γδ T cells: an immunotherapeutic approach for HIV cure strategies

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Introduction

The latent human immunodeficiency virus (HIV) reservoir within resting memory CD4⁺ T cells is the major barrier to efforts to eradicate persistent infection (1–4). Therapeutic approaches that allow the clearance of latent replication-competent HIV are thus needed. Current strategies are based on using latency reversal agents (LRAs) to induce viral antigen expression (5, 6) to allow immunological clearance. This clearance part has been mainly based on CD8⁺ T cells (7, 8). However, CD8-based therapies can be challenging due to insufficient HIV antigen production from infected cells and poor HIV-specific CD8⁺ T cells. γδ T cells represent a unique subset of effector T cells that can traffic to tissues, and selectively target cancer or virally infected cells without requiring MHC presentation. We analyzed whether γδ T cells represent a complementary/alternative immunotherapeutic approach towards HIV cure strategies. γδ T cells from HIV-infected virologically suppressed donors were expanded with bisphosphonate pamidronate (PAM) and cells were used in autologous cellular systems ex vivo. These cells (a) are potent cytotoxic effectors able to efficiently inhibit HIV replication ex vivo, (b) degranulate in the presence of autologous infected CD4⁺ T cells, and (c) specifically clear latently infected cells after latency reversal with vorinostat. This is the first proof of concept to our knowledge showing that γδ T cells target and clear autologous HIV reservoirs upon latency reversal. Our results open potentially new insights into the immunotherapeutic use of γδ T cells for current interventions in HIV eradication strategies.
deserve further investigation to assess their potential in interventions aimed to eradicate HIV infection taking advantage of the knowledge generated from the cancer field (27–29).

In the present study, we demonstrate γδ T cell targeting and elimination of reactivated HIV-infected resting CD4+ T cells ex vivo. To our knowledge, our work constitutes the first study that comprehensively analyzes the potential of γδ T cells to clear persistent HIV infection in ART-treated, aviremic HIV-infected donors. We have performed ex vivo studies demonstrating cytotoxic and antiviral capacities of γδ T cells to specifically target and inhibit viral replication. Further, using autologous systems we demonstrate the specific clearance of latently HIV-infected cells by δ T cells after latency reversal. This study provides rationale to further analyze the immunotherapeutic use of γδ T cells in HIV cure strategies.

**Results**

**Comparison of Vδ2 cell expansion in HIV-infected and uninfected individuals.** We compared different ex vivo experimental conditions to expand Vδ2 cells from ART-suppressed HIV-infected donors (n = 13) and uninfected donors (n = 10). In this first approach we expanded the cells for 6 days and conditions included (a) HMBPP and IL-2, (b) PAM and IL-2, or (c) IL-2 alone. Basal Vδ2 cell percentages within CD3+ cells were analyzed by flow cytometry showing wide interindividual differences in uninfected individuals, and expected (30) profound depletion in HIV-infected donors (mean 4.0% vs. 0.7%, respectively; Figure 1A).

In uninfected individuals, HMBPP was a more potent inducer of Vδ2 cell expansion compared with PAM, while cells from HIV-infected donors expanded better after PAM treatment (Figure 1, B and C, and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.120121DS1). Importantly, the fold change in expansion induced by PAM in HIV-infected donors was comparable to uninfected donors (FDR P = 0.29). Response to HMBPP and PAM in uninfected donors was comparable (FDR P = 0.22), while response to HMBPP in HIV-infected donors was statistically lower (FDR P = 0.04). Uninfected donors (n = 9) are represented with gray circles and HIV-infected donors (n = 11) with pink squares. Uninfected and HIV-infected donors were compared using Mann-Whitney U test. HMBPP, PAM, and IL-2 conditions in uninfected donors and in HIV-infected donors were compared using Wilcoxon’s signed-rank test.
and was variable between subjects (Figure 2A). As we previously reported (24), patients treated in the acute phase of the infection had greater basal Vδ2 cell numbers compared with chronically treated patients (0.7% vs. 0.3%, \( P = 0.007 \)). After expansion, percentages of Vδ2 cells from patients treated in the acute phase of HIV infection were also higher compared with patients treated in the chronic HIV infection (mean 15.0 vs. 6.8, \( P = 0.02 \); Figure 2B), but with comparable fold change in expansion (\( P = 0.56 \), Figure 2C). Overall, mean fold change in expansion was 28.4, ranging from 1.7-fold to a more than 124-fold increase. Altogether, our results show that Vδ2 T cells from suppressed HIV-infected donors were successfully expanded in response to ex vivo exposure to PAM and IL-2.

