry. Concurrent biotin labeling was used to validate the acridine marker and track transfused PR-RBCs in SCD.

Methods: SCD patients ($n = 6$) on chronic transfusion therapy received three aliquots of different (2 μg/mL, 6 μg/mL, and 18 μg/mL) biotin-dose labeled RBCs during one transfusion episode. Aliquots were from one unit labeled before (Pre-PR) and after PR treatment (PR-RBC) and from a conventional RBC unit. The full RBC units (PR and conventional) were transfused, followed by the labeled aliquots from those units. Serial flow cytometry analyses for acridine- and biotin-labeled RBCs were performed on 10 occasions over 16 weeks. Acridine surface density was quantitated using calibrated beads.

Results: Mean acridine surface density was 5062 molecules/PR-RBC at 1–4 h post-transfusion and declined 84.5% within 7 days, remaining detectable (180 molecules/PR-RBC) at 16 weeks. The biotin-labeled PR-RBC aliquots

Abbreviations: Hb, hemoglobin; Hct, hematocrit; IAT, indirect antiglobulin test; MCV, mean cell volume; PR, pathogen reduction; PTR_{24}, 24-h post-transfusion recovery; RBC, Red Blood Cell; RCC, Red Cell Count; SCD, sickle cell disease; T_{50}, half-life.

(initial enrichment 0.6%–1.4%) demonstrated near-identical survival kinetics as the entire acridine-labeled PR-RBC units (initial enrichment 7.5%–13.7%). Pre-PR, PR, and Conventional RBCs revealed non-linear RBC survival kinetics, with similar 24-h post-transfusion recoveries (PTR_{24}) and half-lives (T_{50}), but PR-RBC mean predicted lifespan (mean [SD] 104.4 [4.7] days) was decreased by 9.3% (Pre-PR-RBCs 115.1 [7.2] days, $p = 0.006$).

Conclusions: Survival of amustaline/glutathione PR-RBCs can be tracked in vivo by flow cytometry for RBC surface acridine with similar sensitivity as biotin, without additional processing or radiolabeling.

KEYWORDS

acridine, flow cytometry, pathogen-reduction, RBC recovery and survival, red blood cells

1 | INTRODUCTION

Amustaline (S-303)/glutathione pathogen reduction (PR) of red blood cells (RBCs) is an investigational process designed to reduce the risk of transfusion-transmitted infections and to replace irradiation for the prevention of transfusion-associated graft-versus-host disease.[1–8] Following the discovery of non-clinically significant antibodies with specificity for PR-RBCs with an earlier version of the PR process,[1,9] Geisen et al.[10] described a quasi-quantitative flow cytometric assay to detect and quantitate RBC surface labeling with acridine using an acridine-specific monoclonal antibody (2S197-2MI). Additionally, they validated a sensitive gel-card-based indirect antiglobulin test (IAT) for detecting both natural and treatment-emergent antibodies specific to amustaline/glutathione PR-RBCs. Seventeen of 11,719 screened patients who had never been exposed to PR-RBCs were reactive to PR-RBC-specific natural antibodies. Pilot experiments indicated that the flow cytometric assay could detect circulating PR-RBCs in patient samples.[11,12]

Tracking the recovery and survival of RBCs in vivo has historically been performed in normal volunteers using radiolabeled autologous RBCs. Widespread use of this technique with allogeneic RBCs in patients is hampered by the complexities and risks of handling radionucleotides.[13] More recently, RBC biotinylation has proven to be an informative method to track transfused RBCs in vivo, allowing multiple RBC populations to be tracked simultaneously with different levels of biotin surface decoration. Published studies report results in normal volunteers, neonates, and, more recently, patients with SCD.[14–18] Wider use in patients has been inhibited by the restricted availability of reagent N-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin), the need for cGMP manufacturing facilities to perform labeling, and the emergence of biotin-specific antibodies.[14,15]

The amustaline/glutathione PR process leaves residual surface-bound acridine derived from amustaline, which could offer a way to track PR-RBC survival in patients. This method may prove to be a useful tool for clinical care by identifying shortened RBC survival in patients. In this proof-of-principle study, we used the biotin labeling process to evaluate the utility of the acridine flow cytometric marker to track aliquots and entire units of transfused PR-RBCs in patients with SCD.

2 | METHODS

An observational study, performed in adults with SCD (Table 1) receiving chronic simple transfusion therapy, compared the use of the RBC cell-surface acridine versus biotin as flow cytometric markers for tracking PR-RBCs in vivo and assessed the impact of pathogen reduction on RBC survival. The study was approved by the Institutional Review Board of Emory University, with Food and Drug Administration Investigational Device Exemption (IDE) approval for research use of biotinylation of allogeneic RBC (IDE # 16716: John Roback) and research use of amustaline/GSH pathogen reduction (IDE# BB-IDE 13803: Cerus Corporation). The study was registered with Clinicaltrials.gov (NCT04426591). Study transfusions were conducted in the Georgia Comprehensive Sickle Cell Center at Grady Health Center with patient informed consent.

Conventional, leukocyte-reduced RBCs were supplied by Lifesouth Community Blood Centers (Gainesville, FL). PR-RBCs were supplied by Central California Blood Center (Fresno, CA). Amustaline/glutathione pathogen reduction was performed as previously described[3,4,9] on leukocyte-reduced RBCs suspended in AS-5 (Terumo BCT, Lakewood, CO) additive solution. PR-RBCs were resuspended in SAG-M additive solution (Grifols) and the red cell count (RCC [10^{12}/L] × volume [L]) was

TABLE 1 Demographics, baseline characteristics, and timing of RBC transfusion during the study period.

