Gene Therapy of Canine Leukocyte Adhesion Deficiency Using Lentiviral Vectors With Human CD11b and CD18 Promoters Driving Canine CD18 Expression

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To identify cellular promoters in a self-inactivating (SIN) lentiviral vector that might be beneficial in treating children with leukocyte adhesion deficiency type 1 (LAD-1), we tested lentiviral vectors with human CD11 and CD18 leukocyte integrin proximal promoter elements directing expression of canine CD18 in animals with canine LAD (CLAD). Lentiviral vectors with either the human CD11b (637 bp) proximal promoter or the human CD18 (1,060 bp) proximal promoter resulted in the highest percentages of $CD18^+$ CLAD $CD34^+$ cells *in vitro*. Subsequently, two CLAD dogs were infused with autologous $CD34^+$ cells transduced with the hCD11b (637 bp)-cCD18 vector, and two CLAD dogs were infused with autologous $CD34^+$ cells transduced with the hCD18 (1,060 bp)-cCD18 vector. Each dog received a nonmyeloablative dose of 200 cGy total body irradiation (TBI) before the infusion of transduced cells. The two CLAD dogs treated with the hCD18 (1,060 bp)-cCD18 vector, and one of the two dogs treated with the hCD11b (637 bp)-cCD18 vector, had reversal of the CLAD phenotype. These studies using endogenous leukocyte integrin proximal promoters represent an important step in the development of gene therapy for children with LAD-1.

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INTRODUCTION

Children with leukocyte adhesion deficiency type 1 (LAD-1) suffer from recurrent, life-threatening bacterial infections.[1] The animal counterpart of LAD-1, canine LAD (CLAD), leads to an identical clinical phenotype.[2,3] Both LAD-1 and CLAD result from mutations in the leukocyte integrin CD18 subunit that prevent formation and surface expression of the CD11/CD18 heterodimer.[1,4] This failure to express the CD11/CD18 adhesion complex leads to the inability of leukocytes in both LAD-1 and CLAD to adhere to the endothelium and migrate to the site of infection. Thus, CLAD provides a leukocyte- and disease-specific, large-animal model in which to test new therapeutic approaches for LAD-1.

We previously demonstrated successful gene therapy of CLAD *via* transplantation of CD18-vector transduced, autologous $CD34^+$ cells following a nonmyeloablative dose of 200 cGy total body irradiation (TBI).[5,6] The first study used the murine stem cell virus (MSCV) long terminal repeat to express canine CD18 in a γ-retroviral (RV) vector, and the second study used an internal MSCV promoter/enhancer in a foamy viral vector to transduce CLAD $CD34^+$ cells. Because insertional mutagenesis from viral promoter/enhancers has emerged as a significant genotoxic risk,[7,8] alternative vector designs with self-inactivating (SIN) RV and lentiviral (LV) vectors incorporating an internal cellular promoter have been proposed to reduce the risk of genotoxicity.

In CLAD and LAD-1, the clinical manifestations result primarily from the lack of CD18 on neutrophils.[1,3] Thus, expression of CD18 on differentiated myelomonocytic cells represents the target cell population in CLAD and LAD-1. In previous studies, we and others have reported that the human CD11a, CD11b, and CD18 subunits are expressed in high levels on myeloid leukemia cells that have been induced to differentiate into myelomonocytic cells.[9,10] Moreover, the proximal promoter elements of CD11a, CD11b, and CD18 have been shown to drive expression in myelomonocytic cells and cell lines.[10–15] These observations suggested that the human CD11a, CD11b, and CD18 proximal promoters might be beneficial in directing the expression of CD18 on myeloid cells in CLAD.

Here, we treated four CLAD dogs with *ex vivo* gene therapy using SIN lentiviral LV vectors with either the human CD11b (637 bp) proximal promoter or the human CD18 (1,060 bp) proximal promoter driving expression of canine CD18. Three of four dogs achieved levels of $CD18^+$ neutrophils that were sufficient to reverse the CLAD phenotype. These studies indicate that the human CD11b and CD18 leukocyte integrin promoter elements in SIN LV vectors may be beneficial in treating children with LAD-1.

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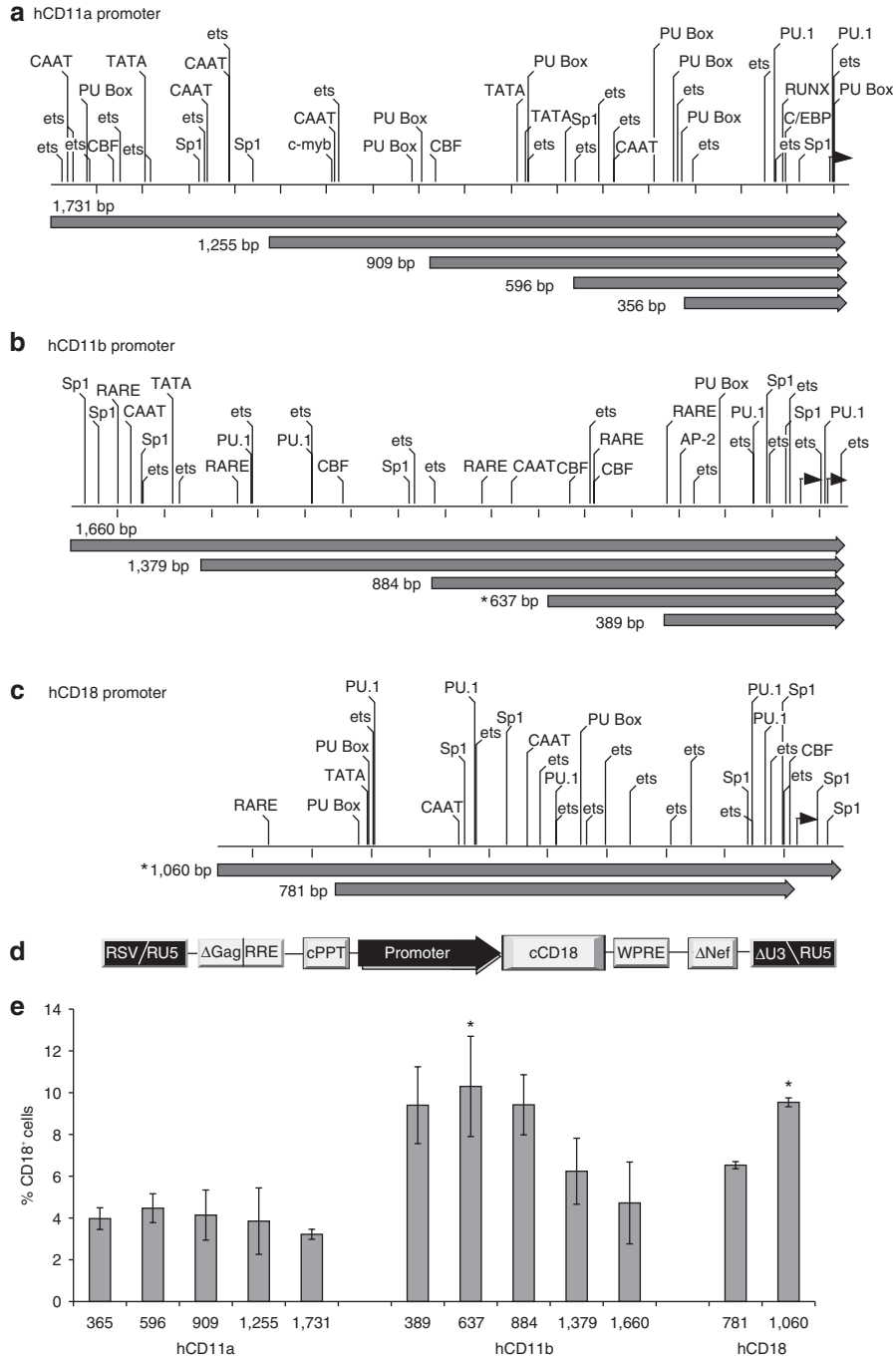


