

Amotosalen-Treated Donor T Cells Have Polyclonal Antigen-Specific Long-Term Function without Graft-versus-Host Disease after Allogeneic Bone Marrow Transplantation

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ABSTRACT

We have previously shown that amotosalen HCl (S-59 psoralen)-treated donor splenocytes, which have limited proliferative capacity *in vitro*, can protect major histocompatibility complex-mismatched bone marrow transplant (BMT) recipients from lethal murine cytomegalovirus infection without causing graft-versus-host disease. In this study, we further investigated the effects of amotosalen-treated donor T cells on immune reconstitution after allogeneic BMT. We were surprised to find that amotosalen-treated donor T cells persisted long-term *in vivo*, comprising 6% to 10% on average of the T-cell compartment of transplant recipients at 4 months after transplantation. Donor T cells derived from amotosalen-treated splenocytes were predominantly polyclonal CD44^{hi/int} CD8⁺ memory T cells and were functionally active, synthesizing interferon γ in response to stimulation with murine cytomegalovirus antigen. Amotosalen-treated donor T cells, reisolated from BMT recipients' spleens ≥ 4 months after transplantation, proliferated *in vitro*, thus indicating repair of amotosalen-mediated DNA cross-links. Compared with infusion of untreated donor splenocytes, amotosalen-treated cells enhanced thymopoiesis by bone marrow-derived stem cells in BMT recipients. However, amotosalen treatment abrogated the thymopoietic activity of lymphoid progenitor cells among the donor splenocytes. Thus, infusion of amotosalen-treated donor T cells produced rapid immune reconstitution after major histocompatibility complex-mismatched BMT by transferring long-lived polyclonal memory T cells with antiviral activity and also by enhancing bone marrow-derived thymopoiesis. This is a novel approach to adoptive immunotherapy in allogeneic BMT.

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KEY WORDS

Donor T cells • Amotosalen • Allogeneic bone marrow transplantation • Immune reconstitution

INTRODUCTION

The therapeutic potential of allogeneic bone marrow transplantation (BMT) and hematopoietic progenitor cell transplantation for treatment of malignant diseases relates both to the effect of the chemoradiotherapy used during conditioning and to the graft-versus-leukemia activity of donor T and natural killer lymphocytes [1-3]. However, graft-versus-host disease (GVHD) is a major complica-

tion after allogeneic BMT that limits the overall success of the procedure. Efforts have been made to reduce the GVHD activity of allogeneic BMT by depleting donor T cells from the graft. However, this approach results in delayed immune reconstitution, which leads to opportunistic infections such as cytomegalovirus (CMV) and aspergillus, as well as the loss of graft-versus-leukemia effects, and this predisposes to leukemia relapse [4,5].

A significant number of hematopoietic progenitor cell transplant recipients experience CMV infection and develop CMV pneumonitis, a fatal clinical syndrome in many affected patients [6]. Drug-resistant herpes simplex infections are also an increasing problem among allogeneic BMT patients [7], and this further highlights the need for more effective antiviral therapeutic approaches after BMT. Using donor T cells with antiviral activity for adoptive immunotherapy is a powerful approach to accelerate immune reconstitution after transplantation; however, existing methods involving the isolation and in vitro expansion of antiviral T-cell clones are labor intensive and have not been widely applied to clinical transplantation [8]. Hence, we have focused on developing rapid approaches to pretreat bulk polyclonal populations of donor T cells to reduce their GVHD potential while preserving their antiviral activity [9].

Recent studies have demonstrated the therapeutic potential of donor T cells treated ex vivo with amotosalen HCl, a novel synthetic psoralen compound that is photoactivated by UV-A light and reacts with pyrimidine bases to form covalent DNA monoadducts; DNA then cross-links, thus preventing DNA replication [10]. Amotosalen-treated donor T cells lack significant proliferative capacity in vitro [11–13]. When transplanted in combination with T cell-depleted (TCD) bone marrow (BM) grafts, pretreated donor T cells suppress allograft rejection and improve survival without causing acute GVHD [14–16]. It is important to note that we have shown that amotosalen-treated donor T cells also rapidly reconstitute antiviral immunity in transplant recipients. Infusion of pretreated donor splenocytes at the time of transplantation protects murine major histocompatibility complex (MHC)-mismatched BMT recipients from lethal murine CMV (mCMV) infection without causing GVHD [17].

The work presented herein was undertaken to evaluate the effects of pretreated T cells on long-term immune reconstitution in survivors of allogeneic BMT. We initially hypothesized that amotosalen-treated donor T cells would persist for only a short period after infusion into BMT recipients. Unexpectedly, we found that amotosalen-treated donor T cells with a memory phenotype persisted for at least 4 months in vivo. By studying the functional properties of these donor T cells reisolated from BMT recipients, we found that amotosalen-treated donor T cells retained the ability to express interferon (IFN)- γ in response to mCMV antigen. Even though pretreated T cells did not produce detectable GVHD, surviving cells had regained the ability to proliferate in vitro in response to mCMV antigen or anti-CD3 stimulation. Additionally, donor BM-derived thymopoiesis, a process that is particularly sensitive to ongoing GVHD

[18], was enhanced in recipients of amotosalen-treated splenocytes as compared with recipients of untreated splenocytes. These data, therefore, demonstrate that this novel form of immunotherapy rapidly improves antiviral immunity by transferring polyclonal memory T cells and enhances long-term immune reconstitution by supporting BM-derived thymopoiesis in allogeneic BMT recipients.

MATERIALS AND METHODS

Mice

CB6F1 (C57BL/6 \times BALB/c; H-2^{b/d}, CD45.2/Thy1.2) mice and PepBoy (B6.SJL-*Ptprc*^a*Pep3*^b/BoyJ; H-2^b, CD45.1/Thy1.2) mice on the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). BA mice (H-2^b, CD45.2/Thy1.1) on the C57BL/6 background (Dr. Miriam Lieberman, Stanford University, Stanford, CA) were bred at Emory. Procedures conformed to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Emory University Institutional Animal Care and Use Committee.

Preparation of Lymphocytes for Adoptive Immunotherapy

Immune splenocytes were harvested from PepBoy donors previously inoculated with 10⁵ plaque-forming units of mCMV (Smith strain; American Type Culture Collection, Manassas, VA) 2 to 4 months earlier. Splenocytes were cultured at 10⁷ cells per milliliter in complete media (RPMI plus 10% heat-inactivated fetal bovine serum, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, 50 mmol/L β -mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin, and 29 mg/mL l-glutamine) at 37°C in 5% CO₂ for 24 hours according to our previously described methods [17]. The cultured cells were resuspended at 20 \times 10⁶ per milliliter of phosphate-buffered saline with 5% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) containing 2 nmol/L amotosalen (S-59 psoralen; Cerus Corporation, Concord, CA). A 25-mL total volume was added per 75-cm² tissue culture flask, and the cells were illuminated with UV-A light (320–400 nm; 3.0 J/cm² UV-A dose; Cole Parmer, Vernon Hills, IL) for a total of 5 minutes divided into 2.5-minute fractions separated by a 1- to 2-minute period of gentle agitation. Treated cells were washed, and live cells were counted again under fluorescence microscopy by using ethidium bromide mixed with acridine orange dye [19]. The viability was usually >80% after photochemical treatment, and an appropriate dosage of viable nucleated cells was used for adoptive immunotherapy. Cells cultured for 24 hours without amotosalen treatment were used as controls.

