Mechanisms of endothelial injury and transplant-associated thrombotic microangiopathy in tandem autologous hematopoietic stem cell transplant for neuroblastoma

Tandem autologous hematopoietic stem cell transplant (auto-HSCT) with cyclophosphamide/thiotepa (Cy/Thio) prior to the first auto-HSCT and carboplatin/etoposide/melphalan (CEM) prior to the second auto-HSCT is now standard therapy for high-risk neuroblastoma patients.[1] This regimen is associated with an increased risk of transplant-associated thrombotic microangiopathy (TA-TMA), reported as 25% in a prospective multicenter TA-TMA screening study.[2] It is unclear why neuroblastoma patients are more prone to this complication than other auto-HSCT recipients who infrequently develop TA-TMA. We hypothesized that increased endothelial injury occurs after the second auto-HSCT and that novel endothelial injury pathways contribute to TA-TMA in this unique patient population. In order to test this hypothesis, we prospectively measured circulating endothelial cells (CEC) in auto-HSCT recipients with neuroblastoma. Moreover, we performed an RNA-sequencing (RNA-seq) analysis using an *in vitro* model of TA-TMA.

Neuroblastoma patients who consented to our HSCT repository and underwent tandem auto-HSCT between July of 2019 and July of 2020 were included in the CEC study. TA-TMA was diagnosed and risk-stratified prospectively using Jodele criteria.[3] Previously published methods for immunomagnetic separation and identification of CEC were used.[4] For our *in vitro* TA-TMA model studies, we used stored serum samples from neuroblastoma patients who consented to our HSCT repository and underwent tandem auto-HSCT between 2017 and 2022. Primary kidney glomerular endothelial cells (CellBiologics, H-6014G) were used in these experiments based on the predominance of kidney involvement in TA-TMA.[5] Glomerular endothelial cells were cultured in two conditions: 20%

