Circulating endothelial cells and the study of vascular injury in children undergoing hematopoietic stem cell transplant

Little is known about the timing or mechanisms of endothelial injury after pediatric hematopoietic stem cell transplant (HSCT) because blood vessels are hard to directly observe. Circulating endothelial cells (CEC) are a marker of endothelial injury.[1] Only one study evaluated CEC in children after HSCT and included only patients with primary immune deficiencies.[2] We prospectively evaluated CEC as a biomarker of outcomes in children undergoing HSCT and determined if CEC could be used to study endothelial injury. We observed that endothelial injury was common, particularly among patients with high-risk thrombotic microangiopathy (TMA). Although CEC are limited as a predictive biomarker by their rapid kinetics, our transmission electron microscopy (TEM), immunofluorescence microscopy (IFM) and RNA sequencing (RNAseq) data confirm CEC are a non-invasive tool to study mechanisms of endothelial injury after HSCT.

CEC were collected from allogeneic or tandem autologous HSCT recipients (for neuroblastoma, associated with TMA[3]) between July 2019 and July 2020. All subjects consented to an Institutional Review Board-approved HSCT repository. Samples were obtained weekly during inpatient admissions and weekly, when feasible, as outpatients. Established protocols were used for CEC isolation.[4] Briefly, invitrogen M450 tosylactivated dynabeads were coupled to a CD146 antibody (FisherSci, 5012898) and combined with patient blood. CEC were isolated with immunomagnetic separation (StemCell Technologies), loaded on a Nageotte cell chamber and imaged using a Zeiss Axio Imager.Z1 microscope. UEA-1 staining (Vector Lab, B1065) was performed to further confirm endothelial origin. CEC were defined as cells having five or more CD146 immunomagnetic beads attached, >10 μm in diameter, a morphology consistent with a single cell, damaged cell, or cluster of cells, and fluorescence with acridine orange (AO) staining.

A total of 642 CEC samples (median, 13 samples/patient) from 53 HSCT recipients (*Online Supplementary Table S1*)

