Concentrated RD114-Pseudotyped MFGS-gp91phox Vector Achieves High Levels of Functional Correction of the Chronic Granulomatous Disease Oxidase Defect in NOD/SCID/β2m−/− Repopulating Mobilized Human Peripheral Blood CD34+ Cells

Running head: RD114 MFGS-gp91phox correction of X-CGD

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ABSTRACT

In previous studies amphotropic MFGS-gp91phox (murine onco-retrovirus vector) was used in a clinical trial of X-linked chronic granulomatous disease (X-CGD) gene therapy to achieve transient correction of oxidase activity in 0.1% of neutrophils. We later showed that transduced CD34+ peripheral blood stem cells (CD34+PBSC) from this trial transplanted into NOD/SCID mice resulted in correction of only 2.5% of human neutrophils. However, higher rates of transduction into stem cells are required. In the current study we demonstrate that the same vector (MFGS-gp91phox) pseudotyped with RD114 envelope in a 4-day culture/transduction regimen results in a 7-fold increase in correction of NOD/SCID mouse repopulating X-CGD CD34+PBSC (14-22% corrected human neutrophils; human cell engraftment 13-67%). This may result from high expression of receptor for RD114 that we demonstrate on CD34+CD38- stem cells. Using RD114-MFGS encoding cyan fluorescent protein to allow similar studies of normal CD34+PBSC, we show that progressively higher levels of gene marking of human neutrophils (67-77%) can be achieved by prolongation of culture/transduction to 6 days, but with lower rates of human cell engraftment. Our data demonstrate the highest reported level of functional correction of any inherited metabolic disorder in human cells in vivo with the NOD/SCID mouse system using onco-retrovirus vector.
INTRODUCTION

Chronic granulomatous disease results from inherited defects of phagocyte oxidase, leading to recurrent infections. Allogeneic transplantation can cure CGD, but treatment related complications remain a problem. Gene therapy is a promising therapeutic alternative, but clinical trials of p47phox-deficient autosomal recessive CGD or gp91phox-deficient X-linked CGD (X-CGD) using amphotropic pseudotyped MFGS onco-retrovirus vector resulted in 0.1% or less of circulating neutrophils demonstrating functional correction with effect lasting less than a year. The NOD/SCID mouse human-xenograft model is used as a surrogate in vivo system for assessment of transduction and engraftment of primitive human hematopoietic stem cells, though there remains controversy whether it is predictive of outcome in humans. More recently with the amphotropic MFGS-gp91phox onco-retrovirus vector used in our clinical study of X-CGD gene therapy we showed that transduced X-CGD CD34+ peripheral blood stem cells (CD34+PBSC) from this trial transplanted into NOD/SCID mice resulted in correction of only 2.5% of human neutrophils in vivo, despite ex vivo transduction of up to 70% of CD34+PBSC.

Low numbers of corrected neutrophils in the clinical trial indicate a need to achieve higher levels of transduction of primitive stem cells. While the newly developed 3rd generation self-inactivating lentivector systems appear to improve stem cell targeting, issues of clinical scale-up of the transient production systems of lentivector production, as well as problems achieving adequate levels of transgene-expression from internal promoters may impede clinical application of lentivector. Not only have MFGS vectors already been used safely in clinical trials, but in the case of gp91phox subunit correction for X-CGD the MFGS-gp91phox achieves high levels of gp91phox transgene protein production that is equal to native protein present in normal neutrophils; a level that could not be achieved even with one of the strongest internal promotors (elongation factor 1 alpha) that has been tried with lentivector. Thus, there are compelling reasons to attempt to improve performance of MFGS vector in the correction of X-CGD, since versions of this vector will have fewer barriers to clinical application.

Recent studies report high level transduction efficiency in hematopoietic stem cells using feline endogenous virus (RD114) envelope, which binds to neutral amino acid transporter (RDR). The current study examines the potential of feline endogenous virus envelope RD114-pseudotyping to significantly improve gene transfer of MFGS-gp91phox into human X-CGD CD34+PBSC to achieve high levels of functional correction of oxidase defect in human X-CGD neutrophils in vivo arising from transduced X-CGD CD34+PBSC transplanted into NOD/SCID mice. In our study we focus on the use of CD34+PBSC as the target because this stem cell source has been used previously for CGD gene therapy trials. However, it is of note that in previously published gene marking studies, the level of engraftment and transduction efficiency of CD34+PBSC in the NOD/SCID model is lower compared to bone marrow (BM) or cord blood CD34+ cells. In our current study we also examine one potential basis for the performance of RD114-pseudotyped vectors by demonstrating for the first time high levels of RDR-expression on hematopoietic stem cells with primitive CD34+CD38 phenotype. Finally
we used RD114-MFGS vector encoding cyan fluorescent protein (CFP) to transduce normal CD34+PBSC to explore the effect on the outcome of transplantation into NOD/SCID mice of prolongation of ex vivo culture to allow time for additional transductions. We will show that prolongation of culture and transduction from 4 to 6 days can greatly increase the observed percent of marked human cells in vivo, though at the cost of reduction in human cell engraftment.

MATERIAL AND METHODS

Collection of normal and X-CGD CD34+PBSC

After obtaining informed consent (Protocols 94-I-0073 and 95-I-0134, approved by the Institutional Review Board of the National Institute of Allergies and Infectious Diseases) one healthy adult volunteer and two patients with X-CGD received five daily subcutaneous injections with 10µg/kg granulocyte colony stimulating factor (G-CSF) [Filgrastim®, Amgen, Thousand Oaks, CA]. PBSC were collected by apheresis on day 5 (CS3000 Plus, Baxter Healthcare, Fenwal Division, Deerfield, IL) and CD34+ selected from the apheresis product (ISOLEX® 300i, Nexell Therapeutics, Inc., Irvine, CA).

