TITLE: Chim3 confers survival advantage to CD4+ T-cells upon HIV-1 infection by preventing HIV-1 DNA integration and HIV-1 induced G2 cell cycle delay

AUTHORS: Simona Porcellini, Francesco Gubinelli, Luca Alberici, Bianca Maria Piovani, Gian-Paolo Rizzardi and Chiara Bovolenta*

INSTITUTION: MolMed S.p.A., Milano, Italy

S.P., F.G., and L.A. contributed equally to this study

The online version of the article contains a data supplement

Short title: Chim3-expressing cells survive to HIV-1 infection

Scientific category: GENE THERAPY

Correspondence: Chiara Bovolenta, MolMed S.p.A., via Olgettina 58 Milano, 20132 Italy;

e-mail: chiara.bovolenta@molmed.com
Abstract

The long-term expression and the ability of a therapeutic gene to confer survival advantage to transduced cells are mandatory requirements for successful anti-HIV gene therapy. In this context, we developed lentiviral vectors (LVs) expressing the F12-Vif derivative Chim3. We recently showed that Chim3 inhibits HIV-1 replication in primary cells by both blocking the accumulation of retrotranscripts, independently of either human APOBEC3G (hA3G) or Vif, and preserving the antiviral function of hA3G. These results were predictive of long-lasting survival of Chim3+ cells after HIV-1 infection. Furthermore, Vif, like Vpr, deregulates cell cycle progression by inducing a delay in G2 phase. Thus, the aim of this study was to investigate the role of Chim3 on both cell survival and cell cycle regulation after HIV-1 infection. Here, we provide evidence that infected Chim3+ T-cells prevail over either mock- or empty-LV engineered cells, show reduced G2 accumulation and, as a consequence, ultimately extend their lifespan. Based on these findings, Chim3 rightly belongs to the most efficacious class of antiviral genes. In conclusion, Chim3 usage in anti-HIV gene therapy based on haemopoietic stem cell modification has to be considered as a promising therapeutic intervention to eventually cope HIV-1 infection.
Introduction

A central issue of anti-HIV gene therapy is the ability of the therapeutic gene(s) to confer genetic resistance against viral infection to target cells. This leads to diminish virally-induced cytopathic effects (CPE) and eventually to extend cell survival. However, not all antiviral genes studied so far possess these positive features\(^1\). In this regard, anti-HIV-1 transgenes have been grouped into three classes depending on the phase of the viral life cycle targeted\(^1\). Class I genes affect the early steps of HIV-1 life-span ultimately preventing integration of proviral DNA, thus keeping the cells free of new infection; class II genes block viral gene expression thereby reducing the overall production of toxic viral proteins and virions from chronically infected cells; class III genes affect the assembly and release of new infectious viral particles\(^1\). According to this classification, mathematical models supported by experimental data predict that class I and class II genes protect from viral CPE and induce prolongation of cell life span, whereas class III genes do not. On this basis, the mode of action of an antiviral gene has to be carefully analyzed to achieve a powerful and life-long therapeutic advantage. In this context, we developed pre-clinical gene therapy technology based on lentiviral vector (LV) expressing a natural mutant of the viral infectivity factor (Vif), named F12-Vif\(^2\). Vif is an HIV-1 key protein, whose major function is to block the antiviral activity of the cellular restriction factor human apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC)-3G (hA3G)\(^3,4\). However, the role of Vif is not limited to counteract hA3G, but it has been proved increasingly complex over time. Although Vif was implicated in reverse transcription process for long time\(^5,6\), only recently, it has been demonstrated to directly interact with both HIV-1 reverse transcriptase (RT) and genomic RNA\(^7\)\(^{11}\). In addition, Vif plays a direct role along with Vpr, though independent of it,
in inducing viral cytopathicity and cell cycle delay in G2 phase by recruiting Cul5-ligase complex and degrading a yet-to-be-identified cell factor. Altogether, Vif is rightly considered an important target for antiviral therapy because it is involved in many key viral and cellular processes linked to HIV-1 pathogenesis. We showed that F12-Vif inhibits HIV-1 replication despite degrading hA3G as efficiently as HIV-1 Vif does. As a consequence of a partial characterization of its mechanism of action, F12-Vif was labeled as a class III antiviral transgene. In contrast, being the F12-Vif derivative Chim3 as lethal against HIV-1 as F12-Vif is, but unable to degrade hA3G, and acting as a dominant negative factor affecting the retrotranscripts accumulation and HIV DNA integration, it has to be categorized as a class I antiviral transgenes.

Once established that Chim3 is a potent intracellular inhibitor of HIV-1 replication operating at the pre-integration step of HIV-1 life cycle, the goal of this study was twofold. First, to verify whether HIV-1 infected Chim3-genetically modified cells prolonged their survival in culture compared to control cells. Second, to analyze the possible contribution of Chim3 on the HIV-mediated deregulation of the cell cycle. To this end, we set up an experimental model in which Chim3-expressing T cells were mixed with control cells at 1:1 ratio and then infected with HIV-1. The end-point analysis was the survival and possible enrichment of Chim3-expressing cells. Furthermore, we examined whether Chim3 played any role in regulating cell cycle either by itself or following HIV-1 infection. Interestingly, we established that Chim3-LV induces survival advantage to both CEM A3.01 cells and primary CD4+ T lymphocytes, and prevents the HIV-1 Vif-mediated cell cycle delay in G2 phase. Taken together, these results suggest that a therapeutic approach based upon the engineering of HSCs with Chim3 can give rise to HIV-1
resistant CD4+ T lineages, which expanding and living long, ultimately repopulate the immune system of individuals living with HIV-1/AIDS.
Materials and methods

Cells

The HEK-293T cells were propagated in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal calf serum (FCS) (EuroClone Ltd, Paignton, United Kingdom) and a combination of penicillin-streptomycin and glutamine (PSG). CEM A3.01 T cell line \(^{15}\) and ACH-2 cells \(^{16}\), were cultivated in RPMI 1640 supplemented with 10% FCS and PSG.