Irradiation, Cell Transfer, and mCMV Infection of BMT Recipients

On day -1, CB6F1 mice received a total of 11 Gy of irradiation divided into 2 doses (5.5 Gy each) 3 hours apart [9]. The following day, BM was flushed from femurs and tibiae of naive BA donor mice, and CD3⁺ T cells were depleted as previously described [9]. A total of 5×10^6 TCD BM cells were then transplanted alone or with different doses (ie, 1×10^6 , 3×10^6 , 10×10^6 , or 30×10^6) of amotosalen-treated or untreated (culture only) donor splenocytes via tail vein injection into irradiated CB6F1 recipient mice. Two hours later, recipient mice were infected intraperitoneally with 5×10^4 plaque-forming units of mCMV or vehicle. The development of clinical GVHD was monitored twice weekly by weight loss and clinical signs of hair loss, ruffled fur, diarrhea, and decreased activity [9]. Moribund mice were killed and considered to have died on the day after death for analysis of posttransplantation survival.

Lymphocyte Isolation

Splenocytes and thymocytes were harvested from BMT recipients by gently crushing tissue between frosted glass slides (Fisher Scientific, Pittsburgh, PA). Single-cell suspensions were prepared by passing the resulting cell suspension through a cell strainer (BD Biosciences, San Jose, CA). Live lymphocytes were quantitated by fluorescence microscopy.

T-Cell Proliferation

To measure mCMV peptide-specific proliferation, 100 000 splenocytes were cultured in 96-well flat-bottomed tissue culture plates with an equal number of irradiated (100 Gy) EL-4 cells previously loaded with the mCMV early peptide HGIRNASFI [20] for 1 hour. After 4 days, cells were pulsed with 1 μ Ci of [³H]thymidine and harvested 16 to 18 hours later, and [³H]thymidine incorporation was measured. To measure proliferation in response to CD3 cross-linking by plate-bound anti-CD3 monoclonal antibody (mAb; clone 145 2C11, functional grade; eBioscience, San Diego, CA), the antibody was diluted with phosphate-buffered saline to a concentration of 10 μ g/mL and used to coat 96-well flat-bottomed tissue culture plates overnight at 4°C. CD45.1⁺ cells were sorted (~99% purity) from total splenocytes by using a FACSVantage (BD Biosciences, San Jose, CA). A total of 100 000 CD45.1⁺ cells in 0.2 mL of complete media were added to anti-CD3-coated wells and incubated for 4 days at 37°C in 5% CO₂. Positive controls were splenocytes harvested from stably engrafted recipients of TCD BM plus 1×10^6 nontreated splenocytes, and cells cultured in uncoated wells served as negative controls.

Proliferation was measured as previously described [21].

Flow Cytometry

Flow cytometry was performed as previously described [9]. The origin of CD8⁺ and CD4⁺ T cells in BMT recipients was determined by staining with mAbs specific for donor BM (Thy1.1⁺), donor spleen (CD45.1⁺), or host T cells (CD45.2⁺ and Thy1.2⁺) in combination with mAbs to CD8 and CD4. V β T-cell receptor (TCR) expression on donor splenic CD8⁺ and CD4⁺ T cells was determined by staining with fluorescein isothiocyanate-conjugated mAbs to 15 V β TCR isotypes (V β 2, 3, 4, 5.1/5.2, 6, 7, 8.1/8.2, 8.3, 9, 10b, 11, 12, 13, 14, and 17a) in combination with CD45.1 phycoerythrin, CD8 α peridinin chlorophyll protein, and CD4 allophycocyanin (APC). Anti-mCMV-specific CD8⁺ T cells were quantitated by using an APC-conjugated HGIRNASFI/H-2D^b tetramer (NIAID Tetramer Core Facility, Atlanta, GA) [20] and by intracellular cytokine staining (ICS). For ICS, surface-stained cells were permeabilized and fixed according to the manufacturer's instructions (Cytofix/Cytoperm; BD Biosciences, San Diego, CA) and then incubated with a mAb against IFN- γ . For quantitation of donor spleen-derived memory and naive CD8⁺ T cells, cells were stained with mAbs for CD44 (fluorescein isothiocyanate) and CD62L (biotin-streptavidin APC), respectively, along with mAbs for CD45.1 (phycoerythrin) and CD8 α (peridinin chlorophyll protein). Isotype-matched antibodies were used as controls. Stained cells were analyzed on a FACSort instrument (BD Biosciences, San Jose, CA) by using Cell Quest software. All antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA).

Statistical Analyses

The Student *t* test was applied as described with differences considered significant when *P* < .05.

RESULTS

Therapeutic Effectiveness of Amotosalen-Treated Allogeneic Donor Splenocytes

By using the parental \rightarrow F1 model of MHC-mismatched BMT, TCD BM from the C57BL/6 congenic strain BA (CD45.2⁺Thy1.1⁺) was transplanted with graded doses (1×10^6 , 3×10^6 , 10×10^6 , or 30×10^6) of splenocytes from mCMV-immune PepBoy (CD45.1⁺Thy1.2⁺) mice. The splenocytes were either amotosalen treated or untreated. Amotosalen-treated splenocytes effectively prevented mCMV lethality over a broad therapeutic range of cell doses. Recipients of 30×10^6 , 10×10^6 , 3×10^6 , and 1×10^6 pretreated splenocytes showed

