
Running Title: GVL effect after TK/GCV gene therapy of GVHD

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Abstract

Clinical data indicate that after allogeneic hematopoietic stem cell transplantation (HSCT) for hematological malignancies, the graft-versus-leukemia (GVL) effect is in large part mediated by the graft-versus-host reaction (GVHR), which also often leads to graft-versus-host disease (GVHD). Controlling alloreactivity to prevent GVHD while retaining GVL poses a true dilemma for the successful treatment of such malignancies. We reasoned that suicide gene therapy, which kills dividing cells expressing the thymidine kinase (TK) “suicide” gene using time-controlled administration of ganciclovir (GCV), might solve this dilemma. We have previously shown that after infusion of allogeneic TK T cells along with HSCT to an irradiated recipient, an early and short GCV treatment efficiently prevents GVHD by selectively eliminating alloreactive T cells while sparing non-alloreactive T cells, which can then contribute to immune reconstitution. Nevertheless, it remained to be established that this therapeutic strategy retained the desired GVL effect. Hypothesizing that a contained GVHR would be essential, we evaluated the GVL effect using different protocols of GCV administration. We were able to show that when the GCV treatment is initiated at or close to the time of grafting, GVHD is controlled but GVL is lost. In contrast, when the onset of GCV administration is delayed until day 6, a potent GVL effect is retained while GVHD is still controlled. These data emphasize that, by a time-optimized scheduling of the administration of GCV, this TK/GCV strategy can be tuned to efficiently treat malignant hemopathies.
**Introduction**

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many hematological malignancies. Following the intensive conditioning of the recipient before transplantation aimed to eliminate malignant cells, donor T cells present in the graft contribute to the graft-versus-leukemia (GVL) effect by eliminating residual leukemic cells expressing disparate MHC antigens and/or tumor-associated antigens. Donor T cells also improve engraftment and provide a graft-versus-infection (GVI) effect for patients who are severely immunodeficient due to the conditioning. Unfortunately, in addition to their ability to provide such beneficial effects, donor T cells are also responsible for the life threatening graft-versus-host disease (GVHD) that is initiated by mature T cells that recognize MHC alloantigens presented by recipient cells. This complication can be circumvented by the removal of mature T cells from the graft, but only to the detriment of engraftment, as well as of the GVI and GVL effects. Thus, the GVHD and GVL effects are closely linked, and controlling alloreactivity to prevent GVHD while retaining the GVL effect represents the challenge for successful HSCT.

We and others have developed a strategy for controlling GVHD that relies on ex vivo transduction of donor T cells carrying a suicide gene encoding *Herpes simplex* type 1 thymidine kinase (TK). The expression of the TK transgene allows the metabolism of the nucleoside analog ganciclovir (GCV) into triphosphated-GCV (the active form of GCV) which blocks DNA elongation and thereby causes cell death. When donor TK-T cells divide after recognizing recipient alloantigens, they become sensitive to, and can be killed by, GCV administration. Suicide gene therapy permits the selective elimination of those T cells that recognize recipient alloantigens, while
preserving the T cells that did not divide during the treatment with GCV.\textsuperscript{12} In preclinical experiments using TK-T cells from transgenic mice, we and others have previously demonstrated that this therapeutic approach (i) efficiently prevents GVHD;\textsuperscript{13,14} (ii) does not impair engraftment after either myeloablative\textsuperscript{13,15,16} or non-myeloablative\textsuperscript{17} conditioning; and (iii) spares a pool of non-dividing donor TK T cells that further contributes to the recipient immune system reconstitution.\textsuperscript{12}

To date, all pre-clinical data using the TK/GCV system in the field of allogeneic HSCT were obtained under conditions where a GVL effect was not required. The clinical relevance of this therapeutic strategy for the treatment of hematological malignancies thus remained to be established by demonstrating that it permits the simultaneous control of GVHD while retaining the desired GVL effect. In this work, using a murine model of leukemia, we show that when the GCV treatment is initiated at, or close to the time of grafting, GVHD is indeed controlled, but the GVL effect is lost. In contrast, when the onset of GCV administration is delayed until day 6, a potent GVL effect is retained while GVHD is still controlled.
Materials and methods

