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Identification and characterization of antigen-specific CD8+ T cells using surface-trapped TNF-α and single-cell sequencing

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Abstract

CD8+ T cells are key mediators of antiviral and antitumor immunity. The isolation and study of antigen-specific CD8+ T cells, as well as mapping of their MHC restriction, has practical importance to the study of disease and the development of therapeutics. Unfortunately, most experimental approaches are cumbersome, due to the highly variable and donor-specific nature of MHC-bound peptide/TCR interactions. Here we present a novel system for rapid identification and characterization of antigen-specific CD8+ T cells, particularly well-suited for samples with limited primary cells. Cells are stimulated *ex vivo* with antigen of interest, followed by live cell sorting based on surface-trapped TNF-α. We take advantage of major advances in single-cell sequencing to generate full-length sequence from the paired TCR alpha and beta chains from these antigen-specific cells. The paired TCR chains are cloned into retroviral vectors and

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used to transduce donor CD8$^+$ T cells. These TCR transductants provide a virtually unlimited experimental reagent, which can be used for further characterization, such as minimal epitope mapping or identification of MHC restriction, without depleting primary cells. We validated this system using Cytomegalovirus-specific CD8$^+$ T cells from rhesus macaques, characterizing an immunodominant Mamu-A1*002:01-restricted epitope. We further demonstrated the utility of this system by mapping a novel HLA-A*68:02-restricted HIV Gag epitope from an HIV-infected donor. Collectively, these data validate a new strategy to rapidly identify novel antigens and characterize antigen-specific CD8$^+$ T cells, with applications ranging from the study of infectious disease to immunotherapeutics and precision medicine.

Introduction

CD8$^+$ T cells are key mediators of antiviral and antitumor immunity (1, 2). During maturation, naïve T cells undergo somatic DNA rearrangement to form a unique T cell receptor (TCR) (3, 4). This receptor is a heterodimer of two unique and variable TCR chains (most commonly alpha and beta chains), which interact with the invariant CD3 chains (5). The alpha/beta chains form a receptor that recognizes antigenic peptides presented by MHC molecules on the antigen presenting cell. The diversity of MHC/HLA genotypes across the population ensures that the identity of antigenic peptides is equally diverse and donor-specific. Determining the identity of these antigenic peptide/MHC pairs can be a laborious and expensive process (6, 7). For these reasons, the study of CD8$^+$ T cells frequently leverages a handful of well-characterized immunodominant epitopes presented by common MHC/HLA alleles, such as Mamu-A1*001:01 (A*01)/SIV Tat SL8 in SIV-infected macaques, HLA*B27/HIV Gag KK10 in HIV-infected humans, or HLA-A*0201/IAV $M1_{58–66}$ in influenza-infected humans (8–11).

Characterizing the CD8$^+$ T cell response to a pathogen does not necessarily require knowing the identity of the antigenic peptides. Upon antigenic stimulation, such as antigen presenting cells (APCs) pulsed with peptide pools or virally-infected targets, activated CD8$^+$ T cells can up-regulate surface markers or secrete cytokines, including interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Flow cytometric or ELISpot assays take advantage of these markers to identify and perform basic characterization of responding cells (12, 13). One common flow-based assay is intracellular cytokine staining (ICS), in which CD8$^+$ T cells are exposed to antigen in the presence of Brefeldin A (BFA), a compound that inhibits protein transport from the endoplasmic reticulum (ER) (14). BFA prevents cytokine secretion, trapping IFN-γ and TNF-α within the cell, thereby providing a highly sensitive flow-based readout of activation (14, 15). It should be noted that ICS requires fixation and permeabilization of the cells, and therefore does not permit isolation of viable cells. If viable cells are required, staining and sorting using fluorescently labeled MHC-I tetramers (or higher-order multimers), remains the gold standard (16, 17). While these tetramers can be highly specific, they are limited to previously characterized MHC/peptide specificities, making them costly to generate and limiting the utility of a given tetramer. Cytokine-capture methods provide an alternative to tetramer staining (18). One form of capture assays uses bi-specific antibodies to tether a secreted molecule, such as IFN-γ, to the cell surface (19–21). Capture methods have the advantage of not requiring knowledge of antigenic peptides, and

they can provide viable cells for downstream characterization; however, they often require species-specific reagents. An alternative cytokine capture method involves incubating $CD8^+$ T cells in the presence of TAPI-0, a metalloprotease inhibitor that blocks the activity of TNF-α converting enzyme (TACE) (22). TACE inhibition prevents the cleavage of TNF-α, thereby tethering it to the cell surface and providing a surface marker that can be used to live-sort viable cells. TCR sequencing has demonstrated that $CD8^+$ T cells sorted using surface-trapped TNF-α can be highly specific and sensitive (23). Sorting cells based on functional attributes, such as TNF-α production, may even have advantages over tetramer-staining because it identifies cells that are functional, and may identify clones with TCRs that bind with low affinity to MHC molecules (24, 25). For these reasons, while TCR sequencing has demonstrated that the set of clones identified by cytokine capture assays broadly recapitulate tetramer staining, the clonal frequencies are not expected to be identical for each method (23, 24).

Identification of antigen-specific TCR sequences would provide an alternative strategy to study $CD8^+$ T cells; however, the identification of TCR sequences can be extremely laborious. A productive alpha/beta TCR is comprised of two independently coded chains (26, 27). Generating the sequence of the paired alpha/beta TCRs from antigen-specific $CD8^+$ T cell is most readily accomplished through single-cell sequencing (28). Single cell sequencing methods have advanced dramatically, with droplet based methods now capable of capturing transcriptomic data from thousands of individual cells in a single experiment (29–31). Further, these methods have been extended to allow PCR enrichment of TCR or B cell receptor (BCR) sequences, providing near-full length sequence data (32). Once the sequence is known, the TCR alpha/beta pair can be synthesized and exogenously expressed in donor $CD8^+$ T cells (33, 34). This exogenous expression has been shown to recapitulate the antigenic potential of the original cell (35, 36). Further, modifications have been published to enhance the surface expression of exogenous TCR, and prevent pairing with any endogenous TCR from the donor cells (37).

We reasoned that the combination of cytokine-capture, massively parallel single-cell sequencing, and exogenous TCR expression in donor cells would provide a novel system to characterize antigen specific $CD8^+$ T cells and identify antigenic peptides. This scheme is especially well suited to situations where primary cells are limited, since it only requires one round of screening and TCR identification from primary patient samples. We tested this system using Cytomegalovirus-specific $CD8^+$ T cell responses in rhesus macaques, validating the sensitivity and accuracy of this approach. We further demonstrate efficacy using cells from an HIV-infected donor, successfully mapping a novel HLA-A*68:02-restricted HIV Gag epitope.