**Phenotype of expanded Vδ2 cells.** The phenotype of expanded Vδ2 cells after 14 days of exposure to PAM was analyzed in a subgroup of 8 ART-treated, suppressed HIV-infected donors (6 patients treated in chronic infection and 2 patients treated in acute infection) by measuring the expression of markers of memory, cytotoxicity, activation, and immune exhaustion by flow cytometry. Vδ2 memory cell populations were defined as follows: central memory (TCM, CD45RA–CD27+CCR7+), transitional memory (TTM, CD45RA –CD27+CCR7–), and effector memory (TEM, CD45RA –CD27– CCR7–). The majority of expanded Vδ2 cells expressed a TTM phenotype (65%), followed by TCM (23%) and finally by TEM (8%) (Figure 3A). Cytotoxic markers including CD8, CD56, and CD16 were expressed by 37%, 32%, and 45% of expanded Vδ2 cells, respectively (Figure 3B). Altogether, 50% of the expanded Vδ2 cells displayed a cytotoxic phenotype (Vδ2+CD56+) and around 30% expressed CD16 (suggesting participation in antibody-dependent cell-mediated cytotoxicity). Activation markers CD69, CD25, and HLA-DR were expressed in a mean of 37.2%, 14.8%, and 16.6% Vδ2 cells, respectively. The expression of the exhaustion marker PD-1 was observed in a mean of 19.8% and CTLA-4 was observed in 4.3% of the expanded Vδ2 cells (Figure 3C).

**PAM-expanded Vδ2 cells inhibit active HIV replication.** We previously reported that γδ T cells from uninfected donors are potent inhibitors of viral replication in vitro (24). To confirm and extend these results, we used an autologous cellular system to measure the capacity of ex vivo–isolated Vδ2 T cells from ART-suppressed HIV-infected donors to inhibit viral replication in CD4+ T cells infected with the JR-CSF strain in vitro. A mean reduction of 85% in HIV p24 production was detected at a 1:1 effector/target cell ratio, 64% at 1:10, and 54% at 1:100 (Figure 4A). HIV p24 production was significantly reduced at 1:1 and 1:10 effector/target cell ratios.
Next, we tested the capacity of PAM-expanded V\(\delta\)2 T cells to inhibit HIV replication, using the same functional assays of viral inhibition. Our results show that PAM-expanded V\(\delta\)2 cells retain their capacity to significantly inhibit viral replication (Figure 4B and Supplemental Figure 2A). Expanded V\(\delta\)2 cells at the 1:1 ratio showed a mean inhibition of viral replication of 71% and 57% at the 1:10 ratio, and 18% at 1:100.

To avoid interindividual differences in the comparison of basal and expanded V\(\delta\)2 cells, we performed a side-by-side analysis using the same donors. Basal mean viral inhibition mediated by ex vivo V\(\delta\)2 T cells was comparable to that measured after expansion with PAM (Figure 4C). We conclude that exposure of V\(\delta\)2 T cells to PAM and IL-2 for 14 days does not impair the antiviral function of these cells. Finally, in 4 patients, we compared the inhibition capacity of V\(\delta\)2 cells with that of CD8+ T cells, showing comparable inhibition capacity between both cell types (Supplemental Figure 2B).

