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14. ABSTRACT

Immune rejection is a major barrier to wider implementation of vascular composite allografts (VCAs) that hold great promise for restoring function in American service members, who have suffered devastating traumatic injuries. Despite systemic immunosuppression, T cell mediated rejection (TCMR) occurs much more frequently in VCA than in solid organ transplants, likely due to the significant number of T donor T cells that survive in the allografts. This study will used banked tissues from VCA patients to comprehensively analyze the contributions of donor versus recipient T cells in VCA rejection. Another question that will be addressed is whether sentinel flaps, transplanted concomitantly with the allograft from the same donor to a distant anatomical site, or circulating levels of clonally expanded T cells, are useful as reliable markers for VCA rejection.

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1. INTRODUCTION:

2.

Immune rejection is a major barrier to wider implementation of vascular composite allografts (VCAs) that hold great promise for restoring function in American service members, who have suffered devastating traumatic injuries. Despite systemic immunosuppression, T cell mediated rejection (TCMR) occurs much more frequently in VCA than in solid organ transplants, likely due to the significant number of donor T cells that survive in the allografts. This study will used banked tissues from VCA patients to comprehensively analyze the contributions of donor versus recipient T cells in VCA rejection. Another question that will be addressed is whether sentinel flaps, transplanted concomitantly with the allograft from the same donor to a distant anatomical site, or circulating levels of clonally expanded T cells, are useful as reliable markers for VCA rejection.

3. KEYWORDS:

Vascular composite allograft, rejection, biomarker, T cell

3. ACCOMPLISHMENTS:

What were the major goals of the project?

We submitted a revised statement of work as part of an extension for the original time frame of the award. Below in red includes the approved revised statement of work.

Major Task 1: Regulatory approval by sponsor and institution. IRB approval estimated at Month 1; Actual approval achieved at Month 3. HRPO approval completed was received 18 Jan 2019. HRPO approval was closed as of 5 Aug 2021. OHRO approval was re-acquired for analysis of additional samples on September 26, 2022 (OHRO Log # E00263.3a).

Major Task 2: Activities for Specific Aim 1
Subtask 1: High throughput TCR sequencing (HTS) of donor and recipient tissues from 6 face transplant patients during rejection and non-rejection.
Subtask 2: Determine functional phenotypes of clonally expanded donor and recipient T cells from face transplant recipients using single nucleus RNA sequencing (sNucSeq x five banked samples), single cell RNA sequencing on fresh biopsies of skin (scRNA-seq, 4 samples total- 3 VCA biopsies (from 2 patients) and 1 healthy donor sample), and digital spatial profiling (DSP x five banked samples).
Subtask 3: Validation of findings (HTS and scRNA-seq) in additional VCA type (eight samples total- 6 VCA biopsies (from 2 upper extremity transplant patients) and 2 samples from healthy controls)
Subtask 4: Data Analyses for Subtask 1-3

Major Task 3: Activities for Specific Aim 2
Subtask 1: HTS of blood samples from 7 face transplant patients to investigate if monitoring T cell clones in the circulation can be used as rejection biomarkers.
Subtask 2: Validation of findings from blood samples from face transplant cohort in additional VCA type (using blood samples from 3 upper extremity transplant patients).
Subtask 3: Data analysis for subtask 1 & 2.

Major Task 4: Activities for Specific Aim 3

Subtask 1: RNA extraction from facial allografts and corresponding sentinel flaps from 7 face transplant patients. Subtask 2: Gene expression profiling of the extracted RNA using NanoString nCounter technology. Subtask 3: Validation of findings using multiplex immunostaining.

What was accomplished under these goals?

Under **Major Task 1**, HRPO approval was received in Jan 2019. HRPO approval was closed as of 5 Aug 2021. OHRO approval was re-acquired for analysis of additional samples on September 26, 2022 (OHRO Log # E00263.3a).

Under **Major Task 2**, **Subtask 2,** we performed digital spatial profiling (DSP) on 3 VCA samples. These three samples are from the same patients at the same clinical encounters as the samples studied previously by single cell RNA sequencing (scRNA-Seq). These samples include:

- Grade 3 Rejection
- Grade 0 Post-Rejection
- Grade 0 Non-Rejection

The DSP was performed on FFPE skin biopsies using the BWH's newly acquired NanoString CosMx Spatial Molecular Imager. This instrument utilizes Nanostring's unique probe sets to directly measure mRNA levels for 1,013 standard genes, 50 customizable add-in genes, expression levels of 100 proteins and spatial information at a subcellular resolution (<100 nm) on FFPE tissue sections.[1] This approach generates true single cell gene expression, protein expression, and spatial information about the proximity of each cell type to others.[1] We carried out our first DSP studies of human face transplant rejection on skin biopsies using this instrument and have confirmed that it generates true single cell data (Fig. 1). A mean 7,700 cells were profiled from each section, with an average of 210 transcripts detected per cell.

