Title
Ectopic expression of anti-HIV-1 shRNAs protects CD8(+) T cells modified with CD4ζ CAR from HIV-1 infection and alleviates impairment of cell proliferation.

Permalink
https://escholarship.org/uc/item/3t50c36j

Journal
Biochemical and biophysical research communications, 463(3)

ISSN
1090-2104

Authors
Kamata, Masakazu
Kim, Patrick Y
Ng, Hwee L
et al.

Publication Date
2015-07-31

Peer reviewed
Ectopic expression of anti-HIV-1 shRNAs protects CD8⁺ T cells modified with CD4ζ CAR from HIV-1 infection and alleviates impairment of cell proliferation

Masakazu Kamata a, *, Patrick Y. Kim b, Hwee L. Ng c, Gene-Errol E. Ringpis b, 1, Emiko Kranz b, Joshua Chan b, Sean O’Connor b, Otto O. Yang b, c, d, e, Irvin S.Y. Chen a, b, d

* Division of Hematology-Oncology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
b Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
c Division of Infectious Diseases, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
d UCLA AIDS Institute, Los Angeles, CA, USA
e AIDS Healthcare Foundation, Los Angeles, CA, USA

A R T I C L E   I N F O

Article history:
Received 5 May 2015
Available online 19 May 2015

Keywords:
HIV-1
CD4ζ
Chimeric antigen receptor (CAR) shRNA
Immunotherapy

A B S T R A C T

Chimeric antigen receptors (CARs) are artificially engineered receptors that confer a desired specificity to immune effector T cells. As an HIV-1-specific CAR, CD4ζ CAR has been extensively tested in vitro as well as in clinical trials. T cells modified with this CAR mediated highly potent anti-HIV-1 activities in vitro and were well-tolerated in vivo, but exerted limited effects on viral load and reservoir size due to poor survival and/or functionality of the transduced cells in patients. We hypothesize that ectopic expression of CD4ζ on CD8⁺ T cells renders them susceptible to HIV-1 infection, resulting in poor survival of those cells. To test this possibility, highly purified CD8⁺ T cells were genetically modified with a CD4ζ-encoding lentiviral vector and infected with HIV-1. CD8⁺ T cells were vulnerable to HIV-1 infection upon expression of CD4ζ, as evidenced by elevated levels of p24 Gag in cells and culture supernatants. Concurrently, the number of CD4ζ-modified CD8⁺ T cells was reduced relative to control cells upon HIV-1 infection. To protect these cells from HIV-1 infection, we co-expressed two anti-HIV-1 shRNAs previously developed by our group together with CD4ζ. This combination vector was able to suppress HIV-1 infection without impairing HIV-1-dependent effector activities of CD4ζ in addition, the number of CD4ζ-modified CD8⁺ T cells maintained similar levels to that of the control even under HIV-1 infection. These results suggest that protecting CD4ζ-modified CD8⁺ T cells from HIV-1 infection is required for prolonged HIV-1-specific immune surveillance.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Autologous T cell-based immunotherapies aim to confer directed and enhanced cytotoxic T lymphocyte (CTL) responses via supplementation of CD8⁺ T cells modified with a desired antigen-specific T cell receptor (TCR) [1–4]. However, TCR-based approaches require a particular human leukocyte antigen (HLA) molecule for proper antigen presentation to the T cells. Chimeric antigen receptors (CARs) are artificial molecules that are able to recognize a desired target molecule in an HLA-independent manner and trigger helper or cytotoxic activity when they are expressed at the surface of CD4⁺ or CD8⁺ T cells, respectively [5–8]. CD4ζ CAR has been developed as a CAR against HIV-1 infected cells and extensively tested for its anti-HIV-1 efficacies in vitro and in clinical trials [9–19]. The CD4ζ contains extracellular domains from the HIV-1 major receptor CD4 and an internal signaling domain derived from a CD3ζ-chain (CD247). When this CAR encounters HIV-1 envelope protein on the infected cell, its target ligand, it signals the cell in a manner similar to a TCR, but in an HLA-independent manner, thus this approach could be used in any HIV-1-infected person. In three clinical trials, this CAR was expressed using a γ-retroviral vector in ex vivo expanded peripheral
T cells and was evaluated [12–14,18]. Treatment was safe, CD4\(z\)-modified T cells were well-tolerated in blood for over a decade with a minimum detection level by flow cytometry, and rectal tissue HIV-1 RNA levels decreased for at least 14 days after infusion of modified T-cells. However, no change in plasma viral load was observed.