Subject	Indication for transfusion	Age (years) Sex (M/F)	Baseline HbS (%)	Spleen Volume (mL)[a]	RBC pit index (%)[b]	Baseline RBC alloantibodies	RBC units transfused during study: Day (# units)
G01	Sickle cell hepatopathy	31 M	49.4[c]	69.1	48.4	–	0 (2)[d]; 23 (2); 48 (2); 76 (1); 104 (2)
G02	Stroke prevention	28 M	32.8	17.8	33.5	–	0 (2)[d]; 34 (2); 69 (2); 97 (2)
G03	Stroke prevention	33 F	50.2[c]	57.4	23.7	DAT (IgG1+, C3-)	0 (2)[d]; 28 (1); 56 (2); 84 (2)
G04	Stroke prevention	28 M	36.6	Splenectomized	34.0	Anti-Kell	0 (2)[d]; 30 (1); 58 (2); 86 (2)
G05	Stroke prevention	32 F	33.1	Not detectable	41.3	–	0 (2)[d]; 27 (2); 55 (2); 83 (2)
G06	Sickle cell ESRD[e]	25 F	7.9	102.6	0.9	–	0 (2)[d]; 35 (2); 70 (2); 105 (2)

[a]Assessed by ultrasound. Normal mean spleen volume is 113–219 mL, varying by height and sex.[23]
[b]Normal RBC pit index <1.5%.[35]
[c]HbS transfusion goal in these patients was </= 50%.
[d]Study transfusions.
[e]ESRD = end stage renal disease with bone marrow failure.

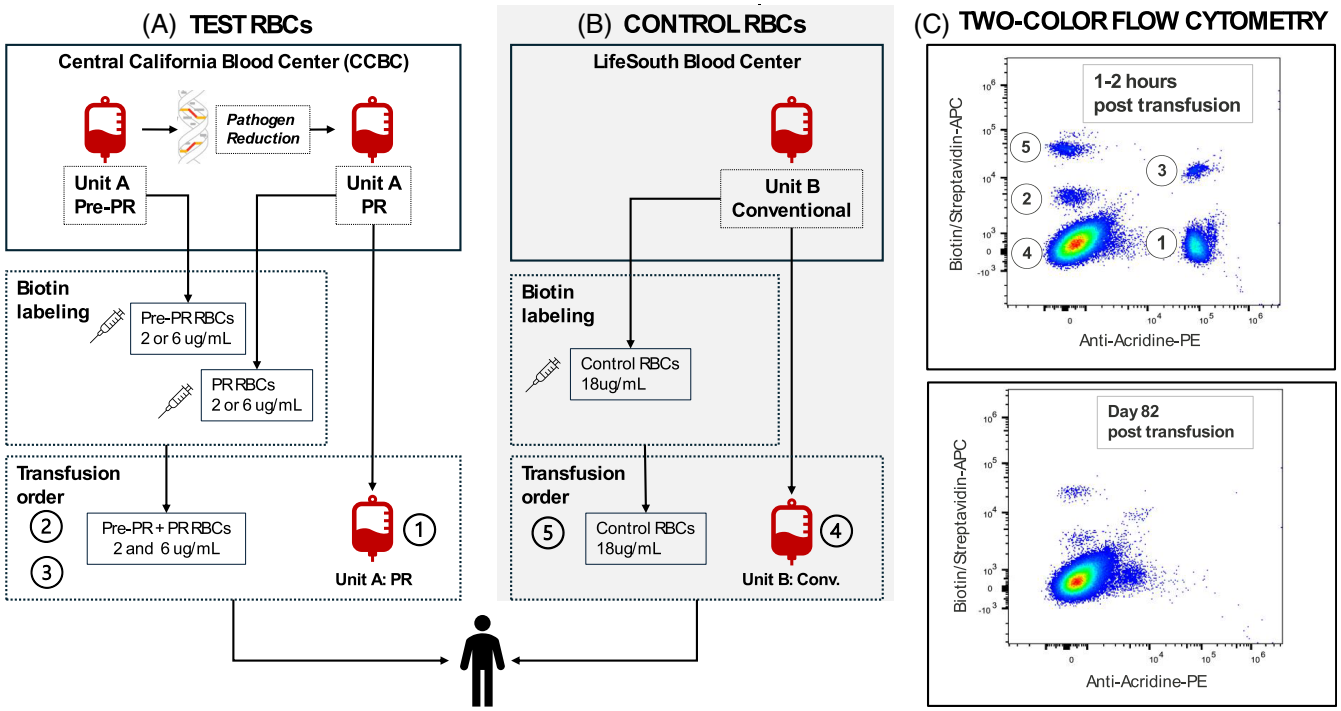

FIGURE 1 Biotinylation and transfusion procedures for (A) Pre-PR and PR-RBCs and (B) Conventional RBCs, and (C) examples of two-color flow cytometry results from subject G01 as revealed in peripheral blood collected and frozen either 1–4 h (Day 0) or 82 days (Week 12) after the study transfusions. In (A and B), the numerals 1–5 designate the order of transfusion, and in (C), the corresponding two-color flow cytometry populations. 1: PR-RBC unit (acridine +, biotin −); 2: Pre-PR-RBC aliquot (acridine −, biotin +); 3: PR-RBC aliquot (acridine +, biotin +); 4: Conventional unit (acridine −, biotin −); 5: Conventional aliquot (acridine −, biotin +). [Color figure can be viewed at wileyonlinelibrary.com]

recorded. The blood centers separated and supplied 50 mL aliquots from the Conventional RBCs and the PR unit before (Pre-PR-RBCs) and after PR treatment (PR-RBCs) for biotinylation (Figure 1). Following cross-match, biotinylation of each aliquot was performed with N-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin) under sterile cGMP conditions as described[14,17,19] to achieve different densities of biotin labeling (2 or 6 μg/mL for Pre-PR and PR-RBCs, alternating with each patient, and 18 μg/mL for Conventional RBCs). Patients were screened for biotin-RBC antibodies and PR-RBC-specific antibodies prior to the study RBC transfusion and at regular intervals up to

6 months thereafter, as previously described.[10,17,18] Baseline antibodies specific for biotin or acridine were exclusion criteria for the study.

