Donor CD8 Cells Prevent Allogeneic Marrow Graft Rejection in Mice: Potential Implications for Marrow Transplantation in Humans
By Paul J. Martin

From the Division of Clinical Research, the Fred Hutchinson Cancer Research Center, Seattle, Washington 98104; and the Department of Medicine, University of Washington, Seattle, Washington 98105

Summary
Numerous experimental models have demonstrated that graft-vs.-host disease (GVHD) does not occur in irradiation chimeras when the graft does not contain mature, immunocompetent T lymphocytes, but clinical studies have shown that T cell depletion of donor marrow can be associated with a greatly increased risk of graft failure. We have developed a model where engraftment of (C57BL/6J × C3H/HeJ)F1 (B6C3) marrow in 800-cGy-irradiated (BALB/cJ × C57BL/6J)F1 (CB6) recipients depends on the presence of donor T cells in the graft. Recipients transplanted with 5.0 × 10⁶ marrow cells depleted of T lymphocytes showed host lymphoid and myeloid reconstitution, whereas recipients transplanted with the same marrow plus 2.5 × 10⁵ purified donor T cells showed donor reconstitution. Adding as few as 0.5 × 10⁵ CD8-enriched donor T cells to marrow grafts containing 5.0 × 10⁶ T cell-depleted donor cells was sufficient to enable donor reconstitution, while surviving recipients transplanted with the same marrow and 0.5–2.5 × 10⁵ CD4-enriched donor cells showed only host reconstitution. To address the question of whether donor CD4 cells could facilitate engraftment under conditions where GVHD would not represent a limiting factor, engraftment of bm1 marrow was tested in major histocompatibility complex (MHC) class I-disparate B6.Ly5² recipients. Results indicated that the donor CD8-enriched population was at least fivefold more active than the CD4-enriched population for facilitating allogeneic marrow engraftment in this strain combination. Thus, the lymphokines and MHC class II-specific cytotoxic T cells generated by CD4 cells were relatively ineffective for enhancing engraftment, possibly reflecting the fact that the host T cells that contain effectors responsible for causing rejection do not express MHC class II antigens. The ability of donor CD8 cells to facilitate engraftment could reflect the activity of a cytokine uniquely elaborated after recognition of an MHC class I disparity. More likely, the graft-enhancing effect of donor CD8 cells may result from the generation of MHC class I-specific or class I-restricted cytotoxic T cells that recognize the host CD4 and CD8 cells responsible for causing rejection. The possibility remains that other mechanisms such as veto inactivation of host T cells by donor CD8 cells may also contribute to the graft-enhancing effect.

Numerous experimental models have demonstrated that GVHD does not occur in irradiation chimeras when the graft does not contain mature, immunocompetent T lymphocytes (reviewed in reference 1). These observations prompted wide-spread clinical trials testing whether removal of T cells from donor marrow could prevent GVHD in humans. Results from these studies have confirmed the expectation that T cell depletion can decrease the incidence and severity of GVHD (reviewed in reference 2). Although there was little evidence from animal experiments that T cell depletion could lead to complications, clinical studies demonstrated that this approach for preventing GVHD can be associated with a greatly increased risk of graft failure (reviewed in reference 2). Thus, with unmodified HLA-identical marrow, the risk of graft failure is <2%, but with T cell depletion, the risk increases to 10–25%. Graft failure after human marrow transplantation usually leads to a fatal outcome because spontaneous reconstitution with host cells seldom occurs and because patients generally cannot tolerate the preparative regimen for another transplant (3, 4).

Precise causes for graft failure associated with T cell depletion of donor marrow in humans remain difficult to define. Deficiency of T cell-derived lymphokines required for proliferation and differentiation of hematopoietic cells and absence
of an immunosuppressive effect mediated by donor T cells represent two possible explanations that are not mutually exclusive. Small numbers of host lymphoid cells are known to survive the chemotherapy and total body irradiation (TBI)\(^1\) regimens used for human marrow transplantation (S). Thus, donor T cells may help to eliminate or inactivate these residual host cells that would otherwise remain capable of causing rejection. In support of this hypothesis are observations that depletion of donor T cells is associated with a high incidence of mixed lymphoid and mixed hematopoietic chimerism (i.e., persistence of host cells) after transplantation (3, 6–10). Moreover, host CD8-positive T cells with specific antidonor cytotoxic or suppressive activity have been identified in the blood of patients with graft failure after T cell–depleted marrow transplantation (11–17). Finally, several studies of T cell–depleted marrow transplantation have shown an inverse relationship between the risk of graft failure and the intensity of the conditioning regimen, implicating an active role for host cells in causing graft failure (3, 18–20).

In the present study, we sought to develop an experimental model that would enable an exploration of the role played by donor T cells in facilitating allogeneic marrow engraftment. Thus, it was necessary to determine conditions in which engraftment depends on the presence of donor T cells. Having done so, we then carried out experiments to determine the phenotype of the cells responsible for this effect and to assess the mechanisms possibly involved. The results suggest that donor T cells enhance engraftment through mechanisms that depend on interactions with host T cells.

