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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

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ABSTRACT

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.

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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 cytokilling 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

Abbreviations: CAR, chimeric antigen receptor (CAR); shRNA, short hairpin RNA; HIV-1, human immunodeficiency virus type 1.

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T cells and was evaluated [12–14,18]. Treatment was safe, CD4ζ-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 CD4ζ-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 CD4ζ renders $CD8^+$ T cells susceptible to HIV-1 infection, and if co-expression of anti-HIV-1 genes together with CD4ζ 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 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 CD4ζ 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 CD4ζ were susceptible to the infection and showed cytopathic effects. By co-expression of two anti-HIV-1 shRNAs, the $CD8^+$ T cells modified with CD4ζ became resistant to both R5-and X4-tropic HIV-1 infection and proliferated as well as control cells.

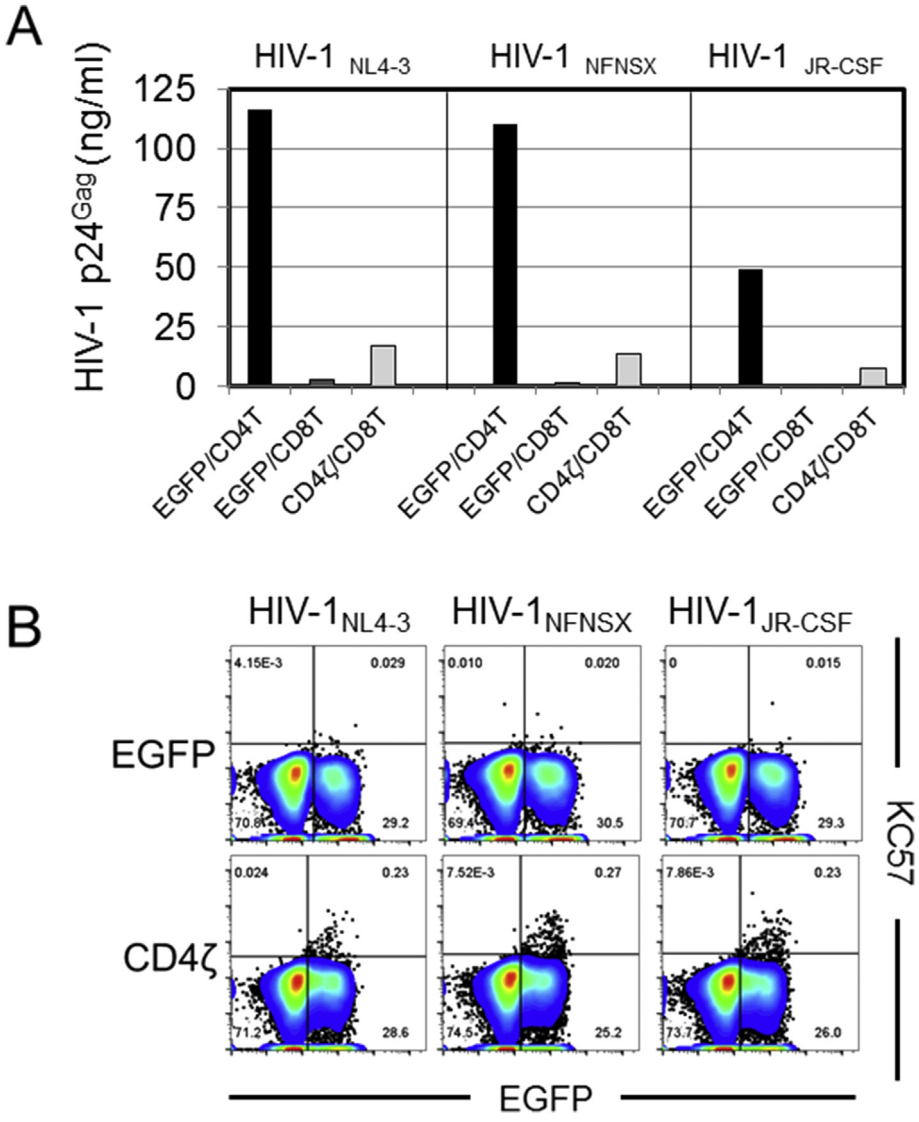

Fig. 1. CD4ζ CAR-transduced $CD8^+$ T cells are susceptible to HIV-1 infection. A) $CD8^+$ T cells were transduced with EGFP-P2A-CD4ζ (CD4ζ) or control EGFP (EGFP) vector. The cells transduced with the vector were sorted based on their EGFP expression and infected with one of three different HIV-1 strains, HIV-1_{NL4-3}, HIV-$1_{NFNSX\ SL9}$ or HIV-1_{JR-CSF} at MOI = 0.2, 1.0 or 1.0, respectively. Cells were plated at 1×10^5 cells/500 μl in a 48-well plate, and HIV-1 $p24^{Gag}$ 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 $p24^{Gag}$ staining by KC57 