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Research paper

A robust and scalable TCR-based reporter cell assay to measure HIV-1 Nef-mediated T cell immune evasion

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ABSTRACT

HIV-1 evades cytotoxic T cell responses through Nef-mediated downregulation of HLA class I molecules from the infected cell surface. Methods to quantify the impact of Nef on T cell recognition typically employ patient-derived T cell clones; however, these assays are limited by the cost and effort required to isolate and maintain primary cell lines. The variable activity of different T cell clones and the limited number of cells generated by re-stimulation can also hinder assay reproducibility and scalability. Here, we describe a heterologous T cell receptor reporter assay and use it to study immune evasion by Nef. Induction of NFAT-driven luciferase following co-culture with peptide-pulsed or virus-infected target cells serves as a rapid, quantitative and antigen-specific measure of T cell recognition of its cognate peptide/HLA complex. We demonstrate that Nef-mediated downregulation of HLA on target cells correlates inversely with T cell receptor-dependent luminescent signal generated by effector cells. This method provides a robust, flexible and scalable platform that is suitable for studies to measure Nef function in the context of different viral peptide/HLA antigens, to assess the function of patient-derived Nef alleles, or to screen small molecule libraries to identify novel Nef inhibitors.

1. Introduction

HIV-1 infection elicits a rapid CD8+ cytotoxic T lymphocyte (CTL) response that targets viral peptide epitopes presented by Human Leukocyte Antigen class I (HLA-I) molecules. While CTL contribute to early control of HIV-1 replication (Borrow et al., 1994; Koup et al., 1994; Safrit et al., 1994), the virus quickly eludes this response to establish a persistent infection. Evasion from CTL is achieved by the selection of CTL escape mutations within or near targeted viral epitopes (McMichael and Phillips, 1997), as well as the actions of HIV-1 Nef, a 27–35 kD myrisoylated accessory protein that enhances plasma viremia and pathogenesis through various mechanisms (Ariën and Verhasselt, 2008; Kestler et al., 1991). In particular, Nef's ability to downregulate HLA-I molecules, specifically HLA-A and -B alleles, reduces CTL recognition and killing of virus-infected cells (Adnan et al., 2006; Collins et al., 1998; Yang et al., 2002). Deletions or mutations in Nef have been identified in HIV-1 long-term non-progressors (Corró et al., 2012; Deacon et al., 1995; Kirchhoff et al., 1995; Learmont et al., 1999) and Nef alleles isolated from elite controllers have decreased in vitro ability to downregulate HLA-I compared to those from progressors (Mwimanzi et al., 2013), indicating that Nef contributes significantly to disease outcome. As such, novel inhibitors of Nef are expected to be of clinical benefit.

The impact of Nef-mediated HLA-I downregulation on antiviral CTL activity has successfully been studied using traditional co-culture methods (Ali et al., 2005; Collins et al., 1998; Shankar et al., 1999; Yang et al., 1996, 2002). In a typical assay, a patient-derived CTL clone is incubated with HLA-expressing target cells that have been infected with HIV-1 harboring a wild type or mutant Nef sequence (e.g. ΔNef or the $M_{20}A$ variant that is defective for HLA-I downregulation (Akari et al., 2000)) and the extent of viral suppression is quantified by measuring HIV-1 p24 antigen levels in the culture supernatant over the course of 7 days. Alternatively, CTL cytolytic activity (or cytokine production) can be measured following co-culture with target cells expressing or not expressing functional Nef (Tomiyama

Abbreviations: TCR, T-cell receptor; CTL, Cytotoxic T lymphocyte; NFAT, Nuclear factor of activated T-cells; HIV, Human immunodeficiency virus; Nef, Negative regulatory factor.

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et al., 2002; Ueno et al., 2008). While these approaches have contributed greatly to our mechanistic understanding of immunological effects of Nef-mediated HLA-I downregulation (Chen et al., 2012; Rajapaksa et al., 2012), the use of primary T cells for such studies presents a number of challenges. First, isolation and maintenance of patient-derived epitope-specific CTL clones can be time consuming and costly. More importantly, in vitro expansion of CTL clones can introduce variability in cell purity and antiviral activity. Finally, the limited proliferative capacity of many CTL clones may preclude assay scale-up.

