Article

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Establishment of Transgenic Mouse Leukemia Cell Lines Expressing Human CD4/CCR5/CyclinT1 Infected with HIV-1

Ya-jing Li[1], Juan Liang[2], Xin-yu Cheng[3], Li-min Zhao[1], Chang-chun Zeng[4,*]

[1]The Scientific Research Department, Shenzhen Longhua District Central Hospital, 518110 Shenzhen, Guangdong, China
[2]School of Science, Westlake University, 310030 Hangzhou, Zhejiang, China
[3]Child Rehabilitation Department, Shenzhen Longhua Maternity and Child Healthcare Hospital, 518110 Shenzhen, Guangdong, China
[4]The Scientific Laboratory Center, Shenzhen Longhua District Central Hospital, 518110 Shenzhen, Guangdong, China
*Correspondence: zengchch@glmc.edu.cn (Chang-chun Zeng)
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Purpose: Establishing a cross-species animal model of human immunodeficiency virus (HIV) infection is crucial for the study of HIV/acquired immunodeficiency syndrome (AIDS). However, due to the species-specific characteristics of HIV-1, the virus can only infect directly humans and a small number of non-human primates. It cannot directly infect mouse cells across species.
Methods: A mouse leukemia cell line with high CD4 (clusters of differentiation 4)/CCR5 (CC-chemokine receptor 5)/CyclinT1 expression was constructed in this study. First, CD4/CCR5/CyclinT1 lentiviral vector was used to infect a murine leukemia cell line L1210 to express the receptor CD4, co-receptor CCR5 and tat protein driving factor CyclinT1, which are required to infect L1210 cells with HIV-1.
Results: The results of sequencing identification and fluorescence expression showed that the plasmid expressing CD4, CCR5, and CyclinT1 vector was successfully constructed and wrapped as the lentiviral vector. Moreover, it was observed that CD4, CCR5, and CyclinT1 proteins were highly expressed in mouse leukemia cells L1210 compared to empty lentiviral vector-transfected cells. Next, viral entry and replication were demonstrated when HIV-1 RNA was detected in body cells and cultured supernatants. Transgenic mice cells L1210 showed significantly greater content of HIV-1 RNA compared to control L1210 cells. Finally, CEMx174 was infected with cell culture supernatants to clarify that the progeny virus is an active virus with infection ability. HIV-1 RNA was highly expressed in CEMx174 cells.
Conclusions: This study made the foundation for future studies evaluating HIV-1 cross-species infection in a murine animal model. The results provided new direction for studies investigating the development of vaccines, antiviral drugs screening, and HIV/AIDS pathogenesis.

Keywords: HIV-1/AIDS; cell model; mouse leukemia cell lines; lentivirus; CD4/CCR5/CyclinT1

Introduction

Human immunodeficiency virus (HIV) is a retrovirus that infects human immune system cells causing acquired immunodeficiency syndrome (AIDS). HIV-1 is the main pathogen related to AIDS global epidemic. Research on HIV/AIDS animal disease models is of great significance to understand the virus pathogenesis, drug efficacy, and vaccine preparation [1].

Currently, there are two types of HIV/AIDS animal models, non-human primate model (NHPs) and humanized rat model. NHPs model is usually made from non-human primates such as macaques, cynomolgus macaques, and pig-tailed macaque monkeys, infected with simian immunodeficiency virus (SIV) [2]. Currently, the NHPs model is very similar to that of humans in terms of organizational structure, immunity, physiology, and metabolism. However, NHPs model clinical manifestations among individuals are quite different from those of humans, besides that they are rare species with scarce resources and high prices. Only a few research institutions can meet the requirements to study HIV-1 infection using a NHPs model, what limits the study of HIV infection and virus replication.

HIV infection humanized rat model consists of transplanting human tissues that can be infected by HIV-1, such as fetal thymus, liver tissues, peripheral blood leukocytes, and hematopoietic stem cells, into mice with severe combined immunodeficiency (SCID), so that SCID mice can infect HIV-1. This model is also known as the chimeric mouse HIV-1 model. The preparation of these model may lead to interspecies rejection and can only be established in SCID mice, resulting in the lack of immune activity *in vivo*. Although there are studies trying to reconstruct the human immune system in SCID mice, the practical application remains a long way off.

