Improved Long-term In Vivo Lentiviral Gene Therapy for Chronic Granulomatous Disease

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Article

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Abstract

Chronic granulomatous disease (CGD) is a congenital immunodeficiency characterized by lack of reactive oxygen species in phagocytes. We developed an in vivo gene therapy strategy based on intravenous (iv) injection of lentiviral vectors (LVs) in X-CGD mice. A non-myeloablative conditioning regimen using busulfan, cyclophosphamide and dexamethasone was developed to improve iv LV gene delivery efficiency. The X-CGD mice received two LVs injections. After the second injection, antibody response to LV particle-associated p24-protein was examined by Western blot. We detected increased gene transfer without anti-p24 antibody response. However, the blood vector copy number (VCN) was gradually reduced after 3–12 months. To improve gene delivery into hematopoietic stem cells (HSCs), the mice were treated with AMD3100 to mobilize HSCs before LV injection. To confirm HSCs gene transfer, we transplanted the HSCs from the LV-CYBB-treated CGD mice into untreated CGD mice. The result showed successful passage of LV-CYBB HSCs to recipient mice. Thus, by combining conditioning and AMD3100 mobilization prior to the iv LV injection, improved in vivo long-term LV gene transfer into HSCs could be established. This improved iv LV gene delivery strategy could reduce both the risk and the cost of CGD gene therapy with great potential in translational applications.

Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by the loss of function of NADPH oxidase in phagocytes (1). NADPH oxidase consists of five subunits, and the loss of function of any subunit can cause CGD. Most patients (~65%) carry a mutation in the CYBB gene on the X chromosome, while the others inherit an autosomal recessive mutation (2–6). Due to the NADPH oxidase deficiency, CGD patients cannot produce reactive oxygen species (ROS) to execute bactericidal effects, and infections occur repeatedly in various parts of the body (5, 6).

Hematopoietic stem cell transplantation (HSCT) is curative for CGD but it requires myeloablative treatment and an HLA-matched donor. Finding a suitable donor is difficult, and HSCT is associated with the risk of graft-versus-host disease (GVHD) and death (7–9). Furthermore, the success rate of HSCT in CGD is lower than in other primary immunodeficiencies (7, 8).

Gene therapy is a promising treatment for CGD. The gene delivery strategies include “in vivo” and “ex vivo” approaches. While “ex vivo” strategy has been successfully used in numerous clinical trials (10–16), it involves collection, genetic modification and expansion of the HSCs. The isolation and culture of HSCs are laborious and have risks of exhaustion and contamination. In addition, ex vivo HSC manipulation is a challenge from a logistic and regulatory standpoint, which demands a high regulatory burden and cost. Furthermore, the myeloablative regimens in CGD patients bear a high risk factor and can result in considerable morbidity (17, 18). In vivo gene therapy, on the other hand, involves direct injection of genetic vehicles into the recipient, which may effectively overcome many of the disadvantages of the ex vivo approach. Richter and Felsburg et al. have investigated iv delivery of foamy virus (FV) vectors in SCID-X1 dogs, and shown corrected CD3 T lymphocytes expressing the therapeutic gene (19). Recent
advances suggest that lentiviral vectors (LVs) could be applied in vivo as well, Richter et al. reported LV-mediated FVIII gene transfer into HSCs, illustrating a persistent therapeutic effect after intra-femoral injection in mice (20–22). Intramuscular or intravenous injection of AAV-mediated factor IX gene therapy has resulted in long-term therapeutic factor IX expression with clinical improvement (23, 24); nevertheless, all of the subjects receiving systemic AAV gene therapeutics developed high-titer AAV neutralizing antibodies (25–31). Thus, innovative vector design and delivery strategy are critical to in vivo gene therapy.