Figure 1 Schematic of predicted transcription factor binding sites and relative fragment sizes for the human CD11a, CD11b, and CD18 promoters in a self-inactivating lentiviral vector and transduction of canine leukocyte adhesion deficiency (CLAD) CD34$^+$ cells. (**a**) pRRLSIN.cPPT.hCD11apro-cCD18.WPRE was generated by replacing the phosphoglycerate kinase (PGK) promoter with the 1,731, 1,255, 909, 596, or the 356 bp human CD11a promoter. (**b**) pRRLSIN.cPPT.hCD11bpro-cCD18.WPRE was generated by replacing the PGK promoter with the 1,660, 1,379, 884, 637, or the 389 bp human CD11b promoter. (**c**) pRRLSIN.cPPT.hCD18pro-cCD18.WPRE was generated by replacing the PGK promoter with the 1,060 or 781 bp fragment of the human CD18 promoter. (**d**) pRRL transfer vector obtained from Addgene (Cambridge, MA) (plasmid: 12252). (**e**) Transduction of CLAD CD34$^+$ cells with vectors containing the five fragments of the human CD11a promoter, the five fragments of the human CD11b promoter, and the two fragments of the human CD18 promoter. The percentage of CD18$^+$ cells was determined by flow cytometry four days after transduction. The results represent an average of at least three experiments with error bars indicated the mean ± SD. cPPT, central polypurine tract; RRE, Rev response element; RSV, Rous sarcoma virus; WPRE, Woodchuck post-transcriptional regulatory element.

RESULTS

Construction and titration of SIN lentiviral vectors

To assess potential regions of the human leukocyte integrin CD11a, CD11b, and CD18 proximal promoters for expressing canine CD18, these three proximal promoters were analyzed for predicted transcription factor binding sites (Figure 1a–c). All three proximal promoters possess Sp1 sites that may tether RNA polymerase II to the transcription start site.[10,13–15] These promoters also

possess numerous *ets* and PU.1 binding sites that appear to play an important role in transcriptional activation.[10,13–15] The transcription factor binding sites were spread over the first 1–2 kb in all three promoters; thus, making it difficult to identify a discrete transcriptional region by analysis (Tfsitescan software; http://www.ifti.org).

Previously, the hCD11a promoter was found to direct expression of high levels of a reporter gene in the Jurkat T-cell line and in the HL-60 leukocyte cell line in transient transfection assays; low levels of the reporter gene were expressed in the nonleukocyte RD rhabdomyosarcoma cell line, the LS180 colonic adenocarcinoma cell line, and the MIA (PaCa-2) human pancreatic carcinoma cell line.[15] Transgenic mice generated using the human CD11a promoter also expressed the reporter gene in all leukocytes.[16]

In this study, five fragments of the human CD11a promoter, designed around regions with predicted transcription factor binding sites, were selected to identify the minimal proximal promoter region required for expression of CD18.[15] The five fragments of the human CD11a promoter—1,731, 1,255, 909, 596, and 356 bp—starting distal to the transcriptional start site and extending in the 5′ direction were cloned upstream of the canine CD18 complementary DNA in the pRRL lentiviral transfer vector[17,18] (**Figure 1a,d**).

We tested the human CD11b and CD18 promoters as the expression of CD11b and CD18 have been shown to increase with myeloid differentiation, and this increase is transcriptionally regulated.[9,13,14] Five fragments of the human CD11b promoter—1,660, 1,379, 884, 637, and 389 bp—were cloned upstream of the cCD18 complementary DNA in the pRRL lentiviral transfer vector[17,18] (**Figure 1b,d**). Previous analysis of the human CD11b proximal promoter indicated that maximal promoter activity was located in a −654 bp fragment.[13] Deletion analyses also indicated the presence of an inhibitory element between −654 bp and −1,287 bp that drastically reduced the promoter activity when this region was included.[13]

Two fragments of the human CD18 promoter—1,060 and 781 bp—were cloned upstream of the cCD18 complementary DNA into the pRRL lentiviral transfer vector[17,18] (**Figure 1c,d**). The additional 279 bp in the 1,060 bp CD18 proximal promoter had been shown to contain retinoic acid response elements that significantly enhanced the activity of the promoter.[10]

To establish relative transduction efficiency of the different vectors, vector-conditioned media was generated for all constructs and titered on a LAD Epstein–Barr virus B-cell line. These studies were feasible because transfer of wild-type human or canine CD18 into LAD Epstein–Barr virus B-cells results in rescue of the human CD11 subunits and expression of the CD11/CD18 complex on the cell surface.[4,19,20] Titers for all vectors used in this study ranged between 1×10^8 and 1×10^9 transducing units per ml.

