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A Novel Real-Time CTL Assay to Measure Designer T Cell Function Against HIV Env⁺ Cells

Andrew G. MacLean¹, Edith Walker¹, Gautam Sahu², Gail Skowron², Preston Marx¹, Dorothee von Laer³, Richard P. Junghans², and Stephen E. Braun¹

¹ Tulane National Primate Research Center, Covington, LA

² Roger William Medical Center, Providence, RI

³ Innsbruck Medical University, Innsbruck, Austria

Abstract

Background—To increase the immunosurveillance in HIV infection, we used retroviral vectors expressing CD4-chimeric antigen receptors (CARs) to genetically modify autologous T cells and redirect CTL towards HIV. The CD4 extracellular domain targets Envelope and the intracellular signaling domains activate T cells. The maC46 fusion inhibitor binds HIV and blocks viral replication.

Methods—We stimulated rhesus PBMCs with αCD3αCD28 and co-transduced T cells with CD4-CAR and maC46 vectors. CD4-CAR transduced T cells were added to Env⁺ 293T cells at E:T of 1:1. Killing of target cells was measured as reduced impedance.

Results—We observed gene expression in 60-70% of rhesus CD3⁺CD8⁺ T cells with the individual vectors and in 35% of the cells with both vectors. CD4-CAR transduced populations specifically killed Env⁺ cells.

Conclusions—In these studies, we showed that designer T cells were redirected to kill Env⁺ cells. Control of viremia without HAART would revolutionize treatment for HIV patients.

Keywords

CTL; dTc; CD4-CAR; maC46; transduced T cells; rhesus macaque

INTRODUCTION

Infection with human immunodeficiency virus-1 (HIV-1) results in CD4⁺ T cell depletion and the subsequent loss of immune function results in AIDS. Because of cytotoxic T lymphocytes (CTL) dysfunction during chronic HIV-1 infection, virus continues to replicate in most people. Antiretroviral therapy (HAART), although effective in controlling plasma viremia, does not purge the latent or persistent reservoirs necessary to eliminate the infection and requires life-long drug therapy. However, a rare minority of HIV infected individuals

naturally suppresses viral replication, below the limits of detection, in the absence of HAART treatment.

One method proposed to increase the number of cytotoxic T lymphocytes has been to transduce T cells with retroviral vectors expressing chimeric antigen receptors. For our CD4-CAR vectors, the extracellular receptor is CD4 fused to intracellular signaling domains (i.e., T cell receptor zeta (TCR ζ) and CD28 signaling domains). Thus, the receptor will redirect these dTc and the intracellular signaling domains will activate the dTc to kill the target cells [8, 29].

As HIV-specific T cells have been found to have higher proviral copy number [6], the CD4-CAR transduced T cells may also be more susceptible to viral infection. To protect dTc from infection, we co-transduced dTc with the membrane-associated C46 (maC46), an extended form of T-20 (enfuvirtide) that is tethered to the dTc membrane. maC46 interferes with folding of the gp41 trimeric coil structures necessary for viral fusion. We had previously demonstrated very potent inhibition of HIV replication in cells transduced with maC46 [10].

To quantitate the cytolytic activity of dTc, several assays have been employed. The traditional assay is a ^{51}Cr release assay, where the radioactive molecule is used to label target cells and cytolytic activity releases the ^{51}Cr from the cytoplasm into the media. Other CTL assays use fluorescently labeled target cells and analyze the killing of these cells with a flow cytometer. In the current studies, we developed a novel assay that directly measures cytotoxicity in real-time by measuring alterations in electrical impedance. CD4-CAR or control-transduced populations were added to HEK 293T cells expressing Env (or control) at effector:target ratio of 1:1 and the change in the electrical impedance compared to baseline impedance was used to measure the Env-specific cytotoxicity. Our results demonstrate that the CD4-CAR transduced rhesus T cells specifically killed Env $^{+}$ target cells, thus verifying the utility of measuring activity with our novel assay.

