Gene therapy/bone marrow transplant in ADA-deficient mice: roles of enzyme replacement therapy and cytoreduction

Denise A. Carbonaro¹*, Xiangyang Jin¹, Xingchao Wang², Xiao-Jin Yu², Nora Rozengurt³, Michael L. Kaufman¹, Xiaoyan Wang⁴, David Gjertson⁴, Yang Zhou⁵, Michael R. Blackburn⁵ and Donald B. Kohn¹

¹ Department of Microbiology, Immunology, and Molecular Genetics, University of California Los Angeles, Los Angeles, California, United States, 90095.

² Division of Research Immunology/BMT, Children’s Hospital Los Angeles, Los Angeles, California, United States 90027.

³ Department of Pathology and Laboratory Medicine, University of California Los Angeles, Los Angeles, California, United States 90095.

⁴ Department of Biostatistics, School of Public Health, University of California Los Angeles, Los Angeles, California, United States 90095.

⁵ Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, Texas, United States 77030.

Running title: ADA-Deficient Mouse HSC Gene Therapy ex vivo

*Corresponding author:
Denise Ann Carbonaro Sarracino, PhD.
Department of Microbiology, Immunology and Molecular Genetics
University of California, Los Angeles
3159 TLSB, 610 Charles E. Young Drive South
Los Angeles CA 90095
Phone: 310-794-1884
Fax: 310-206-0356
e-mail: dsarracino@ucla.edu
Abstract

Gene therapy (GT) for adenosine deaminase-deficient severe combined immune deficiency (ADA-SCID) can provide significant long-term benefit when patients are given non-myeloablative conditioning and ADA enzyme replacement therapy (ERT) is withheld prior to autologous transplantation of gamma-retroviral vector-transduced bone marrow CD34+ cells. To determine the contributions of conditioning and discontinuation of ERT to the therapeutic effects, we analyzed these factors in Ada gene knock-out mice (Ada-/-). Mice were transplanted with ADA-deficient marrow transduced with an ADA-expressing gamma-retroviral vector without pre-conditioning or after 200cGy or 900cGy total-body irradiation and evaluated after 4 months. In all tissues analyzed, vector copy numbers (VCN) were 100-1,000-fold higher in mice receiving 900cGy compared to 200cGy (p-value<0.05). In mice receiving 200cGy, VCN was similar whether ERT was stopped or given for one or four months following GT. In non-conditioned mice, there was decreased survival with and without ERT, and VCN was very low to undetectable. When recipients were conditioned with 200cGy and received transduced lineage-depleted marrow, only recipients receiving ERT (1 month or 4) had detectable vector sequences in thymocytes. In conclusion, cytoreduction is important for engraftment of gene-transduced HSC and short-term ERT after GT did not diminish the capacity of gene-corrected cells to engraft and persist.
Introduction

In humans, adenosine deaminase (ADA) deficiency results in a severe combined immunodeficiency (SCID), and if left untreated, results in infant mortality (1). ADA is responsible for deamination of adenosine (Ado) and 2'-deoxyadenosine (dAdo) to form inosine and 2'-deoxyinosine, respectively, and is essential to purine salvage. Without ADA, Ado and dAdo both accumulate; however, it is primarily the accumulation of dAdo and subsequent dATP formation that cause the cytotoxicity in immature T lymphocytes (2). The preferred treatment for ADA-deficient SCID (ADA-SCID) is hematopoietic stem cell transplantation (HSCT) with a matched sibling donor. Transplants for ADA-SCID using matched-unrelated, or haplo-identical donors have lower survival (3). When a suitable donor is not available or the patient is too ill for transplantation, the patient may be started on enzyme replacement therapy (ERT) with pegylated bovine ADA (Adagen®, Sigma Tau, Gaithersburg, MD). ERT does increase lymphocyte counts and immune function; however, absolute lymphocyte counts remain below normal and immune function decreases over time (4,5).

Since the current clinical options are suboptimal, ADA-deficient SCID is an ideal candidate for gene therapy approaches. It has been hypothesized that correction of a few autologous hematopoietic stem cells (HSCs) could result in corrected T-cell progeny that would have a strong selective advantage and would eventually fill the T-cell compartment (6). The selective advantage of corrected T-cells in ADA-SCID has been observed in patients that had a spontaneous reversion of the mutation in one T-cell precursor where, over time, the T-cell compartment was entirely comprised of corrected T-cell progeny (7,8).

The earliest gene therapy (GT) clinical trials for ADA deficiency used murine gamma-retroviral vectors to transduce peripheral blood T lymphocytes and bone marrow (9,10), CD34+ isolated from
bone marrow (11) and from cord blood (12). In these studies, patients were not given cytoreductive conditioning prior to receiving transduced cells and all patients remained on ERT. These approaches did not provide clinical benefit and the frequency of gene-marked peripheral blood (PB) cells remained very low. However, in some studies, the frequency of gene-corrected PB T-cells increased when the ERT dose was either decreased or discontinued (13,14). It was hypothesized that the survival advantage of gene-corrected cells was blunted by the ERT. Long-term follow-up studies have indicated gene-corrected cells can persist for more than 10 years, contain different vector integration sites and are not the result of insertional oligoclonal expansion (15,16). In a later trial with improved gamma-retroviral vectors and transduction conditions, unconditioned patients remaining on ERT did not have clinical benefit (17). In 2002, investigators in Milan reported that cytoreductive conditioning with a moderate dose of busulfan chemotherapy prior to re-infusion of transduced cells and cessation of ERT, resulted in multi-lineage engraftment of transduced cells and immune reconstitution (18). ADA activity was detected across lineages, which was associated with a decrease in adenine metabolites. A later publication analyzed the long-term follow-up (1.8-10y) of 10 patients treated with this approach, and showed significant, long-term, clinical benefit (19). Since then, groups in the UK and the US have demonstrated efficacy with this approach as well (20-22).