Enrolled patients routinely received two conventional RBC units per transfusion episode with the goal to suppress hemoglobin S (HbS), as part of standard-of-care chronic transfusion therapy. All RBC units transfused were leukoreduced, HbS-negative, and phenotype matched for C/c, E/e, and K; extended phenotype matching for Fya/b, Jka/b, and S was provided for patients with past RBC alloimmunization. On study Day 0, each patient sequentially received a PR-RBC unit; Pre-PR-RBC and PR-RBC biotinylated aliquots (each ~7 mL packed RBCs); a conventional RBC unit; and a biotinylated conventional aliquot (~7 mL packed RBCs), as shown in Figure 1. Initial blood samples were obtained approximately 15 min after the final conventional aliquot transfusion (Day 0), corresponding to 1–4 h after the end of the PR-RBC transfusion. Subsequent samples were drawn on Day 1 (~24 h) post-transfusion and weekly through 16 weeks post-transfusion. Serologic evaluation for biotin and PR-RBC-specific antibodies was performed weekly on fresh samples with end-of-study serology performed at 6 months. Flow cytometry for biotin-labeling was performed locally on fresh samples obtained in EDTA tubes and stained with streptavidin-phycoerythrin (PE) as described.[17,18] RBCs samples were frozen for all early timepoints and on alternate weeks after week 2 according to manufacturers' instructions using Glycerolyte 57 solution (Fresenius Kabi, Lake Zurich, IL) for later biotin and acridine flow cytometry batched analysis by the sponsor. At the end of the study, frozen samples were deglycerolized in patient-specific batches at Cerus Corporation and analyzed in triplicate on a Cytek Aurora CS flow cytometer (Cytek, Fremont, CA). Triplicate results were averaged and background negative-Control values (<0.1%) were subtracted. RBCs ($0.5–1.0 \times 10^6$ total) were gated manually for the analysis with the exclusion of duplexes. RBCs were stained with streptavidin-allophycocyanin (APC) to detect biotin and mouse anti-acridine (2S197-2M1)[10] followed by goat anti-mouse IgG-phycoerythrin (PE) (Becton Dickinson, San Jose, CA). Quantitation of RBC surface acridine was performed using the Quantibrite-PE phycoerythrin fluorescence quantitation kit (Becton Dickinson, San Jose, CA). Patients' total percentage hemoglobin, HbS, reticulocyte counts, and total red cell counts (RCC) were monitored during routine RBC transfusions per institutional protocols (Table 1). The absolute RBC concentration (RBCs $\times 10^{12}$/L) for each population at each time point was calculated as the proportion of biotin- or acridine-positive RBCs (averaged for each unique density) multiplied by the patients' total RCC (RBCs $\times 10^{12}$/L) on that day. RBC survival at each time point was expressed as a proportion (%) relative to the initial Day 0 post-transfusion RBC concentration.

Routine antibody screening was performed as clinically indicated for subsequent transfusions. Monitoring for hemolysis within the first 4 weeks of study transfusion included weekly lactate dehydrogenase (LDH) and direct antiglobulin tests (DAT).[17,18] Reticulocyte counts, complete blood counts, and hemoglobin electrophoresis were performed weekly through 16 weeks. Spleen volumes were estimated using ultrasound techniques,[20] and pitted RBC counts (normal ≤ 1.3%, Erythrocyte Diagnostic Laboratory, Cincinnati Children's) were measured prior to baseline.

3 | STATISTICS

RBC survival was expressed as post-transfusion recovery at 24 h (PTR_{24}) and half-life (T_{50}, time point at which biotinylated RBCs were present at 50% of the concentration immediately post-transfusion). The terminal mean potential lifespan (days) was extrapolated by regression analysis of the Day 28 to Day 112 survival data to approximate the linear portion of the RBC survival curves[21] and avoid the initial highly variable non-linear period. Differences in RBC acridine density and RBC survival on study were compared by Student's *t*-test, using SAS version 9.4 (Cary, NC).

The entire transfused PR-RBC unit was weighed at manufacture, and a complete blood count was performed, allowing an assessment of the total number of PR-RBCs transfused (similar data were not recorded for the aliquots). Given the patients' percentage of acridine-positive PR-RBCs at Day 0 and the patients' RCC, mean cell volume (MCV), and hematocrit (Hct), and assuming 100% RBC recovery, each patient's estimated red cell volume and the volume of distribution of the PR-RBCs could be calculated (Table 3). The following calculations were performed:

- Estimated patient total red cell count was first calculated by dilution as: Total patient RCC = PR-RBC Total RCC × 100/(% of Acridine-positive, biotin-negative RBCs at Day 0 post-transfusion).
- Patient calculated Red Cell Volume = Total patient RCC ($\times 10^{12}$) × MCV (fL).
- Calculated PR-RBC unit volume of distribution = RCV/Hct.

Patient total blood volume (TBV) was estimated based on patient height, weight, and sex, using the Nadler formulas.[22]

4 | RESULTS

The six enrolled adult patients had hemoglobin SS (HbSS) genotype and were receiving chronic transfusion therapy (Table 1) with the goal to maintain HbS% ≤30% (or ≤50% in patients with remote stroke history or SCD hepatopathy with stable brain imaging). One splenectomized patient (G04) and four others with low splenic volumes (Table 1) had RBC pit indices >1.3%, suggestive of compromised or absent splenic function. Patient G04 had 9.8% HbC by electrophoresis at baseline, presumably due to a recent transfusion with heterozygous HbC RBCs. Patient G06 had near-normal splenic volume (102.9 mL: female lower limit 113 mL),[23] a normal RBC pit index (<1.3%), and evidence of endogenous bone marrow suppression with a pretransfusion HbS of 7.9% by electrophoresis (Figure S1). Patients G01 (weeks 3, 6, and 9—severe) and G05 (week 6—moderate) reported vaso-occlusive pain crises during the study. Patient G05 reported a mild febrile non-hemolytic transfusion reaction and a severe viral illness in study week 13. Each patient was nonreactive at baseline for biotin and PR-RBC-specific antibodies. No new autologous or allogeneic, INTERCEPT RBC-specific, or biotin-specific antibodies were detected during the study.

