HIV-Specific T Cells Can Be Generated against Non-escaped T Cell Epitopes with a GMP-Compliant Manufacturing Platform

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INTRODUCTION

Anti-retroviral therapy (ART) for HIV-1 can successfully prevent disease progression in many HIV+ individuals. However, HIV establishes persistent reservoirs, and patients need lifelong ART to prevent viral rebound. These reservoirs, in which proviruses generally remain latent, represent the greatest barrier to HIV cure.1–3 Current cure strategies are directed toward eradicating the reservoir and improving anti-viral immunity.4–10 The so-called kick-and-kill approach exposes reactivated latently infected cells to elimination by cell-mediated immunity, while ART is continued to prevent generation of newly infected cells.

T cells play a critical role in controlling HIV infection, as demonstrated by multiple lines of evidence, including the association of particular human leukocyte antigen (HLA) alleles with viral control, and the ability of HIV-specific CD8+ T cells to destroy infected cells.11–15 The maintenance of highly functional HIV-specific CD4+ T cells has also been associated with HIV control, likely highlighting the importance of CD4+ help and/or direct antiviral activity in viral control.16,17 In HIV+ individuals on ART, HIV-specific T cell (HST) immune responses wane, despite low levels of viremia in patients, suggesting that augmenting or re-directing the natural T cell response against remaining latenly HIV-infected cells might be a promising approach to eliminate residual virus.18–21

Virus-specific T cells have been used successfully to control reactivation and infection from Epstein-Barr virus (EBV) and cytomegalovirus (CMV).22–25 However, T cell immunotherapies against HIV have not achieved comparable success.26–32 The ability of HIV to mutate epitopes to evade T cell attack may contribute to the inability of T cell therapies to control infection. Anti-HIV T cell responses consist of ineffective clones targeting immunodominant but irrelevant epitopes, outnumbering the rare but effective HSTs targeting regions important for viral fitness. Subdominant HST clones in individuals better able to control HIV display anti-viral activity, suggesting that T cell immunotherapy targeting critical conserved HIV epitopes may be required to prevent development of escape variants.33,34

We previously demonstrated the generation of multi-antigen HSTs from both HIV+ and HIV-negative individuals using a Good Manufacturing practice (GMP)-compliant platform, we manufactured HSTs against non-escaped epitope targets (HST-NEETs) from HIV+ and HIV-seronegative donors. HST-NEETs expanded to clinically relevant numbers, lysed autologous antigen-pulsed targets, and showed a polyfunctional pro-inflammatory cytokine response. Notably, HST-NEETs recognized multiple conserved, non-escaped HIV epitopes and their common variants. We propose that HST-NEETs could be used to eliminate reactivated virus from latently infected cells in HIV+ individuals following LRA treatment. Additionally, HST-NEETs derived from HIV-negative individuals could be used post-transplant for HIV+ individuals with hematologic malignancies to augment anti-viral immunity and destroy residual infected cells.

Although anti-retroviral therapy (ART) is successful in suppressing HIV-1 replication, HIV latently infected reservoirs are not eliminated, representing a major hurdle in efforts to eradicate the virus. Current strategies to eradicate HIV involve two steps: (1) the reactivation of latently infected cells with latency reversing agents (LRAs) to expose persisting HIV, and (2) the elimination of these cells with immune effectors while continuing ART to prevent reinfecion. HIV-specific T cells (HSTs) can kill reactivated HIV-infected cells and are currently being evaluated in early-stage immunotherapy trials. HIV can mutate sequences in T cell epitopes and evade T cell-mediated killing of HIV-infected cells. However, by directing T cells to target multiple conserved, non-escaped HIV epitopes, the opportunity for viral escape can be reduced. Using a good manufacturing practice (GMP)-compliant platform, we manufactured HSTs against non-escape epitope targets (HST-NEETs) from HIV+ and HIV-seronegative donors.

