Gene Therapy Studies in a Canine Model of X-Linked Severe Combined Immunodeficiency

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Abstract

Since the occurrence of T cell leukemias in the original human γ -retroviral gene therapy trials for X-linked severe combined immunodeficiency (XSCID), considerable effort has been devoted to developing safer vectors. This review summarizes gene therapy studies performed in a canine model of XSCID to evaluate the efficacy of γ -retroviral, lentiviral, and foamy viral vectors for treating XSCID and a novel method of vector delivery. These studies demonstrate that durable T cell reconstitution and thymopoiesis with no evidence of any serious adverse events and, in contrast to the human XSCID patients, sustained marking in myeloid cells and B cells with reconstitution of normal humoral immune function can be achieved for up to 5 years without any pretreatment conditioning. The presence of sustained levels of gene-marked T cells, B cells, and more importantly myeloid cells for almost 5 years is highly suggestive of transduction of either multipotent hematopoietic stem cells or very primitive committed progenitors.

Introduction

SEVERE COMBINED IMMUNODEFICIENCY (SCID) is a heterogeneous group of diseases characterized by the inability to mount humoral and cell-mediated immune responses and is invariably fatal within the first 2 years of life.^{1,2} X-linked SCID (XSCID) is the most common form of the disease representing approximately 50% of all human SCID,^{1,3} and is caused by mutations in the common gamma chain (γ c) subunit of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.^{4–6} The shared usage of the γ c by receptors for cytokines critical for the proliferation, differentiation, survival, and function of the cells comprising the immune system resulting in a complex of multiple cytokine defects explains the profound immunologic abnormalities and clinical severity of the disease.

Since the first successful HLA-identical bone marrow transplant (BMT) in a boy with XSCID in 1968,⁷ BMT has been the treatment of choice for XSCID. Patients receiving a histocompatible (HLA-identical) BMT have greater than 90% long-term survival rates.^{8,9} However, the majority of patients do not have a histocompatible donor. Advances in BMT techniques, namely, prevention of graft-versus-host

disease by T cell depletion, have permitted the use of haploidentical (half-matched) bone marrow cells for immunologic reconstitution of XSCID patients.^{9,10} Although this development makes possible the treatment of nearly every XSCID patient, survival rates (longer than 1 year) are lower, ranging from 60% to 75%.^{8,9} A potential alternative to bone marrow transplantation, particularly for those patients without an HLA-identical donor, is gene therapy.

The first clinical gene therapy trial for human XSCID was initiated in 1999.¹¹ In this clinical study, XSCID infants received autologous bone marrow CD34⁺ cells transduced *ex vivo* with a γ -retroviral vector carrying the γ c cDNA, resulting in immune reconstitution and reversal of the clinical phenotype in 9 of 10 treated boys.¹² There was evidence of active thymopoiesis with normalization of naive CD45RA⁺CD4⁺ T cells and normal or near-normal number of T cell receptor excision circle (TRECs) in the first 2 years after treatment; however, <1% of the B cells and <0.1% of the myeloid cells were derived from transduced cells. A gradual decline in T cell numbers started approximately 20 months after treatment with a concomitant decrease in CD45RA⁺ (naive) T cells and TREC levels. Long-term follow-up of 8 of the treated boys, approximately 9 years

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following treatment, showed normal or low-normal numbers of T cells persisted in approximately half of the boys, no evidence of transduced B cells or myeloid cells, and no transduced functional progenitors or stem cells persisted in the bone marrow of the treated XSCID boys.¹³

