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ARTICLE

Production and purification of high-titer foamy virus vector for the treatment of leukocyte adhesion deficiency

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Compared to other integrating viral vectors, foamy virus (FV) vectors have distinct advantages as a gene transfer tool, including their nonpathogenicity, the ability to carry larger transgene cassettes, and increased stability of virus particles due to DNA genome formation within the virions. Proof of principle of its therapeutic utility was provided with the correction of canine leukocyte adhesion deficiency using autologous CD34$^+$ cells transduced with FV vector carrying the canine CD18 gene, demonstrating its long-term safety and efficacy. However, infectious titers of FV-human(h)CD18 were low and not suitable for manufacturing of clinical-grade product. Herein, we developed a scalable production and purification process that resulted in 60-fold higher FV-hCD18 titers from ~1.7×10^4 to 1.0×10^6 infectious units (IU)/ml. Process development improvements included use of polyethylenimine-based transfection, use of a codon-optimized *gag*, heparin affinity chromatography, tangential flow filtration, and ultracentrifugation, which reproducibly resulted in 5,000-fold concentrated and purified virus, an overall yield of $19 \pm 3\%$, and final titers of $1–2 \times 10^9$ IU/ml. Highly concentrated vector allowed reduction of final dimethyl sulfoxide (DMSO) concentration, thereby avoiding DMSO-induced toxicity to CD34$^+$ cells while maintaining high transduction efficiencies. This process development results in clinically relevant, high titer FV which can be scaled up for clinical grade production.

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INTRODUCTION

Foamy viruses (FVs), also known as spumaretroviruses, derive their name from the vacuolating foamy-like cytoplasm of productively infected cells and multinucleated syncytia. They are endemic in a number of mammals, including cats, cows, and captive non-human primates, but not found in humans. Despite their highly cytopathic nature in cell culture, they are not associated with any detectable disease in infected hosts.[1,2] The development of leukemia in X-linked severe combined immunodeficiency patients[3,4] and occurrence of myelodysplastic syndrome in chronic granulomatous disease patients[5] caused by gamma-retrovirus vector-mediated insertional mutagenesis after *ex vivo* stem cell gene therapy has stimulated the development of vectors with improved safety profiles for clinical application. FVs have several distinct advantages over other integrating viral vectors such as gamma-retroviruses and lentiviruses as a gene transfer tool.[6,7] These include a large packaging capacity (up to 12 Kb) and a broad host and cell-type tropism.[1,2] Furthermore, FVs can efficiently transduce quiescent cells, since the FV genome can persist in a stable form as cDNA in growth-arrested cells/quiescent cells and can integrate into the host genome when the cells exit the G0 phase of the cell cycle.[8] In addition, as compared to gamma-retrovirus or lentivirus, FV has a safer integration profile with lower risk of insertional mutagenesis.[9–11] FV vectors have been used to correct genetic disorders of hematopoietic stem cells in several animal models, including leukocyte adhesion deficiency (LAD) in dogs,[9,12] and Wiskott–Aldrich syndrome, Fanconi anemia, and X-linked chronic granulomatous disease in mice.[13–15]

Patients with LAD type 1 (LAD-1) and dogs with canine LAD suffer from recurrent and life-threatening bacterial infections.[16,17] Both diseases are caused by mutations in the leukocyte integrin CD18 subunit that prevent the formation and surface expression of CD11/CD18 heterodimeric adhesion molecules resulting in an inability of leukocytes to adhere to the endothelium and migrate toward the sites of infection.[18]

Successful gene therapy of canine LAD was demonstrated in four dogs transplanted with autologous CD34$^+$ cells transduced by FV vectors expressing canine CD18.[9,12] However, the low titers typically obtained with FV vectors[6,7] have precluded their use for clinical application in LAD-1 patients. In addition, processes used previously were not scalable and not compatible with the large-scale manufacturing needed for clinical application. Major obstacles for scale-up of FV vector production and purification include: (i) the low titer of

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calcium phosphate-mediated transfection commonly used in gene transfer vector production,[6,7] (ii) the limited stability of FV vectors in ambient or high temperature, acidic, or basic pH, and high salt concentrations, (iii) their sensitivity to shear forces, and (iv) the necessity to freeze FV vectors in 5% dimethyl sulfoxide (DMSO) and consequently to significantly dilute the vector to minimize toxicity to stem and progenitor cells during transduction.

In this study, we have successfully addressed each obstacle for large-scale manufacturing of FV vectors compatible with current good manufacturing practices. We first improved vector production by optimizing transfection with the use of polyethylenimine (PEI) and by varying parameters of producer cell culture, plasmid concentration, and harvest time. We next improved vector purification with the use of heparin affinity chromatography since heparan sulfate was identified as a receptor for FV,[19,20] and chromatography-based purification methods are scalable and can be performed in a closed system compatible with production of clinical-grade vectors.[21,22] Finally, we used tangential flow filtration (TFF) and ultra-centrifugation for the final step of vector concentration. This optimized process resulted in highly concentrated FV vectors carrying the human CD18 cDNA (FV-hCD18) that can now be scaled up for clinical application.

RESULTS

Optimization of transfection conditions to maximize FV titers

FV vectors were previously produced by calcium phosphate-mediated transient transfection of HEK239T cells with helper (*gag*, *pol*, and *env)* and gene transfer vector plasmids.[7] Unconcentrated titers of FV-GFP were $1.2 \pm 0.2 \times 10^5$ infectious units (IU)/ml as determined on HT1080 cells and those of FV-hCD18 were $1.7 \pm 0.1 \times 10^4$ IU/ml as determined on RAW264.7 cells. We have recently published that PEI-mediated transfection resulted in up to a 50-fold increase in FV vector titers over calcium phosphate transfection.[11] In this study, PEI-mediated transfection was further optimized to maximize FV-GFP and FV-hCD18 vector titers. For both FV-GFP (Figure 1a) and FV-hCD18 (Figure 1b) vectors, titers improved with increasing concentrations of PEI, with a peak titer at 70–80 µg PEI per T75 flask. Further increases in PEI led to reduced titers (Supplementary Figure S1). After optimization, 70 µg of PEI per T75 flask was used during FV vector production in all experiments.

