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Treatment of canine leukocyte adhesion deficiency by foamy virus vectors expressing CD18 from a PGK promoter

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Abstract

Proto-oncogene activation caused by retroviral vector integration can cause malignancies in gene therapy trials. This has led investigators to search for less genotoxic vectors with minimal enhancer activity and a decreased risk of influencing neighboring chromosomal gene expression after integration. We previously showed that foamy virus vectors expressing the canine *CD18* gene from an internal murine stem cell virus promoter could cure canine leukocyte adhesion deficiency. Here we have repeated these studies using a foamy virus vector expressing canine *CD18* from a phosphoglycerate kinase gene promoter. In vitro analysis showed that this vector did not contain an enhancer that activated neighboring genes, and it expressed CD18 efficiently in canine neutrophils and CD34+ cells. However, dogs that received hematopoietic stem cells transduced with the PGK-CD18 vector continued to suffer from leukocyte adhesion deficiency, and sometimes died prematurely of the disease. These studies show that the phosphoglycerate kinase promoter cannot effectively replace the murine stem cell virus promoter in CD18-expressing foamy virus vectors, and they suggest that vectors containing a strong promoter/enhancer may be necessary for the treatment of human leukocyte adhesion deficiency.

Keywords

Foamy virus; retrovirus; gene therapy; immunodeficiency; hematopoietic stem cell; canine

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Introduction

Hematopoietic stem cell (HSC) gene therapy has the potential to cure many genetic diseases and there have been notable successes in the field treating immunodeficiencies [1-3] and metabolic diseases [4]. However, insertional mutagenesis and neighboring gene activation by the viral vectors used can cause clonal expansion and malignancies [5-7]. Thus there is a need for less genotoxic vectors that efficiently transduce HSCs and express transgenes at adequate levels.

Gammaretroviral vectors contain strong viral long terminal repeat (LTR) promoters and enhancers that can activate neighboring genes after integration. These vectors preferentially integrate in actively transcribed genes and near transcription start sites [8], making it likely that the large populations of transduced cells transplanted during HSC gene therapy contain clones with vector proviruses near proto-oncogenes. Self-inactivating (SIN) gammaretroviral vectors may have improved safety due to deletions in the viral LTRs [9,10], but they require internal promoters with their own potential genotoxicity. Similarly, lentiviral (LV) vectors may also lack strong viral promoter/enhancers, but they have a tendency to integrate in actively transcribed genes [11] and depend on internal promoters for transgene expression. These internal promoters should ideally lack enhancer sequences, but still must provide therapeutic levels of transgene expression. For example, the mouse phosphoglycerate kinase (PGK) promoter controls a housekeeping gene and has minimal enhancer activity [12]. Gammaretroviral SIN vectors and LV vectors containing internal PGK promoters have less potential for activating neighboring genes than similar vectors containing murine leukemia virus (MLV) LTR promoters [10,13], and LV vectors containing a PGK promoter did not promote leukemia in a tumor-prone mouse model [14,15]. It remains to be seen if the PGK promoter can provide therapeutic levels of transgene expression during HSC gene therapy.

Our research group and others have developed foamy virus (FV) vectors as an alternative vector system for HSC gene therapy that may have reduced genotoxicity. Foamy viruses, or spumaviruses are non-pathogenic retroviruses found in several mammalian species [16]. High-titer vector stocks can be produced that are free of replication-competent retrovirus [17-19] and efficiently transduce HSCs from mice, dogs and humans [20-26]. FV vectors have a slight preference for integrating near transcription start sites (less than gammaretroviral vectors) but no overall preference for integrating within transcription units or actively transcribed genes [27]. In addition, FV vector proviruses have reduced potential to activate neighboring genes compared to gammaretroviral or LV vectors, due to a lack of enhancer activity and read-through transcription in the viral LTRs [13]. These findings suggest that FV vectors are less likely to integrate near cellular proto-oncogenes, and also less likely to activate whatever genes are present near integration sites. This is supported by HSC transplantation experiments in dogs that showed no enrichment of FV vector proviruses near proto-oncogenes and no post-transplantation expansion of integrants near genes involved in cell proliferation, signal transduction or kinase activity [28].

Leukocyte adhesion deficiency (LAD) is an immunodeficiency caused by mutations in the leukocyte integrin *ITGB2* gene also known as *CD18*. Failure to express the CD11-CD18

adhesion complex on the leukocyte cell surface prevents adhesion to the vascular endothelium and migration to sites of infection, resulting in life-threatening infectious complications [29]. Canine LAD (CLAD) is a large animal model of the human disease caused by a single nucleotide G-to-C transversion that replaces a cysteine with serine at residue 36 (C36S) in the N-terminal extracellular portion of the CD18 protein [30]. Dogs with CLAD suffer from many of the same symptoms as humans, and die prematurely of infections [31]. We previously used an FV vector that expresses the canine *CD18* gene from an internal murine stem cell virus (MSCV) LTR promoter [32] to transduce CLAD HSCs and transplant partially myeloablated, autologous recipients [28]. This produced long-term leukocyte marking rates of 5-10% based on CD18 expression, and the animals remain healthy up to 4 years after gene therapy (unpublished results). Importantly, there was no evidence for clonal expansion of transduced cells based on an extensive integration site analysis [28]. While this study suggests that a similar FV vector expressing human *CD18* from an internal MSCV promoter would be curative for LAD, we had concerns that the strong viral enhancer in the internal MSCV promoter might still lead to neighboring proto-oncogene activation in some situations. Thus we felt it would be preferable to use a vector containing an internal promoter with less enhancer activity. Here we describe experiments in which dogs with CLAD were treated with an FV vector expressing canine *CD18* from a PGK promoter.

