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Engineering Cellular Resistance to HIV-1 Infection *In Vivo* Using a Dual Therapeutic Lentiviral Vector

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We described earlier a dual-combination anti-HIV type 1 (HIV-1) lentiviral vector (LVsh5/C46) that downregulates CCR5 expression of transduced cells via RNAi and inhibits HIV-1 fusion via cell surface expression of cell membrane-anchored C46 antiviral peptide. This combinatorial approach has two points of inhibition for R5-tropic HIV-1 and is also active against X4-tropic HIV-1. Here, we utilize the humanized bone marrow, liver, thymus (BLT) mouse model to characterize the *in vivo* efficacy of LVsh5/C46 (Cal-1) vector to engineer cellular resistance to HIV-1 pathogenesis. Human CD34+ hematopoietic stem/progenitor cells (HSPC) either nonmodified or transduced with LVsh5/C46 vector were transplanted to generate control and treatment groups, respectively. Control and experimental groups displayed similar engraftment and multilineage hematopoietic differentiation that included robust CD4+ T-cell development. Splenocytes isolated from the treatment group were resistant to both R5- and X4-tropic HIV-1 during *ex vivo* challenge experiments. Treatment group animals challenged with R5-tropic HIV-1 displayed significant protection of CD4+ T-cells and reduced viral load within peripheral blood and lymphoid tissues up to 14 weeks postinfection. Gene-marking and transgene expression were confirmed stable at 26 weeks post-transplantation. These data strongly support the use of LVsh5/C46 lentiviral vector in gene and cell therapeutic applications for inhibition of HIV-1 infection.

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Subject Category: siRNAs, shRNAs, and miRNAs Therapeutic proof-of-concept

Introduction

It is estimated that 33 million individuals are currently infected with human immunodeficiency virus (HIV), and the ensuing disease of acquired immune deficiency syndrome remains a major concern for global public health.[1] Advances in treatment for HIV infection, such as the discovery and implementation of highly active antiretroviral therapy have dramatically restored the life expectancy and quality of life for HIV-positive patients.[2] However, multiple complications associated with highly active antiretroviral therapy have been described, including drug toxicities over short- and long-term use, noncompliance to the daily drug regimen, and development of drug-resistant HIV type 1 (HIV-1) viral strains.[3–7] Furthermore, highly active antiretroviral therapy is not a curative approach for the treatment of HIV infection, and there are no vaccines currently effective against HIV.[8–10] Thus, the development of alternative methods is desired to provide a one-time or infrequent treatment that would reduce, if not eliminate, the requirements of highly active antiretroviral therapy to treat HIV-positive patients. Recently, the first documented case of a "functional cure" for HIV-1 infection has been reported, in which the patient received a bone marrow transplant from a donor homozygous for the Δ32 CCR5 deletion.[11–14] Alternatively, autologous cells can be engineered resistant to HIV-1 infection by transduction with lentiviral vectors that express anti-HIV genes that target various aspects of the HIV-1 lifecycle such as the HIV coreceptor CCR5.[15–17]

We previously developed a third generation self-inactivating lentiviral vector that expresses two anti-HIV agents: sh5, a short hairpin RNA (shRNA) specific to human CCR5 that is expressed from the H1 promoter, and C46, a cell membrane-anchored HIV-1 fusion inhibitor that is expressed from the Ubiquitin C promoter.[18] This dual combination vector named LVsh5/C46, or Cal-1, provides two points of inhibition for R5-tropic HIV-1, is active against HIV-1 strains that do not use CCR5 such as X4-tropic HIV-1, and has been shown to protect transduced cells from a broad range of HIV-1 strains including lab adapted and clinical isolates from various clades (B and D) with the three major tropisms (R5-, X4-, and dual-tropic).[18] The two anti-HIV agents, sh5 and C46, inhibit separate immediate-early stages of the viral lifecycle prior to entry, thus preventing accumulation of postintegrated provirus and reduces potential occurrence of escape mutations to a single agent. Sh5, a CCR5-specific shRNA, degrades CCR5 mRNA and prevents protein production thereby inhibiting cell surface expression of CCR5. This particular shRNA to CCR5 (referred to as CCR5 shRNA 1005) has been extensively characterized in primary human cells *in vitro* and in the humanized bone marrow, liver, thymus (BLT) mouse model *in vivo*.[19–22] In addition, a rhesus macaque-adapted analog of this shRNA displayed stable CCR5 downregulation in nonhuman primate hematopoietic stem/progenitor cells (HSPC) transplant studies.[23,24] C46 antiviral peptide is a membrane-anchored C-peptide specific to HIV-1 envelope

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glycoprotein gp41 and prevents the conformational change required for viral envelope fusion with the cellular membrane.[25] C46 has been extensively characterized in preclinical studies which demonstrated significant protection from a broad range of HIV-1 strains with minimal evidence for development of viral escape and has also been tested in a phase 1 clinical trial treating HIV-1 positive patients with gene-modified autologous CD4+ T lymphocytes.[25–29] More recent preclinical efficacy studies using transduced autologous CD34+ HSPC has demonstrated significant C46-mediated protection from SHIV in nonhuman primates.[27,30,31] The combined utility of C46 expression and CCR5 downregulation via RNAi makes the LVsh5/C46 vector a powerful approach for engineering cellular resistance to HIV-1 infection.

Here, we report the first demonstration of LVsh5/C46 vector to engineer human hematopoietic cellular resistance to HIV-1 infection *in vivo* using the NOD/SCID/IL2rγ$^{-/-}$ (NSG) humanized BLT mouse model. The BLT mouse model is the most advanced small-animal model for HIV-1 pathogenesis *in vivo*.[32–34] Preclinical LVsh5/C46 vector, manufactured according to current Good Laboratory Practice, was utilized to genetically modify human CD34+ HSPC prior to generating BLT mice. The safety and feasibility of LVsh5/C46-transduced human CD34+ HSPC to engraft and differentiate *in vivo* were compared to nonmodified human CD34+ HSPC. LVsh5/C46-transduced human CD34+ HSPC differentiated into multiple hematopoietic lineages, including CD4+ T-cells, and LVsh5/C46 marking and transgene expression were confirmed up to 6 months post-transplantation. The ability of the LVsh5/C46 vector to engineer cellular resistance to HIV-1 infection was assessed in *ex vivo* challenge experiments using both R5-tropic and X4-tropic strains of HIV-1, and in *in vivo* challenge experiments with R5-tropic HIV-1 spanning 14 weeks postinfection. Downregulation of CCR5 expression in CD4+ T-cells was observed *in vivo* using a reporter version of LVsh5/C46 (LVsh5/C46/ZsG). These studies provide data that support the efficacy of LVsh5/C46 vector to inhibit R5- and X4-tropic strains of HIV-1, including *in vivo* protection from high-dose R5-tropic HIV-1 challenge, which resulted in protection of CD4+ T-cells and reduced viral load within peripheral blood and lymphoid tissues.

