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Protection of Mice From Methotrexate Toxicity by *Ex Vivo* Transduction using lentivirus vectors expressing Drug-Resistant Dihydrofolate Reductase

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***Ex Vivo* Lentivirus-mediated Chemoprotection**

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NONSTANDARD ABBREVIATIONS:

MTX – Methotrexate

DHFR – dihydrofolate reductase

Tyr22DHFR – tyrosine 22 DHFR

SIN – self-inactivating

PBMC – peripheral blood mononuclear cells

GFP – green fluorescent protein

ABSTRACT

Methotrexate (MTX) dose-escalation studies were conducted in C57BL/6 mice to determine the chemoprotective effect of transplantation using bone marrow transduced with lentivirus vectors expressing a drug-resistant variant of murine dihydrofolate reductase (DHFR). Methotrexate resistant (MTXr) dihydrofolate reductase (tyr22DHFR) and enhanced green fluorescent protein (eGFP) coding sequences were inserted into self-inactivating (SIN) lentiviral vectors as part of a genetic fusion or within the context of a bicistronic expression cassette. MTX-treated animals that received tyr22DHFR-transduced marrow recovered to normal hematocrit levels by three weeks post-transplant and exhibited significant GFP marking in myeloid and lymphoid lineage-derived peripheral blood mononuclear cells (PBMCs). In contrast, MTX-treated animals transplanted with control GFP-transduced marrow exhibited extremely reduced hematocrits, severe marrow hypoplasia and did not survive MTX dose-escalation. To minimize cell manipulation, we treated unfractionated marrow in an overnight exposure and demonstrated that transduction at a multiplicity of infection of 10 resulted in up to 11% vector-modified PBMC in primary recipients and successful repopulation of secondary recipients with vector-marked cells. Experimental cohorts exhibited sustained proviral expression with stable GFP fluorescence intensity. These results demonstrate the effectiveness of lentivirus vectors for chemoprotection in a well-developed animal model, with the potential for further preclinical development toward human application.

INTRODUCTION

Methotrexate (MTX; 4-Amino-10-methylfolic acid) is an anti-proliferative chemotherapeutic that interrupts folate metabolism by inhibition of dihydrofolate reductase (DHFR; EC 1.5.1.3). DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, a required precursor for cofactors involved in macromolecule biosynthesis, one-carbon transfer reactions and other cellular metabolic pathways (Blakely and Benkovic, 1984). MTX has been successfully used to treat a number of malignancies such as acute lymphoblastic leukemia, non-Hodgkin's lymphoma and osteosarcoma (Bertino, 1993). However, the therapeutic dose that can be administered is limited by toxicity to rapidly dividing cells of the bone marrow and gastrointestinal tissues (Sirotnak and Moccio, 1980; Rivera *et al*., 1985).

One potential way to protect against MTX toxicity is by the expression of drug-resistant DHFR in normal drug-sensitive cells and tissues. MTXr-DHFR variants have been shown to mediate protection from antifolate toxicity when expressed in DHFR transgenic hematopoietic cells after transplantation (May *et al*., 1995 & 1996; Morris *et al*., 1996; James *et al*., 1997 & 2000). Improved antifolate chemotherapy has also been shown in mice transplanted with DHFR transgenic marrow or with γ-retrovirus transduced marrow cells expressing drug-resistant DHFR (Sweeney *et al*., 2003; Mayer-Kuckuk *et al*., 2002). Selective expansion of tyrosine-22 (tyr22) DHFR-transduced hematopoietic cells reported in murine and non-human primate models (Allay *et al*., 1997; Persons *et al*., 2004) also supports the potential utility of DHFR gene transfer for protection of normal cells during administration of chemotherapy using antifolates.

Gammaretrovirus vectors transducing MTXr-DHFR cDNAs, sometimes in combination with other drug resistance genes, have been evaluated extensively for gene transfer and drug resistance in mammalian cells and in mouse models (Williams *et al*., 1987; Corey *et al*., 1990; Li

et al., 1994; Budak-Alpdogan *et al*., 2004; Capiaux *et al.*, 2004). However, clinical application of MTXr-DHFR expression will require the most effective means available to achieve gene transfer into hematopoietic cell targets. Lentivirus vectors have been shown to be highly effective in mediating gene transfer into HSCs, particularly within the context of *ex vivo* gene transfer into HSCs of dogs and non-human primates (Horn *et al*., 2004; Hanawa *et al*., 2004). Here, we report the generation of lentivirus vectors expressing murine MTXr-DHFR (tyr22) and demonstrate effective protection of mice from lethal doses of MTX by transplantation with congenic marrow after a single overnight vector transduction. These results suggest the development of a clinically applicable *ex vivo* DHFR gene transfer procedure for reduced toxicity associated with antifolate chemotherapy.

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METHODS

Lentivirus vector construction

Recombinant plasmids were generated using standard molecular cloning techniques. Lentivirus vector plasmids pCSIIEG and pCSII have been previously described (Agarwal *et al.*, 2006). To construct pEFDIG, a tyr22DHFR coding sequence was obtained as a *Xho*I – *Cla*I (blunt) fragment from pLasBD (Zhao *et al.*, 1997) and cloned between *Xho*I and *Bam*HI (blunt) of pCSII-CMV-12G to form pCCDG. A DHFR-IRES-GFP fragment was then generated by polymerase chain reaction (PCR) from pCCDG template plasmid using sense (GCGAATTCTCGAGGGTCCTCTAGAG CAAG) and anti-sense (CGCTGCAGCCTCGATGTTAACTCTAGAGTCG) primers to introduce *Eco*RI and *Pst*I restriction sites at the 5' and 3' fragment ends, respectively, and cloned into pCSII. For construction of DL2G, a DHFR-GFP fusion sequence was first generated by two-step PCR overlap extension. First an anti-sense oligonucleotide (*ACCTCCTCC*tca*TCCACCACCCCT*GTCTTTCTTCTCGTAGACTTCAAACTT) was designed to delete the TGA stop codon (underlined) of the tyr22DHFR cDNA while providing additional sequences encoding eight amino acids of a $[Gly_4Ser]_2$ linker (glycine in italics, serine in lowercase), included to impart three-dimensional flexibility between the tyr22DHFR and eGFP domains of the fusion protein (Lewis *et al.*, 2003). The tyr22DHFR cDNA was amplified using this anti-sense primer along with a sense oligonucleotide (GCGAATTCTCGAGGGTCCTCTAGAGCAAG) to introduce an *Eco*RI site (underlined) at the 5' end. A sense oligonucleotide (*GGTGGA*tca*GGAGGAGGTGGT*tctGCGGTGAGCAAGGGCGA GCTGTTCACCGGG) was designed to provide sequences overlapping the DHFR antisense primer encoding two glycine