LVs have been successfully applied in treating monogenic diseases. The most often used gene therapy LVs have been developed based on HIV-1 (human immunodeficiency virus type I). LV belongs to the retroviral family but can effectively infects both dividing and non-dividing cells (32–34). The major challenges for iv LV gene delivery include exposure to the host immune/complement system and off-target gene transfer. The standard VSVG-pseudotyped LVs have been shown ineffective for direct in vivo injection, since VSVG is rapidly inactivated by human complement (18, 35, 36). In this report, we have developed a chemotherapy conditioning regimen to improve iv LV gene delivery efficiency. Furthermore, to improve gene delivery into HSCs, we applied a pre-treatment using AMD3100 to mobilize HSCs into peripheral blood (37–40). To avoid ectopic off-target toxicity, we engineered LVs carrying a myeloid-specific promoter (miR223) that directed the expression of CYBB only in myeloid cells (41). With all these advancements, we report a safe, efficient and economic iv LV gene therapy strategy for the treatment of CGD.

Materials And Methods

LV preparation - LVs were constructed, packaged and filtration concentrated using the NHP/TYF LV system as described previously (41–43). The green fluorescence protein (GFP) gene mWasabi was a gift of Dr. Jiwu Wang (Allele Biotech). The mWasabi gene and the CYBB cDNA were cloned into pTYF behind the human EF1α promoter or the tissue-specific promoters and packaged in 293T cells as described (41, 44, 45).

Determination of vector copy number (VCN) - The LV VCN was determined using genomic DNA (gDNA) of transduced cells and standardized human and mouse reference cells based on SYBR real-time quantitative PCR (qPCR) as previously described (41, 44).

Animal procedures - All mouse protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Shenzhen Geno-Immune Medical Institute (GIMI, Shenzhen, China). The X-CGD mice were purchased from Jackson Laboratory (X-CGD; B6.129S-Cybb<sup>tm1Din</sup>/J, USA). All mice were housed under specific pathogen-free conditions. Before intravenous injection, the 6–7 week-old male X-CGD mice were conditioned with busulfan (5 mg/kg), cyclophosphamide (100 mg/kg) and dexamethasone (5 mg/kg).
**Western blot (WB) analysis** – For the analysis of anti-HIV-p24 response, standard p24 protein obtained from Boster Biological Technology, USA, was separated by electrophoresis and transfer to a membrane, blocked with 5% BSA buffer (Biofroxx, Germany) for 1 h, and incubated with primary antibodies including a control anti-HIV-1 p24 antibody (Abcam) or sera collected from LV-CYBB iv injected mice, and a control anti-GAPDH antibody (Merck) at 4°C overnight. The membranes were incubated with horse radish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, San Diego, USA) for 1 hr. The signals were detected with a chemiluminescence kit (ECL, Bio-Rad) and exposed and analyzed under the ChemiDoc Touch imaging system. The gray values of WB stripes were analyzed by using Bio-rad Image Lab software. The linear equation between the gray values of WB (Y-axis) and dilution ratio of the standard anti-p24 antibody (X-axis) was generated. Dilution ratio of standard anti-p24 antibody (X) was 5702.45 with the gray values of WB (Y-axis) as 0. The following equation was used:

\[
Y = -5260.9X + 3 \times 10^7, \quad R^2 = 0.9919
\]

(Y-axis is the gray values of WB; X-axis is dilution ratio of standard anti-p24 antibody)

**Dihydrorhodamine 123 (DHR123) assay** - Blood cells were stimulated with phorbol-12-myristate-13-acetate (PMA, Sigma, Beijing, China), stained with DHR123 (Thermo Fisher Scientific, Shanghai, China), and analyzed by flow cytometry as described (41, 46).

**Cell cultures** - The peripheral blood (PB) cells were incubated in RPMI (Hyclone; China), containing 20% FBS, and 1% penicillin and streptomycin. The C57 WT PB cells were placed in a transwell chamber, and the X-CGD PB cells were placed in a transwell insert, and co-cultured together for 18 hours. After washing with PBS, the cells were resuspended in serum-free medium containing PMA for another 4 hours. All cells were cultured at 37°C in 5% CO₂.

**Staphylococcus aureus (S. aureus) challenge** - S. aureus from ATCC were pelleted and prepared as inoculum of 2×10¹⁰ CFU/ml. The mouse was inoculated intranasally with 5×10⁸ CFU twice within 30 min, 3 months after gene therapy. The body weight, death time and CFU counting in the lungs were recorded as described (41, 47, 48).

