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ANTI HIV-1 GENE THERAPY APPROACH COMBINING MULTIPLE siRNAs WITH THE MEMBRANE-ANCHORED FUSION INHIBITOR C-PEPTIDE maC46

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1. SUMMARY

The development of highly active anti-retroviral therapy (HAART) has considerably improved life expectancy of HIV-1 positive patients by transforming this infection, which once was lethal, into a manageable chronic illness. Although a significant suppression of viral replication under undetectable level is guaranteed following a constant therapeutic adherence, this therapy fails to completely eliminate the infection due to the persistence of HIV-1 into reservoirs, which represent therefore the main obstacle to a definite cure. Furthermore, a lifelong adherence to treatment is associated with drug toxicities and persistent immune dysfunction, which can lead to discontinuation of therapy and the onset of drug resistance. These hurdles, together with the high economic costs of providing HAART to more than 35 million people, which are currently affected by HIV-1, contribute to render HIV-1/AIDS pandemic one of the most important global health challenge. In this scenario, the search for a curative strategy is necessary. Recent successes in inherited immune deficiencies treatment and cancer immunotherapy have raised interest in gene and cell modification to treat HIV-1 infection with the final aim of inducing permanent resistance to HIV-1. In particular, anti HIV-1 gene therapy (GT) protocols, based on engineering of autologous T cells or their progenitors, such as the $CD34^+$ hematopoietic stem cells (HSCs), appear a promising approach to repopulate the immune system after a single therapeutic intervention. Long-term HIV-1 remission in the "patient of Berlin", who received an heterologous stem cell transplant for acquired immunodeficiency syndrome-related lymphoma from a CCR5 homozygous null HLA-matched donor (CCR5 -/-), even after discontinuation of conventional therapy, has energized the field. However, due to the limited chance of finding matching Δ32 CCR5 donors, recapitulating this clinical success on a large scale appears to be difficult. Moreover, autologous regimens are potentially less toxic, as they may not require full bone marrow ablation or subsequent immune suppression for engraftment. In this setting, the goal would be to disable the CCR5 gene in enough target cells to confer benefit and transplanted back into the patient. Different GT strategies to artificially disrupt the CCR5 gene or transcript have proved to be successful in primary T cells, HSCs, as well as in humanized mice, and are recently been tested in clinical trials. Inhibitors of entry or of early step of viral replication prior the virus integration are expected to lead an advantage selection of gene modified cells, thus preventing the establishment of chronic HIV-1 infection and limit the continued

replenishment of viral reservoirs. However, a major goal of gene therapy is to target simultaneously multiple viral sites and endogenous host factors, interfering with different steps of viral cycle, hoping to reduce the onset of virus variants. Among antiviral agents, small interfering RNAs (siRNAs) are less immunogenic than protein-based ones and represent a powerful tool to silence gene expression post-transcriptionally in a sequence specific manner. In order to obtain a stable and long expression, necessary for a chronic disease, multiple anti-HIV-1 siRNAs can be accommodated into self-inactivating (SIN) lentiviral vectors, which are currently preferred for their ability to efficiently transduce target cells, and then because they confer a potentially safer integration site profile, compared to gammaretroviral vectors. For these reasons, different combinatorial platforms based on SIN lentiviral vectors were previously developed to express multiple siRNAs against highly conserved regions of cellular and viral genes, including the cellular co-receptor *CCR5*, the *vif* and *tat/rev* viral factors, involved in different phases of HIV-1 replication and pathogenesis. These siRNAs were placed under the control of different human Polymerase III promoters (such as U6, 7SK and H1) either as independent transcriptional units or as extended short hairpin RNA (e-shRNA), able to express the three siRNA under the control of a single promoter. The most potent antiviral activity in transduced human primary $CD4^+$ T lymphocytes was conferred by two effective anti-HIV-1 combinatorial vectors (i.e. pLL3.7 U6shCCR5-7SKshvif-H1lhtat/rev and pLL3.7 H1e-shRNA). However, HIV-1 can use an alternative coreceptor (i.e. CXCR4) for entering into target cells, that is less favored as target of siRNA or novel gene-editing technologies, because its disruption can compromise fundamental physiological functions, especially the maturation of HSCs. At the same time, CXCR4 tropic viruses are relevant in the pathogenesis of AIDS. Thus, to improve the efficacy of this approach, the two selected vectors were optimized by the insertion of a small membrane anchored C-peptide (maC46) fusion inhibitor, which has been shown to protect against a broad range of HIV-1 isolates and it has been tested in a phase 1 clinical trial. When expressed on target cell surface, the maC46 peptide, which derives from the C-terminal heptad repeat (HR2) of the HIV-1 gp41 envelope glycoprotein, blocks the membrane fusion by interacting with the N-terminal coiled coil domain of the gp41 intermediate structure and preventing the six-helix bundle formation. In these constructs, the expression of the peptide, either fused in frame with the enhanced green fluorescence protein (eGFP) or alone, is driven by the human Elongation Factor 1 promoter (EF1), a cellular-derived

enhancer/promoter, which has been shown to confer high level of transgene expression in HSCs and a more safety profile, since decreased cross-activation of nearby promoters. Additionally, the new developed lentiviral vectors carries an optimized version of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE*), which enhances the vector titer and lacks oncogenic properties. Furthermore, control vectors either lacking the maC46 encoding sequence or characterized by a scrambled sequence in place of the siRNA encoding ones were generated. Once transfected into different cell lines, the developed vectors led to the expression of the maC46 peptide, that correctly localized at the plasma membrane, as shown with immunofluorescence cell staining. Furthermore, recombinant lentiviral particles (RLVPs) were produced and titrated either using Reverse Transcriptase (RT) Assay and Fluorescence-activated cell sorting (FACS). The efficacy of the maC46 peptide fusion inhibitor, in combination with the silencing activity of the expressed siRNAs, was evaluated in challenge experiments, in which transduced T lymphoblastoid Jurkat cells were infected with HIV-1 HXBc2 $Vpr^{+}/Vpu^{+}/Nef^{+}$ R4-tropic molecular clone at different multeplicity of infection (M.O.I.). In parallel, in collaboration with the Baum's group at the Department of Experimental Hematology of the Hannover Medical School, the mutagenic potential of these vectors was assessed by means of *in vitro* immortalization assay. The results obtained so far clearly show that the optimized vectors, combining for the first time a potent fusion inhibitor with three short hairpin (sh)RNAs, appear to be extremely promising as anti-HIV-1 approach. Importantly, these vectors showed a strongly reduced insertional transformation potential compared to a positive gammaretroviral control vector. This strategy could be now extended to primary cells and in particular to HSCs, the ultimate target of this gene therapy approach, with the final aim of accomplishing a high level of protection from HIV-1 infection over sustained lengths of time, without cell toxicity, and loss of stemness, proliferation capability and differentiation potential for possible future clinical applications.

2. SOMMARIO

La storia naturale dell'infezione da HIV-1 e il relativo approccio terapeutico hanno stimolato la comunità scientifica a porsi una serie di interrogativi. Negli ultimi anni sono state introdotte diverse classi di nuovi farmaci, tra cui inibitori nucleosidici e nucleotidici della trascrittasi inversa (NRTI), inibitori non nucleosidici della trascrittasi inversa (NNRTI), inibitori della proteasi (PI), dell'integrasi (IN), inibitori di entrata (i.e. antagonisti del corecettore CCR5 e inibitori di fusione), in grado di ridurre notevolmente la carica virale per ristabilire un certo grado di immunocompetenza da parte dell'ospite e sono stati sperimentati protocolli terapeutici caratterizzati da loro combinazioni, come avviene nel regime polifarmacologico della "terapia antiretrovirale altamente attiva" (HAART). Tuttavia, questi trattamenti non sono ancora del tutto esenti da limiti. Infatti, la non eradicabilità dell'infezione, correlata alla persistenza del virus in determinati distretti anatomici e cellulari (reservoir di infezione), obbliga ad un regime terapeutico a tempo indefinito, imponendo, quindi, nuove problematiche quali l'insorgenza di specie virali farmaco-resistenti, la tossicità d'organo, le interazioni farmacologiche dei farmaci somministrati e, non ultimi, gli elevati costi. Ciò ha favorito e incrementato lo sviluppo di strategie alternative e/o complementari per la cura dell'infezione da HIV-1. Il primo importante successo nella cura dell'infezione da HIV-1 è stato ottenuto in un paziente leucemico HIV-1-positivo, in seguito al trapianto allogenico di cellule staminali ematopoietiche (HSCs), intrinsecamente resistenti all'infezione a causa di una delezione a livello del corecettore virale CCR5 (recettore C-C per le chemochine di tipo 5). Tuttavia, il potenziale rischio di rigetto e la difficoltà di reperire donatori compatibili, non ne consentono un'applicazione diffusa. Pertanto, un possibile approccio alternativo è rappresentato dalla terapia genica, finalizzata all'espressione di geni anti-HIV-1 nelle cellule bersaglio dell'infezione, preferenzialmente nelle HSCs, cellule totipotenti in grado di differenziarsi verso tutte le linee emopoietiche coinvolte nella patogenesi dell'infezione, per generare un sistema immunitario permanentemente resistente al virus, a seguito di un singolo trattamento. In particolare, gli inibitori dell'ingresso o delle fasi iniziali della replicazione virale che precedono l'integrazione del genoma virale nel DNA cromosomico della cellula ospite possono prevenire l'instaurarsi di

una infezione cronica, portando ad un vantaggio selettivo delle cellule modificate. D'altro canto, un ulteriore vantaggio consiste nella possibilità di prevenire le mutazioni che insorgono nel processo di trascrizione inversa. Un obiettivo importante della terapia genica consiste nell'agire contemporaneamente nei confronti di più siti virali e fattori cellulari endogeni, in modo da interferire con diverse fasi del ciclo replicativo, mimando il consolidato approccio terapeutico polifarmacologico, con l'intento di ridurre al minimo la probabilità di insorgenza di varianti virali resistenti. A tal proposito, la strategia degli *RNA interference* (RNAi) rappresenta un valido strumento per silenziare l'espressione genica post-trascrizionale. Molteplici short hairpin (sh)RNA diretti verso trascritti virali e cellulari possono essere combinati all'interno di un vettore lentivirale "*self inactivating*" di terza generazione (SIN). Questi vettori sono in grado di conferire un'espressione stabile e duratura nel tempo dei transgeni, grazie all'integrazione nel DNA cromosomico della cellula ospite, caratteristica importante e necessaria ai fini terapeutici di una patologia cronica come l'infezione da HIV-1. Sulla base di tali presupposti, sono state precedentemente sviluppate in laboratorio diverse combinazioni di vettori lentivirali esprimenti *small interfering RNA* (siRNA) diretti contro regioni altamente conservate di geni target cellulari (come *CCR5*) e virali (come *vif*, *tat* e *rev*) allo scopo di interferire con diverse fasi del ciclo replicativo di HIV-1. Nello specifico, due *short hairpin (sh)RNA*, codificanti un singolo siRNA diretto contro il trascritto del gene cellulare *CCR5* (shCCR5) e del gene virale *vif* (shvif) ed un *long hairpin (lh)RNA*, codificante due siRNA diretti contro il trascritto comune del primo esone dei geni *tat* e *rev* (lhtat/rev), sono stati posti sotto il controllo di diversi promotori umani della polimerasi III (tra i quali U6, 7SK, H1), sia come unità trascrizionali indipendenti, sia come *extended short hairpin RNA* (e-shRNA), in grado di esprimere i tre siRNA sotto il controllo di un singolo promotore. Dopo aver verificato che l'attività di silenziamento dei diversi siRNA fosse stabile e funzionale, studi di inibizione della replicazione virale condotti sia in linee cellulari che in cellule umane primarie, hanno portato all'identificazione di due vettori (pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev e pLL3.7 H1e-shRNA), capaci di conferire un'efficiente protezione nei confronti di ceppi di laboratorio di HIV-1. Tuttavia, l'ingresso di HIV-1 all'interno della cellula ospite può essere mediato anche da un altro corecettore, CXCR4, il quale risulta meno favorevole al silenziamento genico da parte di siRNA o alla distruzione ad opera delle emergenti

tecniche di gene editing, essendo coinvolto in importanti funzioni fisiologiche, in particolare nel processo di maturazione delle cellule staminali ematopoietiche. Allo stesso tempo, i virus CXCR4-tropici (X4) sono rilevanti nella patogenesi dell' AIDS. Pertanto, con l'intento di conferire una protezione ad ampio spettro, i vettori precedentemente selezionati sono stati ottimizzati mediante l'inserimento di un inibitore di fusione, appartenente alla classe dei peptidi sintetici C, derivati dalla porzione C-terminale della subunità gp41 della glicoproteina dell'envelope di HIV-1. In particolare, è stato dimostrato che la forma di peptide ancorata alla membrana (maC46), quando viene espressa sulla superficie di cellule geneticamente modificate, è in grado di proteggere queste ultime dall'infezione da parte di un'ampia gamma di isolati clinici e di ceppi di laboratorio di HIV-1, fornendo inoltre un notevole vantaggio selettivo rispetto a cellule non esprimenti il peptide. La cassetta trascrizionale di maC46, inserita all'interno dei vettori selezionati come unità singola o in frame con il gene reporter eGFP, è stata posta sotto il controllo del promotore umano *Elongation Factor 1* (EF1), in grado di indurre elevati livelli di espressione del transgene in cellule staminali ematopoietiche. Inoltre, i nuovi vettori sviluppati presentano una versione ottimizzata del *Wooodchuck Hepatitis Virus post-trascriptional regulatory element* (WPRE*), privato del potenziale oncogeno, per garantire maggiore sicurezza dal punto di vista terapeutico, ma al contempo in grado di mantenere gli stessi livelli di espressione del transgene. Dopo aver testato, mediante tecniche di immunofluorescenza diretta, l'effettiva localizzazione del peptide maC46 a livello della membrana cellulare, sono state prodotte particelle lentivirali ricombinanti in cellule embrionali di rene umano (293T), il cui titolo è stato determinato mediante saggio di attività retrotrascrittasica (*RT Assay*) ed analisi citofluorimetrica (*Fluorescence-activated cell sorting,* FACS). La valutazione del contributo antivirale del peptide maC46, in combinazione con l'attività di silenziamento, dovuta alla presenza di siRNA espressi dai vettori lentivirali, è stata effettuata in esperimenti di challenge, in cui linee cellulari T-linfoblastoidi $CD4^+$, opportunamente trasdotte con le particelle ricombinanti, sono state infettate con il clone molecolare di HIV-1 HXBc2 $Vpr^+/Vpu^+/Nef^+$ X4-tropico, utilizzando diverse molteplicità di infezione. Parallelamente, in collaborazione con il gruppo di ricerca del professor Christopher Baum del Dipartimento di Ematologia Sperimentale della Scuola Medica di Hannover, è stato testato il potenziale rischio di mutagenesi inserzionale dei vettori, valutazione indispensabile prima di poter procedere

all'impiego del modello animale, alla manipolazione di cellule staminali e, in futuro, ad una applicazione clinica. Per tali motivi, i vettori sono stati sottoposti al saggio di immortalizzazione cellulare *in vitro*. I risultati ottenuti fino ad ora hanno chiaramente dimostrato che i vettori sviluppati, in cui è stato combinato per la prima volta un potente inibitore di fusione con tre siRNA, sono estremamente promettenti in termini di attività antivirale nella linea cellulare linfoblastoide impiegata. Inoltre, tali vettori si sono dimostrati incapaci di indurre effetti genotossici. L'obiettivo ultimo del più ampio progetto di ricerca, in cui si inserisce il presente lavoro, consiste nell'accertare l'efficacia e la sicurezza di tali vettori in modelli animali, per giungere infine alla manipolazione genetica di HSCs derivanti da pazienti HIV^+ affetti da linfoma, i quali rappresentano la popolazione ideale in un contesto clinico eticamente accettabile, poiché sono spesso sottoposti a trapianto di HSCs.

3. ABSTRACT

Background HIV-1 infection is normally characterized by sustained viral replication and a progressive loss of $CD4^+$ T cells, leading to AIDS. Combined antiretroviral therapy (cART) suppresses viral replication and drastically reduces mortality. However, cART alone is not able to eradicate the virus, which persists in reservoirs, and it is still associated with considerable comorbidities and the emergence of drug-resistant escape mutants. A cure for HIV-1 infection is needed to bypass the limitations of the current therapy and restore health. Advances in the fields of gene-targeting strategies, based on intracellular immunization of autologous T cells or their progenitors, i.e. the $CD34^+$ hematopoietic stem cells (HSCs), appear encouraging to repopulate the immune system. In particular the transduction of target cells with self-inactivating (SIN) lentiviral vectors encoding short hairpin (sh)RNAs and transplanted back into the patient ensures stable and long expression of antiviral genes in a chronic infection such as the one induced by HIV-1.

Materials and Methods SIN lentiviral vectors were generated in order to express multiple HIV-1 inhibitors. Recombinant particles were produced in HEK 293T cells and titrated by flow cytometry and Reverse Transcriptase (RT) activity assay. To address the antiviral activity of the combinatorial vectors, $CD4^+$ T lymphoblastoid Jurkat cells were transduced with lentiviral particles, then subjected to the cell viability and finally were infected with the HIV-1 HXBc2 $Vpr^+/Vpu^+/Nef^+$ strain using different M.O.I. In order to test the mutagenic potential of this SIN lentiviral platform, the *in vitro* immortalization (IVIM) assay was also employed.

Results The generated vectors were characterized by different combinations of siRNA targeting the *CCR5* cellular gene as well as *vif* and *tat/rev* viral transcripts, along with or without an HIV-1 membrane-anchored C-peptide fusion inhibitor (maC46), which has been shown to efficiently block the entry of both CXCR-4 and CCR5-using viruses. Specifically, the siRNA were either expressed as single transcriptional unit under the control of different human RNA polymerase III promoters (U6, 7SK, H1), or simultaneously, as an extended shRNA. The maC46, which derives from the C-terminal heptad repeat (HR2) domain of envelope glycoprotein (gp41), was cloned under the transcriptional control of the human elongation factor 1 alpha (EF1) promoter. Finally, an optimized version of the Woodchuck Hepatitis Virus Post-transcriptional Regulatory

Element (WPRE*), able to reduce the risk of oncogenicity, was introduced in all vectors. The EF1 alpha promoter led to a high level of maC46 expression, with a correct localization of the peptide at the plasma membrane. The developed lentiviral particles efficiently transduced Jurkat cells, without cytotoxic effect, and potently blocked HIV-1 replication; in particular, the lentiviral combinatorial platform expressing the triple cassette sequence along with the peptide conferred the best antiviral activity under all tested experimental conditions. Furthermore, no impact on the viability or on the proliferation of transduced murine cells was observed in the IVIM assay.

Conclusions The developed vectors, combining a potent fusion inhibitor with three shRNAs, appear to be extremely promising as anti-HIV-1 strategy. Once challenge experiments in human primary $CD4^+$ cells will be completed, the best performing vectors will be assayed in $CD34^+$ HSCs in order to further validate this approach for clinical application in selected HIV-1^+- lymphoma patients.

4. INTRODUCTION

4.1 The Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV) is a member of the *Retroviridae* family and genus *Lentivirus* entirely restricted to vertebrate hosts (*Chiu et al., 1985*). HIV is identified as the etiologic agent of a severe immune deficiency, termed as Acquired Immunodeficiency Syndrome (AIDS) (*Barrè-Sinoussi et al., 1983; Gallo et al., 1984; Blattner et al., 1988*), characterized by a progressive deterioration of the immune system, which is almost uniformly fatal if untreated, because of opportunistic infections, secondary neoplasms, and neurological manifestations. Initial cases of HIV were reported in 1981 to Centre for Disease Control, and the virus was first isolated from patients in 1983; nowadays HIV/AIDS pandemic represents one of the most important global health challenge. More than 35 million people are currently living with HIV and each year approximately 2.1 million of individuals became newly infected (UNAIDS Report on the Global AIDS epidemic 2016), since neither definitive therapy for this disease nor a vaccine to stop the spread of the pandemic are available (*Imami and Herasimtschuk, 2015; Leal et al., 2017)*. *Retroviridae* family reverse transcribes its genome to form copy DNA (cDNA), which integrates into the chromosomal DNA of the host cell as a provirus. Both of these processes are mediated by viral enzymes, reverse transcriptase and integrase, respectively (*Goff, 1990*). *Lentiviruses* are characterized by cytopathicity *in vitro* and enstablishment of chronic infections. Most of the lentiviruses are identified in more than 40 species of African primates (*Castro-Nallar et al., 2012*). The HIV strains arose from cross-species transmission of non-human primate lentiviruses (occurred between chimpanzee (*Pan troglodytes troglodytes*) SIV (SIVcpz) and sooty mangabey SIV (SIVsm) with humans), as revealed by the sequence homology between the two human species and various strains of SIV located in the regions of West Africa (*Barin et al., 1985; Brun-Vezinet et al., 1987*). Two correlated forms of HIV have been isolated from AIDS patients, the HIV-1 and the HIV-2, which are distinguishable by serological and nucleotide sequence differences. While HIV-2 remains almost exclusively localized in some areas of Central and West Equatorial Africa (*Lemey et al., 2003*), HIV-1 is more virulent and it is the protagonist of the pandemic (*Peeters and Sharp, 2000*). One of the major characteristics of HIV-1 worldwide spread is its high genetic variability and extensive heterogeneity among viral

isolates circulating in several individuals or even at different times of the infection in the same individual (the so called viral quasispecies) (*Li et al., 1988; Korber et al., 1998*). This diversity is due to the high number of mutations introduced (little less than a replicated genome error) by the reverse transcriptase enzyme, that lacks proof-reading activity (*Roberts et al., 1988*), combined with a significant turnover of the viral population (over 10^{10} virions produced every day), leading to the development of a viral population adaptable to each dynamic pressure and selection process. Based on the phylogenetic relationships, HIV-1 strains have been classified into distinct genetic groups, designated Major (M), Outlier (O), Non-M, Non-O (N) and P (*Hemelaar, 2012; De Leys et al., 1990; Simon et al., 1998; Plantier et al., 2009*), each of which resulted from an independent cross-species transmission event. Within HIV-1 group M, nine genetically distinct subtypes or clades are recognised (A through D, F through H, J and H), which dominate in different geographical locations, but can also coexist. Co-infection with multiple strains or subtypes can lead to the generation of new circulating recombinant forms (CRFs) or unique recombinant forms (URFs) *(Sharp et al., 1995; Lau and Wong, 2013).* However, the generation of recombinant viruses is a relatively rare event, since simultaneous replication of the two viral variants within the same cell is necessary in the absence of significant competition and only if the new recombinant has a replicative advantage over progenitors. Although the global availability of sequencing techniques has increased, the classification of HIV-1 strains into subtypes and CRFs remains a complex issue and it is subject to evolution as new genetic variants are identified.

4.1.1 Virion morphology, structure and genome organization

Electron microscopic analysis show that mature HIV-1 virions measure 100 to 150 nm in diameter (**Figure 1**). Viral particles are characterized by an electron-dense conical core, which is surrounded by a lipid bilayer membrane (envelope), acquired during the budding from the infected cell (*Gottlinger, 2001*). The envelope contains the transmembrane glyproteins, gp120 and gp41, which project outwards from the virion in the form of spikes. The core contains two long positive sense, single stranded, linear RNA molecules of approximately 9.4 kb in length, which encode the major structural and non-structural proteins common to all replication-competent retroviruses (**Figure 2**). The genome sequence is flanked by two long-terminal repeats (LTRs). The 5' LTR contains the enhancer-promoter sequences for viral transcription and the 3' LTR

contains the polyadenylation signal. From 5' to 3' the viral genome contains the *gag* gene, which encodes the virion structural components; the *pol* gene which encodes the viral enzymes and the *env* gene which encodes the envelope glycoproteins (*Vogt, 1997*). The primate immunodeficiency viruses encode a number of non-enzymatic and non-structural gene products; two of these genes, *tat* and *rev* are essential for viral transcription and nuclear export of viral transcripts respectively (*Karn and Stoltzfus, 2012*). The others (*nef*, *vpr*, *vif* and *vpu* in HIV-1 or *vpx* in HIV-2) are defined accessory genes since they are not absolutely required for viral replication in all *in vitro* systems (*Vicenzi et al., 2013*), although they play an important role *in vivo (Collins and Collins, 2014)*.

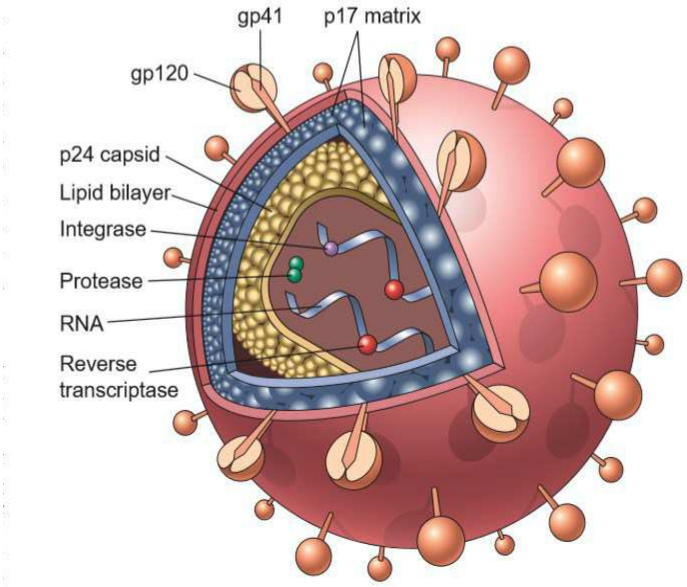

Figure 1. **The structure of the Human Immunodeficiency Virus (HIV)-1 particle.** The viral particle is covered by a lipid bilayer, derived from the host cell, and studded with viral envelope glycoproteins, composed of a surface domain (gp120) and a transmembrane domain (gp41), which are noncovalently associated and form trimeric spikes. The mature virion contains the matrix (MA, p17) protein, the conical core composed of the capsid (CA, p24) protein, the nucleocapsid (NC, p7) protein which binds the genomic RNA, the p6 protein required for budding, the accessory protein Vpr, the reverse transcriptase (RT), the protease, the integrase (IN) and two copies of the genomic RNA. The accessory protein Nef is also virion associated (*Robbins & Cotran, Pathologic basis of disease IX edition*).

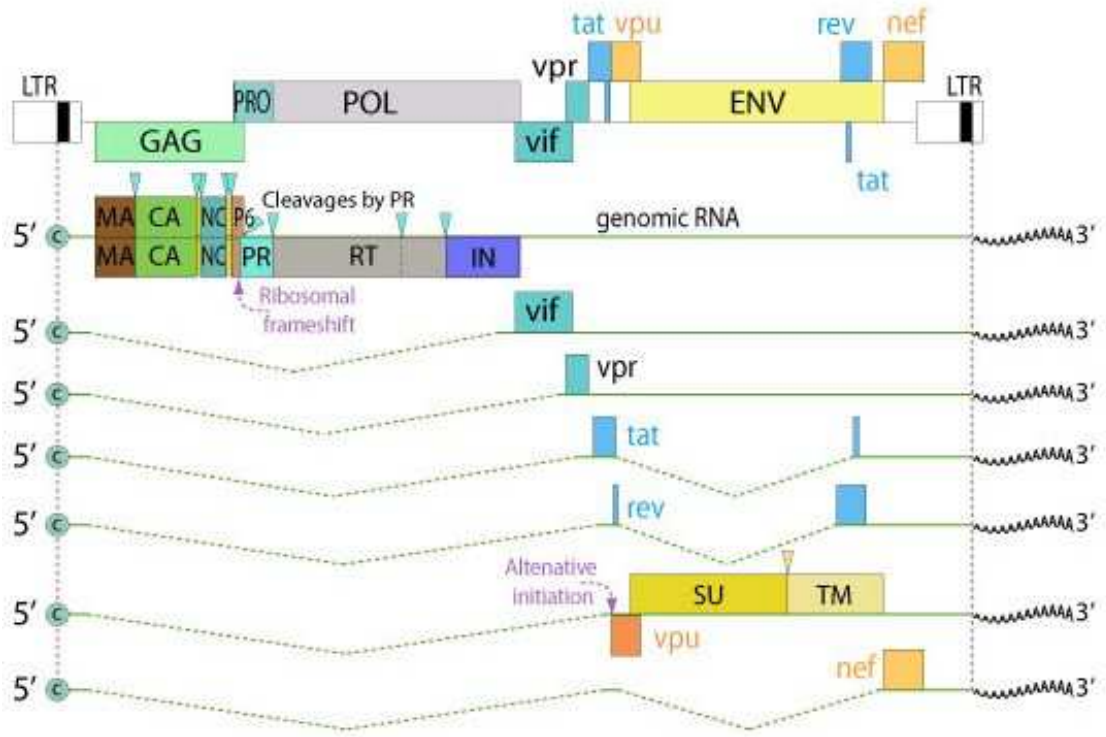

Figure 2. **The HIV-1 genome, transcripts and proteins.** The viral genome of 9,6 kb is flanked by two long terminal repeats (LTR). The integrated provirus utilizes the promoter elements in the 5' LTR to drive transcription. This gives rise to the unspliced full length mRNA that will serve as genomic RNA to be packaged into virions or used as a template for translation of group-specific antigen (gag) and gag-(pro) polymerase (pol) (1 ribosomal frameshift) polyproteins. These polyprotein precursors are processed by the viral protease into nine subunits: protease (PR), reverse transcriptase (RT), which contains RNAse H, integrase (IN), matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1 and p6. The uncompletely spliced mRNAs encode env that is cleaved by cellular proteases, such as furin, into surface (SU) gp120 and transmembrane (TM) gp 41 envelope proteins, and the accessory proteins: viral infectivity factor (Vif), viral protein u (Vpu), and viral protein r (Vpr). Completely spliced mRNAs encode Rev, Tat and Nef accessory proteins. Tat is the main transcriptional regulator of the long terminal repeat (LTR). Its RNA target, the transactivation response (TAR) element, is present at the 5' end of all viral transcripts. Rev is the main nuclear-export protein, which escorts unspliced and uncompletely spliced RNAs out of the nucleus of infected cells; thus it regulates the shift between early and late viral gene expression *(*imagine taken from www.expasy.org/viralzone/).