Transduction of CLAD CD34$^+$ cells *in vitro*

Gene transfer of canine CD18 complementary DNA into CLAD CD34$^+$ cells corrects the CLAD CD18 defect *in vitro*.[4–6] To determine the vector with the highest transduction efficiency for use *in vivo*, CLAD CD34$^+$ cells were transduced overnight with each vector. Transduced cells were incubated for an additional three days and analyzed by flow cytometry (**Figure 1e**). The human CD11b (637 bp)-cCD18 and the human CD18 (1,060 bp)-cCD18 LV vectors displayed the highest transduction efficiency (**Figure 1e**). These two vectors also directed the highest expression per cell of canine CD18 (data not shown). None of the human CD11a promoter vectors achieved levels comparable to the hCD11b (637 bp) and hCD18 (1,060 bp) LV vectors (**Figure 1e**).

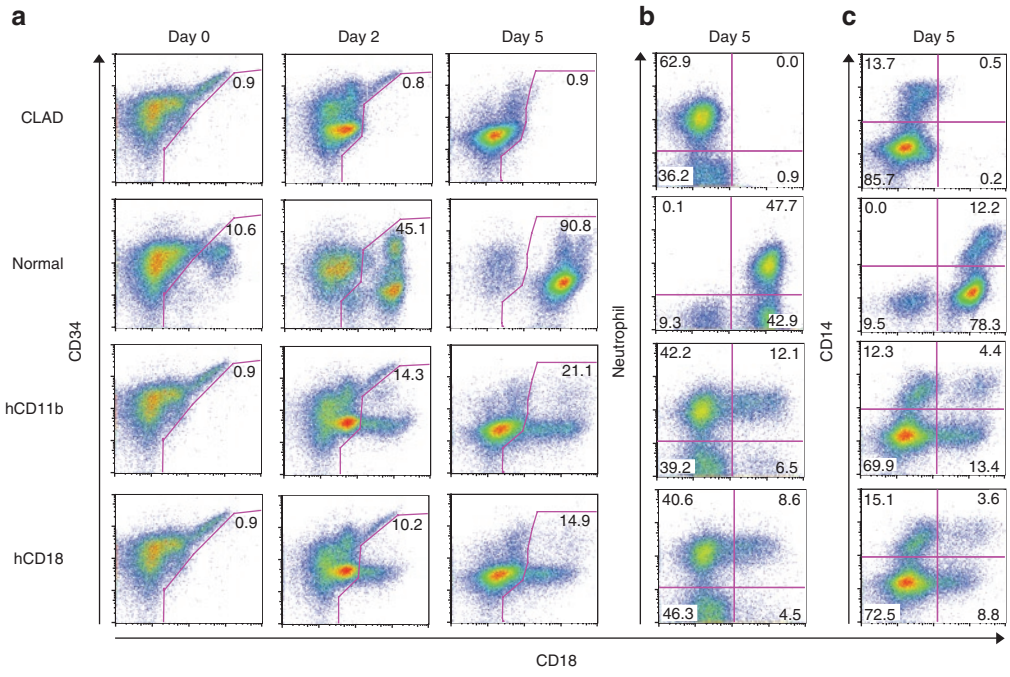
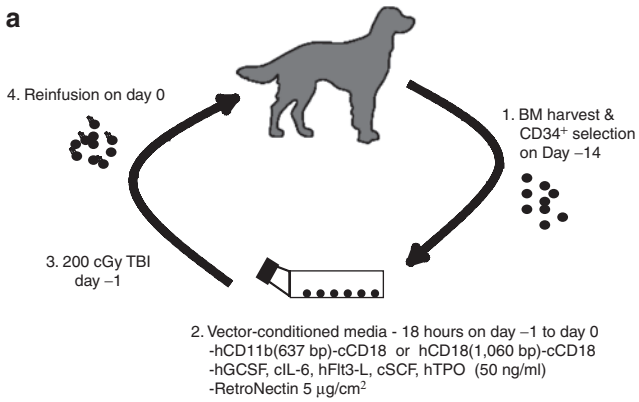


Figure 2 Expression of CD18 on CLAD CD34$^+$ cells following myeloid differentiation. Normal canine CD34$^+$ cells, CLAD CD34$^+$ cells, and CLAD CD34$^+$ cells transduced with the hCD11b (637 bp)-cCD18 vector or the hCD18 (1,060 bp)-cCD18 vector were differentiated in myeloid growth factors for 5 days, and the expression of markers of lineage commitment were analyzed. (**a**) Undifferentiated CD34$^+$ cells and CD34$^+$ cells differentiated for 2 and 5 days. (**b**) Neutrophil (y-axis) versus CD18 expression (x-axis) on CLAD CD34$^+$ cells differentiated for 5 days. (**c**) CD14$^+$ monocytes (y-axis) versus CD18 expression (x-axis) for CLAD CD34$^+$ cells differentiated for 5 days. CLAD, canine leukocyte adhesion deficiency.



b

Dog	Infused $CD34^+$ cells/kg	% $CD18^+$	Transduced cells/kg infused	Outcome
hCD11b-1	12×10^6	6	0.7×10^6	Death 21.7 weeks
hCD11b-2	21×10^6	12.8	2.7×10^6	Alive 52 weeks
hCD18-1	10.8×10^6	4.3	0.5×10^6	Alive 52 weeks
hCD18-2	13.1×10^6	5	0.7×10^6	Alive 52 weeks

Figure 3 Schematic for treatment regimen of canine leukocyte adhesion deficiency (CLAD) dogs. (**a**) Bone marrow (BM) was harvested 2 weeks before treatment and the $CD34^+$ cells were isolated and cryopreserved. At day −1, the cells were thawed, washed, and incubated with vector-conditioned media for 18 hours in the presence of growth factors described above. Also on day −1 the dog received a single, nonmyeloablative dose of 200 cGy TBI. On day 0, the autologous, transduced cells were harvested, washed, and infused intravenously. (**b**) Table of the number and percentage of $CD18^+$ cells transduced with the hCD11b(637 bp)-cCD18 or hCD18(1,060 bp)-cCD18 vectors that were infused into each dog. cSCF, canine stem cell factor; hTPO, human thrombopoietin.

