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SHORT COMMUNICATION

Lentiviral vectors incorporating a human elongation factor 1α promoter for the treatment of canine leukocyte adhesion deficiency

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Canine leukocyte adhesion deficiency (CLAD) provides a unique large animal model for testing new therapeutic approaches for the treatment of children with leukocyte adhesion deficiency (LAD). In our CLAD model, we examined two different fragments of the human elongation factor 1α (EF1α) promoter (EF1αL, 1189 bp and EF1αS, 233 bp) driving the expression of canine CD18 in a self-inactivating (SIN) lentiviral vector. The EF1αS vector resulted in the highest levels of canine CD18 expression in CLAD CD34⁺ cells in vitro*. Subsequently, autologous CD34⁺ bone marrow cells from four CLAD pups were transduced with the EF1αS vector and infused following a non-myeloablative dose of 200 cGy total-body irradiation. None of the CLAD pups achieved levels of circulating CD18⁺ neutrophils sufficient to reverse the CLAD phenotype, and all four animals were euthanized because of infections within 9 weeks of treatment. These results indicate that the EF1αS promoter-driven CD18 expression in the context of a RRLSIN lentiviral vector does not lead to sufficient numbers of CD18⁺ neutrophils* in vivo *to reverse the CLAD phenotype when used in a non-myeloablative transplant regimen in dogs.*
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Introduction

Canine leukocyte adhesion deficiency (CLAD), the canine homologue of leukocyte adhesion deficiency (LAD)-I in humans, is characterized by severe, recurrent bacterial infections leading to death within the first 6 months of life.[1,2] CLAD animals are homozygous for the identical 107G>C nucleotide substitution in the CD18 coding sequence, which results in a C36S amino acid substitution in the CD18 protein.[3]

In previous studies, we used the CLAD model to demonstrate that both γ-retroviral and foamy viral vector-mediated transfer of the canine CD18 cDNA can reverse the CLAD phenotype.[4,5] However, both studies relied on the long terminal repeat (LTR) of the murine stem cell virus (MSCV) promoter to express the canine CD18 cDNA; in the γ-retroviral vector, the MSCV 5′LTR directed the expression of canine CD18, whereas in the foamy viral vector, an internal MSCV LTR promoter fragment directed canine CD18 expression.[4,5]

Recently, insertional mutagenesis from retroviral promoters, particularly the murine leukemia virus LTR promoter/enhancer, has emerged as a serious concern in hematopoietic stem cell gene therapy. In the gene therapy clinical trials for the treatment of X-linked severe combined immunodeficiency disease, and chronic granulomatous disease, insertional activation of nearby proto-oncogenes by a strong retroviral LTR promoter led to leukemia and myelodysplastic syndrome, respectively.[6,7] Subsequent research has demonstrated that replacement of retroviral LTR promoters by cellular promoters may reduce the risk of insertional activation from viral promoters/enhancers.[8]

The human elongation factor 1α (EF1α) has been shown to direct the expression of reporter genes in a variety of cell types, including neural and lymphoid cells.[9] The EF1α promoter (nucleotides 373–1561) has also been shown to be a strong promoter in primary human CD34⁺ cells.[10–13] When used within the context of a self-inactivating (SIN) lentiviral vector, EF1α promoter-driven enhanced green fluorescent protein expression was comparable with that of the MSCV LTR, the phosphoglycerate kinase promoter, and the composite CAG promoter (consisting of the cytomegalovirus immediate early enhancer and the chicken β-actin promoter) in the human myeloid leukemia cell line, KG-1a.[12] In addition, the EF1α promoter has been shown to induce ubiquitous and high-level expression of human CD55 and CD59 in transgenic rabbits.[14]

The studies described above prompted us to test the efficacy of the human EF1α cellular promoter in a SIN lentiviral vector to direct canine CD18 expression in dogs with CLAD.

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Results and discussion

We constructed human immunodeficiency virus-based SIN lentiviral vectors expressing canine CD18 from two different lengths of the human EF1α promoter/enhancer—a 233 bp fragment consisting of nucleotides from 378 to 610 (EF1αS) and a 1189 bp fragment consisting of nucleotides from 373 to 1561 (EF1αL)[12,13] (Figure 1a). As a control, an MSCV promoter driving expression of canine CD18 was also constructed using the same lentiviral vector backbone, pRRLSIN.cPPT.WPRE. Lentiviral vectors were pseudotyped with a vesicular stomatitis virus G-glycoprotein envelope.