**Cell preparation and flow cytometry** - Cells were permeabilized using 0.1% Triton X-100 in PBS for 3 min and incubated with normal rat serum for 30 minutes, and then stained with anti-NOX2 (CYBB) antibody (ab80508, Abcam) at 4°C for 30 min (41). A secondary antibody conjugated with AlexaFluor-488 (Invitrogen, Thermo Fisher) was added for 30 min. Analyses were performed using a NovoCyte Quanteon flow cytometer (ACEA Biosciences, USA) and data were analyzed using the ACEA NovoExpress software.

**Statistical analysis** - Statistical analysis was performed based on the Wilcoxon matched-pairs signed-rank test using GraphPad Prism 8 software (GraphPad Inc., La Jolla, CA). All data were presented as mean ± SEM. Significance of differences between groups was evaluated using student’s t test or ANOVA one-way test (Tukey), and specified as **, p < 0.05; ***, p < 0.03; ****, p < 0.01; n.s., no significant difference.
Results

1. In vivo functional analyses of LV-CYBB after LV iv injection

A challenge for in vivo LV gene delivery is the inhibition by the host immune/complement system against VSVG-pseudotyped LVs. To overcome this, we pre-conditioned the CGD mice with non-myeloablative radiation (6 Gy) before LV iv injection. The CGD mice were tail vein iv injected with 1×10^9 transduction units (TU) of LV-CYBB (n = 5, Fig. 1A), and the expression and function of CYBB gene were analyzed via gp91-phox staining and DHR123 assay 4 weeks post-injection.

The blood cells were collected and intracellularly stained with anti-gp91-phox antibody together with CD11b surface staining for granulocytes followed by flow cytometry analysis. We detected gp91-phox expression in 69.08% of the PBMCs in WT mice, 0.74% in the untreated CGD mice, and 41.46% and 39.28% in the CGD mice treated with LV-EF1a-CYBB and LV-miR223-CYBB, respectively (Fig. 1B and D left panel). The DHR123 assay demonstrated restored ROS production in the treated CGD mice as shown in Fig. 1C and D (right panel). The VCN analysis confirmed LV-CYBB transduction of blood cells in the CGD mice; however, we detected markedly reduced VCN in the iv LV-treated mice three months later (Fig. 1E). Thus, non-myeloablative radiation conditioning substantially increased LV gene delivery efficiency, yet, the iv injection could not establish stable long-term HSC gene transfer.

2. Improved in vivo LV gene therapy in X-CGD mice by repetitive iv injections after chemo-conditioning

The radiation approach improves LV gene delivery but is not clinically translatable. Therefore, we explored a chemotherapy conditioning regimen to improve LV gene delivery efficiency. After exploring different chemotherapy regimens, we established a non-myeloablative condition using busulfan, cyclophosphamide and dexamethasone to pre-treat the CGD mice, which was followed by iv injection of 1×10^9 TU of LV-CYBB. A second injection of 1×10^9 TU of LV-CYBB after repeat chemo-conditioning was given 12 weeks later (n = 5, Fig. 2A). The expression and function of LV-CYBB gene were analyzed 4 weeks post-injection. Furthermore, 14 days after the second injection, we examined anti-p24 antibody response to the LV particles using sera from the LV-injected mice by WB analysis.

We detected expression of gp91-phox after the first versus the second injections as following: LV-EF1a 1.14 ~ 5.18 vs. 5.27 ~ 14.36%, and LV-miR223 1.65 ~ 5.17 vs. 4.59 ~ 17.89% (Fig. 2B and C). The DHR123 assay demonstrated restored ROS production in the iv LV injected CGD mice (Fig. 2B and C). The efficiency of LV gene delivery was illustrated by VCN analysis using blood cells of the treated CGD mice (Fig. 2C). Importantly, we found no HIV-associated p24 antibody response even after a second injection; no WB bands were detected using sera from the LV-injected mice (Fig. 2D).

