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Expression of chimeric receptor CD4 ζ by natural killer cells derived from human pluripotent stem cells improves in vitro activity but does not enhance suppression of HIV infection in vivo

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Abstract

Cell-based immunotherapy has been gaining interest as an improved means to treat HIV/AIDS. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) could become a potential resource. Our previous studies have shown hESC and iPSC-derived natural killer (NK) cells can inhibit HIV-infected targets in vitro. Here, we advance those studies by expressing a HIV chimeric receptor combining the extracellular portion of CD4 to the CD3 ζ intracellular signaling chain. We hypothesized that expression of this CD4 ζ receptor would more efficiently direct hESC- and iPSC-derived NK cells to target HIV-infected cells. In vitro studies showed the CD4 ζ expressing hESC- and iPSC-NK cells inhibited HIV replication in CD4⁺ T cells more efficiently than their unmodified counterparts. We then evaluated CD4 ζ -hESC- and iPSC-NK cells in vivo anti-HIV activity using a humanized mouse model. We demonstrated significant suppression of HIV replication in mice treated with both CD4 ζ -modified and unmodified hESC-/iPSC-NK cells compared to control mice. However, we did not observe significantly increased efficacy of CD4 ζ expression in suppression of HIV infection. These studies indicate that hESC/iPSC-based immunotherapy can be utilized as a unique resource to target HIV/AIDS.

Keywords

human embryonic stem cells; induced pluripotent stem cells; Natural killer cells; HIV-1 infection inhibition; in vitro; in vivo

INTRODUCTION

Highly active antiretroviral therapy (HAART) has significantly decreased the morbidity and mortality of HIV/AIDS, but latent virus still persists in cellular reservoirs. Restoration of cellular immunity in treated patients proceeds slowly and may never return to pre-infection status [1, 2], Cell-based therapy for HIV has gained more attention for its potential of long-term virus control or cure (reviewed in [3, 4]). Initially, studies were designed to inhibit HIV transcription or translation, which is less efficient in controlling the late steps of the viral life cycle [5]. More studies were then focused on early step inhibition before HIV integration into host genome. For instance, several groups have demonstrated the resistance against R5-tropic HIVs in vitro using shRNAs to knock down CCR5 in hematopoietic stem cells (HSC)

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[6-8]. Holt and colleagues [9] used zinc finger nuclease (ZFN) to disrupt the CCR5 gene in human cord blood and fetal liver CD34⁺ cells, which protected reconstituted NSG mice from R5 HIV infection. The use of a CCR5^{-/-} donor for a hematopoietic stem cell transplant of an HIV infected patient with acute myelogenous leukemia has provided a novel impetus for other potential cell-based curative approaches for patients with HIV/AIDS [10, 11].

Alternatively, there are also efforts to help redirect specific immune cell subsets to target and kill HIV. Studies have engineered immune cells with “chimeric antigen receptors” (CAR) to provide enhanced antigen recognition and cellular activation. By fusing the antigen specific portion of an antibody to intracellular signaling domains of the T cell signaling framework, several groups have shown promising results in different cancer clinical trials [12-15]. A similar strategy has also been used on HIV treatment by modifying peripheral T cells with a molecularly cloned T cell receptor (TCR) to redirect cells to HIV targets [16-18]. Using a lentiviral approach, Kitchen et al successfully expressed an HIV-specific TCR into HSCs and developed CD8 T cell with response to HIV in vivo [19]. Other groups have also demonstrated anti-HIV activity by expressing a functional neutralizing antibody in B cells derived from human HSC in vitro [20] and in vivo [21].

Human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs), are becoming an alternative promising source for gene or immunotherapy [22]. Several groups, including our own, have reported that hESCs and iPSCs can give rise to different lymphoid and myeloid lineages [23-29]. Some studies have also demonstrated that hESC- and/or iPSC-derive immune cells are either susceptible to HIV [25, 30] or capable of targeting HIV infected cells [31]. More recently, TCR-specific T cells derived from T-iPS cells [28] further demonstrated the potential of using either hESC or iPSCs-based gene/immunotherapy. We have previously shown that NK cells derived from hESCs and iPSCs have potent anti-HIV activity [31] and these innate immune cell do not possess any antigen specific recognition receptors [1, 32, 33]. Therefore we hypothesized that engineering hESC- and iPSC-derived NK cells with chimeric receptors would enhance their anti-HIV activity. In addition, we have recently demonstrated large-scale production of hESC/iPSC-derived NK cells, which could provide an unlimited cellular therapeutic for off-the-shelf use [34, 35]. We now advance these studies using the CAR strategy to direct NK cell effector function to HIV-infected cells [36].

As the CD4 protein is an absolute requirement for HIV entry, it is plausible to utilize this as an effective “antigen recognition” domain with human leukocyte antigen restriction. June and others pioneered this approach to both basic research and clinical trials [36-40], however this had varying efficacy in vivo when transduced into patients autologous T cells. Here, we modified both hESCs and iPSCs with a CD4 ζ construct to generate NK cells that express the specific HIV CD4 ζ chimeric receptor. We then tested these NK cells for HIV suppression both in vitro and found that both CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells were able to suppress HIV replication more efficiently than their unmodified counterparts. We also determined CD4 ζ -hESC-/iPSC-NK cells mediate in vivo anti-HIV activity in a PBL-NSG mouse xenograft model. We found that CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells were able to inhibit HIV replication and prevent CD4 T cells depletion but no difference compared to regular hESC and iPSC-NK cells. These studies establish a novel system to understand and direct innate immunity against HIV-1 infection. Eventually, hESC- or iPSC-based immune therapy could be utilized as a unique resource for HIV/AIDS treatment.

MATERIALS AND METHODS

Maintenance of hESCs/iPSCs and generation of CD4 ζ expressing hESC (CD4 ζ -hESC) and iPSC (CD4 ζ -iPSC) cells

hESCs (H9) and iPSCs (UCBiPS7, derived from umbilical cord blood CD34⁺ cells) were maintained on mouse embryonic fibroblasts (MEF) as described previously [41]. The plasmid pCCL.PPT.hPGK 1.9.IRES.eGFP containing CD4 ζ chimeric receptor was kindly provided by Drs. Scott Kitchen and Otto Yang from UCLA [36, 38]. Lentiviral production was produced in 293T cells using the Invitrogen ViraPower Lentiviral Expression System (Invitrogen). hESC and iPSC cell lines were then infected with CD4 ζ lentivirus and FACS sorted for GFP⁺ cells. These two lenti CD4 ζ -modified cell lines were used in all in vitro studies. Because the expression of CD4 ζ /GFP in hESCs and iPSCs was readily silenced during differentiation, we generated more stable CD4 ζ -hESC and CD4 ζ -iPSC lines using the sleeping beauty system [42, 43] and developed NK cells for all in vivo experiments.

NK cell derivation from CD4 ζ -hESC and CD4 ζ -iPSC cells

We have been using both stromal-based systems and stroma-free embryoid body (EB)-based systems for hematopoietic differentiation of hESCs and iPSCs. Most recently, we have shifted to using “Spin-EBs” for hematopoietic differentiation of hESCs and iPSCs [34, 44-46]. Briefly, 3000 single cells were seeded per well of 96-well round bottom plates in BPEL media with stem cell factor (SCF, 40ng/ml), vascular endothelial growth factor (VEGF, 20ng/ml) and bone morphogenic protein 4 (BMP4, 20ng/ml). BPEL media was in 200ml volumes and contained Iscove's Modified Dulbecco's Medium (IMDM, 86mL, Invitrogen), F12 Nutrient Mixture with Glutmax I (86mL, Invitrogen), 10% deionized Bovine Serum Albumin (BSA, 5mL, Sigma), 5% Polyvinyl alcohol (10mL, Sigma), Linolenic acid (20uL of 1gm/mL solution, Sigma), Linoleic acid (20uL of 1gm/mL solution, Sigma), Synthecol 500x solution (Sigma), α -monothioglycerol (Sigma, 3.9uL/100ml), Protein-free hybridoma mix II (Invitrogen), ascorbic acid (5 mg/ml, Sigma), Glutamax I (Invitrogen), Insulin-transferrin-selenium 100x solution (Invitrogen), Penicillin/streptomycin (Invitrogen). At day 11 of hematopoietic differentiation, spin EBs were directly transferred into 24-well plates with or without EL08-1D2 stromal cells in NK media supplied with cytokines [34]. After 4-5 weeks of culture, single cell suspensions were stained with APC-, PE-, FITC- and PerCP-cy5.5-coupled IgG or specific antibodies against human blood surface antigens: CD45-PE, CD56-APC, CD56-PE, CD16-PerCP-cy5.5, NKG2D-PE, NKp44-PE, NKp46-PE, CD158b-FITC, CD158e1/2-FITC (all from BD Pharmingen), CD158a/h-PE and CD158i-PE (Beckman Coulter) as shown in Figure 1. All analyses were performed with a FACS Calibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). NK cells isolated from peripheral blood (PB-NK) using an NK negative selection kit (Miltenyi Biotech.) were used as controls for phenotyping characterization and all following experiments.