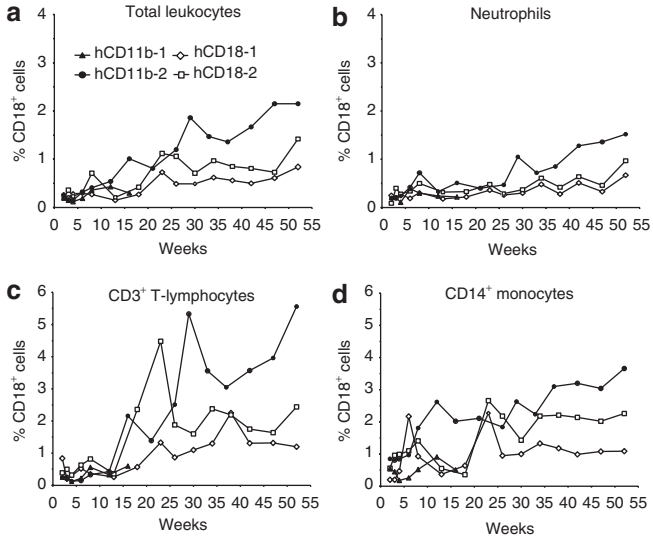


Figure 4 Analysis of percentage of $CD18^+$ peripheral blood leukocytes in lentiviral (LV) vector–treated dogs. The percentage (y-axis) of $CD18^+$ leukocyte subsets in the vector-treated dogs over time (x-axis) is shown. (**a**) Percentage of total $CD18^+$ leukocytes in the blood. (**b**) Percentage of $CD18^+$ neutrophils in the blood. (**c**) Percentage of $CD18^+$ $CD3^+$ T-lymphocytes in the blood. (**d**) Percentage of $CD18^+$ $CD14^+$ monocytes in the blood.

To determine whether the expression of canine CD18 from the two vectors harboring the leukocyte integrin promoters increases with myeloid differentiation, CLAD $CD34^+$ transduced with the two vectors were differentiated along the myeloid lineage and analyzed by flow cytometry (Materials and Methods; **Figure 2**). A low percentage of normal canine $CD34^+$ (10.6%) expressed CD18; however, by 5 days of differentiation 90.8% of the cells were $CD18^+$ (**Figure 2a**). Transduced CLAD $CD34^+$ cells were CD18 negative (**Figure 2a**). However, by 5 days of differentiation the percentages of $CD18^+$ cells were 21.1 and 14.9% for the hCD11b promoter and the hCD18 promoter vectors, respectively (**Figure 2a**). Following 5 days of differentiation, the percentages for the cell populations were as follows: >90% of the cells were CD34 low; >50% of the cells were neutrophils; >12% of the cells were monocytes (**Figure 2a–c**). Less than 1% of the cells were T- or B-lymphocytes following 5 days of differentiation (data not shown).

Ex vivo gene therapy of CLAD

We used the CLAD model to evaluate the efficacy of the hCD11b (637 bp)-cCD18 and hCD18 (1,060 bp)-cCD18 LV vectors for use in in *ex vivo* gene therapy. The schematic for the treatment regimen is shown (**Figure 3a**). Briefly, bone marrow was harvested from CLAD dogs at 6–8 weeks of age, and the $CD34^+$ fraction was isolated and cryopreserved. At day −1, autologous $CD34^+$ cells were thawed and transduced in an overnight incubation with vector-conditioned media containing either the hCD11b (637 bp)-cCD18 LV vector or the hCD18 (1,060 bp)-cCD18 LV vector. The transduction conditions are shown (**Figure 3a**). On day −1, animals received a single, nonmyeloablative dose of 200 cGy TBI. On day 0, transduced CLAD $CD34^+$ cells were harvested and infused. The doses of transduced cells per kilogram that each animal received is shown (**Figure 3b**).

Flow cytometry of $CD18^+$ leukocytes in peripheral blood following treatment

To determine the level of $CD18^+$ leukocytes in the peripheral blood following infusion of the autologous, transduced CLAD $CD34^+$ cells, peripheral blood leukocytes (PBLs) were analyzed at weekly or monthly intervals following treatment. The percentage of total $CD18^+$ leukocytes ranged from 1 to 2% in the three dogs that survived >1 year following treatment (**Figure 4a**). The highest percentage of $CD18^+$ neutrophils (1.5%) at 1 year following treatment was present in the hCD11b (637 bp)-cCD18 vector–treated CLAD dog, designated (hCD11b-2; **Figure 4b**). The percentage of $CD18^+$ neutrophils in the peripheral blood 1 year following treatment in both dogs treated with the hCD18 (1,060 bp)-cCD18 LV vector ranged from 0.7 to 1% (**Figure 4b**). The percentage of $CD18^+$ monocytes in the three dogs that survived 1 year following gene therapy ranged from 1 to 4% (**Figure 4d**). In all dogs, the level of $CD18^+$ leukocytes gradually increased over time during the first year in the neutrophil, monocyte, and lymphocyte populations, then reached a plateau (**Figure 4**).

$CD18^+$ peripheral blood T-lymphocytes versus neutrophils

In all of the treated CLAD dogs, the percentage of $CD18^+$ lymphocytes in the peripheral blood was consistently higher than