Generation of MFGS vector encoding gp91phox and CFP

To generate MFGS-gp91phox and MFGS-CFP transfer vectors, ORF of human gp91phox cDNA or CFP (Clontech, Palo Alto, CA), respectively were inserted into the NcoI-BamHI cloning site of MFGS.16 To obtain RD114-pseudotyped MFGS-gp91phox and MFGS-CFP vectors we transduced FLYRD18-packaging cells17 with amphotropic-MFGS-gp91phox and transfected with MFGS-CFP plasmid, respectively. To obtain GALV-pseudotyped MFGS-CFP vector we transduced PG13-packaging cells with RD114-packaged MFGS-CFP in medium containing tunicamycin.18 For each vector a high titer producer clone was selected.

Virus supernatant production and ultracentrifugation concentration

FLYRD18 MFGS vector producers were plated at 2x10⁶ per 185cm² flask for 48-60hrs and virus supernatant collected in serum-free X-VIVO 10™ (BioWhittaker, Walkersville, MD) with 1% human serum albumin (X-VIVO10/1%HSA). We performed transductions with RD114-pseudotyped vector particles separated from conditioned medium by ultracentrifugation (25,000 RPM [83,000g]; 90 minutes; 4°C).9,19,20 Preliminary experiments showed that biological activity was preserved after ultracentrifugation (data not shown). After ultracentrifugation, virus pellet was suspended in 1% of original volume of fresh X-VIVO10/1%HSA.19 Virus titer of original neat supernatant determined on CD34+PBSC by terminal dilution studies was 2x10⁵ to 4x10⁶ infectious units/ml for both MFGS-CFP and MFGS-gp91phox RD114-pseudotyped vectors, respectively. For GALV-pseudotyped MFGS-CFP vector, PG13 MFGS-CFP producer cells were plated at
2x10^6 per 185cm^2 flask for 60hrs and supernatant was collected after 12hr in X-VIVO10/1%HSA. Virus titer determined by terminal dilution studies on CD34^+PBSC was 6x10^6 infectious units/ml.

Transduction of CD34^+PBSC

Cultures were initiated in Retronectin™ (Takara Shuzo Ltd., Otsu, Japan) pre-coated 6-well plates with 1x10^6 CD34^+PBSC in 3ml growth medium (X-VIVO10/1%HSA; 50ng/ml FLT3-ligand, 50ng/ml stem cell factor, 20ng/ml IL6, 10ng/ml thrombopoietin, 10ng/ml IL3) per well. Beginning 16hrs after culture initiation, CD34^+PBSC were transduced 6 hrs/day times 4 days using dilutions of concentrated virus vector equivalent to 8x10^6 to 1x10^7 infectious units (2-5 fold concentrated from the original neat supernatant) and 5ug/ml protamine. Some CD34^+PBSC were transduced 6 hrs/day times 5 days (table 1, mice III-C and III-D) or 6 days (table 1, mice III-E and III-F). Naïve non-transduced X-CGD CD34^+PBSC served as negative control for gp91^phox^-expression and as one of the negative controls for CFP-expression, while CFP transduced normal CD34^+PBSC served as positive control for native gp91^phox^-expression. Cell number relative to initiation of culture increased 4.4 fold (range, 2.6 to 6.8 fold) over 4 days. For ex vivo comparison of the transduction efficiency of RD114 and GALV-pseudotyped MFGS-CFP vectors, CD34^+PBSC were transduced under optimum conditions for GALV-pseudotyped MFGS-CFP vector using 60% of neat supernatant and RD114-pseudotyped MFGS-CFP vector diluted from concentrated stock to a similar titer (3.6x10^6 infectious units/ml).

Transplantation of CD34^+PBSC into NOD/SCID/β2m^-/- or NOD/SCID mice

NOD/SCID/β2m^-/- and NOD/SCID mice^21,22 (Jackson Laboratory) were housed in micro isolator cages provided with autoclaved food and acidified water. 3-20x10^6 day 4 (and for some mice day 5 and 6, table 1) cultured human transduced CD34^+PBSC cells were injected via tail vein into sub-lethally irradiated (300cGy) 7 week old NOD/SCID/β2m^-/- or NOD/SCID mice. An aliquot of cells was retained in ex vivo culture.

Harvest of BM, peripheral blood (PB) splenocytes and human CD34^+ selection from mice

Mice were sacrificed 6-9 weeks post-transplant and BM from tibias and femurs flushed into X-VIVO10/1%HSA. PB and spleens were collected from some mice. Spleens were minced and filtered (100µm mesh). Red cells in BM, PB and spleen preparations were lysed with ACK lysing buffer (Quality Biological Inc., Gaithersburg, MD).

Engrafted human CD34^+ were positively selected from chimeric mouse BM using the Dynal® CD34^+ Progenitor Cell Selection System (Dynal A.S., Oslo, Norway) following the manufacturer's protocol. A portion of selected cells was cultured in fresh growth
medium (liquid culture) and a portion was plated for colony assays in semi-solid collagen medium (CollagenCult™, StemCell Technologies Inc.). Human growth factors for liquid culture were the same that were used for transductions, except for the addition of G-CSF 10ng/ml. For colony assays IL6 and TPO were omitted, but G-CSF 40ng/ml, GM-CSF 10ng/ml and erythropoietin 3U/ml were added.

**Analysis of human cell engraftment and transgene-expression by flow cytometry**

Anti-human fluorochrome-conjugated monoclonal antibodies were used to identify human hematopoietic cells (CD45-PerCP or CD45-CyChrome), human myeloid cells (CD13-FITC or CD13-PE) and human B-lymphocytes (CD19-PE). Human gp91phox expression was determined by indirect staining with murine monoclonal antibody 7D5 followed by Cy5 or FITC-conjugated goat anti-mouse IgG antibody. For analyses not requiring detection of CFP, a FACSsort (Argon laser, Becton Dickinson) was used, while CFP detection required a Vantage cell sorter (Becton Dickinson) equipped with a Krypton-UV laser (excitation at 413nm).

**NADPH oxidase activity in ex vivo differentiating cultures of human CD34+ cells**

Using the chemiluminescence assay, we measured phorbol 12-myristate 13-acetate (PMA) stimulated superoxide production on day 16 in human myeloid cells differentiating in ex vivo culture from non-transduced and transduced-corrected X-CGD CD34+ PBSC or normal CD34+PBSC.

