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Analysis and dissociation of anti-HIV effects of shRNA to CCR5 and the fusion inhibitor C46.

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Background:

The gene therapeutic Cal-1 comprises the anti-HIV agents: sh5, a short hairpin RNA to CCR5 that down-regulates CCR5 expression; and maC46 (C46), a peptide that inhibits viral fusion with the cell membrane. These constructs were assessed for inhibition of viral replication and selective cell expansion in a number of settings.

Methods:

HIV replication, selective outgrowth and cell surface viral binding were analysed with a single cycle infection assay of 6 pseudotyped HIV strains and a static and longitudinal passaging of MOLT4/CCR5 cells with HIV. Pronase digestion of surface virus and fluorescent microscopy assessed interactions between HIV virions and transduced cells.

Results:

Cal-1 reduced CCR5 expression in PBMC to CCR5Δ32 heterozygote levels. Even low level transduction resulted in significant preferential expansion in MOLT4/CCR5 gene-containing cells over a 3 week HIV challenge regardless of viral suppression (12.5% to 47.0% (C46), 46.7% (sh5), 62.2% (Dual), respectively). The sh5 and Dual constructs at >95% transduction also significantly suppressed virus to day 12 in the passage assay. No escape mutations were present through 9 weeks of challenge. The Dual construct significantly suppressed infection by a panel of CCR5-using viruses, with its efficacy independently determined from the single constructs. Dual and sh5 inhibited virion internalisation, determined through pronase digestion of surface bound virus, by 70% compared to 13% for C46.

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Conclusions:

The use of two anti-HIV genes allows optimal preferential survival and inhibition of HIV replication, with the impact on viral load dependent on the percentage of gene marked cells.

Introduction

Approximately 35 million people worldwide are currently infected with HIV [1]. While antiretroviral therapy (ART) has greatly improved both life expectancy and quality of life for many HIV infected individuals[2-4], it does not provide a cure. ART requires lifelong adherence to daily medications to avoid rebound of virus, decreases in CD4+ T cell counts, the development of drug resistance and treatment failure.

The only documented case of an infected individual clearing HIV has been described by Hutter et al[5-8]; an HIV positive patient with myeloid leukemia received a bone marrow transplant from an allogeneic donor who was homozygous for the 32-base-pair deletion in CCR5, CCR5delta32/CCR5delta32, preventing any surface expression of this HIV co-receptor. Following transplantation, this individual has no detectable virus, in the absence of ART, more than 8 years post-transplant[5-8]. Individuals with the CCR5delta32 mutation exhibit partial (heterozygotes) or complete (homozygotes) protection from HIV infection[9-13]. With 1-2% of the Caucasian population presenting as homozygous for a CCR5delta32 mutation, and showing no notable side-effects other than strong resistance to HIV infection, engineered CCR5 down-regulation is considered as a target for gene therapy[9-11, 13].

The combination of significant risks associated with allogeneic bone marrow transplantation and the low chance of a matched donor being homozygous for CCR5delta32 have led us[14, 15] and other investigators to consider cell-delivered gene therapy whereby protective genes are delivered to target cells[16-18]. Several clinical trials have been conducted using gene therapy to HIV, and have so far indicated both the safety and viability of such a method[19].

HIV virion interactions with host cells begin with the attachment of viral gp120 to the host CD4 receptor, followed by engagement with a co-receptor, typically CCR5 or CXCR4[20]. Once co-receptor attachment has occurred, HIV undergoes conformational changes, exposing gp41 and allowing for fusion through the cell membrane[21, 22]. This first entry stage of the HIV lifecycle is significant in terms of treatment options, especially when aiming to minimise host cell interactions with virions.

Down-regulating CCR5 expression via gene therapy can be achieved in several ways. Zinc-finger nucleases (ZFNs) prevent CCR5 expression by cleaving the CCR5 DNA, thus preventing transcription. ZFNs have shown considerable success both *in vitro* and in mice studies. Recently, clustered regularly interspaced short palindromic repeats (CRISPR) guided by RNA to cleave target sites, have also shown promise as a method of eliminating CCR5 and its consequent expression[23]. Another method, the one used here, is to insert a gene that generates a short-hairpin RNA (shRNA) to cleave the mRNA for CCR5 gene expression. Its safety has been shown both *in vitro* and in non-human primates[24]. Our group has developed an HIV gene therapeutic termed sh5 using this last approach[24].

While a reduction in CCR5 expression is effective in preventing HIV entry for strains that primarily use CCR5 as a co-receptor, other strains able to use the CXCR4 co-receptor would not be blocked for cell entry. In order to protect against this alternate HIV tropism, a second protective mechanism is required. C46 is an HIV-1 entry inhibitor derived from the C-terminal heptad repeat of HIV-1 gp41[25-27]. Cells containing the gene for C46 express the C46 surface protein that blocks HIV-1 fusion to the cellular membrane by interacting with the N-terminal domain of gp41, preventing six-helix bundle formation[25-27]. A variety of tissue culture studies have shown the efficacy of C46 against a range of HIV isolates, including R5, X4, and R5/X4 tropic strains, making it an effective broad-spectrum gene therapeutic[14, 27-29].

Newly acquired HIV infections are almost exclusively CCR5 (R5)-tropic. While targeting CCR5 has proven effective against R5-tropic HIV, it is not expected to significantly impact CXCR4 (X4)-tropic or dual-tropic strains as they utilise the alternative CXCR4 co-receptor for entry. Additionally, X4-tropic HIV is highly virulent and while this tropism emerges in less than half of individuals, its appearance often leads to more rapid progression to AIDS. Approaches capable of inhibiting both R5 and X4-tropic tropic viruses have been explored [27, 28, 30].

CD4+ T cells can down-regulate CD4 receptor expression once infected with HIV, resulting in the cell being unable to take in new virions[31-33]. The inhibition of viral entry into the host cell, either by downregulating CCR5 and/or expressing C46, prevents HIV from reaching a stage of infection whereby it can induce down-regulation of CD4[31-33]. This resulting maintenance of CD4 expression in the gene-protected cells is postulated to allow the host cell to non-productively bind virions. It should be noted that there are approximately 40,000 CD4 receptors on the surface of T cells[34] and the half-life of HIV in peripheral blood is approximately 6 hours[35]. As such, the non-productive binding of HIV would impact the level of circulating virions. In the present report, we assessed the extent of viral trapping on the surface of the construct-containing cells. A protein digesting enzyme (Pronase) was utilised to release viral attachment from the surface of cells in order to differentiate between viral particles bound to the cell surface and those that have entered the cell through the cell membrane.