We also evaluated the effect of poly-L-lysine coating of culture plastic on FV-GFP vector production. Coating of culture plastic with 0.1% of poly-L-lysine prior to seeding HEK293T cells significantly increased FV vector titers (Figure 1c). Although it has been suggested that a 15 minutes PEI-DNA precipitation time is optimal for high-titer FV vector production,[11] our current data showed that a 10 minutes precipitation time yielded the highest titers (Figure 1d). Calcium phosphate-mediated transfection requires a medium change the next day to limit cellular toxicity and increase FV vector titers.[23,24] Similarly, we tested whether a change in medium after PEI-mediated transfection would also increase FV vector titers. Unexpectedly, this actually decreased FV vector titers by twofold to fivefold (Figure 1e). It is not clear whether this is due to a physiological response of the cells or related to a prolonged exposure to PEI and plasmid. Irrespectively, we adopted a protocol in which the transfection medium containing PEI was not removed posttransfection but left with the cells until harvesting the vector. In addition, we optimized the harvest time for FV vectors after transfection of the producer cells. FV-hCD18 vectors were sampled from 24 to 93 hours posttransfection without medium change and titered. In our hands, harvesting of FV vectors around 66 hours posttransfection yielded the highest titers (Figure 1f).

Codon optimized gag plasmid further increased FV titers

We next compared pCiGSΔΨ (original *gag*) and pCiGAGopt (codon optimized gag) plasmids for FV-hCD18 vector production (Figure 2a,b). We previously observed that transfection of HEK293T cells with 10.4 µg of pCiGSΔΨ per T75 flask resulted in optimal FV-hCD18 vector titers (data not shown). However, significant toxicity to HEK293T was observed when the same amount of pCiGAGopt was transfected, resulting in a 10-fold reduction in FV-hCD18 titers (Figure 2a). When amounts of pCiGAGopt were reduced from 10.4 to 1.3 µg per T75 flask in transfection, the titers of FV-hCD18 vectors increased proportionally (Figure 2a). In a follow-up study, the highest FV titer was obtained with 0.65 µg of pCiGAGopt plasmid (Figure 2b). Thus, the use of codon optimized *gag* resulted in doubling of the FV-hCD18 vector titers while using 16-fold less plasmid as compared to the previously optimized amount of pCiGSΔΨ.

Benzonase treatment of cultures posttransfection to reduce residual plasmid

Benzonase endonuclease is commonly used to reduce the amount of residual plasmid and cellular genomic DNA and RNA in the vector product.[25] Treatment of FV vectors for 16 hours with increasing concentrations of Benzonase had only minimal impact on vector titers (Supplementary Figure S2a). Longer exposure (40 hours) with 50 U/ml Benzonase further reduced FV vector titers minimally (Supplementary Figure S2b). While differences were not statistically significant, we chose a 16-hour exposure of Benzonase at 50 U/ml to limit the potential impact of Benzonase on FV titers. Overall, when all optimized conditions are combined, nonpurified, and unconcentrated FV-hCD18 titers of $\sim 1 \times 10^6$ IU/ml were consistently obtained, a 50-fold increase compared to titers obtained with the nonoptimized protocol.

Purification of FV vectors using heparin affinity chromatography

Since membrane-associated heparan sulfate, a heparin-related molecule, is a receptor for FV in cells,[19,20] we hypothesized that FV vector particles could be purified by heparin affinity chromatography. We evaluated the binding, washing, and elution conditions needed for effective purification of FV vector. Prior to chromatography, nuclease-treated FV vector supernatants were filtered through a 0.45 µm filter to remove any coarse cellular debris. Vector supernatants were subsequently loaded onto a 7.9 ml bed volume POROS-OH 50 µm heparin affinity chromatography column at a linear flow rate of 267 cm/hour and a residence time of 2.3 minutes. Faster flow rates and shorter residence time resulted in FV vector into the flow-through fraction (data not shown). After loading, the heparin column was washed with sodium phosphate or Tris–HCl buffer containing 150 mmol/l sodium chloride (pH 7.0). The washing step was continued until the ultraviolet absorbance curve (280 nm) returned to baseline and became stabilized. To evaluate elution conditions, bound virus particles were eluted using a salt gradient from 100 mmol/l to 1.0 mol/l NaCl (pH 7.0). The optimal NaCl concentration for elution was determined based on the presence of infectious FV-GFP particles in individual chromatography fractions as measured on HT1080 cells and sample conductivity which correlated to NaCl concentration (Supplementary Figure S3). We found that most of the FV-hCD18 was eluted at 600 mmol/l of NaCl (Figure 3). In addition, we did not observe any significant loss of FV particles in the flow-through during loading and washing. The average recovery of FV vector in the elution fraction was $69 \pm 6\%$ ($n = 5$) as shown in Table 1.