### Materials and Methods

**Mice.** (C57BL/6J × C3H/HeJ)F1, (B6C3; H-2\(^{a}\)) males, (BALB/cJ × C57BL/6J)F1 (CB6; H-2\(^{b}\)) females, and B6.C-H-2\(^{mi}\) (hm1) males were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-Ly-5.1:pep3 (B6-LyS') females were bred at the Fred Hutchinson Cancer Research Center. Founder B6-Ly-5.1 males and females were kindly provided by Dr. David Myers (Sloan Kettering Institute, New York, NY). Mice were housed in groups of five under microisolated, specific pathogen-free conditions with twice weekly cage changes and were given sterilized food and water (pH 3.5) ad libitum. 4 wk after marrow transplantation, recipients 7–8 wk of age were prepared with 950 cGy, at least 10% of granulocytes were H-2Da negative and therefore originated from the donor H-2D\(^{a}\) negative and therefore originated from the donor

### Results

**Effects of TBI Exposure and Donor T Cells on Engraftment.** Initial experiments were carried out to determine the amount of TBI needed to allow engraftment of T cell–depleted marrow in MHC-disparate recipients. For this purpose, engraftment of T cell–depleted B6C3 marrow was tested in MHC class I and II, and minor antigen-disparate CB6 recipients prepared with single-fraction TBI exposures ranging from 650 to 950 cGy in 50-cGy increments. Noteworthy in this experiment was the finding that all recipients survived for at least 33 d, regardless of the amount of TBI or the number of T cells in the graft. On day 32 after transplantation, chimerism of peripheral blood granulocytes was measured by staining with an H-2D\(^{a}\)-specific antibody. In all recipients prepared with 950 cGy, at least 10% of granulocytes were H-2D\(^{a}\) negative and therefore originated from the donor (Table 1, Exp. 1). With exposures of 850–900 cGy, engraftment of donor cells could be detected in half of the recipients. None of the recipients prepared with 650–800 cGy had ≥10%
donor granulocytes detectable in the peripheral blood. Repeat testing on day 95 after transplantation showed no detectable engraftment of donor granulocytes or T cells in recipients prepared with 800 cGy TBI. Thus, with TBI exposures $<800$ cGy in this strain combination, rejection of the donor marrow occurred uniformly when the graft did not contain T cells, and recipients were reconstituted with hematopoietic cells of the host.

Results were distinctly different when T cells isolated from lymph nodes of the donor were added to the T cell–depleted marrow. With $2.5 \times 10^5$ donor T cells added to the marrow, engraftment of donor granulocytes was observed in some recipients prepared with TBI exposures as low as 700 cGy, and all recipients prepared with TBI exposures $>800$ cGy showed $>55\%$ donor granulocytes on day 32 after transplantation (Table 1, Exp. 1). Retesting on day 95 after transplantation showed $>85\%$ donor granulocytes and T cells in all recipients prepared with 800 cGy TBI. With $10^6$ donor T cells added to the marrow, engraftment of donor granulocytes was observed on day 32 in four of five recipients prepared with 650 cGy, and all recipients prepared with $>700$ cGy showed $>10\%$ donor granulocytes. Most of these recipients had $>50\%$ donor granulocytes and all had $>75\%$ donor T cells. Thus, the presence of donor T cells in the marrow allowed engraftment when the amount of TBI would not otherwise have been sufficient to prevent rejection.

A similar experiment tested the effects of donor T cells on engraftment of bm1 marrow in MHC class I–disparate B6.Ly5a recipients prepared with single-fraction TBI exposures ranging from 700 to 900 cGy. On day 85 after transplantation, chimerism of peripheral blood granulocytes was determined by staining with a Ly5.1-specific antibody. In the absence of donor T cells, rejections ($<10\%$ donor granulocytes) occurred in some recipients prepared with TBI exposures as high as 900 cGy (Table 1, Exp. 2), and no T cells of donor origin were detected in any of the recipients prepared with 700 or 750 cGy TBI. With grafts containing $1.25 \times 10^4$ donor T cells, engraftment ($>10\%$ donor granulocytes) was observed in all recipients given TBI exposures as low as 700 cGy, and all recipients prepared with $>750$ cGy were reconstituted entirely with T cells of donor origin. Taken together, the results of these two experiments demonstrate that under certain circumstances, allogeneic marrow engraftment may require effects mediated by donor T cells.

### Host T Cells Responsible for Marrow Graft Rejection.

In CB6 recipients, 800 cGy represented the highest TBI exposure uniformly followed by rejection of T cell–depleted marrow from B6C3 donors. The radioresistant host cells responsible for rejection in this model were identified by testing the effects of CD4- or CD8-specific antibodies administered to the recipients 5 d before transplantation. Control CB6 recipients transplanted with T cell–depleted B6C3 marrow without prior antibody administration uniformly rejected the grafts (Table 2), consistent with results shown in Table 1. Recipients treated with CD4-specific antibody before transplantation also rejected the grafts. In one experiment, donor cells persisted for $>67$ d in some recipients treated with CD8-specific antibody (Table 2, Exp. 1). In a second experiment, donor granulocytes (but not T cells) persisted for $>45$ d in some recipients treated with CD8-specific antibody, but these donor cells could not be detected on day 69 (Table 2, Exp. 2). In both experiments, donor cells persisted for $>67$ d in recipients treated with a mixture of CD4- and CD8-specific antibodies. These results suggested that either CD4 or CD8 cells of the host can mediate marrow graft rejection in this model.