Human CD4\(^+\) T lymphocytes were purified from umbilical cord blood (UCB), cultivated, expanded by CD3/CD28 beads, and transduced as previously described \(^{17}\).

For survival advantage experiments, cells were analyzed by the Cedex AS20 automated cell culture analyzer (Innovatis AG, GmbH, Bielefeld, Germany) before mixing. For primary CD4\(^+\) T lymphocytes, the experiments were performed only when the different cell types were comparable under cell viability, density, size, morphology and aggregation rate parameters.

Production of lentiviral vectors (LVs), transduction, and immune selection of T cells

The lentiviral vectors (LVs), empty-P\(\Delta\)N (empty-LV) and Chim3-P\(\Delta\)N (Chim3-LV), were described in \(^{2}\) and \(^{17}\), respectively. Chim3 encodes the 45-amino acid region corresponding to amino acids 126-170 of the natural mutant F12-Vif inserted in a NL4-3 HIV Vif backbone together with the constitutive PGK-\(\Delta\)LNGFR selection marker cassette \(^{18}\). The green fluorescent protein (GFP)-LV was obtained by substituting the \(\Delta\)LNGFR with the GFP gene using the \(XbaI\)
and SacII sites in the empty-LV. VSV-G pseudotyped LV stock production, cell transduction, and immune selection were performed as previously described 17. GFP-expressing cells were selected by the BD FACSVantage™ SE Cell Sorter (BD Biosciences, San Diego, CA).

**Fluorescence activated cell sorter (FACS) and cell cycle analysis**

The percentage of LV transduction, measured as ΔLNGFR or GFP expression, was evaluated by flow cytometry analysis (FACS Calibur BD Bioscience, San Jose, CA). NGFR level was measured by using either the anti-NGFR-PE antibody (Ab) (C40-1457, BD Pharmingen™, S. Diego, CA) or the anti-NGFR-biotinylated Ab plus the SAv-PerCP reagent (BD Pharmingen™), the latter only for the detection of ΔLNGFR in the GFP-LV:Chim3-LV mixture. The level of CD4 expression was assessed by either the mouse anti-human CD4 clone SK3 (BD Pharmingen™) or RPA-T4 antibodies (eBioscience Inc., San Diego; CA). FACS results were analyzed by the FlowJo software (Tree Star, Inc., Ashland, OR). The intracellular level of p24Gag was measured using the Kc57 either FITC or PE monoclonal Abs (Beckman Coulter, Fullerton, CA) and the BD Cytofix/Cytoperm™-Fixation/Permeabilization kit following manufacturer’s instructions (BD Biosciences). The TCR Vβ repertoire was determined using the IOTest® Beta Mark kit following the manufacturer’s instructions (Beckman Coulter).

For cell cycle analysis, cells were first synchronized at the G<sub>i</sub>S border by 0.75 μM aphidicolin (Sigma Chemical Corp., St. Louis, MO) treatment for 16 hours. The drug was removed by several washes with PBS and, after 10 hours of release, cells were either mock-
infected or infected with the VSV-G pseudotyped Vif-proficient and Vif-and Vpr-deficient Δenv-
HIV-1 at a multiplicity of infection (MOI) of 3 in the presence of aphidicolin. The double
deficient HIV-1 molecular clone was obtained by removing the SpeI-SalI HXB2 fragment
containing two premature stop codons in the Vif and Vpr genes, respectively. The two viruses
were produced as described in details in 17. After 24 hours of infection, aphidicolin was removed
and following 16 hours of release, cells were permeabilized and stained with p24Gag-FITC Ab.
Then, 1 × 10^6-FITC-labelled cells/ml were resuspended in PBS containing 100 μg/ml propidium
iodide, 0.1% saponin, 5 mM EDTA, and 50 μg/ml RNase A for 30’ at room temperature. Single
cells were analyzed using a double discrimination function; relative cell cycle distribution was
calculated using the FlowJo software, and fitting curves with the Dean-Jett-Fox model.

**HIV-1 infection**

Cells were acutely infected at the indicated MOI with either the X4 NL4-3 or the R5 AD8
HIV-1 molecular clones. Single-round infection experiments were performed using GFP-LV and
Chim3-LV transduced cells infected with VSV-G pseudotyped Vif-Vpr-proficient and Vif-Vpr-
deficient HIV-1. After overnight infection, the virus was removed by several washes with PBS
and fresh medium added. Seventy two hours later, cells were analyzed for GFP, ΔLNGFR and
p24Gag expression by FACS as described in “FACS and cell cycle analysis” section. Reverse
transcriptase (RT) assay was performed as described in 2.
LV and HIV-1 DNA copy number quantification by real-time PCR

The LV copy number and HIV DNA was measured by TaqMan PCR using an ABI PRISM 7900 instrument and analyzed by SDS 2.3 software (Applied Biosystems, Warrington, United Kingdom) as in 17.