An additional 10 XSCID boys have been successfully treated in an English gene therapy trial with similar results observed in the French clinical trial.^{14,15} Unfortunately, the development of T cell leukemia in 5 of the 20 patients approximately 3–5 years following treatment also demonstrates that gene therapy is not without potential serious side effects.^{16–19} Preliminary results have just been published on 9 XSCID patients treated with a self-inactivating γ -retroviral vector incorporating the ELF-1 α promoter.²⁰ One patient died from infection and another failed to engraft gene-corrected cells requiring a cord blood transplant. These 8 patients have been followed for 12–38 months. The kinetics of T cell regeneration was essentially the same as reported for this same time period using the previous γ -retroviral vectors. As in the previous two γ -retroviral gene therapy trials, little marking was present in B cells or myeloid cells. No T cell leukemias have been observed thus far, but the latency period in the previous studies was 2–5½ years.^{16–19}

Our laboratory has identified and characterized a naturally occurring XSCID in two different breeds of dogs, basset and cardigan Welsh corgi, that are caused by two different mutations in the common gamma chain (γ c).^{21–27} The basset mutation is a 4 bp deletion in the signal peptide region resulting in a frame-shift with a premature termination codon

in exon 1,²⁸ and the corgi mutation consists of a insertion of a cytosine following nucleotide 582 resulting in a premature stop codon prior to the transmembrane domain.²⁹ A breeding colony of XSCID dogs was developed from a single carrier female from each breed. Canine XSCID, unlike genetically engineered γ c-deficient mice, has a clinical and immunologic phenotype identical to human XSCID, making it an ideal large-animal preclinical model in which to evaluate strategies for improving gene therapy for XSCID.

Ex Vivo γ -Retroviral Gene Therapy of XSCID Dogs

Twelve XSCID dogs were treated by *ex vivo* γ -retroviral gene therapy using an RD114-pseudotyped MSCV-canine γ c-IRES-GFP vector.³⁰ Four of the 12 treated dogs failed to engraft gene-corrected cells. The remaining dogs developed an average of 68% gene-corrected T cells by 4 weeks following treatment and showed a steady increase in the proportion of gene-corrected T cells during the next 8 weeks that plateaued at an average of 85%. By 2 months post-treatment, all the dogs developed gene-corrected T cells within the normal range (Fig. 1A). Since newly emerging T cells from the thymus express a naïve CD45RA⁺ phenotype, the quantitation of CD45RA⁺ T cells is one assay commonly used to determine whether reconstituted T cells following hematopoietic stem cell transplantation are recent thymic emigrants. During the first 3–4 months posttreatment, >90% of the gene-corrected T cells expressed a CD45RA⁺ (naïve) phenotype (Fig. 1B). The gene-corrected

FIG. 1. Absolute number of GFP⁺ T cells following *ex vivo* or *in vivo* γ -retroviral gene therapy of neonatal X-linked severe combined immunodeficiency (XSCID) dogs (A); comparison of the proportion of CD45RA⁺ T cells between *ex vivo* and *in vivo* γ -retroviral gene therapy-treated XSCID dogs (B); clonal contribution to T cell regeneration following *ex vivo* or *in vivo* γ -retroviral gene therapy of neonatal XSCID dogs as determined by LAM-PCR (C); and proportion of GFP⁺ lymphocytes, monocytes, neutrophils, T cells, and B cells 4 years following *in vivo* γ -retroviral gene therapy of neonatal XSCID dogs (D).

T cells were functional as evaluated by their proliferative response to mitogenic stimulation with phytohemagglutinin (PHA). Although the proportion of gene-corrected T cells remained >80% throughout the study, the number of gene-corrected T cells and the proportion of gene-corrected CD45RA⁺ T cells steadily decreased beginning approximately 4 months posttreatment. By 8–9 months posttreatment, the proportion of CD45RA⁺ T cells averaged $49.8\% \pm 5.6\%$.

