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BIOLOGY

Effect of Ex Vivo Culture of CD34$^+$ Bone Marrow Cells on Immune Reconstitution of XSCID Dogs Following Allogeneic Bone Marrow Transplantation

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Successful genetic treatment of most primary immunodeficiencies or hematological disorders will require the transduction of pluripotent, self-renewing hematopoietic stem cells (HSC) rather than their progeny to achieve enduring production of genetically corrected cells and durable immune reconstitution. Current ex vivo transduction protocols require manipulation of HSC by culture in cytokines for various lengths of time depending upon the retroviral vector that may force HSC to enter pathways of proliferation, and possibly differentiation, which could limit their engraftment potential, pluripotentiality and long-term repopulating capacity. We have compared the ability of normal CD34$^+$ cells cultured in a standard cytokine cocktail for 18 hours or 4.5 days to reconstitute XSCID dogs following bone marrow transplantation in the absence of any pretransplant conditioning with that of freshly isolated CD34$^+$ cells. CD34$^+$ cells cultured under standard γ-retroviral transduction conditions (4.5 days) showed decreased engraftment potential and ability to sustain long-term thymopoiesis. In contrast, XSCID dogs transplanted with CD34$^+$ cells cultured for 18 hours showed a robust T cell immune reconstitution similar to dogs transplanted with freshly isolated CD34$^+$ cells, however, the ability to sustain long-term thymopoiesis was impaired. These results emphasize the need to determine ex vivo culture conditions that maintain both the engraftment potential and "stem cell" potential of the cultured cells.

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INTRODUCTION

A prerequisite for any ex vivo approach to hematopoietic stem cell (HSC) gene therapy is the ability of the transduced HSC to successfully engraft in the recipient following their reinfusion. The major limitation of the ex vivo approach to gene therapy is the need for ex vivo manipulation of HSC with culturing in cytokines for various lengths of time depending upon the retroviral vector. CD34, a cell surface glycoprotein, is an antigen expressed on a subpopulation of hematopoietic cells that contain both stem cells, presumably pluripotent stem cells, and early committed progenitors that are capable of multilineage engraftment in humans, mice, nonhuman primates, and, more recently, dogs [1-8]. It is clear from a large body of clinical and experimental data that a population of cells within the CD34$^+$ population is both pluripotent and capable of self-renewal, and selection based upon CD34 does not deplete the graft of significant numbers of HSCs. Therefore, enumeration of CD34$^+$ cells is the current "surrogate" for determining the stem cell content of a human, nonhuman primate and canine bone marrow grafts and are the current targets for human and canine gene therapy involving diseases of the hematopoietic system.

Because γ-retroviruses require active replication of the target cells for transduction, the typical γ-retroviral ex vivo transduction protocol used in human gene therapy clinical trials consists of a one to two day prestimulation in cytokines followed by three days of transductions to "maximize" the number of transduced

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cells [9-15]. Lentiviral vectors have gained considerable attention as potential vectors for HSC, because they have been shown to be capable of transducing quiescent $CD34^+$ cells, however maximal transduction occurs as the cell enters G_1 of the cell cycle [16,17]. Therefore, most studies use either an 18 hour (overnight) or 48 hour transduction protocol in the presence of various cytokine cocktails. As HSCs are typically quiescent, ex vivo culture in cytokines that allow for gene transfer to occur may force HSCs to enter pathways of proliferation, and possibly differentiation, that could limit their engraftment potential, pluripotentiality, and long-term repopulating capacity. Currently, the most controversial and important issue regarding the clinical use of ex vivo manipulated cells is the question of whether exhaustion of stem cells might result from growth factor stimulation ex vivo, which has considerable significance for both gene therapy and HSC expansion protocols [18].

Over the past few years, evidence has accumulated that cultured HSC have a reduced ability to engraft in murine, xenogeneic, and large animal transplant models. These animal studies were performed in recipients following pretransplant conditioning that is known to damage bone marrow stromal cells [19-21] and decrease HSC homing to or survival in the bone marrow following transplantation [22].

Competitive repopulation studies in mice have revealed significantly reduced engraftment with expanded cells compared with freshly isolated bone marrow (BM) cells [23,24]. Transplantation of ex vivo cultured autologous BM $CD34^+$ cells in myeloablated baboons resulted in delayed short-term engraftment (recovery of normal neutrophil and platelet counts) compared to freshly isolated BM $CD34^+$ cells [25]. Autologous transplantation of irradiated cats with cultured bone marrow cells resulted in an engraftment rate of 40% compared with 100% for noncultured cells [26].