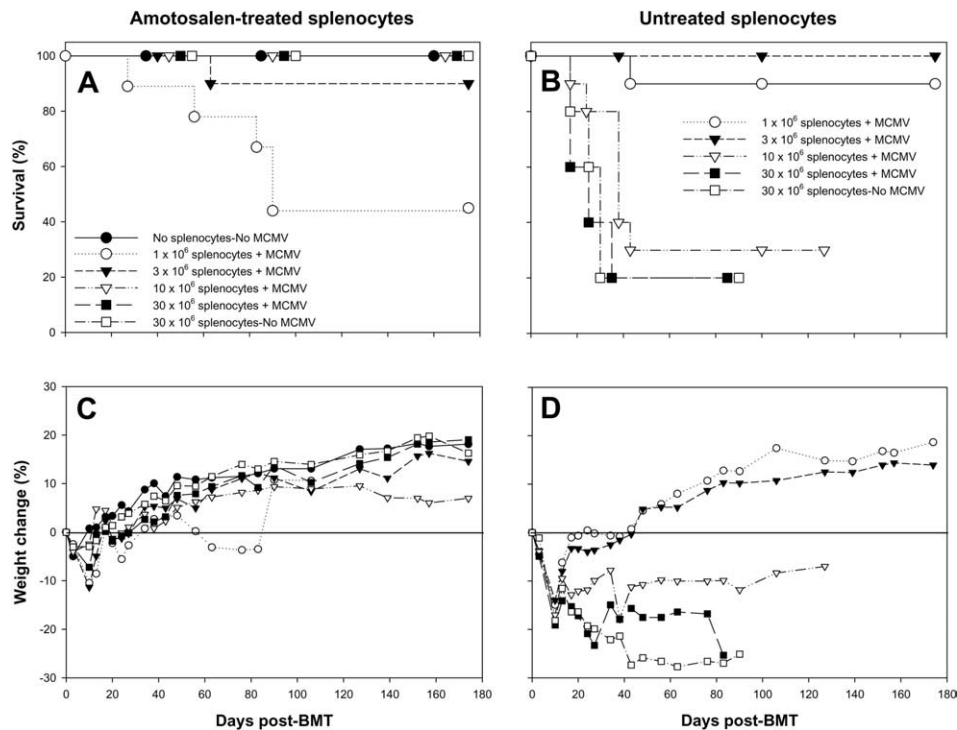


Figure 1. Dose response of amotosalen-treated donor splenocytes in allogeneic BMT. Irradiated F1 mice were injected with 1×10^6 (○), 3×10^6 (▼), 10×10^6 (▽), or 30×10^6 (■) amotosalen-treated (A and C) or untreated (B and D) splenocytes obtained from immunized PepBoy donors in combination with TCD BM from the naive BA donors. Two hours after transplantation, recipients were infected with 5×10^4 plaque-forming units of mCMV. Some groups received 30×10^6 splenocytes without mCMV infection (□) or 5×10^6 TCD BM only (●). A and B, survival; C and D, weight change over the course of the study.

100%, 100%, 90%, and 40% survival to day +100 after transplantation, respectively (Figure 1A). With the exception of the fourth group, all mice gained weight at approximately the same rate as control non-mCMV-infected BMT recipients (no splenocytes/no mCMV; Figure 1C). In contrast, recipients of 30×10^6 untreated splenocytes, with or without concomitant mCMV infection, experienced 80% mortality by day +40 (Figure 1B). In previous studies using this model, we confirmed the antiviral activity of untreated splenocytes and showed that mortality in this setting is due to GVHD [17]. Mice that underwent transplantation with 10×10^6 untreated splenocytes also developed severe GVHD: only 30% survived to day 136. Recipients of 1 to 3×10^6 untreated splenocytes developed transient nonlethal GVHD after transplantation, as manifested by a rapid 20% weight loss and subsequent recovery (Figure 1D). Other clinical signs of GVHD (decreased motion or activity, hunched back, and ruffled fur) were consistent with the weight data (not shown). All mCMV-infected recipients of TCD BM alone died within 20 days of transplantation (not shown), whereas all recipients of TCD BM without splenocytes or mCMV infection survived to the termination of the experiment (no splenocytes/no mCMV; Figure 1A).

Effects of Amotosalen-Treated T Cells on Post-BMT Lymphoid Reconstitution

To investigate the effects of donor T cells on immune reconstitution, long-term-surviving BMT recipients were studied 4 months after transplantation. BMT mice that received 3×10^6 or 10×10^6 amotosalen-treated cells had an average of $140 \pm 39 \times 10^6$ and $117 \pm 38 \times 10^6$ nucleated cells per spleen, respectively, both of which were significantly greater than the average $54 \pm 34 \times 10^6$ splenocytes in mice that received 3×10^6 untreated cells ($P < .05$; Figure 2). Recipients of 30×10^6 amotosalen-treated splenocytes, with and without concomitant mCMV infection, had similar numbers of splenocytes ($92 \pm 28 \times 10^6$ and $96 \pm 49 \times 10^6$, respectively). Because amotosalen treatment blocks lymphocyte proliferation [17], it was unexpected that recipients of amotosalen-treated lymphocytes would have significantly better splenic reconstitution than recipients of untreated cells. Subsequent studies to investigate phenotypic and functional differences in lymphoid reconstitution between recipients of amotosalen-treated and untreated splenocytes focused on recipients of 3×10^6 or 10×10^6 amotosalen-treated and 3×10^6 untreated splenocytes. Recipients of larger numbers ($\geq 10 \times 10^6$) of untreated splenocytes died of GVHD or were severely lymphopenic.

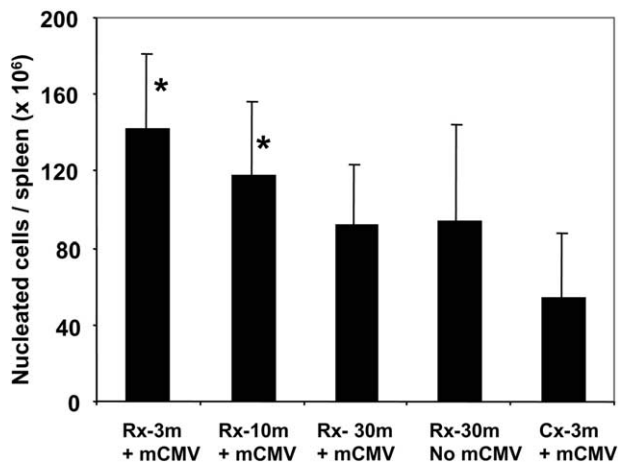


Figure 2. Quantitation of nucleated cells in the spleens of BMT recipients. Splenocytes were harvested from recipients' spleens ≥ 4 months after BMT and mCMV infection and were counted by using fluorescence microscopy. The mean absolute number (with standard error) of nucleated live cells per spleen in each group ($n = 5$) is shown. Rx indicates amotosalen treated; Cx, untreated. *Student *t* test; $P < .05$ as compared with recipients of 3×10^6 untreated splenocytes.

Splenic T-cell chimerism was studied by fluorescence-activated cell-sorting (FACS) analysis with antibodies specific for congenic surface markers (Table 1). We were surprised to find that amotosalen-treated and untreated T cells persisted to a similar extent in vivo. There were no significant differences in the absolute numbers of donor splenocyte-derived $CD3^+$, $CD8^+$, or $CD4^+$ T cells among mice that received 3×10^6 or 10×10^6 amotosalen-treated splenocytes or 3×10^6 untreated splenocytes. Similar numbers were also seen for recipients of 30×10^6 amotosalen-treated splenocytes without mCMV infection. Likewise, there were no differences in the numbers of residual host-derived T cells among these groups. However, the differences in total splenocytes among groups seen in Figure 2 could be accounted for by donor BM-derived lymphocytes. Recipients of amotosalen-treated donor splenocytes and mCMV infection had 3 to 4 times more $CD3^+$, $CD4^+$, and $CD8^+$ T cells derived from donor BM progenitors than did mCMV-infected re-

cipients of 3×10^6 untreated splenocytes ($P < .05$) or mCMV-negative recipients of 30×10^6 amotosalen-treated splenocytes ($P < .05$). It is interesting to note that in all groups, donor splenocyte-derived T cells were predominantly (80%-90%) $CD8^+$, whereas donor BM-derived T cells were mainly (65%-70%) $CD4^+$. Thus, in the presence of mCMV infection, recipients of amotosalen-treated cells had better BM-derived lymphoid reconstitution than recipients of untreated splenocytes.

Persisting Amotosalen-Treated Donor Splenic T Cells Retain a Polyclonal V β TCR Repertoire

Because long-term survival of amotosalen-treated T cells was unexpected, we investigated the possibility that persisting T cells represented an oligoclonal expansion of a limited population of donor T cells that escaped psoralen-mediated DNA cross-linking. TCR V β expression on donor splenocyte-derived T cells was quantitated by FACS. $CD8^+$ (Figure 3) T cells showed similar polyclonal V β expression whether they were derived from amotosalen-treated (3×10^6 or 10×10^6) or untreated (3×10^6) splenocytes. TCR V β diversity was also similar to wild-type C57BL/6 mice with or without mCMV infection. These data indicate that persisting amotosalen-treated T cells are a diverse polyclonal population.