Results

Ex vivo promoter comparison

We previously used FV vector ΔΦMscvCD18 (ΔΦ for "Deleted Foamy" backbone) containing a viral MSCV promoter to successfully treat CLAD animals [28]. Here we compared this vector to others that used either the PGK promoter from the mouse phosphoglycerate kinase 1 gene (*Pgk1*) or the EF1α promoter from the human eukaryotic translation elongation factor 1 alpha 1 gene (*EEF1A1*). Like the PGK promoter, the EF1α promoter controls a ubiquitously expressed gene [33] that functions well in hematopoietic cells [34–36]. We used an intron-deleted version of the EF1α promoter that expresses at high levels [35] and has significantly reduced genotoxicity compared to gammaretroviral LTRs [10] to avoid possible splicing in FV vectors.

FV vectors expressing canine *CD18* from either an internal MSCV or PGK promoter (ΔΦMscvCD18 and ΔΦPCD18 respectively) were compared initially. High titer vector stocks were used to transduce CD34+ cells from CLAD animals at the same multiplicity of infection (based on vector genome-containing particles), and expression levels were analyzed by flow cytometry. In order to assess function in neutrophils (a clinically relevant cell type), the CD34+ cells were differentiated in vitro for 2 weeks down the neutrophil lineage, and then analyzed for expression of CD18. The MSCV promoter conferred high level CD18 expression equivalent to that seen in normal (non-CLAD) cells, while the level of expression from the PGK promoter was much lower on a per cell basis (Figure 1A). In order to improve expression of the PGK-based vector, we codon-optimized the canine *CD18* gene and produced vector ΔΦPCD18opt. This vector showed significantly better expression than the non-optimized vector on a per cell basis, although the percentage of CD18+ cells remained lower than that obtained with the MSCV promoter (Figure 1A). We also tested an

FV vector with the EF1α promoter driving codon-optimized canine *CD18* gene (ΔΦECD18opt). However, expression from this vector was very low in neutrophils and almost indistinguishable from that observed in untransduced CLAD cells (Figure 1A).

We measured CD18 expression levels from the same vectors in CD34+ cells cultured for 5 days in vitro without differentiation down a specific lineage. A similar pattern of gene expression was observed, with CD18 levels decreasing in magnitude in the following order ΔΦMscvCD18 > ΔΦPCD18opt > ΔΦPCD18 > ΔΦECD18opt (Figure 1B). These experiments suggested that the ΔΦPCD18opt vector with a PGK promoter driving a codon-optimized *CD18* gene would be the best choice for achieving potentially therapeutic expression levels from a cellular promoter. Importantly, some of the cells transduced by ΔΦPCD18opt had CD18 expression levels within the range of normal cells. We also tested a slightly modified version of the ΔΦPCD18opt vector called ΔΦPCD18opt-oa containing a *lac* operator sequence tag in the LTR to facilitate the recovery of vector sequences from transduced cells (a feature not used in this study). This vector had similar expression to ΔΦPCD18opt (Figure 1B) and was chosen for transplantation experiments (see below).

Neighboring gene activation

We previously developed a transient transfection assay to measure neighboring gene activation by vector proviruses [13]. In this assay, plasmids containing vector proviruses are nucleofected into human K562 cells, and expression of a linked luciferase gene downstream of an enhancerless, minimal promoter and internal ribosome entry site (IRES) is measured (Figure 2). We compared FV CD18 vector proviruses and found that those with either the PGK or EF1α promoters produced significantly less luciferase expression than ΔΦMscvCD18, as expected. In addition, the ΔΦPCD18opt provirus resulted in slightly lower luciferase levels than the ΔΦECD18opt provirus, suggesting that the EF1α promoter may be more likely to activate neighboring genes after integration. These results also supported our choice of the PGK promoter for CLAD transplantation experiments.

Transplantation and engraftment of transduced CLAD CD34+ cells

Bone marrow CD34+ cells were harvested from four CLAD dogs, and transduced with ΔΦPCD18opt-oa vector stocks. Transduction was by a single overnight exposure to vector at a dose optimized for transduction of CD34+ cells. The dogs ranged in age from 6-8 weeks, and received 2.8-3.3 × 10^6 CD34+ cells/kg (Table 1) after overnight transduction. They also received partial myeloablation with a dose of 200 cGy total body irradiation one day prior to transplantation. The pre-transplant transduction frequencies were determined by measuring CD18 expression in a portion of the cells cultured for 3 additional days after the infusion. These ranged from 6-13%, which was similar to the maximum transduction frequency we were able to obtain with this vector in CD34+ cells. Higher vector doses produced a minimal increase in CD18+ cells (data not shown) and presumably led to multiple integrations per cell. Transduction levels of 10-20% are typical for FV vectors in canine CD34+ cells [23], and the requirement that CD11 also be expressed in cells may have limited our ability to detect CD18 [37].

