The Journal of Immunology

Viral Abrogation of Stem Cell Transplantation Tolerance Causes Graft Rejection and Host Death by Different Mechanisms

Daron Forman, Raymond M. Welsh, Thomas G. Markees, Bruce A. Woda, John P. Mordes, Aldo A. Rossini and Dale L. Greiner

http://www.jimmunol.org/content/168/12/6047

References
This article cites 68 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/168/12/6047.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Viral Abrogation of Stem Cell Transplantation Tolerance Causes Graft Rejection and Host Death by Different Mechanisms

Daron Forman,* Raymond M. Welsh,*§ Thomas G. Markees, ‡ Bruce A. Woda,*§ John P. Mordes,§ Aldo A. Rossini,*‡ and Dale L. Greiner‡‡

Tolerance-based stem cell transplantation using sublethal conditioning is being considered for the treatment of human disease, but safety and efficacy remain to be established. We have shown that mouse bone marrow recipients treated with sublethal irradiation plus transient blockade of the CD40-CD154 costimulatory pathway develop permanent hematopoietic chimerism across allogeneic barriers. We now report that infection with lymphocytic choriomeningitis virus at the time of transplantation prevented engraftment of allogeneic, but not syngeneic, bone marrow in similarly treated mice. Infected allograft recipients also failed to clear the virus and died. Postmortem study revealed hypoplastic bone marrow and spleens. The cause of death was virus-induced IFN-α/β.

The rejection of allogeneic bone marrow was mediated by a radioresistant CD8+ T cell population. We conclude that a noncytopathic viral infection at the time of transplantation can prevent engraftment of allogeneic bone marrow and result in the death of sublethally irradiated mice treated with costimulation blockade. Clinical application of stem cell transplantation protocols based on costimulation blockade and tolerance induction may require patient isolation to facilitate the procedure and to protect recipients.


Stem cell transplantation is a powerful method for inducing donor-specific central transplantation tolerance (1), but its application in clinical medicine has been limited by its inherent risks. Achieving allogeneic hematopoietic chimerism requires preparative conditioning with immunosuppression and at least partial myeloablation. In addition, even in partially ablated recipients, stem cell transplantation almost invariably leads to some degree of graft-vs-host disease (GVHD) (2–5).

To overcome these potential toxicities, newer approaches to the generation of allogeneic hematopoietic chimerism have focused on inhibiting T cell costimulation (6, 7). Successful approaches in mice have included the following: sublethal irradiation combined with CTLA4-Ig and/or anti-CD154 mAb without myeloablative conditioning (8), and costimulation blockade combined with drug-induced myeloablation (11). Allogeneic hematopoietic chimerism in each of these model systems has been documented to generate donor-specific transplantation tolerance in the absence of GVHD. In addition, our laboratory has extended the use of tolerance-based stem cell transplantation to the treatment of autoimmune diabetes; this usage requires not only the generation of allo-tolerance to islet grafts but also the prevention of recurrent islet-destructive autoimmunity (8). The potential clinical utility of allogeneic hematopoietic chimerism for allotransplantation (1), cancer immunotherapy (12), and the treatment of autoimmune disease (13) is clearly enormous.

Despite the promise inherent in stem cell transplantation protocols based on costimulation blockade, significant issues of safety and durability remain to be assessed. In particular, patients treated with partial myeloablation combined with costimulation blockade could be less resistant to viral infection and its associated pathophysiological effects. Many viral infections are known to impair hematopoiesis and to induce bone marrow failure in stem cell transplant recipients treated with conventional procedures; these include EBV (14), CMV (15, 16), human herpesvirus 6 and 7 (17, 18), and HIV (19–21), among others.

In mice, acute lymphocytic choriomeningitis virus (LCMV) infection has been shown to induce transient anemia resulting from the production of type 1 IFNs that suppress hematopoiesis (22). Persistent infection with LCMV has also been shown to lead to aplastic anemia due to chronic CD8+ T cell activation and induction of IFN-γ, TNF, and lymphotoxin-α (23). Moreover, many viral infections (24), including LCMV (25), can enhance allograft rejection (26, 27). The effects of viral infection on human stem cell graft recipients treated with costimulatory blockade are unknown, but recent evidence suggests that viral infection can prevent allogeneic bone marrow engraftment in mice (28).

To analyze this problem, we used a mouse model system of allogeneic hematopoietic chimerism developed in our laboratory (8). This model combines sublethal irradiation and anti-CD154 mAb and generates durable allogeneic hematopoietic chimerism across an MHC mismatch at both major and minor histocompatibility loci. We now report that acute LCMV infection of allogeneic...
bone marrow recipients conditioned with sublethal irradiation, and anti-CD154 mAb prevents the establishment of allogeneic hematopoietic chimera and is fatal. We further demonstrate that a fatal outcome appears to depend on type 1 IFNRs and that the failure to engraft allogeneic stem cells is dependent on a population of radioresistant alloreactive CD8+ T cells.

Materials and Methods

Animals

Female C57BL/6 (H-2b, Ly5.2), C57BL/6-Ly5.1, CBA/JCR (H-2b), and BALB/c (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD) and were housed in a specific pathogen-free facility in microisolator cages, given autoclaved food and acidified water, and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Worcester, MA) and recommendations in the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (National Research Council, National Academy of Sciences).