HIV-1 does not infect mouse cells, but the murine leukemia virus (MuLV) can infect mouse cells. MuLV is similar to HIV-1 in structure and pathogenicity, both are retroviruses. MuLV infection clinical symptoms in mice are similar to those of human AIDS. Some studies have suggested that mice infected with MuLV can mimic HIV/AIDS onset to some extent [3–5]. Therefore, we hypothesize that if we can prepare a type of HIV-1 host cell based on mouse blood cells, such cells transplanted into the rat can avoid the rejection reaction. HIV-1 infected mice model with immune function can do rodent cells cross-species infection and animal models *in vivo*.

HIV-1 virus binding to host cell receptors is the first barrier to conduct an invasive infection. Previous studies illustrated that the HIV-1 envelope glycoprotein GP120 cannot bind to CD4 (clusters of differentiation 4) and CCR5 (CC-chemokine receptor 5) in mice, making HIV-1 unable to enter mouse cells [6]. Human CD4 molecule is the main receptor for HIV-1 to invade cells, and CCR5 is one of the main co-receptors. Researchers have reported that CyclinT1 is a key factor in HIV-1 transcription driven by Tat protein. Transgenic mice CD4/CCR5/CyclinT1 can detect HIV replication in lungs, small intestine, lymph nodes, and other tissues, and can perform HIV infection and viral replication [7,8].

Materials and Methods

Cells

HEK293T cell line and mouse leukemia cell line L1210 were obtained from the ATCC (American Type Culture Collection) library. Cells were cultured in RPMI1640 medium, including 10% fetal bovine serum, 100 U/mL streptomycins, and 100 μg/mL penicillin in a carbon dioxide incubator (37 °C, 5% CO_2). The cells were tested for mycoplasma by mycoplasma detection kit (rep-mys-10, InvivoGen, San Diego, CA, USA) before experiments.

Plasmid Constructs and Virus Production

Lentivirus plasmid pLV [Exp]-EGFP/Neo-CMV > CD4:T2A:hCCNT1:IRES:CCR5 including the genes CD4/CCR5/CyclinT1 (GenBank Accession respectively was No. NM_000616.4, NM_000579.3, and NM_001240.3) were generated using the Gateway technique.

Transfection with Lentiviral Vectors

Transfection started when the cells were in a good growth state, and the cell density was about 1×10^5~1×10^7/mL, then multiplicity of infection (MOI) virus optimal volume was added to the cell suspension. To improve the transfection rate, Polybrene with a final concentration of 5 μg/mL was added (TR-1003, Sigma Chemical, St. Louis, MO, USA) [9,10], and gently mixed. Then, the cellular virus mixture was placed in a carbon dioxide incubator. L1210 cells transfected with lentivirus vectors loaded with CD4/CCR5/CyclinT1 genes were collected for subsequent analysis.

Real-Time PCR

Total cell RNA was extracted using the Trizol reagent method (15596026, ThermoFisher, Carlsbad, CA, USA). RNA content was determined spectrophotometrically. Equal quantities for each factor were used for real-time PCR (RT-PCR). RT-PCR was performed in a SuperScript II System (2690A, Takara Biotechnology, Tokyo, Japan) in a 50 mL reaction volume using 10 pmol of the following specific primers: CD4 (upstream: 5′-TGCCTCAGTATGCTGGCTCT-3′; Downstream: 5′-GAGACCTTTGCCTCCTTGTTC-3′); CCR5 (upstream: 5′-GCTGGTCATCCTCATCCTGATAA-3′; Downstream: 5′-ATGGCCAGGTTGAGCAGGTA-3′); CyclinT1 (upstream: 5′-NNNNGTTACGTAATGGCTCGTCATGGAAAA-3′; Downstream: 5′-NNNNCGACGCGTAATTCTTTTCGACATGTA-3′). GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) (upstream: 5′-TTCACCACCATGGAGAAGGC-3′, downstream: 5′-GGCATGGACTGTGGTCATGA-3′) was used as control. RT-PCR was conducted using the Applied Biosystems (ABI) 7500 Realtime PCR system (ABI, San Diego, CA, USA). The amplification program included an initial denaturation at 95 °C for 5min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The melting curve was obtained with 1 cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. The relative mRNA (messenger ribonucleic acid) expression was determined by $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control. All primers were purchased from Sangon Biotech, Shanghai, China.