Mice

C57BL/6 (B6, H-2^b), DBA/2 (D2, H-2^d) and [B6xD2]F1 (H-2^bd) mice were obtained from Iffa Credo (L'Arbresle, France). ΔTK line 6 (referred to as TK in this report) mice expressing the ΔTK transgene in virtually all CD4 and CD8 T cells, were described previously. Human (h)CD4 transgenic mice line 10 express the hCD4 protein at the cell surface of mouse (m)CD4 and (m)CD8 cells (referred to in the text as CD4 or CD4^+ and CD8 or CD8^+ cells, whereas the transgene is referred to as hCD4). Double transgenic [hCD4xTK] mice were obtained by breeding the line 6 ΔTK and the line 10 hCD4 transgenic mice. All transgenic mice were bred in the animal facility of the Faculté de Médecine Pitié Salpêtrière (Paris, France). Mice were manipulated according to EEC guidelines.

Experimental GVHD and drug administration

Experiments were performed as described, except where otherwise stated. Briefly, hybrid [B6xD2]F1 females (8-12 weeks of age) were lethally irradiated (11Gy). 5 x 10^6 T cell-depleted (TCD)-BM cells from B6 mice plus 8.4 x 10^6 to 9.2 x 10^6 T cells collected from spleen and lymph nodes of TK or [hCD4xTK] transgenic B6 mice were injected intravenously (i.v.) the day after irradiation. GCV (Roche, Neuilly-sur-Seine, France) was administered i.p. at a dosage of 50 mg/kg twice daily. Different schemes of GCV administration were used: a total of 5 injections administered twice daily beginning at day 1, day 3^{1/2} or day 6 post-grafting. The control group received a 7-day GCV treatment using mini-osmotic pumps implanted subcutaneously at the time of BMT under tri-bromo-ethanol anesthesia and delivering 1±0.02 µL/hour. This last group provides an experimental model of lethal GVHD that
is efficiently controlled by early GCV administration as initially described.\textsuperscript{13} Cyclosporin A (CsA, Sandimmun\textsuperscript{TM}, Novartis, Bâle, Swiss) was administered intraperitoneally daily from day -2 before BMT to day 10 at a dose of 50 mg/kg/day, as previously described.\textsuperscript{20}

**Analysis of in vivo T cell proliferation**

T cells from double-transgenic [hCD4\texttimesTK] mice were stained with the 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE). $1 \times 10^7$ cells/mL were incubated during 10 min at 37°C, 5% CO2 in a medium (RPMI 1640) containing CFSE at a concentration of 1.5 µM. Staining was stopped by addition of fetal calf serum to reach a concentration of 20% of the final volume. Labeled cells were washed twice in phosphate buffer saline, numbered and injected intravenously with BM cells in lethally irradiated mice. Splenocytes from grafted animals were collected at day 2,$\frac{1}{2}$, day 3,$\frac{1}{2}$ or day 6,$\frac{1}{2}$ post-transplantation. Cell proliferation was studied by flow cytometry as the sequential loss of CFSE fluorescence upon cell division after gating on donor (hCD4\textsuperscript{+}) CD4 and CD8 populations.

**Flow cytometrical analysis**

Spleens from grafted animals were digested with collagenase and DNase and splenocytes were incubated with 2.4.G2 anti-Fc receptor mAb. Transplanted mature donor T cells and their progeny were identified by their expression of the hCD4 transgene. For analysis of chimerism, cells were stained with combinations of the following mAbs: phycoerythrin (PE) -labeled anti-CD3 (clone 145-2c11, Pharmingen, San Diego, CA); fluoresceine isothiocyanate (FITC)-labeled anti-B220 (clone RA3-6B2, Caltag Laboratories, San Francisco, CA); biotinylated anti-H-2K\textsuperscript{d} (clone SF1-
1.1, Pharmingen) revealed with tricolor–labeled–streptavidin (Caltag); PE-labeled anti-H-2K^b (clone CTKb, Caltag). For CFSE experiments, splenocytes were stained with the following mAbs: APC-labeled anti-hCD4 (clone S3.5, Caltag); PE-labeled anti CD4 (clone RMH-5, Pharmingen) and Tricolor-labeled anti-CD8 (clone CT-CD8a, Caltag). Events were acquired on a FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson).