Abs and flow cytometry

The MR1 hamster anti-mouse CD154 mAb was purified from ascites, diluted in PBS to a final concentration of 1 mg/ml (30), and injected at a dose of 0.5 mg i.p. on days 0 and + relative to bone marrow transplantation. Depleting Abs were used as follows: 1 mg anti-NK1.1 (clone PK-136; Ref. 31) i.p. on days −8, −1, and +6, 0.5 mg anti-CD4 (OK1.5) and anti-CD8 (2.43) i.p. on days −1, −3, 1, and +1. Each mAb was documented by flow microfluorometry to deplete >97% of its target cell population. FITC-conjugated anti-H-2Kb (AF6-88.5) and anti-Ly5.2 (104); PE-conjugated anti-H-2Kd (SFI-1.1), anti-Ly5.1 (A20), anti-CD4 (L3T4), anti-CD8 6-chain (53-6.7), anti-TCR β-chain (H7-597), anti-CD45R/B220 (RA3-6B2), anti-CD11b/Mac1 (M170), anti-NK1.1 (PK136), and anti-GR1 (RB6-8C5); and biotinylated anti-H-2Kd (SF1-1.1) and anti-Ly5.1 (A20) mAbs were all obtained from BD PharMingen (San Diego, CA). Flow microfluorometry was performed as described (8). Briefly, single cell suspensions were labeled with Ab, rinsed, washed, and analyzed on a FACScan (BD Biosciences, Sunnyvale, CA). Forward angle and side scatter were used to distinguish lymphocytes, monocytes, and granulocytes. Dead cells and erythrocytes were excluded by propidium iodide staining. At least 10^4 events were analyzed for each sample. The relative percentages of host- and donor-origin cells in the C57BL/6 (H-2b), Ly5.2 recipients of BALB/c (H-2b) or C57BL/6-Ly5.1 (Ly5.1) bone marrow were determined by flow microfluorometry. In preliminary experiments, known mixtures of donor and host PBMC were analyzed, and it was determined that the lower limit of sensitivity of the assay for detecting either donor (H-2b) or Ly5.1) or host (H-2b or Ly5.2) cells was 0.5%. Because not all hematopoietic cells express MHC class I Ag, the relative percentage of donor-origin cells in chimeric mice recipients was calculated as follows: [percentage of donor cells/percentage of donor cells + percentage of host cells] × 100%. (8)

Cell preparation and bone marrow transplantation

Recipient mice were treated with 6 Gy whole body irradiation using a 137Cs source (Gammarcell 40; Atomic Energy of Canada, Ottawa, Ontario, Canada). This dose was documented in preliminary experiments to be nonlethal for C57BL6 mice. Within 1–3 h of irradiation all recipients received a single i.v. injection of 18–25 × 10^6 donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. Donor femurs and tibias (female BALB/c or C57BL/6-Ly5.1 mice ≥6 wk of age) were flushed with RPMI medium using a syringe and 24-gauge needle. Cells were filtered through sterile nylon mesh (70 μm; BD Biosciences, Franklin Lakes, NJ), counted, and resuspended in RPMI. Recipient mice were females ≥6 wk of age. To assess chimera, blood samples were obtained from mice given donor bone marrow 2–4 wk earlier. Additional samples were obtained periodically as described in Results. Hematopoietic chimera was defined as the presence of ≥2% MHC class I donor-origin cells in peripheral blood.

Suspensions of spleen cells were filtered through sterile nylon mesh (70 μm) and centrifuged. Erythrocytes were lysed with hypotonic NH4Cl, and the spleen cells were resuspended in RPMI and counted using a hemocytometer.

LCMV injection and assay for infectious units of LCMV

Mice were inoculated i.p. with 5 × 10^4 PFU of LCMV, strain Armstrong, propagated in baby hamster kidney cells (25). Mice were inoculated with LCMV immediately after bone marrow injection, or 2 or 7 wk posttransplantation as described. LCMV viral titters were measured by LCMV viral plaque assay as described (32). Results are expressed as geometric mean titters, i.e., the arithmetic mean of the log_{10} values.

Skin transplantation

Full thickness skin grafts ~1 cm in diameter were procured and transplanted as described (30). Grafts were examined three times weekly, and rejection was defined as the first day on which the entire graft surface appeared necrotic (30). Grafts adherent to the bandage or fully necrotic on day 7 were deemed technical failures and were excluded from analysis (33).

Histology

Samples of transplanted skin, host skin, small intestine, large intestine, femur, spleen, and liver were recovered from selected experimental mice, fixed and stored in 10% buffered formalin, embedded in paraffin, processed for light microscopy, and stained with H&E. A qualified pathologist (B. A. Woda), who was unaware of the treatment status of specimen donors, performed histological analyses.

Statistical analysis

Parametric data are given as the arithmetic mean ± 1 SD. Duration of graft survival is given as the median survival time (MST). Graft survival among groups was compared using the method of Kaplan and Meier (34) and the log rank statistic (35). Values of p < 0.05 were considered significant. Analysis of 2 × 2 tables used the Fisher exact statistic (36).

Results

Blockade of CD154 permits establishment of multilineage BALB/c hematopoietic chimera in C57BL6 mice

We first identified doses of sublethal irradiation and anti-CD154 mAb that permitted the generation of mixed hematopoietic chimera in C57BL/6 (H-2b) mice transplanted with either syngeneic (C57BL/6-Ly5.1) or fully allogeneic BALB/c (H-2b) bone marrow. As shown in Table I, mice that received 18–25 × 10^6 syngeneic bone marrow cells plus 6 Gy of radiation uniformly became chimeric. Addition of anti-CD154 mAb treatment was not required for the generation of syngeneic hematopoietic chimera and had no effect on the percentage of syngeneic donor-origin cells present.