The major structural and core proteins of HIV-1 are synthetized from *gag* as a large myristoylated polyprotein precursor, Pr55Gag, which is subsequently cleaved by the viral protease (PR) to the mature Gag proteins matrix (also known as MA or p17), capsid (CA or p24), nucleocapsid (NC or p7). The matrix protein is primarily a peripheral membrane protein located along the inner of the viral lipid envelope, where it directs the incorporation of the envelope glycoprotein (Env) into the forming virion (*Dorfman et al., 1994*). The capsid protein p24 forms the conical core that encloses the viral genome. Cyclophilin A has been demonstrated to interact with the p24 region of p55 leading to its incorporation into HIV-1 particles (*Franke et al., 1994*). The nucleocapside protein p7 is an RNA binding protein required for packaging of the

genomic RNA into the virion (*Lapadat-Tapolsky et al., 1993*). Several smaller cleavage products, p1, p2 and p6 are also generated from the p55 precursor; the p6 protein contains a so-called late or L domain, which is required for viral budding, and it mediates the incorporation of the viral accessory protein Vpr into virions (*Paxton et al., 1993*).

The viral enzymes are also produced by proteolytic cleavage from a large precursor molecule. During translation, a ribosomal frameshift occasionally occurs between the *gag* and *pol* open reading frames, resulting in the synthesis of a *gag-pol* precursor protein (*Jacks et al., 1988).* The ratio of Gag-Pol to Gag produced is approximately 1:20.

The viral protease (Pro), integrase (IN), ribonuclease H (RNase H) and reverse transcriptase (RT) are always expressed within the context of a Gag-Pol fusion protein. The virally encoded protease cleaves the Pol polypeptide away from Gag and further digests it to separate the protease (p10), RT/RNase H (p66/p51), and IN (p32) activities. The aspartyl protease acts as a dimer to cleavage the Gag and Gag-Pol polyprotein precursors during virion maturation. Pharmacologic inhibition of the viral protease prevents the formation of infectious particles (*Ashorn et al., 1990*). RT is an asymmetric heterodimer composed of two related subunits, p66 and p51. This enzyme provides both the RT activity, which allows RNA-dependent DNA polymerization, and RNase H activity, which allows the specific degradation of RNA present in the RNA-DNA hybrid duplex *(Sarafianos et al., 2009*). The smaller subunit (p51) plays a structural role. The IN protein mediates the insertion of the viral DNA into host cellular chromosomal DNA. Preferential integration into regions of open, transcriptionally active, chromatin may facilitate the expression of the provirus. Viral genes are not efficiently expressed from non integrated proviral DNA (*Wiskerchen and Muesing, 1995*).

The protein product of the *env* gene is synthesized in the endoplasmic reticulum as an 88 kDa polypeptide, which undergoes glycosylation in the Golgi network, through the addition of 25 to 30 complex N-linked carbohydrate side chains at asparagine residues. A fraction of the resulting molecule gp160 is cleaved by the cellular furin protease, to generate the transmembrane (gp41) and surface (gp120) subunits (*Stein and Engleman, 1990*). This cleavage event is required for viral infectivity, because it allows subsequent exposure of a sequence within the ectodomain of gp41 that mediates fusion of the viral envelope and target cell plasma membrane. Following proteolytic cleavage, gp 120 remains noncovalently associated with gp41 on the cell surface and the complex is

incorporated into the envelope of budding virions via an interaction between the cytoplasmic domain of gp41 and the p17 matrix protein. The C-terminal subunit, gp41, contains a cytoplasmic domain (ultimately inside the viral membrane), a membrane spanning domain and an extracellular domain, which mediates the conformational change needed for fusion (*Bernstein et al., 1995*). The gp41 mediates the fusion between the viral and cellular membranes through a particular N-terminal fusogenic domain, which can be used to design particular antiviral molecules able to prevent viral entry (*Van Lunzen et al., 2007*). The N-terminal subunit gp120, is completely outside the viral membrane. Interactions between HIV-1 and the host receptor CD4, are mediated through specific domains of gp120 *(Landau et al., 1988)*. Five hypervariable regions are present in gp120, whose amino acid sequences are highly variable among HIV-1 isolates. Among these, one variable region, called the V3 loop, is not involved in CD4 binding, but in the choice of tropism of HIV-1 for different cells, including T cells and macrophages (*Hwang et al., 1991*). The susceptibility of cell types to given viral strains depends on particular sequences within the V3 loop involved in the interaction with the HIV-1 co-receptors CXCR4 and CCR5, belonging to the family of chemokine receptors *(Feng et al., 1996).*

Control of HIV-1 gene expression depends on two viral regulatory proteins, Tat and Rev (*Cullen, 1994*). The HIV-1 Tat protein of 14 kDa is an RNA-binding transcriptional transactivator, which plays a pivotal role in HIV-1 replication (*Ruben et al., 1989*). The 72 and 101 amino acid long forms of Tat are expressed by early fully spliced mRNAs or late incompletely spliced HIV-1 mRNAs, respectively. Both forms function as transcriptional activators and are found within the nuclei and nucleoli of infected cells. Tat binds to a short-stem loop structure, known as the transactivation response element (TAR), that is located at the 5' terminus of HIV-1 RNAs (*Feng and Holland, 1988*). Tat markedly enhances the rate of transcription from the 5’ LTR by recruiting cellular factors that enhance the processivity of the cellular RNA polymerase II complex. Furthermore, Tat can recruit several chromatin-modifying proteins to remodel the promoter region (*Easley et al., 2009*). In the absence of Tat, viral transcription is essentially blocked just after initiation. Tat has been reported to be involved in several other non transcriptional functions (*Romani et al., 2010*), like influencing HIV-1 RNA splicing, capping, translation and reverse transcription, modulating the expression of multiple cellular genes, interacting with a large number of cellular proteins, and inhibiting the cellular RNA interference mechanism.

Rev is a 13-kDa sequence-specific RNA binding protein, which mediates the transport from the nucleus to the cytoplasm of singly spliced and unspliced mRNAs that encode the structural proteins of the virus. Normally, RNAs that contain introns (i.e. unspliced or incompletely spliced RNA) are retained in the nucleus. Rev interacts with a highly structure RNA element referred to as the Rev-responsive element (RRE), (which overlaps the coding sequence of the gp41), through its N-terminal arginine-rich domain (ARD) (*Malim et al., 1989*) and links these RNAs to the nuclear export protein CRM1 (also referred to as exportin 1). The C terminal region of Rev contains a leucine-rich nuclear export signal (NES), which binds to exportin 1 (*Wen et al., 1995*). Rev expression levels are tightly regulated by a negative feedback, whereby high levels of Rev expression can lead to the export of so much intron containing viral RNA up to decreasing the amount of RNA available for complete splicing, which in turn reduces the levels of Rev expression. In the absence of Rev activity, viral mRNAs are inefficienlty exported from the nucleus, and they undergo extensive splicing to yield subgenomic mRNAs encoding only a subset of viral proteins (*Felber et al., 1990*).

The accessory proteins are dispensable for viral replication under certain conditions *in vitro,* but are relevant to *in vivo* pathogenesis. Nef is a 27-kDa myristoylated peripheral membrane protein that is encoded by a single exon that extends into the 3' LTR. It was originally identified as a viral negative factor, that down-regulated transcriptional activity of the HIV-1 LTR; however, the expression of this necessary and enforcing factor in the early stages of the viral life cycle enhances viral replication and pathogenicity promoting the survival of infected cells, by modulating both cellular transduction and membrane trafficking (*Das and Jamel, 2005*). Nef decreases the expression of CD4 and class I major histocompatibility complex (MHC) molecules on the cell surface through endocytosis mediated by clathrin adaptor protein-1 (AP-1) and 2 (AP-2) respectively (*Jia et al., 2012*). Since an excess of CD4 on the cell surface has been found to inhibit Env incorporation and virion budding, the downregulation of this receptor allows the production of more infectious virions and the more efficient release of virions from infected cells (*Lama et al., 1999*). Furthermore, Nef increases the intrinsic infectivity of HIV-1 virions, through the exclusion of SERINC5 and SERINC3 from virion incorporation, thereby preventing the negative effect of these host proteins on virion infectivity (*Rosa et al., 2015*). The 12.7 kDa Vpr (Viral Protein R) virion-associated accessory protein is incorporated into virions and virion cores due to its direct association with the p6 *gag* gene product (*Cohen et al., 1990; Sato et al., 1990*).

This multifunctional protein is involved in different stages of the HIV-1 viral life cycle, including nuclear import of the HIV-1 preintegration complex (PIC), thanks to its affinity with the nuclear pore, particularly in nondividing cells, where this complex must cross an intact nuclear envelope. As a consequence, the protein is essential for viral replication in macrophages *(Heinzinger et al., 1994*). Furthermore, Vpr arrests cells in the G_2 phase of the cell cycle, preventing the activation of the p34cdc2/cyclin B complex, which is a regulator of the cell cycle required for the entry of cells into mitosis increasing the yeald of progeny virions per replication cycle (*He et al., 1995*). The 16-kDa Vpu protein is an integral membrane phosphoprotein that is primarily localized in the internal membranes of the cell and it is expressed from the mRNA that also encodes *env* (*Bour and Strebel, 2003*). Vpu enhances the release of virions from infected cells through its transmembrane domain by antagonizing the tethering effect of BST2/tetherin, an interferon (IFN)-regulated host restriction factor that directly cross-links virions on host cell-surface. Furthermore, Vpu induces a rapid intracellular degradation of CD4 through its cytoplasmatic domain, involving a cellular proteosomal degradation machinery (*Schubert et al., 1996; Dubè et al., 2010*). The viral infectivity factor Vif is a 23-kDa protein that promotes viral replication *in vivo* and is essential for the replication of HIV-1 in non-permissive cells, like peripheral blood mononuclear cells (PBMC). Defective viruses for the *vif* gene are able to replicate correctly only in the so-called "permissive" cells. The protein has the function of blocking a cellular cytidine deaminases called APOBEC3G, recognized as a normal defense mechanism of the body in response to infections (*Rose et al., 2004*). Normally, in the absence of Vif protein, APOBEC3G protein is encapsidated by budding virus particles and converts cytidine into uridine of the negative sense single-stranded DNA, causing hypermutations in the viral genome during reverse transcription. Thus, new viral particles produced in the cells expressing APOBEC3G will not be able to replicate (they will not be infectious). Vif binds to APOBEC3G and triggers its polyubiquitination and rapid degradation, thereby preventing its encapsidation into progeny virions (*Feng et al., 2014*).

4.1.2 The HIV-1 life cycle

The entire replication cycle of HIV-1 (**Figure 3**), from the attachment of virions to target cells to the release of infectious progeny, is completed in approximately 24 hours,

both *in vitro* and *in vivo* (*Kim et al., 1989*). The life cycle begins by binding of the virus to the cell surface, via a high-affinity interaction between the first domain of CD4 and the HIV-1 outer envelope glycoprotein, gp120 (*Sattentau and Moore, 1993*). This explains the selective tropism of the T-cell $CD4^+$ cell or other $CD4^+$ cells, in particular monocytes/macrophages and dendritic cells. However, binding to CD4 is not sufficient for infection. Gp120 also needs to adhere to other cell surface molecules (co-receptors) that render functional adsorption to cytoplasmic penetration: these molecules belong to the family of receptors with seven transmembrane domains coupled with G protein, which perform the physiological function of receptors of a set of chemokines (*Broder and Dimitrov, 1996*). The two major co-receptors are CXCR4 and CCR5. HIV-1 isolates can be divided according to the use of these coreceptors; X4 strains use CXCR4, which is expressed only on T cells; thus, these strains can infect only T cells, both fresh T cells isolated from peripheral blood and T cell lines grown *in vitro*. R5 strains use CCR5, which is expressed by both monocytes and T cells; thus, these strains can infect both monocytes and fresh T cells isolated from peripheral blood, but not T cell lines grown *in vitro*. Dual tropic strains (R5X4) can also be isolated, able to use both coreceptors and therefore can infect macrophages and T cells (*Gorry and Ancuta, 2011*). The natural ligands for CXCR4 and CCR5 are the stromal cell-derived factor 1 (SDF) and the β-chemokines (macrophage inflammatory protein 1α, MIP-1α, MIP-1β, and RANTES) respectively, which can block the infectivity of HIV-1 (*Oberlin et al., 1996; Alkhatib et al., 1996*). Tropism of HIV-1 has been considered to be associated with disease progression. The CCR5 coreceptor is used during the primary or early, asymptomatic stage of HIV-1 infection and R5 viruses are largely responsible for person-to-person transmission. Probably this is due to the fact that dendritic cells in the mucous epithelium express CCR5 but not CXCR4 and are therefore more susceptible to the infection of M-related viruses. In addition, the binding of M-related strains to CCR5 on T cells may induce these cells to produce chemotactic factors for other T cells, thus increasing the population of potential targets near an infected T cell. During the late stage of infection, viral isolates may arise that use CXCR4 for entry (*Verhofstede et al., 2012*). The emergence *in vivo* of X4 or dual-tropic viruses is often associated with a sharp decline in the number of $CD4^+$ T cells in infected individuals and the onset of AIDS-defining symptoms (*Raymond et al., 2010*). The X4 variants seem to be exposed to stronger immune pressure. Evidence suggests that X4 variants have a more potent ability to destroy the immune system because they have higher replication capacity in

some cells and a wider range of host cells than R5 viruses; infact, they are able to infect both native T cells and thymic precursors of T cells (*Fenyo et al., 1989; Naif, 2013*). It has been demonstrated that individuals homozygous for a CCR5 delta 32 mutation (approximately 1% in Caucasians) are naturally resistant to infection and AIDS development from HIV-1 R5 strains. Moreover, heterozygous mutations (which occurs in 4-16% of Caucasians) confer partial resistance to HIV-1 by reducing the CCR5 levels in T cells. In these individuals there is a delayed onset of the disease (*Samson et al., 1996*). After binding of gp120 to the coreceptors, the gp41 molecule undergoes a conformational modification with exposure of a fusogenic motif present in the N-terminal region, allowing the fusion peptide to enter into the double lipid layer of the target cell. The formation of the six-fold folding of gp41, in which anti-parallel interactions are involved between the N-terminal and C-terminal portions of the gp41 helixs, allows fusion of the viral membrane with that of the host cell (*Wu et al., 1996*). Then, viral RNA and some viral enzyme molecules are released into the cytoplasm, where the inverse transcription of the viral genome occurs, with the following formation of a double-stranded DNA molecule containing all information of the viral genome (cDNA). The reverse transcribed viral genome remains associated with several viral proteins as the pre-integration complex (PIC), consisting of p17, Vpr, IN. A flap structure within the middle of the viral plus strand cDNA (which is formed during the process of reverse transcription) is also required for efficient nuclear import. In fact, lentivirus, unlike other retroviruses, has the peculiar feature of replicating also in some non-active proliferation cells. In quiescent T cells, cDNA may remain in the cytoplasm as episomal linear DNA, in T-division cells, the cDNA penetrates into the nucleus and is integrated into the host genome. Thus, the double stranded linear DNA is integrated into the host cell chromosome by the virally encoded integrase in concert with host cell DNA repair enzymes to form the provirus. Viral cDNA is randomly inserted into the host cell genome but prefers the coding regions, probably due to better accessibility (*Schroder et al., 2002*). The proviral DNA remains permanently associated with that of the host cell: it may occur a latent infection if the viral genome remains silent, or a productive infection, with transcription of proviral DNA by the cellular polymerase and viral particle production. Activation of HIV-1 transcription and gene expression is dependent on the activity of both cellular and viral factors. Interaction of several cellular transcription factors (NF-Kb, AP-1, Sp1 and NFAT) with specific recognition sites within the 5' LTR occurs (*Nabel and Baltimore, 1987; Van Lint et al., 1997)*.

Transcription is initiated from a single site in the viral 5'LTR, the 5' boundary of the R region. The primary transcript is alternatively spliced to generate over 30 species of viral mRNAs (*Schwartz et al., 1990*). The early viral transcripts are extensively spliced to form a group of mRNA that are 1.8 to 2 kb in size and encode the proteins Tat, Rev and Nef. Tat markedly increases the level of viral transcription by directing the cellular transcriptional elongation factor P-TEFb to the nascent RNAs, facilitating their elongation. HIV-1 mRNAs are grouped into three size classes: the unspliced 9-kb primary transcript can be expressed to generate the Gag and Gag-Pol precursor proteins or be packaged into virions to serve as the genomic RNA; the incompletely spliced RNA includes heterogeneous mRNAs of 4- to 5-kb which use the splice donor site located nearest the 5' end of the HIV-1 RNA genome in combination with any of the splice acceptors located in the central region of the virus. These RNAs can potentially express Env, Vif, Vpu, Vpr, and the single-exon form of Tat; fully spliced RNA includes mRNAs which have spliced out introns of HIV-1 and can potential express Rev, Nef, and the two-exon form of Tat. These heterogeneous mRNAs do not require the expression of the Rev protein. The Rev protein binds to viral RNAs that retain intron sequences, and directs their export from the nucleus. This export allows the unconventional viral RNAs to bypass the normal "check point" of RNA splicing. The fully spliced viral mRNAs exit the nucleus by using the export pathway followed by the majority of cellular mRNAs. Threshold levels of Rev are necessary for exporting intron-containing HIV-1 mRNAs, explaining why those encode the viral late gene products. In contrast, the proteins encoded by the fully spliced mRNAs, Nef, Tat, and Rev, can be produced immediately, and are thus early viral gene products.

The virion assembles at the plasma membrane. The Gag and Gag-Pol precursor polyproteins are synthesized in the cytoplasm and associate with the plasma membrane via N-terminal myristolation. Dimerization activates the viral protease, which cleaves the precursor proteins to generate the individual structural and enzymatic polypeptides. The Gag polyprotein is sufficient for virion assembly. In contrast to Gag, the Env glycoproteins are synthesized in association with cellular membranes. Env is then recruited into the forming virion at the plasma membrane by association of the cytoplasmic domain of gp41 with p17 MA. The capsid protein p24 forms the core structure, and the nucleocapsid protein p7 binds the genomic RNA. The forming virion buds through the plasma membrane. This budding requires a specific domain (the L domain) in the p6 Gag protein. HIV-1 buds from specialized regions of the plasma

membrane that are enriched in cholesterol and glycolipids (so-called lipid rafts), which presumably favour fusion of the virions with target cells (*Nguyen and Hildreth, 2000*). The life cycle in latently infected cells occurs only after cell activation and, in the case of most $CD4^+$ T cells, viral activation leads to cellular lysis.

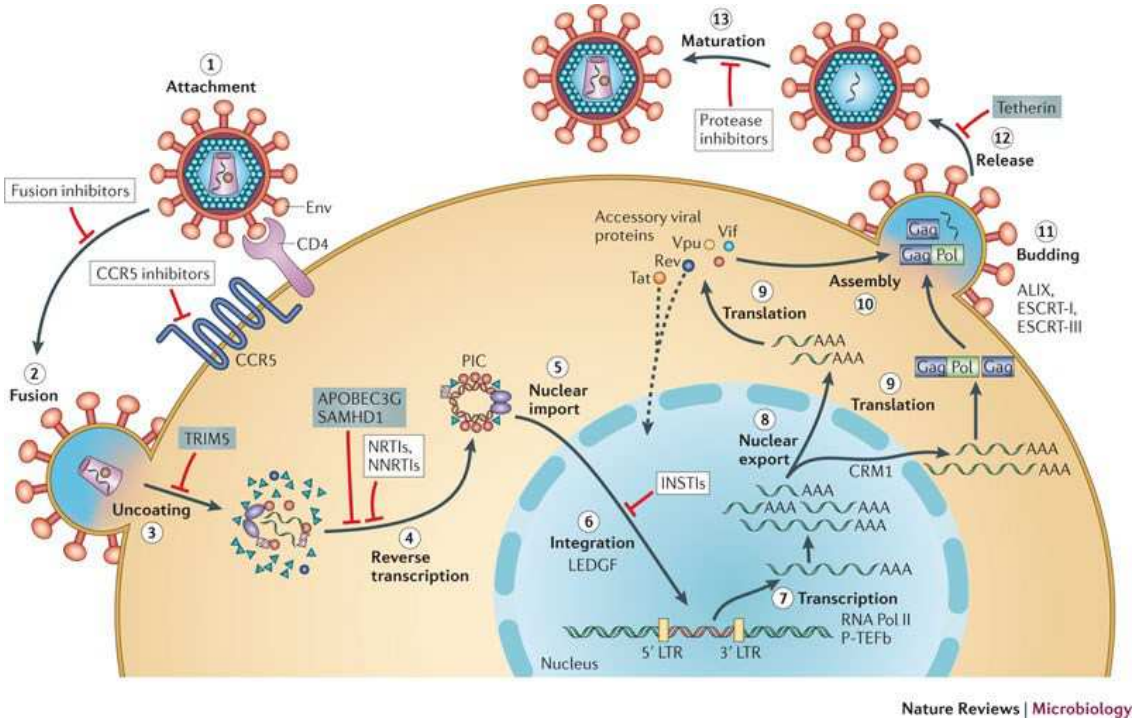

Figure 3. **Schematic depiction of the HIV-1 replication strategy.** The viral cycle proceeds through a series of steps that start when the envelope (Env) glycoprotein spikes engage the surface receptor CD4 and the membrane-spanning co-receptor CC-chemokine receptors (CCR5 or CXCR4), on target cells, mainly represented by T lymphocytes and macrophages (step 1). The fusion of the viral and cellular membranes allows entry of the viral particle into the CD4 cell (step 2). After the partial uncoating, (step 3), HIV-1 releases and uses reverse transcriptase to convert its genetic material in cDNA (step 4), which remains associated with several viral proteins as the pre-integration complex (PIC). After translocation into the cell nucleus (step 5), the virally encoded integrase, in concert with the host chromatin-binding protein lens epithelium-derived growth factor (LEDGF), allows the integration of the double-stranded linear DNA into the host cell chromosome to form the provirus (step 6). The HIV-1 transcription (step 7), which is dependent on the activity of host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), gives rise to different viral transcripts, that are alternatively spliced and exported from the nucleus (step 8). mRNAs serve as templates for proteins production (step 9); genome-length RNA is incorporated into viral particles with protein components (step 10). New HIV-1 protein components and genome move to the cell surface and assemble into an immature (non-infectious) particles (step 10); the viral-particle budding (step 11) and release (step 12) from the cell is mediated by ESCRT (endosomal sorting complex required for transport) complexes and ALIX. Following the protease-mediated maturation (step 13), the infectious viral particle is produced. Antiviral molecoles can interfere with different step of the viral cycle; the sites of action of clinical inhibitors (white boxes) and cellular restriction factors (blue boxes) are reported. INSTI, integrase strand transfer inhibitor; LTR, long terminal repeat; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor (*Engelman and Cherepanov, 2012*).

4.1.3 Natural history and immunopathogenesis of HIV-1 infection

Transmission of HIV-1 occurs through direct contact with infected body fluids, including blood, blood products, semen, vaginal and cervical secretions, amniotic fluid and breast milk. Parental route and sexual contact are major factors for transmittance of HIV-1. In the first case, the likelihood of transmission may be influenced by many factors including the type of needle (hollow versus solid bore), the depth of penetration, the volume of the inoculum and the amount of infectious virus in the inoculum. In the second case, infection may be facilitated by the presence of underlying sexually transmitted diseases including chancroid, herpes genitalis and syphilis, which disrupt the integrity of the skin or mucosal linings (*Shaw and Hunter, 2012*). Whether the infection begins with transmission through direct exposure to infected blood or following mucosal barrier, viral replication occurs in both cases primarily in the lymphoid organs and subsequently in the blood stream.

Approximately two to four weeks following the transmission of the virus, a majority of HIV-1 infected individuals experience a transient illness resembling infectious mononucleosis (*Vergis and Mellors, 2000*). During this early phase (**Figure 4**), known as primary or acute HIV-1 infection, HIV-1 replicates extremely aggressively, reaching levels of plasma viremia as high as 10^{10} copies/mL. Antibodies to HIV-1 are initially absent, and seroconversion usually occurs within a few weeks onset of the acute illness. As the immune response to HIV-1 develops, there is a dramatic reduction in the level of viremia, which falls to a lower plateau level (the set point) ranging from 10^3 to 10^5 copies/mL. The acute phase is usually accompanied by a dramatic reduction of the $CD4^+$ T cell count reflecting both the virus-induced $CD4^+$ T-cell depletion (in peripheral blood and in the gastrointestinal tract, GALT) and the sequestration of circulating $CD4^+$ T cells in lymphoid organs. The lysis of infected cells is due to the activity of specific cytotoxic lymphocytes (CTLs), activated by the presence of chemokines released by the infected cell (*Streeck and Nixon, 2010*). CTLs are responsible, along with humoral defense, for the decrease in the amount of viruses circulating. The number of $CD8^+$ cells is increased with an inversion of the $CD4^+/CD8^+$ ratio that will characterize the course of the infection and it is not able to recover, even in case of complete response to drug therapy. Different mechanisms allow HIV-1 to evade host immune responses leading to a chronic immunodeficiency and making impossible the eradication of the infection; among these, the development of escaping population from neutralizing antibodies (*Wei*

et al., 2003), the mutations in $CD8^+$ T cells epitopes (*Goonetilleke et al., 2009*), and the establishment of persistent resting memory $CD4^+$ T cell resorvoirs (*Chomont et al., 2009*) occur very early in the course of infection.

The second phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency and it is characterized by the gradual loss of $CD4^+$ T cells. Although the asymptomatic period may represent a phase of clinical latency, the virus replicates continuously, especially in lymphatic tissues, leading in some cases to a generalized lymphadenopathy. The higher the set point of plasma HIV-1 RNA, the more rapidly the patient will lose $CD4^+$ T cells and progress to AIDS (*Mellors et al., 1997*). The plasma HIV-1 RNA level (viral load) and the CD4 count are both used in making decisions about when to initiate therapy. The plasma viral load determines how rapidly CD4 cells will be lost, and the CD4 count reflects the degree of impairment of immunologic function and the risk of opportunistic infections. There is considerable individual variation in the length of the asymptomatic period in different infected individuals, which is influnced by the characteristics of the infecting virus (the appearance of X4 viruses is temporally associated with more rapid $CD4^+$ cell decline), host genetic factors influencing virus entry (a 32-bp deletion in CCR5 confers protection against infection with R5 viruses in the homozygous state and it is associated with slower disease progression in heterozygous individuals) and antiviral immune responses and possibly environmental factors related to immune system activation, as well as concurrent infections (*Kumar, 2013*). When CD4 cell count drops to lower than 200 cells/mmc, it leads to several AIDS associated events. Some HIV-1 positive patients progress to AIDS within 2 years of infection, whereas others, referred to as long term non progressors (LTNP), have lived with HIV-1 infection for over 10 years without significant CD4 depletion and with low level of viral load, in the absence of treatment. The final phase is characterized by a significant impairment of host defenses, a dramatic increase in viral load and the appearance of the disease. Worsening fatigue, night sweats and weight loss become more common and the risk of serious, potentially life threatening opportunistic infections (such as visceral toxoplasmosis, cryptosporidiosis, Pneumocystis jirovecii and Cytomegalovirus pneumonia, candidiasis, atypical mycobacteriosis), as well as secondary neoplasms or neurological manifestations (including Kaposi's sarcoma, non-Hodgkin's lymphomas, primitive central nervous system lymphoma) increase significantly, up to culminate with death if the patient is not treated with appropriate pharmacological therapy.

