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Kinetics of In Vivo Elimination of Suicide Gene-Expressing T Cells Affects Engraftment, Graft-versus-Host Disease, and Graft-versus-Leukemia after Allogeneic Bone Marrow Transplantation

Michael P. Rettig,* Julie K. Ritchey,* Julie L. Prior,†‡ Jeffrey S. Haug,* David Piwnica-Worms,†‡ and John F. DiPersio2*

Suicide gene therapy is one approach being evaluated for the control of graft-vs-host disease (GVHD) after allogeneic bone marrow transplantation (BMT). We recently constructed a novel chimeric suicide gene in which the entire coding region of HSV thymidine kinase (HSV-tk) was fused in-frame to the extracellular and transmembrane domains of human CD34 (ΔCD34-tk). ΔCD34-tk is an attractive candidate as a suicide gene in man because of the ensured expression of HSV-tk in all selected cells and the ability to rapidly and efficiently purify gene-modified cells using clinically approved CD34 immunoselection techniques. In this study we assessed the efficacy of the ΔCD34-tk suicide gene in the absence of extended ex vivo manipulation by generating transgenic animals that express ΔCD34-tk in the peripheral and thymic T cell compartments using the CD2 locus control region. We found that ΔCD34-tk-expressing T cells could be purified to near homogeneity by CD34 immunoselection and selectively eliminated ex vivo and in vivo when exposed to low concentrations of GCV. The optimal time to administer GCV after allogeneic BMT with ΔCD34-tk-expressing transgenic T cells was dependent on the intensity of the conditioning regimen, the leukemic status of the recipient, and the dose and timing of T cell infusion. Importantly, we used a controlled graft-vs-host reaction to promote allograft in sublethally irradiated mice and provide a graft-vs-leukemia effect in recipients administered a delayed infusion of ΔCD34-tk-expressing T cells. This murine model demonstrates the potential usefulness of ΔCD34-tk-expressing T cells to control GVHD, promote allografting, and provide a graft-vs-leukemia effect in man. The Journal of Immunology, 2004, 173: 3620–3630.

Graft-vs-host disease (GVHD)3 remains a major cause of morbidity and mortality after allogeneic bone marrow (BM) transplantation (BMT) (1). Because GVHD is initiated by mature donor T cells present in the graft, it can be significantly reduced by pretransplant T cell depletion (TCD) (2). However, such depletion procedures fail to improve the overall survival of transplanted recipients because of increased rates of graft failure, opportunistic infections, EBV-associated lymphoproliferative disorders, and disease relapse (2). These complications arising from TCD have demonstrated the crucial role mature donor T cells have in facilitating engraftment and providing graft-versus-infection and graft-versus-leukemia (GVL) effects after BMT.

One approach to preserve the beneficial effects of allogeneic donor T cells in BMT is to genetically modify the T cells with a drug-inducible suicide gene that can be selectively activated should GVHD develop (3). Both preclinical (4–15) and clinical (16, 17) studies have demonstrated that allogeneic GVHD can be eliminated by in vivo administration of the nucleoside analog ganciclovir (GCV). Recently, we (18) and others (19, 20) developed new chimeric suicide genes by fusing HSV-tk to the extracellular and transmembrane domains of human CD34 (ΔCD34-tk). The ΔCD34-tk chimeric suicide gene strategy offers two distinct advantages over previously used selection systems in HSV-tk/GCV suicide gene therapy of GVHD. First, ΔCD34-tk-modified cells can be rapidly and efficiently selected using a well-established and clinically approved CD34 immunoselection technique (21). Second, in contrast to the previously used dual promoter or internal ribosome entry sequence-based HSV-tk expression systems (16, 17, 22, 23), the ΔCD34-tk fusion gene strategy ensures the expression of the suicide gene in all CD34-selected cells. Because the ΔCD34-tk chimeric suicide gene appears to be an attractive candidate as a cell surface marker/suicide gene in man, in this study we assessed the efficacy of the ΔCD34-tk suicide gene in a murine model of allogeneic BMT. Importantly, we found that murine GVHD could be mitigated by ΔCD34-tk/GCV suicide gene therapy. The optimal time to administer GCV was dependent on several variables, including the intensity of the conditioning regimen, the leukemic status of the recipient, and the dose and timing of T cell infusion. These observations provide an important proof of principle for the use of ΔCD34-tk-expressing T cells in allogeneic BMT.
Materials and Methods

**Mice**

BALB/c (H-2^d, CD45.2^+) and C57BL/6 (B6; H-2^b, CD45.2^+) mice were obtained from Taconic Farms (Germantown, NY). Congenic B6 mice expressing the CD45.1 gene were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal care and euthanasia were approved by the Washington University Medical School animal studies committee. For BMT, donors were 6–12 wk of age, and recipients were 6–8 wk of age.

**Reagents**

GCV (Cytovene; Roche, Nutley, NJ) was dissolved in double-distilled water and stored in 50 mg/ml aliquots at −20°C. Before use, GCV was thawed and diluted to 5 mg/ml in a 5% dextrose saline solution. GCV was administered i.p. at a dose of 50 mg/kg/day.

