

In vitro evidence of complement activation in transplantation-associated thrombotic microangiopathy

Seth J. Rotz,¹ Nathan Luebbering,¹ Bradley P. Dixon,² Eleni Gavriilaki,^{3,4} Robert A. Brodsky,⁴ Christopher E. Dandoy,¹ Sonata Jodele,¹ and Stella M. Davies¹

¹Division of Bone Marrow Transplantation and Immune Deficiency and ²Division of Nephrology and Hypertension, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; ³Bone Marrow Transplantation Unit, Hematology Department, G. Papanicolaou Hospital, Thessaloniki, Greece; and ⁴Division of Hematology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

Key Points

- Transplantation-associated thrombotic microangiopathy is associated with complement activation in vitro.
- This data further supports the use of eculizumab for the treatment of patients with TA-TMA.

Introduction

Hematopoietic stem cell transplantation (HSCT)–associated thrombotic microangiopathy (TA-TMA) significantly affects transplantation-related morbidity and mortality.^{1–5} The cause of TA-TMA has remained unknown over the past several decades, limiting treatment options to nonspecific therapies adapted from other diseases.^{6,7} The role of complement activation in TA-TMA is controversial; moreover, the magnitude of any role is debated. Recent data demonstrating remission of TA-TMA after use of the anti-C5 monoclonal antibody eculizumab suggest a central pathophysiologic role of the complement cascade.^{8–10} The modified Ham test uses *PIGA*-null TF-1 cells, which lack expression of the complement regulatory proteins CD55 and CD59, rendering them increasingly vulnerable to complement-mediated cell death.¹¹ This test has been used in the research setting to distinguish microangiopathic diseases based on the degree of complement activation.^{11,12} We aimed to determine if TA-TMA is associated with complement activation in vitro using a modified Ham test.

Methods

After obtaining institutional review board approval, we assayed 5 samples of pooled normal human serum (NHS; Sigma-Aldrich, St Louis, MO), serum samples from 6 patients diagnosed with TA-TMA, and serum samples from 5 patients post-HSCT without TA-TMA. This study was conducted in accordance with the Declaration of Helsinki. Six replicates of each assay were performed. Patient samples had been collected at weekly intervals during HSCT and stored at -80°C in the Cincinnati Children's Hospital HSCT repository. Samples from patients with TA-TMA were obtained after a diagnosis of TA-TMA was made² but before any anticomplement therapy was administered.

PIGA-null TF1 cells were maintained in culture as previously described.^{11,13} *PIGA*-null TF-1 cells lack expression of the complement regulatory proteins CD55 and CD59, rendering them increasingly vulnerable to complement-mediated cell death.¹¹ We performed the modified Ham test essentially as described by Gavriilaki et al.¹¹ Briefly, we assayed *PIGA*-null TF-1 cells via flow cytometry to confirm they lacked CD59 expression. We plated cells in 96-well V-bottom plates (Corning, New York, NY) until confluent and then washed with phosphate-buffered saline. Serum was diluted in gelatin veronal buffer (Sigma-Aldrich) at a concentration of 1:5 to a total volume of 100 μL . Cells were incubated with serum for 30 minutes at 37°C . Cells were washed with phosphate-buffered saline and incubated with the cell proliferation reagent WST-1 (Roche, Basel, Switzerland) for 2 hours at 37°C . We diluted WST-1 in the cell-culture medium at a concentration of 1:10 and added 100 μL of WST-1 solution per well. The colorimetric assay is based on cleavage of WST-1 by mitochondrial dehydrogenases in viable cells.^{11,14} We measured absorbance in a SpectraMax 190 Reader (Molecular Devices, Sunnyvale, CA) at 440 nm with a reference wavelength at 600 nm.

Heat-inactivated serum was used as a negative control by taking the same patient's serum and incubating at 56°C for 30 minutes. We used lipopolysaccharide 1 mg/mL (Sigma-Aldrich), a potent activator of the complement cascade,^{15,16} as a positive control. We incubated the serum solution at a

Table 1. Patient characteristics

Patient	TA-TMA	Day post-HSCT	LDH (normal range), U/L	Haptoglobin (normal range, 16-200), mg/dL	sC5b-9 (normal range, 72-244), ng/mL	Serum creatinine (current-to-baseline ratio)	Schistocytes	Urine protein-to-creatinine ratio	Quantity of antihypertensives	Treated with eculizumab
1	Yes	+96	436 (135-395)	<7	301	0.5	None	7.2	4	Yes, day +100
2	Yes	+6	1293 (155-450)	9	196	0.9	1+	3.2	2	Yes, day +49
3	Yes	+1 (auto 2 of 2)	573 (135-395)	114	243	2.3	None	7.9	1	Yes, day +98
4	Yes	+44	377 (100-325)	23	NA	2.1	None	0.3	1	No
5	Yes	+61	437 (135-395)	112	369	0.8	None	5.3	4	Yes, day +133
6	Yes	+38	328 (87-246)	22	338	1.9	1+	18.7	4	Yes, day +128
7	No	+28	218 (135-395)	<7	68	0.9	None	0.4	2	No
8	No	+25 (auto 2 of 2)	NA	NA	NA	0.9	None	NA	0	No
9	No	+82	NA	NA	NA	1.2	None	NA	1	No
10	No	+26	203 (155-450)	149	NA	0.8	None	3.8	1	No
11	No	+28 (auto 1 of 4)	264 (100-325)	145	NA	1.3	None	1.2	1	No