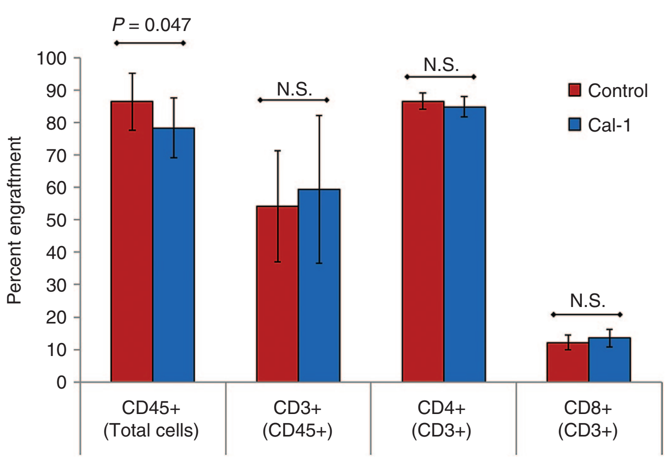

Figure 1 Peripheral blood engraftment at week 12 post-transplantation. FACS analysis of peripheral blood samples was conducted to determine the level of hematopoietic engraftment of LVsh5/C46 treated CD34+ HSPC (Cal-1) compared to nonmodified human CD34+ HSPC (Control). Percentage of each human cell population is displayed and the parental cell population is listed below in parenthesis. The average value of 11 animals per group is plotted with error bars representing the SD. N.S.: nonsignificant difference ($P > 0.05$) between the average means of each group using a two-tailed unpaired *t*-test.

Results

Engraftment of LVsh5/C46 vector transduced human CD34+ HSPC

The humanized BLT mouse model was utilized to characterize the ability of LVsh5/C46 (Cal-1) vector to engineer human hematopoietic cellular resistance to HIV-1 infection *in vivo*. Two groups of BLT mice were generated in parallel: a control group using nonmodified human CD34+ HSPC, and a treatment group using human CD34+ HSPC transduced with LVsh5/C46 vector. Transduction efficiency was determined as 2.86 ± 0.27 vector copies/cell on day 12 post-transduction by qPCR, and we hypothesize that the majority of cells were transduced with at least one vector copy per cell. The ability of LVsh5/C46 modified human CD34+ HSPC to engraft and differentiate *in vivo* was assessed using fluorescence-activated cell sorting (FACS) analysis of

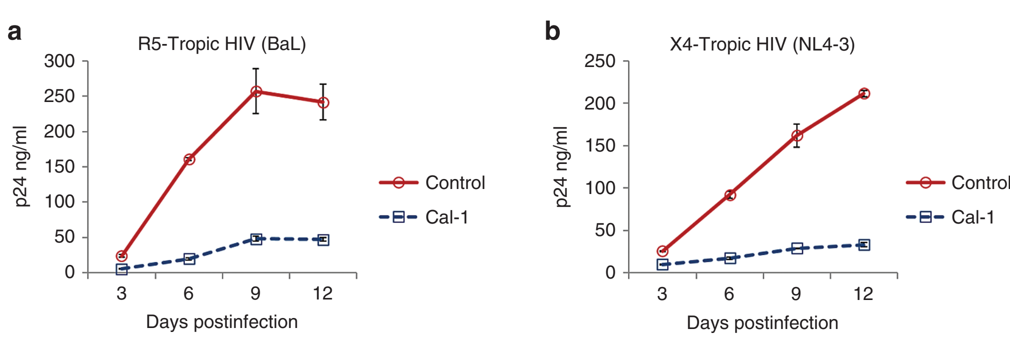

Figure 2 LVsh5/C46-mediated resistance to R5- and X4-tropic HIV-1 infection. *Ex vivo* challenge experiments were performed using bulk splenocytes from BLT mice humanized with LVsh5/C46 transduced CD34+ HSPC (Cal-1) or with mock transduced CD34+ HSPC (Control). Splenocytes were harvested at week 12 post-transplantation from each group of animals and stimulated for 5 days with IL-2 and phytohaemagglutinin prior to challenge with R5-tropic BaL HIV-1 (**a**) or X4-tropic NL4-3 HIV-1 (**b**). At days 3, 6, 9, and 12 postinfection supernatants were collected and analyzed for HIV Gag p24 protein by ELISA to monitor the amount of HIV replication in culture. Error bars represent the SD of triplicate p24 ELISA assays.

peripheral blood samples at week 12 post-transplantation (**Figure 1**). LVsh5/C46-transduced CD34+ HSPC and nonmodified CD34+ HSPC both displayed robust engraftment of human CD45+ cells *in vivo* (control: 87%, treatment: 78%, average of total cell population). Robust development of CD3+ T-cells was observed within the human CD45+ leukocyte population for both groups of animals (control: 54%, treatment 59%, average within CD45+ population). The control and experimental groups displayed similar development of human CD4+ T-cells (control: 87%, LVsh5/C46: 85%, average within CD3+ population) and CD8+ T-cells (control: 12%, LVsh5/C46: 14%, average within CD3+), and CD4+/CD8+ T-cell ratios (control: 7.4, LVsh5/C46: 6.6%, average ratio within CD3+). No statistically significant difference was observed between the groups regarding T-cell engraftment and differentiation. Overall, these data support the safety and feasibility of LVsh5/C46-modified CD34+ HSPC to efficiently engraft *in vivo* and undergo multilineage hematopoietic development, including CD4+ T-cell differentiation.

