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# Role of Transgene Regulation in *Ex Vivo* Lentiviral Correction of Artemis Deficiency

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## Abstract

Artemis is a single-stranded endonuclease, deficiency of which results in a radiation-sensitive form of severe combined immunodeficiency (SCID-A) most effectively treated by allogeneic hematopoietic stem cell (HSC) transplantation and potentially treatable by administration of genetically corrected autologous HSCs. We previously reported cytotoxicity associated with Artemis overexpression and subsequently characterized the human Artemis promoter with the intention to provide Artemis expression that is nontoxic yet sufficient to support immunodevelopment. Here we compare the human Artemis promoter (APro) with the moderate-strength human phosphoglycerate kinase (PGK) promoter and the strong human elongation factor-1$\alpha$ (EF1$\alpha$) promoter to regulate expression of Artemis after *ex vivo* lentiviral transduction of HSCs in a murine model of SCID-A. Recipient animals treated with the PGK-Artemis vector exhibited moderate repopulation of their immune compartment, yet demonstrated a defective proliferative T lymphocyte response to *in vitro* antigen stimulation. Animals treated with the EF1$\alpha$-Artemis vector displayed high levels of T lymphocytes but an absence of B lymphocytes and deficient lymphocyte function. In contrast, *ex vivo* transduction with the APro-Artemis vector supported effective immune reconstitution to wild-type levels, resulting in fully functional T and B lymphocyte responses. These results demonstrate the importance of regulated Artemis expression in immune reconstitution of Artemis-deficient SCID.

## Introduction

Artemis is a hairpin-opening, endonucleolytic enzyme that is a component of the nonhomologous end-joining (NHEJ) DNA double-strand break (DSB) repair pathway.[1] NHEJ is the primary mechanism by which eukaryotes repair genomic insults generated by external damaging agents and by normal cellular processes such as rearrangement of immunoglobulin genes and T cell receptor (TCR) genes mediated by the V(D)J recombination pathway.[2–4] Deficiency of Artemis disrupts both DNA DSB repair and V(D)J recombination, manifested as a radiation-sensitive form of severe combined immunodeficiency (SCID-A) due to the inability to rearrange immunoglobulin and TCR genes.[1,5] SCID-A is effectively treated by allogeneic hematopoietic stem cell transplantation (HSCT) using an HLA-matched donor. However, HSCT carries associated risks of infection, graft rejection, graft-versus-host disease, and 20% mortality, all of which are increased in the absence of a matched donor.[6] In addition, preparative conditioning, necessary for successful B lymphocyte reconstitution in patients with SCID-A undergoing HSCT[7,8] and overcoming natural killer (NK) cell-mediated graft rejection in mismatched transplants, is problematic because of the alkylator and radiation-sensitive nature of Artemis deficiency; there is, therefore, a great need for alternative approaches in the treatment of this disease.

Clinical trials have demonstrated the effectiveness of gene transfer into autologous hematopoietic stem cells (HSCs) for treatment of adenosine deaminase (ADA)-deficient SCID and X-linked SCID.[9–15] The success of these trials demonstrates that *ex vivo* gene transfer can be an effective treatment for genetic deficiency, a compelling argument for genetic correction of other forms of SCID, including SCID-A. Two independent groups reported the correction of murine models of SCID-A by transplantation of genetically modified HSCs.[16,17] In both studies, Artemis-deficient animals

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were transplanted with HSCs that had been transduced with a lentiviral vector encoding human Artemis regulated by the human phosphoglycerate kinase (PGK) promoter, resulting in reconstitution of B and T lymphocyte compartments.[16,17] Surprisingly, Mostoslavsky and colleagues reported lack of lymphoid reconstitution in RAG-1-deficient animals transplanted with SCID-A HSCs that had been transduced using lentiviral vectors encoding human Artemis regulated by the stronger cytomegalovirus (CMV) or elongation factor-1$\alpha$ (EF1$\alpha$) promoter.[16]

We subsequently demonstrated that overexpression of Artemis after lentiviral transduction is associated with cytotoxicity, a halt in cell cycle progression, and fragmentation of genomic DNA ultimately resulting in apoptosis.[18] These results, along with the previous reports demonstrating incomplete immune reconstitution of SCID-A after *ex vivo* transduction with an exogenous promoter,[16,17] emphasize the importance of providing Artemis expression at a level that is nontoxic and yet sufficient to correct the SCID-A $T^-B^-$ phenotype. Accordingly, we isolated and characterized the human Artemis promoter (APro) as a sequence extending 1 kilobase upstream from the human Artemis translational start site on human chromosome 10.[19] *Ex vivo* transduction of murine bone marrow with an APro-regulated green fluorescent protein (GFP) lentiviral vector conferred GFP expression at a significantly reduced level in comparison with control mice transplanted with EF1$\alpha$-GFP-transduced marrow and supported GFP expression in all hematopoietic lineages that persisted in secondary transplant recipients.[19] These results established the usefulness of this promoter for providing reliable, moderate-level gene expression in hematopoietic cells.[19]

In this study, we evaluated the effect of promoter strength on immune reconstitution after *ex vivo* lentiviral transduction of the Artemis coding sequence in a murine model of SCID-A. Previous studies of *ex vivo* lentiviral correction of Artemis deficiency[16,17] used a SCID-A mouse model exhibiting leaky T lymphocyte development, evident from low numbers of single- and double-positive thymocytes and $CD4^+$ T cells in peripheral blood. For our study, we used a murine model of SCID-A that is nonleaky and thus more accurately models the human SCID-A clinical presentation and phenotype.[20] We also bred our SCID-A model onto both CD45.1 and CD45.2 congenic backgrounds, allowing us to quantitatively track donor engraftment at the cellular level. Bone marrow from Artemis-deficient mice was transduced with lentiviral vectors regulating the Artemis coding sequence using the moderate-strength human PGK promoter, the strong human EF1$\alpha$ promoter, or the human Artemis promoter (APro) and then transplanted into congenic, irradiated SCID-A recipients. We found that both APro-Artemis and PGK-Artemis transduction supported effective engraftment of T and B lymphocytes to normal levels. Furthermore, animals treated with APro-Artemis demonstrated a restored response to *in vivo* antigen challenge and *in vitro* mitogen stimulation. In contrast, EF1$\alpha$-Artemis-treated animals exhibited reduced engraftment potential, were unable to repopulate the B lymphoid compartment, and lacked the ability to class switch and display antigen-specific IgG. These results suggest the necessity for near endogenous levels of Artemis to achieve functional immune reconstitution on *ex vivo* complementation of Artemis deficiency, demonstrating the importance of transgene regulation. These studies also validate the effectiveness of the human Artemis promoter in restoring immune function in SCID-A mice and, potentially, in human SCID-A.

## Materials and Methods

### *Plasmids*

Lentiviral vector plasmids pCSIIEG,[21] pCSEPuro,[18] pOK/EF1$\alpha$-Artemis (including the EF1$\alpha$ intron 1 sequence), and pOK/PGK-Artemis[18] have been previously described. For construction of pOK/APro-Artemis, the EF1$\alpha$ promoter was excised from pOK/EF1$\alpha$-Artemis with *Age*I, yielding pOK/Artemis. The APro fragment was excised from pCR2.1/APro[19] with *Age*I and ligated into the *Age*I site of pOK/Artemis directly upstream of the murine Artemis coding sequence (Fig. 1A). Luciferase expression plasmids pAPro-Luc (pGL3-APro[19]) and pPGK-Luc (pGL3-based[22]), and the simian virus 40 (SV40)-regulated plasmid pGL3-C (Clontech, Palo Alto, CA) have been previously described. Plasmid pEf1$\alpha$-Luc was assembled by ligation of a 1102-bp *Xho*I fragment (including the EF1$\alpha$ promoter) isolated from pDL2G[23] into pGL3-Basic at the *Xho*I site upstream of the luciferase coding sequence.