Western Blot Analysis

The CD4/CCR5/CyclinT1 transgenic mice were dissolved and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The proteins were transferred onto the nitrocellulose membrane or polyvinylidene fluoride. After blocking, the membrane was incubated overnight at 4 °C with 1:1000 diluted rabbit anti-human CD4 monoclonal antibody (ERP6855, Abcam, Fremont, CA, USA), CCR5 monoclonal antibody (EPR24589-95, Abcam, Fremont, CA, USA), or CyclinT1 monoclonal antibody (EPR17982, Abcam, Fremont, CA, USA). The membranes were incubated at room temperature for 1 h with 2 mL of goat antirabbit IgG (immunoglobulin G) (ZB-2301, ZSGB-BIO, Beijing, China) diluted at 1:5000. Bands showing protein expression were developed using the ECL (enhanced chemiluminescence) kit (PK10001, ProteinTech, Wuhan, China).

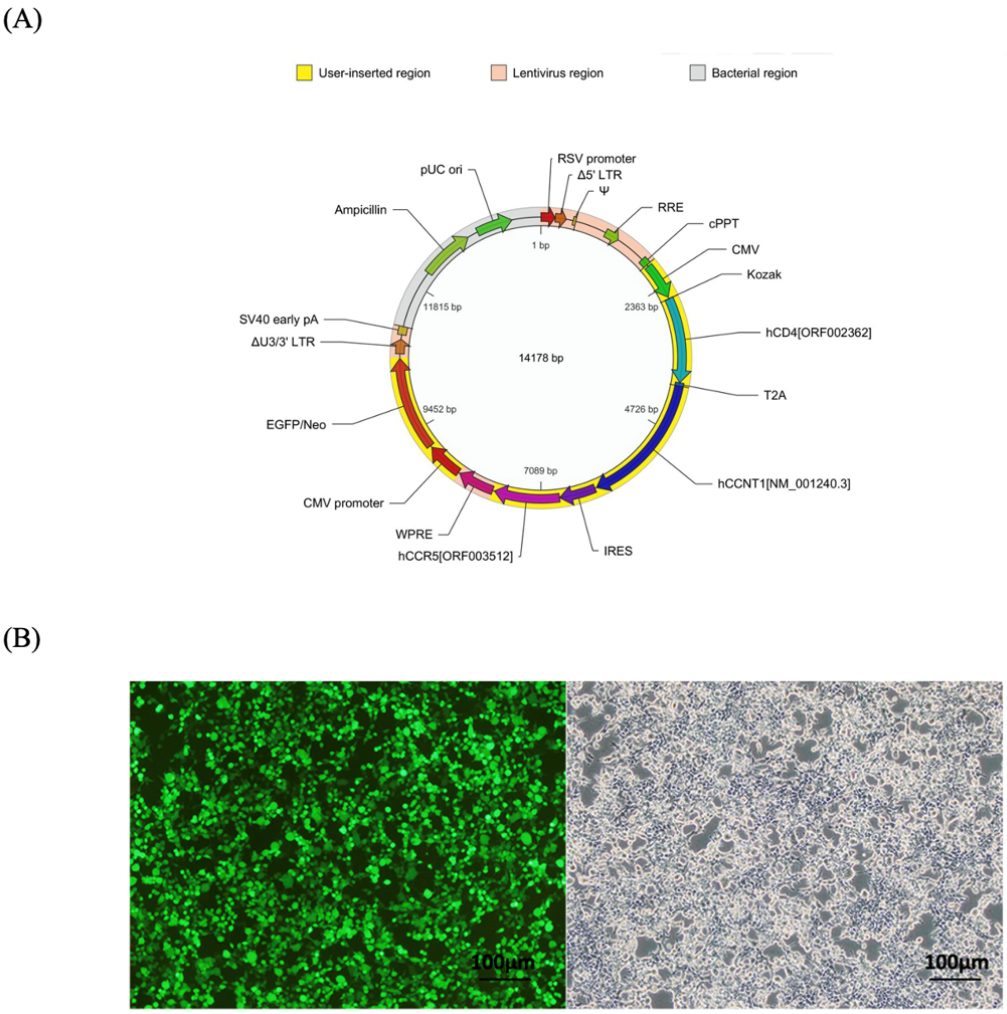

Fig. 1. Lentiviral vectors production. (A) Designed plasmid vectors schema. CMV (cytomegalovirus) is the promoter of CD4; T2A (self-cleaving 2A peptide) used to connect CD4 and CyclinT1; IRES (internal ribosomal entry site) is used to connect CyclinT1 and CCR5; EGFP/Neo is a resistance gene, Pgk is a type of Puro promoter. (B) Lentivirus gene expression vector fluorescence expression.

