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In vivo *protection of activated Tyr22-dihydrofolate reductase gene-modified canine T lymphocytes from methotrexate*

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Abstract

Background—Nonmyeloablative allogeneic hematopoietic stem cell (HSC) transplantation can cure malignant and nonmalignant diseases affecting the hematopoietic system, such as severe combined immunodeficiencies, aplastic anemia and hemoglobinopathies. Although nonmyeloablative is favored over myeloablative transplantation for many patients, graft rejection remains problematic. One strategy to decrease rejection is to protect donor activated T cells in the graft from methotrexate (MTX) by genetically modifying the cells to express MTX-resistant dihydrofolate reductase (Tyr22-DHFR), leaving the immunosuppressive effects of MTX to act solely on activated host T lymphocytes, shifting the balance to favor allogeneic engraftment.

Methods—To evaluate MTX resistance of Tyr22-DHFR$^+$ T lymphocytes *in vivo*, we transplanted dogs with autologous CD34$^+$ cells modified with YFP and DHFR-GFP lentivirus vectors. Dogs were then treated with a standard MTX regimen (days 1, 3, 6, and 11) following immune activation with a foreign antigen as a surrogate assay to mimic early transplantation.

Results—DHFR-GFP$^+$ gene marking was maintained in CD3$^+$CD25$^+$ and CD4$^+$ T lymphocytes after MTX treatment while the level of T lymphocytes that expressed only a fluorescent reporter (YFP$^+$) decreased. These data show that Tyr22-DHFR expression protects T lymphocytes from MTX toxicity in dogs, highlighting a clinically relevant application for preserving donor T lymphocytes during post transplantation immunosuppression.

Conclusions—These findings have implications for clinical translation of MTX-resistant T cells to facilitate engraftment of allogeneic cells following nonmyeloablative conditioning and minimize the risk of rejection. In summary, Tyr22-DHFR expression in T lymphocytes provides chemoprotection from MTX-mediated elimination in the context of immune activation *in vivo*.

Keywords

gene therapy; chemotherapy; viral vector; transplantation; drug resistance; animal model

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INTRODUCTION

Drug resistance gene transfer into hematopoietic stem cells has two important clinical applications. With respect to cancer therapy, expression of drug resistance genes such as the P140K variant of methyl guanine methyl transferase (MGMT) in chemosensitive hematopoietic cells allows for administration a of more intense and/or dose-dense chemotherapy regimen, by decreasing hematopoietic toxicity [1]. When coupled to immunosuppressive chemotherapy that is administered after hematopoietic stem cell (HSC) transplantation to restrict proliferation of recipient T lymphocytes activated by donor alloantigens, drug resistance gene expression has the potential to preserve allogeneic gene-modified donor T cells, while eliminating activated recipient anti-graft T lymphocytes, thereby reducing the likelihood of graft rejection. Given its efficacy and standard clinical use as prophylaxis for graft-versus-host disease (GvHD), the antifolate methotrexate (MTX) is an attractive candidate for this particular drug resistance gene therapy application [2–5].

Methotrexate blocks cell proliferation by inhibiting dihydrofolate reductase (DHFR). DHFR catalyzes the reduction of folate to dihydrofolate and then tetrahydrofolate, a precursor required for nucleoside, purine and DNA synthesis. Although we and others have shown that methotrexate-resistant DHFR (MTXr-DHFR) gene expression in HSCs may support chemoprotection and *in vivo* selection in mice, recapitulating these results in large animal models has been more challenging [6–12]. Although strategies have been developed that improved gene transfer (i.e. mobilization and enrichment of $CD34^+$ cells and use of HIV-1-based lentivirus vectors) and detection of gene-modified cells (i.e. real-time qPCR, fluorescent proteins and flow cytometry) in large animal models [13–16], a feasible clinical application for MTX resistance gene therapy remains to be clarified and tested in a large animal model.

We previously showed that lentivirus-mediated transfer of P140K-MGMT into canine $CD34^+$ cells supports *in vivo* selection and chemoprotection of HSCs from O^6-benzylguanine (O^6-BG) plus 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) or temozolomide (TMZ) in both allogeneic [17] and autologous [18] transplantation settings. Based on the pharmacologic differences between alkylating agents (BCNU, TMZ) and antifolates such as MTX, chemoprotection and *in vivo* selection of true, quiescent HSCs with the Tyr22-DHFR/MTX system is not feasible, as only highly proliferative cells, such as activated T lymphocytes, are sensitive to MTX toxicity. We therefore hypothesized that MTXr-DHFR chemoprotection of donor T lymphocytes has the potential to preserve donor immune function in the context of post hematopoietic stem cell transplantation (HSCT) immunosuppressive chemotherapy while MTX suppresses proliferation of recipient alloantigen activated T lymphocytes.