LVsh5/C46-mediated resistance to R5- and X4-tropic HIV-1 infection

Ex vivo HIV-1 challenge experiments were conducted to evaluate the ability of LVsh5/C46 vector to confer protection to human hematopoietic cells from HIV-1 pathogenesis. BLT mice from the control group and treatment group were sacrificed at week 12 post-transplantation and bulk splenocytes were isolated and stimulated with interleukin-2 (IL-2) and phytohemagglutinin prior to infection with R5-tropic (BaL) or X4-tropic (NL4-3) HIV-1 at a MOI of 1. Splenocytes from control animals displayed robust HIV-1 replication for both R5-tropic and X4-tropic HIV; conversely, splenocytes from the treatment group of animals displayed minimal evidence of HIV-1 replication for both R5-tropic (BaL) and X4-tropic (NL4-3) HIV-1 (**Figure 2**). At day 12 postinfection, the amount of p24 for LVsh5/C46 conditions was reduced 5.2-fold for R5-tropic BaL HIV-1 and 6.4-fold for X4-tropic NL4-3 HIV-1 when compared with nontransduced controls. Overall, these data provide strong evidence for LVsh5/C46-mediated protection from both R5-tropic and X4-tropic HIV-1 infection.

In vivo protection of CD4+ T-cells and reduced HIV-1 viral load

At 12 weeks post-transplantation of human CD34+ HSPC, control BLT mice (N = 8) and treatment BLT mice (N = 8) were intravenously infected with a high dose of R5-tropic BaL HIV-1 (1,600 ng p24 per animal). CD4+ T-cell levels and viral load in peripheral blood were assessed up to week 14 postinfection to determine the pathogenic effect of HIV-1 infection. Within the control animals, human CD4+ T-cell percentages gradually declined over the course of the infection, most severely at weeks 10–14 postinfection when compared with that of the treatment group (**Figure 3a**). In contrast, the treatment group of animals stably maintained human CD4+ T-cells levels within the peripheral blood up to terminal analysis at week 14 postinfection, and this protection was statistically significant when compared with control animals overall (likelihood ratio test $P < 0.05$). HIV-1 plasma viremia analysis was performed to assess the viral load within peripheral blood of animals from control and treatment groups. Control animals displayed high levels of HIV-1 plasma viremia, while the treatment group of animals displayed background levels of detection. Differences in viral load between the control and treatment groups were statistically significant overall (likelihood ratio test $P < 0.05$) and at individual time points starting at week 8 postinfection through terminal analysis at week 14 postinfection (adjusted $P < 0.05$, **Figure 3b**). Recently, this Roche-based HIV-1 viral load assay has been shown to cross-react with lentiviral vector sequences in X-SCID gene therapy trials, which likely accounts for the increased level of background observed in baseline samples (week 0 postinfection) collected from the treatment group of animals prior to HIV-1 infection.[35] Overall, maintenance of CD4+ T-cell levels and reduced viral load provides strong evidence for LVsh5/C46-mediated protection from HIV-1 pathogenesis *in vivo*.

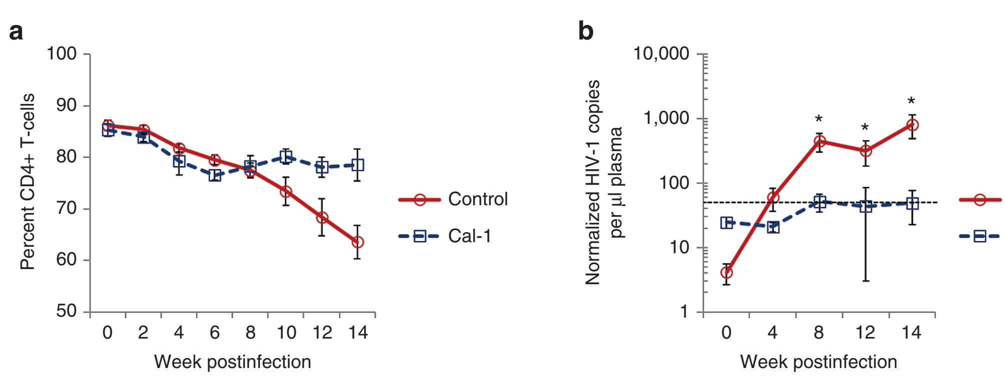

Figure 3 Protection of CD4+ T-cells and reduced HIV-1 viral load within peripheral blood. At 12 weeks post-transplantation of human CD34+ HSPC, eight control animals and eight LVsh5/C46-treated animals were intravenously infected with R5-tropic HIV BaL, and the pathogenic effects within the peripheral blood was monitored every 2 weeks until terminal analysis at week 14 postinfection (p.i.). (**a**) Percentage of human CD4+ T-cells within the CD45+CD3+ population was determined by FACS analysis. (**b**) HIV-1 plasma viremia was quantified via RT-qPCR viral load assay and normalized to input control viral RNA per microliter. The mean values per control group (closed circles) and treatment group (open squares) are plotted with error bars representing the SE of the mean. A statistically significant difference in CD4+ T-cell levels and viral loads was observed between control and treatment groups (likelihood ratio test $P < 0.05$). Asterisk indicates a statistically significant difference in post-hoc tests between the groups per time point (Adjusted $P < 0.05$). Dotted line indicates relative limit of detection of HIV-1 copies per microliter plasma.

At week 14 postinfection, the remaining animals were sacrificed and processed to further characterize the protective effects of LVsh5/C46 on HIV-1 infection *in vivo*. FACS analysis of CD4+ T-cell percentages and qPCR analysis of HIV-1 DNA proviral load was conducted on cellular samples harvested from bone marrow, spleen, and thymus (human thymus/liver implants) of control ($N = 7$) and LVsh5/C46-treated ($N = 6$) BLT mice. Consistent with results observed in the peripheral blood, control animals displayed a reduction in CD4+ T-cells percentages when compared with the LVsh5/C46 treatment group within bone marrow (control: 36%, LVsh5/C46: 61%, average CD4+) and spleen (control: 57%, LVsh5/C46: 77%, average CD4+), and also a reduction of CD4+ thymocytes within the thymus (control: 66%, LVsh5/C46: 91%, average CD4+) ($P < 0.05$ for all tissues, **Figure 4a**). These data confirm significant LVsh5/C46-mediated protection of CD4+ T-cells within the tissue of animals up to 14 weeks postinfection with HIV-1.