For the first 6 months following treatment, the gene-corrected cells demonstrated a polyclonal, diverse T cell repertoire, as determined by TCR V β CDR3-size spectratyping, similar to that of normal dogs. However, between 6 and 9 months posttreatment TCR V β diversity showed signs of decreasing as evidenced by a decrease in the number of polyclonal spectratypes and approximately 50% of the spectratypes exhibiting an oligoclonal or monoclonal profile. LAM-PCR was used to evaluate the diversity of clonal integration sites contributing to the immunologic reconstitution at three different time points in each of the dogs. During the first 4–5 months following treatment, a polyclonal profile was observed; however, as the number of gene-corrected T cells declined, the number of clones contributing to their presence also decreased (Fig. 1C).

Thymic function was assessed in the gene therapy-treated dogs by quantifying TRECs as a measure of thymic output. High levels of TRECs were evident by 2 months following

gene therapy (Fig. 2D); however, the TREC levels started to decline between 3 and 6 months posttreatment, which also correlated with the decrease in CD45RA⁺ T cells.

None of the dogs showed detectable gene marking in myeloid cells, and 2–5% of the peripheral B cells expressed GFP; however, these B cells expressed an immature phenotype. Despite the presence of gene-corrected B cells, serum IgG concentrations remained subnormal and immunization of the dogs with a standard modified-live, multivalent vaccine revealed poor humoral immune reconstitution as determined by their ability to produce protective levels of IgG-specific antibody.

We had the opportunity to evaluate the presence of gene-corrected cells in the thymus and bone marrow in five dogs that were euthanized because of failing health between 8 and 11 months following treatment. Histologically, the thymus had the appearance of a typical small, dysplastic XSCID thymus. The proportion of gene-corrected (GFP⁺) thymocytes ranged from 6.0% to 25.8%. None of the dogs exhibited any GFP⁺CD34⁺ cells in the bone marrow at the time of euthanasia. One dog developed a thymic T cell lymphoma that was shown to have been caused by insertional mutagenesis.

Although treating XSCID dogs by *ex vivo* γ -retroviral gene therapy using standard *ex vivo* culture conditions is capable of reconstituting gene-corrected T cells, this T cell

FIG. 2. Absolute number of gene-corrected T cells following *in vivo* lentiviral and foamy viral gene therapy of neonatal XSCID dogs (**A**); proportion of CD45RA⁺ T cells after lentiviral and foamy viral gene therapy of XSCID dogs (**B**); analysis of T cell receptor excision circles (TRECs) in XSCID dogs treated by *in vivo* lentiviral gene therapy compared with TREC levels of XSCID dogs treated by bone marrow transplantation (BMT) and *ex vivo* γ -retroviral gene therapy (**C**); and proportion of GFP⁺ monocytes and neutrophils following *in vivo* lentiviral gene therapy of neonatal XSCID dogs (**D**).

immune reconstitution is relatively short-lived. The results observed in the XSCID dogs treated by *ex vivo* γ -retroviral gene therapy differ significantly in many respects from those observed in XSCID dogs successfully immune reconstituted by hematopoietic stem cell transplantation (HSCT) with freshly isolated bone marrow CD34⁺ cells. XSCID dogs treated by HSCT, without any pretransplant conditioning, maintain normal numbers of T cells through at least 10 years posttransplant, with the proportion of CD45RA⁺ (naïve) T cells remaining >70%, and a polyclonal, diverse T cell repertoire through 7 years posttransplant.^{31–33}