Although the most relevant clinical application of MTX resistance is gene modification of donor T lymphocytes in the context of an allogeneic HSCT, we decided to evaluate Tyr22-DHFR gene expression using our well-established canine autologous transplantation model system after Tyr22-DHFR lentivirus mediated gene transfer into and transplantation of $CD34^+$ cells. The advantage of testing DHFR mediated chemoprotection of T lymphocytes in this setting is that this model system allows us to evaluate the effect of several different MTX treatment regimens on gene-modified T lymphocyte subsets in single animals over time, in the context of steady state and activated immune function. In contrast to the allogeneic setting, this system also allows assessment of gene-modified lymphocyte persistence without the potential of graft rejection caused by leukocyte antigen disparity. While the allogeneic setting would prevent distinguishing between a transgene-specific and donor-specific immune response, our model paired with an established surrogate immune response assay (i.e. the infusion of the xenogeneic foreign antigen sheep red blood cells

[19]) supports characterization of Tyr22-DHFR lymphocyte chemotherapy resistance and expansion during immune activation.

Furthermore, we chose the canine model because MTX prophylaxis for GvHD prevention has been well characterized in the dog, [20–24]. In this study, we evaluated lentivirus-mediated transgene expression and MTX chemoprotection of gene-modified T lymphocytes in the canine autologous HSC transplantation setting and sought to determine whether MTX mediated chemoprotection supports persistence of Tyr22-DHFR-modified T lymphoid subsets in the context of steady state immune function and acute immune activation.

MATERIALS AND METHODS

Canine animal care

Dogs were housed at the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA) Shared Resource Facility after being born on site or procured from Marshall Farms (North Rose, New York) in compliance with the *Guide for the Care and Use of Laboratory Animals* (ILAR, 1996). Dogs were monitored and maintained in good health as described [25]. The FHCRC hematology laboratory provided complete blood counts (CBC), differentials and serum chemical levels. The protocol was approved by the Institutional Animal Care and Use Committee of FHCRC under Protocol 1289.

Preparation of lentivirus vector stocks

The DHFR-GFP, GFP, and YFP lentivirus vector plasmids used in this study have been previously described [6,14,26]. Lentiviral vector stocks for transduction of canine hematopoietic cells were prepared as described [6,14], concentrated and titered on human HT-1080 fibrosarcoma cells. Briefly, HT-1080 cells were exposed to dilutions of lentivirus vector for 24 hours in the presence of 4 μg/mL protamine sulfate. Three days after the medium change at 24 hours, fluorescence protein expression was assessed by flow cytometry. Cells were also replated into 0.15 μM MTX and MTXr-colony forming units (CFU/mL) and were counted after staining colonies with crystal violet [7]. Titer was also assessed by real-time qPCR for detection of proviral DNA sequences (transducing units/mL) as described [6,25].

Canine CD34$^+$ cell isolation, transduction and transplantation

A total of five individual dogs were used in these studies. CD34-enriched cells were obtained from bone marrow after priming with canine granulocyte colony stimulating factor (cG-CSF) and canine stem cell factor (cSCF). To induce stem cell cycling (priming) in the bone marrow (BM), animals were administered cG-CSF (5 μg/kg twice daily) and cSCF (25 μg/kg once daily) subcutaneously for 5 days. Bone marrow was harvested by vacuum aspiration as described [27]. Harvested cells were treated with hemolytic buffer (155 mM NH_4Cl, 12 mM $NaCO_3$, 0.1 mM EDTA, pH 7.4), washed, collected by centrifugation and resuspended in DNase buffer (2% FBS, 5.1 μg/mL DNase in PBS) and passed through a 70 μm nytex filter. Cells were collected by centrifugation and resuspended in sorting buffer (4% ultra pure BSA, 2 mM EDTA pH 7.0 in PBS and degassed; Miltenyi Biotec). Cells were resuspended in sorting buffer (4% ultra pure BSA, 2 mM EDTA pH 7.0 in PBS and degassed; Miltenyi Biotec) at a density of 1×10^8/mL, incubated with biotinylated monoclonal anti-canine CD34 antibody (clone 1H6) and placed on a tube rotator (MACSmix Model MX001; Miltenyi Biotec) for 30 minutes at 4°C. Cells were washed with sorting buffer, collected by centrifugation, resuspended in sorting buffer and streptavidin-labeled magnetic beads (Streptavidin MicroBeads; Miltenyi Biotec) and placed on a tube rotator (MACSmix Model MX001; Miltenyi Biotec) for 30 minutes at 4°C. Cells were washed, filtered, collected by centrifugation and resuspended in degassed sorting buffer at a

