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Allogeneic T Cells Treated with Amotosalen Prevent Lethal Cytomegalovirus Disease without Producing Graft-versus-Host Disease Following Bone Marrow Transplantation

John D. Roback, Mohammad S. Hossain, Levan Lezhava, David L. Jaye, Christopher D. Hillyer, and Edmund K. Waller

Infusion of donor antiviral T cells can provide protective immunity for recipients of hemopoietic progenitor cell transplants, but may cause graft-vs-host disease (GVHD). Current methods of separating antiviral T cells from the alloreactive T cells that produce GVHD are neither routine nor rapid. In a model of lethal murine CMV (MCMV) infection following MHC-mismatched bone marrow transplantation, infusion of MCMV-immune donor lymphocytes pretreated with the DNA cross-linking compound amotosalen prevented MCMV lethality without producing GVHD. Although 95% of mice receiving 30 x 10^6 pretreated donor lymphocytes survived beyond day +100 without MCMV disease or GVHD, all mice receiving equivalent numbers of untreated lymphocytes rapidly died of GVHD. In vitro, amotosalen blocked T cell proliferation without suppressing MCMV peptide-induced IFN-γ production by MCMV-primed CD8+ T cells. In vivo, pretreated lymphocytes reduced hepatic MCMV load by 4-log10 and promoted full hemopoietic chimerism. Amotosalen-treated, MCMV tetramer-positive memory (CD44high) CD8+ T cells persisted to day +100 following infusion, and expressed IFN-γ when presented with viral peptide. Pretreated T cells were effective at preventing MCMV lethality over a wide range of concentrations. Thus, amotosalen treatment rapidly eliminates the GVHD activity of polyclonal T cells, while preserving long-term antiviral and graft facilitation effects, and may be clinically useful for routine adoptive immunotherapy. The Journal of Immunology, 2003, 171: 6023–6031.

Immuno compromised patients are at risk for morbidity and mortality from opportunistic pathogens, such as CMV. Historically, up to one-third of hemopoietic progenitor cell transplant (HPCT) recipients with CMV infection developed CMV pneumonitis, with a mortality rate of 70% (1). CMV viral load positively correlates with, and is a significant predictor of, CMV disease and death (2). Although PCR-guided pre-emptive ganciclovir (GCV) therapy reduces viral load and the frequency of CMV disease (3), therapy is compromised by drug-resistant CMV mutants (4). Furthermore, prophylactic and pre-emptive GCV therapy is associated with myelosuppression, bacterial and fungal infections, as well as late CMV disease (3, 5), demonstrating the need for alternative approaches to reduce viral load and prevent CMV disease.

CD8+ CTLs and CD4+ T cells are critical for controlling CMV infections (6). HPCT recipients are most vulnerable to CMV infection 30–100 days posttransplantation when CTL counts are low. Although recovery of CMV-specific CTLs can prevent CMV infection and disease, 65% of transplant recipients do not recover CTLs by 40 days posttransplantation, and GCV can further delay CTL reconstitution (7). Failure to reconstitute CMV CTLs, identified using MHC class I tetramers loaded with CMV peptides, is reproducibly observed in patients with CMV disease, while early CTL recovery is associated with short duration of viremia, low viral loads, and protection from CMV disease (8, 9). Patients with low CMV CTLs and concurrent CMV infections also respond poorly to GCV therapy (8).

Infusion of donor antiviral CTLs is a powerful approach for reconstituting cellular immunity in HPCT recipients (10, 11). However, the use of unselected polyclonal lymphocytes can produce lethal graft-vs-host disease (GVHD) if the donor and recipient are MHC mismatched (10). This problem has been addressed by in vitro expansion of virus-specific CD8+ CTLs to eliminate alloreactive T cells before infusion (10, 11). Infused CTL lines persist for at least 12 wk and can clear existing viremia, even with GCV-resistant mutants (11). However, adoptive immunotherapy with CTL lines is rarely used clinically because the culture methodology is lengthy, labor intensive, and costly.

We have sought to develop more rapid and cost-effective methods for adoptive immunotherapy, using the model of murine CMV (MCMV) infection following murine MHC-mismatched bone marrow transplantation (BMT). The present studies demonstrate that infusion of polyclonal donor splenocytes pretreated with amotosalen significantly reduces MCMV viral load and lethality without producing detectable GVHD. Amotosalen-treated MCMV-specific CTLs of the memory phenotype (CD44high) persist in vivo and are functional to at least day +100, and thus may provide...
BMT recipients with long-term antiviral protection without the risk of GVHD.