**Generation of ΔCD34-tk transgenic mice**

The plasmid pCR2.1-ΔCD34-tk(wt) has been described previously (18). The ΔCD34-tk gene was excised from pCR2.1-ΔCD34-tk(wt) with EcoRI and ligated to pgG-CD2 (24) (provided Dr. T. Enver, Institute of Cancer Research, London, U.K.) to generate pgG-CD2-ΔCD34-tk. A KpnI/NheI fragment of pgG-CD2-ΔCD34-tk was injected into (B6×C3H)F1 blastoocytes. Two positive founder lines were established, and the line expressing the highest level of the ΔCD34-tk transgene was backcrossed (F16–F18) onto B6 mice to obtain progeny for these studies.

**Immunomagnetic selection of T cells**

Splenocytes were obtained by disrupting splenic capsules with the blunt end of a syringe, and erythrocytes were removed by hypotonic lysis with 154 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA. Transgenic (Tg) T cells expressing ΔCD34-tk were positively selected as previously described (18). Non-Tg T cells were negatively selected using FITC-conjugated mAbs to CD11b, B220, and Gr-1. Positively selected using FITC-conjugated mAbs to CD11b, B220, and Gr-1.

**In vitro sensitivity to GCV**

Flat-bottom, non-tissue culture-treated, 96-well plates (Falcon; BD Biosciences, Franklin Lakes, NJ) were precoated with 0.07 μg of anti-CD3 mAb and 0.08 μg of anti-CD28 mAb (BD Pharmingen). Cells (5×10^6) were cultured in triplicate at 37°C for 5 days in phenol red-free RPMI 1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 10 mM nonessential amino acids, 10 mM sodium pyruvate, 20 mM L-glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and increasing GCV concentrations. Cell viability was determined using an XTT kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions.

**Immunogenicity of ΔCD34-tk**

Immunoselected Tg (CD45.2^+) and non-Tg (CD45.1^+) T cells from B6 mice were labeled with CFSE as described by others (25). Unconditioned B6 recipient mice (CD45.1^+) were immunized by three injections of a 1:1 mixture of the CFSE-labeled T cells and non-Tg B6 cells within 24 h. Delayed donor lymphocyte infusions (DLI) were injected i.v. at the indicated times post-BMT. Recipients were monitored daily for survival, and total body weights were recorded every 3–4 days.

**Construction of luc-eGFP (luciferase-enhanced GFP) fusion gene**

Luciferase was modified from pFR-Luc (Stratagene, La Jolla, CA) by QuikChange site-directed mutagenesis (Stratagene) using primers (forward, 5′-GGCGGAAAAGCTTGAATTCATGCTAATGCTACTGGAATCTAGCGG-3′ and a complimentary antisense primer) that eliminated the stop codon of the luc gene and introduced a BanHI restriction site. The modified cat-luciferase pFR-Luc was then digested with KpnI and BanHI, and the insert was ligated to the pEFGP-N1 vector (BD Clontech, Palo Alto, CA) to generate the pLuc-EGFP-N1 vector.

**Leukemia model**

BALB/c-derived A20 B cell lymphoma cells (American Type Culture Collection, Manassas, VA) were transfected with the pLuc-EGFP-N1 vector using the Lipofectamine Plus protocol (Invitrogen Life Technologies) and selected with 1 mg/ml G418. Stable transfectants expressing G418 were steriley selected using a MoFlo cell sorter (DakoCytomation, Fort Collins, CO), and a single high expressing clone (A20-luc/eGFP) was expanded. BALB/c recipient mice were conditioned with 900 cGy of TBI and reconstituted with TCD BM (4×10^6 cells) with or without added A20-luc/eGFP cells (1×10^6 cells) within 24 h. Donor lymphocytes (2×10^6 Tg T cells) were administered day 10 post-BMT. Mice were considered leukemic if they exhibited luciferase activity significantly greater than background (see below), lower limb paralysis, or macroscopic signs of tumor at autopsy.

**Bioluminescence imaging (BLI) of animals**

A20-luc/eGFP tumor growth and distribution were assessed noninvasively by BLI with an IVIS CCD camera ( Xenogen, Alameda, CA) as previously described (26, 27). Briefly, mice were injected i.p. with luciferin (150 μg/ml) and imaged after 10 min later with the IVIS (1- to 60-s exposure; binning 8; f-stop 1; field of view, 15 cm). Anesthesia was induced and maintained during imaging by vaporizer delivery of 2–2.5% isoflurane. Total photon flux (photons per second) was quantified on images using a rectangular region of interest encompassing the entire abdomen and thorax. The leukemic status of all surviving animals was evaluated 30–50 days after DLI by BLI, and mice were considered leukemic if they exhibited luciferase activity greater than TCD BM-only controls.

**FACS**

Single-cell suspensions from peripheral blood or spleen were stained on ice for 30 min with FITC-, PE-, or PerCP-conjugated mAbs against CD3, CD4, CD8, B220, CD11b, Gr-1, pan-NK, H-2K^d, H-2K^b, or CD45.2 (BD Pharmingen). Cell surface expression of the ΔCD34-tk chimeric suicide gene was evaluated using PE-conjugated anti-human CD34 Abs (CD34 Pool-PE; Immunotech, Marseilles, France). Control staining was performed using a PE-conjugated isotype control Ab. Cells were analyzed on a FACSscan cytometer (BD Biosciences, Mountain View, CA).