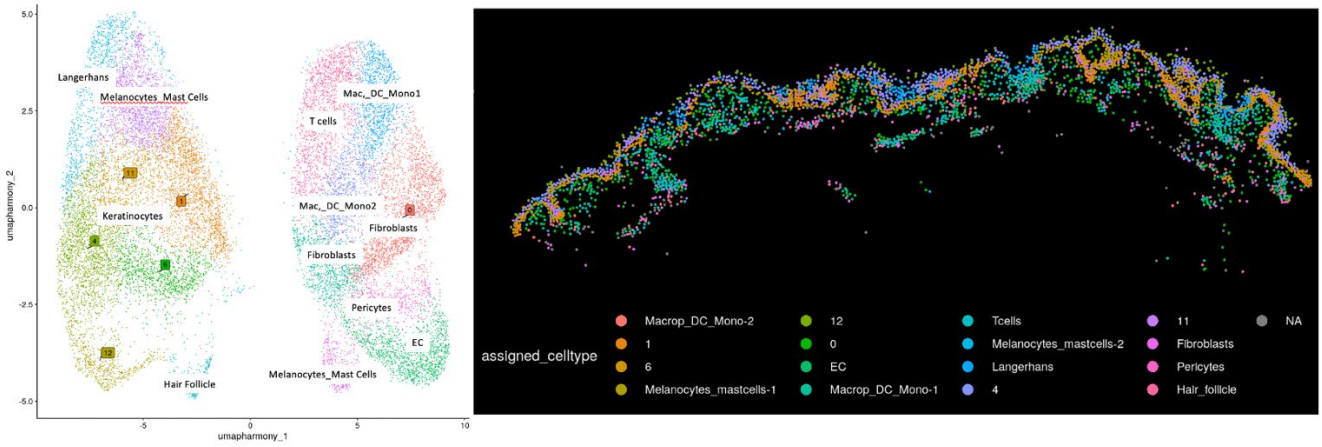

Fig. 1. NanoString CosMx spatial profiling data. These analyses produced true single cell data, with clear delineation of individual immune and stromal cell types. The first panel depicts the integrated clustering from 3 VCA samples: Grade 3 rejection, Grade 0 non-rejection, and Grade 0 post-rejection. A mean 7,700 cells were profiled per section with an average sequencing depth of 210 genes profiled per cell. Cell type clusters are identifiable in the spatial information, with the Grade 3 patient sample shown in the second panel.

We were fortunate to obtain viably frozen skin biopsies to study matched sections from the DSP data by single cell RNA seq (scRNASeq). Ongoing analysis will integrate the deep and unbiased scRNASeq data with the spatial information of DSP. Recently, specialized methods to integrate spatial imaging and transcriptomics data into a single dataset have been developed.[2] These methods rely on commonly expressed genes to measure the similarity between positions and single cells, improving data integration with spatial information. I will utilize SpaOTsc to integrate the data from these two approaches into a single data set.[3] SpaOTsc leverages structured optimal transport, utilizing both gene expression similarities and spatial distances between cells to create a fused, synergistic data set.[3] This will generate information about where each cell is, what other cells it is in contact with, which of the 200 measured proteins it is expressing, and a deep and comprehensive measurement of gene expression and cell state.

Separately, as part of **Subtask 2**, we have studied 4 VCA samples by single nucleus RNA sequencing (sNucSeq). We have previously optimized sNucSeq on OCT-banked frozen skin biopsy samples using 5' 10X Chemistry (Fig. 2). We recently performed nuclear digests of 4 OCT-banked VCA samples (Fig. 3). These samples included two Grade 0 non-rejection skin biopsies and two Grade 2 rejection skin biopsies. were analyzed by 5' 10X chemistry to perform single nucleus RNA sequencing (sNucSeq). We received these data on October 16, 2023, and await the analysis from our bioinformatics collaborative team at the Harvard Bioinformatics Core. This experiment represents first-of-their kind sNucSeq studies of vascular composite allograft skin biopsies.

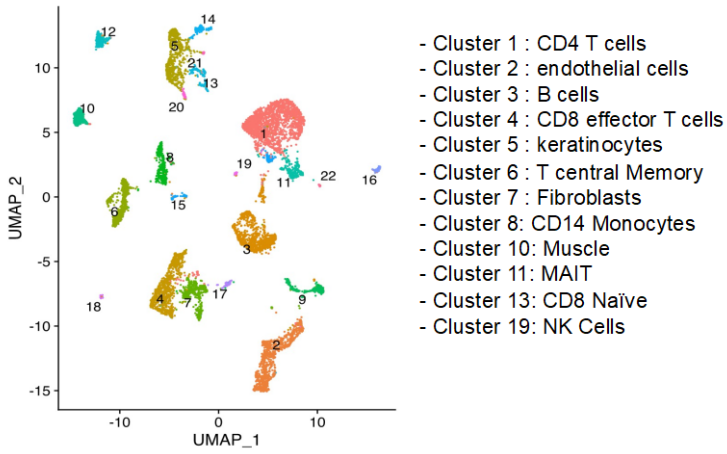

Fig. 2. Cell Type Clustering from pilot single nucleus RNA sequencing (sNuc-Seq). A 4mm OCT-banked skin biopsy was digested and sequenced using the 5' 10X protocol. Among three total pilot experiments, we identified a mean of 8,000 nuclei per sample with over 3,000 transcripts per nuclei.

Fig. 3. VCA samples submitted for single nucleus RNA sequencing (sNucSeq). 4 VCA skin biopsies underwent our optimized nuclear digest protocol. These were delivered to the BWH Single Cell Genomics core for 5' 10X Chemistry sNucSeq. The sequencing data were released on October 16, 2023 to our bioinformatics collaborators.

The updated percent completion of **Major Task 2** (considering the newly revised and approved Statement of Work) is 60%. Estimated completion is 2024.

Subtask 3: Validation of findings (HTS and sNucSeq) from face transplant cohort in additional VCA type (using tissue samples from 3 upper extremity transplant patients). Estimated completion 2022, current percentage of completion is 0%. Estimated completion 2024.

