Post–Hematopoietic Cell Transplantation Control of Graft-versus-Host Disease by Donor CD4$^{+}$25$^{+}$ T Cells to Allow an Effective Graft-versus-Leukemia Response

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ABSTRACT

After allogeneic hematopoietic cell transplantation (HCT), the high inverse correlation between graft-versus-host disease (GVHD) and leukemic relapse requires that calculated measures be taken to reduce GVHD pathology while retaining the graft-versus-leukemia (GVL) effect. We sought to determine whether donor CD4$^{+}$CD25$^{+}$ regulatory T cells could control ongoing GVHD, thereby providing an initial window of time in which the alloreactive anti-host response is permitted to begin, with the intent of most effectively eliminating residual leukemia cells. Prevention of lethal GVHD by infusion of donor CD4$^{+}$CD25$^{+}$ cells early after HCT (day 2) was achieved across a major histocompatibility complex barrier in the haploidentical C3H$^{3}$ (B6xC3H)F1 model. However, in vitro expansion of donor CD4$^{+}$CD25$^{+}$ T cells, stimulated by recipient cells in the presence of high-dose interleukin-2, was required for successful regulation. In contrast, in the major histocompatibility complex–matched, minor histocompatibility antigen–disparate, B10.BR $\rightarrow$ CBA GVHD model, lethal disease could be completely prevented by a single infusion of freshly isolated donor CD4$^{+}$CD25$^{+}$ cells administered as late as 10 days after HCT. Of importance, this late regulatory effect required only a 3:1 ratio of effector CD8:CD4$^{+}$CD25$^{+}$ T cells, indicating a strong potential for the delayed infusion of CD4$^{+}$CD25$^{+}$ cells to control GVHD across minor histocompatibility antigen barriers. Furthermore, this regulation did not interfere with complete and lasting donor engraftment of the hematopoietic compartment. Of most significance, the day 10 infusion of donor CD4$^{+}$CD25$^{+}$ T cells into CBA HCT recipients that had been challenged with the MMCBA6 myeloid leukemia cell line did not block an effective GVL response, despite reducing lethal GVHD. These results suggest that donor CD4$^{+}$CD25$^{+}$ T cells infused soon after transplantation can ameliorate the development of GVHD without sacrificing a sufficient GVL effect.

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KEY WORDS

Regulation  •  Graft-versus-host disease  •  Graft versus leukemia  •  Hematopoietic cell transplantation

INTRODUCTION

The application of allogeneic hematopoietic cell transplantation (HCT) as treatment for a number of otherwise fatal hematologic malignancies is limited by the excessive incidence of graft-versus-host disease (GVHD). The high inverse correlation between GVHD and leukemic relapse, however, requires that very calculated measures be taken to minimize the former without sacrificing the critical graft-versus-leukemia (GVL) effect. A potential approach that may permit a limited and controlled alloreactive anti-host reaction to provide an effective GVL response is the application of CD4$^{+}$CD25$^{+}$ regulatory T cells.

A unique subset of thymus-derived CD4$^{+}$ T cells was found to constitutively express the interleukin (IL)-2 receptor α chain (CD25) and exhibited potent immunoregulatory properties [1]. These CD4$^{+}$CD25$^{+}$
T cells were hypothesized to serve as a safeguard against the activation of autoreactive T cells that escape deletion in the thymus, possibly by blocking their production of IL-2 [2-4]. An interesting feature of CD4+CD25+ regulatory T cells is that despite being classically anergic to T-cell receptor (TCR) stimulation in vitro, this ligation is required to allow them to mediate their immunosuppressive function [5]. Furthermore, once activated, these cells are antigen nonspecific with regard to the T-cell responses that they are able to suppress [5].

Investigations into the parameters that affect the development and diversity of the CD4+CD25+ T-cell repertoire have provided insight into their potential roles in the regulation of the immune system [4,6,7]. Experiments with dual transgenic mice, expressing both a specific TCR and the thymic expression of the cognate peptide suggested that thymic regulatory T-cell generation is dependent on high-affinity interactions between the TCR and the peptide-major histocompatibility complex (MHC) class II complex during T-cell maturation [6]. Another study compared peripheral CD4+CD25+ T-cell numbers across a series of mice expressing MHC class II molecules occupied by a range of different self-peptides and found a direct correlation, supporting the notion that thymic selection of these cells may resemble that of normal CD4+ T cells in repertoire diversity [7]. These results also were consistent with data that showed a diverse involvement of TCR Vα and Vβ gene families in the CD4+CD25+ T-cell repertoire [4]. This broadness of the regulatory T-cell repertoire becomes highly relevant in the context of alloreactivity, a phenomenon driven by cross-reactivity and degeneracy of the T-cell repertoire [8]. Thus, when activated through the TCR by alloantigens, CD4+CD25+ T cells potentially could be used to actively suppress an allosresponse, such as in tissue allograft rejection or in GVHD after allogeneic HCT.

