

1 **Single-nuclear RNA sequencing of endomyocardial biopsies identifies persistence of donor-recipient**
2 **chimerism with distinct signatures in severe cardiac allograft vasculopathy**

3 *Kaushik Amancherla MD¹, Juan Qin PhD³, Michelle L Hulke PhD⁴, Ryan D Pfeiffer BS⁴, Vineet Agrawal MD*
4 *PhD¹, Quanhu Sheng PhD², Yaomin Xu PhD², Kelly H Schlendorf MD MHS¹, JoAnn Lindenfeld MD¹, Ravi V*
5 *Shah MD¹, Jane E Freedman MD¹, *Nathan R Tucker PhD⁴, and *Javid Moslehi MD³.*

6 **Authorship note:** *Nathan Tucker and *Javid Moslehi are co-senior authors.

7 **Affiliations:** Vanderbilt University Medical Center: ¹Division of Cardiovascular Medicine (KA, VA, KHS, JL,
8 RVS, JEF) and ²Department of Biostatistics (QS, YX). University of California San Francisco: ³Section of
9 Cardio-Oncology & Immunology (JQ, JM). ⁴Masonic Medical Research Institute (MLH, RDP, NRT).

10 **Conflicts of interest disclosures:** Dr. Shah is supported in part by grants from the National Institutes of
11 Health and the American Heart Association. In the past 24 months, Dr. Shah has served as a consultant for
12 Myokardia, Cytokinetics, and Best Doctors, and has been on a scientific advisory board for Amgen. Dr. Shah is
13 a co-inventor on a patent for ex-RNAs signatures of cardiac remodeling. Dr. Moslehi has served on advisory
14 boards for Bristol Myers Squibb, AstraZeneca, Myovant, Cytokinetics, Takeda, BeiGene, Kiniksa, Kurome
15 Therapeutics, Pfizer and is supported by National Institutes of Health grants (R01HL141466, R01HL155990,
16 and R01HL156021). The other authors have no conflicts of interest to disclose. All other authors declare no
17 relevant conflicts of interest.

18 **Funding:** Dr. Amancherla is supported by an American Heart Association Career Development Award
19 (#929347). R01HL141466, Team Phenomenal Hope Grant, K01HL140187

20 **Word count:** 789

21 **Figures:** 1

22 **References:** 5

23 **Corresponding author:**

24 Kaushik Amancherla, M.D., Fellow in Advanced Heart Failure & Transplant Cardiology, Vanderbilt University
25 Medical Center, 2220 Pierce Avenue, Nashville, Tennessee 37232, USA. E-mail:
26 kaushik.amancherla@vumc.org.

27

28

29

30

31 **ABSTRACT**

32 Cardiac allograft vasculopathy (CAV) is the leading cause of late allograft failure and mortality after heart
33 transplantation. As current standards of diagnosis and treatment of CAV have significant limitations,
34 understanding cell-specific responses may prove critical for developing improved detection strategies and
35 novel therapeutics. This study is the first to successfully utilize human endomyocardial biopsy (EMB) samples
36 to isolate large numbers of intact nuclei for single-nuclear transcriptomics. These data also lay the groundwork
37 for ongoing experiments to study serial, routinely-collected EMB specimens after heart transplantation to
38 identify novel biomarkers and pathways through which early CAV pathogenesis can be interrupted, thereby
39 prolonging allograft survival.

40 **INTRODUCTION**

41 Cardiac allograft vasculopathy (CAV) is the leading cause of late allograft failure and mortality after heart
42 transplantation. As current standards of diagnosis and treatment of CAV have significant limitations,
43 understanding cell-specific responses may prove critical for developing improved detection strategies and
44 novel therapeutics. This study is the first to successfully utilize human endomyocardial biopsy (EMB) samples
45 to isolate large numbers of intact nuclei for single-nuclear transcriptomics. These data also lay the groundwork
46 for ongoing experiments to study serial, routinely-collected EMB specimens after heart transplantation to
47 identify novel biomarkers and pathways through which early CAV pathogenesis can be interrupted, thereby
48 prolonging allograft survival.

49 Cardiac allograft vasculopathy (CAV) is the leading cause of late allograft failure and mortality after heart
50 transplantation¹. Histologically, CAV is chronic vascular rejection characterized by diffuse intimal thickening of
51 macro- and microvasculature. While *in vitro* cellular models and *in vivo* histologic observations suggest
52 coordinated responses of endothelial, fibroblast, and smooth muscle cells in CAV pathology, cell-specific
53 transcriptional signatures among these in the transplanted human heart have not been studied. As current
54 standards of diagnosis and treatment of CAV have significant limitations, understanding cell-specific responses
55 may prove critical for developing improved detection strategies and novel therapeutics.