Here, we present a reporter T cell method to examine the immune evasion activity of Nef based in part on prior studies (Birkholz et al., 2009; Schaft et al., 2003). This approach uses readily available molecular and cellular reagents to simulate natural T cell recognition. In this assay, a Jurkat T cell line serves as a modifiable "effector" cell population. These cells are transiently transfected with plasmids encoding TCR alpha and beta genes isolated from a primary CTL clone (conferring epitope/HLA specificity), CD8 alpha (to stabilize TCR-peptide/HLA interactions), and an NFAT-luciferase reporter vector (to quantify TCR-mediated signaling). CEM T cell lines stably or transiently expressing specific HLA-I alleles serve as "target" cell populations. These cells can be pulsed with epitopic peptide or infected with HIV-1. Co-culture of effector and target cells triggers antigen-specific TCR-dependent calcium flux in the effector cells, which results in quantifiable NFAT-driven luciferase expression that can be measured by luminescence. This highly flexible system provides a sensitive and scalable method to assess CTL recognition in the presence or absence of Nef.

2. Methods

2.1. Reagents

2.1.1. Plasmids and cell lines

The following reagents were obtained through the NIH AIDS Reagents Program, Division of AIDS, NIAID, NIH: pNL4.3 (Cat #114) from Dr. Malcolm Martin (Adachi et al., 1986); CEM-SS cell line (Cat #776) from Dr. Peter L. Nara (Nara et al., 1987); and pHEF-VSVG (Cat #4693) from Dr. Lung-Ji Chang (Chang et al., 1999).

The CTL clone (5B2) specific for the HLA-A*02-restricted HIV-1 Gag FK10 epitope (FLGKIWPSYK; HIV_{HXB2} amino acid position 433–442) was isolated from an HIV-infected patient following written informed consent. Research ethics board approval was obtained from the University of Toronto, Canada (by M. Ostrowski). The pMSCV-A*02:01 retroviral vector was a gift from Dr. Christian Brander (IrsiCaixa, Spain). The CEM-derived GXR25 cell line containing an HIV-1 Tat-driven GFP reporter construct has been described previously (Brockman et al., 2006). Jurkat clone E6-1 cells (TIB-152) were purchased from the American Type Culture Collection; and RetroPack PT67 and HEK-293 T cells were purchased from Clontech. Plasmids encoding wild type Nef (SF2 strain) and consensus HIV-1 subtype B or subtype C (2004 sequences, available at http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) were described previously (Mann et al., 2013; Mwimanzi et al., 2013). Other expression plasmids were purchased, as follows: pSELECT-GFPzeo and pORF9-hCD8A (InvivoGen); and pNFAT-Luciferase (Affymetrix).

Jurkat, CEM-SS and RetroPack PT67 cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Sigma-Aldrich, Co.) plus 10% calf serum (Life Technologies, Inc.) (R10+). Following HIV-1 infection, T cells were cultured in RPMI-1640 medium containing these supplements plus 20% calf serum (R20+). Cell lines transduced with HLA-A*02:01 or HLA-B*07:02 were maintained in R10+ supplemented with 0.5 μg/mL puromycin to maintain HLA expression. RPMI-1640 medium lacking phenol red (Sigma-Aldrich, Co.) (R10 + _NoPR) was used during co-culture to reduce quenching of luminescent signal. HEK-293 T cells were maintained in DMEM (Lonza) containing 4.5 g/L glucose, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin, plus 10% calf serum (D10+). All cells were maintained, incubated, peptide pulsed, and co-cultured at 37 °C with 5% CO_2.