**Analysis of human CD34+ cells purified from chimeric mouse BM**

We measured PMA stimulated superoxide and H2O2 production by granulocytes arising in culture from human CD34+ cells selected from chimeric mouse BM. Liquid cultures at 20 days were analyzed using the flow cytometry dihydrorhodamine 123 (DHR) assay. Superoxide generation by granulocytes and monocytes in myeloid colonies at 14 days of culture was indicated by formazan precipitate formed from nitroblue tetrazolium (NBT) dye reduction. For the NBT test 0.5ml of 0.1% NBT plus 0.5µg/ml PMA in phosphate buffered saline was layered over colony cultures. After 1hr, 2ml of 1.5% paraformaldehyde in buffered saline was added to halt the reaction and fix cells. After washes, collagen gels were transferred from the wells to slides for drying, counter-stained with safranin to delineate NBT-negative (oxidase negative) colonies. NBT colony assays of human CD34+ cells selected from chimeric BM of mice transplanted with non-transduced X-CGD CD34+ and normal MFGS-CFP CD34+ cells served as negative and positive controls for superoxide generation, respectively. CFP-expression in colony assays of human CD34+ cells selected from chimeric BM of mice transplanted with MFGS-CFP transduced CD34+ cells were assessed with living cultures prior to any staining, fixation or drying using a fluorescence microscope (CFP-excitation peak: 433nm; emission peak: 475nm).
Analysis of vector copy number by real time PCR (TaqMan™)

Vector copy number in genomic DNA of transduced human cells was determined by real time quantitative TaqMan™ PCR (PE Applied Biosystems, Foster City, CA). We used a common forward primer for MFGS vectors that was located just upstream of the respective transgene; a 6FAM-labeled probe that overlapped the start of the transgene sequence; and a reverse primer located within the coding region of the specific transgene close to the 5’ end.

MFGS-CFP:
- forward primer GTGAAGGCTGCCGACCC
- 6FAM-labeled probe TGGACCATCCTCTAGACTGCCATGGC
- reverse primer CTCGCCCTTGCTCACCAT

MFGS-gp91phox:
- forward primer GTGAAGGCTGCCGACCC
- 6FAM-labeled probe TGGACCATCCTCTAGACTGCCATG
- reverse primer CGAAACCAGAATGACAAAAATGG

For TaqMan™ PCR analysis the following incubation periods were applied for all primer sets: 2min at 50°C, 10min at 95°C, 40 cycles of 15sec at 95°C and 60sec at 60°C. Standard curves for the TaqMan™ PCR analysis were obtained by using CD34+ cells or K562 cells transduced with the indicated vector at known copy number.

Reverse transcriptase (RT) real time TaqMan™ PCR analysis of mRNA levels of receptors for RD114 and GALV in human hematopoietic stem cells

Expression of mRNA encoding the receptor for RD114-envelope was determined by reverse transcribing mRNA isolated from unfractionated human mononuclear cells from BM, cord blood and mobilized apheresis product and CD34+ subsets from the mononuclear cells (obtained by flow cytometric cell sorting) containing human CD34+CD38+ or CD34+CD38- cells and analyzing the cDNA by real time PCR under conditions described above. K562 cultured cell mRNA was used as a control with results from all sources normalized to mRNA encoding β2-microglobulin. Primers and probes are shown below.

β2-microglobulin:
- forward primer GGAGGGCATCCAGCGTACTCC
- 6FAM-labeled probe TCAGGTTTACTCAGTCATCCAGCAGAAT
- reverse primer CGGATTGATGAAAAACCCAGACAC
RD114 receptor:
forward primer CCTGGATCATGTGGTACGCC
6FAM-labeled probe ATGTTCCTGGTGGCTGGCAAGATCGT
reverse primer GCGGGCAAAGAGTAAACCC

GALV receptor:
forward primer GCATAGATAGCACCCTGAATGG
6FAM-labeled probe CAGTGCAGTTGCCTAATGGGAACCTTGT
reverse primer GCTGACGGCTTGACTGAAGT

RESULTS

RDR mRNA is highly expressed in human CD34^+CD38^- cells

Previous studies show that mRNA-expression for GALV and amphotropic receptors (Pit1 and Pit2, respectively) are lower in primitive than in more differentiated or lineage positive human cells.11,25,26 Using RT TaqMan™ PCR analyses we found 7 of 9 samples to have the highest levels of RDR mRNA in the primitive (lin^-CD34^+CD38^-) subset of hematopoietic progenitors (Figure 1A). In contrast, Pit1 mRNA levels in all human lin^-CD34^+CD38^- samples analyzed were lower than in lin^-CD34^+CD38^+ cells (Figure 1B).

Figure 1: RDR and GALV mRNA levels relative to β2-microglobulin mRNA in human apheresis product, cord blood and bone marrow. A) RDR mRNA levels were determined by RT TaqMan™ PCR in unfractionated (UF) mononuclear progenitor cells (mean 0.1±0.05), Lin^-CD34^+CD38^+ (mean 0.18±0.05) and Lin^-CD34^+CD38^- (mean 0.20±0.06) cells. B) GALV mRNA levels were determined by RT TaqMan™ PCR in UF mononuclear progenitor cells (mean 0.13±0.07), Lin^-CD34^+CD38^+ (mean 0.21+ 0.14) and Lin^-CD34^+CD38^- (mean 0.08+0.01) cells. In contrast to the RDR mRNA data, with each stem cell source analyzed, the level of GALV mRNA in Lin^-CD34^+CD38^- was lower than in the Lin^-CD34^+CD38^+ cells.
Ultradentification-concentrated RD114-pseudotyped MFGS-gp91phox achieves high transduction of CD34+PBSC ex vivo with functional correction of X-CGD

Ex vivo transduction efficiency of X-CGD or normal CD34+PBSC transduced with RD114-pseudotyped MFGS-gp91phox or MFGS-CFP vector, respectively, ranged from 82-99% on culture day 9 (Figure 2; Table 1, Column 3). By day 30, >70% of cultured cells expressed transgene (Figure 3).

