HIV-1-infected CD4+ T cells present MHCII-restricted epitope via endogenous processing

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Abstract

HIV-1-specific CD4+ T cells (T_{CD4+}) play a critical role in controlling HIV-1 infection. Canonically, T_{CD4+} are activated by peptides derived from extracellular ("exogenous") antigens displayed in complex with major histocompatibility complex class II (MHCII) molecules on the surfaces of "professional" antigen presenting cells (APC) such as dendritic cells (DCs). In contrast, activated human T_{CD4+}, which express MHCII, are not typically considered for their APC potential due to their low endocytic capacity and the exogenous antigen systems historically used for assessment. Using primary T_{CD4+} and monocyte-derived DCs from healthy donors, we show that activated human T_{CD4+} are highly effective at MHCII-restricted presentation of an immunodominant HIV-1-derived epitope following infection and subsequent noncanonical processing and presentation of endogenously produced antigen. Our results indicate that, in addition to marshalling HIV-1-specific immune responses during infection, T_{CD4+} also act as APCs, leading to the activation of HIV-1-specific T_{CD4+}

INTRODUCTION

Despite over forty years of intensive research, human immunodeficiency virus 1 (HIV-1) continues to pose a significant global health threat. HIV-1 attacks the human immune system, eventually leading to a state of profound immune deficiency known as acquired immunodeficiency syndrome (AIDS). In 2020, an estimated 37.7 million people were living

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with HIV, 1.5 million were newly infected, and 680,000 died of AIDS-related illnesses (1). HIV-1 mainly infects activated CD4$^+$ T cells (T_{CD4+}), macrophages, and microglia, and to a lesser extent dendritic cells and osteoclasts (2–4). Infection causes profound immune dysregulation and T_{CD4+} death, leaving infected individuals vulnerable to opportunistic infections and death in the absence of therapeutic intervention (5).

T_{CD4+} play a critical role in the immune response against a wide variety of viral pathogens (6–8). Previous studies indicate that vigorous, HIV-1-specific T_{CD4+} responses play an important role in controlling viremia (9) and are also correlated with broader neutralizing antibody responses in infected individuals (10). Data from chronically infected individuals indicate a link between strong HIV-1-specific T_{CD4+} responses and effective HIV-1-specific CD8$^+$ T cell (T_{CD8+}) responses (11), and IL-21-producing T_{CD4+} have been shown to be important for maintaining HIV-1-specific T_{CD8+} (12). Additionally, HIV-1-specific cytotoxic T_{CD4+} work cooperatively with HIV-1-specific T_{CD8+} to control HIV-1 viremia (13) and expansion of cytolytic T_{CD4+} during acute HIV-1 infection predicts disease outcome (14). T follicular helper cells (Tfh) also play a key role in the anti-HIV-1 immune response. Work by Cohen et al. demonstrated an association between the frequency of Tfh cells during early, untreated infection and the future development of broadly neutralizing antibodies (BNAbs) (15). Additionally, a longitudinal study in rhesus macaques found that continuous HIV-1 envelope antigen production is required for driving Tfh activation, leading to more effective broadly neutralizing antibody responses (16).

Given the importance of T_{CD4+} in host responses to HIV-1, it is crucial to understand the basis for HIV-1-specific T_{CD4+} activation. By convention, T_{CD4+} activation is mediated by professional antigen-presenting cells (APCs) -dendritic cells (DCs), macrophages, and B cells- that that: 1) internalize extracellular antigen, such as whole virions, 2) proteolyze internalized material into within the endocytic network, 3) load the resulting peptides onto major histocompatibility complex class II (MHCII) molecules in the late endosome, and 4) transport resulting peptide:MHCII complexes to the APC plasma membrane, where they are recognized by T cell receptors (TCR) on the surfaces of antigen-specific T_{CD4+} (17). Peptide:MHCII-TCR contact, along with costimulatory signals, leads to T_{CD4+} activation.

Several studies have shown that MHCII-restricted processing and presentation is more complex than this conventional presentation pathway. (18). For example, endogenous processing has been described as a robust alternative to classical processing (19–24), coming into play when an APC becomes productively infected, the nascent viral proteins within the APC providing antigen processing substrates for presentation via MHCII. Endogenous processing has been demonstrated in several viral systems, including measles (25), influenza (24), and, notably, HIV-1 (26). Indeed, we have reported that endogenous processing and presentation is the primary driver of the T_{CD4+} response to influenza infection (24).

The cell types capable of MHCII-restricted presentation may also be more extensive than originally appreciated. Many cell types besides professional APCs constitutively express MHCII (23). In addition, many other cell types express MHCII in response to inflammatory signals such as interferon gamma (IFNγ) (27, 28). This includes human T_{CD4+}, which, unlike their murine counterparts, express MHCII as well as the costimulatory molecule

CD86 upon activation (29). Historically, these cells have not been considered as APCs, likely due to their limited ability to internalize extracellular material (a prerequisite for effective classical processing) and their non-constitutive expression of MHCII. However, the function of MHCII in these cells has been the subject of scrutiny for some time. In addition to their induced MHCII expression, activated human T_{CD4+} are a main cellular target of HIV-1, providing a large intracellular depot of newly synthesized viral antigen that could be accessed for endogenous MHCII processing and presentation. A limited number of studies have shown that, under the right circumstances, activated T_{CD4+} can present HIV-1-derived antigen on MHCII (30, 31), though T_{CD4+} were determined to be inferior to professional APC types, such as B cell lines and monocytes, at stimulating T_{CD4+} responses. However, it is worth noting that these studies were performed using 1) inert purified proteins, not infectious HIV-1, and 2) T_{CD4+} clones as APCs instead of primary *ex vivo* T_{CD4+}, potentially limiting their relevance to the study of endogenous processing in primary T_{CD4+}.

Despite the key role that HIV-1-specific T_{CD4+} responses play, there are very few studies dedicated to the details of MHCII-restricted presentation of HIV-1 epitopes in any cell type. We were intrigued by the possibility of endogenous MHCII-restricted processing of HIV-1 antigens by activated human T_{CD4+}. Infection would obviate the low internalization capacity of T_{CD4+}, and endogenous processing of abundantly produced antigen might lead to potent presentation of HIV-1-derived epitopes. In this study, we asked if activated human T_{CD4+} could present antigen derived from infectious HIV-1 to HIV-1-specific T_{CD4+}, using DCs, the prototypical professional APC, as a comparator.

MATERIALS AND METHODS

Cell Procurement:

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy HLA-DR1, DR11, and DR15$^+$ donors by the University of Pennsylvania Human Immunology Core (HIC) by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, Canada). CD4$^+$ T cell (T_{CD4+}) and monocytes were further isolated by the HIC using the RosetteSep Human CD4$^+$ T Cell Enrichment Cocktail and EasySep Human Monocyte Enrichment kits (Stemcell Technologies). All studies were conducted with the permission of the University of Pennsylvania Institutional Review Board.

T_{CD4+} and Dendritic Cell Culture:

T_{CD4+} were cultured in RPMI 1640 (Invitrogen, Waltham, MA) supplemented with 100 units/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 2 mM L-glutamine (Corning Inc., Corning, NY), 10% heat-inactivated fetal bovine serum (R&D Systems, Minneapolis, MN), and 10 mM HEPES (Invitrogen). Media was further supplemented with 100 IU/mL recombinant human IL-2 (Biolegend, San Diego, CA). T_{CD4+} were activated using K562 based artificial APCs (aAPCs) expressing CD64 and CD86 (32) that were additionally engineered to express a chimeric antigen receptor based on OKT3 antibody irradiated at 100 Gys. T_{CD4+} and aAPCs were plated at a 2:1 ratio at a concentration of 1×10^6 cells/mL in a 24 well plate. On day 2, the media volume was quadrupled, and

cells were moved to a T25 cell culture flask. Cells were counted at days 4 and 6 and fed with fresh media + 100 IU/mL IL-2 to maintain a cell concentration of 2.5×10^5 cells/mL. We confirmed that K562-activated T_{CD4+} expressed typical activation markers using flow cytometry (Fig. S1A). To generate DCs, 5×10^6 monocytes were cultured in 3 mLs of RPMI 1640 (Invitrogen) supplemented with 100 units/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 2 mM L-glutamine (Corning Inc.), 10% fetal bovine serum (R&D Systems) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Media was further supplemented with 100 ng/mL recombinant human GM-CSF (Biolegend) and 50 ng/mL recombinant human IL-4 (Biolegend). On days 3 and 6, fresh media supplemented with the same concentration of GM-CSF and IL-4 was added to DC cultures. Unless otherwise indicated, T_{CD4+} and DCs were used on Day 7.