Xenotransplantation of cultured human cord blood (CB) cells in SCID or NOD/SCID mice have been reported to result in delayed and significantly reduced engraftment compared with fresh $CD34^+$ cells [27-33]. When fresh and expanded human CB $CD34^+$ cells were transplanted together in a competitive repopulation assay, the fresh $CD34^+$ cells significantly out-competed the expanded cells [34]. An important finding from these xenogeneic studies is that the reduced in vivo repopulating capacity was evident as early as 24 to 48 hours in culture [29,30,33].

None of the previous studies have evaluated the effect of ex vivo culture of HSC on engraftment in the absence of pretransplant conditioning or in a large animal model of a relevant human disease, or on the reconstitution of immune function. We have previously shown that freshly isolated bone marrow $CD34^+$ cells from normal dog leukocyte antigen (DLA) identical donors are capable of sustained B and T cell immune reconstitution of XSCID dogs following allogeneic BMT without any pretransplant conditioning [8]. The purpose of this study was to determine the effect of short-term ex vivo culture, similar to that used for γ-retroviral and lentiviral ex vivo transduction, of purified bone marrow $CD34^+$ cells on their ability to reconstitute normal T cell function and long-term thymopoiesis in unconditioned XSCID dogs.

MATERIALS AND METHODS

Dogs

The XSCID dogs used in this study were derived from a breeding colony established from a single carrier female [35,36]. All affected dogs have the same γc mutation, a four bp deletion in exon 1, and were diagnosed shortly after birth by a PCR based mutation detection assay using DNA isolated from whole blood [36,37]. DLA-matched or DLA-haploidentical normal littermate donors for transplantation were determined by PCR assay for highly polymorphic major histocompatibility complex (MHC) class I and class II microsatellite marker polymorphisms [38-40].

Bone Marrow Preparation

Bone marrow cells were collected from normal littermate donors following euthanasia by removing a segment of the femur, flushing the marrow into a sterile petri dish containing HBSS without calcium and magnesium (Mediatech, Fisher Scientific, Philadelphia, PA), and filtered through a fine mesh filter [8,36]. The cells were centrifuged and washed twice in HBSS and resuspended in PBS for isolation of $CD34^+$ cells. Isolation of bone marrow $CD34^+$ cells was performed as previously described [8]. Briefly, unfractionated marrow cells were resuspended at a final concentration of 1×10^8 cells/ml in a PBS solution containing 2% horse serum and incubated with anti-canine CD34 antibody 1H6 at 20 μg/10^8 cells [6]. Cells were incubated with anti-mouse IgG MACS magnetic micro-beads followed by selection on vario-MACS columns according to the manufacturer's protocol (Miltenyi, Auburn, CA). Aliquots of positively selected cells were labeled with phycoerythrin (PE) labeled streptavidin (Jackson Immunoresearch, West Grove, PA) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) to determine the purity of the eluted cells. The purity of the cells was >96%.

$CD34^+$ Cultures and Stimulation

On day 1, purified $CD34^+$ cells were placed in Human Dexter Medium (HDM) containing 72% isocove's DMEM (Mediatech, Fisher Scientific, Philadelphia, PA), 12.5% fetal bovine serum (FBS), 12.5%

horse serum, 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA), 1% sodium pyruvate (Mediatech, Fisher Scientific, Philadelphia,PA), 10 uM β-mercaptoethanol and 1 uM hydrocortisone (Sigma Chemical, St. Louis, MO), stimulated with 100 ng/ml canine granulocye-colony-stimulating factor (G-CSF), 100 ng/ml canine stem cell factor (SCF), 100ng/ml human FLT-3 ligand (FLT3-L) (all kindly supplied by Amgen, Thousand Oaks, CA), and 50 ng/ml human thrombopoietin (TPO; PeproTech, Rocky Hill, NJ) and plated in 75 mm × 117 mm single well plates (Nalge Nunc International, Rochester, NY) at 4×10^5 cells/cm^2. For cells following the γ-retroviral vector transduction protocol, plates were incubated for 2 days at 37 °C, 5% CO_2. On days 3 and 4, cells were washed once, fresh media and cytokines were added, and plates were incubated 4 hours at 37°C, 5% CO_2. After the 4 hour incubation on day 4, cells were washed twice in saline and a cell count was taken. For cells following the lentiviral vector transduction protocol, freshly isolated $CD34^+$ cells were incubated in HDM and the same cytokine cocktail describe above overnight at 37°C, 5% CO_2. After the overnight incubation, cells were washed twice in saline and a cell count was taken. An aliquot of $CD34^+$ cells from each set was labeled with PE-labeled streptavidin (Jackson Immunoresearch, West Grove, PA) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) to determine the purity of the $CD34^+$ cells.