Materials and Methods

Animals

A total of 24 Indian-origin rhesus macaques were used in this study. At assignment, all study macaques were free of cercopithecine herpesvirus 1, D-type simian retrovirus, simian T-lymphotropic virus type 1, and *Mycobacterium tuberculosis*. All animals, except for the enhanced specific-pathogen free macaques, were naturally infected with rhesus CMV. All

study macaques were housed at the Oregon National Primate Research Center (ONPRC) in animal biosafety level 2 rooms with autonomously controlled temperature, humidity, and lighting. Macaques were fed commercially prepared primate chow twice daily and received supplemental fresh fruit or vegetables daily. Fresh, potable water was provided via automatic water systems. Physical examinations including body weight and complete blood counts were performed at all protocol time points. Macaque care and all experimental protocols and procedures were approved by the ONPRC Institutional Animal Care and Use Committee. The ONPRC is a Category I facility. The Laboratory Animal Care and Use Program at the ONPRC is fully accredited by the American Association for Accreditation of Laboratory Animal Care and has an approved assurance (no. A3304–01) for the care and use of animals on file with the National Institutes of Health Office for Protection from Research Risks. The Institutional Animal Care and Use Committee adheres to national guidelines established in the Animal Welfare Act (7 U.S. Code, sections 2131–2159) and the *Guide for the Care and Use of Laboratory Animals, Eighth Edition* as mandated by the U.S. Public Health Service Policy.

Human Subjects

This study includes PBMC samples from a patient enrolled in the well-characterized SCOPE (Study of the Consequences of the Protease Inhibitor Era) cohort. SCOPE is an observational prospective cohort based on the HIV/AIDS clinics at the Zuckerberg San Francisco General Hospital and the San Francisco Veterans Affairs Medical Center (San Francisco, CA). All subjects provided written informed consent and the parent study was approved by the UCSF Committee on Human Research. All subjects were characterized with respect to age, gender, ethnicity, HIV status (serostatus, blood $CD4^+$ and $CD8^+$ T-cell counts, plasma HIV RNA concentrations) and ART regimen.

Tetramer Staining

PBMC from RhCMV-infected Mamu-A1*002:01 (Mamu-A*02) rhesus macaques were isolated from anticoagulant-treated whole blood by Ficoll density gradient centrifugation (GE Healthcare). Tetramers for the Mamu-A*02-restricted RhCMV IE-$2_{313–322}$ AN10 were provided by the NIH Tetramer Core Facility. For tetramer staining, approximately $1–2\times10^6$ cells were placed in 100 μl of RPMI 1640 (with 10% FBS). Tetramer was added at a final concentration of 100nM and cells were incubated in the dark at 37°C for 30 minutes. Subsequently, cells were stained for anti-CD3 (clone: SP34–2, Pacific Blue, BD Biosciences), anti-CD8 (clone: SK1, TruRed, BD Biosciences), anti-CD4 (clone: L200, PE-Cy7, BD Biosciences), anti-CD14 (clone M5E2, FITC, BioLegend), anti-CD16 (clone 3G8, FITC, BioLegend), and LIVE/DEAD Fixable Near Infra-Red Dead Cell Stain (Life Technologies) and were incubated for an additional 30 minutes in the dark at 4°C. Cells were then washed once with 1x PBS and fixed with 2% PFA. Sample collection was performed on LSR-II or FACSymphony instruments (BD Biosciences), and analysis was conducted with FlowJo software (Tree Star).

Antigens and Antigen-Presenting Cells

All peptides utilized in these studies were synthesized by Genscript. RhCMV (Genbank accession MT157325: https://www.ncbi.nlm.nih.gov/nuccore/MT157325) peptide pools

consisted of 15mer peptides overlapping by 11 amino acids corresponding to the non-overlapping exons of RhCMV-IE1, RhCMV-IE-2 (38). Peptide pools from HIV-1 Clade B Gag were provided by the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Germantown MD, USA). PBMC were isolated from anticoagulant-treated whole blood by Ficoll density gradient centrifugation (GE Healthcare). B-lymphoblastoid cell lines (BLCL) were generated by infecting macaque and human PBMC with herpesvirus papio or Epstein Barr virus, respectively, as previously described (39–41). Antigen-presenting cells were pulsed with peptides of interest at a final concentration of 10μM for 90 minutes then washed three times with warm PBS and once with warm R10 (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotic/antimycotic) to remove unbound peptide before combining with effectors. The .221 cell lines expressing single HLA alleles were generated using lentiviral transduction as described (42). HLA fragments were cloned into the modified pLVX-EF1α-IRES-Puro (Clontech) vector, in which EF1α was replaced with the spleen focus-forming virus promoter. The expression cassette encoded ZsGreen linked via self-cleaved P2A peptide to HLA with a FLAG-tag at its N terminus. HLA-positive cells were selected using 0.25 μg/ml puromycin.

Intracellular Cytokine Staining Assays

Antigen-presenting cells were pulsed with peptides of interest at a final concentration of 10μM for 90 minutes, washed three times with warm PBS, and once with warm R10 (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotic/antimycotic) to remove unbound peptide before combining with effectors.

$CD8^+$ T cell responses were measured by flow cytometric ICS. PBMC, isolated CD8β+ T cells, or $CD8^+$ T cell transductants were incubated with peptide-pulsed BLCL as described above or free peptide at a final concentration of 10μM, and the costimulatory molecules CD28 and CD49d (BD Biosciences) for 1 hour, followed by addition of brefeldin A (Sigma-Aldrich) for an additional 8 hours. Co-stimulation with un-pulsed APCs served as background controls. Stimulated cells were stained, collected, and analyzed as previously described (43, 44). Briefly, cells were washed with 1x PBS, surface stained for 30 min, washed with PBS, fixed with 2% paraformaldehyde, permeabilized with Medium B buffer (ThermoFisher), and stained intracellularly for 1 hour. Antibodies used for ICS included: anti-CD3 (clone: SP34–2, Pacific Blue, BD Biosciences), anti-CD8 (clone: SK1, TruRed, BD Biosciences), anti-CD4 (clone: L200, PE-Cy7, BD Biosciences), anti-IFN-γ (clone: B27, FITC, BD Biosciences), anti-TNF (clone: MAb11, APC, BD Biosciences), LIVE/DEAD Fixable Near Infra-Red Dead Cell Stain (Life Technologies) was used to assess cell viability. Sample collection was performed on an LSR-II instrument (BD Biosciences), and analysis was conducted with FlowJo software (Tree Star).