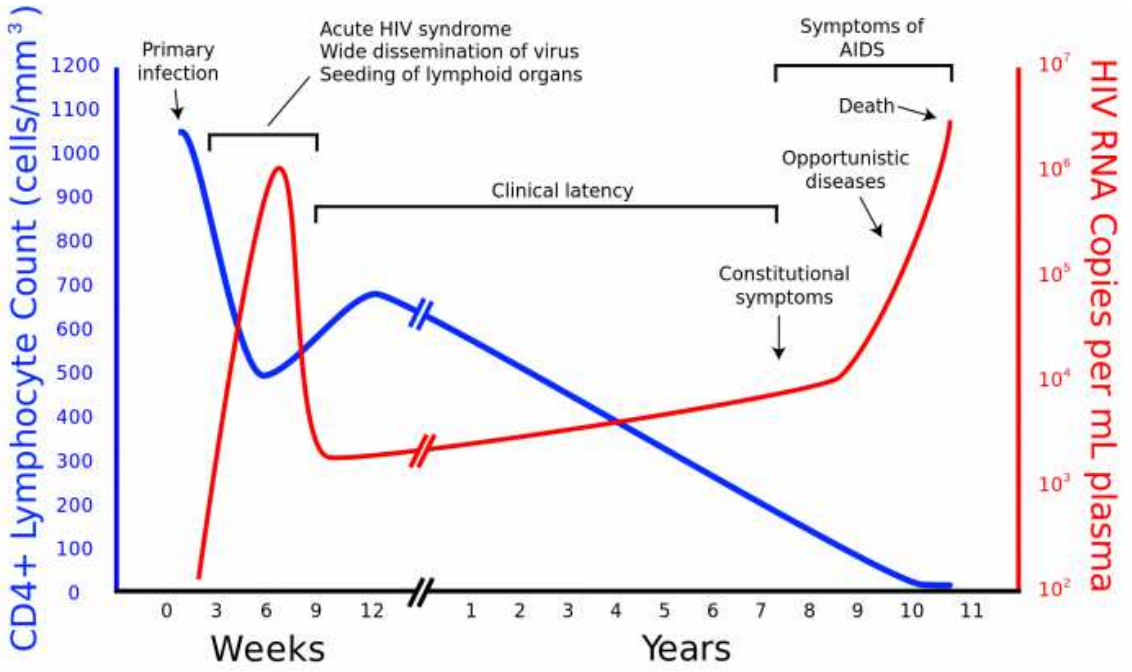

Figure 4. **Time course of infection.** The natural history of HIV-1 infection can be divided into three phases. The higher the set point of plasma HIV-1 RNA (viral load), the more rapidly $CD4^+$ T cells decreases leading to AIDS. The viral load and the CD4 count represent the markers for deciding when to begin therapeutic treatment.

4.1.4 Highly Active Antiretroviral Therapy (HAART)

Given the lack of effective therapies, all AIDS patients lost their life before mid 1990s. With the invention of the high active antiretroviral therapy (HAART), approximately 20 years ago, life expectancy of patients has greatly improved, comparing with the precedent single antiviral drug utilities (*Pomerantz and Horn, 2003*). This combined therapy is characterized by a cocktail of drugs that target different viral proteins and stages of the virus life cycle, in order to reduce the likelihood of onset of resistant viral variants (*Lu and Lu, 2012*). Up to date, more than thirty anti-HIV-1 chemotherapeutic agents have been licensed by the U.S. Food and Drug Administrator (FDA) for formal viral therapeutic utilities worldwide, which have been now divided into six classes based on the chemical nature, the molecular target and the resistance profiles (available at: https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/21/58/fda-approved-hiv medicines).

Initially, HAART commonly combined Nucleoside/Nucleotide analogue Reverse Transcriptase Inhibitor (NRTI) and Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI) agents to inhibit the activity of HIV-1 reverse transcriptase (RT) as a sole target. The NRTIs are analogues of natural RT substrates. Since they lack of the -OH

group at the 3' end, once incorporated by the enzyme for elongation of cDNA in the reverse transcription step, they block cDNA elongation. The NNRTIs have high affinity to an hydrophobic region close to the catalytic site of RT enzyme. The binding of the molecule to its target blocks the enzyme activity hampering cDNA synthesis (*Das and Arnold, 2013*). Afterwards, the combination of NNRTI/NRTI with protease inhibitors (PIs) were introduce to interfere with RT activities and HIV-1 proliferations. The PIs mimic the cleavage sites recognized by the virus protease to process the viral Gag-Pol polyprotein. They bind to the enzyme and block its function (*Lv et al., 2015*). More recently, Integrase Inhibitors (INIs) have been emerged to inhibit the transfer of cDNA into the host genome (*Temesgen and Siraj, 2008*). These drugs have demonstrated efficacy against HIV-1 strains that are resistant to other drug classes (*Dow and Bartlett, 2014*). Now, new types of antiviral drugs such as integrase inhibitors and fusion inhibitors (FIs) are frequently selected in HAART treatments. This last category (FIs) currently includes only one drug (T20 or Enfuvirtide), a 36 amino acid peptide that mimics a gp41 hydrophobic region named HR2 (*Kitchen et al., 2008*). In the HR2 fusion process, it comes into contact with another hydrophobic region of gp41 (HR1), resulting in a change in glycoprotein folding to favor interactions with specific cell membrane receptors. Enfuvirtide destabilizes this process, binding to HR1, and thus blocks the infectivity of the virus. HAART include also chemokine receptor antagonists, among which compounds that block the interaction between the CCR5 co-receptor on the cell surface and the viral particle, namely the gp120 V3 domain (*Rao, 2009*). Since each viral strain presents different tropism for one or more coreceptors, the administration of these compounds is only effective after having ascertained the corresponding tropism of the viral variant in question. The goal of antiretroviral treatment is to indefinitely maintain suppression of plasma viral load under undetectable limits of sensitive HIV-RNA assays (<50 RNA copies/mL), in order to prevent emergence of resistance, facilitate optimal immune recovery and improve the patients' quality of life, as well as decrease the risk of HIV-1 transmission. Actually, there are some unresolved questions about when to begin the therapeutic regimen, whether the HAART should be used immediately after HIV-1 is diagnosed, after AIDS symptoms occurrence or when the cell number counts of CD4 lymphocytes is below 200-250/mmc in patients. Early initiation of HAART limits the establishment of the reservoir and prevents the generation of immune escape in latently infected cells (*Cohen et al., 2011*). For initiating first-line therapy in treatment-naïve patients, several guidelines

recommend preferred regimens. Current preferred regimens in treatment-naïve patients consist of two NRTI plus either a NNRTI, or one of several protease inhibitors, or an integrase inhibitor. If a preferred regimen fails, there are numerous other drugs that can be used in a variety of possible combinations. Continued suppression of HIV-1 RNA can be maintained indefinitely in the majority of individuals who adhere to appropriate HAART regimens. Thus, the selection of drugs to be taken should be guided by the patient's characteristics, expectations of adherence to the therapeutic regimen and stage of the disease. Virological failure of therapy may be the result of an irregular drug intake, a factor that predisposes to the selection of resistant mutants, especially when the drugs in use have reduced genetic barrier, being inactive for variants with single point mutations. Thus, owing to the diversity of genetic/molecular backgrounds and changes in different HIV-1 infected patients, personalized medicine is an important future trend (*Asensi et al., 2015*), even if the therapeutic outcome prediction information needs to be based on modern technically supportive diagnostics. Despite the remarkable successes of HAART in decreasing considerably the morbidity and mortality associated with HIV-1 infection, this therapeutic regimen cannot eradicate the disease, due to the persistence of integrated viral DNA (that is replication competent but transcriptionally silent) in particular cell types (such as cells of the monocyte-macrophage lineage or long lived memory $CD4^+$ T cells and, to a lesser degree, naïve $CD4^+$ T cells) and anatomic districts (renal and central nervous system, lymph nodes, cervical epithelium, bone marrow), not easily accessible by current antiretroviral drugs (*Finzi et al., 1997*; *Alexaki et al., 2008; Dahabieh et al., 2015)*. Multiple cellular mechanisms have been defined, that contribute to the establishment and maintenance of latency (*Cary et al., 2016*). For this reason, the developments of agents to specifically target latency states of HIV-1 might be new therapeutic strategies, among which the so-called ‘shock and kill’ or approach based on silencing HIV-1 provirus permanently (*Archin and Margolis, 2014; Mousseau et al., 2015).* However, a full and clear understanding of these processes, the durability of these states and the steps that allow the latently infected cells to be revealed to the immune system are still lacking and the development of robust assays to measure the impact of therapies on latent HIV-1 reservoirs represents a great challenge. Based on prediction models, more than 30-60 years are necessary so that HIV-1 can be eradicated in patients undergoing continuous suppressive therapy (*Zhang et al., 1999; Ramratnam et al., 2000*). Thus, HAART is used as an indefinite therapy in order to block the progression of the infection. Although greatly improving

patients survival, chronic treatment is correlated both to high costs and to a long-term toxicity (such as diarrhea, lipodystrophy, cardiovascular complications, mitochondrial toxicity, peripheral neuropathy, osteoporosis, persistent immune dysfunction) (*Deeks 2011; Deeks et al., 2013*); for these reasons and due to also complicated drug intake procedures, many patients discontinue therapy with a subsequently rapid viral rebound from reservoirs (*Joos et al., 2008*). The low adherence to the therapy can favor drug resistance mutations selection. Acquiring resistance to a given drug may also frequently involve other compounds of the same category ("cross resistance"), up to a progressive accumulation of mutations, which could compromise alternative therapy regimens (*Wensing et al., 2017*). In this scenario, the search for an innovative strategy to overcome the limits of HAART is now a key priority for the HIV-1 community.

4.2 Gene therapy for HIV-1 infection

Gene therapy (GT) is an increasingly promising alternative for the treatment of HIV-1/AIDS and it may facilitate the sustained inhibition of HIV-1 replication after a single therapeutic intervention, providing a pool of immune cells with sustained intrinsic protection. A single proof of concept, initially considered the “Holy Grail” for the cure of HIV-1, was provided by the so-called “Berlin patient”, who received a heterologous stem cell transplant for acquired immunodeficiency syndrome-related lymphoma from a CCR5 homozygous null HLA-matched donor (CCR5 -/-), and remained free of detectable HIV-1 even after discontinuation of conventional therapy (*Hutter et al., 2009; Allers et al., 2011).* However, another HIV-1 infected patient suffering from anaplastic large cell lymphoma also received a stem cell transplant from a homozygous CCR5-null donor. Unfortunately, in that case, X4-tropic HIV-1 strains emerged that necessitated the re-initiation of HAART (*Kordelas et al., 2014*). With the purpose to eradicate HIV-1, several studies employed autologous or allogeneic Hematopoietic Stem Cell (HSC) transplantation in association with antiretroviral therapy in selected HIV-1 positive patients affected by leukemia and/or lymphomas (*Passaes and Saez-Cirion, 2014*). Nevertheless, in most of these studies, HIV-1 was detected after transplantation, either after the interruption of drug treatment or because the therapeutic regimen was not able to completely eliminate the viral reservoirs; furthermore, in several cases, the patients died after transplantation for graft versus host disease. Thus, due to risky and expensive procedures (recommended only for those who develop cancer) and due to the difficulties of finding compatible donors, being CCR5 Δ32 homozygous donors rare

(*Lucotte, 2002*), this treatment was not applicable on a large scale of HIV-1 infected patients. However, the natural resistance to HIV-1 infection conferred by homozygous CCR5 Δ32 deletion has energized the field for new CCR5-based therapeutic approaches for HIV-1 cure interventions, in addition to the established HAART (*Hütter and Ganepola, 2011; Burke et al., 2013*). Several HIV-1 gene therapy strategies that down regulate/inhibit synthesis of CCR5 (including zinc finger nuclease (ZFN), clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), trascription activator-like effector nuclease (TALEN), shRNA, siRNA, antisense RNA and ribozymes) or prevent its surface expression (including intrabodies and intrakines) have been explored, some of which have been tested also in clinical trials (*Cannon and June, 2011; Hutter et al., 2015).* Even though CCR5 provides a good target, alone it is not ideal for suppressing HIV-1, since its disruption can only prevent infection without effect on already infected cells or on cell-cell transmission; furthermore, the virus can mutate to use alternative co-receptors to infect cells. Thus, a combination of cellular and viral targets appears to be necessary for effective control of HIV-1 replication, focusing attention on highly conserved regions, that encode critical domains of the essential viral proteins, in which mutation comes at a fitness cost to the virus (*DiGiusto et al., 2010)*. The ultimate goal of gene therapy is to achieve an 'intracellular immunization' (*Baltimore, 1988*), through engineering of autologous T cells or their progenitors, i.e. the $CD34^+$ HSC, in order to build a population of cells that are protected against *de novo* HIV-1 infection. In this context, the HSCs are the most attractive target cells in anti-HIV-1 gene therapy protocols, thanks to their capacity of self-renewal and differentiation in all blood cell lineages involved in viral replication/pathogenesis, including monocytes, macrophages and dendritic cells (**Figure 5 and Figure 6**). Thus, their genetic modification should protect the entire spectrum of susceptible cells. However, these cells are rare and technically challenging to isolate, genetically modify and engraft, in particular without the use of preconditioning regimens (radio or chemoteraphy) to ablate the endogenous hematopoietic cells and free the niche. For this reason, such studies are focalized on HIV-1 positive individuals with cancer, for which is ethically acceptable a fully myeloablative treatment, but this patient population is small. Consequently, novel less toxic methods to increase the efficiency of engraftment and the selection of engineered cells *in vivo* need to be developed (*Beard et al., 2010*). Furthermore, other issues concerning the use of HSCs regard the potential risk of malignant transformation, being HSCs more susceptible than mature T cells to

genotoxicity from integrating viral vectors, which represent the main delivery approach to accomodate multiple transgenes (*Newrzela et al. 2008; Montini et al. 2009; Matrai et al., 2010*).

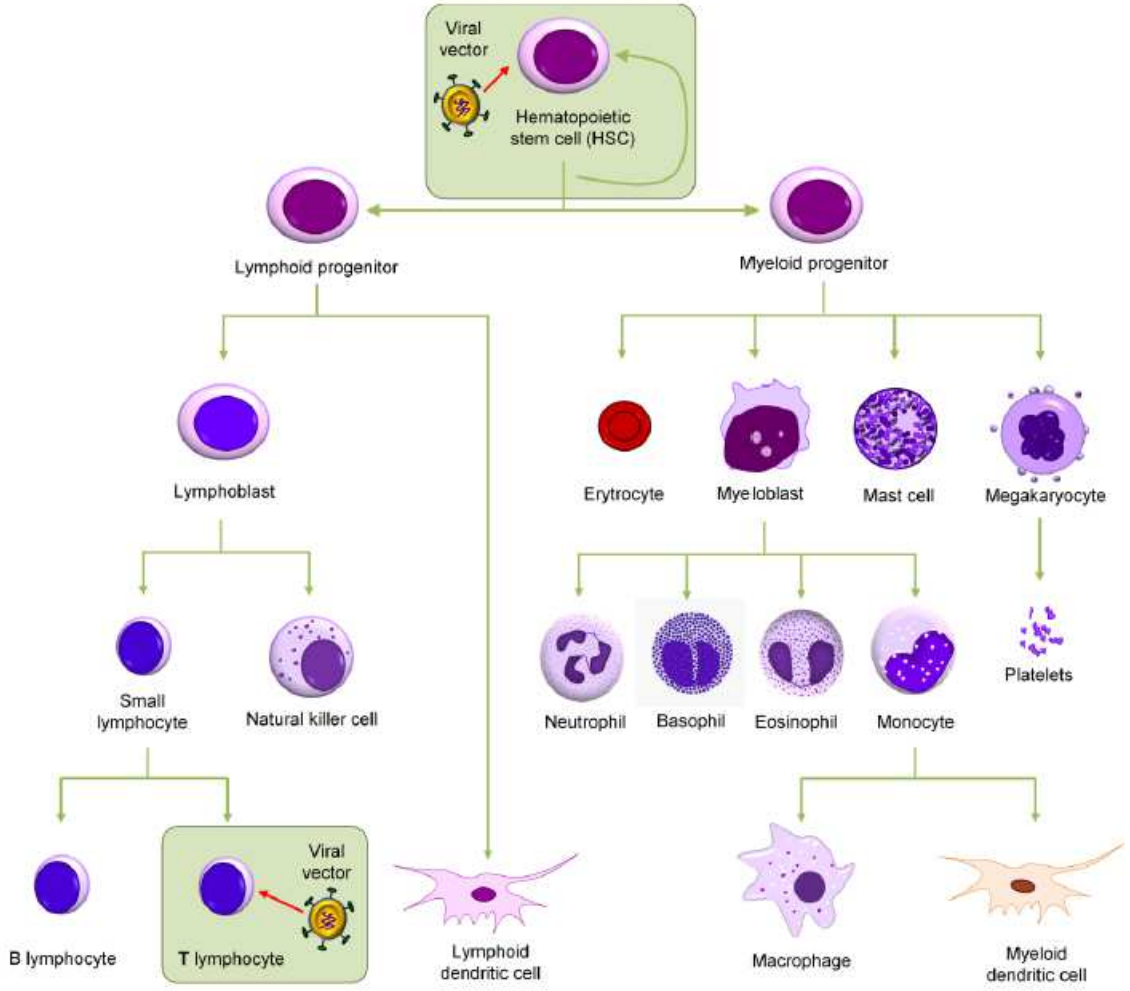

Figure 5. **Target cells for an anti-HIV-1 gene therapy**. The scheme of hematopoiesis is shown in the picture; two main cell populations (either hematopoietic stem cells (HSCs) from bone marrow or the mature $CD4^+$ T cells from peripheral blood), which can be genetically modified in anti-HIV-1 gene therapy protocols, based on lentiviral vectors expressing multiple transgenes, are boxed. In particular, engineered HSCs can self renew and proliferate to provide a pool of differentiated HIV-1 resistant mature immune cells (including T lymphocytes, macrophages and dendtritic cells) over time (*Herrera-Carrillo and Berkhout, 2015).*

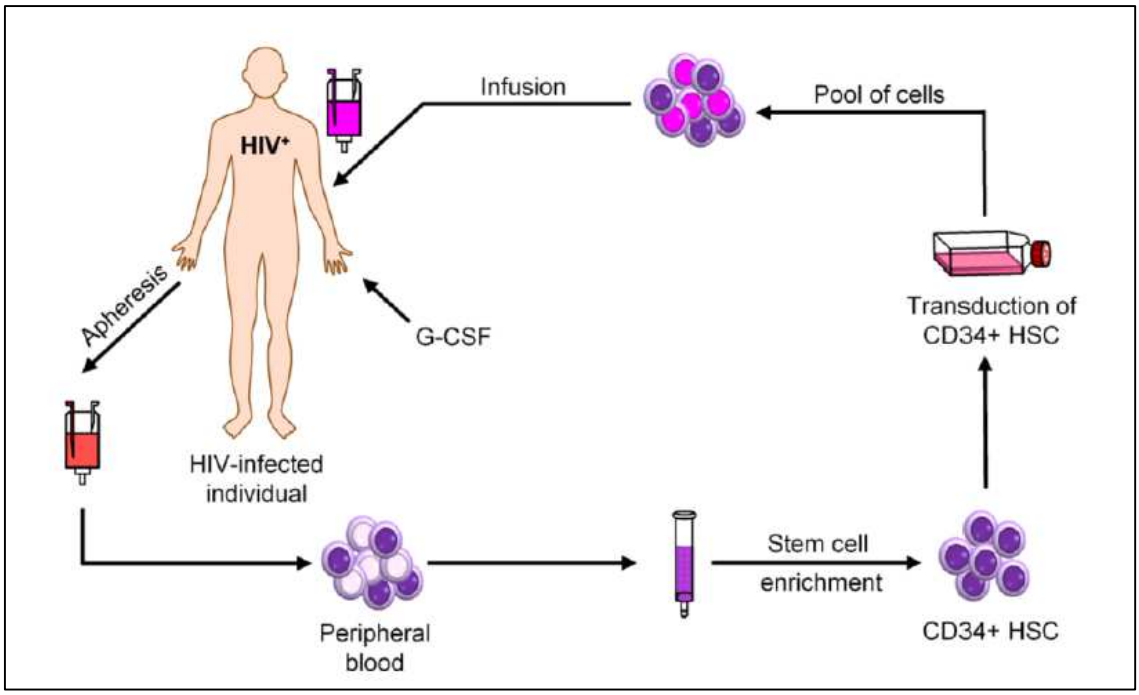

Figure 6. **Schematic of the process for engineering protection from HIV-1 through genetically modified stem cell transplantation into infected patients.** The patient hematopoietic $CD34^+$ stem cells are collected by apheresis after mobilization with several rounds of G-CSF (granulocyte-colony stimulation factor) injection. The mixed cell population containing $CD34^+$ HSC is purified and transduced *ex vivo* with the vector harboring the therapeutic genes. The transduced cells are then transplanted back into the patient and allowed to engraft with the purpose of conferring protection against HIV-1 infection (*Herrera-Carrillo and Berkhout, 2015*).

By targeting different steps of HIV-1 replication (**Figure 7**), gene therapy clinical trials mimic HAART (*Van Lunzen et al., 2011; Joshi et al., 2011; Bennett and Akkina, 2013; Jacobson, 2013*); therefore, for maximum therapeutic benefit, individual components of combinatorial approaches should be designed to confer potent and permanent synergistic inhibitory effects, minimal cytotoxicity and conserved viral sites, hoping to decrease the probability of occurrence of mutant variants (*Anderson et al., 2007*).

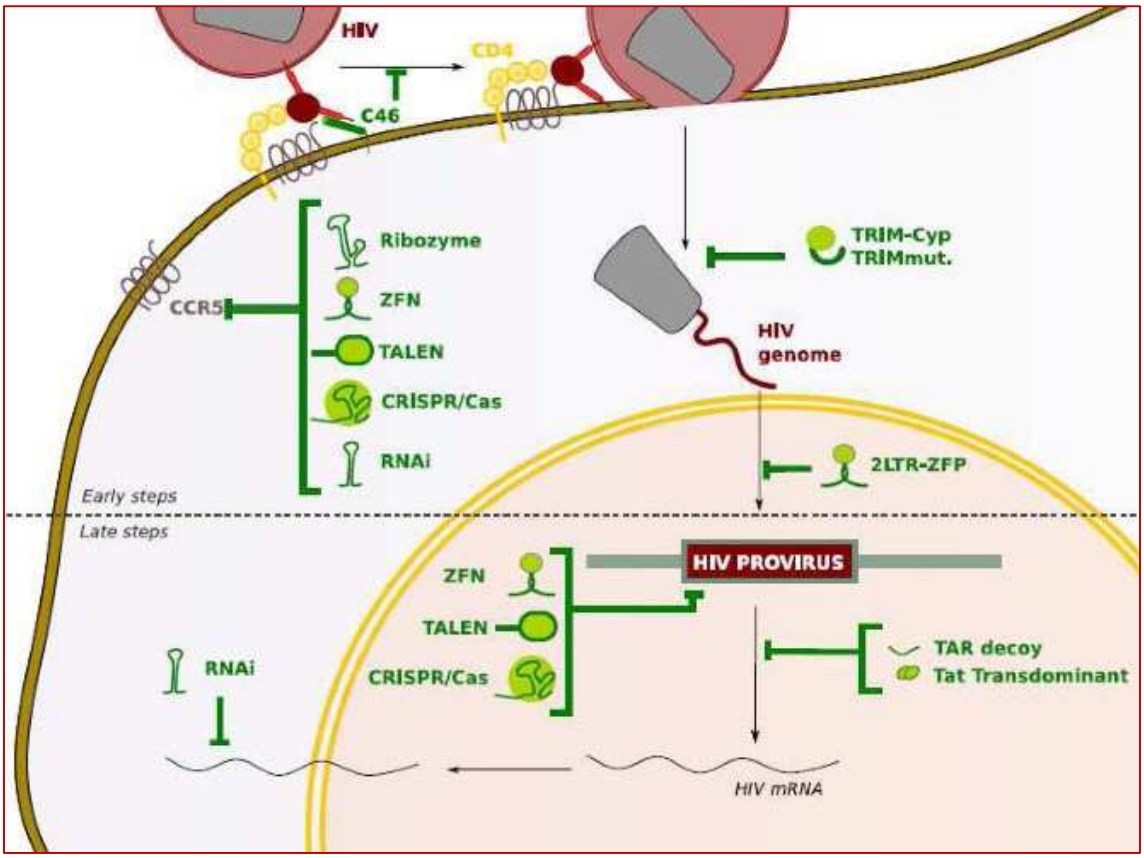

Figure 7. **Gene therapy strategies to engineer HIV-1 resistant cells.** Different steps of HIV-1 replication cycle can be inhibited by anti-HIV-1 genes, either before (early step) or after (late step) integration into host genome. Blocking viral entry may be achieved through the knock out/down of CCR5/CXCR4 co-receptors (by ribozyme, ZFN, TALEN, CRISPR/Cas9 and RNAi), or employing a peptide fusion inhibitor, C46. Genetically engineered versions of the host factor TRIM5α could be applied to block infection shortly after HIV-1 cell entry, by uncoating inhibition. 2LTR ZFP inhibit HIV-1 infection prior to genome integration, by bindind to 2LTR. HIV-1 provirus can be excised from the host DNA of latently infected cells, by gene editing technologies (ZFN, TALEN, CRISPR/Cas9). Gene expression at the post transcription step can be inhibited by RNAi, TAR decoy or Tat negative transdominant (*Pernet et al., 2016*).

Thus, according different criteria (including the potency of inhibition, the lack of immunogenicity and toxicity, the stage in the viral life cycle that they target and the potential for selecting resistant viruses), genetic inhibitors are grouped into three classes (*von Laer et al., 2006*), among which the ones belonging to class I are by far the best inhibitors, according to *in vivo* comparisons (*Kimpel et al., 2010*). Blocking the pre-integration, they suppress virus infection and protect the cells from the associated cytopathic effect, leading to the accumulation of uninfected gene-marked cells, thus preventing the continued replenishment of viral reservoirs (*Egerer et al., 2015*). Antiviral genes, that allow integration of the proviral DNA into the host cell genome, but inhibit subsequent viral protein expression and reproduction of the viral genome, are

grouped into class II. Actually, a relevant protection is conferred by a combination of Classes I and II inhibitors. Class III genes encode late inhibitors interfering with the virus assembly and the release; thus, since they do not confer a selective advantage to the modified cells, they are less favored. However, several studies have emphasized the success of innate host restriction factors, suggesting the importance of Class III inhibitors as well. Host restriction factors, belonging to an intrinsic natural defense mechanism, are able to inhibit HIV-1 replication, thus they are counted among the possible molecular gene therapy approaches. The restriction factors APOBEC3G (A3G) and APOBEC3B (A3B) belong to polynucleotide cytidine deaminase APOBEC3 (A3) family. A3G blocks reverse transcription by deaminating minus-strand viral cDNA, but this activity may be inhibited by the viral Vif protein (*Wissing et al., 2010*), whereas A3B is resistant to Vif. Both proteins can be induced by a synthetic transcriptional activator into human T cells, in which normally they are not express; however only the induced A3B conferred resistant to infection by HIV-1 (*Bogerd et al., 2015*). Another interesting strategy is based on the production of artificial T cells receptors (TCR) expressed by engineered T cells, in order to enhance the recognition of HIV-1 infected cells (*Joseph et al., 2008*). Since the use of this approach is restricted to subjects with the appropriate HLA allele, alternative chimeric antigen receptors (CAR), which combines both antigen-binding and T cell activating functions, have been developed for HIV-1 therapy after the remarkable success obtained in hematological malignancies (*Maude et al., 2014; Liu et al., 2015*). Furthermore, the combination of broadly neutralizing monoclonal antibodies (bNAbs) targeting HIV-1 envelope glycoprotein with CAR signaling domains delivered directly into the CCR5 locus of primary human T cells thanks to gene editing technology have demonstrated encouraging results in term of suppression of replicating virus (*Hale et al., 2017).*

The safety of numerous anti-HIV-1 genes has been demonstrated in many T cell and HSCs clinical trials, and they can be grouped in protein or RNA-based inhibitors. The first include fusion inhibitors (which prevent viral entry by blocking the interaction of gp41 with the host membrane) (*Eggink et al., 2010*), trans-dominant negative proteins (mutants of viral/cellular proteins designed to interfere with the normal activity of its wild-type counterpart to disrupt the replication cycle of the virus) (*Malim et al., 1992; Morgan et al., 2005*; *Lin et al., 2015; Walker et al., 2010*), intrakines (modified intracellular chemokines), intrabodies (recombinant intracellular antibodies) (*Schroers et al., 2002; Zhang et al., 2009*).

The second group of anti-HIV-1 genes include the use of antisense, ribozymes, decoys, aptamers, siRNAs, shRNAs and the related AgoshRNA design recently discovered. In contrast to protein-based inhibitors, these last do not elicite adaptive T-cell responses and lack of immunogenicity. However, RNA approaches can potentially have off-target toxicity because of activation of innate immune responses and competition with endogenous RNA functions (*Lares et al., 2010*). Several short and long antisense RNA transgenes have been designed to target viral transcripts (*Lu* et al*., 2004).* In HIV-1 patients, the infusion of autologous T cells expressing an *env*-specific antisense RNA exerted genetic pressure on the virus, without detecting evidence of clonal selection of lentiviral vector-transduced T cells or integration enrichment near oncogenes *(Tebas* et al*., 2013*). Some ribozymes have been evaluated in clinical trials, including those targeting HIV-1 genes *tat* and *rev* (*Michienzi et al., 2003*), the U5 region of the viral LTR (*Wong-Staal et al., 1998*) and the overlapping reading frames of the viral *vpr* and *tat* genes in unspliced and spliced viral transcripts (*Mitsuyasu et al., 2009*); however, only in the latter trial a significant lower viral load was observed. TAR decoy has been administered to HIV^+ patients with non-Hodgkin's lymphoma in the form of gene-modified autologous HSCs together with two other RNA-based anti-HIV-1 molecoles (an anti-tat/rev shRNA and an anti-CCR5 ribozyme) which were accomplished into a single vector (*DiGiusto* et al*., 2010*). Stable vector expression in human blood cells after transplantation was observed for up to 24 months. This pilot trial has represented the support for the development of a multiple RNA-based cell therapy platform for HIV-1. Overall, the variety of the anti-HIV-1 strategies discussed above were shown to be safe and well tolerated, but very low level of long-term genetic marking was observed in peripheral blood cells *(Younan et al., 2014).* A lower efficiency of gene transfer with HSCs than with T-cell-directed therapies have been observed in several studies (*Rossi et al. 2007; June et al., 2009*), particularly regarding the ability to genetically modify long-term repopulating cells.