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Manufacturing Practice (GMP)-compliant platform. Here, we seek to improve upon the current limited efficacy and persistence of immunotherapy for HIV by generating HSTs against non-escaped epitope targets (HST-NEETs), to decrease viral escape variants after infusion. The HST-NEET PepMix consisted of 40 fifteen-meric peptides spanning HIV Gag and Pol antigens, representing the tHIVconsX immunogen, or conserved regions of HIV associated with protection in natural HIV infection. tHIVconsX is a bivalent mosaic of peptides, containing two versions of each peptide represented in the PepMix, to cover the most common escape variants. Additionally, the entire peptide library of Negative Regulatory Factor (Nef) is included in the HST-NEET PepMix, to increase the potential for HIV-specific clones against multiple antigens in the T cell product. HST-NEET products from HIV+ individuals are denoted as HST-NEETpos, and HST-NEETs derived from HIV-negative individuals are denoted as HST-NEETneg throughout.

We compared the function and specificity of these HSTs against non-escape epitope targets (HST-NEETs) with HSTs generated against the entire peptide libraries of group-specific antigen (Gag), Nef, and DNA polymerase (Pol), without pre-selection for conserved epitopes. These HSTs have been used clinically and demonstrated safety, enabling the rapid translation of HST-NEETs to the clinic.

We manufactured HST-NEETpos and HST in parallel from the same donors with either acute or chronic HIV infection. Here, we show that HST-NEETpos functioned comparably with their HST counterparts, with similar degrees of expansion, phenotype, cytokine secretion, and exhaustion markers profiles. Epitope mapping of HST-NEETpos and HST revealed that HST-NEETpos donors could recognize multiple variants of conserved, non-escaped epitopes of HIV, critical for killing latently infected cells treated with LRAs. Such GMP-compliant HST-NEETs that reduce viral escape in vivo post-infusion could overcome a major hurdle in HIV cure strategies.

### RESULTS

**HST-NEETs Expand to Clinically Relevant Levels and Display HIV Specificity Comparable with HSTs**

HST-NEETpos and HST were generated from the same HIV+ donors in parallel. After 31 days of expansion, HST-NEETpos (median = 118e6 cells; range: 49e6–223e6 cells) displayed similar levels of expansion to HSTs (median = 97e6 cells; range: 70e6–198e6 cells) (Figure 1). HST-NEETpos and HST HIV specificity was measured by interferon-gamma (IFN-γ) spot-forming cells (SFCs) after PepMix stimulation against both PepMixes individually. HST-NEETpos stimulation with HST-NEET PepMix was significant compared with actin (p = 0.001, meanHST-NEET = 874 SFCs/1e5 cells), as was stimulation with HST PepMix (p = 0.001, meanHST = 834 SFCs/1e5 cells) (two-way ANOVA). Similarly, HST stimulation with HST PepMix was significant compared with actin (p < 0.0001, meanHST = 779 SFCs/1e5 cells), as was stimulation with HST-NEET PepMix (p = 0.0002, meanHST-NEET = 700 SFCs/1e5 cells). In both cases, HST-NEETpos and HST produced slightly higher IFN-γ against the actual PepMix they were manufactured with, compared with the other type of PepMix.

### HSTs and HST-NEETs Demonstrate a Skewed CD8+ T Cell Response with Minimal Expression of Markers Associated with Exhaustion

As expected, both HST (median = 85.00%; range: 62.47%–90.10%) and HST-NEETpos (median = 83.97%; range: 52.77%–91.80%) derived from HIV+ individuals with acute or chronic HIV infection
displayed a skewed CD8+ T cell response, with almost negligible CD4+ T cells (Figure 2). In addition, both products displayed a T effector memory phenotype (meanHST = 86.12%; meanHST-NEET = 84.14%). Analysis of markers associated with T cell exhaustion including cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), programmed cell death protein-1 (PD-1), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) revealed minimal expression of these markers on CD3+ cells. However, low-level expression of these markers is associated with T cell activation. As such, we tested the functionality of these HST and HST-NEETpos products, looking at cytokine secretion in response to stimulation and in vitro cytotoxic killing potential.