Surface-Trapped TNF- α Staining Assays

Antigen-specific $CD8^+$ T cells were identified and viable cells sorted using surface-trapped TNF-α staining (23). PBMC, isolated CD8β+ T cells, or $CD8^+$ T cell transductants were incubated with peptide-pulsed antigen-presenting cells, the co-stimulatory molecules CD28 and CD49d (BD Biosciences), anti-TNF (clone: MAb11, PE, BD Biosciences) and TAPI-0 (5uM final concentration, Santa Cruz Biotechnology). Cells were incubated at

37°C for 8 hours. After incubation, cells were stained anti-CD3 (clone: SP34–2, Pacific Blue, BD Biosciences), anti-CD8 (clone: SK1, TruRed, BD Biosciences), anti-CD4 (clone: L200, PE-Cy7, BD Biosciences), anti-CD14 (clone: M5E2, APC, Biolegend), anti-CD69 (FN50, FITC, BioLegend), anti-CD16 (clone: 3G8, APC, Biolegend), and anti-CD20 (clone: 2H7, APC, Biolegend) and LIVE/DEAD Fixable Near Infra-Red Dead Cell Stain (Life Technologies) was used to assess cell viability. Antigen-specific cells were defined as TNF+/CD69+ $CD8^+$ T cell responses 2x the magnitude of the no peptide control, with the no peptide control responses below 0.5%. Viable antigen-specific cells were sorted using a FACSAria Fusion (BD Biosciences), and analysis was conducted with FlowJo software (Tree Star).

ELISPOT Assay

IFN-γ ELISPOT was performed on TCR transductants, similar to previously described methods (12, 22). Briefly, 10,000 BLCL were incubated with 10 μM of the indicated peptide(s) for 90 minutes, washed 3X with 1X PBS, and then combined with 5,000–35,000 effectors. Results are reported as IFN-γ spot-forming cells (SFCs) per 1×10^4 effectors, following subtraction of duplicate wells with media only (negative control).

Single-cell RNA-seq and TCR sequencing

Viable $CD8^+$ T cells were sorted into 20uL chilled R10 media using a FACSAria Fusion (BD Biosciences). Sorted cells were loaded into a 10x Genomics Chromium instrument (10x Genomics) and processed using the Single Cell 5' and V(D)J Enrichment kit Version 1.1 following the manufacturer's protocol. Generation of gene expression libraries was performed using manufacturer's instructions. Generation of VDJ enriched libraries followed manufacturer's instructions with the exception that macaque-specific TCR constant region primers were used in place of human-specific TCR enrichment primers. Enrichment was performed using two nested PCR reactions. The first enrichment PCR used the forward primer 5- AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC-3 at a stock concentration of 1uM, with reverse primers targeting the alpha and beta constant regions: 5- GTCTGCTGGAATAACGCTGTCC-3 (1uM stock conc.), and 5-GCGCTGATCTTTTGGGTGATGG-3 (0.5uM stock conc.). The nested PCR used the forward primer 5- AATGATACGGCGACCACCGAGATCT-3 (1uM stock conc.) and reverse primers targeting the alpha and beta constant regions: 5- ATGCACGTCAGAATCCTTGC-3 (1uM stock conc.) and 5- CAGAAGGTGGCCGAGACC-3 (0.5uM stock conc.). In both cases the concentration of the alpha constant region primer was increased relative to the beta primer to improve capture. PCR conditions for both reactions were as follows: lid temp 105°C, 98°C 0:45, 12 cycles of: 98°C 0:20, 60°C 0:30, 72°C 1:00, followed by 72°C 1:00 and 4°C hold. To multiplex samples, cell hashing was used, using the MULTI-Seq lipid labeling system (45, 46). Sequence libraries were sequenced using Illumina chemistry, on either Novaseq or HiSeq instruments (Illumina).

TCR Sequence Analysis

Raw sequence reads for gene expression and TCR enrichment were first processed using cellranger software, version 3.1 (10x Genomics). For TCR analyses, data were aligned using a custom macaque V/J segment library, developed by our group, available in repseqio

format (47). This library was provided to the cellranger vdj software. The raw clonotype calls produced by cellranger vdj were extracted from the comma-delimited outputs. Cells were demultiplexed and TCR calls were assigned to samples using custom software, made publically available through the cellhashR package (46).

Generation of retroviral transduction vectors

For clones of interest, near full-length V/J sequences were obtained by aligning the consensus sequences from multiple cells. The resulting consensus sequences were trimmed to the V/J open reading frame. Any internal EcoRI or NotI restriction sites were altered with single nucleotide substitutions that preserved the amino acid sequence, but removed the restriction site. For each clone, the macaque TRA and TRB V/J sequences were combined *in silco* with modified murine TRA and TRB constant regions, and separated by a P2A site (37). See Supplemental Table I for the full sequence of each construct. The resulting sequences were synthesized (Genscript), digested with EcoRI and NotI, and cloned into the pMP71 retroviral vector (48). This system has been previously described for T cell transduction (48–50). Murine constant regions are used because they prevent pairing with endogenous TCR chains, and they have been modified to encode additional cysteine residues, thereby increasing the alpha/beta constant region binding affinity and thereby increasing TCR surface expression (37). This vector was used to transfect HEK293-based retroviral packaging cell line 293Vec-RD114 (provided by BioVec Pharma) using TransIT LT1 (Mirus Bio), following the manufacturer's protocol. After 24 hours, the transfection reagent was replaced with fresh medium, and supernatant was harvested 24 hours later, centrifuged to remove debris, aliquoted, and frozen.

Transduction of CD8 T cells with Murinized TCR

$CD8^+$ T cell-enriched PBMC were isolated from rhesus macaque PBMC utilizing a CD4 non-human primate (NHP) bead kit (Miltenyi) according to manufacturer's instructions. Isolated cells were resuspended in X-Vivo-15 medium (Lonza) supplemented with 10% FBS and 500 U/ml IL-2 (Genscript). Cells were then stimulated with a T Cell Activation/ Expansion NHP Kit (Miltenyi) in accordance with manufacturer instructions. Cells were then incubated for 48 hours at 37°C. Activated T cells were separated from T cell activation kit beads by centrifugation on a ficoll (GE Healthcare) gradient. Cells were washed, resuspended in supplemented X-Vivo-15 medium, and plated in 24-well plates at 2×10^6 per ml.