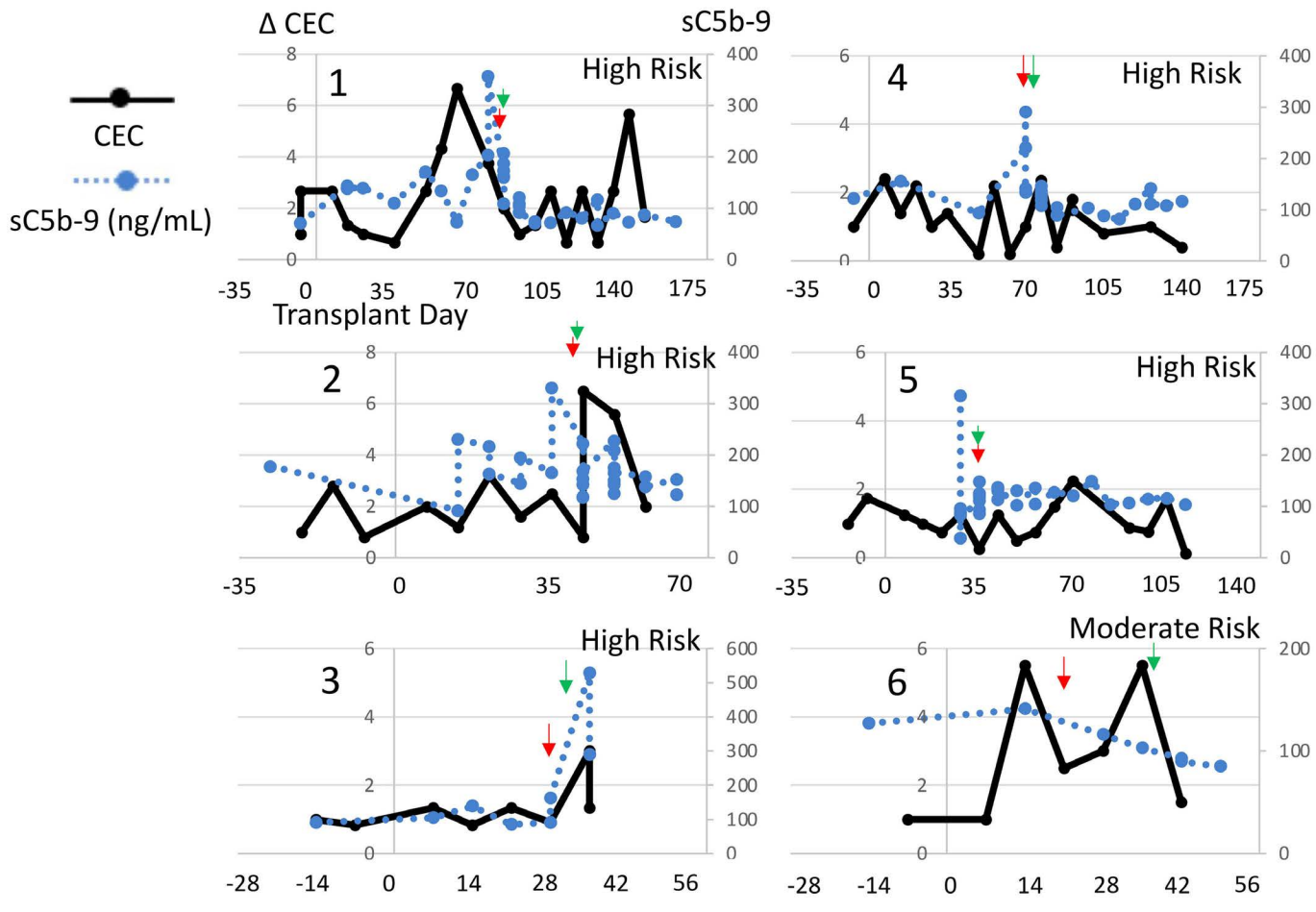

Figure 1. Circulating endothelial cells identify complement-mediated endothelial damage in high and moderate-risk thrombotic microangiopathy treated with eculizumab. (A) Circulating endothelial cell elevations from baseline (Δ CEC) and sC5b-9 levels *vs.* time in patients with thrombotic microangiopathy (TMA) requiring treatment with eculizumab. TMA diagnosis (red arrow) and start of eculizumab therapy (green arrow) are labeled along with TMA risk category using criteria by Jodele *et al.*

were analyzed. Baseline samples were obtained prior to (n=41) or during conditioning (n=12). Peak CEC counts occurred prior to stem cell infusion in 15 of 53 (28.3%) subjects or in the first 30 days after HSCT in 19 of 53 (35.9%) subjects. However, CEC peaks that occurred between days 61-90 after HSCT were significantly higher than peaks that occurred before HSCT (*P*=0.009), in the first 30 days after HSCT (*P*=0.01) or between days 31-60 (*P*=0.03; *Online Supplementary Figure S1)*. There were no associated clinical complications to explain these late CEC elevations. CEC were evaluated based on their increase relative to the first collected pre-HSCT sample for each patient (ΔCEC score). Most HSCT recipients (n=31, 58.5%) had a ΔCEC score >2 at least once after HSCT.

