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Successful treatment of canine leukocyte adhesion deficiency by foamy virus vectors

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Abstract

Recent successes in treating genetic immunodeficiencies have demonstrated the therapeutic potential of stem cell gene therapy^{1–4}. However, the use of gammaretroviral vectors in these trials led to insertional activation of nearby oncogenes and leukemias in some study subjects, prompting studies of modified or alternative vector systems⁵. Here we describe the use of foamy virus vectors to treat canine leukocyte adhesion deficiency (CLAD). Four of five dogs with CLAD that received nonmyeloablative conditioning and infusion of autologous, CD34⁺ hematopoietic stem cells transduced by a foamy virus vector expressing canine CD18 had complete reversal of the CLAD phenotype, which was sustained more than 2 years after infusion. *In vitro* assays showed correction of the lymphocyte proliferation and neutrophil adhesion defects that characterize CLAD. There were no genotoxic complications, and integration site analysis showed polyclonality of transduced cells and a decreased risk of integration near oncogenes as compared to gammaretroviral vectors. These results represent the first successful use of a foamy virus vector to

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AUTHOR CONTRIBUTIONS

T.R.B. designed experiments, performed lymphocyte and neutrophil function assays, performed transductions, analyzed all data and wrote the manuscript. J.M.A. and E.M.O. prepared and characterized foamy virus vector stocks. M.H. performed LAM-PCR studies and LM-PCR studies and participated in the CD34 stem cell collection. L.M.T. collected and analyzed flow cytometry data, participated in CD34 stem cell collection and reinfusion, performed LM-PCR cloning and analysis and participated in clinical care of dogs. I.F.K. performed quantitative PCR. R.L.A. performed LAM-PCR and LM-PCR studies. T.H.B. was responsible for clinical care of dogs. Y.-c.G. participated in CD34 stem cell collection. D.W.R. designed experiments, supervised experimentation, coordinated the foamy virus vector production and wrote the manuscript. D.D.H. designed experiments, supervised experimentation, coordinated the project and wrote the manuscript.

treat a genetic disease, to our knowledge, and suggest that foamy virus vectors will be effective in treating human hematopoietic diseases.

Vectors based on foamy virus have several potential advantages over gammaretroviral vectors, including a wide tropism⁶, a larger packaging capacity⁷ and a cDNA genome that is synthesized before infection^{8,9}, leading to increased stability of the vector in quiescent cells¹⁰. Foamy virus vectors efficiently transduce both mouse and human hematopoietic stem cells (HSCs) after a single overnight exposure^{11–13}. Importantly, foamy viruses are not pathogenic and have not been associated with malignancy¹⁴.

Leukocyte adhesion deficiency is a genetic immunodeficiency caused by mutations in the leukocyte integrin *ITGB2* gene (called *CD18* here) that prevent expression of the CD11-CD18 adhesion complex on the cell surface. Leukocytes lacking CD18 do not adhere to the vascular endothelium and do not migrate to sites of infection, resulting in life-threatening complications¹⁵. Here, we used the CLAD model to determine whether a foamy virus vector can transduce canine HSCs at levels sufficient to reverse the disease phenotype without adverse events. The foamy virus vector Φ MscvCD18 (Fig. 1a) expresses a canine *CD18* cDNA from an internal murine stem cell virus (MSCV) long terminal repeat (LTR) promoter¹⁶, providing high-level expression of the cDNA in all hematopoietic lineages. The deleted foamy virus vector backbone includes only essential *cis*-acting viral sequences, and the vector LTRs are silent owing to deletions in the U3 region and the removal of the essential foamy virus transactivator protein⁷. CLAD CD34⁺ bone marrow cells transduced by the foamy virus vector express CD18 on their cell surface at a level comparable to that of normal canine CD34⁺ cells (Fig. 1b).

We harvested bone marrow CD34⁺ cells from five 4–11-week-old CLAD dogs 2 weeks before infusion. To limit differentiation, CD34⁺ cells were transduced in a single, overnight exposure to foamy virus vector¹⁷. We administered a single, nonmyeloablative dose of 200 cGy total body irradiation 1 d before infusion to facilitate engraftment. Initial, *ex vivo* transduction frequencies ranged from 13.7% to 24.6% (Supplementary Table 1 online). One dog (FD5) died 7 d after infusion from an intussusception, a well-described complication of transplantation regimens in dogs¹⁸. The courses of the four long-term survivors are described here.