Cal-1 comprises the anti-HIV agents: a short hairpin RNA to CCR5 (sh5) that down-regulates CCR5 expression; and maC46 (C46), a peptide that inhibits viral fusion with the cell membrane. We have previously tested a Cal-1 in tissue culture[14], humanized mice[15] and non-human primates[36] showing inhibition of HIV in various systems. This construct is also in clinical testing (ClinicalTrials.gov Identifier: NCT01734850). The questions that we sought to answer in the present study were – 1) is the anti-viral effect mediated rapidly at the entry stage as expected; 2) what degree of selective pressure is provided by Cal-1 and is there generation of escape mutants; 3) how does percentage transduction correlate with inhibition of viral replication; 4) how does the degree of C46 expression impact selection and viral replication; and 5) can we determine a mechanism(s) for enhancement of Cal-1 effect? To answer these questions we examined in detail the effects of these two anti-HIV gene therapeutics to determine the specificity of effect in a variety of assay systems: single cycle

infection, static (non cell passage) and longitudinal (cell passage) HIV challenge and viral-host cell interactions analysed by flow cytometry. We describe the impact of these constructs on viral levels, the outgrowth of cells containing the constructs, their efficacy against a number of HIV pseudotypes, and the ability of gene-containing cells to bind virions and thus provide protection to neighbouring non-gene-containing cells.

Materials and Methods

Ethics Statement

Australian Red Cross Blood Service approved the supply of Buffy coats for PBMC collection and isolation. Blood from volunteers was obtained with written consent, under approval HREC/13/SVH/145 from the St Vincent's Hospital Sydney Human Research Ethics Committee.

Cells and Cell Lines

HEK293T/17. This cell line was used for viral production and was cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% (vol/vol) fetal calf serum (D-10). It was obtained from ATCC (CRL-11268).

Molt4/CCR5. This T-cell line has been transduced with a vector expressing the CCR5 co-receptor. It was obtained from ATCC (CRL-1582). The cultures were supplemented with 1mg/mL geneticin (G418) (Life Technologies) to maintain expression of CCR5 and cultured in Roswell Park Memorial (RPMI 1640) with 10% (vol/vol) fetal calf serum (R-10).

TZM-BL. A HeLa human cervical carcinoma cell line acquired from ATCC (PTA-5659™). The TZM-BL indicator cell line enables simple and quantitative analysis of HIV $TCID_{50}$ using β-gal as a reporter. The TZM-BL cell line is a HeLa clone genetically engineered to express the CXCR4 and CCR5 molecules. This cell line contains the luciferase and β-galactosidase reporter genes under the regulatory control of the HIV LTR. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum (D-10). This cell line was used to titre the mCherry containing HIV viruses.

PM1. PM1 cells (obtained from NIH Aids Reagent Program) were cultured in RPMI + 10% FCS (R-10).

Peripheral Blood mononuclear cells (PBMC) Human peripheral blood was obtained from the Australian Red Cross and voluntary laboratory donors. Mononuclear cells were isolated from human

peripheral blood using Ficoll density gradient centrifugation. Whole blood was centrifuged with the brake off and the mononuclear layer of cells gently aspirated. The mononuclear cells were then transferred to a new tube and washed in Dulbeccos Phosphate Buffered Saline (PBS). The pellet was then resuspended in 10mL PBS and 25mL of 0.8% NH_4Cl, and incubated on ice for 10min in order to lyse red blood cells. Cells were then washed and resuspended in FACS buffer for cell counting, after which purified PBMCs were then cultured in R-20.

Lentiviral vectors. The 3rd generation lentiviral vectors comprised 3 packaging plasmids obtained from Addgene (Plasmid 12253: pRSV-Rev; Plasmid 12251: pMDLg/pRRE; Plasmid 8454: pCMV-VSV-G)) and a unique vector plasmid. The vector plasmids were of two types – those with the anti-HIV genes alone (described previously [14, 15], and designated Cal-C46, Cal-sh5, and Cal-1) and those with the anti-HIV genes plus GFP designated Control (no anti-HIV element), sh5, C46 and Dual. The method of construction and plasmid maps are provided in the supplementary material. All vector plasmids contained cPPT to increase transduction efficiency and gene expression, and WPREmt to also increase gene expression. The lentiviral vectors were produced from plasmid by calcium phosphate transfection using CalPhos Mammalian Transfection kit (ClonTech) on HEK 293T/17 cells. The 3 packaging plasmids were combined with one of the vector plasmids in at a molar transfection ratio of 2:2:1:1 (VSVG, Rev, Gag/Pol, and gene(s) of interest). More detailed information on the lentiviral vectors is in Supplementary Section and Figure S1. The expression of sh5 was determined by flow cytometry via CD195 antibody, the expression of C46 by 2F5 staining, and RT-qPCR (described below).

Lentiviral Transduction.

Single-cycle studies. For these studies, the GFP-containing lentiviral vectors (Control, C46, sh5, Dual) were used to transduce Molt4/CCR5 cells 2x10^6 cells at an M.O.I of 0.5 in T25 flasks with 8µg/mL polybrene. After 2-3 weeks of culture, the cells were sorted for GFP expression via flow cytometry. This process of positive selection was repeated after 3 more weeks of culture in order to ensure pure populations of transduced cells. In addition to cell lines, PBMC were analyzed. Following Ficoll separation and washing, their concentration was adjusted to 2x10^6 cells/mL in R-20, PHA added at 10µL/mL to activate PBMC and culture continued for 48hr at 37^{o}C 5% CO_2. Non-tissue-culture-treated wells of 12-well plates were coated with RetroNectin (diluted in PBS) at 2.5µg/cm^2, and stored at 4^{o}C overnight. Cells were combined in 50mL Falcon Tubes, and washed with RPMI+20% FCS (R-20). They were then resuspended at 2x10^6/mL in R-20+10U/mL Interleukin 2 (IL2) and incubated at 37^{o}C 4hr. Excess/unbound RetroNectin was aspirated from the non-TC plate, which was then rinsed with R-20. Lentiviral vectors were then added to each well and centrifuged at 2200g, 4^{o}C for 2hr. Supernatant was then removed, and 1mL of IL-2 activated cells at 2x10^6/mL were added to each well, incubated overnight at 37^{o}C 5% CO_2. After 24hr, 1mL of R-20 was added, incubated at 37^{o}C, 5% CO_2. After another 2 days incubation, an aliquot of cells was washed with PBS, fixed in 2% paraformaldehyde (PFA) and analysed via flow cytometry for eGFP expression. These cultures were then used for HIV pseudotype infection.

Longitudinal studies. As with the single-cycle studies, Molt4/CCR5 cultures containing the GFP-containing lentiviral vectors (Control, C46, sh5, Dual) were used. These cultures were mixed with untransduced cultures in order to obtain mixed cultures of approximately 12.5%, 25%, 50% gene marked, with untransduced and >95% transduced being pure populations.

HIV binding analysis. PM1 cells were used for this analysis and were also transduced with lentiviral vectors as described above but in this case using an M.O.I of 1. These transduced cultures were then cultured for a maximum of 1 week before HIV challenge with HIV-1_{BaL} and HIV-1_{NL4-3}. For each cell type $1x10^6$ cells of each culture were challenged with HIV-1_{BaL} (M.O.I of 1), or HIV-1_{NL4-3} (M.O.I of 0.01), or R-10 medium alone, performed in 1mL for two hours, rotating at 37°C. Cultures were then washed and resuspended in 3mL R-10 and cultured, expanded and checked regularly for signs of infection (syncytia formation) for up to 10 days. These experiments were conducted with varying time points of sampling, to obtain optimal conditions for observation via fluorescent microscopy.