Coimmunoprecipitation

HEK-293T cells were co-transfected with the Cul5-HA, WT-Vif-Myc and Chim3 vectors at the indicated DNA ratio and after 36 hours were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH7.5, 0.5% NP-40, and a cocktail of protease inhibitors. Five hundreds μg of cell lysates/sample were immunoprecipitated with 5 μg of mouse anti-HA antibody (clone HA-7, Sigma) coupled to 20 μl of protein A-conjugated RMP Protein A Sepharose Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 3 hours rotating at +4°C. The beads were washed four times with the washing buffer (150 mM NaCl, 20 mM Tris-HCl pH7.5, 0.5% NP-40). After elution with 2× Laemmlli buffer and boiling, the co-immunoprecitated complexes were resolved into SDS-PAGE followed by immunoblot using rabbit anti-HA Ab (HA-7, Sigma) at 1:10,000 dilution or HIV-1 HXB2 Vif rabbit antiserum, obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) from Dr. D. Gabuzda, at 1:1,000 dilution.
Southern blot analysis

Genomic DNA (gDNA), isolated by the QIAamp Mini kit according to manufacturer’s instructions (QIAGEN GmbH, Germany), was resolved on 0.8% agarose gels, after overnight digestion with AflII, blotted onto nylon membranes (Hybond–N, Amersham), hybridized to 1 × 10^6 dpm/ml ^32^P-labelled 1-kb ΔLNGFR fragment in PerfectHyb PLUS hybridization buffer (Sigma Chemical Corp.), and visualized by autography.

Statistical analysis

Statistical comparisons were determined by the two-tailed Student’s t-test. The level of significance chosen was P values lower than 0.05. The data are presented as means ± SEM.
Results

CEM A3.01 cells are highly susceptible to HIV-1 infection unless protected by Chim3

Recently, we reported that Chim3 protects primary CD4+ T cells and HSCs-derived macrophages from HIV-1 infection by exploiting a dual mechanism acting at the pre-integration level, ultimately resulting in a drastic reduction of HIV-1 DNA integration 17. These observations prompted us to believe that Chim3-LV should bestow survival advantage on transduced cells respect to control cells following HIV-1 infection. To test this hypothesis, we used the non permissive CEM A3.01 cell line as working model because these cells are highly sensitive to the CPE of HIV-1 and support efficient HIV-1 production 15. Initially, we verified whether Chim3 protected CEM A3.01 cells from HIV-1 infection in a manner similar to that observed in primary cells 17. Cells were engineered with either the empty-LV or the Chim3-LV as detailed in Vallanti et al. (2005) 2 and Porcellini et al. (2009) 17. Both LVs contain the PGK-ΔLNGFR selection marker cassette which allows detection and purification of transduced cells by an anti-NGFR Ab 18. Genetically modified cells were purified (>95% purity), infected with the X4 HIV-1 NL4-3 molecular clone at an MOI of 0.1 and followed over a month for HIV-1 production. In agreement with our previous findings 17, Chim3-transduced, unlike empty-transduced cells were protected from HIV-1 infection (Figure 1A). In empty-LV cells the cytophatic/lethal effect of HIV-1 was detectable at day 21, 5 days after the peak of viral infection (day 16), whereas the viability of infected Chim3-expressing cells remained comparable to that of the uninfected cells (>70%) throughout the entire course of infection (Figure 1B). Moreover, unlike empty-LV, Chim3-LV transduced cells did not show down-modulation of CD4 HIV-1 receptor (Figure 1C), which is a typical feature of mainly X4 HIV-1 infected cells 19,20. This finding excludes CD4 down-
modulation as a possible additional cause of Chim3-mediated antiviral activity. Finally, we verified whether Chim3-expressing cells resist HIV-1 infection because one or few HIV-1 resistant cell clone emerge. To this aim, we carried out Southern blot analysis of gDNA extracted from empty-LV- and Chim3-LV-transduced cells after 28 days of HIV-1 infection using a ΔLNGFR specific probe. Empty-transduced CD4- cells showed a typical clonal genotype, which likely derives from the growth of the few cells outlived to HIV-1 induced cell death (Figure 1D, left panel). In contrast, gDNA of Chim3-expressing cells appeared smeared hybridized, typical of a transduced bulk cell population (Figure 1D, right panel).

Altogether, these findings indicate that Chim3-LV+ CEM A3.01 cells are protected from HIV-1 infection, express CD4 receptor, and do not show signs of viral cytophaticity and cell clonality.

**Chim3-LV confers survival advantage to transduced cells under HIV-1 selective pressure**

On this basis, we assumed that Chim3 expressing cells should prevail over unmodified cells when both cell types were infected within the same cell culture. To test this hypothesis, CEM A3.01 mock-transduced cells were mixed at 1:1 ratio with Chim3-LV transduced cells. The mixture was either left uninfectected (duplicate culture) or infected (6-replicate culture) with NL4-3 HIV-1 at an MOI of 0.01. We measured HIV-1 production by RT activity assay and ΔLNGFR expression, as an estimate of Chim3-LV cells enrichment, by FACS using an anti-NGFR Ab. The mix population was productively infected reaching the peak of virus release after 25 days (RT activity = 8,248 cpm/μl over the value of 481 cpm/μl measured at day 7 post infection).
Therefore, Chim3-expressing cells were exposed for a long time to the cytopathic/lethal effect of the virus derived from mock-transduced infected cells. Of note, despite this challenging condition, ΔLNGFR staining at 49 days of culture indicated that Chim3-LV cells were highly enriched in the infected mixture as compared to the uninfected one (Figure 2A, 88% vs. 41%), demonstrating that under strong HIV-1 selective pressure only Chim3-LV-transduced cells survive for longer than 3 months (Figure 2B). In agreement with the results of Figure 1C, unlike mock-transduced, Chim3-transduced cells did not down-modulate CD4 receptor following HIV-1 challenge (Figure 2C).