Although GT for ADA-SCID has met success with clinical benefit for many patients (~70%), a number of patients in the ongoing clinical trials have experienced events that necessitated the restarting of ERT (19,20,21), suggesting there could be improvements in outcomes. Thus, the primary goal of this study was to delimit the roles of ERT and cytoreductive conditioning in gene therapy ex vivo for ADA-SCID, using the ADA-deficient mouse and a gamma-retroviral vector currently being evaluated in a clinical gene therapy trial. We also investigated the efficacy of a short-term course of ERT post-GT to enhance engraftment of transduced cells. The results of
these experiments clearly demonstrate the importance of cytoreductive conditioning and the potential to enhance thymic engraftment with short-term ERT following gamma-retroviral mediated gene therapy for the clinical treatment of ADA-SCID.

Materials and Methods

Vector, Packaging and Stability. The gamma-retroviral vector, MND-MFG-huAda (MMA), based on the murine leukemia virus (MMLV) retrovirus, as was modified using the MND LTR enhancer/promoter to drive expression of the human Ada cDNA and includes the splice acceptor site from the 5' untranslated region of MMLV envelope gene (env)(23-25). The ATG transcription start site (TSS) of the human Ada cDNA is aligned at the ATG TSS of env to enhance transgene expression (Figure 1A)(26). MMA was packaged by transient transfection in 293T human embryonic kidney cells in D10 for 10-14d (D10-Dulbecco’s Modified Essential Medium, 10% FCS, 100U penicillin/streptomycin), and pseudotyped with vesicular stomatitis virus capsid protein (VSV-G). MMA/VSV-G was used to transduce GP+E+86 (GPE) cells (27) and repackage the vector with the murine ecotropic ENV protein. Cellular GPE clones were made by limited dilution of the transduced pool. Titer was determined on NIH-3T3 fibroblasts and a producer clone was based on the amount of ADA enzyme activity (~120 U/mg/min).

Mice. A two-stage murine model of ADA-deficient SCID was generated and described previously (FVB;129-Ada<sup>tm1Mw</sup>Tg(PLADA)4118Rkmb/J; Jackson Laboratories, Bar Harbor, MA)(28,29). ERT was administered by weekly intramuscular injection of 250U/kg of ADAGEN® (Sigma Tau, Pharmaceuticals, Gaithersburg, MD). Mice were housed in accordance with IACUC (Saban Research Institute at Childrens Hospital Los Angeles) and the National Institutes of Health.
guidelines. All animals were handled in laminar flow hoods and housed in micro-insulator cages in a pathogen-free colony.

**Bone Marrow Harvest, Lineage-Depletion and HSCT.** Ada−/− donor mice (8-10wk) were euthanized and marrow was harvested from the femur, tibia, and humeral bones, pooled and, centrifuged for 10min at 400xg at 10°C. Pooled marrow was plated on fibronectin-fragment CH-296 (20 mg/ml; Retronectin, Takara BIO, Inc., Otsu Japan) coated plates at 1x10^6 cells/ml in basal bone marrow media (BBMM: Iscove’s Modified Dulbecco’s Medium supplemented with 30% fetal calf serum (FCS), 1% bovine serum albumin (BSA; Stem Cell Technologies, Vancouver, Canada), 100uM 2-β-mercaptoethanol, 2mM glutamine, 100U/ml penicillin/streptomycin) freshly supplemented with murine cytokines (IL-3: 10ng/ml; IL-6: 25ng/ml, c-kit ligand: 2.5ng/ml; Biosource International, Inc., Camarillo CA) and cultured at 37°C for 48 hours pre-stimulation. Pooled marrow cells were lineage-depleted using anti-murine, biotin-conjugated, antibodies: CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C, 7-4 and Ter-119) according to the manufacturer’s instructions (Murine Lineage Depletion Kit, Miltenyi Biotec, Auburn, CA). Lineage-depleted cells (lin-) were re-suspended in BBMM at 1x10^6 cells/ml and plated, as described above, for pre-stimulation for 48 hours. Congenic (Ada+/+) female donor mice (8-10wk) were used as controls for transplant of fully ADA-replete marrow, with 5x10^6 unprocessed marrow cells suspended in injectable normal saline (USP; APP, Schaumburg, IL) injected via tail vein into male Ada−/− mice (8-10wk)(30).

**Transduction:** After 48h, one-half of the medium plus non-adherent cells in the flask were collected and centrifuged at 400xg for 10min. The supernatant was discarded and the cells were re-suspended in the same volume of MMA vector supernatant, supplemented with 4ug/ml
polybrene (Sigma, St. Louis MO) and re-added to the flasks. After 24h, the transduction procedure was repeated with fresh vector supernatant.

**Cytoablative Conditioning and Transplantation.** Ada/- recipient mice (8-10wks) were conditioned with total body irradiation (TBI) on the day of bone marrow transplant (0, 200, or 900cGy) from a $^{137}$Cesium source at a dose rate of 95cGy/minute. The non-adherent transduced cells were transferred to a 50ml conical tube and the adherent cells were harvested with cell-dissociation buffer (Invitrogen, Carlsbad, CA), for 15min at 37°C. Transduced marrow cells were re-suspended in 0.9% injectable sodium chloride at 5.0x10^7 cells/ml and transduced lin- marrow cells were re-suspended at 5.0x10^6 cells/ml. Mice were injected via the tail vein with either 5x10^6 whole marrow cells or 5.0x10^5 lin- marrow cells and were maintained on 50mg/ml (in drinking water) Maxim 200 (orthotetracyclin supplied as 200mg/ml, Phoenix Pharmaceuticals, St. Joseph, MO) for 21d.