Bone Marrow Transplantation

Ten XSCID dogs were transplanted with freshly isolated DLA-matched $CD34^+$ bone marrow cells, including 5 from a previous study [8] and 5 unique to this current study, and 6 XSCID dogs were transplanted with freshly isolated DLA-haploidentical $CD34^+$ bone marrow cells. Four XSCID dogs were transplanted with $CD34^+$ bone marrow cells cultured for 4.5 days, and two XSCID dogs were transplanted with $CD34^+$ bone marrow cells cultured for 18 hours (Table 1). All XSCID dogs were <2 weeks of age at the time of transplantation. None of the dogs received any pretransplant conditioning.

Flow Cytometry

Quantitation of peripheral blood lymphocyte subpopulations was determined on EDTA blood samples using a standard whole blood staining and lysis protocol [41]. The murine monoclonal antibody (mAb) used in this study were FITC- or PE-labeled CD3 (CA17.3G9), CD4 (CA13.1E4), CD8 (CA9.JD3), CD21 (CA2.1D6), and CD45RA (CA4.1D3) [42,43]. Analysis gates were adjusted to 1% positive staining with directly labeled isotype controls. For each sample, 10,000 cells were analyzed using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA).

Table 1. Dose of Cultured $CD34^+$ Bone Marrow Cells.

Dog	Donor DLA	CD34 Purity (%)	Dose	Length of Culture
R1789	Matched	96.3	3.2×10^7/kg	4.5 days
X299	Haploidentical	93.9	3.5×10^7/kg	4.5 days
R1824	Haploidentical	97.1	3.0×10^7/kg	4.5 days
R1826	Matched	97.1	3.0×10^7/kg	4.5 days
R1844	Matched	95.6	3.0×10^7/kg	18 hours
R1847	Haploidentical	95.6	3.0×10^7/kg	18 hours

Proliferation Assay

The response of peripheral blood lymphocytes to in vitro mitogenic stimulation with PHA-P (5 μg/ml; Sigma, St. Louis, MO) was performed as previously described using a flow cytometric bromodeoxyuridine (BrdU) assay (BD Bioscience, San Jose, CA) for assessing cell proliferation as per manufacturer's instructions [41]. Secondary staining was performed using an allophycocyanin (APC)-labeled anti-mouse IgG1 antibody for flow cytometric detection.

T Cell Receptor Excision (TREC) Assay

DNA was purified from peripheral whole blood using Generation DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturers instructions. To detect signal joint TRECs, a real-time quantitative PCR method was performed on the 7500 Real-time PCR system (Applied Biosystems, Branchburg, NJ). Each PCR reaction was performed in a 50 μl solution containing DNA from cells of interest, 1.0X Taqman universal master mix (Applied Biosystems, Branchburg, NJ), 900 nM forward and reverse primers, and 250 nM Taqman probe. The sequences of the primers and probe used are the following: forward primer 5'-GACGCCACATGCCTTTCAA-3'; reverse primer 5'-GCGCCAGCTGCAGGGTTTT- 3' and the probe 5'-6FAM-CTCACCTCTGTGCACGGTGATA-TAMRA-3' producing a 131 bp product. As an internal control measurement, to normalize for the input DNA, HPRT was amplified in every sample tested (forward primer 5'- GGATTTGAAATTCCAGACAAGTTTGTTG-3'; reverse primer 5'- GTGAGAAAAAGAAGCAATTACTTACATTC- 3', and the probe 5'- 6FAM-GCCCTTGACTATAATGAATACTTCAGG-TAMRA- 3'). A standard curve was created, by cloning the Sj fragment into a pGEM-T Easy Vector (Promega) and the number of Sj copies present in a given population was calculated by including a dilution series of this standard in each PCR experiment. PCR was performed under the following conditions: 50°C for 2 minutes followed by 95°C for 10 minutes, after which 50 cycles of amplification were carried out (95°C for 15s, 60°C for 1 minute). For each sample, the Ct-value, defined as the minimal number of cycles necessary to exceed the threshold, was measured and applied to the standard curve.

Assessment of Humoral Immune Function

Serum IgG was determined by radial immunodiffusion using canine IgG specific plates (Bethyl Lab. Inc., Montgomery, TX). When serum IgG concentrations reached normal levels, dogs were immunized with a modified-live canine parvovirus (CPV) and canine distemper virus vaccine (CDV) and serum IgG specific antibody titers were determined by either a passive hemagglutination (for CPV) or serum neutralization (for CDV) assay by the Cornell University Veterinary Diagnostic Laboratory.