(italics) and two serine (lowercase) residues of the first glycine-serine repeat, add a second Gly_4Ser repeat, and convert the eGFP start codon to an alanine codon (underlined). This sense oligonucleotide was used along with an antisense oligonucleotide (CGCTGCAGCCTCGATGTTAACTCTAGAGTCG) to amplify the eGFP coding sequence and to introduce a *Pst*I site (underlined) at the 3' end. The amplified tyr22DHFR-Gly_4Ser-Gly_3 and Gly_2Ser-Gly_4Ser-eGFP products were fused by overlap extension (94˚C 5 min, slow cool 30 min in air, add dNTPs and Taq / Tgo DNA polymerase mixture (Expand High Fidelity Enzyme mix; Roche Applied Science, Penzberg Germany), then heat to 72˚C 5 min) and the final product amplified by PCR using the far 5' sense (DHFR) and far 3' antisense (eGFP) oligonucleotides described above. The final PCR product was ligated into pCSII between *Eco*RI and *Pst*I. Correct vector construction was verified by restriction endonuclease mapping and DNA sequencing. All restricytion enzymes were from New England Biolabs (Beverly, MA). pCCDG was used as template for both DHFR and GFP amplifications, with PCR reactions carried out under the following conditions; 94˚C 10 min, 1 cycle; 94˚C 30 s, 55˚C 30 s, 72˚C 3 min, 30 cycles; 72˚C 10 min, 1 cycle. All PCR reactions and overlap extensions were carried out using an Applied Biosystems (Foster City, CA) Thermal Cycler (Model 2720).

Preparation of high-titer vector stocks

Lentiviral vectors were packaged using a 3-plasmid transient transfection procedure as described (Zuffrey *et al*., 1997). Lentivirus vector plasmids (pCSIIEG, pEFDIG, or pDL2G) were co-transfected with pΔNRF to provide the gag, pol and rev proteins and pMD.G for pseudotyping with VSV-G envelope protein. Briefly, 6×10^6 HEK 293T cells were seeded onto poly-L-lysine-coated 15 cm^2 plates 24 hours before transfection in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (P/S) (all from

Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) in humidified atmosphere containing 3% CO_2 in air at 37˚C. For the production of vector particles, 8 μg pM.DG, 30 μg pΔNRF packaging plasmid and 25 μg lentiviral vector plasmid were co-transfected into 293T cells using the DNA-calcium phosphate co-precipitation technique (Tiscornia *et al*., 2006). After overnight transfection, cells were washed with PBS, provided fresh DMEM plus 10% FBS, 1% P/S and 10 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO), and incubated in a humidified atmosphere containing 10% CO_2 in air at 37˚C. Vector supernatants were collected 8 hours later and replaced with DMEM plus 10% FBS and 1% P/S for two additional 12 hour incubations. Supernatants from all three harvests were pooled, concentrated 100-fold by centrifugation at 23,000 x g and resuspended in unsupplemented Iscove's modified Dulbecco medium (IMDM; Invitrogen). Concentrated supernatants were diluted and titrated on NIH 3T3 TK^- (lacking thymidine kinase) murine fibroblasts. Cells and virus were incubated for 48 hours in the presence of 8 μg/mL polybrene (hexadimethrine bromide; Sigma-Aldrich) followed by fluorescence-activated cytometric analysis to determine the percentage of GFP^+ cells using a FACSCalibur instrument (Becton Dickinson Immunocytochemistry Systems [BDIS], San Jose, CA). Titer was also assessed by drug-resistant colony formation after subculturing transduced 3T3 TK^- cells into selective medium containing 0.15 μM MTX (Vinh and McIvor, 1993). After 2 weeks, colonies were stained with crystal violet and the MTXr-DHFR titer calculated as the number of colony forming units/mL. Vector concentrations were increased approximately 100-fold with 70% recovery of transducing units, and final titers ranged between 10^8 and 10^9 transducing units per ml as assessed both by drug resistance (MTXr-DHFR) and flow cytometry (GFP).

Fluorescence microscopy

3T3 TK$^-$ fibroblasts were transduced with EFDIG or DL2G lentiviral vector and subcultured in 100 μM MTX. After two weeks, MTX-resistant clones were isolated and expanded in culture. For fluorescence microscopy, MTX-resistant clones and uninfected cells were subcultured onto coverslips coated with 0.002% poly-L-lysine (Sigma-Aldrich). Confluent monolayers were fixed with 4% paraformaldehyde (Sigma-Aldrich), washed with PBS and mounted onto glass slides with VectaMount (Vector Laboratories, Burlingame, CA). Fluorescence was visualized on an Olympus BX60 upright microscope.

Western blot analysis

MTXr-clones (described in the preceding section) and uninfected cells were harvested by trypsinization, washed with phosphate buffered saline (PBS; Biowhittaker, Walkersville, MD) and lysed with 2% sodium dodecyl sulfate (SDS; Sigma-Aldrich). Cleared lysates were prepared by centrifugation at 15,000 x g for 20 minutes. Protein extracts (25 μg: quantified by Bradford assay; Pierce, Rockford, IL) were boiled for 5 minutes in the presence of sample loading buffer and electrophoresed through 12% polyacrylamide-SDS. Proteins were transferred onto nitrocellulose membranes using the iBLOT™ Dry Blotting System (Invitrogen) and incubated sequentially with either a polyclonal rabbit antibody raised against human DHFR (a kind gift from Dr, B. Dolnick, Roswell Park Memorial Institute, Buffalo, NY)(Morris and McIvor, 1994) or a monoclonal rabbit antibody against eGFP (Clontech, Mountain View, CA) followed by a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (Invitrogen).