Infection of CD4/CCR5/CyclinT Transgenic L1210 Cells with HIV-1

Transgenic L1210 cells CD4/CCR5/CyclinT were inoculated overnight in a 25 cm^2 flask with a canted neck and then infected with HIV-1 virus. HIV-1 was subsequently removed after 4 h, and cells were normally cultured to determine their viability. L1210 supernatants and cells were extracted for HIV RNA analysis at 2-, 4-, 6-, 8-, and 10-days after transfection.

TaqMan Probe PCR for Supernatants HIV-1 RNA Analysis

Quantitative PCR was performed using a 25-microliter volume reaction system [11]. The HIV primer and probe sequences were: HIV probe, 5′-(FAM) CAGCATTATCAGARGGAGCCAC-CCCACA (TAMRA)-3′; HIV sense primer, 5′-STTTTARYCCAGAAGTAATACCCATGTT-3′; And HIV antisense primer, 5′-GCAGCYTCYTCATTGATGGT-3′. The reaction system included 0.2 μL of 250 nM probe, 0.25 μL of 250 nM sense primer, 0.25 μL of 250 nM antisense primer, 2 μL of the cDNA sample, 9.8 μL of deionized water, and 12.5 μL of quantitative PCR master mix. The reaction coil was hatched in an ABI7500 quantitative PCR thermocycling for amplification in the following conditions: 94 °C for 10 min, 40 cycles at 94 °C for 15 s, and 60 °C for 1 min.

SYBR Green PCR for Intracellular HIV-1 RNA Analysis

Total cell RNA was extracted from mouse leukemia cells and then reversed to cDNA according to manufacturers' instructions, RevertAid reverse transcriptase (EP0441, Thermo Scientific, Waltham, MA, USA). After being diluted ten times, the cDNA obtained from reverse transcribing was used as a template for RT-PCR. Empty transfected lentivirus cells were used as a negative

control. The amplification program included an initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The melting curve was obtained with 1 cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Relative mRNA expression was determined by the $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control. The primers were HIV sense primer, 5′-STTTTARYCCAGAAGTAATACCCATGTT-3′, HIV antisense primer, 5′-GCAGCYTCYTCATTGATGGT-3′. GAPDH sense primer, 5′-TTCACCACCATGGAGAAGGC-3′, GAPDH antisense primer, 5′-GGCATGGACTGTGGTCATGA-3′ and GADPH was used as an internal reference control.

Statistics

SPSS 15.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data are presented as mean ± standard deviation (SD). The differences in different groups were determined by one-way ANOVA (analysis of variance) test. p-values < 0.05 demonstrated statistical significance.

Results

Production of Lentiviral Vectors

Vectors loading the genes CD4/CCR5/CyclinT1 using the Gateway technique were generated (Fig. 1A) [12]. The constructed plasmid vector was analyzed by nucleotide sequencing. It was observed that CD4, CCR5, and CyclinT1 were correctly inserted into the plasmid vector. The expression of these three genes in 293 T cells was observed under a fluorescence microscope (Fig. 1B).

Transfection of L1210 Cells Using Lentiviral Vectors

To determine lentiviral transgene vectors transfection rate, we transfected L1210 mouse leukemia cells with MOIs of 1, 5, 10, 50, and 100 (Fig. 2). Five days after transfection, 70% of L1210 cells expressed the green fluorescent protein and were transfected at a MOI of 50 or 100. Then, L1210 cells were plated at 1×10^5 cells per well in a six-well cell culture cluster. Lentiviral vectors containing CD4/CCR5/CyclinT1 were transfected at a MOI of 50. The control was an empty lentiviral vector. L1210 cells were extracted for HIV RNA analysis after transfection.

To confirm the expression of CD4, CCR5, and CyclinT1 genes at mRNA level in L1210 cells, cell line RNA was extracted for reverse transcription (Fig. 3). CD4, CCR5, and CyclinT1 mRNA expression was detected by qRT-PCR at 2-, 4-, 6-, 8-, and 10-days after transduction, with empty lentivirus cells (control) as negative control. If was found that CD4, CCR5, and CyclinT1 cell membrane mRNA levels were expressed on day two and increased with time. L1210 transfected cells showed significantly higher CD4, CCR5, and CyclinT1 mRNA expression levels on day six ($p < 0.001$) and decreased on day

