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Multiparameter Flow Cytometry for the Diagnosis and Monitoring of Small GPI-deficient Cellular Populations

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Abstract

Background—Glycosyl-phosphatidylinositol (GPI)-negative blood cells are diagnostic for Paroxysmal Nocturnal Hemoglobinuria (PNH). Marrow failure states are often associated with GPI negative cell populations. Quantification of small clonal populations of GPI negative cells influences clinical decisions to administer immunosuppressive therapy in marrow failure states (aplastic anemia or myelodysplastic syndrome) and to monitor minimal residual disease after allogeneic blood or marrow transplantation (BMT). We studied the reliability of high resolution flow cytometry markers operating at the limits of detection.

Methods—We performed serial quantification of the PNH clone size in 38 samples using multiparameter flow cytometry. Granulocytes, monocytes and RBCs were gated using forward and side scatter as well as lineage-specific markers. The GPI-linked markers fluorescent aerolysin (FLAER), CD55 and CD59 were comparatively evaluated. We also evaluated CD16 on granulocytes and CD14 on monocytes. The sensitivity of detection by each marker was further defined by serial dilution experiments on a flow-sorted sample. Two patients had quantification of their GPI-negative clones before and after allogeneic BMT.

Results—FLAER was the most discriminant marker and allowed identification of 0.1% of GPI-negative cells despite other markers having superior signal-to-noise characteristics. CD14 and CD16 were inferior to CD55 at lower concentrations and in clinical application.

Conclusions—Multiparameter flow cytometry permits quantification of small GPI-negative clones with a sensitivity limit of about 0.1%. The single most reliable marker to monitor small granulocyte or monocyte PNH clones is FLAER, especially in conditions such as myelodysplastic syndromes or BMT, when traditional GPI-linked surface marker expression can be significantly altered.

Keywords

PNH; flow cytometry; FLAER; sensitivity; specificity; BMT

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic stem-cell disorder caused by acquired somatic mutations in the *PIG-A* gene on the X chromosome (Xp22.1) that encodes glycosyl-phosphatidylinositol (GPI), a structural anchor for several cell-surface proteins. PNH has diverse clinical manifestations, including intravascular hemolysis, bone marrow failure, and thrombophilia. Classic PNH presents with episodes of intravascular hemolysis, often accompanied by hemoglobinuria, while hypoplastic PNH is associated with bone marrow failure. The degree of hemolysis ranges from well-compensated to severe. Complicating the picture is a poorly understood tendency to thrombosis, approaching 50% for patients with active hemolysis. Allogeneic hematopoietic stem cell transplant from a histocompatible donor, the only cure, is indicated in severe cases.

The classic diagnostic method (Ham test) relies upon the sensitivity of GPI-negative erythrocytes to lysis by activated complement (1). In 1996, Hall and Rosse demonstrated that multiparameter flow cytometry was more specific and quantitative and it became the *de facto* standard method to detect PNH (2). Flow cytometry has a far greater sensitivity, the ability to examine multiple GPI-antigens, an advantage in examining multiple blood cell lineages, and provides quantitative information (3–5).

Earlier flow cytometric techniques have focused on detecting the absence of expression of GPI-linked surface markers to define the PNH clone. GPI-linked markers include CD55 (decay accelerating factor [DAF]) and CD59 (membrane inhibitor of reactive lysis [MIRL]), CD16, CD24 and CD52. Further refinement in flow cytometry has used inactive bacterial aerolysin, capable of direct binding to the GPI-anchor (6–8). Fluorescent-labeled aerolysin (FLAER) is more accurate than CD59 expression detection: small GPI-negative granulocyte populations could be detected to a level of about 0.5% and samples from healthy control subjects contained substantially fewer FLAER-negative cells (7). When performed simultaneously with standard markers, FLAER expression correlates well with standard CD55 and CD59 analysis with a detection sensitivity of 1% (9).

High resolution detection of GPI-negative clones has great clinical importance in hypoplastic PNH (associated with marrow failure) and in the monitoring of the PNH clone after allogeneic stem cell transplant. Accurate detection of even very small clones of GPI-negative cells has direct therapeutic relevance in the management of marrow failure states which are most sensitive to immunosuppressive therapy. High sensitivity is also relevant in monitoring minimal residual disease in the setting of a reduced intensity allogeneic stem cell transplant, where a state of mixed chimerism may persist following transplant.