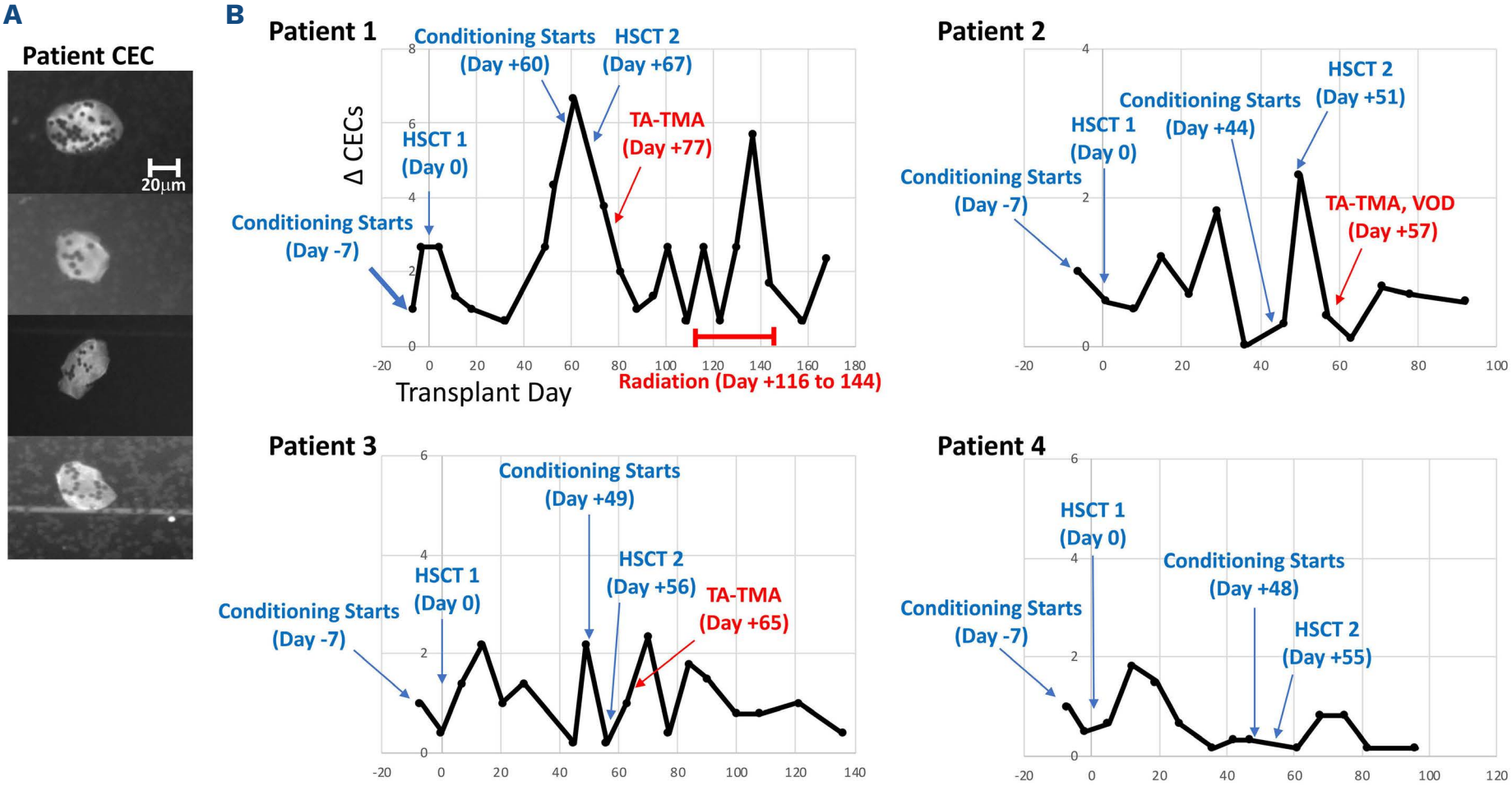

Figure 1. Circulating endothelial cells change from baseline during tandem autologous autologous hematopoietic stem cell transplant for neuroblastoma. (A) Circulating endothelial cells (CEC) isolated from autologous hematopoietic stem cell transplant (HSCT) patients and stained with acridine orange to confirm nuclear material show numerous immunomagnetic beads coupled to a CD146 antibody. (B) CEC kinetics were prospectively measured in 4 pediatric patients with neuroblastoma who underwent tandem auto-HSCT with cyclophosphamide/thiotepa (auto-HSCT 1) and carboplatin/ etoposide/ melphalan (auto-HSCT 2) conditioning. Patients 1-3 suffered from transplant-associated thrombotic microangiopathy TA-TMA and/or veno-occlusive disease (VOD) after their second auto-HSCT and all 3 of these patients more than doubled their baseline CEC count. Patient 4 had no major complications and did not double her baseline CEC count after auto-HSCT.

serum from auto-HSCT recipients with neuroblastoma who developed TA-TMA and 20% serum from auto-HSCT recipients with neuroblastoma who did not develop TA-TMA. Endothelial cells were harvested after 24 and 72 hours of culture and bulk RNA-seq was performed by the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati. RNA-seq data were deposited at Gene Expression Omnibus (GEO) and are publicly accessible (GSE227176). Analysis for differentially expressed genes (DEG) was performed using Basespace Illumina (RNA-Seq Differential Expression version 1.0.1). Enrichment analyses of DEG were performed using MSigDB and Enrichr databases.[6] Adjusted *P* values were calculated using Benjamini-Hochberg correction for false discovery. Adjusted *P* values for DEG and pathway comparisons were considered statistically significant at a cutoff of <0.05. Descriptive statistics were performed and expressed as medians with range or interquartile range (IQR). Differences in sC5b-9 levels and fold changes were analyzed using two-side *t* tests and a *P* value cutoff of <0.05 for statistical significance.

Four neuroblastoma patients were prospectively enrolled in the CEC study, and three were diagnosed with high-risk TA-TMA after their second auto-HSCT (*Online Supplementary Table S1*). TA-TMA diagnosis occurred at a median of 9 days (range, 6-10 days) after CEM conditioning for the second auto-HSCT. Two of three patients with high-risk TA-TMA received therapy with the terminal complement blocker, eculizumab (patients 1 and 3). The remaining patient with high-risk TA-TMA (patient 2) was simultaneously diagnosed with hepatic veno-occlusive disease (VOD) and responded to VOD-directed therapy (defibrotide and methylprednisolone), threfore, did not require eculizumab. CEC were measured in 68 blood samples from the four enrolled patients (median, 16 samples/patient; range, 14-22 samples/patient). All patients diagnosed with TA-TMA more than doubled their CEC count from baseline, and TA-TMA uniformly occurred after CEM conditioning for the second auto-HSCT (Figure 1B). The maximum change in CEC (ΔCEC) scores from baseline for TA-TMA patients similarly occurred during (day -6, patient 1; day -1, patient 2) or after (day 14, patient 3) CEM conditioning for the second HSCT. Patient 4 did not have any significant complications, and the ΔCEC score remained <2 for the duration of transplant (maximum ΔCEC score=1.8-fold on day 12 from Cy/Thio).