NK cell stimulation

CD4 ζ -hESC-, CD4 ζ -iPSC-NK cells and GFP only controls were starved in RPMI 1640 media overnight. As previously described [36], cells were spun down and stimulated with anti-CD4 mAb (OKT4A) for 15 min at 4°C, then washed off unbound Ab and cross linked by goat anti-mouse IgG F(ab')₂ fragments (Jackson ImmunoResearch) for 3 min at 37°C. Cells were then fixed and stained for the tyrosine phosphorylation using mouse anti-human mAb4G10 (Millipore) and PE-donkey anti-mouse IgG (Jackson ImmunoResearch) following the instruction of BD phosflow kit (BD Biosciences).

CD4 ζ -hESC and CD4 ζ -iPSC NK cell anti-HIV activity in vitro

As in our previous studies, CEM-GFP cells infected with HIV-1 NL4-3 were used as targets to test the suppression of HIV of CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells by comparison with their unmodified hESC and iPSC-NK cells. Briefly, CEM-GFP cells were infected with HIV-1 NL4-3 (MOI=0.1) for 4h at 37°C and then were washed twice with fresh medium. 1×10^5 cells were plated with CD4 ζ -hESC-, hESC-, CD4 ζ -iPSC- and iPSC-NK cell at effector: target ratios 1:1 and 5:1 or alone for 14 days in the presence of 100 IU/ml interleukin 2 (IL-2). Cells were collected on day 4, 7, 11, 14 for GFP expression by flow cytometry. Loss of GFP expression in the CEM-GFP cells indicates the suppression of HIV replication. To detect the anti-HIV activities of CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells to HIV-infected primary human cells, CD4 $^+$ T cells enriched from peripheral blood were stimulated with phytohaemagglutinin (PHA from Sigma-Aldrich) in RPMI 1640 with 10% FBS, 2mM L-glutamine supplemented with 100 IU/ml IL-2 for 48-72 hr. At day 3, expanded CD4 $^+$ T cells were infected with a lab-adapted strain HIV-1 SF2 (X4R5, MOI=0.05) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) [47]. At day 10, 1×10^5 of HIV-1-infected CD4 T cells were respectively mixed with same number of CD4 ζ -hESC-, hESC-, CD4 ζ -iPS- and iPSC-NK cells at 37°C for 5 hours, the activation of NK cells were evaluated by CD107a surface expression. PB-NK cells were used as positive controls for all experiments.

Generation of human peripheral blood-reconstituted NSG (PBL-NSG) mouse

Eight- to 10-week-old NSG mice were reconstituted with 1×10^7 freshly isolated human PBLs [48]. Two weeks later, blood was bled from the retro orbital venous plexus for engraftment before HIV NL4-3 infection.

HIV infection and NK cell treatment

HIV-1 NL4-3 virus was grown in 293 T cells. Virus infectivity was determined by limiting dilution titration on 293 T cells. HIV-1 NL4-3 stocks were prepared as described [49]. Two weeks after PBL reconstitution, mice were infected by intraperitoneal injection of 100ul cell-free HIV stocks containing 30,000 50% tissue culture infectious doses (TCID $_{50}$) [50]. Mice were then ip injected with 2×10^6 hESC-, CD4 ζ -hESC-, iPSC-, CD4 ζ -iPSC- or PB-NK cells the day after HIV-1 NL4-3 infection. As previously demonstrated in our lab, mice received ip injection of IL-15 and IL-2 every day for the first 7 days following NK cell treatment and then IL-2 every other day for another week to enhance NK cell proliferation and function [51]. Day 6, 9 and 12 after NK cell injection, blood was collected for human CD4 T cell levels, HIV gag protein p24, viral RNA and proviral DNA detection. Day 13 after NK cell treatment (day 14 of HIV infection), mice were killed and collected for spleens and cells from peritoneal cavity for proviral DNA and intracellular p24 detection.

Measurement of human CD4 T cell levels in PBL-NSG mice

The levels of hCD4 in peripheral blood were monitored every 3 days after NK cell treatment. Whole blood was collected in EDTA-coated tubes and red blood cells were lysed by ammonium chloride for twice, 5mins each time if necessary. Cells were then stained for hCD45-APC, hCD3-PECY7 and hCD4-PE (BD Pharmingen). CD4 $^+$ T cell levels were determined as a ratio of CD4 $^+$ CD3 $^+$ /CD4 $^-$ CD3 $^+$. To establish baseline CD4 $^+$ CD3 $^+$ /CD4 $^-$ CD3 $^+$ ratios, all mice were analyzed prior to HIV infection. To detect hESC-, iPSC- and PB-NK cells in peripheral blood, peritoneal cavity and spleen, cells were stained with hCD45-PE, hCD56-APC (BD Pharmingen) after treatment.

Measurement of HIV viral load

Mouse peripheral blood was collected by facial bleeding in accordance with the university of Minnesota IRB. Plasma was separated by spin at 400rcf/min for 10 mins and frozen at -80°C for viral RNA isolation. Viral RNA was extracted from less than 50ul of EDTA-treated plasma with the QIAamp Viral RNA kit (Qiagen, Valencia, CA). Quantitative RT-PCR was performed using the Taqman one-step RT-PCR Master Mix Reagents kit (Applied Biosystem, Branchburg, NJ) with a set of primers specific for the HIV long terminal repeat (LTR) sequence and an LTR-specific probe as described [52-54]. Viral RNA was expressed as the number of HIVRNA copies per milliliter plasma. To detect integrated provirus, cellular DNA was extracted from peripheral blood, peritoneal cavity and spleens using the high pure PCR template preparation kit (Roche, Mannheim, Germany) and subjected to Q-PCR with the same set of primers above and SYBR Green PCR Master Mix (Applied Biosystems).

Statistical analysis

Experiments were analyzed utilizing prism 5 software the Student's T test and the Wilcoxon Rank Sum Test. Results are shown as means and SD and the value of $P < 0.05$ was determined as significant.

RESULTS

Expression of CD4 ζ in hESCs and iPSCs Using Lentivirus or Sleeping Beauty Transgenesis

The CD4 ζ construct contains the fused extracellular and transmembrane domains of CD4 and the cytoplasmic domain of T-cell receptor CD3 ζ chain, linked to green fluorescent protein (eGFP) by a 2A self-cleaving peptide (Figure 1A). The CD4 ζ lentivirus was made in 293 T cells and infected hESCs and iPSCs. We then performed flow cytometry sorting for GFP positive cells to get pure CD4 ζ expressing hESCs and iPSCs (CD4 ζ -hESCs or CD4 ζ -iPSCs). hESCs and iPSCs expressing GFP-only were used as controls (Figure 1B). During the maintaining of CD4 ζ -hESCs and CD4 ζ -iPSCs, we found that the expression of GFP and CD4 ζ was commonly silenced after 10-15 passages (Figure 1B). Our previous studies and others have demonstrated that the *Sleeping Beauty* (SB) transposon system is a more stable means to transfer genetic information to hESCs [42, 43]. We then re-transduced the CD4 ζ -GFP fused protein into hESCs and iPSCs using the SB system with puromycin antibiotic selection and did not find CD4 ζ -GFP silencing even till passage 37 (Figure 1C). Here we used NK cells derived from CD4 ζ -GFP-SB transduced-hESCs or iPSCs all in vivo studies.