There is, as yet, no standardization of flow cytometry techniques. While most panels of markers against GPI-antigens are uniformly good in detecting small and moderate sized clones, there is no consensus over the best combination of markers to use, gating, sensitivity and specificity. Sample preparation techniques are important and have been reviewed by Hernandez-Campo, et al (10). In addition to FLAER, CD55 and CD59 we ascertained the performance characteristics of CD16 (a GPI-linked granulocyte marker) and CD14 (a GPI-linked monocyte marker).

We describe our efforts in combining FLAER with selected, informative markers, demonstrate our ability to reliably measure exceedingly small GPI-negative clones (0.1%) and show its application in a clinical setting.

PATIENTS, MATERIALS AND METHODS

PATIENTS

Between Nov, 2006 and July, 2009, twelve patients known to have GPI negative populations provided 38 blood samples for PNH flow cytometry at different time points in the course of their evaluation and treatment. In the corresponding time frame, a total of 211 other samples tested negative. Institutional Research Board (IRB) approval was obtained at Roswell Park Cancer Institute to conduct this review of existing data and all individual patient data have been de-identified.

FLOW CYTOMETRY

SAMPLE PREPARATION—All procedures were done on ice or at 4°C. Heparinized blood (35 samples) or bone marrow (three samples) obtained from patients and healthy donors were washed at 300 × g once with phosphate buffered saline (PBS) containing 10 units/ml heparin and a second time with plain PBS. The washed blood and bone marrow were resuspended in PBS to one half its original volume, 200µg/ml mouse IgG (Caltag, Inc., Burlingame, CA) was added, to block Fc antibody binding, and the cells were incubated for 10 minutes on ice. For analysis of PIG linked antigens on WBCs, 50µL of washed, Fc blocked cells was added to tubes containing cocktails of fluorochrome labeled mAbs and FLAER reagent for WBC. All mAbs were pretitered and used at the lowest saturating concentration which gave the highest signal to noise ratio. The sample tubes were mixed and incubated in the dark on ice. After a 20-minute incubation with mAbs, freshly prepared RBC lysing solution (0.155M NH₄Cl, 10mM KHCO₃, 0.089mM EDTA) was added to these tubes. The tubes were inverted three times and held at ambient temperature for 5 minutes to promote lysis of erythrocytes. The tubes were next centrifuged for 5 minutes at 300 × g, washed with PBS, resuspended in 2% methanol free formaldehyde (Polysciences, Inc., Warrington, PA) and stored in the dark at 4°C for no longer than 3 days until analysis. For analysis of PIG linked antigens on RBCs, 10 µL of washed, Fc blocked blood was diluted into 2 mL of PBS and 50 µL of this dilution was added to tubes containing cocktails of fluorochrome labeled mAbs for RBC. The cells were handled as above for WBC except that instead of adding RBC lysing reagent after the 20 minute incubation with mAbs the cells were resuspended in PBS.

FLOW REAGENTS—The lyophilized Alexa 488 proaerolysin (FLAER reagent) was purchased from Protox Biotech (Victoria, BC) and reconstituted in PBS to a concentration of 10⁻⁶M. FLAER reagent was aliquotted and stored frozen at -20°C for no longer than 6 months until use. Antibodies to CD14 PE (MφP9), CD15 APC (HI98), and CD55 PECy5 (IA10) were purchased from BD Biosciences (San Jose CA); CD59 PE (MEM-43) was purchased from eBioscience (San Diego, CA); CD16 (3G8) and fixable-live-dead green reagent were purchased from Invitrogen (Carlsbad, CA); CD64 APC (22.2) was purchased from Trillium Diagnostics (Brewer, ME); glycophorin A (11E4B-7-6 (KC16)) was purchased from Beckman Coulter, (Fullerton, CA). The cocktails routinely used were FLAER A×488 / CD16 PE / CD55 PC5 / CD15 APC for granulocytes, FLAER A×488 / CD14 PE / CD55 PC5 / CD64 APC for monocytes and glycophorin A FITC / CD59 PE / CD55 PC5 for RBC's. In the serial dilution experiments cocktails of FLAER A×488 / CD59 PE / CD55 PC5 / CD15 APC and FLAER A×488 / CD59 PE / CD55 PC5 / CD64 APC were used for granulocytes and monocytes, respectively, in addition to the routine panels.