4.2.1 RNA-interference-based gene therapy approaches to HIV-1 treatment

In order to accomplish a stable and durable anti HIV-1 protection, one of the most promising approaches is represented by RNA interference (RNAi), a conserved mechanism in all eukaryotes, that regulate gene expression post-transcriptionally in a sequence-specific manner (*Ter Brake et al., 2006*) (**Figure 8**). RNAi is a natural cellular mechanism, first identified in 1998 (*Fire et al., 1998*), in which small (~21 nt in length)

double-stranded RNAs are produced endogenously within mammalian cells (microRNA, also known as miRNA) to rapidly regulate gene expression in response to environmental cues. First, double-stranded RNAs in the cytoplasm are loaded into the RNA-induced silencing complex (RISC), consisting of Argonaute family (Ago1-4) and other proteins *(Meister, 2013).* Upon RISC binding, the non targeting (passenger) strand is removed, by cleavage and degradation for Ago2-bound siRNA or unwinding by other Ago proteins, while the "guide" strand directs the RISC to the corresponding messenger RNA (mRNA). The binding of the miRNA to the mRNA target results in post-transcriptional gene silencing (PTGS), through degradation of the mRNA. Generally, when there is a partial homology between the guide strand and the target mRNA, as in miRNAs (usually, between positions 2-8 at the 5′ end of the guide strand, which is called the "seed" sequence), the Ago proteins binding the mRNA and miRNA recruit GW182 protein (also known as TNRC6a, b, and c), which then leads to the recruitment of a series of proteins, including the Caf1-Ccr4 and Pan2-Pan-3 deadenylase complexes and the Dcp-1, Dcp-2 decapping complex, which eventually destabilize the mRNA by inducing deadenylation and decapping, resulting in PTGS (*Jonas and Izaurralde, 2015*). The canonical RNAi pathway can be exploited for therapeutic gene silencing either through chemically synthesized siRNAs that can be transfected into the cells or by expression of short hairpin RNA (shRNA) from a transgene construct in target cells (*Berkhout and Eekels, 2012; Wittrup and Lieberman, 2015)*. shRNAs differ from siRNAs because they comprise a single strand of RNA with two complementary tails connected by a non-homologous stem loop. The shRNA is transcribed in the nucleus as stem loop RNA and transported to the cytoplasm by the Exportin-5-protein to be further processed by Dicer into siRNAs of ~ 21 bp with 2-nt 3' overhangs. The passenger strand of the siRNA is degraded and the guide strand programs RISC to cleave the perfectly complementary target mRNA. The mRNA is then rapidly degraded by exonucleases, leading to PTGS. Antiviral activity of siRNA molecules targeting the HIV-1 RNA genome or the mRNA for important cellular co-factors has been reported (*Eekels et al., 2011*). However, the injection of exogenous siRNA gets rapidly diluted, particularly in dividing cells. Thus, since for a chronic disease like HIV-1, it is necessary to confer long-term protection, endogenously synthesized shRNA is preferable, because it is more stable, particularly if expressed via genome-integrating lentiviral vectors *(Subramanya et al., 2010*).

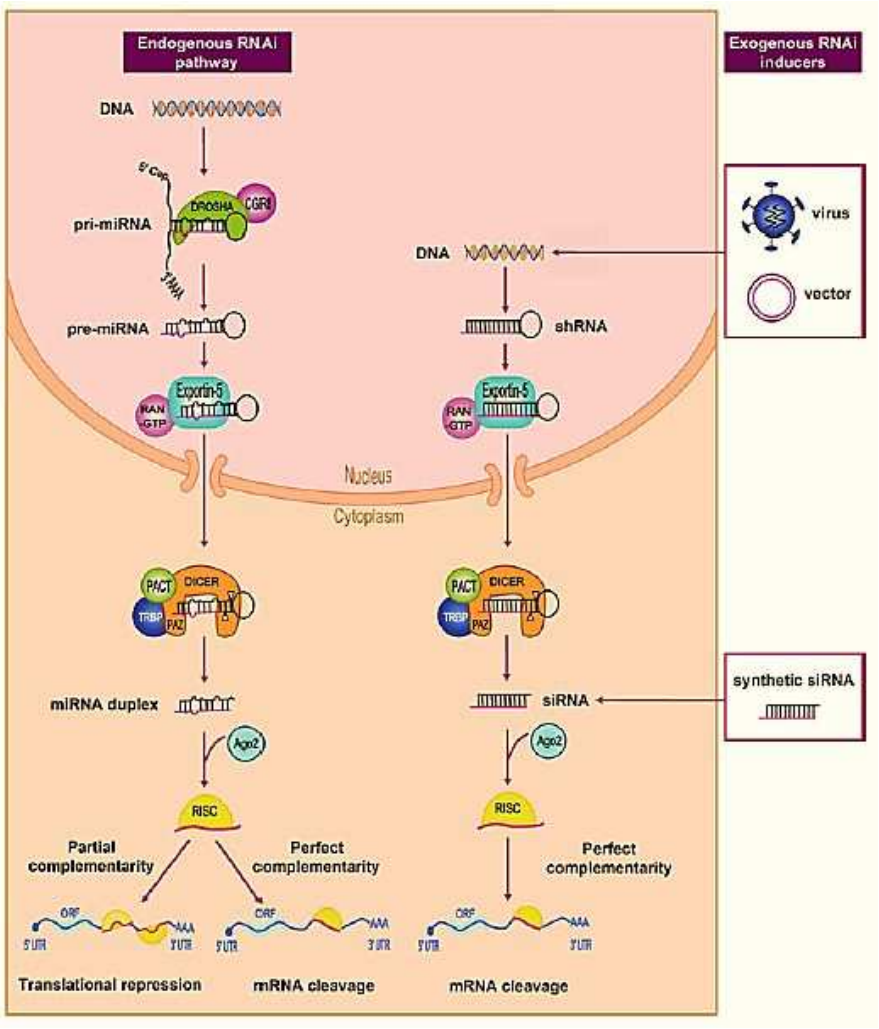

Figure 8. **The RNA interference (RNAi) pathway.** RNAi can be used to silence specific gene expression for therapeutic applications. The endogenous RNAi pathway of mammalian cells is depicted on the left hand side while the man-made exogenous RNAi cascade is shown on the right hand side. Host pri-miRNAs are transcribed in the nucleus, processed by Drosha and DGCR8 into a pre-miRNA and transported to the cytoplasm by Exportin-5. Subsequently, the pre-miRNA is processed by the Dicer/TRBP/PACT endonuclease complex to form the miRNA duplex. The mature miRNA is loaded into the Ago2 enzyme, to yeald the RNA-induced silencing complex (RISC), that induces gene silencing by translational repression or cleavage of the mRNA target. The man-made shRNA gene cassettes are expressed in the nucleus and they are directly processed by Dicer/TRBP/PACT. Synthetic siRNA duplexes (that are ~ 21 nucleotides complementary RNA strands with two-nucleotide 3'-overhangs) can also mediate transient gene silencing by transfection into the cell and will directly instruct RISC for mRNA silencing. The man-made shRNAs and siRNAs are designed to be fully complementary to the target mRNA, which is inactivated by cleavage. The most favored way for expressing shRNA is transduction with self-inactivating (SIN) lentiviral particles, since the integration of vectors in the host cell genome can ensure long-term production of shRNA, that is processed in the cytoplasm into siRNA (*Herrera-Carrillo and Berkhout, 2015).*

In the last few years, several novel gene-editing technologies have emerged, including ZFN, TALEN and CRISPR/Cas9 system, as a powerful tool to knock out/delete genes and also to insert exotic DNA sequences at particular sites (*Manjunath et al., 2013*). All the currently available gene-editing techniques are based on a specific genomic DNA

recognition site, where a nuclease activity induces double-stranded breaks (DSBs). These double stranded breaks are then repaired by cellular repair pathways which depend largely on the cell cycle: error-free homologous recombination occurs particularly during S and G_2 phases of the cell cycle, and results in incorporation of the externally provided DNA at the cleavage site; while, in resting cells, DSBs are common repaired by error-prone non-homologous ends joining (NHEJ) pathway, which is known to induce small nucleotide additions or deletions (indels), that result in disruption of the reading frame and gene expression. In comparison to ZFN and TALEN, which usually requires labor-intensive design and screening, CRISPR/Cas9 has more advantages, among which a simpler design and a single cloning step. ZFN and TALEN use DNA binding motifs fused to the Folk1 endonuclease to mediate sequence-specific DNA cleavage, and require 10 or 34 amino acids, respectively, to recognize a single nucleotide. CRISPR/Cas9 uses a short single guide RNA (sgRNA) molecule for DNA recognition and requires a proto-spacer adjacent motif (PAM) of sequence NGG in the target DNA immediately adjacent to the gRNA homology region. Cas9 DNA endonuclease induce a blunt end DBS 3 bp 5' of the PAM. CRISP/Cas9 can target the newly formed double-strand HIV-1 DNA, either before integration (inducing DNA degradation) or after integration into the cellular genome. Latent provirus can be targeted for cleavage, i.e. excision of viral genome *via* specifically targeting LTR regions (*Saayman et al., 2015*); however, in this last case, the cleavage can induce the NHEJ mutagenic activity, around the predicted cleavage site, helping HIV-1 to replicate further (*Wang et al., 2016*). Thus, if on one hand this CRISPR-mediated therapy can contribute to inactivate HIV-1, on the other side can accelerate a viral escape, producing replication competent viruses resistant to this strategy. Targeting unstructured regions of the LTRs appears to quickly lead to resistance. Targeting two gRNAs to delete relatively short regions of the HIV-1 genome may be more effective then deleting the entire provirus. Better strategies may include targeting multi-plexed gRNAs to RNAs stem loop structures or protein coding regions where single base indels impose a fitness cost on the virus (*Yoder and Bundschuh, 2016*). Overall, these nucleases have been employed to successfully disrupt permanently CCR5 in different cell lines, primary T cells, HSPCs, as well as in humanized mice (*Cornu et al., 2015; Drake and Bates, 2015; Hutter et al., 2015*). A recent clinical trial has employed an adenovirus expressing CCR5 ZFN to transduce autologous CD4 T cells, which were then reinfused into a small cohort of 12 individuals, conferring a survival advantage of modified cells during

treatment interruption (*Tebas et al., 2014*). Human CXCR4 gene has been also efficiently disrupted by CRISPR/Cas9-mediated genome editing, leading to HIV-1 resistance of human primary $CD4^+$ T cells, without significant impairment of these cells (*Hou et al., 2015)*. Recently, an hybrid nuclease platform, which combines a TALE DNA binding domain with a sequence-specific homing endonuclease, was delivered by an adeno-associated virus (AAV) to disrupt CCR5 and replacing it with the gene encoding C46 HIV-1 fusion inhibitor sequence, in order to confer protection against both CCR5 and CXCR4-mediated HIV-1 entry (*Sather et al., 2015*). This protocol conferred 15 to 20% homologous gene targeting in adult human mobilized $CD34^+$ cells. Furthermore, Didigu *et al*. (2014) have observed a relevant protection from infection with CCR5- and CXCR4-using HIV-1 strains in humanized mouse model treated with ZFNs targeting simultaneously both CCR5 and CXCR4 co-receptors, without negative impact on engraft and traffic cells. However, it is not possible to reliably predict long term adverse effects *in vivo* of these emerging strategies, which require additional safety tests concerning the sustained expression of these foreign nucleases in human cells. Furthermore, even if gene editing systems allow permanent disruption/deletion of target gene, like CCR5 host factor, they cannot be used to target viral genes in non infected cells for effective prevention of infection. Hence, considering differences in coreceptor usage and the chances of viral escape, this approach cannot be used in a therapeutic setting based on a simultaneously suppression of host factors and viral genes, like the combinatorial antiretroviral drugs, which is necessary to confer effective resistance to HIV-1 infection. RNAi provides the only practical solution for expression of multiple anti HIV-1 cellular and viral targets in infected and uninfected cells, conferring an effective prevention or treatment of disease. A careful selection of targets represents a crucial step to obtain a long-term therapeutic effect without the emergence of resistance strains (*von Eije et al., 2008*). However, the most important criterion should be finding highly conserved regions of the HIV-1 genome to target, in order to restrict the possibilities for evolution of escape viruses. Single point mutations, deletion of the target sequence and transcriptional up-regulation are the main responsible for RNAi escape (*Berkhout and Sanders, 2011*). Furthermore, multiple shRNAs should be tested because only a minority could be perfectly matched to each viral variant present in patients. Based on mathematical modeling, a combination of four RNAi targets may be sufficient to overcome viral escape. According to these estimates, the simultaneous

expression of seven shRNAs can provide coverage by at least four shRNAs against any given viral strain (*McIntyre et al., 2011).*

One of the potential limitations of using multiple shRNAs can rise from competition with endogenous microRNAs for nuclear-to-cytoplasmic export (*Grimm et al., 2006*) and incorporation into the RNA-silencing machinery. Moreover, shRNAs in the cytoplasm, that are processed by Dicer, can also bind all Ago proteins, further compromising endogenous miRNA function (*Grimm et al., 2010*). Toxicity of RNAi may be due to several factors including improper target recognition resulting from suboptimal annealing of the antisense guide strand to the target strand, improper selection of an intended passenger strand by the RISC complex or after overloading of the silencing machinery. Furthermore, certain motifs may contribute to off-target effects, as through toll-like receptor (TLR) activation, interferon production (GUCCUUCAA, UGUGU) (*Hornung et al., 2005*) or even cytotoxicity (UGGC) (*Fedorov et al., 2006*). Thus, in order to reduce off-target effects of siRNAs, a careful design of the RNA duplex and preferential incorporation of the intended RNA strand into RISC are necessary. Moreover, an important point to consider are the levels of expression for each type of anti-HIV-1 RNA to obtain a sufficient therapeutic effect; since cells infected by HIV-1 exhibit differences in HIV-1 gene expression over maturation and differentiation (*Pimanda et al., 2010*), there is no single promoter that could provide sufficient expression in all stages or cell types. To date, two commonly used Pol III promoters in siRNA expression vectors include the human H1 and U6 promoters (*Lee et al., 2002; Paul et al., 2002*), even though the tolerable therapeutic level should be considered being their activity too high for safe application (*Grimm et al., 2006*). U6 small nuclear RNA plays a central role in processing premature RNA species. The H1 transcript is a component of human nuclear RNase P, an enzyme that cleaves tRNA precursors to produce mature 5′-termini. Different Pol III promoters, such as U6, H1, and 7SK can be used to express multiple anti-HIV-1 shRNAs into a single vector to avoid recombination-mediated deletion of shRNA cassettes, which may occur among repeated promoter sequences (*ter Brake et al., 2008*). Alternatively, another strategy consists on expression of multiple shRNAs as an extended shRNA (e-shRNA), in which two shRNAs on top of each other (total length of 42-43 nt) are expressed under the control of a single Pol III promoter (*Liu et al., 2007*). However, the inclusion of additional shRNAs may lead to a progressive decline of the activity (*Liu and Berkhout, 2009*). Similarly, long hairpin RNA (lhRNA) with a long-stemmed (60-70 nt)

hairpin may be expressed under the control of a single Pol III promoter, with a consequent development of overlapping shRNAs by Dicer processing of the longer stem will. In this case, silencing efficiency progressively declined from the stem base (first position) towards the loop side of the hairpin (*Saayman et al., 2008*; *Saayman et al., 2010*). A recent strategy, called multiplexed miRNA-based shRNAs (shRNA-miRs) is based on multiple shRNAs, which can be expressed from a single vector via tandem repeats of different miRNA-based backbones (*Choi et al., 2015*). The expression of shRNA in an endogenous miRNA backbone may avoid the toxicities resulting from the saturation of RNAi system (*Wu et al., 2013*). Multiplexed shRNAs can be also expressed in the context of polycistronic endogenous miRNA cluster or as multiple pri-mRNAs, each containing the minimal flanking sequence derived from different endogenous miRNAs (*Snyder et al., 2009; Choi et al., 2015*). In this context, a progressive decrease of the expression levels of individual shRNAs with the distance from the promoter has not been observed.

The major advantages and disadvantages of the different combinatorial RNAi approaches are listed in **Figure 9**.

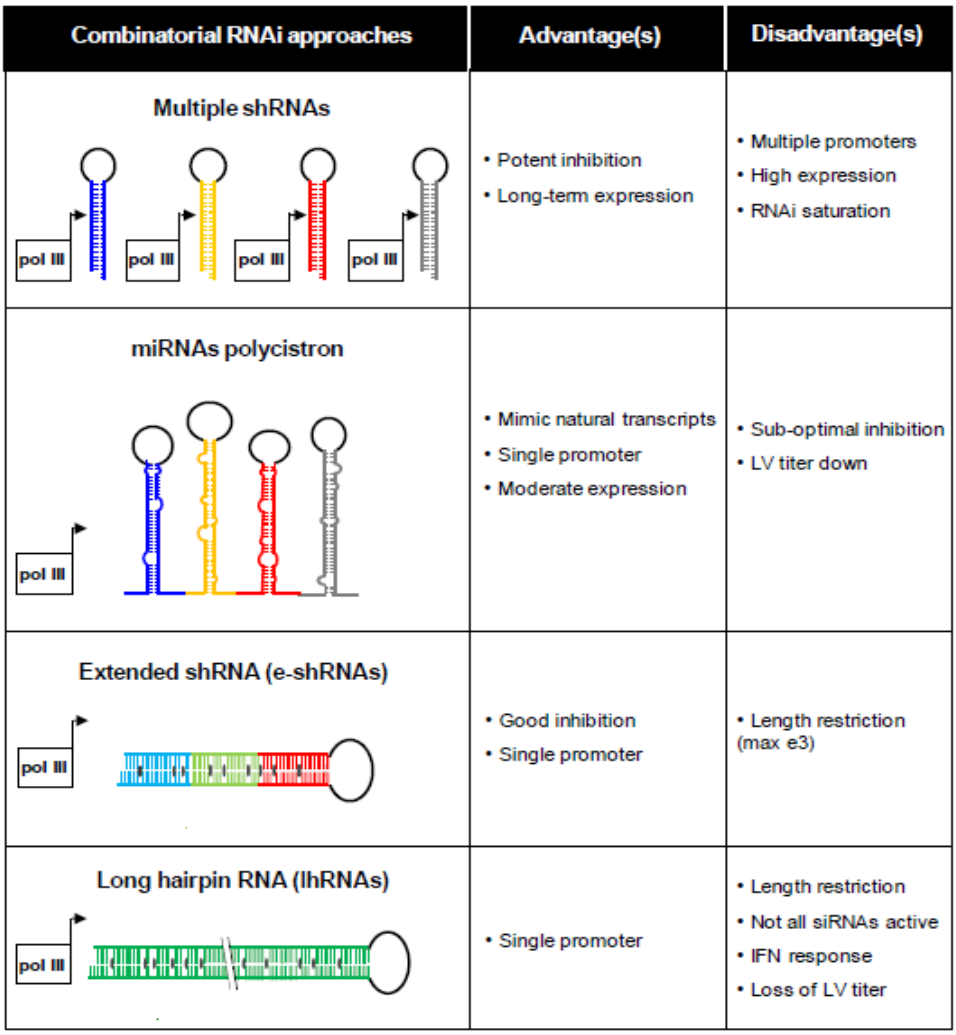

Figure 9. **Combinatorial RNAi strategies**. Advantages and disadvantages of different combinatorial RNAi approaches are shown: expression of multiple shRNAs, as independent cassettes, under the control of separate RNA polymerase III promoters, or expression of a single transcript composed of multiple miRNAs, or the expression of multiple shRNAs as an extended shRNA (e-shRNA), or the expression of many siRNAs from long hairpin RNAs (lhRNAs), under the control of a single RNA polymerase III promoter (*Herrera-Carrillo and Berkhout 2015*).

4.2.2 C- Peptides as HIV-1 fusion inhibitors

The multistep process of HIV-1 entry involves the surface glycoprotein gp120 and gp41. Upon binding to CD4, structural changes within gp120 leads to uncover the binding sites for one of the two coreceptors, CCR5 or CXCR4 (*Berger et al., 1999*). After binding of structural elements of gp120 with co-receptor, conformational changes in the gp41 subunit take place and induce a fusion active state called pre-hairpin configuration necessary for the fusion of virus and cell membrane. The hydrophobic fusion peptide at the N-terminus of gp41 is exposed and penetrates into the plasma membrane of the target cell and subsequently the N-terminal (HR1) and C-terminal

heptaed repeat domains (HR2) of gp41 form the thermostable six-helix bundle. The six-helix bundle is a trimer of hairpins, in which the three HR1-helices form a parallel, coiled-coil core and the HR2 helices are placed in an antiparallel way into grooves of the coiled-coil core. Six helix bundle formation brings the viral and cellular membranes near each other, enabling fusion pore formation and the introduction of the nucleocapsid of HIV-1 into the cytoplasm (*Melikyan et al., 2000*). Several fusion inhibitors and heterologous proteins were designed to mimic one of these domains involved in the intramolecular interaction between envelope viral proteins. In particular, C peptides, derived from the C-terminal HR2 of gp41, competitively block membrane fusion by binding to the coiled-coil of HR1 helices, thus preventing six-helix bundle formation (*Wild et al., 1992*) (**Figure 10**). Up to date, the only sanctioned fusion inhibitor approved as a salvage therapy in patients with multidrug resistant HIV-1 is Enfuvirtide (T20, Fuzeon); T20 is a synthetic soluble peptide of 36 amino acids resembling a portion of the natural sequence of gp41 HR2, which blocks gp41 in a fusion-incompetent state *(Fung and Guo, 2004).* However, the major issues concerning the lack of orally bioavailable, being a peptide, and therefore the need for repeated subcutaneous administrations with common local reactions at the injection sites together with the rapid emergence of resistant virus variants have lead to develop novel anti HIV-1 molecules and therapeutic approaches in order to inhibit the entry of HIV-1 into cell.

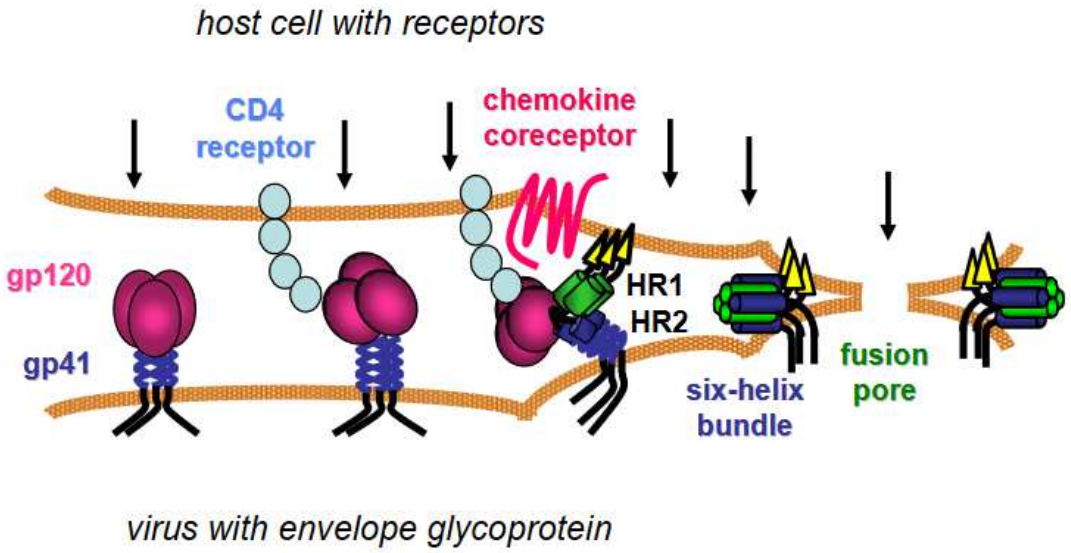

Figure 10. **Model of HIV-1 entry process.** HIV-1 entry into target cells is mediated by the CD4 receptor and chemokine co-receptors (shown on the host cell), with the gp120 surface subunit and gp41 transmembrane subunit of the HIV-1 envelope glycoprotein (shown on the envelope). The binding of gp120 to CD4 induce conformational changes in gp120, allowing its interaction with the chemokine co-receptor. Additional conformational changes in the gp41 transmembrane subunit lead to a fusion active state with the exposure of a fusogenic motif in the amino terminal ectodomain of gp41; subsequently, the N-terminal (HR1) and the C-terminal heptad-repeat domains (HR2) of gp41 self-assemble to form a six-helix bundle structure, which brings the lipid bilayer of the virion with that of the host cell into close proximity, enambling fusion pore formation. Arrows indicate multiple points of vulnerability to inhibit the entry process (*De Feo and Weiss, 2012*).

In gene therapeutic approaches, C peptides may be produced and stable expressed into modified cells after a single treatment, hoping to prevent HIV-1 infection and thus conferring a selective advantage over non-modified cells to repopulate the immune system. Indeed, considering that more than estimated 10^{11} potential target cells for HIV-1 are present in a human subject and considering that only a limited fraction of these cells can be genetically modified, only the antiviral genes belong to class I, such as C-peptides, can lead to the accumulation of gene modified cells (*Lund et al., 1997; von Laer et al., 2006*). Other unprotected cells should gradually decrease due to the cytopathic effect. Two different therapeutic strategies involving C-peptides have been developed, based on antiviral genes encoding either membrane-anchored (ma) or secreted (iSAVE) form of C-peptides. While the former prevents viral entry only in modified cells, which express high local concentration of peptide at the site of action (*Hildinger et al., 2001*), the latter resembles the mode of action of the injected T20 and exerts a bystander effect on unmodified neighboring cells, thus suppressing viral replication even at relatively low levels of gene modification. Thus, secretion of iSAVE peptide in lymphatic tissue, which represents the major site of HIV-1 replication, may

lead to high concentrations of the peptide. However, low level of secretion was observed in primary T cells *in vitro* with consequent failing of the inhibition of viral replication; thus, expression from T cells may result therapeutically insufficient *in vivo* (*Egerer et al., 2011*). Instead, membrane anchored C peptide, in particular a membrane anchored version of 46 amino acids (maC46/M87o), has been observed to confer a strong inhibition of viral replication and a selective advantage following HIV-1 infection both in cultures of primary T lymphocytes as well as in humanized mice model (*Kimpel et al., 2010*). This elongated version of T-20 was developed by the von Laer laboratory (*Hildinger et al., 2001; Egelhofer et al., 2004)* and it contains an N-terminal signal peptide, to mediate transport of the peptide through the endoplasmic reticulum to the cell surface, and a C terminal scaffold consisting of a human IgG2 hinge and a membrane anchor of human CD34 (**Figure 11)**. Like other gp41-derived C peptide, maC46 blocks membrane fusion by interacting with the N-terminal coiled-coil domain of the HIV-1 gp41, thus preventing the six-helix bundle formation.

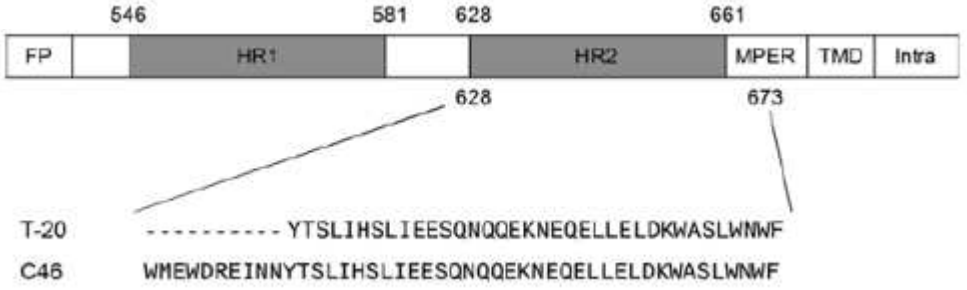

Figure 11. **Structure of gp41 and inhibitory C-peptides derived from the HR2.** The functional regions of the gp41 molecule are an N-terminal fusion peptide (FP), two leucinzipper like heptad repeat regions (HR1 and HR2), and the transmembrane domain (TMD). The amino acid numbering of the individual regions is according to the HIV-1 HxB2 envelope glycoprotein. Inhibitory C peptides, such as T-20 and C46, are derived from the C-terminal heptad repeat domain (HR2) and block membrane fusion by binding to the coiled-coil of HR1 helices, thus preventing six-helix bunble formation (*Egerer et al., 2015*).

