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Control of graft-versus-host disease with maintenance of the graft-versus-leukemia effect in a murine allogeneic transplant model using retrovirally transduced murine suicidal lymphocytes

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Abstract

Objective—Limited clinical trials have validated the hypothesis of controlling graft-versus-host disease (GVHD) arising from stem cell transplant utilizing suicidal T-lymphocytes that have been transduced to express the *HSV-TK* gene. However, clinical utility has been limited by diminished T-cell function arising from the production process. To evaluate strategies for harnessing the graft-versus-leukemia (GVL) effect while improving the safety and function of suicidal lymphocytes, we have developed techniques to produce fully functional, retrovirally transduced, *HSV-TK*–positive murine T cells (TK^+TC).

Methods—Utilizing a murine major histocompatibility complex–matched transplant model, we evaluated the ability of TK^+TC to generate a GVL effect and the ability to control GVHD in experiments where we varied the dose of TK^+TC, ganciclovir (GCV) dose, the start of GCV administration (day 4, 7, 10, 13, 15, or 19) posttransplantation, and the GCV administration route (osmotic pump versus intraperitoneal).

Results—At TK^+TC doses in excess of the standard lethal dose (SLD) of unmanipulated T-cells, GCV administration completely (2 × SLD) and partially (4 × SLD) controlled GVHD. Additionally, GVHD remained reversible despite delaying administration of GCV for a week after GVHD developed. Importantly, GVHD was controlled with a 1-log but not 2-log reduction in GCV dose, and this “partial suicide” preserved more circulating TK^+TC compared with standard-dose GCV. Survival of leukemia-positive mice receiving TK^+TC and GCV was significantly increased compared with control cohorts not receiving GCV or transplanted with unmanipulated T cells, thereby demonstrating a GVL effect.

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Conclusion—Retrovirally transduced suicidal lymphocytes generate a potent GVL effect while simultaneously enabling control of GVHD, which results in improved leukemia and GVHD-free survival.

The risk of graft-versus-host disease (GVHD) in patients with leukemia who undergo allogeneic bone marrow transplantation necessitates finding a suitably matched donor so that these patients can benefit from the graft-versus-leukemia (GVL) effect [1,2]. However, the inability to identify such donors is a barrier to transplantation for most leukemia patients. Although both prophylactic therapeutic strategies and strategies to manipulate the graft are used to prevent GVHD, as many as 40% of patients who receive HLA-matched transplants develop GVHD that requires systemic therapy [2,3]. The interventions required to treat GVHD further compromise an already impaired immune system, and fatal infections are common during GVHD therapy. Consequently, the risks associated with GVHD decrease the curative potential of allogeneic transplantation by either preventing patients from undergoing transplantation in the first place or complicating the course of those who do.

Selectively removing the GVHD-initiating T cells after grafts are established and T cells have been activated might control GVHD with less toxicity than is possible with the current immunosuppressive GVHD therapies. The suicidal lymphocyte strategy attempts to do this by rendering the GVHD-initiating T cells susceptible to controlled killing by the ex vivo introduction of a suicide gene [4–6]. Should these T cells then initiate GVHD, a prodrug is administered, and the suicide gene-bearing T cells are induced into suicide, thereby stopping the GVHD.

Numerous groups, including ours, have demonstrated the ability of retroviruses to stably transduce the herpes simplex virus thymidine kinase (*HSV-TK*) gene into T lymphocytes ex vivo [6–10]. The resultant *HSV-TK*–transduced lymphocytes convert the prodrug ganciclovir (GCV) to the toxic metabolite GCV-triphosphate [11], which interferes with DNA and RNA transcription, culminating in cell death [12]. This strategy has been used in the clinic, and several small trials have provided proof of this concept, with results showing reduced GVHD after the administration of ganciclovir [5,13] in the setting of either donor lymphocyte infusion [5,14–17] or T-cell add-back to T-cell–depleted marrow at the time of the initial transplantation [13]. However, lower than expected rates of GVHD and GVL were observed in these trials, suggesting that the ex vivo manipulations required to produce the T cells diminished their alloreactivity, possibly because of alterations in the composition of the lymphocyte subsets [10], prolonged culture duration and antibiotic selection [18–21], or the detrimental effects of exposure to high-dose interleukin (IL)-2 [7,18,22]. Nevertheless, the use of suicidal lymphocytes remains a promising strategy, and investigators in both the United States and Europe [23] have second-generation clinical trials already accruing patients or near to opening.

To support the development of suicidal lymphocytes and to guide their optimal use, animal models are required. Although several groups have used transgenic mice for the expression of the *TK* gene in every cell [24–28], the TK^+ T cells that were transplanted in their studies were not subjected to the stimulation and culture required to generate retrovirally transduced

HSV-TK–positive T cells (TK^+TC) and were therefore not representative of retrovirally transduced, cultured T cells.

We successfully generated TK^+TC using a production process identical to that used in clinical trials [29] and demonstrated that these TK^+TC survive infusion to cause a GVHD similar to that caused by unmanipulated T cells. Additionally, we showed that early GCV administration preempts the emergence of GVHD [29] in a murine major histocompatibility complex (MHC)–matched B10.BR → AKR/J; $H2^k \rightarrow H2^k$ transplant model. This series of experiments was designed to determine the optimal timing of suicide induction relative to GVHD development; the possibility that a maximally tolerated dose (MTD) of TK^+TC exists in the mouse model, beyond which GVHD is not controllable; the degree to which lymphocyte suicide must occur to suppress GVHD; and the capacity of these heavily manipulated cells to generate a GVL effect.

Materials and methods

Production of HSV-TK$^+$ lymphocytes

Retrovirally transduced murine lymphocytes were produced and selected as previously described using stimulation with Concanavalin A (Sigma, St. Louis, MO, USA) at 2.0 g/mL plus 500 U/mL of IL-2 (Chiron Pharmaceuticals, San Diego, CA, USA) [29]. Transduced $LNGFR^+TK^+$ lymphocyte populations were composed of greater than 90% $LNGFR^+$ cells, and yields were typically three to four $LNGFR^+TK^+$ lymphocytes for each input splenic T cell.

Selection of LNGFR$^+$ murine lymphocytes (TK^+TC)

Forty-eight hours after the final retroviral infection, viable murine lymphocytes were isolated using a Ficoll-Hypaque gradient, washed twice, pelleted, and reacted with anti-LNGFR antibody at a concentration of 5 μg per 10^7 cells. This reaction was performed in AIM-V (no serum) at room temperature. After 30 minutes, the cells were washed once with ice-cold phosphate-buffered saline and reacted with an anti-mouse immunoglobulin G–microbead (Miltenyi Biotech, Auburn, CA, USA), and selected on Miltenyi Vario MACS columns following the manufacturer's protocol. Transduced $LNGFR^+TK^+$ lymphocyte populations were greater than 90% pure cells and yields were typically three to four $LNGFR^+TK^+$ lymphocytes for each starting T cell.