In contrast, mice that received the same number of allogeneic BALB/c bone marrow cells plus 6 Gy of radiation uniformly failed to become chimeric in the absence of anti-CD154 mAb. However, when two doses of 0.5 mg anti-CD154 mAb were added to the conditioning regimen, 9 of 14 recipient mice developed mixed hematopoietic chimera 2 wk after transplantation. One of the nine chimeric mice was selected at random and killed after wk 2 for histologic study, as was a second of these mice that was still chimeric at wk 7. Among the seven mice that were chimeric at wk 2 and observed through wk 9, five maintained high (70–89%) levels of donor-origin cells, whereas in the other two mice the level of donor-origin cells declined progressively to undetectable levels. The five mice that were still allochimeric 9 wk after transplantation, and all of the synchimeric mice, shown in Table I, were reanalyzed at intervals up to 27 wk after transplantation. The percentage of donor-origin cells remained stable and high for the entire period of observation, >80% in 12 of 13 mice analyzed at the end of the experiment.
A subset of the stably allochimeric mice shown in Table I was also analyzed by flow microfluorometry to determine the percentage of donor-origin T cells, B cells, macrophages, and granulocytes present in peripheral blood. As shown in Fig. 1, donor-origin cells representing each of these four lineages were present in allochimeric C57BL/6 recipients throughout the 9-wk period of observation. The percentage of donor-origin T and B cells rose monotonically over time, whereas the percentage of donor-origin granulocytes was maximal 2 wk after transplantation and declined thereafter. As expected, donor-origin cells representing all four lineages were also present in the syngeneic chimeric recipients generated using Ly5 congenic marrow; these lineages exhibited the same temporal changes in percentage (data not shown).

There was no clinical evidence of GVHD in allochimeric mice at any time, in one case up to 340 days after transplantation. Histologic examination of skin, liver, and small and large intestine in the two allochimeric mice selected at random 2 and 7 wk after transplantation also revealed no evidence of GVHD.

Donor-specific skin allografts uniformly survive on mixed chimeric mice that receive anti-CD154 Ab

Having generated mixed hematopoietic chimerism in the absence of GVHD, we next documented the presence of donor-specific transplantation tolerance. To do so, a total of 20 C57BL/6 mice received BALB/c skin grafts 8–17 wk after transplantation of BALB/c bone marrow. Among these bone marrow recipients, nine had been conditioned with sublethal irradiation alone and were nonchimeric. The other 11 had been conditioned with sublethal irradiation and anti-CD154 mAb as described above, and at the time of skin grafting all were chimeric. The percentage of donor-origin PBMC in these recipients was 58–96%. All 11 skin grafts were still intact at the time the allochimeric animals were exclusively killed, 72–251 days after transplantation. In contrast, the MST of skin grafts in the nonchimeric mice was significantly shorter (n = 9; MST, 12 days; range, 10–12 days; p < 0.001 vs allochimeric recipients).

Three of the allochimeric mice with donor-specific (H-2k) skin grafts that had been in place for 30 days were selected at random and given a third party CBA/JCR (H-2k) skin graft on the contralateral flank. Survival of the CBA/JCR skin allografts was brief (MST, 11 days; range, 10–11 days). The BALB/c skin grafts on these mice were still intact at the conclusion of the experiment 127 days after the CBA/JCR skin allografts had been rejected. One additional allochimeric mouse received only a CBA/JCR skin allograft, and survival of that graft was brief (11 days), demonstrating that T cell function was present and that the mice were specifically tolerant to H-2k-expressing cells.

Histological analysis of transplanted skin was performed on a subset of two chimeric mice with healed-in BALB/c skin grafts that had survived intact for 205 and 212 days. In neither instance was there evidence of inflammation suggestive of graft rejection. As expected, the donor-specific (C57BL/6-Ly5.1) skin grafts on chimeric C57BL/6 recipients of syngeneic C57BL/6-Ly5.1 bone marrow survived indefinitely. This was true both for recipients conditioned with radiation alone (MST, 138; range, 32–164 days; n = 5) and for recipients conditioned with both radiation and anti-CD154 mAb (MST, 156; range, 60–297 days; n = 23). These data document a model system characterized by mixed hematopoietic chimerism and donor-specific transplantation tolerance in the absence of GVHD and minimal preparative risk to the recipient. Because the system accurately models an approach that could well be put into clinical practice, it was deemed appropriate for use in analyses of safety and durability in the presence of viral infection, which is a common complication of clinical bone marrow transplantation.

Early but not late LCMV infection abrogates allogeneic hematopoietic chimerism and is fatal

We addressed the issue of safety by studying the effects of viral infection on mice undergoing treatment to induce hematopoietic chimerism. We also addressed issues of both safety and durability by examining the effect of delayed exposure to virus on mice in which mixed chimerism had been successfully established 15 or 50

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Percentage of donor-origin cells in allochimeric mice. Five of the stably allochimeric mice shown in Table I were analyzed by flow microfluorometry as described in Materials and Methods to determine the percentage of donor-origin T cells, B cells, macrophages, and granulocytes present in peripheral blood. Each data point represents the mean ± 1 SD.
days earlier. We first studied the effect of LCMV infection at the time of tolerization and bone marrow transplantation. In two separate experiments, C57BL/6 mice were randomized into two groups and treated with radiation, anti-CD154 mAb, and either syngeneic C57BL/6-Ly5.1 or allogeneic BALB/c bone marrow as described above. The transplanted mice in both groups were then randomly assigned to one of four subgroups. The first subgroup received no further treatment. Mice in the remaining three subgroups were given an i.p. injection of LCMV, strain Armstrong, on the same day as transplantation, or on days 15 or 50 after transplantation.