Static culture. For these studies, the non-GFP vectors (Cal-C46, Cal-sh5, Cal-1) were transduced into Molt4/CCR5 cells to measure C46 expression (M.O.I = 5), as well as in the study of longitudinal infections (M.O.I = 1, 3, 5). Transductions were performed with 8µg/mL polybrene in 12-well tissue-culture plates. Cells were then cultured in RPMI+10% FCS + 1mg/mL G418 prior to HIV challenge.

HIV Challenge

HIV mCherry pseudotyped virus with different envelopes. A total of six HIV envelope plasmids were used to generate the different HIV pseudotypes (Table S1). The pseudotypes were developed through cloning virus isolates from patients, and further examining the plasmid clones [37-39]. These had varying levels of efficiency with regard to co-receptor usage, with Low, Medium and High efficiency at infecting CCR5+ cells, while most had no CXCR4 usage capability. Details on these pseudotypes and their derivation can be found in Supplementary Material including Table S1.

HIV single cycle infection of Molt4/CCR5 and PBMC. For Molt4/CCR5, $5x10^4$ transduced cells in 100µL of R-10 16µg/mL polybrene were transferred to a 96-well plate, with 100µL VCM of HIV pseudotype at M.O.I of 0.5. Cultures were spinoculated at 1,200g, 2hr, 32°C. For PBMC, after concentrating cells to $2x10^6$/mL, $2x10^5$ transduced PBMC were added to a 96-well plate, with 100µL of pseudovirus at M.O.I of 0.1. In both cases, cells were spinoculated at 1,200g for 2hr, 32°C in a flat-bottomed 96-well plate; they were then transferred to a v-bottom plate, and centrifuged at 200g, 5min. Supernatant was then removed and 200µL R-10 added to re-suspend cells, which were then transferred back to the flat-bottomed plate. Culture was incubated for 3 days, 37°C, 5% CO_2. Cultures were washed in PBS, fixed in 2% PFA, and analysed via Flow Cytometry.

HIV Static Challenge. Two types of HIV challenge were conducted using non-GFP lentivirus, in which viral load was measured. Firstly, cultures with varying levels of Cal-1 containing cells were challenged with HIV_{Bal}. And secondly, cultures with varying transduction M.O.I of each non-GFP vector were challenged with each HIV_{Bal} and HIV_{NL4-3}. Molt4/CCR5 cells were prepared for HIV challenge by

adjusting cell concentrations, and mixing Cal-1 gene-containing cells with control cells to a total of $1x10^6$ cells in 3mL at the following percentages: 12.5%, 25%, 50% and >95% gene-positive (measured via 2F5 staining).

In addition to this, cultures of Molt4/CCR5 cells were transduced at M.O.I of 1 and 5 using lentiviral vectors Cal-C46, Cal-sh5 and Cal-1. All these cultures were then challenged with X4-tropic $HIV_{NL4.3}$ or R5-tropic HIV_{Bal} at an M.O.I of 0.2, and extra medium (2mL RPMI+10% FCS + 1mg/mL G418) was added at days 3, 6 and 8.

These cultures were then assayed for p24 (ng/ml) at day 12 (Cal-1 with varying percentage gene marking), and days 8 and 13 (lentiviral constructs at different M.O.I). These cells were challenged with X4-tropic $HIV_{NL4.3}$ or R5-tropic HIV_{Bal} at an M.O.I of 0.2 and assayed for p24 (ng/ml) at day 12. Extra medium (2mL RPMI+10% FCS + 1mg/mL G418) was added at days 3, 6 and 8. In addition, constructs were tested at different M.O.Is (1 and 5) at days 8 and 13, post HIV challenge, with X4-tropic HIV_{NL4-3}, or R5-tropic HIV_{Bal}. p24 protein levels were then assessed.

HIV Longitudinal Challenge. Molt4/CCR5 cell lines were prepared for HIV challenge by adjusting cell concentrations, and mixing GFP and non-GFP cells to a total of $1x10^6$ cells at the following percentages: 12.5%, 25%, 50% and >95% GFP+. These cultures were centrifuged at 200g, 5min in 1.5mL Eppendorf tubes and the supernatant removed. To initiate infection, HIV-1_{Bal} at an M.O.I of 1 of was added to each of the cultures. Following gentle mixing, tubes were incubated at 37°C 5% CO_2 for 2hrs while rotating at 20rpm. Cells were then washed with 1mL of RPMI+10% FCS + 1mg/mL G418, and cultured in 3mL of this medium at 37°C, 5% CO_2. Cultures were maintained for 3 weeks with passaging (1-in-5 dilutions) every 3 days. Sampling for viral load, gene-marking and cell number was performed every 3 days during passaging. All HIV challenges were performed in quadruplicate.

Mutation Testing. This was conducted in the longitudinal challenge and is described in the Supplementary Material**.**

P24/RT Assay for Viral Replication. Aliquots of 200µL of cell culture were taken every 3 days for viral load analysis which was assessed by two assays. P24 ELISA assays were performed on supernatant using the Alliance HIV-1 p24 Antigen ELISA Kit (Perkin Elmer) and a reverse transcriptase (RT) assay developed by Suzuki et al.[40] was used to determine the levels of RT activity in supernatant samples.

Flow cytometric analysis and staining. Flow cytometry was performed using an LSRII SORP (BD BioSciences), and a LSRII Fortessa (BD BioSciences), and analysis was performed using FlowJo software. GFP and mCherry fluorescence were measured and used to determine presence of lentiviral construct (GFP) and pseudotyped HIV (mCherry). For longitudinal infections, phenotypic analysis using antibodies and fluorescent labels was performed on samples collected during passaging. Levels of gene-marking, CCR5 expression and live/dead cell composition were determined using GFP, CD195APC (BD Bioscience) and Live/Dead Violet (Life Technologies) stains of 500µl of culture. Briefly, cells were washed in FACS buffer, before staining with CD195APC for 20min at RT,

washed again, stained with Live/Dead Violet antibody for 30min 4°C, before washing with PBS and adding 2% PFA for a fixative. Cell number was determined by adding 200µL culture to 4% PFA with CountBright beads (BD Bioscience). Cells were fixed at 4°C overnight, then analysed via flow cytometry.