Finally, we wondered whether the HIV-1-resistant CD4⁺ Chim3-LV enriched population maintained the HIV-resistant phenotype for long time. To address this question, an aliquot of both the sestuplicate 4-month-infected culture and the corresponding duplicate uninfected culture were challenged with NL4-3 HIV-1 at an MOI of 0.1. Of note, Chim3⁺-cells were still protected from HIV-1 infection to an extent similar to that of the original ≥ 95% Chim3-expressing cells, which were included as positive control (Figure 2D). Taken together, these results demonstrate that Chim3 is not silenced overtime and its antiviral activity is therefore permanent.

**Chim3-LV, but not empty-LV, confers survival advantage under HIV-1 selective pressure**

To exclude the contribution of any functional cis-acting elements present in the LV backbone itself, we mixed CEM A3.01 mock:Chim3-LV and mock:empty-LV cells at 1:1 ratio and processed them following the same experimental conditions of Figure 2A and B. As expected, Chim3⁺ cells lived on and expanded over mock-transduced cells after HIV-1 infection. In
contrast, the relative percentage of infected empty-LV cells as well as both uninfected cell mixtures remained substantially unchanged throughout the duration of the experiment (Figure 3A). This result points out that there is no involvement of the LV backbone in inducing or reinforcing the therapeutic potential of Chim3-LV.

Next, to establish the level of HIV-1 infection in CEM A3.01 mock:Chim3 compared to mock:empty mixture, we measured the HIV-1 production by RT activity assay on a per cell basis (Figure 3B) along with the integration of HIV-1 DNA on separated mock-transduced and transduced cell populations (Figure 3C). It is interesting to note that, despite the initial contribution of mock cells was equal in both mixtures (ca. 60%), the level of HIV-1 production was overall lower in mock:Chim3-LV than in mock:empty-LV mixture (Figure 3B). Next, we separated the transduced cells from the corresponding mock cells of both mixtures by ΔLNGFR immune selection, and measured the HIV-1 DNA content in each cell types before the peak of HIV-1 production. Mock-transduced (100% HIV-1 DNA) and empty-LV cells derived from the mock:empty-LV mixture contained non-statistically significant different levels of HIV-1 DNA (Figure 3C, white bars, mock vs empty). In contrast, the overall DNA content of mock:Chim3 mixture was lower compared to the mock:empty mixture (Figure 3C, black vs white bars). Of most relevance, mock cells separated from the mock:Chim3 mixture contained 70% less HIV-1 DNA than mock cells separated from the mock:empty control mixture (p<0.01), indicating that less infectious and less numerous viral particles are spreading in the mock:Chim3 than in the control mix culture (Figure 3C, black vs white mock bars). Furthermore, in agreement with our previous studies 17, Chim3-expressing cells carried the lowest content of HIV-1 DNA among the four sub-populations (Figure 3C, black Chim3 bar).
Taken together, these findings demonstrate that the remarkable reduction of HIV-1 production observed in the mock:Chim3-LV cell mixture mostly depends upon the reduction of HIV-1 DNA integration in Chim3-expressing cells.

**Chim3 confers survival advantage to transduced CD4+ T lymphocytes following HIV-1 infection**

We carried out survival advantage experiments on mock:Chim3-LV and mock:empty-LV CD4+ T lymphocytes mixed at 1:1 ratio, and then infected with HIV-1 AD8 at an MOI of 1 (Figure 4A and B). A month after productive infection, uninfected and infected cells were stained for CD4 and ΔLNGFR expression and analyzed by FACS (Figure 4A and B). The percentage of ΔLNGFR+ cells was overall unchanged in uninfected cells of both mixtures compared to the initial 50%:50% ratio (Figure 4A and B), whereas it was greatly increased (85.7%) only in infected mock:Chim3 mix (Figure 4A, right panel). We verified the possible clonality of the Chim3-enriched population by evaluating the TCR Vβ repertoire clonogram of the uninfected and infected cells after 40 days of culture (Figure S1). The expansion of the Vβ2 clone, which is in general the most represented subtype in normal T lymphocytes, was clearly observed in infected cells (Figure S1). This result suggests that, as opposed to CEM A3.01, primary T cells are prone to clonal expansion after HIV-1 infection.

Then, we wanted to estimate the level of HIV-1 proviral integration also in CD4+ T lymphocytes to sustain our hypothesis that Chim3-expressing cells survive longer because contain less HIV-1 DNA (Figure 4C). To this aim, we transduced CD4+ T lymphocytes either
with the Chim3-LV or with the green fluorescent protein (GFP)-LV, instead of the empty-LV, as control. After cell sorting by \( \geq 95\% \Delta \text{LNGFR} \) and GFP positivity, transduced cells were mixed at 1:1 ratio. To avoid cell clonality we performed short-term experiments. We infected the cell mix in single round with a VSV-G pseudotyped \( \Delta \text{env} \)-HIV-1 at an MOI of 3 and after 72 hours, we analyzed the intracellular p24Gag expression as a surrogate marker of HIV-1 DNA integration, as shown in Porcellini et al. (2009) [17]. We observed that Chim3+-infected cells expressed ca. 65% less p24Gag than control cells do (Figure 4C), providing support for the fact that these cells, harboring less HIV-1 DNA are supposed to live longer than control cells.