FLOW CYTOMETER—Cell viability was determined using fixable the fixable-dead green reagent and all samples exhibited more than 95% viable cells after processing. Cytofluorometric analysis was performed using a FACSCalibur (BD BioSciences, San Jose, CA) flow cytometer equipped with 488 nm argon-ion and 635 nm red diode lasers. Forward

scatter (FS), side scatter (SC) and four fluorescent parameters were collected with a threshold set on forward scatter to eliminate debris from list mode data. Fluorescein (FITC), phycoerythrin (PE), and phycoerythrin cyanine 5 tandem (PC5) were excited for the 488 nm laser and detected using logarithmic amplification in the FL1 (530/30 nm bandpass filter), FL2 (585/42 nm bandpass filter), and FL3 (670 nm longpass filter) channels respectively. Allophycocyanin (APC) fluorescence was excited off the 635 nm diode laser and detected in the FL4 (661/16 nm bandpass filter) channel. The data were collected on a Macintosh G4 computer (Cupertino, CA) using CellQuest software. The instrument was set up for daily use by a standard protocol using Spherotech (Lake Forest, IL) microspheres with preset set voltages and gains to confirm instrument performance. Forward, side and fluorescence channels were next checked with a fresh compensation standard consisting of healthy donor blood cells stained separately with CD45-FITC, CD4-PE, CD8-PECy5, and a cocktail of CD3 PerCP and CD45 APC. The locations of these separately stained populations were adjusted to fall within preset regions. Once these adjustments have been made, instrument settings were held constant for data collection. For each tube, a minimum of 20,000 events were routinely collected, although most (60%) had 50,000 events, using a FS threshold set to eliminate debris which gave on average after gating 2,686 monocytes (range 311–8,542), and 19,922 granulocytes (range 959–41,729) for analysis. Data were analyzed using WinList multiparameter analysis software (Verity Software House, Topsham, ME).

ANALYSIS—Different regions were used to gate analysis depending on the population being evaluated. Granulocytes, monocytes, and RBCs were individually identified using a forward and side scatter regions that separately encompassed each population and were further defined using the non-GPI lineage specific marker CD15 for granulocytes, CD64 for monocytes, and glycophorin A for RBCs. (Figure 1) As previously described (11), the anti-CD71 mAb induced slight aggregation of RBCs, however the number of aggregates were usually 1–2% of the total RBC populations and never more than 5% of the RBC population. Cells from a healthy donor control were simultaneously stained with each patient sample and used as an aid in establishing the threshold between negative to dim and positive staining for each GPI linked marker. Type II cells were resolved on RBCs using CD59 PE (Figure 1) but were not seen using any of the mAbs to detect GPI linked antigens on WBCs. The geometric mean fluorescent intensity (MFI) was determined separately for the GPI negative and GPI positive population with each marker. Signal-to-noise (S:N) ratios for each marker were calculated using the following formula: $S:N = (\text{MFI of the GPI positive population}) \div (\text{MFI of the GPI negative population})$. Higher ratios indicate greater separation between populations. The stain index (SI), another normalized functional measure for reagent comparison was also calculated for each marker. SI is defined as D/W , where D is the difference between PNH positive and negative populations and W is equal to 2 S.D. of the PNH negative population. (12)

SERIAL DILUTION ASSAY—Serial dilutions were performed by diluting peripheral blood mononuclear cell sample from a single patient with known PNH into blood from a healthy donor. Monocyte, neutrophil and RBC lineages were identified using the gating strategy outlined above and the numbers of GPI negative cells were compared using the different marker panels. The goal was to identify the panels with the greatest accuracy in reliably detecting small GPI negative populations in order to characterize assay sensitivity. Samples were run in triplicate with standard deviations estimated for each value.