In Vivo γ -Retroviral Gene Therapy of XSCID Dogs

Current *ex vivo* transduction protocols using γ -retroviral vectors require manipulation of hematopoietic stem cells (HSCs) by culture in multiple cytokines for 4–5 days that may force HSCs to enter pathways of proliferation, and possibly differentiation, which could limit their engraftment potential, pluripotentiality, and long-term repopulating capacity. Numerous animal studies have reported HSCs cultured *ex vivo* under similar conditions and cytokine exposure to be at a disadvantage compared with freshly isolated cells in terms of both their short-term and long-term engraftment potential, which was evident as early as 3–6 days of culture. We have recently reported that XSCID dogs transplanted with bone marrow CD34⁺ cells from normal donors that have been cultured *ex vivo* under identical conditions used for γ -retroviral *ex vivo* transduction protocols show the same inability to sustain long-term thymopoiesis as the *ex vivo* γ -retroviral gene therapy-treated dogs.³⁴ A potential reason for this is that the proportion of freshly isolated CD34⁺ bone marrow cells in the “typical lymphocyte” gate averages 79%, which decreases to 22% following a typical γ -retroviral *ex vivo* transduction protocol with the majority of the cells in the more differentiated “blast cell” gate. The importance of this finding is that flow cytometric and functional studies have shown that the most immature CD34⁺ cells are contained in a population of cells consisting of low side scatter characteristics (SSC) and low front scatter characteristics (FSC), the typical lymphocyte gate.^{35–37} An attractive alternative would be to transduce HSCs *in vivo* within their natural environment by direct intravenous injection (*in vivo* gene therapy), thereby eliminating the necessity for *ex vivo* manipulation of the target cell.

Neonatal XSCID dogs were treated by direct intravenous injection using the RD114-pseudotyped MFGS-canine- γ -IRES-GFP-MGMT* retroviral vector that contained a green fluorescent protein (GFP)-mutant methylguanine methyltransferase (MGMT*) fusion protein (GFP-MGMT*).³⁸ The mutant MGMT portion of the fusion protein confers resistance to cytotoxic effects of O⁶-benzylguanine and a DNA alkylating agent such as temozolomide and, although not used in this study, was included for potential future use for *in vivo* selective enrichment of marked marrow cells.

By 4 weeks posttreatment, an average of 65% of the peripheral T cells were GFP⁺, while 0.5–5.6% of the monocytes and 0.5–6.1% of the granulocytes were GFP⁺. The percentage of GFP⁺ T cells and, to a lesser extent, monocytes in the peripheral blood continued to increase through 8 weeks posttreatment, while the percentage of GFP⁺ granulocytes remained relatively constant. The pro-

portion of gene-corrected T cells peaked at an average of >90% by 8 weeks following treatment and remained stable at this level. The majority of the newly emerging gene-marked T lymphocytes in the *in vivo*-treated dogs in this study expressed a CD45RA⁺ (naïve) phenotype, suggesting that they were recent thymic emigrants. The *in vivo* γ -retroviral gene therapy-treated dogs attained normal numbers of gene-corrected peripheral T cells by 8 weeks following treatment (Fig. 1A). The reconstituted peripheral T cells in the treated dogs were functional as assessed by their ability to proliferate and phosphorylate STAT5 in response to cytokine stimulation. They also exhibited a diverse, polyclonal repertoire as determined by TCRV β CDR3 spectratyping by 6 months following treatment.

In contrast to the *ex vivo* gene therapy-treated dogs, the *in vivo* gene therapy-treated dogs showed sustained T cell reconstitution for up to 4 years following treatment. The majority of the gene-corrected T cells maintained a CD45RA⁺ phenotype (Fig. 1B) with multiple clones contributing to the T cell reconstitution for at least 4 years (Fig. 1C). An interesting finding from this study was the sustained presence of gene-corrected monocytes and granulocytes for at least 4 years following (Fig. 1D). This contrasts with the lack of transduced myeloid cells in both XSCID boys and XSCID dogs following *ex vivo* γ -retroviral gene therapy.

Another difference between the *ex vivo* γ -retroviral gene therapy-treated dogs and the *in vivo* γ -retroviral gene therapy dogs is that the XSCID dogs treated by *in vivo* γ -retroviral gene therapy exhibited B cell chimerism as early as 4 weeks posttreatment. More importantly, the B cell chimerism was sustained with proportions of gene-corrected B cells ranging from 15% to 26%. The treated dogs developed normal to low normal concentrations of serum IgG and protective levels of specific IgG antibody production following immunization between 6 and 8 months posttreatment. None of the dogs exhibited any signs of a serious adverse event related to the gene therapy or any evidence of germ-line transduction.