DHFR enzyme assay

3T3 cells were transduced with CSIIEG, EFDIG or DL2G lentivirus vector at an m.o.i. of 1, harvested by trypsinization, washed with PBS, and resuspended in lysis buffer (50 mM Tris buffer [pH 7.5], 150 mM KCl, 25mM $MgCl_2$ (all from Sigma-Aldrich),10 mM β-

mercaptoethanol [BME; BioRad Laboratories, Hercules, CA]). The cells were then sonicated with a Branson 250/450 Sonifier at a setting of 5 for 20 seconds using a microtip, cleared by centrifugation (16,000 x g for 30 min at 4˚C) and extracts diluted into reaction buffer (100 mM Tris buffer [pH7.5], 150 mM KCl, 10 mM BME). When including MTX in the reaction, samples were incubated with drug at room temperature for 10 minutes prior to enzyme assay. Reactions were started by adding NADPH(β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt) and dihydrofolic acid (both from Sigma-Aldrich) to final concentrations of 120 µM and 30 µM, respectively, following the change in absorbance at 340 nm on a Beckman DU40 spectrophotometer. One unit was defined as the amount of enzyme required to reduce 1 nmole of dihydrofolic acid per minute (ε = 12,300 M^{-1} cm^{-1}) (Carr *et al*., 1983).

Mouse bone marrow transplantation, ex vivo transduction and methotrexate administration.

All procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. Six-week old C57Bl/6 CD45.2 and CD45.1 female mice were obtained from the National Cancer Institute (Frederick, MD) and provided food and water *ad libitum*. Donor CD45.2 mice were administered 150 mg/kg 5-fluorouracil (Sigma-Aldrich) two days before marrow harvest. Bone marrow was flushed from the hind limbs of donor mice and processed to a single cell suspension in DMEM plus 10 U/ml heparin and 10% FBS. Cell suspensions were quantitated and assayed for viability by trypan blue exclusion. Following marrow harvest and processing, cells were resuspended in complete Stempro medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin,100 µg/ml streptomycin (Invitrogen), cytokines (20 ng/ml hIL-3, 50 ng/ml hIL-6, 100 ng/ml mSCF; all from R & D Systems, Minneapolis, MN) and 8 µg/ml polybrene (Sigma-Aldrich). All transductions were

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carried out as 12-hour vector exposures following the addition of 100-fold concentrated vector to 2.25 x 10^8 cells plated in three 15-cm^2 tissue culture plates. Concentrated vector was added to a final volume of 15 mL per plate at an m.o.i. of 10 (MTXr CFU/marrow cell). Transduced cells were washed, enumerated, resuspended at a final concentration of 1 to 2 x 10^7/mL DMEM and equal numbers (2 or 3 x 10^6) injected through the lateral tail vein into 12 to 16 week-old sublethally irradiated (700 cGy) congenic recipients (CD45.1). Mice that received MTX (+ Amethopterin, Bedford Laboratories, Bedford, OH) were injected intraperitoneally with 0.25 mg/kg MTX on days 1 to 4, 0.5 mg/kg MTX on days 5 to 8, 1 mg/kg MTX on days 9 to 25 and 1 to 2 mg/kg on days 26 to 45 or until recovery of hematocrit to the normal range. Control mice were injected with equivalent volumes of PBS. For secondary transplant, marrow harvested from primary recipients was injected into lethally irradiated (800-850 cGy) CD45.1 secondary recipients, which were allowed to recover for 4 months prior to analysis of engraftment and transduction.

Hematology and flow cytometry

Blood was obtained from animals at regular intervals for determination of hematocrit and for flow cytometric analysis. Whole blood samples were resuspended in hemolysis buffer (0.15 M NH_4Cl, 1 M $NaHCO_3$, 0.1 M Na_2EDTA, pH 7.2), leukocytes pelleted by low-speed centrifugation, washed with PBS, and stained with fluorochrome-conjugated monoclonal antibodies for determination of immunophenotype. GFP expression was analyzed as previously described (Kurre *et al*., 2004). Engraftment and leukocyte immunophenotyping were conducted using anti-mouse monoclonal antibodies: APC-conjugated CD45.2 and PE-conjugated CD3e (T lymphocytes), B220 (B lymphocytes), CD11b and Gr-1 (myeloid lineages)(all purchased from eBiosciences, San Diego, CA) on a FACSCalibur instrument. Data were collected and analyzed

using CellQuest Pro (BD Biosciences) and FlowJo (Tree Star, Inc., Ashland, OR) software, respectively.

Histopathologic Analysis

Animals were euthanized and tissue samples were harvested, including sternum and ileum. Tissues were fixed in 10% formalin (further decalcifying bone marrow in 1% formic acid; both from Sigma-Aldrich), embedded in paraffin, sectioned, mounted, stained (hematoxylin and eosin), and analyzed microscopically without prior knowledge of sample identity.

Real time qPCR

DNA was extracted from samples using the Puregene® DNA Purification System (Gentra Systems, Minneapolis, MN) and quantitated spectrophotometrically (A_{260}). Reactions were run in duplicate under the following conditions: 60 ng DNA, 200 nM forward (5'-CACATGAAGCAGCACGACTT-3') and reverse (5'-GGTCTTGTAGTTGCCGTCGT-3') GFP-specific primers, 100 nM GFP-specific probe (5'/56-FAM/AGCGCACCATCTTCTTCAAG/3BHQ_1/-3') and 5% DMSO (Sigma-Aldrich) in 10 μL final reaction volume. The Roche Lightcycler 2.0 System was used for amplification of the target sequence with the following cycling parameters: 95˚C 10 min 1 cycle; 95˚C 10 s, 60˚C 20s, 45 cycles, 40˚C 30 s, 1 cycle. Results were analyzed using Lightcycler software, version 3.5 and copy number determined by the second derivative maximum analysis method (i.e. the cycle number at which sample fluorescence is first detected above background fluorescence). A standard curve was generated by first isolating a HeLa cell clone in selective medium containing 0.2 μM MTX after exposure to DHFR lentivirus vector CCDG (see above) at low multiplicity as a source of DNA containing a single copy integrant. CCDG/HeLa DNA was mixed with unexposed HeLa cell DNA in 10-fold serial dilutions to generate a standard curve covering a

range of 10^4 copies to 1 copy of the integrated vector sequence per 60 ng of template DNA. Negative controls for each reaction included no template control, HeLa cell DNA, and DNA extracted from C57BL/6 mouse bone marrow.

Statistical Analysis

Animal survival in response to MTX administration was evaluated by the Kaplan-Meier product limit method (Kaplan and Meier, 1958). The log rank statistic was determined using Prism 4 software (GraphPad Software, Inc., San Diego, CA) to test for differences among recipient cohorts (Peto and Peto, 1972). Data from other experiments were evaluated by unpaired t-test analysis.

RESULTS

Generation and *in vitro* characterization of lentivirus vectors transducing MTXr-DHFR and GFP.