NK Cells Derived from CD4 ζ -hESCs and CD4 ζ -iPSCs

Previous studies by our group to derive NK cells from both hESCs and iPSCs have utilized stromal-based systems [31, 51]. More recently we shifted to use of defined serum-free conditions that can be effectively scaled to produce potentially clinical-scale quantities of NK cells [44, 45, 55]. Briefly, in this system, undifferentiated hESCs or iPSCs are dissociated as single cell suspension and seeded into 96-well round bottom plates by briefly spinning to form embryoid bodies (EBs). After 11 days of culture in serum-free media with defined cytokines, differentiated spin EBs containing hematopoietic progenitors CD34 $^{+}$ /CD45 $^{+}$ were transferred to NK cell differentiation media supplemented with a combination of cytokines with or without EL08 stromal cells routinely generates a lymphocyte population where more than 90% of the cells are CD45 $^{+}$ CD56 $^{+}$ (Figure 2A). Both CD4 ζ -hESC- and CD4 ζ -iPSC-derived CD45 $^{+}$ CD56 $^{+}$ populations expressed the CD4 receptor and GFP. Similar to unmodified hESC-, iPSC- or PB-NK cells [31, 51], these CD45 $^{+}$ CD56 $^{+}$ cell populations are mostly CD117 $^{-}$ CD94 $^{+}$, which has been demonstrated to be a more cytotoxic

subset of NK cells [51, 56, 57]. We have previously demonstrated extensive phenotypic analysis of hESC and iPSC-derived NK cells expressing similar surface makers including the Fc receptor CD16, killer immunoglobulin receptors (KIRs), NKG2A, NKG2D, NKp44 and NKp46 as PB-NK cells [31]. CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells also had a similar phenotype as their unmodified counterparts and PB-NKs (Figure 2B). We then examined chemokine/cytokine receptors expression on CD4 ζ -hESC- or CD4 ζ -iPSC-NK cells. Expression levels of CCR5 and CXCR4, also known as HIV co-receptors [58] were not observed to high levels expression on both CD4 ζ -modified hESC- and iPSC-NK cells compared to their unmodified counterparts or PBNKs (Figure 2B). The chemokine receptors CXCR3, CCR7 and adhesion molecule CD62L are all involved in NK cell homing to second lymphoid organs [59]. We found that CD4 ζ -hESC or iPSCNK cells expressed similar levels of CXCR3 as PB-NKs, but less CCR7 and CD62L (Figure 2B). Next, to evaluate the function of the CD4 ζ chimeric receptor in hESC- and iPSC-NK cells, addition of anti-CD4 mAb OKT4A followed by goat F(ab)' anti-mouse IgG was used to cross-link and stimulate cells. Stimulation of effector function through the CD4 chimeric receptor is dependent on tyrosine phosphorylation [60], which can be determined by phospho-flow cytometry (Figure 2C). We found tyrosine phosphorylation is rapidly induced in both CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells by cross-linking of the CD4 ζ chimeric receptors (Figure 2D), indicating this chimeric receptor is functionally active following differentiation of pluripotent stem cells into NK cells.

CD4 ζ -hESC- and CD4 ζ -iPSC-NK Cell Inhibition of HIV Replication in Vitro

Our previous studies demonstrated that both hESC- and iPSC-NK cells have potent ability to inhibit HIV infection [31]. The CEM-GFP T cell line infected with HIV-1 NL4-3 leads to GFP expression, which provides accurate and reliable quantification of HIV infection and the effects of our NK cell-based inhibition to HIV replication [61]. To determine whether the expression of CD4 ζ enhance anti-HIV activity, CD4 ζ -hESC- or CD4 ζ -iPSC-NK cells and their unmodified counterparts were co-cultured with NL4-3-infected CEM-GFP cells at different effector/target (E/T) ratios and monitored for HIV replication for two weeks [31, 62]. As we have previously demonstrated, unmodified hESC- and iPSC-NK cells both inhibit HIV replication in a dose dependent manner (Figure 3A and 3B). Notably, CD4 ζ modified hESC- and iPSC-NK cells lead to 90% inhibition of HIV replication, significantly greater than unmodified hESC- and iPSC-NK cells ($P < 0.05$). These studies indicate CD4 ζ expression on hESC and iPSC-derived NK cells effectively directs NK cells to better target HIV-infected cells.

As a more rigorous in vitro test, we next studied the ability of each cell population to limit infection of primary CD4⁺ T cells. To compare the anti-HIV activity of CD4 ζ -hESC or CD4 ζ -iPSC-NK cells versus unmodified hESC- or iPSC-NK cells, primary CD4⁺ T cells infected with HIV-1SF2 (X4R5) [47] were used as targets. Again, HIV-infected CD4⁺ T cells were co-cultured with or without NK cells for two weeks. HIV infection was quantified by intracellular staining of CD4⁺T cells for the viral gag protein p24 every 3-4 days over time. Lower percentages of p24⁺ T cells were observed in cultures with all populations of NK cells compared to controls (Figure 3C and 3D). Additionally, the percent of p24⁺ T cells were significantly lower in the co-culture with CD4 ζ -hESC- ($P = 0.034$, $n = 3$) or CD4 ζ -iPSC-NK cells ($P = 0.029$, $n = 3$) compared to unmodified hESC-, iPSC-NK cells respectively. These findings again demonstrate CD4 ζ -modified hESC- and iPSC-NK cells inhibited HIV infection more effectively than unmodified hESC-, iPSC-cells. To further confirm that the inhibition of HIV infection is due to NK cell activation, we monitored NK cell degranulation during co-culture with HIV-1-infected CD4⁺ T cells. Expression of CD107a has been used as a measure of NK cell activity following stimulation [62, 63]. Here, we found CD107a was significantly increased on CD4 ζ -hESC- ($P = 0.045$, $n = 3$) and CD4 ζ -iPSC-NK cells

($P=0.041$, $n=3$) than unmodified hESC- or iPSC-NK cells after co-cultured with HIV-1-infected CD4⁺ T cells at d7 (Figure 3E and 3F). Overall, these data demonstrate that both CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells inhibit HIV replication more efficiently than the unmodified counterparts.

In Vivo Anti-HIV Activity of CD4 ζ -hESC-NK Cells

To evaluate hESC-/iPSC-NK cell anti-HIV activity *in vivo*, we used the hPBL-NSG mouse model of HIV-1 infection [48]. In these studies, 10^7 PBL are injected intraperitoneally (ip) into NSG mice. After two weeks, the mice were then infected with HIV NL4-3 at 30,000 fifty percent tissue culture infectious doses (TCID₅₀) [50]. This leads to productive HIV infection as demonstrated by loss of CD4⁺ cells and production of virions [64]. Then, we injected 2 million CD4 ζ -hESC-, hESC- or autologous PB-NK cells in the day following HIV infection. At days 6, 9, and 12 post-NK cell treatment, blood samples were collected and examined for CD4 T cell levels determined by human CD4⁺CD3⁺/CD4⁻CD3⁺ ratios and HIV infection determined by the percent p24⁺ T cells. A general pattern of higher CD4 T cell level was observed in HIV-infected mice with NK cell treatment (Figure 4), demonstrating suppression of HIV activity [19, 65]. CD4 ζ -hESC-NKs cells, illustrated by their expression of CD45, CD56, CD4 and negative for CD3, were detected in peripheral blood early as day 6 after treatment (Figure 4A). At this time point, CD4⁺CD3⁺/CD4⁻CD3⁺ ratios were seen to decrease less in mice with NK cell treatment than controls (Figure 4B, Figure 4C left panel). By day 12 of NK cell treatment (day 13 of HIV infection), there was a statistically significant difference in CD4⁺CD3⁺/CD4⁻CD3⁺ ratios between mice treated with CD4 ζ -hESC- ($P=0.024$, $n=3$), hESC- ($P=0.028$, $n=3$), PB-NK ($P=0.029$, $n=3$) cells and controls. (Figure 4C, right panel). As CD4 receptor on surface of HIV infected cells is decreased after HIV infection, we used CD3⁺CD8⁻ instead of CD3⁺CD4⁺ to evaluate HIV infection of CD4 T cells. No significant levels of HIV-infected p24⁺CD3⁺CD8⁻ cells were observed (less than 1%) at day 6, but by day 9 of NK cell injection, the percentages of p24⁺CD3⁺ CD8⁻ cells were dramatically increased in control mice than those treated with either CD4 ζ -hESC-, hESC- or PB-NKs (Figure 4D). Notably, we did not observe any statistical difference between CD4 ζ -hESC-NK cells and unmodified hESC-NK cells on suppression of HIV infection and retaining CD4 T cell level, but these data indicate that both CD4 ζ -modified and unmodified hESC-derived NK cells can inhibit HIV replication and prevent CD4⁺ T cells depletion *in vivo*.