Laboratory values are from the day the tested patient sample was collected. Lactate dehydrogenase (LDH) normal range values vary by age. Auto, autologous transplantation; NA, not applicable (laboratory value not obtained in patient at given time).

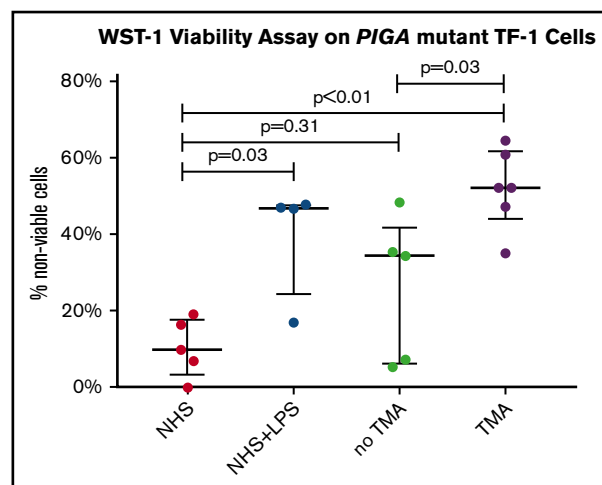


Figure 1. WST-1 viability assay on PIGA-mutant TF-1 cells. Percentage of nonviable cells (circles), with median (wide horizontal line) and interquartile range (narrow horizontal line). LPS, lipopolysaccharide.

concentration of 1:100 with lipopolysaccharide at 37°C for 10 minutes, then at a minimum of 5 minutes on ice before incubation with cells.

We normalized absorbance values of each sample after subtraction of the absorbance value of a blank cell containing 100 μ L of the WST-1 solution. Percentage of viable cells was expressed as a ratio of the absorbance of each sample multiplied by 100 to the absorbance of the same sample's heat-inactivated control. We determined cell nonviability using the following equation: $(100\% - [\text{sample absorbance}/\text{heat-inactivated sample absorbance}])$. We exported data to Prism (GraphPad Software, La Jolla, CA) for analysis. We compared groups using a Wilcoxon rank sum test.

Results and discussion

Samples were obtained a mean of 39.6 days post-HSCT (Table 1; supplemental Table 1). Patients with TA-TMA had higher median levels of lactate dehydrogenase (408 vs 218 U/L; $P = .02$) at the time of sample procurement. Patients with TA-TMA were receiving a median of 3 antihypertensive medications, whereas those without TA-TMA were receiving a median of 1 antihypertensive. Four of 5 patients with TA-TMA were eventually treated with eculizumab (after sample procurement). We identified a greater percentage of nonviable PIGA-negative cells after incubation with serum from patients with TA-TMA than patients post-HSCT without TA-TMA (median, 52.3% vs 34.5%; $P = .03$) or pooled NHS (median, 52.3% vs 9.9%; $P < .01$; Figure 1). Patients without TA-TMA did not significantly differ from pooled NHS (median, 34.5% vs 9.9%, $P = .31$).

Our data further demonstrate that TA-TMA is associated with complement activation in vitro. Our assay results corroborate the role of complement in TA-TMA and support the clinical use of eculizumab for the treatment of patients with TA-TMA. Despite our findings, the mechanism of complement activation in TA-TMA remains unclear. Further investigation into the genetic underpinnings and clinical causes of complement activation post-HSCT may improve risk stratification for TA-TMA and is ongoing in our laboratory.¹⁰ This report is somewhat limited by its small sample

size. Future refinement of this assay may aid in the diagnosis of TA-TMA, but it is not yet ready for clinical use on the basis of this limited sample size. Moreover, comparison with flow cytometry- and immunohistochemistry-based assays in development for atypical hemolytic uremic syndrome may also be helpful.^{11,17} Additionally, we were unable to stratify samples for this assay based on TA-TMA risk because of small sample size. Additional studies including patients with TA-TMA of varying severity (based on levels of sC5b-9 and urine protein)² may help better define who may benefit from complement blockade therapy.