To achieve high efficiency gene transfer in hematopoietic cell targets, we constructed lentivirus vector plasmids pEFDIG and pDL2G, containing a murine MTXr-tyr22DHFR variant cDNA along with the enhanced (e)GFP marker gene under transcriptional control of the human elongation factor 1-alpha (EF1-α) promoter. The tyr22DHFR and eGFP coding sequences were inserted as part of a bicistronic expression cassette (pEFDIG) or as a genetic fusion (pDL2G) (Fig. 1). The EFDIG and DL2G vectors were packaged into VSV-G pseudotyped virions, concentrated 100-fold, and titer assessed in comparison with the eGFP-encoding vector, CSIIEG (Fig. 1). Vector titers ranged between 10^8 and 10^9 IU/mL (Table 1), with reasonably good correlation between titer assessed by flow cytometry for GFP expression and by MTXr colony formation for DHFR expression. Mean fluorescence intensity (MFI) of GFP-positive 3T3 TK^- cells transduced at a multiplicity of infection of 0.2 or less was higher for the DL2G fusion in comparison to the EFDIG-infected cells, indicating that DL2G may be a more reliable vector design to achieve GFP marking of transduced cell populations.

To characterize tyr22DHFR and eGFP protein expressed in transduced cells, clones resistant to a high level of MTX (100 μM, to select for clones expressing a high level of DHFR) were isolated for examination by fluorescence microscopy and extracts prepared for western blot analysis (Fig. 2). Tyr22DHFR and eGFP proteins were clearly identifiable in western blots (Fig. 2A). The presence of a 48 kDa band in MTXr-DL2G lysates immunoreactive with both anti-eGFP and anti-DHFR antibodies confirmed successful generation of the tyr22DHFR-eGFP fusion protein, consistent with the molecular masses of eGFP (27 kDa), DHFR (21 kDa) and the glycine-serine linker (0.8 kDa). In contrast, a 21 kDa band was detected in EFDIG-transduced

cell lysates probed with anti-DHFR antibody, consistent with the molecular weight of endogenous murine DHFR. A 27 kDa band was also detected in GFP-probed EFDIG lysates, consistent with the molecular mass of eGFP. In uninfected and DL2G-transduced cells, endogenous DHFR was detected at greater exposure times (data not shown). Fluorescence microscopy of MTXr-clones showed a higher level of fluorescence in DL2G-positive cells in comparison to EFDIG-positive cells (Fig. 2B).

To determine the effect of the DL2G fusion on DHFR enzyme activity, 3T3 TK^- cells were transduced at a multiplicity of one (based on GFP titer), maintained in culture for 7 days, and then harvested for extract preparation and DHFR enzyme assay. The enzyme activity per mg total protein was approximately four times lower in cells transduced with DL2G compared to EFDIG in the absence of MTX, despite similar levels of GFP expression as determined by flow cytometry and proviral copy numbers (Table 2). When drug was added to the extracts, enzyme activity in EFDIG and DL2G-transduced cells decreased by 27% and 76%, respectively. When U/mg was evaluated within the context of copy number, there was no significant difference between activity/mg per copy number in EFDIG- and DL2G-transduced cells.

Transplantation with EFDIG-transduced marrow protects recipients from lethal doses of MTX.

To determine the level of chemoprotection conferred by lentivirus-mediated tyr22DHFR gene transfer, we conducted dose-escalation studies in animals that had received either CSIIEG or EFDIG-transduced marrow. Following overnight transduction in the presence of IL-3, IL-6 and SCF, 3×10^6 transduced, unfractionated marrow cells were infused into sub-lethally irradiated (700 cGy) recipients. Animals were then administered either PBS or MTX at ascending doses, from 0.25 mg/kg/day on day 1 up to 2 mg/kg/day on day 42. Mice that were transplanted with GFP-transduced marrow and subsequently administered MTX did not survive dose-escalation to

2 mg/kg/day (Fig. 3A). Two of the CSIIEG-marrow recipients administered MTX that survived until day 35 exhibited poor health, as indicated by 25% loss of body weight, lethargy and hematocrits below 15 (Fig. 3B). In contrast, animals transplanted with EFDIG-transduced marrow exhibited a significant survival advantage ($p = 0.0102$) and most of these animals survived escalation of the MTX dose up to 2 mg/kg/day. EFDIG-marrow recipients recovered to healthy hematocrit levels by day 42, exhibiting a similar hematocrit recovery pattern as that observed for the PBS-administered control cohort. Histopathologic analysis of marrow from MTX-treated control (GFP-transduced) recipients revealed severe hypoplasia, consistent with myelosuppression. In contrast, bone marrow from the EFDIG/MTX and CSIIEG/PBS cohorts showed marrow hyperplasia. Histopathologic analysis of samples from the ileum revealed normal crypt:villus ratios and a lack of lesions (data not shown).

Donor cell engraftment levels were assessed in recipient animals by flow cytometric analysis for $CD45.2^+$ leukocytes in peripheral blood at 4 and 8 weeks post transplantation. For PBS-administered control animals, donor engraftment increased from 60% at 4 weeks to 82% at 8 weeks (Table 3). Lower levels of engraftment were observed in MTX-treated EFDIG-marrow recipients 4 weeks post transplantation (30%). By week 8, engraftment increased to 82%. GFP marking was higher in MTX-treated EFDIG marrow recipients (36%) compared to PBS treated animals (15%) during the dose-escalation, but marking decreased to 9-10% in both cohorts 1 month after MTX withdrawal. These data show selective expansion of tyr22DHFR-expressing cells resulting from MTX administration.

<u>MTX dose-escalation studies in DL2G-transduced BMT recipients.</u>

To assess whether the tyr22DHFR-GFP fusion protein protects recipients from MTX-toxicity, we conducted MTX dose-escalation studies in mice transplanted with 2×10^6 DL2G-transduced

marrow cells. Donor CD45.2 marrow cells were harvested and transduced overnight with DL2G or CSIIEG in the presence of IL-3, IL-6 and SCF as described in Materials and Methods. In comparison to the EFDIG-transduced marrow transplant experiment described above, fewer cells were infused due to decreased viable cell yield after overnight transduction. After transplantation, recipient animals were administered either PBS or MTX at an initial dose of 0.25 mg/kg, subsequently increasing the dose to 0.5 mg/kg and then 1.0 mg/kg on day 9 post-transplant. Control CSIIEG-transduced marrow recipients did not survive MTX dose-escalation to 1 mg/kg/day and succumbed by day 16 (Fig. 3C) ($p=0.0398$). Declining health of all animals, including PBS-treated controls, necessitated a 5-day suspension of MTX administration, from day 13 to 17 post-transplant. Once the animals regained their health based on weight, appearance and behavior, treatment was resumed and continued until the end of the 30-day dose-escalation. We observed that DL2G marrow recipients survived MTX dose-escalation to 1 mg/kg/day. In comparison to the MTX treated CSIIEG-marrow recipients, MTX- and PBS-treated DL2G-marrow recipients recovered to normal hematocrit levels by day 21 (Fig. 3D). Histopathologic analysis of marrow and small intestine (ileum) sections from MTX-treated CSIIEG-marrow recipients revealed severe marrow hypoplasia but no evidence of GI atrophy, respectively (data not shown). In contrast, DL2G-transduced marrow recipients treated with MTX or PBS exhibited hyperplastic marrow and normal GI histology.