We hypothesize that CD4\(z\)-modified T cells become susceptible to HIV-1 infection, resulting in a loss of the gene-modified T cells in patients. Indeed, CD8\(^+\) T cells expressing CD4 molecules are known to be infectable by HIV-1 [20–22]. Here we test whether ectopic expression of CD4\(z\) renders CD8\(^+\) T cells susceptible to HIV-1 infection, and if co-expression of anti-HIV-1 genes together with CD4\(z\) is able to protect them from infection and subsequent cytopathic effects. For anti-HIV-1 genes, we chose two shRNAs, sh1005 and sh516, both of which were tested in vitro as well as in vivo using the humanized bone marrow/liver/thymus (BLT) mouse model [23]. sh1005 was found by extensive screening from a shRNA library for CCR5 [23–27] and was able to suppress the expression of CCR5 potently in vitro and in vivo, resulting in protection of the cells from R5-tropic HIV-1 infection, but not X4-tropic HIV-1 infection. sh516 was originally reported by McIntyre et al. via screening from 8846 potential HIV-1 specific siRNAs [28]. The target sequence resides within the \(R\) region of the HIV-1 long terminal repeat (LTR), thus all HIV-1 transcripts contain two sh516 target sequences.

Here we express the two anti-HIV-1 shRNAs together with CD4\(z\) in highly purified primary CD8\(^+\) T cells and test their viability effects on the cells as well as anti-HIV-1 effector functions. As expected, CD8\(^+\) T cells unmodified or modified with control vector were completely resistant to HIV-1 infection, whereas cells expressing CD4\(z\) were susceptible to the infection and showed cytopathic effects. By co-expression of two anti-HIV-1 shRNAs, the CD8\(^+\) T cells modified with CD4\(z\) became resistant to both R5- and X4-tropic HIV-1 infection and proliferated as well as control cells.

**Fig. 1.** CD4\(z\) CAR-transduced CD8\(^+\) T cells are susceptible to HIV-1 infection. A) CD8\(^+\) T cells were transduced with EGFP-P2A-CD4\(z\) (CD4\(z\)) or control EGFP (EGFP) vector. The cells transduced with the vector were sorted based on their EGFP expression and infected with one of three different HIV-1 strains, HIV-1NL4-3, HIV-1NFNSX SL9 or HIV-1JR-CSF at MOI = 0.2, 1.0 or 1.0, respectively. Cells were plated at 1 x 10^5 cells/500 \(\mu\)l in a 48-well plate, and HIV-1 p24\(^\text{Gag}\) production in the culture supernatants was determined by ELISA at day 7 post infection. CD4\(^+\) T cells transduced with control EGFP were used as a positive control for HIV-1 infection. B) Intracellular HIV-1 p24\(^\text{Gag}\) staining by KC57 antibody. CD8\(^+\) T cells transduced with EGFP-P2A-CD4\(z\) (CD4\(z\)) or control EGFP (EGFP) vector were infected with HIV-1NL4-3, HIV-1NFNSX SL9 or HIV-1JR-CSF. Intracellular HIV-1 p24\(^\text{Gag}\) was analyzed by flow cytometry after staining PE-conjugated KC57 antibody 7 days post infection.
2. Materials and methods