3. Increased in vivo LV-CYBB HSC delivery after AMD3100 treatment

To improve LV gene delivery into HSCs, we explored AMD3100 to mobilize HSCs into peripheral blood before iv injection (37–40). The mice were treated with 10 mg/kg AMD3100 and then iv injected with
$2 \times 10^9$ TU of LV-CYBB after chemo-conditioning (n = 5, Fig. 3A). After four weeks, the gp91-phox positive HSCs of total Sca1+ HSCs in blood were examined by flow cytometry. The results of without (-) or with (+) the AMD3100 treatment were as the following: LV-EF1a: (-) 9.52–15.43%, (+) 16.1–29.4%, ($p = 0.022$) and LV-miR223: (-) 4.49–10.35% and (+) 7.06–16.84% ($p = 0.473$), illustrating a significant increase in HSC gene transfer after AMD3100 treatment. Note that the LV-miR223 displayed limited transgene expression in HSCs as compared with the LV- EF1a promoter (Fig. 3B). At 13 weeks, different organs of the treated mice were collected to examine VCN. We found that the LV-CYBB transgene could be detected in multiple organs after iv injection, and significant increase of VCN in bone marrow was detected in the AMD3100-treated mice (** $p < 0.05$ Fig. 3C). Importantly, no VCN was detected in the testes.

While only low VCN was detected in the treated mice, we nevertheless observed marked protective effects based on the S. aureus bacterial challenge experiment. One possible explanation was that the resistance to bacterial infections in multiple organs could be due to the bystander protective effects of ROS or their derivatives affecting the adjacent cells. To test this, we set up a co-culture experiment using different ratios of WT and CGD mouse cells. The results showed that increased DHR123 activities could be observed with increased ratios of WT to CGD cells in the trans-well co-cultures (Fig. S1). These results supported that AMD3100 treatment could improve LV iv gene delivery into HSCs.

To confirm that the LV-CYBB had indeed transduced HSCs, we performed a bone marrow (BM) transplantation experiment (Fig. 3A). The BM cells from LV-CYBB treated CGD mice (donors) were collected and transplanted into CGD mice (recipients) after myeloablation. The VCNs in the donor BM cells and in the recipient mice 4 weeks after transplantation were examined. The results showed the following: LV-EF1a: donor (BM) 4.49% and recipient (HSCT) 1.05–2.57%, and LV-miR223: donor 4.91% and recipient 1.04–2.69% (Fig. 3D). This transplantation study supported that about 25–30% of the LV-CYBB modified Sca-1 positive cells were indeed long-term HSCs, which could be passed onto the recipient CGD mice by HSCT.

**4. Increased phenotypic correction of CGD mice after iv injection of LV-EF1a-CYBB or LV-miR223-CYBB following AMD3100 tretment**

Previous clinical assessment has reported that the DHR123 positive (DHR123+) cells below 10% in the peripheral blood is highly associated with infections (8, 10, 49–51). We showed that the iv LV gene transfer efficiency into HSCs was around 1–3% (Fig. 3B). To see if the iv LV-CYBB gene transfer was sufficient to correct the CGD deficiency, we designed an experiment to test the CGD correction under (+/-) AMD3100 and chemo-conditioning, and examined the ROS recovery ability and resistance to pulmonary bacterial infection. The CGD mice were chemo-conditioned and (+/-) AMD3100 treated as described above before LV iv injection (LV-EF1a and LV-miR223, n = 10).

To assess for resistance to infection, each mouse was intranasally inoculated with $5 \times 10^8$ CFU S. aureus 3 months after gene therapy as illustrated in Fig. 4A (47, 48, 52). We found that the CGD mice treated with AMD3100 (+) were resistant to S. aureus challenge in 7–8 days, with an overall weight loss below 15%,
similar to the challenged WT mice. The maximal weight loss in the AMD3100(+) EF1a and miR223 groups and in the WT mice was around 11% on days 3. The maximal weight loss in the AMD3100(-) EF1a and miR223 groups approached 13–15% on day 3 to 4 (Fig. 4C, and supplementary Table S2). Nevertheless, the AMD3100(-) LV treated mice began to gain weight on day 5, while the AMD3100(+) LV treated mice gained weight on day 4; two of the AMD3100(-) LV treated mice gained weight slowly but gradually, and the EF1a AMD3100(-) mice did better than the miR223 AMD3100(-) mice. The body weight recovery of the two AMD3100(+) LV-treated groups, EF1a and miR223, were similar (Fig. 4C). Several of the AMD3100(-) LV-treated CGD mice died on days 5 to 7. At day 7, the AMD3100(-) LV-treated CGD mice displayed 20% and 30% mortality in the EF1a and miR223 groups, respectively (Fig. 4B). Thus, the AMD3100(+) condition increased the anti-bacterial ability of the LV-treated CGD mice, as quantified by S. aureus CFUs in the lungs (Fig. 4D). The recovery of ROS production under the miR223 promoter was slightly slower than that of the EF1a promoter. Both of these LV promoters converted resistance to infection under the AMD3100(+) treatment.