Persisting Amotosalen-Treated $CD8^+$ T Cells Are Predominantly Memory Cells That Express IFN- γ in Response to mCMV Antigen

At 4 months after transplantation, CD44 and CD62L expression was quantitated on $CD45.1^+$ $CD8^+$ splenic T cells. The vast majority (90%-95%) of $CD8^+$ T cells from mice that received adoptively transferred splenocytes and mCMV infection showed bright or intermediate staining intensity for CD44 ($CD44^{hi}$ and $CD44^{int}$ cells, respectively, in right upper quadrants of Figure 4A). In contrast, approximately 80% of $CD8^+$ T cells were $CD44^{hi/int}$ in BMT mice not infected with mCMV. Approximately 80% of $CD8^+$ splenocytes from naive C57BL/6 mice were also $CD44^{hi/int}$. Qualitative identification of cells as

Table 1. Splenic T-Cell Subsets from Transplant Recipients

Variable	Donor Spleen Derived			Donor BM Derived			Host Derived		
	CD3	CD8	CD4	CD3	CD8	CD4	CD3	CD8	CD4
Rx-10m + mCMV	3 \pm 1.2	2.4 \pm 0.6	0.6 \pm 0.5	27.7 \pm 14.2*	9.3 \pm 4.5*	18.4 \pm 9.7*	0.1 \pm 0.1	0.1 \pm 0.1	0.0 \pm 0.0
Rx-3m + mCMV	1.7 \pm 1.2	1.5 \pm 1.2	0.2 \pm 0.0	27.9 \pm 8.6*	8.4 \pm 2.9*	19.5 \pm 5.8*	1.5 \pm 1.5	0.7 \pm 0.7	0.8 \pm 0.7
Cx-3m + mCMV	2.5 \pm 1.2	2.2 \pm 1.1	0.4 \pm 0.1	7.7 \pm 6.1	2.6 \pm 1.4	5.1 \pm 4.6	0.1 \pm 0.0	0.1 \pm 0.1	0.1 \pm 0.0
Rx-30m/no mCMV	2.5 \pm 1.8	1.4 \pm 0.9	1.0 \pm 0.9	13.1 \pm 10.2	4.7 \pm 4.2	8.4 \pm 6.1	0.2 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.1

Spleens were collected 4 months after transplantation, and $CD3^+$, $CD8^+$, and $CD4^+$ T-cell subpopulations derived from donor splenocytes ($CD45.1^+$), donor BM ($Thy1.2^-CD45.2^+$), or the BMT recipient ($Thy1.2^+CD45.2^+$) were quantitated by FACS analysis. Numbers represent average per spleen \pm SD ($\times 10^6$).

*Student *t* test; $P < .05$ (as compared between the amotosalen-treated and untreated groups).

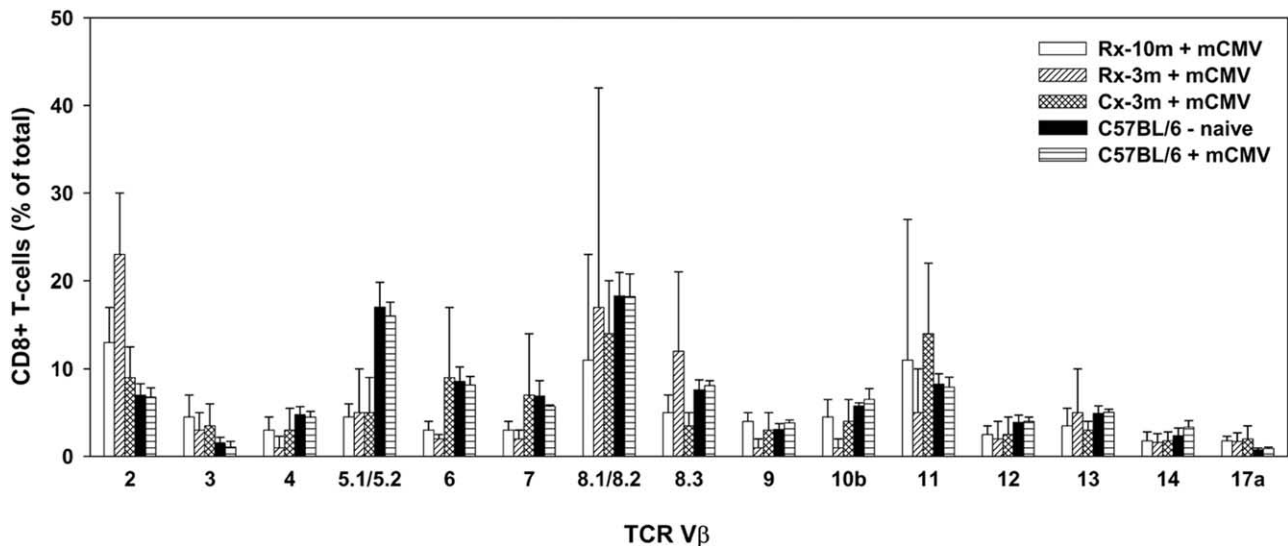


Figure 3. Donor splenocyte–derived CD8⁺ T cells have diverse TCR V β repertoires. Splenocytes were harvested from mCMV-infected BMT recipients (Rx-3m, Rx-10m, and Cx-3m) 4 months after transplantation and from mCMV-infected (60 days) or uninfected wild-type C57BL/6 mice. Cells were stained with anti-TCR V β mAbs along with mAbs against CD45.1⁺ (to select donor splenocyte–derived cells in BMT recipients) and CD8⁺. The percentages (mean \pm SD) of CD8⁺ T cells expressing each TCR V β subset are shown. The sum of all TCR V β ⁺ CD8⁺ subsets represents approximately 70% of the total number of T cells. Five to 6 mice were used per group. Rx indicates amotosalen treated; Cx, untreated.

CD44^{hi}, CD44^{int}, or CD44^{low} is dependent on gating. To better quantify CD44 antigen expression, mean fluorescent intensities (MFIs) were determined for the major populations of CD8⁺ T cells in Figure 4A. Whereas the MFIs varied from 64.3 to 140.3 for BMT mice, the MFI for CD44 staining of CD8⁺ T cells from naive C57BL/6 mice was 46.3. Thus, these findings indicate that CD8⁺ T cells from BMT recipients have reproducibly higher levels of CD44 antigen on their surface than do CD8⁺ T cells from naive C57BL/6 mice. The differences between groups were more impressive for CD62L expression (Figure 4B). The vast majority of CD8⁺ T cells in BMT mice that received adoptive immunotherapy and infection were CD62L^{low} (all approximately 94%). In contrast, 26% of cells were CD62L^{low} in the absence of mCMV infection, and only 10% of CD8⁺ T cells were CD62L^{low} in naive C57BL/6 mice. Thus, donor splenocyte–derived CD45.1⁺ CD8⁺ T cells are predominantly of the memory phenotype [22,23].