In the case of allograft rejection, allogenic/specific regulation was first shown when tolerance to skin grafts was transferred with the injection of CD45RBlow CD4+ and CD4+CD25+ T cells but could only be achieved against grafts from the original tolerating strain [9,10]. Importantly, it also has been shown that CD4+CD25+ T cells can play an active role in maintaining the anergic phenotype of an alloreactive CD8+ T-cell population, thus indicating the immunoregulatory scope of these cells [11].

In the HCT setting, initial observations suggest that de novo–derived donor CD4+CD25+ T cells effectively inhibit the development of GVHD induced by delayed donor lymphocyte infusion in MHC-haploidentical mice [12]. Moreover, CD4+CD25+ T cells generated in vitro by incubation of alloreactive cells in the presence of anti-CD40 ligand antibody are able to block the development of GVHD when coadministered with normal alloreactive donor T cells [13]. In the present study, we confirm that, in the context of an allogeneic MHC-mismatched bone marrow transplant, rapidly lethal GVHD is prevented when CD4+CD25+ T cells from the donor strain are cotransferred along with the GVHD-inducing CD4+ T cells at time of transplantation.

Of primary interest, the HCT setting is unique in the case of patients with leukemia because the same alloreactive donor T cells that mediate GVHD also may mount a GVL effect [14,15]. The important question then becomes whether CD4+CD25+ T cells can not only prevent GVHD, but whether their delayed infusion can be used to ameliorate the development of GVHD after it has been initiated, thus providing an early unrestricted allosresponse for the benefit of a most effective GVL response. Our data indicate that donor CD4+CD25+ T cells may be used to suppress an early GVHD reaction without sacrificing the therapeutic benefit of an effective GVL response.

**MATERIALS AND METHODS**

**Mice**

C3H/HeJ and B10.BR/SgSn (both H2b) strains were purchased from the Jackson Laboratory (Bar Harbor, ME). (B6xC3H)F1 (H2b/k) and CBA mice (either JCr or NCr substrains, as available; H2b) were purchased from the National Cancer Institute Animal Procurement Program (Frederick, MD). For all experiments, sex-matched mice between the ages of 7 and 14 weeks were used as donors and recipients. Mice were housed in a pathogen-free environment in autoclaved microisolator cages and were provided with autoclaved water and food ad libitum.

**Cell Lines and Media**

The MMCBA6 cell line is a c-myc retrovirus-transformed myeloid leukemia cloned from the ascites of CBA mice that had been injected with a c-myc–encoding moloney murine leukemia virus construct, as previously described [16]. The line was grown in complete media: RPMI 1640, supplemented with 2 mmol/L of L-glutamine, 50 IU/mL of penicillin, 50 μg of streptomycin (all from Mediatech, Herndon, VA), 10% fetal calf serum (Sigma Chemical, St. Louis, MO), and 2-mercaptoethanol (Life Technologies, Grand Island, NY), at 37°C in 7% CO2.

**Monoclonal Antibodies**

Ascites fluid containing anti-Thy-1.2 (J1; rat immunoglobulin (Ig)M [17]), anti-CD8 (3.168; rat IgM [18]), or anti-CD4 (RL172; rat IgM [19]) monoclonal antibodies (mAbs) were used along with guinea pig complement (C'; Rockland, Boyertown, PA) for cell
subset depletions. Affinity-purified goat anti-mouse IgG antibody (Cappel-Organon Teknika, West Chester, PA) was used for B-cell panning. For donor chimerism analysis and phenotypic analysis of donor grafts, fluorescein isothiocyanate (FITC) and/or phycoerythrin (PE)-labeled mAbs specific for the following determinants were used: irrelevant isotype control, Ly9.1, H2Kk, CD3, CD4, CD8, B220, CD11b (MAC-1), and CD11c (all from Pharmingen, San Diego, CA). For CD25+ cell selection, FITC- or PE-conjugated anti-CD25 mAbs (clones 7D4 and PC61, respectively; Pharmingen) were used.

**Flow Cytometry**

Between 0.06 to 0.125 µg of each mAb was incubated with ≤1 × 106 cells in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.01% NaN₃ (wash buffer) for 25 minutes at 4°C. After multiple washes in wash buffer, fluorescence analysis was either performed immediately or cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 15 minutes at 4°C, followed by a final wash. Fluorescence analysis was performed on a Beckman Coulter XL-MCL analytic cytometer (Beckman Coulter, Miami, FL). Flow cytometric gates were established by excluding background isotype-control Ab binding. In the case of two-color staining, individual populations of control cells were stained with positive control PE and FITC-labeled antibodies, respectively, to assure no background overlap in fluorescence emission between the two fluorochromes.