V\(\delta\)2 T cells degranulate in the presence of HIV-infected CD4+ cells. The ability of V\(\delta\)2 cells to target HIV-infected CD4+ T cells was measured by analyzing CD107a expression. Total CD4+ T cells from 9 ART-suppressed HIV-infected individuals were isolated and superinfected with the HIV strain JR-CSF to act as targets in cocultures with autologous expanded V\(\delta\)2 cells. In preliminary experiments, isolated CD4+ cells were activated with phytohaemagglutinin (PHA) prior to superinfection with JR-CSF. However, we observed that cytotoxic V\(\delta\)2 cells were activated when CD4+ cells were exposed to PHA, regardless of HIV superinfection (Supplemental Figure 3A). Therefore, we used an alternative protocol to infect isolated CD4+ cells using polybrene rather than PHA. We detected a significant increase of CD107a expression in expanded V\(\delta\)2 cells after coculture with JR-CSF–infected CD4+ cells, but not when cultured with CD4+ cells without superinfection or alone (mean = 13.2%, 8.5%, and 8.3%, respectively, \(P = 0.006\); Figure 5, A and B). Expression of CD107a was also statistically higher in the HIV-CD4+ group activated with PHA (mean = 18.7%, \(P = 0.02\)), but compared with polybrene-infected targets there were not significant differences (\(P = 0.08\)). Finally, we determined if the state of HIV infection (acute or chronic) at the time of ART initiation had an impact on \(\gamma\delta\) T cell effector function. CD107a expression was comparable in cells from acute and chronic treated donors, suggesting that the cytotoxic function of V\(\delta\)2 cells may be recovered after ART initiation (Figure 5C). In summary, we demonstrate that expanded V\(\delta\)2 cells specifically degranulate in the presence of HIV-infected CD4+ cells.

\(\gamma\delta\) T cells can efficiently clear latently HIV-infected cells upon latency disruption. We measured the ability of \(\gamma\delta\) T cells to reduce the recovery of replication-competent HIV after reactivation of resting CD4+ (r-CD4+) T cells with vorinostat (VOR). We made a modification to the previously reported latency clearance assay protocol used for CD8+ T cells (8), which is itself a modification of viral outgrowth assays. This assay provides evidence of the capacity of effector cells to deplete r-CD4+ cells producing replication-competent
HIV following latency reversal. Our modification of the assay, which consists of depleting γδ T cells after 24 hours of coculture, is critical to allow evaluation of the specific clearance by γδ T cells. Adding this modification, we avoid measurement of nonspecific effects on the allogeneic uninfected cells added later for viral outgrowth. Briefly, r-CD4+ cells from 8 ART-suppressed donors (6 treated in chronic infection and 2 treated in acute infection) were isolated, exposed to VOR, and cocultured with or without autologous isolated expanded Vδ2 T cells. After 24 hours of culture, γδ T cells were depleted from the cultures, plated in replicate, and uninfected allogeneic CD4+ cells were added to amplify replication-competent HIV. After 15 out of 19 days of culture, viral outgrowth from r-CD4+ cells cultured alone was detected in 6 out of 8 HIV-infected donors, as measured by the number of HIV p24–positive wells. Interestingly, when γδ T cells were present in the coculture system, no virus was recovered in 4 donors, and viral recovery was reduced from 5 to 2, and 4 to 3 culture wells in the other 2 participants (Figure 6). These results demonstrate that expanded Vδ2 T cells can clear latently infected cells at the time of latency disruption by VOR.

**Discussion**

In this study, we assessed the prevalence, phenotype, function, and ex vivo expansion capability of γδ T cells from ART-suppressed HIV-infected individuals. We demonstrate the cytotoxic capabilities of these cells, as measured by their ability to kill autologous HIV-infected CD4+ T cells. In addition, Vδ2 T cells were able to expand up to 120-fold in response to PAM/IL-2 ex vivo and reduce viral replication up to 80% in autologous coculture systems. Overall, our results support the important finding that γδ T cells possess antiviral capabilities that are maintained in virologically suppressed individuals. Further, such antiviral γδ T cells can be expanded ex vivo to target latently infected cells induced to express HIV. The present work constitutes the first proof-of-concept to our knowledge showing that in the context of durable suppression of HIV infection, γδ T cells are capable of eliminating HIV-infected targets, suggesting that γδ T cells should be explored as a novel effector population to clear HIV infection from latent and active reservoirs.