Murine allogeneic transplant model

As previously described [29], an MHC-matched murine transplant model where splenic lymphocytes and marrow from congenic B10.BR (male) donors are transplanted into lethally irradiated (9 Gy) AKR/J (female) recipients was adapted for use with suicidal lymphocytes [30–33]. On the first day of our experiments (day 0), AKR/J mice were injected in the tail vein with 1×10^7 B10.BR bone marrow cells and varying amounts of either fresh unmanipulated control lymphocytes or TK^+TC. Depending on the cohort and the experiment, some mice also received M1 leukemia cells, GCV, or both, as described below.

Dosing of lymphocytes

To determine the MTD of T cells, mice received 2, 4, or 8 × 10^6 naive T cells or TK^+TC. For the GVL experiments, a dose of 4 × 10^6 lymphocytes was used.

GCV administration

GCV (Cytovene; Syntex, Boulder, CO, USA) was administered either intraperitoneally at 2 mg/kg daily (50 μg/d) for 7 days or with an osmotic pump (Alzet, Cupertino, CA, USA) filled with 25 mg/mL of GCV so that each mouse received 5 mg in 200-μL increments over 7 days. The timing of GCV administration varied in different experiments, beginning on day 4, 7, 10, 13, 15, or 19 posttransplantation. In the experiments testing a partial suicide effect, the dose of GCV was either 5, 0.5, or 0.005 mg administered over 7 days via the pump.

GVL experiments

AKR/J mice can spontaneously develop leukemia, and a cell line derived from an AKR/J mouse named “M1” has been isolated and can be passaged serially from mouse to mouse to generate leukemia. Prior experiments demonstrated that unmanipulated murine lymphocytes can generate a GVL effect against these cells [30–32]. Mice received an intravenous injection of M1 leukemia cells and bone marrow, with or without lymphocytes, on day 0. To determine the dose of M1 leukemia cells that could be controlled by unmanipulated lymphocytes, mice were given transplants as described above of 2 × 10^6 or 4 × 10^6 unmanipulated lymphocytes and 25, 50, 100, 500, 1000, 5000, or 10,000 fresh or frozen leukemia cells. Mice that died underwent autopsy, and the doses at which they died of GVHD instead of leukemia were selected for further experiments.

Monitoring to detect GVHD

Mice were monitored daily, and their weights were recorded. A murine GVHD scoring system developed by Ferrara and colleagues [34] was used to determine a score based on weight loss, fur texture, posture, activity, and skin integrity (GVHD score). Mice with advanced disease were sacrificed following institutional animal care and use policies and were considered a death in our analyses. Autopsies were performed on all mice that were sacrificed because of morbidity and on mice that died during our experiments, provided that necrosis was not advanced. Mice were evaluated for engraftment, for the presence and severity of GVHD by site (skin, gut, liver), and for the presence of leukemia cells, if appropriate. Given the results of a study [35] showing that the truncated form of the LNGFR might be leukemogenic in mice, we immunohistochemically analyzed mice with long survival times (>60 days) that received transplants of TK^+TC, that did not receive M1 leukemia cells, but that had leukemia on autopsy for the presence of the *TK* or *LNGFR* gene in their leukemia cells.

Intracellular cytokine staining and determination of memory vs unmanipulated T-cell status

B10.BR splenocytes were disaggregated and red blood cells lysed using red blood cell lysing buffer (Sigma). Cells were labeled with biotinylated anti-CD3e antibody (BD Pharmingen, San Jose, CA, USA) and then incubated with streptavidin-coated microbeads

(Miltenyi Biotech). $CD3^+$ cells were separated on a MACS Separator according to the manufacturer's instructions. TK^+TC or $CD3^+$-purified B10.BR splenocytes were incubated at 2×10^6/mL in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma), 1 μM ionomycin (Sigma), and BD Golgistop (0.5 μL per milliliter of medium) for 6 hours at 37°C. Cells were washed and stained for CD4 and CD8. Prior to incubation with anti-cytokine antibodies, cell fixation and permeabilization was carried out using the BD Cytofix/Cytoperm Kit (BD Pharmingen), according to manufacturer's instructions. $CD3^+$ unmanipulated splenic lymphocytes or $LNGFR^+$ purified cells were analyzed for memory cell phenotype using the following antibodies: CD4 fluorescein isothiocyanate (FITC), CD8 FITC, CD44 phycoerythrin, and CD62L allophycocyanin. All antibodies used were acquired from BD Pharmingen. All antibodies used were acquired from BD Pharmingen.

Generation of TK T cells expressing luciferase and bioluminescence imaging

To generate *luc*-expressing TK T cells, murine TK T cells expanded by CD3/CD28D beads (Xcyte Technologies, Inc., Seattle, WA, USA) and selected for LNGR/tk expression were infected with a fiber-modified adenoviral vector expressing firefly luciferase. Briefly, B10.Br murine T cells expressing LNGFR/tk were co-incubated with AdLux-F/RGD (MOI 500) for 4 hours at 37°C, as described elsewhere [36]. Twenty-four hours after infection, cells were visualized in vitro to determine *luc* expression levels and used for subsequent in vivo detection. Luciferase-expressing T cells (4×10^6) were injected intravenously via tail vein into Akr/J mice following whole body irradiation, and additional B10.Br bone marrow (1×10^7) was added to rescue the mice. For noninvasive imaging of intravenously injected TK T cell Lux, mice were anesthetized with 3% isoflurane in 100% O_2. On indicated days, mice were injected with 150 mg/kg of D-luciferin (potassium salt; Xenogen Corp., Alameda, CA, USA) and placed for imaging in the In vivo Imaging System (Xenogen) with total imaging time of 2 minutes. Total body bioluminescence was quantified by integrating the photonic flux (photons per second) through a region of interest drawn around each mouse.

Statistical analyses

Kaplan-Meier survival curves were established for each group [37], and differences in survival times were determined with the log-rank test or the Gehan Wilcoxin test, as noted, using the Statistica 6.0 software package (Stat-Soft, Tulsa, OK, USA). Comparisons of GVHD scores and weights of mice were performed using the *F*-test.

Results

Our experiments addressed the following questions:

1. Does the production process affect the subtype composition or the ability of the transduced cells to respond to stimuli?
2. Does the schedule and route of GCV administration affect the ability to control GVHD?
3. Is there an MTD of TK^+TC beyond which GVHD cannot be controlled by GCV administration?

4. Is full TK^+TC suicide induction required to control GVHD?
5. Can GCV administration reverse GVHD once the disease process has started, or can it merely preempt GVHD from emerging?
6. Can TK^+TC generate a GVL effect?
7. Can the GVL effect be maintained while GVHD is simultaneously controlled by administering GCV?