HST-NEETs Demonstrate Cytotoxicity against Autologous Antigen-Pulsed Targets and a Polyfunctional Immune Response to Stimulation

We evaluated the ability of HST-NEETpos and HST to lyse autologous peptide-pulsed phytohemagglutinin (PHA) blast (PHAb) targets in a chromium-51-release cytotoxicity assay (n = 7). At an effector-to-target ratio of 40:1, there was no significant difference (p = 0.3742) in the percentage of specific lysis by HST (mean = 32.67%) versus HST-NEETs (mean = 30.56%) (Figure 4). There was a significant difference compared with the unpulsed PHAb control condition (HST: p = 0.0001; HST-NEET: p = 0.0007), indicating the lysis of autologous targets was due to recognition of peptides presented on the surface of the PHAb.

HST-NEETs Recognize Bivalent Conserved Non-escaped Mosaic Epitopes

Both HST and HST-NEET products were epitope mapped with 15-mer HIV peptides to determine the individual epitopes recognized by each product. Importantly, we wanted to determine whether HST-NEETpos products recognized conserved non-escaped epitope targets from the HST-NEET PepMix based off the tHIVconsvX immunogen.36 Table 1 shows the paired epitope mapping results (n = 5) comparing HST with HST-NEETpos products for each HIV+ donor. Critically, we confirmed that HST-NEETpos products recognize both common variants of the bivalent mosaic epitopes in the HST-NEET PepMix (footnote “a” in Table 1). Notably, despite the differences in PepMix composition, there were commonly recognized epitopes between HST and HST-NEETpos products, indicated by matching colors. Lastly, there were some individual differences in epitope recognition, indicated under unmatched epitopes.

Where cells were available, HLA blocking experiments were conducted to determine class I or II epitope restriction. In a representative analysis (donor 4 shown), all epitopes showed class I restriction, as products, indicating that despite low-level expression of markers associated with T cell activation and exhaustion, these products were highly responsive to HIV PepMix stimulation.

HSTs and HST-NEETs Secrete TNF-α and IFN-γ in Response to HIV PepMix Stimulation

HST and HST-NEETpos were stimulated with their respective HIV PepMix, and tumor necrosis factor alpha (TNF-α) and IFN-γ cytokine secretion were measured by ICS for products generated from the same HIV+ donor (n = 5) (Figure 3). Flow cytometric analysis revealed populations secreting TNF-α, IFN-γ, and cells that were positive for both TNF-α and IFN-γ in both HST (meanTNF-α+ = 15.7%, meanIFN-γ+ = 2.7%, meanTNF-α+IFN-γ+ = 5.9%) and HST-NEETpos (meanTNF-α+ = 19.7%, meanIFN-γ+ = 4.4%, meanTNF-α+IFN-γ+ = 10.2%) of autologous targets was due to recognition of peptides presented on the surface of the PHAb.
expected, based on the primarily CD8\(^+\) T cell composition of the products (Figure 5). There was also a pattern of dominant or high responses (top) with >300 IFN-\(\gamma\) SFCs, mixed with sub-dominant or low responses (bottom) of 50–150 IFN-\(\gamma\) SFCs, seen in both HST and HST-NEET\(^{pos}\) products.

**HST-NEETs Derived from HIV-Negative Donors for Use Post-allogeneic Hematopoietic Stem Cell Transplant (HSCT)**

We wanted to expand the clinical applications of HST-NEETs to allo-geneic hematopoietic stem cell transplantation, because the first case of HIV cure, the Berlin patient, was achieved in this setting. We therefore attempted to generate HST-NEETs from HIV-negative donors, denoted HST-NEET\(^{neg}\). Using the same manufacturing platform, we successfully generated HST-NEET\(^{neg}\) demonstrating robust expansion (median: 161e6 cells; range: 45–376e6 cells) (Figure 6A). HST-NEET\(^{neg}\) displayed significant levels of HIV specificity against both HST (mean: 222 IFN-\(\gamma\) SFCs; \(p = 0.0246\)) and HST-NEET (mean: 362 IFN-\(\gamma\) SFCs; \(p = 0.0011\)) PepMixes compared with the actin control (mean: 3 IFN-\(\gamma\) SFCs) (Figure 6B). These products displayed a mix of CD4\(^+\) (mean: 29.48%) and CD8\(^+\) (mean: 46.10%) T cells, unlike their HIV\(^+\) counterparts, and displayed minimal expression of markers associated with T cell exhaustion (Figures 6C and 6D). To assess polyfunctionality, cytokine secretion was measured in response to PepMix stimulation by both multiplex and intracellular cytokine staining (ICS) (Figures 6E and 6F), and showed secretion of IFN-\(\gamma\), MIP-1b, and TNF-\(\alpha\). Importantly, there were IFN-\(\gamma\)^+\(\times\), TNF-\(\alpha\)^+\(\times\), and IFN-\(\gamma\)^+TNF-\(\alpha\)^+\(\times\) populations by ICS for both CD8\(^+\) and CD4\(^+\) populations, indicating that HST-NEET\(^{neg}\) may recognize both class I and class II-restricted epitopes. The ability to manufacture HST-NEET\(^{neg}\) products from HIV-negative donors could have significant implications in the post-transplant setting, as a method to kill any residual HIV-infected cells.