As previously reported for the N-BP Zol (21), we show that PAM induces expansion of Vδ2 cells in HIV-infected individuals. Although V2 cell numbers were higher in patients treated in the acute phase
Figure 5. Vδ2 cells degranulate in the presence of autologous HIV-infected CD4+ T cells. (A) Flow cytometry plots showing an example of CD107a detection in cocultures of expanded Vδ2 cells with autologous CD4+ cells (left) and with autologous JR-CSF–superinfected CD4+ cells (right). (B) Greater CD107a production in the presence of HIV-infected cells. CD107a production was statistically higher when Vδ2 cells were cocultured with HIV-superinfected CD4+ cells compared with cocultures of autologous isolated CD4+ cells (FDR \( P = 0.006 \)). CD107a production was the highest when Vδ2 cells were cocultured with PHA-activated, HIV-superinfected CD4+ cells (FDR \( P = 0.02 \)) but without statistical differences compared with cells infected using polybrene. Mean ± SEM is represented. \( P = 0.08 \), Wilcoxon’s matched-pairs signed-rank test. (C) Comparable degranulation capacity of Vδ2 cells between donors treated in acute and chronic HIV infection. CD107a production was not statistically different between acute and chronic patients. Both groups of patients showed statistically higher CD107a expression in cocultures of Vδ2 cells and superinfected CD4+ target cells than in cultures of Vδ2 cells cocultured with ex vivo–isolated CD4+ cells. Effector/target ratio (1:1). Mann-Whitney U test.
ance of r-CD4+ infected cells. Our results demonstrate that of effector cells to recognize latent HIV reactivated by LRAs was evaluated. In our modified assay, we formed a modification of the previously reported latency clearance assay (8, 44). In this assay, the capacity term, and perhaps irreversible, changes at the surface of cells that are recognized by increased CD107a production, suggesting that PHA binding to glycosylated surface proteins produces long-

Even when isolated CD4+ cells were allowed to rest for 6 to 7 days after PHA activation, Vδ2 cells were activated by cells treated with PHA independently of HIV infection. CD107a production, although statistically significant, was not very high, suggesting the involvement of other pathways in HIV recognition (42). In addition, as only a fraction of the CD4+ cells used as targets may be infected, CD107a production may be potentially diminished compared with an assay where 100% of targets were activating γδ T cells. Antigen recognition by the γ TCR is generally not restricted to major MHC molecules (14, 15), although γδ T cell recognition of peptides loaded on MHC molecules has been reported (43). Interestingly, our MHC blocking experiments showed a moderate decrease in CD107a expression in Vδ2 cells that needs to be further investigated (Supplemental Figure 3). Our study also highlights the importance of carefully controlling for external factors in culture systems that might provide confounding results — such as the use of PHA to activate prior to infection, as cytotoxic Vδ2 cells were activated by cells treated with PHA independently of HIV infection. Even when isolated CD4+ cells were allowed to rest for 6 to 7 days after PHA activation, Vδ2 cells showed increased CD107a production, suggesting that PHA binding to glycosylated surface proteins produces long-term, and perhaps irreversible, changes at the surface of cells that are recognized by γδ T cells.

To further analyze the role of γδ T cells in a context more relevant to HIV eradication in vivo, we performed a modification of the previously reported latency clearance assay (8, 44). In this assay, the capacity of effector cells to recognize latent HIV reactivated by LRAs was evaluated. In our modified assay, we depleted γδ T cells from the coculture before the addition of uninfected target cells to ensure specific clearance of r-CD4+ infected cells. Our results demonstrate that γδ T cells reduce viral recovery following latency reversal of r-CD4+ cells with VOR. Immunotherapy with ex vivo–expanded γδ T cells has been used in oncology with little toxicity and overall good tolerability (27). Of great significance for future strategies aimed to cure HIV, the use of haploidentical expanded Vδ2 cells from relatives in adoptive transfer strategies has been successful (45, 46). This type of intervention may be valuable for future use in HIV eradication strategies, as γδ T cells can harbor replication-competent HIV (24). In this regard, supernatants from expansions showed a consistent negative HIV p24 detection. However, even if cells are expanded in the presence of antiretrovirals avoiding new rounds of infection, we still do not completely understand the overall importance of defective viruses that may be accumulating, and their modulating effector responses (47).

In summary, γδ T cells from HIV-infected individuals retain their functionality after expansion and constitute an attractive immunotherapeutic alternative or complementary tool to current approaches aimed
to cure HIV. Our work has opened potentially novel and intriguing questions regarding the basic biology, function, and specificities of γδ T cells. Here, we show proof-of-concept of the potential clinical use of γδ T cells in cellular therapy strategies for HIV cure.

**Methods**

**Study participants.** All HIV-infected donors included in this study were on ART and virologically suppressed (<50 copies/ml) for at least 1 year prior to study. Characteristics and inclusion criteria of these donors have been previously described (24, 48). HIV-infected donors treated in the acute phase of HIV infection started therapy within 45 days of the estimated date of infection. Buffy coats from uninfected donor volunteers were obtained from the New York Blood Center.