In our *in vitro* TA-TMA studies, TA-TMA serum samples (n=5) were obtained prior to eculizumab initiation and

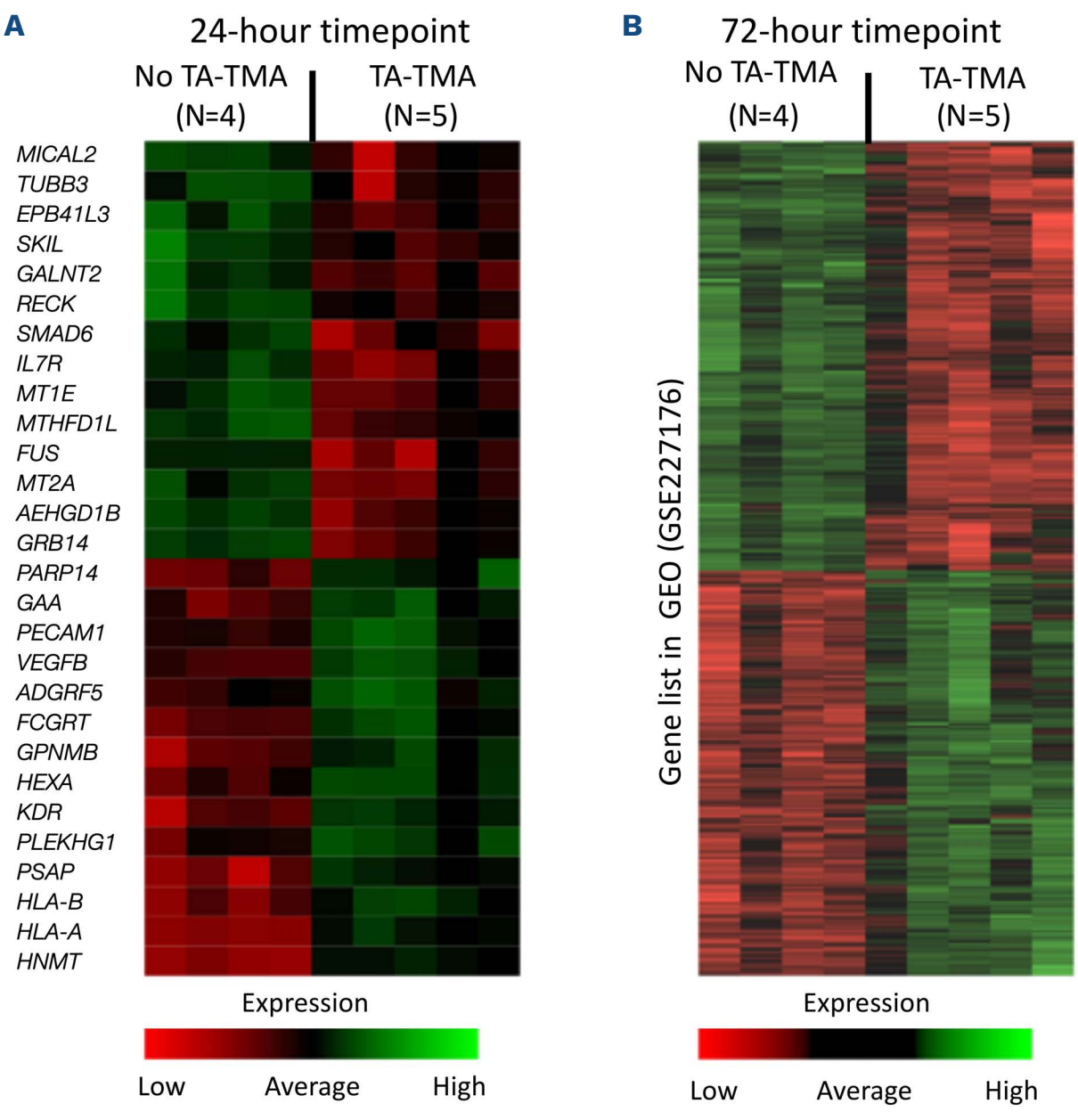

Figure 2. RNA sequencing analysis of glomerular endothelial cells exposed to serum from patients with transplant-associated thrombotic microangiopathy (TA-TMA) *versus* no TA-TMA. Heatmaps showing differentially expressed genes (DEG) in a comparison of cells exposed to serum from patients with TA-TMA and without TA-TMA at (A) 24 hours (N=28 DEG) and (B) 72 hours (N=2,051 DEG).