**Discussion**

*Ex vivo* HSC modification and transplantation has been the main stream LV gene therapy in clinical trials (10–16), yet it involves high-risk myeloablative chemotherapy. Recent advances with AAV and retroviral vectors including foamy virus (FV) vector and LV have illustrated utility for *in vivo* gene therapy (19–31, 53). However, the low *in vivo* transgene delivery efficiency with FV and LV, and the presence of AAV neutralizing antibodies substantially compromise their *in vivo* gene therapy potentials (25–31, 53). Innovative vector design and gene transfer strategies are therefore particularly important for *in vivo* gene therapy.

LV pseudotyped with VSV-G envelope can infect both dividing and non-dividing cells at high efficiencies (32–34). However, major challenges exist for *iv* LV gene delivery due to the inhibitory host immune/complement system and off-target gene transfer. We first developed a non-myeloablative radiation (6 Gy) conditioning regimen to improve *in vivo* LV gene delivery. This was demonstrated by the detection of gp91-phox expression and the DHR123 activities in CGD mice after *iv* LV-CYBB injection (Fig. 1).

Radiation conditioning is not translatable in treating CGD patients and thus, we further developed a chemotherapy conditioning regimen to improve LV *in vivo* gene therapy. After non-myeloablative chemo-conditioning using busulfan, cyclophosphamide and dexamethasone, the *iv* LV-CYBB-injected CGD mice showed restored ROS production (Fig. 2). Importantly, after a second LV injection, we detected increased LV gene transfer efficiencies with no antibody response to the LV particle-associated protein p24. It was plausible that the non-myeloablative chemo-conditioning reduced the host immune/complement inhibitory activities and improved the *iv* LV gene delivery efficiency. These results demonstrate that repeated LV injection is feasible and can increase the *in vivo* LV gene transfer efficiency without antibody induction.
In continued follow-up, we found that the iv injected LVs did not persist in the mouse blood cells. This could be due to the low efficiency of targeting mouse HSCs via the iv route. To improve gene delivery into HSCs, we applied AMD3100 pre-treatment to mobilize HSCs into peripheral blood (37–40). Such treatment substantially increased LV delivery into HSCs as illustrated in Fig. 3, and it was accompanied by increased gp91-phox expression and resistance to bacterial challenge.

With the improved LV iv gene delivery strategy, we could closely evaluate the effects of the two promoters, the universal and the myeloid-specific promoters, on CGD phenotype correction (41). The results showed that both promoters restored the antibacterial activities in the CGD mice. Importantly, without AMD3100 treatment, the universal promoter was better than the myeloid-specific promoter in displaying antibacterial activities in the CGD mice. Furthermore, we did not observe toxicity in the LV-CYBB treated CGD mice even after 70 weeks. The use of a myeloid-specific promoter in the LV-CYBB vector design could improve the safety of systemic iv LV delivery, which may reduce the expression of CYBB in non-orthotopic cells.

In conclusion, we have demonstrated that immune suppressive conditioning is necessary for efficient LV iv gene delivery. Further, repetitive LV iv injection is safe and can increase VCN and transgene expression in vivo without LV-related antibody formation. Importantly, successful HSC transplantation from the LV-CYBB treated CGD donor mice to the recipient CGD mice confirmed increased long-term iv LV gene transfer into HSCs after the AMD3100 treatment, which further improved the survival of CGD mice after pulmonary bacterial challenge.