**Preparation of Donor Cells**

PBS (BioWhittaker, Walkerville, MD), supplemented with 0.1% BSA (Sigma Chemical), was used for all preparative manipulations of the donor bone marrow and lymphocytes. Antibody T-cell-depleted bone marrow (ATBM) was prepared by flushing bone marrow cells from the femurs and tibiae of donor strain mice, followed by incubation with J11-mAb (1:50 dilution) and C’ (1:12 dilution) for 45 minutes at 37°C. T-cell–enriched donor cells were prepared from pooled spleen and lymph node (LN) cell suspensions from donor mice as previously described [20]. Briefly, after red blood cell lysis with Gey’s balanced salt solution containing 0.7% NH₄Cl, B cells were removed by panning the cell suspension over goat anti-mouse IgG-coated plastic petri dishes for 1 hour at 4°C. In some experiments, nonadherent lymphocytes were depleted of CD4+ or CD8+ T cells using incubation with the appropriate mAb (1:50-1:100 dilution) and C’ (1:12 dilution) for 45 minutes at 37°C. Donor T cells were >96% enriched for subset of interest, as determined using flow cytometry. For separation of CD4+CD25+ cells: following the labeling of donor CD4+ T cells with FITC- or PE-conjugated anti-CD25 mAb (0.06–0.1 µg of Ab/10⁶ cells), cells were incubated with immunomagnetic cell sorting anti-FITC or anti-PE microbeads, respectively (Miltenyi). Mean purity of positively selected CD25+ cells ranged between 85% and 93% with >93% being CD4+. Cells were injected intravenously (i.v.) in PBS alone.

**CD4+CD25+ In Vitro Expansion**

Culture conditions were adapted from a previously published protocol [5]. Briefly, after separation, donor CD4+CD25+ or CD25− cells were resuspended in complete media and placed into culture with irradiated (20 Gy) recipient splenocytes (1:2 ratio of responder to stimulator) plus 100 U/mL of recombinant human IL-2 (NIH AIDS Research and Reference Reagent Program, Rockville, MD) for 3 days (37°C, 7% CO₂) and were then cultured in media plus IL-2 alone for an additional 3 to 4 days. After expansion, the CD25+ culture was 84% to 97% CD25+, of which 92% to 98% were CD4+.

**MLR Culture**

Donor CD4+ T cells, fresh, or in vitro expanded CD4+CD25+ or CD25− T cells were isolated as described above. Respective donor T-cell subsets were then placed into at least triplicate culture, in complete RPMI, with irradiated (20 Gy) recipient splenocytes (at 1:1 or 1:2 ratio of responder to stimulator) or were added into mixed lymphocyte reaction culture in titrated numbers to achieve the indicated responder: CD25 ratio. Proliferation was assessed on days 3 to 5 of culture by pulsing with 1 µCi/well [3H]-thymidine (TdR) for 8 hours, and measuring the incorporation levels from harvested cells with a 1205 Betaplate counter (Wallac, Turku, Finland). Data are expressed either as mean [3H]-TdR incorporation counts per minute (CPM) or mean % suppression of cell proliferation, calculated as follows: 100% – (mean CPM MLR with CD25+/− / mean CPM untreated MLR * 100).

**In Vivo Analysis for GVHD and GVL Responses**

C3H (H2k)→(B6xC3H)F1 (H2k/b) and B10.BR (H2k→CBA (H2k) bone marrow transplantations were performed as previously described, with some modifications [20]. (B6xC3H)F1 and CBA recipients were lethally irradiated with 13 Gy, split dose (6.5 Gy at 1.36 Gy/min) from a 137Cs source (Mark-1 Model 68 gamma irradiator; J.L. Shephard, San Fernando, CA) and were injected i.v. 4 to 6 hours later with an appropriate C3H or B10.BR donor inoculum of 1 to 2 × 10⁶ ATBM cells, alone, or in combination with
v}

\[ E_{\text{CD4}} \]

received freshly isolated or \textit{in vitro} expanded donor CD4$^+$CD25$^+$ or CD25$^-$ cells at either 2, 4, or 10 days posttransplantation. In experiments meant to study GVL activity, select groups of CBA recipients were injected intraperitoneally (i.p.) 1 day before transplantation with \(1.5 \times 10^5\) MMCBA6 tumor cells. Animals were monitored for morbidity and mortality and weighed regularly until the termination of the experiment. Death from leukemic relapse was determined by the observation of massive abdominal edema, leukemia cell infiltrated ascites, or solid tumor masses at the time of death, in conjunction with the lack of clinical and/or histological signs of acute GVHD. Observations of acute weight loss, hunched posture, diarrhea, runted appearance, and consideration of histological evidence from tissue harvested at time points leading up to or at the time of death indicated death due to GVHD.

**Histology**

Sequential ear biopsy specimens were collected from mice at specific time points, or collected (along with tongue, liver, and gut) on humane killing of the animal when determined to be moribund (when animals were no longer mobile or were incapable reaching food and water) and prepared for routine histological analysis [21]. For quantitation of dyskeratotic epidermal cells, skin samples were evaluated for features of apoptotic keratinocytes [21] using light microscopy by an experienced pathologist without knowledge of experimental groups. Data is reported as the mean number of apoptotic keratinocytes per linear mm (L/mm).

**Data Analysis**

Median survival times (MST) were calculated as the interpolated 50% survival point of a linear regression through all of the death data points, including zero. Statistical comparisons for survival between experimental groups are based on days of death posttransplantation, and were performed using the non-parametric Wilcoxon signed rank test. Significance for weight comparisons was determined by the \( t \) test at individual time points.