Viruses and Infection:

NL4-R3A (+Nef) has been previously described (33). Nef/Vpu KO NL4-R3A, and K574D NL4-R3A viruses were also generated by the laboratory of Dr. James Hoxie. To make the K574D fusion deficient mutant, HIV-1 R3A gp120/41 Env was mutated at position K574 (34) with QuikChange ‖ XL Site-Directed Mutagenesis (Aligent Technologies) with primers CTCACAGTCTGGGGCATCGATCAGCTCCAGGCAAGAGTC (Fwd) and GACTCTTGCCTGGAGCTGATCGATGCCCCAGACTGTGAG (Rev). Mutations were confirmed by sequencing. The Env was then cloned into the pNL4–3 HIV-1 provirus. This virus was used to inoculate T_{CD4+} cells *in vitro* and no infected cells were detected by microscopy using an anti-p24 antibody. To make the Nef-/Vpu double KO R3A, the R3A Env was cloned into the pNL4–3 vector (which is Nef- by design) then the Vpu gene was mutated with QuickChange XL (Agilent Technologies) at the amino acid L12 with the primer introducing a premature stop codon Fwd 5'-GCAATAGTAGCATAAGTAGTAGCAGCAATAATAGCAATAGTAG-3'. For clarity, the amino acid is underlined: MQSLQILAIVA L VVAAIIAIVVWSIALIEYRKILRQRKIDRIINRIIERAEDSGNESEGDQ EELSALVEMGHHAPWDINDL*. Aldrithiol-2 (AT2) inactivated NL4-R3A (+Nef) was obtained from the University of Pennsylvania Center for AIDS Research (CFAR) Viral and Molecular Core. All viruses were made in HEK 293T cells transfected with Lipofectamine 2000 (Invitrogen) and were titered for p24 content by the University of Pennsylvania CFAR Viral and Molecular Core using Alliance HIV-1 p24 Antigen ELISA kits (PerkinElmer). Viruses were aliquoted and stored at −80°C until use.

On Day 4 of cell culture, T_{CD4+} and DCs were infected in their original media with NL4-R3A or Nef/Vpu KO NL4-R3A (100 ng of p24) per 1×10^6 cells in a 48 well plate. Infection was allowed to proceed for 3 days before cell staining or antigen presentation assays were performed. 100 ng p24 AT2 treated NL4-R3A and 69 ng p24 K574D NL4-R3A per 1×10^6 cells were added to T_{CD4+} and DCs 6 days post culture, approximately 12 hours before antigen presentation assays were performed.

Flow Cytometry:

Expression of antigen processing and presentation machinery was assessed as follows: DCs were stained extracellularly with anti-HLA-DR (1:100 dilution, G46-6, BD), anti-CD86

(1:100 dilution, FUN-1, BD). Activated T_{CD4+} were stained extracellularly with anti-CD3 (1:200 dilution, UCHT1, Biolegend) and anti-CD4 (1:100 dilution, OKT4, Biolegend), anti-HLA-DR (G46-6, BD), anti-CD86 (1:100 dilution, FUN-1, BD) for 20 minutes at 4°C. For intracellular staining, both DCs and T_{CD4+} permeabilized with fixation/permeabilization solution (BD Biosciences) for 15 minutes at room temperature and were then stained with anti-CD74 (1:100 dilution, Pin.1, Biolegend) and anti-HLA-DM (1:100 dilution, MaP.DM1, Biolegend) for 25 minutes at room temperature. To assess the activation state of aAPC-activated T_{CD4+} as compared to anti-CD3/CD28 stimulated T_{CD4+}, cells were stained extracellularly with anti-CD4 (OKT4, Biolegend), anti-CD69 (1:100 dilution, FN50, Biolegend), anti-HLA-DR (G46-;6, BD), anti-CD25 (1:100 dilution, BC96, Biolegend), anti-CD62L (1:100 dilution, DREG-56, Biolegend) for 20 minutes at 4°C. Cells were then stained intracellularly with anti-CD3 (UCHT1, Biolegend) and anti-TNFα (1:100 dilution, MAb1, Biolegend). For DQ-OVA internalization and processing assays, DCs were stained extracellularly with anti-HLA-DR (G46-6, Biolegend), and activated T_{CD4+} were stained with anti-HLA-DR (G46-6, Biolegend), anti-CD4 (OKT4, Biolegend) and anti-CD3 (UCHT1, Biolegend). To validate the HIV-specific TCR expression in transduced T_{CD4+}, cells were stained extracellularly with anti-CD4 (OKT4, Biolegend) and anti-TCR Vβ22 (1:5 dilution, IMMU 546, Beckman Coulter, Brea, CA) for 20 minutes at 4°C. For antigen presentation and viral spread assays, cocultures were stained extracellularly with anti-CD4 (OKT4, Biolegend) and anti-CD69 (FN50, Biolegend) for 20 minutes at 4°C. Cocultures were stained intracellularly with anti-CD3 (UCHT1, Biolegend), anti-TNFα (MAb1, Biolegend), anti-IL2 (1:75 dilution, MQ1–17H12, BD), anti-IFNγ (1:100 dilution, B27, BD Biosciences), and anti-p24 (1:500 dilution, Kc57, Beckman Coulter). All samples were incubated with LIVE/DEAD Fixable Near-IR (Invitrogen) to exclude dead cells. Cells were fixed with and 4% paraformaldehyde prior to analysis. Cells were acquired on a BD LSR Fortessa (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Endocytosis Experiments:

To assess the ability of DCs and activated T_{CD4+} to internalize and process the fluorescent DQ-OVA substrate, 200K DCs or T_{CD4+} were plated in 200 μLs R10 in a 96 well U-bottom plate. DQ-OVA (Invitrogen) was then added at a concentration of 10 μg/mL. After 2 hours at 37°C, cells were washed 3 times in sterile phosphate-buffered saline (PBS) and stained for flow cytometry as described. To assess the ability of DCs and activated T_{CD4+} to internalize FITC-Dextran, 200K DCs or T_{CD4+} were plated in 200 μLs R10 in a 96 well U-bottom plate. FITC-Dextran (Sigma-Aldrich) was then added at a concentration of 1 mg/mL. After 2 hours at either 4°C or 37°C, cells were washed 3 times in sterile phosphate-buffered saline (PBS) and stained for flow cytometry as described.

Cathepsin Activity Assays:

To determine the cathepsin D, L, and S activities of DCs and activated T_{CD4+}, DCs and T_{CD4+} were cultured for 7 days as described above. DCs and T_{CD4+} lysates were then made according to the instructions in the SensoLyte 520 Cathepsin D Assay Kit, SensoLyte 520 Cathepsin L Assay Kit, and SensoLyte 520 Cathepsin S Assay Kit (all by Anaspec). Lysates were then assessed for their cathepsin D and L activities according to the manufacturer's

instructions. Lysate from 100K cells was assayed per well in a 96 well plate. Fluorescence was measured after 30 minutes using an Infinite M200 Pro plate reader (Tecan).