**Declarations**

**Author contributions:**

Engineered the LV constructs and finalized the manuscript: LJC

Designed the study: LJC, HY

Performed the laboratory work: HY, RY, JG, XW, HL, YL

Performed the data analysis: HY, LJC

Drafted and revised the manuscript: HY, LJC

All authors read and approved the final manuscript.

**Conflict of Interests:** The authors declare that they have no financial conflict of interest.

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Data and materials availability: All raw data used for figure generation in this study can be accessed by contacting the corresponding author.

References


**Figures**
Figure 1

Functional analyses of LV-\textit{CYBB} in X-CGD mice after iv injection under radiation conditioning. (A) Study design for iv strategy of the various LV-\textit{CYBB} under non-myeloablative radiation conditioning in X-CGD mice (n=5). (B) Intracellular staining of gp91-phox and flow cytometry analysis of LV-\textit{CYBB}-modified PBMCs at 4 weeks after iv injection. (C) DHR123 flow cytometry analysis of NADPH oxidase activity in PBMCs of WT and X-CGD mice. (D) Statistical analysis of gp91-phox expression in LV-\textit{CYBB}-treated CGD mice 4 weeks after iv injection, $p=0.0493$, ** $<0.05$. (E) Analysis of VCN in control untreated CGD and WT mice, and LV-\textit{EF1a}, or LV-\textit{miR223} treated CGD mice at week 4 or 12 after iv injection. Data are presented as mean $\pm$ SEM. **, $p<0.05$; ***, $p<0.03$; ****, $p<0.01$, n.s., no significant difference.
Figure 2

Functional analyses of LV-CYBB after repeated iv injections under chemo-conditioning in X-CGD mice (A) Study design for iv strategy of the various LV-CYBB under non-myeloablative chemo-conditioning in X-CGD mice (n=5). (B) Intracellular staining of gp91-phox and flow cytometry analysis of LV-CYBB-modified PBMCs at 4 weeks after one and two iv injections. DHR123 flow cytometry analysis of NADPH oxidase activity in PBMCs of WT and X-CGD mice. (C) Statistical analysis of gp91-phox expression in LV-CYBB-treated CGD mice 4 weeks after iv injection. Analysis of VCN in control untreated CGD and WT mice, and LV-EF1a, or LV-miR223 treated CGD mice at week 4 after iv injection. (D) Analysis of anti-P24 antibody by WB. The linear equation between the gray values of WB and dilution ratio of standard anti-HIV-P24 antibody was generated; Y is the gray values of WB; X is dilution ratio of standard anti-HIV-P24 antibody. Data are presented as mean ± SEM. **, p<0.05; ***, p<0.03; ****, p<0.01, n.s., no significant difference.
**Figure 3**

Analyses of AMD3100 treatment prior to LV iv injections of X-CGD mice under chemotherapy conditioning. (A) Study design for LV-CYBB iv injection with or without AMD3100 pre-treatment under non-myeloablative chemo-conditioning in X-CGD mice (n=5) (B) Intracellular staining of gp91-phox and flow cytometry analysis of LV-CYBB-modified blood Sca-1+ cells 4 weeks after intravenous injections. (C) VCN analysis of different organs of the LV-treated mice after 13 weeks. (D) VCN analysis of control untreated CGD, and LV-EF1a, or LV-miR223 treated CGD mice at week 4 after HSC transplantation from the iv LV-treated donor CGD mice. Data are presented as mean ± SEM. **, p<0.05; ***, p<0.03; ****, p<0.01, n.s., no significant difference.
Functional analyses of LV-EF-1a and -miR223 promoters in X-CGD mice after LV iv injections. (A) Diagram of study timeline of the CGD mice challenged with S. aureus to induce pneumonia after LV iv gene therapy. (B) Survival rate of CGD mice after S. aureus challenge at 13 weeks after LV iv gene therapy. (C) The kinetics of body weight loss after S. aureus induced pneumonia. (D) The CFU analysis of bacterial load in the broncho-alveolar lavage fluid of mouse lungs following S. aureus challenge. Data are presented as mean ± SEM. **, p< 0.05; ***, p< 0.03; ****, p< 0.01, n.s., no significant difference.

Supplementary Files

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- figS1.pdf
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