We assessed the marking rates by flow cytometry of peripheral blood leukocytes from each dog following transplantation (Figure 3). Two of the dogs (Venus and Vader) never achieved more than 1% CD18+ cells. Both remained ill and died less than 100 days after transplantation (see below). Two other dogs (Chico and Sonoma) also had fewer than 1% CD18+ leukocytes for the first 3-4 months, but they survived, and their marking rates climbed to 2.3 and 3.6% CD18+ leukocytes respectively 52 weeks after transplantation. Marking in specific cell subsets was also measured by flow cytometry, with the lowest and highest marking rates seen in neutrophils and T lymphocytes respectively (Figure 3). These differences may be due to a combination of factors, including selective extravasation of CD18+ neutrophils from peripheral blood, and a growth advantage for CD18+ lymphocytes. A similar phenomenon was observed previously in CLAD dogs treated with the ΔΦMscvCD18 FV vector [28] or allogeneic bone marrow transplantation [38]. As seen in Figure 4, the CD18+ lymphocytes from Chico and Sonoma proliferated well in response to the mitogen staphylococcal enterotoxin A (SEA), based on their loss of the fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE). In the two surviving dogs, marking rates continued to increase slowly in all cell types.

We also measured transduction frequencies by quantitative PCR (qPCR) of leukocyte DNA from many of the same blood samples (Figure 3). Typically, the number of proviruses detected by PCR was slightly higher than the number of CD18+ cells detected by flow cytometry. This could be due to the presence of multiple provirus copies in some CD18-expressing cells, silencing of transduced cells containing a provirus, or a relative insensitivity of flow cytometry for cells expressing low levels of CD18. In order to address this issue, we flow-sorted CD18+ and CD18- leukocytes from Sonoma 52 weeks after transplantation and found that these cell subsets contained 0.97 and 0.027 provirus copies per cell as measured by qPCR. Therefore most CD18+ cells contained a single copy of the FV vector provirus. The presence of vector proviruses in the CD18- flow-sorted cell population can account for the higher marking rates measured by qPCR and could be due either to inadequate expression for flow-sorting or silencing of the *CD18* transgene.

Integration site analysis

Although the marking rates were low in these animals, it was still possible that a single clone predominated in the transduced cell population. We used LAM-PCR to determine the locations 24 and 28 vector proviruses in the peripheral blood cells of Chico and Sonoma at 18 and 20 months post-transplantation respectively (Supplemental Table 1). Of these 52 integration sites, 18 (35%) were within RefSeq genes, 20 (38%) were within 15 kb of transcription start sites, and 4 (7.7%) were within 30 kb of known cellular proto-oncogenes (*MAML2, FGFR1, MEIS1, RUNX1T1*). These percentages were not statistically different ($P>0.05$) than those observed in the 466 FV vector integration sites previously identified in CLAD dogs treated with an MSCV-CD18 FV vector [28]. In addition, each of the 4 integration sites present near proto-oncogenes was recovered in only a small percentage of all the sequencing reads with mappable junctions obtained for each animal (Supplemental Table 2), suggesting that they did not dominate the population of CD18+ cells. Taken together, these data demonstrate that CLAD dogs treated with the ΔΦPCD18opt-oa vector maintain a polyclonal population of transduced blood cells.

Clinical course of transplanted dogs

All transplanted animals were carefully monitored for signs of infection, and treated according to our standard protocols for CLAD animal veterinary care. As shown in Figure 5A, the 4 animals that received the FV vector were frequently febrile, and required both antibiotics and admission to the intensive care unit (ICU). Venus and Vader were euthanized due to complications of CLAD disease at 75 and 91 days after transplantation respectively, which is similar to the clinical course of CLAD dogs that do not undergo gene therapy but receive equivalent veterinary care in our animal facility [28]. Venus had mandibular swelling due to cranio-mandibular osteodystrophy, and persistent high fever. Vader had lameness due to hypertropic osteodystrophy, otitis externa, high fever, and leukocytosis. Chico and Sonoma survived more than one year after transplantation, which is an unusually long life span for CLAD animals, suggesting that there may have been some therapeutic effect of the gene therapy. In addition the elevated white blood cell (WBC) count typical of CLAD largely resolved in these animals, consistent with improved leukocyte function (Figure 5B). However, both animals continued to suffer from CLAD, experiencing recurrent infections and requiring aggressive antibiotic therapy to survive.

Discussion

In this study we used an FV vector expressing an optimized canine *CD18* gene from a PGK promoter to treat CLAD animals by HSC gene therapy. The PGK promoter was chosen because of its lack of enhancer activity in assays of neighboring gene activation, and its relatively high expression level in neutrophils and CD34+ cells. While there was polyclonal marking and a therapeutic response to the treatment, including a normalization of the WBC count and a restoration of lymphocyte proliferation after antigen stimulation, the treated animals continued to suffer from infections and two out of four died prematurely. These results are markedly different than those obtained when using an internal MSCV promoter to drive *CD18* expression in an FV vector, which resulted in higher marking rates, a complete resolution of infectious symptoms, and a cure of the disease phenotype [28]. Presumably the higher transduction frequency and expression level obtained from the MSCV promoter is required to provide a full therapeutic effect. Our study underscores the importance of using a relevant large animal disease model for testing vector designs, since in vitro testing suggested that the PGK promoter might be therapeutic.

The marking rates of the PGK vector used in this study were lower than those obtained previously with an FV vector containing an MSCV promoter, despite an identical transduction and transplantation protocol. This was true both in cultured cells and in transplanted animals. The reason for the poor in vitro expression is not clear, but was not due to a low multiplicity of infection, since higher vector doses did not appreciably increase the percentage of CD18+ cells. It may be that a higher CD18 expression level is required to provide adequate pairing with CD11 and allow reliable detection of heterodimers on the cell surface by flow cytometry. Other factors could also limit expression from the PGK promoter, since similar transduction rates of 10-20% were observed with an FV vector expressing GFP from the same PGK promoter in canine CD34+ cells [23]. The low levels of CD18+ cells observed in vivo after transplantation may reflect the same phenomenon,

although in this case inadequate CD18 expression could also impact a variety of physiological parameters such as engraftment, proliferation and extravasation. Importantly, a low in vitro marking rate obtained with the MSCV-*CD18* FV vector (13.7%) still produced a cure of CLAD after transplantation [28], so inadequate transduction in vitro cannot fully explain the poor clinical efficacy of the PGK promoter vector. In the case of human CD34+ cells, transduction levels of over 50% can routinely be obtained with FV vectors, even when assaying SCID-repopulating cells [20,21,24]. This may reflect species-specific tropism of wild-type FV, and suggests that the results obtained in a human clinical trial should be superior to those obtained in the dog model.