MATERIALS AND METHODS

Humane Care Guidelines

All animals at the TNPRC are maintained in accordance with the “Guide for the Care and Use of Laboratory Animals” of the Institute of Laboratory Resources, National Research Council. The facility is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Cell lines, retroviral vectors, and antibodies

The packaging cell lines Phoenix (amphotropic) and PG13 (GaLV-pseudotyped) were cultured in DMEM plus 10% FBS, 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine. The retroviral vectors CD4-CAR [5, 15, 23, 25], CD4-CAR2 [21] and maC46:GFP (Figure 1) were shuttled packaged through Phoenix and into PG13 packaging cell lines [3, 4]. Heterogeneous populations of PG13 cells were sorted for expression of CD4 [1], or were used to generate high-titer Phoenix-maC46 clones [2]. These populations of cells were used to generate supernatant containing high quality viruses. Dilutions of viral stocks were used to transduce 293T cells and the percent of CD4 or GFP positive cells was

determined by flow cytometry. Stock titers ranged from 1.0 to 2.6×10^5 transduction units (TU)/ml.

Transduction of rhesus T cells

Blood was collected from rhesus macaques at TNPRC according to Institutional Animal Care and Use Committee guidelines and the NIH Guide for the Care and Use of Laboratory Animals. Rhesus PBMCs were isolated by discontinuous-density gradient centrifugation using LSM (Lymphocyte Separation Media, MP Biomedicals) and resuspended in RPMI-1640 supplemented with 10% FBS; 50 U/ml penicillin; 50 µg/ml streptomycin; 2 mM L-glutamine; 40 U/ml IL-2. Rhesus T cells (1×10^6 cells/ml or 0.5×10^6 cells/cm²) were stimulated using plate-bound anti-CD3 (clone 6G12; BD-Pharmingen) and anti-CD28 (clone CD28.2; BD-Pharmingen) for 3 days at 37°C, 5% CO₂.

Wells on a non-tissue culture treated 24-well plates were coated with Retronectin (RN), a recombinant fragment of fibronectin (Takara, Fisher Scientific), at 10 µg per cm² final concentration for 2-3 hrs at room temperature followed by one wash of PBS with 2% BSA for 30 mins. Plates were washed with PBS and were ready to use. RN-coated plates were preloaded with viral supernatants by centrifugation at 2000 rpm for 30 min at 4°C. T cells were seeded on RN-coated plates and exposed to viral particles that remained bound to the RN. Three days post transduction, cells were analyzed for transduction efficiency by flow cytometry.

Real time measurement of cytotoxic activity

The protocol for cytotoxic killing is described in Figure 2. In brief, to evaluate the CTL activity of the dTc, we developed a novel real-time cytotoxicity assay. This system measures changes in electrical impedance based on cell number, attachment, and spreading. Thus, as adherent target cells are killed, there is a measurable decrease in impedance. Due to the design of the xCELLigence plates, 80% of the well is covered by the electrodes. This allows one to measure very subtle changes, and does not rely on measuring complete monolayers [20, 22].

HEK 293T cells (2.5×10^4 cells), which had been transfected with the Env expression plasmid 99SEnv239gp160, were plated onto xCELLigence E-plates (Acea, San Diego, CA). Cells were allowed to settle at room temperature for 30 minutes and then placed in the xCELLigence RTCA DP device. Independent measures for each well were recorded each minute until the impedance leveled (approximately 24 hours). To measure the cytotoxicity, CD4-CAR and control-transduced dTc were added at effector:target ratio of 1:1 and changes in the electrical impedance were measured over the next 24 hours.

RESULTS

Optimized Co-transduction of Rhesus T cells

As a novel method to generate large numbers of CTL, we transduced rhesus T cells with retroviral vectors expressing CD4-chimeric antigen receptors (CARs) (Figure 1). The extracellular CD4 domain binds to HIV env and redirect “designer T cells” (dTc) towards