**RESULTS**

\textbf{In Vitro Regulation by Naive versus In Vitro Expanded CD4$^+$CD25$^+$ T Cells}

The flow cytometric profile of the CD4$^+$ T-cell population from combined spleen and LN of naive C3H mice indicated that approximately 5% of the gated CD4$^+$ T-cell population expressed the CD25 cell surface protein (Figure 1A), consistent with published observations [4]. After immunomagnetic cell sorting, the mean purity of positively selected CD25$^+$ cells ranged from 85% to 93%, of which >93% were CD4$^+$ (Figure 1B).

We first assessed the ability of donor-derived CD4$^+$CD25$^+$ T cells to inhibit an \textit{in vitro} allogeneic MLR using a haploidentical strain combination. First, either whole CD4$^+$, CD4$^+$CD25$^+$, or CD4$^+$CD25$^-$ T cells from naive C3H mice were placed into culture for 3 days with irradiated (20 Gy) (B6xC3H)F$_1$ splenocytes. Consistent with data from other \textit{in vitro} models [3-5], CD4$^+$CD25$^+$ T cells did not proliferate in response to allogeneic stimulation when compared with the robust responses of whole CD4$^+$ and CD4$^+$CD25$^-$ T cells (Figure 1C). Furthermore, freshly isolated C3H CD4$^+$CD25$^+$ T cells suppressed alloantigen-driven proliferation of CD4$^+$CD25$^-$ T cells by 83.5% at a 2:1 ratio (responder:CD25$^+$), decreasing to only 17.6% inhibition at a 4:1 ratio (Figure 1D). Based on the observation by Thornton and Shevach that CD4$^+$CD25$^+$ cells have more potent suppressor activity after expansion \textit{in vitro} [5], we adapted a similar stimulation protocol by which C3H CD4$^+$CD25$^+$ and CD25$^-$ cells were stimulated with recipient splenocytes (rather than anti-CD3 Ab) in the presence of high-dose IL-2 (100 U/mL). An initial experiment showed that these culture conditions were sufficient to stimulate alloantigen-driven expansion of CD4$^+$CD25$^+$ T cells, as indicated by a 7-fold increase in proliferation over IL-2-only controls on day 3 of culture (measured using 3H-TdR incorporation, data not shown). On average, approximately 100% of the number of CD4$^+$CD25$^+$ T cells initially put into culture were recovered at the end of the 6-day expansion. These numbers were similar to those reported by Taylor et al. [22], who reported a 1.5-fold increase in the CD4$^+$CD25$^+$ T-cell number after a similar method of expansion. These \textit{in vitro} expanded CD4$^+$CD25$^+$ T cells were much more capable of suppression at lower numbers, as indicated by 88.7% and 70.7% suppression of the MLR response at a 4:1 and 8:1 ratio (responder:CD25$^+$), respectively. In contrast, no suppression of the proliferative response resulted from the addition of either naive or \textit{in vitro} expanded CD4$^+$CD25$^-$ cells (Figure 1D).

**Regulation of GVHD Post-HCT in a Haploidentical Model**

Reflecting their inability to proliferate \textit{in vitro} to allogeneic stimulation, \(5 \times 10^5\) freshly isolated C3H CD4$^+$CD25$^+$ T cells were unable to mediate lethal GVHD (MST >52 days) or cause any disease-associated cachexia (\(P > .2\), at all time points) when transferred along with \(2 \times 10^6\) C3H ATBM into lethally irradiated (13 GY, split dose) (B6xC3H)F$_1$ recipients (Figure 2A and 2B). This was in sharp contrast to the very rapid onset of lethal GVHD (MST < 10 days).
that was observed on transfer of the same number of either C3H whole CD4+ or CD4+CD25+ T cells into equivalently irradiated and ATBM-transplanted recipients (Figure 2A). Most importantly, C3H CD4+CD25+ T cells fully suppressed the development of CD4-mediated GVHD (100% survival; MST > 52 days) when cotransplanted (at a 1:1 ratio) with 5 × 10^5 whole C3H CD4+ T cells (Figure 2A). Furthermore, the mean % initial body weights posttransplantation were similar to the control ATBM alone group (P > .4, at all time points; Figure 2B). This inhibitory activity was unique to the CD25+ subset because mice that received CD4+CD25+ T cells at a similar 1:1 ratio with whole C3H CD4+ T cells, at time of transplantation, still succumbed to rapid onset lethal GVHD (MST < 8 days) (Figure 2A).