Effect of the production of TK^+TC on TH1/TH2 cytokine production and memory cell phenotype

The production process required to produce human TK^+TC has been demonstrated to skew the population toward a TH1 subtype, but it is unknown whether similar changes occur during the production of murine TK^+TC. To address this, the ability of stimulated but untransduced $CD3^+$ purified T cells and TK^+TC to produce TH1 cytokines (IL-2 and interferon-gamma [IFNγ]) and TH2 cytokines (IL-4 and IL-10) was measured. In the $CD4^+$ population (Fig. 1A), a significantly higher percentage of the TK^+TC produced IL-2 (61.5% vs 32.7%, $p = 0.046$) and IFNγ (24.7% vs. 7.4%, $p = 0.05$) compared with $CD3^+$ cells, but similar percentages of each produced IL-4 (2.3% vs 0.7%, $p = 0.472$) and IL-10 (3.7% vs 2.1%, $p = 0.61$). In the $CD8^+$ population (Fig. 1B) it again appears that more TK^+TC produce IL-2 (48.1% vs 24.8%), IL-4 (7% vs 0.3%), IL-10 (10.3% vs 0.6%), and IFNγ (61.3% vs 47.5%). However, the only statistically significant difference in the $CD8^+$ population was found in the percentage of cells producing IL-10 (IL-2, $p = 0.053$; IL-4, $p = 0.198$; IL-10, $p = 0.021$; IFNγ, $p = 0.388$). These results indicate that although TK^+TC production results in phenotypic changes to T cells, there is no comprehensive skewing to either a TH1 or a TH2 cell type.

In a similar MHC-matched murine transplant model, memory $CD4^+$ T cells were shown not to induce GVHD [38]. The percentage of T cells in the memory (CD62Llo/CD44hi) and naïve (CD62Lhi/CD44lo) compartments at baseline and after production of TK^+TC was determined. In both $CD4^+$ and $CD8^+$ cell populations, TK^+TC showed an increase in the percentage of memory cells and a decrease in naïve cells compared with CD3-purified cells (Fig. 1C).

Effect of different routes of administration

The pharmacokinetics of GCV in mice are not well described, which raises concerns that the once-daily dosing schedule previously used might be suboptimal and compromise the ability to control GVHD. Continuous infusion of GCV via subcutaneously implanted pumps (Figs. 2B and 3B) proved equally efficacious at controlling GVHD compared with intraperitoneal injection (Figs. 2A and 3A) and was less traumatic, hence all later experiments used osmotic pumps for the delivery of GCV.

Determination of an MTD of HSV-TK^+ lymphocytes

The model developed by Truitt's group [30–33] used a lymphocyte dose of 2×10^6 unmanipulated lymphocytes for GVHD induction. However, other studies have shown that

larger numbers of lymphocytes can facilitate engraftment [39,40] and produce a greater GVL effect [41]. Consequently, we tested higher doses of TK^+TC. As shown in Figure 2A and B, the administration of GCV on days 10 through 16 to mice that had received either 2×10^6 or 4×10^6 TK^+TC led to statistically significant improvements in survival times compared with those of mice receiving either unmanipulated T cells ($p < 0.00001$ for both dose levels) or TK^+TC without GCV ($p < 0.00001$ for both dose levels). Additionally, the survival times of mice receiving either dose level of TK^+TC plus GCV were statistically similar to that of the bone marrow–only controls ($p = 0.15$ for 2×10^6 TK^+TC and $p = 0.66$ for 4×10^6 TK^+TC). The kinetics of the response to GCV administration was rapid, and the GVHD scores of mice receiving TK^+TC and GCV rapidly became indistinguishable from those of mice receiving only bone marrow (Fig. 4A). Control of GVHD was also evident in mice transplanted with HSV-TK^+ T cells that were cotransfected with luciferase as shown in Figure 4B. The signal in mice treated with GCV from days 7 to 13 was significantly less at day 10, midtreatment, and day 19, posttreatment, compared with mice that did not receive GCV. This was true for the signal from the whole mouse, or if individual organs were compared.

Additional experiments were conducted at the highest dose of 8×10^6 TK^+TC (Fig. 2C). Because autopsy results showed that all mice that received unmanipulated lymphocytes at the 2×10^6 or 4×10^6 dose levels died of GVHD, we considered it unnecessary to have a control group that received 8×10^6 unmanipulated lymphocytes. The survival time of mice that received 8×10^6 TK^+TC and GCV was longer than that of mice not receiving GCV, and although their survival outcome was statistically similar ($p = 0.25$) to mice in the bone marrow–only control group, several deaths due to GVHD occurred between days 37 and 58.

The autopsies of the mice that died earlier than 40 days after transplantation with 8×10^6 TK^+TC showed pathologically evident GVHD (skin grade 3 and 4 and gut grade 2 and 2, respectively). In contrast, the autopsies of long-term survivors sacrificed more than 100 days after transplantation showed only grade 1 skin GVHD.

Effect of timing of GCV administration on the ability to control GVHD

Theoretically, the longer that the donor lymphocytes are allowed to persist in the recipient, the greater the potential for a GVL effect. Consequently, it may be desirable to delay the induction of suicide by GCV administration to the latest time possible that would still allow for the control of GVHD and prolong the survival time of mice so it would be comparable to that of the bone marrow–only control groups. Therefore, we tested the effect of starting GCV administration on days 4, 7, 10, 13, 15, or 19 after TK^+TC transplantation on the ability to control GVHD. Our prior studies showed that GCV administration on days 7 through 13, initiated before mice began dying of GVHD, could improve their survival time from GVHD [29]. However, it was unclear from these studies whether GVHD was being preempted or reversed.

In this model, mice begin dying of GVHD at day 10 after TK^+TC transplantation; therefore, delaying the start of GCV until after this time enabled us to determine if GCV administration could reverse GVHD after it was initiated or merely preempt GVHD from developing. We found that administration of GCV as late as day 13 (Fig. 3A) or day 15 (Fig.

3B) after TK^+TC transplantation, when mice were already dying of GVHD, was able to improve the survival time of mice compared with the survival time of mice in either the unmanipulated lymphocyte control group (intraperitoneal route, $p = 0.01$; pump route, $p = 0.002$) or the TK^+TC control group (intraperitoneal route, $p = 0.005$; pump route, $p = 0.001$). Early administration of GCV on day 4 was detrimental to engraftment and survival in comparison to later administration. Lack of engraftment was very rarely observed with other GCV administration schedules. Initiation of GCV administration on day 10 was therefore selected as the preferred time for subsequent experiments.

Effect of GCV dose and duration on the ability to control GVHD

Using lower doses of GCV might still prevent GVHD and simultaneously augment the GVL effect by permitting more lymphocytes to survive suicide induction. To assess whether GVHD could be controlled with lower doses of GCV, reduced GCV doses (0.5 mg or 0.05 mg over 7 days by pump from days 7 through 13 or days 10 through 16) were administered to mice, and outcomes were compared with the results obtained in mice treated with the standard dose. As shown in Figure 5A, a 10-fold reduction of GCV, administered from days 7 through 13, was still able to control GVHD; however, a 100-fold reduction was not. Identical results were obtained with administration of GCV from day 10 through day 16 (data not shown). We also tested whether a shorter course of GCV was sufficient to control GVHD by using a 2-day injection schedule with the standard 2 mg/d dose initiated on days 7, 10, or 13 after transplantation. We found that 2 days of GCV was sufficient to control GVHD (Fig. 5B). The 0.5 mg/wk dose of GCV appeared to preserve the survival of a larger number of circulating TK^+TC on day 21 after transplantation than did the full dose of 5 mg/wk (Fig. 5C). This effect was still present on day 82 after transplantation, with the percentage of circulating $TK^+/LNGFR^+$ cells averaging 81.6% ± 12.9% (SD) in mice (n = 4) that received a 0.5 mg/wk dose of GCV and 59.8% ± 20.6% in mice (n = 5) that received the 5 mg/wk dose.