Generation of HIV-specific TCR-Encoding Lentiviruses:

Gag293-specific TCR (F5) sequences have been previously described (35, 36) and were synthesized by Genscript (Piscataway, NJ). These TCR sequences were cloned into pTRPE transfer plasmid (37) with the TCRβ and TCRα sequences separated by a furin domain (serine-glycine-serine-glycine) + T2A (38). 27 μg pTRPE transfer plasmid was mixed with 3 μg codon-optimized Cocal-g plasmid (ATUM, Newark, California) (39). 18 μg HIV gag-pol packaging plasmid, 18 μg HIV_{REV} expression plasmid (pTRP Rev) and transfected using Lipofectamine 2000 (Invitrogen) into 293T cells. At 24 and 48 hours, supernatants were collected and ultracentrifuged for 2.5 hours at 25000 rpm at 4°C, split into four aliquots, and stored at −80°C until use. A ZnT8-specific TCR was obtained from Roberto Mallone (INSERM, FR) (40), used as a control TCR, and was made according to the protocol above.

Generation of HIV-specific T_{CD4+}:

To generate HIV-specific T_{CD4+}, T_{CD4+} from healthy donors were stimulated with irradiated aAPCs and IL-2 (Biolegend) as described above. 24 hours after stimulation, T_{CD4+} were transduced with lentiviruses encoding either a ZnT8 TCR or the Gag293-specific TCR. Cells were expanded and fed as described above, but were allowed to expand for 10 days, with an additional feeding on Day 8. Successful transduction was validated by flow cytometric analysis for TCR Vβ22 (Fig. S1B) and by measuring T_{CD4+} responses to Gag293 peptide pulsed DCs (Fig. S1C). Transduced T_{CD4+} were then frozen in freezing media consisting of 10% DMSO and 90% FBS and stored at −80°C until needed.

Antigen Presentation Assays:

To assess the ability of DCs and activated T_{CD4+} to present HIV-1-derived, MHCII-restricted epitope, cells were cultured and infected as described above. On the day of the assay, both uninfected and infected DCs and T_{CD4+} were collected, washed with PBS, and incubated with 12.5 μg/mL BFA (Biolegend) in fresh R10 media for a total of 3.5 hours. After an initial 1.5 hours of BFA treatment, 0.5 mg/mL purified Gag293 peptide (FRDYVDRFYKTLRAEQASQE) (Genscript) was added to appropriate wells of uninfected DCs and T_{CD4+}. Cells were then washed with BFA-containing PBS and replated in fresh R10 with 12.5 μg/mL BFA. Lentivirus-transduced T_{CD4+} expressing either a ZnT8 TCR or a Gag293-specific TCR were labeled with CellTrace Violet (Invitrogen) according to manufacturer's recommendations. 1×10^5 transduced T_{CD4+} and 5×10^4 candidate 50K potential antigen presenting cells were then cocultured for 8 hours at 37°C. Cells were then stained for flow cytometry as described above. To confirm MHCII-restriction, DCs and T_{CD4+} were collected, incubated with BFA, and treated with purified Gag293 peptide as described above. However, after 1 hour of peptide incubation, 50 μg/mL MHCII blocking antibody (Tü39, BD Biosciences) or CD71 blocking antibody (CY1G4, Biolegend) was added to relevant wells. After 1 hour, cells were washed with BFA-containing PBS and cocultured with transduced T_{CD4+} as described above. To test the ability of DCs and T_{CD4+} to present epitope derived from NL4-R3A (+Nef)-infected T_{CD4+} cultures, supernatant was collected from WT HIV-1-infected T_{CD4+} cultures 48 hours post infection. This supernatant

was either treated with ultraviolet (UV) light for 1 minute to inactivate any infectious virions (validated by a lack of syncytia formation in Sup R5 cells) or was filtered through a 100 kDa Ultra-0.5 mL Centrifugal Filter (MilliporeSigma, Burlington, MA) to remove any infectious virions and large subcellular particles. This treated supernatant was then mixed at a 1:1 ratio with fresh media supplemented with IL-2 and added to uninfected DCs and T_{CD4+} 12 hours prior to antigen presentation assays.

Statistics:

Fold Change Calculations: For all experiments, except where noted, significance was evaluated by two-tailed paired t test or one-way analysis of variance (ANOVA) test using PRISM software (GraphPad, San Diego, CA). The Tukey method was used to correct for multiple comparisons. A value of $p<0.05$ was considered significant.

Statistical Tests: To convert antigen presentation data from percent cytokine secretion to fold induction, the data were loaded into R (41). Data were grouped by cell type, cytokine, and experiment replicate and summary statistics were calculated. Within each summary statistic group, the mean response rate of the negative condition (DMSO) was determined. Fold changes were calculated as the response to experimental conditions divided by the response to DMSO. The mean and standard deviation of the fold changes were calculated in R.

RESULTS

Activated human T_{CD4+} express antigen processing machinery components

Expression of proteins associated with antigen processing and presentation, such as HLA-DR, CD86, and invariant chain (Ii), have been previously reported in activated human T_{CD4+} (42–44). To verify that activated human T_{CD4+} express these proteins in our experimental system, and to compare their expression in T_{CD4+} vs. DCs from the same donor, activated human T_{CD4+} and DCs were analyzed by flow cytometry. DCs express high levels of HLA-DR, CD86, and HLA-DM (both by percent positive and median fluorescence intensity (MFI)) (Fig. 1A and B, gating strategies shown in Fig. S4A–C), an unsurprising finding given their role as professional APCs. Activated T_{CD4+} also express all these proteins, although at lower levels, supporting the potential for APC function. Since HIV-1 infection has been previously shown to downmodulate antigen processing and presentation machinery in myeloid-derived cells (45, 46), we were also interested to determine whether expression levels of these proteins are maintained in HIV-1 infected T_{CD4+}. To identify HIV-1-infected cells, we gated on CD3+ cells with downregulated levels of surface CD4, as HIV-1 infection induces CD4 downregulation on the cell surface (47). When uninfected and infected T_{CD4+} were compared for levels expression of these proteins, a greater percentage of uninfected T_{CD4+} expressed HLA-DR and CD86, but there were no significant differences in the percentage of cells expressing HLA-DM and invariant chain (Fig. 1C). Furthermore, wildtype (WT) HIV-1 infection did not lead to decreased expression levels of these markers as measured by MFI. Indeed, infection slightly increased HLA-DM expression as assessed by MFI (Fig. 1D). We further confirmed that expression of these markers is upregulated upon activation, as a far lower proportion of freshly thawed, nonactivated T_{CD4+} express

these markers (Fig. 1E) when compared to activated T_{CD4+}, though MFI was high in the few cells that did express these proteins (Fig. 1F). Based on these data, we conclude that activated T_{CD4+}, both uninfected and HIV-1-infected, express well known components of the antigen processing and presentation machinery.

Activated T_{CD4+} have endocytic protease activities but limited internalization capabilities

Endocytic proteolysis is integral to classical antigen processing (48). Therefore, we investigated the activities of representative endosomal proteases, cathepsin D, L, and S in the lysates of DC and activated T_{CD4+} (Fig. 1E–G). Cathepsin D, a lysosomal aspartyl protease, and cathepsin L, a lysosomal endopeptidase, are necessary for intracellular protein breakdown and turnover and have been shown to play a role in antigen presentation (49–51). Cathepsin S is responsible for the degradation of invariant chain associated with mature MHCII molecules, allowing for the binding of antigenic peptides (52). While DCs had significantly greater Cathepsin L activity across 3 different donors, in 2/3 of donors (Fig. 1H), activated T_{CD4+} had greater Cathepsin D activity than did DCs (Fig.1G). DCs and T_{CD4+} had nearly indistinguishable Cathepsin S activities (Figure 1I). These activities, coupled with the expression of antigen processing and presentation components in Fig. 1A through D, suggests that activated human T_{CD4+} have the potential to be effective APCs during an HIV-1 infection.