To begin to determine the capacity of CD4+CD25+ T cells to suppress recently initiated CD4-mediated GVHD in the haploidentical model, (B6xC3H)F1 mice were lethally irradiated (13 Gy, split dose) and initially injected with 2 × 10^6 ATBM cells along with 2.5 × 10^5 C3H whole CD4+ T cells. On day 2 posttransplantation, CD4 recipients were administered i.v. either 1 × 10^6 freshly isolated or in vitro expanded C3H CD4+CD25+ or CD25- T cells to achieve indicated responder:CD25+ ratios, and pulsed with [3H]-TdR for the last 8 hours of culture. Data are expressed as the mean % suppression of MLR proliferation and are based on quadruplicate samples from a single in vitro experiment.
CD4+CD25+ cells on infusion between days 0 and 2 posttransplantation likely reflected the rapid progression of the alloresponse in this haploidentical model. On the other hand, the infusion on day 2 of the same number of in vitro expanded C3H CD4+CD25+ T cells completely prevented GVHD lethality (MST >86 days; P ≤ .005, in comparison with the untreated whole CD4+ T-cell control group; Figure 3A). Significant cachexia (P ≤ .04, compared with ATBM alone) was only noted up to 3 weeks post-HCT, after which point there was further amelioration of disease and no significant weight difference for the remainder of the experiment (P = .1 versus ATBM alone; Figure 3B). The infusion of expanded donor CD4+CD25+ cells also provided a slight prolongation of survival (MST of 23 days; P ≤ .02 versus the untreated CD4+ T control). This regulatory effect was not significantly greater than that of day 2 injected fresh CD4+CD25+ cells (P ≥ .34). Moreover, sustained acute GVHD-associated cachexia (P ≤ .01 versus ATBM, all time points) followed by the eventual 100% mortality of this group indicated much less of a beneficial regulatory effect than expanded CD25+ cells (weight loss, P ≤ .02 expanded CD25+ versus CD25- all time points except for week 1; survival, P ≤ .02, expanded CD25+ versus CD25-) (Figure 3A and Figure 3B). The window of opportunity at

Figure 2. Effect of donor CD4+CD25+ T cells on development of lethal GVHD when infused on day of transplantation. (B6xC3H)F1 mice were lethally irradiated (13 Gy, split dose) and were injected with 2 × 10⁶ C3H ATBM cells alone, or in combination with either 5 × 10⁵ whole C3H CD4+, fresh CD4+CD25+, or CD25- T cells. Additional groups of C3H ATBM + whole CD4+ (5 × 10⁵) recipients also received 5 × 10⁵ freshly isolated C3H CD4+CD25+ or CD25- T cells at the time of transplantation. (A) Survival of transplanted recipients. (B) Body weights for each group normalized as the mean ± SEM % initial body weight during sequential 1-week periods. The data shown are representative of 2 separate experiments that showed similar trends with n = 4 for all groups, except for n = 5 for CD4+CD25+ alone and n = 8 for whole CD4.
the given dose level of in vitro expanded regulatory cells was apparently short, however, because delaying this injection further until day 4 posttransplantation was ineffective, and GVHD developed with similar kinetics as untreated CD4+ T-cell recipients (Figure 3A).

These data show that regulation of acute, severe GVHD post-HCT in this haploidentical model is strongly dependent on the administration of CD4+CD25+ T cells very early posttransplantation, making the management of this alloresponse for the benefit of a sufficient GVL effect a difficult endeavor. Thus, we next focused on a generally less aggressive MHC-matched GVHD model to study the potential of using CD4+CD25+ T cells to regulate GVHD at later times post-HCT.

**Post-HCT Regulation of GVHD across a Minor Histocompatibility Antigen Barrier**

Lethally irradiated (13 Gy, split dose) CBA (H2k) recipients underwent transplantation with MHC-matched, minor histocompatibility antigen (miHA) disparate B10.BR (H2k) ATBM alone, or along with 3 x 10^5 B10.BR CD8+ T cells. On either day 2, 4, or 10 posttransplantation, CD8+ T-cell transplanted recipients were administered 1 dose of 1 x 10^6 freshly isolated B10.BR CD4+CD25+ T cells. Significantly, we found that the lethal CD8-mediated GVHD (MST of 52.4 days) could be suppressed by the donor CD4+CD25+ T cells when injected as late as 10 days posttransplantation (day 2, MST >90 days; data not shown; day 4, MST >90 days, P ≤ .027; day 10, MST.
Figure 4. Regulation of ongoing GVHD by delayed infusion of freshly isolated donor CD4+CD25+ T cells in a MHC-matched model. CBA mice were lethally irradiated (13 Gy, split dose) and were injected with 2 × 10^6 B10.BR ATBM cells alone, or in combination with 3 × 10^6 B10.BR CD8+ T cells. On either days 4 or 10 posttransplantation, CD8+ T-cell transplanted recipients were either left untreated or infused with 1 × 10^6 freshly isolated B10.BR CD4+CD25+, or CD25+ T cells. (A) Survival of transplanted recipients who underwent transplantation. (B) Body weights for each group normalized as the mean ± SEM % initial body weight during sequential 1-week periods. Survival and weight data were pooled from 3 separate experiments (CD8+ T cells alone, n = 19; days 4 and 10 CD4+CD25+ cells, n = 6 and 7, respectively; days 4 and 10 CD4+CD25− cells n = 9 and 9, respectively; ATBM n = 10).

>90 days, \( P \leq .018 \) versus untreated CD8+ T control; Figure 4A). Compiled weight data from pooled experiments showed minor, transient GVHD-associated cachexia in day 4 infused CD25+ recipients (\( P \leq .02 \) compared with the ATBM alone group) during week 5 post-HCT, whereas more pronounced cachexia was noted in the day 10 CD25+ group during weeks 2 through 4 post-HCT (\( P \leq .043 \); Figure 4B). No significant difference in weight loss was noted between day 4 and day 10 CD4+CD25+ recipients (\( P \geq .09 \).