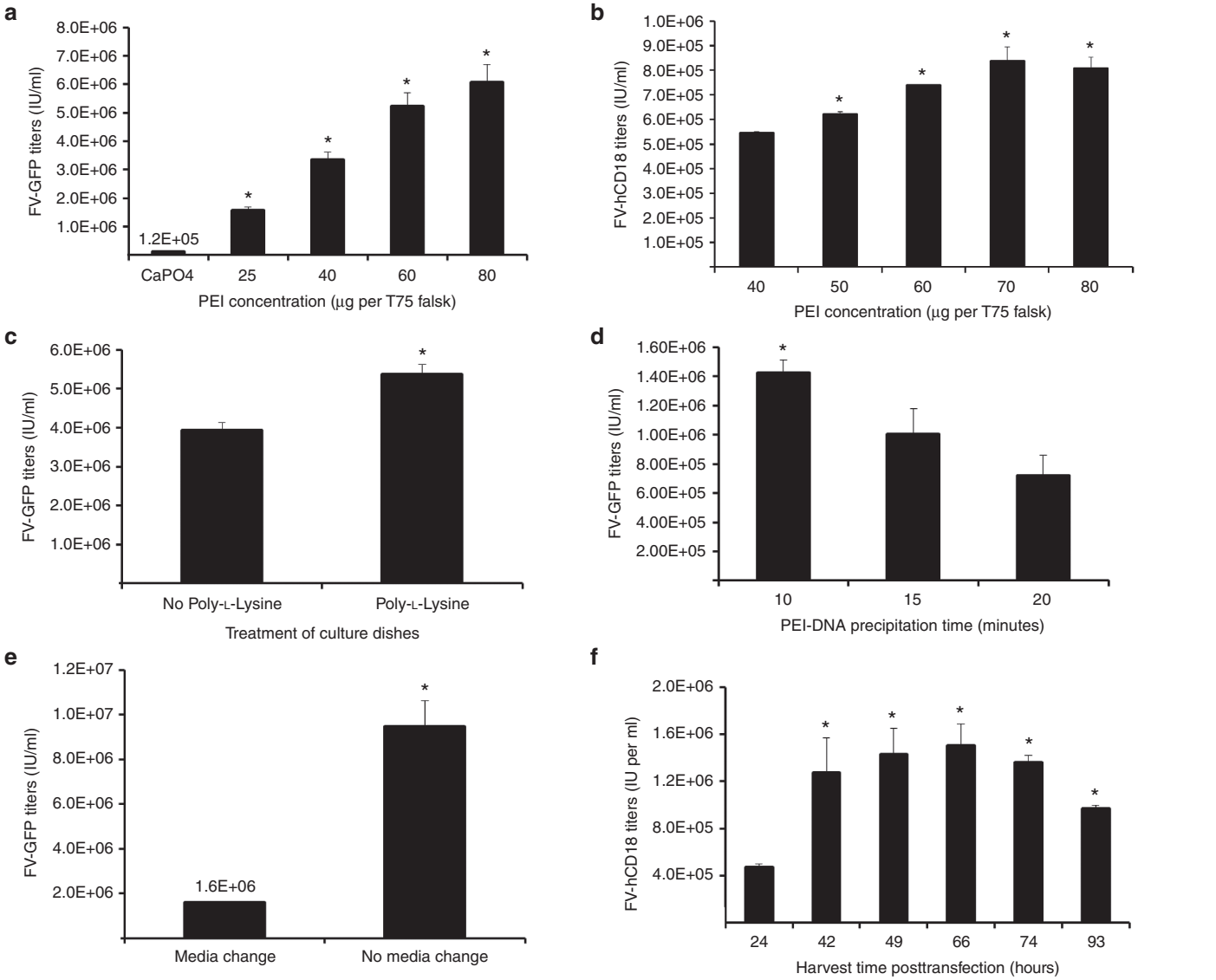

Figure 1 Optimization of FV vector transfection to maximize FV vector titers. HEK293T cells were transfected under various experimental conditions with FV vector plasmids. FV vector supernatants were harvested three days posttransfection and titer (IU/ml) was estimated by infecting HT1080 cells (FV-GFP) or RAW264.7 cells (FV-hCD18). (**a**) FV-GFP plasmid transfection using calcium phosphate or increasing concentrations of PEI, ranging from 25 to 80 µg per T75 flask ($n = 3$, $^*P \leq 0.05$, as compared to CaPO4 transfection). (**b**) FV-hCD18 plasmid transfection using increasing concentrations of PEI ($n = 3$, $^*P \leq 0.05$, as compared to 40 µg PEI). (**c**) FV-GFP plasmid transfection in culture vessels untreated (left bar) or treated (right bar) with poly-L-lysine ($n = 3$, $^*P \leq 0.05$). (**d**) FV-GFP plasmid transfection using various PEI-DNA precipitation times ($n = 3$, $^*P \leq 0.05$, as compared to a 15 minute precipitation time). (**e**) FV-GFP plasmid transfection with or without change of PEI containing transfection media from producer cells ($n = 3$, $^*P \leq 0.05$). (**f**) Optimization of harvest time after FV-hCD18 plasmid transfection ($n = 3$, $^*P \leq 0.05$, as compared to the 24-hour time point).

Concentration of FV vectors

TFF is a rapid, efficient, and scalable method for concentration of small and large volumes of biological samples. Here, we used TFF as a method to concentrate heparin affinity chromatography purified FV vector. Ultrafiltration was performed by recirculating the sample at 280 ml per minute through a TFF cartridge with a 750 KDa nominal cut off using a trans-membrane pressure between 5 and 6 psi. Vector particles were retained within the membrane, whereas proteins smaller than 750 kDa were removed resulting in concentration and further purification of the vector. Vector was subsequently diafiltered using 100 ml of 150 mmol/l NaCl, 25 mmol/l Tris–HCl (pH 7.4) buffer. This step changed the concentration of salt to a physiological level. Using TFF, vectors were concentrated 20- to 30-fold with an average recovery of 89 ± 13% ($n = 5$) as shown in Table 2. The material was subsequently concentrated by ultracentrifugation at 50,000*g* for 2 hours. Pellets were resuspended in final formulation buffer consisting of X-VIVO 10, 1% human serum albumin, and 5% DMSO. This last step concentrated the vector an additional 60-fold with 48 ± 14% ($n = 5$) recovery (Table 2). Overall, using the optimized conditions established for heparin affinity chromatography, TFF, and ultracentrifugation, the FV vectors were concentrated ~5,000-fold with a net recovery of 19 ± 3.1% ($n = 5$).