antibody. $CD8^+$ T cells transduced with EGFP-P2A-CD4ζ (CD4ζ) or control EGFP (EGFP) vector were infected with HIV-1_{NL4-3}, HIV-$1_{NFNSX\ SL9}$ or HIV-1_{JR-CSF}. Intracellular HIV-1 $p24^{Gag}$ 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). CD4ζ-transduced $CD8^+$ T cells were enriched with $CD4^+$ microbeads (Miltenyi Biotech Inc., Auburn, CA). All cells were incubated at 37 °C in 5% CO_2. Chromium release assays were performed as previously described using T2 cells (ATCC CRL-1992) infected with HIV-1_{NL4-3} as target cells [10].

Lentiviral vector and HIV-1 stocks were generated and infected as previously described [23]. HIV-1 $p24^{Gag}$ 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 LSRFortessa 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 Biotec 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 cytokine 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 cytokine production, the cells were incubated with HIV-1_{NL4-3}-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-1_{NL4-3}) or R5-tropic (HIV-$1_{NFNSX\ SL9}$ and HIV-1_{JR-CSF}) HIV-1 strains. $CD4^+$ T cells expressing EGFP served as a positive control of HIV-1 infection (Fig. 1A, EGFP/CD4T). Extracellular and intracellular $p24^{Gag}$ production were analyzed by ELISA and KC57 staining, respectively. ELISA for $p24^{Gag}$ in culture supernatants indicated that ectopic expression of CD4ζ mediated a productive infection of $CD8^+$ T cells (Fig. 1A, CD4ζ/CD8T). Furthermore, intracellular $p24^{Gag}$ 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 through 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 cytokine 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 magnetic beads (CD4ζ only: 99.2%, Triple CD4ζ: 98.0%) and infected with X4-tropic (HIV-1_{NL4-3}) or R5-tropic HIV-1 (HIV-$1_{NFNSX\ SL9}$). The amounts of $p24^{Gag}$ 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