**Chim3 itself does not affect cell cycle**

In addition to the well-recognized role of HIV Vpr on cell cycle disruption [14], it has been recently reported that also Vif itself induces a delay in G2, which is independent of the action of hA3G and, interestingly, is exerted through the recruitment of the same E3 ubiquitin ligase complex that is involved in hA3G degradation [12-14]. Based on this notion, we decided to verify whether, similarly to Vif, Chim3 can deregulate the cell cycle. To this aim, we transduced HEK-293T cells with empty-, WT-Vif- and Chim3-LV at equal copy number (5 copies/cell). Transduced cells were then transfected with Tat and Rev plasmids to trigger transgene expression. We then assessed both the induction of the Vif proteins by Western blot (Figure 5B) and the possible effect of Chim3 on cell cycle perturbation by incubating the cells with propidium iodide (P.I.) to visualize DNA content as indicator of cell distribution into the cell cycle phases (Figure 5A). To determine the amplitude of the delay in G2, the G2:G1 ratio was
calculated only on live gated cells. We confirmed the results of Wang et al. (2007) on Vif and, of interest, we established that, in contrast, Chim3 has no effect, suggesting that it is likely defective in recruiting the Cul5-E3 ligase complex and thereby unable to affect the cell cycle. This hypothesis was supported by our finding showing that Chim3 binds poorly Zn which is required for Cul5/Vif interaction.

**Chim3 poorly interacts with Cul5**

To confirm this idea biochemically, we carried out co-immunoprecipitation analysis between Cul5 and Vif proteins (Figure 5C and D). HEK-293T cells were transfected with a fix amount of Cul5-HA expression vector and increasing amount of either WT-Vif-Myc (Figure 5C) or Chim3 vectors (Figure 5D). Quantification of the bands intensity, normalized by the level of the input extract used (WCE) (Figure S2A), indicated that Chim3 interacts with Cul5 from 3.5 to 9.3-fold less than WT-Vif (Figure S2B).

**Large amount of Chim3 affects HIV-1 Vif/Cul5 interaction**

Based on this finding, we wondered whether Chim3 could also interfere with the Cul5/WT-Vif interaction and therefore prevent the recruitment of the E3-ligase complex to destroy the yet-to-be-identified protein. To answer this question, we transfected fixed amount of Cul5-HA and WT-Vif-Myc and increasing amount of Chim3 vectors, coimmunoprecipitated the protein complexes with anti-HA Abs, and finally revealed them with specific anti-Vif and anti-anti-HA
Abs (Figure 5E). Interestingly, quantification of the bands intensity, normalized by the level of the input extract used (WCE) (Figure S2C), established that 4-fold increase of Chim3, produced a decrement of WT-Vif-Myc interaction to Cul5 of ca 50% and an increment in Chim3 interaction of ca. 50% (Figure S2D, columns 13 vs 8). This result indicates that large amount of Chim3 competes with WT-Vif for Cul5 interaction of at least 50% and suggests the formation of possibly non-functional heterodimers or Chim3 homodimers interacting with Cul5.

**Chim3 prevents HIV-1 Vif-induced cell cycle delay at the G2 phase in CD4+ T cells**

Next, we wished to investigate whether the weak Chim3-Cul5 interaction and the interference of Chim3 on Cul5-Vif binding translated into a direct effect on cell cycle progression in CD4+ T cells. As we showed that transduced T cells, unlike transduced HEK-293T cells, accumulated Vif proteins even in the absence of Tat and Rev \(^{17}\), we first verified whether Chim3 itself alters the cell cycle of uninfected CD4+ T cells. Similarly to transduced HEK-293T, the G2:G1 ratio was comparable in uninfected empty-LV and Chim3-LV transduced cells (data not shown), indicating that Chim3 itself does not affect cell cycle in the absence of HIV-1 infection also in T cells. Next, we single-round infected empty-LV and Chim3-LV CEM A3.01 and CD4+ T lymphocytes with VSV-G pseudotyped Vif-Vpr-proficient and Vif-Vpr-deficient (ΔvifΔvpr) HIV-1 molecular clones at an MOI of 3 and then analyzed cell cycle profile in both p24Gag+ and p24Gag− live gated cells. To mimic the effect of HIV-1 particles emerging from empty-LV and Chim3-LV transduced cells after the first round of infection, both viruses were produced from HEK-293T cells expressing either the empty-LV or Chim3-LV in equal copy number. Following
infection with Vif-Vpr-proficient HIV-1 produced from empty-LV transduced HEK-293T cells, the G2:G1 ratio was highest in empty target cells (Figure 6A), and slightly lower in Chim3-LV target CEM A3.01 cells (Figure 6C). In contrast, after infection with Vif-Vpr-proficient HIV-1 generated from Chim3-LV producer cells, Chim3-target cells showed the lowest ratio among the four conditions (Figure 6D). Interestingly, when the cells were infected with Vif-Vpr-deficient HIV-1, the G2:G1 ratios were similar in all conditions (Figure 6E-H), indicating a dominant negative function of Chim3. Similarly, the p24Gag- cells showed a cell cycle profile similar to that Δvif-Δvpr HIV-1 infected cells (Figure S3). Comparable results were obtained also with purified CD4+ T lymphocytes (Figure S4 and S5). Taken together, these results not only provide support for the previous findings on the involvement of Vif in affecting the cell cycle\textsuperscript{12-14}, but also indicate that Chim3 prevents the G2 delay induced by HIV-1 Vif protein, when it is expressed either in target or producer cells. A cumulative effect is appreciated when Chim3-expressing target cells are infected with HIV-1 generated from Chim3-producer cells (Figure 6D).
Discussion