To determine whether regulation of GVHD by donor CD4+CD25+ cells affected the establishment of lasting donor chimerism, spleens were harvested at the conclusion of the experiment (day 90) from recipient mice. Flow cytometric analysis was performed for expression of Ly9.1, the presence of which denotes cells of host CBA origin (Table 1). Importantly, animals that were rescued from lethal GVHD by injection of donor regulatory T cells had a higher percentage of donor-derived T and B cells compared with equivalently irradiated recipient mice that received B10.BR ATBM alone (Table 1). This was consistent with the notion that infused donor T cells could still enhance donor chimerism by targeting residual recipient hematopoietic cells.

**Regulation of Ongoing GVHD with Concomitant Leukemia Burden**

Experiments were performed to determine whether the delayed infusion of donor CD4+CD25+ T cells, to suppress the full pathological development
of GVHD, compromised an effective GVL response. CBA mice were challenged i.p. with 1.5 × 10⁵ MMCBA6 cells (a myeloid leukemia cell line of CBA origin) 1 day before exposure to lethal irradiation (13 Gy, split dose), followed by i.v. injection with 2 × 10⁶ B10.BR ATBM cells alone, or in combination with 15 × 10⁶ B10.BR CD8⁺ T cells. All of the MMCBA6 challenged recipients who were transplanted with ATBM alone succumbed to leukemia burden by day 28 posttransplantation (Figure 5A), indicated by the presence of massive abdominal edema, hemorrhagic ascities, and, in some animals, solid tumor masses observed at time of death (all hallmarks of tumor cell burden in this myeloid leukemia model [23]). All mice injected with only ATBM and B10.BR CD8⁺ T cells succumbed to GVHD, with an MST of 19.6 days.

Table 1. Regulation of GVHD by the Delayed Infusion of Donor CD4⁺CD25⁺ T Cells Does Not Sacrifice Stable Donor Engraftment

<table>
<thead>
<tr>
<th>Recipient Group</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>B220</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATBM (n = 3)</td>
<td>75.4</td>
<td>76.4</td>
<td>80.1</td>
<td>92.2</td>
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<tr>
<td>CD8 + day 4 CD25⁺ (n = 4)</td>
<td>96.0</td>
<td>98.2</td>
<td>97.5</td>
<td>97.9</td>
</tr>
<tr>
<td>CD8 + day 10 CD25⁺ (n = 3)</td>
<td>98.5</td>
<td>99.4</td>
<td>98.6</td>
<td>98.9</td>
</tr>
</tbody>
</table>

NOTE. At the conclusion of the experiment (day 90), spleens were harvested from lethally irradiated CBA mice that received B10.BR ATBM alone or in combination with 3 × 10⁶ CD8⁺ T cells followed by 1 × 10⁶ B10.BR CD4⁺CD25⁺ T cells on either days 4 or 10 posttransplantation. Dual color flow cytometry was performed for the lymphocyte subset expression of Ly9.1, the presence of which denoted cells of host origin. Data is expressed as mean % donor chimerism ± SEM and is representative of 2 individual experiments.
whereas those additionally challenged with MMCBA6 leukemia cells also died of apparent GVHD (MST 29.6 days, with no significant difference between these groups; $P = 0.06$). In contrast, the day 10 infusion of donor CD4$^+$CD25$^+$ T cells allowed for a prolonged survival with an MST of 64.8 days. Reduced GVHD target tissue injury was shown by lower apoptotic keratinocyte counts per Lmm of epidermis from skin samples harvested on day 35 post-transplantation from CD4$^+$CD25$^+$ T cells allowed for a prolonged survival with an MST of 64.8 days. Reduced GVHD target tissue injury was shown by lower apoptotic keratinocyte counts per Lmm of epidermis from skin samples harvested on day 35 post-transplantation from CD4$^+$CD25$^+$ infused mice compared with untreated CD8 plus MMCBA6 controls (Figure 6). Further qualitative histological analysis of skin samples also showed a greater degree of epithelial hyperplasia and epidermal T-cell infiltration in untreated animals compared with CD25$^+$ recipients (Figure 6). Gross pathological examination of surviving mice at the conclusion of the experiment (day 100) showed restricted leukemia growth, as indicated by qualitative observations of either early-stage (low-volume) peritoneal edema collection or solid tumor growth localized to the area of the original tumor injection. Furthermore, these remaining mice showed no signs of clinical GVHD (ie, cachexia, runted appearance) and had mean % initial body weights similar to the control ATBM group by 10 weeks post-HCT ($P = 0.09$; Figure 5B).