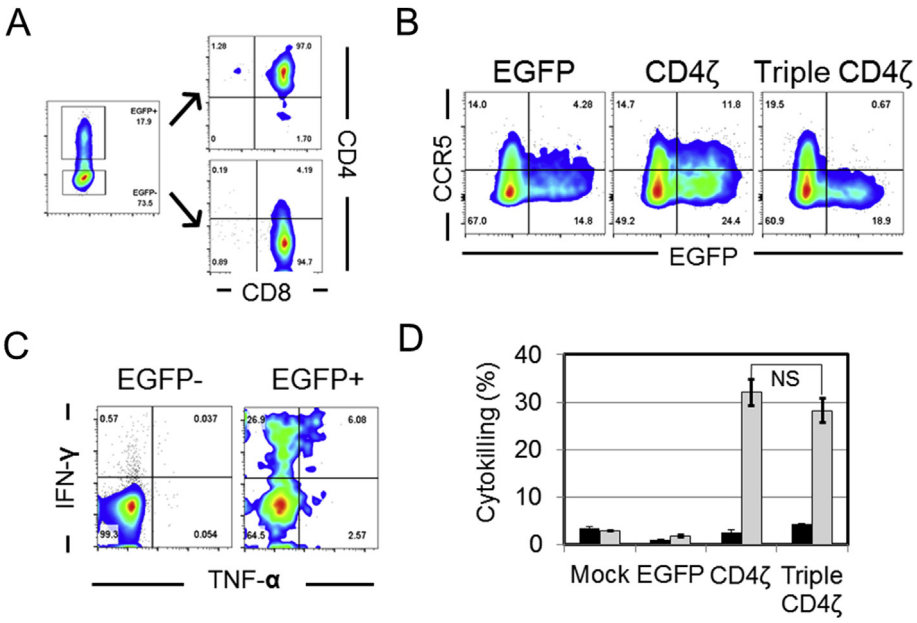

Fig. 2. Triple combination vector exerts identical anti-HIV-1 effect with prototype CD4ζ CAR vector. A & B) Expression profile of Triple combination vector in $CD8^+$ T cells. PBMC-derived $CD8^+$ T cells were stimulated by anti-CD3 and anti-CD28 antibodies for 3 days, and transduced with EGFP-P2A-CD4ζ (CD4ζ), Triple combination vector (Triple CD4ζ) or control EGFP vector (EGFP). Cells were then cultured for 4 days and stained with anti-CD4 antibody (A) and anti-CCR5 antibody (B). C) HIV-1-dependent cytokine production by CD4ζ CAR. Cells transduced with Triple combination vector were incubated with T2 cells infected with or without HIV-1_{NL4-3} for 16 h at a 10:1 E:T ratio. Intracellular cytokines (IFN-γ and TNF-α) were analyzed by flow cytometry. D) Triple CD4ζ induces HIV-1-dependent T2 cell killing. Cells transduced with EGFP-P2A-CD4ζ (CD4ζ), Triple combination vector (Triple CD4ζ) or control EGFP (EGFP) vector were flow cytometry-sorted based on their EGFP expression, and incubated with T2 cells infected with or without HIV-1_{NL4-3} labeled with $Na_2(^{51}CrO_4)$ for 3.5 h at a 10:1 E:T ratio. Cytolytic activity was determined by analysis of ^{51}Cr release. Numbers are mean ± SD from 3 independent reactions. Total incorporated ^{51}Cr was set as 100%. Two-tailed paired t test was used to calculate statistical significance. NS: not significant (p value greater than 0.1).

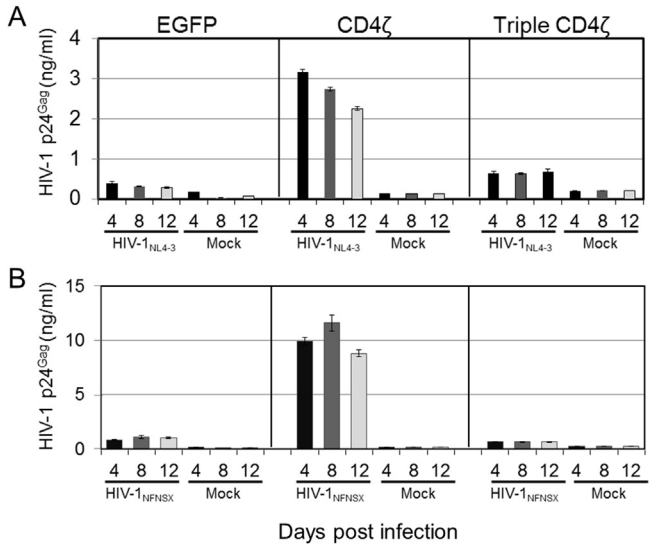

Fig. 3. Triple combination vector protects $CD8^+$ T cells from HIV-1 infection. PBMC-derived $CD8^+$ T cells were stimulated by anti-CD3 and anti-CD28 antibodies for 3 days and transduced with EGFP-P2A-CD4ζ (CD4ζ), Triple combination vector (Triple CD4ζ) or control EGFP vector (EGFP). On day 4 post vector transduction, CD4ζ-positive populations were purified using anti-CD4 microbeads, and infected with HIV-1_{NL4-3} or HIV-$1_{NFNSX\ SL9}$ at MOI = 0.2 or 1.0, respectively. Cells were plated at 0.2 × 10^5 cells/200 μl in a 96-well plate, and the amount of HIV-1 $p24^{Gag}$ production in culture supernatant was measured at days 4, 8, and 12 post infection (A. HIV-1_{NL4-3}, B. HIV-$1_{NFNSX\ SL9}$). (A&B) HIV-1 $p24^{Gag}$ production in the culture supernatants was determined by ELISA. Numbers are mean ± SD from 3 independent wells.