In conclusion, our findings raise important concerns about the use of alternative promoters in HSC gene therapy. While, the decrease in genotoxicity expected from promoters with minimal enhancer activity is a desirable goal, they must still provide a therapeutic transgene expression level. In the case of CLAD, the PGK promoter does not appear to produce a therapeutic expression level in vivo, even when the transgene is codon-optimized. A related study also showed that LV vectors expressing *CD18* from the EF1α promoter failed to cure CLAD dogs [39]. Thus we are left with a difficult decision when translating the results obtained in the dog model to a human clinical trial: whether to use a strong viral promoter and enhancer such as MSCV that can provide a therapeutic expression level, or to continue testing alternative promoters until one can be found that provides adequate expression. This decision is further complicated by the fact that CLAD dogs treated with an FV vector containing an MSCV promoter remain cured more than 4 years after transplantation with no sign of leukemia or clonal expansion (our unpublished results), and because many patients with LAD currently die from their disease or complications resulting from allogeneic HSC transplantation. Therefore an FV vector expressing *CD18* from an internal MSCV promoter may be a safe enough option to treat critically ill LAD patients, despite a theoretical risk of proto-oncogene activation due to insertional mutagenesis. It is possible that this theoretical risk could be further reduced by the inclusion of insulator elements or other modifications of the vector backbone [40], but this remains to be demonstrated in the FV vector system.

Materials and Methods

Animal procedures

All animal protocols were approved by the NCI Institutional Animal Care and Use Committee. CLAD animals were treated prophylactically with oral amoxicillin / potassium clavulanate upon diagnosis of CLAD. More intensive treatment with parenteral antibiotics, fluids, and analgesics were provided when necessary. Bone marrow CD34+ cells were isolated and stored as described previously [28]. Transplant recipients received 200 cGy of total body irradiation delivered from a ^{60}Co source one day before infusion of autologous transduced cells. Peripheral blood samples were obtained at multiple timepoints, and cell counts and differentials were performed by Antech Diagnostics (Lake Success, NY) or the NIH Clinical Center laboratory.

Plasmids and vectors

All FV vectors were based on the pΔΦ backbone [17] and prepared by four plasmid transfection as described [17]. Plasmids used in the neighboring gene activation assay (Figure 2) contained 2-LTR proviral forms of each vector tested in the pACT5 backbone [13]. Sequences of the plasmids used are available upon request. When comparing vectors in vitro (Figure 1), MOIs were based on vector-genome containing particles. These were determined by isolating vector DNA from stock preparations by sequential extraction with phenol, phenol:chloroform and chloroform, precipitation with ethanol, and quantitative Southern blot analysis.

Transduction conditions and measurements

CD34+ bone marrow cells were transduced by a single overnight infection as described [28], after first determining the optimal vector dose on cultured CLAD CD34+ cells to ensure maximal transduction frequencies were obtained prior to transplantation. CD34+ cells were cultured for three additional days after infusion in Stemspan SFEM media (Stemcell Technologies, Vancouver, BC, Canada) containing 10% fetal bovine serum and 50 mg/mL human G-CSF (Neupogen; Amgen), canine SCF (R&D Systems), and human Flt3-L (Miltenyi Biotec) before measuring ex vivo transduction frequencies. CD18 expression was measured by flow cytometry with a mouse antibody to canine CD18 (CA1.4E9; Serotec). Neutrophil and monocyte populations, as well as lymphocyte subsets, were analyzed as described using specific antibodies [38]. In vitro analysis of neutrophils derived from CD34+ cells was performed by culturing transduced CD34+ cells as above for 4 days, then adding 50 ng/mL canine IL-6 (R&D Systems), canine GM-CSF (R&D Systems), and human TPO (R&D Systems) for an additional 10 days to promote myeloid differentiation. After culture, neutrophil populations were assayed by immunostaining with an anti-canine neutrophil antibody (CADO48A; VMRD, Inc). FV vector provirus copy numbers were determined by isolating genomic DNA from blood cells as described [28]. Lymphocyte proliferation assays were performed as described [28].

LAM-PCR

Genomic DNA was purified from peripheral blood leukocytes by using a Wizard genomic DNA purification kit (Promega Corporation, Madison, WI). Vector junction sequences underwent two rounds of linear amplification using 100 ng of genomic DNA, 0.25 pmol of the FV vector-specific 5′ biotinylated primer [5′–GAACCTTGTGTCTCTCATCCC–3′] and 2.5 units of *Taq* polymerase (Qiagen Inc. Valencia, CA) with cycling conditions of initial denaturation at 95 °C for 3 min, 50 cycles of amplification (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds), and a final extension at 72°C for 3 min. Double-strand synthesis was performed by using random hexanucleotide primer extension with Klenow (Roche Diagnostics Corporation, Indianapolis, IN) and then digested with *Fat* I (New England Biolabs, Ipswich, MA) or *Tas* I (Fermentas Inc, Glen Burnie, MD). Restriction enzyme-digested DNA was ligated to enzyme specific double-stranded linkers (5′-GACCCGGGAGATCTGAATTCAGTGGCACAGCAGTTAGG-3 / 5′-CATGCCTAACTGCTGTGCCACTGAATTCAGATC-3′ for *Fat* I and 5′-GACCCGGGAGATCTGAATTCAGTGGCACAGCAGTTAGG-3′ / 5′-