concentration of 3×10^8 cells/mL. The cell suspension was added to equilibrated cell separation columns (1 column per 6×10^8 cells; LS Columns; Miltenyi Biotec) attached to a magnetic holder (*Midi*MACs Separator; Miltenyi Biotec). After washing the cells in the column, the column was removed from the magnet and $CD34^+$ cells eluted in IMDM 10/1 (Iscove's Modified Dulbecco's Medium plus 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin). The positive and negative fractions of the selection were washed, resuspended in IMDM 10/1. The CD34-enriched cell fraction was kept as a single preparation or split into two equal volumes (for competitive repopulation) and incubated with lentivirus vector in non-tissue-culture-treated 75-cm^2 flasks coated with 2 $\mu g/cm^2$ CH-296 (RetroNectin; Takara Bio). Cells were transduced for up to 36 hours at a multiplicity of infection between 3 and 15 in the presence of 8 μg/mL protamine sulfate, canine and human cytokines (50 ng/mL each of cG-CSF, cSCF, hFlt-3, and hMGDF) as described [28] and harvested for infusion. Four to 6 hours before cell transplantation, the dogs received a single myeloablative dose of total body irradiation (920 cGy). To ensure hematopoietic reconstitution, both the transduced CD34-enriched fraction and untransduced CD34-negative fractions were infused into the dogs. Dogs were treated with cyclosporine (CSP, up to 15 mg/kg orally, twice daily) and mycophenolate mofetil (MMF, 5 mg/kg orally, twice daily) for immunosuppression up to 100 days after transplantation. Transplant recipients were also administered cG-CSF (5 μg/kg subcutaneously twice daily) and given platelet transfusions, as needed until hematopoietic recovery (i.e. until platelet counts returned to normal range). Complete blood counts were monitored daily until hematologic recovery based on absolute neutrophil (ANC) and platelet counts (PLT) or until GFP marking decreased below the background detection level (<0.1%).

Canine *ex vivo* colony-forming cell cultures

Transduced CD34-enriched cell preparations were cultured in a double-layer agar system as described [14]. After transduction, $CD34^+$ cells were plated at a density of 3,000 cells per plate. The total numbers of colonies and GFP/YFP^+ colonies were scored 14 days after plating by fluorescence microscopy.

Flow cytometric analysis of canine *ex vivo* cultures and peripheral blood

For *ex vivo* studies, transduced and untransduced CD34-enriched cell populations were maintained in liquid culture (IMDM 10/1 supplemented with 50 ng/mL each of cG-CSF, cSCF, hMDGF, hFlt3-L) and analyzed 4 and/or 10 days after transduction. Canine peripheral blood was analyzed by flow cytometry every week after transplantation, starting as early as 9 days post transplantation. If gene marking decreased below the level of detection by flow cytometry, then routine analysis was suspended. Gene marking (GFP and/or YFP) was assessed in peripheral blood subsets including lymphocytes, granulocytes, platelet and red blood precursors. Cell subsets were distinguished based on forward and side scatter gating and dead cells excluded from analysis by propidium iodide staining (1 μg/mL). Hemolyzed blood was used for white blood cell analysis and anti-coagulated whole blood samples were used for marking of platelet and red blood cell precursors. To distinguish between GFP and YFP subsets in some dogs, BD FACSVantage SE or LSR-II flow cytometers (BD Biosciences) were used. In some cases, gene marking and immunophenotyping were assessed by staining leukocytes with labeled with phycoerythin (PE)-conjugated antibodies to detect lymphoid (CD3, CD21) and myeloid (CD13, CD14, DM5) subsets (all antibodies from Abd Serotec). To track T lymphocyte subsets after stable long-term engraftment was achieved, peripheral blood leukocytes were stained with the following antibodies: Allophycocyanin (APC)-conjugated rat anti-dog CD4, eFluor660 conjugated mouse anti-dog CD25 (eBioscience Clone P4A10), mouse anti-dog CD3 (followed by PE-conjugated goat anti-mouse IgG secondary antibody, Dako), and PE-conjugated rat anti-dog CD4 and CD8 (all primary antibodies were from Adb Serotec unless

otherwise indicated). In some experiments, leukocytes were also stained with APC-conjugated anti-human FoxP3 antibody (Biolegend Clone 150D) after fixation and permeabilization of cells according to the manufacturer's protocol.