For analysis of viral trapping, aliquots of cells were washed in PBS, and supernatant was removed. 0.5mL of pronase (1mg/mL) was added and cells incubated at 37°C for 10min. Cells were then washed with 1mL R-10 and the supernatant gently aspirated before washing twice more with R-10. The FCS in R-10 was a necessary supplement to ensure limited pronase digestion. After washing with FACS buffer, cells were resuspended in Medium A Fix+Perm Kit and incubated for 15min at RT. The cell suspension was then washed once more with FACS buffer (200g, 5min) and resuspended in a solution of 100µL Medium B Fix+Perm Kit and 5µL anti-p24 antibody (anti-p24-RD1, Beckman Coulter, Clone KC57) and incubated for 15min at RT. After two more washes with FACS buffer, cells were resuspended in PFA (400µl) and stored at 4°C. From this point, samples were used for two methods of analysis; flow cytometry and fluorescent microscopy, with half of the sample used for each.

Fluorescent Microscopy. Cell Tak mixture was prepared fresh before each use. 27µL of Cell Tak was added to 759µL of 0.1M sodium bicarbonate, followed by the addition of 13.5µL of 1M NaOH. The solution was mixed and immediately added to circular coverslips and incubated for 20min at RT. The coverslips were left to dry and then rinsed with dH_2O and allowed to dry again. Coverslips were then transferred to a 24-well plate and the prepared cells (see flow cytometric staining) were transferred to the coverslip and the plate centrifuged at 200g for 3min, followed by gentle aspiration of the supernatant. After carefully removing the coverslips from the wells they were placed on to a mounting slide, fixed to the slide with nail polish to prevent any movement and stored in the dark at RT overnight followed by storage at 4°C until analysis. Cells were analysed on a Deltavision Fluorescent microscope and microscope images were analysed using Softworx software (Softworx Inc. SA). Images were taken with the focus at several different depths of the cell (using z-stacks), and the middle stack was chosen to view the full diameter of the cell.

RT-qPCR measuring C46 expression. RNA was extracted from cell pellets with PureLink RNA MiniKit (Invitrogen, Cat#12183018A) followed by DNase Treatment (Ambion DNase Treatment Kit, Cat# AMI1906), and Reverse Transcription (Invitrogen, Superscript Vilo© cDNA Synthesis Kit, Cat# 11754-010). RT qPCR was performed using Agilent Brilliant II QPCR Master Mix (Agilent, Cat#600804) in a MX3000/MX3005P Thermocycler, using Stratagene MxPro MX3000P software. RT-qPCR conditions were 1x 2min 50°C, 1x 10min 95°C, 40x (15sec 95°C, 1min 60°C). Primers used were: C46-F 5'-CACAGCCTGATCGAGGAGAG-3', C46-R 3'-GTCCTGCCACTGGTGGTG-5', C46-Probe- 5' 6-FAM™, Int ZEN™ 3' Iowa Black FQ 5'-CACTCCACGCAGCACTTCCGCTCG-3'. β2m-F 5'-GCTGGCGCTACTCTCTCTTTCT-3', β2m-R 3'-GGATGGCGTGAGTAAACCTGAA-5', β2m-Probe-5' HEX, Int ZEN™ 3' Iowa Black FQ 5'-CCTGGAGGCTATCCAGCGTACTCCAAAG-3'.

An additional qPCR was used to determine lentiviral copy number in gene-marked populations was determined using a qPCR assay specific for the WPRE sequence within the vectors. The assay simultaneously quantifies both the total number of lentiviral vector sequences and the total number of sequences of an endogenous control gene (Albumin) within a DNA sample. Since each diploid cell has two copies of the endogenous gene sequence, we could determine the number of lentiviral vector copies per cell in this manner (copies/cell). This assay has been fully validated, and has been optimised in-house and provides accurate and reproducible results. Samples were analysed in triplicate, and performed twice, with consistent results.

Statistical Analysis. FlowJo analysis was reported as mean +/-Standard Error of Mean (SEM). In single cycle infections comparisons between constructs were tested for significance using a 1-way ANOVA and Tukey's multiple comparison test. For longitudinal analysis comparisons between constructs and cell cultures were tested for significance using a 2-way ANOVA and Sidak's multiple comparison test A P value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Results

Expression of sh5 and C46

We first analyzed expression of the anti-HIV elements. It is known that Individuals with the CCR5delta32 mutation exhibit partial (heterozygotes) or complete (homozygotes) protection from HIV infection[9-13]. We assessed CCR5 expression inhibition by each of the GFP- containing constructs in a T cell line that had been modified to express CCR5, Molt4/CCR5 cells. As outlined in Material and Methods, cultures were sorted by GFP expression, ensuring that cultures were pure populations of gene-containing cells. There was some dimming of GFP signal over time with the expansion of the cultures, especially for cultures infected with the C46 containing vectors, so that after 2-3 weeks of expansion of these gene-containing cells, the cultures had shifted slightly to the left relative to the original gating for GFP (Figure 1). This reduced signal was likely a result of lower expression of the eGFP gene due to it being located further downstream of the UbC promoter for both the C46 and Dual construct-containing Molt4/CCR5 cells. Despite the GFP dimming, the cultures maintained the same purity for gene-containing cells as the originally sorted cultures.

As expected, no difference in CCR5 expression was seen between the Control (cells containing only GFP and no gene therapeutic), and the C46 cultures (Figure 1A, a-d). By contrast cells transduced with either the sh5 gene or the Dual (C46 and sh5) combination showed decreased CCR5 expression - from 99.2% in the Control vector, to 11.5% for the sh5 and 40.6% for the Dual construct, as determined by both upper quadrants (Q1 and Q2) (Figure 1A, e, f). The average lentiviral copy number measured via qPCR, was 73% higher for cells containing the sh5 compared to the Dual construct, which may explain the greater impact on CCR5 expression with sh5 (data not shown).

In terms of expectation of effect, the sh5 gene construct provided a level of CCR5 suppression similar to CCR5delta32 heterozygosity. When delivered to PBMC, sh5 reduced CCR5 expression from more than 30% to approximately 10% (Figure 1B, e-g), less than the CCR5 expression level of 13% for a CCR5delta32 heterozygote (Figure 1B, a-c), but not as low as the 2% CCR5 expression for a CCR5delta32 homozygote (Figure 1B, d).

Having determined relative expression of sh5 we next sought to analyse expression of C46 as determined by RT-qPCR. Molt4/CCR5 cells transduced with the GFP containing constructs, C46 and Dual, expressed C46 at approximately 1/3 and 1/7 the levels (respectively) compared to expression of C46 in Cal-1 containing cells which do not contain GFP (Figure 2). This lesser expression of C46 is likely due to the fact that these cells were selected for high GFP expression, resulting in cells that express lower levels of C46, due to pol II promoter (UbC and Ef1α) competition.

Therapeutic genes provide protection against single-round infection from a range of CCR5 efficiency viruses

We assessed how effectively each of these constructs inhibited infection by 6 pseudotyped HIV viruses modified to only permit a single round of infection. The panel was chosen to represent viruses with a range of abilities to infect cells via the CCR5 coreceptor, from those that resulted in

low levels of infection in CD4/CCR5 cells [39] which we label as "low CCR5 efficiency", to those that achieve higher infection levels, "high CCR5 efficiency". A clade G R5 tropic and a clade A dual tropic virus were also included. The total level of infection for each culture was calculated relative to the average level for Control cultures and described as 'Relative Infectivity'. Comparisons were made relative to the Control construct as these cells were transduced as for the therapeutic constructs, and since levels of HIV infection were equivalent to untransduced cultures in Molt4/CCR5 cells (data not shown) and marginally lower in PBMC (Figure S2). In this set of experiments, cell cultures consisted of transduced Molt4/CCR5 cells with at least 95% gene-containing cells.