**Necropsy, Tissue Harvest, and Immunohistochemical Analysis.** Mice were euthanized and perfused via cardiac puncture with 15ml of PBS. The thymus, spleen, liver and right lungs were harvested into ice-cold PBS with 1.0% fetal calf serum (PBS-1%FCS) in a six-well plate. Fragments of thymus, spleen, and liver were fixed and the left lung was perfused and fixed with 2% para-formaldehyde. Immunohistochemistry was performed as described using anti-human ADA monoclonal antibody (N-19; Santa Cruz Biotechnology, Santa Cruz, CA)(31).

**Immunophenotype and Function Assays.** All immunophenotype and functional assays were performed as previously described (30,32). Single cell suspensions were made from thymus, spleen and bone marrow and immunophenotyped by flow cytometry. T-cell function was assessed by an *in vitro* mitogenic stimulation assay, using concanavalin A (ConA). B-cell function
was assessed in vivo, by vaccination with Pneumovax 23 (Merck, Whitehouse Station, NJ; 10ul in 100 ul of injectable saline) followed by measurement of serum antibodies after 10d by ELISA (32).

**VCN Determination.** DNA was extracted with phenol/chloroform, re-suspended in TE and quantified using DNA-specific fluorometry (Hoechst dye; Sigma, DNA-QF, St. Louis). Vector copy number (VCN) was determined by real-time quantitative PCR (qPCR). Primer/probe set was vector specific to MND-MFG-huAda: sense primer (5’-tcaatgcggccaatctagtt), anti-sense primer (5’-tggactaatcgataccgtcgac-3’) and the TAMRA probe sequence (6FAM-tgacctgtcttataaagctatggatgc-TAMRA). The final concentration of the primers was 400nM and the probe was 50nM. All reactions utilized Universal Master Mix (Applied Biosystems, Inc. (ABI), Fullerton, CA) and were run under default conditions in the 7900 Sequence Detector System (ABI). For each sample, 350ng of DNA were interrogated for vector sequence (in duplicate) and compared to a standard curve produced by serially diluting (2.0-0.00002 vector copies) the DNA of a cellular clone that contained 2 copies of the MMA gamma-retroviral vector as determined by Southern blot.

**Engraftment.** The TSYR gene, located on the Y chromosome, was used to determine the amount of chimerism or engraftment in sex-mismatched transplants. To quantify the percentage of male DNA, qPCR was performed using a standard curve constructed from male murine tail DNA diluted into female murine tail DNA to form mixtures between 0.3-100% male DNA (33).

**ADA Enzyme Activity and Substrate Concentration in Tissues.** Tissues were harvested as parts of whole organs and flash frozen and store at -80C until processed. ADA enzyme assay was performed as described (29). Perchloric acid was used to extract adenine nucleosides from frozen
tissue, and adenosine (Ado) and deoxyadenosine (dAdo) were separated and quantified using reversed-phase high-pressure liquid chromatography as described (34).

**Statistical Analysis.** Descriptive statistics of continuous outcome variables such as the means and standard errors by experimental groups are presented in figures. For each experiment, overall group difference was identified by analysis of variance (ANOVA) approach (35). If normality assumption is violated, non-parametric Krusal-Wallis analysis of variance was used (36). Within the ANOVA/Krusal-Wallis test framework, we performed estimation and hypothesis testing comparing group differences under various experimental conditions. For all statistical investigations, tests for significance are two-tailed, with a statistically significant p-value threshold of 0.05. Statistical analyses were carried out using SAS version 9.2 (37).

**Results**

In mice, ADA-deficiency results in profound multi-system defects, including severe combined immunodeficiency (SCID). If untreated, Ada-/− pups succumb to a non-infectious, pulmonary insufficiency by postnatal d20; thus, survival after gene therapy (GT) is a potent test for efficacy. In a series of sequential experiments, bone marrow from Ada-/− mice was transduced *ex vivo* with the MMA gamma-retroviral vector and transplanted into syngeneic Ada-/− recipients. The first study assessed the role of cytoreduction prior to GT and the role of ERT after GT, the second study assessed efficacy of a short-term course of ERT after GT compared to a long-term course, and the third study assessed differences between transplanting transduced whole marrow (wm) compared to transduced lineage-depleted (lin-) marrow cells, with and without mild cytoreductive conditioning and/or ERT.
Study 1. Cytoreductive Conditioning and ERT Cessation

Transduction Efficiency and Survival. Marrow was harvested from Ada/- male donors (8w) and transduced with the MMA gamma-retroviral vector (Figure 1A). Recipient Ada/- mice (8-10w) were conditioned with 200 or 900cGy total body irradiation (TBI) and then each received 5x10^6 transduced whole marrow cells. Recipient mice were maintained on ERT (200+ERT and 900+ERT) or not maintained on ERT (200-NoERT or 900-NoERT) and analyzed at 4m. Vector copy number (VCN) at the end of the transduction period was determined by qPCR, and ranged from 0.1-1.2 copies/cell in five different experiments (Figure 1B). There were no significant differences in survival between mice receiving 900cGy or 200cGy, regardless if they were maintained on ERT after GT or not (Figure 2A).