To further determine if the NK cell treatment could suppress HIV replication, the levels of viral RNA in mouse peripheral blood plasma and proviral DNA in different tissues were evaluated. We isolated viral RNA from plasma collected on day 12 of NK cell treatment and performed quantitative reverse PCR was performed to measure the RNA level of long terminal repeat (LTR) sequence [54, 65]. Standard LTR cDNA was used to calculate RNA copy numbers [19, 65]. The viral RNA in HIV-infected mice had approximately 2000 copies/ml, whereas the RNA levels were significantly lower in all mice treated with either CD4 ζ -hESC-, hESC- or PB-NK cells at day 12 (Figure 5A). This confirms NK cell-mediated suppression of HIV replication *in vivo*. To test integrated proviral DNA, peripheral blood was collected from mice at day 6, 9 and 12 post-NK cell treatments. DNA was extracted and proviral elements were detected by specific amplification of HIV-1. We were not able to detect proviral DNA from blood samples by QPCR until day 9 (Figure 5B). Similar to the viral RNA level, the proviral DNA was decreased in peripheral blood in mice receiving NK cells. We did not observe detectable DNA levels in peripheral blood at day 12, which might be due to low levels of CD4⁺ T cells. To test proviral DNA in other peripheral tissues/organs, mice were then sacrificed on day 13 of NK cell treatment (day 14 of HIV infection) and mouse peritoneal washes and spleens were collected for the quantity of LTR sequences. Again, the DNA levels of HIV were also found to be significantly lower in the

peritoneal cavity (Figure 5C) and spleen (Figure 5D) of HIV-1-infected mice treated with CD4 ζ -hESC-NK cells, hESC-NK cells or PB-NK cells as compared to control mice. Thus, these findings suggest that NK cell treatment resulted in significant suppression of HIV replication in several tissues/organs in this hPBL NSG mouse model.

In Vivo Anti-HIV Activity of CD4 ζ -iPSC-NK Cells

Next, we investigated in vivo anti-HIV activities of both CD4 ζ -modified and unmodified iPSC-NK cells. Using the PBL-NSG mouse model, we treated HIV-infected mice with CD4 ζ -iPSC-, iPSC- or autologous PB-NK cells as above. CD4⁺ T cell levels (measured as CD4⁺CD3⁺/CD4⁻CD3⁺ ratios again) were examined at days 6, 9 and 12 post NK cell treatment (Figure 6A). Consistent with CD4 ζ -modified and unmodified hESC-NK cells, CD4 ζ -iPSC- and iPSC-NK cells were able to retain CD4⁺CD3⁺/CD4⁻CD3⁺ ratios at higher levels in mice up to two weeks after HIV infection (Figure 6B). The viral load was suppressed to a significantly lower level in the plasma of mice treated with all NK cells compared to controls (Figure 6C). All of these results suggest that NK cells derived from iPSCs are also capable of inhibiting HIV replication in vivo.

DISCUSSION

Recent studies based on genetic modification of HSCs or mature lymphocytes have begun to use combined gene and immune therapy against HIV/AIDS. However, this approach typically needs to be done on a patient-specific basis, which could be challenging to apply in a broad clinical setting. In contrast, hESC and iPSCs derived cells could provide a novel “universal” and “off-the-shelf” cell population for anti-HIV treatment without concerns for immune rejection. In fact, studies have demonstrated that hESC and iPSCs are able to differentiate into different hematopoietic lineages including recently TCR specific-T cells derived from direct reprogramming or T-iPSCs [23-29], indicating the potential of hESC- or iPSC-based innate and adaptive immunotherapy for HIV.

CARs have been designed and targeted to a wide-array of cancers and virally-infected targets. This system has been suggested as a highly effective means to increase immunity in cancer therapy [12-15]. Investigators have also adapted this strategy to HIV treatment by genetic engineering primary T/NK cells with specific anti-HIV TCR or HIV CD4 receptor (reviewed in [4]). The CD4 ζ construct has been extensively tested in multiple systems such as primary T and NK cells against HIV [36-40]. Some of these trials transduced CD4 ζ into primary T cells of HIV-infected patients and turned out with varying effects [37, 39]. Here, we combined the unique advantages of the hESCs/iPSCs system and the specificity and efficacy of CARs together to genetically modify hESCs/iPSCs. We initially expressed CD4 ζ in hESCs and iPSCs by lentivirus, which was commonly silenced after 10-15 passages (Figure 1C). Our previous studies and others have demonstrated that the *Sleeping Beauty* (*SB*) transposon system is a more stable means to transfer genetic information to hESCs [42, 43]. Therefore, we used the *SB* system to achieve more stable CD4 ζ expressing hESC and iPSC cell lines for our in vivo studies.

By using “spin EB” system, we were able to develop NK cells from CD4 ζ -modified hESCs and iPSCs that express high levels of CD4 and a panel of surface markers as unmodified hESC-, iPSC- and PB-NKs (Figure 2). We found that both CD4 ζ -modified hESC-NK and iPSC-NK cells are able to inhibit HIV replication more efficiently in vitro than their unmodified counterparts respectively (Figure 3). Although KIR expression is associated with NK licensing through interaction with HLA class I molecules, the activation of NK cells is ultimately determined by the balance between inhibitory and activating receptors including but not limited to KIRs [66]. Thus higher KIR expression may not necessarily lead to increased functional responses at a single cell level [67]. Here we noticed that CD4 ζ -iPSC-

NKs express higher levels of KIRs (Figure 2b), but their response (measured by CD107a) to HIV-infected targets was similar to CD4 ζ -hESC-NKs that had lower KIRs expression (Figure 3B). However, fewer infected T cells (as evidenced by p24⁺CD4⁺) in CD4 ζ -hESC-NKs and HIV co-culture (Figure 3C) might be secondary to other mechanisms such as increased apoptosis through TNF-related apoptosis-inducing ligand (TRAIL) pathway [68].

During the *in vivo* assays, we also observed both CD4 ζ -modified hESC- and iPSC-NK cells and their unmodified cells were able to suppress HIV infection in PBL-NSG mice (Figure 4, 5 and 6). However, we did not find a significant difference in HIV inhibition from CD4 ζ -modified NK cells as *in vitro*. In this case, we treated HIV-infected mice with 2×10^6 NK cells, which may be insufficient cell number for CD4 ζ -hESC-/iPSC-NK cells to induce significant inhibition on viral infection *in vivo*. The lack of additional co-stimulatory domains present on the intracellular portion ζ chain may limit the activity [69]. As has been demonstrated in several pre-clinical cancer models, CARs containing additional signaling molecules (2nd and 3rd generation CARs) have enhanced *in vivo* persistence and activity [70]. Additionally, other investigators have found that addition of membrane bound cytokines can also enhance the *in vivo* activity of CAR containing effectors [67, 71]. Collectively, the CD4 ζ receptor may not be the best choice for HIV therapy as its varying effect has been suggested in preclinical studies [37, 39]. Recently, a broadly neutralizing anti-HIV antibody b12, which recognizes gp120 CD4 binding site, has been successfully engineered into human HSPCs and produced functional IgG in culture system [20]. Interestingly, several neutralizing Abs have been identified and characterized (reviewed in [72]). It may be beneficial to engineer them as “CAR” into hESC/iPSC system and test their *in vivo* efficacy.