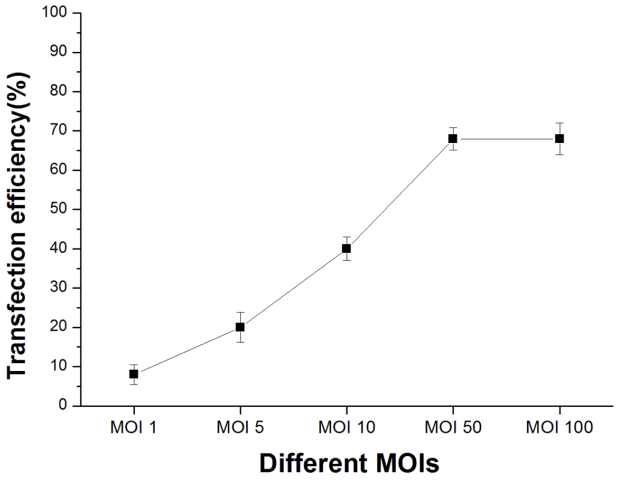

Fig. 2. Transfection efficiency at different MOIs transfection. L1210 cells were transfected with different lentiviruses MOIs. The transfection efficiency was determined by a fluorescence microscope.

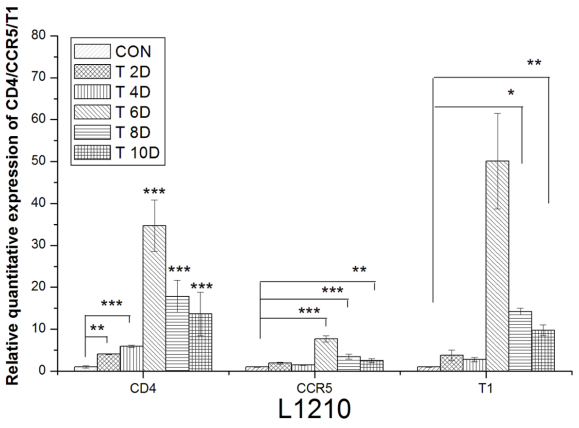

Fig. 3. CD4, CCR5, and CyclinT1 levels in transgenic cells qRT-PCR analysis. L1210 cell was transfected with different lentivirus vectors containing CD4/CCR5/CyclinT1. Control represents the cell transfected with empty lentivirus. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

eight compared to those of the control group. Thus, CD4, CCR5, and CyclinT1 genes were successfully transfected into cells and their biological function was maintained.

To further verify CD4, CCR5, and CyclinT1 expression, CD4, CCR5, and CyclinT1 protein analysis was performed in mouse leukemia cells using Western blots [13] (Fig. 4). Western blot data demonstrated that lentiviral vectors containing CD4/CCR5/CyclinT1 were stably integrated and expressed in L1210 cells and CD4, CCR5, and CyclinT1 protein expression in transfected L1210 cells first increased and then decreased, compared to β-actin stable expression.

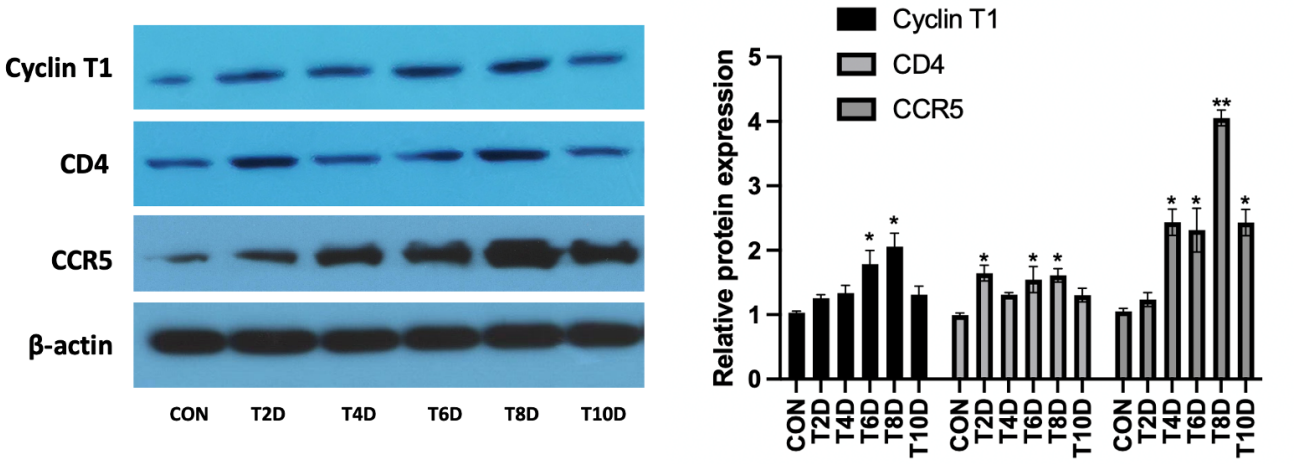

Fig. 4. CD4, CCR5, and CyclinT1 protein expression in transgenic L1210 cells. L1210 cells transfected with lentivirus containing CD4/CCR5/CyclinT1 expressed CD4, CCR5, and CyclinT1. $^{*}p < 0.05$ and $^{**}p < 0.01$.