qPCR analysis of canine peripheral blood

GFP marking in peripheral blood samples was assessed by qPCR during gene-modified cell engraftment and MTX administration as described [6,17].

Sheep red blood cell administration

To induce immune activation, sheep red blood cells (SRBC) were administered to the dogs as previously described [19]. After three treatments with SRBCs, each treatment separated by several months, dogs received a fourth SRBC treatment, this time followed by MTX treatment regimen on days 1, 3, 6, and 11 after SRBC infusion (see MTX dosing below). Lymphocyte counts, gene marking and T lymphocyte phenotype were assessed by CBC and flow cytometry analyses, respectively.

MTX administration to dogs

Animal G675 was treated with three separate single bolus intravenous infusions of MTX at doses of 200, 400 or 600 mg/m^2. Twenty-four to 48 hours after MTX treatment, leucovorin (3 mg/kg) was administered. Later, G675 was treated with 0.4 mg/kg MTX daily for one week without leucovorin rescue. Dogs H001 and G236 were treated with 0.4 to 0.6 mg/kg of MTX on a days 1, 3, 6, 11 with or without SRBC infusion on day 0. Animal health, CBC and gene marking by qPCR was monitored or assessed during and after MTX treatments.

RESULT

Stable low level gene marking and hematopoietic recovery after engraftment with Tyr22DHFR-GFP-modified hematopoietic cells

In this study, we first wanted to determine whether CD34$^+$ hematopoietic cells transduced with the MTX resistance gene Tyr22-DHFR would give rise to MTXr-lymphocytes *in vivo*. To this end, 4 dogs were transplanted with autologous CD34$^+$ cells transduced with DHFR-GFP lentivirus vectors with or without YFP transduced cells at a low infection multiplicity (Table 1). Despite the moderate levels of DHFR-GFP gene transfer detected *ex vivo* (2–6%), initial gene marking in the peripheral blood *in vivo* was relatively low (<3%) compared to a control animal that was transplanted with GFP and YFP transduced cells (G236). This lower level of gene marking observed in the DHFR-GFP transduced cell recipients, compared to GFP or YFP transduced cell recipients, is likely due less efficient gene transfer caused by the larger genetic cargo of the bicistronic expression cassette. Stable, low level DHFR-GFP gene marking was achieved in two of the four DHFR-GFP transplanted dogs (Table 1 and Figure 1A). Two of the four dogs infused with both DHFR-GFP and YFP transduced CD34$^+$ cells had low gene marking in both GFP and YFP arms after hematopoietic reconstitution, indicating that the low level of gene marking achieved is likely due to inefficient gene transfer into long-term repopulating hematopoietic stem cells (Table 1 animals G670, G977). Consistent with the flow cytometry data, transgene marking by qPCR in animals competitively repopulated with DHFR-GFP and YFP cells (G670, G977) was transient (data not shown). In contrast, gene marking by qPCR for one of the two dogs with long-term engraftment of DHFR-GFP modified cells (G675) ranged between 2 and 6% in peripheral blood mononuclear cell DNA extracts (data not shown). After hematopoietic recovery from myeloablative conditioning and hematopoietic cell transplantation, peripheral blood counts, including neutrophils and platelets, remained in the normal range for all transplanted dogs

(Figure 1B and data not shown). After transplantation, absolute neutrophil counts (ANC) were less that 100 per μL of blood for less than one week (Table 1).