Authorship

Contribution: S.J.R., S.J., C.E.D., S.M.D., and B.P.D. designed research; S.J.R. and N.L. performed research; R.A.B., E.G., and B.P.D. contributed vital new reagents or analytical tools; S.J.R. and N.L. collected data; S.J.R., N.L., and C.E.D. analyzed and interpreted data; S.J.R. and N.L. performed statistical analysis; and S.J.R., N.L., S.J., and S.M.D. wrote the manuscript.

References

1. Gavriilaki E, Sakellari I, Anagnostopoulos A, Brodsky R. Transplant-associated thrombotic microangiopathy: opening Pandora's box [published online ahead of print 13 March 2017]. *Bone Marrow Transplant*. doi:10.1038/bmt.2017.39.
2. Jodele S, Davies SM, Lane A, et al. Diagnostic and risk criteria for HSCT-associated thrombotic microangiopathy: a study in children and young adults. *Blood*. 2014;124(4):645-653.
3. Rotz SJ, Dandoy CE, Davies SM. ST2 and endothelial injury as a link between GVHD and microangiopathy. *N Engl J Med*. 2017;376(12):1189-1190.
4. Jodele S, Laskin BL, Dandoy CE, et al. A new paradigm: diagnosis and management of HSCT-associated thrombotic microangiopathy as multi-system endothelial injury. *Blood Rev*. 2015;29(3):191-204.
5. Laskin BL, Goebel J, Davies SM, Jodele S. Small vessels, big trouble in the kidneys and beyond: hematopoietic stem cell transplantation-associated thrombotic microangiopathy. *Blood*. 2011;118(6):1452-1462.
6. Batts ED, Lazarus HM. Diagnosis and treatment of transplantation-associated thrombotic microangiopathy: real progress or are we still waiting? *Bone Marrow Transplant*. 2007;40(8):709-719.
7. George JN. Hematopoietic stem cell transplantation-associated thrombotic microangiopathy: defining a disorder. *Bone Marrow Transplant*. 2008;41(11):917-918.
8. Jodele S, Fukuda T, Mizuno K, et al. Variable eculizumab clearance requires pharmacodynamic monitoring to optimize therapy for thrombotic microangiopathy after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2016;22(2):307-315.
9. Jodele S, Dandoy CE, Myers KC, et al. New approaches in the diagnosis, pathophysiology, and treatment of pediatric hematopoietic stem cell transplantation-associated thrombotic microangiopathy. *Transfus Apheresis Sci*. 2016;54(2):181-190.
10. Jodele S, Laskin B, Zhang K, et al. The genetic fingerprint of susceptibility to transplant associated thrombotic microangiopathy. *Biol Blood Marrow Transplant*. 2015;2(21):S98.
11. Gavriilaki E, Yuan X, Ye Z, et al. Modified Ham test for atypical hemolytic uremic syndrome. *Blood*. 2015;125(23):3637-3646.
12. Vaught AJ, Gavriilaki E, Hueppchen N, et al. Direct evidence of complement activation in HELLP syndrome: A link to atypical hemolytic uremic syndrome. *Exp Hematol*. 2016;44(5):390-398.
13. Savage WJ, Barber JP, Mukhina GL, et al. Glycosylphosphatidylinositol-anchored protein deficiency confers resistance to apoptosis in PNH. *Exp Hematol*. 2009;37(1):42-51.
14. Berridge MV, Tan AS, McCoy KD, Wang R. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochimica*. 1996;4(1):14-19.
15. Grossman N, Leive L. Complement activation via the alternative pathway by purified Salmonella lipopolysaccharide is affected by its structure but not its O-antigen length. *J Immunol*. 1984;132(1):376-385.
16. Morrison DC, Kline LF. Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). *J Immunol*. 1977;118(1):362-368.
17. Noris M, Galbusera M, Gastoldi S, et al. Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. *Blood*. 2014;124(11):1715-1726.

Conflict-of-interest disclosure: S.M.D. discloses a consultancy with Novartis AG. S.J. and S.M.D. have a US provisional patent application for methods and compositions related to transplantation-associated thrombotic microangiopathy. E.G. is supported by a European Haematology Association clinical research grant. R.A.B. serves on the advisory boards of Alexion Pharmaceuticals, Apellis Pharmaceuticals, and Achillion Pharmaceuticals. (R.A.B. has not received any honorarium from them in the past year. R.A.B. does receive grant funding for his laboratory from Alexion Pharmaceuticals.) B.P.D. received honoraria from Alexion Pharmaceuticals and has been in consultancy for both Alexion Pharmaceuticals and Achillion Pharmaceuticals and has provided expert testimony within the past 2 years. The remaining authors declare no competing financial interests.

ORCID profiles: S.J.R., 0000-0003-2896-1113; E.G., 0000-0002-8883-8208; R.A.B., 0000-0001-5741-1255; C.E.D., 0000-0002-4001-9203; S.J., 0000-0002-1791-7718.

Correspondence: Seth J. Rotz, 3333 Burnet Ave, Cincinnati, OH 45229; e-mail: rotzs@ccf.org.