The presence of sustained levels of gene-marked T cells, B cells, and more importantly myeloid cells for 4 years is highly suggestive that *in vivo* γ -retroviral gene therapy by direct intravenous administration is capable of transducing long-lived, multipotent HSCs and/or primitive committed progenitors in their natural environment, resulting in the correction of the XSCID phenotype and sustained immune reconstitution without any pretreatment conditioning.

In Vivo Lentiviral and Foamy Viral Gene Therapy of XSCID Dogs

Since the occurrence of T cell leukemias in the human XSCID patients, strategies for increasing the safety of gene therapy have been proposed, including the use of lentiviral vectors in place of γ -retroviral vectors since they appear to have a “safer” integration profile. γ -Retroviral vectors have a preference for integrating near genes with a strong preference for integration near the transcriptional start site (promoter).^{16,39–43} Lentiviral vectors also have a preference for integrating in genes; however, they do not have a preference for transcriptional start sites but are distributed throughout the coding region of the gene.^{39–41,43,44} Foamy viral vectors have a unique integration profile with less

frequent integration near promoters than γ -retroviral vectors and they do not preferentially integrate within genes.^{40,45} They also have a reduced potential to activate neighboring genes compared with γ -retroviral and lentiviral vectors. Therefore, in principle, foamy viral vectors, because of their nearly random integration pattern, may be safer vectors than γ -retroviral and lentiviral vectors. Other advantages of lentiviral vectors are that they are relatively amenable to modification for potentially further improving the safety of the vector and have the potential to transduce relatively quiescent cells such as HSCs that will be required for providing sustained production of genetically corrected hematopoietic cells and immune reconstitution in XSCID.^{46,47}

Neonatal XSCID dogs were treated by *in vivo* lentiviral gene therapy using either an SIV-based (RD114/TR SIV-MSCV-h γ c-dCI) or a HIV-based (VSV-G CL20-4i-EF1 α -h γ c-OPT) vector without any pretreatment conditioning.^{48–50} Both vectors are self-inactivating and contain an insulator, but the SIV vector contains the viral MSCV promoter, whereas the HIV vector contains the endogenous EF1 α promoter. The HIV-based lentiviral vector also contains a codon-optimized version of the γ c that allows for more stable mRNA. We chose to use the *in vivo* delivery approach in the lentiviral studies based upon the results obtained in the *in vivo* γ -retroviral gene therapy studies and the *in vivo* approach precludes the necessity for *ex vivo* manipulation of the host's CD34⁺ cells.

By 4 weeks following treatment, the proportion of γ c⁺ T cells averaged approximately 50% in both the SIV- and HIV-lentiviral-treated dogs and continued to increase during the next 6 months to between 80% and 90% and were sustained that level for at least 57 months following treatment. Normal numbers of γ c⁺ T cells in both groups were evident by 2 months following treatment and remained in the normal range for at least 57 months (Fig. 2A). These cells were functional based upon their ability to phosphorylate STAT5 in response to stimulation with either IL-2 or IL-7 and proliferate in response to mitogenic stimulation. The regenerated gene-corrected T cells expressed the naïve CD45RA⁺ phenotype in both the SIV- and HIV-treated dogs, which was sustained for up to 4½ years (Fig. 2B). Further evidence for long-term thymopoiesis was the sustained presence of high levels of TRECs (Fig. 2C).

As in the *in vivo* γ -retroviral gene therapy-treated XSCID dogs, the lentiviral gene therapy-treated dogs showed B cell chimerism and the sustained presence of gene-corrected myeloid cells (Fig. 2D). Serum IgG concentrations at 7 months posttreatment in the SIV-treated dogs ranged from 400 to 1000 mg/dl and in the HIV-treated dogs ranged from 420 to 680 mg/dl (normal range in 7–11-month-old dogs = 420 to 1236 mg/dl). The dogs were vaccinated at 6–7 months following treatment with a multivalent modified-live viral vaccine, and all dogs developed protective titers of IgG-specific antibody to canine distemper virus and canine distemper virus. Although integration site analysis remains to be performed in the lentiviral gene therapy-treated XSCID dogs, no serious adverse events associated with the gene therapy have been observed in the treated dogs.