**DISCUSSION**

In this study, we demonstrated that it is possible to generate HSTs against non-escaped conserved epitopes (HST-NEETs) from both
negative donors. HST-NEETneg products showed robust expansion to be safe and well tolerated, with minimal adverse events. However, in or chronic HIV infection who remain on ART. These products appear in vivo phase I clinical trial (NCT02208167) in HIV+ individuals with acute measurable reduction in the HIV reservoir. HSTs have been used in a setting, for HIV+ individuals with hematologic malignancies, we to combining this immunotherapy with an LRA.

To explore this, HST-NEET products are being evaluated in a phase I clinical trial (IND17562) in participants with acute or chronic HIV infection. The products will be tested for safety prior to use in combination with an LRA, to discover whether HST-NEETpos can produce a measurable reduction in the HIV reservoir. HSTs have been used in a phase I clinical trial (NCT02208167) in HIV+ individuals with acute or chronic HIV infection who remain on ART. These products appear to be safe and well tolerated, with minimal adverse events. However, in the absence of an LRA to reactivate and expose latently infected cells to PepMix stimulation. Importantly, HST-NEETpos displayed the ability to recognize both variants of the epitopes in the bivalent mosaic HST-NEET PepMix. The recognition of multiple variants of conserved epitopes may be critical in preventing viral escape in vivo.

HIV+ and HIV-negative donors. Similar to HSTs, HST-NEETpos showed robust expansion, the ability to lyse autologous antigen-pulsed targets, and IFN-γ and TNF-α secretion in response to HIV PepMix stimulation. Importantly, HST-NEETpos displayed the ability to recognize both variants of the epitopes. The recognition of multiple variants of conserved epitopes may be critical in preventing viral escape in vivo.

To explore this, HST-NEET products are being evaluated in a phase I clinical trial (IND17562) in participants with acute or chronic HIV infection. The products will be tested for safety prior to use in combination with an LRA, to discover whether HST-NEETpos can produce a measurable reduction in the HIV reservoir. HSTs have been used in a phase I clinical trial (NCT02208167) in HIV+ individuals with acute or chronic HIV infection who remain on ART. These products appear to be safe and well tolerated, with minimal adverse events. However, in the absence of an LRA to reactivate and expose latently infected cells to infused T cells, the antigen burden remained low during ongoing ART, without measurable reduction in the number of infected CD4+ T cells. Thus, we plan to assess the safety and tolerance of HST-NEETpos, prior to combining this immunotherapy with an LRA.

To expand the applications of this therapy to the allogeneic transplant setting, for HIV+ individuals with hematologic malignancies, we demonstrated the ability to generate HST-NEETpos from five HIV-negative donors. HST-NEETpos products showed robust expansion and significant levels of IFN-γ and TNF-α secretion in response to HIV PepMix stimulation. Significantly, HST-NEETpos products were composed of both CD4+ and CD8+ T cell subsets, and both subsets secreted cytokine in response to PepMix stimulation, indicative of both class I and class II recognition of HIV epitopes. CD4+ T cells in immunotherapy have been linked to improved persistence, suggesting that the mixed HST-NEETpos phenotype may be important in providing long-term anti-HIV immunity in patients after hematopoietic stem cell transplant, particularly when they are most susceptible to viral reactivation. These HST-NEETpos products could be used in a potential cure strategy to target the low levels of residual HIV-infected cells that remain post-transplant.