The Pre-PR and PR-RBC units were stored at a mean [SD] of 8.1 [3.6] days, and the conventional RBCs had a mean [SD] of 17.6 [6.2] days before transfusion. Flow cytometry results performed on the fresh samples for the biotin markers were highly correlated with the batch results from the frozen samples performed at the end of the study (correlation coefficient $R^2 = 0.91–0.98$). Two-color flow cytometry analysis of the frozen RBC samples revealed five distinct populations, as numbered in Figure 1C. On Day 0, each biotinylated aliquot comprised 0.6%–1.4% of the circulating RBCs and the entire PR-RBC unit comprised between 7.5% -13.7% of the circulating RBCs using the acridine marker. The fluorescent intensity of acridine staining was noted to decline in vivo, in both the entire PR-RBC unit [Figure 1C: population 1] and in the biotinylated PR-RBC aliquot [Figure 1C: population 3], but at different rates (Figure 2). The intensity of biotin staining was more stable over time (Figure S2). Quantitation using calibrated reference beads demonstrated that both populations expressed mean RBC surface acridine at ~5000 PE equivalents on Day 0. The entire PR-RBC unit lost 48.3% of surface acridine expression within 24 h and 84.5% by Day 7 (Figure 2). There was a significantly slower acridine loss in the dual-labeled (acridine positive, biotin positive) aliquot compared with the entire

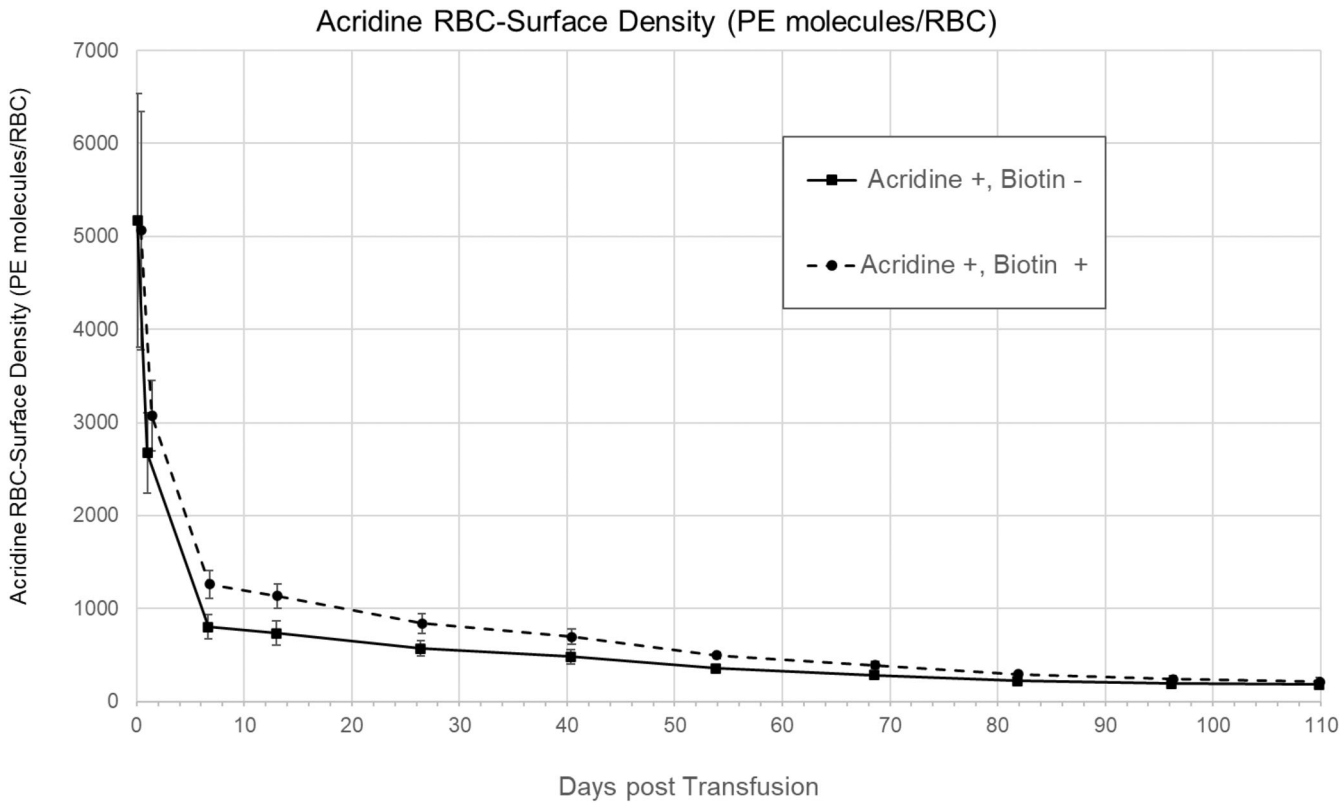

FIGURE 2 RBC surface acridine mean density as a function of time since transfusion (days). Data shown for the entire PR-RBC unit (solid line) and the dual-labeled biotinylated PR-RBC aliquot (dashed line).

PR-RBC unit, 39.3% of surface acridine expression within 24 h and 68.4% by Day 7. Both populations continued to express low levels (~180–209 molecules/RBC) of surface acridine at week 16, allowing quantitation of the number of residual circulating PR-RBCs using the acridine marker.