RESULTS

Six XSCID dogs were transplanted with allogeneic $CD34^+$ bone marrow cells from normal donors that were cultured for either 4.5 days or 18 hours in a cytokine cocktail used for ex vivo retroviral transduction. Table 1 illustrates the dose of cells and haplotype of the donor for each of the XSCID recipients. The purity of the isolated $CD34^+$ cells ranged from 94% to 97%. The dose of cells chosen was based on studies using freshly isolated allogeneic $CD34^+$ cells from both DLA-matched and DLA-haploidentical donors that result in sustained engraftment of both donor-derived B and T cells with reconstitution of normal B and T cell function. Fig. 1 illustrates that T cell reconstitution as measured by $CD45RA^+$ (naïve) T cells is similar in XSCID dogs transplanted with freshly isolated $CD34^+$ cells from both DLA-matched and DLA-haploidentical donors at doses of $\geq 5 \times 10^6$ cells/kg (range 5 to 40×10^6/kg). None of the dogs in either experiment received any pretransplant conditioning.

Prior to transplantation, all six dogs had the typical XSCID phenotype of low to absent peripheral T cells and normal or elevated numbers of peripheral B cells. During the first 3 months following transplantation, all 6 dogs demonstrated an increase in the proportion of peripheral T cells (Fig. 2A). Some XSCID dogs, like some XSCID boys, can develop autologous phenotypically mature, but nonfunctional, T cells with age because of peripheral expansion through a γc-independent pathway. However, these T cells have an activated ($CD45RA^-$) phenotype with the dogs remaining severely T cell lymphopenic and not surviving past 3 months of age. Because T cells derived from transplanted stem cells or T cell progenitors would be expected to develop through a thymic-dependent pathway and have a naïve ($CD45RA^+$) phenotype, we examined the CD45 isoform usage in the transplanted dogs as a measurement of T cell reconstitution. Between 1 and 1.5 months post transplant the majority of peripheral T cells in all the dogs expressed a naïve ($CD45RA^+$) phenotype (Fig. 2B). However, 2 of the 4 dogs transplanted with cells cultured for 4.5 days showed a rapid conversion to a $CD45RA^-$ phenotype typical of untreated XSCID dogs. These same two dogs failed to develop normal numbers (>1000/μl) of $CD45RA^+$ T cells (Fig. 2C). The other two dogs in this group, R1789 and X299, maintained normal proportions and numbers of $CD45RA^+$ peripheral T cells through 8 months post transplant, although the absolute numbers of $CD45RA^+$ T cells remained less than normally observed in XSCID dogs transplanted with freshly isolated $CD34^+$ cells. At 8 months post transplant, the proportion and number of $CD45RA^+$ peripheral T cells started to decline. The kinetics of T cell reconstitution differed in these two dogs with one dog, X299, showing a rapid increase in the number of $CD45RA^+$ peripheral T cells, whereas the other dog, R1789, showed a more gradual increase in the number of $CD45RA^+$ peripheral T cells. Both of the dogs transplanted with cells cultured for 18 hours developed and sustained numbers of $CD45RA^+$ peripheral T cells similar to dogs transplanted with freshly isolated cells. The CD4/CD8 ratio in the four dogs that successfully engrafted followed a similar kinetics as seen in XSCID dogs transplanted with freshly isolated $CD34^+$ cells (data not shown).

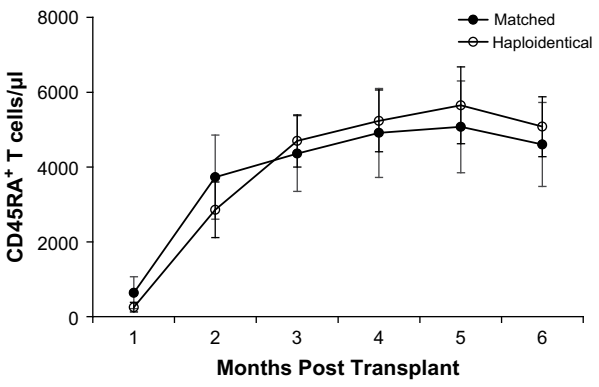

Figure 1. T cell reconstitution following DLA-matched or haploidentical bone marrow transplantation of XSCID dogs. Absolute number of peripheral $CD45RA^+$ T cells in XSCID dogs following transplantation with $\geq 5 \times 10^6$ $CD34^+$ bone marrow cells from normal matched (n = 10) or haploidentical (n = 6) donors.

Proliferation in response to mitogenic stimulation was used to assess T cell function in the dogs that successfully engrafted. Fig. 3 illustrates that the two dogs transplanted with cells cultured for 4.5 days, R1789 and X299, and both of the dogs transplanted with cells cultured for 18 hours demonstrated normal proliferation.