56 Here, we used single-nuclear RNA sequencing (snRNA-seq) to elucidate the transcriptomic landscape of CAV.
57 Importantly, we establish the feasibility of performing snRNA-seq from human endomyocardial biopsy (EMB)

58 specimens (3-10 mg in size) obtained at the time of right heart catheterization, enabling high-resolution
59 molecular profiling of samples collected during routine clinical practice. We compared tissue obtained at the
60 time of re-transplantation from 4 individuals with severe CAV to EMB specimens from 3 individuals post-
61 transplant without CAV (**Figure 1A**). For all 7 individuals, samples were obtained from the right ventricle (RV).
62 In 3 out of 4 patients with severe CAV, left ventricular (LV) samples were also obtained (for a total 10
63 samples).

64 METHODS

65 After nuclear isolation with modifications to account for low tissue mass, libraries were generated, sequenced,
66 quality-controlled, and analyzed as previously described². Raw FASTQ files are deposited at the NIH NCBI
67 GEO data repository (GSE203548) and code used for these analyses are deposited at
68 <https://github.com/learning-MD/CAV>. This study was approved by the Vanderbilt University Medical Center's
69 Institutional Review Board.

70 RESULTS

71 We successfully isolated 62,465 nuclei and identified 17 major cell types with heterogenous distribution across
72 the ten different samples (**Figures 1B, 1C**). When comparing RV samples, endothelial cells and fibroblasts in
73 CAV exhibited increased expression of *SERPINE1*, which promotes neointimal hyperplasia and fibrosis³.
74 Endothelial cells were enriched for pathways involved in angiogenesis, cell migration, and extracellular matrix
75 (ECM) organization (**Figures 1D, 1E**). Fibroblasts in CAV exhibited increased expression of genes involved in
76 ECM deposition and fibrosis (e.g., *MMP2*, *CCN1*, *THBS1*) while also highly expressing *IL6ST*, involved in IL-6
77 signaling. As expected, macrophages in CAV showed increased expression of genes associated with
78 inflammation (e.g., *TLR2*, *IFNAR2*). While no significant differences in T cells were noted between conditions,
79 subclusters included CD4 central memory T cells (*IL7R*, *TCF7*), CD4 T regulatory cells (*FOXP3*, *CTLA4*,
80 *IL2RA*), and CD4 T cells exhibiting markers of exhaustion (*LAG3*, *CTLA4*, *PDCD1*), along with CD8 memory T
81 cells (*CCL5*). No major differences in gene expression were noted between RV and LV CAV samples.

82 We repurposed a genotype-free demultiplexing tool to infer donor- and recipient-derived nuclei from each
83 individual CAV sample. Using 5 of the 7 combined CAV samples (including both LV and RV tissue), 2,827
84 nuclei were confidently called as donor- or recipient-derived in the absence of genotyping (**Figure 1F**).

85 Endothelial cells exhibited significant donor-recipient chimerism (21.8% recipient-derived). Donor-derived
86 endothelial cells were enriched for markers of endothelial-to-mesenchymal transition (EndoMT; *SERPINE1*,
87 *VIM*, *COL3A1*; **Figure 1G**). In contrast, immune cells were largely replaced by those originating from the
88 recipient (91.1% of macrophages/monocytes, 92.6% of NK cells, 88% of T cells). Recipient-derived
89 macrophages included both *CCR2*⁺ monocyte-derived macrophages and *CCR2*⁻ *MRC1*⁺ tissue resident
90 macrophages, traditionally thought to be involved in cardiac repair⁴ (**Figure 1H**). Macrophages exhibited
91 markers of activation, including *HLA-DRA* and *CD74*, and increased expression of *TGFB1*, a potential driver
92 for the EndoMT observed in donor-derived endothelial cells.

93 **DISCUSSION**

94 This study is the first to successfully utilize human EMB samples to isolate large numbers of intact nuclei for
95 single-nuclear transcriptomics. As expected from an ischemic allograft, we see enrichment for genes and
96 pathways involved in inflammation, fibrosis, and tissue healing. We highlight several unique findings enabled
97 by this approach: 1) cell composition amongst EMB samples is highly heterogeneous, suggesting that bulk
98 RNA-seq approaches may exhibit high levels of variability due to sampling bias; 2) there are unique
99 transcriptomic signatures of donor- versus recipient-derived cells, particularly endothelial cells, highlighting
100 putative novel avenues for investigation; and 3) the presence of recipient-derived *CCR2*⁻ macrophages
101 warrants further study, as only a small percentage would be expected to be recipient-derived⁴. However,
102 recent single-cell data have implicated partial replacement of *MHC-II*^{hi}*CCR2*⁻ cardiac macrophages by
103 monocytes, suggesting a still evolving understanding of macrophage subsets⁵.