RD114 TCR supernatants were thawed and centrifuged at 20,000 × g at 4°C for 1 hour on a 20% sucrose (w/v) gradient. The supernatants were removed and residual volume and pellets were incubated with ViroMag beads (OzBiosciences) for 15 minutes at room temperature. Beads/RD114-pseudotyped retrovirus were then added to activated T cells and the plate was briefly spun at 1600 × g. The plate was then placed on top of the manufacturer's magnet and incubated at 37°C with 5% CO_2 for 15 minutes. Transduction efficiency was assessed two days post-infection by staining with anti-murine TCR β chain antibody (clone: H57–957, PE, Biolegend). Cells were then expanded for one-week before assays were performed.

Results

Rhesus cytomegalovirus-specific TCR Clonotypic Hierarchies

To validate whether the combination of surface-trapped TNF-α sorting (STTS), single-cell sequencing, and TCR transductants can rapidly identify and characterize antigen-specific $CD8^+$ T cells, we first examined responses against rhesus cytomegalovirus (RhCMV) in two rhesus macaques. Virtually all rhesus macaques are naturally infected with RhCMV at a young age, generally mounting robust $CD8^+$ T cell responses (51, 52).

PBMC from each animal was stimulated *ex vivo* with peptide pools for CMV intermediate/early (IE) proteins 1 and 2, along with a no peptide control, using 1 million PBMC per assay. We performed standard intracellular cytokine staining (ICS) in parallel with STTS. Incubation time and TAPI-0 concentration were determined using a separate time course experiment (Figure S1). The responses are shown in Figure 1A–B, with the magnitude of the responding cells indicated. The Rh-204C/IE-1 response was not above background (data not shown). It should be noted that while the frequency of CD69+/TNF-α+ cells detected using STTS was consistently lower than that of the corresponding ICS assay, which may be due to inefficient or incomplete capture of TNF-α, sufficient CD69+/TNF-α+ cells were detected to allow sorting. An evaluation of STTS sensitivity is shown in Figure S2.

Sorted cells were single-cell sequenced using the 10x Genomics platform. Prior to sorting and concurrent with fluorescent antibody staining, we labeled each PBMC sample with nucleotide-barcoded lipids (termed “cell hashing”) (45, 53). This allows samples to be pooled, which can then be multiplexed and processed using a single 10x lane. This multiplexing capability dramatically reduces the cost per sample, and is critical for throughput and feasibility of this approach. The resulting sequence data provided the TCR clonotypic hierarchies for each response (Figure 1C–D). In each case, the response to a given protein was characterized by a small number of dominant clones. Critical for this system, TCR sequence data can be linked back to individual cells, thereby identifying the pairing of alpha/beta chains (Figure 1E).

Generation and Validation of Anti-CMV Transductants

We selected the three most common IE-2 TCR clones for further characterization. The full-length macaque V/J regions of each TCR alpha/beta pair were synthesized and cloned into a previously described retroviral expression system (48–50). In this system, the macaque V/J regions are fused to modified murine constant regions (Figure 2A). This provides two advantages: the murine constant regions prevent pairing between the exogenous and endogenous TCRs present on the donor cells, and the murine constant regions have point mutations to create additional disulfide bonds, increasing binding affinity and thereby increasing TCR surface expression (37). The complete nucleotide sequence of each TCR construct is shown in Supplemental Table I. $CD8^+$ T cells from a macaque donor were transduced with these constructs to stably express either TCR51, TCR54, or TCR55. Surface expression of exogenous TCR can be monitored using antibodies against the murine TCRβ constant region, a further advantage of this approach (Figure 2B).

Next, we sought to map the RhCMV epitopes recognized by each clone, using these TCR transductants. Because both animals express the high-frequency allele Mamu-A*02, we selected a pre-existing BLCL from a Mamu-A*02+ donor (animal Rh-A02) to use for antigen presentation. We stimulated each transductant using these BLCLs pulsed with CMV IE-2 overlapping 15mer pools, followed by IFN-γ ELISPOT. For each transductant, we detected the strongest response to peptide Pool H (Figure 2C). We then performed similar experiments in which each transductant was stimulated using the same BLCLs pulsed with each of the ten individual 15mer peptides from Pool H, followed by IFN-γ ELISPOT. All three TCR transductants responded most strongly to two overlapping 15mers, IE-$2_{313–327}$ and IE-$2_{309–323}$ (Figure 2D). To map the minimal optimal epitope, we next stimulated TCR51 and TCR54 transductants using BLCLs pulsed with the overlapping 9 and 10mer peptides from this region, using decreasing concentrations, measured by IFN-γ ELISPOT. Peptide AN10 (ATTRSLEYKN, IE-$2_{313–322}$) showed the highest affinity throughout the dilution series (Figure 2E and F). Finally, in order to verify the restricting MHC-Ia allele, transductants expressing TCR51, TCR54, or non-transduced cells were incubated with a panel of APCs, each encoding a different MHC-Ia allele, pulsed with AN10 peptide (Figure 2G). Only Rh-A02 BLCL (a Mamu-A*02+ animal), and K562s expressing Mamu-A*02 induced a response, measured by ICS (Figure 2G). No response was detected in the control MHC-Ia negative K562 cells, the no peptide control, or in K562s expressing either Mamu-A*01 or Mamu-B*008:01 (Mamu-B*08). From these data we concluded that the dominant CMV IE-2 $CD8^+$ T cells clones from both Rh-204C and Rh-200A each recognize the peptide IE-2 AN10, presented by Mamu-A*02.

Validation of AN10 Using Primary Cells

We next sought to verify whether the *in vitro* transductant results mirrored the endogenous $CD8^+$ T cells. We constructed an MHC-I tetramer using Mamu-A*02 and AN10. We used this tetramer to stain PBMC from Rh-204C and Rh-200A (Figure 3A). Tetramer positive cells were live sorted, followed by single-cell sequencing. Tetramer sorting identified the same dominant clones, TCR51, TCR54, and TCR55, demonstrating that the *in vitro* transductant experiments identified the correct epitope. To further validate our results, PBMC from Rh-204C and Rh-200A were stimulated *ex vivo* with AN10 peptide using STTS. Responding cells were sorted and single-cell sequenced. These experiments identified the expected TCR clones (TCR51, TCR54, and TCR55), demonstrating that primary cells are capable of cytokine production following AN10 stimulation (Figure 3B). It should be noted that in this assay TCR51 was detected at a much lower frequency than that detected by tetramer sorting, with TCR55 representing a greater fraction of cytokine-producing cells. While this could represent assay-to-assay variation, it has been reported that not all tetramer positive cells have the potential to produce TNF-α. Tetramer staining can therefore identify a superset of cells relative to TNF-α based detection methods (23). Of note, while the Rh-200A IE-2 response was dominated by TCR54, the minor clone CASSSRPGLPGQETQYF (denoted with ** in Figure 3A) was also detected in the previous IE-2 ORF STTS (Figure 1), which suggests AN10 dominates the CMV IE-2 response in this animal. Collectively, these data demonstrate that the AN10 epitope, which was identified *in vitro* using TCR transductants, is the biologically relevant antigen and that AN10-specific

CD8 T cells represent the majority of the CMV IE-2 $CD8^+$ T cell response in both Rh-204C and Rh-200A.