**Leukemia model**

P815 mastocytoma cells which are of DBA/2 mice origin express H-2^d MHC molecules. 2 x 10^3 P815 cells were injected i.v. in the retro-orbital sinus to recipients at the time of BMT. Clinical signs of leukemia were, chronologically: macroscopic tumor at the site of leukemic cell injection followed in most cases by lower-limb paralysis. Leukemic cells were also identified in peripheral blood by the surface expression of H-2^d (recipient-type) but not H-2^b (donor-type) histocompatibility antigens.

**Histopathological examination**

Liver samples were prepared in Bouin's fixative, embedded in paraffin and sections were stained with hematoxylin and eosin. A pathologist unaware of treatment evaluated slides. GVHD lesions were scored as described previously.21

**Statistical analysis.**

Statview software (Abacus Concepts, Berkeley, CA) was used for statistical analysis. Kaplan-Meier survival curves were established for each group. Mice suffering from advanced stage of GVHD were sacrificed for histopathological examination and
considered as dead in the Kaplan-Meier analysis. Survival differences between two groups were determined using the log-rank test. ANOVA was used to compare GVHD scores. $P$-values are indicated only when differences between the two groups were statistically significant.
Results

Optimizing the GCV treatment protocol according to the kinetics of T cell proliferation following allogeneic transfer.

We have previously shown that a 7-day GCV treatment initiated immediately after grafting provides a good prophylaxis against GVHD.\textsuperscript{13} Based on experimental as well as clinical data, we hypothesized that preserving the GVL effect would require letting the GVHR proceed for a limited period of time, and then stopping it before GVHD appears and becomes uncontrollable. Since the TK/GCV approach is based on the selective killing of dividing cells, we reasoned that a careful examination of T cell division would help define the optimal GCV protocol to achieve this goal.

Using CFSE-cell staining, we analyzed T cell division kinetics after allogeneic BMT, as well as after GCV administration. CFSE-stained TK-T cells were infused together with BM cells from wild type B6 mice into lethally irradiated semi-allogeneic [B6xD2]F1 recipients. To unambiguously identify transplanted donor T cells, we used T cells obtained from double-transgenic mice expressing the hCD4 marker molecule on both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in addition to the TK transgene.\textsuperscript{15,19} Spleen cells from transplanted mice were collected at different time points after BMT, and donor T cell division was assessed by the sequential loss of CFSE fluorescence intensity after gating on hCD4\textsuperscript{+}CD4\textsuperscript{+} and hCD4\textsuperscript{+}CD8\textsuperscript{+} double positive populations. Using a similar approach, we recently demonstrated that after injection of allogeneic donor T-cells (without BM cells) into lethally irradiated mice, alloreactive and non-alloreactive T-cells can be distinguished on the basis of their division rate and phenotypic differences.\textsuperscript{22} In the current experiments, we observed that CD4\textsuperscript{+} and CD8\textsuperscript{+} donor T cells present in the spleen of grafted animals had already started to divide by day 2 \textsuperscript{1/2}, and that at day 3 \textsuperscript{1/2} the vast majority of donor T cells had divided several times.
(Figure 1A). We thus reasoned that a short GCV treatment administered from day 1 to day 3 post BMT should be sufficient to eliminate most alloreactive T cells and, therefore to control GVHD. Additionally, shortening the duration of the GCV-treatment from 7 (as initially described\textsuperscript{13}) to 2 days should affect fewer non-alloreactive T cells,\textsuperscript{12} including those that recognize tumor-associated antigens and might participate in the non allogeneic part of the GVL effect. When GCV-treatment was administered from day 1 to day 3 post BMT, we did not observe any cell division in CD4\textsuperscript{+} and CD8\textsuperscript{+} donor T cells during the period of GCV administration (Figure 1A). At day 3\textsuperscript{1/2} we observed a ~10-fold decrease of hCD4\textsuperscript{+}CD4\textsuperscript{+} and hCD4\textsuperscript{+}CD8\textsuperscript{+} T cell counts as compared to untreated animals, illustrating the cytolytic effect of GCV on dividing TK-T cells (Figure 1B). At day 6\textsuperscript{1/2} (3\textsuperscript{1/2} days after discontinuation of GCV), numerous cell divisions had occurred in both CD4\textsuperscript{+} and CD8\textsuperscript{+} donor T cells. Thus, these data suggest that a short GCV administration should suffice to eliminate alloreactive T cells while sparing at least a portion of the non alloreactive donor TK-T cells.
Short and early GCV treatment protects against GVHD but also prevents the GVL effect