We next investigated the ability of DCs and activated T_{CD4+} to internalize and proteolyze the fluorescent protease substrate DQ-OVA and the endocytic tracer FITC-Dextran, as internalization of extracellular antigen has been functionally linked to conventional MHCII-restricted processing and presentation (17, 53–56). DQ-OVA is internalized through receptor-mediated endocytosis (57) and fluid-phase micropinocytosis (58), and subsequent endocytic hydrolysis results in a fluorescent signal. Both DCs and activated T_{CD4+} internalize and proteolyze DQ-OVA (Fig. 1J and K). However, DCs internalize and proteolyze DQ-OVA at far greater levels than activated T_{CD4+}, an unsurprising result given the well-documented ability of DCs to sample the external environment at a high rate (17, 53, 55, 56). Given that the fluorescent signal from DQ-OVA requires both internalization and proteolysis, and we can detect protease activity in activated T_{CD4+}, the low signal likely reflects the modest internalization capabilities of this cell type. We confirmed these findings by examining the internalization of FITC-Dextran, another fluorescent substrate (59), by these two cell types. Similar to our DQ-OVA results, we found that while both DCs and activated T_{CD4+} are capable of internalizing FITC-Dextran (Fig. 1L), DCs do so at a much greater level (Fig. 1M). Taken together, these data suggest that activated T_{CD4+} have a limited ability to internalize and process exogenous antigens.

Activated T_{CD4+} present HIV-1-derived antigen to Gag293-specific T_{CD4+}

Results thus far suggest that T_{CD4+} are limited in conventional processing of exogenous antigens due to their modest ability to internalize exogenous antigen. However, we reasoned that, because activated T_{CD4+} are highly susceptible to HIV-1 infection, they might be effective at endogenous processing following synthesis of viral proteins (19–24). To explore this possibility, we needed to establish a reliable system to read out epitope production. In initial experiments, we attempted to use activation of HIV-1-specific T_{CD4+} clones as

the readout; however, we were unable to generate the cell numbers needed for sufficient biological and technical replicates and the experimental variations outlined below. We therefore turned to a lentiviral transduction system similar to that described by Benati et al. (35), in which we attained HIV-1-specificity by transducing T_{CD4+} from HIV-1-negative donors with an HIV-1-specific TCR. Extensive searches yielded only three full-length, MHCII-restricted HIV-1-specific TCR sequences, all specific for the immunodominant Gag293 epitope located within the HIV-1 capsid protein. The HIV-1-specific public TCR used in these studies, termed F5 (35), recognizes the Gag293 epitope in complex with HLA-DR1, DR11, and DR15 molecules. HLA-DR1$^+$, DR11$^+$, or DR15$^+$ DCs and activated T_{CD4+} were infected with WT HIV-1 3 days prior to the assay. To minimize any nonspecific cytokine secretion from uninfected and HIV-1-infected T_{CD4+} over the course of infection, these cells were treated with brefeldin A (BFA) for 4 hours prior to coculture with HIV-1-specific T_{CD4+} and subsequently washed to remove any secreted cytokines. Synthetic Gag293 peptide was added to DCs and activated T_{CD4+} 2 hours prior to coculture (2 hours into the BFA pretreatment) and was washed out prior to coculture. HIV-1-specific T_{CD4+} were labeled with Cell Trace Violet dye to differentiate between the two T_{CD4+} populations. Candidate APCs (DCs and activated T_{CD4+}) and HIV-1-specific T_{CD4+} were cocultured for 8 hours in the presence of BFA. Cocultures were then stained for flow cytometric analysis to detect production of interleukin-2 (IL-2), tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) by responding T_{CD4+}. Notably, we did not observe any differences in presentation patterns depending on identity of the presenting molecule (HLA-DR1, 11 or 15), though we did note occasional variability in the magnitude of responses depending on the donor and experiment. We measured three different indicators of activation (IL-2, TNFα, and IFNγ) to maximize the chances of detecting T_{CD4+} activation, as it has been observed that T_{CD4+} cytokine profiles may differ depending on the activating conditions used (60).

Results reproducibly showed that DCs present synthetic Gag293 peptide but are unable to present the epitope derived from infectious HIV-1 (Fig. 2A). Conversely, activated T_{CD4+} present the Gag293 epitope derived from infectious HIV-1 but are unable to present synthetic Gag293 peptide (Fig. 2B, representative flow plots shown in Fig. S4D), even at much higher concentrations of synthetic peptide (Fig. S3C) despite high levels of MHCII expression. This phenomenon has been previously observed in other antigen systems (23). Two potential explanations are limited peptide exchange at the cell surface (61–63), or the need for peptide to be internalized by the cell for successful presentation (64), a mechanism that is likely severely compromised in T_{CD4+} considering the results shown in Fig. 1.

We confirmed that the activation of T_{CD4+} was epitope specific, as T_{CD4+} transduced with a TCR specific for ZnT8, a zinc transporter implicated in Type 1 diabetes (40) demonstrated either marginal or undetectable activation in response to infected T_{CD4+} (Fig. S2A–B). Additionally, we used an MHCII blocking antibody to confirm that presentation to HIV-1-specific T_{CD4+} is MHCII-dependent (Fig. 2C). Treatment with a control antibody against the transferrin receptor CD71 did not block presentation by infected T_{CD4+} (Fig. S2C). Notably, HIV-1 infection leads to CD4 downregulation at the infected T_{CD4+} surface, preventing superinfection (47) and likely preventing infected T_{CD4+} from presenting material derived from newly internalized virions. These results indicate that T_{CD4+}, but not DCs, are capable of presenting Gag293 from infectious HIV-1 in an MHCII-restricted manner, leading to the

activation of HIV-1-specific T_{CD4+}. These data strongly suggest that activation is a direct consequence of MHCII-restricted epitope presentation.

Activated T_{CD4+} do not present inactivated or fusion-deficient HIV-1

Given the weak internalization capabilities of activated T_{CD4+} (Fig. 1G and H) and their susceptibility to productive HIV-1 infection, presentation seemed most attributable to endogenous processing, in which nascent viral antigen, not input virions, constitutes the major processing substrate (24, 26, 65). Thus, we hypothesized that activated T_{CD4+} would be unable to present epitope derived from structurally intact, chemically inactivated HIV-1. The same experimental setup as in Fig. 2 was used in these experiments, but a new experimental condition was introduced: aldrithiol-2 (AT2)-inactivated HIV-1. AT2-treated HIV-1 was added to uninfected DCs and activated T_{CD4+} 12 hours prior to the start of the assay. AT2-treated HIV-1 is still capable of fusing to its receptor, CD4, and its coreceptor, CCR5 or CXCR4, at the cell surface and inserting its nucleocapsid into the cytoplasm (66). However, the virus is unable to uncoat and initiate productive infection. If input virus was responsible for the presentation observed in Figure 2, then activated T_{CD4+} should be able to present this inactivated virus. As shown in Fig. 3A and B (representative flow plots shown in Fig. S4F–G), neither DCs nor activated T_{CD4+} can detectably present the Gag293 epitope derived from AT2-treated HIV-1, even at a much higher dose (Fig. S3A–B), supporting the notion that activated HIV-1-infected T_{CD4+} employ endogenous presentation to activate HIV-1-specific T_{CD4+}.

To reinforce this finding, we tested the abilities of DCs and activated T_{CD4+} to generate epitope from infectious, but fusion-deficient, HIV-1 (K574D HIV-1). This virus was tested for two main reasons. First, chemical inactivation might have negatively impacted virion processibility, and thus use of a fusion-deficient virus provided an orthogonal approach to reinforce the finding. Second, since HIV-1 fuses at the target cell surface and injects its viral material directly into the cytoplasm (67), HIV-1 proteins may have limited opportunity to access endocytic compartments where conventional MHCII antigen processing occurs (17). Thus, absence of fusion at the cell surface may drive more input virions into endocytic compartments. Again, the same experimental setup was utilized in these experiments as in Fig. 2, except that fusion deficient HIV-1, quantified by p24 content, was compared to fusion-competent HIV-1, both being added to DCs and activated T_{CD4+} 12 hours prior to addition of Gag293-specific T_{CD4+}. As indicated in Fig. 3C and D (representative flow plots shown in Fig. S4H–I), activated T_{CD4+} are unable to present fusion deficient HIV-1. DCs are able to present fusion deficient HIV-1, though the resulting HIV-1-specific T_{CD4+} responses are not robust. These data, combined with the lack of presentation of AT2-inactivated HIV-1, strongly suggest that HIV-1 must 1) fuse at the cell surface and 2) initiate productive infection in order to be processed and presented by activated T_{CD4+}. However, in our hands, DCs are reproducibly unable to present the Gag293 epitope from infectious or chemically inactivated HIV-1 and are capable of only modest presentation of fusion deficient virus.