To provide additional validation of the AN10 epitope and to measure the frequency of this response in the Oregon National Primate Research Center (ONPRC) colony, we screened three additional cohorts: RhCMV+/Mamu-A*02+ animals, RhCMV+/Mamu-A*02- animals, and RhCMV-/Mamu-A*02+ animals. The latter were selected from the ONPRC Enhanced Specific Pathogen Free (SPF) colony, and have been verified to be RhCMV-negative (23). As expected, all RhCMV+ animals had a robust ELISPOT response to CMV lysate, relative to RhCMV- animals (Figure 3C). When stimulated with AN10 peptide, only PBMC from RhCMV+/Mamu-A*02+ animals responded, as would be expected for an epitope restricted by Mamu*A*02. In addition to validating our system as a rapid method for epitope identification, it is worth noting that virtually all Mamu-A*02+ animals we tested (8/9) responded to AN10. Because such a high fraction of Mamu-A*02+ animals mount this response, it may be of experimental interest either in the study of RhCMV, or as a control $CD8^+$ T cell response for use in other disease studies.

Identification, Characterization, and Validation of a novel HIV Gag Response

We next turned to an HIV-infected human donor, representing a sample with limited primary cells. Following a similar strategy as the macaque experiments, we initially stimulated PBMC *ex vivo* with HIV-1 Clade B Gag peptide pools, followed by IFN-γ ELISPOT (Figure 4A–B). These experiments consumed 1 million primary PBMC per pool. While multiple peptide pools produced responses above background, we selected Pool E for further characterization. As before, we performed STTS using 1 million PBMC, live-sorted the responding cells, and performed single-cell sequencing (Figure 4B). The TCR repertoire to this peptide pool was dominated by one major clone: 1247-TCR-1 (Figure 4C–D). We synthesized this clone and inserted it into the same expression vector as before, expressing the human V/J segments fused to modified murine constant regions (Figure 2A). This construct was used to generate retrovirus and transduce $CD8^+$ T cells from an HIV-naïve donor (Figure 4E). We next sought to map the minimal epitope for this clone, following the same approach as the rhesus macaque experiments. TCR transductants expressing 1247-TCR-1 were stimulated *in vitro* using autologous BLCL peptide-pulsed with the individual 15mer peptides from Gag Pool E, identifying $Gag_{165–179}$ (SA15) as the strongest response (Figure 4F). We next performed two sets of experiments in parallel. To identify the minimal optimal epitope, we stimulated 1247-TCR-1 transductants with autologous BLCL pulsed with the 9–10mer peptides overlapping SA15, followed by IFN-γ ELISPOT (Figure 4G). This identified EL9 (EVIPMFSAL, $Gag_{167–175}$) as the minimal optimal peptide. Finally, we sought to identify the restricting HLA allele. We performed flow cytometric ICS with a panel of MHC-I null .221 cells, with each expressing a single HLA allele present in the donor: HLA-A*32:01, HLA-A*68:02, HLA-B*39:10, HLA-B*53:01, HLA-Cw*04:01, and HLA-Cw*14:03. These APCs were pulsed with the SA15 peptide and incubated with 1247-TCR-1 transductants, followed by ICS (Figure 4H). The only APCs to elicit a response above background were cells expressing HLA-A*68:02, identifying HLA-A*68:02 as the presenting HLA allele. We subsequently peptide pulsed .221s expressing HLA-A*68:02 with either SA15 or the minimal epitope EL9, followed by incubation with 1247-TCR-1

transductants and ICS (Figure S3). As expected, the minimal optimal EL9 peptide elicited a nearly 2-fold higher response.

Of interest, the EL9 peptide has been described in prior studies as an HLA-A*26-restricted response, and this peptide has been included in a commonly used ELISPOT screening panel (10, 54–58). To test whether 1247-TCR-1 could recognize EL9 bound by HLA-A*26, we generated .221 cells expressing either HLA-A*68:02 or HLA-A*26:01. These APCs were pulsed with EL9 peptide and incubated with 1247-TCR-1 transductants, followed by ICS (Figure S3). The HLA-A*26:01 expressing cells did not elicit a response above background, indicating that even if EL9 can be presented by HLA-A*26:01, it is not recognized by 1247-TCR-1. Our data nonetheless demonstrate this Gag epitope can be presented by multiple HLA alleles.

Discussion

Here we present a novel scheme to characterize antigen-specific $CD8^+$ T cells and their cognate MHC antigens. This system combines three components: 1) high sensitivity TNF-α capture to identify and isolate viable antigen-specific cells, 2) massively parallel single-cell resolution sequencing to identify the complete clonotypic hierarchy of each response, and 3) exogenous expression of the TCR alpha/beta pair on donor cells. The latter produces a nearly unlimited experimental reagent that can be used for *in vitro* validation and characterization, without depleting primary cells. We demonstrate this system accurately identifies and characterizes antigen specific $CD8^+$ T cells in both CMV-infected rhesus macaques and an HIV-infected donor, identifying the antigenic peptides and restricting MHC alleles for each. We validated our results using primary cells, demonstrating the *in vitro* system recapitulates endogenous TCR/MHC interactions.

This system has a wide range of applications. It provides a clear advantage whenever patient/donor samples are limited. This approach requires as little as one experiment with primary cells: the STTS screen to identify antigen-specific TCR sequences. Even when samples are not limited, such as most studies using animal models, the transductant system may have benefits. For example, it could facilitate epitope mapping when the host response is weak or rare. In some contexts, the primary $CD8^+$ T cell response to a given antigen may be difficult to culture *in vitro*, such as the terminally differentiated $CD8^+$ T cell responses elicited by CMV vaccine vectors (39, 43). The identification of the TCR sequences, followed by expression in transductants may facilitate study of these T cells. The rapid generation and characterization of patient-specific T cell responses and their cognate antigens has clear applications for the creation of personalized immunotherapeutics as well.

The system is premised on $CD8^+$ T cell antigen specificity being conferred nearly exclusively through the TCR and cognate MHC-bound peptide. Exogenous expression of the specific TCR α/β pair on donor cells is generally sufficient for antigenic recognition, as we demonstrate in this study and has been previously published (35, 37, 59–62). There are nonetheless examples of other receptors and signaling pathways that modulate $CD8^+$ T cell specificity and activation (63, 64). Potentially interesting extensions of the system we present in this study could involve careful selection or experimental manipulation of the

donor $CD8^+$ T cells, either to control the genetic background, or to perform overexpression or knock-out of other signaling molecules.