The CD18 subunit does not become surface expressed without the CD11 subunit; therefore, we used an Epstein-Barr virus (EBV)-transformed B-cell line derived from a LAD patient (ZJ) to determine the expression of CD18 from the three vectors. The ZJ LAD EBV B-cell line expresses the endogenous human CD11a leukocyte integrin subunit, but does not express the human CD18 subunit.[15] Transduction of normal human or canine CD18 into LAD EBV B-cells has been shown previously to rescue the CD11a subunits and result in surface expression of the CD11a–CD18 complexes.[3,16] When analyzed on day 5 after a 16 h transduction with serial dilutions of each 240 × concentrated vector, the EF1αS vector resulted in a higher percentage of $CD18^+$ cells compared with the EF1αL vector at all concentrations tested. The MSCV promoter within the same vector backbone resulted in the highest percentage of $CD18^+$ cells (Figure 1b). The titers, represented as transduction units per ml (TU ml^{-1}), were calculated for each vector in the linear range where up to 30% of the cells were $CD18^+$. The EF1αS vector yielded titers three times higher than the EF1αL vector (4.3×10^8 vs 1.3×10^8 TU ml^{-1}). All three vectors yielded comparable levels

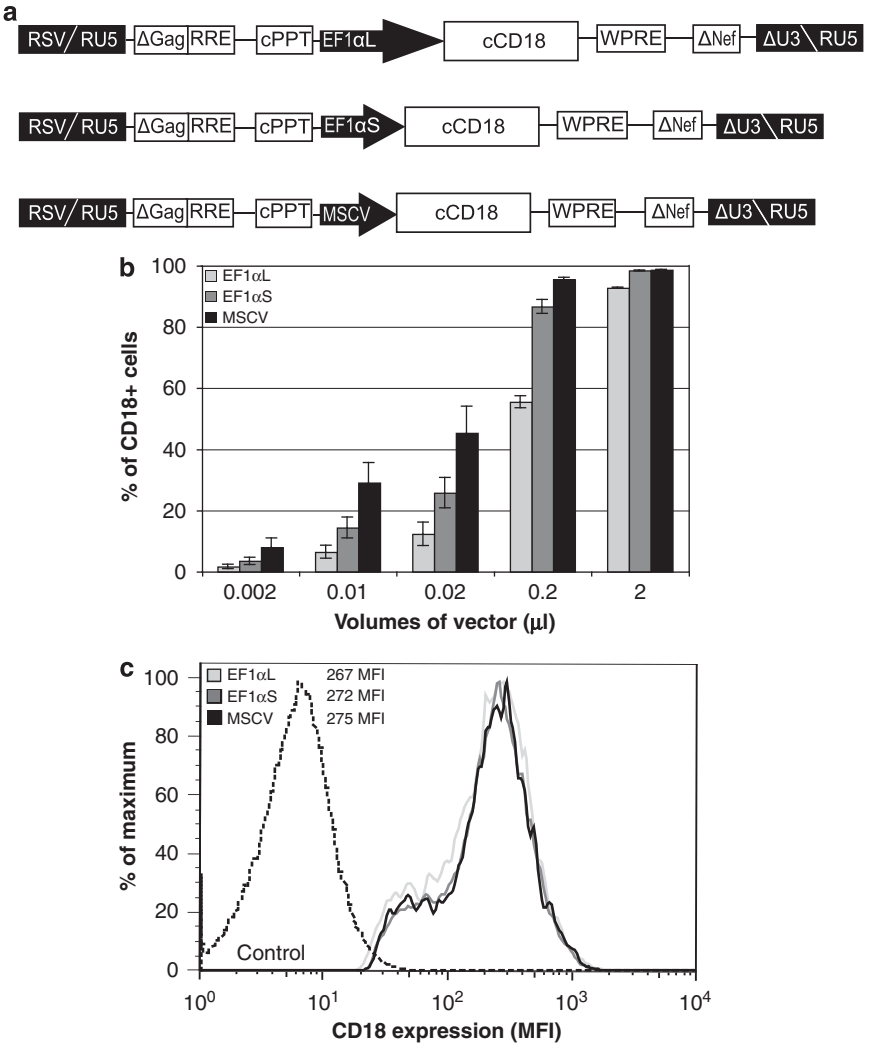

Figure 1 Construction, and testing of lentiviral vectors. (**a**) Schematic of the vector constructs. EF1α, elongation factor 1α; MSCV, murine stem cell virus; cPPT, central polypurine tract; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; RRE, Rev-responsive element; RSV, Rous sarcoma virus. **(b)** Transduction efficiency in LAD EBV B-cells (ZJ). ZJ cells (2.5×10^5 per well) were incubated with increasing volumes of each vector (240 × concentrated) in RetroNectin coated, non-tissue culture treated, 24-well plates at 37 °C. After transduction, cells were analyzed for CD18 expression by flow cytometry on day 5. The volumes of each concentrated vector (μl per well) are shown on the *x* axis. The percentage of $CD18^+$ cells is indicated on the *y* axis. **(c)** Comparison of CD18 expression in ZJ cells. Mean fluorescence intensities (MFIs) of the three vectors transduced at similar percentages are shown. The MFI of an untransduced control is shown for comparison.

of CD18 expression based on the mean fluorescence intensity values, although the MSCV vector had the highest titer (9.8×10^8 TU ml^{-1}). Thus, all three vectors expressed equivalent levels of CD18 in ZJ cells on a per cell basis (Figure 1c).

To determine the ability of the lentiviral vectors to transduce CD34$^+$ cells, CLAD bone marrow CD34$^+$ cells were incubated for 16 h with each of the vectors at multiplicities of infection of 10 and 100, and analyzed on day 5 after transduction. Representative dot plots