DCs, but not activated T_{CD4+}, indirectly present subviral material

We have so far observed presentation by activated T_{CD4+} only when infectious HIV-1 was used. This could be due to direct, endogenous processing and presentation by the infected

T_{CD4+} or via "indirect presentation" (68, 69), in which infected T_{CD4+} release viral material that is taken up and presented by uninfected, activated T_{CD4+}. We subjected supernatant from HIV-1 infected T_{CD4+} to either 1) UV light to render the supernatant non-infectious or 2) 100 kDa filtration to remove whole virions and larger subcellular material. DCs and activated T_{CD4+} were incubated with treated supernatant for 12 hours prior to the assay. We found that DCs were able to present epitope from UV-treated supernatant, indicating that they are capable of MHCII-restricted indirect presentation (Fig. 4A, representative flow plots shown in Fig. S4J–K). This result is consistent with previous studies showing DCs are capable of indirect presentation (68, 69). Interestingly, DCs were unable to present filtered supernatant (Fig. 4A), and thus may require relatively large subcellular material, such as exosomes (69) or apoptotic bodies (70) for successful indirect presentation of the Gag293 epitope. In contrast, activated T_{CD4+} are capable of only modest presentation of UV-treated supernatant (Fig. 4B), indicating that indirect presentation does not play a major role in the presentation of HIV-1-derived epitope by activated T_{CD4+}.

In addition to addressing the question of indirect presentation, the results provide additional support for our conclusion that activated T_{CD4+} carry out endogenous processing. Supernatants were collected from activated T_{CD4+} 72 hours post-infection, the same duration of infection prior to addition of responding T_{CD4+}. Lack of a robust level of presentation of the UV treated supernatant by activated T_{CD4+} (Fig. 4) argues against the presentation of exogenous material in the form of extracellular virions or subviral material and in favor of *bona fide* endogenous processing and presentation.

Nef and Vpu do not impact T_{CD4+}-mediated presentation of HIV-1 antigen

The viral accessory proteins Nef and Vpu have been reported to disrupt MHCII-restricted antigen presentation in myeloid-derived cells (45, 71, 72). We investigated whether these proteins contribute to the lack of detectable presentation of live HIV-1 by DCs. To test this, and to determine the impact of these proteins on MHCII-restricted presentation by activated T_{CD4+}, we utilized a Nef/Vpu double knockout (KO) HIV-1. Infection with the Nef/Vpu double KO virus was performed using the same method as in Fig. 2. Using this virus, we could not detect any impact of Nef or Vpu on presentation of the Gag293 epitope by either DCs or activated T_{CD4+} (Fig. 5A and B, representative flow plots shown in Fig. S4L–M). DCs were still unable to present the Gag293 epitope from infectious Nef/Vpu KO virus (Fig. 5A), and there were no significant differences in the presentation of WT and double KO HIV-1 by activated T_{CD4+} (Fig. 5B).

DISCUSSION

Our study reveals that activated T_{CD4+} express several key components of the antigen processing and presentation machinery (Fig. 1A and B), and this expression remains intact in HIV-1-infected cells (Fig. 1C and D). We also show that activated T_{CD4+} express functional endosomal proteases (Fig. 1E and F), though their ability to internalize exogenous antigen is restricted, as demonstrated by their limited ability to unquench DQ-OVA (Fig. 1G and H) and internalize FITC-Dextran (Fig. 1L–M). Taken together, these data suggest that activated T_{CD4+} have the ability to process and present antigen on MHCII but are limited

with respect to classical processing and presentation by their modest ability to internalize extracellular antigen.

Importantly, we observed that activated T_{CD4+} are able to present antigen derived from infectious HIV-1 to HIV-1-specific T_{CD4+}, eliciting a strong cytokine response (Fig. 2A and B). This presentation is MHCII-dependent, as the stimulation of IL-2 secretion by HIV-1-specific T_{CD4+} can be inhibited with MHCII blocking antibody (Fig. 2C), and is antigen-specific, as ZnT8 TCR-transduced T_{CD4+} experienced either far weaker or undetectable activation in response to HIV-1-infected T_{CD4+} (Fig. S2A–B). We further confirmed that T_{CD4+} can present HIV-1-derived epitope only when they are productively infected, as these cells are unable to present chemically inactivated virus (Fig. 3B) or fusion-deficient virus (Fig. 3D) and are unable to indirectly present epitope from supernatants of HIV-1-infected cells (Fig. 4B). We also note that activated T_{CD4+} are unable to present synthetic peptide, even at high concentrations (Fig. S3C). This phenomenon has been previously observed in other antigen systems (23). Two potential explanations are limited peptide exchange at the cell surface (61–63), or the need for peptide to be internalized by the cell for successful presentation (64), which T_{CD4+} appear to be incapable of performing. These data lead us to conclude that activated T_{CD4+} employ endogenous processing to stimulate HIV-1-specific T_{CD4+}. Thus, cell types with only a marginal capacity for classical processing and presentation can nevertheless be highly functional APCs via viral infection and endogenous processing and presentation. How far this paradigm extends beyond HIV-1 and T_{CD4+} remains to be seen. To date, the vast majority of endogenous processing studies in primary cells focus on professional APCs. However, Toulmin et al. recently demonstrated that type II alveolar cells, epithelial cells located in the distal lung, express MHCII and utilize endogenous processing to present influenza proteins and the endogenous E protein (23). These findings, along with ours, suggest that non-professional APCs may play key roles in MHCII-restricted presentation via endogenous antigen processing during viral infections.

Previous studies have reported that activated T_{CD4+} are capable of presenting HIV-1 gp120-derived epitope (30, 31). However, ours appears to be the first demonstration of MHCII-restricted presentation by activated T_{CD4+} of epitope derived from viable HIV-1 virions. These results suggest a novel function for T_{CD4+} during the immune response to HIV-1, though these findings will need to be confirmed *in vivo*. In addition to orchestrating the adaptive immune response to HIV-1, our data suggest that T_{CD4+} may also act as APCs, leading to the activation of HIV- 1-specific T_{CD4+}, though this presentation could potentially come at the price of enhanced viral spread to HIV-1-specific T_{CD4+} (73).

Previous studies have shown that monocyte-derived DCs are capable of presenting the Gag293 epitope from infectious HIV-1 (36); however, our results are not necessarily in conflict with this work. Galperin et al. focused on T_{CD4+}-mediated killing of infected DCs as a readout of T_{CD4+} activation, while we focused on cytokine secretion. It is possible that the presentation of infectious HIV-1 by DCs leads to cytotoxicity and not cytokine production in responding T_{CD4+}, which could explain the differences in our results. Of note, DCs can present the Gag293 epitope when exposed to UV-treated, HIV-1-infected supernatant (Fig. 4A), indicating that indirect presentation is a viable production route for this particular epitope in DCs. We focused on the Gag293 epitope due to the limited

number of available full length, MHCII-restricted HIV-1-specific TCR sequences. It will be interesting to determine whether our observations extend to other MHCII-restricted HIV-1 epitopes. Based on our previous work with influenza (24) (in which 6 out of 6 MHCII-restricted epitopes are endogenously presented), we anticipate that Gag293 is representative of other epitopes, though the processing pathways could be quite different.