2.1. Cells and viruses

Peripheral blood mononuclear cells (PBMCs) from healthy human donors were obtained from the CFAR Virology core at UCLA without personal identifying information. CD4+ or CD8+ T cells from fresh human PBMCs were negatively isolated with EasySep Human CD4+ T cell or CD8+ T cell enrichment kit (StemCell Technologies, Inc., Vancouver, Canada) and maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 1% GlutaMAX supplement and Antibiotic-Antimycotic (Life Technologies, Grand Island, NY), 20% FCS (ThermoFisher Scientific, Waltham, MA), and 0.1 mM 2-mercaptoethanol (Sigma–Aldrich St.Louis, MO) (T-cell medium). Prior to lentiviral vector transduction or HIV-1 infection, isolated CD4+ or CD8+ T cells were incubated with 0.1 μg/ml anti-CD3 (Hit3a; BioLegend, San Diego, CA) and 2 μg/ml anti-CD28 antibodies (CD28.2; BioLegend) for 48–72 h, then maintained in T-cell medium supplemented with 5 ng/ml IL-7 and IL-15 (R&D systems, Minneapolis, MN). CD4+-transduced CD8+ T cells were enriched with CD4+ microbeads (Miltenyi Biotech Inc., Auburn, CA). All cells were incubated at 37 °C in 5% CO2. Chromium release assays were performed as previously described using T2 cells (ATCC CRL-1992) infected with HIV-1NL4-3 as target cells [10].

Lentiviral vector and HIV-1 stocks were generated and infected as previously described [23]. HIV-1 p24Gag ELISA assays were performed by the CFAR Virology core at UCLA. Lentiviral vector information used in this research can be provided upon request.

2.2. Antibodies and flow cytometry

The following antibodies were used in flow cytometry: CD3, CD4, CD8, IFN-γ, TNF-α (BioLegend), CCR5 (2D7; BD Biosciences, San Jose, CA) and anti-HIV-1 core antigen clone KC57 (Beckman Coulter, Brea, CA). Flow cytometry was performed using LSRII flow cytometer using FACSdiva software (BD biosciences). Data were analyzed using Flowjo software (Flow Jo, LLC, Ashland, OR). Absolute cell counts were determined using MACSQuant analyzer (Miltenyi Biotech Inc.). Cell sorting was performed by the CFAR Flow Cytometry Core Facility at UCLA.

3. Results and discussion

3.1. Expression of CD4ζ, CAR confers HIV-1-dependent effector functions to CD8+ T cells

CD4ζ CAR is a fusion molecule of human CD4 extracellular domain with the TCR-CD3 complex ζ-chain. This employs CD4 as a recognition receptor for the HIV-1 gp120 envelope on the surface of infected cells; its subsequent engagement then triggers T cell recognition of infected cells through ζ-chain signaling. This CAR has been characterized extensively and shown to exert potent antiviral efficacy in vitro, but no apparent effect on plasma viral loads.

We first constructed a lentiviral vector expressing CD4ζ as a P2A peptide-EGFP fusion to monitor gene-modified cells (Supplementary Fig. 1), and assessed its effector functions by HIV-1-dependent cytolytic production and cytokilling activity (Supplementary Fig. 2). The vector was transduced into highly enriched primary CD8+ T cells using magnetic beads (>99.0%), and expression was confirmed by flow cytometry (Supplementary Fig. 2A). To test HIV-1-dependent cytolytic production, the cells were incubated with HIV-1NL4-3-infected T2 cells for 16 h at a 1:1 E:T ratio and subjected to intracellular staining of two-inflammatory cytokines, IFN-γ and TNF-α (Supplementary Fig. 2B). Cytokine production in response to HIV-1 infected T2 cells was observed with CD8+ T cells modified with CD4ζ, but not with EGFP-modified or unmodified cells. HIV-1-dependent cytokilling activity mediated by CD4ζ was also confirmed with cells derived from two independent donors (Supplementary Fig. 2C).