Studies by Bernstein et al showed that stimulated CD4⁺ NK cells had a low percentage of p24⁺ when infected by HIV-1-Bal (R5) [73]. As we expressed HIV CD4 receptor in hESCs and iPSCs, it is reasonable to question whether the CD4 expression would make hESC- and iPSCs-NK cells susceptible to HIV infection. Fortunately, these hESC- or iPSC-NK cells did not express the HIV co-receptor CXCR4, which is required for HIV-1 X4 or X4R5 entry into targets [74] (Figure 2B). Although a small proportion of CD4 ζ -hESC-NK cells are positive for another co-receptor CCR5 (Figure 2B), NK cells were not infected when they were co-cultured with HIV-1 (SF2, X4R5)-infected CD4 T cells (Fig.3E).

CXCR3, CCR7 and CD62L are three major receptors involved in NK cells homing to secondary lymphoid organs [59]. We examined their expression on hESC- or iPSC-NK cells (Figure 2B) and found that CXCR3 was comparably expressed on both CD4 ζ modified and unmodified hESC- and iPSC-NK cells as PB-NKs. However, CD62L and CCR7 are expressed at a lower level on hESC- and iPSC-NK cells, which may explain why hESC- or iPSC-NK cells were not detected in second lymphoid organs such as spleen in NSG mice after injection (data not shown). It is also pertinent to note that the mouse model does not recapitulate the extent of human physiology and could alter the *in vivo* trafficking of the infused cells. Subsequent work will aim to improve the homing and trafficking activity of hESC- or iPSC-derived NK cells by enforced expression of defined homing receptors [67, 71], which could also potentially help to clear viral reservoirs.

CONCLUSION

CD4 ζ - hESC- and CD4 ζ - iPSC-derived NK cells are capable of suppressing HIV replication *in vitro* with higher efficacy than their unmodified counterparts. Both CD4 ζ - modified and unmodified hESC- and iPSC-NK cells demonstrate their inhibition of HIV inhibition *in vivo*, indicating the feasibility of using hESC/iPSC as a cellular source for combined

immune/gene therapy of HIV treatment in vivo. These studies also provide a foundation and a model system to further investigate innate immune responses during viral infection.

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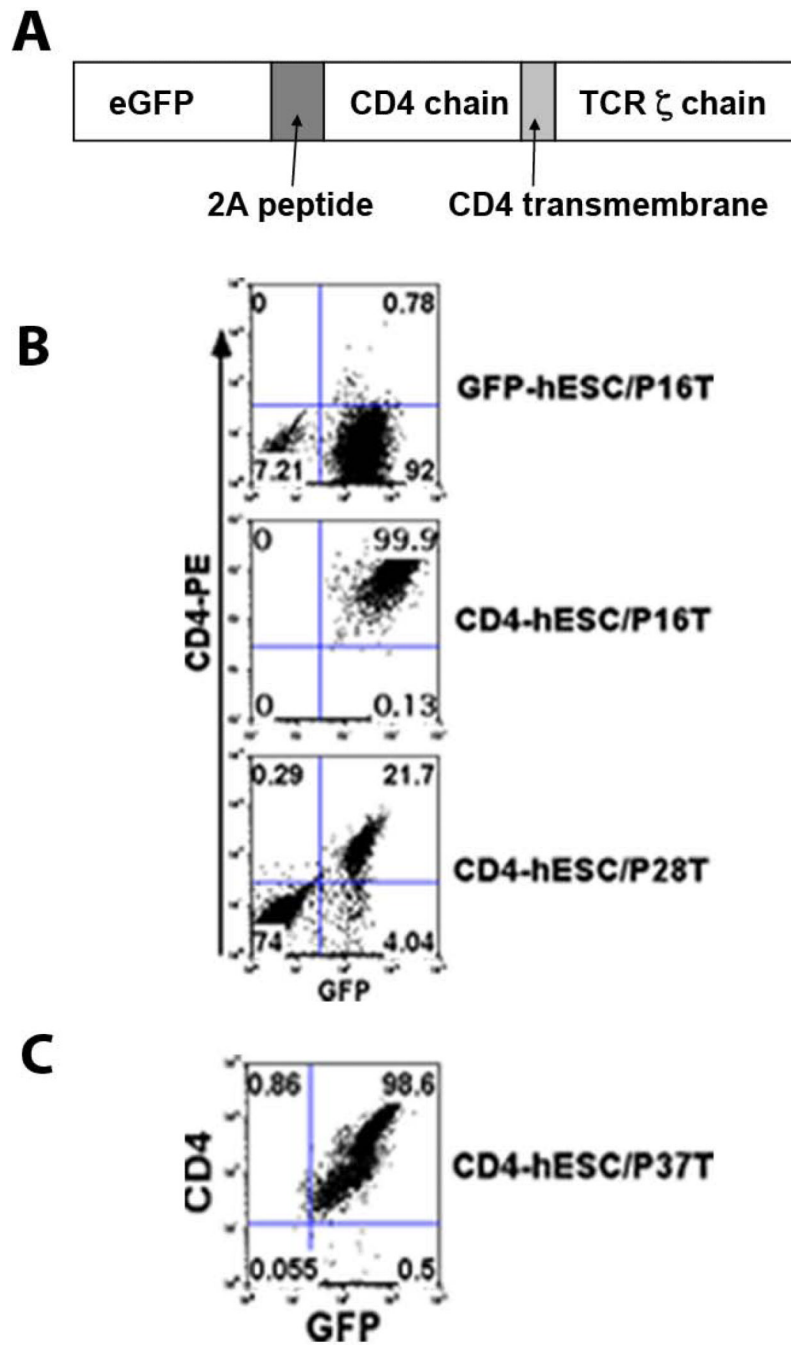


Figure 1. Expression of CD4 ζ chimeric receptor in hESCs and iPSCs
(A) Diagram of CD4 ζ cloned in lentiviral vector or Sleeping Beauty transposon vector. **(B)** Transduced hESC cells were analyzed by flow cytometry for expression GFP and CD4 ζ receptor (upper and middle lanes). Both GFP and CD4 ζ expression getting silenced during culture maintain (lower lane). **(C)** SB-transduced hESCs stably express GFP-CD4 ζ .