We assessed PMA-stimulated superoxide production (chemiluminescence assay) by myeloid cells differentiating from ex vivo cultured naive (non-transduced) and transduced-corrected X-CGD CD34+PBSC or normal CD34+PBSC. Cultures of naive X-CGD CD34+PBSC had oxidase activity <0.5% of cultures of normal CD34+PBSC. MFGS-gp91phox transduced X-CGD CD34+PBSC cultures demonstrated supra-normal levels of superoxide production equivalent to 5 fold that of normal controls. It is important to note that in cultures of normal CD34+PBSC at day 16 only 14-27% of cells in culture expressed native gp91phox (not shown) while over 85% of MFGS-gp91phox transduced X-CGD CD34+PBSC expressed gp91phox transgene (see results for day 9 in Table 1, Column 3). Furthermore, the mean fluorescence intensity (MFI) of anti-gp91phox labeling in cultured transduced X-CGD CD34+PBSC was consistently 3 fold higher than the MFI of labeling of native gp91phox in the positive cells arising from normal CD34+PBSC in similar culture conditions.

**Figure 2:** Flow cytometric analyses of transgene expression in human CD34+PBSC on day 9 of ex vivo culture. Panels 2A and 2B, respectively, show representative histogram analyses of RD114-MFGS-gp91phox and MFGS-CFP transgene expression of transduced human X-CGD CD34+PBSC and normal human CD34+PBSC, where the gp91phox and CFP transgene expression is 96% and 97%. Non-transduced X-CGD CD34+PBSC served as negative control for their respective histograms as represented by the continuous black lines in Panel 2A and 2B, respectively.
CFP-transgene-expression persists after prolonged ex vivo culture

*Ex vivo* cultured MFGS-CFP transduced normal CD34⁺PBSC that expressed CFP in 95% of cells were sorted at day 15 into CFP-positive and CFP-negative populations. Two weeks later CFP-positive sorted cells continued to express CFP in greater than 99% of cells (Figure 3), while cells sorted negative for CFP demonstrated CFP-expression in less than 1% of cells (not shown). Vector copy number on day 23 in the CFP-positive versus CFP-negative sorted populations was 5.9 versus 0.1, respectively, indicating that transgene integration was highly associated with expression of transgene product.

**Figure 3: Flow cytometric analyses of CFP transgene expression after prolonged* ex vivo *culture.** Panels 3A, 3B and 3C show dot plot analyses of MFGS-CFP transduced normal CD34⁺PBSC on different days. (Day 9 of *ex vivo* culture for the same sample is shown in Figure 2B). Cells sorted for CFP-transgene expression on day 15 maintain CFP expression.

Relationship between length of *ex vivo* culture, transduction efficiency and engraftment in NOD/SCID mice

A culture initiated with 18x10⁶ CD34⁺PBSC was transduced daily from culture day 1 to culture day 6 with RD114-MFGS-CFP. One third of the culture, respectively, was transplanted into 2 NOD/SCID mice on day 4 (culture expanded to 15x10⁶ cells per mouse; Table 1, mice III-A and B), day 5 (30x10⁶ cultured cells per mouse; Table 1, mice III-C and D), and day 6 (55x10⁶ cultured cells per mouse; Table 1, mice III-E and F). The *ex vivo* transduction rates at days 4, 5 and 6 were 97%, 98% and 99% analyzed on day 9 of *ex vivo* culture (Table 1, Column #3). When chimeric BM was analyzed 6 weeks later, the 4, 5 and 6 day cultured cells demonstrated a highly significant drop in total human cell engraftment on day 5 and 6 (Figure 4, dashed line), while the percent transgene-expression in the engrafted human granulocytes progressively increased (Table 1, Column 6). Therefore for all subsequent experiments with NOD/SCID mice the 4 day culture and transduction regimen was used.
Human CD34⁺ PBSC engraftment in NOD/SCID/β2m⁻/⁻ and NOD/SCID mice

NOD/SCID/β2m⁻/⁻ mice were transplanted with either naïve cultured X-CGD CD34⁺ PBSC; RD114-MFGS-gp91phox transduced X-CGD CD34⁺ PBSC; or RD114-MFGS-CFP transduced normal CD34⁺ PBSC. The last group of cells also was transplanted into NOD/SCID mice for comparison with experiments using NOD/SCID/β2m⁻/⁻ mice. Human hematopoietic cell engraftment of the 4 day ex vivo cultured cells at 6-9 weeks post-transplant averaged 35% (range: 13-67%) in BM (see Table 1, Column 4 for BM), 15% (range: 7-33%) in spleens; and 9% (range: 4-16%) in peripheral blood.

The gp91phox transgene-expression and oxidase correction in X-CGD CD34⁺ PBSC isolated from chimeric NOD/SCID/β2m⁻/⁻ mice

Naïve cultured human X-CGD CD34⁺ PBSC transplanted into NOD/SCID/β2m⁻/⁻ mice gave rise to human CD45⁺ CD13⁺ high side scatter cells (‘human granulocyte gate’) that did not express human gp91phox (Non-filled overlay in Figures 5E and 5F). In mice transplanted with MFGS-gp91phox transduced X-CGD CD34⁺ PBSC, 14-22% of cells from chimeric BM in the human granulocyte gate expressed gp91phox-transgene (Table 1, Column 5; and Figure 5E). Mice transplanted with MFGS-CFP transduced normal
CD34⁺PBSC served as a control for native gp91phox-expression where 94-98% of chimeric BM cells in the human granulocyte gate expressed native gp91phox (representative analysis shown in Figure 5F). CFP transgene-expression in normal CD34⁺PBSC did not influence the native-expression of gp91phox relative to that seen in the same cells not transduced (data not shown). The gp91phox-positive populations in Figures 5E and 5F were distinct from the gp91phox-negative cells. However, in contrast to observations ex vivo, the mean fluorescence intensity was higher for native gp91phox-expression in normal granulocytes (Figure 5F) than for gp91phox-transgene-expression in corrected X-CGD cells (Figure 5E).