3.2. Ectopic expression of CD4ζ on CD8+ T cells renders them susceptible to HIV-1 infection

We assessed whether the expression of CD4ζ confers HIV-1 susceptibility to CD8+ T cells. CD8+ T cells were transduced with the vector encoding CD4ζ or EGFP as a control (Supplementary Fig. 1, CD4ζ or EGFP, respectively), and infected with X4-tropic (HIV-1NL4-3) or R5-tropic (HIV-1 NLNX SL9 and HIV-1 R-CRF) HIV-1 strains. CD4ζ+ T cells expressing EGFP served as a positive control of HIV-1 infection (Fig. 1A, EGFP/CD4T). Extracellular and intracellular p24Gag production were analyzed by ELISA and KC57 staining, respectively. ELISA for p24Gag in culture supernatants indicated that ectopic expression of CD4ζ mediated a productive infection of CD8+ T cells (Fig. 1A, CD4ζ/CD8T). Furthermore, intracellular p24Gag staining showed that the cells modified by CD4ζ, but not EGFP control or unmodified cells, were positive for KC57 staining upon infection with all three HIV-1 strains (0.23–0.27%, Fig. 1B, CD4ζ) at levels which were around 10 fold lower than those on CD4+ T cells (2–5%, data not shown).

3.3. HIV-1 infection of CD8+ T cells modified by CD4ζ, CAR can be inhibited by co-expressing anti-HIV-1 shRNAs

We have previously developed two unique anti-HIV shRNA reagents which can protect CD4+ T cells from both X4- and R5-tropic HIV-1 infection [23,24,26,29,30]. The first shRNA, sh1005, inhibits R5-tropic HIV-1 at the point of virus entry downregulation of the CCR5 co-receptor. The second shRNA, sh516, is directed to HIV-1 itself, which unlike sh1005, will protect from both X4- and R5-tropic HIV-1 infection. The target sequence is highly conserved in 96.1% (1262/1313) of clade B HIV-1 sequences found in the Los Alamos National Lab HIV Sequence Database. It resides within the R-region of the HIV-1 LTR and since both the 5’ and 3’ LTRs of HIV possess this region, all HIV-1 transcripts, including all spliced transcripts [31], contain two sh516 targets. A vector co-expressing those two shRNAs within a single vector, designated as a Dual sh1005/sh516, was able to protect cells from both R5- and X4-tropic HIV-1 infection in vitro as well as in humanized BLT mice.

We introduced this dual shRNA expression cassette architecture into the CD4ζ CAR lentiviral vector (Supplementary Fig. 1, Triple CD4ζ). The expression of these shRNAs had no effect on CD4ζ expression (Fig. 2A). This combination vector, unlike vectors encoding only EGFP or CD4ζ, suppressed CCR5 expression on the surface of CD8+ T cells as previously described (Fig. 2B) [23,24,26,29,30]. Importantly, those shRNAs did not affect HIV-1-dependent cytolytic production (Fig. 2C) nor cytokilling activity of CD4ζ (Fig. 2D).

Using this vector, we tested whether those shRNA expressions have an impact on HIV-1 infection via CD4ζ CAR (Fig. 3). CD8+ T cells were transduced with vectors encoding CD4ζ only, CD4ζ plus the two anti-HIV-1 shRNAs (Triple CD4ζ), or EGFP as a control. CD4ζ-modified CD8+ T cells were enriched using anti-CD4 magnet beads (CD4ζ only: 99.2%, Triple CD4ζ: 98.0%) and infected with X4-tropic (HIV-1NL4-3) or R5-tropic HIV-1 (HIV-1 NLNX SL9). The amounts of p24Gag in culture supernatants were determined by ELISA on days 4, 8, and 12 after HIV-1 challenge. As seen in Fig. 1, CD8+ T cells became susceptible to infection by X4- and R5-tropic...
HIV-1 strains when they were modified by CD4ζ only (Fig. 3A and B, CD4ζ), but not EGFP control (Fig. 3A and B, EGFP). HIV-1 susceptibility induced by CD4ζ was suppressed to levels of the control by co-expressing two anti-HIV shRNAs (Fig. 3A and B, Triple CD4ζ).