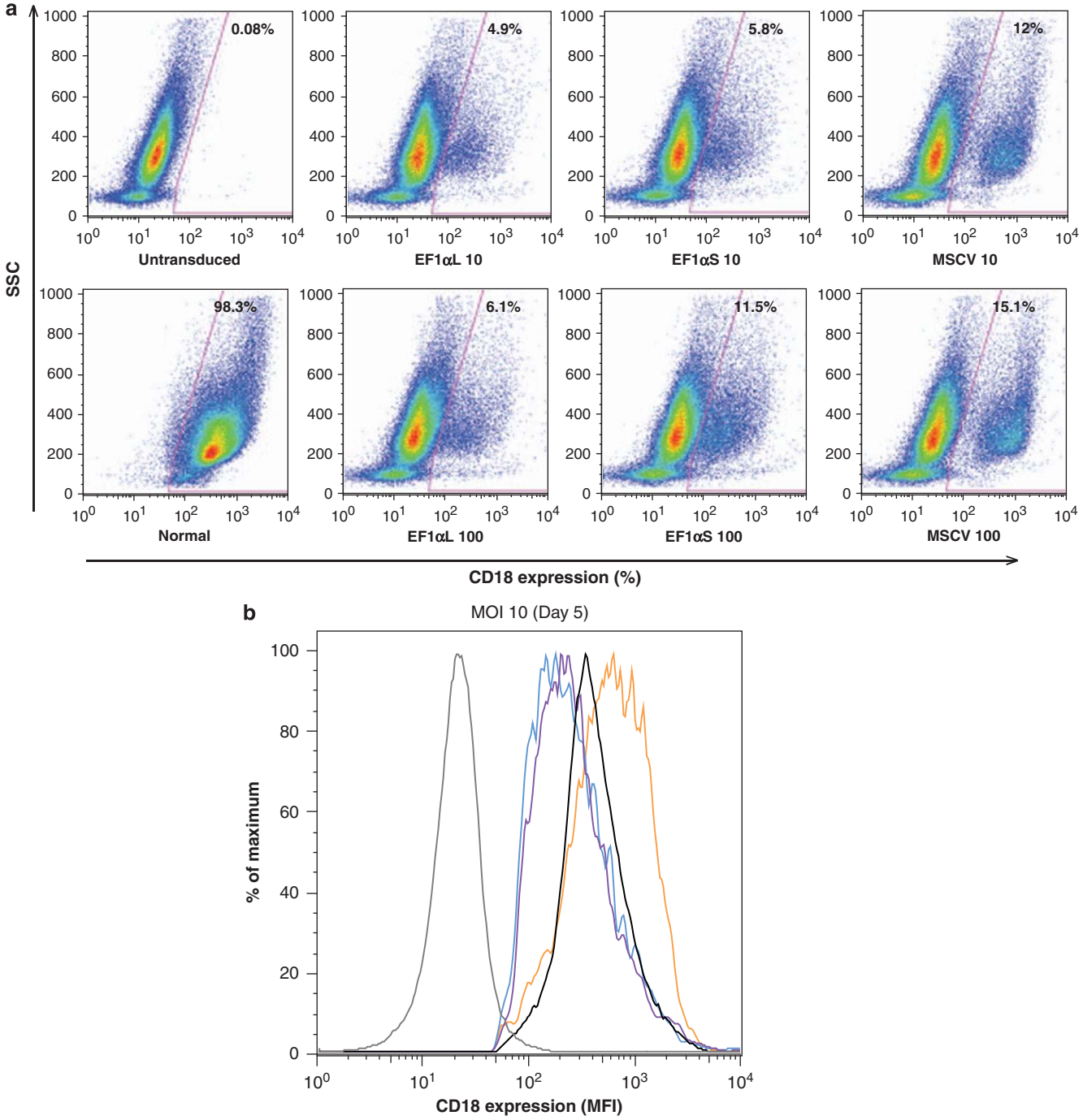

Figure 2 Transduction of CLAD CD34$^+$ cells with SIN lentiviral vectors expressing canine CD18. (**a**) CD34$^+$ bone marrow cells (5×10^5 per well) from CLAD pups were added to RetroNectin coated, non-tissue culture treated, 24-well plates. StemSpan Serum-Free Expansion Medium+10% fetal bovine serum (FBS), along with 5 μg ml^{-1} of protamine sulfate, and a cytokine cocktail consisting of 50 ng ml^{-1} each of canine IL-6, canine stem cell factor (SCF), human Flt3-L, human thrombopoietin (TPO), and human granulocyte colony-stimulating factor (G-CSF) were also added per well. Vectors were added at multiplicities of infections of 10 and 100. Plates were spinoculated (2500 r.p.m., 32 °C, 30 min) and incubated at 37 °C. After transduction, cells were analyzed for CD18 expression by flow cytometry on day 5. The *x* axis indicates CD18 expression, whereas the *y* axis indicates the side scatter (SSC). The percentages of CD18$^+$ cells are shown in the upper right-hand corner of each dot plot. **(b)** The mean fluorescence intensity (MFI) of CD18 expression in CLAD CD34$^+$ cells after transduction with the three vectors (day 5). CD18$^+$cells were gated as indicated by the selected areas in (**a**). Each vector is shown as follows: EF1αL (purple), EF1αS (blue) and MSCV (orange). MFIs corresponding to an untransduced control (gray), and that of a normal dog (black) are shown for comparison.

from a CLAD CD34$^+$ transduction experiment are shown (Figure 2a). Untransduced CLAD CD34$^+$ cells and normal canine CD34$^+$ cells served as negative and positive controls, respectively. The EF1αS vector yielded higher transduction efficiencies (5.8 and 11.5%, at multiplicities of infections of 10 and 100, respectively) than the EF1αL vector (4.9 and 6.1%). Again, the MSCV vector resulted in the highest percentage of CD18$^+$ cells (12 and 15.1%) (Figure 2a). Analysis of the mean fluorescence intensity based on CD18 expression in vector-transduced CD34$^+$ cells indicated that the expression of CD18 from both the EF1α promoters were comparable, and similar to the CD18 expression in CD34$^+$ cells from a normal dog (Figure 2b). The MSCV vector generated higher levels of CD18 expression than that observed on normal canine CD34$^+$ cells (Figure 2b).