Thymic function was assessed in all 4 of the dogs that successfully engrafted using a TREC assay to evaluate thymic output. All the dogs showed high levels of TRECs during the first four months following transplantation (Fig. 4A). The number of TRECs in the two dogs transplanted with cells cultured for 4.5 days, dogs 1789 and X299, started declining between 4 to >6 months post transplant. The dogs transplanted with cells for 18 hours, dogs 1844 and 1847,

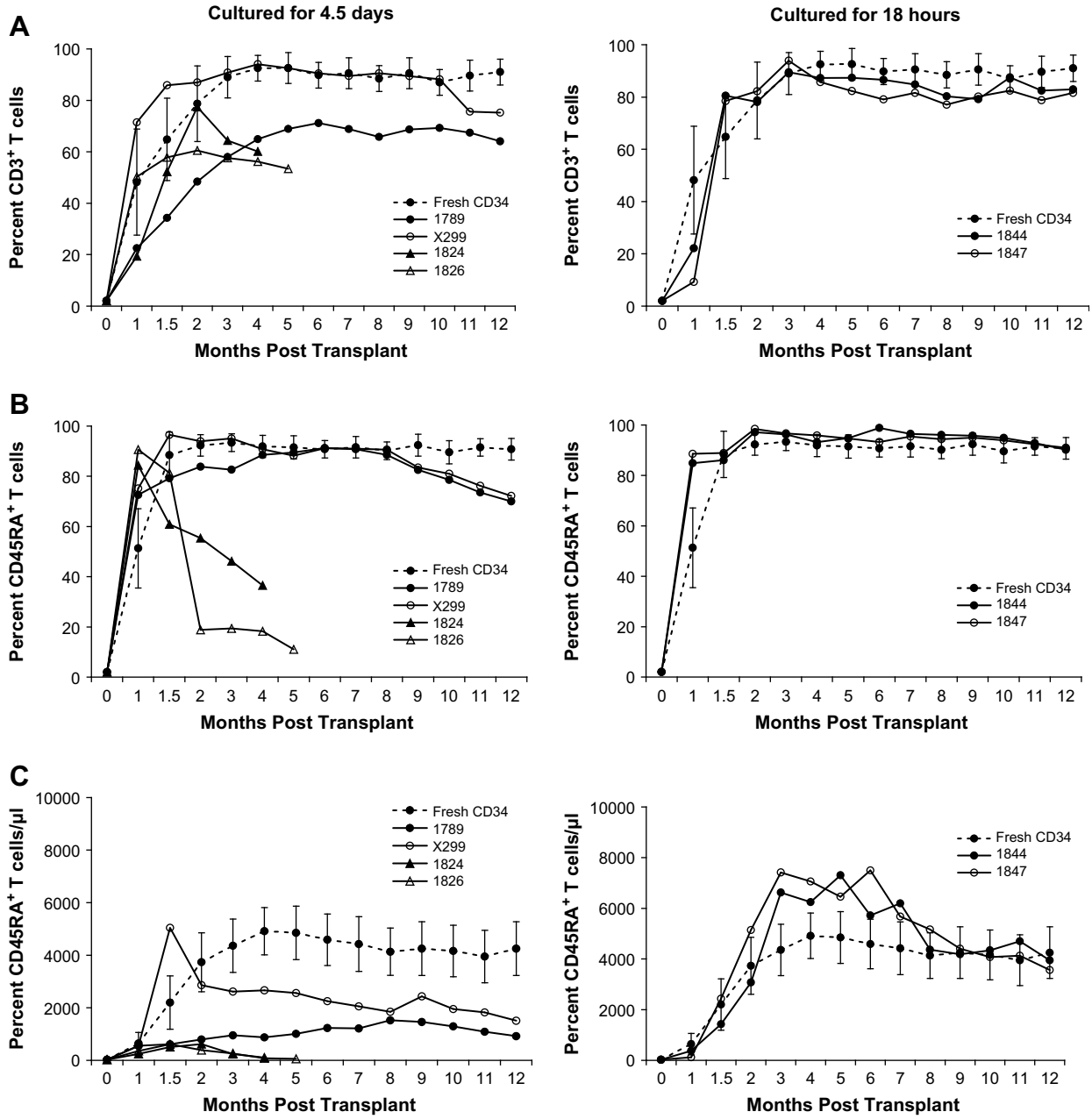

Figure 2. Longitudinal analysis of T cell reconstitution following bone marrow transplantation of XSCID dogs with cultured bone marrow $CD34^+$ cells. Proportion of peripheral T cells (A), and proportion (B) and absolute number (C) of peripheral T cells expressing a naïve ($CD45RA^+$) phenotype in XSCID dogs following transplantation with $CD34^+$ bone marrow cells following culture for 18 hours or 4.5 days in comparison with 8 dogs transplanted with freshly isolated $CD34^+$ cells in Fig1, which that were incorporated into our breeding program.