2.1.2. Viral stocks

Viruses were generated by transfection using Lipofectamine 3000 (Life Technologies, Inc.) according to the manufacturer's directions. To prepare retroviral vectors, lipid complexes (0.5 mL) containing 5 μg pMSCV-HLA-A*02:01 and 1 μg pHEF-VSVG in Opti-MEM (Life Technologies, Inc.) were added to RetroPack PT67 cells in a 25 cm^2 flask containing 4.5 mL R10+. To prepare VSV-g pseudotyped HIV-1 stocks, lipid complexes (1.5 mL) containing 40 μg of pNL4.3 (containing the HIV-1 subtype B reference strain NL4.3) or pNL4.3ΔNef (containing a Nef-deleted NL4.3 variant) plus 4 μg pHEF-VSVG in Opti-MEM were added to HEK-293 T cells in a 75 cm^2 flask containing 13.5 mL R10+. Cells were incubated for 6 h, after which media was replaced with fresh R10+. Supernatant was collected at 48 h post-transfection, cell debris was removed by centrifugation at 500 ×*g* for 10 min, and aliquots were stored at −80 °C until use. The infectivity of HIV-1 stocks was determined using GXR25 reporter cells as described previously (Brockman et al., 2006).

2.2. Preparation of Jurkat effector T cells

2.2.1. Isolation and cloning of TCR alpha and TCR beta transcripts

Total RNA was extracted from 1×10^6 5B2 T cells using the RNeasy Mini kit (Qiagen) and 125 ng RNA was used to prepare cDNA according to the 5′ RACE protocol included with the SMARTer cDNA Synthesis Kit (Clontech). TCR cDNAs were amplified by nested PCR using Hi-Fidelity DNA polymerase (Roche) with the following gene-specific reverse primers: 1st round, TCRalpha_RevOutA: 5′-TGT CAG GCA GTG ACA AGC AG, TCRB1_1230: 5′-CCT GAC TGA ATG GGG AGA GTC ACA GGG, or TCRB2_1550: 5′-GAC ACT CCT GAA ATG CAA CCA GGC CC; 2nd round, TCRalpha_RevIn: 5′-CAG CAG TGT TTG GCA GCT CT, TCRB1_1090: 5′-AGA TTT CAG CCG TGA GTG TGC AGG, or TCRB2_1390: 5′-GGA ACA CAG ATT GGG AGC AGG TAC AGG AG. Thermocycler conditions for touchdown PCR were: 5 cycles at (95 °C for 30 s, 72 °C for 3 min); 5 cycles at (95 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min); 30 cycles at (95 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min); 72 °C for 7 min. TCR alpha and beta products were confirmed by Sanger sequencing using the BigDye Terminator v3.1 kit on an 3130xl Genetic Analyzer (Applied Biosystems, Inc.) and cloned into the SgrAI and NheI restriction sites of pSELECT-GFPzeo to generate pSELECT-5B2alpha (TRAV12-2*02; GenBank # KT207830) and pSELECT-5B2beta (TRBV7-2*01; GenBank # KT207831). The alpha and beta genes for TCR clone 868, which is specific for the A*02-restricted HIV-1 Gag SL9 epitope (SLYNTVATL; HIV_{HXB2} amino acid position 77–85) (Varela-Rohena et al., 2008), were synthesized (Integrated DNA Technologies) according to their reported sequences and cloned similarly into pSELECT-GFPzeo.

2.2.2. Transfection of effector cells

Jurkat T cells were pelleted and resuspended at a concentration of 50×10^6 cells/mL in Opti-MEM. A total of 10×10^6 cells (in 200 μL) were used for electroporation as follows. Cells were transferred to a 0.4 cm cuvette (Bio-Rad Laboratories, Inc.) containing 3 μg pSELECT-5B2alpha, 3 μg pSELECT-5B2beta, 5 μg pORF9-hCD8A and 10 μg pNFAT-luciferase and transfected using a Gene Pulser MXcell™ or a Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Inc.) with the following square-wave protocol: 250 or 500 V, 2000 μF, 3 ms, and 1 pulse. After a 10 min recovery at room temperature, cells were transferred into 10 mL R10 + _NoPR and incubated 16–20 h prior to use. Identical transfection conditions were used to produce effector cells expressing the 868 TCR (against HIV-1 Gag SL9). Transfection efficiency was assessed by flow cytometry to detect GFP (co-expressed with TCR) and surface expression of CD8 alpha (allophycocyanin (APC)-Cy7; clone SK1, BD Pharmingen).

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