We next evaluated the efficacy of GVHD control in allo-grafted mice receiving a day 1 to day 3 GCV-treatment. This short treatment prevented GVHD as efficiently as the
day 0 to day 7 GCV-treatment\textsuperscript{13,15,16}. 85\% of the treated mice were still alive at day 60 (Figure 2A) with a histopathological score (Figure 2B) comparable to that observed with the 7-day GCV-treatment. In contrast, all untreated mice died (Figure 2A) with characteristic clinical signs (weight loss, skin lesions and hunching), and histological signs (lymphocyte infiltration and parenchymal injury of liver) of GVHD (Figure 2B). More than 95\% of the splenocytes from GCV-treated animals were of donor H-2\textsuperscript{b} and not of recipient H-2\textsuperscript{d} origin in both the B and T cell compartments, attesting a good engraftment (not shown). Thus, a 2-day GCV treatment started at day 1 post-grafting is efficient in preventing GVHD.

We next investigated whether a GVL effect could be observed using such GCV treatment. Leukemia was induced in lethally irradiated mice by injecting 2x10\textsuperscript{3} P815 cells i.v. at day 0. This led to 100\% mortality in mice grafted with TCD-BM (Figure 2A), death being preceded by the appearance of leukemic cells in peripheral blood, of a retro-orbital tumor at the site of injection, and, in most cases, of a hind-limb paralysis. Similar mortality was observed in TCD-BM grafted mice treated with GCV, indicating that GCV was not toxic for the leukemic cells (not shown). We then assessed whether the addition of TK-T cells to the TCD-BM and the P815 cells, under our GCV treatment scheme, provided a GVL effect. In this experiment, all mice died with characteristic biological and clinical signs of leukemia, with the same kinetics as those observed for control mice not receiving the TK T cells (Figure 2A).
The presence of leukemic cells in the peripheral blood of mice was unequivocally confirmed by their expression of recipient-type H-2K^d and not donor-type H-2K^b MHC class I molecules (Figure 3). Thus, when alloreactive T cells are eliminated by GCV-treatment early after BMT, GVHD is controlled but there is no GVL effect.
Delayed GCV treatment permits GVL effect without altering protection against GVHD

We reasoned that if GCV administration was delayed, alloreactive donor T cells might be maintained for a longer time after BMT, thus allowing a time-limited GVHR that might favor the GVL effect. When mice received the same 2-day GCV treatment from day 3 1/2 or day 6 rather than from day 1, they all survived without any clinical signs of GVHD (Figure 4A). As compared to mice receiving a GCV treatment starting at day 0 or day 1, histological signs of GVHD were either absent when the treatment was delayed to day 3 1/2 or slightly increased when the treatment started at day 6 (Figure 3B). Splenocytes of GCV-treated animals were of donor H-2b, and not of recipient H-2d origin (>97%), in the B and T cell compartments, attesting satisfactory engraftment (not shown). This indicates that GCV administration can be delayed without losing therapeutic efficacy.

When GCV was administered at day 3 1/2 to mice challenged with P815 leukemic cells, leukemia-associated mortality was significantly delayed as compared to mice treated at day 1 (p<0.05), although 7 out of 10 mice still died of leukemia (Figure 4A). The 3-surviving mice had neither clinical signs of leukemia, nor clinical or
histopathological signs of GVHD (Figure 4B).

When we further delayed GCV
administration to day 6, only 2 out of 10 mice died of leukemia. The 8 mice that survived until day 60 (p<0.001 as compared with mice treated by GCV at day 1), had no detectable leukemic cells in the peripheral blood at the end of the experiments (Figure 5).

