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Development of single-cycle replicable human immunodeficiency virus 1 mutants

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Summary. – Non-infectious but antigenic human immunodeficiency virus 1 (HIV-1) particles are essential tool for the research on many topics associated with this virus. Here we report the construction of plasmid containing the HIV-1 genome mutated in the *pol* gene, which was co-transfected with plasmids expressing the *pol* gene products reverse transcriptase (RT) and integrase (IN), and the glycoprotein G of vesicular stomatitis virus (VSV-G). The virions produced in HEK 293 T cells were antigenic, but able to replicate only for one cycle, e.g. first generation single-cycle replicable (SCR) virions. The presence of VSV-G in the envelope of these virions had to ensure a wider spectrum of susceptible cell types for the replication of SCR. Replication of the first generation SCR virions in HEK 293T, MT-2, and mouse spleen cells was examined by p24-capture ELISA, syncytium formation assay, and electron microscopy (EM). HEK 293T and MT-2 cell lines showed a similar replication capacity, while primary cultures of mouse spleen cells were much less effective. The infection of MT-2 cells with the first generation of SCR virions yielded the second generation SCR virions, which were non-infectious. Summing up, the HIV-1 SCR virions represent the useful tool for HIV-1 research facilitating a better biological safety. Moreover, considering their antigenic composition and limited replication, SCR virions may be a promising candidate for the vaccine studies.

Keywords: human immunodeficiency virus 1; mutants; single-cycle replicable virions; AIDS vaccine

Introduction

HIV-1 that infects the human T lymphocytes is the etiologic agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al*., 1983). Worldwide 33 million people are infected with this virus and 5,700 people die each day (Anglaret, 2008). However, no promising vaccine or effective therapy against HIV-1 is available. HIV-1 infection is becoming a serious health problem in the developing as well as Middle East countries (Cheemeh *et al*., 2006). This infection is the subject of a huge number of studies looking for the suitable therapeutics or vaccines. However, HIV-1 virus propagated *in vitro* is needed for these studies, what involves major safety concerns regarding the infectivity of replication-competent viruses that restrict thoroughgoing research. In this frame, recombinant HIV-1 virions with the restricted replication seem to be a valuable tool that avoids the risk of infectious virus spread.

Several elements within the HIV-1 genome such as LTRs contain TATA box and binding sites for the transcription

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Abbreviations: EM = electron microscopy; HIV-1 = human immunodeficiency virus 1; IN = integrase; p.i. = post infection; RT = reverse transcriptase; SCR = single-cycle replicable; SIN = self-inactivating; SIV = simian immunodeficiency virus; VSV-G = vesicular stomatitis virus-glycoprotein G

factors. In addition, the *gag-pol* gene that encodes reverse transcriptase (RT) and integrase (IN) enzymes takes part in the virus replication (Freed, 2001).

HIV-1-based self-inactivating (SIN) lentiviral vectors have been already introduced as a model of such replication-deficient virions that contain a large deletion within the U3 region of their 3′LTR (Miyoshi *et al.*, 1998; MiyBayer *et al.*, 2008). This mutation is transferred to the 5′LTR during the reverse transcription, what leads to the transcriptional inactivation of LTR in the provirus (Miyoshi *et al.*, 1998; Mukherjee *et al.*, 2007). This mutation reduces the chance of producing the replication-competent virions and prevents the occurrence of recombination between vector and wild type HIV-1 within the infected cells. These SIN virions capable of efficient transduction of target cells with desirable genes are generally accepted as the safe lentiviral vectors for gene therapy experiments (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998; Mukherjee *et al.*, 2007;). However, a screening of drugs against HIV-1 requires the examination of a number of novel compounds with the potential replication inhibitory effect. Obviously, the replication-competent but safe HIV-1 virions are needed for this assessment.

SCR virions are defective in certain viral components that are required for the infectious particle assembly (Dudek and Knipe, 2006). These viruses undergo one complete cycle of replication that results in the production of a second generation virions. However, these viruses are non-infectious and unable to replicate and produce a third generation (Dudek and Knipe, 2006). Hence, SCR virions represent the replication-competent safe viruses. It is also noteworthy that these particles are capable of strong induction of the immune system and consequently, they may be considered as the good candidates for the vaccine development studies.