We then evaluated clinical associations with ΔCEC scores (*Online Supplementary Table S2*). HSCT recipients were prospectively screened for TMA and assigned to risk groups using Jodele criteria.[5,6] Moderate- or high-risk TMA occurred in 14 subjects during CEC collection. CEC elevations were temporally correlated with terminal complement (sC5b-9) activation in subjects who required eculizumab therapy (Figure 1) and in those with high-risk TMA. All subjects with high-risk TMA had a ΔCEC score >2 after HSCT. Similarly, all subjects with TMA who required treatment with eculizumab had a ΔCEC score >2 after HSCT. One patient developed high-risk TMA immediately after being diagnosed with hepatic VOD, received defibrotide, and their TMA improved without eculizumab. One patient with moderate-risk TMA was treated with eculizumab for persistent proteinuria. Three subjects required defibrotide for hepatic VOD (grade 4 [n=2], grade 2 [n=1)][7]) and all three had ΔCEC scores >2. Seven subjects were diagnosed with acute or chronic graft-*versus*-host disease (GvHD) during the study period. GvHD diagnosed outside of the collection period was excluded. ΔCEC scores were not different in subjects with GvHD compared to those without GvHD (*P*>0.99). There were also no observed differences in ΔCEC scores or absolute CEC values in MAC *versus* RIC regimens (*P*>0.99) or total body irradiation (TBI) *versus* no TBI regimens (*P*=0.64).

Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus 6 (HHV-6), herpes simplex virus (HSV) and adenovirus viremia were not temporally associated with CEC elevations. In contrast, BK polyomavirus (BKPyV) viremia was temporally associated with endothelial injury. BKPyV viremia occurred in 16 subjects (30.2%) and occurred during CEC collection in 11 subjects. Plasma BKPyV copy number was commonly, although not always, associated with absolute CEC counts and ΔCEC scores (Figure 2), perhaps reflecting a limitation of weekly testing. Subject 1 in Figure 2 had two separate episodes of BKPyV viremia and cystitis, both of which were associated with a rise in CEC and plasma BKPyV copy number. Eight of 11 subjects with BKPyV viremia had a ΔCEC score >2, and six of seven subjects with BKPyV viremia and cystitis had a ΔCEC score >2.

Given the observed association between CEC and BKPyV viremia, we hypothesized that BKPyV directly infects the endothelium and causes cellular injury. We therefore per-