Together, this study validates a novel system to rapidly identify $CD8^+$ T cells and their peptide/MHC antigens. We demonstrate that limited experiments with primary cells can accurately identify the TCR sequences of antigen-specific $CD8^+$ T cells, these sequences can be cloned into donor cells to recapitulate antigen specificity of the primary cells, and this *in vitro* system provides a surrogate to accurately characterize the primary $CD8^+$ T cell response. This scheme is well suited to any situation where primary cells are rare or not amenable to *ex vivo* functional studies, with a range of practical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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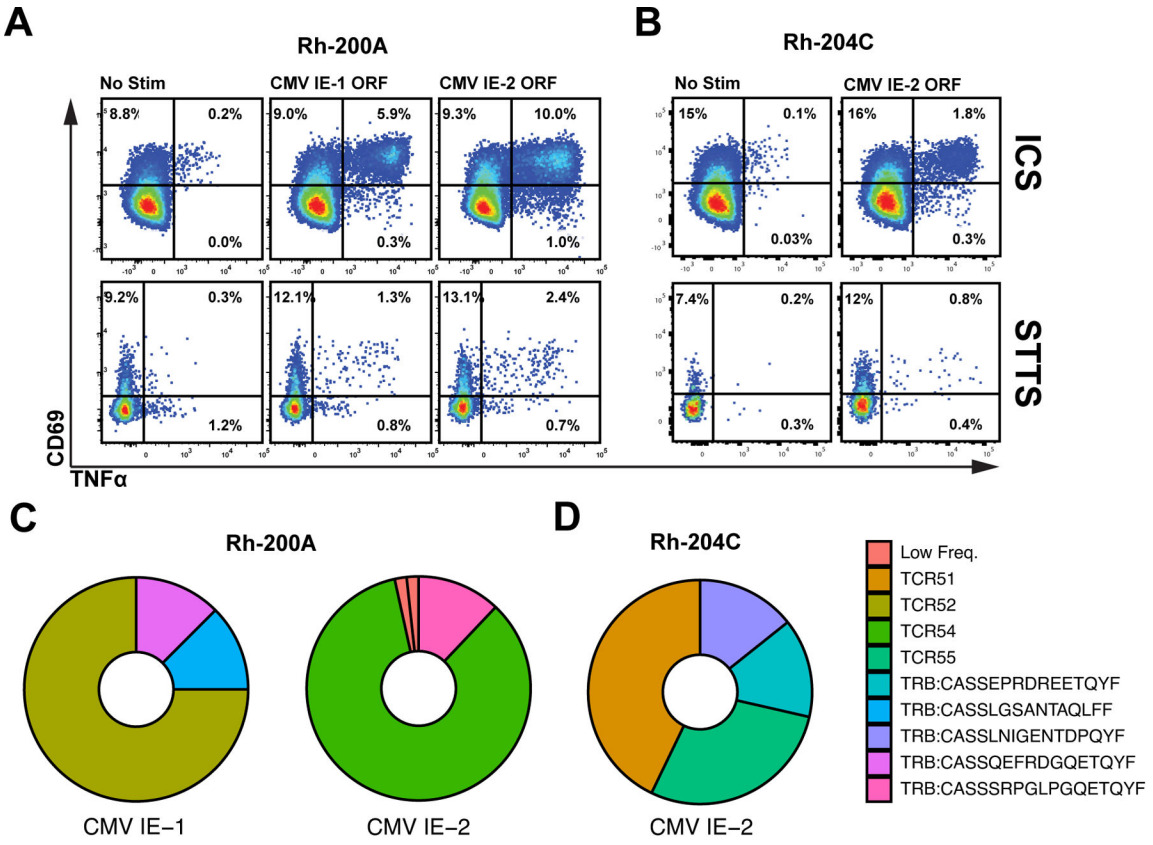
Key Points:

- Surface-trapped TNF-α and single-cell sequencing can identify antigen specific TCRs
- Exogenous TCR expression in donor cells allows characterization and epitope mapping



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Name	Alpha				Beta				
	CDR3a	V	J	C	CDR3b	V	D	J	C
TCR51	CATFSTDSWGKLQF	TRAV17*01	TRAJ24*01	TRAC*01	CASTWGGANTAQLFF	TRBV5-6*01	TRBD1*01	TRBV5-6*01	TRBC1*01
TCR52	CAMRASSGGGYVLTF	TRAV14-2*01	TRAJ6*01	TRAC*01	CASSEDRRNEKLFF	TRBV6-8*bw02	TRBD1*01	TRBJ1-4*01	TRBC1*01
TCR54	CAGRDNFNKFYF	TRAV25*01	TRAJ21*01	TRAC*01	CASSPREDSNYDYTF	TRBV27*w02	TRBD2*01	TRBJ1-2*01	TRBC1*01
TCR55	CVLRDYYGQNFIF	TRAV18*01	TRAJ26*w02	TRAC*01	CASSWRPDANYDYTF	TRBV27*01	---	TRBJ1-2*01	TRBC1*01

Figure 1. Identification of RhCMV-specific CD8$^+$ T Cell Clonotypes.

A-B) PBMCs from two RhCMV+ rhesus macaques were stimulated *ex vivo* with 15mer peptide pools for either CMV IE-1 or IE-2, measuring using ICS (top) or STTS (bottom). The Rh-204C IE-1 response was not above background (data not shown). C-D) CD69+/ TNF-α+ cells were live-sorted from each response, followed by single-cell sequencing. Pie charts indicate the TCR clonotypic hierarchies of each response. E) Table shows the V/J usage and CDR3 sequence for the paired alpha/beta chains of the four most common TCR clonotypes. The identity of the TRBD segment for TCR55 could not be unambiguously determined from the 9-NT region between the V- and J-segments.