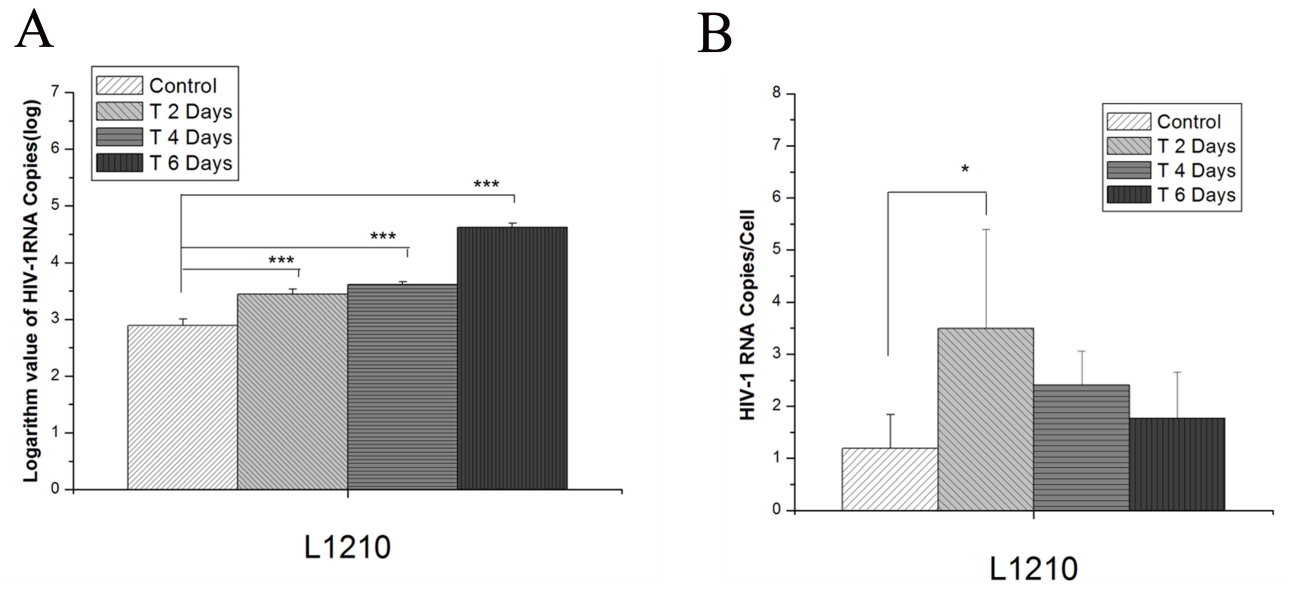

Fig. 5. HIV-1 RNA levels in transgenic L1210 cells. L1210 cells transfected with the lentivirus vector containing CD4/CCR5/CyclinT1 expressed at high levels of HIV-1 RNA copies (A) and HIV-1 RNA copies/cell (B) than the empty lentivirus transfection group. $^{*}p < 0.05$ and $^{***}p < 0.001$.

Transgenic L1210 Cells Alow Efficient HIV-1 Entry and Replication

To confirm that the CD4/CCR5/CyclinT1 transgenic L1210 cells can support efficiently HIV-1 infection and replication, supernatants were collected at 2-, 4-, and 6-days after HIV-1 infection for quantitative real-time PCR analysis (Fig. 5A). HIV-RNA expression in L1210 were observed in the supernatant on the fourth day after HIV-1 infection and reached the maximum HIV-RNA load ($p < 0.001$) after the eighth day compared to the control group. The results illustrated that HIV-1 could effectively enter, integrate and replicate in CD4/CCR5/CyclinT1 L1210 cells.

L1210 cells were infected with HIV-1 after lentivirus transfection (Fig. 5B). HIV-RNA expression was observed cells on the second day after HIV-1 infection, but there was no HIV-1 RNA expression on the 6th day. No HIV-1 RNA expression was observed in the control group infected with empty lentivirus. The results showed that L1210 cells transfected with lentivirus were infected by HIV-1 and then they replicated HIV-1 virus.