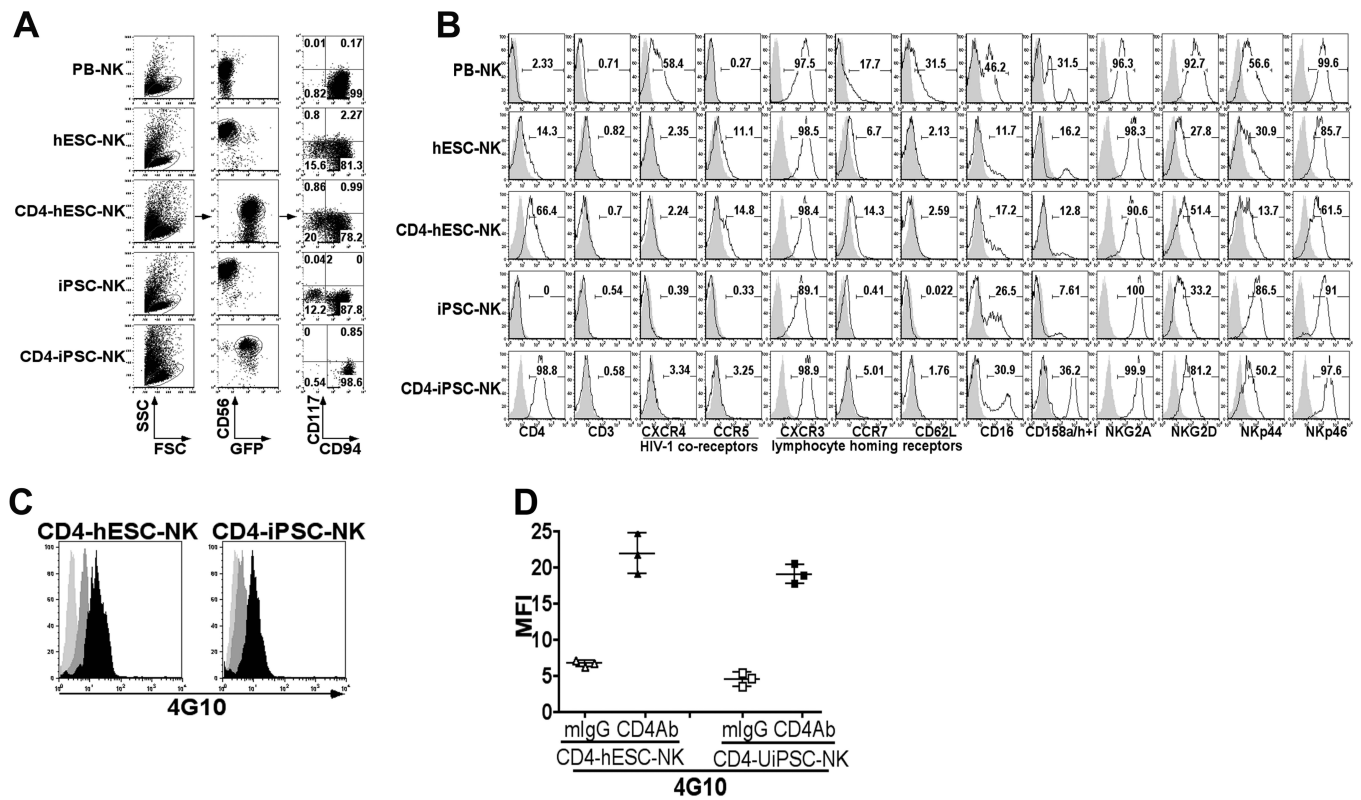


Figure 2. Generation of NK cells from CD4 ζ -hESCs and CD4 ζ -iPSCs

(A) Flow cytometric analysis of CD56⁺CD45⁺ NK cells derived from hESC, CD4 ζ -hESC, iPSC and CD4 ζ -iPSC. Expression of lymphocyte activating receptors and homing receptors on NK cells as indicated. These cells are compared to NK cells isolated from peripheral blood (PB-NK). (B) CD56⁺ NK cell from hESC, CD4 ζ -hESC, iPSCs, CD4 ζ -iPSCs are all CD3⁻ as are PB-NKs. Expression of surface marker CD16, KIRs, NKG2A, NKG2D, NKp44, NKp46, HIV co-receptor CCR5, CXCR4 and homing receptor CXCR3, CCR7 and CD62L. (C) Activity of CD4 ζ in NK cells derived from CD4 ζ -hESCs and CD4 ζ -iPSCs. Both CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells were stimulated with (■) or without (▒) anti-CD4 and goat anti-mouse IgG F(ab')₂ to initiate receptor cross-linking. Cells were then intracellularly stained by tyrosine phosphorylation Ab 4G10 followed by PE- anti-mouse IgG. Cross-linked cells were stained with mouse IgG and PE- anti-mouse IgG were used as isotype controls (■). Flow cytometry plots represented 1 of at least 3 independent experiments. (D) Tyrosine phosphorylation measured by flow cytometry for the mean fluorescent intensity (MFI). The solid lines represent the mean \pm the SD.

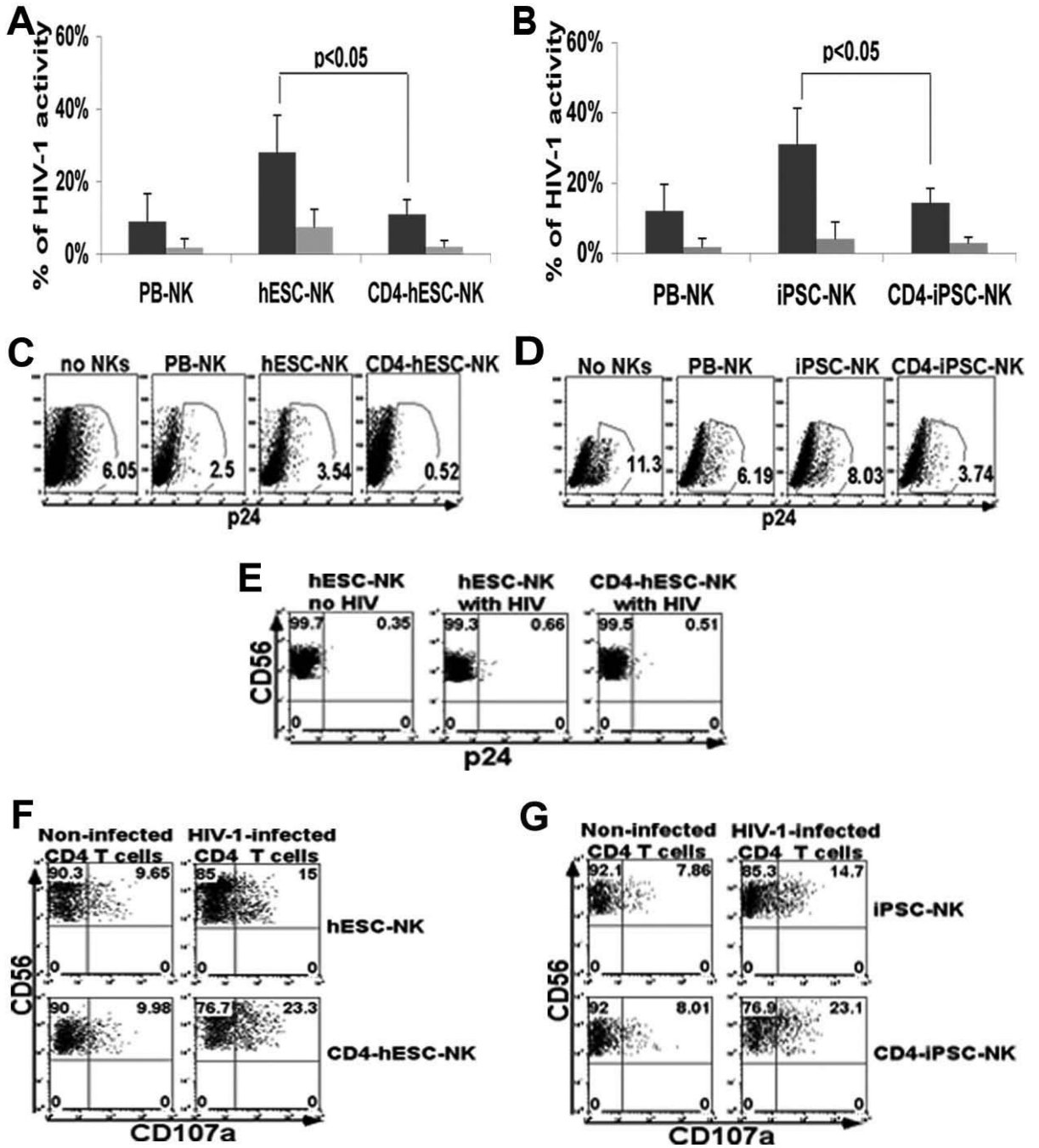


Figure 3. CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells inhibit the replication of HIV in vitro (A to B) CEM-GFP cells were incubated with HIVNL4-3 virus for 4 hours. Cells were then co-cultured with CD4 ζ -modified hESC-, CD4 ζ -iPSC- or PB-NK cells for 14 days. HIV infection was assessed by flow cytometry for GFP expression. Activity of HIV-1 was measured by the percent GFP⁺ of CEM-GFP cells co-cultured with (A) PB-, hESC- and CD4 ζ -hESC-NK cells or (B) PB-, iPSC-, and CD4 ζ -iPSC-NK cells at day 11 with E:T ratios of 1:1 (■) and 5:1 (▒). Cells were CEM gated. The error bars represent the mean \pm the standard deviation (SD). Statistical comparison of % GFP⁺ between CD4 ζ -hESC-/iPSC-NK vs. hESC-/iPSC-NK cells was performed using the Student's t test. (C-F) NK cells function