Ability of TK-positive lymphocytes to generate a GVL effect

As discussed previously, the GVL effect of suicidal lymphocytes used in the previous clinical trials was diminished compared with the results of donor lymphocyte infusion trials using unmanipulated lymphocytes. It was therefore imperative to determine if suicidal lymphocytes could maintain their ability to produce a GVL effect. Additionally, it was important to determine if GVHD could be controlled without losing the GVL effect. To determine if TK^+TC maintained the ability to mount a GVL effect similar to that of unmanipulated lymphocytes, we first had to determine what dose of leukemia cells could be controlled by unmanipulated lymphocytes; therefore, the ability of unmanipulated lymphocytes to control different doses of the AKR/J-derived M1 leukemia was tested. The results of autopsies of mice receiving 50 fresh leukemic cells and 4×10^6 unmanipulated lymphocytes revealed death from GVHD in six of six mice; leukemia was only found in one of six mice. However, mice that received 100 leukemia cells all died of leukemia, as shown by autopsy. These findings define the lethal leukemia cell dose as 100 M1 cells and the unmanipulated lymphocyte controllable dose as 50 M1 leukemia cells.

The ability of TK^+TC to generate a GVL effect was tested in mice that received doses of 50 or 100 leukemia cells, bone marrow, and either TK^+TC or unmanipulated lymphocytes on day 0. The results from three experiments are shown in Figure 6. As with unmanipulated lymphocytes, mice receiving TK^+TC and 100 M1 leukemia cells all died of leukemia (data not shown). However, when mice received 50 M1 leukemia cells, the cohort that received GCV on days 10 through 16 had a significantly longer survival time than did mice with similar transplants that did not receive GCV ($p = 0.002$; Fig. 6).

Among mice that died earlier than 50 days after transplantation, the frequency of the GVL effect, defined as the absence of leukemia on autopsy, was similar to that in mice that received normal lymphocytes (6/11), TK^+TC (6/10), and TK^+TC and GCV (3/5). By comparison all 25 mice receiving bone marrow + 50 leukemia cells died of leukemia (eight of eight on autopsy) with a significantly shorter median survival of 27 days (range 11–48). None of the mice that received TK^+TC, leukemia cells, and GCV and that died beyond day 50 after transplantation had leukemia on autopsy. The GVL effect was also maintained when the reduced dose (0.5 vs 5 mg) of GVL was administered over 7 days.

To determine if the challenge with leukemia cells affected the number of circulating TK^+TC over the long term, we performed fluorescence-activated cytometry analysis for *LNGFR* on the peripheral blood of mice on day 82 and day 134 after transplantation. The percentage of *LNGFR*-positive cells (i.e., TK^+TC) in the circulation was significantly lower on day 82 in four mice that had survived the leukemic challenge than it was in five mice that had received TK^+TC and GCV but no leukemia cells (32% ± 10.5% vs 59.8% ± 20.5%, $p = 0.03$). However, by day 134, this difference had narrowed (Fig. 7).

No evidence of leukemogenicity of LNGFR

In these experiments, there were six long-term survivors that had not received leukemia cells but had leukemia on autopsy. In all cases, immunohistochemical analysis showed that these leukemias were of recipient origin, which suggested that they were the spontaneously arising type of leukemia observed in AKR/J mice and not a leukemia induced by the insertion of the *LNGFR* transgene.

Discussion

Our data demonstrate that suicidal lymphocytes maintain the ability to generate a GVL effect equal to that of unmanipulated lymphocytes. The murine suicidal lymphocyte production process used in this model was designed to avoid the shortcomings that may have contributed to the decreased rates of GVHD and GVL observed in the first generation of suicidal lymphocyte clinical trials in humans [19]. Notably the $CD4^+$ TK^+TC produced statistically higher levels of TH1 cytokines, whereas the $CD8^+$ TK^+TC produced both TH1 and TH2 cytokines at statistically similar frequencies compared with stimulated but untransduced T cells, a result partially different from the shift toward TH1 observed after the production of human TK^+TC [42]. We did observe a shift toward a higher percentage of memory T cells in both the CD4 and CD8 compartments. We are aware of several groups that have clinical trials using suicidal lymphocytes that are close to opening, including a trial of ours that has received Regulatory Activities Committee approval. The implication of our

findings is that suicidal lymphocytes produced under conditions designed to maintain lymphocyte function are more likely to produce a GVL effect in these forthcoming second-generation clinical trials. In contrast, the TK^+TC used in transgenic mouse models, which have all been MHC-mismatched compared with our MHC-matched model, have not undergone any of these manipulations and are therefore not as likely to provide relevant guidance, although these cells have been shown to generate a GVL effect [26–28]. Similar to our findings, and highlighting the difference between transgenic and retrovirally transduced TK^+TC, the administration of GCV administration had to be delayed in one study from that time usually used for optimal control of GVHD until the same period used in our study to successfully control GVHD and maintain a GVL effect [28].

Our second major finding is that it is possible to maintain the GVL effect while simultaneously controlling GVHD. Because GVHD and the GVL effect are likely to arise concurrently in patients, the ability to maintain the latter effect is crucial to the ultimate success of this treatment strategy. In humans receiving donor lymphocyte infusions for recurrent chronic myelogenous leukemia, the appearance of GVHD often occurs earlier than does the disappearance of Bcr-abl positivity, according to the results of polymerase chain reaction. It is unclear whether GVHD precedes the GVL effect or whether they are simultaneous, but the efficacy of the GVL effect takes longer to be manifested.

Next, we demonstrated that it is possible to increase the number of TK^+TC administered compared with unmanipulated T cells and still control GVHD. At a dose four times the lethal dose of unmanipulated T cells, GVHD was still controlled in half the mice. However, the level of control was not as complete as that observed when we used a lower dose of TK^+TC, which suggests that there is a maximally controllable dose of TK^+TC. The reason for an MTD is currently under investigation. One possibility is that the *TK* vector used in this model is known to have a cryptic second splice acceptor site that results in the production of GCV-nonresponsive *TK* transcripts in approximately 10% of transduced cells [4]. Stochastically, as the dose of TK^+TC increases, the number of cells nonresponsive to GCV would also increase. At the 8×10^6 dose, the number of cells nonresponsive to GCV may have exceeded a tolerable threshold. We are currently developing a new vector with this second acceptor site removed to alleviate this potential problem. We observed that the TK^+TC population had more memory $CD4^+$ T cells, which one group has shown to not induce GVHD [38], and fewer unmanipulated $CD4^+$ T cells. Because we see GVHD of similar magnitude with similar doses of TK^+ or unmanipulated T cells, this decrease in unmanipulated $CD4^+$ cells would not explain the MTD on a stochastic basis. The value of giving higher doses of lymphocytes is that engraftment is thereby improved, and the GVL effect may be stronger because of the introduction of a higher number of leukemia-reactive T cells. Thus, the ability to safely increase the number of T cells delivered could improve outcome.