Cytoreduction. After 4m, the mean VCN was 100-1000 fold higher in mice conditioned with 900cGy (0.11-1.2 copies/cell) compared to mice conditioned with 200cGy (0.0007-0.006 copies/cell) for all tissues analyzed (p-values<0.05; Figure 2B). Mean thymus VCN was approximately 1000 fold higher with 900cGy (0.9 +ERT, 1.2 NoERT) compared to 200cGy (0.002 +ERT, 0.0005 NoERT) and the mean spleen VCN was 100 fold higher with 900cGy (0.9 +ERT, 0.62 NoERT) compared to 200cGy (0.02 +ERT, 0.006 NoERT). To assess the effect of TBI dosage on the numbers of gene-corrected cells, the absolute numbers of gene-marked cells were calculated by multiplying the absolute numbers of cells by the VCN (expressed as percent transduced)(Figure 2C-D). By this measure, the mean absolute numbers of gene-marked lymphocytes were significantly higher in mice conditioned with 900cGy compared to mice conditioned with 200cGy regardless of ERT treatment (p-values<0.05). When there was a sex-mismatched donor, qPCR was used to quantify donor male Y-chromosome sequences in the recipient marrow. Donor engraftment was 30% in mice conditioned with 900cGy compared to <1%
in those receiving 200cGy indicating that cytoreductive conditioning is very important for donor cell engraftment.

**ERT.** Ada-/- mice conditioned with 900cGy or 200cGy had no significant differences in VCN or in the absolute numbers of gene-marked cells in the thymus, whether they received ERT or not (Figures 2B-D). However 200+ERT mice had higher numbers of lymphocytes (CD4+, CD8+, CD19+) compared to 200-NoERT mice (p-values<0.05), presumably due to rescue of uncorrected lymphocytes. However, because neither VCN nor the absolute numbers of gene-marked cells decreased with continued ERT, ERT is not detrimental to the survival and expansion of gene-corrected cells despite dilution of the vector sequences with uncorrected cell DNA. As seen in our previous studies with Ada-/- mice, mitogenic proliferation assays showed that mature lymphocytes (corrected or uncorrected) were functional in vitro (Supplemental Figure 1)(30,32).

**Study 2. Long Course ERT and Short Course ERT**

**Experimental Design.** Since long-term ERT after GT did not appear to be detrimental to the engraftment, survival and/or expansion of the corrected cells, we evaluated the effects of short-term ERT compared to long-term ERT. Recipient mice were conditioned with 200cGy TBI and received γ-retrovirally transduced ADA-deficient whole marrow (wm) cells and maintained on ERT for 4m after GT (200+4mERT), or for only the first month following GT (200+1mERT), or not at all after GT (200-NoERT)(Figure 3A). Treated mice were analyzed at 2, 4, 10 and 16 weeks.

**Vector Marking.** VCN was similar across all time-points in all tissues analyzed, independent of ERT treatment (Figure 3B). When compared to mice not on ERT, the absolute numbers of gene-marked thymocytes from mice on ERT was significantly higher only at the earliest time-point (2w) immediately after GT but not at subsequent time-points (p-values<0.05) probably due to the loss of
uncorrected cells without ERT (Supplemental Figure 2A-D). Immunohistological staining of thymi with an antibody specific for human ADA, demonstrated the presence of stained (brown) lymphocytes distributed throughout the thymic cortex and medulla, indicative of human ADA expression at 4 and 10 weeks, regardless of ERT treatment (Figure 4A-B). Thus, ERT after gene therapy with mild cytoreduction (200cGy) did not affect the survival of Ada gene-corrected thymocytes.

**ADA Enzyme Activity and Substrate Concentration.** ADA enzyme activity and deoxyadenosine concentrations measured in tissues at 10 weeks were not significantly different in mice maintained on ERT or not (Figure 5A-B). Lung adenosine concentrations were lower in mice maintained on ERT (200+4mERT) compared to mice not continuously maintained on ERT (200+1mERT and 200-NoERT)(p-values<0.05). Ada-/- mice are particularly sensitive to the accumulation of adenosine in lung tissue and perhaps extracellular ADA (ERT) is more efficacious than intracellular (GT) as extracellular adenosine mediated signaling through G-coupled adenosine receptors has been implicated in the pulmonary phenotype (39).

**Study 3. Cytoreduction, ERT and Target Cell.**

In Studies 1 and 2, whole bone marrow was transduced and infused into recipients. However, in the clinical setting, patients are infused with transduced autologous CD34+ marrow cells, a population enriched ~50-fold for human HSC. The following study was designed to simulate conditions closer to approaches used in current clinical trials for ADA-SCID. While the CD34 marker is not useful for enrichment of murine HSC, lineage-depleted murine bone marrow cell preparations are similarly enriched ~50-fold for murine HSC. Ada-/- mice were conditioned with 0cGy (0) or 200cGy (200) TBI, and transplanted with either transduced whole marrow (wm) or
transduced lineage-depleted bone marrow (lin-), and maintained on ERT for 4m (+4mERT), 1m (+1mERT) or ERT was stopped after GT (-NoERT).

**Survival.** Unlike ADA-deficient patients, survival can be used as a measure of efficacy (engraftment of gene-marked HSC) in the Ada−/− mice, since ADA-deficiency is a lethal condition unrelated to the SCID phenotype. Overall, mice conditioned with 200cGy TBI had higher survival than non-conditioned mice, regardless of transduced cell type (wm or lin-) or ERT course of treatment (p-values<0.01)(Figure 6A). When non-conditioned mice were not maintained on ERT, survival was significantly reduced compared to non-conditioned mice that were maintained on a short course of ERT (p-values<0.01)(Figure 6B). In the condition closest to current clinical gene therapy protocols for ADA-SCID (non-myeloablative conditioning and no ERT), survival was also significantly reduced in mice conditioned with 200cGy and transplanted with lineage-depleted marrow and not maintained on ERT compared to mice maintained on ERT for 1 or 4 months (p-values<0.01). As seen previously, for mice conditioned with 200cGy and transplanted with whole marrow, there were no differences in survival with or without ERT.