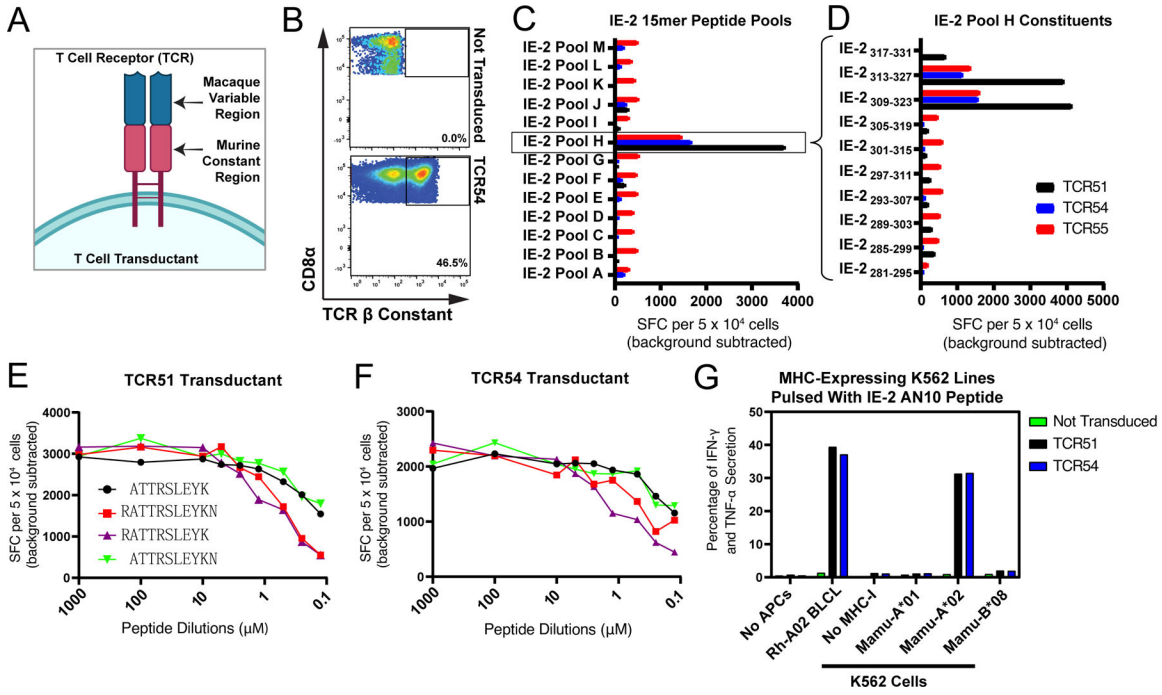

Figure 2. Characterization of RhCMV-specific TCRs using transductants.

A) Schematic of the chimeric macaque V/J, murine constant region (“murinized”) TCR receptor, B) Representative flow plot of $CD8^+$ T cells from a donor macaque, with or without transduction of TCR54, illustrating the detection of surface murine TCRβ in transduced cells. C) Transductants expressing TCR51 (black), TCR54 (blue), or TCR55 (red) were incubated with BLCL pulsed with the indicated 15mer IE-2 peptide pools, followed by IFN-γ ELISPOT. Bars indicate the spot forming cells per 5×10^4 cells, after background subtraction. D) Similar graph as C showing transductants stimulated with the ten individual 15mer peptides from IE-2 Pool H. E-F) Peptide titration experiments were performed to determine the minimal/optimal peptide sequence, using overlapping 9–10mer peptides spanning IE-2 residues 313–322. BLCLs were pulsed with each of the peptides indicated in F, at varying concentrations, followed by incubation with transductants expressing either TCR51 (E) or TCR54 (F). Graphs indicate the SFC per 5×10^4 cells, measured by IFN-γ ELISPOT. G) Transductants expressing either TCR51 (black), TCR54 (blue), or non-transduced (green) were incubated with the indicated APCs, pulsed with AN10 peptide ($IE\text{-}2_{313–322}$). Rh-A02 BLCL is from a Mamu-A*02 positive animal. No MHC-I denotes the parent MHC-null K562 cells. A*01, A*02 and B*08 denote K562 cell lines expressing the indicated MHC-Ia allele. Graph indicates the percentage of murine TCRβ+ cells positive for TNF-α and IFN-γ, measured by flow cytometric ICS.

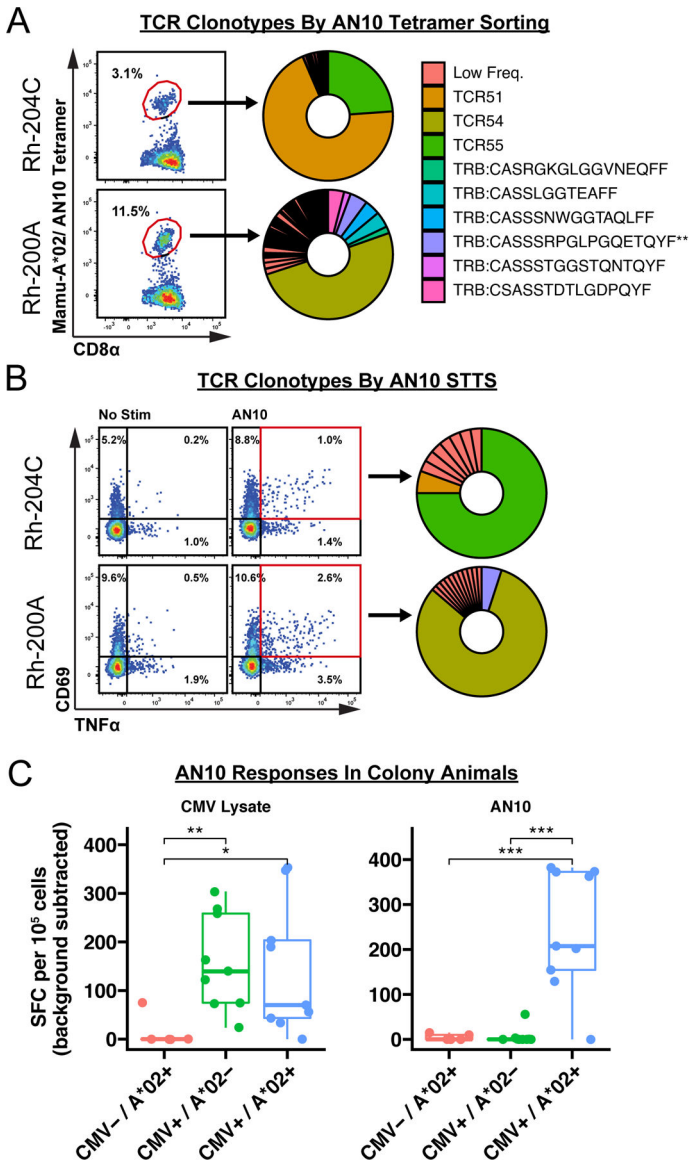

Figure 3. *Ex vivo* validation of IE-2 AN10.