To assess the ability of the EF1αS promoter within the context of a SIN lentiviral vector to direct canine CD18 expression on leukocytes *in vivo*, we used this vector in an *ex vivo* gene therapy protocol that we had used previously to test foamy viral vectors in the treatment of CLAD.[5] We selected the EF1αS vector because of its higher titer and higher transduction efficiency in CLAD CD34$^+$ cells compared with the EF1αL vector. Autologous bone marrow-derived CD34$^+$ cells from four CLAD pups were transduced in a 16 h exposure to the RRLSIN.cPPT.EF1αS.cCD18. WPRE lentiviral vector plus cytokines on RetroNectin. After transduction, cells were harvested and infused into animals that had received a single, non-myeloablative dose of 200 cGy TBI on the day before infusion to facilitate engraftment. All four CLAD pups were treated at approximately 7 weeks of age. Initial *ex vivo* transduction efficiency ranged from 4.1 to 8.9%, leading to an estimated range of 0.41×10^6–1.41×10^6 CD18$^+$CD34$^+$ cells kg^{-1} at the time of infusion (Table 1).

Table 1 Comparison of cell doses and outcomes with lentiviral vector (LV), foamy viral vector (FV) and γ-retroviral (RV) vector transduced CD18$^+$CD34$^+$ cells infused into CLAD dogs

Dog	*CD18$^+$ cells (%)*	*CD18$^+$CD34$^+$ cells per kg*	*CD18$^+$ PBL (% at 8 weeks)*	*Outcome*
LV1	7.5	0.71×10^6	0.3	Death at 8.4 weeks
LV2	8.9	1.41×10^6	0.2	Death at 9.0 weeks
LV3	6.8	0.71×10^6	0.2	Death at 7.7 weeks
LV4	4.1	0.41×10^6	0.2*	Death at 3.4 weeks
FV1	13.7	0.32×10^6	1.4	Alive >1 year
FV2	24.6	0.42×10^6	2.4	Alive >1 year
FV3	23.2	0.72×10^6	2.2	Alive >1 year
FV4	22.2	0.75×10^6	0.8	Alive >1 year
RV1	21.1	0.17×10^6	0.5	Alive >1 year
RV2	11.6	0.61×10^6	0.3	Alive >1 year

Abbreviations: CLAD, canine leukocyte adhesion deficiency; EF, elongation factor; LTR, long terminal repeat; MSCV, murine stem cell virus; PBL, peripheral blood leukocyte.
Lentiviral vector used the EF1αS promoter to express canine CD18 cDNA; foamy viral vector used the MSCV internal promoter to express canine CD18 cDNA; and γ-retroviral vector used the MSCV LTR to express canine CD18 cDNA. *Timepoint before death.