The therapeutic constructs greatly reduced infection from the lower CCR5 efficiency HIV strains, NB2_C1 and NB24_C3. In both cases, the sh5 and Dual therapeutics provided significantly greater protection than both the Control and C46 constructs (Figure 3A, a-f). Against the HIV strains with a higher CCR5 efficiency, NB2_C4 and NB23_C3 [39], the Dual therapeutic resulted in significantly lower infection. The sh5 and C46 gene constructs significantly inhibited infection by NB23_C3 but not by NB23_C4, so that these strains appeared to be less sensitive to the therapeutics than their less CCR5-efficient counterparts. Infections from both the dual-tropic clade A (92RW020.5) and the R5-tropic clade G (92UG975.1) strains were also significantly reduced by the therapeutics, with Dual showing the strongest efficacy against both strains.

As may be expected, sh5 showed greater protection against HIV strains that were inefficient at infecting cells through the CCR5 co-receptor [39, 41]. Interestingly, while the clade A 92RW020.5 strain is dual-tropic, it is still very efficient at using CCR5 and heavily relies on it [42], and as such, the sh5 construct provided significant protection. Due to C46's mode of action of competitively binding to HR-1, it was capable of providing similar levels of protection against most strains of HIV, regardless of the virus co-receptor usage or its CCR5 efficiency.

Similar profiles of inhibition occurred in experiments with this panel of viruses when applied to PBMC (Figure 3B). Once again, the Dual construct showed the greatest level of protection against each of the HIV pseudotypes. While sh5 provided protection against four of the strains (Figure 3B, c-f), it did not significantly inhibit the HIV strains where there was low infectivity of CCR5+ cells (NB2_C1, NB24_C3) (Figure 3B, a, b). All three therapeutic constructs were shown to protect against the HIV strains with a higher efficiency of infecting CCR5+ cells (NB2_C4, NB23_C3), as well as against both the 92RW020.5 and 92UG975.1 strains.

This evidence that the Dual construct efficacy in cell lines translates to PBMC is of great importance for further studies of these gene therapies. These results also showed that the impact of the Dual construct (eff_{dual}) is approximately what would be expected if the sh5 and C46 acted collectively but independently ($eff_{dual} = 1 - (1 - eff_{sh5})(1 - eff_{C46})$). This indicates that not only do both genes provide protection, but those protections act independently and do not interfere with or impair one another in any way.

Static Infection

In order to analyse further the protective effects against both HIV tropisms, the Cal-1 construct was tested at day 12 in a challenge assay (HIV Static Challenge, see Materials and Methods) using both R5-tropic HIV_{Bal}, and the X4-tropic $HIV_{NL4.3}$. Much higher levels of infection were observed for HIV_{Bal} - approximately 2-logs higher at each level of gene marking (Figure 4a). For both viral challenges the greatest impact was observed at >95% gene-marking of culture, at which reductions in p24 of approximately 1-log were achieved. Since up to 5% of cells in culture remained unmodified, there were up to 50,000 susceptible cells capable of sustaining ongoing HIV infection. A decrease in p24 levels was also observed for 50% gene-marking (one half-log). Single constructs (sh5, C46) without GFP were also tested in the HIV Static Challenge and were shown to be less effective than Cal-1 in terms of HIV inhibition. Similar to what has been described previously by Wolstein *et al.*[14], Cal-1 showed 2-log inhibition at day 8, (compared to untransduced cultures), with 91% (M.O.I 1) and 98% (M.O.I 5) gene marking, against both X4 and R5 tropic virus Figure 4b. As expected sh5 was effective against only R5-tropic virus (approximately 0.5 log at day 8), while C46 alone was effective against both strains of HIV (approximately 2 logs at day 8) with 85% gene-marking (M.O.I 5). Together with previously published results [14] these data show that the Cal-1 construct can be administered at lower and safer M.O.I while still providing a strong impact, and that the increased levels that will occur over time provides increasing degrees of impact on HIV levels.

Selective advantage of cells containing therapeutic genes in a longitudinal HIV challenge

The previous experiments assessed the efficacy of these gene constructs against single round infections and static infection. Part of the rationale of gene therapy for HIV is that cells containing the therapeutic will not only be less susceptible to infection and act to decrease its levels but will also be able to expand as a percentage of the total CD4+ population due to a selective advantage. We therefore investigated these aspects in a 3-week longitudinal challenge with the R5-tropic HIV_{BaL} against Molt4/CCR5 cells.

The experiments compared infection dynamics and cell survival when the cultures were commenced with different percentages of cells containing each single construct and HIV infection at an M.O.I of 1. When HIV challenge was initiated at 12.5% gene-marking, C46, sh5 and Dual containing cells expanded to 47.0% (3.8 fold), 46.7% (3.7 fold), and 62.2% (5 fold) of culture respectively (Figure 5 a). The expansion for the Dual construct was greater than either the C46 ($P=0.002$) or sh5 ($P=0.002$) marked cells, while all constructs showed significant expansion compared to the control vector ($P<0.0001$), the latter showing no change in gene-marking levels.

For cultures that initially contained gene-marking of 25% of the cells, there was expansion of the gene-marked cells to 63.4% (C46), 63.7% (sh5), and 72.6% (Dual) (Figure 5 b) ($P<0.0001$ for all relative to Control). Initial gene-marking of 50% resulted in an increase to 77.1% (C46), 80.5% (sh5), and 80.6% (Dual) (Figure 5 c) ($P<0.0001$). While therapeutic constructs increased significantly compared to the Control, there was no significant difference in expansion between the non-Control therapeutics (Day 21: C46 vs sh5 $P>0.99$, Dual vs C46 $P=0.29$, Dual vs sh5 $P=0.32$). Levels of gene-marking were still increasing when the culture was terminated, as such, we believe that further

positive selection would continue until cultures had reached almost 100% transduced. In addition to this preferential expansion, >95% dual-marked cultures (Figure 5 d) were protected from HIV-mediated cell death (Figure S3). Therapeutic gene-marked cells showed no preferential expansion in HIV-negative cultures, indicating that any observed expansion in these experiments was due to selective pressure from HIV, from the non-proliferation and/or death of HIV-infected cells.