All four dogs showed a progressive increase in CD18⁺ leukocyte abundance after infusion (Fig. 2a). The percentage of peripheral blood leukocytes (PBLs) expressing CD18 reached a plateau approximately 12 months after infusion and remained stable at 5–10% through the latest (24-month) time point (Fig. 2a). The expression of CD18 on transduced PBLs was very similar to that on normal PBLs, reflecting the limiting level of the CD11 partner chain required for heterodimerization, except for a small population of lymphocytes that expressed slightly higher levels (Fig. 2b). The presence of a small population of CD8⁺CD18⁺ T lymphocytes in the peripheral blood is most likely indicative of a normal, physiological, antigen-driven process rather than a pathological state¹⁹. CD18⁺ myeloid cells in the peripheral blood were consistently present at lower levels than were CD18⁺ lymphocytes, possibly owing to selective extravasation of functional CD18⁺ myeloid cells²⁰ or to a slight growth advantage of transduced lymphocytes. In three of the four vector-treated dogs, the

majority of the CD18⁺ lymphocytes were CD4⁺, as seen in normal dogs, and there was a tendency toward a loss of CD45RA⁺ naive cells, which is consistent with an enhanced activated or memory phenotype for the CD18⁺ lymphocytes (Supplementary Table 2 online). One year after infusion, 3–7% of CD34⁺ bone marrow cells were CD18⁺ (data not shown).

The average vector copy number ranged from 0.83–1.25 provirus copies per diploid cell in CD18⁺ cells to 0.01–0.04 copies in CD18[−] cells, as determined by quantitative PCR 1 year after transplantation (Fig. 2c). Thus, most of the transduced cells contained a single copy of the vector provirus that actively expressed CD18. Given the low copy number in the CD18[−] fraction, there was no evidence of silencing of the vector transgene.

To determine whether the adhesion defect of CLAD cells was reversed, we employed an *in vitro* adhesion assay²¹. Stimulation by phorbol-12-myristate-13-acetate (PMA) led to enhanced adhesion and spreading by CD18⁺ PBLs of both foamy virus vector-treated and normal dogs, but not the CD18[−] PBLs of the treated or untreated CLAD dogs (Fig. 2d). Overall, there was a 2–8-fold enhancement of adherence in CD18⁺ cells from all four treated dogs after PMA stimulation (Supplementary Fig. 1a online). This adhesion was CD18 specific and was blocked by preincubation with an antibody to CD18 (ref. 22).

The higher percentage of transduced lymphocytes in the peripheral blood (Fig. 2a) may reflect a selective growth advantage due to CD18 expression. A similar pattern occurs in CLAD animals treated by allogeneic transplantation²³. To determine whether CD18⁺ transduced lymphocytes showed a proliferative advantage, we used an *in vitro* proliferation assay in which lymphocyte-enriched mononuclear cells were stimulated by varying doses of staphylococcal enterotoxin A (SEA) mitogen. CLAD CD18[−] lymphocytes proliferated poorly, even at a dose of 10 ng of mitogen (Fig. 2e). In contrast, CD18⁺ lymphocytes from a foamy virus vector-treated CLAD dog (FD3), as well as CD18⁺ lymphocytes from a normal dog, proliferated well in response to a low, 1-pg dose (Fig. 2e). Similar results were obtained with the other three treated dogs (Supplementary Fig. 1b).

The clinical course of the foamy virus vector-treated dogs showed a correction of the CLAD phenotype (Fig. 3a). Before treatment, all four dogs showed signs of CLAD, including omphalitis (FD2, FD3, FD4) or fever with hypertrophic osteodystrophy (FD1), a non-neoplastic inflammatory bone disease observed in CLAD (ref. 24). After infusion of transduced cells, there were mild febrile episodes for the first few months followed by the complete absence of signs of CLAD. All four treated dogs remain healthy without antibiotics more than 24 months after infusion. In contrast, four CLAD dogs that did not receive gene therapy experienced severe, recurrent infections and died by 6 months of age, despite antibiotic treatment. The foamy virus vector-treated CLAD dogs also had normalized white blood cell (WBC) counts after infusion, which were consistent with infection abatement (Fig. 3b). In contrast, untreated CLAD dogs had persistently elevated WBC counts, a diagnostic sign of CLAD disease (Fig. 3c).