Materials and Methods

Animals

C57BL/6 (H-2b) (CD45.2/Thy-1.2), BALB/cJ (H-2d) (CD45.2/Thy-1.2), F1 (C57BL/6 × BALB/cJ) (H-2b), and PepBoy (B6.3LJ-PimpcPep3/BoyJ; H-2d) (CD45.1/Thy-1.2) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), BA mice (CD45.2/Thy-1.1) on the C57BL/6 background (M. Lieberman, Stanford University, Stanford, CA) and BA-PepBoy mice (H-2d) (CD45.1/Thy-1.1) were bred at Emory. Mice weighed 25-30 g at the time of transplantation. Procedures conformed with 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

Polyclonal splenocytes were harvested from donors inoculated with 105–106 PFU MCMV (Smith strain; American Type Culture Collection, Manassas, VA) 2-4 mo earlier, divided into four sets, and cultured at 107 cells/ml in complete medium (RPMI plus 10% heat-inactivated FBS) at 37°C in 5% CO2 for 24 h. One set was incubated with fludarabine (20 μg/ml; Research Diagnostics, NJ) and uracil (50 μM; Sigma, St. Louis, MO) for the entire 24-h period. A second set was exposed to 7.5 μg/g from a 131I source after 24 h. A total of 2 nM amotosalen (S-59; Cerus, Concord, CA) was added to the third set for H-2b or H-2d MHC, Thy-1.1, Thy-1.2, and/or isotype-specific Ab (BD PharMingen). ICS was performed similarly for peripheral blood samples, except peptide-pulsed EL4 cells (H-2b) were used for Ag-specific detection as described (12).

Irradiation, reconstitution, adoptive transfer, and MCMV infection of BMT recipients

On day −1, recipient F1 mice received two 5.5 Gy irradiation doses, 3 h apart (12). The following day, bone marrow (BM) was flushed from femora and tibia of donor mice and depleted of mature T cells (12), and 5 × 106 of the resulting T cell-depleted (TCD) BM cells was injected into the tail vein of irradiated F1 mice. In adoptive immunotherapy experiments, donor splenocytes were simultaneously injected. Two hours later, mice were injected i.p. with MCMV or vehicle. The development of clinical GVHD was monitored by weight loss and daily observation for alopecia, ruffled fur, diarrhea, and decreased activity. Moribund mice were euthanized.

T cell proliferation

Lymphocytes were treated with varying doses of amotosalen and exposed to UVA light; 2 × 107 cells from each group were plated in triplicate into 96-well plates precoated with anti-CD3 (clone 145-2C11; BioXience, San Diego, CA). Uncoated wells served as controls. After 4 days, cells were harvested and proliferation was determined, as previously described (13).

Flow cytometry

Lymphocyte chimerism was studied by flow cytometry, as described (12). Abs included those specific for H-2b or H-2d MHC, Thy-1.1, Thy-1.2, CD45.1, and CD45.2 Ags (BD Pharmingen, San Diego, CA). Propidium iodide was added to exclude dead cells. MCMV-specific CD8+ CTLs were identified using an APC-conjugated H-2Db tetramer containing the MCMV immunodominant peptide HGRNASFI (14) (National Institute of Allergy and Infectious Diseases Tetramer Core Facility, Atlanta, GA) and by intracellular cytokine staining (ICS). For ICS, splenocytes incubated with 10-6 M HGRNASFI for 5 h in the presence of brefeldin A (GolgiPlug; BD Pharmingen) were surface stained with appropriate Abs, fixed, permeabilized (Cytofix/Cytoperm; BD Pharmingen), and stained with an IFN-γ Ab (BD Pharmingen). ICS was performed similarly for peripheral blood samples, except peptide-pulsed EL4 cells (H-2b) were used for Ag presentation. No anti-MCMV CTLs were detectable when uninfected mice and/or isotype-specific Abs were used as controls.

Quantitative PCR amplification of the MCMV DNA

Liver DNA was isolated (EZNA kit; Omega Bio-Tek, Doraville, GA) and quantitated (PicoGreen dsDNA Quantitation Kit; Molecular Probes, Eugene, OR). MCMV DNA was quantitated by real-time PCR in a 96-well plate (iCycler; Bio-Rad Laboratories, Hercules, CA). Each 15-μl reaction contained 7.5 μl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and primers IE1.1983 and IE1.2348 (15). For each sample, 10-fold serial dilutions of purified DNA, starting with 40 ng, were assayed in quadruplicate. Amplification conditions were 95°C × 10 min, followed by 50 cycles of 95°C × 15 s, 62°C × 15 s, and 72°C × 60 s. PCR were scored as positive or negative based on the amplion’s melting profile. In initial studies, the accuracy of the melt curve in identifying positive and negative samples was confirmed by gel electrophoresis. The number of MCMV genomes in each sample was calculated using the method of Kartel (16). This assay can routinely detect 1-5 MCMV genome equivalents.

Histologic assessment of viral replication and GVHD

Active MCMV infection in liver was identified by immunocytochemistry for the MCMV early gene product pp65 using mAb 25G11 (J. Shanley, University of Connecticut Health Center, Farmington, CT) (17). Acetone-fixed frozen sections of liver were incubated with biotinylated Ab (1:50), washed, and developed using the ABC system (Vector Laboratories, Burlingame, CA).