### *Mammalian cell culture and transfection*

HEK 293T and murine NIH 3T3 thymidine kinase $(TK)^-$ cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic at 37°C and 5% $CO_2$. HEK 293T cells ($3 \times 10^6$/6-cm plate) were transfected using Lipofectamine 3000 (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA) with 2 $\mu$g of test firefly luciferase-encoding plasmid along with 25 ng of phRL-CMV standardizing plasmid encoding *Renilla* luciferase. Two days later cells were harvested and lysates assayed for both firefly and *Renilla* luciferases, using a Berthold Lumat luminometer (Berthold Detection Systems, Bad Wildbad, Germany) and the Dual-Luciferase reporter assay system (Promega, Madison, WI).

### *Preparation and titering of lentiviral vectors*

Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vectors were generated as described.[23,24] Briefly, $1.4 \times 10^7$ HEK 293T cells were seeded into poly-L-lysine-coated 15-cm$^2$ plates and cultured overnight in DMEM supplemented with 1% penicillin–streptomycin (Pen Strep) and 8% FBS. Lentiviral vector plasmid constructs were cotransfected with p$\Delta$NRF, encoding lentiviral structural and enzymatic proteins, and pMD.G, encoding VSV-G envelope protein, and incubated at 37°C with 10% $CO_2$. At 12 hr posttransfection, the medium was replaced with DMEM supplemented with 4% FBS and then viral supernatants were collected 24, 36, and 48 hr posttransfection, pooled, and concentrated 100-fold by centrifugation at $23{,}000 \times g$ in a Sorvall RC-5B centrifuge. Lentiviral vectors were resuspended in Iscove's modified Dulbecco's medium (IMDMEM), aliquoted, and stored at −80°C for future use.

Vector titers were determined by transduction of NIH 3T3 $TK^-$ cells in the presence of Polybrene (8 $\mu$g/ml). Forty-eight hours later, DNA was extracted from the exposed cells and vector copy number was determined by quantitative PCR for the integrated lentiviral strong stop sequence or for the GFP sequence as previously described.[23] Concentrated vector titers ranged from $10^8$/ml to $10^9$/ml. CSIIEG was also titered for GFP expression by flow cytometry as previously described.[23]

### *Ex vivo lentiviral transduction and bone marrow transplantation*

All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. A murine model of Artemis deficiency backcrossed onto C57BL/6 background and exhibiting no leakiness[20] was maintained under specific pathogen-free conditions at the University of Minnesota and further bred onto both CD45.1 and CD45.2 congenic backgrounds. CD45.2 and CD45.1 C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Animals were provided food and water *ad libitum*.

Bone marrow was flushed from the hind limbs of donor mice into DMEM supplemented with heparin (10 U/ml), 10% FBS, and 1% Pen Strep (Invitrogen, Carlsbad, CA). Red blood cells were lysed with ammonium chloride hemolysis buffer (0.8% $NH_4Cl$ with 0.1 m*M* EDTA) (StemCell Technologies, Vancouver, BC, Canada), and then the nucleated cells were washed with 1 × phosphate-buffered saline (PBS) and rendered into a single-cell suspension in transduction medium (complete StemPro-34 SFM medium with supplements [Invitrogen]) supplemented with 2 m*M* L-glutamine (Invitrogen), 1% Pen Strep (Invitrogen), murine interleukin (IL)-3 (100 ng/ml), murine IL-6 (100 ng/ml), murine thrombopoietin (TPO, 100 ng/ml), murine stem cell factor (SCF, 100 ng/ml) (all cytokines from R&D Systems, Minneapolis, MN), and Polybrene (8 μg/ml; Sigma-Aldrich, St. Louis, MO). The marrow was transduced twice with lentiviral vector at a multiplicity of infection (MOI) of 30, once immediately after marrow harvest and a second time 20 hr after the initial exposure. Transduced cells were washed, and resuspended in IMDMEM, and 3.3–5.2 × $10^6$ cells were injected via the lateral tail vein into sublethally irradiated (500 rads, X-irradiation source) congenic recipients.

For secondary transplantation, marrow samples were collected from individual primary recipients as described previously and the whole marrow sample was divided equally and infused into three secondary irradiated C57BL/6 (800 rads, X-irradiation source) recipients.

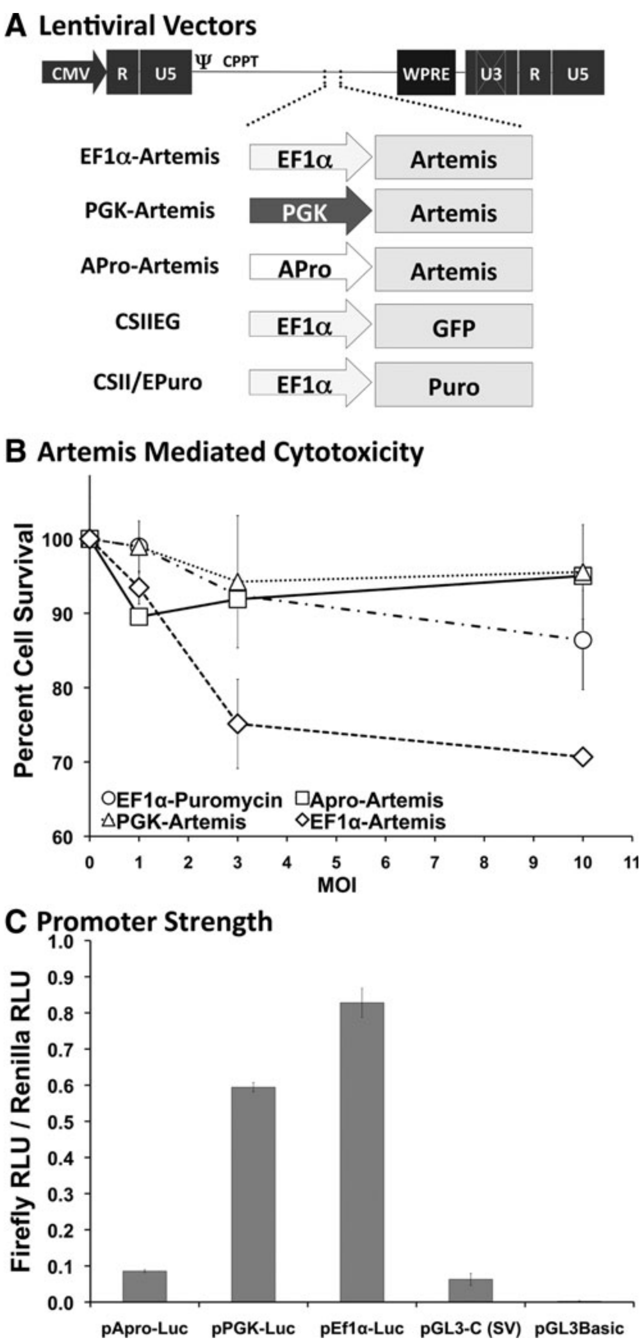


**FIG. 1.** Lentiviral vector constructs and Artemis-associated cytotoxicity. **(A)** Lentiviral vectors were used for analysis and complementation of Artemis deficiency. Experimental lentiviral vectors were engineered to express the Artemis coding sequence under the transcriptional regulation of either the human elongation factor 1α promoter (EF1α), the human phosphoglycerate kinase promoter (PGK), or the 1-kilobase endogenous human Artemis promoter (APro). Lentiviral vectors serving as transduction controls expressed either green fluorescent protein (CSIIEG) or the puromycin resistance gene (CSII/E-Puro) regulated by EF1α. Abbreviations: CMV, cytomegalovirus early promoter/enhancer region; U3/U5/R, unique 3′/unique 5′/repeat regions of the HIV long terminal repeat; ψ, packaging signal; cPPT, central polypurine tract; WPRE, woodchuck posttranscriptional regulatory element. Arrows indicate sites and direction of transcript initiation. **(B)** Artemis regulation by APro avoids cytotoxicity. Murine NIH 3T3 cells were transduced at increasing MOIs using CSII/E-Puro, EF1α-Artemis, PGK-Artemis, or APro-Artemis lentiviral vectors as indicated. Cell survival was assessed 5 days posttransduction by MTT assay and plotted as the percentage of cells surviving in control, untreated populations. Each value represents the mean of three replicates + SD. **(C)** Low-level expression conferred by the Artemis promoter. Luciferase expression constructs were transfected into human 293T cells and then lysates were assayed for luciferase activity. The mean relative level of expression versus a cotransfected *Renilla* luciferase standard is shown ± SD.