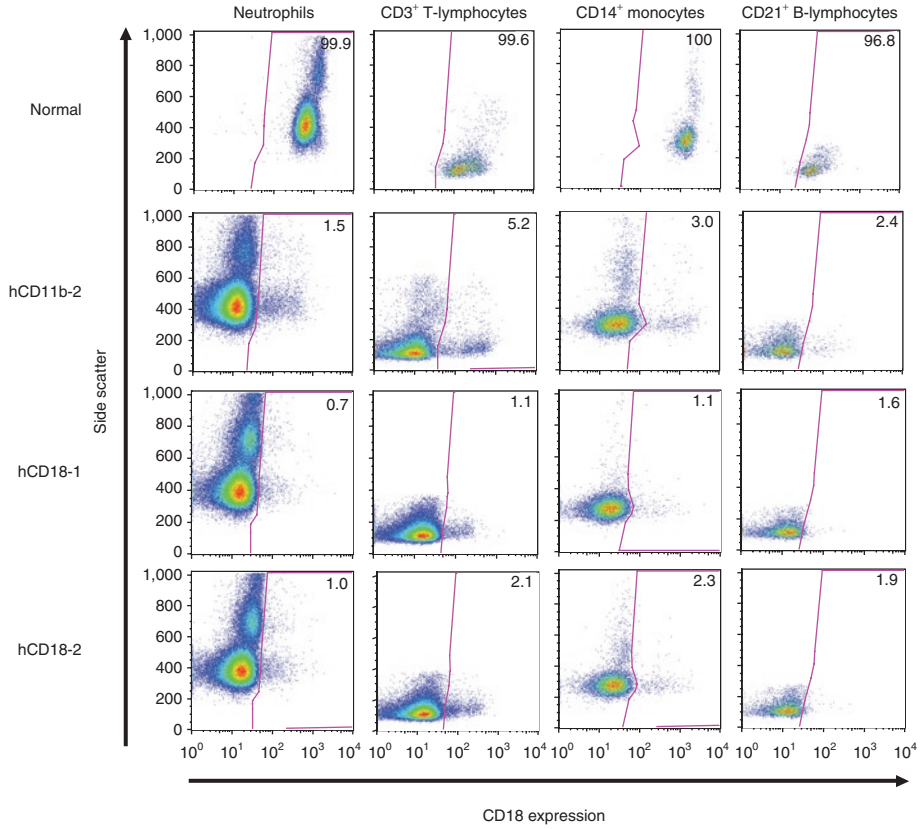


Figure 5 Flow cytometry of CD18 expression on leukocyte subsets of the treated dogs compared to CD18 expression on normal canine leukocytes. One-year follow-up after gene therapy, peripheral blood neutrophils, $CD3^+$ T-lymphocytes, $CD14^+$ monocytes, and $CD21^+$ B-lymphocytes were analyzed by flow cytometry for CD18 expression.

the $CD18^+$ neutrophil levels (**Figure 4b,c**). The relative increase in $CD3^+$ T-lymphocytes is consistent with our previously published data demonstrating a selective proliferation of $CD18^+$ T-lymphocytes in response to mitogens in both allotransplant and gene therapy treated CLAD dogs.[5,6,21] To confirm that this proliferation is a normal, antigen-driven process rather than a pathologic process, we carried out cell proliferation assays in normal, CLAD, and vector-treated animals. The $CD18^+$ lymphocytes displayed a markedly enhanced proliferative response to low doses of Staphylococcal enterotoxin A (**Supplementary Figure S1**).

Analysis of levels of CD18 expression on PBLs in vector-treated dogs

Flow cytometry of PBL 1 year following gene therapy demonstrated that not only were low percentages of $CD18^+$ neutrophils present in the vector-treated dogs, but that the levels of CD18 expression per neutrophil were also low compared to CD18 expression on neutrophils from a normal dog (**Figure 5**). Low levels of $CD18^+$ cells were present in the $CD3^+$ T-lymphocyte, $CD14^+$ monocyte, and $CD21^+$ B-lymphocyte populations (**Figure 5**).

CD18 expression per cell in PBLs of LV vector–treated dogs

To quantitate the levels of CD18 expression per cell, the mean fluorescence intensity on neutrophils, $CD3^+$ T-lymphocytes, $CD14^+$ monocytes, and $CD21^+$ B-lymphocytes was assessed. This addresses an important issue as considerable clinical evidence from children with LAD-1 indicates that the levels of CD18 expression per myelomonocytic cell correlates with the clinical phenotype.[1] Neutrophils, $CD3^+$ T-lymphocytes, $CD14^+$ monocytes, and $CD21^+$ B-lymphocytes from LV vector–treated dogs were analyzed at 1 year for levels of CD18 expression per cell. These levels were compared to the levels of CD18 expression per cell in a normal dog. In the hCD11b (637 bp)-cCD18 vector–treated dog 24.3% of the neutrophils expressed normal levels of canine CD18, whereas in the two hCD18 (1,060 bp)-cCD18 vector–treated dogs 12 and 7% of the neutrophils expressed normal levels of CD18 (**Figure 6**). In contrast, in both the hCD11b (637 bp)-cCD18 dog, and in the two hCD18 (1,060 bp)-cCD18 vector–treated dogs, 98.8, 99.9, and 100% of the $CD18^+$ $CD3^+$ T-lymphocytes expressed normal levels of CD18 (**Figure 6**). This finding was not surprising for the CD18 promoter as CD18 is expressed on all leukocytes. However, CD11b is expressed on monocytes and neutrophils, and the fact that its promoter-directed expression on $CD3^+$ T-lymphocytes and $CD21^+$ B-lymphocytes indicated "leakiness" of the promoter (**Figure 6**).

Specificity of CD18 expression on leukocytes

To determine whether the human CD11b or human CD18 promoter led to aberrant expression of CD18 on the surface of platelets or red blood cells, we stained whole peripheral blood with a platelet-specific antibody (CD61) or leukocyte-specific antibody