FV-hCD18 vector transduction

We next tested the ability of purified FV-hCD18 vectors to transduce granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood $CD34^+$ cells obtained from two subjects diagnosed with LAD-1, using two independent FV-hCD18 vector pilot batches

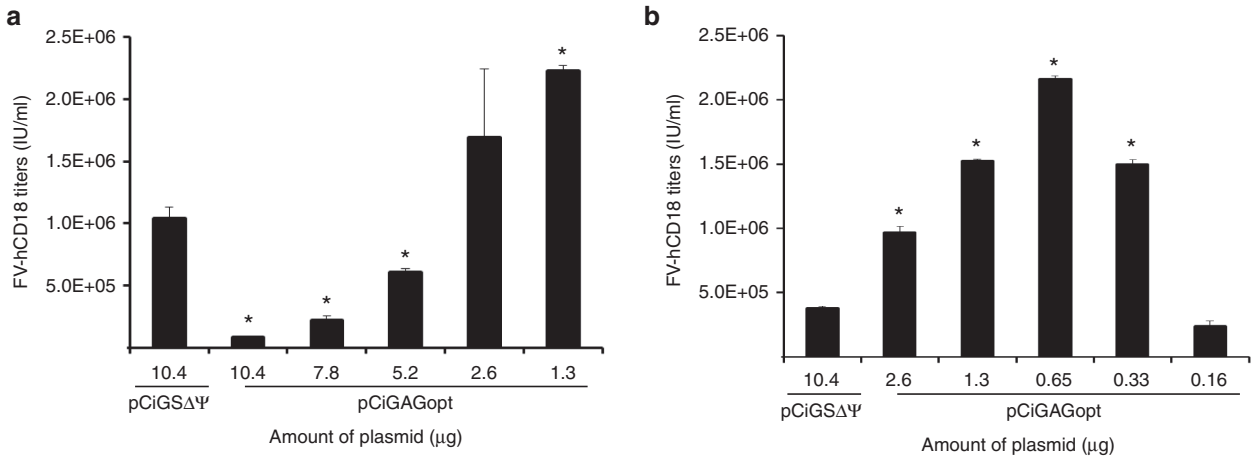

Figure 2 Codon-optimized gag plasmid for FV vector production. FV-hCD18 vectors were produced by PEI-mediated transfection of HEK293T cells with FV vector packaging plasmids, including either the previously optimized amount of the original *gag* plasmid (pCiGS 10.4 μg) or various amounts of the codon-optimized *gag* plasmid (pCiGAGopt). Vector supernatants were harvested three days post-transfection and titers were estimated using RAW 264.7 cells. (**a**) Transfection of HEK293T cells with amounts of pCiGAGopt ranging from 10.4 to 1.3 μg per T75 flask ($n = 3$, $^*P \leq 0.05$, as compared to 10.4 μg of pCiGSΔΨ). (**b**) Transfection of HEK293T cells with amounts of pCiGAGopt ranging from 2.6 to 0.16 μg per T75 flask ($n = 3$, $^*P \leq 0.05$, as compared to 10.4 μg of pCiGSΔΨ).

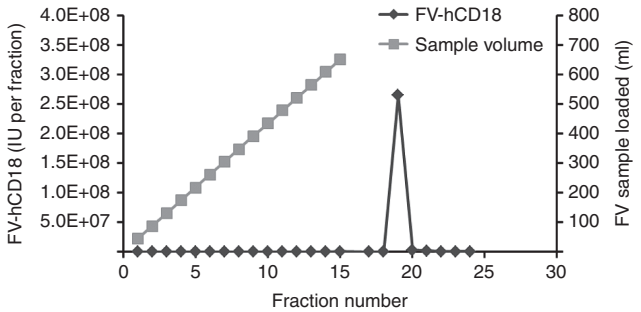

Figure 3 Purification of FV-hCD18 vector supernatants with heparin affinity chromatography. FV-hCD18 vectors were purified using optimal conditions of sample loading and washing. The elution was carried out with 600 mmol/l NaCl. Infectious unit (IU) of FV vectors was estimated after infecting cells with the diluted fractions of FV vector samples. Line with gray squares shows the volume of FV sample loaded; the line with dark diamonds shows the total infectious units of FV-hCD18 vector in each fraction (43.5 ml).

Table 1 Recovery of FV-hCD18 vector after each step of heparin affinity chromatography run

Step	*% of recovery (average ± SEM)*[a]	*n*
Pre-load	100 ± 0	5
Loading	4 ± 1.8	5
Washing	0 ± 0	5
Elution	69 ± 2.7	5

[a]Data represent mean and SEM of five independent experiments.

(Figure 4). CD34$^+$ cells were cultured in the presence of cytokines on Retronectin-coated plates and transduced for 16 hours with concentrated and purified FV-hCD18 vector at various dilutions. Cells were washed and continued in culture for an additional 3 days to allow maximal detection of CD18 expression by flow cytometry. Since DMSO must be added for optimal recovery of FV vectors after cryopreservation, the highly concentrated FV-hCD18 vector was

Table 2 Estimated recovery from 1 l of FV-hCD18 vector after each step of purification

Step	*Volume of vector (ml)*	*Processing time*	*% of step recovery (average ± SEM)*[a]	*n*
Heparin column	333.3	5 hours	69 ± 2.7	5
TFF	11.9	45 minutes	89 ± 5.8	5
0.2 m filter	11.9	15 minutes	84 ± 4.5	5
Ultracentrifugation	0.2	2 hours	48 ± 6.3	5
Net recovery	0.2	8 hours[b]	19 ± 3.1	5

[a]Data represent mean and standard error of mean (SEM) of five independent experiments. [b]Total processing time.