Another means to possibly improve the GVL effect of TK^+TC would be to increase the number of T cells remaining after suicide induction for the control of GVHD. Experiments using lower doses of GCV demonstrated that it was possible to control GVHD with a 1- but not 2-log reduction in the dose. This log-reduction strategy did appear to spare greater

numbers of TK^+TC and to maintain the GVL effect. Experiments are required to determine whether the persistence of a greater number of TK^+TC enhances the GVL effect.

Finally, our experiments demonstrating control of GVHD with only 2 days of full-dose GCV therapy, initiated as late as day 13 after transplantation, show that control can be achieved with shorter courses of GCV. The potential advantage of this would be decreased toxicity from GCV.

In summary, our experiments confirm the ability of suicidal lymphocytes to generate a GVL effect and simultaneously control GVHD in a murine model using retrovirally transduced T cells. We were also able to safely increase the number of delivered T cells and to control GVHD through a partial suicide. These observations will provide guidance for second-generation suicidal lymphocyte trials in the clinic.

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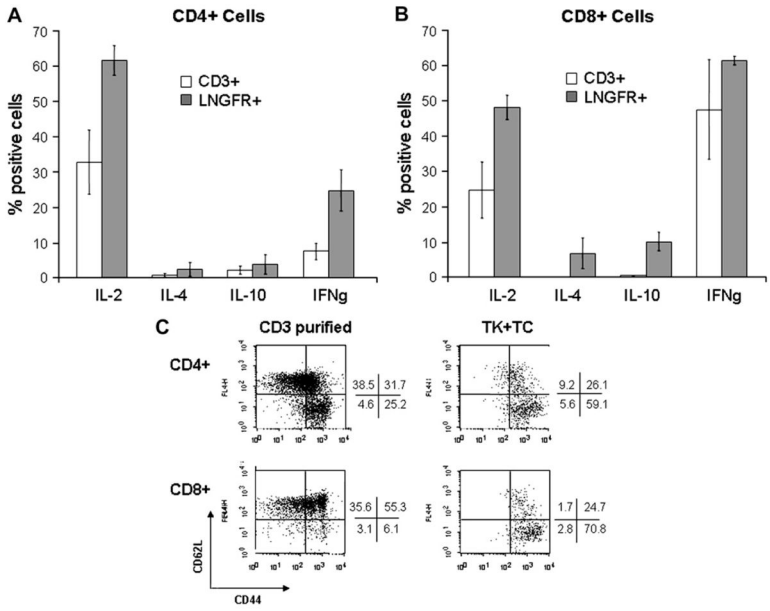

Figure 1.

TH1 and TH2 cytokine expression in CD3 purified and TK^+TC CD4 and CD8 T cells. T cells retrovirally transduced to contain the HSV-TK^+ gene, as described, or CD3 cells purified from splenic lymphocytes were stimulated with 20 ng/mL PMA, 1 μM ionomycin, and BD Golgistop for 6 hours. The percentage of **(A)** $CD4^+$ cells and **(B)** $CD8^+$ cells producing TH1 cytokines; IL-2 and IFNγ, or TH2 cytokines; IL-4 and IL-10 was measured by flow cytometry. A greater percentage TK^+TC were found to produce cytokines compared with CD3-purified cells. However, results were only statistically significant when comparing $CD4^+$ IL-2 levels and $CD8^+$ IL-10 levels. Means and standard errors of three experiments are displayed. **(C):** CD3 purified and TK^+TC $CD4^+$ and $CD8^+$ cells (unstimulated with PMA, ionomycin, or Golgistop) were analyzed for expression levels of surface CD44 and CD62L. $CD4^+$ and $CD8^+$ TK^+TC populations were found to contain more cells expressing a memory phenotype (CD62Llo/CD44hi) than CD3-purified cells. Results from one of three representative experiments are displayed.

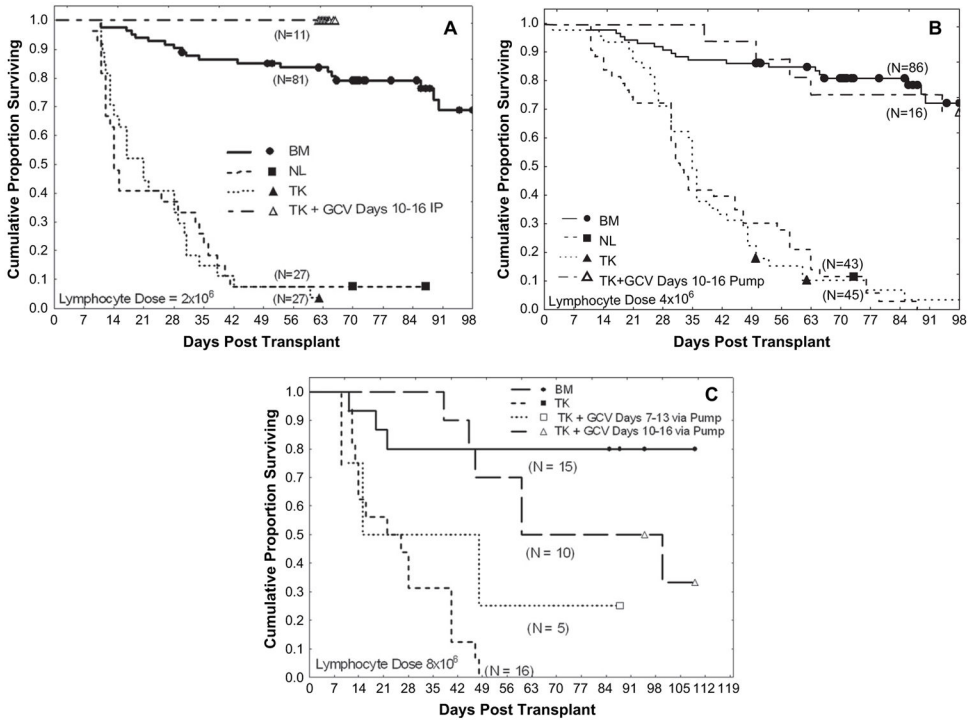

Figure 2.