104 Our study is limited by a small sample size and the use of samples derived from severe CAV. However, these
105 data demonstrate feasibility of performing snRNA-seq using frozen EMBs, presenting a unique opportunity that
106 may have broad ramifications on the fields of heart transplantation and cardio-oncology/immunology. These
107 data also lay the groundwork for ongoing experiments to study serial, routinely-collected EMB specimens after
108 heart transplantation to identify novel biomarkers and pathways through which early CAV pathogenesis can be
109 interrupted, thereby prolonging allograft survival.

110

111

112 **REFERENCES**

113 1. Pober JS, Jane-wit D, Qin L, Tellides G. Interacting mechanisms in the pathogenesis of cardiac allograft
114 vasculopathy. *Arterioscler Thromb Vasc Biol.* 2014;34(8):1609-1614.

115 2. Tucker NR, Chaffin M, Fleming SJ, et al. Transcriptional and Cellular Diversity of the Human Heart. *Circulation.*
116 2020;142(5):466-482.

117 3. Ji Y, Weng Z, Fish P, et al. Pharmacological Targeting of Plasminogen Activator Inhibitor-1 Decreases Vascular
118 Smooth Muscle Cell Migration and Neointima Formation. *Arterioscler Thromb Vasc Biol.* 2016;36(11):2167-2175.

119 4. Bajpai G, Schneider C, Wong N, et al. The human heart contains distinct macrophage subsets with divergent
120 origins and functions. *Nat Med.* 2018;24(8):1234-1245.

121 5. Dick SA, Macklin JA, Nejat S, et al. Self-renewing resident cardiac macrophages limit adverse remodeling
122 following myocardial infarction. *Nat Immunol.* 2019;20(1):29-39.

123

124

125

126

127

128

129

130

131

132

133

134

135

136

137

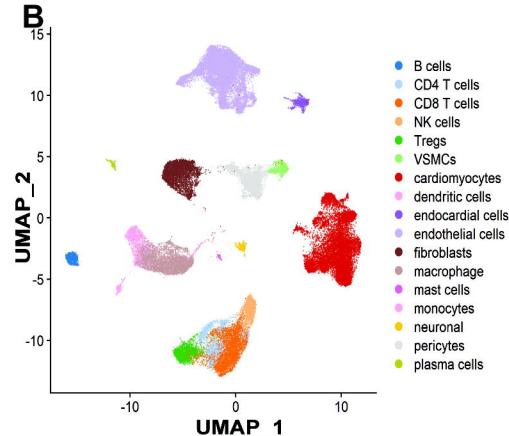
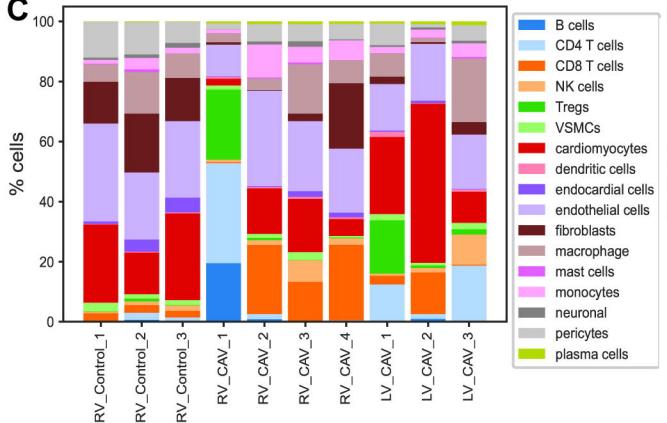
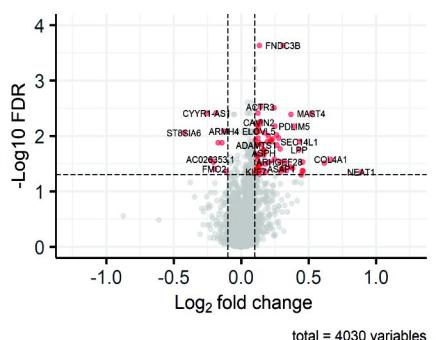
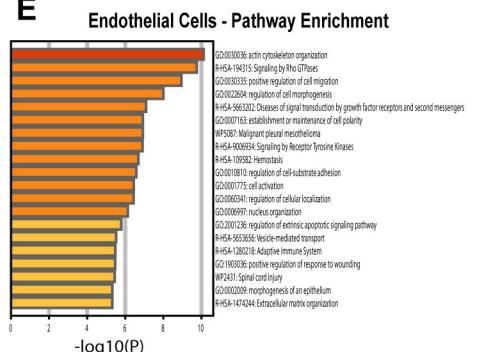
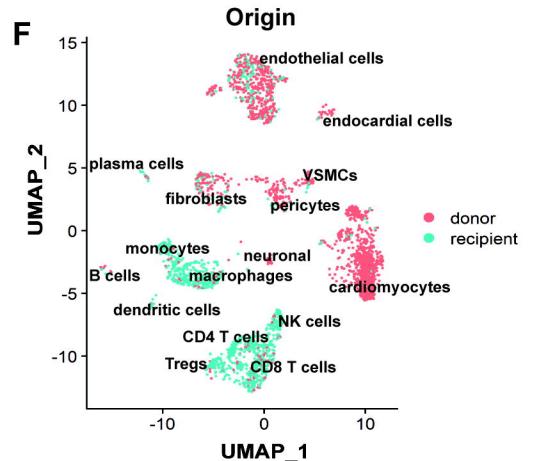
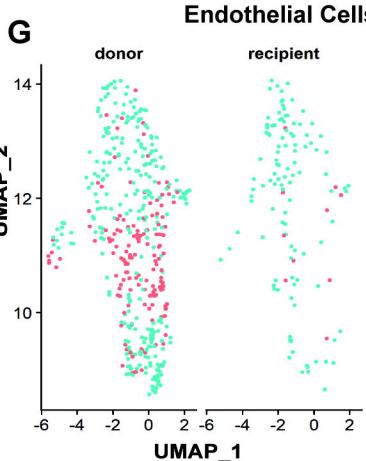
138