diluted to reduce DMSO concentration to ≤0.1% to limit the toxicity to CD34$^+$ cells during transduction. Increasing doses of DMSO, especially with a prolonged exposure are well known to be toxic to murine and human hematopoietic cells and other types of stem cells, including human embryonic stem cells.[26–28] We confirmed these results and observed reduced viable CD34+ cells when DMSO concentrations exceeded 0.1% (Supplementary Figure S4). For both subjects, percentages of transduction in bulk CD34$^+$ cells increased proportionally with increasing volumes of FV vector. Subject 1 has a moderate clinical phenotype and 18.7% of CD34+ cells expressed CD18 at baseline; CD18$^+$ cells increased to 77.4% (*i.e.*, 59% over baseline CD18 expression) after transduction at the highest MOI of FV vector tested. This level was similar to baseline CD18$^+$ cells (87.3%) measured in mobilized peripheral blood CD34$^+$ cells from a healthy subject (Figure 4, upper panel). In subject 2 with a severe phenotype, no CD18$^+$ cells were detected at baseline. Up to 26.4 and 21.2% of LAD-1 CD34+ cells expressed CD18 after transduction with FV vector batch 1 (Figure 4, middle panel) and 2 (Figure 4, lower panel), respectively. For both subjects, FV-hCD18 vector had negligible impact on cell viability and cell growth, as measured 3 days after transduction, compared to untransduced LAD-1 CD34+ cells. Overall, these experiments provide proof of principle that clinical-grade high-titer FV vectors can be produced and purified

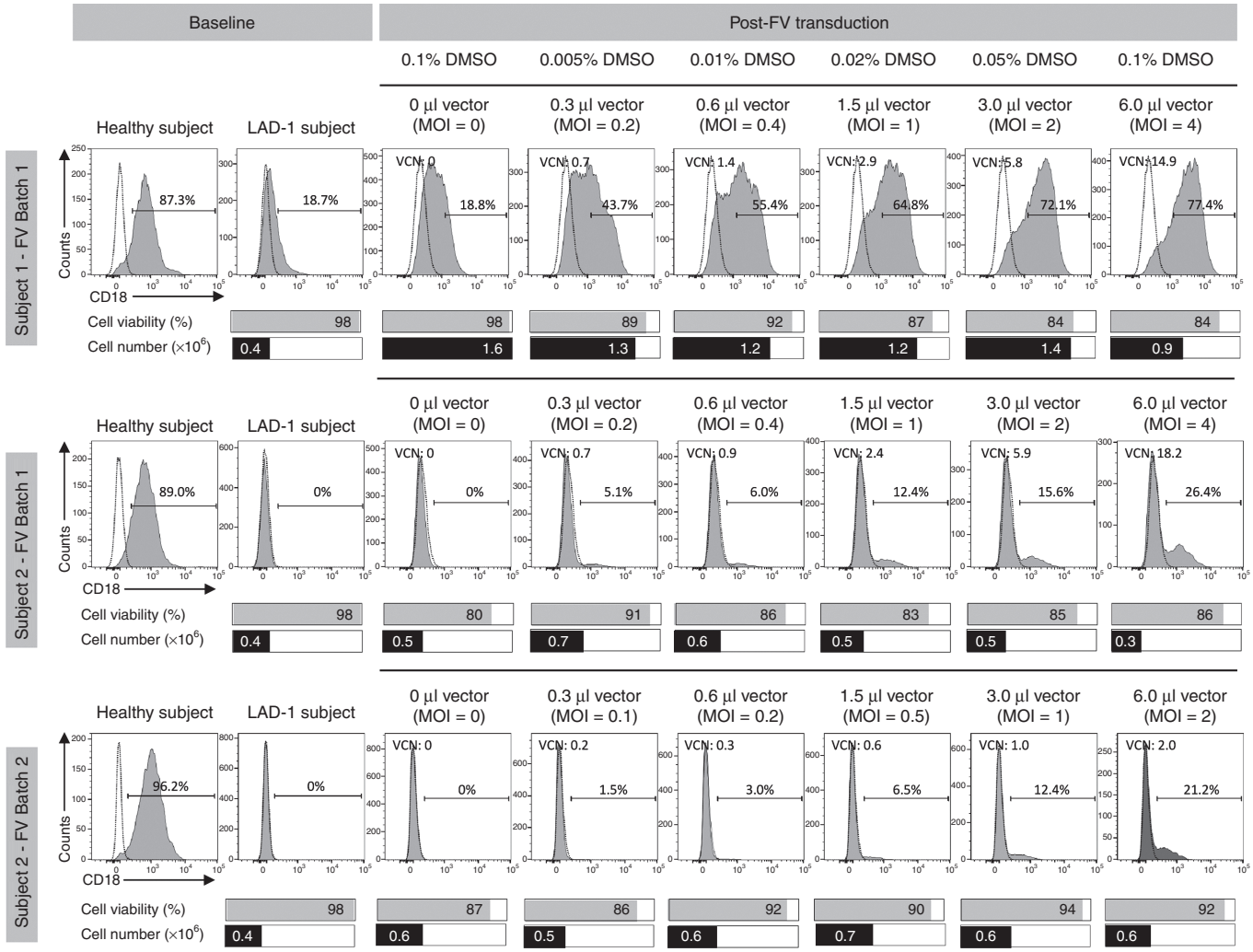

Figure 4 Transduction of LAD-1 CD34+ cells with concentrated FV-hCD18 vector. G-CSF mobilized CD34+ cells from two subjects with LAD-1 were transduced with various MOI of FV-hCD18 vectors produced, purified, and concentrated using an optimized protocol in two independent pilot batches. MOIs are based on titers of FV-hCD18 vector which were measured on Raw264.7 cells. CD18 expression (solid histograms), cell viability (gray bars), and cell number (black bars) were determined for subject 1 (upper panel) and subject 2 (middle and lower panels) at baseline (left panel) and after transduction (right panel) with FV-hCD18 vector pilot batch 1 (subject 1) or batches 1 and 2 (subject 2). CD18 expression is shown in healthy subjects for comparison (left panel). Dashed histograms: isotype controls; VCN: vector copy number per diploid genome.

for efficient transduction of LAD CD34+ cells with minimal DMSO-related toxicities.

DISCUSSION

FV vectors represent a potentially safer alternative to currently used integrating viral vectors for gene therapy application. However, approaches customarily used to manufacture large-scale lentiviral vector for clinical application have resulted in low titers for FV vectors,[6,7] hampering their clinical development. In this study, we have presented process development with a step-by-step optimization of FV vector production and purification (Figure 5).