The ability of both CD4 and CD8 cells to mediate allogeneic marrow graft rejection was confirmed by testing the effects of host CB6 T cells added in graded numbers to grafts containing $5.0 \times 10^4$ T cell–depleted donor B6C3 marrow cells transplanted into heavily irradiated (1,100 cGy) CB6 recipients. Controls transplanted with no host T cells added to the graft all survived for $>96$ d after transplantation, and all were durably engrafted with donor cells (Table 3). When as few as $5.0 \times 10^4$ CD4- or CD8-enriched host T cells were added to the graft, none of the recipients survived. With $2.5 \times 10^5$ CD4-enriched host T cells added to the grafts, three deaths occurred 10 d after transplantation, and the remaining two recipients died, respectively, on days 15 and 16. With $2.5 \times 10^5$ CD8-enriched host T cells added to the grafts, four deaths occurred, respectively, on days 15, 18, 22, and 22, and the remaining recipient died between 62 and 95 d after transplantation. Some recipients transplanted with grafts containing $2.0 \times 10^4$ or $1.0 \times 10^4$ CD4- or CD8-enriched host cells survived for $>96$ d after transplantation. All four surviving recipients transplanted with grafts containing host CD4–enriched T cells were durably engrafted with donor cells. Three of the eight surviving recipients transplanted with grafts containing host CD8–enriched T cells had rejected the donor marrow and were reconstituted with host T cells and granulocytes. When these rejections were taken into account together with the deaths before day 96, the results of this

### Table 1. Effects of Donor T Cells and TBI Exposure on Engraftment

| TBI (cGy) | Exp. | No. of T cells |
|-----------|------|---------------|
|           | 650  | 700 | 750 | 800 | 850 | 900 | 950 |
| 1         | None | 0   | 0   | 0   | 2   | 3   | 5   |
|           | $2.5 \times 10^5$ | 0   | 1   | 3   | 5   | 5   | 5   |
|           | $1.0 \times 10^4$ | 4   | 5   | 5   | 5   | 5   | 5   |
| 2         | None | 2   | 0   | 1   | 3   | 3   |
|           | $1.25 \times 10^4$ | 5   | 5   | 5   | 5   | 5   |

Groups of five irradiated recipients were transplanted with $5.0 \times 10^4$ T cell–depleted marrow cells with or without the indicated numbers of donor T cells added to the graft. Exp. 1 tested B6C3 donors and CB6 recipients, while Exp. 2 tested bm1 donors and B6.Ly5a recipients. Chimerism was assessed by staining with H-2 class I (Exp. 1) or Ly5 (Exp. 2) allele-specific antibodies. Data indicate the numbers of recipients in each group with $>10\%$ donor granulocytes in the peripheral blood on day 32 (Exp. 1) or day 85 (Exp. 2) after transplantation.

* One of the five recipients in this group died before day 85.
Table 2. In Vivo T Cell Subset Depletion before Transplantation

| Exp. | Day | Antibody Treatment | T cells (mean) | Granulocytes (mean) |
|------|-----|-------------------|----------------|---------------------|
| 1    | 67  | None              | 0,0,0,0,0      | 6,4,3,5             |
|      |     | CD4               | 2,0,0,0,0      | 7,2,1,2,4           |
|      |     | CD8               | 12,5,3,3,2     | 12,5,84,87          |
|      |     | CD4 + CD8         | 68,23,5,76,92  | 47                  |
| 2    | 45  | None              | 3,0,0,0,0,0,0  | 15,11,5,1,10        |
|      |     | CD4               | 1,2,4,2        | 2,2                |
|      |     | CD8               | 53,8,42,50     | 38                  |
|      |     | CD4 + CD8         | 88,88,68,78,72 | 79                  |
| 69   | None| None              | 0,0,0,0,0      | 0,1,0,1,1           |
|      |     | CD4               | 1,0,2,1,0      | 2,2                |
|      |     | CD8               | 1,0,3,6        | 3,3                |
|      |     | CD4 + CD8         | 95,92,89,58,15 | 70                 |

Groups of five CB6 recipients were injected intravenously with the indicated antibodies (50 μg each), exposed to 800 cGy TBI 5 d later, and transplanted on the following day with 5.0 × 10⁶ T cell-depleted B6C3 marrow cells. On the indicated days after transplantation, the percent donor T cells and granulocytes in the peripheral blood of survivors was determined by two-color staining with CD3 and H-2 class I allele–specific antibodies. Data indicate results for individual recipients.

experiment indicate that CD4- and CD8-enriched populations had equivalent abilities to mediate allogeneic marrow graft rejection.

**Donor T Cells Responsible for Marrow Graft Enhancement.** The number of donor T cells required to facilitate engraftment of B6C3 marrow in 800-cGy-irradiated MHC class I and II, and minor antigen-disparate CB6 recipient was determined in a titration experiment. Controls transplanted with T cell-depleted marrow uniformly rejected the grafts (Table 4). In four of five recipients, as few as 5.0 × 10⁴

Table 3. Rejection Mediated by Host CD4 Cells and CD8 Cells

| Host T cells added | Deaths before day 96 | Percent donor cells (day 96) | Total rejections |
|-------------------|----------------------|------------------------------|-----------------|
|                   |                      | T cells | Granulocytes |                      |
| None              | 0                    | 81,79,70,74,83 | 95,88,79,93,89 | 0                 |
| 2.0 × 10⁴ CD4⁺    | 2                    | 90,80,88   | 81,43,89     | 2                 |
| 1.0 × 10⁴ CD4⁺    | 4                    | 81         | 95           | 4                 |
| 5.0 × 10⁴ CD4⁺    | 5                    |            | 5            |                   |
| 2.5 × 10⁵ CD4⁺    | 5                    |            | 5            |                   |
| 2.0 × 10⁵ CD8⁺    | 1                    | 81,85,1,77 | 89,78,1,85   | 2                 |
| 1.0 × 10⁵ CD8⁺    | 1                    | 0,54,15,39 | 1,85,86      | 3                 |
| 5.0 × 10⁵ CD8⁺    | 5                    |            | 5            |                   |
| 2.5 × 10⁶ CD8⁺    | 5                    |            | 5            |                   |

Groups of five irradiated (1,100 cGy) CB6 recipients were transplanted with 5.0 × 10⁶ T cell-depleted B6C3 marrow cells with or without the indicated numbers of host (CB6) T cells added to the graft. The CD4-enriched population used for this experiment contained 95% CD4 cells and 1.5% CD8 cells, while the CD8-enriched population contained 87% CD8 cells and 2.4% CD4 cells. The percent donor T cells and granulocytes in the peripheral blood of recipients surviving on day 96 was determined by two-color staining with CD3 and H-2 class I allele–specific antibodies. Data indicate results for individual recipients. Recipients with <10% donor T cells or granulocytes on day 96 were considered as having rejected the marrow graft. One other experiment also showed that both CD4 and CD8 cells of the host could mediate allogeneic marrow graft rejection.