Over the 16-week analysis period, the absolute number of circulating RBCs (RCC × % marker positive) in each population was calculated, and RBC survival was expressed as a percentage of the Day 0 value. A comparison of the RBC survival of the entire PR-RBC unit in six patients assessed using the acridine marker and the corresponding dual-labeled aliquot assessed using the biotin marker (Figure 3) revealed almost identical survival curves (correlation coefficient $R^2 = 0.96–0.98$), demonstrating that

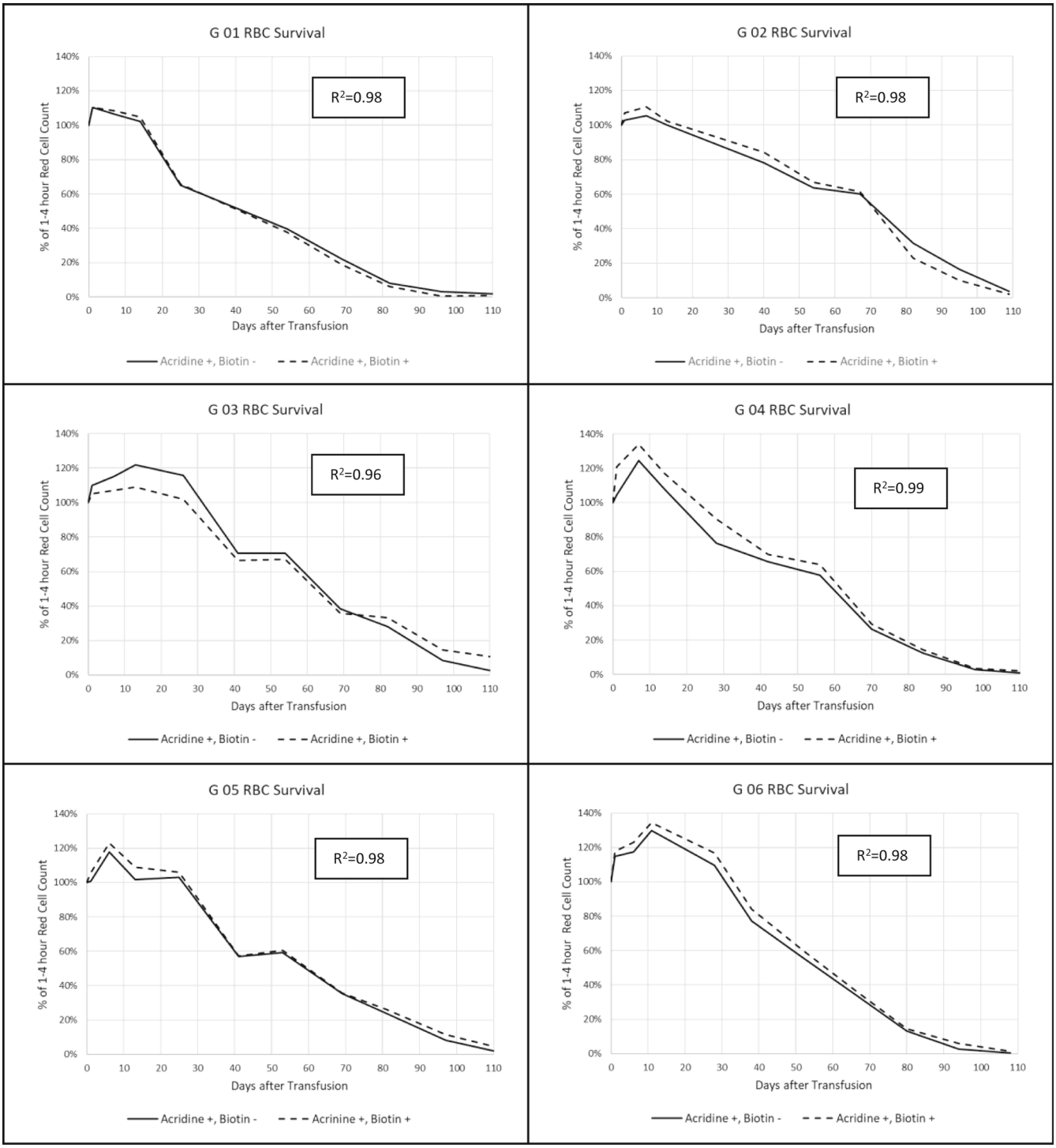

FIGURE 3 RBC survival curves of an entire unit of PR-RBCs (acridine +, biotin −: Solid line) tracked using the RBC surface acridine marker and a dual-labeled (acridine +, biotin +: Dashed line) PR-RBC aliquot tracked using the biotin marker. RBC survival is shown as the proportion of Day 0 marker (biotin or acridine) positive RCCs. Correlation coefficients (R^2) are shown.

the acridine marker was equivalent to the biotin marker for assessing PR-RBC survival. There was no impact of subsequent RBC transfusions on the study RBC survival (Figure S1).