We also showed that the HIV-1 accessory proteins Nef and Vpu do not impact presentation of the Gag293 epitope by either DCs or activated T_{CD4+} (Fig. 5A and B). Again, the different processing requirements for different epitopes (24, 65, 74, 75) may explain the apparent contradiction with previous studies, which showed that Nef and Vpu disrupt MHCII-restricted presentation in DCs (45, 71, 72). Additionally, while we can conclude that Nef and Vpu do not interfere with the presentation of the Gag293 epitope in DCs and activated T_{CD4+}, we are unable to speculate on the effects of these proteins on the presentation by other APC types.

In summary, our results indicate that activated, HIV-1 infected T_{CD4+} are highly effective at MHCII-restricted presentation of HIV-1-derived epitopes due to the tropism of HIV-1 for T_{CD4+} and the potency of endogenous MHCII-restricted processing and presentation. Given the critical importance of HIV-1-specific T_{CD4+}, these results could significantly alter our understanding of the MHCII-restricted processing and presentation landscape that develops during HIV-1 infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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COMPETING INTERESTS

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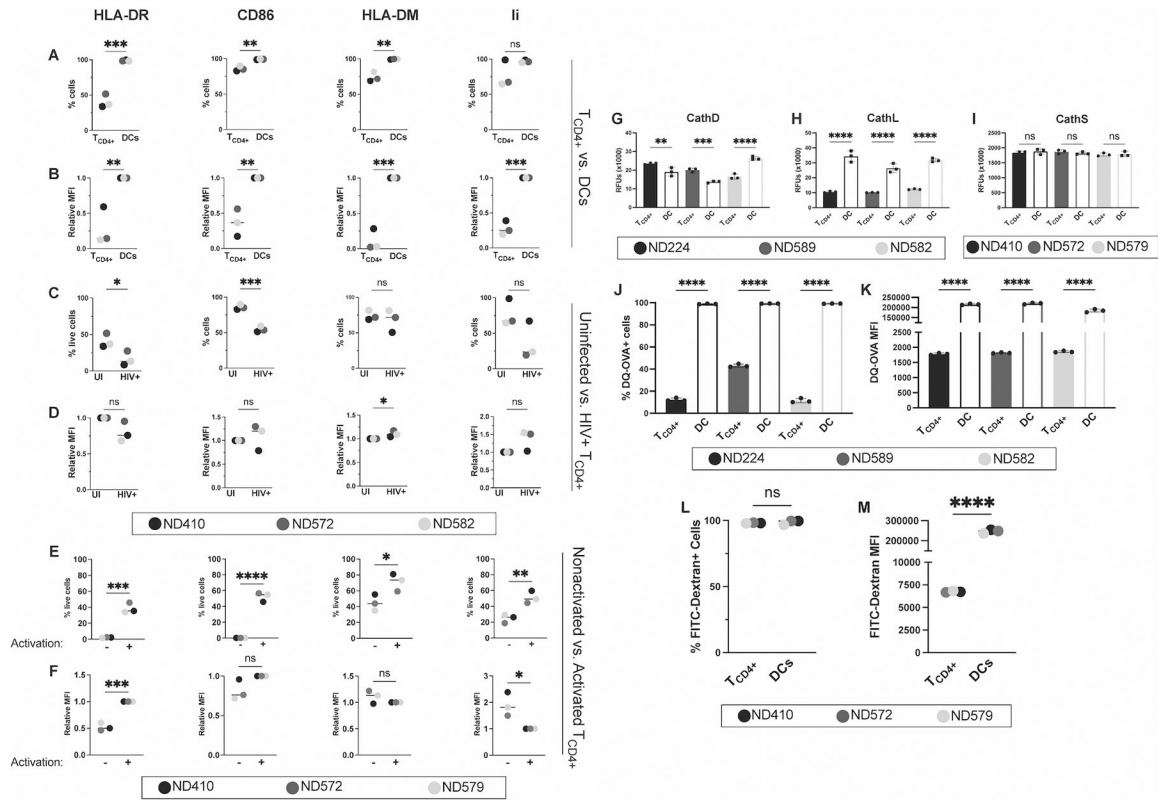
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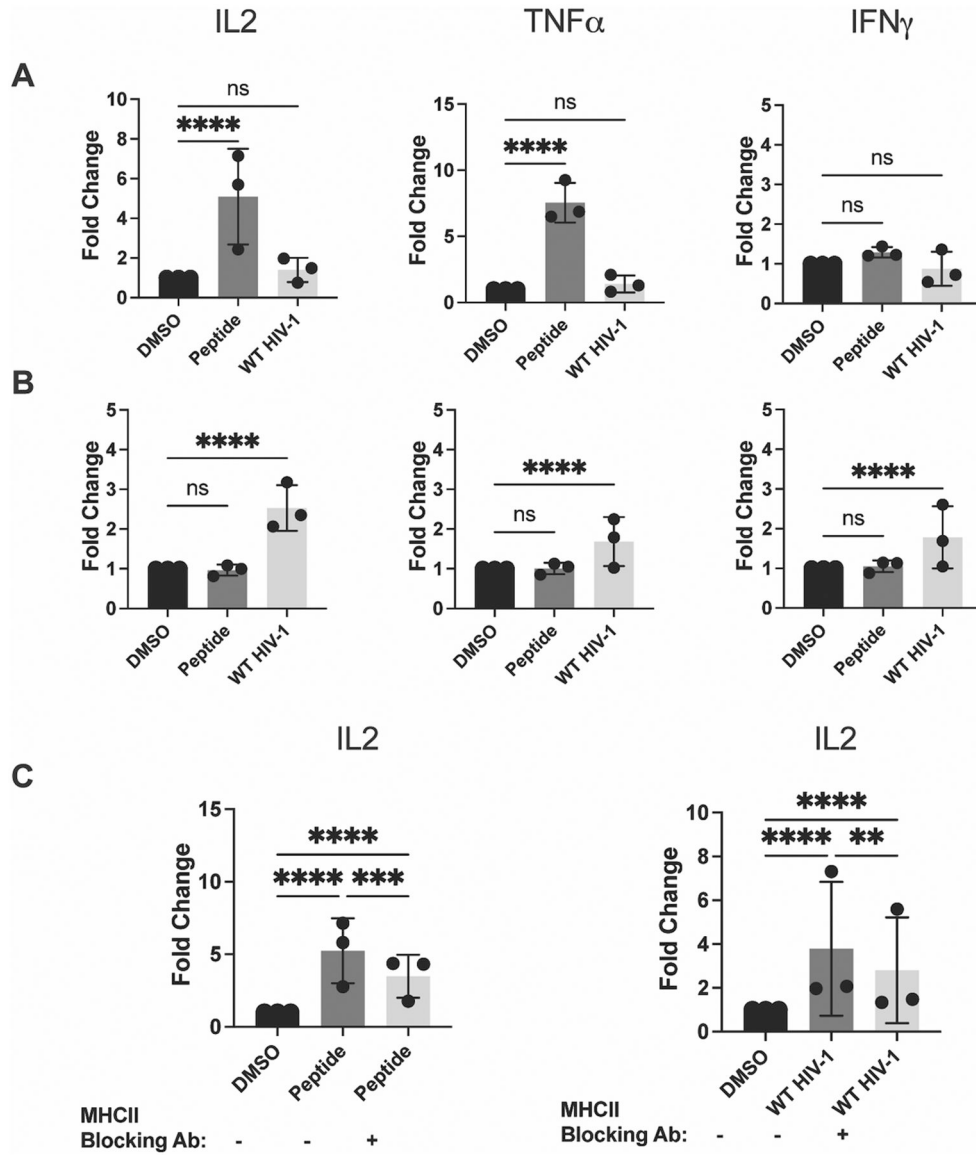
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KEY POINTS

- Activated T_{CD4+} can present MHCII-restricted antigen derived from infectious HIV-1.

- Activated T_{CD4+} use endogenous processing to process and present HIV-1 antigen.