706 Donor CD8 Cells Prevent Allogeneic Marrow Rejection in Mice
Table 4. Graft-enhancing Effects of Donor T Cells and T Cell Subsets: Donor/Recipient Disparity for MHC Class I and II Antigens and Minor Histocompatibility Antigens

| Donor T cells added | Lymphocytes (mean) | Granulocytes (mean) |
|---------------------|-------------------|---------------------|
| None                | 0,0,0,0,0 (0)     | 2,1,2,2,3 (2)       |
| 0.5 × 10⁶ CD3⁺      | 29,0,0,0,5 (7)    | 83,10,4,24,74 (39)  |
| 2.5 × 10⁶ CD3⁺      | 81,88,86,88,74 (83)| 99,98,99,99,99 (99)|
| 12.5 × 10⁶ CD3⁺     | 70,66,89,79 (76)  | 99,100,99,97 (99)   |
| 0.5 × 10⁶ CD8⁺      | 96,99,99,99,99 (98) | 97,96,94,98,99 (97) |
| 2.5 × 10⁶ CD8⁺      | 88,90,24,81,67 (70)| 99,99,83,99,96 (95)|
| 12.5 × 10⁶ CD8⁺     | 44,49,49,49 (47)  | 82,94,97 (91)       |
| 0.5 × 10⁶ CD4⁺      | 0,0,0,0,0 (0)     | 2,1,2,9,1 (3)       |
| 2.5 × 10⁶ CD4⁺      | 0,0,0,0,0 (0)     | 2,2 (2)             |

Groups of five irradiated (800 cGy) CB6 recipients were transplanted with 5.0 × 10⁶ T cell-depleted B6C3 marrow cells with or without the indicated numbers of donor (B6C3) T cells added to the graft. On day 39 after transplantation, the percent donor lymphocytes and granulocytes in the peripheral blood of survivors was determined by staining with an H-2 class 1 allele-specific antibody. Data indicate results for individual recipients. One other experiment with B6C3 donors and CB6 recipients also showed that donor CD8 cells could facilitate allogeneic marrow engraftment, while CD4 cells could not.

Donor T cells were sufficient to enable engraftment, but these recipients remained mixed myeloid chimeras at 39 d after transplantation. One of these recipients died on day 38, and the others subsequently rejected the grafts and were reconstituted with host T cells and granulocytes. Full myeloid chimerism was seen on day 39 in all recipients when the grafts contained 2.5 × 10⁶ or 1.25 × 10⁶ donor T cells. Despite full myeloid chimerism, host lymphoid cells persisted in these recipients. 6 of the 10 recipients transplanted with grafts containing 2.5 × 10⁶ or 1.25 × 10⁶ donor T cells survived to 117 d after transplantation, and all were reconstituted with >95% donor T cells and granulocytes.

In the same experiment, the effects of donor CD4- and CD8-enriched lymphoid subsets were tested by similar titration. Full myeloid chimerism was seen in all recipients when the grafts contained as few as 5.0 × 10⁴ CD8 cells. Paradoxically, the extent of donor lymphoid chimerism appeared to decrease as the number of donor CD8 cells in the graft was increased. Recipients transplanted with grafts containing 5.0 × 10⁴ donor CD8 cells were reconstituted entirely with donor T cells and granulocytes when retested on days 77 and 117. Those transplanted with grafts containing larger numbers of donor CD8 cells all died by day 71. Donor CD4 cells caused severe GVHD and early deaths (see below). Three of five recipients transplanted with grafts containing 2.5 × 10⁵ donor CD4 cells and all five recipients transplanted with grafts containing 1.25 × 10⁶ donor CD4 cells, died before day 39. None of the recipients surviving after administration of 5.0 × 10⁴ or 2.5 × 10⁵ donor T cells showed detectable numbers of donor granulocytes or lymphocytes. Thus, in this model, donor CD8 cells can facilitate allogeneic marrow engraftment, while donor CD4 cells administered in numbers that did not cause lethal GVHD did not facilitate engraftment.

Acute GVHD appeared to be more severe in recipients transplanted with CD4- or CD8-enriched T cells than in recipients transplanted with unseparated CD3-positive T cells. Recipients transplanted with 1.25 × 10⁶ CD4-enriched cells died between 6 and 9 d after transplantation, whereas the initial deaths in groups transplanted with 1.25 × 10⁶ CD8-enriched cells or unseparated CD3-positive cells did not occur until days 31 and 16, respectively. Recipients transplanted with 1.25 × 10⁶ CD8-enriched cells all died before day 50, whereas four of the five recipients transplanted with 1.25 × 10⁶ unseparated CD3-positive T cells survived beyond 50 d. Weight loss was somewhat greater in the group transplanted with 5.0 × 10⁴ CD8-enriched cells than in the group transplanted with 5.0 × 10⁴ unseparated CD3-positive T cells (Fig. 1). Separate experiments showed that recipients transplanted with 1.25 × 10⁵ CD4 cells had pulmonary vasculitis, acute necrotizing enteritis, and cellular marrows on day 6 after transplantation (pathologic review by Dr. Denny Liggitt, University of Washington). Thus, GVHD rather than graft rejection was the likely cause of death in the experiments described above.