### Flow cytometry

Whole blood was collected from the submandibular vein, treated with ammonium chloride hemolysis buffer (0.8% $NH_4Cl$ with 0.1 m*M* EDTA) (StemCell Technologies), washed, and then the leukocytes were pelleted and resuspended in staining buffer (1×PBS, 1% FBS, and 0.002% sodium azide) plus fluorochrome-conjugated monoclonal antibodies recognizing CD45.1, CD45.2, B220 (B lymphocytes), CD3e (T lymphocytes), CD4 (helper T lymphocytes), CD8α (cytotoxic T lymphocytes), NK1.1 (natural killer cells), Gr-1 and CD11b (myeloid lineages). Fluorochrome staining and GFP expression (for CSIIEG-transduced cells) were assayed on an LSRII instrument. Isotype staining was done to provide an internal control and to determine appropriate gating. Data were collected using CellQuest Pro (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR) software. Total lymphocyte number was determined by Hemavet (Drew Scientific, Dallas, TX) analysis of whole blood. Total B cells and T cells were calculated as the product of the percentage of B cells, $CD4^+$ T cells, and $CD8^+$ T cells times the total number of donor lymphocytes.

### Determination of lentiviral integrant copy number in peripheral blood

At various time points, DNA was extracted from whole blood, using Whatman FTA Elute Micro Cards (Fisher Scientific, Pittsburg, PA) according to the manufacturer's instructions. Cells stained with anti-IgM (B lymphocytes) and anti-CD3e (T lymphocytes)–fluorochrome were sorted on a BD FACSDiva instrument into IMDMEM plus 10% FBS. Isotype staining was used to determine appropriate gating. DNA was extracted from the sorted cells or from total peripheral blood mononucleocytes upon sacrifice, using a ZymoBead genomic DNA kit (Zymo Research, Cambridge Bioscience, Cambridge, UK). Whole genome amplification was performed on DNA extracts from the sorted cells, using an illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ). Vector copy number was determined by quantitative PCR for the lentiviral strong stop sequence as described.[23]

### In vivo *KLH challenge*

Test animals were immunized by intraperitoneal injection of 100 μg of NP-KLH (4-hydroxy-3-nitrophenylacetyl hapten-conjugated keyhole limpet hemocyanin) (Biosearch Technologies, Novato, CA) and boosted 5 weeks later. Before immunization and 1 week after the boost injection, sera were collected, aliquoted, and stored at −20°C for analysis by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc, Rochester, NY) were coated with 0.5 μg of NP-BSA (4-hydroxy-3-nitrophenylacetyl hapten-conjugated bovine serum albumin) (Biosearch Technologies) in coating buffer (0.05 *M* carbonate–bicarbonate, pH 9.6) and incubated overnight at 4°C. Coating solution was aspirated from the plate and each well was washed three times in Tris–saline (50 m*M* Tris-buffered saline [TBS], pH 8.0; 0.05% Tween 20), incubated with blocking solution (50 m*M* TBS, pH 8.0; 1% BSA) for 30 min, washed three times, and then incubated for 1 hr with test serum samples serially diluted in sample buffer (50 m*M* TBS, pH 8.0; 1% BSA, 0.05% Tween 20). The wells were washed five times and then supplemented with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in sample buffer (1:100,000 dilution of anti-IgM–HRP and 1:150,000 dilution anti-IgG–HRP; both antibodies from Bethyl Laboratories, Montgomery, TX). After 1 hr, the plates were washed five times and then supplemented with HRP enzymatic reaction components, using a TMB 20-component microwell peroxidase substrate kit (Kirkegaard & Perry, Gaithersburg, MD). Reaction products were quantified with a Biotech SpectraMax plate reader at 450 nm.

### Splenocyte stimulation

Spleens were harvested from mice on sacrifice and brought to a single-cell suspension in RPMI plus 10% FBS. The spleen cell suspension was treated with ammonium chloride hemolysis buffer, washed twice in RPMI plus 10% FBS, and resuspended in complete RPMI medium (supplemented with 1% Pen Strep and 10% FBS). Microtiter plates were treated with anti-CD3e antibody diluted in 1×PBS to 0, 2, 5, and 10 μg/ml, added to wells in triplicate, and incubated at 37°C for 4 hr. The wells were washed twice with 1×PBS and then $4\times10^5$ splenocytes were added to each well and incubated at 37°C and 5% $CO_2$ for 48 hr. For concanavalin A (Con-A) stimulation, splenocytes were plated at $5\times10^5$ cells per well. The plate was supplemented with Con-A (Amersham Biosciences, Piscataway, NJ) diluted in complete RPMI medium to yield final concentrations of 0, 2.5, 5, and 10 μg/ml, and then incubated at 37°C and 5% $CO_2$ for 48 hr. After incubation, proliferation was assayed with a CellTiter 96 nonradioactive cell proliferation assay (MTT) kit (Promega) and quantified with a Biotech SpectraMax plate reader at 570 nm. Results are reported as proliferation index, calculated as the absorbance observed in the presence of mitogen divided by absorbance observed for samples incubated without mitogen.

### Statistical analysis

Data were statistically evaluated either by unpaired Student *t* test when variance was compared between two groups or by analysis of variance (ANOVA) when measuring three or more levels for variability, using Prism 4 software (GraphPad Software, San Diego, CA), with $p<0.05$ considered significant.

## Results

### Innate regulation of Artemis prevents Artemis-mediated cytotoxicity

Previously, we reported that overexpression of Artemis results in apoptosis due to DNA fragmentation and a halt at $G_1$ in the cell cycle.[18] We subsequently characterized the 1-kb APro segment[19] as a weak promoter both *in vitro* and *in vivo* with the intention of employing this endogenous element to regulate Artemis expression in lentiviral vector-transduced cells. To determine the effect of promoter strength on Artemis-mediated cytotoxicity and the potential for correction of SCID-A by lentiviral transduction, an APro-regulated Artemis vector was compared with other vectors previously generated and characterized that contain the Artemis coding sequence regulated by the strong EF1α

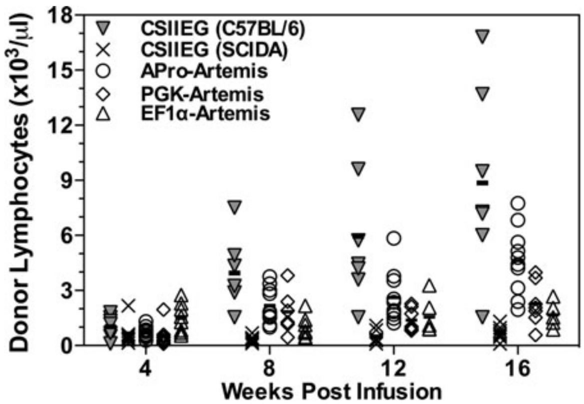


**FIG. 2.** Donor engraftment in SCID-A mice: Time course. After infusion of transduced donor marrow, peripheral blood was collected over a period of 16 weeks to monitor donor lymphoid engraftment and repopulation, and plotted as the number of donor lymphocytes ($\times 10^3$) per microliter. Each symbol represents the results from a single animal for the groups indicated in the key. Bars represent mean values.