Anti-mCMV cytotoxic T lymphocytes (CTLs) derived from donor splenocytes (CD45.1⁺) were quantitated by both ICS and tetramer staining (Figure 4C). Isolated splenocytes were stimulated *in vitro* by EL-4 cells loaded with the HGIRNASFI mCMV peptide and subsequently stained for CD45.1, CD8, and intracellular IFN- γ [20]. Unstimulated splenocytes were stained with anti-CD8 and an H-2D^b/HGIRNASFI tetramer. Similar numbers of splenocyte donor–derived anti-mCMV CTLs were identified by both techniques, with averages ranging from 1.0 to 3.5 \times 10⁴ cells per spleen. There were no significant differ-

ences between mCMV-infected mice receiving amotosalen-treated (Rx) or untreated (Cx) splenocytes ($P > .05$). However, in the absence of mCMV infection in recipients of 30 \times 10⁶ (30m) Rx splenocytes, antiviral CTLs were present at significantly lower levels ($P < .05$). Similar results were obtained for peripheral blood lymphocytes (Figure 4D). Anti-mCMV CTLs derived from the BM donor or BMT recipient were only rarely identified (not shown). When splenocytes (Figure 4E) or peripheral blood lymphocytes (Figure 4F) from mCMV-infected BMT recipients were subjected to antigen-nonspecific stimulation with phorbol myristate acetate/ionomycin, there were once again similar numbers of CD45.1⁺CD8⁺ T cells expressing IFN- γ . Cell numbers were significantly lower ($P < .05$) in the absence of mCMV infection (amotosalen-treated-30m; Figure 4F). CD4⁺ T cells were only rarely IFN- γ ⁺ after phorbol myristate acetate/ionomycin stimulation (not shown). Thus, in mCMV-infected BMT recipients, persisting amotosalen-treated donor T cells were primarily of the memory phenotype and were capable of expressing IFN- γ in response to viral antigen or nonspecific stimulation.

Amotosalen-Treated T Cells Regain Limited Proliferative Potential after Infusion into BMT Recipients

We previously reported that immediately after treatment with amotosalen, T cells failed to proliferate *in vitro* when stimulated with plate-bound anti-CD3 mAb [17]. Because we found that a substantial number of amotosalen-treated donor T cells persisted

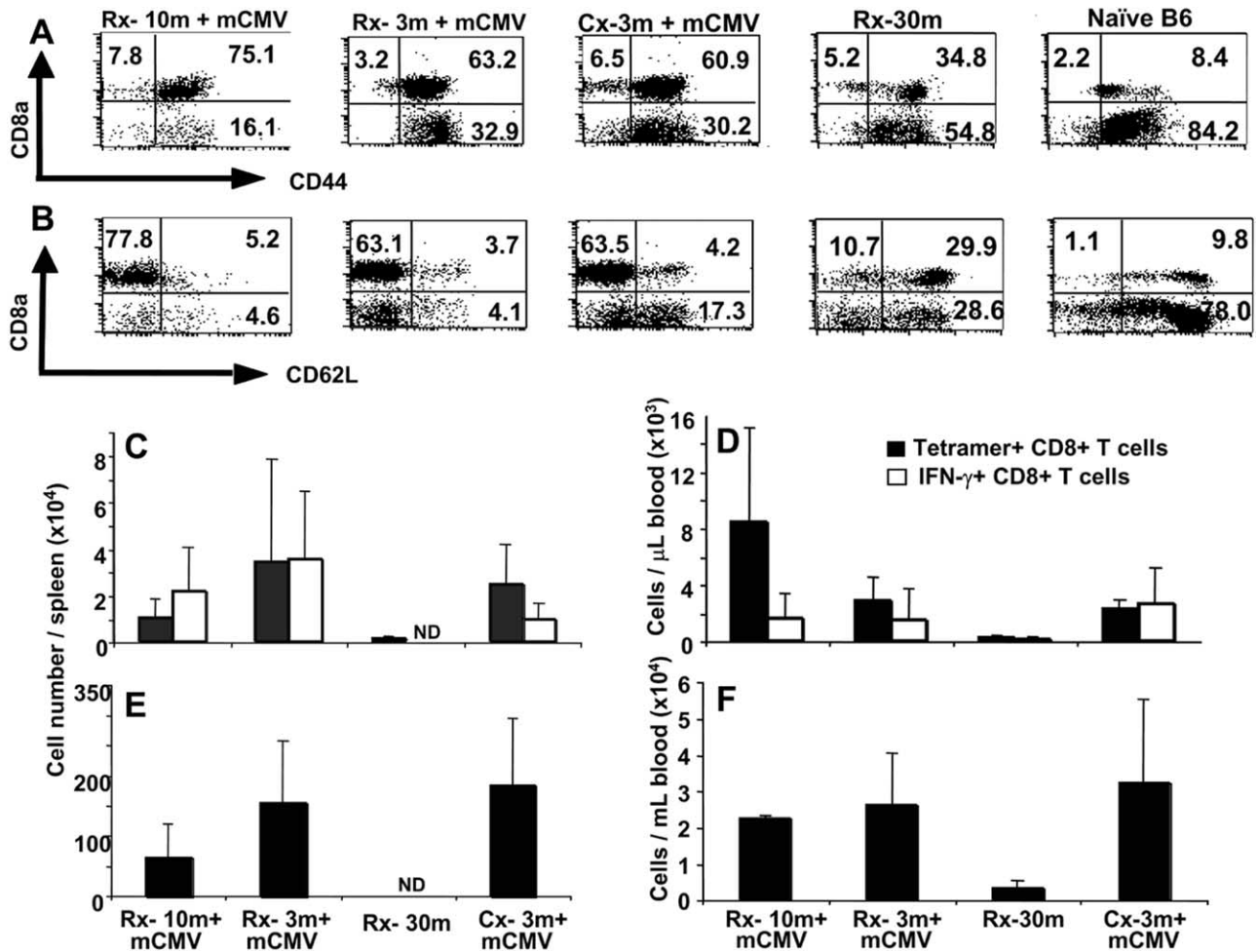


Figure 4. Surviving amotosalen-treated T cells are predominantly CD44^{hi} memory T cells that produce IFN- γ in response to stimulation. Splenocytes obtained from BMT recipient mice 4 months after transplantation and naive C57BL/6 mice were stained with mAbs against CD45.1, CD3, CD8, CD44, and CD62L. Data from representative mice show the percentage of CD45.1⁺ gated cells (from BMT recipients) or CD3⁺ gated cells (for C57BL/6 mice) that stain with CD8 and/or CD44 (A) and CD8 and/or CD62L (B). Antiviral T cells were quantitated as the number (mean \pm SD) of IFN- γ ⁺ CD8⁺ T cells after stimulation with mCMV peptide or HGIRNASFI/H-2D^b tetramer⁺ CD8⁺ T cells in the spleen (C) or peripheral blood (D). The numbers (mean \pm SD) of IFN- γ ⁺ CD8⁺ T cells in the spleen (E) and peripheral blood (F) are shown after stimulation with phorbol myristate acetate/ionomycin. Five to 6 mice were used per group.