**Isolation of cell populations.** PBMCs from HIV-infected individuals were isolated from leukapheresis products, and cells from uninfected individuals were isolated from buffy coats, all by Ficoll-gradient centrifugation. Vδ2 cells and CD8+ T cells were isolated by fluorescence-activated cell sorting (FACS) using a FACS Aria II (BD). PBMCs were stained with monoclonal antibodies against CD3 (clone SK7), Vδ2 (clone B6), CD4 (clone SK3), and CD8 (clone SK1) (all from Biolegend). V2 T cells were defined as CD3+Vδ2+ and CD8+ T cells were defined by CD3+Vδ1+Vδ2+CD4-CD8+. CD4+ T cells were isolated from the same donor using a commercially available enrichment kit that contains antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCR-γ/δ, and glycophorin A (StemCell Technologies). r-CD4+ cells were isolated using a custom StemCell Technologies cocktail that contained antibodies against the following proteins: CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, γδ TCR, glycophorin A, CD66b, CD25, HLA-DR, and CD69. Purity of the FACS-isolated populations and resting CD4+ T cells was greater than 99%, and magnetically isolated CD4+ T cells showed a purity of greater than 96%.

**Expansion of Vδ2 cells.** To compare Vδ2 cell response to pyrophosphates and N-BP from HIV-infected and uninfected donors, 1 × 10^6 PBMCs were incubated in the presence of 100 nM HMBPP (provided by H. Jomaa, Justus-Liebig University, Giessen, Germany) and 100 μU/ml IL-2, or 25 μg/ml PAM and 100 μU/ml IL-2, or 100 μU/ml IL-2 alone. After 6 days, cells were stained with monoclonal antibodies against CD3 and V2 to analyze γδ T cell frequency by flow cytometry. Briefly, cells were harvested, washed, resuspended in staining buffer, incubated on ice in the dark for 20 minutes and finally washed and resuspended in 2% paraformaldehyde solution. Acquisition and analysis was performed on the Attune NxT flow cytometer (Applied Biosystems).

Fifty to 70 million PBMCs from HIV-infected individuals were incubated in complete IMDM containing antiretrovirals (10 mM raltegravir and either 10 mM abacavir or 1 μM efavirenz, depending on the patient’s regimen), 25 μg/ml pamidronate (PAM, Sigma-Aldrich), and 200 U/ml IL-2 during 14 days. Every 3–4 days, medium containing 100 U/ml IL-2 was refreshed. HIV p24 quantification (ABL, Inc.) at days 7 and 14 was consistently under the limit of detection of the assay.

**Phenotypic analysis of expanded Vδ2 cells.** At day 14 of expansion, an aliquot of PBMCs was harvested, washed, and resuspended in staining buffer to analyze the expression of different markers within expanded Vδ2 cells by flow cytometry. (a) Memory: CD45RA (clone HI100), CD27 (clone MT271), and CCR7 (clone G043H7); (b) cytotoxic: CD8 (clone SK1), CD56 (clone 5.1H11), and CD16 (clone 3G8); (c) activation: CD69 (clone FN50), CD25 (clone BC96), and HLA-DR (clone L243); and exhaustion: PD-1 (clone EH12.2H7) and CTLA-4 (clone BNI3). Cells were stained for 20 minutes on ice in the dark, washed, fixed in a 2% paraformaldehyde solution, acquired on a FACSAria II, and analyzed using FlowJo v.10.1 (Tree Star).