HIV-1 DNA proviral load analysis was then performed to compare the level of infection between the control and LVsh5/C46 treated groups of animals using genomic DNA extracted from the bone marrow, spleen, and thymus of each animal at terminal analysis. HIV-1 DNA was detected within all control animals and the level of proviral load was significantly higher within the bone marrow, spleen, and thymus when compared with LVsh5/C46-treated animals ($P < 0.05$ for all tissues, **Figure 4b**). Interestingly, HIV-1 DNA was not detected within any of the tissues of LVsh5/C46-treated animals when using a qPCR assay that has a limit of detection of ~1 HIV DNA copy per 1,000 cells. These data confirm LVsh5/C46-mediated reduction of HIV-1 proviral load within the tissue of animals up to 14 weeks postinfection. These data provide compelling evidence that LVsh5/C46-modified human CD34+ HSPC engrafted and differentiated normally *in vivo* and that the progeny of LVsh5/C46-modified CD34+ HSPC conferred protection from HIV-mediated pathogenesis and significantly reduced viral load within peripheral blood and tissues up to 6 months post-transplantation.

Stable LVsh5/C46 gene-marking and transgene expression *in vivo*

To confirm the presence and stability of LVsh5/C46-modified cells, LVsh5/C46 gene-marking analysis was performed at week 26 post-transplantation (week 14 postinfection). LVsh5/C46 gene-marking was detected within the peripheral blood (0.60 ± 0.22 average vector copies/cell), bone marrow (1.30 ± 0.62 average vector copies/cell), spleen (1.23 ± 0.30 average vector copies/cell), and thymus (0.39 ± 0.29 average vector copies/cell) of each animal in the LVsh5/C46 treatment group ($N = 6$) with a total average of 0.88 ± 0.45 LVsh5/C46 vector copies/cell within the tissues analyzed at week 26 post-transplantation (**Figure 5a**). Gene-marking was not measured over time to determine if LVsh5/C46-modified cells had a selective advantage during *in vivo* HIV-1 challenge. The vector copy number values of samples obtained post-transplantation were slightly less when compared with

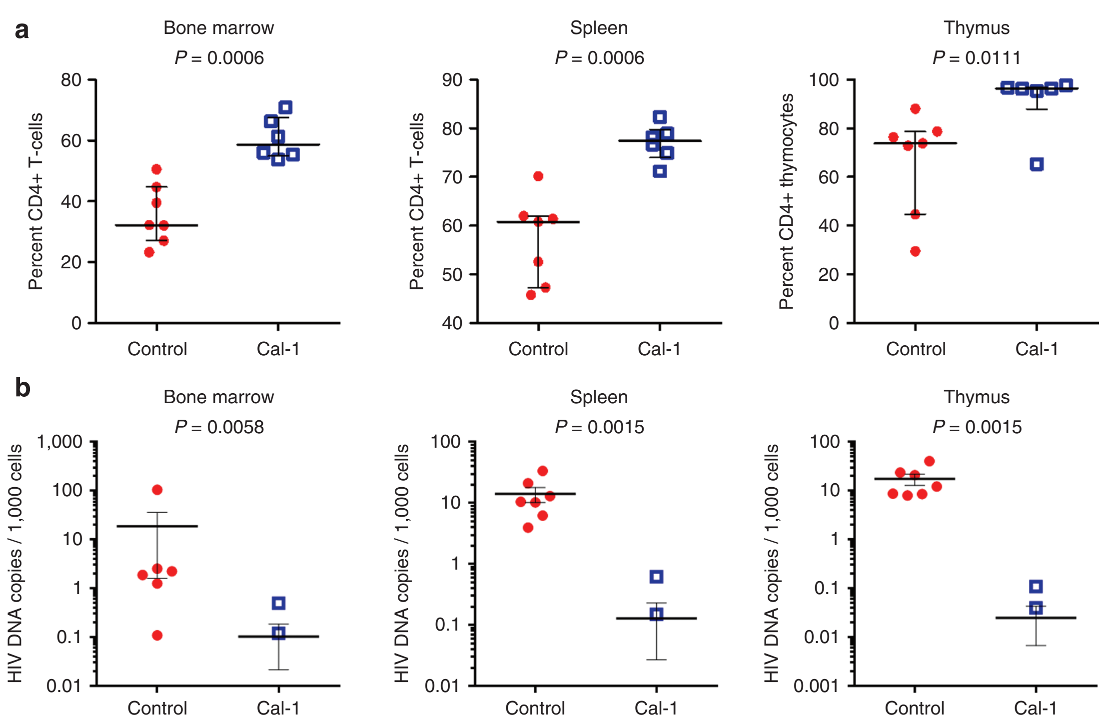

Figure 4 Protection of CD4+ cells and reduced HIV-1 proviral load within lymphoid tissues. Terminal analysis was performed on remaining animals at week 14 postinfection. (**a**) Percentage of human CD4+ T-cells within the bone marrow and spleen, and percentage of CD4+ thymocytes within the thymus was determined by FACS analysis of the human CD45+CD3+ parental population. (**b**) HIV-1 DNA proviral load was quantified via qPCR analysis of genomic DNA extracted from bone marrow, spleen, and thymus. Values are displayed for individual control animals ($N = 7$; closed circles) and treatment animals ($N = 6$; open squares). The median for each group is displayed as a horizontal line, and error bars represent the interquartile range. Mann–Whitney tests confirmed significant differences between the distributions of each group for all tissues ($P < 0.05$).

the transduction efficiency assessment of CD34+ cells from extended culture. The difference in these values can be attributed to the transduction efficiency assessment being from a bulk culture of CD34+ cells and that the hematopoietic stem cells that engrafted likely had a reduced vector copy number value when compared with that of further differentiated hematopoietic progenitors within culture. As expected, the LVsh5/C46 vector sequence was not detected within control animals. These data demonstrate effective engraftment of LVsh5/C46 vector-modified HSPC and the persistence of gene-modified cells at significant levels *in vivo* for up to 6 months.