for >4 months after transplantation, we investigated whether they had repaired DNA cross-links and regained the ability to proliferate. First, we reasoned that stimulation of CD8⁺ T cells with HGIRNASFI peptide should be relatively specific for HGIRNASFI/H-2D^b tetramer-positive cells, which in BMT recipients were found only among donor spleen-derived T cells and not donor BM-derived or residual recipient T cells (Figure 5A). When splenocytes from BMT recipients were stimulated with mCMV peptide-loaded irradiated EL-4 cells for 4 days, there was a significant peptide-specific proliferative response when compared with control cultures stimulated with EL-4 cells in the absence of peptide loading (Figure 5B). Proliferation of splenocytes from recipients of amotosalen-treated donor cells was comparable to proliferation of T cells from mice that received cultured splenocytes. To unequivocally demonstrate that the progeny of amotosalen-

treated T cells regained proliferative capacity, we next sorted donor spleen-derived CD45.1⁺ cells to >99% purity (not shown) from mice that originally underwent transplantation with 30×10^6 amotosalen-treated splenocytes 6 months earlier. The sorted cells were then stimulated with plate-bound anti-CD3 mAb for 4 days, and proliferation was compared with that of cells cultured in the absence of anti-CD3 (Figure 5C). Unfractionated splenocytes from recipients of 1×10^6 untreated splenocytes were used as a positive control. The T-cell progeny of the amotosalen-treated splenocytes strongly proliferated in response to anti-CD3 stimulation, as did untreated splenocytes. The results of these studies show that amotosalen-treated T cells that survive long-term in BMT recipients regain the ability to proliferate in response to stimulation through the TCR, which may account for their persistence [24,25].

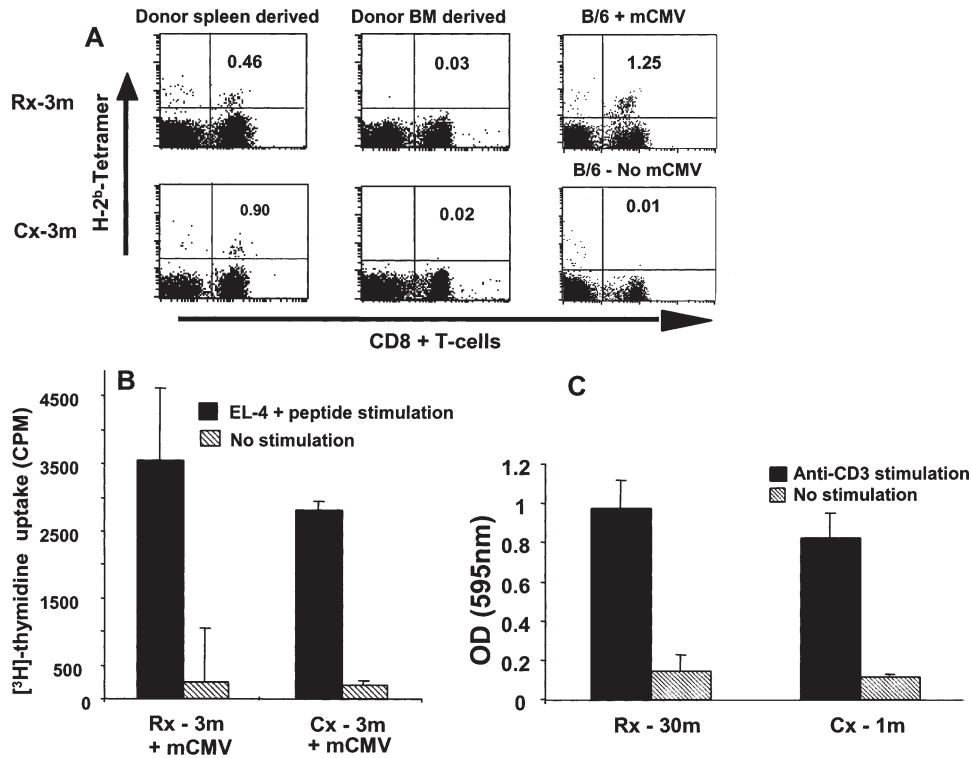


Figure 5. In vitro proliferation of T cells derived from amotosalen-treated splenocytes. Amotosalen-treated and untreated T cells were harvested 4 months after transplantation and stained with mAbs for CD45.1, Thy1.1, and CD8, as well as the HGIRNASFI/H-2D^b tetramer. Splenocytes from naive and mCMV-infected (7 days) C57BL/6 mice were prepared similarly (A). Isolated splenocytes (100 000) from BMT recipients were stimulated with an equal number of irradiated EL-4 cells loaded with mCMV peptide. Control wells contained splenocytes and irradiated EL-4 cells that were not loaded with peptide (no stimulation). After 4 days, proliferation was measured by [³H]thymidine incorporation. Mean and standard deviation for each group are shown (B). Donor spleen-derived CD45.1⁺ cells were FACS-sorted from BMT recipients of 30 × 10⁶ amotosalen-treated splenocytes (Rx-30m) 170 days after transplantation. A total of 100 000 cells were stimulated with plate-bound anti-CD3 mAb for 4 days, and 100 000 splenocytes from BMT recipients of 1 × 10⁶ untreated splenocytes (Cx-1m) were used as positive controls. After 4 days of culture, the cells were pulsed with methylthiazoletetrazolium, and incorporated methylthiazoletetrazolium was determined by the outer diameter measured at 595 nm (C). Rx indicates amotosalen treated; Cx, untreated.

Amotosalen-Treated T Cells Promote BM-Derived Thymopoiesis

Because recipients of amotosalen-treated T cells showed significantly better splenic lymphoid reconstitution by BM-derived T cells than recipients of untreated splenocytes, we investigated whether similar effects would be seen in thymopoiesis. Thymocytes were isolated from long-term-surviving BMT recipients, and T-cell chimerism was studied by FACS analysis. Mice that received 3 × 10⁶ or 10 × 10⁶ amotosalen-treated splenocytes had an average of 50 × 10⁶ cells per thymus, almost all of which (>98%) were derived from the donor BM (Figure 6A). In contrast, in BMT mice that received 3 × 10⁶ or 10 × 10⁶ untreated splenocytes and had GVHD, there were fewer total cells per thymus, and a higher percentage of thymocytes were derived from the splenocyte donor. In mice that received 30 × 10⁶ amotosalen-treated splenocytes without mCMV infection, essentially all thymocytes were derived from the donor BM. However, these mice had significantly fewer total thymocytes than the mCMV-infected recipients of treated splenocytes.

An orderly pattern of BM-derived thymopoiesis was observed in the mice that received amotosalen-treated splenocytes, including thymic intermediates (CD4⁺ CD8⁺; Figure 6B). In contrast, these mice had only rare thymic intermediates derived from the amotosalen-treated splenocytes, thus indicating that expansion of amotosalen-treated T cells after BMT is likely to be thymus independent. Infusion of untreated splenocytes resulted in thymopoiesis from both the donor marrow and donor splenocytes. Thus, amotosalen treatment largely abolished lymphoid progenitor cell activities in the donor splenocytes, although these animals displayed greater BM-derived thymopoiesis than recipients of identical numbers of untreated splenocytes and mCMV infection.