Under **Major Task 4**:

Vascular composite allografts (VCAs), including face and limb transplants, hold the promise of functional and aesthetic restoration for patients whose injuries cannot be adequately addressed by conventional reconstructive surgical techniques.[4-6] Yet, VCAs have not reached widespread implementation because of challenges associated with diagnosing and treating frequent immune rejection episodes that occur despite long-term multi-drug immunosuppression.[7] Moreover, repeated rejection episodes can become chronic, which can lead to the potentially fatal complication of graft loss.[8,9] Reliable methods for detecting VCA rejection early and accurately are thus critical for the management of VCA patients.

Currently, VCA rejection surveillance requires histological evaluation of allograft skin biopsies according to the Banff 2007 classification.[10] This presents several limitations. First, some allografts, like partial face transplants, provide only small areas of skin available for biopsies; next, recurrent allograft biopsies may induce scarring and compromise aesthetic outcomes; and lastly, skin is prone to dermatologic conditions that can be mistaken for changes associated with rejection.[11-13] To address these limitations, sentinel flaps are often transplanted concomitantly from the same donor, as a remote site clinical and histologic indicator of rejection.[14,15] However, the cellular and molecular correlation of rejection in sentinel flaps and VCA allografts is not well established. Unlike face or extremity allografts, sentinel flaps are fasciocutaneous (contains skin and fascia), and do not include muscle or bone, tissues known to be less immunogenic than skin.[16] It is unknown whether the different compositions of sentinel flaps and VCA allografts result in diverging manifestations of rejection.

In the below studies under **Major Task 4**, we used gene expression profiling, immunostaining, and T cell receptor (TCR) sequencing to compare paired banked skin biopsies from VCA facial allografts and sentinel flaps. We studied concomitantly acquired biopsies from both tissue sites during clinical presentations of rejection and non-rejection from our cohort of VCA patients. Our study indicates that sentinel flaps and facial allografts exhibit corollary expression of pro- and anti-inflammatory genes that we previously identified as mediators of VCA rejection.[17] We also found shared T cell clones as putative drivers of rejection at both tissue sites. Our study offers new insights that sentinel flaps accurately reflect inflammation and rejection in VCA.

We studied five patients who received face transplants with sentinel flaps at Brigham and Women's Hospital. 3 patients received full face transplants and 2 received partial face allografts (Figure 4A). There were 4 male and 1 female, all were white. Sentinel flaps were surgically inset on patients' right groin (n = 1), right hand (n = 2), left hand (n = 1), or left axilla (n = 1) (Figure 4A). In this patient cohort, there has been 1 graft loss and 1 death. 4mm skin punch biopsies were taken from the facial allograft and sentinel flap of the same patient at the same time, forming a "matched pair." 14 matched paired biopsies were studied using histologic examination, multiplex gene expression profiling, immunostaining, and high-throughput TCR sequencing. The current standard for diagnosing acute T cell mediated rejection (ACMR) is histological evaluation of biopsies according to the Banff classification, which categorizes rejection into four grades: grade 0 (no rejection), grade 1 (mild rejection), grade 2 (moderate rejection), and grade 3 (severe rejection).[10] For this study, "non-rejection" included grades 0 and 1 since grade 1 does not differ significantly from grade 0.[17] "Rejection" included grades 2 and 3, and biopsies graded "2 or 3" ("2/3," Table 1) were treated as grade 3. Of the matched pairs, 8 included pairs with both tissues graded as rejection, 5 pairs with both tissues graded as non-rejection, and 1 pair with mixed sentinel flap rejection and facial allograft non-rejection (Figure 4B, Table 1). 9 matched pairs received the same grade in both tissues

(Figure 4B, green), 2 pairs scored a higher grade in the sentinel flap (Figure 4B, red), and 3 pairs scored a higher grade in the facial allograft (Figure 4B, blue).

Table 1. Histological Grade of Sentinel Flap and Facial Allograft Matched Pairs

Matched Pair ID n=14	Patient	BANFF Histological Grade		Rejection Status R=Rejection (n=7); NR=Non-Rejection (n=6); Mixed (n=1)
		Sentinel Flap	Facial Allograft	
1	A	2	2	R
2	B	2	2	R
3	B	1	1	NR
4	B	1	0	NR
5	B	2/3	2/3	R
6	B	3	3	R
7	B	0	0	NR
8	C	1	1	NR
9	D	2/3	2/3	R
10	D	2/3	3	R
11	D	2/3	3	R
12	E	2	1	Mixed
13	E	0	0	NR
14	E	0	1	NR

Clinical and histological features of rejection in facial allografts and sentinel flaps. Representative images of a full-face transplant and right hand sentinel flap illustrate clinical presentation of grade 0 and grade 3 allografts (Figure 4C and D). Grade 0 exhibits little or no signs of edema or erythema (Figure 4C). Clinical signs of grade 3 rejection include skin edema and erythema at both tissue sites (Figure 4D). H&E stains of matched paired facial allografts and sentinel flaps distinguish grade 0 and grade 3 (Figure 4E and F). The grade 0 biopsy from both the sentinel flap and face exhibit no histological signs of rejection with minimal inflammatory infiltrate (Figure 4E). The grade 3 face biopsy exhibits focal epidermal infiltration by lymphocytes and vacuolar degeneration of basal keratinocytes with occasional dyskeratotic cells (arrow) (Figure 4F). In the grade 3 sentinel flap biopsy, there is interface alteration with dyskeratotic cells (arrows) (Figure 4F).