In vivo chemoprotection of DHFR-GFP expressing granulocytes and lymphocytes after methotrexate chemotherapy

For the two dogs that exhibited stable DHFR-GFP gene marking *in vivo*, we next wanted to determine whether gene-modified peripheral blood subsets were protected from methotrexate chemotherapy. First, we assessed the effect of high-dose, single bolus injections of MTX on DHFR-GFP expressing white blood cells (animal G675). Single doses of MTX transiently increased GFP marking in granulocytes and lymphocytes after the first and second MTX treatments (200 and 400 mg/m^2, respectively; Figure 1A, left panel). The third MTX dose (600 mg/m^2) increased marking 2.5-fold in lymphocytes with no reduction in blood counts. However, there was some evidence of GI toxicity at this dose, despite leucovorin rescue (data not shown). Heightened and variable GI toxicity to MTX prevented us from treating all animals with the MTX treatment regimen. In all cases, while the increase in GFP marking was transient after single high dose injections, peripheral blood counts remained in the normal range during and after chemotherapy administration (Figure 1B, left panel). We next wanted to determine whether a low dose MTX standard treatment regimen (0.4 mg/kg on days 1, 3, 6, 11) without leucovorin rescue would increase gene marking in peripheral blood subsets of animal G675. Although gene marking did not increase after chemotherapy, complete blood counts remained in the normal range and the percentage of DHFR-GFP marked cells was maintained.

Given that this MTX regimen was well-tolerated by G675, we next wanted to determine whether MTX standard treatment regimen would increase GFP marking in H001, an animal which initially had a higher level of DHFR-GFP modified cell engraftment compared to the other transplanted dogs (3% vs. 1%, Table 1). One cycle of 0.4 mg/kg on days 1, 3, 6, and 11 caused a decrease in YFP-expressing granulocytes and lymphocytes shortly after chemotherapy, while the DHFR-GFP expressing cells were unaffected (Figure 1, middle panels). To determine whether we could increase gene marking with a higher dose treatment regimen, cycle 2 of MTX was increased to 0.6 mg/kg (days 1, 3, 6, 11). Following this dose escalation, the percentage of DHFR-GFP expressing granulocytes and lymphocytes nearly doubled (from ~5% to ~10%). In contrast, the YFP marked cells transiently decreased after cycle 2 of chemotherapy. Blood counts remained in the normal range after chemotherapy. In order to show that the transient increase in DHFR-GFP marking was transgene dependent, we next evaluated the effect of a standard MTX treatment regimen (0.6 mg/kg, days 1, 3, 6, 11) on GFP marking in a control animal (G236, engrafted with GFP and YFP transduced cells) in comparison to dog H001 (DHFR-GFP and YFP gene modified cell engraftment). Importantly, while DHFR-GFP marking increased in H001 after cycle 3, the level of GFP and YFP expressing cells both decreased in the control animal (Figure 1, compare middle and right panels). These data show that that the Tyr22-DHFR transgene provides chemoprotection to gene-modified peripheral blood subsets *in vivo*.

Methotrexate-resistant DHFR-GFP activated T lymphocytes are protected from MTX during immune system activation

In order to assess the effect of methotrexate on the survival of MTX-resistant (DHFR-GFP) and MTX-sensitive (YFP) T lymphocytes in the context of immune activation *in vivo*, we next primed the immune system of animals H001 (DHFR-GFP vs. YFP) and G236 (GFP vs. YFP). Animals were primed three times by infusion of sheep red blood cells [19]. Within 1 week after SRBC treatment, CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T lymphocyte counts increased and CD4/CD8 ratios decreased, compared to an untreated control dog and pretreatment lymphocyte counts. After the fourth SRBC infusion, both animals were treated with 0.6 mg/

kg MTX on days 1, 3, 6, and 11 relative to infusion. During and up to three weeks after MTX treatment, the total number of YFP^+ and GFP^+ $CD3^+$ lymphocytes in the control dog (G236) and YFP^+ cells in animal H001 decreased. In contrast, there was a 2-fold increase in the number of $CD3^+$, $CD8^+$, and $CD8^+CD25^+$ DHFR-GFP^+ lymphocytes, while total $CD3^+CD25^+$ lymphocyte counts were maintained during and after chemotherapy in dog H001 (Figure 2A). Importantly, the absolute number of lymphocytes with a regulatory T cell phenotype ($CD3^+CD25^+FoxP3^+$) in the dog [29] were unaffected by MTX chemotherapy (data not shown). In both animals, lymphocyte counts were maintained within the normal range during MTX chemotherapy (data not shown). In summary, DHFR-GFP T lymphocytes with an activated phenotype ($CD3^+CD25^+$) were protected from MTX treatment, while the level of MTX-sensitive gene marked lymphocytes decreased in the context of immune activation.