promoter and the more moderate-strength PGK promoter (Fig. 1A). Dose-dependent cell survival was assessed in 3T3 cells 5 days posttransduction with increasing amounts of APro-, PGK-, and EF1α-regulated Artemis lentiviral vectors, with an EF1α-regulated puromycin resistance vector serving as a transduction control (Fig. 1B). Similar to our previous report,[18] in a 5-day survival study mouse 3T3 cultures transduced with both EF1α-Puro and PGK-Artemis remained viable at all vector doses ($p > 0.05$ at an MOI of 10); however, a dose-dependent decrease in cell survival was observed for cultures transduced with EF1α-Artemis at increasing multiplicities of infection ($p < 0.05$ vs. all other groups; Fig. 1B). In contrast, we observed that cultures transduced with the APro-Artemis vector remained viable even at increasing multiplicities of infection, demonstrating that innate regulation of Artemis obviates a cytotoxic response ($p < 0.05$ vs. EF1α-Artemis; $p > 0.05$ vs. all other groups; Fig. 1B). In a side-by-side comparison with the EF1α and PGK promoters, we found that the level of expression conferred by the Artemis promoter was reduced by 6- to 8-fold after transfection of luciferase-encoding constructs into human 293T cells (Fig. 1C).

### *Engraftment after lentiviral transduction of the Artemis coding sequence*

To evaluate the comparative effectiveness of lentiviral transduction in the correction of murine SCID-A, whole bone marrow was harvested from donor CD45.1 SCID-A animals, exposed twice (once overnight and once 20 hr postharvest) to either APro-Artemis, PGK-Artemis, or EF1α-Artemis at an MOI of 30, and then transplanted into CD45.2 recipient SCID-A animals preconditioned with 500 cGy of X-irradiation. This was the lowest effective sublethal dose resulting in donor cell engraftment that did not give rise to clinical signs of irradiation toxicity in control animals not receiving a transplant. In animals preconditioned at lower doses of irradiation (100, 200, or 300 cGy), donor mononuclear cells were observed by 8 weeks posttransplantation in all groups, but lymphocyte repopulation was not observed at 16 weeks posttransplantation (data not shown). Donor-derived lymphocytes emerged in recipient animals preconditioned with 500 cGy by 4 weeks posttransplantation, excluding the control group receiving CSIIEG-transduced SCID-A marrow (Fig. 2). Subsequently, donor cell populations increased over time so that by 16 weeks posttransplantation 100% of animals receiving APro-Artemis-transduced marrow demonstrated donor-derived lymphocytes, whereas 83% of PGK-Artemis-treated animals and 80% of EF1α-Artemis-treated animals demonstrated donor lymphocyte engraftment (Fig. 2). Of the total lymphocyte compartment, PGK-Artemis- and EF1α-Artemis-treated animals contained a lower percentage of donor-derived lymphocytes (77.7 and 87.4% donor lymphocytes, respectively) than animals treated with APro-Artemis (95.1%) ($p < 0.01$; Table 1). In addition, by 16 weeks posttransplantation 80% of animals receiving APro-Artemis-transduced marrow demonstrated a repopulated lymphocyte compartment equivalent to wild-type animals ($> 2 \times 10^3/\mu l$), whereas only 66% of PGK-Artemis-treated animals and 40%

TABLE 1. ENGRAFTMENT, LENTIVIRAL INTEGRATION, AND IMMUNE RECONSTITUTION

| Vector | Donor | Recipient | *Total lymphocytes*[a] ($\times 10^3/\mu l$) | *Animals per group* (n) | *Donor engraftment*[b] (%) | *Lentiviral integration* *PBMCs*[c] (%) | *Lentiviral integration* $CD3^+$ *(VC/GE)*[d] | *Lentiviral integration* $IgM^+$ *(VC/GE)*[d] |
|---|---|---|---|---|---|---|---|---|
| None | None | C57BL/6 | 6.5 (±1.2) | 8 | — | 0.01 (±0.01) | — | — |
| None | None | SCID-A | 0.5 (±0.3) | 8 | — | 0.02 (±0.02) | — | — |
| CSIIEG | C57BL/6 | SCID-A | 10.1 (±4.1) | 8 | 96.6 (±2.5) | 0.6 (±0.3) | — | — |
| CSIIEG | SCID-A | SCID-A | 1.4 (±0.8) | 8 | 55.7 (±22.7) | 3.2 (±2.5) | — | — |
| APro-Artemis | SCID-A | SCID-A | 4.9 (±2.0) | 10 | 95.1 (±7.7) | 6.8 (±4.4) | 2.1 (±1.9) | 2.1 (±2.2) |
| PGK-Artemis | SCID-A | SCID-A | 2.9 (±1.2) | 8 | 77.7 (±12.3) | 2.0 (±1.7) | 1.7 (±1.2) | 2.8 (±1.7) |
| EF1α-Artemis | SCID-A | SCID-A | 1.9 (±0.7) | 8 | 87.4 (±12.9) | 2.7 (±2.5) | 0.42 (±0.29) | 0.12 (±0.12) |

GE, genome equivalent; PBMCs, peripheral blood mononuclear cells; VC, vector copies.

[a]Total lymphocyte number exhibited by animals 16 weeks posttransplantation.

[b]Sixteen weeks posttransplantation, donor engraftment was determined.

[c]Quantitative PCR for the lentiviral strong stop sequence was used to determine lentiviral integration frequency in peripheral blood collected 14 weeks posttransplantation and is presented as percent strong stop copies per genome equivalent in peripheral blood mononucleocytes.

[d]Quantitative PCR for the lentiviral strong stop sequence was used to determine vector copy number in peripheral blood cells sorted for $CD3^+$ or $IgM^+$ cells and is presented as the number of viral copies per genome equivalent.

of EF1α-Artemis-treated animals reached wild-type cellular levels (Fig. 2). Animals transplanted with CSIIEG-transduced SCID-A marrow demonstrated low levels of donor mononuclear cell engraftment, whereas animals transplanted with CSIIEG-transduced wild-type C57BL/6 marrow engrafted to wild-type levels by 8 weeks posttransplantation (Fig. 2).

All groups were found to have significant levels of gene marking by qPCR of DNA extracted from peripheral blood 16 weeks posttransplantation (Table 1). APro-Artemis-treated animals exhibited marking at 6.8%, whereas EF1α-Artemis- and PGK-Artemis-treated animals demonstrated 2.7 and 2.0% gene marking, respectively (Table 1). Control SCID-A animals transplanted with CSIIEG-transduced wild-type marrow displayed only 0.6% gene marking, demonstrating a selective advantage for engraftment and repopulation of Artemis-transduced cells (Table 1).

### *Immune reconstitution after* ex vivo *Artemis transduction*

After transplantation with transduced SCID-A marrow cells, the B lymphocyte compartment ($B220^+NK1.1^-$ cells) achieved wild-type levels in groups receiving both PGK- and APro-regulated Artemis (Fig. 3A). On the other hand, there was an absence of B lymphocytes in animals transplanted with EF1α-Art-transduced marrow throughout the 16-week time course of the experiment (Fig. 3A). The majority of treated animals in all three groups had $CD3^+CD4^+$ helper and $CD3^+CD8^+$ cytotoxic T lymphocyte levels in the normal range, with APro-Artemis-treated animals exhibiting levels close to those achieved by transplantation with wild-type marrow ($p > 0.05$) (Fig. 3B and C).