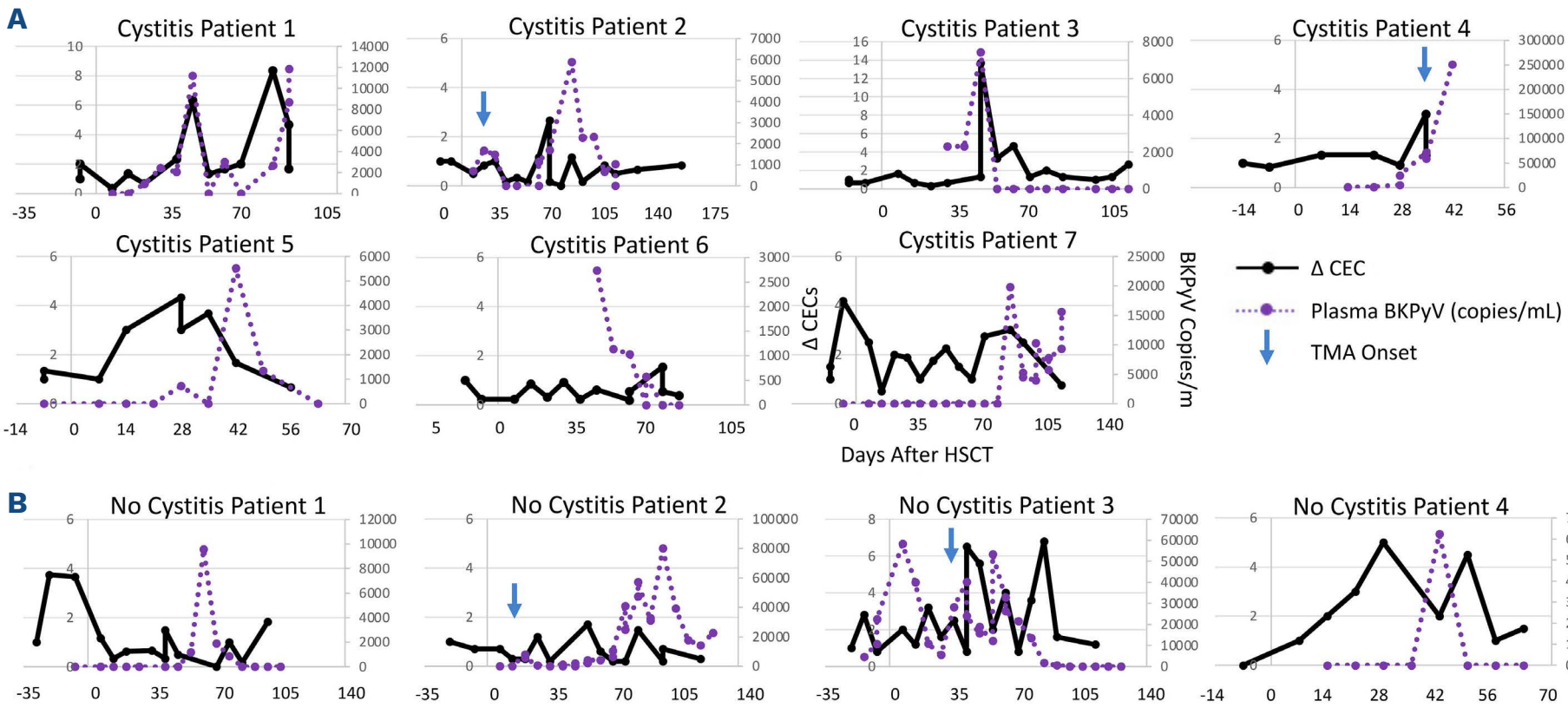

Figure 2. Circulating endothelial cell elevations are closely associated with rising BK polyomavirus viremia. Circulating endothelial cell elevations from baseline (Δ CEC) and plasma BK polyomavirus (BKPyV) copy number are shown *vs.* time in patients with cystitis (A) and without cystitis (B). Timing of hrombotic microangiopathy (TMA) onset is marked with an arrow in patients who developed TMA. Plasma BKPyV levels were obtained clinically.

formed IFM of CEC from "cystitis patient 4" in Figure 2 at two different time points using a polyomavirus-specific marker (VP1) and an endothelial marker (UEA-1). CEC in both samples co-stained with VP1 and UEA-1, confirming BKPyV infection of CEC (Figure 3A). In order to better characterize CEC morphology, TEM[8] was performed on CEC isolated from a subject with chronic high-level BKPyV viremia after HSCT. These CEC had nuclear inclusions and abnormal, fragmented mitochondria (Figure 3B). The latter indicates mitochondrial injury which is reported as a key mechanism of cellular infection with BKPyV.[9]

RNAseq (Takara SMART-Seq v4 full-length transcriptome analysis) was performed on CEC isolated from a HSCT recipient with BKPyV viremia and a HSCT recipient without an active viral infection. Genes were ranked based on the fold change between subjects. Gene set enrichment analysis (GSEA) was performed with a focus on hallmark gene set and cell type signature analysis.[10-12] CEC from the BKPyV viremia patient had upregulated KRAS (P=0.008, false discovery rate [FDR]=0.15), TNF α (P=0.002, FDR=0.11) and interferon γ (P=0.008, FDR=0.11) signaling on hallmark gene set analysis compared to the patient without any viral infection. Cell type signature analysis showed multiple cell signatures upregulated in the subject with BKPyV viremia. These included cardiac endothelial cells (P<0.001, FDR=0.14), large intestine mesenchymal cells (P<0.001, FDR=0.04), and hepatic NK/T cells (P<0.001, FDR=0.04), among others. Multiple cell signatures were upregulated in CEC from the subject without BKPyV viremia as well. These included kidney endothelial cells (P<0.001, FDR<0.001), pulmonary capillary intermediate cells (P<0.001, FDR<0.001), and pulmonary NK/T cells (P<0.001, FDR<0.001). The overall composition of cell signatures in these samples confirmed i) CEC originate from multiple organ systems and ii) mesenchymal cells and lymphocytes were present in the analysis.