**Figure 5: Flow cytometric analyses of engraftment and transgene expression in human cells in chimeric NOD/SCID and NOD/SCID/β2m⁻/⁻ mice.** Panels 5A-D indicate how flow cytometry gates were set to analyse human granulocytes arising in transplanted mice. The gate shown in Panels 5A (control untransplanted mouse) and 5B (human CD34⁺PBSC transplanted mouse) confines human cells (human CD45⁺) with high side scatter characteristics, while the gate in Panels 5C and 5D confines human CD45⁺/CD13⁺ cells. The analyses shown in Panels 5E-G are delimited to cells that satisfy the characteristics confined to both gates, and define a ‘human granulocyte gate’. Panels 5B and 5D demonstrate representative analyses of bone marrow from a mouse transplanted with human RD114-MFGS-gp91phox transduced X-CGD CD34⁺PBSC, where total human cell engraftment (all human CD45⁺ irrespective of side scatter) was 36% (Panel 5B) and the percent of CD45⁺/CD13⁺ cells was 17% (Panel 5D). Panels 5A and 5C, respectively, show representative analyses of bone marrow from a non-transplanted mouse, indicating that the combined gating eliminates almost all murine cell background signal. Panels 5E and 5F, respectively, show representative analyses of gp91phox expression in the ‘human granulocyte gate’ in the bone marrow of mice transplanted with RD114-MFGS-gp91phox transduced X-CGD CD34⁺PBSC or RD114-MFGS-CFP transduced normal CD34⁺PBSC (the latter used to demonstrate expression of native gp91phox in normal human cells). Panel 5G shows a representative analysis of CFP-transgene expression in the ‘human granulocyte gate’ in the bone marrow of a NOD/SCID mouse transplanted with RD114-MFGS-CFP transduced normal CD34⁺PBSC. Naïve human X-CGD CD34⁺PBSC transplanted into NOD/SCID/β2m⁻/⁻ mice gave rise to human ‘human granulocytes’ that did not express gp91phox or CFP (Non-filled overlays in Panels 5E-G).
The flow cytometry gating for human granulocytes in chimeric mouse PB probably included cells other than human granulocytes, since native gp91$^{\text{phox}}$ expression was observed in only 89% of PB cells in the human granulocyte gate from a chimeric mouse transplanted with normal human CD34$^{+}$PBSC. Mice transplanted with MFGS-gp91$^{\text{phox}}$ transduced human X-CGD CD34$^{+}$PBSC expressed human gp91$^{\text{phox}}$ transgene in 10-25% of PB cells in the human granulocyte gate (Table 1, Column 8).

Transduction efficiency of MFGS-CFP transduced normal CD34$^{+}$PBSC engrafted in chimeric mice

In NOD/SCID/β2m$^{-/-}$ or NOD/SCID mice transplanted with 4 day MFGS-CFP transduced normal CD34$^{+}$PBSC, 20-42% of cells from chimeric BM in the human granulocyte gate expressed CFP-transgene (Table 1, Column 6; and Figure 5G). In the PB of 4 of these mice, CFP-expression was detected in 35-77% of cells in the human granulocyte gate (Table 1, Column 9); and in 19-50% of human CD45$^{+}$/CD19$^{+}$ cells (human B-lymphocyte gate; Table 1, Column 10), demonstrating high CFP-transgene expression in both the B-lymphoid and myeloid lineages in PB. In spleens from these same 4 mice, 9-13% of human B-lymphocytes expressed CFP transgene (Table 1, Column 7). Inspection of the results shown in Table 1, Column 6 suggests that when sufficient cells are transplanted to achieve greater than 10% human cell engraftment in NOD/SCID mice, there is no difference in either the human engraftment nor in the high transduction efficiencies seen in NOD/SCID/β2m$^{-/-}$ (6 mice: I-4a, I-4b and II-4a through II-4d) versus the NOD/SCID mice (6 mice, IIIA-IIIF, in Table 1, Column 2). In NOD/SCID mice transplanted with normal CD34$^{+}$PBSC transduced with MFGS-CFP for 5 and 6 days, respectively, 42-48% and 67-77% of cells from chimeric BM in the human granulocyte gate expressed CFP-transgene (Table 1, Column 6; and Figure 4).

Analysis of cultured human CD34$^{+}$ cells selected from chimeric mouse BM

Human CD34$^{+}$ cells that were isolated from the chimeric mouse BM were cultured in liquid medium or plated in semi-solid collagen as outlined in the Methods. At 14 days of ex vivo liquid culture after human CD34$^{+}$ isolation from chimeric BM of mice transplanted with MFGS-gp91$^{\text{phox}}$ transduced X-CGD CD34$^{+}$PBSC, 7-10% of differentiating cultured cells expressed gp91$^{\text{phox}}$ transgene (Table 1, Column 11 and Figure 6B). For the non-transduced X-CGD CD34$^{+}$ group of chimeric mice, no expression of gp91$^{\text{phox}}$ was detected (Figure 6A). For comparison, in a culture of human CD34$^{+}$ cells isolated from chimeric BM from one mouse transplanted with MFGS-CFP transduced normal CD34$^{+}$PBSC, 76% of the cells in culture expressed native gp91$^{\text{phox}}$ (Figure 6C). Similarly, CFP-expression by flow cytometry of this group of cultures of human CD34$^{+}$ isolated from chimeric BM of mice transplanted with MFGS-CFP transduced normal CD34$^{+}$PBSC ranged from 6-24% (Table 1, Column 12; and Figure 6E).
Figure 6: Transgene expression by human CD34+ cells that were cultured in vitro for 14 days after immuno-bead selection from the chimeric mouse bone marrow. Immuno-bead selected and cultured non-transduced X-CGD CD34+ cells served as negative control for gp91phox and CFP expression, respectively (Panel 6A and 6D). Panels 6B and 6C show representative analyses of gp91phox expression of transduced MFGS-gp91phox X-CGD CD34+PBSC and MFGS-CFP transduced normal CD34+PBSC, respectively, where gp91phox transgene expression was detected in 10% of cells and native gp91phox expression was detected in 76% of cells. Panel 6E shows a representative analysis of CFP-transgene expression by MFGS-CFP transduced normal CD34+PBSC in culture, where CFP expression was detected in 24% of cells.