Paraffin-embedded segments of liver were stained with H&E and examined microscopically by an anatomic pathologist (D. Jaye) blinded to treatment group. Typical eosinophilic intranuclear cytomegalic inclusion bodies were counted in 10 contiguous ×400 high-power fields. For GVHD, two liver sections per mouse were graded according to criteria previously described (12). The results were decoded, tabulated, and analyzed by another coauthor (J. Roback).

Statistical analyses

Mantel-Cox log-rank test, χ2 test, and Student’s t test were applied, as described in the text. Significance was demonstrated at the 0.05 level.

Results

Increased MCMV lethality in MHC-mismatched BMT recipients

F1 recipients of syngeneic F1 (Fig. 1A) or allogeneic C57BL/6 (Fig. 1B) BM displayed 100% survival in the absence of MCMV infection. Following MCMV infection, mortality of C57BL/6→F1 allogeneic BMT recipients was significantly greater than for F1→F1 syngeneic recipients at MCMV inocula of 103 (p < 0.001), 5 × 103 (p < 0.001), 103 (p < 0.005), and 5 × 105 PFU (p < 0.025). Infection with 106 or more PFU MCMV was uniformly fatal in all mice. Thus, murine BMT accurately models human transplantation in which recipients of allogeneic transplants are more susceptible to lethal CMV disease than recipients of syngeneic (autologous) BMT (18).

Adoptive immunotherapy with pretreated splenocytes from MHC-mismatched MCMV-immune donors prevents MCMV lethality without producing GVHD

Because allogeneic C57BL/6→F1 recipients were highly susceptible to MCMV lethality, they were used to develop rapid and effective methods for adoptive immunotherapy. These investigations used polyclonal splenocytes from MCMV-immune C57BL/6 or congenic donors because in initial studies immune splenocytes were significantly more effective than naive cells in preventing MCMV lethality (data not shown). In two separate experiments (n = 10 per group), C57BL/6→F1 BMT mice were infected with 2 × 104 PFU MCMV with or without preceding infusion of 30 × 106 splenocytes. MCMV was lethal in 85% of mice that did not receive adoptive immunotherapy (Fig. 2A). However, adoptive transfer of untreated splenocytes resulted in 100% lethality (p > 0.05) due primarily to GVHD (see below). To prevent GVHD, we attempted to abrogate the alloreactivity of donor splenocytes by pretreating with amotosalen (19), γ-irradiation (12), or fludarabine (20). All treatments significantly improved survival (Fig. 2A; p < 0.001). Remarkably, 95% of BMT mice receiving amotosalen-treated C57BL/6 splenocytes survived the lethal MCMV challenge, although their survival was not statistically better than mice receiving irradiated cells (p > 0.05).

Although infusion of pretreated MCMV-immune splenocytes significantly improved survival, it was unclear whether the residual
mortality was due to MCMV disease or GVHD. The above experiments were repeated without MCMV infection so that observed lethality could be attributed to GVHD from adoptive immunotherapy (Fig. 2B). Although only 10% of BMT mice that did not receive splenocytes died, the mortality was 100% for mice receiving 30 × 10⁶ untreated cells (p < 0.001) consistently with uniformly lethal GVHD. In contrast, all mice survived following infusion of 30 × 10⁶ splenocytes pretreated with amotosalen or γ-irradiation (p > 0.05, vs mice that did not receive adoptive immunotherapy). Fifty percent of mice receiving fludarabine-treated splenocytes died by day +80. Weight loss following BMT is a surrogate measure for GVHD in mice (12). Mice infused with untreated cells displayed a marked continuous decline in weight, and all died by day +30 (Fig. 2C). In contrast, mice receiving amotosalen-treated or γ-irradiated splenocytes showed minimal decline in weight, similar to control BMT mice. Thus, lethality associated with infusion of untreated splenocytes was due to GVHD and could be completely prevented without compromising anti-MCMV activity by pretreating with amotosalen or γ-irradiation. In subsequent studies, we focused on amotosalen treatment because it was more uniformly effective throughout early investigations.

**Amotosalen-treated T cells do not proliferate, but retain ability to up-regulate IFN-γ production**

One possible explanation for the decreased GVHD activity of amotosalen-treated splenocytes is nonspecific cell inactivation leading to infusion of fewer functional cells. This hypothesis would suggest that the antiviral efficacy of treated splenocytes should decrease in parallel with their proliferative capacity. To investigate this possibility, immune splenocytes were treated with increasing doses (0–20 nM) of amotosalen, followed by UVA light. Stimulation of untreated lymphocytes with anti-CD3ε produced a significant increase in proliferation compared with untreated cells cultured without anti-CD3ε (p < 0.001). A total of 2 nM amotosalen, the concentration used for adaptive immunotherapy, is in a range of doses previously shown to block T cell proliferation (19). Likewise, in the present studies, 2–20 nM amotosalen reduced anti-CD3ε-mediated T cell proliferation to baseline levels seen in unstimulated cultures (p > 0.05; Fig. 3A). Exposure of splenocytes from acutely infected mice (10 days) to HGIR-NASFI, an H-2Db-restricted MCMV immunodominant peptide (14), stimulated IFN-γ production in ~3% of CD8⁺ T cells. In