Upon sacrifice, whole blood was drawn from vector-treated animals and from C57BL/6 control animals, sorted for $CD3^+$ and $IgM^+$ populations, and assayed for vector integrants by qPCR analysis of the strong stop sequence. APro-Artemis-treated animals contained an average of 2.1 vector copies per genome equivalent in both $CD3^+$ and $IgM^+$ populations, and PGK-Artemis-treated animals contained an average of 1.7 and 2.8 vector copies per genome equivalent in the $CD3^+$ and $IgM^+$ populations, respectively, demonstrating that all resulting B and T lymphocytes were generated from transduced prelymphoid targets containing at least one lentiviral integrant. EF1α-Artemis-treated animals contained 0.42 vector copies per genome equivalent in the CD3-sorted population, and essentially undetectable vector copies in the IgM-sorted population.

### *Restored immune function in APro-Artemis-treated animals but aberrant function in EF1α-Artemis-treated animals*

At 18 weeks posttransplantation, all animals were challenged *in vivo* with two injections of 4-hydroxy-3-nitrophenylacetyl hapten-conjugated keyhole limpet hemocyanin (NP-KLH) to evaluate humoral immune response function. One week after the boost injection, all treated groups of animals mounted a significant IgM response against NP-KLH, comparable to wild-type animals ($p > 0.05$) (Fig. 4A). APro-Artemis- and PGK-Artemis-treated animals also exhibited effective class switch and generated an IgG response against the antigen, with only the APro-Artemis-treated group exhibiting a significant response comparable

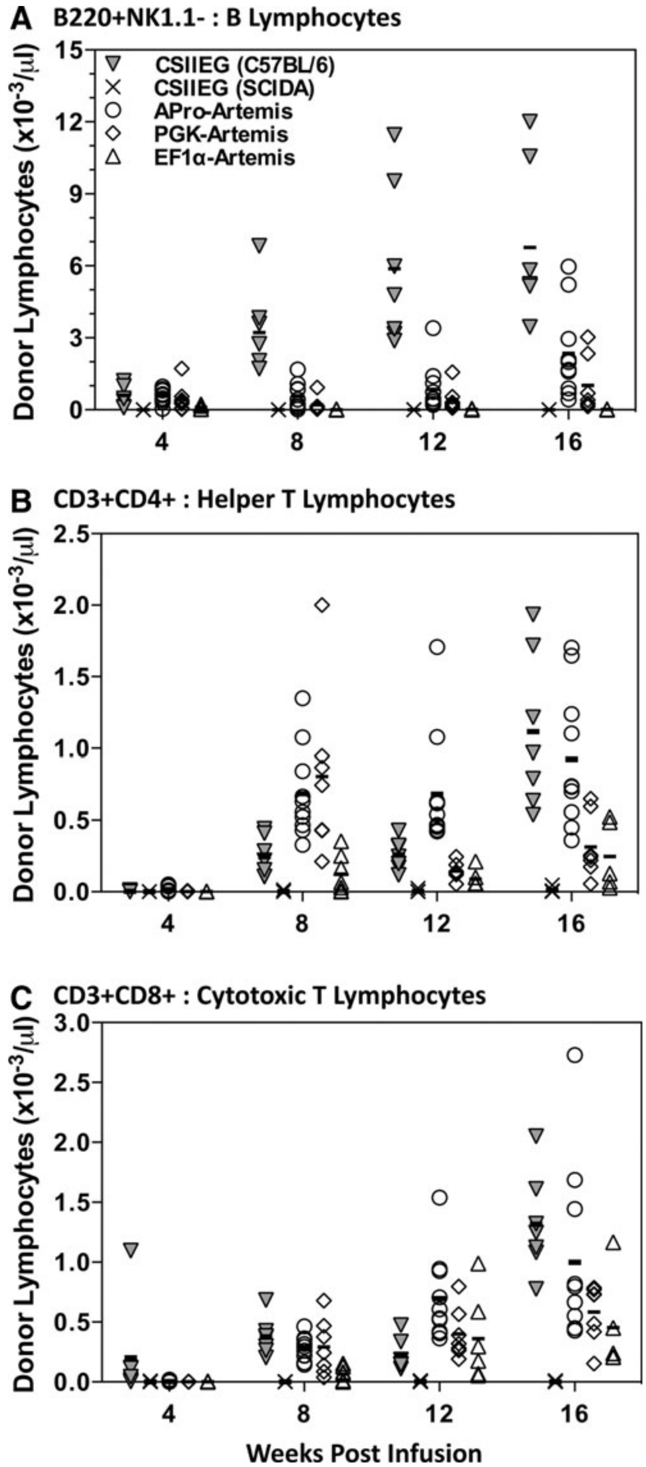


**FIG. 3.** Repopulation of circulating lymphocyte subsets in transplanted SCID-A mice. Peripheral blood was collected over a period of 16 weeks posttransplantation for analysis of lymphocyte populations. **(A)** Circulating B lymphocytes ($B220^+NK1.1^-$), **(B)** helper T lymphocytes ($CD3^+CD4^+$), and **(C)** cytotoxic T lymphocytes ($CD3^+CD8^+$) are plotted over time as the number of lymphocytes $\times 10^3$ per microliter within the donor lymphocyte ($CD45.1^+$) population. Percentages were obtained by flow cytometry and cell counts were obtained by Hemavet analysis of whole blood.