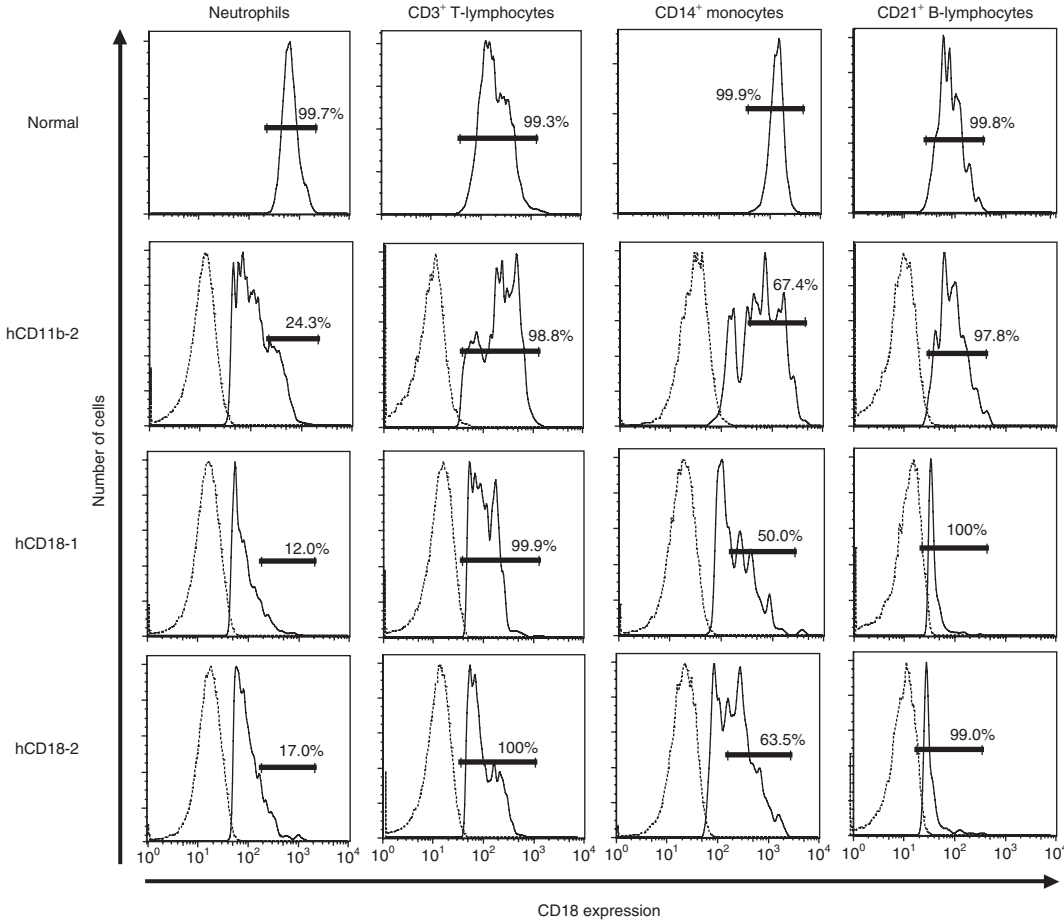


Figure 6 CD18 expression per cell in peripheral blood leukocytes of normal and vector-treated canine leukocyte adhesion deficiency (CLAD) dogs. The levels of CD18 expression per cell on neutrophils, $CD3^+$ T-lymphocytes, $CD14^+$ monocytes, and $CD21^+$ B-lymphocytes at 1-year follow-up were assessed using flow cytometry. Levels were compared to the levels of CD18 expression per cell in a normal dog. The normal level of CD18 expression per cell is shown as a dark horizontal line for each population. The percentage indicates the percentage of cells with normal levels of CD18.

(CD45). Platelet populations were identified for all three dogs by scatter profiles and CD61 reactivity. However, no platelet population showed reactivity with the CD18 antibody, indicating that CD18 was not aberrantly expressed on platelets (**Figure 7**). Because no antibody detecting canine red blood cells is available, red blood cells were identified by a lack of reactivity to CD45, which stains all leukocytes, and by a scatter profile similar to that of leukocytes. The leukocytes present in the peripheral blood (about 0.4–1%) stained with CD45, whereas the lack of staining indicated red blood cells (**Figure 7**, middle row). The lack of CD18 staining for the dogs treated with the hCD11b or hCD18 promoter indicates that CD18 is not aberrantly expressed on red blood cells.

Clinical outcome

CLAD is a lethal disease with death occurring from infection within the first 6 months of life[2,3] In previous studies, we have shown that life expectancy in CLAD depends upon the absolute number of $CD18^+$ neutrophils.[22,23] One of the hCD11b (637 bp)-cCD18 vector–treated dogs failed to achieve sufficient levels of $CD18^+$ neutrophils to reverse the CLAD phenotype and succumbed to infection 22 weeks after infusion (**Figure 8**). The three surviving dogs had reversal of the phenotype with no infectious

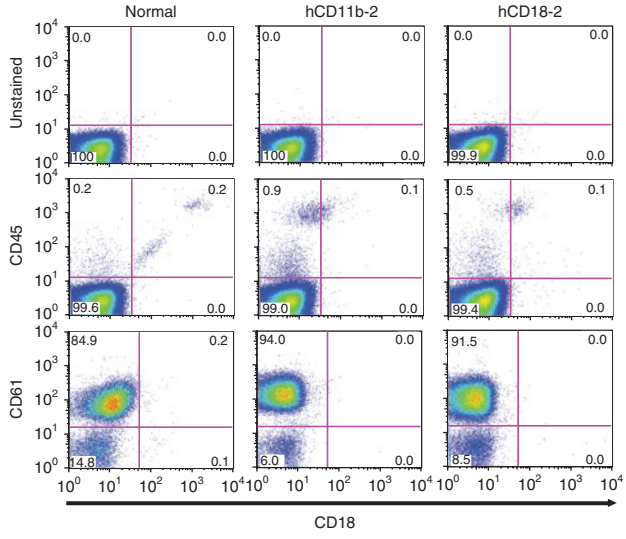


Figure 7 Analysis of CD18 expression on red blood cells and platelets. Peripheral blood was obtained from a normal dog, hCD11b-2 dog, and hCD18-2 dog 1 year following treatment. Dot plots represent CD18 expression (x-axis) versus unstained (top row), CD45 (middle row), or CD61 (bottom row) populations (y-axis).

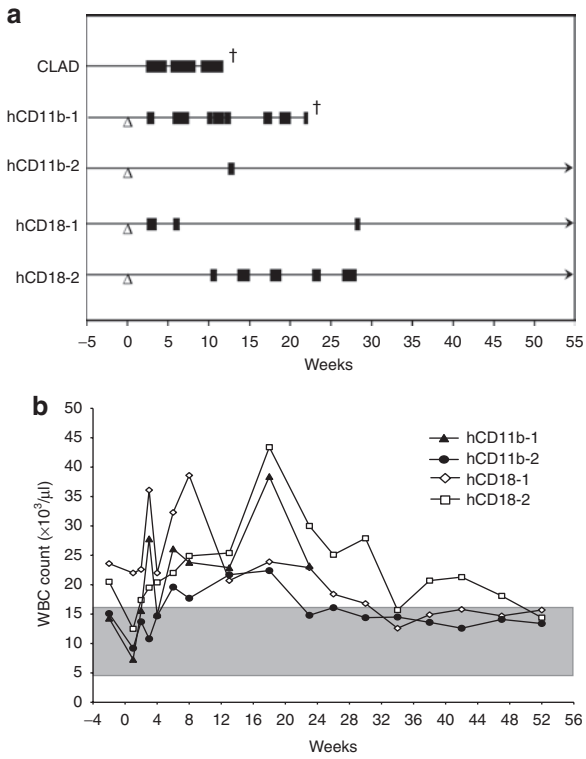


Figure 8 Clinical course of the hCD11b(637 bp)-cCD18 and the hCD18(1,060 bp)-cCD18 vector–treated dogs. (**a**) Individual dogs are listed on the y-axis and the time from birth appears on the x-axis. The horizontal lines represent the life of each animal. Triangles indicate the time of infusion. Boxes represent episodes of fever requiring parenteral antibiotics. Crosses indicate death. (**b**) WBC counts of the vector-treated CLAD dogs before and up to 52 weeks after gene therapy. Time is shown on the x-axis and the WBC counts on the y-axis. The shaded box indicates a normal range of WBC. CLAD, canine leukocyte adhesion deficiency; WBC, white blood cells.

episodes after 28 weeks following gene therapy (**Figure 8a**). The white blood cell count, a surrogate marker for CLAD, returned to near normal levels by 34 weeks following treatment in the three surviving dogs (**Figure 8b**).