MTX and PBS-treated DL2G-marrow recipients exhibited 9 and 14% CD45.2 donor cell marking, respectively, in peripheral blood at the endpoint of drug administration (31 days). By 8 weeks after marrow transplantation, donor cell engraftment increased to 59% and 60% for these cohorts, respectively (Table 3). In PBS-administered control animals, GFP marking in peripheral blood was stable between 4 and 8 weeks (3% GFP-positive PBMCs). In contrast, GFP marking

in MTX-treated DL2G-marrow recipients decreased by 8 weeks (from 13% to 2% GFP-positive PBMCs). GFP marking was observed in donor myeloid and lymphoid lineages at mean frequencies from 10% to 60% (Table 4). The greater level of GFP marking in MTX treated animals implies a selective advantage for tyr22DHFR-expressing donor hematopoietic cells over untransduced donor cells during MTX administration. Although antifolate administration alone is not sufficient for *in vivo* selection of hematopoietic stem cells (Allay *et al.*, 1998), tyr22DHFR-myeloid and lymphoid progeny appear to be less sensitive to MTX toxicity and therefore may have a survival advantage over their untransduced counterparts.

Proviral integrant frequencies (determined by real-time qPCR) in the bone marrow of primary transplant recipients were similar among those animals within each experiment (Table 3). The higher proviral integrant frequency in experiment 1 compared to experiment 2, in combination with the reduced number of marrow cells transplanted in experiment 2, may account for the decreased recovery time and higher level of chemoprotection achieved in MTX-treated EFDIG-marrow recipients (2 mg/kg/day vs. 1 mg/kg/day).

Repopulation of secondary recipients with lentiviral vector transduced hematopoietic stem cells. To confirm transduction of long-term repopulating stem cells, bone marrow was harvested from primary recipients and $5x10^6$ cells transplanted into each of 3 lethally irradiated CD45.1 secondary recipients. Peripheral blood from the secondary recipients was analyzed for CD45.2 donor and GFP^+ marking by flow cytometry at 8 and 16 weeks post transplantation. By 16 weeks, the mean engraftment level of $CD45.2^+$ cells in the peripheral blood of secondary recipients was not significantly different between cohorts ($p<0.05$). GFP marking was stable over the 4-month period and similar to the GFP-marking level observed for EFDIG-transduced primary marrow transplant recipients (Fig. 4A). By comparison, engraftment of $CD45.2^+$ cells in

DL2G-marrow recipients was lower, likely due to the fact that 1 million fewer cells were transplanted into primary recipients. GFP marking in secondary DL2G-marrow recipients was substantially lower (1% GFP^+), likely due to reduced stem cell marking (Fig. 4B). The level of GFP expression persisted in secondary recipients, as revealed by stable mean fluorescence intensity over time observed in secondary recipients of EFDIG, DL2G and CSIIEG-transduced marrow. These results allow us to conclude that: (i) EFDIG and DL2G treatment of unfractionated marrow supported the transduction of hematopoietic stem cells; (ii) primitive hematopoietic cells engrafted, repopulated and gave rise to MTX-resistant progeny in primary recipients sufficient to provide significant chemoprotection and prolonged animal survival during MTX dose-escalation; and (iii) genetically marked hematopoietic stem cells reconstituted the bone marrow of lethally irradiated secondary recipients, giving rise to progeny that persisted in the peripheral blood.

DISCUSSION

Previous studies have shown that transplantation with drug-resistant DHFR transgenic marrow or with marrow transduced using a γ-retrovirus encoding human MTXr-DHFR conferred significant chemoprotection to transplant recipients (May *et al*., 1995 &1996; James *et al*., 1999; Budak-Alpdogan *et al*., 2004). The primary goal of the studies described in this paper was to test the effectiveness of lentivirus vectors for tyr22DHFR gene transfer into murine bone marrow and protection of transplant recipients from MTX toxicity. We constructed both a tyr22DHFR-IRES-eGFP bicistronic vector and a tyr22DHFR-eGFP genetic fusion for comparative *in vivo* chemoprotection studies, with the rationale that the eGFP coding sequence located 3' to the IRES may not always be expressed to the same degree as the upstream tyr22DHFR (Mitzuguchi *et al*., 2000; Yu *et al*., 2003). In two separate *in vivo* chemoprotection studies, EFDIG- and DL2G-transduced marrow recipients recovered to healthy hematocrit levels and had a significant survival advantage over control GFP transduced marrow recipients during MTX administration ($p<0.05$). Animals that received GFP-transduced bone marrow exhibited severe myelosuppression (hct < 20 and marrow hypoplasia upon histopathologic analysis) and did not survive MTX-dose escalation to 1 mg/kg/day. We conclude that both EFDIG and DL2G lentiviral vectors confer a substantial chemoprotective effect in mouse marrow transplant recipients administered MTX.

To facilitate vector titering and the tracking of DHFR-transduced hematopoietic cells, we included a GFP coding sequence in both of the vectors tested in our study. For vector EFDIG, expression of the downstream eGFP sequence relies on internal translation from a picornavirus IRES sequence positioned between the DHFR and eGFP coding sequence. While there is ample evidence in the literature for the effectiveness of this strategy for expression of two genes from

the same vector, maintenance of expression can be problematic, particularly for the downstream coding sequence. Persons *et al*. (1997) solved this problem for DHFR-expressing MoMLV vector by placing the GFP coding sequence in the upstream position of a GFP-IRES-DHFR transcript, thereby forcing expression of both genes due to the selective pressure exerted on DHFR in the downstream position. In this study, we generated a tyr22DHFR-eGFP fusion protein so as to more intimately link GFP expression to drug-resistant DHFR function. Similar use of a human Phe22/Ser31 DHFR-GFP fusion protein was previously reported by Mayer-Kuckuk (2002). We verified the predicted size of the DHFR-GFP fusion protein by western blot using both anti-GFP and anti-DHFR antibodies, as well as maintained GFP fluorescence and DHFR enzyme activity in cell extracts. Direct comparison of cells transduced with EF1α-regulated vectors (EFDIG vs. CSIIEG, Table 1) revealed a 1.8-fold reduction in GFP mean fluorescence intensity resulting from the DHFR-GFP fusion (175 vs. 322, respectively). However, GFP fluorescence of the IRES regulated construct EFDIG was reduced 3.3-fold (MFI = 98) compared to CSIIEG, thus exemplifying our motivation in seeking a more reliable strategy for GFP co-expression in these vectors. The effectiveness of the fusion design was also apparent in the maintenance of MFI in DL2G transduced peripheral blood cells of secondary transplant recipients (Fig. 4B), the low level of stem cell transduction in this experiment notwithstanding.