**Statistics**

Kaplan-Meier survival curves were compared using the log-rank test. Comparison between groups was performed using unpaired Student’s t test. All statistical analyses were performed using StatView software (Abacus Concepts, Berkeley, CA).

**Results**

**Characterization of ΔCD34-tk transgenic mice**

Previous studies by others have shown that ex vivo manipulation of T cells compromises their in vivo function (12, 28–30). Therefore, to assess the efficacy of the ΔCD34-tk suicide gene in the absence of extended ex vivo manipulation, we generated Tg animals in which ΔCD34-tk was targeted to the peripheral and thymic T cell compartment using the CD2 locus control region. Greater than 95% of peripheral blood CD3^+ T cells expressed the ΔCD34-tk transgene, with equal expression in both the CD4^+ and CD8^+ subsets (Fig. 1A). In contrast, no ΔCD34-tk expression was observed in B220^+ B cells, Mac-1^+ macrophages, Gr-1^+ myeloid cells, Pan-NK^+ NK cells, or c-Ki^+ lineage marker−/low, Sca-1^+ hemopoietic stem cells (Fig. 1A and data not shown). Similar to Tg mice expressing human CD34 under the control of the CD2 locus control region (24), we observed no alterations in the development, maturation, CD4 and CD8 subset composition, or distribution of Tg T cells. In addition, the Tg mice exhibited normal life spans, with no evidence of leukemia in >500 evaluable animals. Importantly, the ΔCD34-tk Tg T cells could be efficiently purified to...
GCV in vitro. Demonstrates that cycling H9004B using a human CD34 cell isolation kit (Fig. 1/H11022C, Unconditioned magnetic cell separator (Miltenyi Biotech). The percentage of CD34-expressing cells in the isolated Tg T cells (positive sort) was determined by FACS. The Tg mice were labeled with a PE-conjugated Ab to human CD34. Samples were then split and labeled with FITC-conjugated mAbs specific for T cells (CD3, CD4, and CD8), B cells (B220), macrophages (Mac-1), granulocytes (Gr-1), or NK cells (Pan-NK) and analyzed by FACS. The percentage of cells in each quadrant is indicated. B, ΔCD34-tk-expressing T cells from the spleen of a Tg mouse were isolated by CD34 immunoisolation using a VarioMACS magnetic cell separator (Miltenyi Biotech). The percentage of CD34-expressing cells in the isolated Tg T cells (positive sort) was determined by FACS. C, Unconditioned ΔCD34-tk Tg (▼ and ●) and non-Tg (▼ and ○) mice were injected i.p. with a solution of either dextrose (▼ and ▼) or GCV (50 mg/kg/day; ● and ○) for 7 days. The percentage of T cells in the spleens of mice was determined by FACS on the indicated days post-GCV treatment (n = 2–3/day). D, Immunelected ΔCD34-tk Tg (●) and non-Tg (○) T cells were stimulated with immobilized anti-CD3 and anti-CD28 mAbs for 7 days in the presence of GCV. Cell viability was determined using an XTT kit, and the absorbance for each well was recorded and expressed as a percentage of the value for control wells with no GCV added. Each point represents the mean ± SD of a single experiment, in which each sample was assayed in triplicate.

>97% using a human CD34 cell isolation kit (Fig. 1B). This observation further demonstrates that murine T cells process the human CD34 molecule similarly to human hemopoietic cells.

GCV sensitivity of ΔCD34-tk Tg T cells

To evaluate the GCV sensitivity of the Tg T cells in vivo under steady state conditions, we treated 12- to 16-wk-old Tg and non-Tg mice with GCV for 7 days and determined the percentage of T cells in the spleen 1–3 wk post-GCV administration by FACS. As shown in Fig. 1C, ΔCD34-tk Tg T cells were largely unaffected by in vivo exposure to GCV. Therefore, similar to normal mice, the majority of mature T cells in Tg mice are in a quiescent state. The nonspecific 10–15% reduction in the percentage of splenic T cells 3 wk post-GCV treatment of Tg and non-Tg mice was most likely caused by the immunosuppressive effects of GCV (31).

To confirm HSV-tk activity in the Tg T cells, ΔCD34-tk-selected spleen cells were activated in vitro with immobilized anti-CD3 and anti-CD28 mAb for 7 days in the presence of IL-2 and increasing GCV concentrations. Non-Tg T cells were largely unaffected by GCV exposure, except at high concentrations, at which GCV is nonspecifically toxic (Fig. 1D). In contrast, treatment of Tg T cells with GCV resulted in a progressive and selective reduction in the number of viable cells, with >90% of the killing occurring in the presence of 0.1 μM GCV. This observation demonstrates that cycling ΔCD34-tk Tg T cells are highly sensitive to GCV in vitro.

Evaluation of ΔCD34-tk immunogenicity

To evaluate whether ΔCD34-tk was immunogenic, we immunized unconditioned B6 recipient mice with three injections of a 1/1 mixture of CFSE-labeled T cells from Tg and non-Tg B6 mice. Peripheral blood samples were collected at different time points after immunization, and the percentage of CFSE-labeled cells was assessed by FACS (Fig. 2A). We observed no difference between the in vivo persistence of the Tg and non-Tg T cells in two of the three immunized mice (Fig. 2B, mice 1 and 2). In contrast, Tg cells disappeared rapidly after the second infusion of cells in mouse 3. However, these cells were not rapidly eliminated after the third injection of cells into mouse 3 (Fig. 2B). Therefore, this mouse must not have generated an immune response to the ΔCD34-tk protein. Furthermore, splenocytes obtained from the vaccinated mice did not show any ΔCD34-tk-specific cytotoxicity, indicating the absence of cytotoxic T cell responses to the ΔCD34-tk protein (data not shown). These results suggest that the ΔCD34-tk protein is not immunogenic in B6 mice.