To our knowledge, this is the largest analysis of CEC in pediatric HSCT patients. We observed that CEC were commonly elevated during conditioning and early post transplant. This supports that early endothelial injury occurs from release of toxic intracellular molecules as a consequence of massive cell lysis of host hematopoietic tissue during conditioning.[13] Interestingly, CEC peaks between days 61-90 were frequent and of greater magnitude than earlier peaks. These peaks were not associated with any specific clinical complication, such as TMA, VOD

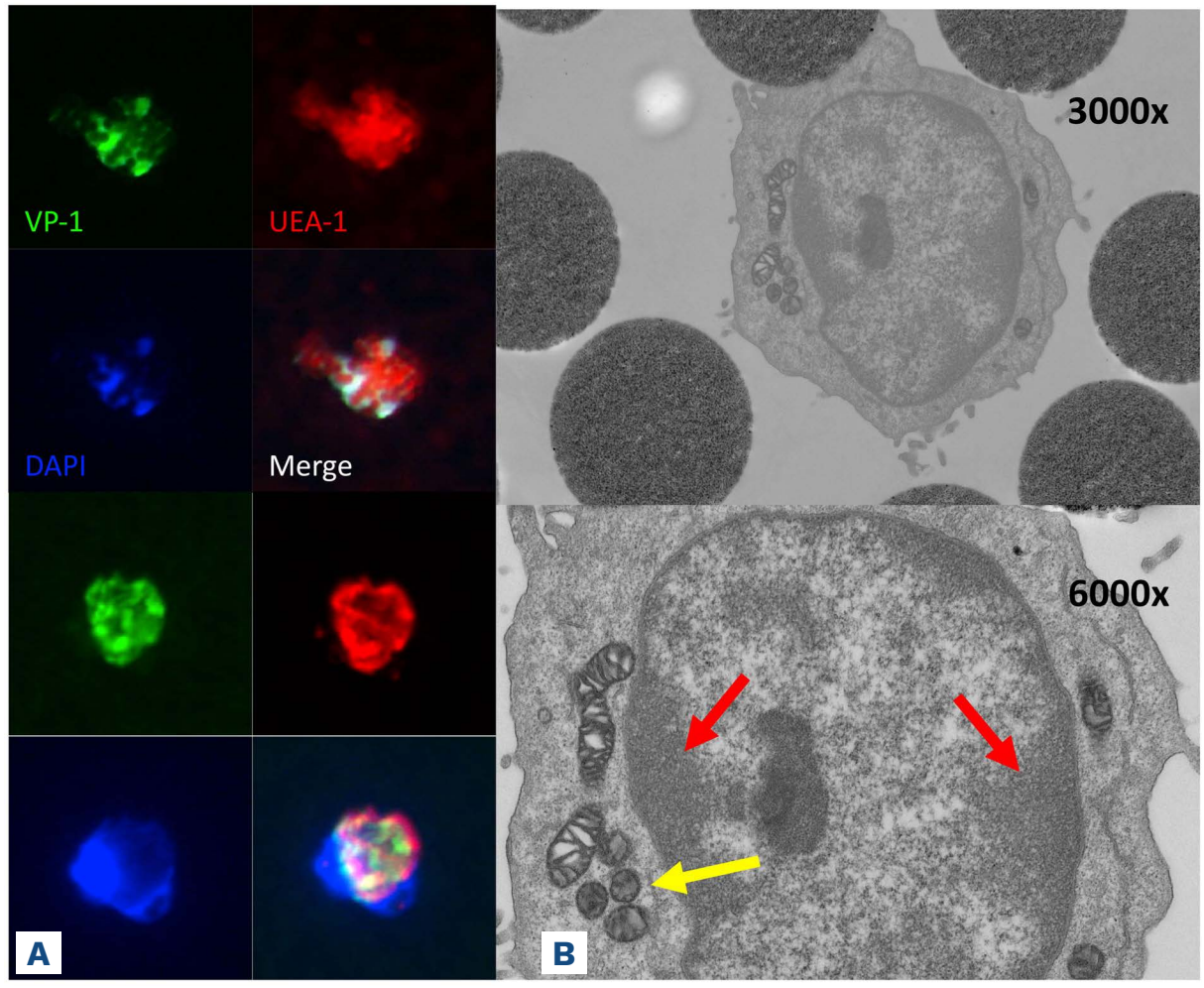

Figure 3. Multimodal imaging of circulating endothelial cells isolated from hematopoietic stem cell transplant patients with BK polyomavirus viremia. (A) Immunofluorescence microscopy (IFM) imaging of 2 circulating endothelial cells (CEC) (top, bottom) from a patient with BK polyomavirus (BKPyV) viremia shows positive staining for polyomavirus (VP-1, green), endothelial (UEA-1, red) and nuclear (DAPI, blue) markers. These CEC were isolated at two separate time points. The CEC in the top image was isolated when this subject had a plasma BKPyV copy number of 1,200 copies/mL. The CEC in the bottom image was isolated when the subject had a plasma BKPyV copy number of 251,264 copies/mL. (B) Transmission electron microscopy of a CEC from a HSCT patient with BKPyV viremia shows nuclear inclusions (red arrows) and an abnormal, fragmented mitochondrion (yellow arrow). The plasma BKPyV copy number in this subject was 7,006,235 copies/mL on the day of sample collection.

or infection. A prior longitudinal study of CEC in adults showed that CEC could remain elevated for at least 1 year after HSCT.[14] We speculate that the later CEC peaks reflect cumulative vascular injury from multiple "hits" throughout transplant (e.g., conditioning, infection, TMA).

TMA and VOD injure the endothelium and were specifically evaluated. All subjects with high-risk TMA, TMA requiring eculizumab, and VOD requiring defibrotide more than doubled their baseline CEC. CEC elevation also correlated with terminal complement activation in most, but not all patients treated with eculizumab. These findings suggest that CEC increase in children with severe HSCT-related endothelial injury but are too non-specific to serve as a biomarker. While we performed weekly CEC testing, more frequent testing would be needed to capture all early CEC peaks.