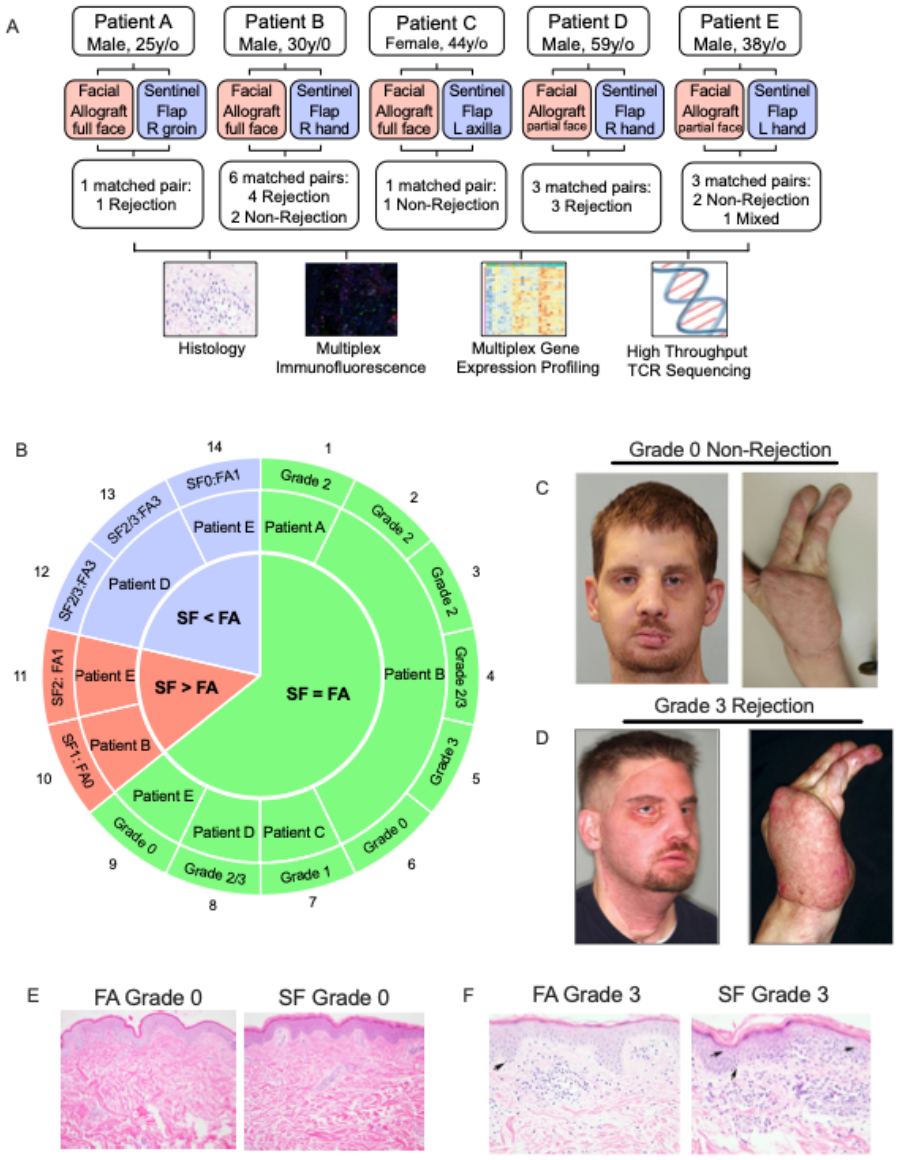

Figure 4. Design of study, samples studied, and clinical and histological characteristics of rejection. (A) Design of study. Matched paired skin biopsies were taken from facial allografts and sentinel flaps of 5 face transplant patients during episodes of rejection (grades 2,3) and non-rejection (grades 0,1). 3 patients received full facial transplants and 2 received partial facial allografts. Sentinel flaps were inset on each patient on their right groin ($n = 1$), right hand ($n = 2$), left hand ($n = 1$), or left axilla ($n = 1$). 14 matched paired biopsies were used in the study, with both sentinel flap and facial allograft diagnosed as rejection ($n = 8$), non-rejection ($n = 5$), and mixed sentinel flap rejection with facial allograft non-rejection ($n = 1$). Matched pairs were analyzed by histology, immunostaining, multiplex gene expression profiling, and high throughput TCR sequencing (HTS). (B) 9 matched paired biopsies received the same Banff histological grade (green), 2 matched pairs had a higher grade in the sentinel flap (red), and 3 matched pairs had a higher grade in the facial allograft. Numbers outlying pie chart indicate identifier for each matched pair used in these studies. (C, D) Clinical images of a full face and right hand sentinel flap allograft recipient during grade 0 non-rejection (C) and grade 3 rejection (D). (E,F). H&E stains of matched paired facial allografts and sentinel flaps during grade 0 and grade 3 rejection.

Facial allografts and sentinel flaps share upregulated genes, activated pathways, upstream regulators, and infiltrating cell types in grade 3 rejection.

We studied gene expression in the same archived formalin-fixed paraffin-embedded (FFPE) skin biopsies used for histologic diagnosis of rejection using Nanostring profiling.[18] Unsupervised principal component analysis (PCA) clustered non-rejection (Figure 5A) and rejection (Figure 5B) biopsies from both tissue types according to similarities in their patterns of expression in 730 genes. We compared differentially expressed genes (DEG) from grade 3 versus grade 0 in facial allografts and sentinel flaps to determine whether similar genes were upregulated during rejection. In the FA biopsies, a total of 126 genes were differentially expressed in grade 3 biopsies versus grade 0 ($\log_2$FC >|1|; P value <0.05) (Figure 5C). In the SF biopsies, a total of 171 genes were differentially expressed in grade 3 biopsies versus grade 0 ($\log_2$FC >|1|; P value <0.05) (Figure 5E). 89 of these DEG were shared by both tissue types, including critical pro-inflammatory (*GZMA*, *PRF1, HLA-DPB1*, *CASP8, STAT1*) and immunoregulatory (*SOCS1, CTLA4, TIGIT, PTPRC*, *HLA-DOB*) molecules we previously identified as mediators of VCA rejection [17].