**Marking.** Overall, mice receiving transduced whole marrow had 10-100x higher bone marrow VCN compared to mice receiving lineage-depleted marrow regardless of ERT course (p-values<0.05) (Figure 6C). For most tissues analyzed, ERT did not affect VCN or numbers of gene-marked cells when mice received transduced whole marrow. Mice transplanted with transduced whole marrow had higher gene-marked thymocytes (CD8+ and CD4-CD8-) compared to mice transplanted with lineage-depleted marrow (p-value<0.05). Although, spleen VCN was significantly higher in mice not maintained on ERT (200-NoERTwm) compared to mice maintained on ERT (200+4mERTwm), there were no differences in the number of gene-marked cells (p-values<0.05), probably because the absolute numbers of splenocytes were significantly increased...
with ERT indicating uncorrected and corrected splenocyte survival was supported with ERT. Mice transplanted with transduced whole marrow with no ERT had higher numbers of gene-marked splenic lymphocytes (total, sCD4+, and sCD19+) compared to mice transplanted with transduced lineage-depleted marrow with no ERT (p-values<0.05) suggesting that the target cell population is an important factor in the engraftment of transduced cells in the absence of ERT.

In mice transplanted with lineage-depleted marrow without conditioning (0+1mERTlin- or 0+4mERTlin-) there were few gene-marked lymphocytes present (Figure 7A-D). Furthermore, there was a complete lack of detectable vector sequences in the thymi of mice receiving non-myeloablative conditioning and no ERT (200-NoERTlin- mice), the condition most similar to current clinical gene therapy protocol. However, if the mice were maintained on ERT for 1 or 4m (200+1mERTlin- or 200+4mERTlin-), vector marking was detected. These results demonstrate that cytoreduction is very important in the engraftment of transduced cells and that a short course of ERT may be beneficial, especially when transplanting a transduced, enriched HSC population.

**Discussion**

Initial gene therapy trials for ADA-deficient SCID patients used enriched populations of bone marrow CD34+ cells, and patients were not given cytoreductive conditioning and they remained on ERT (10-13). These studies did not demonstrate clinical efficacy, as the frequency of gene-corrected cells was low. It was hypothesized that omission of cytoreductive marrow conditioning limited HSC engraftment and the continuous administration of ERT blunted any selective survival advantage the corrected lymphocytes may have had over the uncorrected cells. Thus, in subsequent clinical trials, patients are being treated with non-myeloablative doses of busulfan or melphalan and ERT is discontinued prior to re-infusion of the autologous, transduced CD34+ cells (14,19-22). The premises for these protocol changes were two-fold: cytoreductive conditioning would ‘make space’ in the marrow compartment and
ERT cessation would allow the transduced, corrected lymphocytes to realize their survival advantage. This approach has resulted in a high frequency of gene-marked cells, especially in the T-cell compartment, and has had a clear clinical benefit for the majority of patients (19,22). However, despite the clinical benefits achieved, it was not possible to determine the individual roles of cytoreduction and ERT, as both changes were implemented together. While both factors may add to efficacy, they also add to potential risks, from chemotherapy-related toxicities and from an increased risk of infection due to lymphopenia that occurs following ERT withdrawal. In one study, ADA-deficient SCID patients receiving HSC GT, with ERT cessation, but without conditioning, had delayed immune reconstitution (39), supporting the role for cytoreduction. As gene therapy becomes a standard of care for ADA-deficient SCID, it will be important to understand the roles of conditioning, with enhanced engraftment of transduced HSC, and the cessation of ERT, with increased selective advantage for gene-corrected cells for achieving best clinical outcomes. We explored these two issues in gene transfer/BMT studies in ADA gene knock-out mice.

**Cytoreductive Conditioning.** ADA-deficient mice die postnatal d20-21 unless treated with ERT, HSCT or GT. If treatment is discontinued (ERT; less than 14 days) or ineffective (HSCT/GT) the adult mice will eventually die of pulmonary insufficiency, (pathogen-free housing), thus survival can be a measure of efficacy of a treatment. In our studies, higher intensity conditioning consistently led to higher levels of gene-marked cells in lymphoid and non-lymphoid organs. Mice receiving 900cGy always had higher VCN (10-1000 fold) in the thymus, spleen, marrow, lung and liver than mice receiving 200cGy, regardless of whether ERT was continued or not. Also, the absolute numbers of gene-marked cells in the thymus and spleen were 100-1000 fold higher in the 900cGy mice compared to the 200cGy mice. Furthermore, mice not conditioned (0cGy), had poor survival without short (1m) or long (4m)
term ERT and VCN was dramatically reduced. These results indicate that the TBI dose of cytoreductive conditioning is critical for determining the amount of engraftment and expansion of transduced HSC and progenitors, as has been shown in other murine models of gene therapy (40). Similarly, in clinical trials of gene therapy for ADA-deficient SCID, patients with the longest period of neutropenia following conditioning with busulfan had the highest frequency of gene-marked cells (19) and when two protocols were used, one with and without busulfan, marking is greatly improved with busulfan (21). It is important to note that radiation is not equivalent to busulfan, and that the possible effects of radiation should be considered when interpreting these results. Mice receiving 900cGy did have lower total numbers of immature and mature lymphocytes than those receiving 200cGy, suggesting that the higher dose of radiation may damage the lymphoid organ stroma and impair immune reconstitution (41).