time point matched samples were selected as controls from patients who did not develop TA-TMA (n=4; *Online Supplementary Table S2*). Soluble C5b-9 levels were significantly higher (*P*=0.008) in TA-TMA patient serum (median, 324 ng/mL; IQR, 295.5-403 ng/mL) compared to serum from patients without TA-TMA (median, 185 ng/mL; IQR, 158.3-261.3 ng/mL; *Online Supplementary Figure S1*). RNA-seq from cells cultured for 24 hours with TA-TMA patient serum compared to control patient serum showed 28 DEG (27,914 potential genes annotated and 11,453 genes assessed for statistical significance; Figure 2A). Pathway analysis using MsigDB and Enrichr software identified four differentially expressed pathways at this time point: hypoxia, KRAS signaling, interferon-γ response, and TGF-β signaling (Table 1A).

A comparison of cells cultured for 72 hours with TA-TMA patient serum *versus* control patient serum identified 2,051 DEG (27,914 potential genes annotated and 13,338 genes assessed for statistical significance; Figure 2B). Pathway analysis of the top 500 differentially expressed genes using MsigDB and Enrichr software identified 18 differentially expressed pathways at this time point (Table 1B). The most significant pathway differences are related to cell cycle regulation (E2F targets; *P*=1E-12 and G2-M checkpoint; *P*=1E-10)). Complement (*P*=7E-6) and coagulation (*P*=8E-7) pathways were also significantly different. KRAS signaling and hypoxia pathways remained significant at 72 hours, while interferon-γ and TGF-β pathways were no longer significantly different. This perhaps suggests an early contribution of interferon-γ and TGF-β to TA-TMA initiation events.

This study measured CEC in a cohort of pediatric neuroblastoma patients undergoing tandem auto-HSCT, a population at increased risk of TA-TMA. CEC doubled from baseline in all TA-TMA patients and elevations were observed early after the second auto-HSCT, during conditioning chemotherapy. This supports our hypothesis that peak endothelial injury occurs after the second auto-HSCT for neuroblastoma leading to TA-TMA. Dvorak *et al.* describe a "three-hit hypothesis" of TA-TMA initiation where endothelial injury from conditioning chemotherapy is the second hit.[7] In tandem auto-HSCT for neuroblastoma this third hit could reasonably be CEM conditioning for the second transplant. TA-TMA onset following CEM is also earlier than typical TA-TMA onset after allogeneic HSCT, which further supports the hypothesis that CEM conditioning itself, or release of toxic intracellular molecules as a consequence of lysis of hematopoietic cells by conditioning, acts as a third endothelial hit for these patients.[3,8]

TA-TMA risk factors are well-described but the mechanism of TA-TMA initiation and maintenance remain elusive. Complement and coagulation pathways have a known role in TA-TMA and we observed differential expression of these pathways in our *in vitro* model comparing endothelial cells cultured with TA-TMA serum with endothelial cells cultured with serum from auto-HSCT recipients without TA-TMA. These complement and coagulation pathway observations support the relevance of this *in vitro* model and suggest valuable mechanistic information can be derived from these experiments. Differential expression of several novel pathways was observed in our *in vitro* TA-TMA model and likely contributes to both TA-TMA initi-

Table 1. Pathway analyses using Enrichr and MsigDB Hallmark 2020 show differentially expressed pathways in cells exposed to serum from patients with transplant-associated thrombotic microangiopathy (TA-TMA) *versus* no TA-TMA.