AATTCCTAACTGCTGTGCCACTGAATTCAGATC-3′ for *Tas* I) using a Fast-Link DNA ligation kit (Epicentre Biotechnologies, Madison, WI). Exponential amplification of ligated products was performed using 12.5 pmol each of FV vector-specific primer [5′–GTCTATGAGGAGCAGGAGTA–3′] and linker cassette-specific primer [5′–GACCCGGGAGATCTGAATTC–3′]. Eight percent of the first exponential PCR reaction was then used as template for a second, nested PCR amplification with 12.5 pmol each of nested FV vector-specific primer [5′-CCTCCTTCCCTGTAATACTC–3′] and nested linker cassette-specific primer [5′-AGTGGCACAGCAGTTAGG–3′] using conditions identical to the first PCR. DNA sequencing was performed on products from the second, nested PCR amplification that had been cloned into a pCR4-TOPO vector by using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA). FV vector integration sites were mapped to the dog genome as described [28]. Statistical comparisons to prior integration site datasets were performed by Fisher's Exact Test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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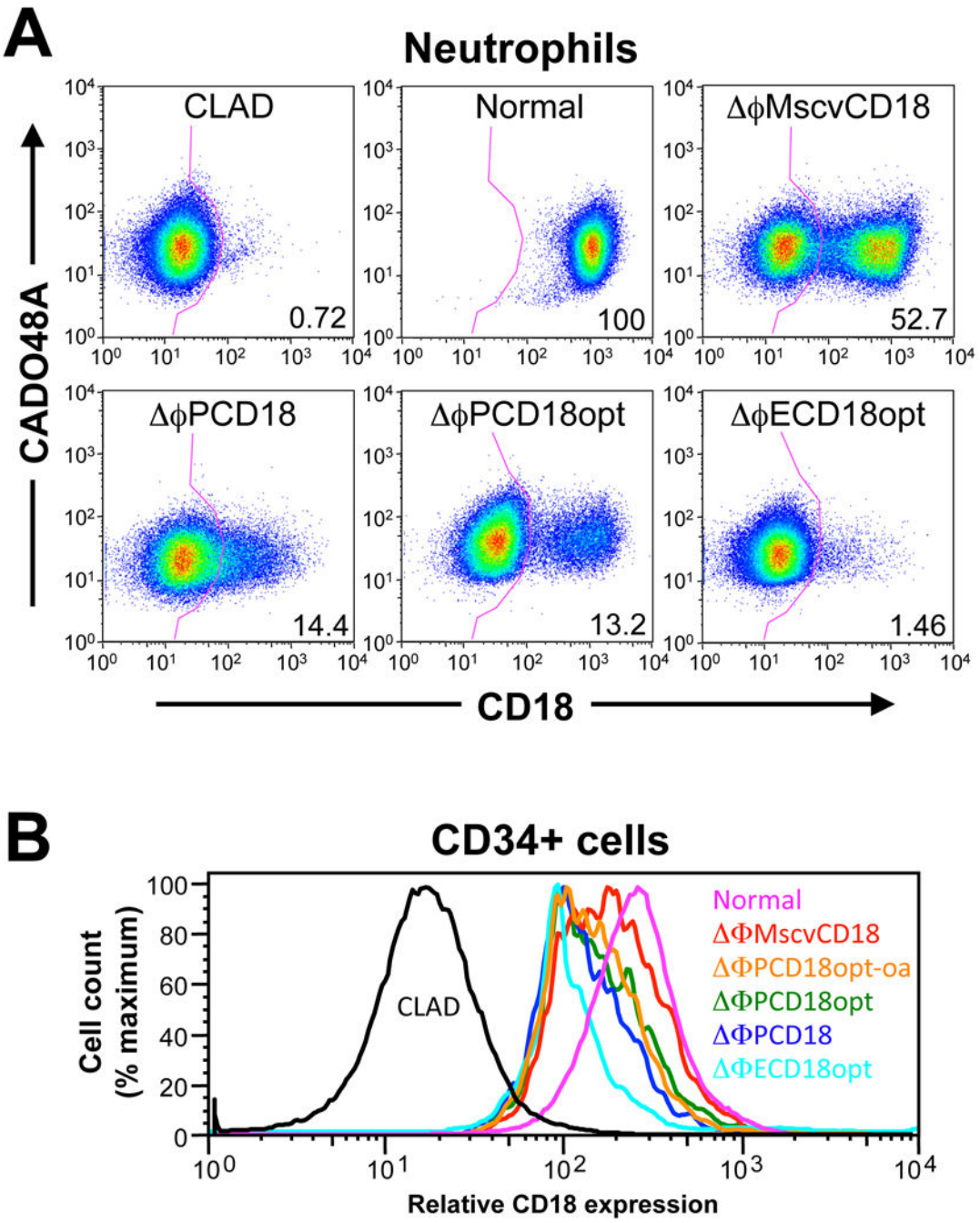

Figure 1. CD18 expression from different FV vectors

A) Expression in neutrophils was determined by transducing CLAD CD34+ cells with the indicated vector at a multiplicity of infection (MOI) of 10 vector genome-containing particles/cell, culturing the cells for 2 weeks to promote differentiation into neutrophils, and performing flow cytometry to detect CD18 surface expression. Antibody CADO48A (from VMRD, Pullman, WA) is specific for canine neutrophils. Numbers on the lower right quadrant of each panel indicate the percentage of CD18+ cells detected. Controls include untransduced CLAD CD34+ cells and normal canine CD34+ cells. B) Expression in CD34+

cells was measured by transducing CLAD CD34+ cells with the indicated vector at an MOI of 1 vector genome-containing particle/cell, culturing the cells for 4 days and measuring CD18 expression levels by flow cytometry. Results for the CD18+ cell fraction are shown, except for the untransduced CLAD control sample, which represents the total cell population.