**ERT.** In the early clinical trials, ERT was reduced in a few patients at various times after GT and this resulted in an increase in the measured frequency of gene-marked cells in peripheral blood T lymphocytes (13,14). In one ADA-deficient patient who experienced a spontaneous reversion of a mutant Ada allele in a progenitor cell, ADA-replete T-cells that arose were obscured when the patient resumed ERT (42). From these results, it was hypothesized that continued ERT may blunt the selective advantage of the gene-corrected cells, resulting in a lower number of corrected cells and vector sequence detection (43). However, when only the average VCN is measured (e.g. qPCR for vector sequence in tissue DNA), it would be difficult to distinguish between a decrease in vector detection from reduced selective pressure from a decrease in VCN from dilution of vector sequence by the increased amount of un-corrected cell DNA. Therefore, to understand if there is a true diminution of the absolute numbers of gene-corrected cells, we calculated the absolute number of gene-marked cells in tissues when ADA-deficient mice remained on ERT or not. It is of interest that we found no difference in the
absolute numbers of gene-marked cells in any analyzed tissue when mice received 900cGy prior to receiving transduced whole marrow cells, with or without ERT. Likewise, we found no difference in the absolute numbers of gene-marked lymphocytes from mice receiving cytoreduction with 200cGy, with or without ERT, despite increases in the absolute numbers of lymphocytes with ERT and apparent dilution of vector sequences in gene-corrected cell DNA by uncorrected cell DNA. Likewise, a few subjects in ADA gene therapy clinical trials have been restarted on ERT after GT and PBMC DNA VCN did not decrease (19-22). Thus, it could be argued that the frequency/survival of gene-corrected cells actually increased on ERT since VCN remained the same despite the putative dilution of vector sequence with continued ERT.

We cannot say for sure how ERT improves survival of gene-corrected cells, but we hypothesize ERT may augment the ADA supplied by the gene-corrected thymocytes by providing a good ‘soil’ for thymopoiesis to further expand the T-cell population since it has been shown that CD4-CD8- thymocyte differentiation is disrupted and apoptosis is initiated when ADA substrates accumulate in ADA-deficient fetal thymic organ culture (44). Study 3 also supports this hypothesis. When mice received lineage-depleted marrow with 200cGy and no ERT (the closest condition to current clinical trial approaches), vector sequences were only detected in the thymus when mice remained on ERT for 1 or 4m.

**Whole Marrow vs. Lineage-depleted Marrow.** Mice transplanted with transduced whole marrow (5.0x10^6 total cells) had engraftment and detectable gene-marking in thymus, spleen, marrow, lung and liver, regardless of ERT course. However, mice transplanted with transduced lineage-depleted marrow (5.0x10^5 total cells), and a theoretically higher HSC dose, there was no detectable vector in thymus and lower numbers of gene-marked lymphocytes detected without
ERT compared to mice transplanted with whole marrow without ERT. It is not clear what mechanism(s) is responsible for the enhanced engraftment of transduced HSC and/or development of gene-corrected lymphocytes when transplanting whole marrow instead of lineage-depleted marrow, or how ERT post GT enhances engraftment with lineage-depleted marrow. Transplanting transduced whole marrow may provide one or more of the following: 1. a more effective HSC dose, 2. cells that enhance the bone marrow niche and support hematopoiesis, 3. transient ADA expression from transduced mature marrow cells, creating a detoxified microenvironment and/or improving the function of other cells residing in the niche.

Transduced whole marrow may represent a more effective HSC dose because there is virtually no processing/enrichment of HSC and their stromal support remains intact. In matched sibling HSCT, ADA-deficient patients receive un-manipulated, whole marrow where 100% of the cells are ADA replete, with 2 functional copies of the ADA gene per cell. Even non-engrafting cells may provide enough ADA early in the engraftment period to detoxify the microenvironment for engraftment and for other facilitating cells residing in the bone marrow niche. Indeed, Sauer et al, found that within the bone marrow niche, ADA-deficient mice have decreased osteoblast function in situ, suggesting an altered bone marrow microenvironment (45). Furthermore, they found decreased colony formation when Ada-/- lineage-depleted cells were co-cultured with Ada-/- stromal feeder layers compared to ADA+/- stromal feeder layers. These parameters were all reversed with ERT, HSCT and GT in Ada-/- mice.

Likewise, transduced whole marrow will also contain many types of transduced cells expressing ADA, albeit not all are transduced with 2 copies/cell as in HSCT. We hypothesize that many transduced cells will not achieve long-term engraftment, but could provide ADA for detoxification of the niche, as well as transduced stromal cells that may be important for HSC localization within the
bone marrow niche and other tissues. Unexpectedly, we found VCN was higher in liver and lung from recipients of transduced whole marrow compared to lineage-depleted marrow cells, regardless of ERT treatment. Both lung and liver exhibit significant pathology in the ADA-deficient mouse and transduced whole marrow may have other hematopoietic and mesenchymal cells that facilitate “liver/lung engraftment” (45). Although it is not clear how relevant this observation is to the human experience, a recent study showed that many ADA-deficient patients, unlike X-linked SCID patients, presented with lung pathology not associated with an infectious agent and with histopathology similar to that observed in ADA-deficient mice (46).

Although the mechanism for improved engraftment is not fully elucidated, these studies suggest early ADA expression from transduced whole marrow or from exogenously supplied ERT combined with transduced lineage-depleted marrow cells, may act to enhance the marrow and thymic micro-environments to allow for the engraftment and development of gene-corrected cells. These results, combined with the clinical experience in HSCT and GT in ADA-deficient patients, strongly suggest a positive role for ERT during the early engraftment period after GT, especially with transplant of HSC-enriched transduced cells and non-myeloablative conditioning.