In order to tease out the dynamics of viral replication, we analyzed impact on RT levels, knowing that each 3 day passage provided fresh viral replication over the course of the 3-week HIV-1 challenge (Figure 5, e-h). As expected, construct-negative cultures showed no impact on viral load and only high levels of gene-containing cells (95% sh5 and dual) impacted on viral load. Higher starting percentages of the C46 construct produced minor reductions in viral load: day 6 RT levels for >95% C46 gene-marking compared to construct-negative cultures was closest to being significantly different ($P=0.075$). Initial levels of the sh5 construct of 12.5% resulted in little change to viral load, however a level of 25% sh5 resulted in peak RT levels at day 9 of roughly 500pg/µL which was 3 times lower than for the construct-negative control ($P=0.0001$). Similar levels of viral suppression were observed with 50% sh5 gene marking, with a delay in peak viral load to day 12. Cultures with >95% sh5 containing cells greatly suppressed viral load, with RT levels 2-3 logs lower than construct-negative control at days 9 and 12 ($P<0.0001$). The Dual construct showed slightly reduced RT for initial gene marking of approximately 12.5%, 25%, and 50%. However, >95% Dual gene marking suppressed viral load by 2-3 logs compared to construct-negative control for days 6-12. Surprisingly RT increased from day 9 onwards in the >95% sh5 and Dual cultures, reaching levels comparable to cultures with fewer therapeutic cells. This indicates a likely delayed infection of the few remaining unprotected cells still present in the culture.

No Sign of HIV Mutation to Escape Therapeutic Constructs

An extended longitudinal HIV challenge over 9 weeks was additionally performed to determine likelihood of mutation in the V3 loop and HR1 and HR2 regions that might indicate the development of resistance to the sh5 and C46 constructs respectively. Only one mutation could be detected, a single nucleotide A-G substitution in the HR-1 region resulting in the amino acid change I48V. This nucleotide switch appeared and reversed in almost all cultures, even without the presence of therapeutics (Supplementary Figure S4).

Impact on Viral Entry – and Virus-Host Interactions

The therapeutic genes used here prevent co-receptor binding from R5-tropic HIV (sh5) and membrane fusion from both R5 and X4-tropic HIV (C46). As these therapeutics still allow for primary receptor, and in the case of C46, co-receptor attachment, we sought to analyze the virus-host interactions occurring at the cell surface, and the potential impact these may have on the efficacy of these entry inhibitors. p24 staining indicates a cell to be HIV-positive, but it is not possible to know if p24 is located on the cell surface or inside the cell. However, after pronase digestion only virions which have fused with the cell membrane or were internalised remained, allowing for a comparison

of HIV within the target cell relative to particles on the cell surface. This enabled relative quantification of internalised virus (membrane fused/internalised) versus surface associated virus (Figure 6, a-d), Figure 7, a-d).

Challenge with HIV-1_{BaL} showed that almost all PM1 cells in C46-containing cultures presented as p24-positive (28.4/(28.4+3.9)×100%=88% for C46-containing (those in GFP+ quadrants) and 58.7/(58.7+9.06)×100%=87% for gene-negative cells (those in GFP- quadrants)) (Figure 6 b, left-hand figure). After removal of surface virions with pronase, p24 levels were only reduced by 13% for C46 containing cells (76% were p24-positive vs 86% for gene-negative cells leading to a (1-76/88/(86/87))×100%=13% reduction with C46, Figure 6 b, middle figure). All calculations regarding inhibition of viral entry are shown in Table S2 in Supplementary material. Therefore, C46 seemingly provided minimal protection against internalisation of HIV-1_{BaL}. Dual-containing and gene-negative cells registered similar levels of p24 (78% and 79%, respectively, Figure 6 d). However, substantially less of this was internalised for cells containing the Dual vector (7% vs 24%, Figure 6 d, middle figure), resulting in a 70% reduction of HIV internalisation. Similarly, the sh5-construct, reduced internalisation by 69% (Figure 6 c). These data indicate that the therapeutic constructs did not impair the initial attachment of HIV, but they did reduce internalisation of bound virus into the host cell.

Challenges with the X4-tropic HIV-1_{NL4-3} virus showed a greater impact on internalisation with the C46 construct. C46 resulted in a 53% reduction of internalisation of HIV-1_{NL4-3} into host cells (Figure 7b). The Dual construct produced a weaker effect with only a 24% reduction in virion internalisation (Figure 7d). As expected, no impact on internalisation was observed in sh5-containing cells (Figure 7c, Table S2).

Visualisation of constructs inhibiting virion internalisation

Fluorescent microscopy was used to visualise what was occurring in these virus-to-host interactions. The GFP-expression of gene-marked cells, and the staining of p24, enabled visualisation of not only HIV infected cells, but also where on a cell those particles were located. HIV located in the cellular cytoplasm shows as large numbers of fluorescent particles (shown in red via p24 staining), typically with well-defined borders at the cellular membrane, and excluding the nucleus (right hand panels in Figures 6, 7). HIV infected many gene-negative cells, with large numbers of virions apparent in the cellular cytoplasm. However, with cells containing the therapeutic constructs (shown as green-filled cells through GFP expression) the virus was seen only on the surface of the cell.

Discussion

In previous work by members of our group, the Cal-1 construct containing the two anti-HIV genes, sh5 and C46, had been shown effective in tissue culture [30] as well as murine [15] and non-human primate studies [36]. These previous reports had not conducted longitudinal studies of HIV challenge in tissue culture nor had they assessed the impact of single cycle infections with a panel of

HIV pseudotypes, nor HIV surface-trapping. In the present study, we sought to further define the anti-HIV effects of these entry blocking agents both separately and in combination by characterising the effects in single-cycle, longitudinal and cell/virus interaction assay systems enabling the assessment of multiple aspects of cell/virus interactions. In the cell/virus staining experiments we specifically determined viral entry dynamics (surface versus internalised virus).

The sh5 vector reduced CCR5 expression in PBMC to levels similar to those seen in individuals who are heterozygous for the CCR5delta32 mutation (Figure 1). It was therefore surprising that sh5 had no significant impact on HIV infection in PBMC by strains that are inefficient at using CCR5, compared to the strong protection in Molt4/CCR5 cells with the same HIV strains. The lesser impact of sh5 in PBMC is unlikely to be the result of generally reduced infection in these cells, as the total infection in PBMC was approximately equal across all pseudotyped HIV (data not shown). This suggests these HIV strains, that are inefficient at using CCR5 and do not infect through CXCR4, may be capable of utilising other receptors for attachment to CD4+ T cells. This potential for usage of minor receptors would explain the consistent efficacy of C46, with the apparent loss of efficacy of sh5 against an R5-tropic HIV. The ability for some R5-tropic HIV to utilise CCR3 has been described previously, with some research indicating inhibition of CCR3 can have as great an impact as inhibiting CCR5 against some HIV strains [43]. This effect was only observed in the two strains that are naturally inefficient at infecting CCR5+ cells (NB2_C1, NB24_C3) so that they may regularly rely on usage of other minor receptors such as CCR2 and/or CCR3, as some HIV strains have shown susceptibility to changes in these receptors [43-45].