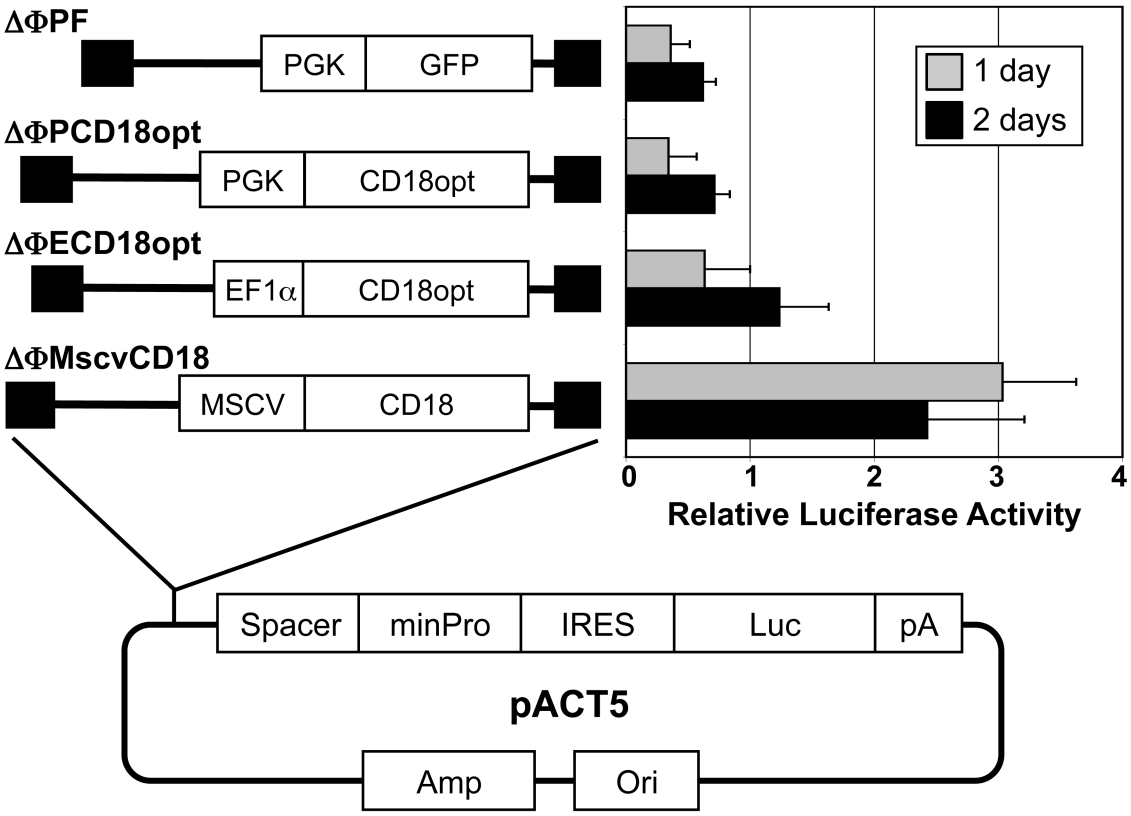

Figure 2. Neighbouring gene activation by vector proviruses

The proviral form of each FV vector shown was cloned into the pACT5 plasmid backbone, nucleofected into K562 cells, and luciferase expression was measured 1 and 2 days later as indicated. The mean and standard deviations of 3 independent measurements are shown to the right of each vector map. The pACT5 plasmid contains a spacer sequence from intron 2 of the human *HPRT1* gene, a minimal cytomegalovirus immediate early promoter (minPro), an internal ribosome entry site (IRES), a luciferase reporter gene (Luc), a polyadenylation signal (pA), an ampicillin resistance gene (Amp) and a bacterial plasmid replication origin (Ori).