DISCUSSION

To facilitate translation of MTX resistance gene therapy to an appropriate clinical application, namely chemoprotection of donor T lymphocytes, here we studied Tyr22-DHFR transgene expression in peripheral blood lymphocytes after engraftment of lentivirus transduced canine $CD34^+$ cells in the autologous setting. Although chemoprotection of donor T lymphocytes is more clinically applicable to the preservation of donor immune function immediately after allogeneic HSCT, we initiated these pilot studies in the autologous setting to determine Tyr22-DHFR functionality independent of immunity caused by leukocyte antigen disparity. We have previously shown that lentivirus-mediated Tyr22-DHFR gene transfer into mouse HSCs protects recipients from myelotoxicity during a low-dose daily MTX treatment regimen administered immediately after marrow transplantation [6]. However, the translation of MTX resistance gene therapy to a large animal model with a clinically relevant application has met with several challenges. Several years ago when drug resistance gene therapy was first identified as a useful tool for improving HSCT, barely detectable levels (<0.5%) of DHFR marking and only transient low-level chemoprotection were achieved in dogs [30] and rhesus macaques [11,13] after transplantation with transduced autologous hematopoietic cells. Physiologic scale-up to large animal models with the limited options of gene delivery (γ-retrovirus vectors) and cell culture systems available at the time revealed challenges in translating and scaling up a gene therapy protocol from the mouse to a large animal model.

More importantly, the findings in mice and the early large animal studies also strongly suggested that the DHFR/MTX system may be better suited to the protection/selection of highly proliferating cells, such as donor T lymphocytes, in the context of short-term MTX chemotherapy (i.e. post HSCT GvHD prophylaxis) as opposed to the initially proposed application, which was the protection of gene-modified HSC derived progeny long after transplantation (i.e. to protect the bone marrow from MTX toxicity in the context of chemotherapy treatment after leukemia relapse). We anticipate that MTX resistance gene therapy is most appropriate for prevention of donor T lymphocyte elimination during MTX mediated elimination of alloreactive recipient T cells to prevent graft rejection in the context of allogeneic HSCT. Toward testing the feasibility of this application, we therefore initiated studies to determine whether Tyr22-DHFR expression in T lymphocytes is sufficient to maintain gene-modified T cells during and immediately after MTX chemotherapy.

First, we show lentivirus-mediated delivery of Tyr22-DHFR in canine hematopoietic cells results in stable expression. Second, a low level of gene marking (2%) is sufficient to prevent elimination of gene-modified lymphocyte $CD3^+$ $CD25^+$ subsets in a chemotherapeutic regimen that causes a decrease in T cells that do not express Tyr22-DHFR. This finding indicates that a low level of DHFR expression is sufficient to protect

gene modified T lymphocytes from MTX toxicity during a standard treatment regimen used in GVHD prophylaxis (days 1, 3, 6, 11). Importantly, we also show that in the same dog gene-modified activated T lymphocytes ($YFP^+CD3^+CD25^+$) are highly sensitive to the standard MTX treatment regimen, as this subset decreased by 50%, while the gene-modified activated T lymphocytes ($DHFR^+GFP^+CD3^+CD25^+$ and $DHFR^+GFP^+CD8^+CD25^+$) increased despite MTX treatment after immune activation with SRBC treatment. Finally, the level of lymphocytes with a T regulatory phenotype (Tregs) was not affected by MTX administration. Given that lower levels of Tregs detected early (2 weeks) after allogeneic HSCT are more likely to occur in patients that later go on to develop acute GVHD [31], the finding that the MTX dose and regiment tested would allow preservation of both donor and recipient Tregs, implies that MTX treatment will not interfere with Treg-mediated prevention of GVHD [32].

The responsiveness of canine T lymphocytes to MTX chemotherapy in the context of immune activation indicates that Tyr22-DHFR gene transfer into T lymphocytes for infusion in the context of allogeneic HSCT in combination with post HSCT MTX immunosuppression provides an effective method to prevent expansion of alloreactive recipient T lymphocytes, while simultaneously preserving donor T lymphocyte function, which will likely reduce the risk of graft rejection. In summary, we have made significant progress toward establishing a canine model of chemoprotection from antifolate toxicity by DHFR expression in T lymphocyte subsets for the purpose of preventing graft rejection.