Previous studies have investigated whether the virus sequestered on the surface of maC46-expressing cells was still infectious and if could be transmitted to non-protected, maC46-negative cells. The obtained results have demonstrated that the maC46-expressing T cells were able to trap HIV-1 virions at the cell surface and they did not efficiently transfer virus to non-transduced neighboring T-cells; furthermore, they did not contain any intracellular viral antigen (*Kimpel et al., 2010).* Several studies have investigated the possibility of development of mutations to maC46 and have

demonstrated that this peptide confers effectively protection against a broad range of HIV-1 isolates, resistant instead to T20 (*Hermann et al., 2009*); indeed, no mutations in the domain around the motif in HR1, which is generally associated with resistance to C peptide fusion inhibitors, were observed; rather, after nearly 200 days of *in vitro* infected cell passages, in the presence of gradually increasing concentrations of maC46, the emerging mutations enhanced the binding affinity between gp41 HR1 and HR2, but did not reduce the binding affinity to maC46. Thus, the development of resistance to maC46 requires multiple mutations engaging highly conserved regions of the envelope glycoproteins. Furthermore, C46 peptide has been successfully tested in non human primate model of AIDS (*Younan et al., 2013*) and was found to be tolerated in HIV-1 infected patients with advanced disease and multidrug resistant virus treated with *ex vivo* gene modified T cells (*Lohrengel et al., 2005; Van Lunzen et al., 2007*; *Zahn et al., 2008*). However, low level of maC46 gene positive cells was observed in peripheral blood of these patients (<0,1 %) after 10 years of treatment, because gene protected cells cannot accumulate and prevail in patients with reduced proliferative capacity of T-cells. Instead, targeting hematopoietic stem cells might represent a powerful alternative to confer a stronger therapeutic effect, thanks to the possibility of generating on one hand a pool of gene protected T cells and macrophages and on other hand of regenerating the T cell repertoire in HIV-1 infected patients. A relevant percentage of modified cells (reaching 90% of $CD4^+$ cells in the blood) was observed in non-human primates transplanted with maC46-gene modified autologous HSCs (*Younan et al., 2015*). These results are promising to move to human clinical setting.

4.2.3 Combination of RNAi and C-peptides as anti-HIV-1 gene therapy strategy

The possibility to block viral entry may represent a strong point to prevent chronic HIV-1 infection and thus the establishment of reservoirs. The potent strategy of RNAi can be employed only to silence CCR5, one of the two main coreceptors of HIV-1, because it has been observed not to be necessary for the development of T-lymphocytes. By contrast, CXCR4 receptor is less favored as target because of its involvement in important cellular processes, being essential for hematopoiesis and colonization of bone marrow by hematopoietic cells during ontogeny and in maintaining the blood cells in the niches; permanent ablation of CXCR4 may result in side effect of HSC egress from the bone marrow into the peripheral blood and compromise T cell trafficking (*Burger and Peled, 2009*; *Nagasawa, 2007*). At the same time, CXCR4 tropic viruses are

relevant in the progression of AIDS, since they are associated with higher replication rates and higher cytopathic potential (*Langford et al., 2007*). Thus, in order to confer a long protection against a broad range of HIV-1 isolates, it is possible to accomodate into the same vector short hairpin RNA and small peptide inhibitors of HIV-1 entry. To this end, a SIN lentiviral vector was recently developed; this vector expresses a combination of shRNA against CCR5 and the potent fusion inhibitor C46 peptide (LVsh5/C46) in order to inhibit separate immediate-early stages of the viral life cycle prior to entry and reduce the potential occurrence of escape mutations to a single agent. The dual therapeutic vector has displayed a synergistic effect in the inhibition of HIV-1 replication both in T cell lines, peripheral blood mononuclear cells as well as in humanized bone marrow liver thymus (BLT) mice model *in vivo* (*Wolstein et al., 2014; Burke et al., 2015*). The BLT mouse represents the main small animal model for HIV-1 pathogenesis *in vivo*, since the development of human T cells occurs only in the context of a human thymic microenvironment (*Karpel et al., 2015*). A phase 1/2 of clinical trial is ongoing to evaluate the antiviral activity of this construct administered *ex vivo* using both autologous $CD4^+$ T lymphocytes and $CD34^+$ HSC in HIV-1^+ subjects, without malignancy. To overcome the common issue of low frequency of gene modified cells, which frequently occurs in stem cell based gene therapy, modified cells were selected using a robust system (O^6-BG/BCNU) based on the expression of a modified enzyme (MGMT) only in gene modified cells; this modified enzyme is not sensitive to O^6 benzylguanine (O^6-BG) treatment, thus conferring to gene modified cells a survival advantage after treatment with an alkylating agent (BCNU) (*Zielske et al., 2003*).

5. AIM OF THE STUDY

This work is part of a wider research project, aimed to develop and evaluate the anti-HIV-1 activity of a combinatorial platform that expresses multiple siRNAs, targeting both cellular (*CCR5*) and viral factors (*tat*, *rev* and *vif*) along with the membrane-anchored form of C-peptide (*maC46*) fusion inhibitor, by means of a SIN lentiviral vector, to inhibit simultaneously different steps of HIV-1 entry and replication in order to confer protection against a broad range of isolates and reduce the occurrence of resistant viral species. Furthermore, a careful analysis of the potential genotoxicity of these new developed vectors is necessary before moving to clinical application. The ultimate goal of the research project would be to assess the efficacy and safety of these optimized vectors in animal models, in order to select the best performing one for the following manipulation of HSCs derived from selected HIV^+-lymphoma patients. HSCs represent an attractive target in anti-HIV-1 gene therapy approach, since their modification could repopulate the immune system. Furthermore, AIDS-related lymphoma (ARL) patients represent the ideal population in a clinical setting ethically acceptable, since they are often subjected to transplantation of HSCs, including a full myeloablative conditioning regimen before the re-infusion of autologous gene-modified cells.

6. MATERIALS AND METHODS

6.1 Plasmids

For this experimental work, the following plasmids were employed:

Constructs available in the laboratory:

pLentiLox3.7 (pLL3.7): third-generation, replicative-defective, self-inactivating (SIN) lentiviral vector (LV), previously described by Rubinson (*Rubinson et al., 2003*). This 7650 bp vector (referred to as the empty vector), was used to develop the anti HIV-1 constructs. The internal expression cassette containing the transgene (s) can be inserted upstream of the LoxP site, which preceded the cytomegalovirus (CMV) - enhanced (e) GFP expression region in the vector, between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter. The antibiotic bacterial selection is conferred by the AmpR gene.

The packaging system (*kindly provided by T. Friedman, University of California, San Diego, USA*):

pMDLg/pRRE: this 8895 bp vector contains the *gag* and *pol* genes as well as the RRE sequence from HIV-1 HxBc2 molecular clone under the control of the CMV promoter and the polyadenylation signal from the human β-globin gene (pA) (*Dull et al., 1998)*.

pMD2.VSVG: this 5824 bp vector contains the Vesicular stomatitis virus (VSV)-G glycoprotein encoding sequence under the control of the CMV promoter and the polyadenylation signal from the human β-globin gene (pA). The transfection of this construct in mammalian cells produces a high concentration of VSV-G which can be used for pseudotyping lentiviral vector particles, after co-transfection with defective viral vectors for *env*, conferring a wide tropism both *in vitro* and *in vivo,* since its phospholipid receptor is ubiquitously expressed in mammalian cells (*Dull et al., 1998).*

pRSV-Rev: this 4174 bp vector contains the Rev encoding sequence under the control of the Rous Sarcoma Virus (RSV) promoter and the polyadenylation signal from the human β-globin gene (pA) (*Dull et al., 1998*).

A schematic diagram of the tranfer plasmid and packaging plasmids is reported in **Figure 12**.

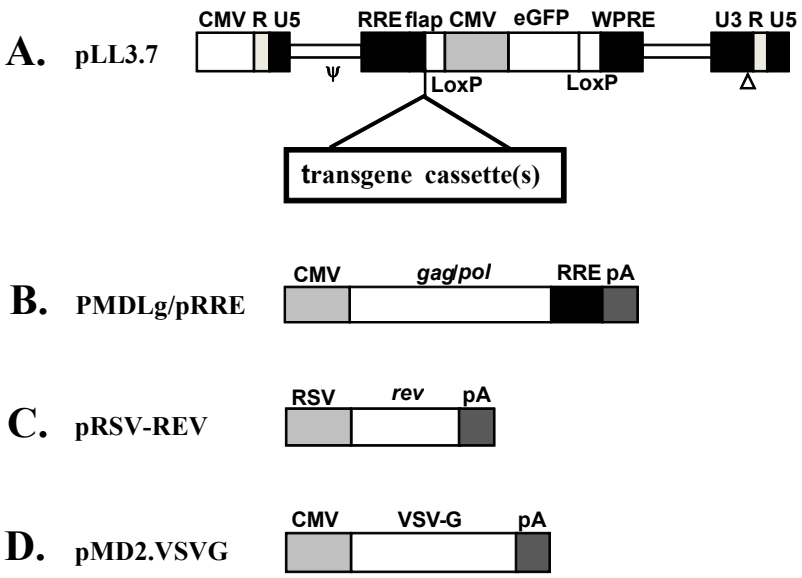

***Figure 12*. The pLL3.7 lentiviral vector and the packaging system. A**. The transfer vector pLL3.7 carries a chimeric 5'LTR in order to render the LV promoter Tat-indipendent, with the CMV promoter and enhancer sequence replacing the U3 region; the packaging signal (Ψ), the Rev response element (RRE), the Flap sequence, the eGFP reporter gene driven by CMV promoter, flanked by LoxP sites, the Woodchuck post-transcriptional regulatory element (WPRE) sequence, to increase the overall levels of transcripts both in producer and target cells; the 3' LTR in which the U3 region has been deleted to remove all transcriptionally active sequences, creating the so-called self inactivating (SIN) LTR; the SV40 origin of replication. The transgene cassette was inserted directly upstream of the LoxP sites which precedes the CMV-eGFP cassette in the vector. **B**. pMDLg/pRRE carries the *gag* and *pol* genes driven by the CMV promoter and the RRE sequence from HIV-1. **C**. pRSV-Rev carries the Rev encoding sequence driven by the RSV promoter. **D**. pMD2.VSVG carries the VSV-G glycoprotein encoding gene driven by the CMV promoter. pA indicates the polyadenylation signal from the human β-globin gene.

pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev: third-generation, replicative defective, SIN lentiviral vector derived from the pLL3.7 plasmid, previously described by Spanevello (*Spanevello et al., 2016*). This 8399 bp vector encodes two short hairpin RNA (shRNA) against the cellular co-receptor CCR5 (i.e. shCCR5) and the viral gene *vif* (i.e. shVif) and a long hairpin RNA (lhRNA), which gives rise to two different siRNAs against contiguous sequences in the *tat/rev* common transcript (i.e. lhTat/Rev). These siRNA are under the control of three independent Polymerase III human promoters, respectively: the human U6 small nuclear RNA promoter (U6), the 7SK small nuclear RNA promoter (7SK) and the human RNase P RNA H1 promoter (H1). The transgene cassette was subcloned into the pLL3.7 plasmid between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter.

pLL3.7 H1e-shRNA: third-generation, replicative-defective, SIN lentiviral vector derived from the pLL3.7 plasmid, previously described by Spanevello *(Spanevello et*

al., 2016). This 7729 bp vector encodes a single hairpin of 64 bp in the stem under the control of the single Polymerase III promoter, H1. The e-shRNA gives rise to three distinct siRNAs targeting the CCR5 cellular gene and the *tat/rev* and *vif* viral transcripts. In order to attenuate the innate immune response to long dsRNAs and to facilitate the propagation of this plasmid in *E. coli*, G:U wobble parings were included at regular intervals in the sense strand of the lhtat/rev (*Saayman et al., 2008*). The transgene cassette was subcloned into pLL3.7 between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter.

pLL3.7 H1*scrambled*CCR5 (H1scrCCR5): third-generation, replicative-defective, SIN lentiviral vector, derived from the pLL3.7 plasmid, previously described by Spanevello *(Spanevello et al., 2016)*. This vector carries a sequence expressing a shRNA, under the control of the H1 promoter, which gives rise to a siRNA that is three nucleotides different from the one produced by the shCCR5 (5′-GAGCAAGCTCTCGTTACACC-3′) and it is used as control. The transgene cassette was subcloned into pLL3.7 between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter.

The shRNA sequences feature a 9 nt loop situated between the sense and the reverse complementary sequences and a pol-III termination signal (polyT) at the 3' end.

Constructs kindly provided by Professor von Laer's laboratory, Innsbruck, Austria:

pM809: this SIN lentiviral vector of 5545 bp vector contains the SV40 origin of replication; the hybrid 5' LTR in which the U3 region is replaced with the RSV promoter and enhancer sequences and a SIN 3' LTR; the AmpR gene confers the bacterial antibiotic selection. This vector carries the membrane-anchored form of C-peptide (maC46) encoding sequence, under the transcriptional control of the human Polymerase II Elongation Factor 1 (EF1) promoter (short version after removal of its intron) and an optimized version of the Woodchuck hepatitis virus Post-transcriptional Regulatory Element oPRE (referred to as WPRE*) between the *NotI-HindIII* restriction sites.

pT385: this SIN lentiviral vector of 6331 bp was created by ligation of M589-*EcoRI*-blunt-*BstEII* into M809-*SbfI*-blunt-*BstEII*. This vector contains the same backbone of the M809, but carries the maC46 encoding sequence fused in frame with the eGFP reporter gene.

pT392: this SIN lentiviral vector of 5861 bp contains the same backbone of the M809 and T385 vectors along with the eGFP reporter gene, driven by the EF1 promoter.

6.2 Vector development

In order to develop lentiviral vectors expressing multiple siRNA and a membrane-anchored peptide (maC46) able to inhibit entry and replication, the two vectors already available in the laboratory (pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev and pLL3.7 H1 e-shRNA) (*Spanevello et al., 2016*) were optimized by inserting the maC46 peptide along with or without the eGFP reporter gene (*derived from constructs kindly provided by von Laer's laboratory, Innsbruck, Austria*). The following constructs were obtained:

- pLL3.7 H1e-shRNA EF1maC46eGFPWPRE* and **pLL3.7 H1e-shRNA EF1maC46 WPRE***

In order to obtain these constructs, the CMV-eGFP-WPRE region in the pLL3.7 H1eshRNA vector was replaced with the EF1maC46eGFPWPRE* and EF1maC46WPRE* cassettes of the T385 and M809 vectors respectively. To this end, the EF1maC46eGFPWPRE* and EF1maC46WPRE* cassettes were excised with *NotI-HindIII* enzymes. The pLL3.7 H1eshRNA was digested with *NotI* and *KpnI* enzymes, followed by a PCR reaction (94° C 10', (94°C 30",60°C 30", 72°C 30") x 35 cycles, 72°C 7', 4°C ∞) with a couple of primers (CLA1(forward): 5'-***A/AGCTT***ATCGATACCGTCGACC-3', which generated a *HindIII* binding site, CLA2(reverse): 5'-***GGTAC/C***TGAGGTGTGACTGG-3', which generated a *KpnI* binding site), able to amplify the portion from the end of the WPRE region until the *KpnI* binding site. The PCR product was cloned into the commercial pCR 2.1-TOPO plasmid vector (TOPO TA Cloning, Invitrogen, Carlsbad, CA), which was *HindIII-KpnI* digested to obtain the first portion of the 3' LTR. The purified fragments (i.e. *NotI-HindIII* EF1maC46eGFPWPRE* or EF1maC46WPRE* fragment, *NotI-KpnI* pLL3.7 H1eshRNA backbone and *HindIII-KpnI* 3'LTR fragment) underwent a ligation reaction.

- pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFPWPRE* and **pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 WPRE***

For the construction of these lentiviral vectors, the H1e-shRNA cassette was excised from either the pLL3.7 H1e-shRNA EF1maC46eGFPWPRE* or the pLL3.7 H1e-

shRNA EF1maC46 WPRE* vector by digestion with *XbaI* and *XhoI* enzyme and replaced with the U6shCCR5-7SKshVif-H1lhTat/Rev cassette.

- pLL3.7 H1e-shRNA EF1eGFP WPRE*

In order to obtain this construct, the T392 vector was digested with *ClaI*, treated with Klenow fragment to obtain blunt ends and, after a *HindIII* digestion, the EF1eGFP WPRE* cassette was obtained. The pLL3.7 H1eshRNA was digested with *NotI*, treated with Klenow fragment and finally digested with *KpnI*. The *KpnI-NotI*-blunt pLL3.7 H1eshRNA, *ClaI*-blunt-*HindIII* EF1eGFPWPRE* and the *HindIII-KpnI* 3'LTR fragments underwent a ligation reaction.

- pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFPWPRE*

To obtain this construct, the pLL3.7 H1e-shRNA EF1eGFP WPRE* vector, previously cloned, was digested with the enzymes *XbaI* and *XhoI*, to remove the H1e-shRNA cassette and replace it with the U6shCCR5-7SKshVif-H1lhTat/Rev insert. In this case, there are two *XbaI* restriction sites, one upstream of the H1eshRNA, and another downstream of the eGFP sequence, located in the T392 cassette. The *XhoI- XbaI* 5'-EF1eGFPWPRE* and the *XbaI- XbaI* pLL3.7 backbone bands were purified and used for the following three-fragment ligation reaction, together with the *XbaI-XhoI* pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev insert.

- Scrambled vectors

Three *scrambled* (scr) vectors to be used as controls were developed. These constructs contain a sequence expressing a shRNA, driven by the human Polymerase III H1 promoter, which, once introduced into the cells, generates a siRNA that is three nucleotides different from the one produced by the shCCR5. The fusion inhibitor was cloned into the *scrambled* vector either alone or fused in frame with the eGFP, under the transcriptional control of the human Polymerase II EF1 promoter (i.e. EF1maC46, i.e. EF1maC46eGFP); in addition, a negative control for the maC46 expression was developed, by cloning the eGFP reporter gene, driven by the same promoter (i.e. EF1eGFP), into the *scrambled* vector. In order to obtain these constructs, the H1e-shRNA region was replaced in the pLL3.7 H1e-shRNA EF1maC46eGFP, pLL3.7 H1e-shRNA EF1maC46 or pLL3.7 H1e-shRNA EF1eGFP vector, respectively, with the H1*scrambled*CCR5 (H1scrCCR5) region of the pLL3.7 H1*scrambled*CCR5 vector by

digestion with *XbaI* and *XhoI* enzymes. Since the pLL3.7 H1e-shRNA EF1eGFP vector carries an additional *XbaI* site downstream of the eGFP cassette, three fragments were obtained after this digestion (*XbaI-XbaI* backbone fragment, *XbaI-XhoI* H1e-shRNA fragment and *XhoI-XbaI* EF1eGFP fragment). The purified fragments (i.e. pLL3.7 ΔH1e-shRNA EF1maC46eGFP backbone and H1scrCCR5 insert) (i.e. pLL3.7 ΔH1e-shRNA EF1maC46 backbone and H1scrCCR5 insert) (i.e. pLL3.7 ΔH1e-shRNA ΔEF1eGFP backbone, the EF1eGFP insert and H1scrCCR5 insert) underwent to a ligation reaction.

The developed vectors were confirmed by restriction enzyme reaction, gel electrophoresis and DNA sequencing. The primer oligonucleotides employed in the PCR reaction before Sanger sequencing (*Sanger et al., 1977*) are: 3.7 Forward (F) (5'-CAGGGGAAGAATAGTAGAC-3') and 3.7 Reverse (R) (5'-CGGCCGCTTAAGCTTGGAAC-3'), which were employed to discriminate the cassette placed between *XbaI* and *XhoI* enzyme restriction sites (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev; H1 e-shRNA; H1scrCCR5), and Cla2 R (5'-GGTACCTGAGGTGTGACTGG -3') and WPRE R (5'-CATAGCGTAAAAGGAGCAACA-3'), which were employed to discriminate the cassette carrying the maC46 peptide and the WPRE* (i.e. EF1maC46WPRE*; EF1maC46eGFPWPRE*).

A schematic representation of the SIN LV vectors based on the combination of the triple cassette and e-shRNA expressing vectors previously developed (*Spanevello et al., 2016*), along with the maC46 expressing cassettes as well as control vectors is depicted in **Figure 13.**

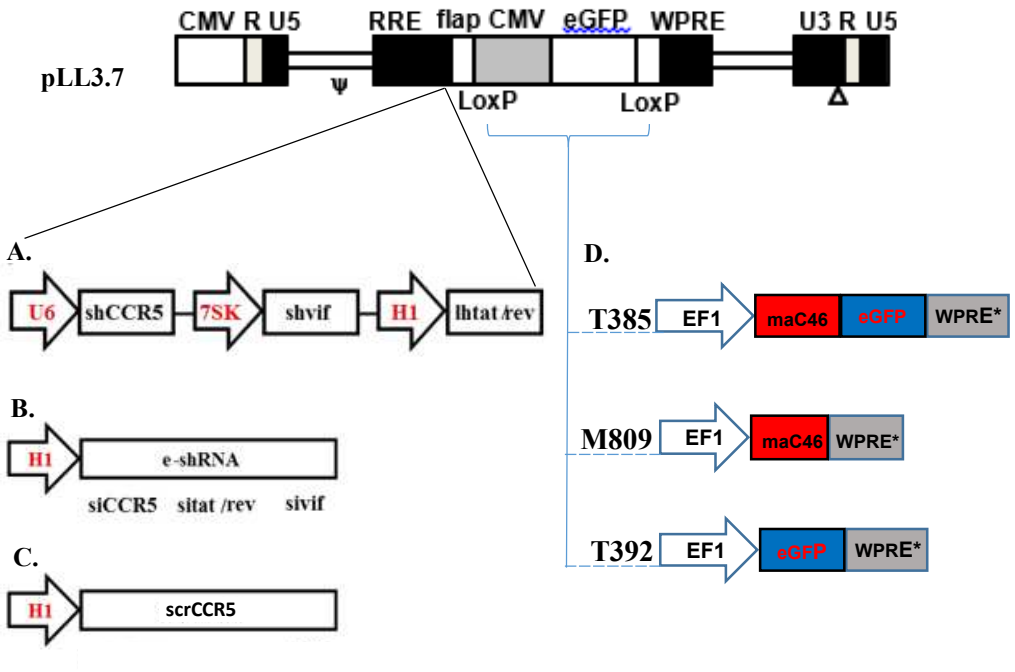

Figure 13. **Schematic representation of the combinatorial vectors and the maC46 cassettes.** The third-generation pLentiLox3.7 (pLL3.7) SIN lentiviral vector, which contains an eGFP reporter gene, was used to develop the anti-HIV-1 constructs. **A**. The triple cassette vector expresses the shCCR5, the shvif and the lhtat/rev (which generates two siRNAs targeting the first overlapping exon of the *tat* and *rev* viral transcripts) as independent transcriptional units, under the control of Polymerase III U6, 7SK and H1 promoter respectively. **B**. The H1e-shRNA vector encodes for a single hairpin under the control of Polymerase III H1 promoter. This e-shRNA gives rise to three distinct siRNAs targeting the CCR5, the *tat/rev* and the *vif* transcripts. G:U wobble parings were included at regular intervals in the sense strand of the lhtat/rev, to attenuate the innate immune response to long dsRNAs. **C**. Control vector, driven by the Polymerase III H1 promoter, based on a scrambled sequence. **D**. Cassettes derived from constructs kindly provided by von Laer's laboratory, Innsbruck, Austria: T385 carries the maC46 gene fused in frame with eGFP; M809 carries the maC46 peptide alone; T392 carries the eGFP. The expression of these genes is under the control of human Polymerase II EF1 promoter (short version after removal of its intron). The optimized version of WPRE (WPRE*) enhances the expression of the genes while ensures a reduced risk of oncogenesis.

6.3 Cell lines

For this experimental work, the following cell lines were used:

The 293T cell line is a highly transfectable derivative of human embryonic kidney (HEK) 293 cells, with stellar morphology, and constitutively expresses the T antigen of the simian virus 40 (SV40), thus enabling efficient replication of vectors carrying the SV40 region of replication. This cell line gives high titers when used to produce retroviral stocks. The cellular line was kindly provided by Dr. D. Baltimore (Rockfeller University, New York) (ATCC® CRL-3216™).

The Human T lymphoblastoid Jurkat cell line (Clone E6-1) was established from the peripheral blood of a 14 year old boy with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. These cells are common targets of CXCR4-using HIV-1 strains (ATCC® TIB-152™).

The C8166 cell line (Clone of C63/CRII-4) derived by fusion of primary umbilical cord blood cells with HTLV-1 producing line from adult T cell leukemia lymphoma patient. It contains defective HTLV-1 genome. In the presence of HIV, cells fuse to form syncytia.
The 293T cell line grew in adhesion and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Monza, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies).

The Human T lymphoblastoid Jurkat and C8166 cell lines grew in suspension and were maintained in Roswell Park Memorial Institute's 1640 medium (RPMI) (Gibco, Life Technologies) supplemented with 10% heat-inactivated FBS. All cell lines were cultured in an incubator (Heraeus BBD 6220) at constant temperature of 37 ° C in 5% of carbon dioxide humidified atmosphere.

6.4 Immunofluorescence cell staining

To ascertain the correct expression of the maC46 fusion inhibitor, immunofluorescence cell staining on HEK 293T cells transfected with the developed vectors was performed. Briefly, 24 hours before transfection, HEK 293T cells were seeded in 6-well plate, previously covered by a glass coverslip treated with a solution of Poly-L-Lysine (0.1 mg/mL, Sigma), at a density of 3.5 x 10^5 cells per well (to achieve a 90-95% confluence at the time of transfection). The next day, cells were transfected with 10 µL of Lipofectamine 2000 (1 mg/mL Invitrogen, Life Technologies), diluted in 250 µL of DMEM Serum free, and 4 µg of DNA, diluted in 250 µL of DMEM Serum free, and incubated for 20 minutes at room temperature. The medium was replaced 6 hours after transfection in order to reduce the cytotoxic effect of the liposomes. 24 hours after transfection, cells were washed with room temperature-phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and fixed in a solution of 4% Paraformaldehyde (PFA) in PBS for 10 minutes at room temperature. After two washing in PBS, cells were first incubated for 30 minutes at room

temperature with a solution of 0.5% Bovine Serum Albumine (BSA) in PBS and subsequently for 2 hours at 4°C with 40 µL of the phycoerythrin-labeled recombinant human monoclonal antibody to HIV-1 gp41 epitope ELDKWA (2F5-PE, Polymun Scientific), diluted either 1:50 or 1:100. After a washing in PBS, cells were incubated for ten minutes at room temperature with 40 µL of nuclear fluorescent staining DRAQ5 (5 mM, Life Technologies), diluted 1:1000 in MQ water, followed by another wash in PBS. To preserve fluorescence, 8 µL of Mounting Medium (VECTASHIELD H-1000, Vector Laboratories) were dispensed on the glass slide before applying the coverslip.

Next, 1 x 10^6 human T lymphoblastoid Jurkat cells were transduced with RT equivalent units (16000 and 64000 cpm) of recombinant lentiviral particles and three days after transduction, Jurkat cells transduced with the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and pLL3.7 H1e-shRNA EF1maC46 vectors were pelleted by centrifugation at 1100 rpm for 7 minutes at 4°C and resuspended in 200 µL of PBS. The cell suspension was loaded in the proper cytospin cuvette and spinned at 800 rpm for 3 minutes using a cytospin centrifuge (Cytospin 3 SHANDON). Cells were subsequently fixed in 4% PFA in PBS for 10 minutes at room temperature and, after two washing in PBS, were firstly incubated for 30 minutes at room temperature with a solution of 0.5% BSA in PBS and secondly for 2 hours at 4°C with 40 µL of the 2F5-PE antibody, diluted 1:50. Samples were, then, washed twice in PBS and 8 µL of Mounting Medium were dispensed on the glass slide before applying the coverslip.

Samples were analyzed by confocal microscopy with an immersion objective (LEICA DM Irbe).

6.5 Production/titration of recombinant lentiviral particles

Vesicular stomatitis virus (VSV)-G pseudotyped vector stocks were produced by calcium phosphate transfection of HEK 293T cells. Briefly, cells were seeded at 12 × 10^6 per T150 tissue culture flask 24 hours before transfection, and, once the 80% confluence was reached, they were co-transfected with 20 µg of the appropriate gene transfer vector, 10 µg of pMDLg/pRRE, 10 µg of pRSV-Rev and 10 µg of pMD2.VSVG (*kindly supplied by T. Friedman, University of California, San Diego, USA*), which were previously diluited in TE 1:10 (TE: Tris-HCl 1 mM pH 8, EDTA 0.5 mM pH 8) up to a final volume of 1800 µL. Subsequently, 200 µL of 2.5 M $CaCl_2$ was added to the DNA and then 2 mL of 2X HPB buffer pH 7.1 (5M NaCl, 0.5 M HEPES

pH 7.1, 0.15 M Na_2HPO_4 $7H_2O$) was added dropwise into the precipitate. Finally, the mixture was added dropwise to the cell culture and flasks were then returned to the incubator; 3 to 4 hours later, cells were gently washed twice with 10 mL of PBS and fresh growth medium was replaced. The culture supernatants were collected on day 2 post-transfection, filtered with a 0.45-µm-pore-size membrane (Millipore), concentrated 100X by ultracentrifugation (27000 rpm, 2 h, 4°C in a Beckman SW28 rotor) and stored at -80° C until use. The viral titer of all stocks was measured by the Reverse Transcriptase (RT) activity assay and expressed in counts per minute (cpm), according to the conditions reported in the paragraph**;** the infectious titer of vectors that carried the reporter gene was determined by transducing 2.5 x 10^5 HEK 293T cells with 10-fold serial dilutions of the lentiviral stocks in 6-well plates; 72 hours later eGFP expression was assessed by flow cytometry (Fluorescence-Activated Cell Sorter Calibur, Becton Dickinson, with 488 nm excitation laser and green filter) and the data analysis was performed with CellQuest (Berton Dickinson). The infectious titer was expressed as transducing units/mL (TU/mL) and was calculated according to the following formula, using the percentage of positive cells: Transducing units (TU/ml) = (% positive cells ($eGFP^+$/100) x transduced cell number x dilution factor)/lentiviral particle volume (mL).