A	
MsigDB hallmark pathway analysis (24 hours, TA-TMA *vs.* no TA-TMA)	
Term	**Adjusted *P***
Hypoxia	9.33E-04
KRAS signaling up	9.33E-04
Interferon-γ response	9.33E-04
TGF-β signaling	0.01098898
B	
MsigDB hallmark pathway analysis (72 hours, TA-TMA *vs.* no TA-TMA)	
Term	**Adjusted *P***
E2F targets	1.07E-12
G2-M checkpoint	1.05E-10
Epithelial mesenchymal transition	1.05E-10
Coagulation	8.22E-07
Complement	7.08E-06
Apoptosis	1.1E-04
Cholesterol homeostasis	0.0033116
Myc targets V1	0.0033116
KRAS signaling Up	0.00903304
UV response down	0.01445485
Mitotic spindle	0.01445485
Hypoxia	0.01445485
Estrogen response late	0.01445485
Apical junction	0.01445485
Inflammatory response	0.01445485
Xenobiotic metabolism	0.01445485
Myogenesis	0.0357266
Myc targets V2	0.04109434

Differentially expressed pathways at (A) 24 hours (N=4 pathways) and (B) 72 hours (N=18 pathways) are shown. Adjusted *P* value calculated using Benjamini-Hochberg correction for multiple hypotheses testing. UV: ultraviolet.

ation and persistence. Hypoxia pathways were differentially expressed in cells exposed to TA-TMA serum compared to non-TA-TMA serum, consistent with recent studies that concluded hypoxia-inducible factor-1α (HIF-1α) is a key contributor to complement activation. Hypoxia pathways may be a valuable therapeutic target in TA-TMA.[9,10] Future studies can identify whether the differential expression of hypoxia pathways is related to localized or systemic hypoxia, or both.

Hypoxia pathways may also be related to mesenchymal transition which occurs when stressed endothelial cells lose their endothelial cell phenotype and acquire mesenchymal cell morphology and function.[11] This is driven primarily through TGF-β signaling[11] and we found that TGF-β signaling was differentially expressed in cells exposed to TA-TMA serum after 24 hours incubation. This early time point observation suggests TGF-β signaling occurs early in TA-TMA-mediated endothelial injury and is, therefore, an attractive therapeutic target for TA-TMA prevention and treatment. Mauro *et al*. showed that TGF-β production in microvascular cells exposed to TMA patient sera varied significantly based on the microvascular organ system of origin.[12] Pulmonary microvascular cells produced significantly more TGF-β compared to other organ systems, which may be clinically relevant to organ injury patterns in various TMA.[12] Future studies should, therefore, incorporate endothelial cells from other organ systems in this *in vitro* model to investigate tissue-specific differences in endothelial injury mechanisms from TA-TMA.

We previously reported that upregulation of interferon-responsive complement genes occurs at the onset of TA-TMA and returns to baseline after resolution of TA-TMA.[13] In the current study of our *in vitro* TA-TMA model we observed significant interferon pathway activation after 24 hours of culture with serum from patients with TA-TMA. Interestingly, interferon pathway activation preceded complement and coagulation pathway activation in our model. It is possible that genetic predispositions (e.g., interferon pathway polymorphisms) contributed to the increased interferon activation in this study, however, if if this timeline occurs *in vivo* in TA-TMA patients, early intervention with interferon blockade may prevent pathologic complement activation and the development of TA-TMA.

In conclusion, the early timing of CEC peaks and early TA-TMA diagnosis after CEM conditioning supports the hypothesis that CEM conditioning might serve as the "third hit" for TA-TMA initiation in these patients. Differences in hypoxia, interferon-γ response, TGF-β signaling and mesenchymal transition pathways may further contribute to the high incidence of TA-TMA in neuroblastoma patients who received tandem auto-HSCT. This *in vitro* human model will be useful for studying other aspects of TA-TMA biology, including novel mechanisms of TA-TMA initiation and strategies for TA-TMA treatment and prevention.

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Contributions

AS wrote the manuscript, designed the study, performed the experiments, analyzed the data, performed statistical analyses and performed chart reviews. SA, NL, BA and CC collected specimens, performed the experiments and analyzed the data. KL, AD, LS, WB and LL collected, processed and stored patient samples. SD performed statistical analyses and chart reviews. CED and AES analyzed data and reviewed and edited the manuscript. EAO, JTB

and AZ contributed to study design, reviewed and edited the manuscript and provided transcriptomic expertise. BLL analyzed data, performed statistical analyses, and reviewed and edited the manuscript. SJ analyzed the data, contributed to study design, performed chart reviews and reviewed and edited the manuscript. SMD designed and supervised the study, wrote and edited the manuscript, and analyzed the data.

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Data-sharing statement

All data presented in this manuscript will be shared upon email request. RNA-sequencing data were deposited at Gene Expression Omnibus (GEO) and are publicly accessible (GSE227176).

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