Ability to control GVHD with GCV after infusion of 2×10^6, 4×10^6, or 8×10^6 TK^+TC. **(A):** Mice were given transplants as described in Materials and methods of 1×10^7 bone marrow cells (BM, ●) alone (to serve as a GVHD control in this model as the marrow has insufficient lymphocytes to initiate GVHD) or bone marrow cells plus 2×10^6 unmanipulated T cells (NL, ■) or TK^+TC (TK, ▲) or TK^+TC followed by intraperitoneal (IP) administration of GCV on days 10 through 16 (TK^+GCV, Δ). Results from several experiments are combined for the three control groups and from two experiments for the TK^+GCV group. The number of mice treated is shown in parentheses by each curve. The survival time of mice receiving either bone marrow only or TK^+TC plus GCV was significantly longer compared with mice receiving either unmanipulated T cells or TK^+TC without GCV ($p < 0.00001$ for all four comparisons). **(B):** Mice were transplanted as described in the Material and methods with 1×10^7 bone marrow cells alone (BM, ●) or along with 4×10^6 unmanipulated T cells (NL, ■), or TK^+TC (TK, ▲) or TK^+TC with administration of GCV via an Alzet pump on days 10 through 16 (TK^+GCV, Δ). Results from several experiments are combined for the three control groups and from two experiments for the group receiving GCV. The number of mice treated is shown in parentheses by each curve. The survival of mice receiving bone marrow only or TK^+TC plus GCV was significantly longer than that of mice receiving either unmanipulated T cells or TK^+TC without GCV ($p < 0.00002$ for all four comparisons). **(C):** Mice were transplanted as described in Material and methods with 1×10^7 bone marrow cells alone (BM, ●) or along with 8×10^6 or TK^+TC (TK, ▲) or TK^+TC with administration of GCV via Alzet pump on days 7 through 13 (TK^+GCV Days 7–13, □) or on days 10 through 16 (TK^+GCV Days 10–16, Δ). Results from several experiments are combined for the three control groups and from one experiment for the groups that received GCV. The number of mice treated is

shown in parentheses by each curve. The survival time of mice receiving TK^+TC + GCV on days 10 through 16 was significantly longer than that of mice receiving TK^+TC without GCV ($p < 0.0001$) and was statistically similar to that of bone marrow only controls ($p = 0.25$). The survival time of mice receiving TK^+TC plus GCV on days 7 through 13 was statistically similar to that of mice receiving TK^+TC without GCV ($p = 0.63$) or those receiving TK^+TC + GCV on days 10 through 16 ($p = 0.17$) but was inferior to that of the bone marrow–only controls ($p = 0.05$).

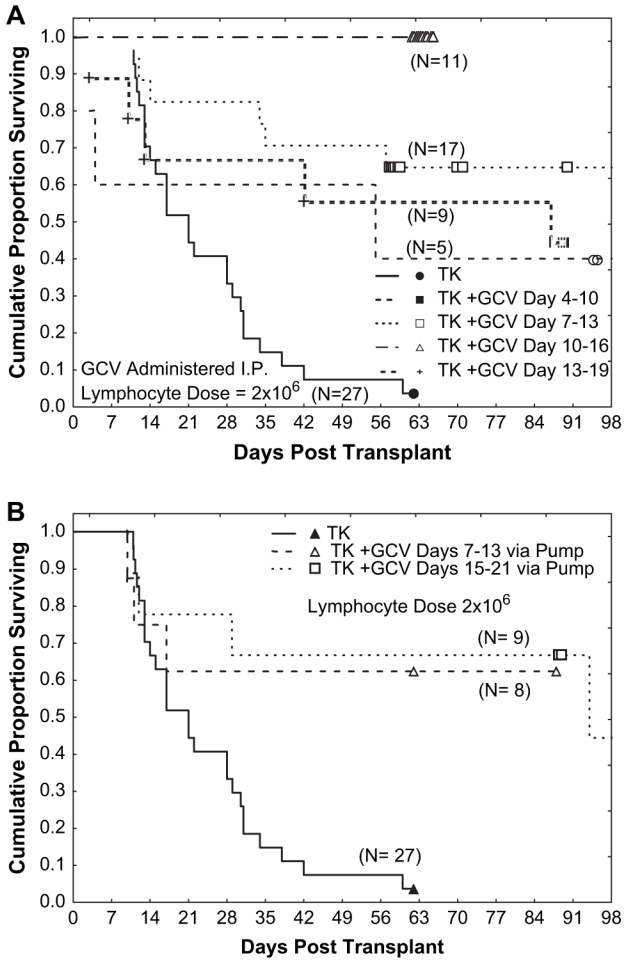

Figure 3.

Effect of route of administration and timing on the ability to control GVHD. **(A):** Mice were given transplants as described in Materials and methods of 1×10^7 bone marrow cells plus 2×10^6 TK^+TC (TK) or 2×10^6 TK^+TC followed by the intraperitoneal administration of GCV daily for 7 days starting on days 4 (○), 7 (□), 10 (Δ), or 13 (+). The survival time of all treated groups was significantly longer than that of the untreated group ($p = 0.005$ [day 4], $p = 0.00002$ [day 7], $p < 0.00001$ [day 10], or $p = 0.005$ [day 13]), even though some mice in the day 13 group had died of GVHD before the initiation of GCV. **(B):** Mice were given transplants as described in Materials and methods of 1×10^7 bone marrow cells plus 2×10^6 TK^+TC (▲) or TK^+TC followed by administration of GCV via an Alzet pump for 7 days beginning on day 7 (Δ) or day 15 (□). The survival time of both treated groups was significantly longer than that of the untreated control ($p = 0.005$ for day 7 and $p = 0.001$ for day 15). For the sake of clarity, the survival times of mice that received either bone marrow alone or unmanipulated lymphocytes are not shown here but are shown in Figure 1. Results from several experiments are combined. The number of mice treated is shown in parentheses by each curve.