To confirm HIV-1 virus activity, CEMx174 cells were co-cultured with virus supernatants collected from the CD4/CCR5/CyclinT1 transgenic L1210. CEMx174 cells infected with normal HIV-1 were considered positive control, and HIV-1 from the transgenic L1210-infected empty lentiviral vectors were considered negative control. Cell

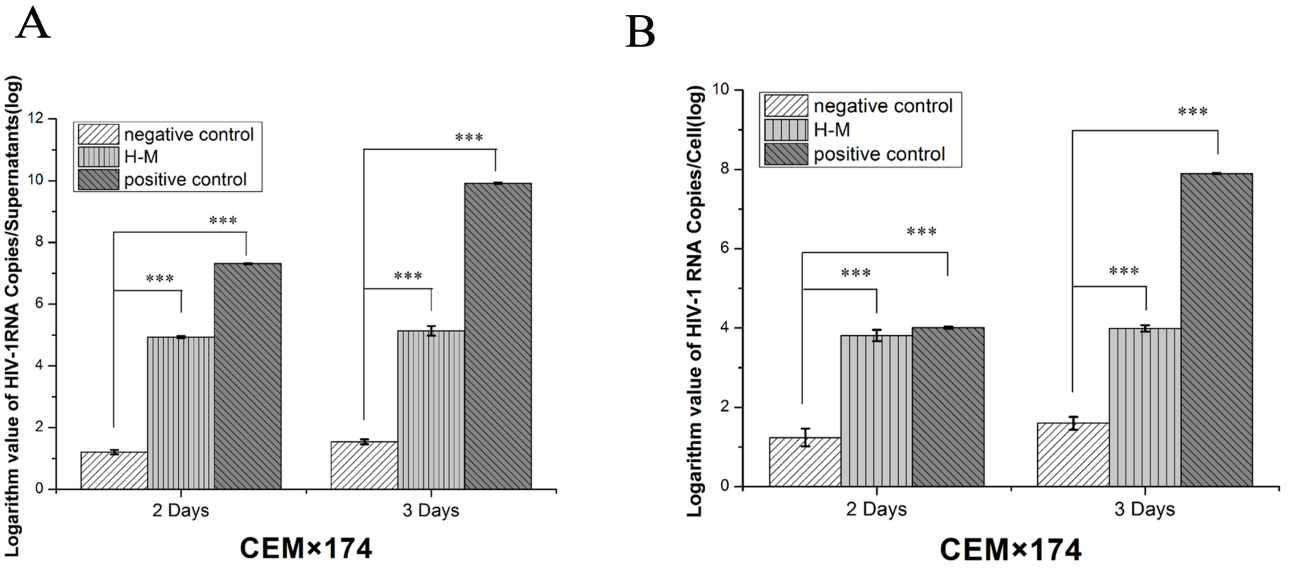

Fig. 6. HIV-1 RNA expression level in CEMx174 cells. (A) The HIV-1 RNA level in the supernatant. (B) Cellular HIV-1 RNA expression. H-M represents cells infected with the HIV-1 collected from transgenic L1210, negative control represents cells infected with the HIV-1 collected from empty-transgenic L1210, and positive control represents cells infected with normal HIV-1. HIV-1 RNA expression was expressed in two days and increased at three days in the H-M group and positive control, in both cell lysates and supernatants. Data are expressed as mean ± SD of three samples. $^{***}p < 0.001$ compared to the empty lentiviral vector group.

lysates and supernatants were evaluated at two and three days after HIV-1 RNA transduction (Fig. 6). HIV-1 RNA expression in the H-M group was lower compared to the positive control group and higher compared to the negative control group, which conforms normal HIV-1 infected lymphocytes characteristic, both cell lysates, and supernatants. These results show that HIV-1 collected from the CD4/CCR5/CyclinT1 transgenic L1210 has strong biological activity and can be transmitted via co-cultured human lymphocytes.

Discussion

HIV is a retrovirus divided into two types, HIV-1 and HIV-2, of which HIV-1 is the main pathogen leading to AIDS. HIV-1 is highly selective on host cells. It can only directly infect humans and a few non-human primates cells, such as T4 lymphocytes, mononuclear-macrophage, and dendritic cells. HIV-1 cannot cross species barriers to infect other animals due to receptor and virus replication restriction. Therefore, non-HIV-1 host cells must overcome receptor and virus replication limitations to be infected by the virus.