As shown in Table II, there was no effect of LCMV infection at any time point on the recipients of syngeneic C57BL/6-Ly5.1 bone marrow with respect to the number of mice becoming chimeric or the percentage of donor-origin cells present 2–9 wk after transplantation. The percentage of donor-origin cells at each time point was comparable to that observed in the uninfected control mice (Table I, line 2). None of the mice in any group appeared sick or died during the period of observation.

In contrast, all C57BL/6 recipients of allogeneic BALB/c bone marrow that were infected with LCMV immediately after transplantation died 13–18 days later (n = 15). Among these 15 mice, 12 survived long enough to be tested for chimerism on days 12–13; no peripheral donor-origin cells were detectable in any of them (Table II), indicating that the viral infection had blocked the development of allogeneic hematopoietic chimerism.

To determine to what extent these strikingly different outcomes were due to deleterious effects of allogeneic bone marrow vs rescue by syngeneic marrow, an additional cohort of control C57BL/6 mice was treated with radiation, anti-CD154 mAb, and LCMV but no bone marrow; 13 of 19 mice (68%) survived for >4 wk. This rate of survival was significantly higher than that of the LCMV-infected allogeneic bone marrow recipients described above (0%; n = 15; p < 0.0001) and statistically similar to the survival rate in the LCMV-infected recipients of syngeneic bone marrow (100%; n = 10; p = 0.07, Fisher exact statistic). In addition, nearly all control mice survived >4 wk (eight of nine treated with radiation and LCMV, five of five treated with radiation alone, five of five treated with radiation plus anti-CD154 mAb, and five of five treated with anti-CD154 mAb plus LCMV). These data indicate that the allogeneic bone marrow cells have a role in the LCMV-induced death of sublethally irradiated hosts treated with anti-CD154 mAb.

### Durability of the chimeric state following delayed infection with LCMV

We next studied the durability of our chimeric state by delaying LCMV infection until 2 or 7 wk after the establishment of mixed allogeneic hematopoietic chimerism. Among the mice randomized to receive LCMV 15 days after transplantation, one died before infection. Subsequent to infection, the percentage of donor-origin PBMC declined by 17% on day 28 and remained at approximately this same level on days 49 and 63 (Table II). None of these mice appeared ill and none died. At each time point after infection, the percentage of donor-origin PBMC in the LCMV-infected mice (Table II) was somewhat lower than in the uninfected controls (Table I, line 4). This experiment indicates that the deleterious effects of LCMV infection on host and graft survival are confined to a narrow window of time during the tolerization and transplantation process.

Among the mice randomized to receive LCMV 50 days after transplantation, 9 of 10 were chimeric on the day before infection. After infection, the percentage of donor-origin PBMC in the chimeric mice declined by ~11% on day 63 (Table II). None of the 10 mice appeared ill and none died. At corresponding time points after infection, the percentage of donor-origin PBMC in the LCMV-infected mice (Table II) was again lower than in the uninfected controls (Table I, line 4).

### Clearance kinetics of LCMV in infected hematopoietic chimeras

We next hypothesized that the differential survival of syngeneic vs allogeneic bone marrow recipients was due to differential ability to clear LCMV. To test this hypothesis we measured LCMV titers in chimeric mice infected with LCMV at different times after transplantation. As shown in Table III, all recipients of allogeneic bone marrow infected on the day of transplantation had failed to clear virus during the first 2 wk and died soon after. In contrast, syngeneic chimeras infected on the day of transplantation survived, but thereafter they were persistent carriers of virus. Viral titers 2 wk after transplantation and infection were similar in the allogeneic bone marrow recipients that died and in the syngeneic bone marrow recipients that survived. The data suggest that neither viral load per se nor ability to completely clear virus was the determinant of differential survival.

Syngeneic chimeras infected 15 days after transplantation cleared the virus, whereas allogeneic chimeras infected on day 15

### Table II. Percentage of donor-origin PBMC in C57BL/6 recipients of syngeneic and allogeneic bone marrow infected with LCMV at various times

<table>
<thead>
<tr>
<th>Bone Marrow Donor</th>
<th>LCMV Infection Day</th>
<th>Frequency of Chimerism on Days 12–13</th>
<th>Donor-Origin PBMC in Chimeric Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td>Days 12–13</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>0</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>15</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>50</td>
<td>10</td>
<td>10/10</td>
</tr>
<tr>
<td>BALB/c</td>
<td>0</td>
<td>15</td>
<td>0/15</td>
</tr>
<tr>
<td>BALB/c</td>
<td>15</td>
<td>10</td>
<td>0/99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* C57BL/6 mice were given 6 Gy of radiation. Within 1–3 h of irradiation all recipients received a single i.v. injection of 18–25 × 10⁹ donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. Anti-CD154 mAb (0.5 mg) was injected i.p. into all mice on the day of irradiation and on day +3. Mice were injected with LCMV on the days indicated as described in Materials and Methods. The percentage of H-2ª or Ly5.1 donor-origin PBMC was determined by flow microfluorometry 2–9 wk after bone marrow transplantation. Chimerism was defined as the presence of >2% MHC class Iª donor-origin cells 2 wk after transplantation. The one mouse with <2% donor-origin cells at the 2-wk time point had <2% donor cells at all other time points. All mice chimeric at wk 2 remained chimeric throughout the duration of the experiment. Each data point represents the mean ± 1 SD.

* Three additional mice in this cohort died on day 12, before they could be tested for chimerism.
became persistent carriers. Both allogeneic and syngeneic chimeras infected with LCMV 50 days after successful bone marrow transplantation cleared the virus within 2 wk, documenting the immune competency of the hematopoietic chimeras.