also showed a decline in TREC levels, however, at 8 months post transplant the TREC levels were higher than those observed in the dogs transplanted with the cells cultured for 4 ½ days at 6 months post transplant. These results contrast with those in two dogs recently transplanted with with freshly isolated cells, dogs R1751 and R1752, in whom TREC levels have remained stable for at least 12 months post transplant. Fig. 4B illustrates the histology of the thymus at 12 months post transplant, showing that dogs transplanted freshly isolated cells possess a normal thymic histology characterized by numerous thymocytes and a well defined cortex and medulla. The dogs transplanted with cells cultured for 4.5 days had a thymic morphology very similar to that of an untreated XSCID, whereas the thymic morphology of dogs transplanted with cells cultured for 18 hours, although not normal, still had an appearance of a normal thymus with a well defined cortex and medulla.

Serum IgG concentrations in dogs that successfully engrafted reached low normal values between 6 to 8 months post transplant (data not shown). These dogs were immunized with a modified, live vaccine to CPV and CDV and, in contrast to untreated XSCID

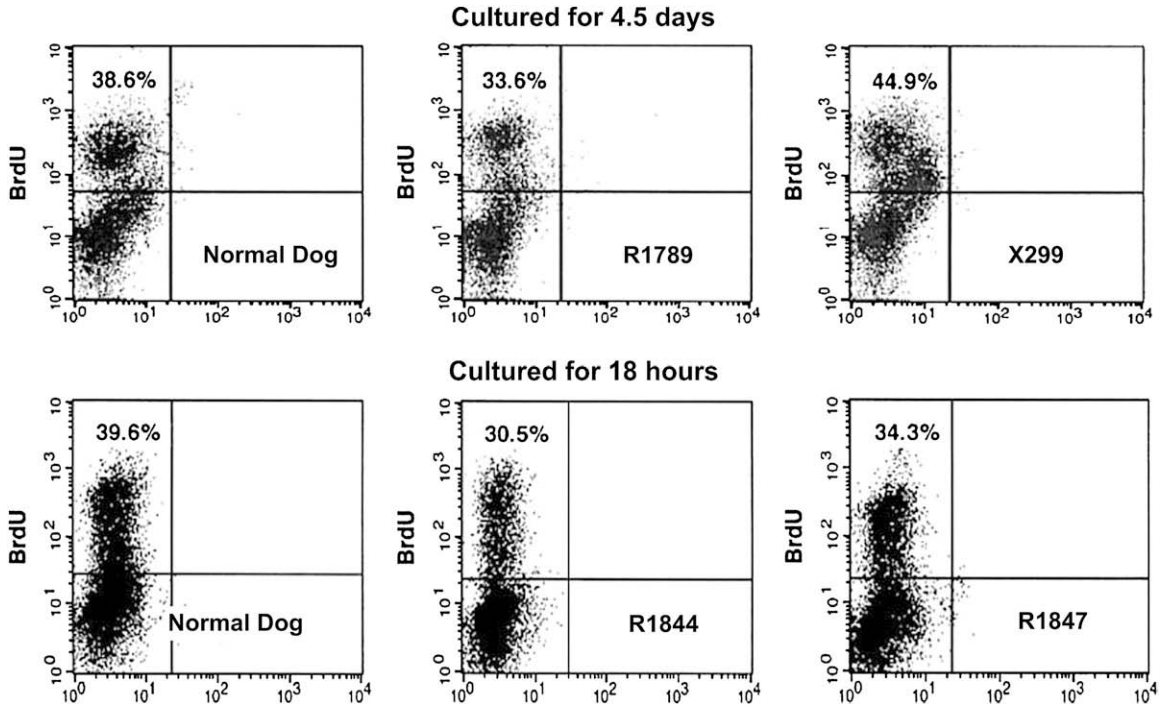

Figure 3. T cell function following bone marrow transplantation. Proliferative response of peripheral blood T cells following PHA stimulation in dogs R1789, X299, R1844, and R1847 at 4 months post BMT. The results of a normal dog analyzed at the same time are also presented. Percentages represent the proportion $BrdU^+$ cells.

dogs, demonstrated a significant increase in IgG specific antibody titers to each virus. All the dogs had pre-immunization specific IgG antibody titer to CDV of < 1:10 and to CPV of <1:4. Following two doses of vaccine, the IgG specific antibody titers to CDV ranged from 1:160 to 1:1280 CDV and from 1:128 and 1:640 for CPV. No adverse reaction to the vaccine was observed.