The overall pattern of proviral integration was assessed by linear amplification-mediated PCR (LAM-PCR). DNA samples isolated from PBLs 6, 12 18, and 24 months after infusion

showed persistent polyclonality, with multiple LAM-PCR bands representing distinct integration events for all four dogs (Supplementary Fig. 2a,b online). DNA from sorted T lymphocytes and neutrophils also showed multiple bands by LAM-PCR (Supplementary Fig. 2c). Additionally, we determined the chromosomal positions of 1,141 foamy virus vector integration sites isolated from PBLs, sorted T lymphocytes and neutrophils from 2-year postinfusion and preinfusion CD34⁺ cells (Supplementary Table 3 online and Fig. 4a). Polyclonality was observed in DNA from each source, with 38 integrations in common between the 1- and 2-year samples, demonstrating persistent repopulation by the same transduced clones, and 2 sites in common between the neutrophil and lymphocyte subsets, suggesting transduction of HSCs with myeloid and lymphoid potential. Only three sites were found to be shared between the postinfusion and preinfusion samples. All chromosomes contained integrants. Unlike with gammaretroviral or lentiviral vectors²⁵, there seemed to be an inverse correlation between gene density and integration frequency (Supplementary Fig. 3a,b online). In comparison to the insertion sites in CLAD dogs treated with the gammaretroviral vector PG13/MSCV-cCD18 (ref. ²¹; M.H., unpublished data), the foamy virus vector showed less bias toward integration near transcriptional start sites, and no bias toward integration within genes ($P = 0.001$; Fig. 4b). Of note, the foamy virus vector integrated significantly less in or near oncogenes than the gammaretroviral vector ($P = 0.005$), and the foamy virus vector integration pattern was not different from a random pattern (Fig. 4b). *In vivo* foamy virus vector integration sites were not found near the *LMO2* oncogene, and only two were present in the *MDS1-EVII* gene locus (Supplementary Table 4 online). We performed gene ontology analysis to examine overexpressed classes of genes found near postinfusion insertion sites compared to a random canine gene background. A comparison of over 880 gene classes in the postinfusion gene set to randomly generated gene datasets revealed only a single class, catabolism, for which the data were suggestive of over-representation ($P = 0.03$; Fig. 4c), arguing against *in vivo* selection for clones in which the provirus had activated neighboring genes.

These results indicate that foamy virus vectors represent a promising vector system for HSC gene therapy. Therapeutic long-term PBL-marking rates of 5–10% were obtained with a single overnight exposure to vector and a nonmyeloablative conditioning regimen, and no genotoxic complications have yet been observed. Our findings suggest that foamy virus vectors may be safer than gammaretroviral vectors, as there was a more favorable polyclonal integration site profile at all time points examined and no evidence for the expansion of clones with proviruses near genes involved in cell proliferation. Lentiviral vectors may also be safer than gammaretroviral vectors, but we have not tested this directly in the CLAD model. In our experiments, most transduced cells contained a single provirus that was not silenced over time. Thus, it may be possible to achieve a therapeutic effect with a minimal number of integration events, further reducing the likelihood of activating a nearby oncogene. The foamy virus vector we used might be improved upon by substituting the internal MCV LTR promoter with one that has less enhancer activity.

METHODS

Dogs

All animal protocols were approved by the US National Cancer Institute Institutional Animal Care and Use Committee. We treated CLAD dogs prophylactically with oral amoxicillin and potassium clavulanate upon diagnosis of CLAD. We provided more intensive treatment with parenteral antibiotics, fluids and analgesics when necessary.