We used ingenuity pathway analysis (IPA) to study DEG from grade 3 versus grade 0 biopsies to determine if rejection at both tissue sites involved induction of similar pathways, upstream regulators. Grade 3 rejection in FA and SF both upregulated pathways we previously identified as mediators of VCA rejection.[17] Pathways induced during grade 3 rejection included those involving T cell activation (T cell receptor signaling, Th1 signaling, cytotoxic T cell-mediated apoptosis, and

PKCθ signaling in T lymphocytes), innate immune activation (classical and alternative macrophage activation, dendritic cell maturation, NK cell signaling, crosstalk between DC and NK cells), interferon signaling, and immunogenic cell death signaling (Figure 5F). IPA upstream regulator analysis predicted that FA and SF similarly induce signaling by pro-inflammatory cytokines (IFNA2, IFNG, IL4, TNF), innate danger signals (RIGI, NFκB complex, STING), and antigen-specific T cell activation (CD40L). We used Nanostring nSolver cell type profiling to identify infiltrating cell types in grade 3 rejection compared with grade 0 in facial allografts (Figure 5H) and sentinel flaps (Figure 5I). FA and SF both demonstrated an increased presence of CD8+ T cells, total T cells, cytotoxic cells, and recirculating T cells.

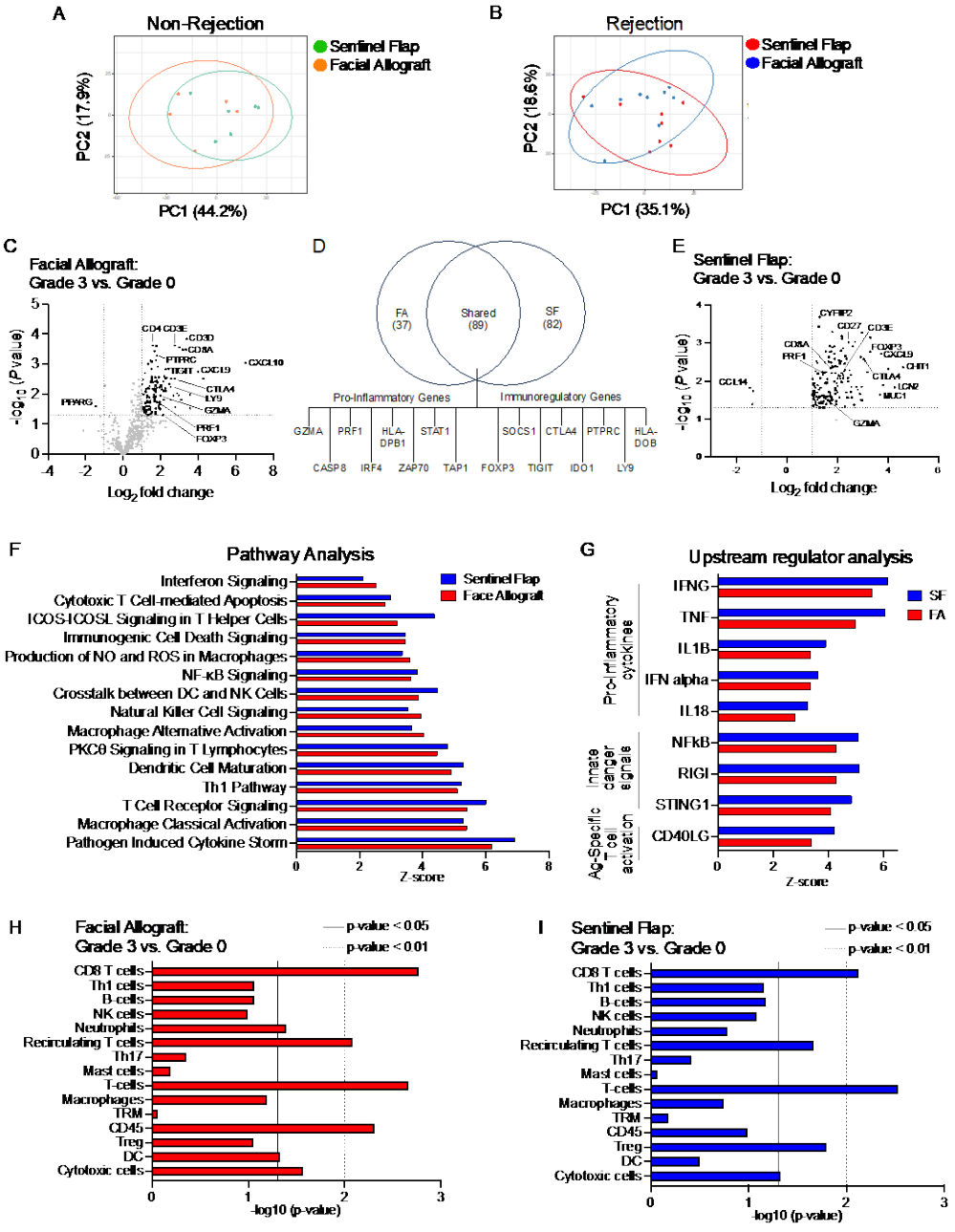

Figure 5. Facial allografts and sentinel flaps share upregulated genes, activated pathways, upstream regulators, and infiltrating cell types in grade 3 rejection. (A) Unsupervised principal component analysis (PCA) of non-rejection skin biopsies (grade 0, grade 1) overlapped clustering of SF (green) and FA (orange). (B) PCA of rejection skin biopsies (grade 2, grade 3) overlapped clustering of SF (blue) and FA (red). (C) DEG of FA grade 3 (n=4) vs. grade 0 (n=3), with 126 DEG at $\log_2$ fold change >1; *P* value <0.05. (E) DEG of SF grade 3 (n=4) vs. grade 0 (n=3), with 171 DEG at $\log_2$ fold change >1; *P* value <0.05. (D) Venn diagram illustrates that 89 of the DEG were shared by both tissue types, including known pro-inflammatory and immunoregulatory mediators of VCA rejection. Upregulated pathways (F) and upstream regulators (G) in SF (blue) and FA (red) as measured by Ingenuity Pathway Analysis. (H-I) Nanostring cell type profiling of grade 3 vs. grade 0 biopsies