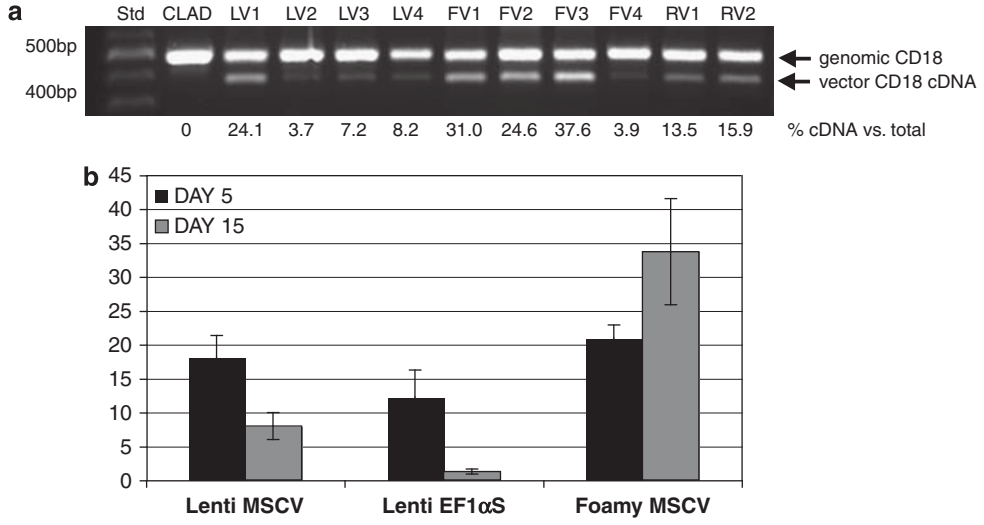

Figure 3 (**a**) Genomic PCR from PBLs for canine CD18 cDNA integration. Genomic DNA was isolated from PBLs 8 weeks (3 weeks for dog (LV4) after infusion of vector-transduced cells for the LV1−4 and at 12 weeks after infusion for the foamy viral vector (FV1−4) and the γ-retroviral vector (RV1,2). In all, 100 ng of genomic DNA was used as a template to assess the integration of canine CD18 cDNA by PCR. Std, 100-bp size standard; CLAD, untreated CLAD dog. **(b)** Change in the percentage of CD18$^+$ cells between day 5 and day 15 after transduction. CLAD CD34$^+$ cells transduced with the previously stated cytokine cocktail in RetroNectin coated 24-well plates, analyzed for CD18 expression on day 5 or after an additional 10 days in growth factors to differentiate the cells down the myeloid lineage (day 15).

Surface expression of CD18 was assessed on peripheral blood leukocytes before infusion, and at weekly intervals following infusion. We had previously demonstrated that even low levels (0.5%) of $CD18^+$ peripheral blood leukocytes (PBLs) could reverse or attenuate the CLAD phenotype.[17,18] However, none of the EF1αS promoter lentiviral vector-treated dogs had greater than 0.3% $CD18^+$ PBLs at any time after infusion, and all four succumbed to infection by 9 weeks after treatment.

Despite the very low levels of $CD18^+$ leukocytes in the peripheral blood after the infusion of vector-transduced autologous $CD34^+$ cells, vector-integrated CD18 cDNA could be amplified readily from the genomic DNA extracted from the PBLs of all four dogs, suggesting that the amount of the vector DNA present in the PBLs of the treated dogs did not correlate with very low levels of surface CD18 expression (Figure 3a).

Previously, we reported that *ex vivo* gene therapy in CLAD $CD34^+$ cells using a γ-retroviral vector with the MSCV LTR, and a foamy virus vector incorporating the MSCV internal promoter, resulted in sufficient surface expression of canine CD18 to reverse the CLAD phenotype using the same non-myeloablative conditioning regimen of 200 cGy TBI used in this study.[4,5] In this study, the human EF1αS promoter (EF1α promoter without intron 1) within the context of a SIN lentiviral vector did not result in sufficient numbers of $CD18^+$ neutrophils *in vivo* to reverse the CLAD phenotype.

To investigate this failure of the EF1αS promoter in a lentiviral vector to reverse the CLAD phenotype, we compared the differences of the lentiviral vector with the foamy viral vector and the γ-retroviral vector (Table 1). Although the transduction efficiency of the foamy viral vector and the γ-retroviral vector were higher than the lentiviral vector, comparable numbers of $CD18^+CD34^+$ cells per kg were infused because of the higher numbers of $CD34^+$ cells used with the lentiviral vector-treated animals (Table 1). Also, the percentages of $CD18^+$ PBLs were only slightly higher in the γ-retroviral vector treated dogs compared with the lentiviral vector-treated animals. The amount of DNA copies of CD18 cDNA were nearly commensurate with the levels seen in the γ-retroviral vector-treated animals in three lentiviral vector-treated dogs, and actually exceeded the levels seen in the γ-retroviral vector-treated dogs in one case namely, LV1 (Figure 3a).

To pursue this question of whether the EF1αS promoter in the lentiviral vector might lose activity over time, we compared CLAD $CD34^+$ cells transduced with the EF1αS promoter in the lentiviral vector to CLAD $CD34^+$ cells transduced with the MSCV promoter in the same lentiviral vector backbone, and to CLAD $CD34^+$ cells transduced with the foamy viral vector incorporating an internal MSCV promoter (Figure 3b). $CD18^+$ expression at two time points was compared: on day 5 after the 16 h transduction, and on day 15 after transduction and further incubation with growth factors (cG-CSF, c-SCF and Flt3 ligand). There was a marked decrease in CD18 expression in $CD34^+$ cells transduced with the EF1αS vector compared with the MSCV lentiviral vector after the 2-week expansion (Figure 3b). This raises the question as to whether the EF1αS promoter in the lentiviral vector is being silenced. There is evidence that the EF1αS promoter in a lentiviral vector is prone to transcriptional gene silencing.[19]

Our future studies are directed towards improving vector design and efficiency of transduction, as well as identifying other cellular promoters capable of directing consistent, stable and therapeutically relevant levels of CD18 expression *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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