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**FIGURE 1.** MCMV lethality following BMT. F1 mice received BMT from either syngeneic F1 (A) or allogeneic C57BL/6 mice (B), followed by MCMV infection at inoculae of 10⁴ (○), 5 × 10⁴ (▲), 10⁵ (△), 5 × 10⁵ (■), 10⁶ (●), or 5 × 10⁶ (▲) PFU (n = 10 mice per inoculum). All control BMT recipients not infected with MCMV survived (●). Mortality of allogeneic BMT recipients (B) was significantly higher than syngeneic recipients (A) at inoculae of 10⁴ (p < 0.001), 5 × 10⁴ (p < 0.001), 10⁵ (p < 0.005), and 5 × 10⁵ PFU (p < 0.025). Representative data from one experiment are shown. This study was performed three times with similar results.

**FIGURE 2.** Adoptive immunotherapy to prevent MCMV lethality. Following allogeneic BMT, mice were injected with 2 × 10⁵ PFU MCMV (A) or vehicle (B). A. Compared with MCMV-infected BMT mice that did not receive adoptive immunotherapy (■), infusion of amotosalen-treated (●), γ-irradiated (▲), or fludarabine-treated (○) immune splenocytes significantly improved survival (p < 0.001). Survival was not improved by infusion of untreated lymphocytes (p > 0.05). B. In the absence of MCMV infection, the survival of BMT mice that did not receive adoptive immunotherapy was similar to survival following infusion of treated lymphocytes (p > 0.05), but was significantly better than in mice infused with untreated lymphocytes (p < 0.001). C. Body weight change of infected BMT mice (○) was similar to MCMV-infected mice protected by amotosalen-treated (●) or γ-irradiated (▲) splenocytes. In contrast, MCMV infection alone (■) produced a rapid initial decrease in weight, as did MCMV infection and concomitant adoptive immunotherapy with untreated (△) or fludarabine-treated (○) splenocytes. Cumulative data from two experiments (20 mice per group) are shown.
CD8

GVHD do not suppress IFN-γ that amotosalen concentrations that block cell proliferation and the absence of HGIRNASFI stimulation. These data demonstrate –

CD8 T cells and those pretreated with 0.2 μM amotosalen reduced this proliferative response to levels indistinguishable from that in unstimulated cultures (p > 0.05). A 10-fold increase in amotosalen concentration to 20 nM did not further affect proliferation. B, Similarly pretreated splenocytes were cultured with or without the H-2D β-restricted MCMV immunodominant peptide HGIRNASFI (HGIR) and then immunostained for intracellular IFN-γ. Only rare CD8+ T cells expressed detectable IFN-γ in the absence of HGIRNASFI, while ~5% of CD8+ cells had detectable IFN-γ after HGIRNASFI stimulation. There were no significant differences in the percentage of cells expressing IFN-γ between cultures treated with amotosalen at various concentrations and untreated cultures (p > 0.05), demonstrating that doses of amotosalen that block T cell proliferation do not affect peptide-stimulated IFN-γ production. Representative data from one experiment are shown. This study was performed three times with similar results.

Dose response to amotosalen-treated splenocytes

To investigate the therapeutic window for adoptive immunotherapy with amotosalen-treated splenocytes, BMT mice with or without MCMV infection (5 × 10⁴ PFU) received 1–30 × 10⁶ treated or untreated splenocytes. BA and PepBoy congenic mice were used as BM and splenic lymphocyte donors, respectively, to trace T cell origin. Ten animals were used per group (Table I), except for groups 11 and 12, in which five animals were used because these controls were highly reproducible in previous studies (Fig. 2A). Infusion of amotosalen-treated splenocytes over a broad range of doses (3–30 × 10⁶) resulted in 90% or greater day +100 survival of MCMV-infected BMT mice (Fig. 4). Surviving mice gained weight appropriately without evidence of GVHD. In contrast, untreated splenocytes were only effective over the narrow range of 1–3 × 10⁶ cells. With 10 × 10⁶ and 30 × 10⁶ untreated splenocytes, mortality was 70 and 100%. Furthermore, mice that survived following infusion of 10 × 10⁶ and 3 × 10⁶ untreated splenocytes had evidence of GVHD. The group receiving 10 × 10⁶ had ruffled fur and reduced weight gain, while mice receiving 3 × 10⁶ untreated cells had small fibrotic spleens containing significantly fewer lymphocytes (average ~53.9 × 10⁶) than mice receiving 30 × 10⁶ amotosalen-treated splenocytes (141.4 × 10⁶; p < 0.006). Thus, compared with the narrow therapeutic window for untreated splenocytes, amotosalen pretreatment expanded the effective range of lymphocyte doses for adoptive immunotherapy.