Facial allografts and sentinel flaps exhibit similar cytotoxic T cell numbers and T cell-mediated cytotoxic events during grade 3 rejection.

We immunostained FFPE biopsies from facial allografts (FA) and sentinel flaps (SF) by to compare expression of $CD3^+$, $CD8^+$, and granzyme B (GZMB) during non-rejection (grades 0 or 1) and grade 3 rejection (Figure 6A). We previously showed that T cells are the major source of cytotoxic injury in grade 3 rejection of facial allografts [17]. Quantification of immunostaining indicated there was no statistically significant difference between FA and SF regarding number of $CD3^+$ T cells,

number of $CD8^+$ cytotoxic T lymphocytes (CTL), or percent of $CD8^+$ CTL in either the non-rejection or grade 3 biopsies (Figure 6B and C). GZMB is a primary cytotoxic molecule that mediates tissue damage during grade 3 rejection of VCA [17]. There was no significant difference in the number of $CD3^+GZMB^+$ T cells in FA and SF samples in either non-rejection or grade 3 rejection (Figure 6D). The percentage of $CD3^+$ T cells expressing GZMB was not significantly different between tissue types in non-rejection biopsies but reached slight significance in grade 3 rejection ($P = 0.0495$) (Figure 6D). GZMB has been implicated in acute rejection of VCA [17] and many types of acutely rejecting solid organs [19]. One mechanism of CTL GZMB-induced programmed cell death is through proteolytic cleavage and activation of caspase-3 [20]. There was no statistically significant difference between the number of T cell cytotoxic events (cleaved caspase-3^+ cells adjacent to $CD3^+GZMB^+$) in FA and SF in non-rejection or grade 3 biopsies (Figure 6E).