This study was conducted to investigate whether Chim3 extends the life-span of transduced cells following HIV-1 infection. Owing to the fact that Chim3 belongs to group I anti-HIV-1 transgenes for its mechanism of action \(^1,17\), we expected it was endowed with the ability to confer survival advantage. Here, we provide evidence that the weak, long-term expression of Chim3 prolongs the existence of Chim3-LV-transduced cells compared to mock- and empty-LV-transduced CEM A3.01 cells and primary CD4\(^+\) T lymphocytes after HIV-1 infection. We observed that primary T lymphocytes are more prone to cell clonality compared to CEM A3.01 cell line in comparable experimental settings. This likely derives from the different culture conditions used for the two cell types. Primary cells are subjected to multiple CD3/CD28 stimulations that, in addition to the further restriction induced by HIV-1 infection, might explain the restriction of the V\(\beta\) repertoire. In this context, the largest sample of LV integration sites analysis in CD4\(^+\) T cells isolated from patients undergoing a phase I/II clinical trial with the VIRxSYS anti-HIV vector VRX496, yielded no LV integration close to either proto-oncogenes or tumor suppressor genes known to contribute to lymphoid specific cancers. Moreover, the patients, followed for 4 years after infusion with VRX496-modified autologous CD4\(^+\) T cells, developed no abnormal cell expansions. This finding is consistent with the observation that no T-cell malignancies have ever been associated to HIV-1 integration in millions of HIV-1\(^+\) individuals \(^23\).

Interestingly, Chim3 expression affects neither the survival nor the cell cycle progression of uninfected cells, suggesting that the antiviral and surviving functions of Chim3 strictly depend
on the presence of HIV-1 Vif, which is a typical dominant negative feature. In contrast to WT-Vif, Chim3 does not induce a delay in G2 phase after infection with a Vif-proficient, but not with a Vif-Vpr-deficient HIV-1. It is well documented that Vpr-induced cell cycle arrest in G2 promotes HIV LTR transcriptional activity, viral production, and the onset of apoptosis in infected cells. Similarly, Vif has been lately implicated in the cell cycle delay in G2, independently of Vpr, by recruiting the same Cul5-E3-ligase complex responsible of the degradation of hA3G and then targeting a yet-to-be-identified cellular protein, different from hA3G, to the proteasome. In this context, we established that Chim3 alone interacts with Cul5 from 3.5- to 9-fold less efficiently than WT-Vif. This result gives credit to our previous data demonstrating that Chim3 binds less efficiently than WT-Vif to a Zn affinity column and to the findings of Giri et al. (2009) showing that the presence of Zn enhances the strength of the interaction of Cul5 with a Vif HCCH peptide by 8-fold. Thus it is conceivable that the F12-Vif specific 5 amino acid mutations of Chim3 located in the Zn-finger motif hinders Zn binding and consequently Cul5 interaction. Most importantly, we have shown that an excess of Chim3 halves the WT-Vif-Cul5 interaction and, at the same time, increases the binding of Chim3-Cul5 suggesting the formation of Chim3 homodimers and/or WT-Vif-Chim3 heterodimers interacting with Cul5. This is supported by the notion that the molar ratio between an HCCH peptide and Cul5 is 2:1 indicating that Vif binds to Cul5 as homodimers. We propose that the inhibitory function of Chim3 on the deregulation of the cell cycle, might derive indirectly from the inhibition of HIV-1 DNA integration, that ultimately results in a reduced total amount of newly synthesized HIV-1 Vif, but also to a direct effect of Chim3 on preventing the degradation of the still unknown cellular protein involved in cell cycle perturbation. We show here that upon HIV-1 infection, the level of proviral DNA is decreased in Chim3-expressing cells even when
these cells are embedded in an environment much more challenging than that represented by the pure (≥ 95% Chim3+) CD4+ T lymphocyte and macrophage populations 17. Indeed, Chim3-expressing cells directly in contact with non protected HIV-1+ cells represent an environment closer to the in vivo physiological one, where transduced cells are expected to be, at least initially, a minority. We have also recently reported that Vif acts as an auxiliary factor for HIV-1 reverse transcriptase increasing its rate of association to RNA or DNA templates 22. Thus collectively, a growing body of evidence sustains the concept that Chim3 acts at multiple levels, some of which, such as the block of HIV-1 DNA integration, the lower binding to a Zn-affinity column, and the prevention of G2 delay of the cell cycle, are all independent of the presence of hA3G.

Based on the results we recently published 17,22 and those presented in this manuscript, we draw the following model of action of Chim3 (Figure 7). During the first round of HIV-1 infection, the percentage of cells delayed in G2-phase (Figure 6) and the number of retrotranscripts 17 are lower in Chim3-cells than in control cells. Consistently, viral particles produced during the first round are identical to the input virus if budded out from mock cells, but less infectious and in lower number if budded out from Chim3-expressing cells because Chim3 preserves the antiviral action of hA3G, which is, in turn, accumulated into virions 17. Thus during the second round of infection the virus emerging from mock cells shows the same infectivity on target cells as during the first round, whereas the virus budded out from Chim3+ cells, being less infectious for carrying hA3G, integrates with lower efficiency in both mock- and Chim3-LV transduced cells. During the first round of infection, Chim3 plays a role at the level of target cells, whereas during the second round of infection, at the level of both target and producer cells.
The amplitude of the effect observed in a single cycle infection experiments, which is reiterated during a long-term infection, cannot be other than small. However, a small effect can eventually give rise to an exponentially amplified outcome after several rounds of infection and cell doublings.

Altogether, our preclinical findings are encouraging and warrant the investigation of this approach in an appropriate animal model \(^{26}\) paving the way towards clinical testing. Potentially, HSCs transduced with Chim3-LV once re-infused into patients might give rise to CD4\(^+\) T lymphocytes, which not only resist to HIV-1 infection, but also survive and prevail on the untransduced HIV-1 target cells. In the long term, these genetically-modified functionally active cells might overcome chronically infected cells.
Acknowledgments

We wish to thank Anna Stornaiuolo for helpful suggestion on plasmid vector construction and Ermanna Rovida (CNR - Istituto di Tecnologie Biomediche, Milano) for helpful discussion. This work was supported, in part, from TaKaRa Bio (Otsu, Japan).