**Future Clinical Protocol Development.** The data presented here indicate that cytoreduction conditioning probably plays a larger role in the success of gene therapy in current clinical trials than the cessation of ERT. Post-GT ERT did not diminish the absolute numbers of gene-marked cells and may even have improved engraftment and development of gene-corrected cells. These findings raise important questions about the clinical benefit of ERT cessation prior to gene therapy for ADA-SCID. There may be a role for short-term ERT in the early engraftment period after cytoreductive conditioning and infusion of gene-corrected cells, with the understanding that the goal of GT is to be curative and not to have patients remain on ERT.
At a minimum, there appears to be no detriment to the development and survival of gene-corrected cells from providing ERT for 1m after GT. ADA-deficient patients may have a quicker emergence of gene-corrected T-cells and may experience fewer infections if a short course of ERT is continued after GT.

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Author Contributions

Contribution: D.A.C., X.J., X-J.Y., M.L.K., Y.Z., M.B. performed experiments, X.W. prepared the immunohistological specimens, N.R. performed immunohistological/pathological analysis, X.W. and D.G. performed statistical analysis; D.A.C. and D.B.K. designed the research and wrote the paper.

Conflict Of Interest Disclosures

The authors declare no competing financial interests.
References


Figure Legends

Figure 1. Gamma-Retroviral Mediated Gene Transfer into Murine ADA-Deficient Marrow Cells. Bone marrow cells from Ada/- donor mice were transduced with the MMA gamma-retroviral vector and administered to age matched ADA-deficient recipients conditioned either with 200 or 900cGy total body irradiation (TBI). Following transplant, the recipients were either maintained on PEG-ADA ERT (+ERT) or not (-ERT).

A. Map of the MND-MFG-huAda (MMA) Gamma-retroviral vector construct carrying a normal human Ada cDNA (HuAda). EcoRV restriction sites in the long-terminal repeats are indicated. SD=splice donor site; SA= splice acceptor site and residual sequences from the Moloney Murine Leukemia Virus gag and env 5’ untranslated region (env utr) are indicated.

B. VCN by qPCR of bone marrow harvested from Ada/- donors after transduction with the MMA vector for five separate transplant experiments.

Figure 2. Survival, Vector Copy Numbers (VCN), Absolute Numbers of Lymphocytes (Total and Gene-Marked) after Conditioning with 200cGy and 900cGy TBI.

Tissues were harvested 4m after recipients received transduced cells. VCN was determined using qPCR. Thymic sub-populations and splenic sub-populations were determined by flow cytometry. The absolute numbers of lymphocytes were determined by multiplying the percentage of cells in a sub-population by the total number of lymphocytes in the lymphoid tissue. The absolute numbers of gene-marked cells were determined by multiplying the absolute numbers of thymocytes or splenocytes by the average VCN in thymus or spleen, respectively.

A. Survival of recipients. Mice were euthanized and analyzed at day 120. Total mice in five experiments.
B. VCN in tissue cell suspensions and isolated cell populations from the thymus (CD4/8), spleen (CD19), and marrow (CD11b) determined by qPCR for vector sequence. (900+ERT, n=14; 900-ERT, n=12; 200+ERT, n=12; 200-NoERT, n=8)(Means +/- SEM). *Significantly higher marking with 900cGy compared to 200cGy (p-values<0.05). **Significantly higher marking with ERT compared to without ERT (p-values<0.05).

C. Absolute numbers of thymocyte and splenocyte populations (Mean+/SEM). *Significantly higher number of cells with 200+/ERT compared to 900+/ERT (p-values<0.05).

D. Absolute numbers of gene-marked thymocyte and splenocyte populations (Mean+/SEM). *Significantly higher number of marked cells with 900+ERT compared to 200+ERT and 900-NoERT compared to 200-NoERT (p-values<0.05). ** Significantly higher number of gene-marked cells with 900+ERT compared to 200+ERT only (p-values<0.05).

Figure 3. Effects of ERT on Survival, VCN, Absolute Numbers of Lymphocytes and Gene-Marked Lymphocytes and their Function after GT.

A. Experimental Schema: Ada-/- were conditioned with 200cGy TBI, transplanted with transduced marrow, and either remained on ERT for the duration of the experiment (200+ERT-solid line), remained on ERT for 1 month after GT (200+ERT-solid line to dotted line) or did not remain on ERT after GT (200-NoERT-dotted line). Recipients were analyzed at 2, 4, 10 and 16 weeks (Per arm: 2w n=2, 4w n=3, 10w n=4, 16w n=4). See Figure 2 for lymphocyte subpopulation analysis and calculation of absolute values (Means +/- SEM).

B. Vector copy number (VCN). VCN in tissue cell suspensions were measured by qPCR for vector sequence (Mean+/SEM). *Significantly lower at 4w than at 2w (p-values<0.05).
Figure 4. Immunohistochemical Analysis of Thymus from ADA-Deficient mice after GT with Long and Short Term ERT. Perfused thymi were fixed, sectioned and stained with anti-human ADA antibody (N-19; Santa Cruz Biotechnology, Santa Cruz, CA) that is specific to human ADA and does not cross react with murine ADA. Cells containing immune-reactive protein are stained brown. Micrographs of stained sections were made using an Olympus BX40 Microscope (Olympus America Inc., Melville, NY) and an Olympus DP11 Camera (Olympus America Inc., Melville, NY). All micrographs were made at room temperature, without the use of imagine, medium, fluorochromes or acquisition software. Magnification: (optical x10) total magnification x200.

A. Thymi from two different Normal Control (Ada+/+) mice, 200+1mERT mice and 200-NoERT mice at 4 weeks.

B. Thymi from two different 200+ERT mice, 200-NoERT mice, and 200+1mERT mice at 10 weeks.

Figure 5. Effect of ERT on ADA Enzyme Activity and Adenine Substrate Concentrations.