 Taken together, these results indicate that by adjusting the modalities of GCV administration, a potent GVL effect can be obtained without compromising the protection against GVHD.

 We also tested whether donor T cells with anti-leukemic activity persisted after GCV treatment administered at day 6. Mice protected from GVHD and leukemia by the GCV-controlled GVHR were rechallenged with P815 cells at day 15. 4/4 such mice developed and eventually died of leukemia.

**Cyclosporin A impairs the GVL effect mediated by TK-T cells after allogeneic BMT**

Cyclosporin A (CsA) administered alone or in combination with methotrexate is the standard preventive treatment of GVHD.23 This immunosuppressive agent may, however, interfere with the antileukemic effect of donor TK-T cells, by inhibiting T cell proliferation24,25. We thus evaluated whether the presence of CsA before and during GCV administration had an impact on the GVL effect. To answer this question,
CsA was administered from day 2 before transplantation until day 10 post-BMT to mice receiving a 2-day GCV administration schedule started at day 6 post-BMT. Under these experimental conditions, we have recently demonstrated that CsA does not alter the capacity of GCV to control GVHD.\textsuperscript{26} In the present experiment, the GVL effect was dramatically altered in mice challenged with P815 cells, when compared to mice not receiving CsA. Indeed, 60\% of mice treated with GCV and CsA developed typical signs of leukemia and died between day 23 and day 26 (Figure 4A). These results indicate that combining a CsA prophylactic treatment to the TK/GCV approach might result in the loss of the GVL effect.
Discussion

The GVL effect is one of the main benefits brought by donor T cells after allogeneic HSCT. The antileukemic activity of allogeneic donor T cells has been directly evidenced by remissions observed after donor lymphocyte infusions into patients with hematological malignancies relapsing following allogeneic BMT. More recently, such a role for alloreactive T cells has also been shown in solid tumors after partial or complete regression of metastatic renal-cell carcinoma was obtained in patients receiving allogeneic HSCT. These alloreactivity-based approaches have proven to be promising for the treatment of leukemia as well as solid tumors, although their usefulness remains limited by the life-threatening GVHD. Here, we tested for the first time the efficacy of the TK/GCV strategy in pre-clinical conditions where a GVL effect is required. One of the main advantages of this TK/GCV strategy is its versatility, and in particular the time-controlled delivery of the ablative signal, i.e. GCV. Although few experimental data suggest that some GVL effect can be obtained independently of alloreactivity, it is clear from numerous studies that, in most cases, the GVHR is likely to be the main driving force behind the GVL effect. We thus anticipated that an optimal GVL effect would only be achieved by allowing a limited GVHR to proceed. However, we have previously shown that delaying GCV administration beyond a certain time point can result in the appearance of an uncontrollable GVHD. We thus assessed the consequences of delaying and reducing GCV administration on both the GVHD and GVL effect. We first observed that a 2-day GCV treatment administered at day 1 post-transplantation was as efficient in eliminating alloreactive donor T cells and control GVHD as our previous 7-day long treatment was. Starting this 2-day long treatment at day 3 or even at day 6, had no or minimal effect on its ability to control GVHD. Interestingly, when GCV was started at day 1, no GVL effect
was observed. In contrast, when GCV was administered at day $3^{1/2}$, a significant GVL effect was observed, which was increased if the GCV administration was further delayed to day 6. These data indicate that in this model, the GVL effect is mainly due to donor T cells dividing within the first 6 days following transplantation, and is thus most probably mediated by alloreactive T cells. This is further demonstrated by the inability of remaining donor T-cells to control leukemia relapse after a second challenge with P815 cells. It is thus possible that, after the GCV treatment, the immune repertoire contains reduced numbers of allo- or leukemic-antigens specific GVL-mediating T cells capable to prevent late leukemia relapse. In this case, it will still be possible to perform delayed donor TK-T cells infusion to mediate a GVL effect while controlling GVHD, as already evaluated in a clinical trial.\textsuperscript{32}