Vectors

We made the foamy virus vector Φ MscvCD18 (sequence available on request) by four-plasmid transient transfection as described previously⁷. We resuspended foamy virus vector in Dulbecco's DMEM containing 10% heat-inactivated FBS and 5% DMSO and stored it frozen before use. Vectors made by this method are free of replication-competent helper virus as determined by a sensitive marker-rescue assay²⁶. We titered vector stocks by exposing dilutions to CLAD CD34⁺ cells and performing flow cytometry 4 d later to determine the percentage of CD18⁺ cells. The gammaretroviral vector PG13/MSCV-cCD18 has been described previously²¹.

CD34⁺ cell collection and transduction

We harvested bone marrow cells, enriched them for CD34⁺ cells and cryopreserved them as previously described²⁷. For transductions, we thawed CD34⁺ cells and resuspended them in StemSpan serum-free expansion medium (SFEM; StemCell Technologies) containing 10% heat-inactivated FBS and 50 ng/ml of canine granulocyte colony-stimulating factor, canine stem cell factor and human Flt3 ligand (Amgen). We rapidly thawed foamy virus vector and added it to cells in non-tissue culture-treated T-75 flasks precoated with RetroNectin (BioWhittaker) at a concentration of 5 μ g/cm². After approximately 16 h, we washed the cells and resuspended them in a solution of Plasma-Lyte A Injection pH 7.4 (Baxter Healthcare) with 1% heat-inactivated autologous serum and 2 U/ml heparin for infusion. To determine transduction frequencies *in vitro*, we cultured an aliquot of transduced cells for an additional 3 d in StemSpan SFEM medium before flow cytometry or for 2 additional weeks for integration site analysis.

Flow cytometry

We measured CD18 expression with either a mouse antibody to human CD18 (MHM23; Dako) for PBLs or a mouse antibody to canine CD18 (CA1.4E9; Serotec) for leukocyte subsets. We analyzed neutrophil and monocyte populations, as well as lymphocyte subsets, as previously described with specific antibodies²⁸.

Lymphocyte proliferation assays

We isolated lymphocyte-enriched mononuclear cells by gradient separation of peripheral blood cells on Nycoprep 1.077A (Axis-Shield) at 800g for 30 min at 25 °C. After washing the cells twice in PBS containing 0.1% BSA, we incubated them with 1–5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE), using a CellTrace CFSE Cell proliferation kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

We added 400,000 cells to each well of a round-bottom 96-well plate with varying concentrations of SEA (Sigma) and cultured them for 4 d before performing flow cytometry for CD18 and CFSE as described above.

Cell adhesion assays

We performed cell adhesion assays as previously described²¹ with a minor modification. We removed nonadherent cells by washing them with an EL401 manual microplate washer (Bio-Tek Instruments) using PBS with 1% BSA.

Linker-mediated PCR and linear amplification-mediated PCR

We purified genomic DNA from foamy virus vector-treated dogs at 11–12 months after infusion (1 year), from flow-sorted lymphocyte or neutrophil samples at 22–26 months after infusion (2 years), or from preinfusion foamy virus vector-treated CD34⁺ cells expanded *in vitro* for 2 weeks (preinfusion) with the blood and cell-culture DNA kit (Qiagen) or Wizard genomic DNA purification kit (Promega). We performed linker-mediated (LM)-PCR and LAM-PCR as previously described^{21,29} with minor modifications (see Supplementary Methods online).

Integration-site analysis

We examined provirus junctions from LM-PCR and LAM-PCR clones for the presence of the PCR primer and 5' LTR sequences. We considered the integrations valid if the end of the 5' LTR was no more than three base pairs from the matching genomic sequence within the integration and if there were at least 90% homology to the canine genome and 20 base pairs of matching genomic sequence. We analyzed the integration sites with custom Perl script programs.

Statistical analysis

We performed statistical analysis comparing differences between the insertion sites near transcription start sites, within genes or near oncogenes from the foamy virus and RV vectors with a two-tailed Fisher's exact test for significance ($\alpha = 0.05$). We performed statistical analysis for over-representation of ontological gene classes against the canine background by using a modification of a one-tailed, right-sided Fisher's exact test, with one hit removed from the term hits as a conservative adjustment³⁰. When comparing the classes of genes near foamy virus vector insertion sites to random sites from the canine genome, the analysis was performed only with those gene classes with hits in all three random datasets. Only those gene classes near foamy virus insertion sites with $P < 0.05$ ($\alpha = 0.05$) against all three random datasets were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