**DISCUSSION**

A number of different approaches have shown promise in preclinical studies as a means to achieve the separation of GVHD development from GVL responses. These approaches include selective lymphocyte subset depletion (CD4$^+$, CD8$^+$, NK1.1$^+$) [23-25]; *in vivo* cytokine manipulation [26-30]; treatments
based on effector phenotype differences between GVHD- and GVL-mediating T-cell populations [31-34]; and in vivo T-cell depletion posttransplantation [35,36]. In addition, we have used CDR3-size spectra-type analysis to identify TCR Vβ families involved in the anti-tumor but not the anti-host allogeneic response, allowing for the successful transfer of T cells capable of mediating GVL without GVHD [37]. Arguably, however, the most effective GV response occurs in the context of GVHD [38-42], taking advantage not only of tumor allantigen expression but also the inflammatory environment generated by the allogeneic reaction. Experimentally, this can be appreciated in the work of Johnson et al. and Drobski et al, where they note more effective GVL and increased donor engraftment after protocols targeted for GVHD therapy rather than prevention [35,36]. These data suggest that by controlling the progression of the allogeneic response posttransplantation, the positive effects of a GVH reaction can be separated from its pathological effects. Our current results support this notion by showing that delayed infusion of donor-derived CD4+CD25+ regulatory T cells to the recipient posttransplantation can be used as a means to ameliorate an ongoing GVHD response without sacrificing lasting donor engraftment or a protective GV effect.

The use of CD4+CD25+ T cells in tissue transplantation models has high clinical relevance. Not only have CD4+CD25+ cells been found in the thymus and peripheral blood of humans, but they also appear to have immunoregulatory properties similar to those observed in experimental models [43-45]. Furthermore, the difficulty of isolating significant numbers of these cells from human peripheral blood (about 3% of total peripheral blood mononuclear cells) can be overcome by in vitro expansion, a process that does not forfeit their regulatory function [5,22,43].

We have shown that freshly isolated donor CD4+CD25+ T cells themselves are not capable of mediating GVHD in the C3H (H2k)→(B6xC3H)F1 (H2k/b) haploidentical, lethal irradiation model. However, they can prevent lethal GVHD and most associated cachexia when administered at a 1:1 ratio (effector cell:CD25) at the time of transplantation. Although these findings support published results showing prevention of GVHD by infusion of freshly isolated donor CD4+CD25+ T cells at the time of transplantation [46,47], these data contrast with the findings of Taylor et al. [13], who reported only a modest GVHD protective effect mediated by these cells at a similar 1:1 ratio in the B6→bm12 MHC class II-disparate model. These different observations are most likely because of variation in the GVHD models used because their system involved sublethal irradiation without the use of donor bone marrow rescue. B6 T cells were transplanted to bm12 recipients and may have been able to destroy host stem cells before CD25 regulation could take full effect [13]. In contrast, in the present study, GVHD-related target organ injury may occur at a slower pace and, therefore, could come under better control by the CD25+ cells.

Because suppression of the allogeneic response at the time of transplantation could endanger engraftment facilitating GVH reactions as well as an optimal GVL effect, we sought to discern whether donor CD4+CD25+ cells could still temper GVHD development when injected post-HCT. In contrast to infusion at the time of transplantation, we found that freshly isolated donor CD4+CD25+ cells were incapable of preventing lethal GVHD in the C3H→(B6xC3H)F1 model when injected on day 2 posttransplantation, even at a 1:4 ratio (effector cell:CD25). However, the same number of in vitro expanded CD4+CD25+ cells completely prevented lethal GVHD, likely reflecting the markedly enhanced frequency and regulatory properties of these cells, as first described by Thornton and Shevach [5]. These data are consistent with the previous findings of Taylor et al. in the injection of in vitro expanded regulatory T cells at the time of transplantation to prevent GVHD [22].

A small prolongation in overall survival was observed in lethally irradiated (B6xC3H)F1 transplanted recipients underwent transplantation with C3H CD4+ T cells and infused with donor CD4+CD25+ cells on day 2 posttransplantation. Some degree of regulatory activity by CD25+ cells also has been observed in murine experimental autoimmune encephalomyelitis models [48]. In the context of allogeneic HCT, this small effect might be related to the observed GVHD ameliorating properties of exogenous IL-2 and IL-12 [49,50] because significant amounts of these cytokines may be produced after the alloactivation of the infused C3H CD4+CD25+ cells. Interestingly, B10.BR CD4+CD25+ cells infused on either day 2, 4, or 10 into recipients of allogeneic CD8+ T cells did not show any regulatory properties and, in fact, hastened GVHD kinetics. This is consistent with the lack of inhibitory effects of IL-2 administration in CD8-mediated GVHD models [51,52].

Despite their enhanced regulatory function, expanded CD4+CD25+ cells used at a 1:4 ratio (effector cell:CD25) could not prevent lethal CD4-mediated GVHD in the C3H→(B6xC3H)F1 model when injected beyond day 2 posttransplantation. In stark contrast, lethal GVHD was prevented with a day 10 posttransplantation infusion of freshly isolated donor CD4+CD25+ T cells in the MHC matched, CD8-mediated B10.BR (H2k)→CBA(H2k) model. Clearly, there are differences in the intensity and severity of GVHD development between the 2 models, which could account for the capacity to control disease at a
later stage in the miHA disparate CD8-mediated model. On the other hand, in the CD4-mediated haploidentical model, the most critical period of T-cell involvement is likely to include their activation, expansion, and early release of cytokines, such as INF-γ and IL-2 [53]. This sets the stage for the rapid priming and activation of donor/host accessory cells, such as macrophages, which quickly escalate the inflammatory response with the production of large amounts of TNF-α, IL-1, nitric oxide, and IL-12 [53]. Death of the animal soon follows rapid weight loss and massive diarrhea. In such a scenario, regulatory T cells would only make a difference in the progression of the disease early in its development, because it is unlikely that they would have as significant an effect once accessory cells take on a larger effector cell role. Support for the concept of a larger accessory cell role in the C57→B6×C3H)F1 model comes from data showing greater suppression of GVHD lethality by TNF-α neutralizing mAb treatment in this model than in the B10.BR→CBA strain combination (unpublished data, R. Korngold). Furthermore, the notion of required sustained donor T-cell involvement during miHA-elicited GVHD is supported by data showing amelioration of disease across an MHC-matched barrier after disruption of the alloreactive T-cell response with anti-CD3 mAb at a late time point (18 days) post-HCT [35]. Therefore, it is in models, such as the B10.BR→CBA, where the efficacy of delayed therapeutic approaches that target alloreactive T cells is likely to be realized.