We performed a DHR analysis of oxidase activity of granulocytes maturing at day 20 in liquid cultures of human CD34+ cells isolated from BMs of chimeric mice. With normal CD34+ cell cultures, 17.5% of cells were DHR positive, consistent with our previously published data that at 2-3 weeks, cultures of normal CD34+PBSC contain 10-20% oxidase positive cells. Only mature myeloid cells in the culture have all the oxidase subunit components required for demonstrating a positive DHR response. At day 20 in liquid cultures of RD114-MFGS-gp91phox transduced human X-CGD CD34+PBSC isolated from chimeric BM, an average of 1.8 % of cells were DHR positive. When compared to the 17.5% DHR positive cells in the normal control, it suggested that the level of oxidase correction was about 10% of the similarly cultured normal human CD34+ cells isolated from the chimeric mouse BM as noted above. No DHR positive cells were seen in cultures from human CD34+ cells selected from chimeric mice transplanted with non-transduced X-CGD CD34+PBSC. Aliquots of human CD34+ cells selected from BM of chimeric mice transplanted with MFGS-gp91phox transduced X-CGD CD34+PBSC, naïve X-CGD CD34+PBSC, or MFGS-CFP transduced normal CD34+PBSC were plated in semi-solid collagen cultures. Colonies were assessed for stimulated superoxide production at 14 days of culture using the NBT assay or examined for CFP-expression as indicated in the Methods. 11-16% of all colonies from the MFGS-gp91phox X-CGD group were NBT positive (Table 1, Column 13; and Figure 7A). No NBT positive colonies were seen in the naïve X-CGD group (negative control for NBT), while 75-97% of all colonies from the MFGS-CFP normal group (normal positive control for NBT) were NBT positive (not shown). With the MFGS-CFP transductions, CFP-expression was
detected in 3-22% of colonies from human normal CD34\(^+\) cells isolated from chimeric mice (Table 1, Column 14; and Figure 7B).

Figure 7: Photomicroscopic images demonstrating oxidase correction (NBT positive staining in gp91\(^{phox}\) transduced X-CGD cells) and CFP-transgene expression (fluorescence of CFP transduced normal cells), panels A and B respectively, in colonies derived from transduced CD34\(^+\) purified from chimeric mice. (A) NBT positive colony from RD114-MFGSgp91\(^{phox}\) transduced X-CGD CD34\(^+\)PBSC cells, 14 days after immuno-bead selection of human CD34\(^+\) cells from mouse bone marrow. Original magnification was X60. NBT negative colonies do not form formazan precipitate, but can be delineated by safranin counter staining (not shown). (B) CFP positive colony from RD114-MFGS-CFP transduced normal CD34\(^+\)PBSC cells, 14 days after immuno-bead selection of human CD34\(^+\) cells from mouse bone marrow. Original magnification was X100.

TaqMan\textsuperscript{TM} PCR analysis of genomic vector insert copy number in transduced CD34\(^+\)PBSC ex vivo and in human CD34\(^+\) cells isolated from chimeric mouse BM

In two experiments in which X-CGD CD34\(^+\)PBSC were transduced with RD114-MFGS-gp91\(^{phox}\) (transduction rates were 92% and 96%, see Table 1, Column 3), the vector insert copy number in the ex vivo transduced cells was 7.2 and 8.1 respectively, calculated per gp91\(^{phox}\) transgene positive cell by flow cytometry. Similarly in four experiments in which normal CD34\(^+\)PBSC were transduced for 4 days with RD114-MFGS-CFP (transduction rates were 82-97%, see Table 1, Column 3), the vector insert copy number in the ex vivo transduced cells was 7.1-8.4, respectively, calculated per CFP-transgene positive cell by flow cytometry.

We have noted above how we purified the human CD34\(^+\) cells that had engrafted in vivo in the chimeric mouse BM. When these cells were differentiated in liquid cultures for up to 3 weeks, the resultant populations consisted of only human CD45\(^+\) cells. In addition to assessing expression of transgene and oxidase activity, we also analyzed genomic insert copy number. The average copy number of MFGS-gp91\(^{phox}\) in human CD34\(^+\) cells isolated from the chimeric mice engrafted with transduced X-CGD CD34\(^+\)PBSC was 2.53±0.45 copies per transgene-expressing cell. Similarly, the average copy number of MFGS-CFP in human CD34\(^+\) cells isolated from the chimeric mice engrafted with transduced normal CD34\(^+\)PBSC was 1.65±0.24 copies per transgene-expressing cell. Thus, we note that the average insert copy number per transgene expressing positive cell in ex vivo transduced human CD34\(^+\)PBSC before transplant into the mice was 3 to 4 fold
higher than the copy number in the transduced human CD34+ cells engrafted in vivo in the mice.

With two of the cultures of human CD34+ cells isolated from chimeric BM of mice transplanted with MFGS-CFP transduced normal CD34+PBSC (I-4a and I-4b in Table 1) the cells were sorted into CFP-positive and CFP-negative populations at day 17. Subsequent post-sort analyses revealed a purity of 93% and 95% CFP-positive cells in the positive groups and no CFP-positive cells in the negative groups. Vector copy number in genomic DNA isolated from the sorted cell populations was determined by real time TaqMan™ PCR. Analyses of the sorted, cultured human cells derived from chimeric marrow of the two mice showed that CFP-positive sorted cells had 2.0 and 2.1 vector copies per cell, while the CFP-negative sorted cells had 0.025 and 0.031 copies per cell. These data are consistent with the vector copy numbers determined without sorting as described in the previous paragraph. The results demonstrate that the great majority of CFP-negative cells have no integrated copies of the vector, suggesting minimal vector silencing of integrated MFGS-CFP vector in the human cells that had engrafted in the mice.