HST-NEETs recognize conserved HIV epitopes, which may be critical in vivo in preventing viral escape. It will be important to epitope map clinical products and sequence autologous virus isolated from participants, to determine whether infusion of T cells that recognize these epitopes can generate a measurable reduction in the number of HIV-infected CD4+ T cells. Ultimately, this therapy has several potential applications, including use in both the autologous setting for HIV+ individuals with acute or chronic HIV infection and the allogeneic setting in HIV+ individuals with hematologic malignancies.

**MATERIALS AND METHODS**

**Isolation of Peripheral Blood Mononuclear Cells**

Peripheral blood mononuclear cells (PBMCs) were isolated from HIV+ adult donors on ART (Maple Leaf Medical Clinic, Toronto, ON, Canada; Whitman Walker Health Clinic, Washington, DC, USA) and from HIV-negative donors (NIH, Blood Bank). All donations were obtained under informed consent protocols, approved at the respective institution’s institutional review board (IRB). PBMCs were isolated from leukopheresis samples and buffy coats through dilution with 1× PBS, and layered on top of 10–15 mL of Lymphocyte Separation Medium (MP Biomedicals, CA, USA). Samples were spun for 30 min at 600 × g at room temperature (RT) for density separation. PBMCs were harvested from the lymphocyte layer and washed three times with 1× PBS.

**Generation of Dendritic Cells and Phytohemagglutinin Blasts (PHAbs)**

PBMCs were plated in dendritic cell (DC) medium (CellGenix, Germany) for 2–4 h on tissue culture plates at 37°C. Non-adherent cells were washed off and cryopreserved. Adherent cells were cultured with interleukin-4 (IL-4: 1,000 U/mL; R&D Systems, MN, USA) and granulocytemacrophage colony-stimulating factor (GM-CSF: 800 U/mL; R&D Systems, MN, USA), and day 5 DCs matured as previously described.5,35 DCs were harvested 24–48 h after maturation and loaded with peptide antigens for stimulation 1. PHAbs were generated for antigen presentation as previously described,5,35 for stimulations 2 and 3.

**Generation of HIV-Specific T Cells (HSTs)**

Matured DCs were incubated with either HST-NEET PepMix (custom order; JPT Technologies) or HST PepMix (spanning the entirety of Gag and Pol; JPT Technologies). The HST-NEET PepMix consisted of 402 fifteen-meric peptides spanning non-escaped, conserved HIV Gag and Pol epitopes, based on the tHIVconsX

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**Figure 4. Functionality of HST and HST-NEETpos Cell Products**

(A) 51Chromium-release cytotoxicity assay was used to assess the ability of HST and HST-NEETpos cell products to kill autologous PHAb pulsed with HIV PepMix. At an effector-to-target ratio of 40:1, both products killed autologous targets, displaying similar levels of specific lysis (n = 14). Each condition was plated in triplicate. (B) Multiplex demonstrating the range of IFN-γ, MIP-1β, and TNF-α secretion by HST and HST-NEETpos products in response to HIV PepMix (n = 14).
Both HST-NEET and HST PepMixes included the peptide library spanning the length of Nef (JPT Technologies). DCs were incubated with the respective PepMix for 1 h in cytotoxic T lymphocyte (CTL) medium (Click's Medium; Irvine Scientific, CA, USA; 10% FBS, GE Healthcare; GlutaMAX, GIBCO). Autologous PBMCs or non-adherent cells from DC generation were thawed and subsequently stimulated according to our established protocol. HSTs derived from HIV+ individuals were grown in a combination of anti-retrovirals (ARVs) (indinavir, raltegravir, and enfuvirtide) throughout the expansion process. HSTs derived from HIV-negative donors were grown according to the methods described above, without ARVs.