Peripheral blood T cell chimerism was quantitated in BMT recipients surviving past day +100 (Table I). Using flow cytometric

Table I. Analysis of T cell chimerism following adoptive immunotherapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Amotosalen Treated</th>
<th>Untreated</th>
<th>MCMV</th>
<th>Origin of Peripheral Blood T Cells at Day +100 Following BMT (% of Total T Cells)</th>
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<td>Recipient Derived</td>
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<td></td>
<td>5 × 10⁶</td>
<td>14.0 ± 3.2</td>
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<td>5.8 ± 5.0</td>
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<td>1 × 10⁶</td>
<td></td>
<td>5 × 10⁶</td>
<td>0.9 ± 1.5</td>
</tr>
<tr>
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<td>3 × 10⁶</td>
<td></td>
<td>5 × 10⁶</td>
<td>0.9 ± 1.5</td>
</tr>
<tr>
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<td>10 × 10⁶</td>
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<td>0.5 ± 0.9</td>
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<tr>
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<td></td>
<td>5 × 10⁶</td>
<td>ND</td>
</tr>
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</table>
phenotyping for congeneric surface markers, the T cells could be
categorized as BMT recipient derived (Thy-1.2/CD45.2), BM
donor derived (Thy-1.1/CD45.2), or splenocyte donor derived (Thy-
1.2/CD45.1). As expected, increasing the number of adoptively
transferred untreated splenocytes resulted in more persisting T
cells. Surprisingly, despite amotosalen pretreatment sufficient to
block proliferation in vitro (2 nM; Fig. 3), untreated splenocytes sur-
vived long-term after infusion in vivo, accounting for 5–12% of
peripheral blood T cells in MCMV-infected recipients (groups
3–6). Infusion of either untreated or amotosalen-treated splen-
cytes promoted full T cell chimerism, with less than 1% recipient-
derived T cells remaining at day +100, except for mice receiving
1 × 10^6 amotosalen-treated cells that had 6% recipient T cells.
Interestingly, in mice receiving 30 × 10^6 amotosalen-treated lymph-
cytes, concurrent MCMV infection significantly increased their
contribution to the overall T cell pool from 1.3% in uninfected
mice (group 11) to 11.9% (group 6; p < 0.001).

Amotosalen-treated splenocytes significantly decrease viral load
and replication without histologic GVHD

To more specifically examine the antiviral activity of amotosalen-
treated T cells in vivo, viral load and replication were examined in
the liver, an organ dependent on CD8+ T cells for control of
MCMV (21). C57BL/6→F1 BMT mice received 30 × 10^6 donor
spleenocytes and 5 × 10^6 PFU MCMV. Surviving mice were sac-
crificed at day +14. Only 6 of 10 mice survived to day +14 without
adoptive immunotherapy, and the surviving mice had viral loads
averaging 10^7 copies/40 ng DNA (Fig. 5A). In contrast, adoptive
immunotherapy improved survival to 90% and significantly re-
duced average hepatic MCMV DNA load by 4 log_{10} or more (p <
0.05). The difference in viral loads between mice receiving un-
treated or amotosalen-treated splenocytes was not significant (p >
0.05). Liver samples were also immunostained for the MCMV
pp56 protein, which correlates with viral replication (17). Without
adoptive immunotherapy, at least 50% of hepatocytes expressed
pp56 (Fig. 5B), while no pp56-positive cells were detectable in
mice receiving amotosalen-treated splenocytes (Fig. 5C). Quanti-
tation of MCMV intranuclear inclusions produced similar results
(data not shown).

Hepatic inflammatory infiltrates were quantitated as a histologic
correlate of GVHD (Fig. 6). Uninfected BMT mice had normal
hepatic architecture with an average inflammation score of 1.1 (of
a maximum of 5) (A). In contrast, MCMV infection in the absence
of adoptive immunotherapy produced minimal inflammation
(score of 1.4), but marked necrosis (B). Mice that received adopt-
ive immunotherapy with untreated lymphocytes in the absence
of GVHD (C) or presence (D) of MCMV infection showed marked portal
lymphocytic infiltration with an average inflammation score of 4.5.
In contrast, mice infused with amotosalen-treated lymphocytes had
significantly less inflammation (p < 0.01) whether uninfected (E;
score of 2.3) or inoculated with MCMV (F; 3.0). MCMV-infected
mice that received amotosalen-treated lymphocytes showed no he-
patic necrosis, MCMV inclusions, or viral cytopathic effect.