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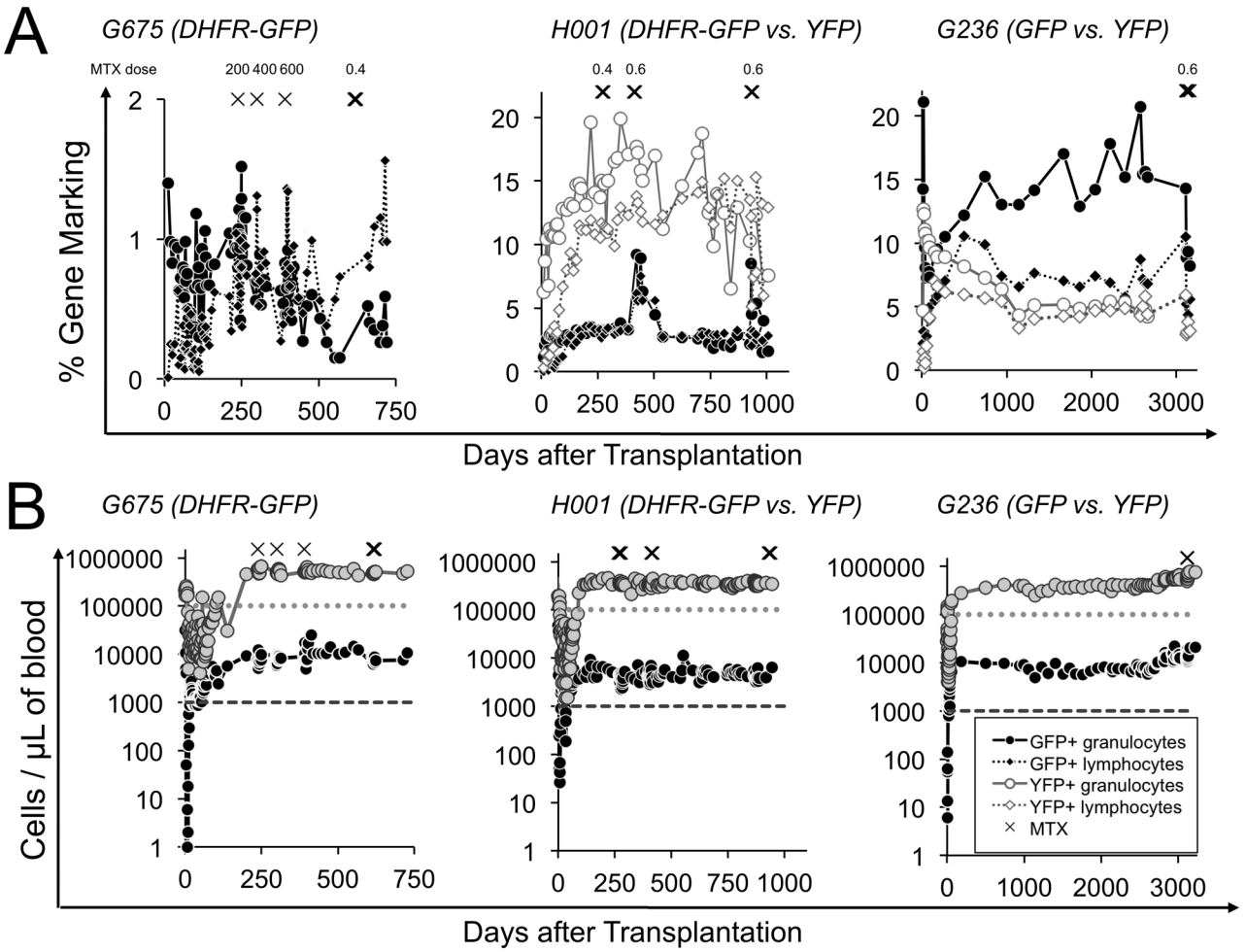

Figure 1. Gene marking and chemoprotection of dogs transplanted with DHFR-GFP or GFP and YFP transduced CD34$^+$ cells

(A) GFP and YFP marking was monitored at regular intervals by flow cytometry in peripheral blood granulocytes (black circles) and lymphocytes (diamond) for the indicated dogs. Open symbols correspond to YFP subsets and closed symbols correspond to GFP subsets. Methotrexate treatments in dogs represented in the top panel are indicated by an 'x'. (B) Hematopoietic reconstitution and chemoprotection of dogs after gene-modified CD34$^+$ cell autologous transplantation. To monitor hematopoietic reconstitution, complete blood counts including neutrophils (black circles) and platelets (gray circles) were evaluated at regular intervals after CD34$^+$ cell transplantation and during MTX chemotherapy. The lower levels (subset thresholds) associated with the normal range are indicated by dashed lines.