ΔCD34-tk Tg T cells induce lethal GVHD

To determine whether ΔCD34-tk Tg T cells retain their GVHD potential, BALB/c recipients were lethally irradiated (900 cGy) and given TCD BM supplemented with either Tg or non-Tg purified T cells. Untreated animals receiving 5 × 10^7 or 2 × 10^6 Tg T cells died of GVHD in a T cell dose-dependent manner, with
median survivals of 77 days (Fig. 3A) and 23 days (Fig. 3B), respectively. Consistent with GVHD, untreated mice lost >30% of their pretransplant body weight (Fig. 3, C and D), were complete donor chimeras (Fig. 3, E and F), and exhibited impaired lymphoid reconstitution (Fig. 3G and data not shown). Importantly, mice transplanted with 2 × 10^6 non-Tg T cells exhibited weight loss and overall survival similar to the Tg T cells (Fig. 3, B and D). These data indicate that expression of the ΔCD34-tk suicide gene in murine T cells does not alter their ability to induce lethal GVHD in a fully MHC-mismatched BMT model.

Increased survival of 900 cγy-conditioned mice treated with GCV after BMT with ΔCD34-tk Tg T cells

To evaluate the ability of GCV to prevent GVHD after BMT with ΔCD34-tk-expressing T cells, we administered GCV beginning 1, 4, or 10 days post-BMT. GCV treatment significantly prolonged the survival of all groups compared with untreated mice (Fig. 3, A and B). Animals treated from days 1–7 had an overall survival rate of ~85% (Fig. 3, A and B), gained weight at a rate comparable to controls receiving BM alone (Fig. 3, C and D), remained mixed chimeras (Fig. 3, E and F), and exhibited normal lymphoid recovery (Fig. 3, G and H). We observed a similar prevention of GVHD when GCV was administered from days 1–3 or 1–5 post-BMT (Fig. 3, B, D, F, and H). Importantly, mice transplanted with non-Tg T cells and treated with GCV from days 1–7 post-BMT were not protected from GVHD. Taken together, these results indicate that GVHD initiated by allogeneic T cells expressing the ΔCD34-tk transgene is prevented by the early addition of GCV.

Although there were no statistically significant differences in survival between the different GCV administration schedules after conditioning with 900 cGy (Fig. 3, A and B), delaying GCV administration until day 4 or 10 post-BMT increased the severity of GVHD in a T cell dose- and GCV schedule-dependent manner. Mice receiving the lower dose of Tg T cells (5 × 10^6 cells) and GCV from days 4–10 exhibited normal body weight recovery (Fig. 3C) and lymphoid reconstitution (Fig. 3G). In contrast, day 4–10 treated mice receiving the higher T cell dose (2 × 10^7 cells) exhibited signs of ongoing GVHD, as evidenced by significantly impaired body weight (Fig. 3D; p < 0.05 from day 20 post-BMT onward compared with BMT-only control). Interestingly, these mice initially exhibited significant weight gain after GCV administration. However, the protective effect of GCV on weight loss was not sustained, as indicated by the >10% loss of pretransplant body weight between days 20 and 35 post-BMT (Fig. 3D). A similar pattern of weight loss was observed in animals treated with GCV from days 10–16 regardless of the T cell dose (Fig. 3, C and D).

At 30 days post-BMT, the percentage of donor T cells that were CD45.2+ (ΔCD34-tk+) in untreated mice transplanted with 2 × 10^6 Tg T cells ranged from 0.6–15.8% and averaged 4.8% (data not shown). Although treatment with GCV decreased this percentage to <1.5%, the numbers of circulating Tg T cells at 30 days post-BMT were too small to accurately evaluate the effectiveness of the different GCV administration schedules to eliminate these cells. Therefore, we killed mice 1 day after the final dose of GCV was administered and determined the percentage of CD45.2+ donor T cells in the spleen by flow cytometry. As shown in Fig. 4, the percentage of Tg T cells in the spleen of untreated mice decreased from 50 to 24% between days 8 and 17 post-BMT. Treatment with a 7-day course of GCV beginning 1, 4, or 10 days post-BMT reduced the percentage of CD45.2+ donor T cells 30-, 4.6-, and 2.4-fold, respectively (Fig. 4). These results confirm that GCV administration can selectively eliminate ΔCD34-tk Tg T cells mediating a GVH reaction and that early administration of GCV is more effective in eliminating cells that cause GVHD.
Early GCV administration prevents donor engraftment in reduced intensity conditioning regimens

Because the intensity of the conditioning regimen affects the severity of GVHD (32), we reduced the TBI dose by increments of 200 cGy and evaluated the abilities of various GCV administration schedules to prevent GVHD and graft rejection after BMT with \( \Delta CD34 \)-tk-expressing Tg T cells. Eight of 14 recipients conditioned with 700 cGy of TBI and transplanted with TCD BM completely rejected the grafts, whereas the six remaining mice all exhibited 25% donor cell engraftment (Fig. 5E). Supplementing the TCD BM with \( 2 \times 10^6 \) Tg T cells facilitated complete donor cell engraftment (Fig. 5E), but all mice died from GVHD (Fig. 5A). Overall engraftment 30 days post-BMT. Results are pooled from three experiments. The Non-Tg + GCV control was performed in only a single experiment in the absence of a Non-Tg only control. Survival and weight change for irradiation controls (XRT only) are shown in A–D. *p < 0.01 compared with mice that received \( \Delta CD34 \)-tk Tg T cells but were not treated with GCV (No GCV control). **p < 0.01 compared with BMT only control. Values of \( p \) in C and D were determined at 30, 60, and 90 days post-BMT.