Achieving reduced GVHD lethality without sacrificing a high level of donor engraftment or an effective GVL response underscores the importance of being able to control the progression of the anti-host-specific T cells posttransplantation. Importantly, the requirements for regulation of the alloresponse at later time points may be less stringent than at the time of HCT because the latter situation involves the suppression of a large bulk of alloreactive cells at once in the lymphoid compartment, and, perhaps mostly in the spleen, after intravenous coadministration. In contrast, by day 10 in the miHA model studied here, many of the alloreactive cells may have already undergone activation in the lymphoid compartment and subsequent activation-induced cell death, or have left to traffic to other peripheral tissue sites, thereby reducing the number of cells in situ to be regulated by the entering CD4⁺CD25⁺ T cells, and, therefore, requiring less of them. Another consideration is that by day 10 post-HCT, most of the lymphoid compartment antigen-presenting cells (APCs) have been killed by the cytolytic anti-host CD8⁺ T-cell response, thus preventing the activation of MHC class II restricted CD4⁺CD25⁺ T cells in the spleen and LNs, and possibly allowing for more direct trafficking of these cells to peripheral tissue sites. Stimulation by class II expressed on activated epithelial cells, endothelial cells, and professional APCs that have not yet been targeted, may then follow, leading to direct regulation of the tissue-lytic alloresponse. We are currently investigating whether in vitro expanded CD4⁺CD25⁺ T cells could be of even greater use in the miHA system than freshly isolated cells. Preliminary results suggest that the frequency of anti-miHA T cells may still be too low after primary in vitro allo + IL-2 expansion to make a significant difference, thus suggesting that, in this case, stimulation with anti-CD3 and IL-2 may be a more effective means of expansion.

During the initiation of GVHD, not all alloreactive mature donor T cells respond at once to alloantigen [54]. Therefore, protocols that limit GVHD by targeting the alloreactive T-cell response and that involve pharmaceutical or biochemical agents with short biological half lives must be administered multiple times to be continuously present throughout the development of the alloresponse. The administration of CD4⁺CD25⁺ T cells to control GVHD has important advantages over such protocols. First, the extended survival and proliferation of CD4⁺CD25⁺ cells in vivo, without the loss of regulatory function, has been shown in lymphopenic hosts [55]. This extended presence means that regulation of GVHD can be maintained over the full development of the response with one administration of CD4⁺CD25⁺ cells. Second, the ability of CD4⁺CD25⁺ cells to regulate the function of activated, as well as naive, T cells has been shown in an experimental autoimmune model [56]. Therefore, mature donor T cells that have already responded to donor alloantigen also may be subjected to regulation by a subsequent CD4⁺CD25⁺ cell injection. Our data support this observation by showing control of GVHD development with a day 10 posttransplantation injection of CD4⁺CD25⁺ cells, by which point, one would expect that most donor CD8⁺ T cells had already been activated by host alloantigens. Indeed, in vivo observations of the initial anti-miHA proliferative response of CD4⁺, as well as CD8⁺ T cells in the MHC-matched B6→BALB.B strain combination, have indicated that as many as 7 rounds of cell division can occur within 5 days after transplantation (unpublished data, S. C. Jones and R. Korngold).

We have yet to fully address questions surrounding how the infusion of donor CD4⁺CD25⁺ T cells might affect reconstitution of general immunity because the finding that these cells are capable of antigen-nonspecific regulation [5] makes this a valid concern. Although the extended survival of CD4⁺CD25⁺ T cells in a lymphopenic environment has been shown [55], their longevity in MHC-disparate recipients that have converted to full donor chimerism is questionable because their survival also has been shown to be dependent on recognition of cognate antigen
[57,58]. In the miHA-disparate situation, however, there is the potential for continuous allo-stimulation because even with 100% donor chimerism, radioresistant host stromal tissue is continuously scavenged and thus class II restricted miHAs can still be presented by donor-derived APCs to remaining donor CD4\(^+\)CD25\(^+\) cells.

In conclusion, although a number of uncertainties remain surrounding the application of CD4\(^+\)CD25\(^+\) regulatory T cells, the current results provide the impetus for further investigation into their delayed infusion to regulate the full pathological development of GVHD while not sacrificing an effective GVL response.

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