6.6 Transduction of target cells with recombinant lentiviral particles

To transduce HEK 293T cells with vectors carrying reporter gene, 2.5 x 10^5 cells/well were seeded in 6-well plates in 2 mL of DMEM 10% FBS 24 hours prior to transduction. The next day, medium was replaced with 1 mL of DMEM 10% FBS containing 10-fold serial dilutions of the lentiviral stocks. 1 mL of fresh culture medium was added to the cells approximately 8 hours later. Three days after transduction, cells were detached from the tissue culture dish and transferred into 15 mL Falcon tubes, washed twice by centrifugation at 1200 rpm at 4°C for 7 minutes and resuspended in 500 µL of cold PBS to evaluate the eGFP expression by flow cytometry.

HEK 293T cells transduced with lentiviral vectors lacking the eGFP reporter gene (i.e. pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and pLL3.7 H1e-shRNA EF1maC46) were initially analyzed by flow cytometry. To this end, 48 hours after transduction, up to 2 x 10^5 cells were transferred into 15 mL Falcon tubes and washed twice by centrifugation at 1200 rpm at 4°C for 7 minutes and resuspended in 500 µL of FACS buffer (0.05% NaAzide, 1% FBS in PBS). Then, cells were incubated for 30

minutes at 4°C with 5 µL of the 2F5-PE antibody, washed twice with FACS buffer and fixed in 500 µL of 4% PFA in PBS for 10 minutes at room temperature. After another wash with FACS buffer, samples were analyzed. Transduction efficiency was determined by calculating the percentage of C46-PE positive cells.

To ascertain the correct localization of the peptide on $CD4^+$ T lymphoblastoid cell line plama membrane, 1 x 10^6 Jurkat cells were transduced with pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and pLL3.7 H1e-shRNA EF1maC46 recombinant lentiviral particles, using 16000 count per minute (cpm) of equivalent Reverse Transcriptase **(**RT) units for non-concentrated stocks and 64000 cpm for concentrated ones. 72 hours post transduction, cells were spotted on a glass slide by means of cytospin and analyzed by immunofluorescence cell staining. Moreover, these same conditions of transduction were adopted to compare the transduction efficiency between the previously developed vectors (*Spanevello et al., 2016*), carrying the CMV promoter, and the new optimized vectors, carrying the EF1 promoter.

For transduction of Jurkat cells to be used in the following challenge experiments, 1 x 10^6 cells were incubated in 12-well plates with equivalent RT units of lentiviral supernatant (700000 and 35000 cpm) in a total volume of 1 mL. After three days of culture, 5 x 10^4 cells, transduced with 700000 equivalent RT units, underwent cell viability assay. The transduction efficiency was ascertained by FACS analysis on the basis of eGFP expression and cells were then used for HIV-1 challenge. Jurkat cells were prepared for FACS analysis using the same protocol employed for transduced HEK 293T cells.

6.7 Reverse Transcriptase (RT) activity assay

This method allows to determine the RNA-dependent DNA polymerase activity of the viral Reverse Transcriptase as incorporation of 3H-dTTP (radioactively labeled triphosphate deoxytidine with tritium atoms) on a synthetic polyA template in the presence of an oligo-dT (*Rho et al., 1981*). Briefly, viral particles were pelleted from 500 µL of the filtered culture supernatants by centrifugation at 13000 rpm for 2 hours at 4°C. The precipitate was resuspended in 10 µL of a Suspension buffer containing 50 mM Tris-HCl pH 7.5, 1mM dithiothreitol (DTT), 20% glycerol, 250 mM KCl and 0.25% Triton X-100, transferred in dry ice and lysed through three cycles of freezing and thawing. The sample was added to a reaction mixture containing 10 µL of 5X RT

assay buffer (250 mM Tris-HCl pH 7.5, 37.5 mM $MgCl_2$, 0.25% Triton X-100), 1.2 µL of 200 mM DTT, 5 µL of 100 µg/mL oligo-dT-polyA (Roche), 1 µL of 84 Ci/mmol ^{3}H-dTTP (Perkin Elmer) and water in a final volume of 50 µL. After 1 hours of incubation at 37°C, the mix was spotted on a positively charged nylon membrane (Amersham Hybond ^{TM}-N$^+$) and, to remove the excess of ^{3}H-dTTP, membranes were immediately washed three times in Saline Sodium Citrate (SSC) buffer (0.3 M NaCl, 0.03 M sodium citrate pH 7.2) for 10 minutes each, twice in absolute ethanol for 10 seconds each and, then, dried. After that, filters were soaked into 4 mL of liquid scintillation cocktail (ULTIMA Gold, Perkin Elmer). The radioactivity was measured by using a benchtop liquid scintillation analyzer (TRI-carb 2810 TR, Perkin Elmer) and expressed in counts per minute (cpm).

6.8 Cell viability assay

Human T lymphoblastoid Jurkat cells were seeded at a density of 1 x 10^6 cells/well and transduced with 700000 cpm of recombinant lentiviral particles. Three days after transduction, 5 x 10^4 transduced cells were plated in 96-well plate in 100 µL of RPMI 10% FBS. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay was performed, according to the manufacturer instructions (Roche). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The formazan crystals formed were solubilized and the resulting colored solution was quantified using a scanning multiwell spectrophotometer (ELISA reader) at a wavelength of 620 nm. The value obtained for control cells was set to 100% and relative cell viability for other samples was calculated accordingly.

6.9 HIV-1 stock production/titration and infection

The HIV-1 stock was produced by transient transfection of 5 x 10^6 Jurkat cells with 10 µg of the pSVC Vpr$^+$/Vpu$^+$/Nef$^+$ construct (*kindly provided by Heinrich Göttlinger, University of Massachusetts Medical School, Massachusetts*) by the Diethylaminoethyl (DEAE)-dextran method (*Smale, 2010*). The construct is a derivative of the pSVC21 plasmid (*Sodroski et al., 1984*), containing the HIV HXBc2 molecular clone (*Ratner et al., 1985*), where the *vpr*, *vpu*, and *nef* sequences were substituted with those derived from the pNL4-3 (*vpr*/*vpu*) (*Adachi et al., 1986*), and pLAI (*nef*) (*Peden et al., 1991*) molecular clones, in order to introduce functional *vpr*, *vpu*, and *nef* genes, respectively. 48 hours post-transfection, Jurkat cell supernatants were harvested, filtered (pore size,

0.45 μm) and stored at −80 °C until use. The viral titer was determined as 50% tissue culture infective doses ($TCID_{50}$)/ml on C8166 cell culture by cytopathic effect, according to the Reed and Muench end point dilution method (*Reed and Muench, 1938*), as well as by measuring the RT activity assay, as previously described.

Three days post-transduction with recombinant lentiviral particles, Jurkat cells were seeded in a 12-well plate at the density of 1×10^6/well, and infected with HIV-1 HXBc2 $Vpr^+/Vpu^+/Nef^+$ R4-tropic molecular clone using different M.O.I., as reported in the results, in a total volume of 300 μL. After 1 hour of incubation at 37 °C, the cultures were washed three times and cultured in RPMI 10% FBS medium. On various days post infection (d.p.i.), cell-free culture supernatants were collected to monitor virus replication by RT activity assay.

6.10 *In Vitro* Immortalization (IVIM) Assay

To assess the mutagenic potential of the developed SIN lentiviral platform, the IVIM assay was performed, in collaboration with the Baum' s group at the *Department of Experimental Hematology of the Hannover Medical School (Modlich et al., 2006*). Only the vectors lacking the eGFP have been selected (U6shCCR5-7SKshVif-H1lhTat/RevEF1maC46 and H1e-shRNAEF1maC46 referred to as U6-EFS-C46 and H1-EFS-C46, respectively) to be tested along with the scrambled vector (H1*scrambled*CCR5 referred to as H1-scrambled). A schematic illustration of this assay is shown in the **Figure 14**.

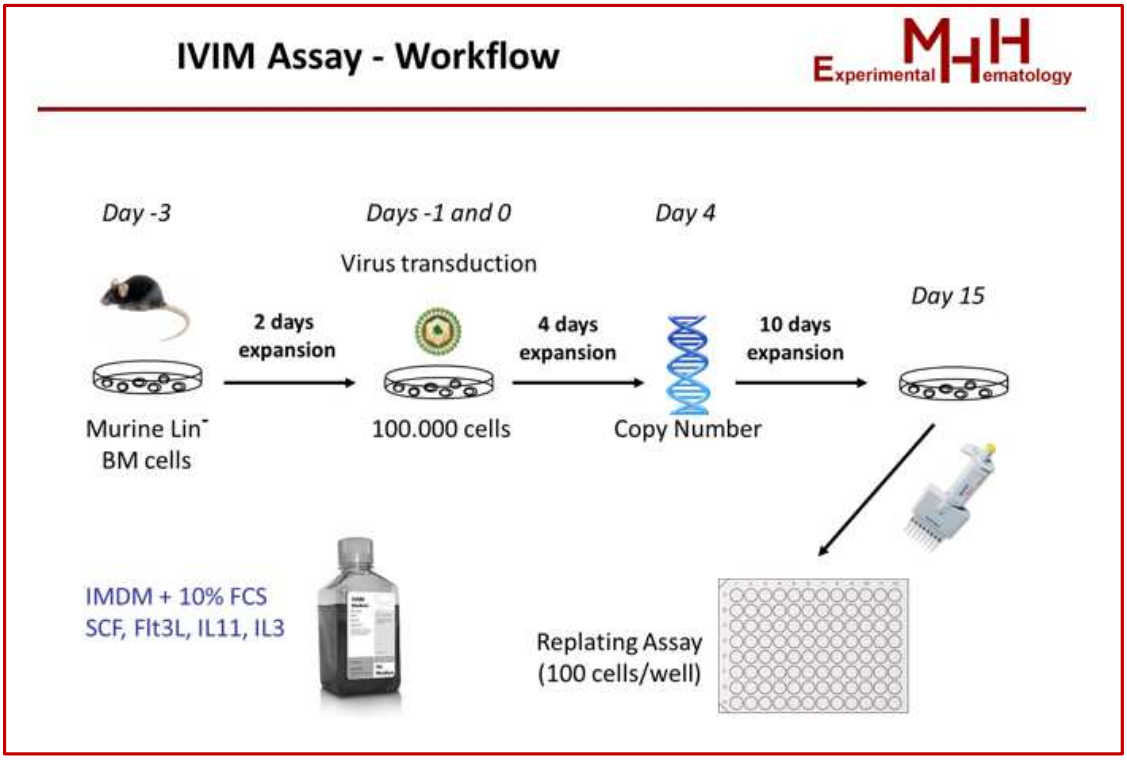

Figure 14. **Workflow of the IVIM-assay.** Lineage-negative (Lin$^-$) bone marrow (BM) cells of untreated C57B16/J mice were isolated, prestimulated for 2 days before retroviral transduction, using a protocol that allows efficient and dose-controlled retroviral gene transfer, cytokine-supplemented expansion cultures. Two rounds of transduction were performed (on days -1 and 0) at a defined multiplicy of infection (M.O.I.) and the transgene expression was monitored by flow cytometry (on day 4 and 15). The mean vector copy number per cell (VCN) was determined on day 4. The cells were grown in cytokine-supplemented media for another 2 weeks, before being replated in 96-well plates (on day 15) at a very low density of 100 cells/well. Under these conditions, mock cells usually cannot proliferate anymore, apart from cases of spontaneous cell growth. Two weeks later the positive wells were counted and the frequency of replating cells was calculated. The ratio of replating frequency (determined by limiting diluition cloning) per VCN (detected by real-time PCR) 4 days after transduction, is a measure of the degree of transformation.

To examine whether the potential insertional activation of cellular proto-oncogenes represented the driving force of the enhanced fitness detected in the replating assay, primary murine hematopoietic cells (Lin$^-$) were transduced at a cumulative (c) M.O.I. of 60 and 140 and expanded for two weeks under particular myeloid culture conditions, before being replaced on 96-well plates (100 cells/well). A mock infected sample cultured without a viral vector served as a measure of spontaneous immortalization and routinely scored negative. Test vectors were compared to a positive control vector shown previously to induce *in vitro* immortalization by insertional mutagenesis (*Modlich et al., 2009*), pRSF91.GFPgPRE (RSF91), a gammaretroviral vector, with internal long terminal repeat – contained spleen focus forming virus promoter sequences. Under these conditions of very low cell concentrations, mock-treated cells

hardly survived, whereas insertional mutants could still grow. Thus, after additional two weeks of cell culture, the positive wells were counted to quantify the incidence of cell transformation as replating frequency (RF, the number of positive wells on a plate as a measurement of clonal fitness) per vector copy number (VCN). As an example in case of 17 positive wells (100 cells seeded), the RF based on Poisson distribution is 1.95 x 10^{-3}. Thus the VCN normalization will be:

$$\frac{1.95\ x\ 10^{-3}}{3} = 6.5 \text{ x } 10^{-4}$$

7. RESULTS

7.1 Background

In the context of an international consortium for the development of an innovative gene therapy protocols for AIDS, our research group generated combinatorial vectors based on a SIN lentiviral HIV-1 platform, simultaneously expressing anti-HIV-1 molecules against viral entry, infectivity and gene expression (*Spanevello et al., 2016*). Initially we focused our attention on small interfering RNAs (siRNAs), targeting both the *CCR5* cellular gene (one of the 2 main HIV-1 co-receptors) as well as the *vif*, *tat*, and *rev* viral transcripts. The siRNA were either expressed as single transcriptional unit under the control of different human RNA polymerase III promoters (*Ter Brake et al., 2008*): U6, 7SK, H1 (*Chung et al., 2013*), or simultaneously, as an extended shRNA (e-shRNA) (*Liu et al., 2009*), under the transcriptional control of a single pol-III promoter: once introduced into the cells, it gives rise to more than one siRNA and it was optimized to reduce the activation of the cellular interferon response. The silencing and antiviral activity of the developed vectors were firstly analyzed in T lymphoblastoid $CD4^+$ Jurkat cells (**Figure 15**) and the most potent triple cassette and e-shRNA expressing vectors were selected to be further tested in human primary $CD4^+$ T lymphocytes against 2 different HIV-1 strains (**Figure 16**).

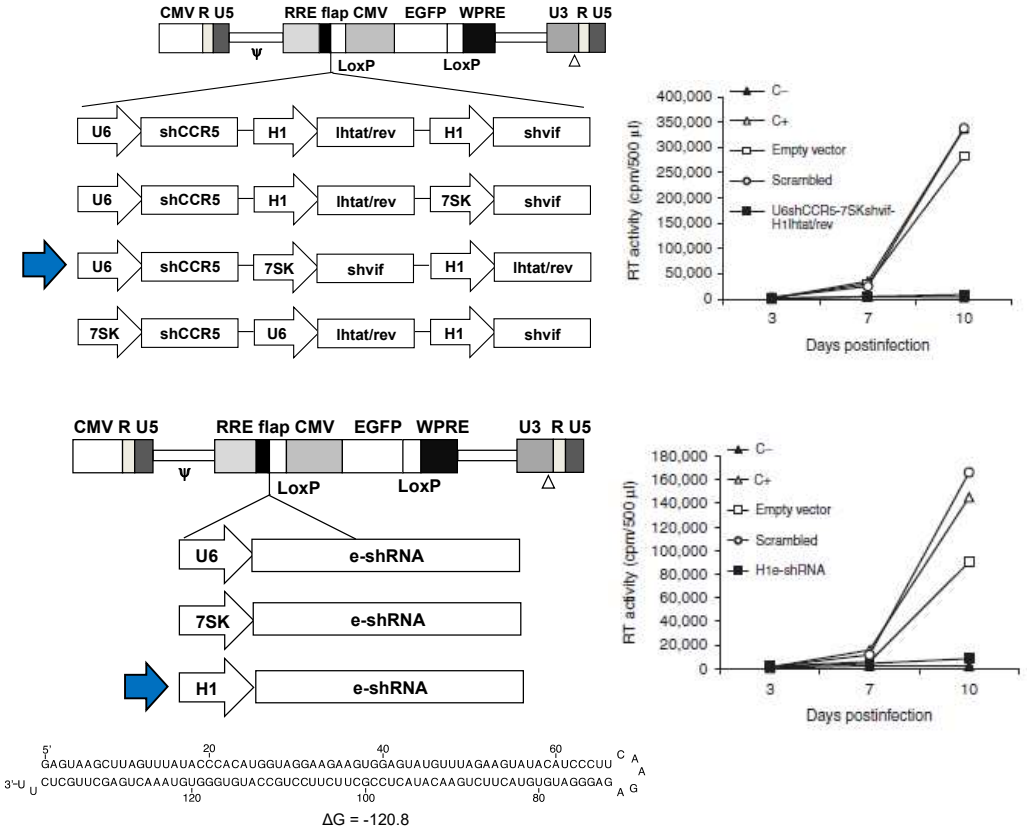

Figure 15. **Schematic representation of the triple cassette and e-shRNA SIN lentiviral vector according to different combinations of promoter-RNAi trigger and evaluation of the antiviral activity in human CD4$^+$ T lymphoblastoid Jurkat cell line.** The blue arrows indicate the best combination of promoter/siRNA selected in term of inhibition of HIV-1 replication in human Jurkat T-cells, which were transduced with the triple cassette vectors, the e-shRNA expressing vectors or the control empty and scrambled vectors and infected with the HIV-1 HXBc2 Vpr$^+$/Vpu$^+$/Nef$^-$ R4-tropic molecular clone (M.O.I. = 0.1 $TCID_{50}$/cell). Culture supernatants were collected at the indicated time points and assayed for RT activity (*Spanevello et al., 2016*).

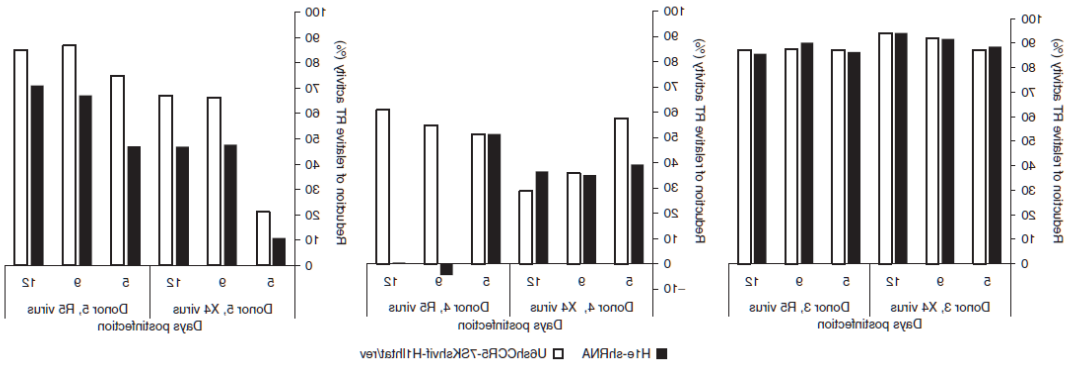

Figure 16. **Inhibition of HIV-1 replication in human primary CD4$^+$ T lymphocytes transduced with the combinatorial vectors.** CD4$^+$ T cells transduced with either the empty vector, the H1e-shRNA or the U6shCCR5-7SKshvif-H1lhtat/rev vector were challenged with the CXCR4 coreceptor-using HIV-1 HXBc2 Vpr$^+$/Vpu$^+$/Nef$^-$ or the CCR5 coreceptor-using HIV-1 NL4-3-ADA at a M.O.I. of 0.1 $TCID_{50}$/cell and the cell culture supernatants were collected at the indicated time points post-infection and processed by RT assay. The indicated percentages represent the reduction of the relative RT activity calculated with respect to the value obtained for the empty vector-EGFP-positive selected-transduced cells (*Spanevello et al., 2016*).

During the course of 12 days of infection, inhibition of HIV-1 replication in $CD4^+$ T cells transduced with the triple cassette expressing vector was higher than in cells transduced with the e-shRNA expressing vector. Despite of the donor variability, no significant differences in terms of inhibition were observed between the CXCR4 and CCR5 coreceptor-using HIV-1 strains, allowing to conclude that the main contribution in term of antiviral activity in these challenge experiments was likely due to the silencing of viral transcripts targeted by the *tat/rev*-specific siRNAs, regardless the tropism.

In this context, we have to take into account few considerations. Later during infection, HIV-1 can use alternative CXC chemokine receptor 4 (CXCR4) to enter into target cells, which is associated with rapid progression to AIDS. X4-variants of HIV-1 can re-emerge if the R5 viral suppression is incomplete and/or the reservoir size of HIV-1 has not been suppressed to a critical level. While CCR5 can be deleted without major problems for the cells, because it is not required for the normal development and function of T-lymphocytes, CXCR4 receptor is less favored as target, because it plays fundamental physiological roles especially for the maturation of hematopoietic stem cells; thus, its disruption must be targeted only in mature T cells. Furthermore, genes that inhibit prior to integration of the provirus are therapeutically most effective, since only these genes are expected to lead to an accumulation of non-infected, gene-protected cells (*von Laer et al., 2006*). Post-integration inhibitory genes will confer a selective advantage to cells harboring a suppressed provirus, leading to expansion of a latent virus reservoir. Accordingly, work done by von Laer's laboratory, has clearly demonstrated that entry of both CXCR4 and CCR5 using viruses can be efficiently blocked by expressing, on the target cell surface, fusion inhibitors which are small membrane-anchored peptides derived from the C-terminal heptad repeat of the HIV-1 Envelope glycoprotein (gp41). C peptides interact with the trimeric coiled coil structure formed by the gp41 N-terminal heptad repeat and thereby lock gp41 in a fusion incompetent state. Blocking viral entry through the knock down of CCR5 by siRNA, or through fusion inhibitors such as C-peptides, has the advantage of leading to the accumulation of uninfected gene-protected cells, thus preventing the continued replenishment of viral reservoirs.

7.2 Development of recombinant lentiviral vectors expressing the specific shRNAs along with the maC46 peptide

To further increase the overall protection against a broad range of viral isolates, the two vectors reported above were optimized by the insertion of the maC46 fusion inhibitor encoding sequence either alone (maC46) or fused in frame with the enhanced Green Fluorescence Protein (maC46eGFP) under the transcriptional control of the human Polymerase II Elongation Factor 1 (EF1) promoter, which has been shown to confer high level of transgene expression in human hematopoietic progenitors as well as in differentiated blood lineages after transduction with lentiviral vectors (*Salmon et al., 2000*). Moreover, in order to discriminate the contribute of the peptide in comparison with the genes silencing by RNAi in the inhibition of viral replication, control vectors either lacking the maC46 encoding sequence or characterized by a scrambled sequence in place of the siRNA encoding ones were also generated. In addition, the new recombinant vectors included an optimized version of the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE*), able to reduce the risk of oncogenicity while ensuring high level of transgene expression (*Schambach et al., 2006*). A schematic diagram of the developed vectors is reported in **Figure 17**.

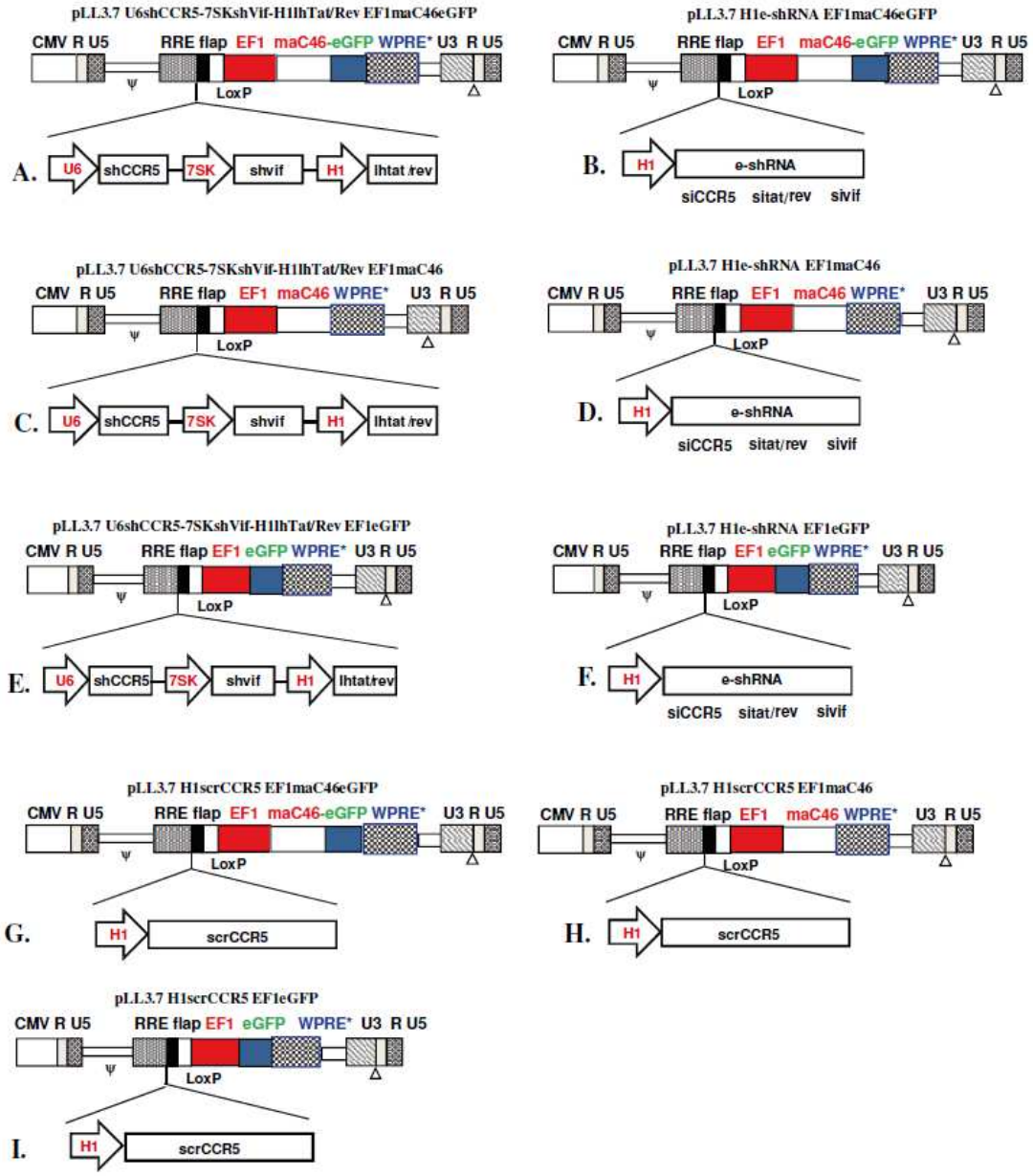

Figure 17. **Schematic diagram of the optimized plasmid vectors.** The new SIN-lentiviral platform is based on the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev or the pLL3.7 H1eshRNA vector, previously developed (*Spanevello et al., 2016*). The maC46 fusion inhibitor was inserted either fused with eGFP reporter gene (**A** and **B**) or alone (**C** and **D**), under the transcriptional control of the EF1 promoter in place of the CMVeGFP cassette (Fig.16). Furthermore, control vectors either lacking the peptide and expressing the triple or the e-shRNA cassette along with the reporter gene driven by EF1 promoter (**E** and **F**), or characterized by a scrambled sequence in place of the siRNA encoding ones along with (**G-H)** or without **(I)** the peptide were generated. All vectors contain an optimized version of the Woodchuck post-transcriptional regulatory element (WPRE*).

7.3 Localization of the maC46 peptide on the cellular membrane

To verify the maC46 peptide expression on the cell membrane, HEK 293T cells were transfected with the developed vectors, fixed and analyzed by confocal microscopy (**Figure 18**). The results obtained demonstrated that the peptide was correctly localized at the plasma membrane, as expected, not only when expressed as a fusion protein (with eGFP reporter gene) (**EF1maC46eGPF**), but also when expressed alone (**EF1maC46)**. The vectors expressing only eGFP were used as controls (**EF1eGFP**).