Loss of the infectivity of HIV-1 virions due to the introduction of mutations in either RT or IN genes has been already shown (Nakamura *et al.*, 1997; Cristofaro *et al.*, 2002; Rinke *et al.*, 2002; McBurney *et al.*, 2006). These viral particles contain the structural features of wild- type HIV-1, but their genome does not code for the functional RT or IN proteins. After the first replication they produce non-infective virions that are unable to integrate their genomes into the DNA of the cell. However, a major concern about the SCR virions is the occurrence of reverse mutations that may compensate for the viral non-functionality (Logan *et al.*, 2004).

In the present study, we report the design and construction of novel SCR HIV-1 virions by introducing either a frameshift mutation or a large deletion across the HIV-1 *pol* gene, encoding RT and IN enzymes. We further provide evidence for the reliability of these strategies to develop irreversible and safe SCR HIV-1 virions that may be used for the drug discovery and vaccine development.

Materials and Methods

Plasmid constructs. pNL4-3 plasmid containing the chimeric fragments of NY5 and LAV HIV-1 strains as a replication-competent full-length DNA was obtained from the AIDS Research and Reference Reagent Program (NIH, USA) (Adachi *et al.*, 1986). To knock out the RT gene, pNL4-3 was digested with *Age*I restriction enzyme (Fermentas). The sticky ends were filled using DNA polymerase Klenow fragment (Fermentas) and the created blunt ends were re-ligated in such a way that four nucleotides were inserted into the position of nt 3,485 what consequently disrupted the reading frame in the down stream

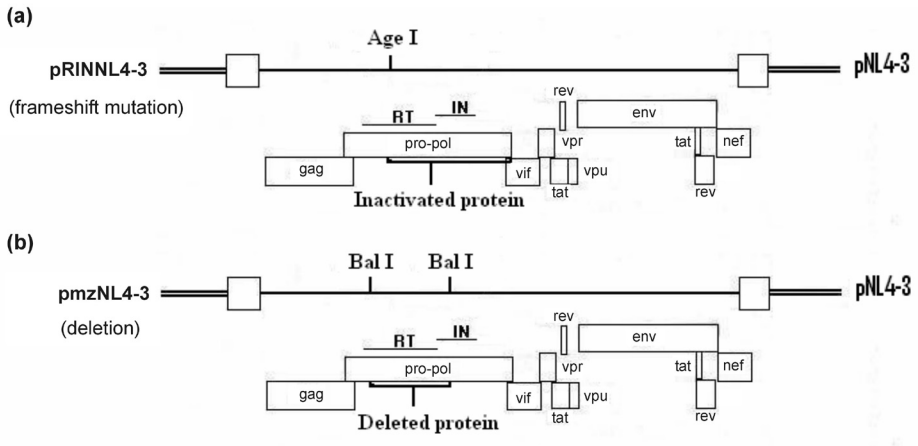

Fig. 1

Plasmids containing mutated HIV-1 genome

part of the gene. This strategy led to the construction of pRINNL4-3 plasmid (Fig. 1a). In another approach, a large part of RT-IN segment (1,931 nts) was removed from the pNL4-3 plasmid (Rezaei, 2007). Briefly, pNL4-3 was double-digested using *Bal*I restriction enzyme (Fermentas) at the positions of nt 2,620 and 4,551 and the sequence between the two cutting sites was removed. Re-ligation of digested pNL4-3 resulted in the construction of pmzNL4-3 plasmid (Fig. 1b). Possessing the packaging signal (ψ), both pRINNL4-3 and pmzNL4-3 plasmids could produce viral mRNA transcripts capable of being packaged into the viral particles.

The eukaryotic plasmids of psPAX2, pMD2.G, and pLOX-CWgfp were obtained from Addgene (www.addgene.org). psPAX2 plasmid encodes for the HIV-1 *gag* and *gag-pol* polyproteins, in addition to the viral accessory proteins, while lacks the packaging signal (ψ). pMD2.G plasmid encodes for the VSV-G and pLOX-CWgfp produces mRNA transcripts that are translated to GFP protein and carry the packaging signal as well.

Cells. The human embryonic kidney (HEK 293T) and MT-2 cell lines were obtained from the National Cell Bank of Iran. Single-cell suspension of mouse splenocytes was prepared in a cell homogenizer (Memarnejadian and Roohvand, 2010). Cells were cultured in either DMEM (293T) or RPMI 1640 (MT-2 and spleenocytes) media (Chemicon), supplemented with 15% FBS (Gibco), L-glutamine (2 mmol/l), penicillin G (100 U/ml), streptomycin (100 mg/ml), HEPES (25 mmol/l), and maintained in 5% CO_2 and 37°C. In the case of spleen cells, the medium contained 10 μg/ml of phytohemagglutinin A.