**IFN-γ Enzyme-Linked Immune absorbent SPOT (ELISPOT) Assay and Epitope Mapping**

Staphylococcal enterotoxin B (SEB) was used as a positive control (Sigma-Aldrich, MO, USA), and either cells alone (negative control, no peptides) or actin (JPT Technologies, Germany) was used as an irrelevant antigen control. HST-NEETs and HSTs were plated at 1e5/well on anti-IFN-γ-coated ELISPOT plates (Millipore, NJ, USA). Positive responses were defined as having more than four times the SFCs obtained in the negative control actin. For epitope mapping, peptides were mixed into pools of 10–15 peptides prior to T cell stimulation on an ELISPOT as described previously. Using matrices, cross-reactive pools were analyzed for common epitopes, and these peptides were then individually tested on ELISPOT to confirm specificity.

**HLA Class I and II Restriction Determination**

HSTs and HST-NEETs were tested for HLA specificity to individual HIV peptides. For HLA blocking, 1e5 cells/well were treated with irradiated PHAbs. The table below shows the epitope mapping of HST-NEETs and HSTs for different donors, highlighting matched and unmatched epitopes. The table also indicates the diversity of epitope breadth among cell products, with most products recognizing multiple HIV epitopes. Additionally, HST-NEET cell products demonstrated the ability to recognize bivalent mosaic epitopes, or multiple escape variants of the same HIV epitope. **Bivalent** mosaic epitopes were recognized (both variants). Epitope sequences recognized in both HST-NEET and HST peptide pools by each donor cell product are preceded by the same footnote letter. The number of footnotes per donor cell product emphasizes the multi-epitope specificity generated with this manufacturing strategy.

### Table 1. Epitope Mapping of HST-NEETpos and HST Products

| Donor 1 | Matched Epitopes: |
|---------|------------------|
| MOS-19: INEEAAEWDRVHPVH | GAG-54: TINEEAAEWDRLPY |
| MOS-20: AAEWDRVHPVHAGP | GAG-55: EAAEWDRLHPVHAGP |
| MOS-218: INEEAAEWDRLHPVH | NEF-23: DLSHFLKEGGLEGL |
| MOS-219: AAEWDRLHPVHAGP | NEF-24: FLKEGGLEGLGYSK |
| GAG-53: MLKDTINEEAAEWDR | NEF-35: PGIRYPITGFVCFL |
| GAG-54: TINEEAAEWDRLPV | NEF-19: RIQVPFLRMTYKAAL |
| GAG-55: EAAEWDRLHPVHAGP | NEF-21: MTKAALDLHFLK |
| NEF-23: DLSHFLKEGGLEGL | NEF-36: YPLTFGWCFKLVVP |
| NEF-24: FLKEGGLEGLGYSK | Unmatched Epitopes |
| NEF-35: PGIRYPITGFVCFL | NEF-17: EEVGFVPVRQVPLR |

| Donor 2 | Matched Epitopes: |
|---------|------------------|
| MOS-3: QMVHQAISPRTLNAW | GAG-38: GQMVHQAISPRTLNA |
| MOS-202: QMVHQALSPRTLNAW | NEF-30: LWYHTQGYFPDQWN |
| NEF-30: LWYHTQGYFPDQWN | NEF-31: HTQGYFPDQWNYTIPG |
| NEF-31: HTQGYFPDQWNYTIPG | POL-242: VQDNSDKVPPRRK |
| POL-242: VQDNSDKVPPRRK | POL-243: NSDKVPPRRKAKI |
| POL-243: NSDKVPPRRKAKI | Unmatched Epitopes |
| POL-99: KTVTPQVPVLPEKDSW | GAG-63: GTTSLQEQIAWMTS |
| POL-218: MNKELKKIQVYRDQ | NEF-17: EEYGVPPVQRQVPLR |
| NEF-35: PGIRYPITGFVCFL | POL-148: IVGAETFYDVDAANR |

| Donor 3 | Matched Epitopes: |
|---------|------------------|
| MOS-194: KVVPVRKKVIIKDY | POL-244: KVVPVRKKIIRIDY |
| MOS-195: PRKRVRDDYKGM | POL-245: RRKAKIRDDYKGM |
| MOS-398: KVVPVRKKIIRIDY | POL-246: RRKAKIRDDYKGM |
| MOS-399: PRKRAKIRDDYKGM | POL-247: RRKAKIRDDYKGM |
| POL-244: KVVPVRKKIIRIDY | Unmatched Epitopes |
| POL-53: NPYNTPVAFIKKDS | POL-148: IVGAETFYDVDAANR |