HIV env⁺ target cells while the intracellular signaling domains from the TCR ζ and the CD28 activate the T cells. To protect dTc from infection, we co-transduced dTc with M341, a vector expressing the membrane-associated C46 (maC46, an extended form of T-20 [enfuvirtide]) tethered to the dTc plasma membrane and fused to GFP. maC46 is derived from the C-peptide of gp41 (aa 628-673 of HIV-1_{HXB2} env) and interferes with folding of the gp41 trimeric coil structures necessary for membrane fusion [7, 14]. The CD4-CAR1 vector is the original 1st generation vector used in human trials [5, 15, 25]. The CD4-CAR2 is the next generation vector containing the T-cell receptor zeta (TCR ζ) and CD28 co-stimulation receptor. The CD4-CARs were GaLV-pseudotyped in PG13 packaging cells, while the LZRS-GFP and M341 vectors were pseudotyped in Phoenix amphotropic packaging cell line. High-titer packaging cell lines were isolated.

Based on our previous data [4], we showed that rhesus T cells were susceptible to high levels of transduction in a single round with gamma-viral vectors. To explore the potential for dTc to be co-transduced with the maC46 inhibitor, we evaluated the optimal kinetics of stimulation and viral exposure by stimulating rhesus PBMCs with immobilized anti-CD3 and anti-CD28 antibodies. The control vector LZRS-GFP was preloaded to RN-coated plates and exposed to stimulated cells on days three or four. As shown in Figure 2A, transduction and expression of GFP was significantly greater in both the CD4 and CD8 populations when vector was exposed to stimulated T cells on day 3 post-stimulation. This confirms what we had observed previously [4]. To evaluate co-transduction with CD4-CAR and maC46, we pre-loaded RN-coated plates with PG13-pseudotyped CD4-CAR and amphotropic enveloped M341 viral supernatant by centrifugation. Stimulated PBMCs were exposed to pre-adsorbed viral particles and analyzed by flow cytometry for expression of human CD4 and GFP. As shown in Figure 2B, the single vectors were able to transduce approximately 60-70% of the CD3⁺ T cells. When pre-absorbed together, the combination of vectors were able to transduce 56% and 51% of the CD4-CAR and the maC46:GFP, respectively. In combination, 35% of the rhesus CD3⁺ T cells were co-transduced and expressed both vectors, while 16% and 21% of the cells were transduced with only one vector. Thus, similar proportions of the maC46:GFP vector (68%) and of the CD4-CAR vector (62.5%) were co-transduced as were transduced singly with each vector. These data demonstrate strong transduction and co-transduction of rhesus T cells with the CD4-CAR and maC46 vectors.

HIV Env-specific killing with CD4-CAR transduced T cells

To measure the cytotoxicity of the CD4-CAR transduced dTc, we developed a novel real-time CTL assay measuring changes in the electrical impedance with the xCELLigence RTCA DP system to quantitate cytolytic killing of the target cells (Figure 3). To generate target cells, we transfected HEK 293T cells with the Env expression plasmid 99SEnv239gp160 [11]. The HEK 293T cells were plated onto the E-plates after 24 hours and baseline impedance was measured for 24 hours. On day 5 after transduction, dTc populations were counted and plated in the E-plates at 1:1 effector to target ratio. Changes in the electrical impedance were measured every minute for the first 2 hours and every 5 minutes thereafter (Figure 4). The dTc populations were transduced with the control vector (LZRS-GFP), or with vectors expressing the maC46:GFP fusion inhibitor (M341), CD4-

CAR1 (Hege) or CD4-CAR2. Three transduced populations of each CD4-CAR vector were measured independently and compared with control-transduced cells. As shown in Figure 4, the relative changes in electrical impedance of the CD4-CAR1 (purple trace) and CD4-CAR2 (blue) vectors were increased compared with the LZRS GFP (red trace, to which everything else was referenced) and M341 fusion inhibitor (green trace). With dTc expressing only the fusion inhibitor, CTL activity produced a small effect on the target cells and the duration of the effect was limited, perhaps indicating a recovery by the target cells. The percent CTL activity were 34%, 24% and 19% for the CAR, CAR2, and maC46-transduced populations. Killing was demonstrated to be specific to Env⁺ cells (data not shown). Overall, these data demonstrate the utility of the real-time CTL assay to measure CTL functional activity in CD4-CAR transduced dTc with the E-plates.