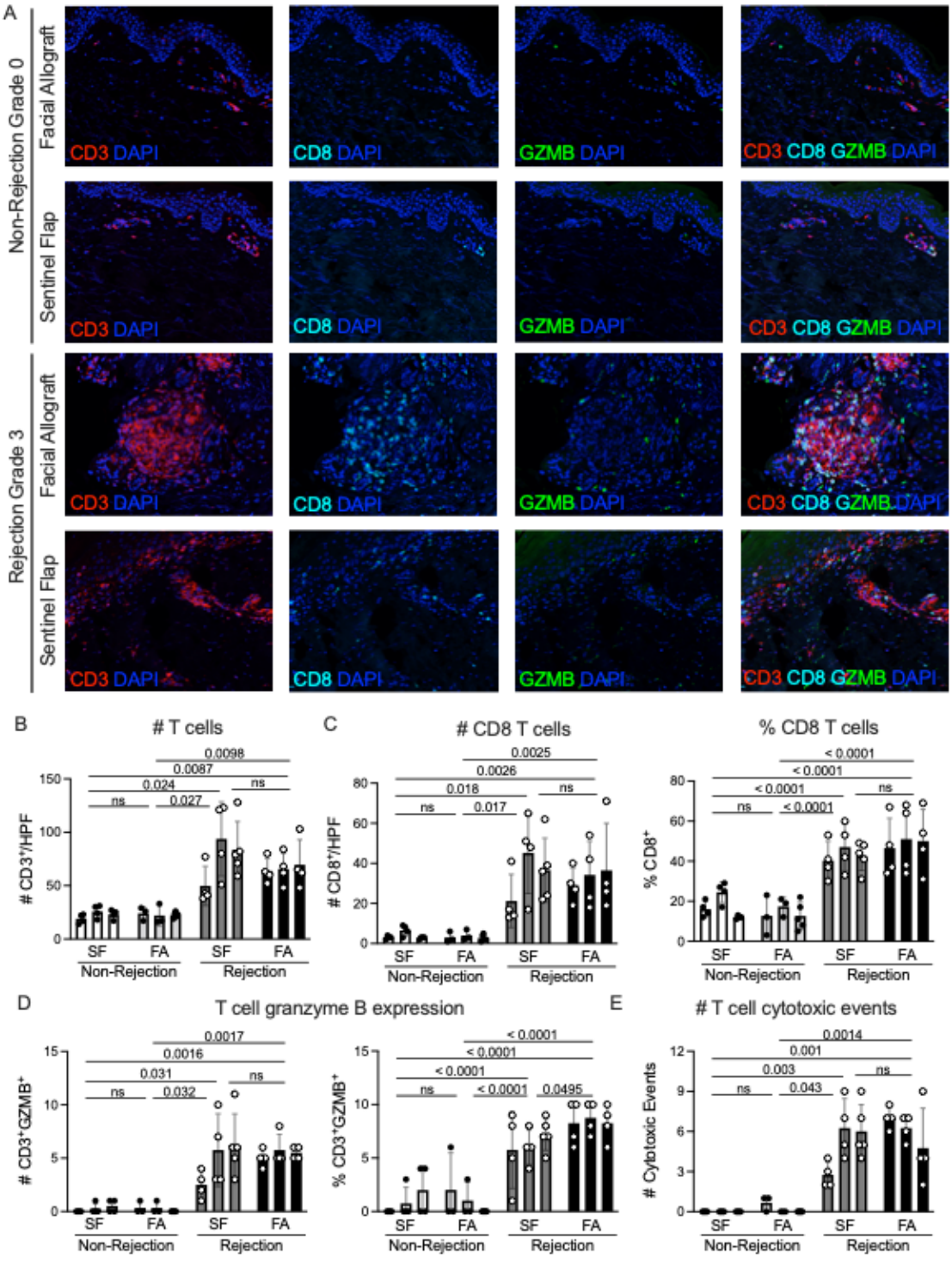

Figure 6. Cytotoxic T cell numbers and T cell-mediated cytotoxic events are similar sentinel flaps and facial allografts during grade 0 and grade 3 rejection. (A) Matched paired facial allografts and sentinel flaps were stained for $CD3^+$, $CD8^+$ granzyme B (GZMB), and DAPI during grade 0 non-rejection (bottom two rows) and grade 3 rejection (top two rows). (B-E) Graphs depict quantification of $CD3^+$ T cells, number and percentage of $CD8^+$ T cells, number and percentage of $CD3^+$granzyme B^+, and number of T cell cytotoxic events (cleaved caspase-3^+ cell adjacent to $CD3^+GZMB^+$). Bars represent individual donors, and error bars represent the mean and SEM of at least 3 separate measurements per donor. Dots represent counts per ×200 high-power field (HPF). Adjusted P values were calculated using nested 1-way ANOVA and Tukey's post hoc test for comparison between groups.

Shared T cell clones drive rejection in sentinel flaps and facial allografts.
We used high throughput sequencing (HTS) of CDR3 regions of TCR-β genes to determine if the same T cell clones mediated rejection at both tissue sites. The hypervariable CDR3 region represents the antigen recognition domain of the TCR.[21] Thus, T cell clones with shared CDR3 nucleotide sequences recognize the same antigen. HTS of four matched pairs of FA and SF biopsies revealed shared CDR3 nucleotide sequences at both tissue sites (Figure 7A). The absolute number of shared clones for each of the matched pairs was 18, 38, 48, and 28, respectively (Figure 7B). Since sentinel flaps are intended to provide remote site monitoring for rejection in the facial allograft, we identified the top 30 most frequent T cell clones in each FA and found shared clones present in all four matched sentinel flaps at an absolute number of 6, 25, 21, and 21, respectively (Figure 7C). Of the top 30 most frequent T cell clones in the facial allograft, the percentages of these same clones in matched sentinel flaps were 20%, 66.67%, 70%, and 70%, respectively.