DISCUSSION

These studies were designed to identify human leukocyte integrin proximal promoters in a SIN lentiviral vector for *ex vivo* gene therapy of children with LAD-1. Children with severe deficiency LAD-1 typically die from bacterial infections within the first few years of life due to the inability of their neutrophils to adhere to the endothelium and migrate to the site of infection.[24] Heterogeneous mutations in the leukocyte integrin CD18 gene result in the inability to express the CD11/CD18 leukocyte integrin heterodimers on the leukocyte surface in LAD-1.[25] There is a genotype–phenotype correlation in LAD-1 with the most severe clinical phenotype occurring in children with the lowest levels (e.g. <1%) of CD18 expression on leukocytes.[1]

In previous studies, we demonstrated that *ex vivo* RV-mediated gene therapy in CLAD with an RV vector expressing canine CD18 from the MSCV long terminal repeat resulted in therapeutic levels of $CD18^+$ leukocytes with 1.2–8.3% $CD18^+$ PBLs by 1 year following treatment. However, insertion site analysis demonstrated the presence of a number of integrations in proximity to oncogenes.[26]

In recent studies, we used a foamy viral vector with an internal MSCV promoter/enhancer driving canine CD18 expression to treat CLAD and demonstrated that infusion of autologous foamy viral vector–transduced cells into CLAD animals following 200 cGy TBI resulted in 3–7% $CD18^+$ neutrophils 2 years following infusion. This number of $CD18^+$ neutrophils reversed the CLAD phenotype.[6]

The development of MDS in two patients in a chronic granulomatous disease gene therapy clinical trial, and the development of leukemia in five patients in an X-linked severe combined immunodeficiency gene therapy clinical trial, has led to increasing concerns regarding the use of vectors in which expression of the therapeutic transgene is driven from murine leukemia virus long terminal repeats.[8,7] These viral long terminal repeats normally possess strong enhancer activity and insertional activation of genes in proximity to the vector appears to underlie the genotoxicity of these vectors. One approach to circumvent the genotoxicity of conventional RV vectors consists of using SIN vectors harboring deletions in the U3 promoter/enhancer region in combination with nonviral internal promoters.

In a murine model of Glanzmann's thrombesthenia resulting from a mutation in the CD61 subunit of the platelet integrin receptor, a 900-bp fragment of the human CD41 promoter was used to drive expression of human CD61 in an LV vector.[27] Human CD61 formed a human CD61/murine CD41 cross-species heterodimer. The CD41 promoter restricted transgene expression to the platelet lineage with 34% of transduced megakaryocytes expressing normal levels of CD41/CD61.[27] When levels of CD41/CD61 receptor expression were >7%, a shortening of the bleeding time was observed, and the bleeding phenotype was reversed.[28]

In this study, we tested the human leukocyte integrin CD11a, CD11b, and CD18 proximal promoters to express canine CD18. In a previous study, we demonstrated that the 1.7-kb fragment of the hCD11a promoter–directed functional activity of a heterologous reporter gene in the Jurkat T-lymphocyte cell line and the HL-60 myeloid cell line, but not in nonleukocyte cell lines.[15] The human CD11a promoter was subsequently shown to have *cis*-acting regulatory elements within 140 bp of the transcriptional start site.[12] The human CD11b subunit has been shown to be expressed in the highest levels in mature myelomonocytic cells, and the human CD11b proximal promoter directs myeloid-specific and developmentally regulated expression.[12,13,29] When the human CD18 promoter was isolated,[10,14] a region encompassing the first 900 bp was found to contain strong promoter activity. A number of known transcription factors were subsequently found to bind to this 900-bp region including Sp1, PU.1, and GA-binding proteins.[30,31] In several reports, a region of only 302 bp was shown to possess the ability to confer PMA-induced expression of CD18.[32]

In this study, we screened human CD11 and CD18 leukocyte integrin proximal promoters in a SIN lentiviral vector as candidate promoter/enhancers to treat animals with CLAD, a large-animal model of LAD-1. *In vitro* studies supported the testing of two SIN LV vectors—hCD11b (637 bp)-cCD18 and hCD18 (1,060 bp)-cCD18—for *ex vivo* gene therapy of CLAD. Three of the four CLAD dogs treated with these vectors had 0.7–1.5% $CD18^+$ neutrophils

1 year following gene therapy, a level sufficient for reversal of the CLAD phenotype. The highest level of CD18 expression in neutrophils was observed with the hCD11b (637 bp)-cCD18 vector. The short hCD11b promoter fragment (637 bp) used in this study appears to lack inhibitory regulatory elements resulting in high-level expression of CD18 in all leukocytes, rather than just myelomonocytic cells. The high-level expression of CD18 in the lymphoid and monocyte populations with these promoters may enable them to be used in SIN LV vectors for gene therapy of lymphoid and monocytic diseases. Of note, expression of CD18 from these promoters appears to increase with myeloid differentiation.

There are two outcomes that lead to successful treatment of CLAD with gene therapy. First, allogeneic transplantation in CLAD indicates that a very low number or percentage of normal neutrophils (e.g. 1%) results in reversal of the CLAD.[23] These neutrophils are derived from a normal donor, and thus have a normal level of CD18 expression per cell. Second, gene therapy of CLAD in which a low percentage of $CD18^+$ neutrophils (1–2%) with normal levels of CD18 expression per cell from an MSCV promoter results in reversal of CLAD.[5,6]

This study demonstrates that a low number or percentage of $CD18^+$ neutrophils (1–2%) with a low expression level of CD18 molecules per neutrophil (15–24% of normal CD18 expression per cell) result in reversal of CLAD in three of four CLAD dogs. Thus, the lowest number of $CD18^+$ neutrophils along with the lowest level of CD18 expression per cell remains to be defined.