**FIGURE 3.** GCV treatment mitigates GVHD in lethally irradiated mice transplanted with \( \Delta CD34 \)-tk Tg T cells. BALB/c mice were conditioned with 900 cGy of TBI and reconstituted with TCD B6 BM (CD45.1\(^+\)) supplemented with \( 5 \times 10^5 \) (upper panels; \( n = 8/\text{group} \)) or \( 2 \times 10^5 \) (lower panels; BMT only, \( n = 25 \); no GCV, \( n = 20 \); days 1–7, \( n = 20 \); days 1–5, \( n = 6 \); days 1–3, \( n = 13 \); days 4–10, \( n = 16 \); days 10–16, \( n = 6 \); non-Tg, \( n = 18 \); non-Tg and GCV, \( n = 7 \); irradiation (XRT) only, \( n = 6 \)) purified B6 \( \Delta CD34 \)-tk Tg or non-Tg T cells (CD45.2\(^+\)). Animals receiving \( \Delta CD34 \)-tk Tg T cells were then either left untreated (no GCV) or were treated with GCV (50 mg/kg/day i.p.) as indicated. Animals receiving non-Tg T cells were either left untreated (Non-Tg) or treated with GCV (50 mg/kg/day i.p.) from days 1–7 post-BMT (Non-Tg + GCV). Surviving animals and age-matched unconditioned (No TBI) BALB/c mice were bled at 30 and 100 days post-BMT and analyzed by FACS. A and B, Kaplan-Meier survival curves. C and D, Percent change in pretransplant body weight. E and F, Overall engraftment 30 days post-BMT. G and H, Lymphoid chimerism 100 days post-BMT. Results are pooled from three experiments. The Non-Tg + GCV control was performed in only a single experiment in the absence of a Non-Tg only control. Survival and weight change for irradiation controls (XRT only) are shown in A–D. *p < 0.01 compared with mice that received \( \Delta CD34 \)-tk Tg T cells but were not treated with GCV (No GCV control). **p < 0.01 compared with BMT only control. Values of \( p \) in C and D were determined at 30, 60, and 90 days post-BMT.

**FIGURE 4.** Early GCV administration is more effective in eliminating cells that cause GVHD. Lethally irradiated (900 cGy) BALB/c mice were transplanted with TCD B6 BM supplemented with \( 2 \times 10^6 \) purified \( \Delta CD34 \)-tk Tg T cells. Animals receiving T cells were then either left untreated (No GCV) or were treated with GCV (50 mg/kg/day i.p.) as indicated. Animals were killed 1 day after the final dose of GCV was administered, and the percentage of \( \Delta CD34 \)-tk Tg T cells in the spleen was analyzed by FACS (\( n = 1 \) for untreated; \( n = 2 \) for GCV treated).
treated with GCV from days 1–7 failed to engraft. This failure to engraft was dependent upon the expression of /H9004CD34-tk, because similarly treated mice transplanted with non-Tg T cells fully engrafted and developed lethal GVHD.

Although delayed administration of GCV facilitated engraftment and improved overall survival, mice conditioned with 700 cGy of TBI and treated with GCV from days 4–10, 10–16, or 20–26 exhibited significant weight loss compared with animals receiving BM alone (Fig. 5C; p < 0.01 from days 23–72 post-BMT for day 10–16 GCV-treated group compared with BMT-only control). Similar to mice conditioned with 900 cGy of TBI, this weight loss was progressively more severe as GCV treatment was delayed. It should be noted that the >30% increase in body weight of day 10–16 treated animals between days 50 and 120 post-BMT was primarily due to the death of three mice with severe GVHD. In addition to the weight loss, day 20–26 GCV-treated mice exhibited significantly impaired lymphoid reconstitution (Fig. 5G).

These results further demonstrate that GVHD is not completely prevented after delayed administration of GCV.

Animals conditioned with 500 cGy of TBI failed to engraft unless the TCD BM was supplemented with 2 × 10⁶ Tg T cells (Fig. 5, F and H). Reducing the donor T cell dose 4-fold, to 5 × 10⁵ cells, resulted in graft failure (data not shown). Interestingly, not all untreated mice receiving 500 cGy of TBI and 2 × 10⁶ Tg T cells died from GVHD (Fig. 6B). In fact, compared with mice conditioned with 900 or 700 cGy of TBI, untreated animals irradiated with 500 cGy of TBI exhibited significantly delayed mortality (Fig. 5B; p < 0.0001 compared with other TBI doses) and enhanced body weights (Fig. 5D) after ΔCD34-tk Tg T cell transfer.