PEI-mediated transfection of FV plasmids into HEK293T cells significantly increases the titers over those achieved with calcium phosphate.[11] PEI has the ability to avoid trafficking to degradative lysosomes and its buffering capacity leads to osmotic swelling and rupture of endosomes, resulting in release of the vector particles into the cytoplasm and subsequently to the culture medium.[29] PEI has a high cationic charge density at physiological pH due to partial protonation of the amino groups in every third position. These amino groups form noncovalent complexes with negatively charged DNA, which leads to condensation and shielding of the negative charges, thereby allowing endocytosis into the cells, resulting in efficient transfection of vector producer cells.[30]

Substantial plasmid DNA contamination is carried over in vector supernatants produced by transient transfection.[31] Plasmid DNA present in vector supernatants artificially increases the PCR-based titer of vectors and may be toxic to primary cells such as hematopoietic stem and progenitor cells exposed to the concentrated vectors. Nucleic acids also result in increased supernatant viscosity which interferes with purification steps and reduces vector titers. Addition of benzonase endonuclease during FV vector production allowed complete digestion of all forms of DNA and RNA to 5′-monophosphate terminated oligonucleotides 2 to 5 bases in length.[32] It is effective over a wide range of temperature and pH and has no proteolytic activity, providing a simple approach to enhance FV vector production. Our data supports that Benzonase endonuclease can be safely used in the manufacture of FV vector without significant loss of infectious titer.

Commonly used purification methods such as ultracentrifugation can precipitate FV particles along with cellular debris and serum

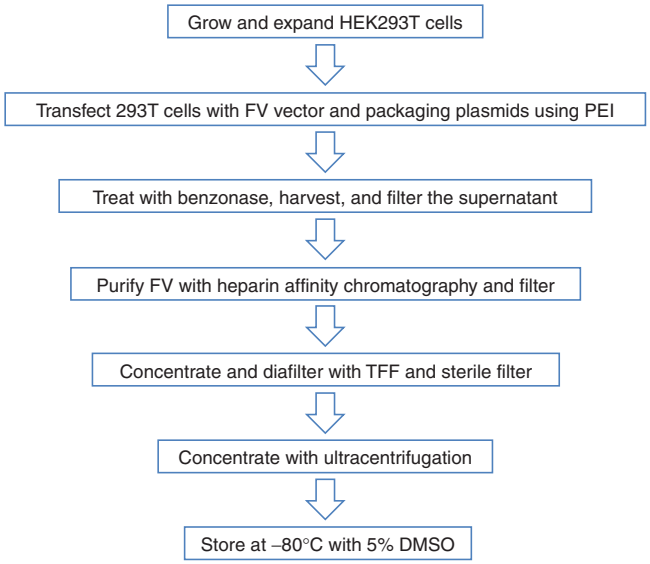

Figure 5 Flow diagram of FV vector production and purification. HEK293T producer cells were seeded in cell culture vessels treated with poly-L-Lysine and FV vector plasmids were transfected into the cells with PEI. Cultures were treated with Benzonase for 16 hours prior to vector harvest. FV supernate was filtered, purified with heparin affinity chromatography and filtered, concentrated, and diafiltered with TFF, sterile filtered, and concentrated aseptically using ultracentrifugation. FV vector supernatants were stored at −80 °C in the presence of 5% DMSO.

proteins[33] which can be toxic to the target cells. Heparin affinity medium strongly binds only those particles that have affinity for heparin molecules.[34,35] Unbound and loosely bound material present in FV supernatant, including cellular debris and serum proteins, elute in the flow-through during sample loading and washing with low salt containing buffer. The FV-heparin interaction is stable but reversible, requiring relatively low salt concentrations for dissociation as demonstrated here. This is important in considering the susceptibility of retroviruses to osmotic shock[36] and limited stability of FV vectors in high salt (data not shown).

In contrast to conventional heparin affinity chromatography medium, POROS perfusion chromatography medium is engineered to have two discreet classes of pores. Large "through pores" allow convection flow to occur through the particles themselves, quickly carrying sample molecules to short "diffusive" pores inside. By reducing the distance over which diffusion needs to occur, the time required for sample molecules to interact with interior binding sites is also reduced. Diffusion is no longer limiting and flow rates can be dramatically increased without compromising resolution or capacity. Separation can be achieved at speeds up to 100-fold faster as compared to conventional heparin medium.[37] We have carefully optimized the binding conditions and found POROS-Heparin to be superior in its ability to effectively capture FV particles as compared to Heparin-Sepharose medium such as Hi-Trap Heparin (data not shown).

The stability of vectors is strongly dependent on ultrafiltration parameters such as trans-membrane pressure, shear, and process run duration.[38] These parameters were optimized to maximize the concentration and recovery of FV vector. Although higher shear forces were helpful in reducing membrane fouling, these reduced vector titer (data not shown). Shear values between 2,000 and 3,000 s^{-1} resulted in an 89% recovery of infectious virus particles in our study. Membrane fouling was not an issue since most of the proteins were removed during the chromatography run. Since TFF is a closed system and ultracentrifugation tubes are sealed prior to the centrifugation step, both are compatible with clinical grade vector production.[21,22]