Examining the biotin-labeled aliquot survival curves (Figure 4), all six patients showed non-linear kinetics of RBC clearance that varied by patient and by RBC component, as previously described.[17,18] The number of circulating RBCs (both PR and untreated RBCs) increased in all six patients from Day 0 to Day 1, as reflected in the mean PTR_{24} of >100% (Table 2), and remained increased in some cases for as long as 7–28 days after transfusion

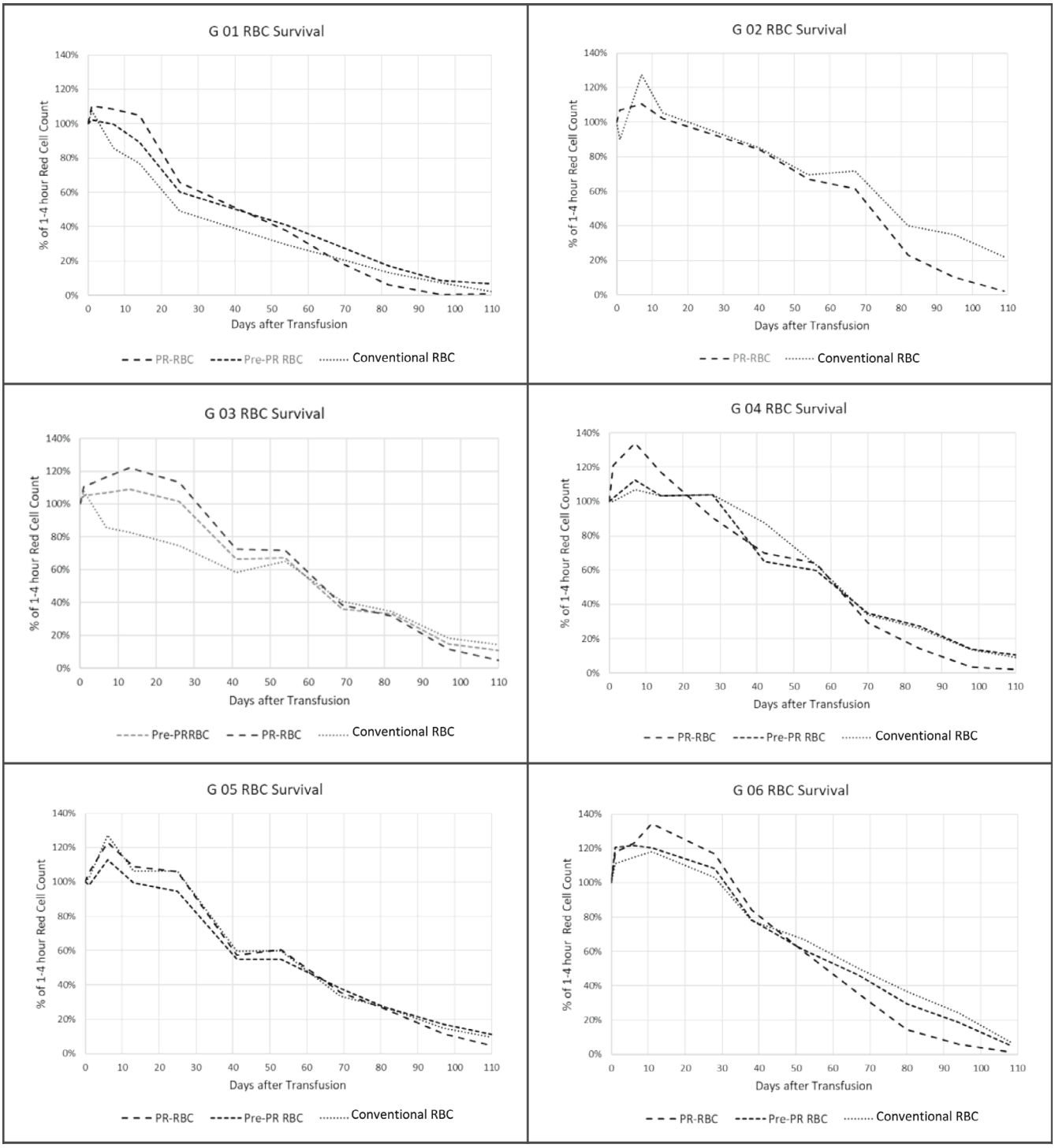

FIGURE 4 RBC survival curves of RBC aliquots tracked using RBC surface biotin markers. Results shown for each patient (G01–G06) for PR-RBC (dashed line); Pre-PR-RBC (fine dashed line) and the Conventional RBCs (dotted line). RBC survival is shown as the proportion of Day 0 marker-positive RCCs. The Conventional RBCs in patient G01 were noted to be microcytic (MCV =64.7 fL).

TABLE 2 24-h RBC recovery (PTR_{24}), half-life (T_{50}) and lifespan using biotin and acridine flow cytometric markers.

Product transfused (flow cytometric marker assessed)	Post-transfusion recovery at 24-h (PTR_{24}) % [SD]	RBC half-life (T_{50}) Days [SD]	Lifespan[a] Days [SD]
PR-RBC entire unit (acridine)	107.2 [5.3]	58.3 [10.0]	104.8 [5.7]
Pre-PR-RBC Aliquot (biotin)	106.7 [9.0]	57.6 [10.3]	115.1 [7.2]*
PR-RBC Aliquot (biotin)	111.1 [6.7]	59.2 [10.5]	104.4 [4.7]*,**
Conventional RBC Aliquot (biotin)	103.1 [7.7]	59.3 [18.3]	115.1 [9.8]**

[a]Lifespan estimated using Day 28 to Day 112 survival data.
*$p = 0.006$ paired sample students' t-test.
**$p = 0.045$, non-paired sample students' t-test.

TABLE 3 Volume of distribution of entire PR-RBCs units.

Subject	RBC unit total red cell count ($\times 10^{12}$)	Measured patient % acridine + RBCs on Day 0	Calculated patient total red cell count ($\times 10^{12}$)	Calculated volume of distribution (L)	Total blood volume (L) (Nadler)	Volume of distribution/total blood volume (%)
G01	1.126	9.7	10.5	4.4	4.8	91.7
G02	1.084	9.8	11.0	3.5	4.3	81.4
G03	1.026	9.5	10.8	2.9	4.2	69.0
G04	0.951	7.5	12.6	3.4	4.8	70.8
G05	1.225	9.7	12.6	4.6	4.7	97.9
G06	1.162	13.6	8.5	3.4	4.4	77.3

(Figure 4). After the first 28 days, during which transfused RBCs were potentially redistributing between circulatory and organ/tissue compartments, the survival curves showed a linear decline through the last assessed time-point (~Day 112). RBC survival, as reflected by the T_{50} (days), was not different between any of the biotinylated or acridine-labeled RBC populations within each patient (Table 2); however, in four of six patients (Figure 4: G01, G02, G04, and G06), a greater decrease in survival of PR-RBCs compared with the conventional or Pre-PR-RBCs after study Day 60 was noted. Linear regression of the Day 28–112 phase of the survival curves showed a significant (~9.3%) decrease in overall PR-RBC mean predicted lifespan compared with the pre-PR-RBC aliquot or the conventional RBC aliquot due to the late decline after Day 60.