| Donor 4 | Matched Epitopes: |
|---------|------------------|
| MOS-3: QMVHQAISPRTLNAW | GAG-38: GQMVHQAISPRTLNA |
| MOS-202: QMVHQALSPRTLNAW | NEF-30: LWYHTQGYFPDQWN |
| NEF-30: LWYHTQGYFPDQWN | NEF-31: HTQGYFPDQWNYTIPG |
| NEF-31: HTQGYFPDQWNYTIPG | POL-242: VQDNSDKVPPRRK |
| POL-242: VQDNSDKVPPRRK | POL-243: NSDKVPPRRKAKI |
| POL-243: NSDKVPPRRKAKI | Unmatched Epitopes |
| POL-99: KTVTPQVPVLPEKDSW | GAG-63: GTTSLQEQIAWMTS |
| POL-218: MNKELKKIQVYRDQ | NEF-17: EEYGVPPVQRQVPLR |
| NEF-35: PGIRYPITGFVCFL | POL-148: IVGAETFYDVDAANR |

| Donor 5 | Matched Epitopes: |
|---------|------------------|
| MOS-194: KVVPVRKKVIIKDY | POL-244: KVVPVRKKIIRIDY |
| MOS-195: PRKRVRDDYKGM | POL-245: RRKAKIRDDYKGM |
| MOS-398: KVVPVRKKIIRIDY | POL-246: RRKAKIRDDYKGM |
| MOS-399: PRKRAKIRDDYKGM | POL-247: RRKAKIRDDYKGM |
| POL-244: KVVPVRKKIIRIDY | Unmatched Epitopes |
| POL-53: NPYNTPVAFIKKDS | POL-148: IVGAETFYDVDAANR |

| Donor 6 | Matched Epitopes: |
|---------|------------------|
| MOS-217: LKETINEEAAEWDR | GAG-53: MLKDTINEEAAEWDR |
| GAG-53: MLKDTINEEAAEWDR | NEF-19: RIQVPFLRMTYKAAL |

### Table 1. Continued

| Donor 7 | Matched Epitopes: |
|---------|------------------|
| MOS-125: GSPAIJQSSMTKILE | GAG-5: EKIRLRPGGKKKYRL |
| POL-124: TYQIYQEPFKNLKTG | Cell products were mapped by ELISPOT to determine their epitope restrictions. As shown on the table, the epitope breadth was diverse among cell products, with most products recognizing multiple HIV epitopes. Additionally, HST-NEET cell products demonstrated the ability to recognize bivalent mosaic epitopes, or multiple escape variants of the same HIV epitope. **Bivalent** mosaic epitopes were recognized (both variants). Epitope sequences recognized in both HST-NEET and HST peptide pools by each donor cell product are preceded by the same footnote letter. The number of footnotes per donor cell product emphasizes the multi-epitope specificity generated with this manufacturing strategy. |
monoclonal mouse anti-human HLA class I or HLA class II antibody (Dako, Agilent, CA, USA) in a 96-well round-bottom plate for 1 h at 37°C. Treated cells were transferred to ELISPOT plate, stimulated with peptide, and developed as previously described.6,35