To address the question of whether donor CD4 cells could facilitate engraftment under conditions where GVHD would not represent a limiting factor, engraftment of bm1 marrow was tested in B6.Ly5⁻ recipients prepared with 750-cGy TBI. In this strain combination, both rejection and GVHD are caused by MHC class I disparity. Controls transplanted with T cell-depleted marrow uniformly rejected the grafts (Table 5). In each of five recipients, 2.5 × 10⁵ CD8-enriched donor...
T cells were sufficient to enable engraftment, and few, if any, host T cells or granulocytes remained in these recipients when tested on days 34 and 64 after transplantation. When the experiment was terminated on day 86, there was no overt evidence of GVHD in the recipients transplanted with grafts containing 2.5 \times 10^5 CD8 cells. In contrast, rejection occurred by day 64 in four of five recipients transplanted with grafts containing 2.5 \times 10^5 CD4-enriched donor T cells and in two of five recipients transplanted with 12.5 \times 10^5 CD4-enriched donor T cells. Results of this experiment indicate that the CD8-enriched population was at least fivefold more efficient than the CD4-enriched population for facilitating allogeneic marrow engraftment in this strain combination.

**Discussion**

This study was designed to explore the possible mechanisms by which donor T lymphocytes can facilitate marrow engraftment in allogeneic recipients. In both strain combinations tested, it was possible to define conditions under which engraftment of allogeneic marrow reproducibly depended on the presence of donor T cells in the graft. As might have been expected, the amount of TBI represented a critical determinant of donor engraftment. Differences in sensitivity to irradiation were not strikingly apparent among the strain combinations tested, and single-fraction \(^{60}\)Co exposures in the range of 750–800 cGy delivered at \(\sim 20\) cGy/min were sufficient for engraftment when donor T cells were present in the graft but not when they were absent.

In both humans and in mice, an inverse relationship between the amount of TBI exposure and the frequency of rejection after T cell-depleted marrow transplantation has implicated an active role for host cells in causing rejections (3, 18–20). In mice, rejection has been correlated with the number of clonable T cells remaining after TBI (30). Furthermore, rejection can be prevented by administration of a CD3-specific antibody or immunotoxin before transplantation (31, 32).

### Table 5. Graft-enhancing Effects of Donor T Cells and T Cell Subsets: Donor/Recipient Disparity for MHC Class I Antigens

| Donor T cells added | Percent donor cells (day 64) | Granulocytes (mean) | (mean) |
|---------------------|-----------------------------|---------------------|-------|
| None                | 1,0,0,0                     | 4,1,1,1             | (2)   |
| \(0.5 \times 10^5\) CD8* | 0,0,0,0                     | 2,1,1,1,1           | (1)   |
| \(2.5 \times 10^5\) CD8* | 100,100,100,100,100        | 97,97,99,84,96      | (95)  |
| \(0.5 \times 10^5\) CD4* | 0,0,0,0                     | 1,4,2,1,3           | (2)   |
| \(2.5 \times 10^5\) CD4* | 67,0,0,0,0                 | 82,5,2,1,1         | (18)  |
| \(12.5 \times 10^5\) CD4* | 48,50,44,0,0             | 78,81,47,2,1       | (42)  |

Groups of five irradiated (750 cGy) B6.Ly5 recipients were transplanted with 5.0 \times 10^6 T cell-depleted bm1 marrow cells with or without the indicated numbers of donor (bm1) T cells added to the graft. The CD8-enriched population used for this experiment contained 88% CD8 cells and 0.6% CD4 cells, while the CD4-enriched population contained 95% CD4 cells and 0.7% CD8 cells. On day 64 after transplantation, the percent donor lymphocytes and granulocytes in the peripheral blood of survivors was determined by staining with an Ly5 allele-specific antibody. Data indicate results for individual recipients.
Additional evidence implicating T cells in causing rejection has come from observations demonstrating that T cells in irradiated mice can be sensitized by immunization with allografts (33). A specific role for CD8 cells has been demonstrated by observations that rejection can be overcome by administration of CD8-specific antibody before transplantation (34) and that host CD8 T cell clones specific for MHC class I antigens of the donor can mediate rejection when coadministered with an allogeneic marrow graft (35).

Only limited evidence has previously suggested that host CD4 cells can mediate allogeneic marrow graft rejection in mice. In experiments similar to those described in this paper, it has been shown that allogeneic marrow engraftment in sublethally irradiated (600 cGy) mice can be facilitated by pretreatment administration of both CD4- and CD8-specific antibodies but not by either antibody alone (36). These results demonstrate that failure of donor engraftment after transplantation of T cell–depleted marrow cannot be explained simply by competitive repopulation from radioresistant hematopoietic stem cells of the host. Given the preponderance of evidence implicating CD8 cells in causing rejection both in mice (34, 35) and in humans (11–17), we did not expect to find that CD4 and CD8 cells of B6 mice have equivalent abilities to reject T cell–depleted B6C3 marrow. The CD4-enriched populations tested in our experiments contained >50-fold fewer CD8 cells than the CD8-enriched population. Thus, small numbers of contaminating CD8 cells cannot explain the ability of the CD4-enriched population to mediate marrow graft rejection. Given that pretreatment of recipients with both CD4- and CD8-specific antibodies could prevent rejection, it follows that rejection mediated by host CD4– and CD8-enriched populations cannot be explained by contaminating cells that lack both markers. Therefore, in this strain combination with disparity for MHC class I and II antigens as well as minor histocompatibility antigens, both CD4 and CD8 cells of the host can cause marrow graft rejection, although rejections mediated by CD4 cells occurred earlier than those mediated by an equivalent number of CD8 cells.