Therapeutic application of antifolates such as MTX is limited by systemic toxicity and tumor-acquired drug resistance (Bertino, 1993). Low and intermediate dose MTX therapy causes cytopenia among hematopoietic lineages, thereby compromising overall patient health. The primary acute toxicities associated with high dose MTX treatment are myelosuppression and mucositis (Moe *et al*., 2000). This is especially limiting in applications where GI sensitivity to MTX precludes dose-escalation to a level that would improve the likelihood for patient relapse-

free survival. As an approach to protect against antifolate toxicities, *ex vivo* gammaretroviral transduction studies in mice have shown that virus-mediated MTXr-DHFR gene transfer supports higher-dose antifolate administration while limiting myelotoxicity. Protection of animals from lethal doses of methotrexate by *ex vivo* retroviral vector mediated transduction of hematopoietic stem cells was demonstrated by Williams (Corey *et al*., 1990) and also by Bertino (Capiaux *et al*., 2003). Our laboratory reported similar protection against lethal doses of MTX in animals transplanted with transgenic marrow expressing one of several MTX resistant DHFR variants (May *et al*., 1995 & 1996, Morris *et al*., 1996, James *et al*., 1997 & 2000). Sorrentino's group demonstrated selective protection against the antifolate trimetrexate mediated by tyr22DHFR transduction at the stem cell level (Allay *et al*., 1998). Here, we have extended these chemoprotection studies to include a lentiviral vector-mediated approach with the intention of providing a more potent means of achieving tyr22DHFR gene transfer into hematopoietic targets.

There are several hematologic observations that were made as a part of this study. (i) First, the evidence from our study supports transduction of hematopoietic stem cells. GFP^+ donor leukocytes of both myeloid and lymphoid lineages observed at 8 weeks post-transplant could have resulted from transduction of stem cells or of more committed myeloid and lymphoid progenitors. Drug resistance gene expression in committed progenitors is important, in fact, to confer chemoprotection in the period soon after bone marrow transplant (in mice, the first 2 – 3 weeks) (James *et al*., 1999). The data from our secondary transplant recipients, however, indicate that gene transfer into primitive stem cells was achieved in the primary transplanted animals, most likely giving rise to at least some transduced cells in the peripheral blood starting 3 to 4 weeks post-transplant. (ii) We also observed that transgene expression based on GFP

marking in the peripheral blood was significantly increased during MTX treatment and then subsided 1 month after drug withdrawal ($p<0.05$). In contrast, GFP marking did not decrease significantly in PBS-treated animals after cessation of injections ($p>0.05$). This is consistent with a survival advantage imparted on more differentiated cells during MTX administration, but not on more primitive stem cells. This is also consistent with previous reports that selection of DHFR transduced stem cells requires the use of a more specific DHFR inhibitor (i.e. trimetrexate) in combination with a nucleoside transport inhibitor (Allay *et al*., 1997). Temporary expansion of more differentiated cell types has also been reported for non-human primates transplanted with DHFR transduced marrow upon administration of trimetrexate (Persons *et al*., 2004). Such temporary expansion of $DHFR^+$ PBL nonetheless implies a certain degree of chemoprotection achieved, with potential therapeutic benefit. (iii) Gene transfer was achieved after a simple overnight exposure of marrow cells to DHFR lentivirus vector at an infection multiplicity of 10, resulting in a moderate overall proviral copy number in peripheral blood cells (0.5 copies/cell in expt1, 0.2 to 0.3 copies/cell in expt 2). Nonetheless, the low gene transfer frequency into stem cells observed in these experiments (11% in expt 1, and 1% in expt 2) was sufficient to protect transplant recipients from myelosuppression and support MTX dose-escalation to 2 mg/kg and 1 mg/kg per day, respectively. Such chemoprotection was previously observed in mice engrafted at low levels (down to 1%) with transgenic marrow expressing drug-resistant DHFR (James *et al*., 2000).

Clinical testing of drug-resistance genes has included introduction of the MDR1 gene as a means of protecting against the toxicity of paclitaxel chemotherapy (Abonour *et al.*, 2000 and others) and introduction of the methylguanine methyltransferase gene to confer resistance to alkylating agents (Cornetta *et al.*, 2006). These studies have targeted human $CD34^+$

hematopoietic stem cells using retroviral vectors for introduction of the MDR1 or MGMT genes. In particular, Abonour *et al*. reported successful MDR gene transfer into $CD34^+$ cells engrafted in recipient patients, with a transient increase in MDR1-positive cells observed in the peripheral blood after a course of paclitaxel chemotherapy. These results imply protection against the toxicity of chemotherapy brought about by introduction of the MDR gene. We suggest that effective protection against antifolate toxicity may similarly be brought about by introduction of DHFR genes that encode antifolate-resistant enzyme activity, and at the higher frequency afforded by the use of lentivirus rather than murine retrovirus vectors. The studies described herein show that *ex vivo* lentiviral transduction of mouse bone marrow supports DHFR gene transfer into HSCs and more differentiated hematopoietic lineages, providing short- and long-term chemoprotection to marrow transplant recipients during MTX dose-escalation. These studies also demonstrate the effectiveness of lentivirus vectors for this purpose, using *ex vivo* transduction conditions (i.e., short-term exposure) that are relevant to the clinical setting. Systemic chemoprotection coupled with antifolate chemotherapy may thus provide more effective treatment of antifolate responsive tumors.

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FOOTNOTES

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Gori JL, Podetz-Pedersen K, Swanson D, Karlen AD, Somia NV, and McIvor RS (2006) Chemoprotection of mouse marrow transplant recipients by *ex vivo* lentiviral transduction of murine tyr22 dihydrofolate reductase conferring resistance to methotrexate. *Mol Ther* **13**:S299.