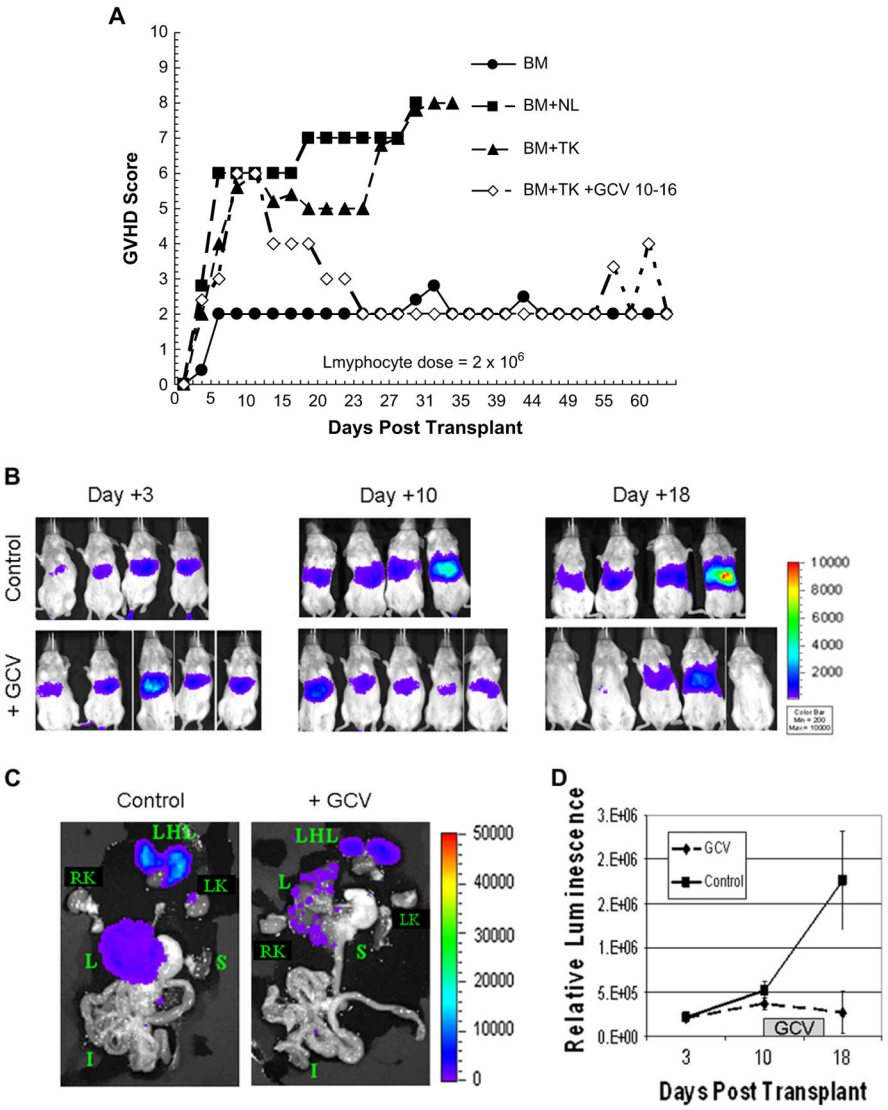

Figure 4.

Control of GVHD by GCV administration using the GVHD score and luciferase imaging. **(A):** Mice were scored for GVHD as described in Materials and methods. The daily score for one of the experiments described in Figure 2 is shown. A rapid decline in the GVHD score is apparent in the cohort treated with $TK^{+}TC$ (TK) that received GCV on days 10 through 16 by intraperitoneal injection. BM, bone marrow; NL, unmanipulated T cells. **(B):** Mice were transplanted as in the other experiments, but with 4×10^6 HSV-TK^{+} T cells cotransduced with fiber-modified adenoviral vector expressing firefly luciferase. Representative control (top row) and GCV-treated days 7 to 13 (bottom row) mice were imaged as described on days 3, 10, and 18, corresponding to early GVHD pre-GCV, mid-GCV, and post-GCV treatment time points and sacrificed on day 19. **(C):** Imaging of the internal organs from two representative mice sacrificed on day 19. Organs are labeled as follows: I, intestines; LHL, lung–heart–lung; L, liver; LK, left kidney; RK, right kidney; S, spleen. **(D):** Relative luminescence of the GCV-treated mice compared with the control mice

at days 3, 10, and 18 is shown. The period of GCV administration is shown by the gray rectangle. Signal intensity of the GCV-treated mice relative to the control mice was 1.97, 0.11, and 0.115 at days 3, 10, and 18 days, respectively, for the whole body images and 0.14 (liver), 0.17 (lungs), 0.26 (spleen) 0.30(kidneys) 0.55(intestines) for the organs.

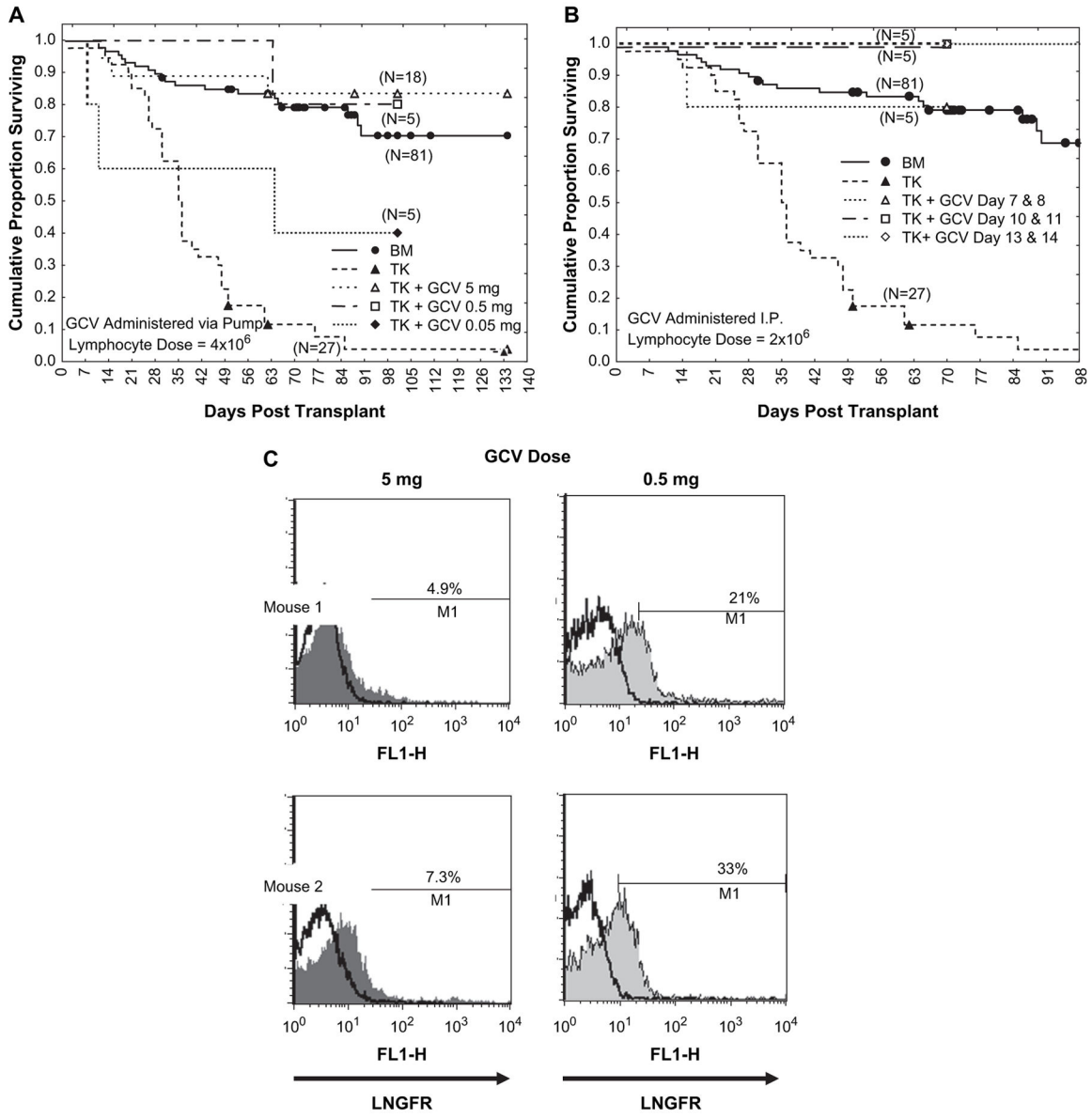

Figure 5.