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-$1_{NFNSX\ SL9}$ compared to those infected by HIV-1_{NL4-3}. This is likely due to the fact that HIV-$1_{NFNSX\ SL9}$ 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-1_{NL4-3} 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-1_{NL4-3} or HIV-$1_{NFNSX\ SL9}$, 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-1_{NL4-3} and HIV-$1_{NFNSX\ SL9}$, respectively. In contrast, cells modified by Triple CD4ζ were protected from HIV-1 mediated cytopathic effects; cell numbers infected with HIV-1_{NL4-3} were significantly recovered up to 80% of those of control, whereas the numbers infected with HIV-$1_{NFNSX\ SL9}$ 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.

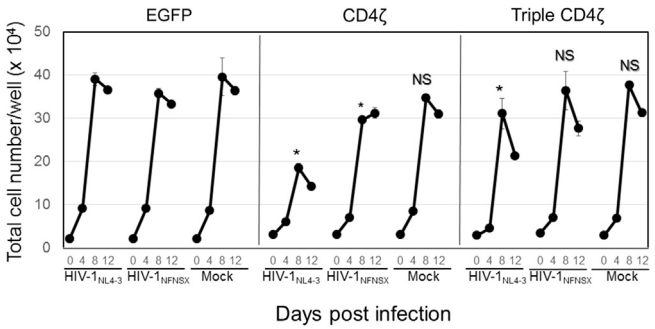

Fig. 4. Protecting CD8$^+$ T cells from HIV-1 infection minimizes impairment of cell proliferation mediated by HIV-1 infection via CD4ζ CAR. CD8$^+$ T cells modified with EGFP-P2A-CD4ζ (CD4ζ), Triple combination vector (Triple CD4ζ) or control EGFP vector (EGFP) described in Fig. 3 were infected with HIV-1_{NL4-3} or HIV-$1_{NFNSX\ SL9}$ at MOI = 0.2 or 1.0, respectively. The cells were plated at 0.2 × 10^5 cells/200 μl in a 96 well plate, and total cell numbers in the wells were determined by MACSQuant analyzer. Numbers are mean ± SD from 3 independent wells. A two-tailed paired t test was used to assess a significant difference in CD4ζ or Triple CD4ζ group versus EGFP control group. *: statistically significant (p value less than or equal to 0.05). NS: not significant (p value greater than 0.1). p values: HIV-1_{NL4-3}/EGFP vs HIV-1_{NL4-3}/CD4ζ = 0.005, HIV-1_{NL4-3}/EGFP vs HIV-1_{NL4-3}/Triple CD4ζ = 0.0026, HIV-1_{NFNSX}/EGFP vs HIV-1_{NFNSX}/CD4ζ = 0.006, HIV-1_{NFNSX}/EGFP vs HIV-1_{NFNSX}/Triple CD4ζ = 0.798, Mock/EGFP vs Mock/CD4ζ = 0.233, Mock/EGFP vs Mock/Triple CD4ζ = 0.606.

Here we developed a novel combination vector encoding HIV-1-specific CD4ζ 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 CD4ζ rendered CD8$^+$ T cells susceptible to HIV-1 infection (Figs. 1 and 3), resulting in a decreased cell number of CD4ζ-modified CD8$^+$ T cells (Fig. 4). Those negative impacts on CD8$^+$ T cells by ectopic expression of CD4ζ were mostly abrogated by protecting the cells from HIV-1 infection with anti-HIV-1 shRNAs. Importantly, those shRNAs did not impair effector function of CD4ζ-modified CD8$^+$ T cells nor their growth, suggesting that this combination vector is able to overcome the one drawback of CD4ζ.

CD4ζ-modified CD8$^+$ T cells can efficiently eliminate HIV-1-infected cells *in vitro* and can maintain HIV-1-dependent effector function safely for decades in patients. Some effector activities were observed in rectal tissue, but there was no impact on HIV-1 viral load or improving clinical status [12]. CD4ζ expression on CD8$^+$ T cells, which should be the same as that on CD4$^+$ T cells, can only be effective if HIV-1 susceptibility is addressed. Our studies demonstrate the potential of CD4ζ CAR-based therapies when HIV-1 protection is conferred.

Conflict of interest

None declared.

Acknowledgments

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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.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.026.

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