The schedule of GCV administration also affected donor cell engraftment in mice conditioned with 500 cGy of TBI. Mice transplanted with ΔCD34-tk Tg T cells and treated with GCV from days 1–7 or 4–10 failed to engraft, whereas all the day 10–16 and

FIGURE 5. Early GCV administration prevents alloengraftment in reduced intensity conditioning regimens. BALB/c mice were conditioned with 700 cGy (upper panels; BM only, n = 16; no GCV, n = 16; days 1–7, n = 17; days 4–10, n = 8; days 10–16, n = 8; days 20–26, n = 8; non-Tg and GCV, n = 8; irradiation (XRT) only, n = 3) or 500 cGy (lower panels; BM only, n = 16; no GCV, n = 17; days 1–7, n = 16; days 4–10, n = 8; days 10–16, n = 8; days 20–26, n = 8; non-Tg and GCV, n = 7; XRT only, n = 3) TBI and reconstituted with TCD B6 BM (CD45.1⁺) supplemented with 2 × 10⁶ purified B6 ΔCD34-tk Tg or non-Tg T cells (CD45.2⁺). Animals receiving ΔCD34-tk Tg T cells were then either left untreated (No GCV) or were treated with GCV (50 mg/kg/day i.p.) from days 1–7 post-BMT (Non-Tg + GCV). Surviving animals and age-matched unconditioned (No TBI) BALB/c mice were bled at 30 and 100 days post-BMT and analyzed by FACS. A and B, Kaplan-Meier survival curves. C and D, Percent change in pretransplant body weight. E and F, Overall engraftment 30 days post-BMT. G and H, Lymphoid chimerism 100 days post-BMT. Results are pooled from two experiments. Data from the irradiation controls (XRT only) are shown in A–F. *, p < 0.01 compared with mice that received ΔCD34-tk Tg T cells but were not treated with GCV (No GCV control). **, p < 0.01 compared with BMT only control. Values of p in C and D were determined at 30, 60, and 90 days post-BMT.
day 20–26 GCV-treated mice had >85% donor cell engraftment (Fig. 5F). Again, the failure of mice to engraft after early GCV treatment was dependent upon the expression of ΔCD34-tk, because all but one mouse transplanted with non-Tg T cells and treated with GCV from days 1–7 post-BMT exhibited complete donor engraftment (Fig. 5F). It is important to note that the predominantly host lymphoid chimerism displayed in Fig. 5H for the non-Tg group treated with GCV is caused by the survival of the single mouse that failed to engraft.

Because reducing the irradiation dose to 500 cGy significantly reduced the severity of GVHD, we failed to observe a significant difference in survival upon treatment of engrafted recipients with GCV (Fig. 5B). However, both day 10–16 and day 20–26 GCV-treated mice exhibited significantly improved body weight recovery compared with the untreated mice (Fig. 5D; p < 0.05 from days 27–64 post-BMT for day 10–16 and day 20–26 GCV-treated groups compared with no GCV control). These results indicate that the severity of GVHD is reduced after delayed administration of GCV to 500 cGy TBI-conditioned mice.

**Prevention of GVHD after delayed DLI of ΔCD34-tk Tg T cells**

Delayed DLI are used to enhance GVL activity and increase the level of donor chimerism after allogeneic BMT (33). To evaluate the effectiveness of various GCV administration schedules to prevent GVHD after delayed DLI, lethally irradiated (900 cGy) BALB/c recipients were given TCD BM and DLI of 2 x 10^6 or 10^7 purified B6 ΔCD34-tk Tg T cells (CD45.2^+) on day 10 (upper panels; BMT only, n = 24; 2e6, no GCV, n = 20; 2e6, days 4–10, n = 13; 2e6, days 10–16, n = 8; 1e7, no GCV, n = 17; 1e7, days 4–10, n = 8; 1e7, days 10–16, n = 7) or day 20 (lower panels; BMT only, n = 21; 2e6, no GCV, n = 15; 2e6, days 4–10, n = 8; 2e6, days 10–16, n = 8; 1e7, no GCV, n = 12; 1e7, days 4–10, n = 8; 1e7, days 10–16, n = 8) post-BMT. Animals receiving T cells were then either left untreated (No GCV) or were treated with GCV (50 mg/kg/day i.p.) as indicated. Surviving animals and age-matched unconditioned (No TBI) BALB/c mice were bled at 30 and 100 days post-BMT and analyzed by FACS. A and B, Kaplan-Meier survival curves. C and D, Percent change in pretransplant body weight. E and F, Overall engraftment 30 days post-BMT. G and H, Lymphoid chimerism 100 days post-BMT. Results are pooled from three experiments. *, p < 0.01 compared with mice that received ΔCD34-tk Tg T cells but were not treated with GCV (No GCV control). **, p < 0.01 compared with BMT only control. Values of p in C and D were determined at 30, 60, and 90 days post-BMT.
higher dose of cells (10^7) on day 20 post-BMT exhibited a median survival of 24 days, similar to day 10 DLI recipients, and lost >20% of their pretransplant body weight (Fig. 6D). However, mice receiving the lower dose of cells (2 × 10^6) on day 20 post-BMT exhibited an overall survival (Fig. 6B) and lymphoid reconstitution (Fig. 6I) similar to those in the TCD BMT-only controls (p > 0.9 compared with BMT-only control). All untreated day 10 and day 20 DLI recipients converted from mixed T cell chimerism to full donor chimerism within 30 days post-DLI (Fig. 6, E and F). In contrast, we observed mixed T cell chimerism and no GVHD in recipients that received DLI 70 days post-BMT (data not shown).