against HIV-1-infected human CD4⁺ primary T cells. (C and D) NK cells were co-cultured with SF2-infected CD4⁺ T cells at E:T ratios of 5:1 for two weeks. HIV infection was evaluated by intracellular staining for gag p24 in all CD4 T cells. The percentage of p24⁺ CD4⁺ in the co-cultures of (C) no NKs, PB-, hESC-, and CD4 ζ -hESC-NK cells or (D) no NKs PB-, iPSC- and CD4 ζ -iPSC-NK cells with HIV-infected CD4 T cells at day 11. Cells were CD56⁻ gated. (C) and (D) demonstrate statistically lower % p24⁺ in CD4 ζ -hESC-/iPSC-NK culture compared to hESC-/iPSC-NK cells respectively. (E) NK cells were evaluated for HIV infection in all CD56⁺ cells at day 11 of co-culture. Either CD4 ζ -hESC-NKs or hESC-NKs were negative for p24 staining. hESC-NKs with no HIV as negative controls. (F and G) Surface expression of CD107a was evaluated to measure NK cell cytolytic activity. Flow cytometric analyses of CD107a expression on (F) hESC- and CD4 ζ -hESC-NKs or (G) iPSC- and CD4 ζ -iPSC-NKs following stimulation with HIV-1-infected CD4⁺ T cells for 5 hours. Uninfected CD4⁺ T cells were used as controls. Cells were all CD56⁺ gated. Both CD4 ζ -hESC- and CD4 ζ -iPSC-NK cells populations stimulated by HIV-1-infected CD4⁺ T cells show significantly increased CD107a expression compared to hESC- and iPSC-NK cells (P<0.05). The data represent one of at least 3 independent experiments.

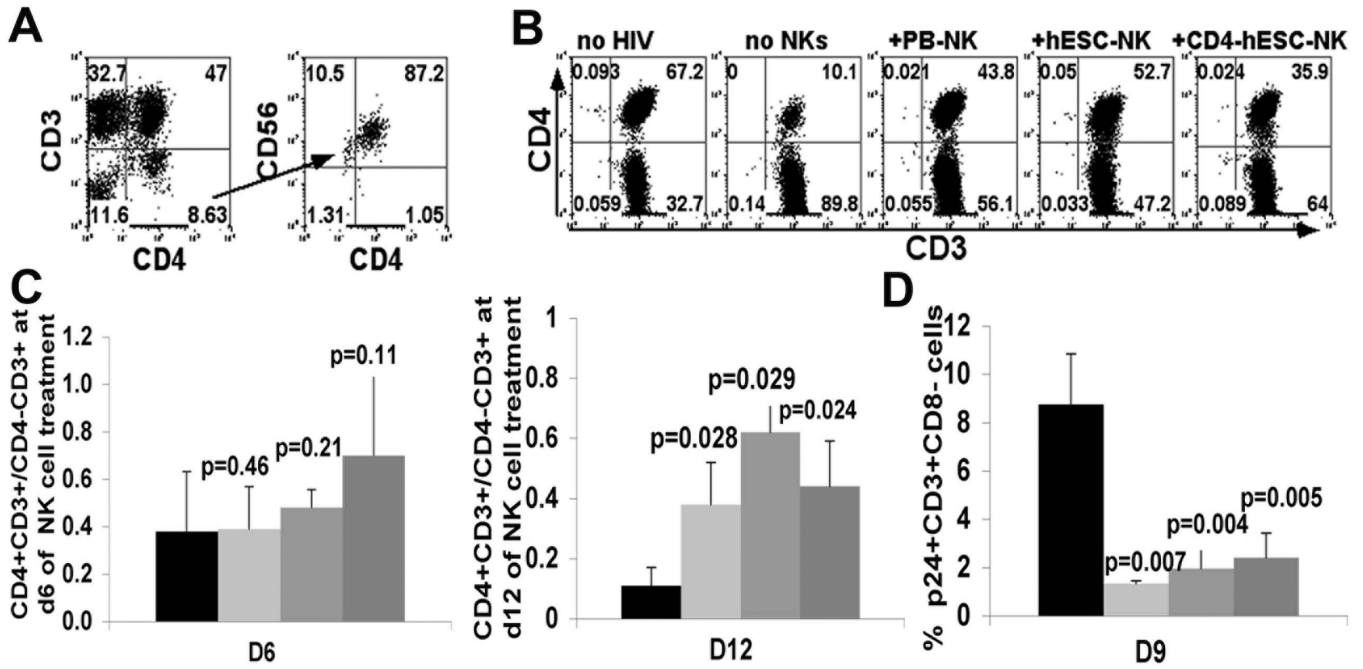


Figure 4. hESC-NK cells and CD4 ζ -hESC-NK cells suppress HIV replication in peripheral blood
 Two weeks after PBL reconstitution, mice were infected with HIV NL4-3 and treated with NK cells next day. Peripheral blood was then collected at day6, 9 and 12 with or without NK cell treatment. HIV infection was evaluated by CD4⁺ T cell depletion and HIV⁺ cell percentage in peripheral blood. CD4⁺ T cell level was determined by flow cytometry for CD4⁺CD3⁺/CD4⁻CD3⁺ ratios. HIV infected human cells were evaluated by intracellular staining for gag p24⁺. **(A)** CD45⁺CD3⁻CD4⁺ cells that were CD56⁺ and GFP⁺ detected in peripheral blood of mice treated with CD4-hESC-NK cells after day 6. **(B)** Human CD45⁺ cells that express CD3 and CD4 were assessed in peripheral blood of HIV-1-infected NSG mice treated with or without NK cells at day 6. All cells were hCD45⁺ gated. Flow cytometry plots are representative of 1 mouse of each condition in at least 3 independent experiments with a minimum of 3 mice in each experimental group. **(C)** CD4⁺ T cell levels in peripheral blood of HIV infected mice determined by CD4⁺CD3⁺/CD4⁻CD3⁺ ratios at day 6 (left panel) and day 12 (right panel) of NK cell treatment. All mice were analyzed prior to HIV infection to set up baseline CD4⁺CD3⁺/CD4⁻CD3⁺ratios. **(D)** Suppression of HIV infection was evaluated by the percentages of p24⁺ cells in all hCD3⁺CD8⁻ from peripheral blood at day 9 of NK cell treatment. Data in **(C)** and **(D)** represent the average of one of three separated experiments with at least 3 mice in each group, the error bars indicate the mean \pm the SD. Statistical comparison of CD4⁺ T cell level and % p24⁺CD3⁺CD8⁻ in NK treated mice to untreated mice was performed using Student's t test. P values are provided for each indicated comparison.