139 **Figure 1. A)** Clinical characteristics of all seven patients studied. **B)** Uniform Manifold Approximation and
140 Projection (UMAP) of 62,465 nuclei identified 17 major cell types using canonical marker genes. **C)** Cell
141 compositional analyses were performed using scCODA v0.1.6. Labels correspond to patients described in
142 *Figure 1A*. No significant difference in cell composition was noted using an automated reference cluster. **D)**
143 Differential gene expression was performed using MAST. Volcano plot representing right ventricular CAV vs.
144 control samples for the endothelial cell cluster. **E)** Biological pathway enrichment analysis of differentially
145 upregulated genes in RV CAV endothelial cells using Metascape. **F)** Genotype-free inference of donor- versus
146 recipient-derived nuclei was performed using souporcell v2.0. UMAP of donor- vs. recipient-derived nuclei. The
147 clusters correspond to the same clusters annotated in *Figure 1B*. **G)** Donor-derived endothelial cells are
148 enriched for markers of endothelial-to-mesenchymal transition. **H)** The monocyte/macrophage cluster is largely
149 recipient-derived. Presence of distinct *CCR2*⁺ monocytes and *CCR2*⁺*MCR1*⁺ macrophages is highlighted using
150 Nebulosa. *LVEF* = left ventricular ejection fraction; *HTN* = hypertension; *DM2* = type 2 diabetes mellitus; *CKD*
151 = chronic kidney disease; *DSA* = donor-specific antibodies; *ACR* = acute cellular rejection; *AMR* = antibody-
152 mediated rejection; *ER* = extended release; *MMF* = mycophenolate mofetil; *RV* = samples from right ventricle;
153 *LV* = samples from left ventricle; *CAV* = cardiac allograft vasculopathy; *EndoMT* = endothelial-to-mesenchymal
154 transition.

155

A

	Control 1	Control 2	Control 3	CAV 1	CAV 2	CAV 3	CAV 4
Age	56	44	59	52	54	45	54
Gender	M	M	F	F	F	F	M
Days After Transplant	304	188	146	2045	615	2531	2450
Dual-Organ Transplant	No	No	Yes	No	No	Yes	Yes
LVEF	65	80	55	50	55	55	40
HTN	Yes	Yes	No	Yes	No	No	Yes
DM2	Yes	Yes	Yes	Yes	No	No	Yes
Dyslipidemia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CKD	Yes	Yes	Yes	Yes	Yes	Yes	Yes
DSAs	No	No	No	No	Yes	Yes	Yes
Prior ACR \geq 2R	Yes	Yes	No	No	No	Yes	Yes
Prior AMR	No	No	No	No	Yes	Yes	Yes
Current ACR	1R	1R	OR	Yes	OR	OR	Yes
Current AMR	No	No	No	No	No	No	No
Stanford Classification	III	IV	n/a	n/a	n/a	n/a	n/a
Specimen Source	Biopsy	Biopsy	Biopsy	Explant	Explant	Explant	Explant
Immunosuppression Regimen	Tacrolimus, Sirolimus	Tacrolimus ER, azathioprine	Tacrolimus, MMF, Prednisone	Cyclosporine, MMF, Prednisone	Tacrolimus, MMF	Tacrolimus, Prednisone	Tacrolimus, MMF

B**C****D Endothelial Cells - RV CAV vs RV Control****E****F****G Endothelial Cells****H CCR2**