Figure 1. Activated T_{CD4+} and DCs both express antigen presentation machinery components and have endocytic protease activities but T_{CD4+} have weak internalization capabilities.
(**A**) percent positive and (**B**) relative mean fluorescence intensity (MFI) of HLA-DR, CD86, HLA-DM, and invariant chain in uninfected T_{CD4+} and DCs. T_{CD4+} and DCs were cultured as described. Cells were then stained for flow cytometry. HLA-DR and CD86 positive populations were gated on live singlets. HLA-DM and invariant chain expression was determined by pre-gating HLA-DR and CD86 double positive cells. (**C**) Percent positive and (**D**) relative MFI of these markers in uninfected (UI) and WT HIV-1 infected (HIV+) T_{CD4+}. Where indicated, activated T_{CD4+} were infected with 100 ng p24 HIV-1 3 days prior to staining. Activated T_{CD4+} and DCs lysates were then analyzed for (**E**) cathepsin D, (**F**) cathepsin L, and (**G**) cathepsin S activity using fluorometric cathepsin substrates. Lysates from 1×10^5 cells were analyzed per replicate. Measurements were taken after 30 mins at 37°C. Activated T_{CD4+} and DCs were then tested for their ability to internalize and proteolyze DQ-OVA. All values are background subtracted. (**H**) Percent DQ-OVA positive cells and (**I**) MFI in DQ-OVA+ cells. T_{CD4+} and DCs were cultured as previously described and incubated with DQ-OVA fluorescent substrate for 2 hours at 37°C. Uptake and proteolysis were measured by flow cytometry on live singlets. Gates were drawn based on no-substrate controls. Percent FITC-Dextran positive cells (**J**) and (**K**) MFI in FITC-Dextran+ cells. T_{CD4+} and DCs were cultured as previously described and incubated with FITC-Dextran fluorescent substrate for 2 hours at 37°C. Uptake and proteolysis were measured by flow cytometry on live singlets. Gates were drawn based on no-substrate controls. Each color represents a unique donor, as indicated in the figure legend. For **G-K**, each dot represents a technical replicate. Representative of 3 independent experiments with

the exception of **L** and **M**, which are representative of 2 independent experiments. For **A-F** and **L-M**, data were analyzed with an unpaired t test. For **G-K**, data were analyzed with a One-way ANOVA. Bars represent mean ± SD. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

Figure 2. Activated T$_{CD4+}$, but not DCs, can present epitope derived from infectious HIV-1 in an MHCII-dependent manner.

DCs and activated T$_{CD4+}$ were cultured and infected as described in Materials and Methods. (**A**) DCs and (**B**) T$_{CD4+}$ were assessed for their ability to present peptide and WT HIV-1 after 8 hours of coculture with HIV-1-specific T$_{CD4+}$. IL-2, TNFα, and IFNγ expression was evaluated by flow cytometry. (**C**) DCs (left) and activated T$_{CD4+}$ (right) were treated with 50 μg/mL MHCII blocking antibody 2 hours prior to the beginning of the assay. IL-2 expression was assessed by flow cytometry. Fold induction of each cytokine is shown, using DMSO as a baseline. Each dot represents an independent experiment. Bars represent mean ± SD. One way ANOVA, **p<0.01, ****p<0.001.
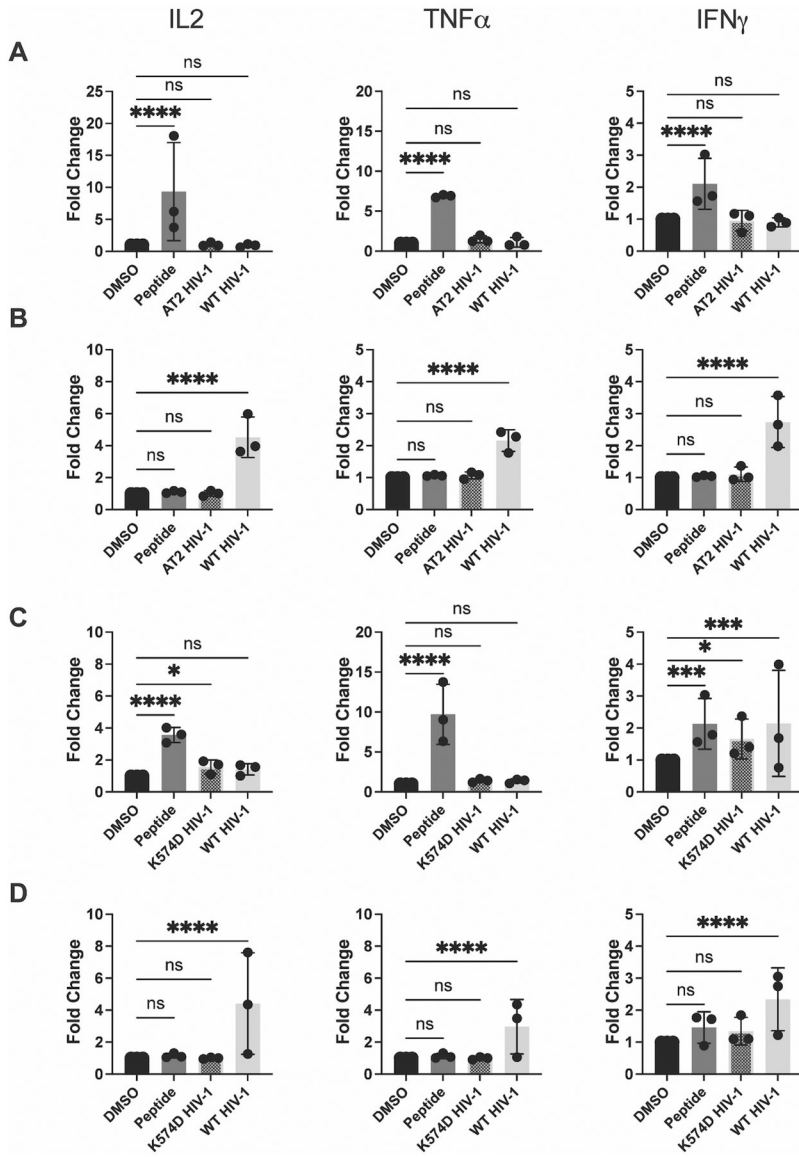
Figure 3. DCs and activated T$_{CD4+}$ are unable to present epitope derived from AT2-inactivated and K574D fusion-deficient HIV-1.