GvHD may injure the endothelium, although we did not identify a difference between CEC levels in patients with or without GvHD. The association between GvHD and CEC is complex and a prior study reported lower CEC counts in patients who develop GvHD.[15] Any associations between CEC and GvHD are limited by sample size since few patients developed GvHD during sample collection.

In addition to studying CEC kinetics, we evaluated the use of CEC as a liquid biopsy of the vascular wall. We observed a novel association between BKPyV viremia and CEC and have previously associated BKPyV viremia with a higher risk of TMA after HSCT. We hypothesized that BKPyV directly injures the endothelium and used CEC as a source of vascular tissue. IFM and TEM analyses confirmed that BKPyV can infect CEC. RNAseq of CEC was feasible and identified differences in inflammatory pathways and endothelial cell origin. Larger studies are needed to test if patterns of organ-specific endothelial cell enrichment correlate with clinical disease.

In summary, we observed that endothelial injury commonly occurs in pediatric HSCT recipients and CEC elevations correlate with endothelial injury. Although there are limitations to the use of CEC as a clinical biomarker, CEC can serve as a liquid biopsy and are a tractable, non-invasive tool to study vascular biology. This type of application led to the novel association between BKPyV and endothelial injury, which merits further study to establish clinical relevance.

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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. SD performed statistical analyses. KL, AD, LS, WB and LG collected, processed and stored patient samples. CED and AES analyzed data and reviewed and edited the manuscript. EAO and JTB reviewed and edited the manuscript and provided virologic expertise. AZ reviewed and interpreted RNAseq data. 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.

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