After optimization of the process, two pilot batches of FV vectors produced showed 21–59% transduction efficiencies in G-CSF mobilized $CD34^+$ cells derived from two LAD-1 subjects. In a preclinical gene therapy study of canine LAD, clinical benefit was observed with CD18 gene marking of 14–25% in bulk canine hematopoietic stem and progenitor cells after transduction,[9,39] suggesting clinically relevant transduction efficiencies were achieved. FV vector cryopreservation necessitates 5% DMSO[1][40] and, therefore, further escalation of FV vector volumes during transduction was not feasible due to DMSO-induced toxicity on target $CD34^+$ cells (Supplementary Figure S4). Despite nearly identical vector copy number between subjects 1 and 2, expression of CD18 was quite different. The timing of flow cytometry for optimal CD18 gene expression in bulk $CD34^+$ cells after transduction may vary between patients. For consistency, we have chosen a period of 72 hours for both subjects but this may not be optimal for subject 2. Given the scarcity of LAD CD34+ cells, kinetic expression studies are impractical. Other explanations related to molecular differences (different mutations), phenotypic differences (subject 1: moderate; subject 2: severe), age differences (subject 1:19YO; subject 2:33 YO), or technical differences (widely different duration of cryopreservation of CD34+ cells, 4 years versus 1 month) between subjects 1 and 2 cannot be entirely ruled out. Given that transduction differed between the two patients tested here, it may be helpful to examine transduction efficiencies of patients $CD34^+$ cells prior to gene therapy to optimize clinical transduction and even attempt correlating with heparan sulfate expression. If differences in transduction correlate with heparan sulfate levels, heparan sulfate expression may be used as a marker to predict transducibility. Based on the average FV titers using this methodology and the data in Figure 4, where 450,000 cells transduced at 21.2% with 6 µl FV vector, transduction of 250 million cells (to treat a 50 kg individual with 5×10^6 transduced cells/kg) will require approximately 3 ml of 5,000-fold concentrated vector. This represents the equivalent of approximately 15 l of initial culture volume per patient, which is feasible from the manufacturing standpoint. In addition, canine data and some of our unpublished results show that transduction efficiencies of ~20% are sufficient for long-term correction of LAD. Therefore, the FV vector production process described in this study paves the way to scale-up FV production for clinical manufacturing of FV-hCD18 vectors for a clinical trial in LAD-1 patients.

MATERIALS AND METHODS

Plasmids

Self-inactivating FV gene-transfer vector plasmids pΔΦ-MSCV-green fluorescent protein (GFP) and pΔΦ-MSCV-huCD18, as well as packaging gene plasmids pCiGSΔΨ (*gag*), pCiGAGopt (codon optimized *gag*), pCiPS (*pol*), and pCiES (*env*) (Supplementary Figure S5) were constructed by Dr David Russell.[7] FV gene transfer, *gag*, *pol*, and *env* vector plasmids were used at a ratio of 14:14:2:1. When *gag* plasmid pCiGAGopt was used instead of pCiGSΔΨ, a 16-fold lower concentration of the plasmid was used for optimal titer. Plasmids were manufactured by Puresyn (Malvern, PA).

Cell culture

Human embryonic kidney cell line HEK293T, mouse macrophage cell line RAW 264.7, and human fibrosarcoma cell line HT1080 were grown in Dulbecco's modified Eagle's medium, high glucose, (DMEM; Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum, 1 mmol/l L-glutamax, 1 mmol/l sodium pyruvate, and 1 mmol/l nonessential amino acids (Invitrogen, San Diego, CA). Human $CD34^+$ cells from two LAD-1 patients were cultured in

StemSpan Serum-Free Expansion Media (SFEM) II (StemCell Technologies, Vancouver, BC, Canada) containing penicillin–streptomycin and cytokines (hereafter referred to as $CD34^+$ cell culture medium), including 300 ng/ml human stem cell factor, 100 ng/ml human thrombopoietin and 300 ng/ml human FLT3 ligand (all from PeproTech, Rocky Hill, NJ). All cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Vector production

In some experiments, FV vectors were produced by calcium phosphate-mediated transient transfection, as described previously.[6,7,41] In most experiments, FV vectors were produced by PEI (Polyplus-Transfection, France)-mediated transient transfection. HEK293T cells were seeded in growth media in tissue culture treated flasks or CellSTACKS pre-coated with poly-L-lysine (Sigma-Aldrich) at 0.01% for 10 minutes at ambient temperature. For transfection, FV plasmids and PEI solution were diluted each in serum-free DMEM, combined, and mixed by swirling. The mixture was incubated for 10–20 minutes (with 10 minutes being optimal) at ambient temperature to allow for the formation of a DNA-PEI precipitate. The used medium was removed from the cells and fresh growth medium containing the transfection reaction mixture was added. Transfected cells were cultured for ~48 hours and subsequently treated with 50 Units/ml of Benzonase endonuclease (Millipore, Bellerica, MA) in media containing 10 mmol/l $MgCl_2$ at 37 °C for ~16 hours to digest residual plasmid, genomic DNA, and RNA. FV supernatants were harvested and clarified by passing through a leukocyte reduction filter (LRF; Pall) and 0.45 µm Gamma Gold filter (Millipore, Bellerica, MA). FV supernatants were stored at −80 °C with 5% DMSO or purified immediately.

Vector purification and concentration

Since FV reversibly binds heparin molecules, heparin affinity chromatography was used for the capture of FV vectors from media derived from transfected cultures. The resin, POROS Heparin (Applied Biosystems, San Diego, CA) contains an immobilized heparin functional group designed for high-throughput purification of proteins or viruses with specific affinity for heparin. Filtered FV vector supernatants were loaded onto a POROS-OH 50 µm heparin column using an AKTAvant 150 (GE Healthcare, Piscataway, NJ) chromatography system running with Unicorn 6.2 software, with a linear flow rate of 267 cm/hour and residence time of 2.3 minutes. After loading, the column was washed with 20 mmol/l sodium phosphate (pH 7.4) or 25 mmol/l Tris–HCl (pH 7.4) buffer containing 150 mmol/l sodium chloride. Washing continued until the ultraviolet absorbance curve (280 nm) returned to baseline and stabilized. Bound FV vector particles were eluted from the heparin column in 25 mmol/l Tris–HCl containing 600 mmol/l NaCl. Upon collection, vector was immediately diluted to a final concentration 150 mmol/l NaCl using 25 mmol/l Tris–HCl (pH 7.4). Post-chromatography, vector was clarified using a 0.45 µm filter and concentrated 25 to 30-fold using a 750 kDa TFF column (UFP-750-C-3MA, GE Healthcare, Piscataway, NJ). Vector was diafiltered with a 10-fold excess of 25 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl. The concentrated retentate was sterile filtered through 0.22 µm pore size filter and subjected to ultracentrifugation in pre-sterilized ultra-centrifuge tubes (Beckman Coulter, Indianapolis, IN) at 50,000*g* for 2 hours at 11 °C using aseptic technique. The pellet containing the vector was resuspended by pipetting up and down in X-VIVO 10 (Lonza, Allendale, NJ) containing 1% human serum albumin. The vector was resuspended in 5% DMSO (Sigma, St Louis, MO) to obtain a concentration factor of ~5,000-fold as compared to the starting material. Vector was frozen on dry ice, and stored at −80 °C.