DISCUSSION

In this study, we show that ultracentrifugation concentrated RD114-pseudotyped MFGS-gp91phox and MFGS-CFP routinely achieves unprecedented levels of ex vivo transduction of human CD34+PBSC greater than 95% resulting in 14-77% transgene-expression (gp91phox or CFP) in vivo in human cells engrafted in NOD/SCID mice.

For this study we concentrated the RD114 vector by ultracentrifugation without loss of activity. While highly concentrated vector was not toxic to the cells, use of vector concentrations greater than 10 fold over neat culture supernatant did not result in higher levels of transduction of NOD/SCID repopulating cells. Another reason for vector concentration was that culture of human CD34+ cells in conditioned medium from the FLYRD18 line appears to induce loss of NOD/SCID repopulating cells.20 This effect appears to be eliminated when ultracentrifugation concentrated RD114-pseudotyped vector that is re-suspended in fresh medium is used for transduction.9

The monoclonal antibody we use to detect expression of human gp91phox does not bind to mouse gp91phox. This allowed straightforward assessment of expression of gp91phox transgene in the human neutrophils arising in NOD/SCID mice. However, in our study we have for the first time also measured the level of functional correction of oxidase activity of the corrected, engrafted human cells. However, this assessment required the isolation of the human cells from the chimeric mouse marrow, because murine neutrophils in NOD/SCID animals have normal oxidase which would interfere with the functional assay. Since we also wanted to assess the number of transduced progenitors present six weeks after transplantation, we isolated human CD34+ cells from the chimeric BM. Isolated human CD34+ cells from mice transplanted with RD114-MFGS-gp91phox transduced X-CGD CD34+PBSC gave rise to oxidase normal neutrophils in
NBT colony assays (12% NBT positive for transduced X-CGD cells versus 86% NBT positive for control normal cells). The DHR assay was performed on the same cells grown in liquid culture, demonstrating a similar level of correction relative to cultures of normal CD34+ cells isolated from transplanted mice. Furthermore, up to 25% of human granulocytes circulating in the PB of mice transplanted with MFGS-gp91phox transduced CD34+PBSC expressed gp91phox transgene. Similar observations are made with the experiments where normal CD34+PBSC were transduced with RD114-pseudotyped MFGS-CFP and transplanted into both NOD/SCID/beta2m−/− and NOD/SCID mice, where 20-42% and 35-77% of human granulocytes in the chimeric BM and PB, respectively, were CFP positive. When CD34+ cells are isolated from the chimeric BM of these mice, we observe that 13.3±2.3% of colonies arising from these cells express CFP.

In normal human PB, flow cytometry detection of native gp91phox is limited to granulocytes and monocytes, where the mean fluorescence intensity for granulocytes is 3-4 fold higher than that for monocytes. The cytochrome b558 component of the phagocyte NADPH oxidase is a heterodimer of p22phox and gp91phox, where protein synthesis and stability of both subunits are tightly linked. Thus transgene gp91phox protein-expression may be limited to myeloid cells as well. In fact, it was only in the granulocytes that we were able to detect expression of both human native gp91phox and human transgene gp91phox in cells from chimeric mice. We were not able to detect high-level expression of gp91phox in the human B-lymphocyte gate by flow cytometry. With CFP-expression there is no lineage restriction, making it possible to assess and compare expression of this transgene not only in human granulocytes, but also in the human B-lymphocytes arising in chimeric mice. We found that 19-50% of human CD45+CD19+ cells from the PB and 9-13% of human CD45+CD19+ cells from spleens of these chimeric mice were CFP positive.

Most studies of human CD34+ stem cell engraftment in NOD/SCID mice are performed using the ontologically younger cord blood CD34+ cells that have a relative advantage for engraftment in this in vivo model. In studies where cord blood was compared to BM or mobilized peripheral blood as the source of human transduced CD34+ cells transplanted into NOD/SCID mice, cord blood cells demonstrated significantly higher levels of transgene-expression in human cells in vivo. Thus, the high level of transgene-expression in NOD/SCID repopulating cells reported in this study is notable for the fact that we used CD34+PBSC, a cell source that would have much broader clinical relevance for autologous stem cell gene therapy than the use of cord blood CD34+.

Prior to our current studies it had been reported that infusion of up to 20x10^6 freshly isolated CD34+PBSC appeared to be required to achieve human blood cell engraftment levels of greater than 20% in sub-lethally irradiated NOD/SCID mice. For the NOD/SCID/β2m−/− model, higher human cell engraftment potential was reported due to elimination of NK cell activity. In preliminary studies, we found that engraftment of 4 day cultured human CD34+PBSC in NOD/SCID/β2m−/− mice was superior to NOD/SCID mice, when the cell inoculum was less than 1x10^6 (data not shown). However, when more than 3-5x10^6 of 4 day cultured cells per mouse were injected, the engraftment
observed with both immunodeficient strains of mice became similar. Because we are only able to obtain limited numbers of CD34⁺PBSC from our patients with X-CGD for pre-clinical studies, we chose the NOD/SCID/β2m⁻/⁻ mouse model for studies with X-CGD CD34⁺PBSC, but found as we continued our studies that we did not see substantial difference between the two models as observed in the data shown in Table 1.