Authorship contributions and conflict of interest disclosures:

S.P. performed most of the experiments of the research and contributed to the conception and design of the experiments and to the interpretation of the results.

F.G. performed most of the experiments of the research and contributed to the conception and design of the experiments and to the interpretation of the results.

L.A. performed most of the experiments of the research and contributed to the conception and design of the experiments and to the interpretation of the results.

B.P. performed Southern blot analyses.

G-P.R. supervised the conception and design of the experiments, the interpretation of the results and the writing of the manuscript.

C.B. conceived and designed the experiments, supervised the crude data, interpreted the results and wrote the manuscript.
References


Figure Legends

Figure 1. CEM A3.01 cells are highly susceptible to HIV-1 infection unless protected by the therapeutic gene Chim3. (A) Kinetic of HIV-1 infection. Empty-LV and Chim3-LV-transduced cells were infected with the NL4-3 HIV-1 at an MOI of 0.1. Virus production was measured on the culture supernatants by RT activity assay at the indicated time points. Values represent mean±SEM of n=3. (B) Cell viability. The empty-LV and Chim3-LV-transduced infected cells of panel A and uninfected cells were analyzed using forward scatter (FSC) and side scatter (SSC) profiles by FACS at the indicated time points. Values represent the percent of alive cells on the total events acquired expressed as mean±SEM of n=3. (C) CD4 expression. The level of CD4 receptor expression was measured by FACS in empty-LV and Chim3-LV-transduced cells, either uninfected or infected at two time points of the cell culture. (D) Chim3-LV cells do not show clonal phenotype following HIV-1 infection. Southern blot analysis of gDNA isolated from 3 independent wells of empty-LV and Chim3-LV transduced cells of panel A after 28 days of infection. The filter was hybridized with a 1-kb fragment ΔLNGFR probe.

Figure 2. Chim3-LV confers survival advantage to transduced CEM A3.01 cells under HIV-1 selective pressure. (A) Chim3-transduced CEM A3.01 cells are enriched over mock-transduced cells after HIV-1 infection. Chim3-transduced cells were mixed at 1:1 ratio with mock-transduced cells and either infected with NL4-3 HIV-1 at an MOI of 0.01 or left uninfected. FACS staining of ΔLNGFR+ cells was performed after 49 days of cell cultivation.
The results are representative of a sestuplicate culture for infected and of a duplicate culture for uninfected cells. (B) Percentage over time of $\Delta$LNGFR$^+$ cells in the mock:Chim3-transduced cell mixture of panel A. Results are the mean±SEM of sestuplicate and duplicate cultures for infected and uninfected cells, respectively. (C) CD4 expression. The 12:88% mock:Chim3 cell mixture of panel A was separated by an anti-$\Delta$LNGFR Ab, and CD4 levels in either Chim3 (88% $\Delta$LNGFR$^+$ cells) or mock cells (12% $\Delta$LNGFR$^-$ cells) were analyzed by FACS. (D) Chim3-cells that survive to HIV-1 infection maintain their resistant phenotype after new viral challenge. HIV-1 resistant mock:Chim3 (12:88%) mixture of panel A was challenged with HIV-1 NL4-3 at an MOI of 0.1. The uninfected mock:Chim3 (59:41%) cell mixture, along with the original starting population (mock and original ≥ 95% Chim3-expressing cells) were used as control cells.

Figure 3. Chim3-LV, but not empty-LV, confers survival advantage to transduced cells upon HIV-1 infection and precludes HIV-1 DNA integration. (A) The 60:40% mock:empty and mock:Chim3 CEMA3.01 cell mixtures were either left uninfected or infected with NL4-3 HIV-1 at an MOI of 0.01. The expression of $\Delta$LNGFR, indicating the enrichment of transduced cells, has been evaluated by FACS for 35 days. (B) The infected 60:40% mock:empty and mock:Chim3 cell mixtures of panel A were analyzed for viral production by RT activity assay. (C) The HIV-1 DNA copy number was calculated by quantitative TaqMan PCR analysis in either mock- or LV-transduced cells which were separated using the anti-$\Delta$LNGFR Ab from the respective mixtures after 11 days of NL4-3 HIV-1 infection at an MOI of 0.01. Results are mean±SEM of n=9.
Figure 4. Chim3-LV confers survival advantage to transduced CD4+ T lymphocytes under HIV-1 selective pressure. (A) Empty-LV- and (B) Chim3-LV-transduced CD4+ T lymphocytes were selected to > 95% ΔLNGFR by anti-ΔLNGFR Ab and then mixed at 1:1 ratio with mock-transduced cells. Mixtures were either infected with the AD8 HIV-1 molecular clone at an MOI of 1 or left uninfected. FACS staining of ΔLNGFR+ cells of infected and uninfected cultures was performed after 40 days of cell cultivation. The results are representative of a triplicate culture for infected cells and of a duplicate culture for uninfected cells. (C) GFP-LV- and Chim3-LV-transduced CD4+ T lymphocytes were infected with VSV-G pseudotyped Vif-proficient NL4-3-based HIV-1 at an MOI of 3. Seventy-two hours after viral challenge, intracellular p24Gag level was evaluated by FACS using an anti-p24Gag Ab on fixed and permeabilized cells. Values express mean±SEM percent of p24Gag expression (n=16).