LVsh5/C46 transgene expression was determined within animals at terminal analysis to confirm that both sh5 and C46 transgenes were stably expressed within the peripheral blood and lymphoid tissues of treatment group animals up to 6 months post-transplantation of transduced human CD34+ HSPC (14 weeks postinfection with HIV-1). RT-qPCR assays confirmed expression of both sh5 and C46 transgenes within the peripheral blood, bone marrow, spleen, and thymus of all treatment group animals at week 26 post-transplantation; as expected, sh5 and C46 target sequences were not detected within control animals (**Figure 5b**,**c**). Overall, these data strongly support the safety and feasibility of LVsh5/C46-transduced human CD34+ HSPC to engraft *in vivo* and produce progeny with stable LVsh5/C46 gene-marking and persistent expression of both sh5 and C46 transgenes within the peripheral blood and lymphoid tissues of treated animals for up to 6 months post-transplantation.

In vitro CCR5 downregulation by LVsh5/C46/ZsG reporter lentiviral vector

A reporter vector version of LVsh5/C46 vector which incorporates an EF1α promoter-driven ZsGreen expression cassette (LVsh5/C46/ZsG) was generated to allow characterization specifically of transduced cells modified to express sh5 and C46. PBMC were transduced with LVsh5/C46/ZsG, ZsGreen reporter vectors that express sh5 or C46 alone, or a control vector (ZsGreen alone). Significant downregulation of CCR5 cell-surface expression was observed in cells transduced with vectors containing sh5 (LVsh5 and LVsh5/C46/ZsG) (**Figure 6a**,**b**). LVsh5/C46/ZsG dual vector achieved similar levels of CCR5 downregulation as with the LVsh5 single vector, demonstrating that downstream addition of the C46 expression cassette does not interfere with shRNA activity. The expression of CXCR4, which is the second most commonly utilized HIV-1 coreceptor (the first one being CCR5 (ref. 36)), was not affected by any of the vectors

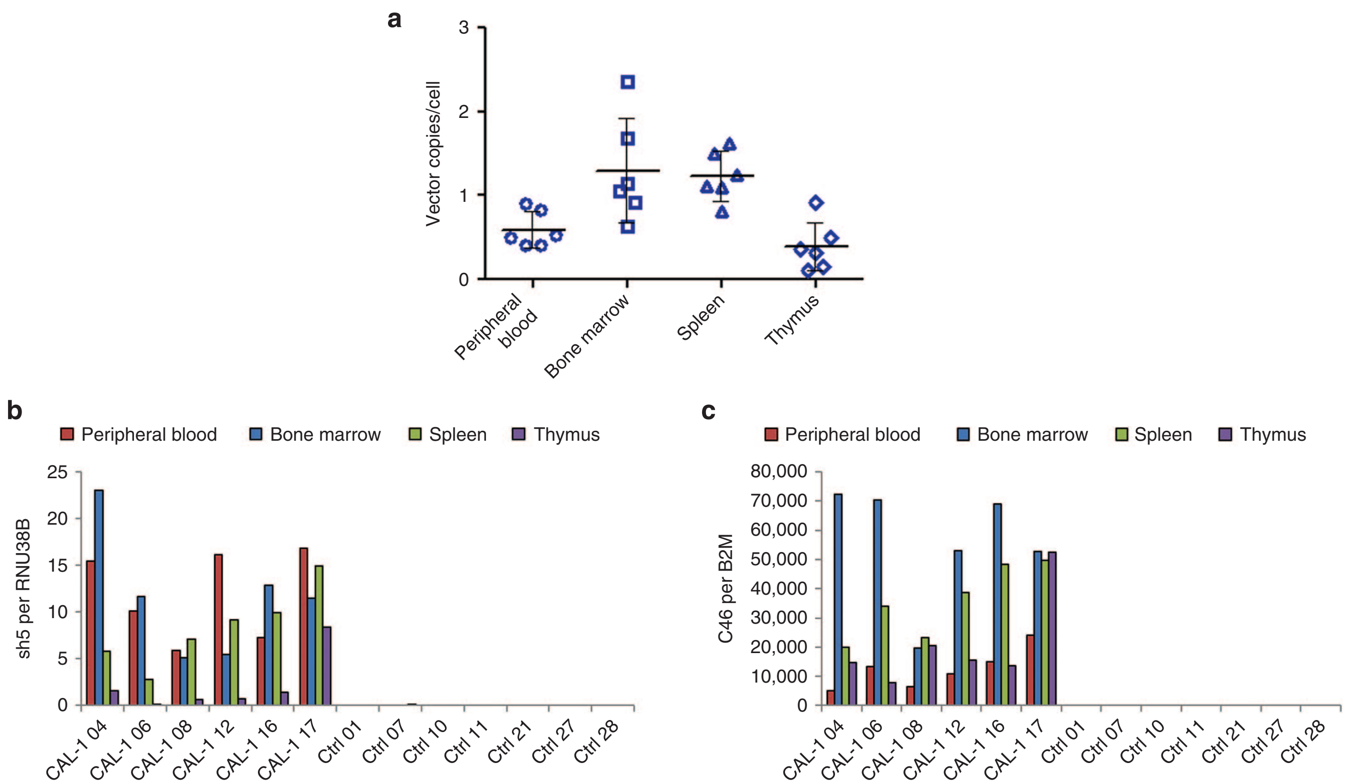

Figure 5 Stable LVsh5/C46 gene-marking and transgene expression at week 26 post-transplantation (week 14 p.i.). (**a**) LVsh5/C46 gene-marking was determined by performing qPCR analysis of DNA extracted from peripheral blood and tissues of animals at terminal analysis. LVsh5/C46 vector copies per cell are displayed for each treated animal (*N* = 6) within each tissue analyzed. The mean for each tissue is displayed as a horizontal line, and error bars represent the SD. LVsh5/C46 vector was not detected within any samples of Control animals. (**b**) sh5 and (**c**) C46 transgene expression was determined by performing RT-qPCR analysis of RNA extracted from peripheral blood and tissues of animals at terminal analysis. Expression of sh5 was normalized to the expression of human small RNA reference gene RNU38B, and expression of C46 mRNA was normalized to human β-2 microglobulin (B2M) mRNA relative expression. Results are displayed as the average value of duplicate sample analysis.