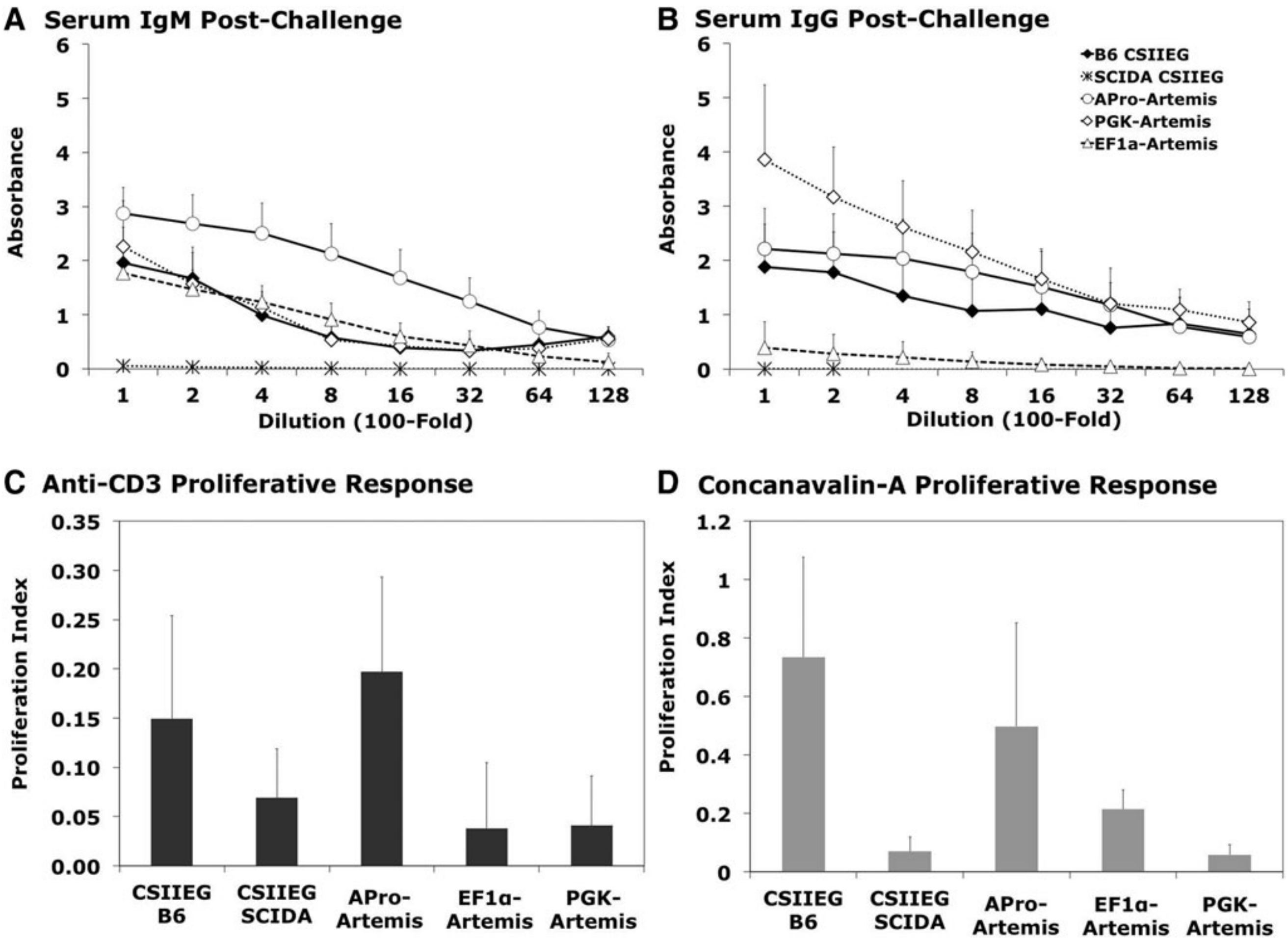


**FIG. 4.** Functional *in vivo* immune response. **(A** and **B)** Artemis-treated animals as well as control C57BL/6 and SCID-A untreated animals were challenged with NP-KLH and boosted 5 weeks after the initial challenge. One week after the final boost, sera were collected and analyzed by ELISA for the presence of **(A)** IgM and **(B)** IgG elicited against NP-KLH as compared with naive sera collected prechallenge. Immunoglobulin levels are plotted as absorbance (450 nm) versus serum dilution. **(C** and **D)** Upon sacrifice of Artemis-treated, C57BL/6 control, and SCID-A control animals, splenocytes were prepared and then plated in the presence of increasing amounts of either **(C)** anti-CD3 or **(D)** concanavalin A. Proliferative indices were calculated by dividing the MTT absorbance acquired in the presence of mitogen by absorbance acquired from samples without mitogen stimulation.

to wild-type animals ($p > 0.1$) (Fig. 4B). Interestingly, EF1α-Artemis-treated animals were unable to generate anti-NP-KLH IgG, demonstrating a failure to class switch and indicating an aberrant immune response against antigen challenge (Fig. 4B).

After the *in vivo* NP-KLH challenge, all animals were killed for further immunophenotyping of cell populations within lymphoid organs. Both APro-Artemis- and PGK-Artemis-treated animals demonstrated wild-type levels of $B220^+IgM^+$ B lymphocytes and $CD3^+CD4^+$, $CD3^+CD8^+$ T lymphocytes in both primary and secondary lymphoid organs: bone marrow (Fig. 5A), thymus (Fig. 5B), spleen (Fig. 5C), and lymph nodes (Fig. 5D). Representative flow plots are shown in Supplementary Fig. S1 (supplementary data are available online at www.liebertpub.com/hum). B lymphocytes observed in APro-Artemis- and PGK-Artemis-treated bone marrow included cells in the pro-B stage ($B220^+IgM^-$), as well as immature B lymphocyte stage ($B220^+IgM^+$), comparable to wild-type C57BL/6 animals, whereas untreated SCID-A animals displayed a characteristic arrest in B lymphocyte development at the pro-B stage ($B220^+IgM^-$)[20] (Fig. 5A). APro-Artemis- and PGK-Artemis-treated animals also displayed developmental patterning of single-positive ($CD4^+$ or $CD8^+$) as well as double-positive ($CD4^+CD8^+$) T lymphocytes within the thymus at levels comparable to wild-type untreated C57BL/6 control animals, whereas untreated SCID-A animals displayed background levels of these populations (Fig. 5B). *Ex vivo* lentiviral transduction with either APro-Artemis or PGK-Artemis vectors thus restored development of B and T lymphocytes within the bone marrow and thymus, respectively, and the ability to generate subpopulations within peripheral lymphoid organs. In contrast, animals treated with EF1α-Artemis demonstrated aberrant immune maturation and phenotype. Few $B220^+IgM^+$ lymphocytes were detected either in the bone marrow or in secondary immune organs (Fig. 5). In addition, the thymus displayed abnormally low levels of $CD4^+$ and $CD8^+$ single-positive cells as well as $CD4^+CD8^+$ double-positive cells, indicating aberrant T lymphocyte development (Fig. 5B). Normal levels of both $CD3^+CD4^+$ and