CHIMERISM ANALYSIS

Chimerism analysis was performed to quantify the proportion of recipient cells in the two patients who underwent allogeneic hematopoietic stem cell transplant using the AmpFISTR Profiler Plus PCR Amplification Kit [Applied Biosystems, Foster City, California]. Briefly,

multiplex PCR was used to amplify nine microsatellite loci and a X/Y chromosome marker to quantify the level of engraftment after allogeneic transplantation. One primer of each locus-specific primer pair was dye-labeled. PCR products generated from a sample were sized by capillary electrophoresis on the ABI PRISM 3130xl Genetic Analyzer and the loci and alleles identified by color and size. After the germline DNA patterns of the patient and donor were identified, loci with alleles that differ between the two individuals were selected (informative loci). Post transplant (follow up) samples underwent PCR in duplicate, electrophoresed by capillary electrophoresis, and 2 or more informative loci were used to calculate percent engraftment. The assay is capable of reliably quantifying 5% of one individual's DNA mixed into another individual.

STATISTICAL ANALYSIS

Descriptive statistics were used to describe patient demographics. Pairwise correlations between the proportions of the GPI-negative clone defined by each marker for a given patient were determined by the Pearson product moment correlation (R). Nonparametric comparisons of S:N ratios and the Stain Index for three sets of GPI-linked markers (FLAER vs CD55 vs CD14 or 16) were performed with the Kruskal-Wallis test ($\alpha=0.05$; two-tailed); the Mann Whitney test ($\alpha=0.05$; two-tailed) was used to compare S:N ratios and the Stain Index for two sets of GPI-linked markers (CD55 vs CD59 on RBCs). Statistical analyses were performed with GraphPad Prism version 5.0 a, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS

Patients

Demographics of patients identified as having a PNH clone using flow cytometry are listed in [Table 1](#). These twelve patients provided 38 samples over time. There were eight males and four females with a median age at diagnosis of 41. All patients had cytopenias and eight were pancytopenic. Five had severe aplastic anemia, six had myelodysplastic syndrome and only one had classic hemolytic PNH. Nine had evidence of intravascular hemolysis by elevations in LDH and reductions in haptoglobin. One patient had cerebrovascular thrombosis. Two patients (#2 and 7) underwent unrelated donor allogeneic stem cell transplants, with subsequent follow up flow cytometric testing (eight samples) until elimination of all recipient cells. The gating strategy and a representative histogram are shown in Figure 1.

Measures of marker separation (Signal:Noise and Stain Index)

The signal to noise (S:N) ratio, a measure of separation between GPI positive and negative populations, was acceptable (~ 10 , i.e. $1-\log_{10}$) for all markers (Figure 2). Interestingly, only FLAER had a normal distribution of S:N ratios (Kolmogorov-Smirnov, $\alpha=0.05$). There were no statistically significant differences in the S:N ratios of the different markers on granulocytes ($p=0.12$) and red blood cells ($p=0.42$). However, CD16 and CD55 had higher S:N than FLAER on monocytes ($p<0.001$). The stain index showed similar findings to the S:N.

Serial dilution analysis

The performance of various marker panels in serial dilution analysis is shown in Figure 3. CD55 had excellent correlation to FLAER at all dilutions but CD59 correlated poorly at higher dilutions. CD59 was equivalent to CD55 on granulocytes, inferior on monocytes and superior for RBCs in this subject. CD14 and CD16 were found to have unacceptable characteristics, with the GPI-negative population not dropping below a plateau of 1% with

increasing dilution. FLAER and CD55 were the most sensitive marker in granulocytes and monocytes at concentrations < 1%; the limit of reliable detection using FLAER was found to be 0.1%.

Diagnostic accuracy in special clinical situations

Since FLAER labels the GPI-anchor itself, it is superior in situations where one or more GPI-linked surface markers might be down-regulated to falsely suggest the presence of a GPI-negative population. We utilized our high resolution technique to analyze samples in the typical clinical situation of clone sizes >1% by FLAER as well as special clinical situations associated with low clone sizes (<1% by FLAER) or in cases of discordance between FLAER vs. other GPI-linked markers.

Panel characteristics in subjects with GPI-deficient population size >1% by FLAER—Samples (n=28) from 10 patients were unambiguous with *GPI-deficient population* size were >1% by FLAER in granulocytes and/or monocytes. Correlation coefficients (R) were calculated for the percentage of GPI negative cells for each pair of markers (Table 2). In this group all the GPI-linked markers were tightly correlated with the exception of CD16 on granulocytes. Interestingly, the correlation between CD55 with CD59 on RBCs was lower than expected at 0.61.