DISCUSSION

DNA-targeted photochemical treatment of allogeneic donor lymphocytes with amotosalen limits the proliferation of donor T cells in vitro and has been

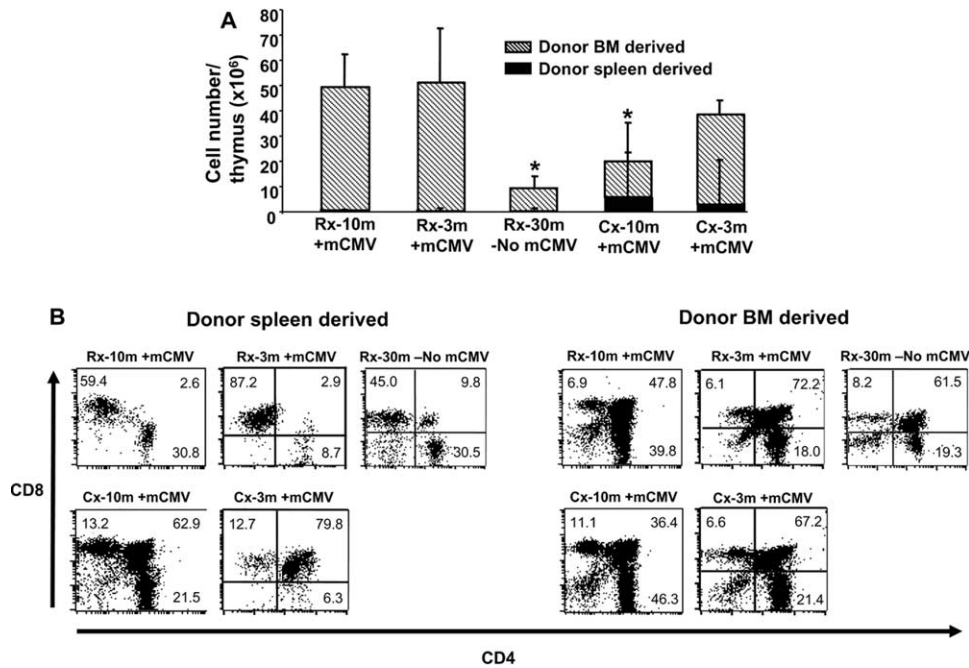


Figure 6. Amotosalen-treated splenocytes promote donor marrow–derived thymopoiesis. Thymocytes were harvested 4 months after transplantation from the designated BMT recipients. Cells were stained with mAbs for CD45.1⁺ and Thy1.1⁺, in combination with mAbs for CD4⁺ and CD8⁺ T cells. A, The overall mean chimerism per thymus. B, Representative FACS dot plots. Five to 6 mice were used in each group except for Cx-10m recipients, for which 3 mice were used. *Student *t* test; *P* < .05 as compared with recipients of 10×10^6 treated splenocytes. Rx indicates amotosalen treated; Cx, untreated.

used to promote BM engraftment with a limited risk of GVHD in BMT recipients [16,26]. Using the C57BL/6 → F1 murine BMT model with concomitant mCMV infection, we recently extended these findings and reported the following. First, 90% to 100% of BMT mice survive if they do not receive mCMV infection or donor splenocytes. Second, mCMV infection (5×10^4 plaque-forming units) of BMT mice in the absence of splenocyte infusion produces 85% to 100% mortality by day +30. Thus, without adoptive immunotherapy, mCMV infection is almost universally lethal in this model. Third, adoptive transfer of 30×10^6 untreated splenocytes to BMT mice (in the absence of mCMV infection) produces 100% mortality by day +35. In contrast, the same number of amotosalen-treated cells leads to $\leq 5\%$ mortality up to day +80. These data showed that infusion of untreated donor splenocytes leads to universally lethal GVHD in this model and that this can be prevented by amotosalen pretreatment of the splenocytes before infusion. Fourth, most importantly, amotosalen-treated lymphocytes retain potent antiviral activity in mCMV-infected BMT mice, on the basis of reductions in mortality from mCMV disease, reductions in viral loads, and persistence of donor spleen–derived anti-mCMV CTLs [17]. Although the splenocyte preparations contained approximately 40% CD3⁺ T cells and although we have shown that anti-mCMV CTLs in the preparations survive and

retain antiviral activity after amotosalen treatment [17], we have not yet ruled out the contribution of a minor cell population to control of mCMV infection in this model. Thus, amotosalen pretreatment effectively dissociates the GVHD and antiviral activities of donor splenocytes: treated splenocytes prevent otherwise lethal mCMV disease without causing GVHD. These findings and the accompanying observation that amotosalen-treated T cells persisted after infusion in vivo led us to investigate the effects of adoptive immunotherapy with amotosalen-treated donor splenocytes on long-term reconstitution of antiviral immunity in MHC-mismatched BMT recipients.

There are 2 major findings of these investigations. First, amotosalen-treated T cells persist and retain long-term functional antiviral activity after infusion into BMT recipients. Although some surviving T cells reacquire proliferative capacity, likely by repairing amotosalen cross-links, there is no evidence of chronic GVHD. Second, compared with recipients of untreated T cells, BMT mice that received amotosalen-treated cells showed significantly improved lymphoid reconstitution from donor BM–derived hematopoietic progenitors. These findings indicate that adoptive immunotherapy with amotosalen-treated lymphocytes can limit immune deficiency after BMT by providing functional donor T cells immediately after transplantation and by accelerating engraftment of the lymphoid system by donor hematopoietic progenitors. In this respect, our findings

differ from those of Arber et al. [27], who demonstrated that protection against lethal mCMV infection can develop after adoptive transfer of common lymphoid progenitors but did not test for antiviral activity sooner than 14 days after BMT.

Although mCMV-infected recipients of 3×10^6 amotosalen-treated versus untreated splenocytes showed similar survival and weight gain, there are quantifiable differences between the groups. For example, recipients of 3×10^6 pretreated splenocytes had more BM-derived lymphocytes in their spleens (Figure 2 and Table 1), including slightly higher numbers of anti-mCMV CTLs (Figure 4C), and improved thymopoiesis from BM-derived progenitors (Figure 6). Additionally, it is important to note that almost all mice receiving 3 to 30×10^6 amotosalen-treated splenocytes survived past 4 months and were available for analysis. Surviving mice gained weight appropriately without evidence of GVHD. By comparison, there was a much smaller therapeutic window for mice receiving untreated splenocytes in this study. Infusion of untreated splenocytes was effective only over the narrow range of 1 to 3×10^6 cells. Furthermore, mice that survived after infusion of 10×10^6 and 3×10^6 untreated splenocytes had evidence of GVHD. The group receiving 10×10^6 had ruffled fur and reduced weight gain, whereas mice receiving 3×10^6 untreated cells had small fibrotic spleens containing few lymphocytes (Figure 2). Thus, compared with the narrow therapeutic window for untreated splenocytes, amotosalen pretreatment is advantageous in that it expands the effective range of lymphocyte doses for adoptive immunotherapy. The broad therapeutic range for amotosalen-treated lymphocytes may allow the use of multiple escalated lymphocyte infusions to control viral infections with minimal risks that the accumulated cells would produce GVHD.