Immunocompromised mice are known to become persistent virus carriers due to clonal exhaustion of LCMV-specific T cells (37). The persistent carrier state in allograft recipients infected on day 15 suggests that they may be immunocompromised at that time point.

The bone marrow and lymphoid compartments of allogeneic bone marrow recipients given anti-CD154 mAb and LCMV infection are markedly hypoplastic

To determine the cause of death in allogeneic bone marrow recipients infected with LCMV on the day of bone marrow transplantation, cohorts of control and infected bone marrow recipients were killed 7 or 14 days after transplantation. Light microscopic analysis of sections of spleen and femurs of infected mice (n = 8) revealed severe reductions in the number of all hematopoietic populations (Fig. 2). Reductions in marrow cellularity averaged 86 ± 12% (range, 60–97%). Histologic examination of the spleens revealed lymphoid depletion in all cases, and, with the exception of a single splenic nodule in one mouse that showed regenerative activity, there was no evidence of extramedullary hematopoiesis.

In additional studies, total cell counts were performed on both spleen and femoral bone marrow recovered 4–35 days after transplantation. As shown in Fig. 3, cell counts in both tissues were depressed immediately after irradiation and transplantation. In syngeneic bone marrow recipients, cell counts thereafter recovered, irrespective of LCMV infection at the time of transplantation. Recovery in the spleen cell compartment of the LCMV-infected mice was somewhat slower than in the bone marrow compartment. Control uninfected allogeneic bone marrow recipients also showed cellular recovery in both spleen and bone marrow. However, in contrast to all other groups, cell counts in recipients of allogeneic bone marrow that were infected with LCMV on the day of transplantation never recovered, and all mice died before day 21.

Host IFN-αβR expression is required for LCMV-induced hypoplasia and death in allogeneic bone marrow recipients given anti-CD154 mAb

Having discovered severe hypoplasia restricted to recipients of allogeneic bone marrow and LCMV infection, we next sought to determine its cause. We first hypothesized that the cause was related to cytokine release. Reversible depression of hematopoiesis is known to occur early in the course of LCMV infection and has been reported to be a direct effect of IFN-αβ (22). To determine the role of IFN-αβ in our model system, we repeated our experiments using 129/Sv IFN-αβR knockout mice (29). 129/Sv IFN-αβR knockout mice were irradiated (600 rad) and given 25 million BALB/c bone marrow cells and two injections of anti-CD154 mAb on days 0 and +3. Half were then infected with LCMV at the time of transplantation. As shown in Table IV, control 129/Sv +/+ and 129/Sv IFN-αβR knockout recipients readily accepted BALB/c bone marrow and were chimeric. As expected, LCMV infection of control 129/Sv +/+ recipients led to failure of engraftment and death. In contrast, LCMV infection of 129/Sv IFN-αβR knockout recipients also led to failure of the bone marrow allograft, but all of the mice survived. As was the case for LCMV-infected C57BL/6 recipient mice (Fig. 2), histologic study of LCMV-infected SV129 +/+ recipients revealed bone marrow hypoplasia and splenic lymphopenia. In contrast, the spleen and bone marrow of SV129 IFN-αβR knockout mice treated in a similar way showed normal cellularity. This experiment suggests that death of the host was the consequence of a type 1 IFN-mediated process, but rejection of the allogeneic marrow graft was due to a different mechanism.

CD8+ TCRαβ NK1.1− cells prevent allogeneic bone marrow engraftment in recipients treated with LCMV infection and anti-CD154 mAb

Although interference with the function of IFN-αβRs prevented death in LCMV-infected allogeneic bone marrow recipients treated with anti-CD154 mAb, the allogeneic marrow still did not engraft and survival depended on the recovery of the host marrow. Therefore, we questioned whether failure of allogeneic bone marrow engraftment in the presence of LCMV infection was the result of cell-mediated rejection. To identify the cell type responsible for graft failure we conducted a series of cell deletion studies focused on T cell subsets and NK cells. NK cells were of particular interest because they reportedly play a pivotal role in rejection of murine allogeneic bone marrow transplants in irradiated hosts (38–40).

Studies in mice treated with cell-depleting reagents

We first studied cell-depleting reagents in uninfected mice. As shown in the upper half of Table V, pretransplantation administration of anti-NK1.1 mAb, anti-CD4 mAb, or anti-CD8 mAb had little or no effect on subsequent hematopoietic chimerism or survival in C57BL/6 recipients of BALB/c bone marrow and anti-CD154 mAb in the absence of LCMV.

The effects of these reagents on survival and chimerism in mice infected with LCMV on the day of bone marrow transplantation are shown in the lower half of Table V. The principal finding was that treatment with anti-CD8 mAb completely prevented the deleterious effect of LCMV infection, as the great majority of treated

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Day of Virus Infection</th>
<th>n</th>
<th>2 wk</th>
<th>5 wk</th>
<th>7 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>C57BL/6</td>
<td>0</td>
<td>6</td>
<td>3.5 ± 0.3</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>0</td>
<td>12</td>
<td>3.3 ± 0.8</td>
<td>Dead</td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>15</td>
<td>9</td>
<td>2.8 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>50</td>
<td>8</td>
<td>&lt;2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>0</td>
<td>10</td>
<td>3.4 ± 0.3</td>
<td>3.3 ± 0.4</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>15</td>
<td>10</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>50</td>
<td>9</td>
<td>&lt;2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Peripheral blood from mice shown in Table II were assayed for LCMV titers as described in Materials and Methods at 2–7 wk after LCMV infection. Results are expressed as geometric mean titers, i.e., the arithmetic mean of the log_{10} titer values.
mice both survived and exhibited allogeneic hematopoietic chimerism. In contrast, treatment with anti-NK1.1 mAb had no effect on LCMV-infected mice, as all tested mice failed to engraft \((n/11005)\) and most (eight of nine) died. LCMV-infected mice treated with anti-CD4 mAb displayed improved survival vs untreated LCMV-infected controls, but (with the exception of a single mouse) allogeneic bone marrow still did not engraft. As expected, and consistent with the results in Table I, control recipients treated only with sublethal irradiation, anti-CD154 mAb, and LCMV showed no evidence of allogeneic bone marrow engraftment \((n/13)\), and the great majority (16 of 17) died.