Amotosalen-treated Ag-specific T cells persist in vivo

At day +100 or later after BMT and MCMV infection, flow cy-
mometry was used to identify anti-MCMV CTLs in the peripheral
blood and spleens of surviving mice. Gates were set to collect the
CD45.1+ lymphocytes derived from adoptively transferred sple-
cytes (Fig. 7A), or the CD45.2+ lymphocytes derived from the BM
donor or BMT recipient. At each splenocyte dose (1–30 × 10^6),
0.5–2% of CD45.1+ lymphocytes stained with the H-2D^b-HGIR-
NASFI tetramer (Fig. 7B). The peripheral blood concentration of
CD8+ tetramer + T cells ranged from 1 to 16 × 10^3 per ml (Fig.
7E), which is comparable to levels in human HPCT recipients
following adoptive immunotherapy (11). Similar numbers of tet-
ramer-positive cells were seen whether lymphocytes were amoto-
salen treated or not. In contrast, CD45.2+ tetramer-positive CTLs
derived from the donor BM or F1 recipient were rarely observed
(data not shown). Similarly, 0.5–2% of CD45.1 lymphocytes ex-
pressed IFN-γ when stimulated with the HGIRNASFI peptide pre-
sented by EL4 cells (Fig. 7C), demonstrating that even 100 days
after amotosalen treatment, CD8+ T cells could still up-regulate
IFN-γ expression in response to viral peptide. CD45.1+ tetramer-
positive CTLs derived from amotosalen-treated donor splenocytes

FIGURE 4. Amotosalen broadens the cell dose for effective adoptive
immunotherapy. C57BL/6→F1 BMT mice received selected doses of un-
treated (○) or amotosalen-treated splenocytes (●) from MCMV-immune
donors, followed by MCMV infection. Survival at day +100 is displayed.
In contrast to the narrow range of doses over which untreated lymphocytes
improved survival, 90% or more of mice receiving 3 × 10^6–30 × 10^6
amotosalen-treated splenocytes survived lethal MCMV infection without
developing lethal GVHD. Ten animals were used per group, except for
groups 11 and 12, which had five (Table I).

FIGURE 5. Adoptive immunotherapy decreases hepatic MCMV load.
A, Compared with allogeneic BMT mice that did not receive adoptive
immunotherapy (None), infusion of untreated or amotosalen-treated
splenocytes (●) or amotosalen-treated splenocytes (○) from MCMV-immune
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hepatic architecture with an average inflammation score of 1.1 (of
a maximum of 5) (A). In contrast, MCMV infection in the absence
of adoptive immunotherapy produced minimal inflammation
(score of 1.4), but marked necrosis (B). Mice that received adopt-
ive immunotherapy with untreated lymphocytes in the absence
of GVHD (C) or presence (D) of MCMV infection showed marked portal
lymphocytic infiltration with an average inflammation score of 4.5.
In contrast, mice infused with amotosalen-treated lymphocytes had
significantly less inflammation (p < 0.01) whether uninfected (E;
score of 2.3) or inoculated with MCMV (F; 3.0). MCMV-infected
mice that received amotosalen-treated lymphocytes showed no he-
patic necrosis, MCMV inclusions, or viral cytopathic effect.

Amotosalen-treated Ag-specific T cells persist in vivo

At day +100 or later after BMT and MCMV infection, flow cy-
mometry was used to identify anti-MCMV CTLs in the peripheral
blood and spleens of surviving mice. Gates were set to collect the
CD45.1+ lymphocytes derived from adoptively transferred sple-
cytes (Fig. 7A), or the CD45.2+ lymphocytes derived from the BM
donor or BMT recipient. At each splenocyte dose (1–30 × 10^6),
0.5–2% of CD45.1+ lymphocytes stained with the H-2D^b-HGIR-
NASFI tetramer (Fig. 7B). The peripheral blood concentration of
CD8+ tetramer + T cells ranged from 1 to 16 × 10^3 per ml (Fig.
7E), which is comparable to levels in human HPCT recipients
following adoptive immunotherapy (11). Similar numbers of tet-
ramer-positive cells were seen whether lymphocytes were amoto-
salen treated or not. In contrast, CD45.2+ tetramer-positive CTLs
derived from the donor BM or F1 recipient were rarely observed
(data not shown). Similarly, 0.5–2% of CD45.1 lymphocytes ex-
pressed IFN-γ when stimulated with the HGIRNASFI peptide pre-
sented by EL4 cells (Fig. 7C), demonstrating that even 100 days
after amotosalen treatment, CD8+ T cells could still up-regulate
IFN-γ expression in response to viral peptide. CD45.1+ tetramer-
positive CTLs derived from amotosalen-treated donor splenocytes
likely to be due to augmentation of GVHD, as was previously reported (24). Rather, preliminary results demonstrate that the increased lethality in allorecipients correlates with significantly higher viral loads (data not shown). The mechanisms underlying this observation are currently being investigated. This allotransplant model appears ideal for developing methods for allogeneic adoptive immunotherapy because infusion of unmanipulated donor splenocytes produces lethal GVHD. These mice may, in fact, be more susceptible to GVHD than most patients because the one-way MHC mismatch does not allow rejection of donor lymphocytes. In contrast, previous murine adoptive immunotherapy studies could not assess the risk of GVHD because syngeneic BALB/c mice were used as BMT recipients, BMT donors, and lymphocyte donors (6). Thus, this is a sensitive system for monitoring both antiviral activity and GVHD potential of adoptively transferred T cells.