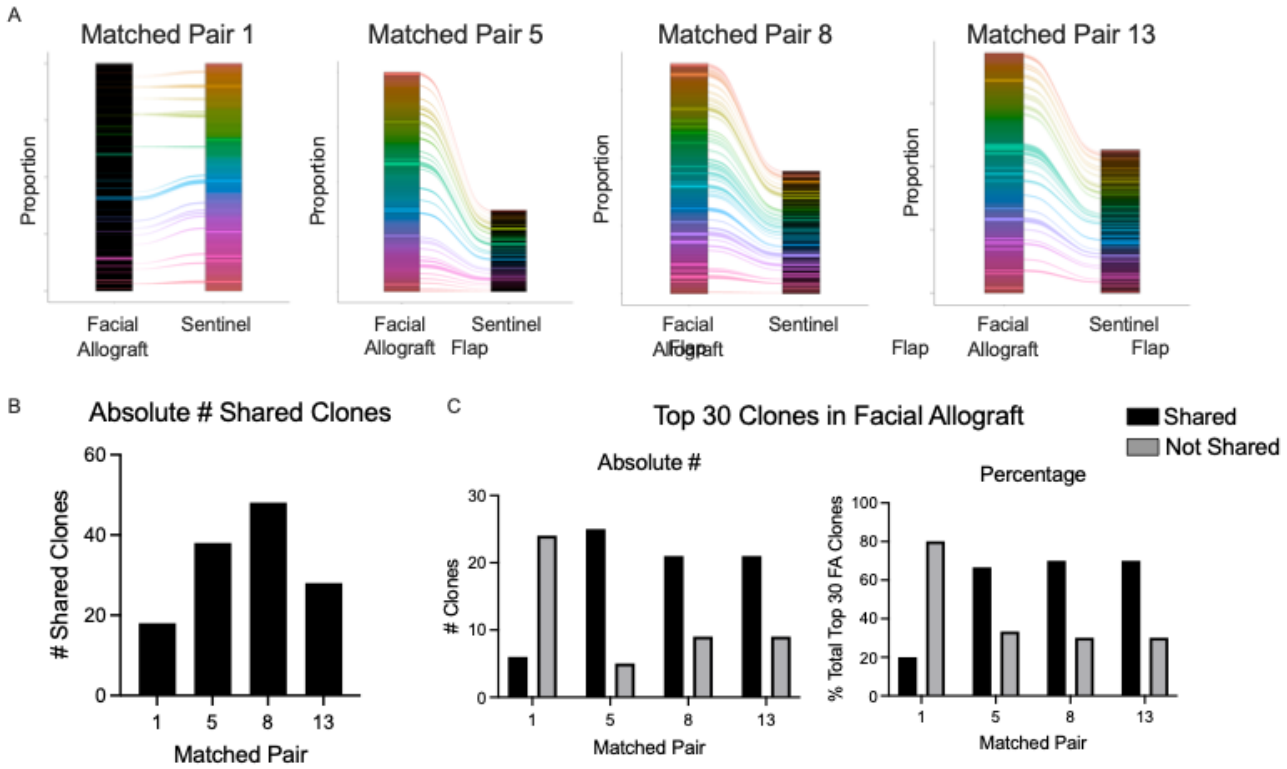

Figure 7. Sentinel flaps and facial allografts exhibit shared T cell clones in matched rejection skin biopsies. High throughput sequencing (HTS) of CDR3 of TCR-β genes from 4 matched pairs during rejection revealed shared clones between tissue types. (A) Alluvial plots of shared CDR3 nucleotide sequences between facial allograft (left bar) and sentinel flap (right bar) are shown. CDR3 sequences unique to one tissue type are not shown. (B) Bar graphs depict absolute number of shared T cell clones for each matched pair. (D-E) Of the top 30 T cell clones found in the facial allograft by productive frequency, bar graphs depict the absolute number (D) and percentage (E) of clones shared with the sentinel flap (black bars) and not shared (gray bars).

Our findings suggest that sentinel flap biopsies could serve as a valuable clinical metric for monitoring rejection in facial allografts. To our knowledge, this study provides the first insights into the overlapping molecular manifestations of rejection between sentinel flaps and facial allografts. These findings also suggest that there are indeed shared mechanisms of rejection between tissues, lending credence to the consensus that skin is the primary site and driver of rejection in VCA since skin is shared between the tissues. The percentage of completion of **Major Task 4** is 95%, and we are now preparing to submit these studies for publication in the *American Journal of Transplantation*.

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20 Pardo, J. *et al.* Granzyme B-induced cell death exerted by ex vivo CTL: discriminating requirements for cell death and some of its signs. *Cell Death Differ* **15**, 567-579, doi:10.1038/sj.cdd.4402289 (2008).

21 Glanville, J. *et al.* Identifying specificity groups in the T cell receptor repertoire. *Nature* **547**, 94-98, doi:10.1038/nature22976 (2017).

What opportunities for training and professional development has the project provided?

This project has provided training for junior faculty William J. Crisler to develop a novel sequencing approach to studying human skin, which will be critical for understanding VCA rejection. Furthermore, this tool can be applied to other human skin samples from many different types of diseases from cancer to autoimmunity and represents a niche for Dr. Crisler's future independent line of inquiry. Dr. Crisler was able to leverage studies funded by this DOD award to secure a competitive Dermatology Foundation Career Development Award. Additionally, Dr. Crisler's work in VCA rejection from these studies granted him the opportunity to serve as a member of Harvard Medical School's teaching faculty in the Immunity in Defense and Disease course. Dr. Crisler gave two lectures to Harvard medical students: one on skin immunology and another on transplant rejection. This experience is valuable for Dr. Crisler's development toward a higher ranking faculty position. Furthermore, Dr. Crisler presented data from this Statement of Work at the 2023 American Association of Immunologists conference in Washington, D.C. and also at the 2023 American Transplant Society Congress in San Diego, CA.

How were the results disseminated to communities of interest?

No new publications, though we are nearing submission of the sentinel flap studies to the *American Journal of Transplantation*. We are also planning a publication of the scRNASeq and DSP data from Major Task 2 as these data are analyzed. Dr. Crisler presented data from this Statement of Work at the 2023 American Association of Immunologists conference in Washington, D.C. and also at the 2023 American Transplant Society Congress in San Diego, CA.

What do you plan to do during the next reporting period to accomplish the goals?

For Major Task 2, we will perform further DSP analysis on other samples. We will continue to analyze these data, as well as analyzing the scRNASeq and sNucSeq data by leveraging our robust collaboration with the Harvard Bioinformatics Core. Under Major Task 4, we will finalize and publish our data comparing sentinel flaps and facial allografts.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

This project inspired the development of a novel protocol to perform single nucleus RNA sequencing on cryopreserved samples of human skin. During this reporting period, we performed the first sNucSeq studies of OCT-banked, cryopreserved skin biopsies from vascular composite allograft patients. This assay has already sparked interest with potential collaborations including the application of the assay on cryopreserved human skin from recurrent/remitting psoriasis skin lesions.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The only changes were submitted as updates to the statement of work, which were approved by the DOD and our award period was extended.

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

IRB (Protocol # 2018P003007): Approved 18 January 2019

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Conference lecture at the University of Copenhagen's 2022 Skin Immunology Conference in April, 2022.

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Rachael Clark, MD PhD
Project Role: Principal Investigator
Nearest person month worked: 1.2
Contribution to Project: Dr. Clark provided scientific oversight and provided feedback and support on regulatory and protocol submissions.

Name: William J. Crisler, PhD
Project Role: Research Fellow
Nearest person month worked: 10.8
Contribution to Project: Dr. Crisler has worked on regulatory submissions as well as scientific experiments. He has also performed the analysis described in this report. Dr. Crisler is responsible for collecting and analyzing data under Major tasks 2, 3, and 4.

Has there been a change in the active other support of the PD/PI(s) or senior/ key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: Harvard Bioinformatics Core
Location of Organization: Boston, MA
We have a robust collaboration with bioinformatics experts to help us identify the phenotype of donor vs. recipient cells in single cell RNA sequencing data using the cutting-edge Freemuxlet technology. The core has also provided deeper insights in our Nanostring data shown in Major Task 4.

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS: *N/A*
   QUAD CHARTS: *N/A*

9. **APPENDICES:** *N/A*