Panel characteristics in subjects with clone sizes <1% by FLAER—Samples with FLAER clone sizes of <1% or great discrepancy (where deficiency of 2 GPI-linked markers could not be demonstrated in 2 or more cell lineages) were found in patients post transplant or with MDS (n=10). Eight samples were post-transplant (cases #2 and 7) and two samples were from subjects with MDS (cases #11 and 12). There was no significant difference between the S:N ratios for each marker in this group compared to the “large clone” population. In this group of “post transplant/MDS” there was no correlation among the GPI-linked markers (Table 2).

Complete results for these post transplant and MDS samples are shown in Table 3. Analysis of the post transplant samples was aided by the chimerism analysis that independently confirmed the extent of recipient cells by molecular techniques; in all post transplant samples this proportion was <5%. In this analysis of minimal residual disease, the persistence of GPI-negative RBC clones was not considered given the long half-life ($t_{1/2}$) of RBCs in favor of analysis of granulocytes and monocytes which have faster turnover kinetics. GPI-negative granulocytes could not be detected post transplant in either of our two patients; however, residual GPI-negative monocytes could be detected by FLAER in a proportion that declined with time as expected. The identification of GPI-negative granulocytes or monocytes by the GPI-linked markers CD55, CD16 or CD14 was extremely erratic in the post transplant setting (Figure 4). Two subjects (#11 and 12) with MDS were found to have great discrepancy in the proportions of GPI-negative granulocytes by CD55 (patient #11) and CD16 (patient #11, 12) or GPI-negative monocytes by CD55 (#11, 12) and CD14 (patient #11, 12) compared to FLAER. It is possible that these patients had a very small PNH population.

DISCUSSION

This study extends previous work showing the utility of FLAER alone (7) and in combination with other GPI-linked markers (9). Sutherland, et al. conducted a direct comparison between FLAER on monocytes (gated with CD14) versus a CD59-based assay for RBCs and showed that PNH clones were detected with the FLAER assay in 63 (11.8%) of 536 samples tested, whereas PNH RBCs were detected in only 33 (6.2%), and always

with a smaller clone size. (11) Overall, studies with FLAER have described a sensitivity ranging between 0.5 and 1% in identifying GPI negative WBCs in samples from aplastic anemia patients (9,13). There is often clinical necessity to obtain reliable information on even smaller clone sizes for purposes of offering immunosuppressive therapy to marrow failure state patients and to monitor minimal residual disease after allogeneic transplant for PNH. In the era before FLAER, high background noise made unambiguous detection of GPI negative populations difficult below the 1% threshold.

Scheinberg, et al used a threshold of 1% GPI deficient cells to identify a “PNH clone” in patients with SAA. (14) Using this threshold, only 37% of SAA patients had PNH populations >1%, and this failed to demonstrate an association of the presence of a PNH clone with response to immunosuppression. In contrast, the strategy of defining a GPI-negative cell only if both CD55 and 59 were simultaneously negative allowed lowering of the threshold of detection to 0.003% for granulocytes and 0.005% for RBCs. (15) (16) This improved the identification of PNH populations in 68% of Japanese patients with SAA and allowed the identification of a “PNH clone” to predict response to immunotherapy. Our gating strategy differs from the Japanese technique in that we labeled a cell as GPI-deficient based on the presence or absence of single markers in order to aid comparison. We show that flow cytometry incorporating FLAER can reliably identify small clones of GPI-negative cells. The limits of detection were 0.1% by FLAER and to a lesser extent with other markers. While we utilized the lyophilized formulation of FLAER, it is possible that the newer liquid formulation may have even greater sensitivity. We showed that FLAER continued to be reliable while other markers were erratic in the setting of MDS, a disease of disordered expression of surface molecules and in the inflammatory environment of an allogeneic transplant. The extreme nature of the inflammatory environment after allogeneic transplant is demonstrated by significant increases in sCD4, sCD8, sCD25, G-CSF, and IL-6 (17). Matsuda et al. also demonstrated alterations in adhesion molecules after allogeneic transplantation (18). By binding to the GPI-anchor directly rather than client surface proteins, FLAER consistently stains all normal leukocytes and is more reliable, whereas CD14, CD16, CD55 and possibly CD59 expression appear more susceptible to modulation by the developmental state of the cell or to inflammatory stimuli (19). Interestingly, FLAER produced more discriminant results despite fairly comparable S:N ratio and SI to other GPI-linked markers. The S:N ratios depend upon the characteristics of the fluorophore bound to the GPI-linked marker: Alexa 488 for FLAER, PE for CD14, PC5 for CD55 and PE for CD59. The lower S:N ratio for FLAER (8.6 in monocytes and 13.9 in granulocytes) was still adequate for fine discrimination and the higher S:N ratio for CD14 or 16 resulted not only in a high false-positive rate but also failed to identify small populations by limiting dilution. The performance characteristics of CD14 in this study differ from the experience of other groups with expertise in flow cytometry for PNH (9,11). While heterogeneous expression of CD14 may be the culprit, it is also possible that technical differences may have given rise to our discordant findings. Variability with staining CD55 or 59 on white cells may be confounded by the variable numbers of red cells and by the choice of stain/lyse/wash versus a lyse then stain approach.