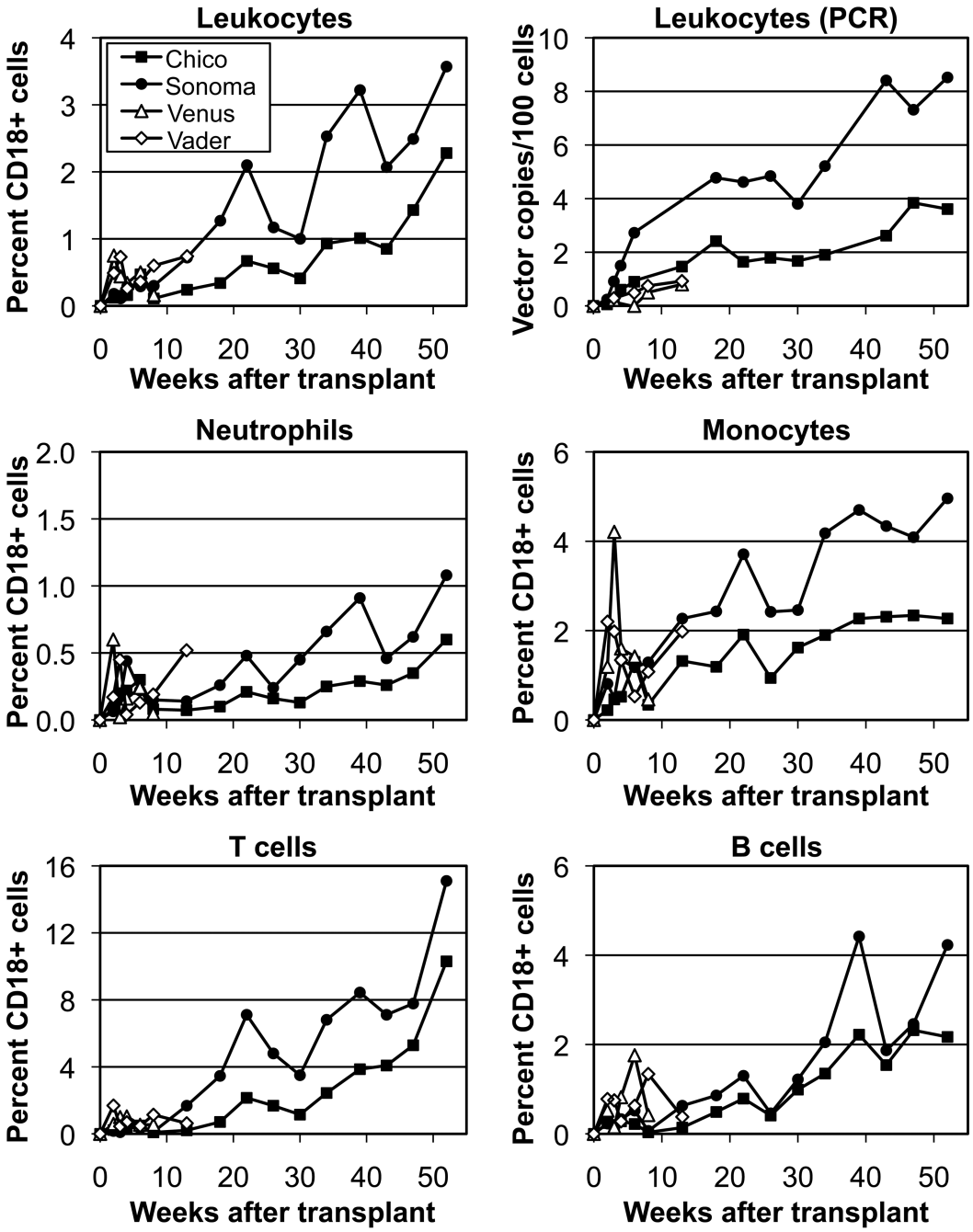

Figure 3. Transduction levels

The percentages of CD18+ cells were determined by flow cytometry (all panels except the upper right) in the peripheral blood cells of each of the 4 transplanted dogs at the indicated times after transplantation. Samples were analyzed as total leukocytes, neutrophils, monocytes, B lymphocytes or T lymphocytes. The upper right panel represents the vector provirus copy number in total leukocytes as determined by quantitative PCR.

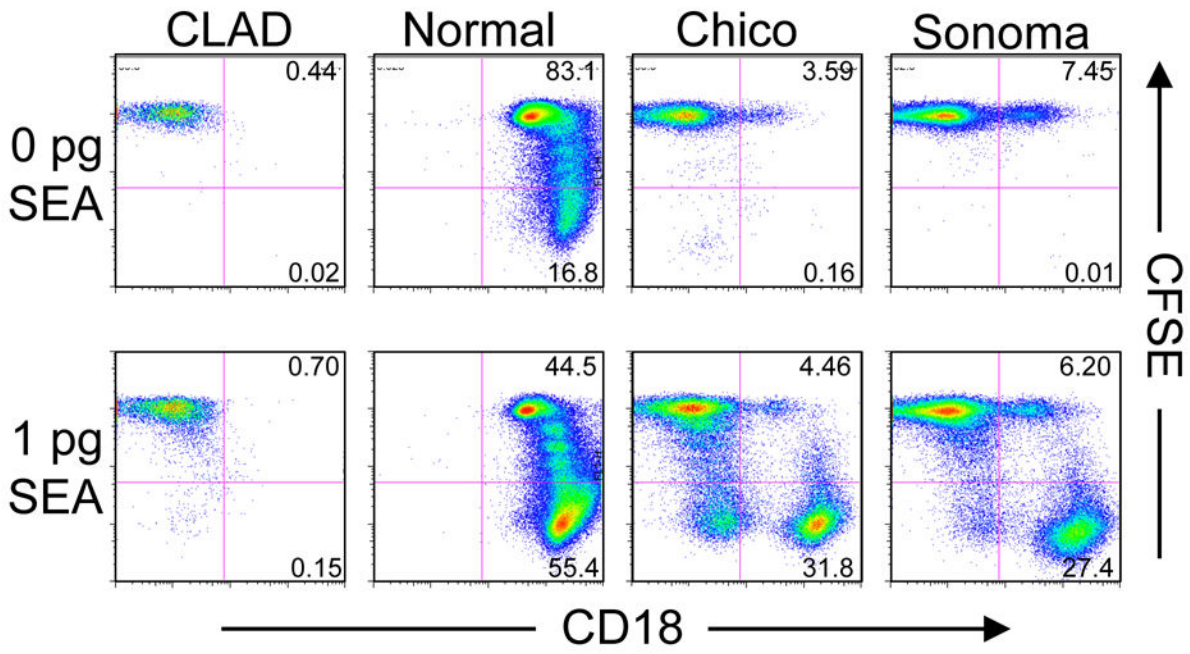

Figure 4. T cell proliferation in transplanted animals

CD18 expression and CFSE fluorescence were measured by flow cytometry in lymphocyte proliferation assays of cells from an untreated CLAD animal, a normal dog, and two FV vector-treated CLAD animals (Chico and Sonoma, 44 weeks after transplantation) at the indicated doses of SEA mitogen (0, and 1 pg/ml). Proliferation results in decreased fluorescence of the CFSE label. The percentages of CFSE-high (non-proliferating) and CFSE-low (proliferating) $CD18^{+}$ cells are indicated in the upper and lower right quadrants of each panel respectively.