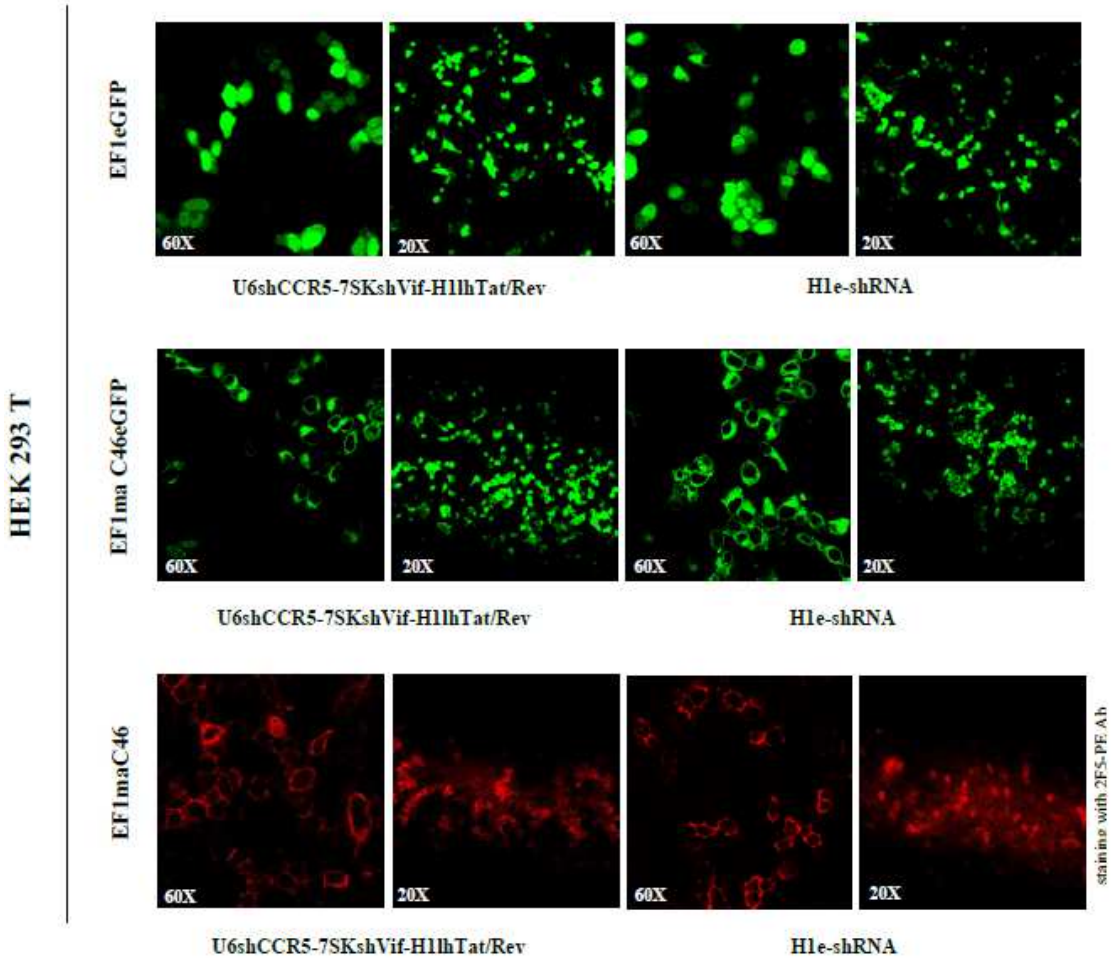

Figure 18. **Localization of the maC46 peptide on the plasma membrane of HEK 293T cells.** 3.5 x 10^5 HEK 293T cells were seeded in 6-well plates and, the next day, transfected by liposomes with the developed lentiviral vectors, expressing the maC46 peptide fused with eGFP or alone, along with the triple siRNA cassette or the H1e-shRNA. The vectors expressing only eGFP were used as controls, (EF1eGFP, upper panel). In the case of the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and of the pLL3.7 H1eshRNA EF1maC46 (lower panel), the localization of the maC46 peptide was evaluated by staining with the 2F5-PE antibody. Immunofluorescence analysis was performed by confocal microscopy with an immersion objective.

7.4 Production and titration of recombinant lentiviral particles

In order to obtain a suitable high titer for the following experiments, conditions of recombinant lentiviral particles (RLVPs) production were optimized, as reported in **Table 1**.

Table 1. Optimization of protocol for RLVP production

HEK 293T cells	**A.** 4 x 10^6	**B.** 2.5 x 10^6	**C.** 2.5 x 10^6	**D.** 12 x 10^6
Plasmid type (µg)				
PMDLg/pRRE	5	10	15	10
pRSV-REV	3	5	5	10
pMD2.VSVG	1.5	6	5	10
Transfer vector	15	20	15	20
Infectious Titer (TU/mL)	1.5 x 10^{6*} 5.4 x 10^{6**}	1.36 x 10^{6*}	2.81 x 10^{5*}	1.11 x 10^{7*} 2.1 x 10^{8**}

HEK 293T cells were transfected with lentiviral transfer vector pLL3.7, together with the packaging plasmids at a different ratio as reported above, using the $Ca_3(PO_4)_2$ method. Titers were determined prior and after stock concentration, based on the percentage of eGFP positive cells and expressed as transducing units/mL (TU/mL). The values are averages from three independent experiments performed in triplicate (protocol A. and D.) and two independent experiments performed in duplicate (protocol B. and C.). *non-concentrated stock, ** concentrated stock.

The infectious titer of the RLVP stocks, was determined by flow cytometry in HEK 293T cells, transduced with serial diluitions of lentiviral supernatants and expressed in TU/mL, considering reliable only the percentages ranging from 1 to 20 (*Salmon and Trono, 2007*). A titer ranging from 2.81 x 10^5 to 1.11 x 10^7 TU/mL for the non-concentrated lentiviral stock and from 5.4 x 10^6 to 2.1 x 10^8 TU/mL for the concentrated one was achieved. Thus, all recombinant lentiviral particles were produced and appropriately concentrated according to the selected conditions and reported in the materials and methods session. In three independent experiments, a suitable high titer was obtained for all developed recombinant lentiviral particles, ranging from 1.69 x 10^8 to 3.01 x 10^8 TU/mL (**Table 2**). Furthermore, since two vectors (i.e. H1e-shRNA EF1maC46 and U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46) do not encode the eGFP, in order to provide a valid and comparable indication regarding the presence of

recombinant lentiviral particles in all samples, the Reverse Transcriptase (RT) activity was measured in the lentiviral lysates; equivalent RT units were obtained for all stocks, ranging from 3.03 x 10^5 to 4.98 x 10^5 cpm/25 µL (**Table 3**), to be employed in the next challenge experiments.

Table 2. Transduction efficiency of RLVPs[a]

Infectious Titer (TU/mL)	MEAN ± SD
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP	1.54 x 10^8 ± 0.30
H1e-shRNA EF1maC46eGFP	2.10 x 10^8 ± 0.53
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	1.96 x 10^8 ± 0.39
H1e-shRNA EF1eGFP	2.89 x 10^8 ± 1.50
H1scrCCR5 EF1eGFP	3.01 x 10^8 ± 0.89
H1scrCCR5 CMVeGFP	1.69 x 10^8 ± 0.54

[a] HEK 293T cells were transduced with the lentiviral vectors shown above as described in the materials and methods session. The titers were obtained with FACS analysis and expressed as the number of eGFP-positive cells transduced with 1 mL of viral supernatant. The values are averages from three independent experiments performed in duplicate.

Table 3. Results of the reverse transcriptase (RT) activity assay[a]

Equivalent RT unit	(cpm/25 µL)
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP	3.03 x 10^5
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46	4.22 x 10^5
H1e-shRNA EF1maC46eGFP	4.08 x 10^5
H1e-shRNA EF1maC46	4.50 x 10^5
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	4.26 x 10^5
H1e-shRNA EF1eGFP	4.98 x 10^5
H1scrCCR5 EF1eGFP	4.84 x 10^5
H1scrCCR5 CMVeGFP	4.25 x 10^5

[a] Viral particles were precipitated from cell-free culture supernatants and underwent to RT assay as described in the materials and methods session. The amount of recorded RT activity was measured using a scintillator and expressed in count per minutes (cpm/25 µL). Each experiment was performed in duplicate and the shown data represent the average values.

Next, $CD4^+$ T lymphoblastoid Jurkat cells were subjected to different conditions of transduction; FACS analysis was carried out three days post-transduction and the percentage of eGFP positive cells were determined, for vectors carrying the reporter gene, by flow cytometry. Even taking into account the transduction variability and the different amount of equivalent RT units employed, the developed lentiviral particles were clearly able to efficiently transduce Jurkat cells, obtaining a percentage of eGFP positive cells, ranging from 91% to 99% and 49% to 73%, when equivalent RT units (700000 and 35000 cpm) of lentiviral particles were respectively employed, as reported in the **Table 4**.

Table 4. % eGFP positive Jurkat T-cells transduced with RLVPs[a]

	Equivalent RT unit (cpm/25 µL)	
RLVPs	**700000**	**35000**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	91.66 ± 10.12	-
H1e-shRNA EF1eGFP	91.33 ± 8.62	-
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP	98.67 ± 0.58	72.88 ± 12.17
H1e-shRNA EF1maC46eGFP	99.33 ± 0.58	48.9 ± 10.30
H1scrCCR5 CMVeGFP	94 ± 5	-
H1scrCCR5 EF1eGFP	-	70.98 ± 9.79

[a] Jurkat T-cells were transduced with the lentiviral vectors shown above as described in the materials and methods session. The titers were obtained with FACS analysis and expressed as the number of eGFP-positive cells transduced with equivalent RT units (700000; 35000 cpm) of viral supernatant. The values are averages from three independent experiments performed in duplicate.

7.5 Efficiency of recombinant lentiviral particles to express the transgenes

To determine maC46 cell surface expression for vectors lacking the reporter gene (i.e. pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; pLL3.7 H1e-shRNA EF1maC46) HEK 293T transduced cells were stained with the monoclonal antibody directed against HIV-1 gp41, conjugated to PE, and analyzed both by FACS analysis (**Figure 19**).

A titer of 3×10^5 and 3.2×10^4 TU/mL was obtained for cells transduced, respectively, with the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and pLL3.7 H1e-shRNA EF1maC46 lentiviral stocks. The lower titer observed for these two vectors, compared

to ones carrying the peptide fused in frame with the eGFP, might be due to the different experimental settings, rather than to a lower production of the relative lentiviral particles or to a lower expression of the peptide.

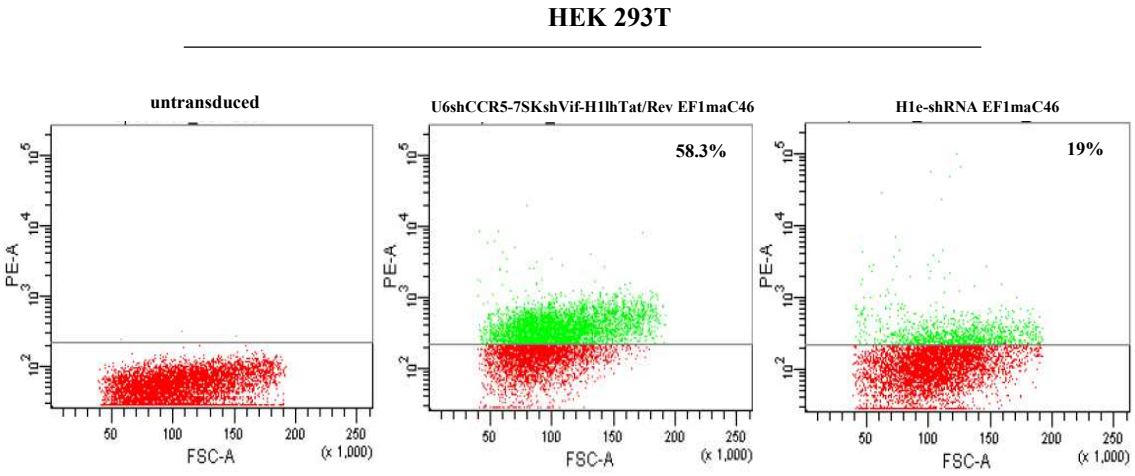

Figure 19. **Expression of the maC46 peptide in transduced HEK 293T cells.** Cell-surface expression of the maC46 peptide was detected by staining with 2F5-PE antibody of 2 x 10^5 HEK 293T cells transduced with recombinant lentiviral vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and H1e-shRNA EF1maC46) and assessed by flow cytometry. The percentage of cells considered maC46-PE^+ cells was referred to 10000 events corresponding to living cells.

To ascertain the correct localization of the peptide on cellular membrane, T lymphoblastoid Jurkat cells were initially transduced with small-scale preparations of the reported two vectors (i.e. pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; pLL3.7 H1e-shRNA EF1maC46) and the expression of the fusion inhibitor was evaluated by means of cytospin centrifugation and immunofluorescence analysis (**Figure 20**). A clear fluorescently stained signal was evident on the cellular membrane, after staining of cells with the antibody.

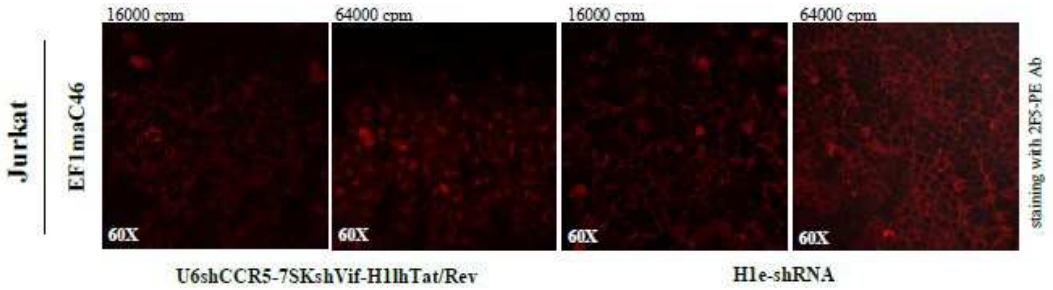

Figure 20. **Localization of the maC46 peptide on the plasma membrane of transduced T lymphoblastoid Jurkat cells.** 1 x 10^6 Jurkat cells were transduced with equivalent RT units of the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/RevEF1maC46 and the pLL3.7 H1eshRNA EF1maC46 lentiviral supernatants (16000 and 64000 cpm) and, after three days of culture, 1 x 10^5 cells were spotted on glass slides by cytospin centrifugation and the localization of the maC46 peptide was evaluated by staining with the 2F5-PE antibody. Immunofluorescence analysis was performed by confocal microscopy with an immersion objective.

In parallel, the transduction efficiency of Jurkat cells transduced with 64000 cpm of both the previously developed vectors (*Spanevello et al., 2016*) and the relative new optimized ones (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP; H1e-shRNA EF1eGFP), was evaluated by flow cytometry, in order to compare the expression of the reporter gene driven by either the CMV or the EF1 promoter. As shown in **Figure 21**, a shift of fluorescence signal towards the higher intensity region (right side) of the plot was observable for vectors carrying the EF1 promoter in comparison to the one obtained with those carrying the CMV promoter.

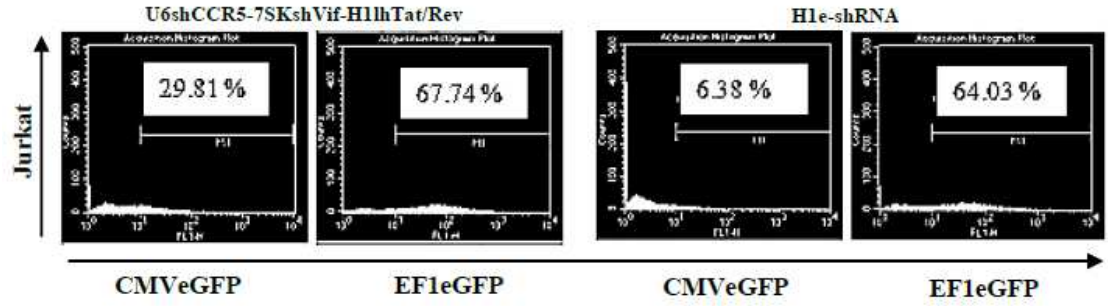

Figure 21. **EF1 versus CMV eGFP-driven expression.** $CD4^+$ T lymphoblastoid Jurkat cells were transduced with equivalent RT units (64000 cpm) of the indicated and selected recombinant vectors. The results were displayed as number of events (*counts*, vertical axis) *versus* eGFP fluorescent signal value (forward side scatter or *FL1*, horizontal axis). The Marker (M) indicated the population of $eGFP^+$ cells, among all analyzed events corresponding to living cells (which value was set to 10000).

7.6 Antiviral activity of siRNA/maC46 combinatorial vectors

With the purpose to discriminate the ability of the developed vectors in suppressing viral entry and replication, transduced human $CD4^+$ T lymphoblastoid Jurkat cells were challenged with the CXCR4-coreceptor using laboratory-adapted HXBc2 $Vpr^+/Vpu^+/Nef^+$ strain of HIV-1 at different M.O.I. Cell-free culture supernatants were collected at the indicated time points up to 25 days post infection and assayed for RT activity.

To this end, high equivalent RT units (700000 cpm) were employed in order to firstly evaluate the influence of lentiviral transduction on cell viability. Comparable activities in untransduced and transduced cells were observed by MTT cell viability assay, performed 72 hours post - transduction (**Figure 22**).

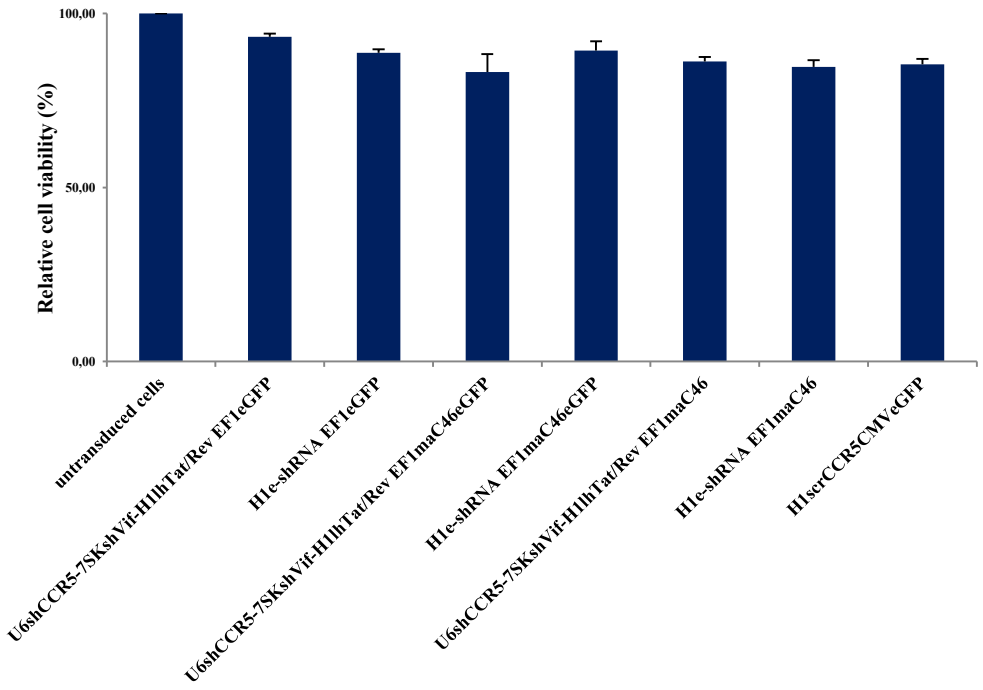

Figure 22. **Analysis of combinatorial vector-related cytotoxicity.** Three days after transduction with equivalent RT units (700000 cpm) of recombinant lentiviral particles, $5x10^4$ transduced Jurkat cells were plated in 96-well plate and subjected to MTT assay. Cell viability was calculated by measuring the absorbance at 620 nm and the value obtained for control cells (untransduced cells) was set to 100%. Relative cell viability for other samples was calculated accordingly. All error bars indicate ± s.d.

Thus, Jurkat cells transduced with the developed lentiviral vectors (expressing the triple cassette or e-shRNA sequence along with the maC46 sequence) and the corresponding control vectors (lacking the peptide) were infected with the HIV-1 R4-tropic molecular strain using a M.O.I. of 0.02 and 0.1 $TCID_{50}$ per cell (**Figure 23**). The results obtained in these both experiments clearly showed that the developed vectors, carrying the combination of multiple siRNAs coding sequence along with the fusion inhibitor, potently blocked viral replication, compared to the ones lacking the peptide. In particular, three vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP; U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; H1e-shRNA EF1maC46eGFP) conferred major protection, up to 25 days post-infection, both in the challenge performed with a M.O.I. of 0.02 $TCID_{50}$/cell (**Figure 23 A**) and in the one performed with a M.O.I. of 0.1 $TCID_{50}$/cell (**Figure 23 B**), while the control vectors displayed antiviral activity up to 7 or 4 days post infection (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP) and 11 or 7 days post infection (i.e. H1e-shRNA EF1eGFP) in the respectively challenges. By contrast, one of the new developed vector (i.e. H1e-shRNA EF1maC46) did not seem to be so potent in inhibiting viral replication in these challenge experiments. Indeed, in the first challenge (M.O.I. = 0.02 $TCID_{50}$/cell), the virus started growing in transduced cells 18 days post infection; while, using nearly 10-fold higher inputs (M.O.I. = 0.1 $TCID_{50}$/cell), the protection was conferred up to 7 days post infection. Several factors may explain the variability of these results, including the potential different levels of transduction efficiency and the level of the peptide expression, not being able to accurately detect the efficiency of transduction of constructs lacking the reporter gene.

Overall, taking into account its relevant role, only vectors carrying the peptide along with the multiple siRNAs were selected for the following challenge experiments (**Figure 24**), in order to identify, this time, the best combination. Thus, Jurkat cells were transduced with a much lower amount of lentiviral particles (35000 cpm), to obtain a single integrated LV per cell, to avoid the shRNA overexpression and saturation of the RNAi machinery. Furthermore, the negative control for the expression of the peptide (i.e. H1scrCCR5 EF1eGFP) has been selected as control, in place of the scrambled vector carrying the CMV promoter, employed in the previously experiments, in order to include a valid control and evaluate unspecific effects. When Jurkat cells were infected with 0.01 $TCID_{50}$/cell of HIV-1 R4-tropic molecular strain, two vectors (i.e.

U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP; U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46) were able to protect up to 25 days post infection (**Figure 24 A**); on the other hand, in less favorable conditions, at an higher M.O.I., a more pronounced antiviral effect was observed for the vector carrying the triple cassette sequence along with the peptide fused to eGFP (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP), probably due to the greater peptide stability when expressed as a fusion protein along with the reporter gene (**Figure 24 B**).

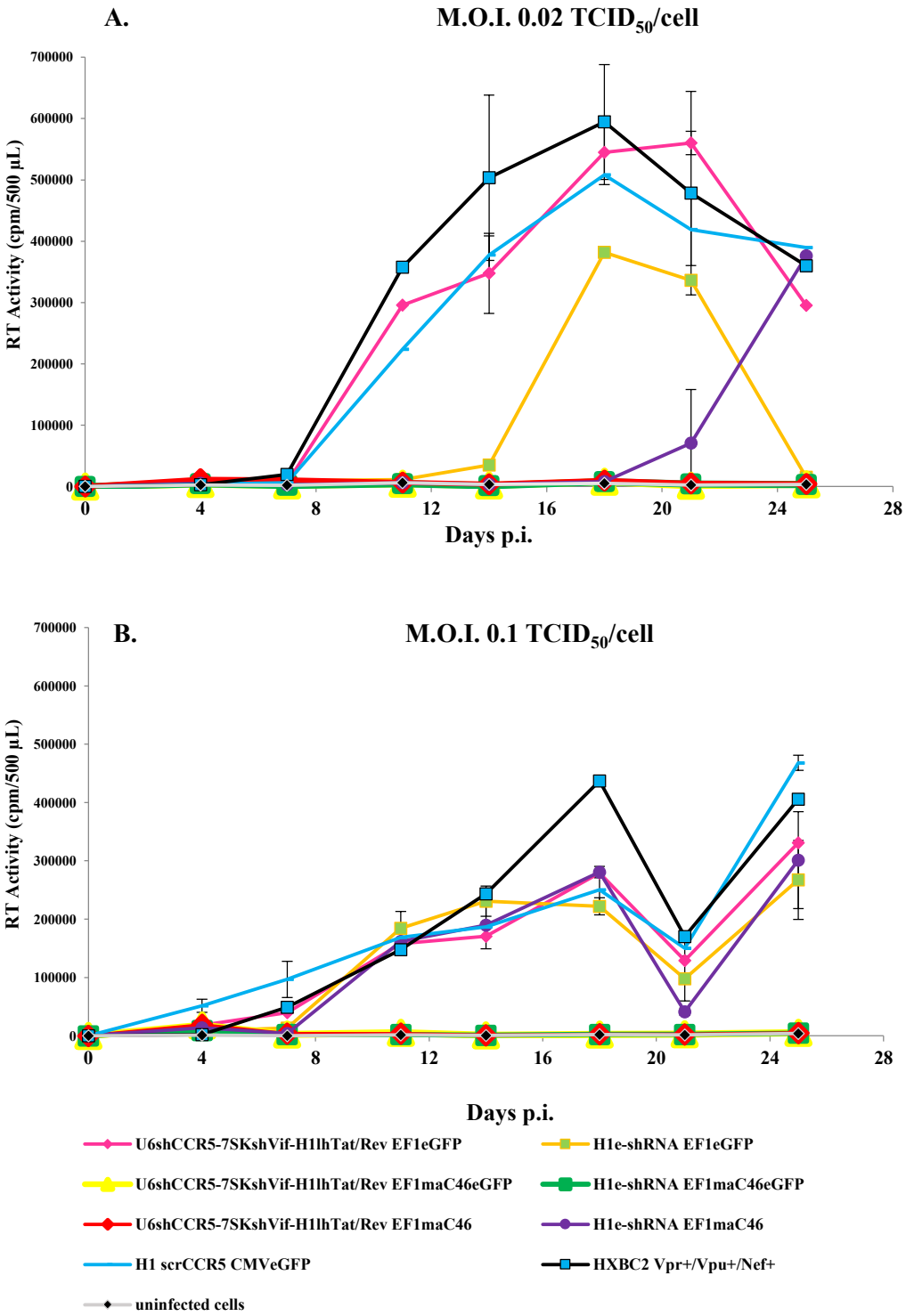

Figure 23. **Inhibition of HIV-1 replication by the maC46 fusion inhibitor and multiple siRNA.** Untransduced Jurkat cells and Jurkat cells transduced with equivalent RT units (700000 cpm) of vectors expressing the triple cassette or e-shRNA along with the maC46 peptide alone or fused in frame with eGFP and control vectors (either lacking the maC46 encoding sequence or characterized by a scrambled sequence in place of the siRNA encoding ones, i.e. H1scrCCR5 CMVeGFP) were infected with the HIV-1 HXBc2 $Vpr^{+}/Vpu^{+}/Nef^{+}$ R4-tropic molecular clone at M.O.I. of 0.02 (**A**) and 0.1 (**B**) $TCID_{50}$/cell. Viral replication was assessed by measuring the RT activity in cell free culture supernatants harvested on day 4, 7, 11, 14, 18, 21, 25 post infection (p.i.). Each experiment was performed in duplicate for each sample and the average value was calculated. All error bars indicate ± s.d.