The importance of these findings from a clinical standpoint is that incorporating insulating elements as a safety feature does not have an adverse effect on transgene expression in a large animal model of a relevant human disease and that normal γ c expression can be achieved when

driven by an endogenous promoter. The presence of sustained levels of gene-marked T cells, B cells, and more importantly myeloid cells for almost 5 years is highly suggestive of transduction of either multipotent HSCs or very primitive committed progenitors by lentiviral vectors, especially when administered by direct intravenous injection.

We have recently initiated studies to evaluate the efficacy of foamy viral gene therapy for treating XSCID. Five neonatal XSCID dogs were treated by *in vivo* gene therapy using the foamy vector pFV-EF1 α -GFP-2A- γ c containing GFP and the coding sequence for the human common gamma chain (γ c) linked by a 2A element, expressed under control of the elongation factor 1 promoter (EF1 α).⁵¹ Transgene-positive lymphocytes were detected in peripheral blood beginning at 4–6 weeks posttreatment and peaked at approximately 7 months. Three dogs recovered lymphocyte counts to the normal range within 16 weeks of treatment although the rate of reconstitution was somewhat delayed compared with the lentiviral-treated dogs (Fig. 2A). The reconstituted T cells expressed a naïve CD45RA⁺ phenotype, suggesting that they were recent thymic emigrants (Fig. 2B), and were functional as determined by proliferation and STAT5 phosphorylation assays. TCRV β spectratyping revealed a generally diverse T cell repertoire, with some evidence of TCR oligoclonality. Retroviral insertion site analysis of peripheral white blood cells 3 months following vector administration shows that between 200 and 600 unique clones contributed to hematopoiesis in two dogs examined. Furthermore, B cell chimerism was observed in the treated dogs and a small population of GFP-positive gene-marked cells could be detected in myeloid lineages. These preliminary results demonstrate that a foamy virus vector can be used safely in a preclinical model for *in vivo* gene therapy. We are currently evaluating multiple parameters of vector design for improving the efficacy of foamy viral gene therapy for canine XSCID using the *in vivo* approach.

Concluding Remarks

Canine XSCID provides a unique large animal preclinical model for evaluating methods for improving the efficacy and safety of gene therapy for human XSCID. Studies can be performed in the canine XSCID model that would be difficult to perform in human patients. For example, competitive repopulation studies can be performed to directly compare vector backbones, promoters, or delivery methods to determine which works best in the same animal. Testing of improved *ex vivo* transduction protocols that preserve both short-term and long-term engraftment potential, while maintaining sufficient transduction efficiency that results in sustained immune reconstitution, can be evaluated in this animal model. The XSCID dog is also an ideal preclinical model for further evaluating the efficacy and safety of the *in vivo* approach to gene therapy, particularly the extent of any off-target transduction that follows an intravenous viral injection, which will be necessary before entertaining this approach as alternative to the current *ex vivo* approach in human patients. In addition, other potential delivery strategies aimed at targeting true hematopoietic progenitors, such as intra-osseous injection, can readily be tested in this model.

Canine XSCID also has many advantages as a large animal model for addressing important questions concerning

HSC gene therapy for other hematopoietic diseases. It has a cell lineage with a strong selective developmental and proliferative advantage (T cells) and cell lineages with little, or no, selective developmental advantage (B cells and myeloid cells). Therefore, not only will results obtained in the XSCID dog apply directly to gene therapy for XSCID, but many of the findings may be applicable for gene therapy of other immune and/or hematologic diseases as well.

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Author Disclosure Statement

No competing financial interests exist.

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