The final question we studied in this work was whether the TK/GCV strategy should be associated with the standard prophylactic treatment of GVHD based on CsA. Indeed, there is theoretically a putative antagonism between the cytostatic effect exerted by CsA\textsuperscript{24,25} and the cell division-dependent destruction of alloreactive T cells mediated by the TK/GCV system. We recently observed that the efficacy of GCV to control GVHD was not affected by CsA administration which could only inhibit T cell division for 2 days. Thereafter, grafted T cells proliferate despite CsA and could be eliminated by GCV.\textsuperscript{26} The consequence of CsA administration on GVL when GVHD is controlled by the TK/GCV system remained to be determined. Here, we demonstrate that if CsA is administered during the first 6 days after HSCT before GCV is started, GVL is dramatically reduced. This indicates that the CsA mediated inhibition of alloreactive T cell activation during the first 2 days significantly reduced the potency of GVHR, and thus GVL. Likewise, CsA administration, although not
interfering with GCV-mediated control of GVHD, should preferably be delayed until
the end of GCV administration.

Our data emphasize that, by a time-optimized scheduling, this therapeutic approach
can be tuned to efficiently treat malignant hemopathies. The TK/GCV strategy for the
control of GVHD has already been evaluated in clinical trials. One study concerned
patients who received ex vivo transduced donor TK-T cells infusion for the treatment
of relapse or EBV-induced lymphoproliferation occurring after TCD-allogeneic
BMT. In another study, transduced T cells were administered with a TCD-
allogeneic BMT. In both cases, GCV was administered to patients after they had
developed GVHD. Altogether, in 6 out of 7 patients, GCV administration was
associated with complete or partial remission of GVHD. We have previously
observed in mice that a course of GCV treatment sufficient to prevent GVHD was not
able to completely reverse an advanced GVHD. We demonstrate here that an
intermediate strategy can be proposed for the treatment of leukemia involving no
GCV administration at the time of grafting to improve the GVL effect, followed by a
systematic GCV treatment before the occurrence of clinical signs of GVHD. These
data obtained in our experimental mouse model should now be translated to the
clinical setting of allo-HSCT. Here, GVHR and GVL effects occurred in a MHC class I
mismatch setting. Allo-reactivity is therefore robust and probably has a more rapid
onset than in HLA matched situations. Given the variability of major and minor
antigen discrepancies encountered in human HLA matched or mismatched
transplantation, it is presumable that a unique time frame for GCV administration may
not apply to all donor-recipient pairs. Also, in our experiments we used
unmanipulated TK-T cells from transgenic mice that presumably retain better immune
function than T cells transduced ex vivo with a retroviral vector. Altogether, we have
proved the concept that with the TK/GCV system, a time-controlled GVHR permits to solve the dilemma of allo-HSCT: retaining GVL while controlling GVHD. The definition of appropriate therapeutic scheme(s) for human allo-HSCT can now only be investigated in clinical trials. We indeed are about to test different therapeutic schemes in HLA matched or haplo-mismatched human HSCT, for the treatment of leukemia or severe auto-immune diseases.
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Figure legends

Figure 1. Kinetics of cell division after TK T cells infusion in lethally irradiated hosts. (A) [B6xD2]F1 irradiated mice received B6 BM cells supplemented with 1 x 10⁷ CFSE-labeled mature T cells from [hCD4xTK] double-transgenic B6 mice. Control groups received no GCV treatment (no GCV). In treated groups, GCV treatment consisted in 5 injections beginning 24 hours after grafting and administered each 12 hours (GCV d1-3). † indicates time of sacrifice on the experimental scheme. CFSE intensity of hCD4⁺ in CD4⁺ and CD8⁺ T cells was analyzed at different time points post-grafting. Each histogram is representative of two mice. The peak of highest intensity on the log scale identifies parent generation of infused donor T cells. Peaks with decreased CFSE intensity represent daughter generations that have undergone cell-division. (B) The number of CD4⁺ and CD8⁺ donor T cells in spleens of grafted animals (n=2 for each group) was evaluated at day 3 ¹/₂ by their expression of the hCD4 marker. The coefficient of variation of duplicates was <10%.