DISCUSSION

The disappointing results in attempting to duplicate the γ-retroviral gene transfer experiments in mice to large animals and humans in the 1990s were often attributed to "low" transduction efficiencies. Therefore, considerable effort was devoted to increasing transduction efficiencies in $CD34^+$ cells from large animals and humans that resulted in the first successful human clinical gene therapy trial in 1999 [44]. Current protocols for ex vivo transduction of HSC with γ-retroviral vectors require ex vivo manipulation and culture of the cells in various cytokine cocktails for up to 5 days. Numerous murine transplant studies, including xenotransplant studies using human $CD34^+$ cells in NOD/SCID mice, and nonhuman primate transplant studies have shown that ex vivo culture of HSC for as little as 24 to 48 hours may have a detrimental effect on their short-term and long-term engraftment potential, however these studies were performed in myeloablated recipients [reviewed in 45]. Although the NOD/SCID xenotransplant model has become the "gold standard" for evaluating human hematopoiesis in vivo, this model is limited because of it's short life span precluding long-term studies [46], and does not support thymopoiesis so T cell development cannot be studied [47].

In this study we addressed the effect of ex vivo culturing of normal canine $CD34^+$ bone marrow cells under conditions similar to those used for ex vivo gene transfer on their ability to reconstitute normal T cell function and sustain long-term thympoiesis in nonconditioned XSCID dogs following allogeneic BMT. Although the focus of this study was to address ex vivo culture conditions used for ex vivo gene therapy, we chose to perform these studies in an allogeneic BMT setting as it eliminates one major variable associated with gene therapy studies, namely the transduction efficiency of the γ-retroviral or lentiviral vector. This is the first study to address this question in a large animal model of a relevant human disease and in the absence of any pre-transplant conditioning, and is also the first to demonstrate the effect of ex vivo culture on thymopoiesis.

The dose of cells used in this study was the upper limit used in our previous studies using freshly isolated $CD34^+$ bone marrow cells [8,48]. Only two of the four XSCID dogs transplanted with $CD34^+$ cells cultured for 4.5 days successfully engrafted with each dog showing a different kinetics of T cell reconstitution. Although both of these dogs developed numbers of $CD45RA^+$ (naïve) T cells within the range for age-