Flow Cytometry
Samples for intracellular staining (ICS) were incubated at 37°C + 5% CO2 in the presence of brefeldin A (1/100; GolgiPlug; BD Biosciences) for 5 h prior to staining. Unstimulated and SEB-stimulated cells were plated as negative and positive controls for IFN-γ and TNF-α production. Following incubation, all samples were washed and resuspended in PBS with 5 μL Fc receptor (FcR) block to control against non-specific binding. A total of 100 μL Fixable Live Dead Aqua viability dye (1/1,000; Thermo Fisher Scientific) was added for 15 min at RT in the dark. The cells were washed and resuspended in 50 μL of pre-prepared antibody cocktails. Antibody cocktails were prepared in Brilliant Stain Buffer using optimally titrated antibody concentrations. The following antibodies were used across three panels, all from BioLegend, unless otherwise indicated: CD16 FITC, CD127 phycoerythrin (PE), CD14 peridinin-chlorophyll-protein complex (PerCP) (Miltenyi), CD19 PerCP Cy5.5, CD57 PE-Vio770 (Miltenyi), CD25 Alexa Fluor 700, CD3 APC-Fire750, CD8 BV421, CD4 BV605, CD95 PE-Dazzle594, CD28 PE-Cy5 (BD Biosciences), CD45RO PE-Vio770 (Miltenyi), CCR7 Alexa Fluor 700, CD62L BV650, CTLA-4 FITC (eBioscience), TNF-α PE, Perforin PerCP Cy5.5, LAG3 (CD223) PE-Cy7, PD-1 APC (BD Biosciences), IFN-γ Alexa Fluor 700 (Invitrogen), and TIM3 (CD366) BV650. All samples were acquired on a BD FACSCalibur flow cytometer with a Cytek upgrade to 15 parameters (407-, 488-, 561-, and 637-nm lasers). Data were analyzed with FlowJo software. Background autofluorescence was calculated off unstained cells; OneComp eBeads (Invitrogen) were used for single-color compensation controls.

Cytotoxicity Assay
The cytotoxic ability of HST-NEETs and HSTs was determined with a chromium-51-release assay. Autologous PHAb targets were pulsed with nothing (negative control), HST-NEET PepMix, or HST PepMix, and incubated with chromium-51 for 1 h. Targets were washed three times and co-cultured with autologous effector T cells at varying effector-to-target ratios. Cr51 release assay was set up as previously described.6 Specific lysis % was measured as: (Experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Multiplex Assay
Polyfunctionality of HST and HST-NEET products was assessed with a Bio-plex Pro Human 17-plex Cytokine Assay kit (Bio-Rad, CA, USA). HST-NEETs and HSTs were washed and plated at 1e6 cells/well with
1 μL of corresponding PepMix: actin, HST or HST-NEET, or SEB. The next day, supernatants were harvested from the wells and assessed based on the Bio-Rad multiplex protocol. Samples were analyzed for concentrations of cytokines based on the standard curves produced.

Statistical Analysis
Two-way ANOVA with Holm-Sidak correction was used to determine the statistical significance of IFN-γ release on ELISPOT in response to HIV antigens compared with the irrelevant control, actin. Means, medians, and ranges were provided where applicable.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.10.001.

Figure 6. Characterization of HST-NEET<sup>neg</sup> Cell Products
Using the same manufacturing process, we generated HSTs from HIV-negative donors, using HST-NEET PepMix. (A and B) HST-NEET<sup>neg</sup> products demonstrated (A) expansion and (B) significant specificity against HIV PepMixes by ELISPOT. (C and D) The phenotype of HST-NEET<sup>neg</sup> was more diverse compared with their HST-NEET<sup>pos</sup> counterparts (C), as shown by the mixed composition of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with minimal expression of exhaustion markers (D). (E and F) HST-NEET<sup>neg</sup> cell products secreted IFN-γ, MIP-1b, and TNF-α in response to HIV PepMix stimulation, demonstrating CD4<sup>+</sup> and CD8<sup>+</sup> polyfunctionality (n = 6) (E). The ICS plots show representative data from two HIV-negative donor-derived HST-NEET<sup>neg</sup> cell products (F). p values represent significance of Two-way ANOVA between actin and the HIV peptide pools.

AUTHOR CONTRIBUTIONS
S.P. designed experiments, conducted formal data analysis, and wrote the manuscript. S.P., R.H., M.G., D.S., and S.V.P. conducted the experiments. S.P., G.S., M.K., P.J.H., D.F.N., R.B.J., and C.M.B. contributed to the protocol development and provided expertise on experimental approaches. C.M.B. supervised the experiments, provided funding acquisition, and wrote the manuscript. All authors contributed to the editing of the manuscript.

CONFLICTS OF INTEREST
P.J.H. is a founder and director of Mana Therapeutics. C.M.B. is on the scientific advisory boards for Cellectis, has stock options in Neximmune and Torque Therapeutics, and has stock or ownership in Mana Therapeutics. R.B.J. is on the scientific advisory board of...
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