Figure 5. Chim3 affects WT-Vif-Cul5 interaction. (A) HEK-293T cells transduced with equal number (5 copies/cell) of empty-, WT-Vif- and Chim3-LVs were either transfected with Tat and Rev plasmids or left untransfected. Forty eight hours after transfection, cells were analyzed for cell cycle distribution by determining the intracellular P.I. content. Results are mean±SEM of n=4. (B) Aliquots of the cells of panel A were used to determine the expression of Vif in the absence or presence of Tat/Rev proteins as indicated. (C and D) Left panels, 40 μg of whole cell extracts (Input, WCE), derived from HEK-293T cells transfected with a fix amount of Cul5-HA plasmid DNA (670 ng/1 × 10⁶ cells) and increasing amount of either WT-Vif-Myc (C) or Chim3
DNA vectors (D) (lanes 1 and 7 = 80 ng, lanes 2 and 8 = 170 ng, lanes 3 and 9 = 330 ng, lanes 4 and 10 = 670 ng, lanes 5 and 11 = 1330 ng/1 × 10^6 cells) were analyzed by Western blot to determine the relative amount of Cul5 and Vifs. Right panels, 500 μg of the same WCEs were coimmunoprecipitated with the anti-HA Abs and then immunoblotted with either the anti-HA or the anti-Vif Abs, as indicated. Lanes 6 and 12, containing the same amount of WT-Vif-Myc and Chim3 of lanes 5 and 11, but no Cul5-HA, correspond to negative controls. (E) Competition experiments were performed using HEK-293T cells transfected with a fixed amount of Cul5-HA (670 ng/1 × 10^6 cells) and WT-Vif-Myc (170 ng/1 × 10^6 cells) and increasing amount of untagged Chim3-expressing vector (lanes 2 and 9 = 80 ng, lanes 3 and 10 = 330 ng, lanes 4 and 11 = 670 ng, lanes 5 and 12 = 1,330 ng/1 × 10^6 cells). Coimmunoprecipitations were carried out using the anti-HA Abs and the blots were revealed with the anti-HA and the anti-Vif specific Abs. Lanes 7 and 14, containing the same amount of WT-Vif-Myc of lanes 6 and 13, but no Cul5-HA, correspond to negative controls. For the left and right panel apply the same description as for panels C and D.

**Figure 6. Chim3 prevents HIV-1 Vif-induced cell cycle arrest in G2.** CEM A3.01 cells transduced with empty-LV or Chim3-LV (target cells) were synchronized with aphidicolin and then infected in a single round infection with VSV-G pseudotyped Vif-proficient (panels A-D) and Vif-deficient (Δvif) (panels E-H) NL4-3-based HIV-1 molecular clones produced from either HEK-293T cells expressing the empty-LV or Chim3-LV (producer cells). After intracellular p24Gag staining, P.I. content was evaluated only in electronically gated p24Gag^+ cells. The G2:G1 ratio values are indicated on the right upper corner of each cell cycle profile panel.
Figure 7. Schematic model of Chim3-induced survival advantage following HIV-1 infection. The model illustrates the effect of Chim3 in inducing prolongation of Chim3-cell survival. After n rounds of infection and cell duplication, the Chim3 population prevails over mock-transduced cells.
Figure 1
Figure 2

A. Day 49 post infection

B. Mean±SEM % NGFR: cells

C. Mock vs Chim3

D. Days post infection

Legend:
- Infected cells
- Uninfected cells
- Negative control
Figure 3

A

B

C

Means±SEM HIV-1 DNA (%)
Figure 4
Figure 5
**Figure 6**

**HIV-1 p24Gag**

<table>
<thead>
<tr>
<th>Producer cells (HEK-293T)</th>
<th>Target cells (CEM A3.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Empty</td>
<td>Chim3</td>
</tr>
<tr>
<td>28%, 41%</td>
<td>41%, 38%</td>
</tr>
<tr>
<td>52%, 42%</td>
<td>42%, 42%</td>
</tr>
<tr>
<td>1.81</td>
<td>1.02</td>
</tr>
</tbody>
</table>

| C                         | D                        |
| Empty                     | Chim3                    |
| 31%, 42%                  | 42%, 42%                 |
| 44%, 36%                  | 0.84                     |

| E                         | F                        |
| Empty                     | Chim3                    |
| 59%, 38%                  | 55%, 35%                 |
| 22%                       | 25%                      |
| 0.36                      | 0.45                     |

| G                         | H                        |
| Empty                     | Chim3                    |
| 59%, 41%                  | 55%, 38%                 |
| 23%                       | 21%                      |
| 0.38                      | 0.39                     |

**Δvif-Δvpr-HIV-1 p24Gag**

<table>
<thead>
<tr>
<th>Producer cells (HEK-293T)</th>
<th>Target cells (CEM A3.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>Empty</td>
<td>Chim3</td>
</tr>
<tr>
<td>59%, 38%</td>
<td>55%, 35%</td>
</tr>
<tr>
<td>22%</td>
<td>25%</td>
</tr>
<tr>
<td>0.36</td>
<td>0.45</td>
</tr>
</tbody>
</table>

| G                         | H                        |
| Empty                     | Chim3                    |
| 59%, 41%                  | 55%, 38%                 |
| 23%                       | 21%                      |
| 0.38                      | 0.39                     |

DNA Content (P.I.)

For personal use only. on November 12, 2017. by guest

www.bloodjournal.org From
Figure 7
Chim3 confers survival advantage to CD4+ T cells upon HIV-1 infection by preventing HIV-1 DNA integration and HIV-1-induced G2 cell cycle delay

Simona Porcellini, Francesco Gubinelli, Luca Alberici, Bianca Maria Piovani, Gian-Paolo Rizzardi and Chiara Bovolenta