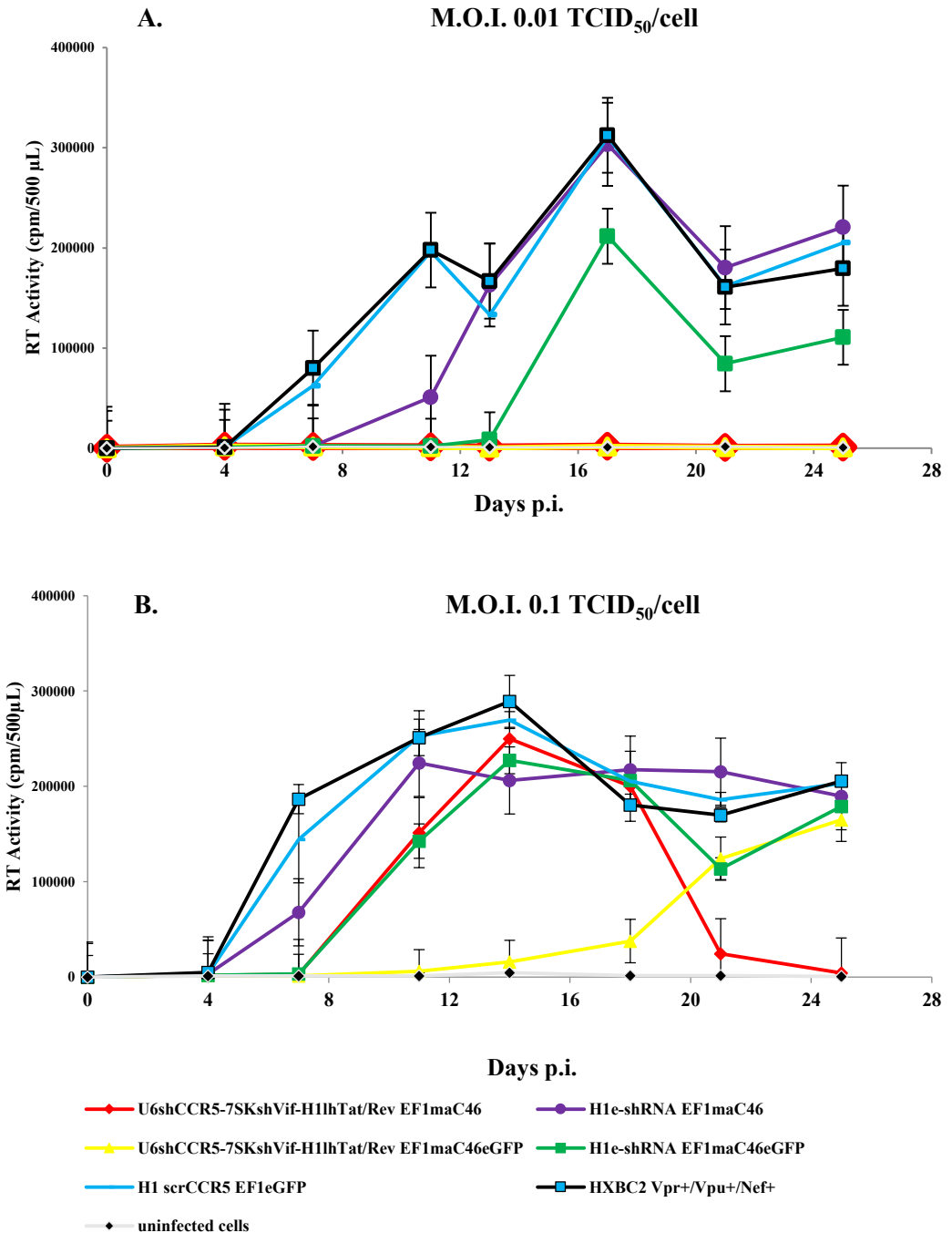

Figure 24. **Inhibition of HIV-1 replication by the maC46 fusion inhibitor and multiple siRNA.** Untransduced Jurkat cells and Jurkat cells transduced with equivalent RT units (35000 cpm) of vectors expressing the triple cassette or e-shRNA along with the maC46 peptide alone or fused in frame with eGFP and a control vector (H1 scrCCR5 EF1eGFP) were infected with the HIV-1 HXBc2 $Vpr^{+}/Vpu^{+}/Nef^{+}$ R4-tropic molecular clone at M.O.I. of 0.01 (**A**) and 0.1 (**B**) $TCID_{50}$/cell. Viral replication was assessed by measuring the RT activity in cell free culture supernatants harvested on day 4, 7, 11, 14, 18, 21,25 post infection (p.i.). Each experiment was performed in duplicate for each sample and the average value was calculated. All error bars indicate ± s.d.

7.7 Analysis of the genotoxicity potential of the developed combinatorial lentiviral platforms

Considering random vector insertion, the possible activation of cellular proto-oncogenes as a result of clonal transformation is a potential limitation in a therapeutic approach involving integrating gene transfer vectors. In comparison to gammaretroviral vectors (GV), with strong viral promoter/enhancer sequences ("LTR-driven"), employed in the early days of gene therapy, lentiviral vectors present a potentially safer insertion profile, with their preferred integration in transcribed genes than GV, which prefer instead integration next to transcriptional start sites and regulatory gene regions (*Zychlinski et al., 2008*). Thus, we set up a collaboration with the Baum's group at the Department of Experimental Hematology of the Hannover Medical School, in order to carry on experiments to test the mutagenic potential of our vectors. In particular, the vectors lacking the eGFP have been selected (U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and H1e-shRNA EF1maC46 referred to as U6-EFS-C46 and H1-EFS-C46 respectively), along with the H1*scr*CCR5 CMVeGFP referred to as H1-scrambled vector) to be tested in *in vitro* immortalization assay. Test vectors were compared with the pRSF91.GFPgPRE positive control vector referred to as RSF91, with a documented potential to induce *in vitro* immortalization by insertional mutagenesis. To this end, a large amount of selected lentiviral supernatants were produced and titrated by anti-p24 immunoassay (H1-EFS-C46 and U6-EFS-C46 105.7 µg/mL and H1scrambled 115.16 µg/mL), and also by flow cytometry in the vector carrying eGFP reporter gene ($1.8x10^8$ TU/mL). Thus, to evaluate the mutagenic potential by retroviral gene transfer in a relevant target cell type, murine Lin^- bone marrow cells were harvested with a purity of greater than 90% from steady state hematopoiesis of C57Bl6 mice and treated with retroviral particles, as described under Materials and Methods. Two rounds of transduction were performed on days -1 and 0 at a defined M.O.I.; the mean vector copy number (VCN) was measured on day 4; this is the first time point when proliferation reduces the number of episomal elements enough to yield reproducible mean copy numbers and early enough to exclude potential plasmid contaminations (resulting from the use of supernatants produced by transient transfection of packaging cells), influencing the overall VCN and transduction efficiency. The transgene expression (where applicable, for the vector containing a reporter gene) was determined on day 4 and 15 as reported in the **Table 5**.

Table 5. Vector copy number and transgene expression in transduced Lin- cells.

IVIM ID	Sample ID	Vector	c.MOI*	VCN **	% eGFP	MFI***	% eGFP
160803	160803-1	**Mock**	-	0.00	-	-	-
160803	160803-2	**Mock**	-	0.00	-	-	-
160928	160928-1	**Mock**	-	0.00	-	-	-
160928	160928-2	**Mock**	-	0.00	-	-	-
160928	160928-3	**Mock**	-	0.00	-	-	-
160803	160803-3	**RSF91**	60	7.74	98.00%	6096	99.90%
160803	160803-4	**RSF91**	60	9.01	97.40%	6285	93.00%
160928	160928-4	**RSF91**	60	8.37	98.70%	6423	99.50%
160928	160928-5	**RSF91**	60	7.69	97.80%	6152	97.50%
160928	160928-6	**RSF91**	60	8.09	98.60%	6392	99.10%
160803	160803-14	**H1 scrambled**	140	4.18	6.69%	154	32.80%
160803	160803-15	**H1 scrambled**	140	4.63	6.87%	137	24.00%
160928	160928-10	**H1 scrambled**	140	5.48	19.80%	145	41.10%
160928	160928-11	**H1 scrambled**	140	7.85	23.00%	170	35.80%
160928	160928-12	**H1 scrambled**	140	5.59	21.60%	172	49.30%
160803	160803-18	**H1-EFS-C46**	140	15.48	-	-	-
160803	160803-19	**H1-EFS-C46**	140	23.89	-	-	-
160928	160928-16	**H1-EFS-C46**	140	25.57	-	-	-
160928	160928-17	**H1-EFS-C46**	140	24.24	-	-	-
160928	160928-18	**H1-EFS-C46**	140	20.25	-	-	-
160803	160803-16	**U6-EFS-C46**	140	19.27	-	-	-
160803	160803-17	**U6-EFS-C46**	140	13.81	-	-	-
160928	160928-13	**U6-EFS-C46**	140	13.15	-	-	-
160928	160928-14	**U6-EFS-C46**	140	10.45	-	-	-
160928	160928-15	**U6-EFS-C46**	140	11.56	-	-	-

Mock: control sample for which no retroviral transduction was performed
* Cumulative MOI (2 rounds of transduction were performed on day -1 and day 0)
** **Vector Copy Number** (for the assay to be valid, the mean VCN on day 4 must be above 1)
*** **GFP Mean Fluorescent Intensity** (The transgene expression was determined on day 4 and 15,where applicable)
The IVIM assay and the VCN determination were performed under the standard operating procedure (SOP) version 151123 and 120724 - Taqman respectively.

Our vectors showed a good transduction efficiency, measured as VCN, up to 25 VCN/cell. According to this assay, when 1 x 10^5 lineage negative cells are transduced and the efficiency is above 80%, with samples having a VCN of at least 3, as determined on day 4, 90% of all assays are expected to trigger immortalized insertional mutants together with a significant correlation between an increase of the VCN and immortalization phenotype (IP). However, although the extremely high VCN levels obtained for test vectors, having used a M.O.I. higher than that used for the positive

control vector RSF91, no impact on cell proliferation and on cell viability was observed, as well as no vector supernatant associated toxicity was evident. Indeed, the potential unspecific toxic effects of vector preparations were determined by documenting the viability and growth rate of the cells during an early phase after transduction (day 1-6) and a later phase (day 8-15) (**Figure 25**). A reduced proliferation rate and viability could reveal cytotoxic effects of the supernatant. A meta-analysis (MA) of Mock samples for day 1-6 (n=45) was used to determine the expected proliferation rate during the early phase (mean = 1.87 ± 0.09 division per day).

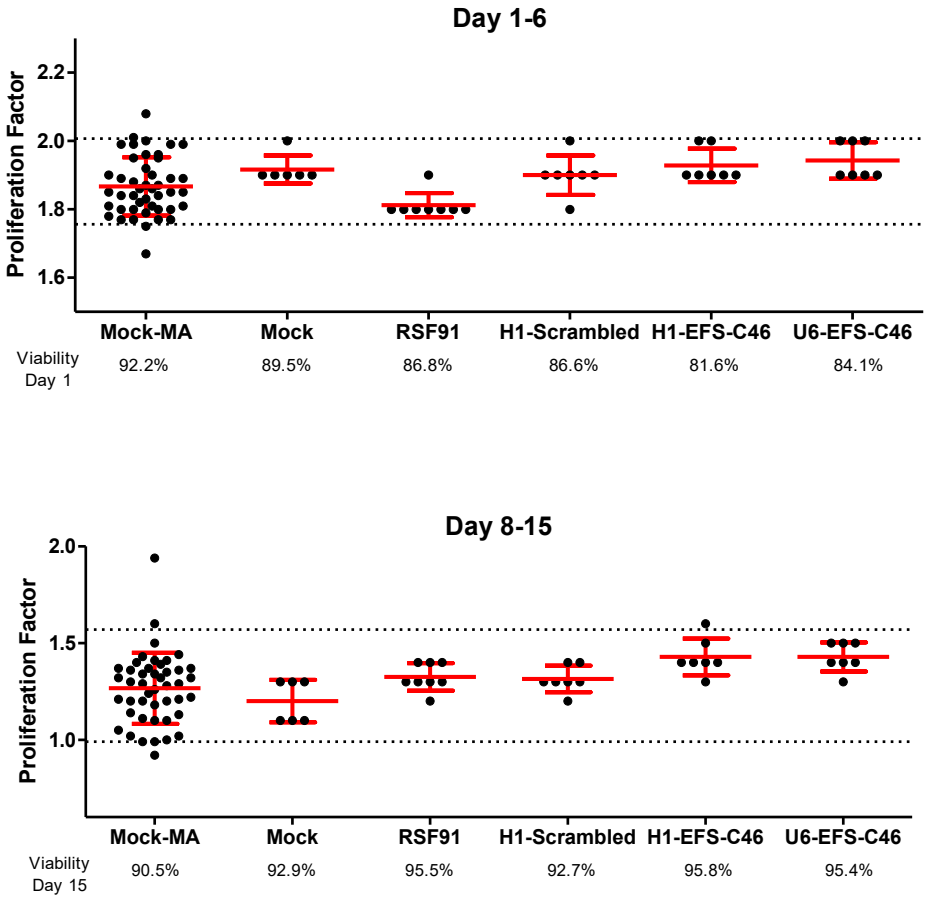

Figure 25. **Proliferation rate during first six days and during days 8-15 of the IVIM assay.** The Lin$^-$ cell number and viability were measured during the transduction procedure and the subsequent expansion phase (days 1, 4, 6, 8, 11, 13 and 15). The values from a meta-analysis (MA) of various Mock samples are shown on the left. The dotted lines indicate the 5% and 95% percentile of the MA-Mock samples and serve as an expectation range of normal proliferation. The read bars indicate means ± standard deviation. Mean viability on day 1 and 15 is shown below in percent. Test vectors are referred to as U6-EFS-C46, H1-EFS-C46 and H1scrambled; the positive control vector is referred to as RSF91.

One day after transduction, a slightly lower viability was observed for all transduced samples (statistically indistinguishable from Mock), which was recovered already on day 4. Overall, both RSF91 and the test vectors showed a normal proliferation rate within the expectation range, both in the early and the late phase of the assay.

Cells were then grown in cytokine-supplemented medium for another two weeks before being replated in 96-well plates. Under these conditions, mock-treated cells barely survived. With a constant number of target cells exposed to the gene transfer vectors, the incidence of cells with a transformed (replating) phenotype was determined (**Figure 26**).

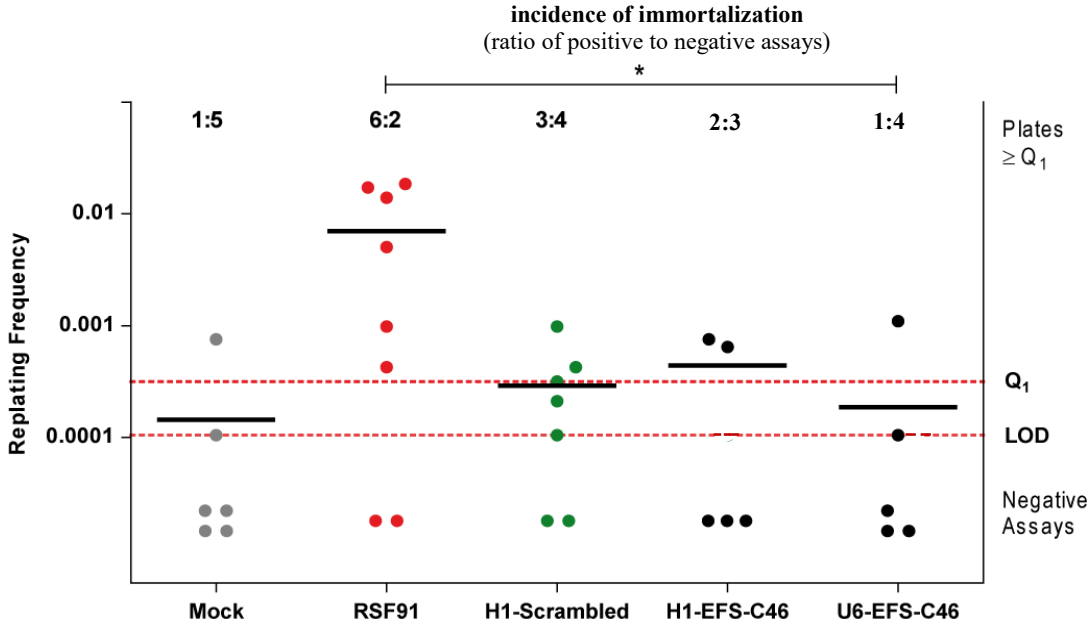

Figure 26. **Replating Frequency (RF) of Mock (grey), RSF91 (red) and test vector transduced samples (green dots for variants encoding eGFP, black without eGFP).** The ratio of replating frequency (determined by limiting diluition cloning) per vector copy number (detected by RT-PCR) is a measure of the degree of transformation. Ratios above the graph indicate the number of assays with cell growth above (left number) and below (right number) the Q1-MTT-threshold. Black bar for RSF91 indicates the mean replating frequency. The limit of detection (LOD) according to Poisson statistics (using 100 cells per well in a 96-well plate) is 1.05 x 10^{-4}, which corresponds to 1 positive well in the MTT-readout. All replating frequencies between the LOD and Q1 level cannot be distinguished from spontaneous cell growth. The filled circles for negative assays below the LOD were manually inserted into the graph. Every absorbance reading from the positive control or the test vectors above the MTT-threshold was counted as a positive well. The difference in incidence of positive to negative assays for RSF91 to U6-EFS-C46 was significant (Fisher's Exact; p = 0.0406).

In three independent IVIM assays, the transformation frequency per vector copy number in cells treated with our test vectors was strongly lower than that of the positive

control, in particular for the test vector U6-EFS-C46, suggesting a significantly reduced risk of insertional mutagenesis and genotoxicity.

8. DISCUSSION

HIV-1 still remains one of the most widespread infection which affects millions of people worldwide. After the introduction in 1996 of highly active antiretroviral therapy (HAART), the quality and life expectations of HIV-1 infected individuals have significantly improved. However, current treatment fails to achieve a complete eradication of infection due to the persistence of integrated viral DNA in reservoirs. This "cryptic" virus replication forces to a constant therapeutic regimen, often associated with several side effects, which creates difficulties to fully adhere to treatment and consequently a viral load rapidly rebounds after interruption, together with the onset of drug resistant viral variants. To overcome these limitations, current researches are mainly focused on alternative therapeutic strategies for a life-long remission of HIV-1/AIDS. Based on clinical and experimental evidence **(***Lopalco 2010; Allers and Schneider, 2015; Wang and Cannon, 2016***)**, approaches aimed at rendering the entire cell repertoire susceptible to HIV-1 infection and or replication resistant to the virus might represent a successful option. This result might be achieved by genetically modifying hematopoietic stem cells (HSCs), that are going to give rise to all cells involved in viral replication (including $CD4^+$ T and myeloid cells); once modified, these cells could be transplanted back safely and efficiently into HIV-1 infected patients, who should be now protected against de novo infection (*Kiem et al., 2012; Pernet et al., 2016*).

In this context, our research group has recently developed combinatorial platforms, based on self-inactivating (SIN) lentiviral vectors, expressing multiple small interfering RNAs (siRNAs), targeting both cellular and viral transcripts involved in HIV-1 replication, with the final aim of genetically modifying HSCs (*Spanevello et al. 2016*). SIN lentiviral vectors provide an efficient and safe gene delivery system for multiple anti HIV-1 genes, particularly when expressed as shRNA from the integrated vector into host cell genome, in order to induce long term stable gene silencing in both dividing and non-dividing cells, including HSCs (*Anderson et al., 2007; DiGiusto et al., 2010; Ringpis et al., 2012; Wolstein et al., 2014*). Furthermore, it has been demonstrated that blocking early steps of HIV-1 life-cycle, in particular viral entry into target cells, represents the most active strategy for interfering with viral infection (*Digigu and Doms, 2014*). On the other side, the combination of molecules targeting different

cellular/viral factors essential for viral replication/pathogenesis of infection, mimicking the HAART approach, ensures a better protection against emergence of viral strains resistant to therapy (*Li et al., 2005*). Thus, as mentioned above, in our vectors, we combined in a single construct siRNAs targeting different steps of HIV-1 life cycle (entry, transcription, nuclear export of viral RNAs, and production of infectious particles). In particular, *CCR5*, *tat*, *rev*, and *vif* were selected as target transcripts (S*panevello et al., 2016*). Two of the developed vectors, namely one containing multiple pol-III promoter (H1, U6 and 7SK)/shRNA cassettes within the same vector (pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev) and one expressing a single sequence (e-shRNA) that, once introduced into the cells, gives rise to more than one siRNA (pLL3.7 H1e-shRNA), were proved to efficiently block HIV-1 replication in human primary CD4 positive cells (*Spanevello et al., 2016*). Even though the achieved results were promising, this strategy suffers of an issue common to all genetic/pharmacological approaches targeting CCR5. Indeed, some HIV-1 isolates, as well as laboratory adapted strains, use the chemokine receptor CXCR4 as main coreceptor for entry into target cells. While these viral isolates are relevant to HIV-1 infection pathogenesis *in vivo*, pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev and pLL3.7 H1e-shRNA vectors cannot interfere with the entry of X4-tropic viruses into cells.

Thus, the first aim of my PhD project has been to further improve the two selected vectors by conferring them the capacity to inhibit cell infection by CXCR4 using HIV-1 strains/isolates. CXCR4 has been considered a potential target for anti HIV-1 gene therapy. Both RNAi and genome editing technologies have been successfully applied to also knockdown/out CXCR4 in cell line and in primary T cells (*Anderson et al., 2003; Yuan et al., 2012; Hou et al., 2015*). Moreover, recent "knock in" application of CRISPR/Cas9 system, based on the possibility of integrate anti HIV-1 genes into a defined locus, has been applied to disrupt CXCR4 gene locus in human T cells (*Schumann et al., 2015*). However, if the inhibition of CCR5 expression does not cause apparent adverse effects in the immune system, the knockdown/out of CXCR4 could compromise the physiological HSCs growth, migration and differentiation into mature functional cells, since its expression is necessary for HSCs migration and quiescence (*Tu et al., 2016; Lapidot et al., 2001*). Therefore, CXCR4 can be used as target of anti HIV-1 gene therapy only in the mature $CD4^+$ T cells against infection supported by X4-tropic viruses. Recent studies have investigated the possibilities to confer protection

against both R5-tropic and X4-tropic HIV-1 strains by potent fusion inhibitors, among which small synthetic peptides derived from the C-terminal heptad repeat of HIV-1 glycoprotein (gp) 41 (*Hildinger et al., 2001; Zhan et al., 2008*). In particular, a membrane anchored form of 46 amino acids (maC46), which is an elongated version of the FDA approved soluble peptide drug Enfuvirtide (T20, Fuzeon), when expressed on the surface of genetic modified cells, has been proven to be safe and confer a selective survival advantage over infected cells. MaC46 peptide interacts with the N-terminal hydrophobic α-helix of HIV-1 gp41, preventing the six-helix bundle formation of HIV-1 and subsequently the fusion to the host cellular membrane.

Thus, the maC46 coding sequence, either alone or fused in frame with the eGFP reporter gene, was introduced within pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev and pLL3.7 H1e-shRNA backbones in place of the CMV-eGFP cassette present in these vectors. Of note, in the new developed vectors, the transgene expression was driven by the human Polymerase II elongation factor 1 (EF1) promoter (EF1p). The reason why we selected EF1p resides on previous literature data showing that this promoter drives the expression of transgenes in lymphoblastoid cell lines as well as in HSCs more efficiently than other promoters, including the CMV one (*Salmon et al., 2000*; *Sumiyoshi et al., 2009; Norrman et al., 2010*). We were able to demonstrate that this is the case also in our experimental conditions. Indeed, when $CD4^+$ T lymphoblastoid Jurkat cells were transduced with the new developed vectors, carrying the triple cassette or the e-shRNA sequence along with the reporter gene, driven by the EF1 promoter (i.e. pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev EF1eGFP; pLL3.7 H1e-shRNA EF1eGFP), and with the starting vectors carrying the CMVeGFP cassette (i.e. pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev CMVeGFP; pLL3.7 H1e-shRNA CMVeGFP) we observed increased levels of eGFP gene expression, when driven by EF1p. In addition, the new developed vectors were improved in their safety profile thanks to the insertion of an optimized version of the Woodchuck hepatitis virus (WHV) post transcriptional regulatory element (PRE), into 3' UTR, lacking the X protein coding sequence and the X ORF promoter, which has been observed to contribute to the risk of tumor induction after lentiviral vector transduction of hepatocytes (*Themis et al., 2005*). In particular, we generated four new vectors, namely: pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46eGFP; pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46; pLL3.7 H1e-shRNA EF1maC46eGFP; pLL3.7 H1e-shRNA EF1maC46).

Since previous data indicate that the level of protection conferred by the maC46 peptide correlates with the amount of protein expressed on the cell surface, once developed the new vectors, a first analysis was conducted to ascertain the correct surface localization of the maC46 in both transfected HEK 293T and transduced $CD4^+$ T lymphoblastoid Jurkat cells. The obtained results demonstrated that the peptide was correctly processed into the cells and was localized at the plasma membrane, not only when expressed as eGFP fusion protein, but also when expressed *per se*. The expression of this fusion inhibitor was also evaluated by flow cytometry, in which HEK 293T cells were transduced with recombinant lentiviral particles and the expression of the synthetized protein was observed either by recording its own fluorescence, when fused with the reporter gene, or by means of a PE-conjugated antibody, respectively.

Once demonstrated that the developed vectors were able to drive the expression of the maC46 and that the peptide localization was correct, recombinant lentiviral particles (RLVPs) were produced and titrated. In particular, RLVPs were quantified by measuring the reverse transcriptase (RT) enzyme activity. Next, in order to evaluate the antiviral effects of the developed vectors, T lymphoblastoid Jurkat cells were transduced with different equivalent RT units of RLVPs. First of all we demonstrated that cells could be efficiently transduced, achieving a good percentage of eGFP positive cells (ranging from 91 to 99%), without any major cytotoxic effects. Then, Jurkat cells transduced with 700000 cpm of RLVPs, were infected with HIV-1 HXBc2 $Vpr^+/Vpu^+/Nef^+$ R4-tropic molecular clone (M.O.I. = 0.02 and 0.1 $TCID_{50}$/cell). The results obtained in this first challenge clearly demonstrated the strong contribution of the maC46 peptide in interfering with HIV-1 replication. Indeed, three of the optimized vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46eGFP; H1e-shRNA EF1maC46eGFP) potently blocked viral replication up to 25 days post infection, independently from the employed M.O.I. By contrast, one of the new developed vectors combining siRNAs with the maC46 (i.e. H1e-shRNA EF1maC46) inhibited viral replication up to18 days post infection, only when the lower M.O.I. was adopted; by contrast, at the M.O.I. of 0.1 $TCID_{50}$/cell, it conferred protection against HIV-1 replication only up to 7 days post infection. Several factors may this finding, including the potential different levels of transduction efficiency and the level of the peptide expression, that we are currently investigating.

In any case, this first challenge clearly indicated that at least three of the developed vectors displayed a very potent inhibitory effect toward HIV-1 replication. Thus, while performing new challenge experiments, as reported below, we also decided to test the genotoxic potential of the developed vectors. Indeed, if on one hand the integration of lentiviral vectors into host cell genome guarantees a stable expression of transgenes, on the other side the insertional transformation remains a concern, due to the possible alterations of proto-oncogenes or tumor-suppressor genes. Thus, in collaboration with the Baum's group at the Department of Experimental Hematology (Hannover Medical School), the genotoxic risk of our integrating gene transfer vectors was investigated, by adopting a cell culture assay (*Modlich et al., 2006*), that reflected the transforming potential of insertional mutagenesis in primary murine bone marrow cells (more prone to immortalization, since derived from genetically defined mouse strains that harbor transforming lesions). Despite the high vector copy numbers obtained for our tested vectors, no impact on cell proliferation and on cell viability was observed. Furthermore, no vector supernatant associated toxicity was evident, thus allowing us to conclude that our vectors do not have mutagenic potential on hematopoietic murine cells. In particular, in three independent experiments, the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 vector showed a significantly lower incidence of insertional mutants compared to the positive control of the experiment.

In parallel to these experiments, in order to further select the best performing vector/s to be employed in the next steps of the project, we decided to investigate the antiviral activity of the developed platforms combining siRNAs with the maC46 in less favorable experimental conditions. In particular, we decreased the input of transducing RLVPs from 700000 cpm to 35000 cpm. Our idea was that a lower amount of RLVPs not only could allow us to select the best combinatorial platform, among the developed ones, but should also contribute to the overall increased biosafety of the approach. Indeed, the identification of a minimal amounts of RLVPs, which ensures an acceptable transduction efficiency, while preventing potential side effects, is one of the goal when developing a gene therapy strategy (*Herrera-Carrillo and Berkhout, 2015*). This is particularly critical in the case of shRNA-based approaches since a large number of integrated lentiviral vectors per cell could lead to the saturation of the RNAi machinery (*Herrera-Carrillo and Berkhout, 2015*). Importantly, in our experimental conditions, we achieved a suitable transduction efficiency, ranging from nearly 50% to 73% eGFP

positive cells, also when 35000 cpm were employed. Finally, in this challenge experiment, a negative control vector characterized by a scrambled sequence in place of the siRNA encoding cassettes along with the EF1p-eGFP unit was performed. In agreement with the results obtained with the previous challenge experiment, even under less favorable conditions, vectors combining the triple cassette sequence along with the peptide, either alone or fused in frame with the reporter gene, conferred the best antiviral activity, in the challenge performed with the M.O.I of 0.01 $TCID_{50}$/cell. By contrast, at the higher M.O.I. (0.1 $TCID_{50}$/cell), an antiviral effect was observed only in the case of the vector carrying the peptide fused to the eGFP (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP). This finding, confirmed by the overall data constantly showing an highest antiviral activity when maC46-eGFP was expressed, suggests that eGFP fusion to the peptide confers to the latter an higher stability. This important aspect is currently under investigation. On the other hand, the data, once again supported by the results of the first challenge, indicate that the triple cassette is more efficient than the e-shRNA in conferring anti-HIV-1 protection to transduced Jurkat cells. It is important to note that, while our results clearly indicate that the maC46 has an important role in protecting the cells against HIV-1 replication (first challenge), at the moment, we cannot discriminate between the contribution of the two antiviral molecules (the three selected siRNAs on one side and the maC46 on the other) to the achieved level of protection against HIV-1 replication in transduced Jurkat cells. In order to address this point, we already developed two ad hoc control vectors (i.e. H1scrCCR5 EF1maC46eGFP; H1scrCCR5 EF1maC46, which are described in the Materials and Methods of this thesis) that we are currently testing in appropriate challenge experiments.

In conclusion, our data indicate that the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46eGFP and U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46, even though the latter to a less extend, represent the best performing vectors, at least under the experimental conditions we adopted here. Taking into account the protection conferred by these two vectors in the challenge experiments (up to 25 days post-infection at lower M.O.I.), combined with the lack of genotoxic potential, we can conclude that the developed combinatorial platforms represent a promising strategy to render HSCs, and, as a consequence, all the HIV-1 susceptible cell types, resistant to viral infection.

9. REFERENCES

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