In vitro analysis of foamy virus vector Φ MscvCD18. **(a)** Schematic diagrams of foamy virus vector Φ MscvCD18 and wild-type foamy virus (FV). The locations of *gag*, *pol*, *env* and *bel* gene sequences are labeled in the wild-type FV and are indicated by shading in the vector. All viral genes are deleted or truncated in the vector, and the LTR contains a deletion in the U3 region (Δ U3). The packaging signal (Ψ), translation termination codons (asterisks), central polypurine tracts (cPPTs; filled circles), MSCV promoter and canine *CD18* cDNA are also shown. **(b)** CD18 expression levels in FV vector–transduced CLAD CD34⁺ cells (middle) compared to normal CD34⁺ cells (right) and untransduced CLAD CD34⁺ cells (left). The percentage of CD18⁺ cells within the stained population is indicated in the upper right corner of each panel. CD18⁺ cells appearing in the CLAD populations represent background staining due to cytokine expansion.

Figure 2.

Expression and function of transduced CLAD peripheral blood cells after infusion. **(a)** The percentages of CD18⁺ PBLs, neutrophils, monocytes and lymphocytes determined by flow cytometry for the first 24 months after treatment are shown for each animal. The y axis has a different scale for the lymphocytes. **(b)** CD18 expression in FV vector-transduced PBLs compared to PBLs from a normal dog, as determined by flow cytometry. The percentage of CD18⁺ cells is indicated in the upper right corner of each panel. **(c)** Comparison of FV vector provirus copy number in CD18⁻ and CD18⁺ PBLs from FV vector-treated CLAD dogs 12 months after infusion, as determined by quantitative PCR. **(d)** PMA-stimulated adhesion of CD18⁺ leukocytes. PBLs from an untreated CLAD dog, an FV vector-treated CLAD dog (FD3) and a normal dog were added to fibrinogen-coated wells with or without stimulation by PMA or blocking by an antibody to CD18. Representative images are shown with blue fluorescence for Hoechst-stained nuclei and green fluorescence for CD18 expression. **(e)** CD18 expression and CFSE fluorescence in lymphocyte proliferation assays of cells from a CLAD dog, an FV vector-treated CLAD dog (FD3) and a normal (carrier) dog, at the indicated dose of SEA. Proliferation results in decreased fluorescence of the CFSE label. The percentages of CD18⁻ and CD18⁺ proliferating cells are indicated in the lower left and right quadrants, respectively.

Figure 3.

Correction of the CLAD phenotype *in vivo*. **(a)** The clinical course of the four FV vector-treated CLAD dogs is compared to that of four CLAD dogs that did not receive FV vector-treated cells (UD1–4). Each horizontal line represents the life of a dog (arrow represents ongoing follow-up), with the day of infusion indicated by the filled triangle. Evidence of fever and infection are indicated by the shaded boxes. A dagger indicates the death of the dog. **(b)** WBC counts for the four FV vector-treated CLAD dogs. **(c)** WBC counts for four untreated CLAD dogs until the time of death.

Figure 4.

Integration sites in FV vector-treated dogs. **(a)** The chromosomal positions (distance from the centromere) of FV vector integration sites are shown for every chromosome except the Y chromosome. Each dot is a different integration site from four different cell populations (the number of sites for each group is shown in parentheses). **(b)** Percentage of all integration sites (IS) within 15 kilobases (kb) of transcriptional (Tx) start sites, within genes, and within 30 kb of human oncogenes is shown for a PG13/MSCV-cCD18 gammaretroviral vector used to treat CLAD dogs²¹ (RV), the FV vector integration sites from PBLs 1 year after transplant (FV) and computer-generated random sites (RND). Significant P values are shown ($**$) as ascertained by a two-tailed Fisher's exact test. **(c)** Ontologies of genes within 30 kb of FV vector integration sites are shown for preinfusion and postinfusion (PBLs, lymphocytes and neutrophils combined) cell sources and an averaged set of random sites. Percentages represent the number of genes for each particular gene class divided by the total number of genes found in or near insertion sites. The asterisk (*) indicates those classes overrepresented after analysis with a modified one-tailed Fisher's exact test.