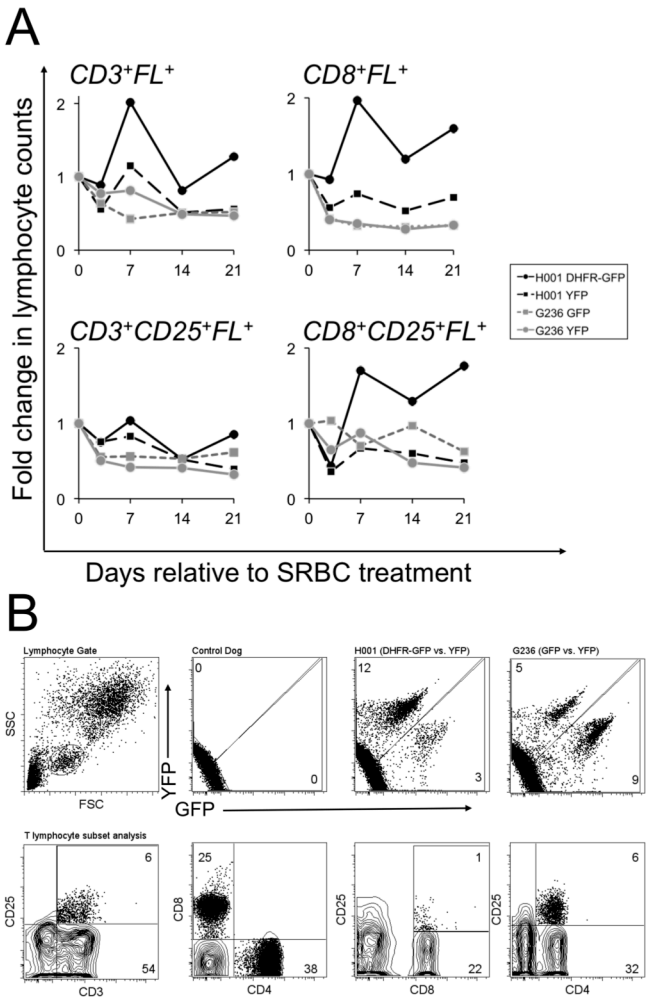

Figure 2. MTX-resistant DHFR expressing T lymphocytes are protected from methotrexate

The immune system of dogs H001 (DHFR-GFP versus YFP) and G236 (GFP versus YFP) was primed by three treatments with SRBCs. After the fourth treatment, animals were treated with 0.6 mg/kg MTX on days 1, 3, 6 and 11 relative to SRBC infusion. (A) Fold change in the absolute lymphocyte counts (ALC) of the indicated GFP and YFP T lymphocyte subsets. The fold change was calculated relative to day 0 ALC (before SRBC or MTX treatment). (B) Representative flow cytometry data. Upper panel: Gene marking in total lymphocytes of a control, H001 and G236 dogs. Lower panel: Representative T lymphocyte subset flow cytometry analysis.

TABLE 1

Summary of lentivirus mediated gene transfer and $CD34^+$ cell transplantation in dogs.

Dog	Lentivirus vector	Transduction (total MOI)	Initial Gene Transfer (%)[a]	$CD34^+$ Cells infused × 10^6/kg	Days ANC <100/μL	Day of Initial Gene Marking Assessment[c]	Initial Gene Marking[d] (%)
G675	DHFR-GFP	6.4	2	2.7	6	64	0.6
G670	DHFR-GFP/YFP	4	6/8[b]	2	2	63	0.02/0.1[b]
H001	DHFR-GFP/YFP	10 × 2	3/16[b]	2.2	5	64	2.1/10.3[b]
G977	DHFR-GFP	7.5	1	1.2	0	64	0.1
G236	GFP/YFP	100	25/26[b]	10	5	79	9.5/7.5[b]

[a] flow cytometry analysis on cells maintained *ex vivo* for 10 days after transduction

[b] %GFP+ cells/%YFP^+ cells

[c] *In vivo* flow cytometry analysis of gene marking on day 60 post transplantation (ANC >100/μL)

[d] Gene marking in peripheral blood white blood cells captured in a single gate established based on FSC and SSC flow cytometry profile to include granulocytes, monocytes and lymphocytes.