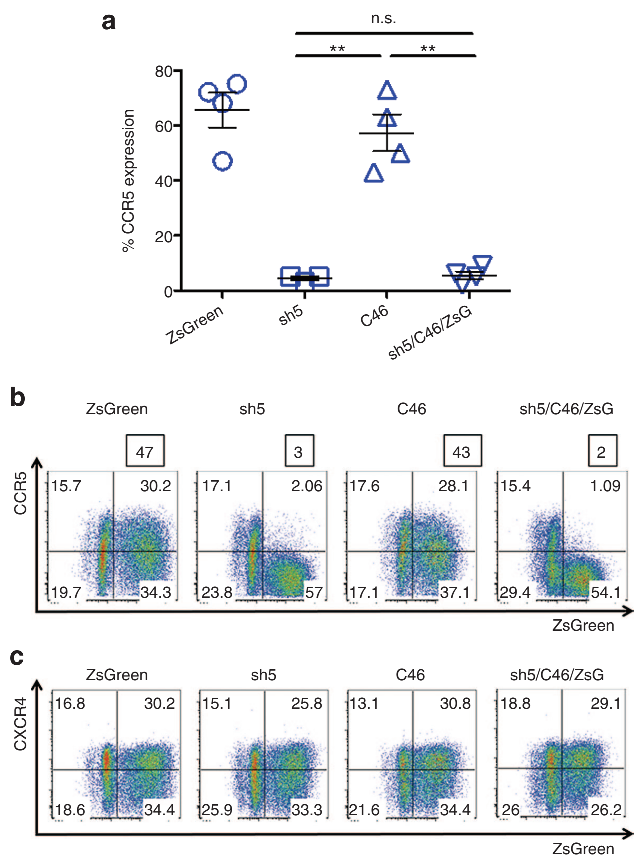

Figure 6 CCR5 downregulation by LVsh5/C46/ZsG reporter vector in human PBMC *in vitro*. Human PBMCs were stimulated with phytohemagglutinin then IL-2 for 2 days and transduced with four different lentiviral vectors (ZsGreen vector does not express anti-HIV genes but express only ZsGreen: open circles; sh5 vector expresses sh1005 and ZsGreen: open squares; C46 vector expresses C46 and ZsGreen: open triangles; sh5/C46/ZsG express sh1005, C46 and ZsGreen: open inverted triangles). CCR5 expression was examined 12 days postlentiviral vector transduction by monoclonal antibody staining of CCR5 and flow cytometric analysis. (**a**) The percentage of CCR5-expressing cells within the ZsGreen positive population was calculated from four independent experiments. n.s. = not significant, $*P< 0.05$, $**P < 0.005$ (Unpaired *t*-test with welch's correction). (**b**) A representative data from one of the four experiments graphed in **a**. The percentage of CCR5-expressing cells within the ZsGreen positive population is shown above each plot. (**c**) Representative data from the same experiment as in **b**. displaying CXCR4 expression.

(**Figure 6c**). Furthermore, transduction of PBMC with LVsh5/C46/ZsG provided resistance to infection by both R5-tropic and X4-tropic reporter strains of replication competent HIV (**Supplementary Figure S1**).

In vivo CCR5 downregulation by LVsh5/C46/ZsG reporter lentiviral vector

Human fetal liver derived CD34+ HSPC were transduced with either LVsh5/C46/ZsG vector (MOI 5–20) or a control vector (MOI 1–3) that expresses the mCherry reporter gene. The average transduction efficiency was ~40% for the LVsh5/C46/ZsG vector and 55% for the control vector. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were then transplanted with a 50 : 50 mixture of therapeutic vector and control vector transduced cells along with thymus pieces to generate hu-BLT mice, as described earlier.[22] In this manner, the impact of the therapeutic and control vectors can be measured independently within the same animal. At week 20 post-transplantation, both ZsGreen and mCherry expressing cells were detected in human CD45+ populations of CD3+ T lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, CD19+ B lymphocytes, and CD14+ monocyte/macrophages in peripheral blood, bone marrow, spleen, and the gut-associated lymphoid tissue (**Supplementary Table S1**). These results demonstrate that LVsh5/C46/ZsG vector-transduced human CD34+ HSPC can differentiate into multilineage hematopoietic cells in peripheral blood and multiple lymphoid tissues.

We next examined CCR5 downregulation in LVsh5/C46/ZsG vector-transduced CD4+ T lymphocytes in lymphoid tissues at 20 weeks post-transplantation. CCR5 expression was efficiently downregulated in ZsGreen+ CD4+ T lymphocytes in peripheral blood, spleen, bone marrow, and the gut-associated lymphoid tissue (**Figure 7**). In contrast, CCR5 expression remained elevated in mCherry+ control CD4+T lymphocytes, showing the specificity of CCR5 downregulation by the LVsh5/C46/ZsG vector. Furthermore, *ex vivo* challenge of purified ZsGreen- or mCherry-expressing splenocytes demonstrated that LVsh5/C46/ZsG vector engineered cellular resistance to infection from both R5-tropic and X4-tropic strains of HIV-1 (**Supplementary Figure S2**). Overall, these data demonstrate that LVsh5/C46/ZsG vector-transduced human CD34+ HSPC can support reconstitution of multilineage hematopoietic engraftment and differentiation in the humanized BLT mouse model, efficiently downregulate CCR5 expression in human CD4+ T lymphocytes in multiple lymphoid tissues including the gut-associated lymphoid tissue, and engineer cellular resistance to both R5-tropic and X4-tropic HIV infection.

Discussion

We have developed a third generation lentiviral vector (LVsh5/C46) that engineers cellular resistance to HIV-1 infection by expressing two active anti-HIV agents: sh5, a siRNA that downregulates CCR5 expression, and C46, a cell surface membrane-anchored fusion inhibitor peptide. The sh5 transgene has been proven safe and effective in delivering stable CCR5 downregulation in multiple studies using human cells, humanized mouse models, and nonhuman primate models[19,20,22,23,37], and the C46 transgene has also been proven effective at inhibiting HIV-1 infection in numerous preclinical studies and was recently shown to be safe for clinical use.[25–28] The LVsh5/C46 vector is unique in that it expresses both of these potent anti-HIV agents within a single lentiviral vector and is thus powered by its ability to confer cellular resistance to HIV-1 infection by targeting two immediate-early stages of HIV-1 infection. The combination of these two highly effective agents allows for several advantages compared to either transgene alone, such as providing two separate mechanisms that protect against R5-tropic HIV-1 to reduce potential development of single agent escape mutations and also providing active protection from a broad range of HIV-1 variants that includes X4-tropic HIV-1. Previous reports also indicate