Production of the first generation SCR virions. To produce the first generation SCR virions, HEK 293T cells were separately co-transfected with different plasmid combinations using the PolyFect reagent (Qiagen). Briefly, 5 x 10^5 cells/well were plated in a 6-well plate and the next day a transfection mixture containing DNA plasmids (total amount of 2 μg) and PolyFect reagent (20 μl) was added to each well.

Combination of psPAX2 with pmzNL4-3 and pRINNL4-3 was considered to produce the first generation mzNL4-3 and RINNL4-3 SCR virions, respectively. To produce the VSV-G pseudotyped virions (referred to as G-mzNL4-3 and G-RINNL4-3) pMD2.G plasmid was also included to the mentioned mixtures. Finally, combination of pLOX-CWgfp with psPAX2 and pMD2.G was used to produce the GFP reporter control virions.

Virus stocks were prepared according to the previous protocol (Cavrois *et al.*, 2004; Svarovskaia *et al.*, 2004). In brief, culture supernatants of the transfected cells were harvested at 24, 48, and 72 hrs post transfection, clarified by 15 mins centrifugation at 10,000 x g, and further subjected to 120 mins centrifugation at 60,000 x g. The pelleted virions were resuspended in 1/20 volume of RPMI 1640 by overnight gentle mixing at 4°C, quantified by p24 ELISA assay kit (Cell Biolabs) and stored at -70°C until use.

Production of the second generation SCR virions. Virions produced by replication of the first generation SCR HIV-1 viruses (from virus stock) were named as the second generation SCR virions. To produce them, HEK 293T, MT-2 (6 x 10^4 cells) and mouse spleen cells (10^6 cells) grown on a 24-well plate were separately infected with 600 ng (HEK 293T and MT-2 cells) or 6 μg (mouse spleen cells) of p24 antigen in the volume of 240 μl from the first generation virus stock for 5 hrs. Cells were washed two times with 10% FBS medium and resuspended in 500 μl of fresh medium (Morgan *et al.*, 1995). Supernatants were then harvested at 24, 48, and 72 hrs post infection (p.i.) and assayed for p24 antigen level using the quantitative p24 ELISA assay kit (Cell Biolabs). Replication

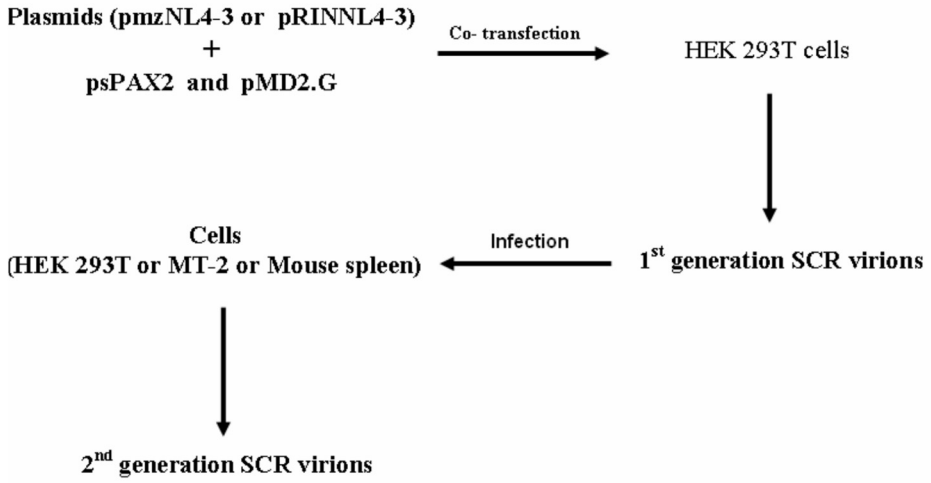

Fig. 2

Production of the SCR HIV-1 virions in cultured cells

potency of the second generation SCR virions in MT-2 cells was also evaluated using the same protocol.

Syncytium formation assay. MT-2 cells cultured in 24-well plates (130 x 10^3 cells/well) were infected with the first generation SCR virions equivalent to 5 μg of p24 antigen as mentioned above. The produced syncytia were observed in comparison with uninfected MT-2 cells and counted under a light microscope, as described before (Madani *et al.*, 2007; Wang *et al.*, 2008).