Figure 2. Absence of GVL effect after early GCV treatment. A. Experimental GVHD was induced by B6 TK T cells injected together with B6 TCD-BM cells in lethally irradiated [B6xD2]F1 recipient mice. Leukemic challenge was performed by intravenous injection of 2 x 10^³ P815 (H-2d) mastocytoma cells at the time of grafting. In the absence of treatment (black circle, [n=10]) GVHD is lethal. GCV is administered by continuous subcutaneous infusion from day 0 until day 7 after graft (white square, [n=4]) or by 5 intraperitoneal injections each 12 hours beginning 24 hours after BMT (white triangle, [n=6]). For the leukemia control group, [B6xD2]F1 mice were grafted with B6 TCD-BM plus P815 cells (white circle, [n=5]). The experimental group is represented by [B6xD2]F1 mice grafted with B6 TCD BM plus
B6 TK T cells plus P815 cells and treated by GCV from day 1 to day 3 after graft (black square, [n=8]). Results are presented as Kaplan-Meier survival.

**B. Histopathologic score of liver after semi-allogeneic BMT.** Histopathologic examination and grading of GVHD of liver from mice were performed 65-75 days after transplantation. GVHD control mice did not receive GCV treatment (no GCV; [n=2]) and were sacrificed at day 19 with clinical signs of GVHD (body weight <13 g, hunching). GCV treatment consisted of continuous subcutaneous administration from day 0 to day 7 after graft (GCV d0-7; [n=4]) or of 5 intraperitoneal injections each 12 hours beginning 24 hours after graft (GCV d1-3; [n=5]). Histograms indicate the mean histopatological score for each group. The Fisher test was used for the analysis of variance (ANOVA); p-value<0.05 for GVHD group versus all other groups.

**Figure 3. Detection of P815 leukemic cells in the peripheral blood of mice.** P815 mastocytoma cells were identified by flow cytometry by the expression of recipient type (H-2d) but not donor type (H-2b) MHC class I molecules. P815 cells were identified on 3/3 mice having clinical leukemic signs after BM-grafting plus TK T cell infusion and GCV treatment from day 1 to day 3, as illustrated here.

**Figure 4. GVL effect obtained by delayed GCV administration. A.** Experimental GVHD was induced by B6 TK T cells injected together with B6 TCD-BM cells in lethally irradiated [B6xD2]F1 recipient mice. Leukemic challenge was performed by intravenous injection of 2 x 10^3 P815 cells at the time of grafting. GCV was administered from day 6 to day 8 after graft (black square; [n=10]); GVL effect was induced in GVHD–mice receiving P815 cells and treated with GCV from day 3 1/2 after
graft (white square [n=10]) or from day 6 after graft (white circle; [n=10]). One group of mice also received CsA treatment from day –2 until day 10 after the graft and GCV treatment from day 6 to day 8 (white triangle; [n=5]). Results are presented as Kaplan-Meier survival curves of cumulative data of 2 independent experiments. B. **Histopathologic score of liver after semi-allogeneic BMT.** Histopathologic examination and grading of GVHD of liver from mice were performed 65-75 days after transplantation. GCV was administered at day 6 (GCV d6-8; [n=10]). Two groups of mice received P815 leukemia cells at the time of transplantation and were treated by GCV starting on day 3 1/2 (P815 + GCV d3 1/2-d5 1/2; [n=3]) or day 6 (P815 + GCV d6-8; [n=8]) after the graft, respectively. Histograms indicate the mean histopatological score for each group. The Fisher test was used for analysis of variance (ANOVA); p-values<0.05 for P815 + GCV d3 1/2-5 1/2 versus P815 + GCV d6-8.

**Figure 5. Detection of P815 leukemic cells in the peripheral blood of mice.** In mice protected from GVHD and which did not develop clinical signs of leukemia, peripheral blood cells were systematically collected and tested for the presence of P815 cells. On 3/3 and 8/8 protected mice treated at day 3 1/2 or day 6, respectively, P815 cells were never detected more than two months after P815 cells injection, as illustrated here in a mouse treated at day 6.
Graft-versus-leukemia effect after suicide-gene-mediated control of graft-versus-host disease

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