As previously noted, amphotropic-pseudotyped onco-retrovirus vectors yield low transduction of NOD/SCID repopulating CD34⁺PBSC cells. In a marking study comparing amphotropic and RD114-pseudotyped onco-retrovirus vectors in NOD/SCID repopulating human cord blood CD34⁺ cells, the RD114-pseudotyped vector greatly outperformed the amphotropic vector. Compared to amphotropic-pseudotyped vectors, GALV-pseudotyped vectors have achieved higher transduction in some human, non-human primate and dog transplant models, though in one report of a dog model of gene transfer, GALV and RD114-pseudotyped vectors appeared to be equivalent. In preliminary studies, transduction of CD34⁺PBSC under optimal conditions with our GALV-pseudotyped MFGS-CFP vector resulted in 38-60% ex vivo marking. This was in contrast to our ultracentrifugation concentrated RD114-pseudotyped MFGS-CFP vector that, even when diluted to a titer similar to the GALV-pseudotyped vector (as determined on a K562 human erythro-leukemia cell line) achieved transduction rates of 80-97% in human CD34⁺ cells under the same conditions. In a direct comparison of GALV and RD114-pseudotyped vectors by van der Loo et al., the RD114-pseudotyped vector achieved a 20.9 fold higher level of gene transfer into NOD/SCID repopulating CD34⁺PBSC than the GALV-pseudotyped vector. The higher ex vivo transduction rates with RD114 versus amphotropic and GALV-pseudotyped vectors seen by us and in the above mentioned studies and the relative lower marking of GALV and amphotropic vector transduced human cells engrafted in the NOD/SCID mice, suggest a correlation between the level of ex vivo transduction of the bulk population of CD34⁺PBSC and that of the more primitive NOD/SCID repopulating cells. However, the one to two order of magnitude increase in targeting of the NOD/SCID repopulating cells with the RD114 vector is far out of proportion to the percent increase in overall bulk transduction, and likely relates to better targeting of the more primitive cell population. This high transduction level of RD114-pseudotyped vectors in NOD/SCID repopulating cells might relate in part to high expression levels of mRNA for the neutral amino acid transporter (RD114 receptor) on primitive lin⁻CD34⁺CD38⁻ cells, as demonstrated in Figure 1. However, other physiologic or biochemical factors such as receptor-envelope affinity might greatly influence transduction efficiency and overshadow the influence of receptor-expression.

Although the 14-77% transgene-expression of human cells engrafted in chimeric BM of NOD/SCID/β2m⁻/⁻ or NOD/SCID mice in our studies targeting human CD34⁺PBSC with RD114-pseudotyped vectors is encouraging, this represents a marked decrease from the initial ex vivo bulk transduction of 82-97% transgene-expression (7.1-8.4 transgene copies per transgene expressing cell ex vivo). To evaluate if gene silencing was a reason for this decrease, we analyzed the vector copy number in MFGS-CFP transduced human CD34⁺ cells selected from chimeric mice and sorted into CFP positive and negative populations. We found that vector copy number in the CFP negative population was
0.03, while that in the CFP-positive population was 2.05 copies per cell. This suggests that silencing is not the major cause of the difference between *ex vivo* and *in vivo* transgene-expression rates, but rather the higher engraftment potential of the non-transduced cells. The lower insert copy number in the engrafted CFP expressing human CD34⁺ cells versus the *ex vivo* insert copy number before transplantation most likely represents the lower efficiency of transduction of primitive NOD/SCID repopulating cells that divide late and thus less frequently during the 4 day *ex vivo* transduction period than more mature cells that, while going through several cell divisions acquire several vector inserts, but do not engraft this mouse model.

When we examined the effect of additional days of transduction, we found that extending the transductions from day 4 to day 6 almost doubled the observed percent of human cells expressing transgene in NOD/SCID mice *in vivo* as seen in Figure 4. It is important to note that even on day 4 the observed *ex vivo* transduction for this experiment was 97%, while on day 6 it had increased to 99%. Thus, the extraordinarily high level of *ex vivo* transduction on both days was barely distinguishable, yet the outcome *in vivo* in the mice was quite different indicating that substantial changes were occurring with the very small number of stem cells that mediate engraftment in the NOD/SCID mouse. Again as noted above, it is likely that substantial numbers of stem cells do not go into the cell division required for onco-retrovirus integration until after day 4 of culture. However, while the extension of the time for culture and transduction enhances integration of vector, it also reduces the engraftment potential of the stem cell compartment. Thus, we conclude that for our transduction conditions a four day culture with 3 daily transductions is the right compromise to achieve both goals. Alternative culture conditions might be found that allow preservation of engraftment potential during the time required for additional transductions. These observations emphasize the importance of considering preservation of total engraftment potential when evaluating methods that maximize transduction efficiency of primitive human hematopoietic stem cells.
References:


14. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood
as measured by the quantitative in vivo SCID-repopulating cell assay. Blood. 1997;89:3919-3924.


Table 1: Human cell engraftment in NOD/SCID/beta2m⁻/⁻ and NOD/SCID mice, and gene transfer efficiency ex vivo, in vivo and in human CD34+ cells selected from mice

<table>
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<tr>
<th>Group</th>
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<th>% transgene+ cells ex vivo D9</th>
<th>Human cell engraftment in BM (%)</th>
<th>% transgene positive engrafted human cells (%)</th>
<th>% transgene+ in chimeric BM NOD34+ cultures</th>
<th>CFU assay of human CD34+ selected cells at day 14</th>
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</tr>
<tr>
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<td>97</td>
<td>52</td>
<td>60</td>
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<td>33</td>
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<td>48</td>
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Data in Column #1 3-12 were derived from flow cytometry analysis. Analysis was performed without gating or limited to gates as indicated in the Column #5: human granulocyte gate (CD45+/CD14-), high SSC) for Column #5 5, 6, 7, 9 or human B-cell gate (CD45+/CD19+) for Column #5 7, 10; Data in Column #11-14 were derived from cultures of human CB34+ cells isolated from chimeric mouse BM (liquid culture for Column #1 11, 12; semi-solid Colony Forming Unit, CFU assays for Column #1 5-14). Data in Column #1 1-14 were derived by microscopic analysis. Mice III-C, III-D and mice III-E, III-F received CD34+PBSC cultured and transduced for 5 and 6 days, respectively. All other mice were transplanted with 4 day cultured cells. The 4 mice marked with an asterisk (*) in column #2 were NOD/SCID mice, all others were NOD/SCID/beta2m⁻/-. Mice were sacrificed after 6 weeks, except group IV that were sacrificed after 8 weeks. Background values for human CD45 staining in non-transplanted mice (not shown) were <2% and subtracted from the given human engraftment levels. Similarly, background levels from cells that served as negative control were subtracted from values indicated for transduced cells.
Concentrated RD114-pseudotyped MFGS-gp91phox vector achieves high levels of functional correction of the chronic granulomatous disease oxidase defect in NOD/SCID/β2m−/− repopulating mobilized human peripheral blood CD34+ cells

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