The consistently greater protection provided by the combination of both the C46 and sh5 therapeutics, demonstrated the beneficial effect of using multiple therapeutic genes in a single construct. The Dual construct inhibited infection in the single-cycle experiments by approximately 70% (In Molt4/CCR5 cells) and 60% (in PBMC) across all viral pseudotypes. The protection offered by the Dual construct against most HIV strains was approximately equal to the product of protection offered by the individual C46 and sh5 constructs, suggesting no cross-therapeutic interference.

The single cycle experiments allowed calculation of the immediate inhibition of infection by the therapeutic constructs but could not determine what other benefits they provided over time. The longitudinal challenge experiments over 3 weeks saw a significant survival advantage for cells containing the therapeutics. Cells containing the Dual construct expanded from an initial 12.5% to 62.2%, while the other constructs also exhibited similar expansion, despite, but possibly because of, having only minor impact on viral levels. This significant expansion over time is important for practical implementation of HIV gene therapy since it is difficult to transduce more than 20% of CD4+ T cells or hematopoietic stem cells without performing myeloablation, which has its own risks. When the Dual and sh5 constructs were at maximal levels, viral levels were also significantly reduced verifying the inhibitory effects of these constructs determined from the single cycle experiments.

Cultures with maximal sh5 and Dual containing cells suppressed viral levels until approximately day 12 after which it steadily increased, while C46 inhibited viral levels to day 9. This decrease in HIV

inhibition after these times was likely due to the presence of a minor population of susceptible cells that did not contain the gene therapeutics, and which allowed virus to spread. Previous research has shown decreased HIV virus replication in 97% C46 gene-containing cultures, and delays in peak viral load[26]. The small untransduced populations of HIV susceptible cells in both our study (approximately 5%) and that of Von Laer (approximately 3%), may have partly benefitted from "herd immunity" provided by the more prevalent gene-containing cells within these cultures, resulting in a slower replication of HIV infection.

While many therapeutic approaches can prevent productive infection of HIV, the majority allow for entry of virions and the subsequent HIV-mediated down-regulation of CD4[31-33], and while this has little impact for the individual cell, it can impact on virus-host cell interactions. The therapeutics in this study allow virions to attach to the CD4 receptor but inhibit internalisation. Once entry has occurred, the viral proteins Vpr and nef can down-regulate CD4 expression without integration of the viral genome [46]. Hence inhibiting stages later than entry can reduce the number of CD4 receptors available to circulating virions and allow these virions to attach to and infect uninfected cells. On the other hand, cells containing the therapeutics studied here, maintain CD4 expression and allow substantial levels of virion attachment (Figure 6d). So not only do these therapeutics protect cells containing them, but by removing virions from circulation they also protect some of the neighbouring unmodified cells.

This study demonstrates that the combination of C46 and sh5 in a gene therapy provides a stable protective benefit to cells against HIV-1 infection, and a consequent selective advantage of gene-containing cells, without observable risk of encouraging resistance mutations. In addition to this, we have also shown protective effects against multiple HIV pseudotypes, indicating broad-spectrum protection when compared to single therapeutic genes. They provide the basis for more extensive work on these gene therapies, their interactions with HIV, and their potential for use as a clinical treatment option.

Acknowledgements

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Conflict of Interest Statement

The authors declare that they have no competing interests. MB, MM, GS are full-time employees of Calimmune.

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Figure Legends

Figure 1: A: CCR5 and GFP Expression. Each image shows flow cytometry results for a culture of Molt4/CCR5 cells with or without their respective lentiviral constructs measured by GFP expression. (a) Isotype stained to indicate CCR5-ve/GFP-ve gating; (b) No construct with normal CCR5 expression levels; (c) Control vector with eGFP alone; (d) C46 vector; (e) sh5 vector; (f) Dual vector. **B: Comparison of CCR5 levels in natural CCR5delta32 mutations and sh5 in PBMC.** Background CCR5 levels in isotype stained controls (a) and those in delta32 wild-type individuals (b) are shown. (c) delta32 heterozygous cells. (d) delta32 homozygous cells. We also assessed the impact of the sh5 construct on CCR5 expression in PBMC, showing isotype (e) and untransduced PBMC (f). PBMC transduced with the sh5 construct (g) are shown in a mixed culture with gene-marked cells expressing GFP.

Figure 2: Expression of C46 as measured by RT-qPCR. RT-qPCR was performed on extracted RNA from cell cultures containing the lentiviral vectors and C46 expression was normalised to β2-microglobulin. Molt4/CCR5 cells containing the Cal-1 vector were used as the positive control (solid black histogram), while Molt4/CCR5 cells provided the negative control. The other cultures measured were >95% gene-containing cultures for the lentiviral vectors: Control, C46, sh5, Dual.

Figure 3: Construct inhibition of infection from virus with different CCR5 efficiency, in MOLT4/CCR5 (A) cells and PBMC (B). Results are the mean ± SEM of at least 6 replicates using six different enveloped pseudotyped HIV. Values are given as Relative Infectivity, whereby level of infection is compared to that achieved in the Control cultures (for each replicate). Significance of differences in Relative Infectivity of the construct compared to the Control cultures, is displayed using asterisks, NS=not significant, *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001. Cell cultures consisted of MOLT4/CCR5 cells (A) or PBMC (B), which had been purified so that at least 95% of cells contained the gene constructs. The pseudotyped HIV used in each panel were (a) NB2_C1, (b) NB24_C3, (c) NB2_C4, (d) NB23_C3, (e) 92UG975.1, (f) 92RW020.5. CCR5 efficiency refers to the efficacy of HIV to infect CCR5+ cells.

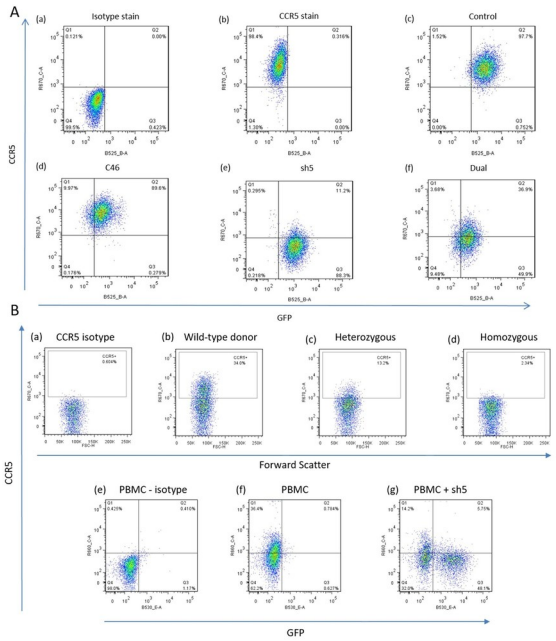
Figure 4: p24 levels in Molt4/CCR5 cultures containing non-GFP lentiviral vectors challenged with HIV_{Bal} and HIV_{NL4-3}. Cells were either untransduced, or were transduced with the Cal-C46, Cal-sh5 or Cal-1 lentiviral vectors, and subsequently challenged with X4 tropic HIV_{NL4-3} or R5 tropic HIV_{Bal}. (a) Data were obtained using Molt4/CCR5 cultures with varying levels of gene-marking with the Cal-1 vector. p24 levels were assessed 12 days after viral challenge as a measure of HIV infection; (b) Data were obtained using Molt4/CCR5 cultures transduced with different non-GFP containing lentiviral constructs at an M.O.I of 1 or 5, with p24 analysis at day 8, challenged with X4 tropic HIV_{NL4-3} or R5 tropic HIV_{Bal}.