Electron microscopy. Negative staining of concentrated first generation SCR virions for electron microscopy was performed as described elsewhere (Biel and Gelderblom, 1999). Briefly, pioloform F-coated, carbon reinforced 400-mesh copper grids were floated on 30 μl drops of SCR virions suspended in RPMI 1640 for 10 mins. Subsequently, the grids were washed twice with double distilled water and then negatively stained with 2% aqueous phosphotungstic acid (pH 7.2) for 2 mins using the droplet technique and dried for 45 mins. The samples were analyzed with a Philips 3000 transmission electron microscope operating at 140 kV.

Results

Production of the first generation SCR virions

The goal of this study was to establish SCR HIV-1 virions as a safe tool for the drug discovery and vaccine studies. This was achieved by a deletion/disruption in RT or RT-IN genes within the HIV-1 full genome that led to the construction of pmzNL4-3 and pRINNL4-3 novel plasmids. Since the active RT and IN enzymes are required for the production of HIV-1 particles (Nakamura *et al.*, 1997; Rinke *et al.*, 2002), the first generation of SCR virions were recovered from HEK 293T cell line by its co-transfection with psPAX2 (encoding the intact RT-IN genes, but with the deletion of *env* gene), pMD2.G (encoding the VSV-G protein) and either of the mutant plasmids (Fig. 1). Of note, VSV-G protein provided the possibility of attachment of pseudotyped infectious virions to the various cell types (Yu *et al.*, 2009). Accordingly, G-mzNL4-3 and G-RINNL4-3 virions containing *gag* and *gag-pol* from psPAX2, RNA from either pmzNL4-3 or pRINNL4-3 and surface VSV-G from pMD2.G were recovered. Quantification of the relative production of the first generation SCR virions by HIV-1 p24 measurement in the culture supernatants of transfected cells indicated the highest production rate for G-mzNL4-3 virions (200 ng/ml) in comparison with mzNL4-3 (100 ng/ml) and GFP control virions (28 ng/ml). This result showed the capability of pmzNL4-3 plasmid to ensure the efficient production of the first generation HIV-1 particles in the presence of trans-acting psPAX2 and pDMG.2 plasmids.

Production of the second generation SCR virions

In the next step we analyzed infectivity of the first generation SCR virions in different cell lines. HEK 293T, MT-2, and mouse spleen cells were separately infected with the first generation SCR virions and the production of second generation SCR virions in culture supernatants was monitored by the measurement of p24 at different time-points p.i. In HEK 293T cells, SCR virions indicated a sharp increase in the production at 48 hrs p.i. and a decrease afterwards. In the MT-2 cells production of SCR virions had a slighter slope and reached the considerable level only after 96 hrs p.i. (Fig. 3a). Apart from the rate of virion production, no difference in the total amount of virus recovery was observed in HEK 293T and MT-2 cells.

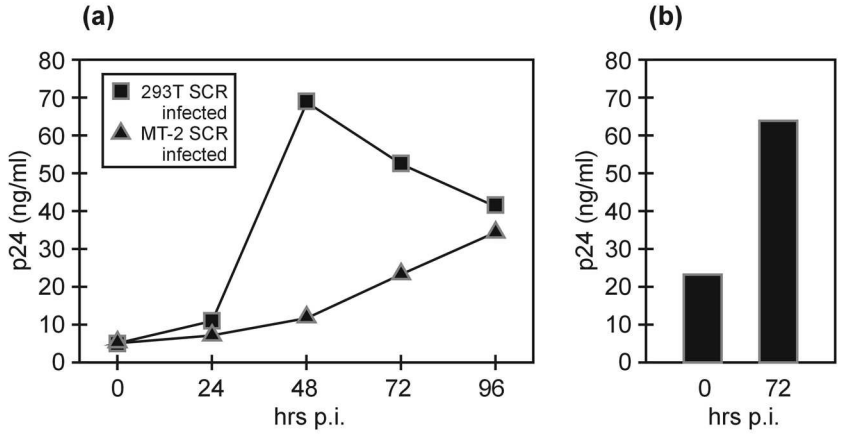

Fig. 3

Production of the second generation SCR HIV-1 virions in cultured cells

HEK 293T and MT-2 cells (a), mouse spleen cells (b).