Based on calculations using the number of acridine-positive PR-RBCs transfused and the percentage detected post-transfusion on Day 0, assuming 100% RBC recovery, the patients' estimated red cell volume and volume of distribution are shown in Table 3, and compared to Nadler formula estimates. The calculated volume of distribution was considerably lower than patients' estimated TBV using the Nadler formula, varying from 69.0% to 97.9% (mean 81.6%).

5 | DISCUSSION

We observed that in SCD patients, most with poor splenic function, PR-RBCs could be tracked in vivo for up to 16 weeks post-transfusion using the acridine marker, with sensitivity comparable with biotinylated RBCs. The survival kinetics of the relatively fresh Pre-PR and PR-RBCs (storage age mean [SD]: 8.1 [3.6] days) were non-linear, with an initial period of redistribution where high circulating concentrations of transfused RBCs were maintained for 7 to 28 days, followed by a more conventional linear decreasing survival curve.[21] PR-RBCs had similar initial recovery (PTR_{24}) and half-lives (T_{50}) as untreated RBCs, but a 9.3% shorter terminal lifespan (mean [SD] lifespan PR-RBCs of 104.4 [4.7] days vs. Pre-PR-RBCs 115.1 [7.2] days, $p = 0.006$). These data can be contrasted with published radiolabeling studies analyzing autologous PR-RBCs after 35-days of storage in normal volunteers that showed similar PTR_{24} and survival "area under the curve," but a 15.6% decrease in half-life (T_{50}) (PR-RBCs 33.5 [7.1] days vs. Control 39.7 [10.2] days) and a 16.4% decrease in lifespan (PR-RBCs 62.8 [10.6] days vs. Control 75.1 [13.7] days) when compared with conventional autologous control RBCs.[3] A pilot study ($n = 6$)

utilizing biotinylated autologous PR-RBCs at 35-day outdated in normal volunteers suggested a 17.4% decrease in half-life (T_{50}) and a 12.0% decrease in lifespan compared with the conventional autologous control.[16] These data emphasize the importance of considering real-world RBC storage age conditions and using allogeneic RBCs in patients when considering the clinical impact of PR processes on RBC survival. For example, a small change in terminal lifespan[21] is unlikely to impact patients receiving acute transfusions or undergoing red cell exchange, but may affect the number of transfusions needed by transfusion-dependent subjects undergoing regular repeated chronic transfusions. To this point, Aydinok et al. reported the non-inferiority of transfused amustaline/GSH PR-RBCs with respect to hemoglobin consumption in transfusion-dependent thalassemia patients utilizing a 15% non-inferiority margin.[24] The results suggest that if there is a consequence to a decreased PR-RBC terminal lifespan, it is small and within the bounds of current medical practice.

The INTERCEPT System for RBCs uses amustaline (Figure 5), a modular compound that targets and modifies nucleic acids to prevent nucleic acid replication.[7,9] Amustaline passes rapidly into cells and pathogens and intercalates into helical regions of nucleic acids via its anchoring acridine moiety. The bis-alkylating effector group crosslinks and forms adducts, while the frangible ester linker hydrolyses more slowly, releasing a negatively charged, nonreactive acridine-based byproduct, S-300.[9] The bis-alkylating pathogen inactivation function is essentially complete within hours at room temperature, and there is no measurable alkylating activity or native amustaline compound in solution after a terminal volume exchange step at 20–24 h.[7] Amustaline may react with other nucleophiles including proteins and phospholipids. Glutathione (GSH), a naturally occurring antioxidant, is included in the process to quench extracellular

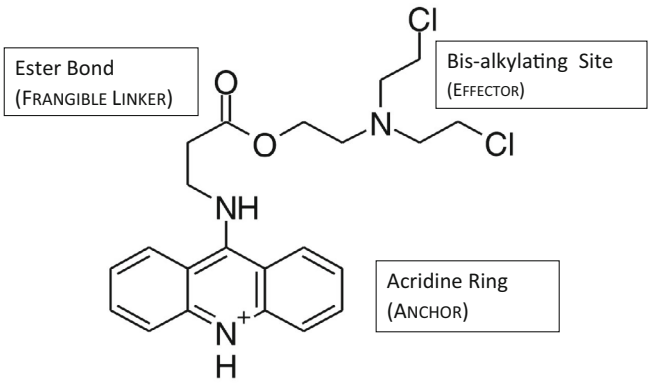

FIGURE 5 Chemical structure of amustaline (S-303) showing the bis-alkylating effector moiety, the frangible ester bond, and the acridine ring anchoring structure.[7]

reactions without a significant impact on pathogen inactivation.[25] Nevertheless, immediately after processing, PR-RBCs express ~9000–13,000 acridine molecules on their cell surfaces, although this decreases with storage at 2–6°C, resulting in 5000–7000 acridine molecules/RBC after 35 days.[9,10,26]

In this study, we found that in vivo, the cell surfaces of PR-RBCs (mean age of 8.1 days) were decorated with ~5000 acridine molecules/RBC at 1–4 h after transfusion and that this decreased exponentially to ~800–1260 molecules/RBC at 7 days and to ~180–209 molecules/RBC after 16 weeks. It is presumed that this process represents accelerated ester-bond hydrolysis at physiological temperatures and pH with the release of S-300. The macromolecular points of cell-surface attachment and the reason that the biotinylated aliquot revealed differing kinetics are not yet fully understood; however, it is notable that the biotinylation process included 8 five-volume washes in a pH 7.4 saline-dextrose-bicarbonate-phosphate buffer.[27] These washes did not alter the survival kinetics (Figure 3) or reduce the level of RBC surface acridine expression (Figure 2) relative to the unwashed entire PR-RBCs, but may potentially have altered the RBC membrane binding chemistry.