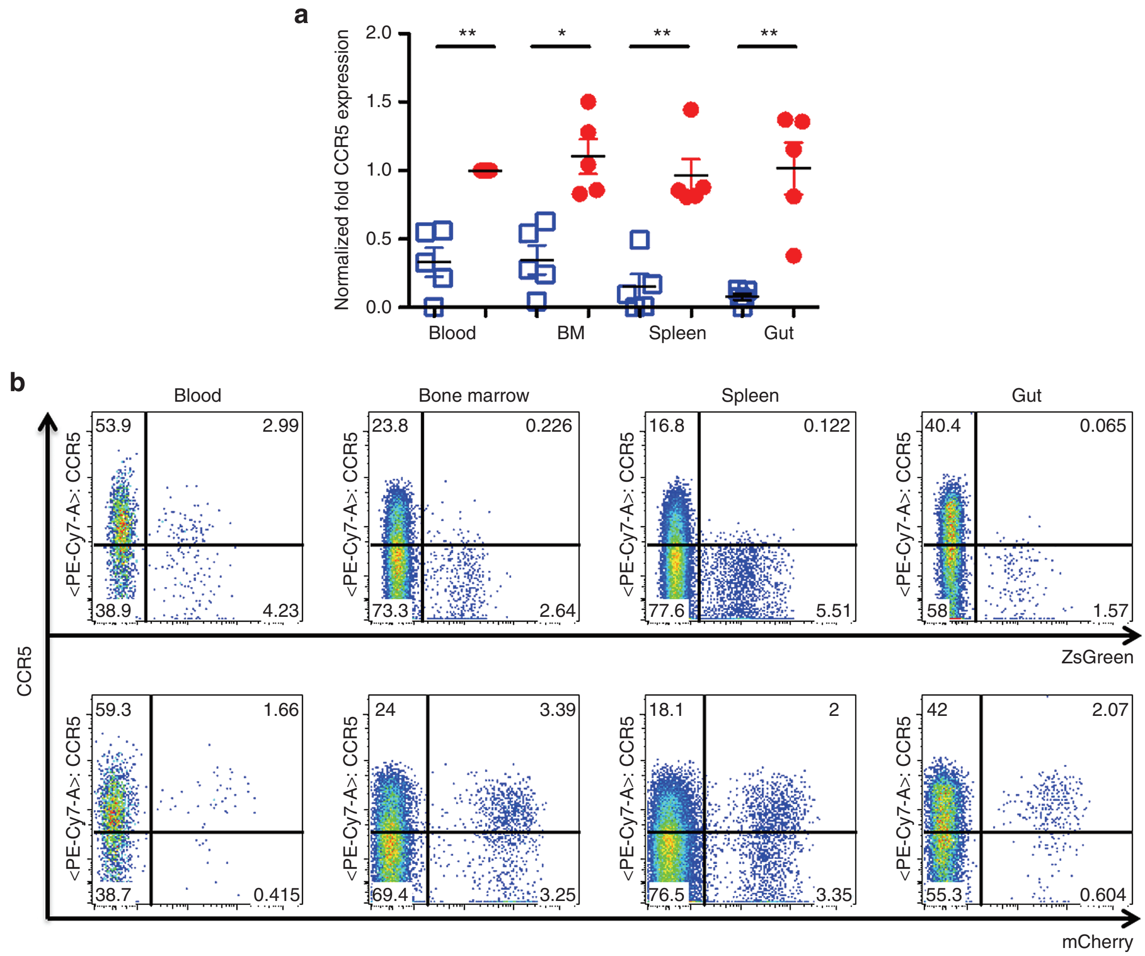

Figure 7 CCR5 downregulation in LVsh5/C46/ZsG reporter vector marked human CD4+ T lymphocytes *in vivo*. (**a**) The level of CCR5 expression was compared in ZsGreen-marked (LVsh5/C46/ZsG reporter vector: open squares) and mCherry-marked (control vector: filled circles) human CD4+ T lymphocytes in lymphoid tissues from the hu-BLT mice. Samples were obtained between 11 and 25 weeks post-transplantation. CCR5 expression level was normalized using the mean CCR5 expression in mCherry-positive cells from peripheral blood in order to effectively analyze CCR5 downregulation with different levels of CCR5, ZsGreen, and mCherry+ expression in tissues of each mouse. Each dot represents a mouse. Error bars show SE of the mean. ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.005$ (student *t*-test) (**b**) Representative data from a single mouse showing CCR5 reduction in ZsGreen but not mCherry transduced CD4+ T-cells in lymphoid tissues.

potential for a synergistic effect of the combined mechanisms by theoretically extending the therapeutic window of C46-related fusion inhibitors during instances of reduced coreceptor availability.[38–40]

Here we utilized the humanized BLT mouse model during preclinical studies designed to characterize the ability of LVsh5/C46 vector to protect human hematopoietic cells from HIV-1 infection *in vivo*. Human CD34+ HSPC were transduced with preclinical LVsh5/C46 vector at a MOI of 10 in a 24-hour transduction protocol resulting in 2.86 ± 0.27 vector copies/cell. No differences in engraftment were observed between control and experimental groups, which included robust CD4+ T-cells development. *Ex vivo* challenge experiments with splenocytes from the treatment group displayed protection from both R5-tropic and X4-tropic HIV-1 infection, with inhibition of HIV-1 replication as measured by p24 ELISA of culture supernatant. Twelve weeks after human CD34+ HSPC transplantation, high-dose intravenous infection of R5-tropic HIV-1 exists. Treatment group animals maintained CD4+ T-cell levels and reduced viral loads within the peripheral blood and lymphoid tissues. Interestingly, the treatment group of animals displayed background levels of HIV-1 detection within the peripheral blood and lymphoid tissues tested at 14 weeks postinfection. The lack of substantial evidence of HIV-1 detection within the treatment group suggests that LVsh5/C46-modified cells were able to systemically control the infection or potentially completely inhibit the initial viral challenge infection. Furthermore, LVsh5/C46 gene-marking and transgene expression was stable up to 6 months post-transplantation of transduced CD34+ HSPC, and a reporter version of the construct was able to demonstrate significant downregulation of CCR5 in vector-modified cells *in vivo*. In total, these data support the use of LVsh5/C46 vector as a single dose treatment that can constitutively deliver a therapeutic advantage to cells and allow for long-term engraftment and cellular resistance to HIV-1 pathogenesis *in vivo* by downregulating CCR5 expression and inhibiting HIV-1 fusion via C46. LVsh5/C46 lentiviral vector is built on decades of HIV-1 gene therapy experience and adds to the developing technologies designed to stably engineer cellular protection from HIV-1 infection such as CCR5-specific nucleases and immunoprophylaxis.[16,17,41,42] Currently, underway is a phase 1/2 clinical trial designed to test the safety and feasibility of infusing LVsh5/C46-modified CD34+ HSPC and CD4+ T-cells to treat HIV-1 infection without the use of antiretroviral drugs, and includes the use of busulfan as conditioning to enhance engraftment of LVsh5/C46-modified CD34+ HSPC.[43]