The survival of MCMV-infected BMT mice was significantly improved by adoptive immunotherapy, provided that the splenocytes were pretreated with amotosalen, or to a lesser extent with γ-irradiation or fludarabine (Fig. 2). Several lines of evidence demonstrated that amotosalen can abrogate the GVHD activity of allogeneic T cells without impairing their antiviral effectiveness. In all experiments, at least 95% of mice receiving 3 × 10⁶ amotosalen-treated lymphocytes reproducibly survived an MCMV challenge without developing GVHD (Fig. 2). In vitro, amotosalen treatments that blocked mitogen-stimulated T cell proliferation (2–20 nM) did not reduce MCMV peptide-induced IFN-γ production by CD8⁺ T cells (Fig. 3). In vivo, amotosalen-treated splenocytes significantly reduced MCMV load and replication 14 days after BMT as quantitated by PCR for MCMV DNA, enumeration of MCMV inclusions, and immunocytochemistry for the viral pp56 protein (Fig. 5). In the same studies, amotosalen-treated T cells produced significantly less hepatic inflammation than untreated T cells (Fig. 6).

The effectiveness of amotosalen in dissociating the antiviral and GVHD activities of polyclonal T cells was unexpected, given previous investigations. Truitt and colleagues (25) examined the GVHD and graft-vs-leukemia (GVL) activities of amotosalen-treated T cells in a similar murine MHC-mismatched BMT model. A narrow range of pretreatment conditions was identified (optimum of 10 nM amotosalen/0.42 J/cm² UVA) that preserved an estimated 10% of the initial GVL activity, but with varying degrees of residual GVHD (19). However, treatment regimens that best preserved GVL activity were generally associated with the greatest amount of GVHD, probably because GVL activity was directed against allogeneic MHC class I determinants and was thus a component of GVHD activity.

There are most likely multiple overlapping mechanisms through which amotosalen treatment successfully separated the antiviral and GVHD activities of T cells in our study. First, amotosalen appeared to stabilize the infused T cell repertoire by preventing clonal expansion without inhibiting T cell function. In the presence of UVA light, 1 nM amotosalen covalently cross-links dsDNA every 10⁷–10⁶ bp or 3,000–30,000 times per cell (26), effectively blocking T cell proliferation (19, 27). In the present study, concentrations of 2–20 nM likewise prevented proliferation, but did not inhibit production of the antiviral cytokine IFN-γ by CD8⁺ T cells (Fig. 3), probably because the density of DNA cross-links under these conditions is insufficient to inactivate the IFN-γ gene in the majority of treated cells. Thus, the net antiviral and GVHD activities of amotosalen-treated lymphocytes may in part reflect the numbers of anti-MCMV and alloreactive T cells present at the time of treatment, respectively. Consistent with this mechanism,
splenocytes from MCMV-immune mice, which have an expanded antiviral compartment including memory CTLs (28), were more effective at preventing viral lethality than lymphocytes from naive mice (data not shown). Other investigators have also noted that amotosalen-treated T cells from immune donors are more effective than cells from naive donors, and that effector CTLs retain potent cytolytic activity following amotosalen treatment (19, 27). A second mechanism is suggested by the long-term in vivo survival of memory-type (CD44hi) T cells following amotosalen treatment (Fig. 7). Memory T cells are known to be more resistant to irradiation- and Fas-mediated apoptosis than naive cells (29, 30). We hypothesize that amotosalen-treated memory CTLs are relatively resistant to apoptosis, while treated naive cells, including the alloreactive CD8+ T cells that would cause GVHD, may preferentially undergo apoptosis when they encounter Ag. This mechanism, which would further skew the T cell repertoire toward antiviral activity and away from allostereactivity, is currently under investigation. There is also support in the literature for a third possibility. The polyclonal lymphocytes used in the present studies were obtained from mice that had recovered from MCMV infection 2–4 mo earlier, but were never exposed to recipient alloantigen. This population should include memory antiviral T cells, which are competent for cytokine expression, as well as naive alloreactive T cells that must undergo priming (28). Because cell division is a critical factor in priming naive T cells for IFN-γ and IL-4 synthesis (31), naive alloreactive cells with DNA cross-links produced by amotosalen may not be efficiently primed because they cannot divide. Further work is necessary to determine the relative contributions of each mechanism to the observed amotosalen effect.

Other approaches have been studied for prevention of GVHD following adoptive immunotherapy. CMV-specific CD8+ CTL clones have not produced significant GVHD in clinical studies (10, 32), but have not been widely applied because the in vitro expansion process is lengthy, expensive, and labor intensive. In vitro retroviral transduction of T cells with a suicide vector activated by GCV or other drugs has been used to delete cells that produce GVHD (33, 34). Although this approach is potentially powerful, the transfection protocol requires a 12-day culture period, the transgene may be eliminated or inactivated, and not all suicide gene-containing cells are deleted when the activating stimulus is applied (33, 34). Furthermore, in mice, lethal GVHD could not be completely prevented using this system (35).