A) MHC-tetramers were folded using Mamu-A*02 and IE-2 AN10, and then used to stain PBMC from Rh-204C and Rh-200A. Flow plots indicate the fraction of $CD8^+$ T cells stained by AN10 tetramers for each animal. The tetramer-positive cells were live sorted, followed by single-cell sequencing. Pie charts indicate the clonotypic hierarchies for each, demonstrating that the clones previously identified by IE-1/2 ORF stims are present. B) PBMC from Rh-204C and Rh-200A were stimulated *ex vivo* with AN10 peptide, followed by STTS. Pie charts indicate the clonotypic hierarchies, demonstrating that primary cells stimulated with AN10 recapitulate the transductant experiments. C) To further validate the AN10 response, and to evaluate its prevalence in the broader rhesus macaque population, we performed IFN-γ ELISPOT using cells using animals in three groups: RhCMV-/Mamu-A*02+, RhCMV+/Mamu-A*02-, and RhCMV+/Mamu-A*02+. Cells were stimulated with

either CMV lysate (left), or AN10 peptide (right). Graphs indicate spot forming cells per 5×10^4 cells (*, p <= 0.05; **, p <= 0.01; ***, p <= 0.001).

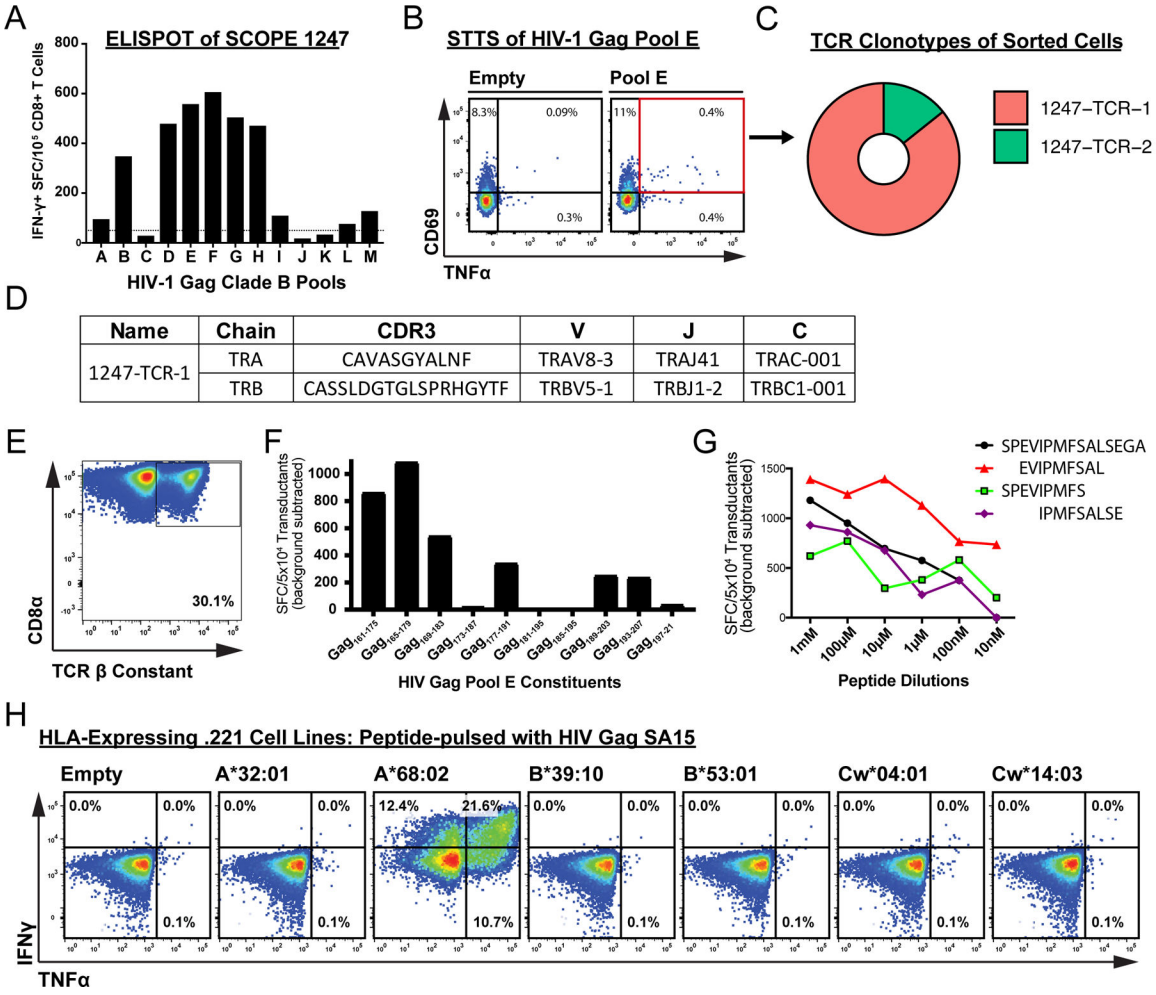

Figure 4. Identification and characterization of a novel HIV Gag epitope.

A) PBMCs from an HIV-infected donor, SCOPE 1247, were stimulated *ex vivo* with HIV Gag 15mer peptide pools. Graph indicates the frequency of responding cells, measured by IFN-γ ELISPOT. Dotted line indicates the limit of quantitation (LOQ; 50 SFC per 10^5 $CD8^+$ T cells). B) Flow plot for STTS using SCOPE 1247 PBMCs, pulsed with HIV-1 Gag Clade B Pool E. The CD69+/TNF-α+ cells were live cell sorted, followed by single cell sequencing. C) Pie chart indicates the TCR clonotypic hierarchy of CD8 T cells activated by Pool E peptide, demonstrating one major clone, 1247-TCR-1, comprises the majority of the response. D) Table shows the paired alpha/beta chains from clone 1247-TCR-1. E) The alpha/beta chains from 1247-TCR-1 were synthesized and used to transduce donor $CD8^+$ T cells. The flow plot shows the fraction of cells stained for murine TCRβ, indicating cell surface expression of exogenous 1247-TCR-1. F) IFN-γ ELISPOT was performed using 1247-TCR-1 transductants stimulated with autologous BLCL, pulsed with each of the peptides comprising HIV Gag Pool E (LOQ = 5 SFC per 10^4 cells). The strongest response was detected from $Gag_{165–179}$ (SA15). G) ELISPOT was performed using autologous BLCL pulsed with the indicated 9 and 10mer peptides, which overlap SA15. The strongest response was detected from EVIPMFSAL (EL9). H) To determine HLA-restriction, 1247-TCR-1 transductants were incubated with .221 cell lines individually

expressing each of the HLA alleles encoded by the patient, pulsed with SA15 peptide. Cells expressing HLA-A*68:02 were the only APCs that produced a response above background, measured by ICS.