Richards, et al have commented on the occurrence of false positive and negative results in flow cytometric screening for PNH, and efforts have been made to develop an external quality assessment program (20). Others have commented on the necessity of adding a lineage marker to forward versus side scatter gating because of aberrant cell structures in myelodysplastic syndromes and post transplant (21). We have applied this gating strategy consistently through our analysis using a non-GPI lineage specific marker: CD15 for granulocytes, CD64 for monocytes, and glycophorin A for RBCs.

Flow cytometry for PNH continues to evolve from a simple diagnostic test to one which can discriminate GPI-negative cells at high resolution. FLAER techniques are superior in defining small PNH populations and should be the preferred technique in the setting of bone marrow failure states and after allogeneic stem cell transplant. Caution is required in the interpretation of flow cytometry results using GPI-linked markers other than FLAER in subjects post transplant and with MDS or systemic inflammation. This study raises the question if it is time to completely abandon the other traditional GPI-linked markers and use FLAER alone for identification of small populations of granulocytes and monocytes.

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Figure 1A

Figure 1B

Figure 1. Flow cytometric gating and analysis strategy to quantify GPI deficient populations

A: GPI deficient cells in the RBC population were enumerated using an unlysed tube of washed blood that had been stained with CD71 FITC, CD59 PE and CD55 PC5. RBCs were separated from debris by defining regions (R1) around the RBC population using forward and side scatter and (R2) around glycophorin A positive cells. Analysis of CD55 and CD59 was done using histograms gated on R1 and R2. Markers M1 and M2 define the percentage of GPI deficient cells. Clearly seen in the single parameter CD59 histogram are the Type I (CD59 bright), Type II (CD59 dim) and Type III (CD59 negative) cells.

B: Enumeration of GPI deficient cells in the WBC population. Granulocytes were analyzed using a tube stained with FLAER A×488, CD16 PE, CD55 PC5, and CD15 APC and defined by creating regions (R3) around the high side scatter population on a forward versus side scatter plot and then on the CD15 positive cells (R5). The number of FLAER, CD16 and CD55 negative to dim cells were determined using histograms gated on R3 and R5. M3, M4, and M5 define the percentage of GPI deficient cells for each marker. Monocytes were analyzed using a separate tube containing FLAER A×488, CD14 PE, CD55 PC5, CD64 APC. They were defined as cells exhibiting high forward scatter and moderate side scatter (R4) that were CD64 positive (R6). The number of FLAER, CD14 and CD55 negative to dim cells were determined using histograms gated on R4 and R6 using the markers M6, M7, and M8. Note in the CD14 versus FLAER dual parameter histogram the population of monocytes which is negative to dim for CD14 but positive for FLAER. This heterogeneous staining for CD14 is common in both healthy donors and patients with GPI deficiencies and may account for the poorer correlation of CD14 with FLAER.

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FIGURE 3.

Serial dilutions ($\log_{10}$ -scale) were performed on a peripheral blood mononuclear cell sample from a patient with a large PNH clone in triplicate (mean $\pm$ SD) to study assay characteristics at a low clone size. Granulocytes were gated on CD15 and scatter, monocytes on CD64 and scatter, RBCs on glycoporphin A and scatter. GPI negative cells were identified by FLAER, CD55, CD59 and CD16 (granulocytes), FLAER, CD55, CD59 and CD14 (monocytes) and CD55 and CD59 (RBCs).

FIGURE 4.