Compared with these approaches, amotosalen treatment of donor lymphocytes has the following advantages: it is rapid, requiring less than 1 h; it prevents the development of GVHD, rather than providing a mechanism to treat GVHD as with suicide gene transfer; it could be used in conjunction with these other methods, as well as with conventional antiviral drugs; and amotosalen is nearing FDA approval for ex vivo pretreatment of blood components before transfusion (36). Additionally, amotosalen can be used with polyclonal lymphocyte populations containing memory CTLs against multiple infectious agents, allowing the simultaneous adoptive transfer of broad spectrum immunity. This approach may be particularly effective against viral mutants that do not express immunodominant epitopes and can evade adoptively transferred CTL clones with narrow Ag specificities (37).

Amotosalen-treated splenocytes prevented viral lethality without causing GVHD at doses between $3 \times 10^6$ and $30 \times 10^6$ (Fig. 4), while the therapeutic window for untreated cells was very narrow. 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. It should be possible to procure comparable numbers of lymphocytes for clinical use. Before peripheral blood progenitor cell transplantation, clinicians typically attempt to collect $2–6 \times 10^8$ PBMC/kg recipient body
weight (12–36 × 10^6 total PBMC for a 60 kg recipient). If these collection goals are achieved, CD34+ stem cells (usually 1% or less of PBMC) could be separated and transplanted, while the remaining PBMC could be amotosalen treated and then infused. At the upper end of the collection target range, the use of 30 × 10^9 amotosalen-treated PBMC in a 60 kg patient would be approximately equivalent to the use of 10 × 10^9 amotosalen-treated lymphocytes in a mouse, a dose that was very effective at preventing lethal MCMV disease in the present study.

Amotosalen-treated lymphocytes including MCMV-specific CTLs persisted for at least 100 days after infusion, consistent with previous observations (19). Surprisingly, despite evidence that lymphocytes cannot divide immediately following amotosalen treatment (Fig. 3A), these cells appear to retain at least limited proliferative capacity after infusion. Mice that received 3 × 10^6 treated splenocytes (30% or 0.9 × 10^6 of which were T cells) had an average of 1.7 × 10^6 CD45.1 T cells of donor splenocyte origin at 100 days after BMT, indicating that a 2-fold expansion of amotosalen-treated T cells had occurred in vivo (data not shown). It seems unlikely that proliferating T cells represent a small fraction that escaped DNA cross-linking. Rather, they may be cells that repaired DNA damage and regained proliferative potential. In preliminary investigations, amotosalen-treated donor T cells, isolated from BMT recipients 100 days after transplant, proliferate to a limited extent in vitro. Extensive TCR V-β diversity suggests that proliferating cells do not represent a small clonal population that has escaped amotosalen treatment (manuscript in preparation).

At 100 days after BMT, anti-MCMV CTLs were readily detected by tetramer staining (Fig. 7). These CTLs stained for CD45.1, demonstrating that they were derived from the adoptively transferred splenocytes, including splenocytes pretreated with amotosalen before infusion. Anti-MCMV CTLs derived from the BM donor or F1 BMT recipient were only rarely observed. At each lymphodepe, similar absolute numbers of tetramer-positive CTLs were present whether the cells were amotosalen treated or not, providing further evidence against nonselective toxicity of the treatment regimen. Importantly, ICS showed that the amotosalen-treated cells retained the capacity to synthesize IFN-γ in response to viral peptide 100 days after infusion. The anti-MCMV CTLs were brightly stained for CD44, which is usually associated with long-lived memory CD8+ T cells that may confer persistent viral protection (28). The peripheral blood concentration of anti-MCMV CTLs at 100 days after transfer approximates that in patients receiving adoptive immunotherapy. Among 8 HPCT recipients that received CMV-specific T cell lines, CMV-specific CTLs in peripheral blood typically ranged from 0 to 4.01 CTLs/μl, although one patient had 41.58 CTLs/μl (11). Notably, in another study, patients with >5 peptide-specific CD8+ T cells/μl did not develop either plasma viremia or CMV disease (9). These findings indicate that infusion of amotosalen-treated lymphocytes can lead to persistent clinically relevant levels of antiviral CTLs that may protect against both early and late CMV disease after transplant.

In conclusion, we have demonstrated in a mouse model that pretreatment of MHC-mismatched polyclonal donor lymphocytes with amotosalen before adoptive immunotherapy abrogates their potential to produce lethal GVHD while still preserving sufficient antiviral activity to prevent lethal MCMV infection. Given that treatment with amotosalen is simple, rapid, and reproducibly effective at improving the therapeutic index of adoptive immunotherapy, this methodology may allow more widespread clinical application of adoptive immunotherapy to prevent viral disease in immunocompromised patients.

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References