DCs and activated T$_{CD4+}$ were cultured and infected with WT HIV-1 as described in Materials and Methods. AT2-treated HIV-1 and K574D fusion-deficient HIV-1 were added to DCs and activated T$_{CD4+}$ 12 hours prior to the beginning of the assay. (**A**) DCs and (**B**) T$_{CD4+}$ were assessed for their ability to present AT2-treated HIV-1 after 8 hours of coculture with HIV-1-specific T$_{CD4+}$. IL-2, TNFα, and IFNγ expression was assessed by flow cytometry. (**C**) DCs and (**D**) activated T$_{CD4+}$ were assessed for their ability to present fusion-deficient HIV-1 in a similar manner. Fold induction of each cytokine is shown, using DMSO as a baseline. Each dot represents an independent experiment. Bars represent mean ± SD. One way ANOVA, *p<0.05, **p<0.01, ****p<0.001.
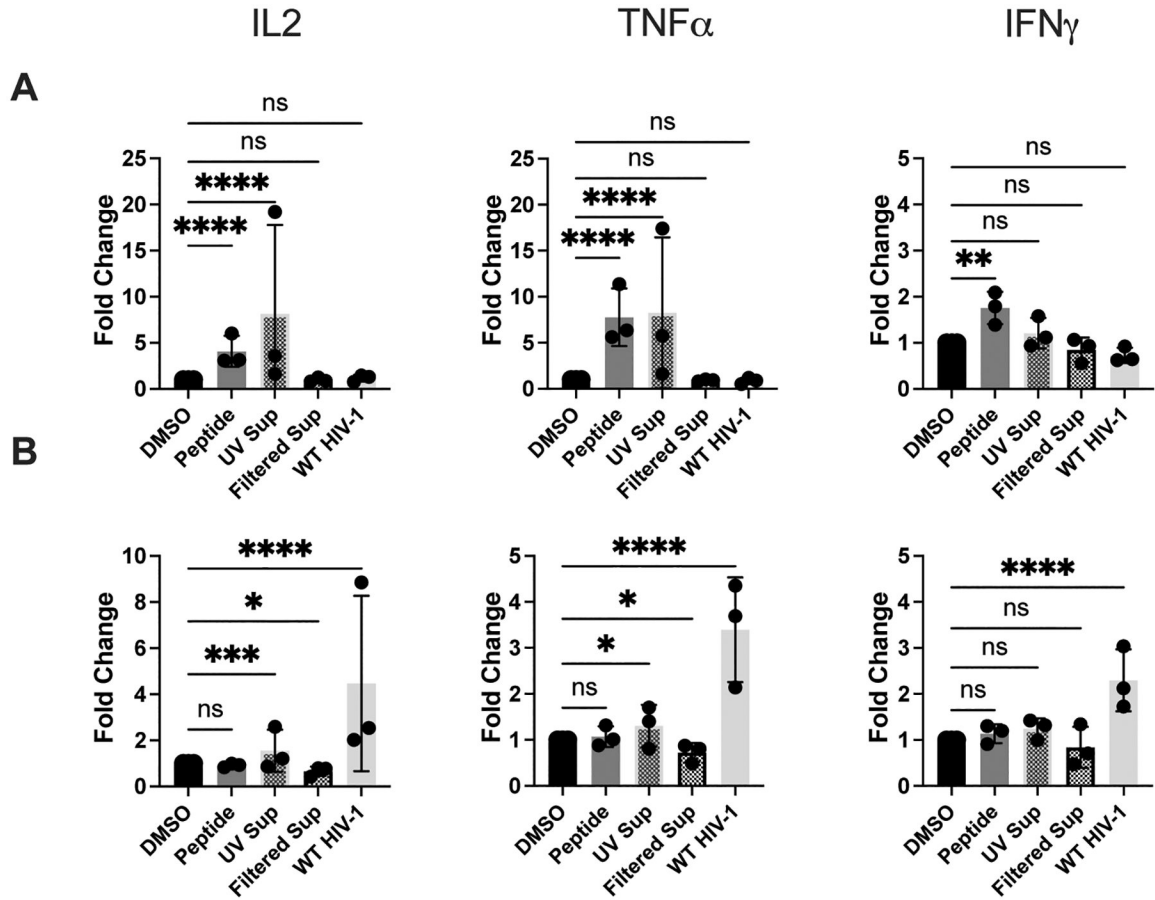
Figure 4. DCs, but not activated T_{CD4+}, indirectly present HIV-1-derived epitope.
Presentation of UV-treated and filtered HIV-1$^+$ supernatant by DCs and activated T_{CD4+} to HIV-1-specific T_{CD4+}. DCs and activated T_{CD4+} were cultured and infected with WT HIV-1 as described in Materials and Methods. Supernatant from WT HIV-1-infected T_{CD4+} was collected and treated with UV light or filtered through a 100 kDa filter and then added to DCs and activated T_{CD4+} 12 hours prior to the beginning of the assay. (**A**) DCs and (**B**) T_{CD4+} were cocultured with HIV-1-specific T_{CD4+} for 8 hours and IL-2, TNFα, and IFNγ expression was assessed by flow cytometry. Fold induction of each cytokine is shown, using DMSO as a baseline. Each dot represents an independent experiment. Bars represent mean ± SD. One way ANOVA, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.
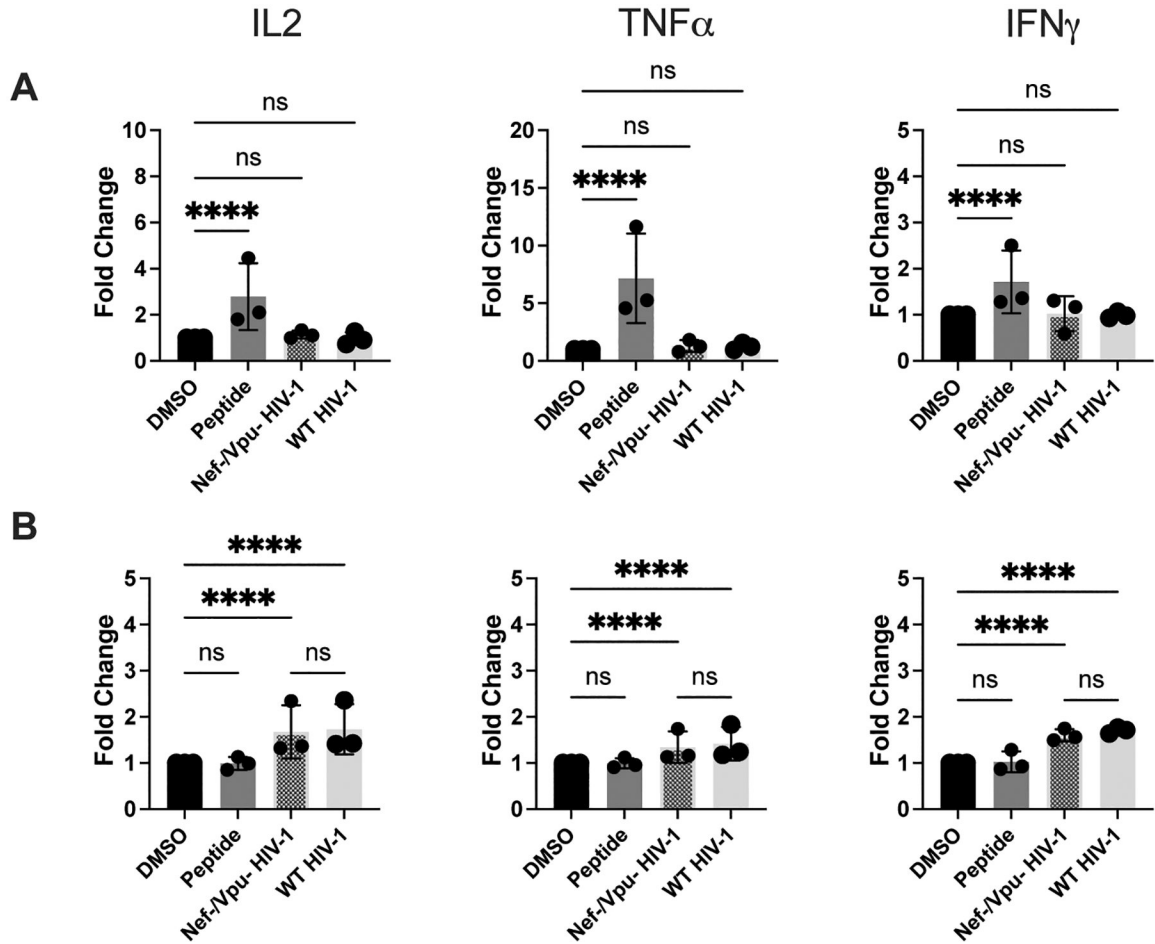
Figure 5. The viral accessory proteins Nef and Vpu do not impact the presentation of the Gag293 epitope by DCs and activated T_{CD4+}.

DCs and activated T_{CD4+} were cultured and infected with WT HIV-1 and Nef-/Vpu- HIV-1 as described in Materials and Methods. (**A**) DCs and (**B**) T_{CD4+} were cocultured with HIV-1-specific T_{CD4+} for 8 hours and IL-2, TNFα, and IFNγ expression was assessed by flow cytometry. Fold induction of each cytokine is shown, using DMSO as a baseline. Each dot represents an independent experiment. Bars represent mean ± SD. One way ANOVA, ****p<0.001.