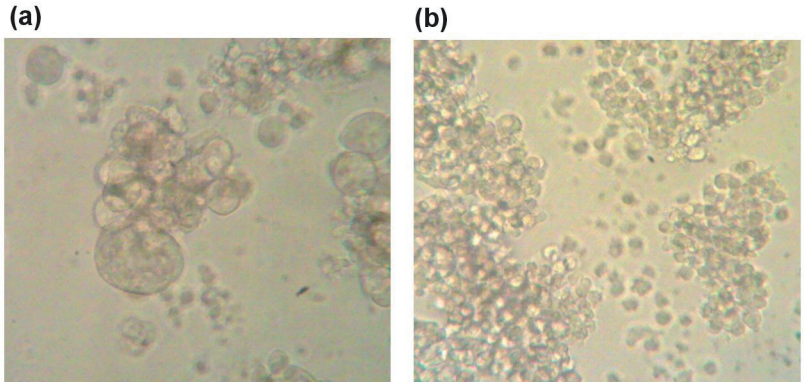

Fig. 4

Syncytium formation in MT-2 cells infected with the first generation SCR HIV-1 virions

Infected (a) and non-infected (b) cells.

As expected, the primary cell culture – mouse spleen cells – was not so efficient in the virion production by 72 hrs p.i., even when they were infected with the G-mzNL4-3 (Fig. 3b). In comparison with 293T and MT-2 cell lines, 10 times more first generation virions (6 μg) were required to produce similar particle amounts in splenocytes. Unexpectedly, we were not able to detect the p24 raise in the supernatant of MT-2 cells that were infected with the first generation mzNL4-3 virions, even after 20 days p.i.

In order to confirm the production of HIV-1 virions, we also monitored the formation of syncytia in MT-2 cells, which were able to produce the cytopathic effect (Madani *et al.*, 2007). MT-2 cells infected with the first generation G-mzNL4-3 virions began to form syncytia at 48 hrs p.i. After 5 days, the average number of formed syncytia (in 200x microscopic field) was 80 for the infected cells and around 0.5 for the non-infected cells (Fig. 4). This observation showed that G-mzNL4-3 virions preserved the intact and functional HIV-1 env protein.

Finally, the viral particles were directly observed by EM. The presence of rounded particles with heterogenic morphology and size of about 120–150 nm verified the production of second generation G-mzNL4-3 particles in the culture supernatant of infected MT-2 cells at 72 hrs p.i. (Fig. 5).

Non-infectivity of the second generation SCR virions

To evaluate replication potency of the second generation virions, MT-2 cells were infected with the second generation virions (equivalent to 600 ng of p24) and 72 hrs p.i. the supernatant was analyzed by EM and assayed for the load of p24 antigen. As expected, neither viral particles nor p24 antigen were detected in the supernatant. To verify these results, the load of p24 in culture supernatant was monitored at 20 days p.i. and similarly to the non-infected cells no positive signal was detected. To confirm this result, the experiment was repeated by the application of 10 times more inoculation dose (6 μg of p24) and the production of virions was

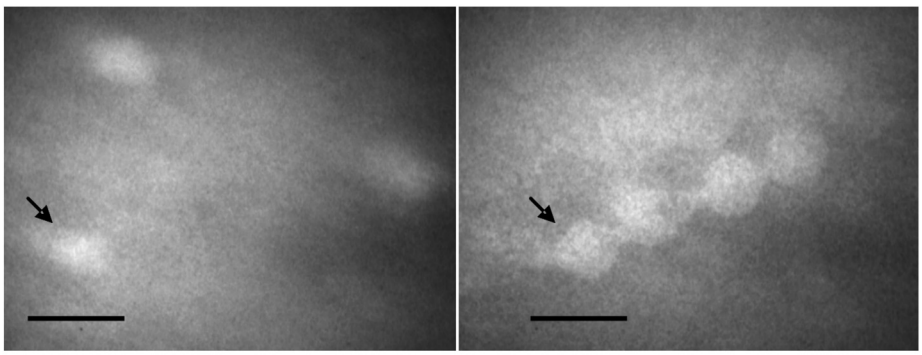

Fig. 5

Electron microscopy of MT-2 cells infected with the first generation SCR HIV-1 virions

72 hrs p.i., the arrows show viral particles, the bars indicate 200 nm.

assessed at different time points p.i. Similarly, the obtained results demonstrated the inability of the first generation of HIV-1 to produce virions (Fig. 6). Taken together, these results confirmed the conclusion that the second generation virions were replication-deficient and could not produce the viral particles.