To evaluate the effectiveness of GCV in mitigating delayed DLI-induced GVHD, we treated BMT recipients with a 7-day course of GCV beginning 4 or 10 days post-DLI. Day 10 DLI recipients (2 × 10^6 or 10^7 T cells) treated with GCV from days 4–10 post-DLI exhibited overall survival (Fig. 6A), weight gain (Fig. 6C), and lymphoid reconstitution (Fig. 6G) that were similar to those of the TCD BMT-only controls. Importantly, the level of donor chimerism increased in these mice, indicating that a controlled GVH reaction was achieved (Fig. 6E; p < 0.01 for both T cell doses compared with BMT-only control). Delaying GCV administration for 10 days after the day 10 DLI resulted in mortality (Fig. 6A; p < 0.0001 for both T cell doses compared with BMT-only control), weight loss (Fig. 6C; p < 0.01 from day 20 post-BMT onward for both T cell doses compared with BMT-only control), and impaired lymphoid reconstitution (Fig. 6G) similar to those in untreated mice.

In contrast to the day 10 DLI recipients, both GCV administration schedules reduced the severity of GVHD in day 20 DLI recipients infused with 10^7 T cells (Fig. 6, B and D). However, early administration of GCV (days 4–10 post-DLI) to day 20 DLI recipients prevented the conversion to full donor chimerism (Fig. 6F). Taken together, these observations suggest that the optimal GCV administration schedule in a DLI setting using HSV-tk-modified T cells is dependent upon the T cell dose and timing of DLI.

Delayed administration of GCV is required for maintenance of a GVL effect after suicide gene therapy of GVHD

We next evaluated whether delayed DLI of ΔCD34-tk-expressing T cells and treatment with GCV could provide a GVL effect in the absence of GVHD. To induce leukemia, BALB/c mice were lethally irradiated and reconstituted with TCD BM and A20-luc/egfp cells. Ten days after BMT, we administered a DLI of 2 × 10^9 Tg T cells and assessed tumor growth at various time intervals by BLI. Eight of 10 recipients who received TCD BM and A20-luc/egfp cells exhibited tumor engraftment and growth (data not shown). Importantly, GCV had no inhibitory effect on the growth of A20-luc/egfp cells in vivo (data not shown). As previously described by others (39, 40), we observed homing of the A20 cells to the BM, spleen, liver, mesenteric lymph nodes, and spinal cord, with no evidence of luciferase or EGFP immunogenicity (Fig. 7A and data not shown).

In contrast to leukemic recipients who received only TCD BM, we found no evidence of leukemia in untreated mice that received a DLI 10 days after BMT. However, these animals developed GVHD with high mortality (Fig. 7B; p = 0.033 compared with BMT-only control) and significant weight loss (Fig. 7C; p < 0.05 from days 8–65 post-BMT compared with BMT-only control). To evaluate the ability of GCV to prevent this DLI-induced GVHD and maintain a GVL effect, we administered GCV beginning 1, 4, or 10 days post-DLI. As expected, both day 1–7 and day 4–10 treated leukemic animals were protected from GVHD (Fig. 7, C–E). However, 60% of the day 1–7 treated mice and 58% of the day 4–10 treated animals developed leukemia, with an overall survival rate not significantly different than the leukemia controls (Fig. 7B). In contrast, only two of 10 mice developed leukemia if GCV treatment was delayed until day 10 post-DLI (Fig. 7, A and B). Importantly, this GVL effect was obtained in the absence of GVHD, as evidenced by the improved survival (Fig. 7B) and weight gain (Fig. 7B) of the day 10–16 GCV-treated mice compared with the untreated controls (A20+ DLI).

Discussion

In this report we demonstrated, for the first time, that murine GVHD could be mitigated by ΔCD34-tk/GCV suicide gene therapy. To date, clinical trials using HSV-tk-modified T cells to control GVHD in allogeneic BMT have selected gene-modified cells using either the neomycin phospho transferase gene and G418, or ΔLNGFR and immunomagnetic selection (16, 17, 41). Unfortunately, both these selection strategies have disadvantages that limit their clinical usefulness. G418 selection of neomycin phospho transferase-transduced T cells requires prolonged culture periods and impairs T cell alloreactivity (16, 17, 41–43). Although both these limitations were overcome with the development of the ΔLNGFR selection marker (23), this approach is still limited by the independent expression of ΔLNGFR and HSV-tk and the lack of a clinically approved isolation system for ΔLNGFR-modified cells. As mentioned previously, the ΔCD34-tk chimeric suicide gene strategy offers two main advantages over the neomycin phospho transferase and ΔLNGFR selection approaches. First, fusing HSV-tk to ΔCD34 ensures the expression of the suicide gene in all selected cells. Second, ΔCD34-tk-modified cells can be rapidly and efficiently selected using a well-established and clinically approved CD34 immunoselection selection technique (21).