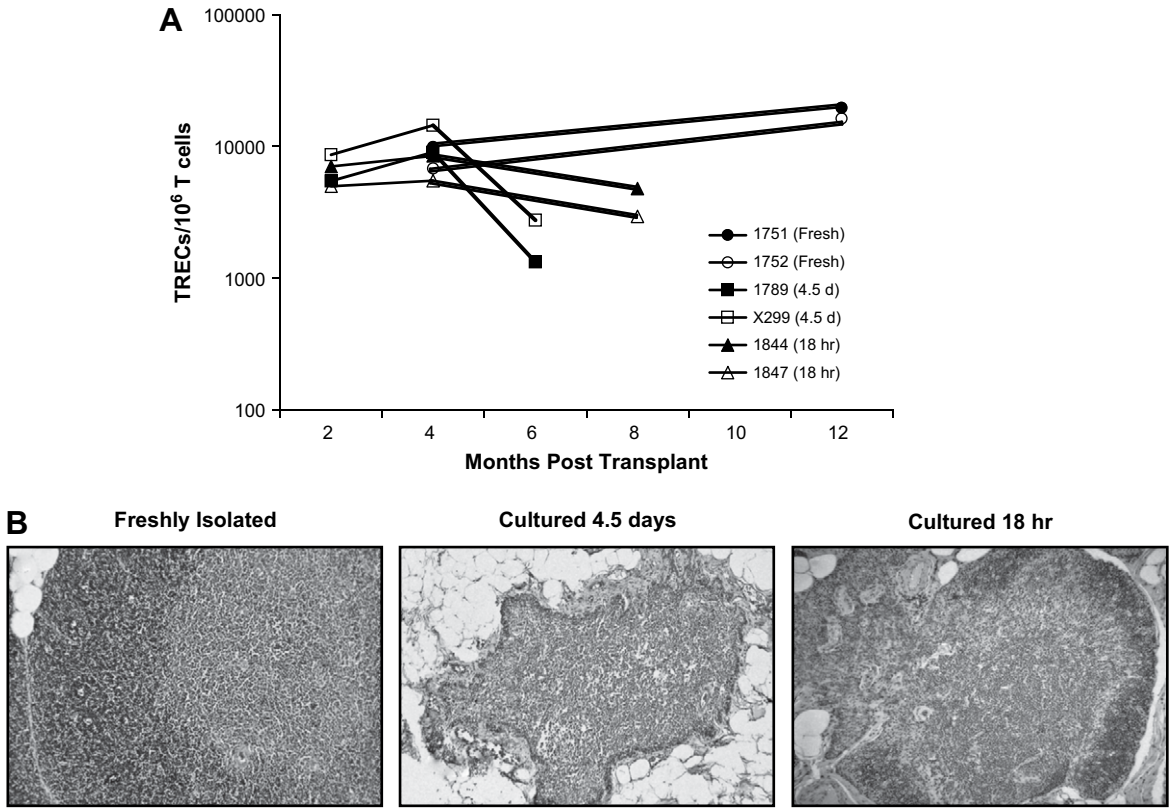

Figure 4. Thymus output following bone marrow transplantation. TREC levels in XSCID dogs following transplantation with freshly isolated $CD34^+$ cells or $CD34^+$ cells cultured for 18 hours or 4.5 days (A). Histologic appearance of the thymus in XSCID dogs one year following transplantation with freshly isolated bone marrow $CD34^+$ cells or $CD34^+$ cells cultured for 18 hours or 4.5 days (B).

matched normal dogs, they were substantially lower than that observed in XSCID dogs transplanted with freshly isolated $CD34^+$ bone marrow cells. Both dogs showed a gradual decline in the proportion and absolute number of peripheral $CD45RA^+$ T cells to the lower limits of age-matched normal dogs (>750/μl) by 12 months following transplantation. These results differ substantially from those observed in XSCID dogs successfully immune reconstituted with freshly isolated $CD34^+$ bone marrow cells from both DLA-matched and haploidentical donors that maintain normal numbers of peripheral T cells ranging from 1950/μl to 2190/μl through 5 to 7.5 years post transplant [8,48]. In contrast, both XSCID dogs transplanted with $CD34^+$ cells cultured for 18 hours successfully engrafted with a robust T cell reconstitution with sustained numbers of peripheral $CD45RA^+$ T cells similar to that observed using freshly isolated $CD34^+$ cells.

The dogs that successfully engrafted donor cells exhibited T and B cell function as demonstrated by a normal T cell proliferative response following mitogenic stimulation and the ability to produce antigen-specific IgG antibody following immunization.

Evaluation of thymopoiesis by TREC analysis and the histologic appearance of the thymus, however, demonstrated differences between both groups of XSCID dogs treated with cultured $CD34^+$ cells compared with XSCID dogs transplanted with freshly isolated $CD34^+$ cells. XSCID dogs transplanted with freshly isolated $CD34^+$ cells exhibit a thymus that has a normal histiologic appearance and increasing numbers of TRECS at 12 months post transplant, suggesting active thymopoieis is still occurring. XSCID dogs transplanted with $CD34^+$ cells cultured for 4.5 days showed a rapid decrease in TRECs and a thymus that had a histologic appearance of a dysplastic XSCID thymus 12 months post transplant. In contrast, the XSCID dogs transplanted with $CD34^+$ cells cultured for 18 hours showed a more gradual decrease in TRECs with a thymus that, although not normal, had a well-defined cortex and medulla. These results suggest that the ability to sustain long-term thymopoiesis is impaired under culture conditions used for ex vivo gene therapy, and that ex vivo culturing of the HSC under conditions used for γ-retroviral also affects their engraftment potential.

Our results are consistent with the results of 21 XSCID boys treated by ex vivo γ-retroviral gene therapy [10,13,14,49-52]. Two patients failed to engraft gene-corrected cells and an additional 4 patients had suboptimal levels of T cell immune reconstitution. Long-term follow-up of the 15 patients who reconstituted normal or low normal gene-corrected T cells

have shown a gradual decline in T cell numbers starting approximately 2 years after treatment with a concomitant decrease in CD45RA$^+$ (naïve) T cells [50-52], suggesting that the ability to sustain long-term thymopoiesis is impaired under current conditions used for ex vivo γ-retroviral gene therapy. These results can be best explained by the engraftment of transduced committed T cell progenitors, and probably few, if any, transduced HSC capable of self-renewal. If this were the case, the continual production of new T cells would be expected to decrease over time as appears to be the case.

Central to optimizing ex vivo gene therapy, is determining culture conditions that maintain both the engraftment potential and "stem cell" potential of the transduced (cultured) cells. The true test, however, of whether these culture conditions preserve stem cell activity and engraftment potential is evaluating their performance in vivo, preferably in large animal models under conditions of no conditioning [16]. For over 50 years, the dog has historically been the pre clinical animal model for BMT with many of the advances made in this model being directly transferable to human clinical BMT protocols [53-57]. A major advantage of using the XSCID dog model to test ex vivo culture conditions associated with gene therapy is that BMT can be performed in the absence of any pre transplant conditioning, thereby eliminating any potential effects of the conditioning regimen itself on either the bone marrow or thymic microenvironments.

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