First, CD4 and CCR5 molecular structures in mouse cell membranes are different from those of human cell membranes. The envelope glycoprotein GP120 of HIV-1 cannot bind to CD4 and CCR5 molecules in mouse cells [14], so it cannot produce HIV-1 infection in murine cells. Second, HIV-1 Tat can lose its potency in mouse cells because it cannot bind to CyclinT1 mouse cells, and then affects HIV-1 positive transcription elongation factor b (P-TEFb) complex recruitment to activate HIV-1 transcription [15–17]. This is one of the main reasons why humanized mouse methods and transgenic mice can only produce HIV-1 infection mouse models. Murine cells have several barriers against retroviral replication, such as SAMHD1 (SAM domain and HD domain-containing protein 1), APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), TRIM5 (tripartite motif protein 5), and other cell-specific limiting factors [18].

In this study the cited problems were overcame by preparing a murine cell model with trans-species susceptibility and efficient HIV-1 viral replication. A lentiviral vector successfully transferred CD4, CCR5, and CyclinT1 genes to L615 and L1210. GP120 envelope glycoprotein of HIV-1 recognizes and binds to CD4 molecules on L1210 cell membrane, causing conformational changes in GP120 and exposing gp41, which then binds to the co-receptor CCR5 on the surface of target cells [19]. CD4-GP120-CXCR4/CCR5 complex is mediate in viral capsule and cell membrane fusion [20]. Thus, HIV-1 can invade mice cells successfully. CyclinT1 is a key factor in HIV-1 transcription process driven by the Tat protein and is widely expressed in human tissues [21]. Tat protein is one of the regulatory proteins encoded by viral genes. Its main function is to transactivate the initiation and extension of viral genome transcription and initiate viral replication in cells infected with HIV. CyclinT1 to Tat protein binding significantly increase HIV-1 expression.

After transfection, qRT-PCR results showed that L1210 cells could positively express CD4, CCR5, and cyclin T1 mRNA (Fig. 3). L1210 cells protein was extracted and determined by Western blot. The results showed that CD4, CCR5, and CyclinT1 proteins were expressed in L1210 cells (Fig. 4). The results confirmed that the lentivirus vector can transfer foreign genes into cells, thus achieving positive CD4, CCR5, and CyclinT1 expression in L1210 cells.

After HIV-1 virions enter the cells, virus replication, assembly, maturation and budding are inhibited by some restriction factors in mouse cells, which hinder virus replication. In this study, HIV-1 low replication capacity was addressed by selecting appropriate mouse cells. Murine leukemia virus (MuLV) is a retrovirus that can infect mouse leukocytes and cause leukemia in mice. Mouse leukemia structure and pathogenesis are similar to HIV-1. MuLV-infected mice can be used as HIV/AIDS animal model to a certain extent [22–25].

MuLV-induced murine leukemia cell line L1210 was selected as HIV-1 host cell, considering that after MuLV successfully induced L1210 cells, the barrier against cells retroviral replication was largely removed, which was conducive to HIV-1 virus replication promotion. Therefore, L1210 cells have certain advantages as host cells for HIV-1 cross-species infection. HIV-1 RNA expression was determined by qPT-PCR in culture supernatant and cell lysates (Fig. 5) of transgenic mouse cells. Moreover, HIV-1 virus activity in transgenic L1210 infected HIV-1 was observed by HIV-1 RNA detection from the co-cultured CEMx174 cells (Fig. 6). The results showed that HIV-1 infection and virus replication were observed in CD4/CCR5/CyclinT1 transfected L1210 cells.

Our study has some limitations. Although the cell model established in this experiment can be used as a model for evaluating HIV infection, whether this model is susceptible to all HIV subtypes remains to be further verified. At the same time, compared to the natural host, the innate immune regulation caused by this model also needs to be further confirmed by future research.

Conclusions

In summary, we efficiently transferred CD4, CCR5, and CyclinT1 genes to L1210 using lentiviral vectors. HIV1 susceptibility assays confirmed that the transgenic mouse leukemia cells prepared in our study could support efficient entry, integration, and full HIV-1 replication. Therefore, this study successfully constructed a mouse HIV-1 infected cell model that can stably express CD4/CCR5/CyclinT1. As a mouse host cell for HIV-1 cross-species infection, this cell model can be transplanted into mice to prepare a HIV-1 infection mouse animal model, which provides the basis for HIV/AIDS pathogenesis research. This is a new platform for the evaluation of antiviral drugs and vaccine research.

Availability of Data and Materials

Data will be available from the corresponding author upon reasonable request.

Author Contributions

YJL and CCZ—designed the research study; XYC, LMZ and JL—performed the research; YJL and XYC—analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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