Studies in knockout mice

Because our data suggested that a CD8\(^+\) non-NK cell was responsible for the failure of bone marrow engraftment in LCMV-infected mice, we next retested this hypothesis using appropriate knockout mice as graft recipients. As shown in the upper half of Table VI, the absence of cell surface expression of CD4, CD8, TCR-αβ, or TCR-γδ had little or no effect on hematopoietic chimerism or survival in C57BL/6 knockout recipients of BALB/c bone marrow and anti-CD154 mAb in the absence of LCMV infection.

In contrast to LCMV-infected control mice, which failed to develop allogeneic hematopoietic chimerism and died, allogeneic bone marrow transplantation into either LCMV-infected CD8 or TCR-αβ knockout mice resulted in greatly enhanced survival and robust hematopoietic chimerism. Transplantation into LCMV-infected CD4 and TCR-γδ knockout mice resulted in slightly but not statistically significantly improved survival, but there was no engraftment of donor bone marrow (Table VI).

Discussion

The data presented in this work document that viral infection can prevent allogeneic bone marrow engraftment and is fatal for allogeneic stem cell recipients treated with sublethal irradiation and costimulation blockade. Furthermore, the data document distinct and independent mechanisms for the failure of engraftment in virus-infected recipients (a radioresistant CD8\(^+\) T cell) and for the fatal outcome in these recipients (host response to viral induction of IFN-αβ).

These studies clearly document the risk posed to stem cell graft recipients by viral infection when it occurs at the time of the procedure. Although there have to date been no reports of adverse virus-associated events in clinical trials of costimulation blockade therapy of autoimmune diseases, adaptation of costimulation blockade to clinical stem cell transplantation will require careful attention to this risk and documentation of safety (41). Viral infection is one of the most important risks faced by allogeneic stem cell recipients. It may arise from infected transplanted tissue, from
reactivation of latent host viruses as a consequence of an allogeneic stem cell graft recipients is alteration of the host cellular milieu leading to host-vs-graft reaction and graft failure. Tolerance induced by a donor-specific transfusion and a short course of anti-CD154 mAb (47), like other costimulation blockade protocols that induce tolerance (48), is dependent on the deletion of host alloreactive CD8+ T cells. Consistent with concerns that LCMV infection could activate the host immune system and abrogate tolerance, we previously documented that infection with LCMV at the time of transplantation induced skin allograft rejection in mice treated with donor-specific transfusion and anti-CD154 mAb (25). We subsequently demonstrated that skin graft rejection in those LCMV-infected mice was due to the ability of LCMV infection to abrogate host alloreactive CD8+ T cell deletion (49). Others have shown that mice conditioned with busulfan and treated with anti-CD154 mAb, CTLA-4-Ig, and a bone marrow allograft lose that graft (but do not die) if infected with LCMV (28). In that report, the mechanism of allogeneic stem cell graft failure was not identified, but evidence was presented to suggest that it was dependent on dendritic cell activation. In this work we report that allograft failure is due to a radiosensitive alloreactive CD8+ T cell that appears to be activated by the viral infection and that is not deleted by costimulation blockade in LCMV-infected hosts.

With respect to underlying mechanisms, our data set provides insight into the distinct and independent factors that determine whether or not the allogeneic stem cell graft will survive and whether or not the recipient will survive irrespective of the success or failure of the stem cell allograft. Our data show that allogeneic bone marrow is unable to engraft in recipient mice treated with sublethal irradiation, anti-CD154 mAb treatment, and an LCMV infection at the time of transplantation. The mice appear to die as the result of bone marrow hypoplasia 2–3 wk after transplantation. In contrast, syngeneic bone marrow readily engrafts in identically

Table IV.

<p>| Table IV. Chimerism and survival in 129/Sv wild-type and 129/Sv IFN-αβR knockout mice* |
|----------------------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Virus</th>
<th>n</th>
<th>Chimerism at 2 wk</th>
<th>Survival at 7 wk</th>
<th>Donor-Origin PBMC in Chimeric Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv +/+</td>
<td>None</td>
<td>15</td>
<td>15/15</td>
<td>15/15</td>
<td>65 ± 23</td>
</tr>
<tr>
<td>129/Sv +/+</td>
<td>Day 0</td>
<td>15</td>
<td>0/2*</td>
<td>0/15</td>
<td>&lt;2</td>
</tr>
<tr>
<td>129/Sv IFN-αβR KO†</td>
<td>None</td>
<td>15</td>
<td>14/15</td>
<td>15/15</td>
<td>52 ± 23</td>
</tr>
<tr>
<td>129/Sv IFN-αβR KO</td>
<td>Day 0</td>
<td>15</td>
<td>0/15</td>
<td>15/15</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* Groups of 129/Sv wild-type and 129/Sv IFN-αβR knockout mice were treated with radiation, 25 × 10⁶ BALB/c bone marrow cells, and anti-CD154 mAb according to our standard transplantation protocol as described in Materials and Methods. Immediately after transplantation, mice in each of these two groups were randomized, and half of each group was injected with LCMV. The percentage of donor-origin PBMC was determined by flow microfluoroscopy 2 and 4–7 wk after transplantation as described in Materials and Methods. Each data point represents the mean ± 1 SD.
† Thirteen mice in this cohort died before they could be tested for chimerism.
†† KO, knockout.
treated LCMV-infected mice. The simplest hypothesis to explain these findings was that LCMV-infected mice receiving allogeneic bone marrow died due to the complete lack of donor cell engraftment. We believe this hypothesis is not correct for two reasons. First, we showed that 89% of LCMV-infected mice that received irradiation but no bone marrow transplant survived. Second, we showed that BALB/c stem cells were unable to engraft in IFN-αR knockout 129/Sv mice, yet these knockout mice survived for the duration of the experiment (up to 7 wk). These data clearly indicate that simple failure of allogeneic stem cells to engraft is, in and of itself, insufficient to kill sublethally irradiated, LCMV-infected mice. The data further document that the allogeneic bone marrow graft itself was important in the death of the host.