**HIV infection of isolated CD4+ T cells.** Isolated CD4+ T cells from HIV-infected donors were superinfected with the viral strain JR-CSF using 2 different approaches. (a) CD4+ cells were activated with 4 μg/ml PHA and 100 U/ml IL-2 for 24 hours, washed twice, and superinfected by spinoculation at 2,500 rpm for 2 hours. Cells were then extensively washed to remove free virions and then used for further experiments. (b) Isolated CD4+ cells were spinoculated at 2,500 rpm for 4 hours in the presence of 8 μg/ml polybrene. Cells were resuspended in complete media containing 20 U/ml IL-2 and without washing the virus were further cultured for 7 days. Then, cells were extensively washed, resuspended in suitable media and experiments were performed. As a control, isolated CD4+ cells were mock infected following the same 2 approaches. Level of infection of isolated CD4+ cells was routinely tested, quantifying HIV p24 production in culture supernatants by ELISA. Results showed a consistent efficacy of infection with a mean of 264.1 ng/ml for cells activated with PHA and 128.9 ng/ml for cells infected with polybrene. HIV p24 production was below the limit of detection of the assay for non-superinfected CD4+ T cells.
Viral inhibition assays. Viral inhibition assays using Vδ2 cells from HIV-infected individuals as effectors were performed as previously described for uninfected individuals (24), with some modifications. Fifty thousand infected CD4+ T cells were cocultured in triplicate at different effector/target ratios of 1:1, 1:10, and 1:100, unless otherwise noted. Infected CD4+ cells alone were also cultured in triplicate as control for HIV production. In some experiments, ex vivo–isolated CD8+ T cells were used as effector cells. Supernatants were harvested at day 7 and stored at –20°C until HIVp24 ELISA quantification (ABL, Inc.) was performed. Results are expressed as percentage of viral inhibition normalized to HIV p24 production when target CD4+ T cells were cultured alone.

Degranulation assays. CD107a was used as a functional marker of cytotoxicity. FACS-isolated, expanded Vδ2 T cells were cocultured with JR-CSF–infected autologous CD4+ cells as targets. CD4+ T cells were infected following the 2 different approaches described above. At least 100,000 effector cells were cocultured at a 1:1 ratio with CD4+ target cells in 96-well plates for 5 hours in the presence of GolgiStop (BD) and an anti-CD107a monoclonal antibody (clone H4A3, BD). In some experiments, MHC expression was blocked by incubating the cells with a pan-HLA monoclonal antibody (clone W6/32, Biolegend). Cells were then harvested, washed with staining buffer, stained with Vδ2-FITC (Biolegend) for 20 minutes on ice in the dark, washed twice, resuspended in staining buffer, and analyzed in the Attune Acoustic Focusing Cytometer (Applied Biosystems).

Assays to analyze clearance of latent HIV after reactivation. These experiments are based on the latency clearance assay reported for CD8+ T cells (8) and more recently for NK cells (44). Briefly, r-CD4+ cells from HIV-infected donors were isolated as described above, and exposed to 0.5 μM VOR for 18 hours. After washing, r-CD4+ cells were split into 2 conditions, one cultured alone and the other cocultured with isolated expanded γδ T cells at a 2:10 ratio (γδ cell/r-CD4+ cell). After 24 hours of culture, γδ cells were removed using a depletion magnetic bead–based kit according to the manufacturer’s instructions (Miltenyi Biotec). The same treatment was applied to both conditions in parallel. This γδ T cell depletion after the initial 24 hours of culture constitutes our modification of the original latency clearance assay reported for CD8+ T cells, providing definitive proof that the effect observed at the end of the culture is due to clearance of the reactivated r-CD4+ cells by δ T cells. After depletion, r-CD4+ cells were plated at 1 million cells/well. The same number of wells (from 7 to 23 replicates) were assayed for the condition of r-CD4+ cells alone and r-CD4+ cells cocultured with γδ T cells. As target cells to amplify viral signal, we added isolated, PHA-activated total CD4+ cells from uninfected donors at days 3 and 8 of culture. Supernatants were harvested at day 15 and 19 by HIV p24 ELISA. Depletion of γδ T cells after coculture was analyzed by flow cytometry, showing greater than 99.9% efficacy. Results are expressed as number of positive wells for HIV p24, comparing cultures of r-CD4+ cells with and without γδ T cells.

Study approval. All patients provided written informed consent prior to inclusion in the study, and studies were approved by the University of North Carolina (UNC) Institutional Review Board.

Statistics. Nonparametric tests were used. Comparisons between different groups were performed using the Mann-Whitney U test and repeated measures within same groups were analyzed using Wilcoxon’s matched-pairs signed-rank test. Where indicated, multiple comparisons were accounted for using FDR-adjusted P values (49). Statistical significance was considered for P < 0.05.

Author contributions
DMM and NSS conceptualized the study, provided resources, and acquired funding. CG and NSS developed the methodology. CG, MLC, CPW, and NSS performed the investigation. CG, DMM, and NSS wrote the draft of the manuscript. MH and NSS performed statistical analyses. NSS supervised the study.

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