Biotinylation of RBCs has been used to investigate the recovery and survival of autologous RBCs in neonates and normal volunteers, and in the presence of anti-biotin and allogeneic and autoimmune RBC antibodies.[14,18,28–31] We previously explored the impact of donor hemoglobinopathies and showed decreased survival of allogeneic RBCs from a donor with alpha thalassemia trait. In subject G01 in our study, we observed that Conventional RBCs with microcytosis (MCV 64.7 fL) had decreased initial survival as compared to Pre-PR-RBCs (T_{50} 25 days vs. 42 days) (Figure 4). We were not able to investigate this donor further to confirm alpha thalassemia trait.

We also noted from electrophoresis studies (Figure S1 and data not shown) that Patient G04 had 9.8% HbC at baseline, presumably from prior allogeneic transfusions. The absolute amount of circulating HbC increased from 1.25 g/dL at 1–4 h post-transfusion to 1.28 g/dL at 24 h, in keeping with our finding of >100% PTR_{24} with the biotin and acridine markers on study transfusions. Patient G06 acquired 10.5% HbC at Day 73, following a routine, standard-of-care RBC transfusion. The patient's HbC level increased from a Day 73 concentration of 0.92 g/dL to a Day 80 concentration of 0.93 g/dL, again suggesting >100% PTR_{24}. That three of the 47 (6.4%) RBC study and non-study units (Table 1) that patients received during this study were taken from donors with likely hemoglobinopathies speaks to the risks of focusing blood donor recruitment efforts on African American donors who have a higher prevalence of hemoglobinopathy traits that typically do not preclude blood donation.

Transfusion of an entire PR-RBC unit allowed careful measurement of the in vivo volume of distribution based on the dilution of an absolute number of transfused RBCs and assuming 100% initial recovery. We found that the average volume of distribution was 81.6% (range 69.0% to 97.9%) of the TBV estimated using the Nadler formula (Table 3) based on height and weight.[22] These data may suggest an impact on the volume of distribution from reduced splenic volume and function; however, patient G06, who had near-normal splenic function and volume, had a volume of distribution of 77.3%, and patient G05, with no detectable splenic volume and a highly abnormal RBC pit index, demonstrated a volume of distribution of 97.9%, suggesting a lack of correlation with splenic volume. Furthermore, the PTR_{24} for all four RBC populations averaged >100% (Table 2), indicating an increase in the circulating number of transfused RBCs at 24 h, which would imply an even smaller volume of distribution. Similar increases in circulating biotinylated RBCs at 24 h after transfusion were reported in 5 of 6 patients with SCD and 6 of 6 patients with β-thalassemia by Gerritsma et al.[32] and in SCD patients by Yee et al.[17,18] Yee et al.[17] further suggested that transfused biotinylated RBCs may be transiently retained in the liver and/or spleen[33] at the initial post-transfusion timepoint and then released into the circulation at 24 h, resulting in >100% 24-h recovery. If so, the initial Day 0 PR-RBC concentration would be less than predicted by simple dilution; however, we found a higher-than-expected concentration of circulating RBCs and the initial volume of distribution of PR-RBCs to be lower than expected using the Nadler estimate of TBV. These data, and our finding that some RBC components, both PR and untreated, demonstrated >100% recovery compared with the Day 0 RBC concentration on Day 1 and for up to 28 days, suggest that we have a poor understanding of the circulation of transfused RBCs in SCD patients.

Our study was limited by a small number of patients and a lack of an independent means to assess each patient's TBV for quantitative comparisons. Nevertheless, the ability to track multiple RBC populations in each patient provided substantial hypothesis-generating data. Furthermore, the study clearly demonstrates that the acridine marker for flow cytometry offers a new tool to explore transfusion biology when using amustaline/glutathione PR-RBCs. In this vein, we recently reported persistent circulating PR-RBCs with low surface acridine concentrations in five patients with treatment-emergent INTERCEPT RBC-specific antibodies, demonstrating the utility of the acridine marker to assess the clinical significance of RBC alloantibodies.[34]

ACKNOWLEDGMENTS

Marianne Yee received funding from the National Heart, Lung, and Blood Institute of the National Institutes of Health under award number K23HL146901. The authors thank Lifesouth Community Blood Centers and Central California Blood Center for their support and assistance with this study. Thank you to Dr. Tom Gniadek for reviewing the manuscript and providing useful insights.

FUNDING INFORMATION

The study was funded by the US Department of Health and Human Services Biomedical Advanced Research and Development Authority (BARDA) under contract HHS010020160009c and supported in part by an NIH/NHLBI award (K23HL146904 PI: Yee).

CONFLICT OF INTEREST STATEMENT

RJB, JPP, CK, SH, AE, LC, and NM are employees of Cerus Corporation, the sponsor of the study. RMF serves on a medical advisory board and receives research funding from Cerus; he serves as a consultant for REDSIV-Pediatric, which is funded by the NIH/NHLBI. JR, MEMY, and PEZ have no conflicts to declare.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Benjamin RJ, Pitman JP, Karim C, Huang S, Erickson A, Corash L, et al. A novel acridine flow cytometry marker to track post-transfusion amustaline/glutathione pathogen-reduced red blood cell survival in sickle cell disease patients. Transfusion. 2025;65(5):909–20. https://doi.org/10.1111/trf.18245