We hypothesize that the donor allogeneic bone marrow contributed to a fatal outcome in LCMV-infected recipients by initiating a graft-vs-host reaction (GVHD) mediated by alloreactive T cells in the donor bone marrow inoculum, thereby activating the host immune system (50–52). The allogeneic marrow may also have induced a host response against donor alloantigens. Host immune systems activated by GVHD or allogeneic cells plus LCMV infection would be expected to induce high levels of inflammatory cytokines in the host. Our data show that IFN-αβR knockout mice that were given allogeneic marrow, costimulation blockade, and LCMV infection did not die. It is known that LCMV-infected mice produce high levels of IFN-αβ (53–56), and in a preliminary study we confirmed that IFN-αβ can readily be detected in the serum of both wild-type and IFN-αβR knockout C57BL/6 mice 2 and 4 days after LCMV infection of recipients transplanted with either syngeneic or allogeneic bone marrow (D. Forman, unpublished observations). IFN-αβ is produced by several cell types including dendritic-like cells (57, 58) and monocytes/macrophages (59). It inhibits the generation of both CFU and burst-forming units in long-term human bone marrow cultures (60) and leads to bone marrow dysfunction in vivo in both mice and humans (22, 61).

### Table V. Chimerism and survival in recipients of bone marrow allografts treated to deplete T cell subsets or NK cells

<table>
<thead>
<tr>
<th>LCMV Infection</th>
<th>Ab</th>
<th>n</th>
<th>Chimerism at 2 wk</th>
<th>Survival at 6 wk</th>
<th>Donor-Origin PBMC in Chimeric Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>9</td>
<td>7/9</td>
<td>9/9</td>
<td>56 ± 25</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td>9</td>
<td>7/9</td>
<td>9/9</td>
<td>57 ± 20</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>7</td>
<td>7/7</td>
<td>7/7</td>
<td>65 ± 32</td>
<td>79 ± 18</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>8</td>
<td>8/8</td>
<td>8/8</td>
<td>69 ± 16</td>
<td>84 ± 9</td>
</tr>
<tr>
<td>LCMV (day 0)</td>
<td>None</td>
<td>17</td>
<td>0/13</td>
<td>1/17</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td>9</td>
<td>0/4</td>
<td>1/9</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>7</td>
<td>1/4</td>
<td>3/7</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>9</td>
<td>6/8</td>
<td>8/9</td>
<td>89 ± 8</td>
<td>76 ± 12</td>
</tr>
</tbody>
</table>

* C57BL/6 mice intended for bone marrow transplantation were randomized into four groups. The first received no treatment. The remaining groups were treated with injections of the listed depleting reagents. Treatments were begun on days 1–6 as described in Materials and Methods. Treatment schedules were based on preliminary studies and documented to deplete >97% of targeted cells. On day 0 all mice received 6 Gy of radiation, 25 million BALB/c bone marrow cells, and the first of two doses of anti-CD154 mAb as described in Materials and Methods. Mice in all four treatment groups were then randomized a second time, and half received LCMV immediately after bone marrow transplantation. The remaining half received no virus. The percentage of donor-origin PBMC was determined by flow microfluorometry 2 and 4–6 wk after transplantation as described in Materials and Methods. Each data point represents the mean ± 1 SD.

<table>
<thead>
<tr>
<th>LCMV Infection</th>
<th>Recipient</th>
<th>n</th>
<th>Chimerism at 2 wk</th>
<th>Survival at 6 wk</th>
<th>Donor-Origin PBMC in Chimeric Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>CD4 KO C57BL/6</td>
<td>12</td>
<td>6/12</td>
<td>12/12</td>
<td>54 ± 14</td>
</tr>
<tr>
<td></td>
<td>CD4 KO C57BL/6</td>
<td>9</td>
<td>8/9</td>
<td>9/9</td>
<td>64 ± 15</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-γδ KO</td>
<td>8</td>
<td>8/8</td>
<td>8/8</td>
<td>65 ± 24</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-αβ KO</td>
<td>8</td>
<td>8/8</td>
<td>8/8</td>
<td>88 ± 11</td>
</tr>
<tr>
<td></td>
<td>CD8 KO C57BL/6</td>
<td>10</td>
<td>10/10</td>
<td>10/10</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>LCMV (day 0)</td>
<td>C57BL/6 wild type</td>
<td>12</td>
<td>0/8</td>
<td>0/12</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>CD4 KO C57BL/6</td>
<td>9</td>
<td>0/4</td>
<td>2/9</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-γδ KO</td>
<td>8</td>
<td>0/6</td>
<td>5/8</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-αβ KO</td>
<td>9</td>
<td>8/9</td>
<td>8/9</td>
<td>82 ± 26</td>
</tr>
<tr>
<td></td>
<td>CD8 KO C57BL/6</td>
<td>10</td>
<td>9/9</td>
<td>9/10</td>
<td>81 ± 12</td>
</tr>
</tbody>
</table>