Future studies to improve gene therapy of CLAD and LAD-1 will include both increasing the transduction efficiency and CD18 expression per cell to ensure reliable reversal of the disease phenotype. Increased transduction efficiency may be achieved with higher titer vectors, including a prestimulation step before transduction, or using improved vector designs. Increasing CD18 expression per cell may be achieved by the addition of additional regulatory elements for the human leukocyte integrin promoters. Thus, these studies represent an important step toward the design of vectors with nonviral promoter elements for use in gene therapy of LAD-1.

MATERIALS AND METHODS

Construction of SIN lentiviral vectors. The hCD11a(1731bp) promoter fragment was obtained form the CD11aPro_BS2SKp plasmid[15] and cloned into the pRRLSIN.cPPT.PGK-cCD18.WPRE, originally obtained from Addgene (Cambridge, MA) as plasmid 12252 pRRLSIN.cPPT.PGK.GFP.WPRE, by replacing the PGK promoter to make pRRL.hCD11a(1731bp)-cCD18. The remaining hCD11a promoter fragments were PCR amplified from pRRL.hCD11a(1731bp)-cCD18 and cloned into the pRRL plasmid by replacing the PGK promoter.

The hCD11b(389 bp) promoter fragment was excised from the LN11BPA plasmid[33] and cloned into pRRLSIN.cPPT.PGK-cCD18.WPRE to make pRRL.hCD11b(389 bp)-cCD18. The remaining hCD11b promoter fragments were PCR amplified from CD11bpro(1.7 kb)-pØGH[34] and cloned into pRRLSIN.cPPT.PGK-cCD18.WPRE by replacing the PGK promoter.

The hCD18(781 bp) promoter fragment was excised from the LN18PA plasmid and cloned into the pRRLSIN.cPPT.PGK-cCD18.WPRE by replacing the hPGK promoter to make pRRL.hCD18(781bp)-cCD18. The hCD18(1,060 bp) promoter fragment was PCR amplified from the pVZSK-hCD18pro(4.7 kb) plasmid and cloned in place of the hPGK promoter to make the pRRL.hCD18(1,060 bp)-cCD18 construct. The specific primer pairs used for each construct are shown in **Supplementary Table S1.**

Viral production, titration determination, and* in vitro *transduction of CLAD $CD34^+$ cells. A four-plasmid transient transfection protocol,[35] titration experiments, and *in vitro* transduction of CLAD $CD34^+$ cells were performed as previously described.[36]

Transduction and differentiation of CLAD $CD34^+$ cells. Normal, CLAD, and transduced CLAD $CD34^+$ cells were incubated on RetroNectin coated, non-tissue-culture-treated, 24-well plates with StemSpan serum-free expansion medium and 10% fetal bovine serum, along with 5 μg/ml of protamine sulfate, and a cytokine cocktail consisting of 50 ng/ml each of canine interleukin-6, canine stem cell factor, human Flt3-L, human thrombopoietin, and human granulocyte colony–stimulating factor for 24 hours before transduction. Before the transduction and post-transduction on days 2 and 5, cells were analyzed for CD18 expression by flow cytometry.

Animals. Dogs were housed in National Institutes of Health facilities in Bethesda, Maryland and Poolesville, Maryland in accordance with National Institutes of Health guidelines. These facilities are approved by the American Association for Accreditation of Laboratory Animal Care and Use Committees of the National Cancer Institutes, National Institutes of Health, Bethesda, Maryland. The studies were performed in accordance with the principles outlined in the Guide for Laboratory Animals Facilities and Care of the National Academy of Sciences, National Research Council.

Bone marrow harvest and $CD34^+$ isolation. Bone marrow was collected and $CD34^+$ cells were isolated as previously described.[6,36]

Transplantation of autologous hematopoietic stem cells. One day before transplant autologous CLAD $CD34^+$ cells were thawed, resuspended in X-VIVO 15/1% human serum albumin, and transduced overnight as described for the *in vitro* studies. On the day before infusion all dogs received a single, nonmyeloablative dose of 200 cGy TBI delivered from a ^{60}Co source. The following morning the $CD34^+$ cells were harvested, washed, resuspended in a solution of Plasmalyte A and 1% heat-inactivated autologous serum, and infused intravenously over 15 minutes.

Clinical monitoring of CLAD animals. All CLAD animals were monitored daily by physical exam and temperature. CLAD dogs were treated prophylactically with oral amoxicillin/potassium clavulanate as described.[5] Treatment with intravenous antibiotics was used when needed. White blood cell counts and differentials were performed commercially (Antec Diagnostics, Lake Success, NY).

Flow cytometry of $CD18^+$ levels following infusion. To assess the percentages of circulating $CD18^+$ leukocytes after infusion, PBLs were collected weekly, biweekly, or monthly from each recipient and analyzed for $CD18^+$ surface expression as described.[5,6] Leukocyte subsets including neutrophils, monocytes, T-cells, and B-cells were determined using subset-specific antibodies as described.[6]

Flow cytometry of CD18 expression on nonleukocyte lineages. Whole peripheral blood from three dogs (normal, hCD11b-2, and hCD18-2) was incubated with a fluorescein isothiocyanate–conjugated mouse anti-pig CD61 monoclonal antibody (cross-reactive with dog platelets; AbDSerotec, Oxford, UK) and a phycoerythrin-conjugated rat anti-dog CD45 monoclonal antibody (AbDSerotec). Platelet populations were identified by low forward-scatter and side-scatter profiles and by reactivity with CD61. Red blood cell populations were identified by a forward- and side-scatter profile similar to that of leukocytes and by a lack of reactivity to CD45 (all leukocytes bind CD45). After washing of the cells with phosphate-buffered saline/1% bovine serum albumin (red blood cells: 2× 400*g* 6 minutes; platelets: 2× 800*g* 5 minutes), cells were incubated with 1 μg/ml 7-aminoactinomycin D for dead cell discrimination and analyzed on a Becton Dickinson FACSCalibur instrument (San Jose, CA).

Lymphocyte proliferation assay. Proliferation assays were performed as described.[6]

SUPPLEMENTARY MATERIAL

Table S1. Primer sequences used to amplify various promoter fragments for the hCD11a, hCD11b, and hCD18 promoters.

Figure S1. *In vitro* lymphocyte proliferation assay of the peripheral blood of hCD11b (637 bp)-cCD18 and the hCD18 (1,060 bp)-cCD18 vector-treated dogs.

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