Protection from HIV-1 infection appeared more complete for cells infected by HIV-1NFNSX SL9 compared to those infected by HIV-1NL4-3. This is likely due to the fact that HIV-1NFNSX SL9 infection can be inhibited by both sh1005 and sh516 at the levels of viral entry as well as viral mRNA expression, respectively, whereas infection of HIV-1NL4-3 is only inhibited by sh516 at the level of the latter.

3.4. HIV-1-induced cytopathic effect on CD4ζ-modified CD8+ T cell growth is attenuated by co-expressing anti-HIV-1 shRNAs

It has been well-studied that HIV-1 infection induces cytopathic effects by various mechanisms [32–38]. We therefore tested whether HIV-1 infection also has a negative impact on CD4ζ-modified CD8+ T cells by monitoring the cell growth rate post-HIV-1 infection. The modified CD8+ T cells enriched with anti-CD4 microbeads were infected with HIV-1NL4-3 or HIV-1NFNSX SL9, and cytopathic effects were monitored by counting absolute cell numbers (Fig. 4).

As shown in Figs. 1 and 3, ectopic expression of CD4ζ on CD8+ T cells renders them susceptible to HIV-1 infection. Concurrently, the numbers of CD4ζ-modified CD8+ T cells decreased significantly by 47% and 88% at day 8 compared to those of EGFP control upon infection of HIV-1NL4-3 and HIV-1NFNSX SL9, respectively. In contrast, cells modified by Triple CD4ζ were protected from HIV-1 mediated cytopathic effects; cell numbers infected with HIV-1NL4-3 were significantly recovered up to 80% of those of control, whereas the numbers infected with HIV-1NFNSX SL9 were not significantly different from those of control. These results indicate that protection of CD4ζ-modified CD8+ T cells from HIV-1 infection abrogates cytopathic effects of HIV-1.
Here we developed a novel combination vector encoding HIV-1-specific CD4⁺ CAR and two anti-HIV-1 shRNAs, which we previously screened and extensively tested in vitro and in a humanized mouse model. Ectopic expression of only CD4⁺ CAR rendered CD8⁺ T cells susceptible to HIV-1 infection (Figs. 1 and 3), resulting in a greater (p value less than or equal to 0.05). NS: not significant (p value greater than 0.1). values: HIV-1NL4-3/EGFP vs HIV-1NL4-3/CD4⁻ ¼ 0.005, HIV-1NL4-3/EGFP vs HIV-1NL4-3/Triple CD4⁺ ¼ 0.006, HIV-1NL4-3/E0 vs HIV-1NL4-3/Triple CD4⁺ ¼ 0.006, HIV-1NL4-3/E0 vs HIV-1NL4-3/Triple CD4⁺ ¼ 2, Mock/EGFP vs Mock/Triple CD4⁺ ¼ 0.006.

Fig. 4. Protecting CD8⁺ T cells from HIV-1 infection minimizes impairment of cell proliferation mediated by HIV-1 infection via CD4⁺ CAR. CD8⁺ T cells modified with EGFP-P2A-CD4⁺ (CD4⁺), Triple combination vector (Triple CD4⁺) or control EGFP vector (EGFP) described in Fig. 3 were infected with HIV-1NL4-3 or HIV-1Nemo at an MOI of 0.2 or 1.0, respectively. The cells were plated at 0.2 × 10⁵ cells/200 μl in a 96 well plate, and total cell numbers in the wells were determined by MACSQuant analyzer. Numbers statistically significant (p < 0.05).

Conflict of interest
None declared.

Acknowledgments
This work was supported by California HIV/AIDS Research Grants Program Office of the University of California grant ID3-LA-563 (to M.K.), U.S. National Institutes of Health (NIH) grant AI10297 (to I.S.Y.C). I.S.Y.C. is a founder of Calimmune, Inc., a company developing genetic therapies for HIV disease. Cell sorting was performed in the UCLA CFAR Flow Cytometry Core Facility (NIH grants P30 CA016042/SP30 AI028697). We would also like to thank the CFAR Virology Core lab for supplying PBMCs and p24⁺ ELISA.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.026.