Several case reports have described the appearance of host CD8 cells with specific antidonor cytotoxic activity in patients with graft failure after T cell–depleted marrow transplantation (11–17), but only a single case has been described where host CD8 cells with cytotoxic activity specific for donor MHC class II antigens were detected shortly after rejection of a T cell–depleted marrow graft (37). Rejection mediated by CD4 cells might not have been observed if CD4 cells have more sensitivity to irradiation than CD8 cells. Evidence has suggested, however, that human CD4 cells and CD8 cells have equivalent radiation sensitivity as measured in T cell cloning assays (38). An alternative explanation comes from observations that in vitro cytotoxic responses against MHC class II disparities are diminished or preduded when T cells are stimulated simultaneously by MHC class I and II disparities as opposed to stimulation by class II disparity alone (39). The ability of CD4 cells to mediate marrow graft rejection does not necessarily imply that the most primitive hematopoietic stem cells responsible for long-term reconstitution must express MHC class II molecules. The expression of MHC class II antigens by more mature committed hematopoietic progenitors or by accessory cells necessary for hematopoiesis would allow effects functionally indistinguishable from rejection of hematopoietic stem cells.

Using short-term assays of hematopoiesis, Murphy et al. (40) showed that an absence of T cells in murine marrow grafts leads to an increased susceptibility to rejection by host NK cells and T cells. Rejection by either mechanism could be overcome by adding 20 × 10⁶ donor thymocytes to the marrow grafts. Similarly, Lapidot et al. (41) showed that engraftment of MHC-incompatible marrow could be facilitated by as few as 8 × 10⁶ immunocompetent donor thymocytes. The present study showed that lymph node CD8 cells were much more effective than CD4 cells in facilitating durable engraftment of donor hematopoietic cells. While the experiment with bm1 donors and B6.Ly5a recipients suggested that CD4 cells may have limited graft-enhancing activity, it should be noted that the CD4-enriched population used for this experiment contained 0.7% CD8 cells and ~4% double-negative (CD4-“CD8-“) cells. Thus, the 12.5 × 10⁶ CD4-enriched cells that enabled partial engraftment in three of five recipients contained ~0.09 × 10⁵ CD8 cells and 0.5 × 10⁵ double-negative cells. The 2.5 × 10⁵ CD8-enriched cells that enabled full enlargement in all five recipients contained ~11%, or 0.28 × 10⁵, double-negative cells. Although double-negative cells cannot explain the degree of graft facilitation mediated by the CD8-enriched population, they could have contributed to the limited activity seen with the CD4-enriched population. Alternatively, subset interactions could have increased the activity of relatively small numbers of CD8 cells within the CD4-enriched population.

Results from subset analysis in the present study may provide important new insight into the mechanisms responsible for the marrow graft–enhancing effect mediated by donor T cells. Previous in vitro studies (42) have demonstrated that CD4 cells can generate primary T helper responses against MHC class II alloantigens and against MHC class I alloantigens presented by self-MHC class II molecules. CD4 cells can also generate primary T cytotoxic responses against MHC class II alloantigens but not against MHC class I alloantigens. CD8 cells can generate primary T helper responses against MHC class I alloantigens and T cytotoxic responses against both MHC class I and II alloantigens. Although CD8 cells mediate T cytotoxic responses against MHC class II antigens, this activity appears not to be sufficient for a graft-facilitating effect because CD4 cells that also generate T cytotoxic responses against MHC class II antigens were relatively ineffective for enhancing engraftment. Therefore, results of the present study implicate either T helper or T cytotoxic (or both) responses against MHC class I alloantigens in the mechanism(s) responsible for facilitation of allogeneic marrow engraftment. The relative inability of CD4-derived T helps to facilitate engraftment argues against the possibility that CD8-derived T helper responses are sufficient for allogeneic marrow graft facilitation unless it is postulated that this effect is mediated by a cytokine uniquely elaborated by CD8 cells.
Thus, the generation of T cytotoxic responses against MHC class I alloantigens or class I-restricted peptides most likely represents the mechanism that accounts for much of the marrow graft-facilitating effect of donor T cells. This activity may be mediated optimally by dual-function CD8 cells having both T cytotoxic and T helper responses (43).

The host CD4 and CD8 populations that contain the effectors responsible for causing rejection are known to express MHC class I antigens. Therefore, donor CD8 cells may facilitate engraftment simply by recognizing and eliminating the residual host T cells that survive the TBI administered before transplantation. The relative inability of donor CD4 cells to facilitate engraftment in mice may be caused by the absence of MHC class II antigens on murine T lymphocytes (44). Although generation of donor cytotoxic T cells against host class I antigens may represent a highly potent mechanism for enhancement of allogeneic marrow engraftment, mechanisms that do not depend on alloreactivity may also facilitate engraftment. For example, IL-2-activated donor cells can prevent rejection through veto inactivation of host T cells (45). Alternatively, T cells may elaborate cytokines that promote engraftment. Lapidot et al. (46) showed that mature CD8-positive thymocytes from donors genetically tolerant of host alloantigens could accelerate hematopoietic reconstitution during the first 2 wk after transplantation. Statistically significant effects were observed with as few as 1.5 x 10^6 CD8 cells, while similar numbers of CD4 cells had no effect. These experiments were carried out using recipients that had been treated with 8.0 Gy TBI and dimethylmyleran, a regimen that enables durable engraftment of T cell-depleted marrow in nonsensitized allogeneic recipients (47), unlike the pretransplant regimens used in our experiments. Thus, the experiments of Lapidot et al. (46) were not designed to determine whether donor CD8 cells tolerant of host alloantigens can prevent rejection, and this issue remains an open question. As shown in the present study, CD8 cells from nontolerant donors clearly do have this ability.