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LEGENDS FOR FIGURES

Figure 1. Lentivirus vectors. In each vector, the promoter (U3) region of the long terminal repeats (LTRs) flanking the viral genome has been deleted. The cytomegalovirus (CMV) promoter regulates transcription of the modified viral genome in transfected cells. The repeat (R) and U5 elements are retained for polyadenylation, reverse transcription and integration of viral vector genomes. Other features include the rev response element (rre), woodchuck post-transcriptional regulatory element (wpre), internal ribosomal entry site (ires) and the elongation factor-1 alpha promoter (EF-1α). A, EFDIG, tyr22DHFR-ires-eGFP bicistronic vector. B, DL2G, tyr22DHFR-eGFP fusion with a flexible glycine-serine linker encoded between the two transgenes. C, CSIIEG, control eGFP vector.

Figure 2. Characterization of DHFR / GFP fusion protein expressed in transduced NIH3T3 TK$^-$ fibroblasts. Transduced cells were selected in 100 μM MTX for two weeks. Drug-resistant colonies were isolated and expanded under non-selective conditions for subsequent analyses. A, *Western blot analysis.* 25 μg protein from each of the specified clones or from uninfected cells (indicated across the top of the blot) was electrophoresed through 12% SDS-PAGE gel and blotted onto nitrocellulose. Blots were probed with (right) rabbit anti-eGFP or (left) anti-DHFR antibodies. Molecular weight markers are shown on the right. B, *Fluorescence microscopy*. MTX-resistant clones or uninfected cells were seeded onto glass cover slips, fixed onto slides and photographed.

Figure 3. MTX dose escalation after transplantation with tyr22DHFR-transduced marrow. Animals transplanted with EFDIG (n=8), DL2G (n=8) or CSIIEG (n=6) transduced marrow were

administered MTX daily starting at 0.25 mg/kg/day, escalating to 2 mg/kg/day (dose indicated in variably shaded bar across the top of A-D) over a thirty-five day period or until the animal succumbed. A third cohort for each experiment that received CSIIEG (n=6) or DL2G (n=3)-transduced marrow was administered PBS daily. A and C, Kaplan-Meier plots of the fraction of animals surviving at the indicated times after initiation of MTX administration (A, p=0.0102; B p=0.0398). Animal groups are labeled in the figure. B and D, Average weekly hematocrit for surviving animals. Error bars = S.D.

Figure 4. Persistent transgene expression in secondary transplant recipients. Sixteen weeks after secondary marrow transplantation, GFP marking and mean fluorescence intensity (MFI) were assessed by flow cytometry. Dot plots depicting %GFP positive cells (on the left) and MFI (on the right) associated with 5 representative primary recipient mice (black diamonds) and their three respective secondary transplant recipients (open circles) are shown. A, experiment 1 (EFDIG). B, experiment 2 (DL2G).

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TABLES

Table 1. Lentiviral vector titers based on MTXr-CFUs and GFP fluorescence[a]

	Crude supernatant		*Concentrated vector*		
LV	CFU/mL[b]	GTU/mL[c]	CFU/mL	GTU/mL	MFI
EFDIG	1.5×10^6	1.6×10^6	1.0×10^8	9.8×10^7	98
DL2G	2.4×10^6	5.2×10^6	6.0×10^7	9.9×10^7	175
CSIIEG	0	1.8×10^6	0	1.3×10^8	322

[a]Mean tyr22DHFR and GFP titers determined by transduction of MTX-sensitive NIH3T3 TK^- cells with vectors carrying the indicated expression cassettes. Titer was assessed by drug resistant colony formation in MTX and flow cytometry for GFP expression. Mean fluorescence intensities (MFI) of GFP^+ cells are indicated in the far-right column of the table.

[b]Methotrexate-resistant colony forming units per mL virus supernatant

[c]GFP transducing units per mL virus supernatant

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Table 2. DHFR enzyme activity in transduced 3T3 TK$^-$ cells[a]

			-MTX		*+MTX*	
	% GFP$^+$	copy #/cell	U/mg	U/mg/copy #	U/mg	U/mg/copy #
DL2G-3T3	91.9	2.3	17.74 ± 3.48	7.71	4.26 ± 2.13	1.85
EFDIG-3T3	97.1	28.3	64.23 ± 9.76	2.27	46.67 ± 6.64	1.65
3T3	0	0	8.08 ± 0.57	NA	0	NA

[a]Cell lysates from transduced or untransduced 3T3 TK$^-$ cells were assayed for DHFR activity in the presence and absence of 0.25 μM MTX. One unit of enzyme activity is defined as 1 nmol/min. Results shown are the mean of three assays ± S.D.

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Table 3. Mean engraftment and GFP marking in primary recipients

			1 month post BMT[a]		2 months post BMT[b]		
LV/Rx	BMT	No. BMT/ followup	Engraftment (% CD45.2$^+$)	GFP (%)	Engraftment (% CD45.2$^+$)	GFP (%)	Copy #/ cell
CSIIEG/PBS	3 x 10^6	6/6	60 ± 13	15 ± 8	82 ± 5	10 ± 5	0.5
CSIIEG/MTX	3 x 10^6	6/0	NA	NA	NA	NA	NA
EFDIG/MTX	3 x 10^6	8/7	30 ± 5	36 ± 8	82 ± 4	9 ± 2	0.5
DL2G/PBS	2 x 10^6	3/2	14 ± 16	3 ± 0	59 ± 3	3 ± 1	0.2
CSIIEG/MTX	2 x 10^6	5/0	NA	NA	NA	NA	NA
DL2G/MTX	2 x 10^6	7/5	9 ± 4	13 ± 10	60 ± 12	2 ± 1	0.3

[a]plus Rx

[b]1 month post Rx removal

NA – Not available due to mortality prior to followup

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Table 4. GFP marking in myeloid and lymphoid lineages 2 months post BMT[a]

LV/Rx	Gr-1$^+$ (%)	Mac-1$^+$ (%)	B220$^+$ (%)	CD3$^+$ (%)
CSIIEG/PBS	7 ± 4	8 ± 3	59 ± 15	41 ± 19
EFDIG/MTX	10 ± 3	7 ± 4	50 ± 12	44 ± 20
DL2G/PBS	18 ± 9	20 ± 6	21 ± 22	35 ± 30
DL2G/MTX	31 ± 24	28 ± 16	18 ± 21	41 ± 18

[a]Values are expressed as % cells of a given phenotype that were GFP$^+$ +/- S.D.