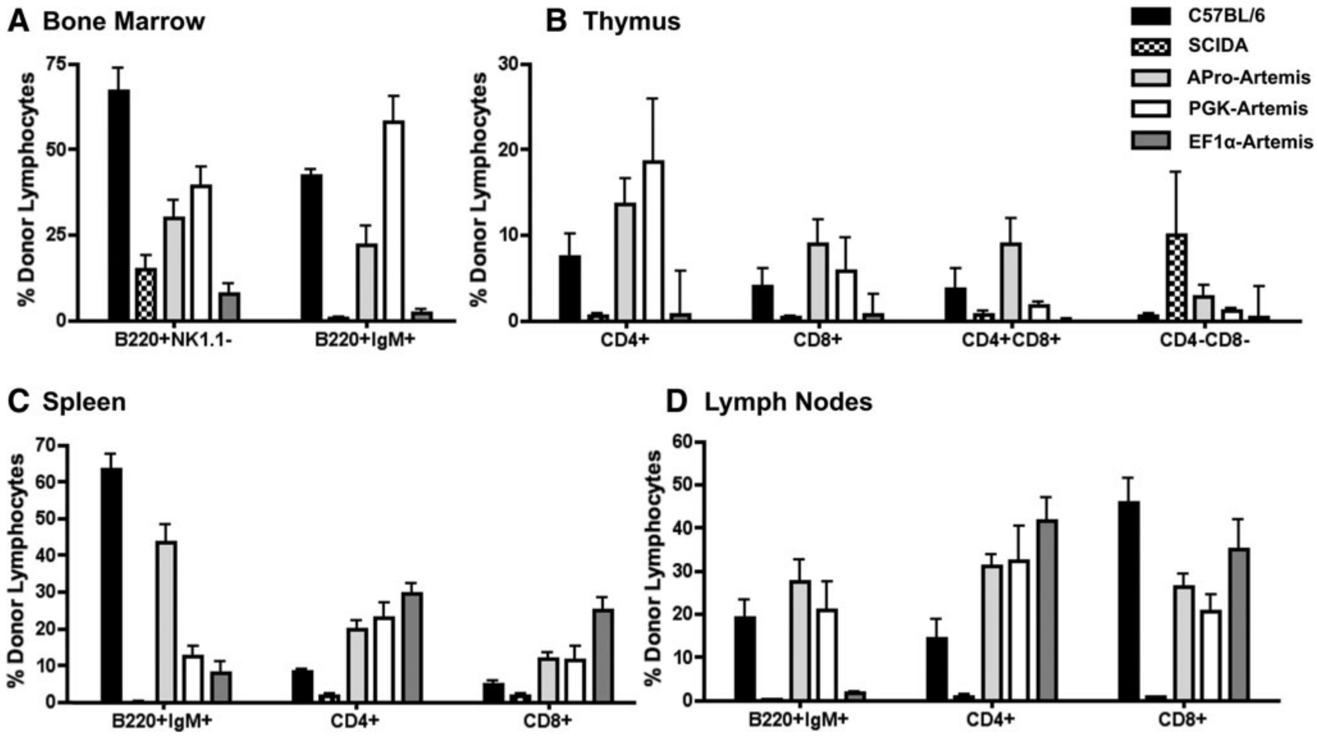


**FIG. 5.** Repopulation within lymphoid organs. Upon sacrifice, primary lymphoid organs were harvested and single-cell suspensions were analyzed by flow cytometry for the presence of B, helper T, and cytotoxic T donor lymphocyte populations of Artemis-treated animals, control C57BL/6 animals, and control SCID-A animals. **(A)** B lymphocyte development was characterized in the bone marrow, identifying both immature ($B220^+NK1.1^-$) and mature ($B220^+IgM^+$) B lymphocytes. **(B)** T lymphocyte development was monitored in the $CD3^+$ donor lymphocyte compartment within the thymus, detecting the presence of helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), double-positive T lymphocytes ($CD4^+CD8^+$), and double-negative T lymphocytes ($CD4^-CD8^-$). **(C)** Spleen and **(D)** lymph nodes were analyzed for the presence of B ($B220^+IgM^+$), helper T ($CD3^+CD4^+$), and cytotoxic T ($CD3^+CD8^+$) cells. Results are plotted as the percentage of total lymphocytes in control C57BL/6 animals and control untreated SCID-A animals, and as the percentage of total donor lymphocytes in animals receiving transduced bone marrow.

$CD3^+CD8^+$ T lymphocytes observed in the spleen and lymph nodes of these animals may have resulted from homeostatic proliferation within the lymph nodes triggered by lymphopenia and ultimately allowing low numbers of T cells to expand and fill the compartment.[25]

To evaluate proliferative responses, splenocytes were prepared from animals on sacrifice and cultured in the presence of either anti-CD3 or Con-A. Splenocytes from APro-Artemis-treated animals exhibited a proliferative response to both stimuli that was similar to that of wild-type C57BL/6 animals ($p > 0.1$), whereas there was essentially no proliferative response of splenocytes from Artemis-deficient control animals (Fig. 4C and D). Animals treated with either PGK-Artemis or EF1α-Artemis demonstrated a markedly reduced proliferative response to either anti-CD3 or Con-A as compared with APro-Artemis-treated animals ($p < 0.0028$ and $p < 0.028$, respectively) (Fig. 4C and D).

### *Normal immunodevelopment of APro-Artemis- and PGK-Artemis-transduced cells after secondary transplantation*

Secondary transplantation was carried out for all treatment groups (APro-Artemis, PGK-Artemis, and EF1α-Artemis) 6 months after primary transplantation of transduced SCID-A marrow. Sixteen weeks later, the majority of animals transplanted with marrow from animals treated with either APro-regulated (Fig. 6A) or PGK-regulated (Fig. 6B) Artemis demonstrated repopulation of both B ($B220^+NK1.1^-$) and T ($CD3^+CD4^+$ and $CD3^+CD8^+$) donor lymphocyte compartments (100% of animals treated with APro-Artemis and 83% of animals treated with PGK-Artemis vectors). Reduced levels of donor $CD3^+CD8^+$ cells in secondary recipients of PGK-Artemis-treated animals could be the result of Artemis toxicity. The sustainability of these lymphoid compartments after secondary engraftment demonstrates the capability to maintain Artemis gene expression after transduction into primitive hematopoietic stem cells. In contrast, only 20% of secondary animals receiving EF1α-Artemis-transduced marrow demonstrated repopulation of B lymphocytes and $CD3^+CD8^+$ T lymphocytes (Fig. 6C). The failure to repopulate lymphocyte populations in EF1α-Artemis secondary-transplanted animals may be due to Artemis-associated toxicity, either in developing lymphoid cells or in more primitive lymphohematopoietic progenitors.

One set of secondary transplant recipients (established from a single EF1α-Artemis-treated primary recipient) exhibited massively enlarged spleens, a condition subsequently characterized histologically as lymphoma with infiltration of several organs. DNA extracted from the marrow and spleen of these animals exhibited only 0.003 to 0.04 lentiviral vector copies per genome equivalent, however, so