Analysis of lymphocytes in the spleens of BMT recipients 4 months after transplantation showed that approximately 6% to 10% of T cells were derived from the 3×10^6 or 10×10^6 amotosalen-treated splenocytes (containing $0.6\text{--}2 \times 10^6$ donor T cells) infused at the time of transplantation (Table 1). Thus, the number of T cells derived from the amotosalen-treated splenocytes was approximately equal to that present in the original splenocyte graft. Because the spleen contains approximately $\leq 10\%$ of all T cells in the body (M.S.H., unpublished data), these data imply that amotosalen-treated donor T cells expanded by an average of approximately 10-fold after transplantation. The phenotype of surviving donor T cells was primarily (80%–90%) that of memory $CD8^+$ T cells: $CD3^+$ $CD8^+$ $CD44^{hi/int}$ $CD62L^-$ [28] (Figure 4, A and B). The surviving amotosalen-treated donor T cells were functional, as illustrated by production of IFN- γ in response to mCMV antigen exposure (Figure 4). Quantitation of anti-mCMV CTLs by ICS and tetramer staining produced

comparable results of 1 to 3.5×10^4 antigen-specific donor spleen-derived $CD8^+$ T cells per spleen, on average, in recipients of amotosalen-treated splenocytes. Thus, a functional population of donor memory $CD8^+$ T cells survives amotosalen treatment and persists in MHC-mismatched BMT recipients, thus resulting in long-term transfer of antigen-specific immunity without GVHD [19]. Survival seems to be dependent in part on concomitant mCMV infection, on the basis of nearly undetectable levels of anti-mCMV CTLs in uninfected BMT recipients.

The polyclonal TCR V β repertoire of donor spleen-derived T cells (Figure 3) argues against selective survival of a small oligoclonal population of pretreated T cells and suggests the presence of T cells reactive to other antigens. Despite the polyclonality of persisting donor T cells, these mice did not experience GVHD, thus indicating that alloreactive donor T cells were either eliminated or inactivated after amotosalen pretreatment. This illustrates a significant difference between the use of amotosalen-treated lymphocytes for adoptive immunotherapy and the earlier approach of using antiviral CTL clones expanded *in vitro* [8]. Transfer of broad-spectrum polyclonal immunity with amotosalen-treated lymphocytes may be particularly effective against viral mutants that do not express immunodominant epitopes and can evade adoptively transferred CTL clones with narrow antigen specificity [29].

Amotosalen-pretreated T cells, which do not proliferate immediately after treatment [16,17], survived for at least 4 months *in vivo* at high levels. Two approaches were used to demonstrate that they had regained proliferative capacity after infusion. First, antigen-specific donor spleen-derived $CD8^+$ T cells proliferated in response to mCMV peptide loaded on EL-4 cells. Second, FACS-sorted donor spleen-derived T cells proliferated in response to antigen-nonspecific stimuli (Figure 5). These findings, along with evidence for a net 10-fold expansion of amotosalen-treated donor T cells, likely indicate that pretreated donor T cells have repaired their DNA cross-links. This process may possibly be due to co-existing viral infection or other *in vivo* stimuli, such as interleukin 2 and lipopolysaccharide, mainly produced from the intestines of irradiated recipients [30].

Although proliferation of mature pretreated T cells may in part explain the long-term persistence of a polyclonal donor T-cell population, it is also possible that some of these cells had developed from lymphoid progenitors among the pretreated splenocytes. However, FACS analysis of thymocytes from BMT recipients clearly shows that amotosalen pretreatment largely abolishes thymus-dependent T-cell expansion (Figure 6). In contrast to recipients of untreated splenocytes, in which donor spleen-derived $CD4^+$ $CD8^+$ T-cell progenitors were prevalent, there was a complete absence of these donor spleen-derived

thymic (common lymphoid) progenitors in recipients of amotosalen-treated cells.

BM-derived immune reconstitution was significantly better in recipients of amotosalen-treated versus untreated splenocytes. Although there were no significant differences in donor spleen-derived or host-derived CD3⁺ T cells between mice that received 3 to 10 × 10⁶ amotosalen-treated splenocytes or 3 × 10⁶ untreated splenocytes, recipients of amotosalen-treated donor splenocytes had 3 to 4 times more CD3⁺ T cells derived from donor BM than did recipients of untreated splenocytes. Improved BM-derived lymphoid reconstitution is reflected in thymic lymphoid development, including the presence of large numbers of BM-derived CD4⁺ CD8⁺ T-cell progenitors (Figure 6B).

The effects of amotosalen-treated splenocytes on BM lymphopoiesis are unlikely to be due to abrogation of graft rejection (because in the parental → F1 model there is no graft rejection vector) or to the ability of pretreated splenocytes to directly promote thymopoiesis. Rather, we favor the hypothesis that the reduced GVHD in BMT mice that received amotosalen-treated cells, as compared with untreated cells (Figure 1), provides an improved environment for BM-derived lymphoid reconstitution. In the presence of mCMV infection, BM-derived thymopoiesis is reduced in approximate proportion to the degree of GVHD (amotosalen-treated-10m = amotosalen-treated-3m < untreated-3m < untreated-10m). However, in the absence of mCMV infection and GVHD (amotosalen-treated-30m/no mCMV), lower levels of thymopoiesis are observed. Thus, mCMV infection may also have an important effect on thymopoiesis, which we have started to investigate. A role for GVHD is consistent with previous investigations demonstrating that chronic GVHD inhibits thymopoiesis and causes thymic dysfunction [31]. GVHD also induces thymic dysplasia associated with thymic involution, depletion of cortical and medullary thymocytes, epithelial cell destruction, and loss of Hassall bodies, which collectively result in T-cell lymphopenia, immunodeficiency, and a failure of negative selection of potentially autoreactive T cells [32-36]. Thus, reducing GVHD through the use of amotosalen-treated splenocytes likely protects thymic function and supports the generation of BM-derived T cells.

In this experimental model, splenocytes were isolated from donor mice that had recovered from sublethal mCMV infection 2 to 4 months earlier. The donors had completely cleared active mCMV infection, and antiviral CTLs were present at significantly lower levels than at peak infection (data not shown), consistent with contraction to a long-term stable memory CTL population such as is seen after experimental lymphocytic choriomeningitis virus infection [28]. Thus, this approach is likely to be effective clinically by using CMV-seropositive donors

with remote infections, where anti-CMV CTLs can represent ≥4% of the peripheral CD8⁺ T-cell population in the absence of acute infection [37]. Because 60% to 80% of the population is seropositive, this method should be broadly applicable.

In summary, amotosalen-treated polyclonal donor splenocytes persist long-term after infusion into MHC-mismatched BMT recipients and protect against lethal mCMV infection. Pretreated cells have a memory phenotype and remain functional on the basis of IFN-γ expression after stimulation. Despite the observation that these cells regain proliferative capacity, they do not induce clinically detectable GVHD. It is interesting to note that adoptive immunotherapy with amotosalen-treated splenocytes leads to improved thymopoiesis and immune reconstitution by BM-derived progenitors as compared with the use of untreated lymphocytes. Although we have shown that the administration of amotosalen-treated donor splenocytes at the time of allogeneic TCD BM eliminates early viral disease [17], this approach should also prevent late viral disease because pretreated T cells persist for longer than 4 months and accelerate donor BM-derived immune reconstitution. Thus, this is a potentially powerful approach to reduce the window for opportunistic infections and disease relapse after transplantation. The use of amotosalen-treated polyclonal donor T cells would be significantly more rapid and less expensive than current approaches involving the isolation and expansion of CMV-specific T-cell clones [8].

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