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Figure 1

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Figure 2

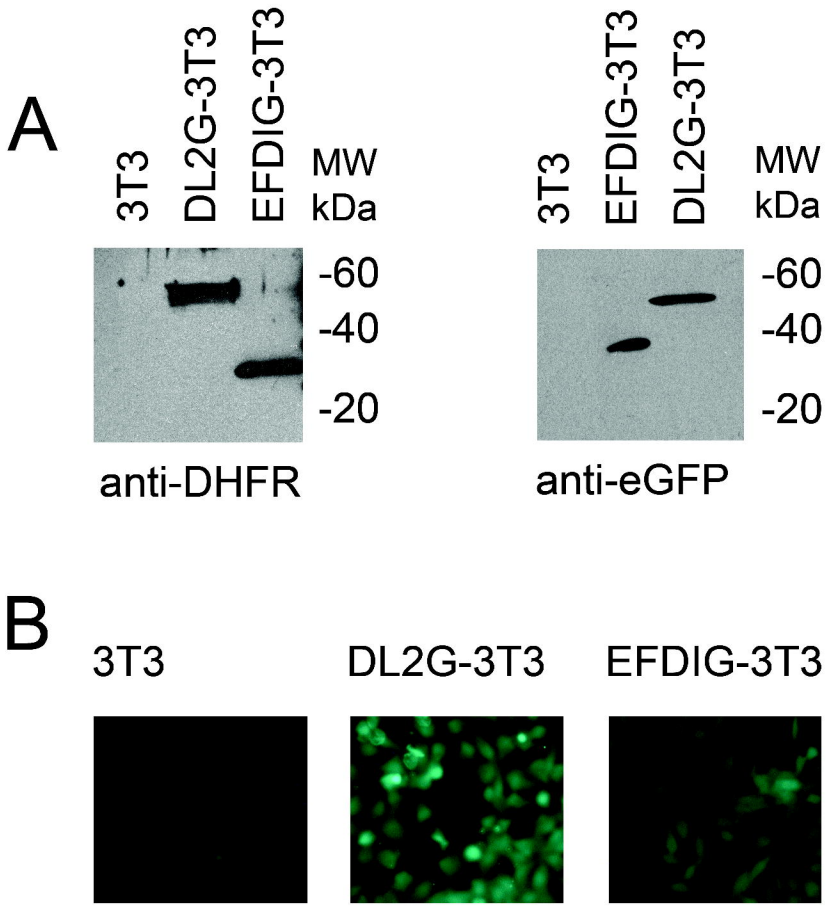

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Figure 3

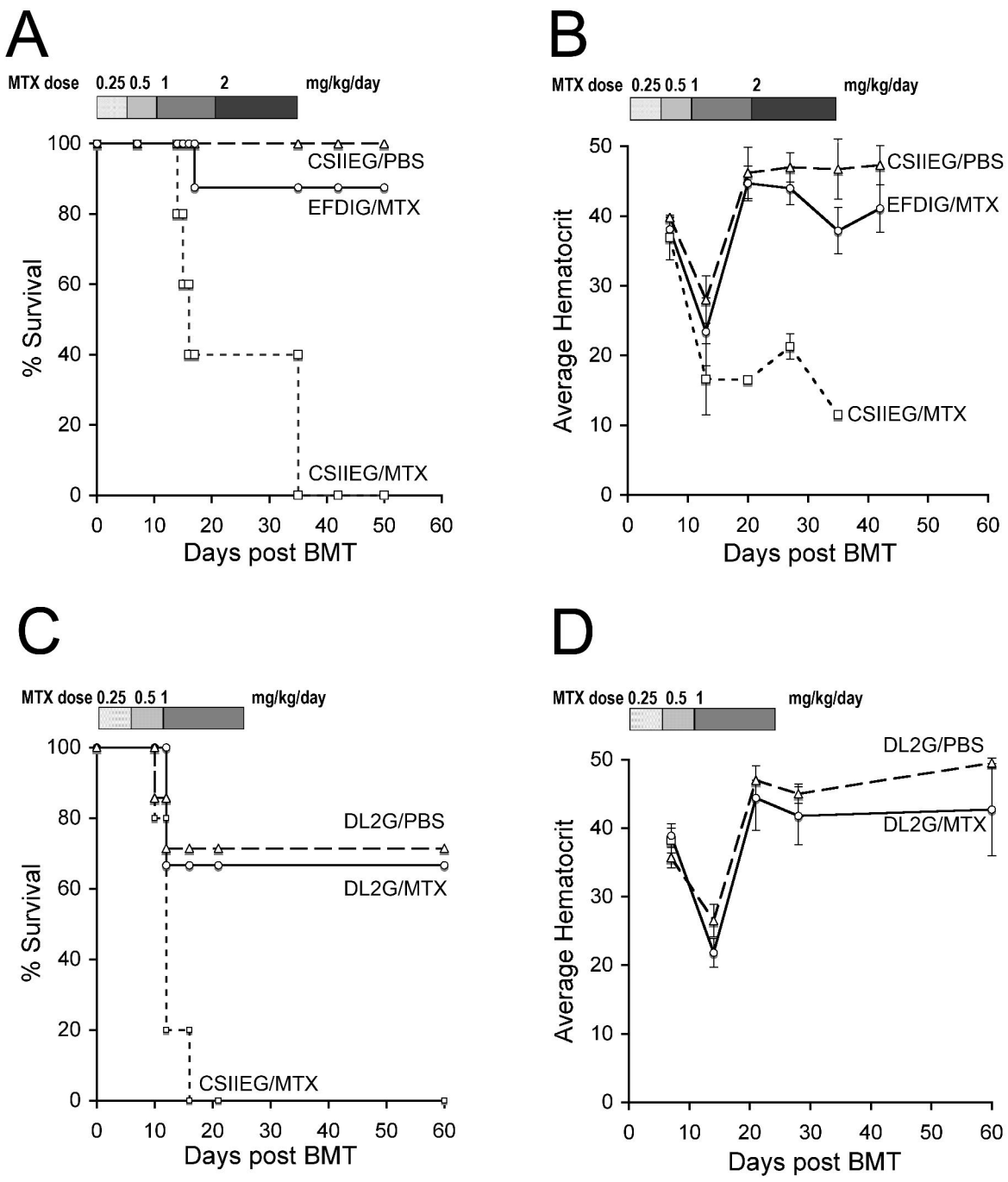

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Figure 4