Discussion

Several studies have focused on the development of self-inactivating or live attenuated viruses by deleting or truncating the genes required for the viral replication and spread (Baba *et al.*, 1995, 1999; Zufferey *et al.*, 1998; Alexander *et al.*, 2003) . Herein, we addressed this concern by the development of novel single-cycle replicable HIV-1 virions that were prepared by the partial deletion/disruption within the *pol* gene. These mutant virions maintained the native structure of HIV-1 particles and possessed the effective infection machinery that allowed them to completely replicate in the first cycle and produce the infective first generation viruses. However, their replication in the second cycle led to the production of non-infective HIV-1 virions that lack RT and IN enzymes.

Concept of SCR is similar to the SIN lentiviral vectors that have been designed for the safe lentiviral gene delivery (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998). SIN vectors contain a large deletion within their U3 sequence that inhibits their RNA to be packaged into the budding virions (Miyoshi *et al.*, 1998; Mukherjee *et al.*, 2007). However, the ability of one-cycle replication is in fact a major advantage of SCR HIV-1 virions that presents them as a valuable tool for the discovery of replication inhibitory compounds.

In addition to the gene delivery, SIN lentiviral vectors carrying HIV-1/SIV antigens have been successfully applied for the protective and therapeutic vaccination purposes (Buffa *et al.*, 2006; Negri *et al.*, 2007). These findings showed the potency of SIN lentiviral vectors for triggering the immune response against the antigens that are carried by the vector.

Here, we showed that the designed SCR HIV-1 virions could represent a better alternative for the use in vaccine studies. The reason may lie in the facts that these virions present the native forms of HIV-1 antigens to the immune system and additionally, their replication potency provides a more potent stimulation of the immune cells. These features put forward SCR HIV-1 virions not only as the potential HIV-1 vaccine candidates, but also as the general vaccine vehicle with the capacity to accommodate other heterologous antigens instead of the deleted parts. It is believed that the live replicating viruses have a higher capability for the induction of immune response (Baba *et al.*, 1995, 1999) and therefore, attenuated HIV-1 virions may be considered as the alternate vaccine candidates. However, these virions continue their replication in a restricted rate and still meet the safety concerns (Baba *et al.*, 1995, 1999; Alexander *et al.*, 2003). On the other hand, the replication of SCR HIV-1 virions is definitely blocked at the second generation and consequently, they seem to be completely safe.

It is noteworthy that SCR HIV-1 virions can be produced by almost any RT and/or IN-negative molecular clone. In this study, we aimed at the deletion of a large part of RT to reduce significantly the chance of compensating reverse mutations. As a result, the second generation SCR virions were completely replication-deficient even during a long period of time.

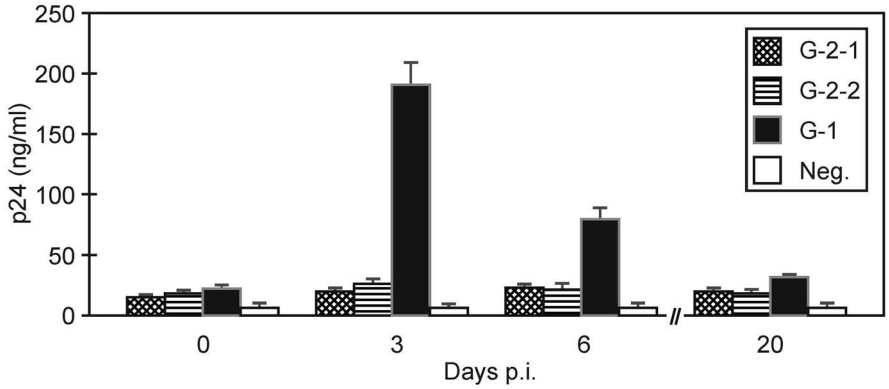

Fig. 6

Evaluation of infectivity of the second generation G-mzNL4-3 virions in cultured MT-2 cells

Cells were infected with either 600 ng (G-2-1) or 6 μg (G-2-2) of the second generation virions or 600 ng of the first generation virions (G-1). Production of the new virions was assessed by measurement of p24 in different time points p.i. Non-infected cells (Neg.) and the cells infected with G-1 served as negative and positive control, respectively.

Pseudotyping of the SCR HIV-1 with VSV-G was mainly designed to expand the host range of the G-mzNL4-3 virions and to facilitate further virological studies.

Taken together, in this study we generated replication-competent single-cycle HIV-1 virions and provided the additional data in respect to their non-infectivity after one replication cycle. These SCR virions are in the frame for successful HIV-1 vaccine studies and also in the safe screening of HIV-1 replication inhibitory compounds.

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