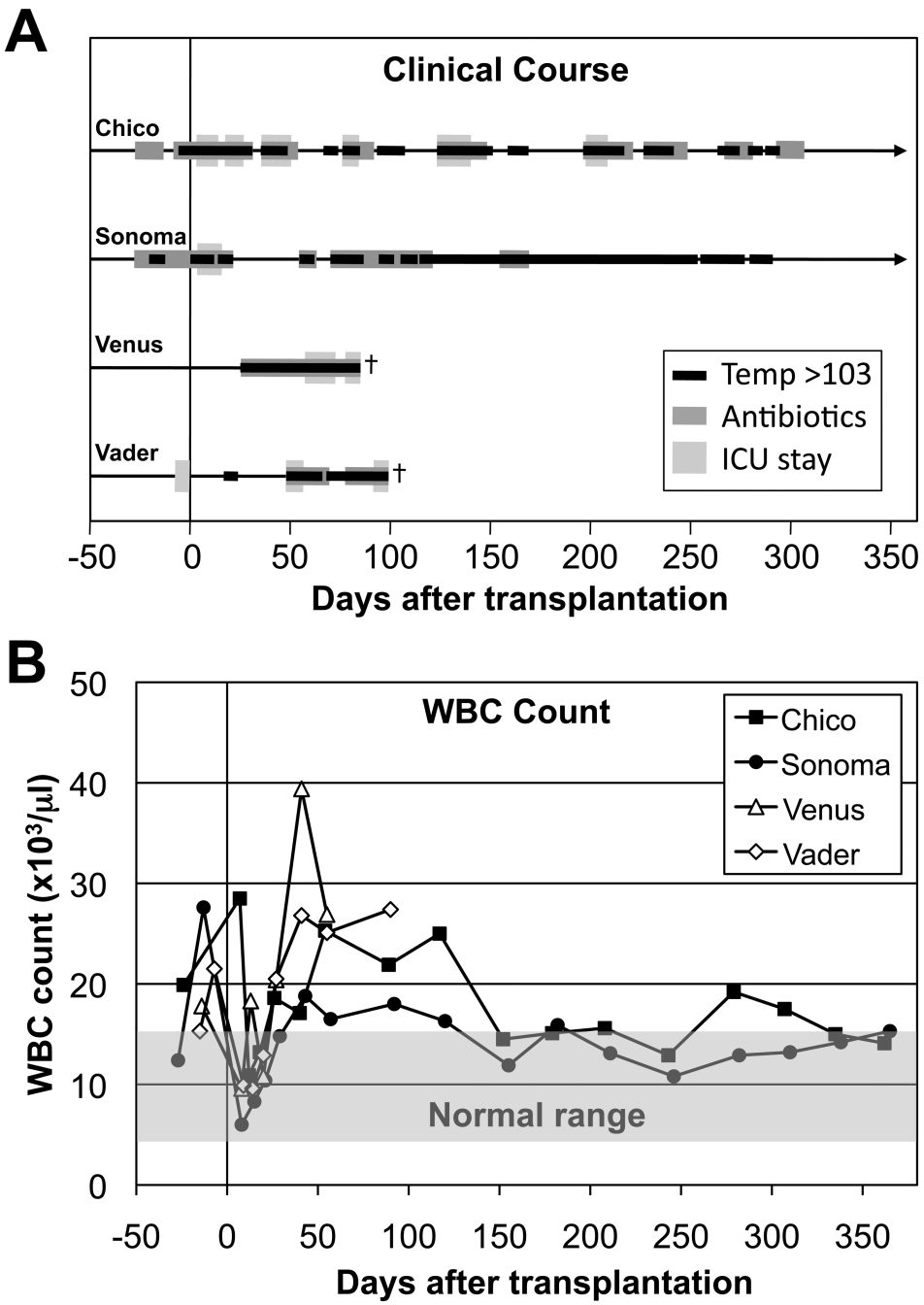

Figure 5. Clinical courses and blood counts of treated animals

A) The times at which each transplanted animal had febrile episodes, received antibiotic treatments, or required hospitalization in an intensive care unit (ICU) are shown from 50 days prior to receiving gene therapy until one year later or the animal's death (indicated by a cross). B) The peripheral blood white blood cell (WBC) counts are shown for each transplanted animal over the same time period. The normal WBC range for dogs is shaded light grey in the figure.

Table 1
Transplantation conditions and transduction of CLAD CD34+ cells

Name	Age at Infusion	Initial % CD18+[a]	Infused CD34+ cells/kg
Vader	6.7 weeks	6.40%	3.3E+06
Venus	7.7 weeks	6.13%	2.8E+06
Chico	8.3 weeks	8.62%	3.0E+06
Sonoma	8.0 weeks	12.53%	3.3E+06

[a] Determined by flow cytometry after 3 days of *ex vivo* culture of a sample of transduced cells.