Data from murine models cannot address whether CD4 cells could facilitate engraftment more effectively in species whose T cells express MHC class II molecules after activation, as is the case in humans (46). To some extent, this question has been addressed in clinical studies. Champlin et al. (47) found that 4 of 36 patients (11%) transplanted with CD8-depleted HLA genotypically identical marrow and given cyclosporine after transplantation had either failure of initial engraftment (n = 3) or late graft failure (n = 1). In their study, 28% of patients developed grades II-IV acute GVHD, suggesting that many donor/recipient pairs had disparity for minor histocompatibility antigens presented by MHC class II molecules. At least five immunologic explanations could have accounted for the graft failures: (a) the patients with graft failure may not have had disparity for minor histocompatibility antigens that are presented by MHC class II molecules on activated T cells; (b) MHC class II molecules on human T lymphocytes may not be able to present minor histocompatibility antigens; (c) donor CD4 cells might not mediate the effector functions necessary to facilitate engraftment through recognition of minor histocompatibility antigens presented by MHC class II molecules on activated host T cells; (d) cyclosporine might have interfered with the ability of donor CD4 cells to facilitate engraftment; or (e) graft failure might have been caused by a population of MHC class II-negative host cells and not by T lymphocytes.

Data from our experiments demonstrate that under highly controlled conditions, it was possible to adjust the number and types of T cells in the graft in a way that allowed reliable donor hematopoietic reconstitution in mice for at least 4 mo while minimizing the severity of acute GVHD. With B6.C3 donors and CB6 recipients, the minimum number of CD3 cells needed to produce durable engraftment (2.5 x 10^6) was also sufficient to cause GVHD that did not show improvement with time after transplantation. The minimum number of CD8 cells needed to produce durable engraftment (5.0 x 10^6) also caused GVHD, but subsequent weight gain in these recipients suggested that GVHD caused by small numbers of CD8 cells in this strain combination may be a self-limited process. Findings were similar in B6.Ly5a recipients transplanted with bm1 marrow containing 2.5 x 10^6 donor CD8 cells. The paradoxical effect of reduced lymphoid chimerism in recipients transplanted with large numbers of donor CD8 cells could reflect an impairment of donor T cell development caused by GVHD-induced thymic atrophy. In humans with variable degrees of unmeasurable disparity for minor histocompatibility antigens and with differences in the extent of pregnancy- and transfusion-induced alloimmunization of the donor and recipient, it seems unlikely that any given number of donor CD8 cells could enable engraftment without GVHD in all patients. Nonetheless, it might be possible to adjust the number and types of donor T cells in the graft in ways that substantially reduce the risk of GVHD without increasing the risk of rejection.

The experiments described in this study were carried out by Tim Axtelle, Len Vanderbosch, and Kelli McIntyre. This manuscript was prepared with assistance from Alison Sell. Drs. E. Donnall Thomas, Suzanne Ildstad, and Susan McCarthy reviewed the manuscript and provided helpful comments.

This research was supported by U.S. Public Health Service grant AI-27951, awarded by the Department of Health and Human Services, National Institutes of Health.
References

1. Korngold, R., and J. Sprent. 1987. T cell subsets and graft-versus-host disease. *Transplantation (Baltimore).* 44:335.

2. Martin, P.J., and N. Kernan. 1990. T cell depletion for GVHD prevention in humans. In *Graft Versus Host Disease: Research and Clinical Management.* S. Burakoff, H.J. Deeg, J. Ferrara, and K. Atkinson, editors. Marcel Dekker, Inc., New York, NY. 371–387.

3. Martin, P.J., J.A. Hansen, B. Torok-Storb, D. Dormam, D. Przezioika, J. O’Quigley, J. Sanders, K.M. Sullivan, R.P. Witherspoon, H.J. Deeg, F.R. Appelbaum, P. Stewart, P. Weiden, K. Doney, C.D. Buckner, R. Clift, R. Storb, and E.D. Thomas. 1988. Graft failure in patients receiving T cell-depleted HLA-identical allogeneic marrow transplants. *Bone Marrow Transplant.* 3:445.

4. Kernan, N.A., C. Bordignon, G. Heller, I. Cunningham, H. Castro-Malaspina, B. Shank, N. Flomenberg, J. Burns, S.Y. Yang, P. Black, N.H. Collins, and R. O’Reilly. 1989. Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: I. Analysis of risk factors and results of secondary transplants. *Blood.* 74:2227.

5. Butturini, A., R.C. Seger, and R.P. Gale. 1986. Recipient immune-competent T-lymphocytes can survive intensive conditioning for bone marrow transplantation. *Blood.* 68:954.

6. Bretagne, S., M. Vidaud, M. Kuentz, C. Cordonnier, T. Henni, G. Vinci, M. Goosens, and J.P. Vernant. 1987. Mixed blood chimerism in T cell-depleted bone marrow transplant recipients: evaluation using DNA polymorphisms. *Blood.* 70:1692.

7. Bertheas, M.E, D. Maraninchi, M. Lafage, J. Fraisse, D. Blaise, A.M. Stoppa, G. Michel, C.P. Brizard, M.H. Gaspard, G. Novakovitch, P. Monmapi, P. Viens, and Y. Carcassone. 1988. Partial chimerism after T-cell depleted allogeneic bone marrow transplantation in leukemic HLA-matched patients: a cyto-genetic documentation. *Blood.* 72:89.