* Groups of C57BL/6 wild-type and C57BL/6 CD4, CD8, TCR-αβ, or TCR-γδ knockout mice were treated with 6 Gy of radiation, 25 × 10⁶ BALB/c bone marrow cells, and anti-CD154 mAb according to our standard transplantation protocol as described in Materials and Methods. Immediately after transplantation, mice in each of the three groups were randomized, and half of each group was injected with LCMV. The percentage of donor-origin PBMC was determined by flow microfluorometry 2–4 and 7 wk after transplantation as described in Materials and Methods. Each data point represents the mean ± 1 SD.

* Some mice did not survive long enough to be tested for chimerism at wk 2.

* KO, knockout.

* Value of p <0.001 vs untreated control (Fisher exact statistic).
In addition, the transient pancyclopenia that follows LCMV infection in normal mice (22) does not occur in IFN-α/βR knockout mice (22).

If IFN-αβ is the agent of lethality but not allograft rejection in LCMV-infected mice, another mechanism must prevent allogeneic stem cells from engrafting in mice that receive irradiation, anti-CD154 mAb, and LCMV infection. Because NK cells are activated by LCMV infection (62) and play a key role in allogeneic bone marrow graft rejection (63), we examined their role in our system. We observed that depletion of NK1.1+ cells had no effect on allogeneic bone marrow engraftment or on survival of LCMV-infected recipients of allogeneic bone marrow. These data suggest that the cell type responsible for preventing allogeneic bone marrow engraftment is a NK1.1+ cell.

To identify the cell that prevents allogeneic bone marrow engraftment in LCMV-infected mice, we used C57BL/6 knockout mice and C57BL/6 mice that had been depleted of various cell types. The critical observation was that, after infection with LCMV, only three types of recipients survived and became chimeric: mice depleted of CD8+ T cells, CD8 knockout mice, and TCR-αβ knockout mice. These data indicate that the mediator of bone marrow allograft destruction in LCMV-infected mice treated with costimulatory blockade is a radiosensitive CD8+ NK1.1+ TCRαβ+ T cell. This conclusion is supported by the observation that anti-CD8 mAb treatment facilitates the induction of mixed hematopoietic chimerism in sublethally irradiated mice given anti-CD154 mAb plus allogeneic bone marrow grafts (64), indicating an important role for alloreactive CD8+ T cells in allogeneic bone marrow rejection. In this work we show that this host alloreactive activity can be greatly amplified by virus infection to overcome costimulation blockade and prevent allogeneic marrow engraftment. The infection of donor bone marrow cells by LCMV may also have contributed to this effect by rendering them highly susceptible to a host CD8-mediated antiviral immune response. Interestingly, the data suggest that a bone marrow CD8+ T-cell facilitator cell (65, 66) may not be required for allogeneic bone marrow engraftment in this model.

Finally, in a previous report using nonobese diabetic and BALB/c mice as recipients (8), we showed that sublethal irradiation and costimulation blockade led to the generation of stable but complete allogeneic hematopoietic chimerism. The present study demonstrates that the same methods can be used to generate mixed hematopoietic chimerism in sublethally irradiated mice given anti-CD154 mAb plus allogeneic bone marrow grafts (64), indicating an important role for alloreactive CD8+ T cells in allogeneic bone marrow rejection. In this work we show that this host alloreactive activity can be greatly amplified by virus infection to overcome costimulation blockade and prevent allogeneic marrow engraftment. The infection of donor bone marrow cells by LCMV may also have contributed to this effect by rendering them highly susceptible to a host CD8-mediated antiviral immune response. Interestingly, the data suggest that a bone marrow CD8+ T-cell facilitator cell (65, 66) may not be required for allogeneic bone marrow engraftment in this model.

We conclude that viral infection at the time of transplantation in mice treated with anti-CD154 Ab can prevent the engraftment of allogeneic bone marrow and lead to the death of recipients. The mechanism of graft destruction appears to be mediated by a radiosensitive alloreactive CD8+ T cell. Graft loss is associated with a fatal outcome despite the fact that recipients received sublethal conditioning. The mechanism of death appeared to depend on IFN-αβR expression on host cells. Finally, it is important to note that both graft loss and a fatal outcome occurred after challenge with a virus that is noncytopathic. It is of concern that more virulent agents might have similar adverse consequences in the context of less stringent conditioning, or at later time points after bone marrow transplantation. Clinical application of stem cell transplantation protocols based on costimulation blockade and tolerance induction may require patient isolation to facilitate the procedure and to protect recipients.

Acknowledgments
We thank Linda Paquin, Stephanie Gibbons, Keith Daniels, Amy Cuthbert, and Jean Leif for technical assistance.

References
with the noncytopathic lymphocytic choriomeningitis virus. J. Exp. Med. 185: 517.


56. Singer, A., K. S. Hartcock, and M. Sykes. 2001. CD4 T cell-mediated allorejection to fully MHC-mismatched allogeneic bone marrow engraftment is dependent on CD40-CD40 ligand interactions, and lasting T cell tolerance is induced by bone marrow transplantation with initial blockade of this pathway. J. Immunol. 166:2970.