Figure 5: Longitudinal Analysis shows selective outgrowth by therapeutic gene-containing cells in the presence of HIV, as well as impact on viral reverse transcriptase levels. Results at each time point depict mean ± SEM of 4 replicates, using Molt4/CCR5 cells and HIV-1_{Bal}. Panels a-to-d display the GFP expression results of the four lentiviral constructs. Varying initial levels of gene marking are

(a) 12.5%, (b) 25%, (c) 50%, (d) >95%. Similarly, panels e-to-h display the reverse transcriptase results in cultures of the four lentiviral constructs.

Figure 6: PM1 cell line challenged with HIV-1_{BaL}. Flow cytometry and fluorescent microscopy data showing GFP expression from gene-containing cells, and HIV stained with p24 antibody (KC57RD1). Flow cytometric analysis was performed 8 days after challenge where half of the samples were digested with pronase to remove surface particles. In fluorescent microscopy images, gene-marked cells express GFP (green). HIV is stained with a p24 antibody (red). HIV can be seen in the cytoplasm of untransduced cells, yet only on the surface of those with constructs. The first column of panels displays PM1 cell cultures transduced with their respective lentiviral vectors, challenged with HIV-1_{BaL}, with standard p24 staining. While the second column of panels show those same cultures, but with p24 staining performed after pronase treatment to remove surface-associated particles.

Figure 7: PM1 cell line challenged with HIV-1_{NL4-3}. Flow cytometry and fluorescent microscopy data showing GFP expression from gene-containing cells, and HIV stained with p24 antibody (KC57RD1). Flow cytometric analysis was performed 5 days after challenge where half of the samples were digested with pronase to remove surface particles. In fluorescent microscopy images, gene-marked cells express GFP (green). HIV is stained with a p24 antibody (red). HIV can be seen in the cytoplasm of untransduced cells, yet only on the surface of those with constructs. The first column of panels displays PM1 cell cultures transduced with their respective lentiviral vectors, challenged with HIV-1_{NL4-3}, with standard p24 staining. While the second column of panels show those same cultures, but with p24 staining performed after pronase treatment to remove surface-associated particles.



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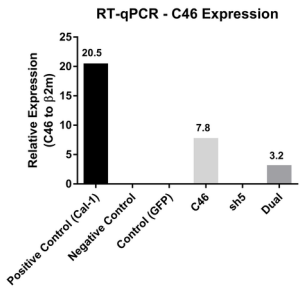

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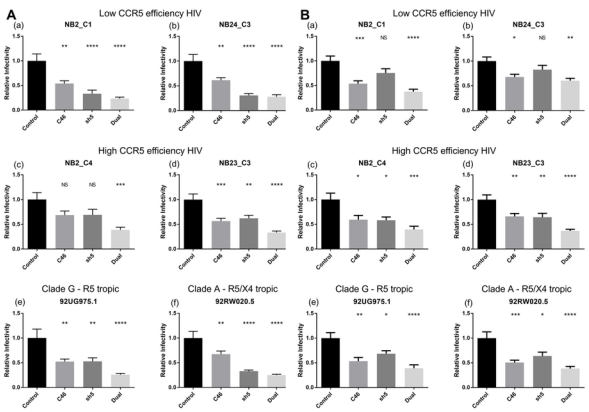

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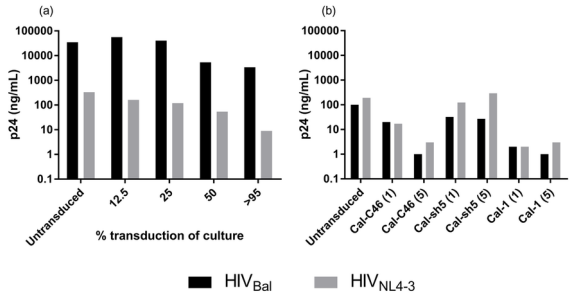

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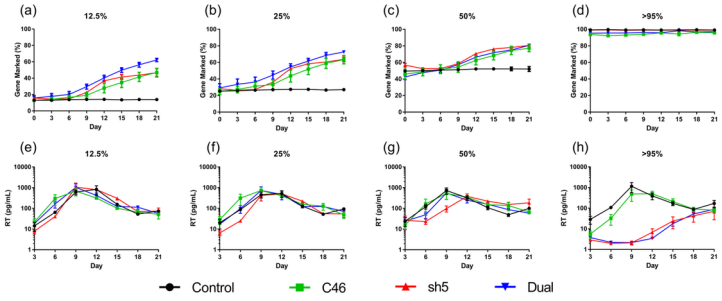

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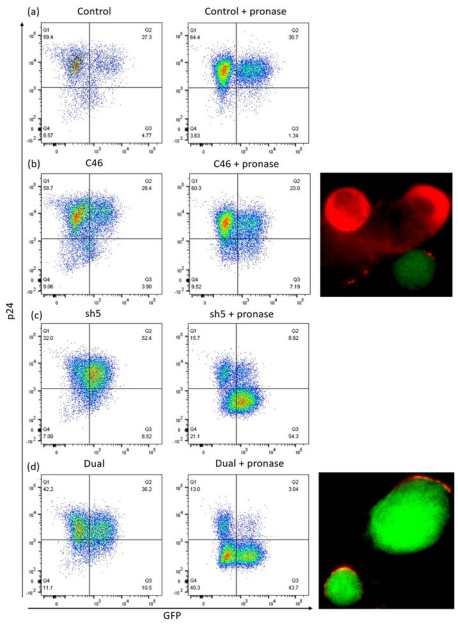

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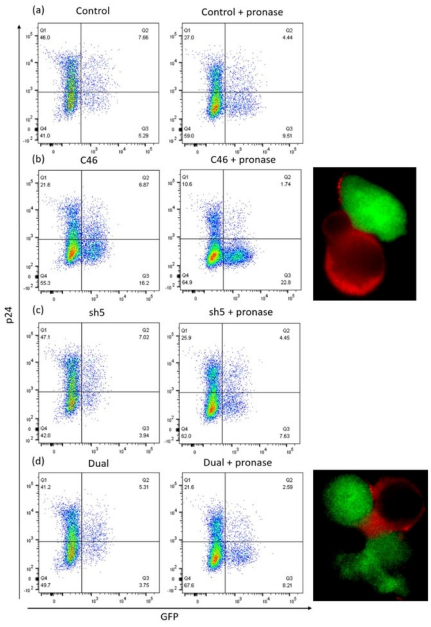

JGM_3006_F7.jpg