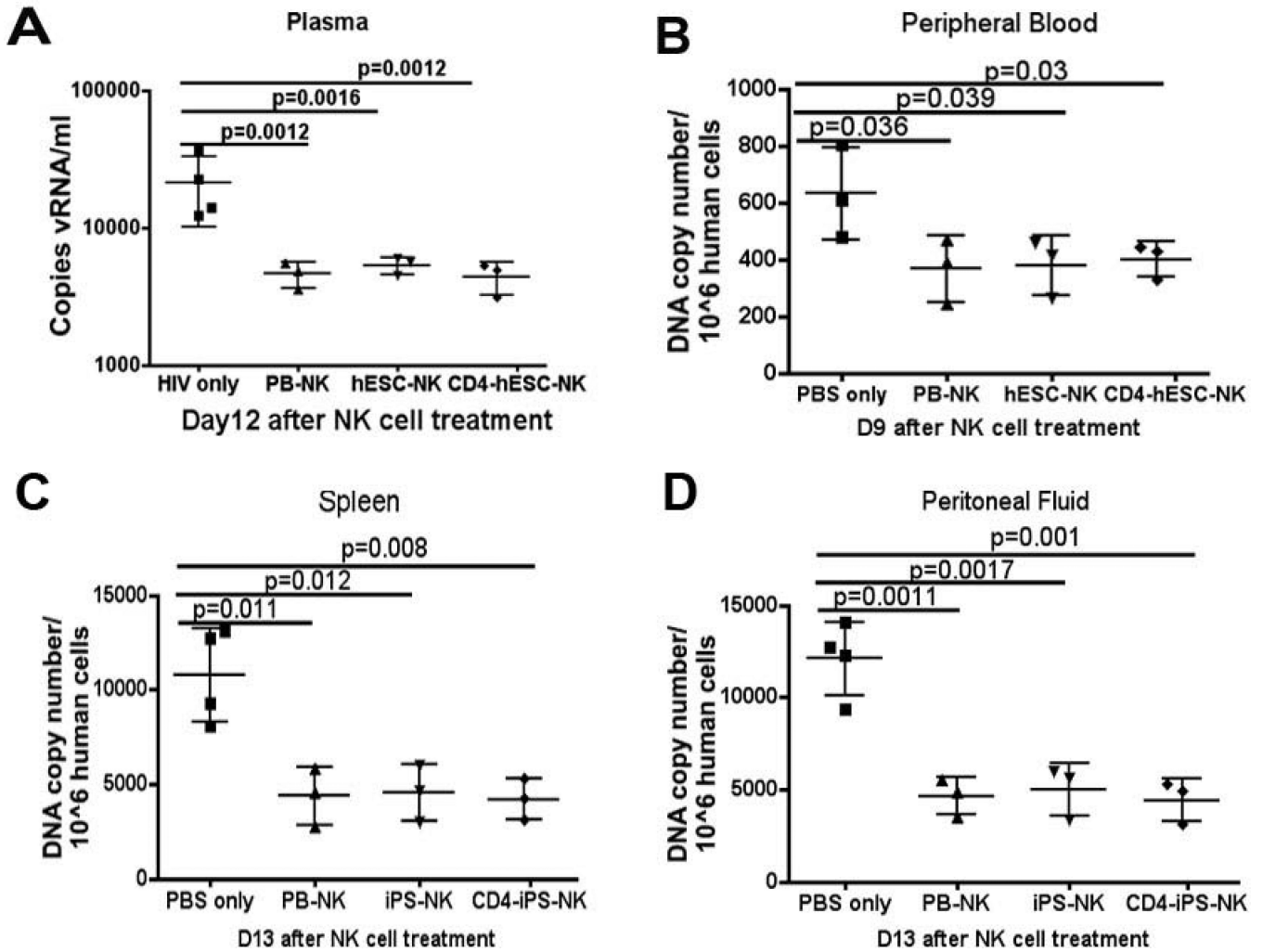


Figure 5. CD4 ζ - modified and unmodified hESC-NK cells suppress HIV replication in the plasma and tissues of PBL-NSG mice
 (A) Blood plasma from HIV infected mice was collected 12 days after NK cell treatment. Viral RNA levels per sample were determined by quantitative reverse transcriptase (Q-RT)-PCR and results were calculated based on the standard LTR cDNA copy numbers. The points represent the copies of HIV RNA per milliliter of blood and the solid line represents mean per group. HIV proviral DNA was quantitatively assessed in human cells from peripheral blood collected on day 6, 9, 12 day after NK cell treatment. The DNA level was detected by Q-PCR at day 9 (B). Mice were sacrificed at day 13 of NK cell treatment and cells were collected from spleen (C) and peritoneal fluid (D) for Q-PCR. The points represent the copies of HIV proviral DNA per 10⁶ human CD45⁺ cells and the solid line represents mean per group. Statistical comparison was performed using prism 5 between NK cell treated groups vs. non-treated group. The solid lines represent the mean \pm the SD. The data are representative of one of 3 experiments with at least 3 mice each group.

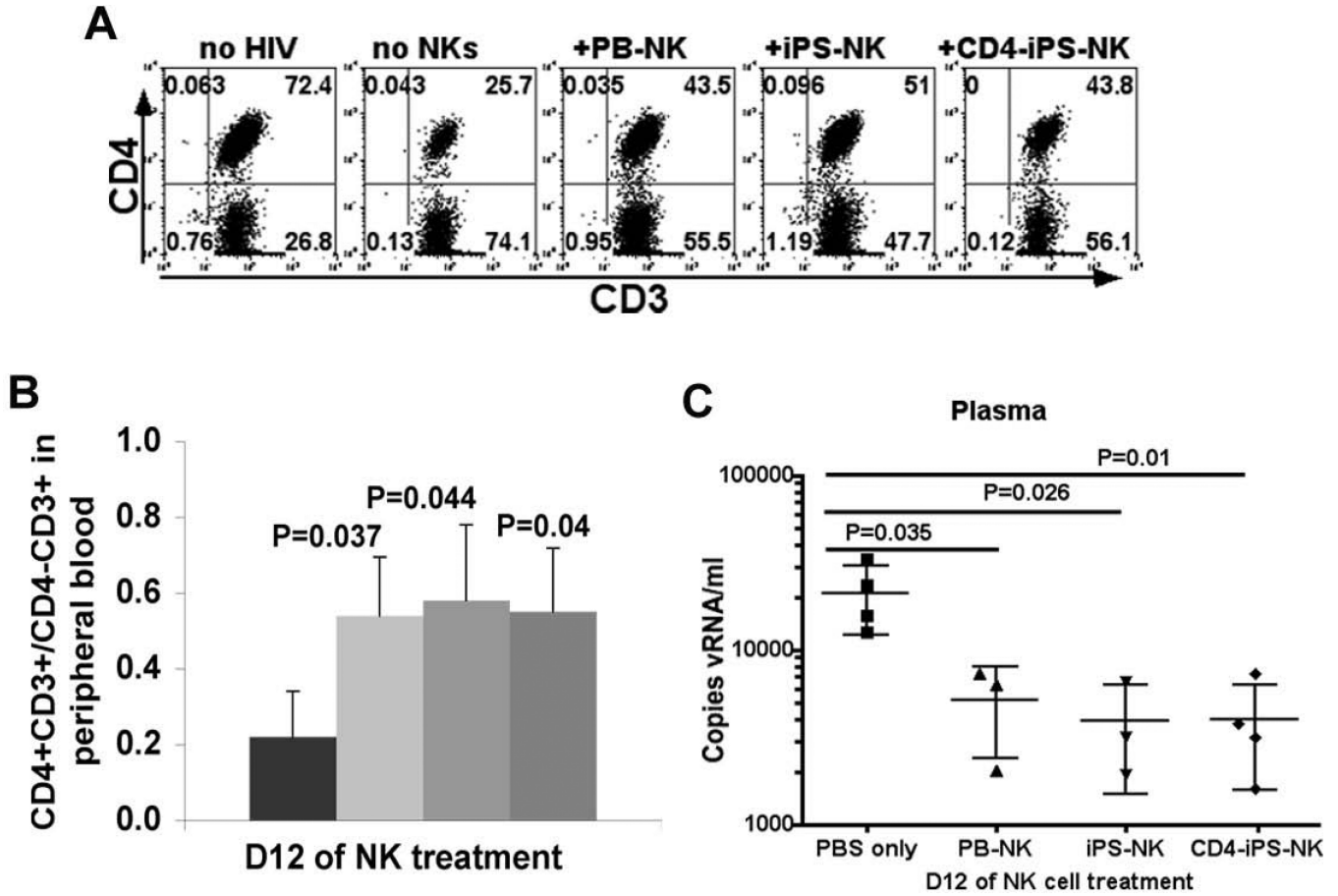


Figure 6. iPSC- and CD4 ζ - expressing NK cells suppress HIV replication in peripheral blood in HIV-infected mice

Peripheral blood was collected from HIV-infected NSG mice after NK cell treatment. CD4⁺ T cell levels were determined by flow cytometry for CD4⁺CD3⁺/CD4⁻CD3⁺. HIV infected human cells were evaluated by gag p24⁺. **(A)** Human CD45⁺ cells that express CD3 and CD4 were assessed in peripheral blood of HIV-1-infected NSG mice treat with or without NK cells at day 12. All cells were hCD45⁺ gated. The flow cytometry plots are representative of 1 mouse of each group in at least 3 independent experiments with a minimum of 3 mice. **(B)** CD4⁺ T cell levels in peripheral blood of HIV infected mice determined by CD4⁺CD3⁺/CD4⁻CD3⁺ ratios after NK cell treatment at day 12. **(C)** HIV infection was evaluated by the percentages of p24⁺ cells in CD3⁺CD8⁻. Data in **(B)** and **(C)** represent the average of one of three separated experiments with at least 3 mice in each group, the error bars indicate the mean +/- the SD.