Multiple preclinical studies by others have demonstrated that GCV administration can prevent acute GVHD induced by allogeneic HSV-tk-expressing T cells after myeloablative BMT (4–15). The level of protection from GVHD in these studies has been primarily dependent upon the degree of MHC incompatibility between the donor and the recipient and the GCV administration schedule. In general, early administration of GCV (before day 3) prevented GVHD, whereas delayed GCV treatment reduced the severity of the disease. We observed a similar trend in GVHD protection in this study. Lethally irradiated (900 cGy) mice transplanted with ΔCD34-tk Tg T cells and treated with GCV beginning 4 or 10 days post-BMT exhibited significant and prolonged weight loss and impaired lymphoid reconstitution. This failure of delayed GCV treatment to prevent GVHD is most likely caused by multiple factors, including the inability of GCV to 1) cure recipient tissue (skin, intestine, and liver) damage that occurred before prodrug administration, 2) kill mature donor effector T cells that are not dividing or expressing sufficient levels of HSV-tk at the time of GCV administration, and 3) prevent ongoing tissue damage caused by inflammatory cytokines (IFN, TNF, IL-1, and IL-2) and secondary immune effector cells (i.e., mononuclear phagocytes and NK cells). Whatever the explanation for the failure of delayed GCV treatment to prevent GVHD, the most important observation in this study is that murine GVHD could be mitigated by ΔCD34-tk/GCV suicide gene therapy.

One potential limitation with the use of ΔCD34-tk-modified T cells in allogeneic BMT may be the development of a host immune response to the ΔCD34-tk chimeric protein. Because of its human origin, the ΔCD34 Ag should not be immunogenic. However, some patients have developed CD8-mediated immune responses to HSV-tk (16, 23, 44, 45). Interestingly, the development of immunity to HSV-tk-modified donor T cells has been inconsistent in allogeneic transplant recipients. In one study, eight of 24 patients...
Donor lymphocytes (CD45.2/H11001 left untreated (A20/H11001 administered 10 days post-BMT. Animals receiving T cells were then either

GVL effect obtained by delaying GCV administration after delayed DLI was dependent upon the timing of the DLI and the leukemic status of the recipient. In nonleukemic mice, treatment with GCV from days 4–10 post-DLI protected from GVHD. These data further support the need for clinical evaluation of HSV-tk-expressing allogeneic T cells in reduced intensity conditioning regimens.

In contrast to preclinical studies in mice (34–38), GVHD remains the most significant complication after delayed administration of donor lymphocytes in humans (33, 53, 54). Although initial clinical data from the study by Bonini et al. (16) demonstrated the ability of the HSV-tk/GCV suicide gene system to control GVHD after delayed DLI, no preclinical studies have evaluated the effectiveness of various GCV administration schedules to prevent GVHD and preserve a GVL effect after delayed administration of HSV-tk-expressing donor lymphocytes. In this study we found that the optimal GCV administration schedule for prevention of GVHD after delayed DLI was dependent upon the timing of the DLI and the leukemic status of the recipient. In nonleukemic mice, treatment with GCV from days 4–10 post-DLI protected both day 10 and day 20 DLI recipients from lethal GVHD, whereas day 10–16 GCV administration failed to prevent GVHD in day 10 DLI recipients. In contrast, we observed no GVHD in leukemic recipients who were treated with GCV from days 10–16 after a day 10 DLI. Why donor T cells preferentially targeted the A20 cells as opposed to host tissues in leukemic recipients is an interesting question that requires further investigation.

Two recent reports demonstrated that HSV-tk/GCV suicide gene therapy could be used to dissociate GVL activity from GVHD after allogeneic BMT (14, 15). Although the kinetics of GCV treatment required to obtain a GVL effect were slightly different between the two studies, both reports showed that the GVL

indicated in the color scale bars. Mice 1 and 2 in the day 10–16 GCV treatment group had tumor signal above background. Mouse 3 did not exhibit significant tumor signal and is representative of the remaining seven mice in the day 10–16 GCV treatment group. B, Kaplan-Meier survival curve. C, Percent change in pretransplant body weight. D, Overall engraftment 30 days post-BMT. E, Lymphoid chimerism 100 days post-BMT. Results are pooled from two experiments. *p < 0.01 compared with A20 and DLI control. **p < 0.01 compared with BMT only control. Values of p in C were determined at 30, 60, and 990 days post-BMT.
effect was lost if GCV treatment was initiated at or close to the time of BMT. Our results have extended these observations by demonstrating that appropriately timed administration of GCV after delayed DLI of HSV-tk-modified T cells retains a GVL effect while controlling GVHD. Importantly, this was the first study to use in vivo BLI to assess disease burden after HSV-tk/GCV suicide gene therapy. Using BLI we found that eight of 10 mice treated with GCV from days 10–16 post-DLI were free of disease. In contrast, ~60% of mice treated with GCV from days 1–7 and 4–10 developed leukemia. This ability to noninvasively and repeatedly image residual disease using each animal as its own control should facilitate the development of therapeutic interventions to treat relapse after suicide gene therapy of GVHD.

In summary, this study demonstrated that ΔCD34-tk expressing Tg T cells function similar to HSV-tk-expressing Tg T cells after allogeneic BMT. This demonstration that the ΔCD34-tk suicide gene is functional in vivo represents an important proof-of-principle for the development of clinical trials evaluating ΔCD34-tk-expressing T cells in allogeneic BMT.

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References