Vector titration

Infectious titers of FV-GFP were determined using human HT1080 cells. Infectious titers of FV vector expressing CD18 cDNA were determined on RAW264.7 murine monocytic cell line. RAW264.7 cells express mouse but not human CD11/CD18. When transduced with FV vector expressing human CD18 cDNA, the human CD18 cross-heterodimerizes with mouse CD11. Transduced cells expressing the mouse/human hybrid CD11/CD18 are identified by flow cytometry using a fluorescently labeled mouse anti-human CD18 monoclonal antibody (Supplementary Figure S6). Since titers vary with the cell type, FV-GFP infectious titers were compared on both HT1080 and RAW cells and found to be an order of magnitude higher in HT1080 cells, in general. Briefly, cells were seeded at 5×10^4 cells/well in a 24-well plate 1 day before infection. FV vector supernatants were added to the cells at limiting dilution. Medium was replaced with fresh growth medium the next day. Three days post-transduction, cells were harvested, and analyzed for GFP expression or stained with mouse anti-human CD18-APC (Clone 6.7, BD Biosciences, San Diego, CA) diluted in 1% bovine serum albumin (BSA) in PBS. Cells were washed with 1% BSA in PBS and analyzed on a flow cytometer (LSR-Fortessa, BD Biosciences). Titers (IU per ml) were calculated based on the number of cells at the time of infection, the dilution factor, and percentage of GFP^+ or $CD18^+$ cells.

Mobilization, apheresis, and purification of human LAD-1 CD34+ cells

Two subjects with LAD-1 received G-CSF 10 µg/kg (Amgen, Thousand Oaks, CA) for 5 days, given as a single daily s.c. injection. Large volume (15 l) leukapheresis was initiated on the morning of day 5 of G-CSF administration, using a blood cell separator (Cobe Spectra, Terumo BCT, Lakewood, CO). The mononuclear cell (MNC) concentrates were enriched in $CD34^+$ cells using a semi-automated CliniMACS Plus instrument (Miltenyi Biotec, Auburn, CA) and cryopreserved prior to transduction. All subjects gave written informed consent on treatment protocols approved by the Institutional Review Board (IRB) of the National Heart, Lung and Blood Institute (NHLBI), National Institutes of Health (NIH), in accordance with the Declaration of Helsinki.

Transduction of human LAD-1 CD34+ cells

Human LAD-1 $CD34^+$ cells (450,000 cells/well) were transduced with different volumes of FV-hCD18 in 300 µl $CD34^+$ cell culture medium in 24-well tissue culture plates coated with RetroNectin 5 µg/cm^2 (TaKaRa, Shiga, Japan). Plates were subjected to spinoculation at 300*g* for 5 minutes and incubated overnight (16–17 hours) at 37 °C. The following morning, FV vector supernatant was removed and fresh $CD34^+$ cell culture medium was added. Three days posttransduction, cells were collected by gentle scraping, stained with anti-human CD18-FITC antibody (clone 6.7, BD Biosciences, San Jose, CA), and analyzed by flow cytometry using a LSR Fortessa instrument (BD Biosciences).

Real-time PCR for vector copy number determination

The presence of CD18 proviral sequences in genomic DNA isolated from CD34+ cells after transduction was determined using the ABI PRISM 7500 Real-Time PCR System (Life Technologies, Grand Island, NY). Briefly, primers MSCV-F (5′-AGTCCTCCGATAGACTGC GT-3′), and CD18-R (5′-CTTCGTGCACTCCTGAGAGA-3′) amplified a vector-specific 123-bp fragment spanning the MSCV promoter and hCD18 cDNA. Amplification was detected with the MSCV-CD18 probe (5′- /56-FAM/TCTCCACCA/ZEN/TGCTGGGCCTG/3IABkFQ/ - 3′). The human albumin gene was used as an endogenous control for data normalization. Primers Hs Albumin-F (5′-GCT CTC CTG CCT GTT CTT TA -3′) and Hs Albumin R (5′- GGATTCTGTG CAGCATTTGG -3′) amplified a 204-bp fragment spanning the intron 11-exon 12 junction of the human albumin gene. Amplification was detected with the Hs Albumin probe (5′- /56-FAM/CCGTGGT CC/ZEN/TGAACCAGTTAT GTGT/3IABkFQ/-3′). Amplification of plasmids containing cloned target sequences of MSCV-hCD18 or Hs Albumin intron 11-exon 12 junction was used to prepare a standard curve to quantify the number of FV-hCD18 vector integrations per diploid genome. For multiplex pPCR reactions, the FV- and albumin-specific amplicon primers were used in combination with the FAM-labeled, vector-specific TaqMan probe (MSCV-CD18) described above and the following albumin gene-specific TaqMan probe: 5′-/56-JOE NHS/CCGTGGTCC/ZEN/TGAACCAGTTATGTGT/3IABkFQ/-3′. Samples underwent denaturation at 95 °C for 10 minutes, followed by 40 cycles of amplification (15 seconds at 95 °C, 1 minute at 60 °C).

Statistical analysis

Statistical analysis was done using a two-tailed Student's *t*-test. A *P* value of ≤ 0.05 was considered statistically significant.

CONFLICT OF INTEREST

We declare that there is no financial conflict of interest in our work.

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