DISCUSSION

To evaluate *in vitro* killing activity of HIV infected cells by designer T cells (dTc), we developed a novel CTL assay using changes in electrical impedance to measure the killing of Envelope expressing target cells in real time. The dTc were rhesus CD3⁺ T cells transduced with retroviral vectors expressing CD4-chimeric antigen receptors (CAR). We established conditions for efficient co-transduction of dTc with the membrane-associated C46 (maC46) fusion inhibitor. In the real-time CTL assay, we observed Env-specific targeting by the transduced dTc. These data demonstrate the functional activity of the CD4-CAR transduced dTc and the utility of measuring CTL activity with this real-time assay.

In the xCELLigence RTCA DP system, adherent cells increase the resistance of the E-plates by spreading and forming junctions between cells [20, 22]. The more cells that are attached the larger the increase in electrical impedance. Additionally, the impedance is a function of the quality of the cell interaction with the electrode (ie, increased cell adhesion and spreading will increase impedance). In contrast, cellular toxicity or killing will decrease impedance. Thus, electrode impedance can be used to monitor cell viability, number, morphology, and adhesion degree in a number of cell-based assays. As T cells have minimal adherence to the E-plates, there is essentially no change in the electrical impedance when T cells are added to the plates [9]. These studies demonstrate the feasibility of using the E-plates to detect cytolytic activity in viral-infected subjects in real time.

Several lines of evidence suggest that cytotoxic T lymphocytes (CTL) inhibit viremia. 1) Potent CTL-mediated suppression has been reported in many natural elite controllers. 2) Temporal increases in cellular responses in acute infection correspond to declining viral load. 3) Inversely, depletion of CD8⁺ CTL leads to increased viral load. 4) Consistently, viral evolution is driven by CTL (and antibody) responses. A novel method to generate large numbers of CTL is to transduce T cells with retroviral vectors expressing chimeric antigen receptor (CARs) fused to intracellular T cell signaling domains [8, 29]. The CAR redirects “designer T cells” (dTc) towards a new target, while the signaling domains stimulate T cell activity. For our CD4-CAR vectors, the extracellular receptor is CD4 fused to intracellular signaling domains (i.e., T cell receptor zeta (TCR ζ) and CD28 signaling domains). The advantages of using CD4 for the extracellular binding domain are: 1) Envelope binding to CD4 is essential for HIV infection. Therefore, it will be difficult for the virus to develop

resistance. 2) HIV envelope binding to CD4 is strong ($K_d = 5$ nM, [13]). 3) CD4 binds broadly to all HIV clades and subtypes. 4) HIV envelope binding is independent of MHC class I restriction and resistant to Nef-mediated downregulation of MHC. CD4-CARs that maintain CTL functional activity *in vivo* have the potential to control viral replication as is observed in elite controllers.

To protect the dTc from infection, we explored co-transducing dTc with a retroviral vector expressing the peptide fusion inhibitor maC46. Therefore, we explored co-transducing T cells with both the CD4-CAR and maC46 vectors. We demonstrated that stimulated T cells are more susceptible to transduction with a single vector on day 3 than day 4, as described previously [4]. Additionally, we achieved between 60-70% transduction with the CD4-CAR and the maC46:GFP vectors singly and 35% co-transduction when dTc were co-transducing dTc with the maC46 fusion inhibitor. Given these levels of gene transfer, we would expect adoptive transfer of 100×10^6 transduced T cells could lead to approximately 1-5% gene marking in the peripheral blood lymphocyte compartment and potentially 1% of T cells that co-express the CD4-CAR and the maC46. These levels of CTL effectors cell would compare to any vaccine strategy.

Poly-functional CTL activity (ie, proliferation, cytokine production, and activation by target cells) would provide positive selection pressure for the CAR-transduced cells; infection would exert negative selection pressure on these cells. Co-transduction of the maC46 fusion inhibitor would protect T cells from HIV infection, as we previously observed. In those studies, we showed strong selection advantage of maC46-transduced cells, which expanded from [10] 1% of the population up to >95% of the population *in vitro* [10]. Additionally, Younan *et al* showed positive selection of maC46-expressing CD4⁺ T cells and maintenance of viral specific immunological responses in animals with maC46-transduced hematopoietic stem cells [30]. Thus, generating distinct dTc subpopulations with positive and negative selection pressures *in vivo* may contribute to our understanding of T cell dynamics after viral infection. The small subpopulation of CAR⁺/maC46⁻ transduced dTc in the context of 1% gene marking *in vivo* would not significantly alter T cell dynamics.