Effect of GCV dose and duration on ability to control GVHD and the number of circulating TK^+TC. **(A):** Mice were given transplants as described in Materials and methods of 1×10^7 bone marrow cells alone (●) or 1×10^7 bone marrow cells and 4×10^6 TK^+TC, with or without GCV (▲) and administered over 7 days via an Alzet pump from days 7 through 13 after transplantation at a total dose of 5 mg (Δ), 0.5 mg (□), or 0.05 mg (◆). The survival times of groups treated with a total dose of 5 mg or 0.5 mg were significantly longer than that of the untreated group ($p < 0.00001$ and $p = 0.0002$, respectively) and similar to that of the bone marrow–only control group ($p = 0.74$ and $p = 0.76$ respectively). For the cohort receiving a total dose of 0.05 mg, the survival time was similar to that of the untreated control group ($p = 0.18$) and worse than that of the bone marrow–only control group ($p = 0.04$). **(B):** Mice were given transplants as described in Materials and methods of 1×10^7

bone marrow (BM) cells alone (●) or 1×10^7 bone marrow cells with 4×10^6 TK^+TC (TK) without GCV (▲) or with intraperitoneal (I.P.) administration of 2 mg/d of GCV on days 7 and 8 (Δ), days 10 and 11 (□), or 13 and 14 (◇). The survival times of all three treatment groups were significantly longer than that of the untreated control group ($p \leq 0.005$ for all comparisons) and were statistically similar to that of the bone marrow–only controls ($p \geq 0.23$ for all comparisons). **(C):** Approximately 100 μL of peripheral blood was collected on day 21 after transplantation from the tail vein of two mice given transplants as described elsewhere and treated with 5 or 0.5 mg of GCV via an Alzet pump from days 7 through 13 after transplantation. The percentage of circulating cells expressing LNGFR (shaded contour) was assessed by flow cytometry, and the percentage positive cells was determined by comparison with the immunoglobulin G isotype control (unshaded contour).

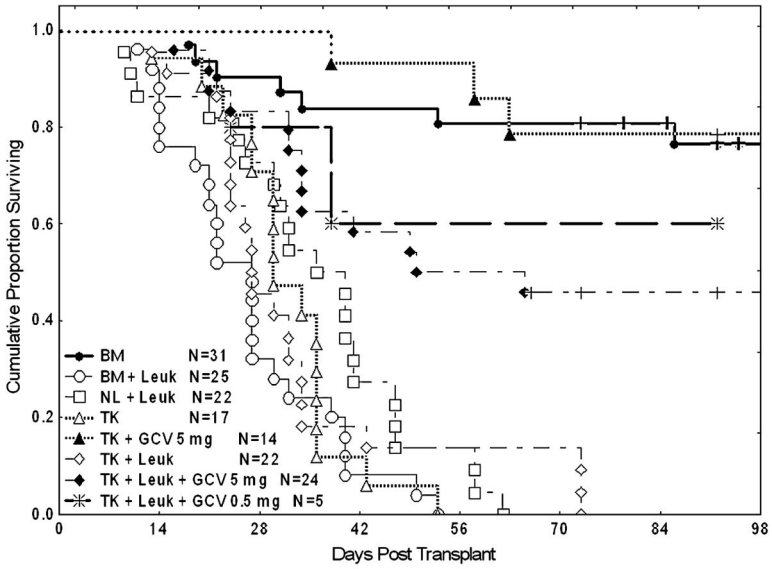

Figure 6.

TK^+TC generate a GVL effect. All mice were given transplants as described in Materials and methods with 1×10^7 bone marrow (BM) cells alone (●) or with 50 M1 leukemia cells (Leuk) (O) or with 4×10^6 normal lymphocytes and 50 M1 leukemia cells (NL + Leuk, □) or with TK^+TC (TK) without GCV (Δ), with GCV administered via an Alzet pump on days 10 through 16 (▲) after transplantation, with 50 M1 leukemia cells (Leuk) (◇) or with 50 leukemia cells and GCV administered via an Alzet pump on days 10 through 16 at a dose of 5 mg/wk (◆) or 0.5 mg/wk (*). Mice receiving TK^+TC, leukemia cells, and GCV had a significantly longer survival times than did mice treated with TK^+TC and leukemia cells without GCV ($p = 0.002$) but significantly shorter survival times than did mice receiving bone marrow alone ($p = 0.02$) or TK^+TC and GCV without leukemia cells ($p = 0.02$). Results from seven experiments are combined. The number of mice treated is shown in stated in the legend. In the TK + Leuk + GCV 5-mg group, two mice are censored at 63 and 73 days and seven others survived >100 days.

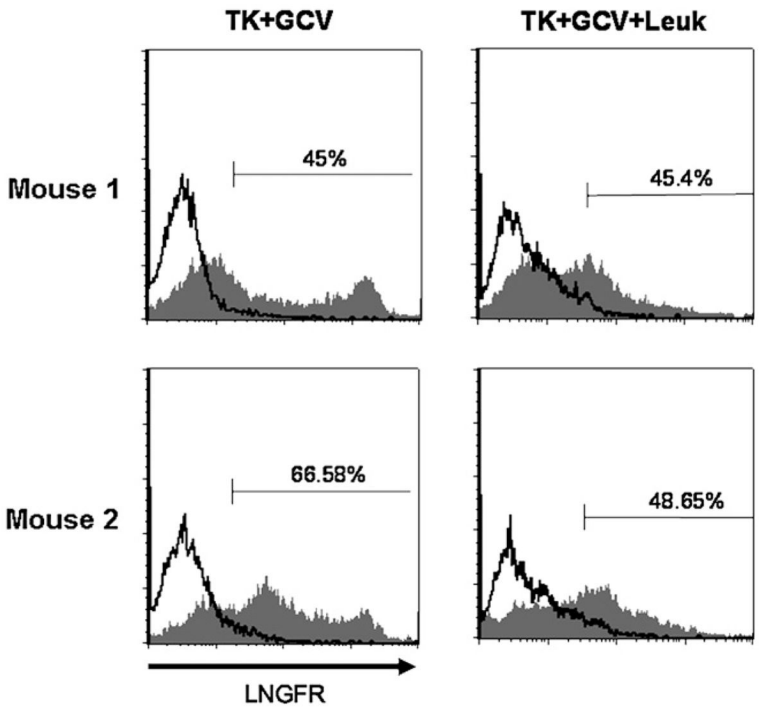

Figure 7.

Long-term expression of TK^+TC is not altered by a leukemic challenge. Approximately 100 μL of peripheral blood was collected on day 134 after transplantation from the tail vein of two mice given transplants of bone marrow and 4×10^6 TK^+TC (TK) ± 50 M1 leukemia cells (Leuk) and treated with 5 mg of GCV via an Alzet pump from days 10 through 16 after transplantation. The percentage of circulating cells expressing *LNGFR* (shaded contour) was assessed by flow cytometry, and the percentage of positive cells was determined by comparison with the immunoglobulin G isotype control (unshaded contour). There were no control mice (TK^+TC but no GCV) that survived a similar length of time for comparison.