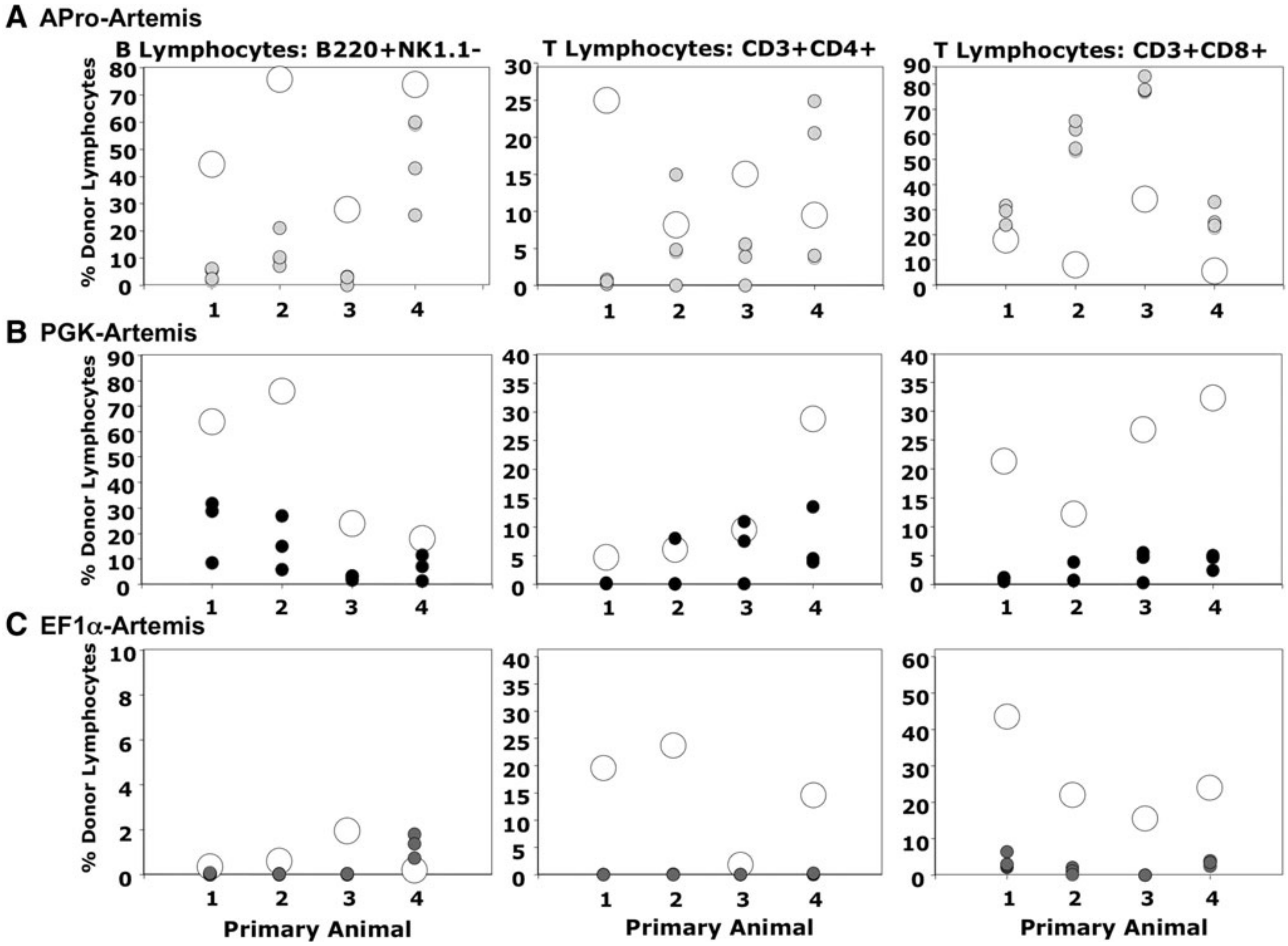


**FIG. 6.** Lymphoid reconstitution after secondary transplantation of transduced marrow. Bone marrow was collected individually from primary recipients treated with **(A)** APro-Artemis, **(B)** PGK-Artemis, and **(C)** EF1α-Artemis ($n=4$) and infused into each of three secondary irradiated (800 rads, X-irradiation source) C57BL/6 recipient animals. Peripheral blood was collected at monthly time points posttransplantation. B lymphocyte ($B220^+NK1.1^-$) as well as helper T ($CD3^+CD4^+$) and cytotoxic T ($CD3^+CD8^+$) lymphocyte repopulations were found to persist in secondary transplant recipients, plotted as percentage of the donor lymphocyte compartment. Shaded circles represent lymphocyte percentages in secondary transplant recipients 16 weeks posttransplantation; open circles represent lymphocyte percentages in respective primary donors at week 16 after primary transplantation.

this was most likely a naturally occurring tumor that arose in the primary Artemis$^{-/-}$ recipient animal that was not associated with lentiviral integration.

## Discussion

Regulation of human Artemis expression by its endogenous promoter effectively complemented the Artemis-deficient phenotype, solving the problem of cytotoxicity associated with Artemis overexpression. In contrast, animals treated with an EF1α-Artemis construct exhibited repopulation of the T lymphocyte compartment but an absence of circulating B lymphocytes. In addition, these animals demonstrated an aberrant response to NP-KLH antigen, representative of a dysfunctional immune system. Treatment of SCID-A animals with either PGK-Artemis or APro-Artemis provided complete reconstitution of both B and T lymphocyte compartments. Most importantly, APro-Artemis-treated animals mounted *in vivo* immune responses against NP-KLH antigen and displayed *in vitro* mitogenic responses of splenocytes more effectively than PGK-Artemis-treated animals and similar to wild-type animals, thus demonstrating the effectiveness of lentiviral transduction using the natural human Artemis promoter for correction of SCID-A.

Gene transfer is emerging as a promising approach for the treatment of genetic disorders, exemplified by results from clinical trials demonstrating the effectiveness of transplantation using autologous HSCs after *ex vivo* genetic correction by retroviral transduction for two severe combined immunodeficiencies caused by genetic deficiency of adenosine deaminase and the common $\gamma$ chain receptor.[10–14] These studies report long-term engraftment of corrected stem cells in a majority of patients, ultimately resulting in reconstitution of cellular and humoral immunity. However, two independent studies reported adverse events after *ex vivo* genetic correction of X-linked SCID,[10,12,15] in which 5 of 20 patients developed clonal T cell outgrowth resulting in a leukemia-like syndrome.[26,27] Although insertional oncogene activation was reported in three of the leukemic cases it has also been demonstrated that overexpression of the common $\gamma$ chain induces cellular proliferation and may have contributed to the T lymphocyte clonal outgrowth.[26–28] Tighter regulation of the common $\gamma$ chain gene may thus reduce the risk of oncogenesis resulting from aberrant overexpression.

Achieving expression of Artemis for the correction of SCID-A may present a challenge in transgene regulation similar to what was encountered during the X-linked SCID trial, considering the cytotoxicity associated with Artemis overexpression. We found that overexpression of Artemis upon lentiviral transduction results in genomic shearing, cell cycle arrest, and apoptosis.[18] Considering the endonucleolytic activity of Artemis[29–32] these results are not surprising, yet they emphasize the importance of providing Artemis expression at a level that is nontoxic and yet sufficient to correct the $T^-B^-$ phenotype in preclinical studies and in clinical application to human SCID-A. We subsequently reported the isolation and characterization of the endogenous human Artemis promoter as a sequence that effectively mediates gene expression at levels substantially lower than that mediated by the strong EF1$\alpha$ promoter both *in vitro* and *in vivo*.[19] Here we extend these results to show lack of cytotoxicity after transduction using a lentiviral vector in which Artemis is naturally regulated. These data establish the effectiveness of APro as a proficient promoter for gene expression in the hematopoietic system, specifically for the regulation of Artemis expression but potentially applicable to other gene products as well.

Consistent with previous reports, we demonstrate that Artemis expression regulated by the moderate-strength PGK promoter resulted in a functional lymphoid compartment; however, it was not reconstituted to wild-type cellular levels and was unresponsive to splenocyte proliferation stimulus. Treatment of SCID-A animals with EF1$\alpha$-Artemis led to aberrant immune reconstitution with an absence of B lymphocytes detected in the circulation, failure to generate an effective IgG response to NP-KLH, and unresponsiveness of splenocytes to proliferative stimulus. These data suggest that Artemis dysregulation or overexpression may be effecting improper lymphocyte development and repopulation, especially within the B lymphocyte lineage. The presence of B lymphocytes in primary lymphoid organs coupled with the absence of these cells in the peripheral organs and blood of EF1$\alpha$-Artemis-treated animals suggests an arrest in B cell maturation. One possible mechanism to explain such an arrest would be Artemis-mediated apoptosis during B cell development and maturation. During immunodevelopment, B lymphocytes undergo genomic alteration in the context of V(D)J recombination, activation-induced cytidine deaminase-mediated somatic hypermutation, and class switch recombination,[33,34] all of which require highly active and effective DNA repair machinery.[35–38] Immature B lymphocytes acquiring an excess number of nonproductive mutations undergo Fas-mediated apoptosis.[39,40] It is tempting to speculate that the coupling of these natural DNA DSB repair-inducing events with the global DNA damage accrued upon Artemis overexpression further sensitizes the cell to apoptotic stimuli. Consistent with this argument, the T lymphocyte population, which does not undergo somatic hypermutation or class switch recombination, was repopulated in EF1$\alpha$-Artemis-treated mice. In addition, the observed abundance of T cells and absence of B cells may be expected considering that T cells possess a greater propensity to fill a void lymphocyte compartment via homeostatic cytokine signaling than do B cells. What's more, upon sacrifice of the EF1$\alpha$-Artemis-treated mice, characterization of the splenic B lymphocytes revealed a predominant $IgM^{high}/IgD^{low}$ population (Supplementary Fig. S2), which may suggest an early transitional or marginal B lymphocyte population.[41–43] Lack of class switch recombination along with the observed B lymphocyte surface marker repertoire indicates incomplete repopulation within the EF1$\alpha$-Artemis-treated animals by an early transitional thymic-independent B lymphocyte as opposed to a mature thymic-dependent B lymphocyte population.[43,44]

Overall, these results underscore the requisite of providing levels of Artemis that avoid cytotoxicity yet allow for successful reconstitution of the lymphoid lineages. We provide an example of how Artemis overexpression results in aberrant lymphoid reconstitution and dysfunctional immune response, and corroborate previously reported results demonstrating complete correction by using a moderately regulated Artemis vector.[16,17] Moreover, we report the complete reconstitution of a functional lymphoid population in SCID-A animals by *ex vivo* lentiviral gene transfer using a vector in which Artemis expression is regulated by the natural human promoter. These results suggest that the natural levels of Artemis expression achieved via *ex vivo* lentiviral transduction into hematopoietic stem cells will serve as a clinically relevant and feasible treatment of human SCID-A.

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## Author Disclosure Statement

The authors declare no competing financial interests.

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