Proportion of GPI negative cells measured serially by flow cytometry on peripheral blood or marrow performed on patient #2 before (day -72) and after allogeneic stem cell transplant.

Panel A: monocytes stained with FLAER, anti-CD55 and anti-CD14. Panel B: granulocytes stained with FLAER, anti-CD55 and anti-CD16.

Residual recipient cells by chimerism analysis (accuracy $\pm 5\%$) is shown below each time point. Of the GPI-linked markers, FLAER corresponds best with the declining trend in residual recipient cells, whereas other markers show aberrant expression at multiple time-points.

Patient characteristics

Table 1

ID	Sex	Age	Reason for referral	Diagnosis	Intravascular hemolysis	Hypocellular marrow	Previous immunosuppression	Thrombosis	BMT
1	M	40	pancytopenia	SAA	Yes	Yes	ATG	No	
2	F	21	pancytopenia	MDS	Yes	Yes	ATG	No	Yes
3	M	55	anemia	Classic PNH	Yes	No	No	No	
4	F	28	pancytopenia	SAA, PNH	Yes	Yes	ATG	Yes	
5	M	41	pancytopenia	SAA	Yes	Yes	ATG	No	
6	M	16	pancytopenia	SAA	Yes	Yes	ATG, cyclosporine	No	
7	F	40	thrombocytopenia	MDS	Yes	Yes	No	No	Yes
8	F	22	pancytopenia	SAA	Yes	Yes	ATG, cyclosporine, MMF	No	
9	M	60	thrombocytopenia	MDS	No	No	No	No	
10	M	53	thrombocytopenia	MDS	No	Yes	No	No	
11	M	51	anemia	MDS	Yes	No	No	No	
12	M	66	pancytopenia	MDS	No	NA	6 MP for ulcerative colitis	No	

6MP, 6-mercaptopurine; ATG, antithymocyte globulin; BMT, blood or marrow Transplant; F, female; M, male; MDS, myelodysplastic syndrome; MMF, Mycophenolic Acid; NA, not available; PNH, paroxysmal nocturnal hemoglobinuria; SAA, severe aplastic anemia.

Table 2

Correlations of PNH clone size with different GPI-linked markers

Comparison	GPI-def > 1%	Post transplant/MDS
	correlation	correlation
Granulocyte FLAER vs. CD55	0.98	0.02
Granulocyte FLAER vs. CD16	−0.13	−0.27
Granulocyte CD16 vs. CD55	−0.16	0.52
Monocyte FLAER vs. CD55	0.97	−0.18
Monocyte FLAER vs. CD14	0.85	0.38
Monocyte CD14 vs. CD55	0.78	0.07
RBC CD55 vs. CD59	0.61	0.53

Table 3

Characteristics of PNH clone size in post-transplant and MDS patients

Patient	Days post BMT	Granulocytes			Monocytes			% Recipient Chimerism
		FLAER%	CD55%	CD16%	FLAER%	CD55%	CD14%	
#2 (PB)	32	0.6	3	8.5	0.5	0.6	9.6	0
#2 (BM)	32	0.4	14.4	44.7	5.2	3.3	21.9	1.5
#2 (BM)	95	0.6	10.5	34.7	8.2	1.7	19.3	0
#2 (PB)	118	0.7	0.6	16.8	0.9	2.7	13.2	ND
#2 (PB)	129	0.7	8.5	31.9	12.8	3.3	47.5	0
#2 (PB)	160	0.1	0.5	5.4	0.1	0	1.2	ND
#2 (PB)	216	0.3	0.9	42.4	0.2	1	3.9	2.5
#7 (PB)	37	1.7	28.4	46	33.4	1.4	14.4	1
#11 (PB)	NA	0	49.5	60	0	47.7	14.9	NA
#12 (PB)	NA	0.1	0	68.7	0.4	3.1	3.8	NA

Patients #2 and #7 underwent allogeneic BMT and were subsequently monitored by flow cytometry for minimal residual disease. Chimerism analysis has an accuracy of +/-5%. Patients #11 and #12 with MDS meet criteria for presence of a PNH clone on the basis of two-GPI linked markers but this is negated by FLAER data.

BM, bone marrow sample; BMT, blood or marrow transplantation; NA, not applicable; ND, not done; PB, peripheral blood sample