We have previously shown in maC46-transduced cells that HIV binds to the plasma membrane, but does not enter the cell [10]. CD4 binding triggers a conformational change in gp120 exposing chemokines binding sites [16, 26]. Chemokine coreceptor binding then triggers the membrane fusion machinery in the gp41 subunit to insert the fusion peptide into the target cell membrane. Association of C-heptad repeat (CHR) peptide with the N-heptad repeat (NHR) trimer forms the pre-hairpin fusion intermediate and, subsequently, a stable six-helix bundle core, which bringing the viral envelope and target cell membrane into close proximity for fusion [16, 26]. Expression of the maC46 on CD4⁺ T cells is thought to mimic the CHR binding to the NHR, preventing further conformation changes, and inhibit fusion of HIV to target cells. In the context of the CD4-CARs, binding of maC46 to the NHR domain in HIV Env could potentially increase the avidity of the dTc to HIV infected cells. Additional studies would be necessary to confirm the increased function of the co-transduced dTc.

Adaptation of this assay to other measures of viral replication and cytotoxicity has been shown [9, 12, 17-19, 24, 27, 28], including cytotoxic T lymphocytes [9, 17], complement-dependent antibody cytotoxicity assay [18, 19, 28], virus induced CPE [24], viral titration and antibody neutralization [24, 27]. These studies, along with our results, support the continued development of this technology for evaluating cellular cytotoxicity in other viral infections.

In conclusion, we demonstrated that the CD4-CAR vectors, when transduced and expressed in rhesus T cells, mediate cytolytic activity towards HIV Env⁺ cells in our *in vitro* assay. Generation of large numbers of HIV specific CTL, and adoptive transfer of these dTc back into their autologous host, should kill actively infected cells *in vivo*. If these cells maintain their proliferation potential and kill HIV Env⁺ cells, they could potentially target reactivated HIV reservoirs and ultimately control viral replication long-term without HAART.

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Figure 1. Schematic Diagram of the Retroviral Vectors

The gamma-retroviral vectors LZRS-GFP, M341, HEGE, and CD4-CAR2 express GFP, the maC46 fusion inhibitor, or CD4-CARs (respectively). Abbreviations include MuLV: murine leukemia virus; LTR: long terminal repeat; Ψ : Psi packaging element; MPSV: myeloproliferative sarcoma virus; MESV leader: mouse embryonic stem cell leader; wPRE: woodchuck hepatitis post-transcriptional regulatory element; maC46: HIV fusion inhibitory peptide derived from the C-peptide of gp41 (aa 628-673 of HIV-1_{HXB2} env); TCR ζ ; T-cell receptor zeta. Vectors are packaged in amphotropic or GaLV packaging cells.

Figure 2. Optimized Co-transduction of Rhesus T cells

A) Transduced rhesus CD4 (upper row) and CD8 (bottom row) T cell populations were gated with CD3 and CD8 prior to analysis of GFP expression. B) Transduced rhesus T cells were gated on CD3 and analyzed for expression human CAR-specific CD4 and maC46:GFP.

Figure 3. Novel cytolytic T lymphocyte Assay

Schematic diagram of the novel CTL assay targeting Env⁺ cells. Env transfected HEK 293T cells are plated onto the E-plates after 24 hrs and baseline impedance recorded for 24 hours. CD4-CAR and control-transduced populations are added at effector:target ratio of 1:1 and changes in the electrical impedance are independently measured for each well every minute.

Figure 4. CD4-CAR specific killing of Env⁺ cells

The graph shows the relative change in electrical impedance of transfected 293T cells over time when mixed with maC46- (green), HEGE- (purple), and CD4-CAR2 (blue) transduced cell populations. The impedance of the GFP control (red) mixture was used as the reference.