Materials and methods

Generation of humanized BLT mice. A total of 28 NOD/SCID/IL2rγ $^{-/-}$ (NSG) mice were used in the study to generate humanized BLT animals according to UCLA Humanized Mouse Core Laboratory procedures.[44] In brief, NSG animals were conditioned with total body irradiation at a dose of 270 rads, and then 14 animals per group received a cellular product consisting of 1×10^6 human fetal liver-derived CD34+ HSPC per mouse from a single human donor. CD34+ HSPC were either nonmodified (control group) or transduced with LVsh5/C46 vector at MOI of 10 (treatment group) during overnight culture in media supplemented with thrombopoietin, stem cell factor, and Flt3 ligand (50 ng/ml each) using RetroNectin (20 µg/ml) coated six-well plates. Each group of animals received 5×10^5 CD34+ HSPC transplanted with thymic stromal tissue and matrigel under the mouse kidney capsule, and an additional 5×10^5 CD34+ HSPC infused retro-orbitally on the same day. At week 12 post-transplantation, 11 animals per group survived with >10% human CD3+ T-cells, and these animals were then designated for balanced *ex vivo* and *in vivo* HIV-1 challenge analysis.

Ex vivo HIV-1 challenge. Animals were sacrificed at week 12 post-transplantation of human CD34+ HSPC, spleens were harvested, splenocytes were stimulated for 3 days in RPMI +10% FBS + IL-2 (20 U/ml) + phytohemagglutinin (5 µg/ml), then cryopreserved. Cryopreserved splenocytes were thawed and combined per group, then stimulated for 2 days with RPMI +20% FBS +100 U/ml IL-2 prior to HIV-1 challenge. A total of 2×10^6 cells per condition were challenged with R5-tropic BaL and X4-tropic NL4-3 strains of HIV at a MOI of 1. After 2-hour challenge, cells were cultured in RPMI + 20% FBS + 20 U/ml IL-2 for 12 days postinfection. Supernatant from cultures was collected on days 3, 6, 9, and 12 postinfection, and HIV-1 replication was monitored by performing p24 ELISA in triplicate for each sample. p24 ELISA was performed by the UCLA Virology Core Laboratory according to the Perkin Elmer p24 kit protocol.

Molecular and cellular analysis. HIV-1 strain BaL was prepared by infecting PM-1 cells and titre was determined using Perkin Elmer HIV-1 P24 ELISA. HIV-1 strain NL4-3 was grown in CEMx174 from plasmid-derived stock, and viral infectivity was determined by limiting dilution titration on CEMx174 cells. FACS analysis was performed as described earlier according to UCLA Humanized Mouse Core Laboratory procedures.[44] Quantitation of HIV-1 plasma viremia was performed by the UCLA Virology Core Laboratory according to the earlier published methods.[45] Results were reported using the following formula: normalized HIV-1 copies per µl of plasma = (HIV-1 copies/control HIV copies)*(number of control HIV copies spiked to plasma sample/volume of plasma sample). Quantitation of HIV-1 DNA proviral load was performed by UCLA Humanized Mouse Core Laboratory according to the earlier published methods.[46] The following primers were used to detect HIV-1 sequences not present within lentiviral vectors: Sense Primer: 5′-CAATGG CAGCAATTTCACCA-3′, Anti-Sense Primer: 5′-GAATGCC AAATTCCTGCTTGA-3′, Probe: 5′-(6-FAM)-CCCACCAACA GGCGGCCTTAA CTG-(Tamra-Q)-3′. LVsh5/C46 vector copy number per cell and expression of transgenes were determined as described earlier.[18]

Statistical analysis. For data measured at a single time point, two-tailed *t*-tests or Mann–Whitney tests (for skewed data) were performed to compare groups. Linear mixed effects models were used to compare the %CD4+ T-cells and the normalized HIV copy number per µl of plasma outcomes, where both models included a time–group interaction. *P* values for the group effect and time–group interaction effect were calculated using likelihood ratio tests of the model with the term of interest against the model excluding the term. *Post hoc* Wilcoxon rank sum tests were conducted at each time point and adjusted *P* values were calculated using Storey's false discovery rate. Mixed effect regression analyses were conducted using the lme4 (ref. 47) and qvalue[48] packages in R.[49] Additional analyses were conducted using GraphPad Prism (version 5.04) for Windows. Significance was assessed at the 0.05 level (for both adjusted and unadjusted *P* values) and all tests were two-tailed.

Ethics statement. Human fetal tissue was purchased from Advanced Biosciences Resources and the UCLA CFAR Gene and Cellular Therapy core laboratory, was obtained without personal identifying information, and did not require Institutional Review Board approval for use. Animal research described in this manuscript was performed under the written approval of the UCLA Animal Research Committee (ARC) in accordance to all federal, state, and local guidelines. These studies were conducted in strict accordance to The Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the accreditation and guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care (AALAC) under the UCLA ARC Protocol Number 2010-038-02B. All surgeries were performed under anesthesia using ketamine/xylazine and isofluorane, and all efforts were made to minimize pain and discomfort.

Supplementary material

Figure S1. Inhibition of R5-tropic and X4-tropic HIV infection by LVsh5/C46/ZsG reporter vector in PBMC in vitro.
Figure S2. Inhibition of R5-tropic and X4-tropic HIV infection by LVsh5/C46/ZsG reporter vector in splenocytes ex vivo.
Table S1. In vivo multi-lineage reconstitution of LVsh5/C46/ZsG reporter lentiviral vector transduced cells.

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