A. ADA enzyme activity (nmoles/mg/min) was measured in tissues 4m after GT with 1m, 4m or 0m ERT (Mean+-SEM).

B. Deoxyadenosine concentration (nmoles/mg of protein) in tissues 4 months after GT with 1m, 4m or 0m ERT (Mean+-SEM).

C. Adenosine concentration (nmoles/mg protein) in tissues 4 months after GT with 1m, 4m or 0m ERT (Mean+-SEM). *Significantly lower adenosine levels in ERT-NoGT mice compared to mice in other conditions (p-values<0.05). **Significantly lower adenosine levels in ERT-NoGT compared 200+1mERT and 200+4mERT (p-values<0.05). ***Significantly lower in 200+4mERT compared to 200+1mERT.
**Figure 6. Survival and Vector Copy Number (VCN 4 Months after GT with Transduced Lineage-Depleted (lin-) Cells or Transduced Whole Marrow (wm) Cells.** The recipients were conditioned with either 0cGy or 200cGy and either remained on ERT long term (4m) or short term (1 m) or not at all.

A. Survival. **SOLID lines are -NoERT:** 0-NoERT lin- (n=9), 0-NoERTwm (n=7), 200-NoERT lin- (n=13), 200-NoERTwm (n=8), **DASHED lines are +1mERT:** 0+1mERTlin- (n=7), 0+1mERTwm (n=4), 200+1mERTlin- (n=7), 200+1mERTwm (n=4). **DOTTED lines are +4mERT:** 0+4mERTlin- (n=7), 200+4mERTlin- (n=11), 200+4mERTwm (n=10). Survival was decreased with no conditioning compared to 200cGy TBI (p-value<0.0001).

B. Percent survival at day 120. *Survival with no conditioning and no ERT (0-NoERT) after infusion of transduced lin- or transduced wm cells was significantly decreased compared to all others (p-values<0.01). **Survival in 200-NoERTlin- mice was significantly less than 200+1mERTlin- or 200+4mERTlin- mice (p-values<0.05).

C. VCN in cell suspension of tissues analyzed four months after GT: 200-NoERTlin- (n=5), 200+1mERTlin- (n=6), 200+4mERTlin- (n=11), 200-NoERTwm (n=8), 200+1mERTwm (n=4) or 200+4mERTwm (n=10)(Means+/ SEM). *Spleen VCN is significantly higher in 200-NoERTwm compared to 200-NoERTlin-, 200+1mERTlin-, 200+4mERTlin- and 200+4mERTwm (p-values<0.05). **Marrow VCN is significantly higher in 200+1mERTwm compared to 200+1mERTlin- (p-values<0.05).
Figure 7. Absolute Numbers of Lymphocytes and Gene-Marked Lymphocytes and their Function 4 Months after GT with Transduced Lineage-Depleted (lin-) Cells or Transduced Whole Marrow (wm) Cells.

A. Absolute numbers of thymocytes (Mean+/−SEM). *Significantly higher in 200+4mERTwm mice compared 200-NoERTwm, 200-NoERTlin- and 200+1mERTlin- (p-values<0.05). **Significantly higher in all mice receiving transduced whole marrow compared to transduced lineage-depleted marrow (p-values<0.05).

B. Absolute numbers of splenocytes (Mean+/−SEM). *Significantly higher in 200+4mERTwm mice compared to all other conditions (p-values<0.05). **Significantly higher in 200+4mERTwm mice compared to all other conditions except 200+4mERTlin- (p-values<0.05).

C. Absolute numbers of gene-marked thymocytes (Mean+/−SEM). *Significantly higher than 200-NoERTlin- (p-values<0.05).

D. Absolute numbers of gene-marked splenocytes (Mean+/−SEM). *Significantly higher than 200-NoERTlin- (p-values<0.05).
Figure 1. Gamma-Retroviral Mediated Gene Transfer into Murine ADA-Deficient Marrow Cells.

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A. Experimental Schema.

B. Vector copy number (VCN) in tissue cell suspensions.

A. 

GT@ 10 wks  

+4mERT  

+1mERT  

-NoERT

B. 

Thymus  

Lung  

Spleen  

Liver  

Marrow
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A. Thymi from Normal Control (Ada+/+) mice, 200+1mERT mice and 200-NoERT mice at 4w.

B. Thymi from two different 200+ERT mice, 200-NoERT mice, and 200+1mERT mice at 10w.
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A. ADA enzyme activity (nmoles/mg/min) in tissues after GT with 1m, 4m or 0m ERT.

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Figure 6. Survival and Vector Copy Number (VCN 4 Months after GT with Transduced Lineage-Depleted (lin-) Cells or Transduced Whole Marrow (wm) Cells

A. Survival.

B. Percent survival at day 120.

C. VCN in cell suspension of tissues analyzed four months after GT.
Figure 7. Absolute Numbers of Lymphocytes and Gene-Marked Lymphocytes and their Function 4 Months after GT with Transduced Lineage-Depleted (lin-) Cells or Transduced Whole Marrow (wm) Cells.

A. Absolute numbers of thymocytes.
B. Absolute numbers of splenocytes.
C. Absolute numbers of gene-marked thymocytes.
D. Absolute numbers of gene-marked splenocytes.
Gene therapy/bone marrow transplant in ADA-deficient mice: roles of enzyme replacement therapy and cytoreduction

Denise A. Carbonaro, Xiangyang Jin, Xingchao Wang, Xiao-Jin Yu, Nora Rozengurt, Michael L. Kaufman, Xiaoyan Wang, David Gjertson, Yang Zhou, Michael R. Blackburn and Donald B. Kohn