8. Schouten, H.C., W. Sizoo, M.B. Van't Veer, A. Hagenbeek, and B. Lowenberg. 1988. Incomplete chimerism in erythroid, myeloid and B lymphocyte lineage after T cell depleted allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 3:407.

9. Roy, D.C., R. Tantravahi, C. Murray, K. Dear, B. Gorgone, K.C. Anderson, A.S. Freedman, L.M. Nadler, and J. Ritz. 1990. Natural history of mixed chimerism after bone marrow transplantation with CD6-depleted allogeneic marrow: a stable equilibrium. *Blood.* 75:296.

10. Offit, K., J.P. Burns, I. Cunningham, S.C. Jhanwar, P. Black, N.A. Kernan, R.J. O’Reilly, and Chaganti, R.S. 1990. Cyto genetic analysis of chimerism and leukemia relapse in chronic myelogenous leukemia patients after T cell-depleted bone marrow transplantation. *Blood.* 75:1346.

11. Sondel, P.M., J.A. Hank, M.E. Trigg, P.C. Kohler, J.L. Finlay, J. Blank, L. Meisner, W. Borchering, R. Hong, R. Stieves, R. Billing, B. Flynn, and M.J. Bozdech. 1986. Transplantation of HLA-haploidentical T cell-depleted marrow for leukemia: autologous marrow recovery with specific immune sen sitization to donor antigens. *Exp. Hematol. (NY).* 14:278.

12. Bunjes, D., W. Heit, R. Arnold, T. Schmeiser, M. Weisneth, R. Carbonell, R. Porzsolt, A. Raghauacher, and H. Heimpel. 1987. Evidence for the involvement of host-derived OKT8-positive T cells in the rejection of T-depleted, HLA-identical bone marrow grafts. *Transplantation (Baltimore).* 43:501.

13. Kernan, N.A., N. Flomenberg, B. Dupont, and R.J. O’Reilly. 1987. Graft rejection in recipients of T-cell depleted HLA-nonidentical marrow transplants for leukemia. *Transplantation (Baltimore).* 43:842.

14. Sandell, L., G. Johnson, D. Przezioika, and B. Torok-Storb. 1988. Phenotype and function of T-cells associated with marrow graft failure and rejection. In *T-Cell Depletion in Allogeneic Bone-Marrow Transplantation.* M.F. Martelli, Grignani, and Y. Reisner, editors. Ares-Serono Symposia, Rome, Italy, 49–56.

15. Bierer, B.E., S.G. Emerson, J. Antin, R. Maziarz, J.M. Rappeport, B.R. Smith, and S.K. Burakoff. 1988. Regulation of cytotoxic T lymphocyte-mediated graft rejection following bone marrow transplantation. *Transplantation (Baltimore).* 46:835.

16. Bocserman, L.D., C. Murray, T. Takvorian, K.C. Anderson, A.S. Freedman, J. Fitzsimmons, F. Coral, L.M. Nadler, S.F. Schlossman, and J. Ritz. 1989. Mechanism of graft failure in HLA-matched and HLA-mismatched bone marrow transplant recipients. *Bone Marrow Transplant.* 4:239.

17. Bordignon, C., C.A. Keever, T.N. Small, N. Flomenberg, B. Dupont, R.J. O’Reilly, and N.A. Kernan. 1989. Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia. II. In vitro analyses of host effector mechanisms. *Blood.* 74:2237.

18. Patterson, J., H.G. Prentice, M.K. Brenner, M. Gilmore, G. Janossy, K. Ivory, D. Skegg, H. Morgan, J. Lord, H.A. Blacklock, A.V. Hoffbrand, J.F. Apperley, J.M. Goldman, A. Burnett, J. Bribben, M. Alcorn, C. Pearson, I. McVickers, I.M. Hamn, C. Reid, D. Wardle, F.J. Gravett, A. Bacigalupo, and A.G. Robertson. 1986. Graft rejection following HLA matched T lymphocyte depleted bone marrow transplantation. *Br. J. Haematol.* 63:221.
23. Sarmiento, M., A.L. Glasebrook, and F.W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing LYT2 antigen block T cell-mediated cytolysis in the absence of complement. J. Immunol. 125:2665.

24. Young, J.W., and IL.M. Steinman. 1990. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. J. Exp. Med. 171:1315.

25. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. Proc. Natl. Acad. Sci. USA. 84:1374.

26. Ozato, K., N. Mayer, and D.H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. Transplantation (Baltimore). 34:113.

27. Ozato, K., N. Mayer, and D.H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J. Immunol. 124:533.

28. Shen, F.W. 1981. Monoclonal antibodies to mouse lymphocyte differentiation antigens. In Monoclonal Antibodies and T Cell Hybridomas: Perspectives and Technical Advances. G.J. Hammerling, U. Hammerling, and J.F. Kearney, editors. Elsevier Science Publishers B.V. Amsterdam. 25–31.

29. Morse, H.C., F.-W. Shen, and U. Hammerling. 1987. Genetic nomenclature for loci controlling mouse lymphocyte antigens. Immunogenetics. 25:71.

30. Schwartz, E., T. Lapidot, D. Gozes, T.S. Singer, and Y. Reisner. 1987. Abrogation of bone marrow allograft resistance in mice by increased total body irradiation correlates with eradication of host clonal T-cells and alloreactive cytotoxic precursors. J. Immunol. 138:460.

31. Blazar, B.R., R. Hirsch, R.E. Gress, S.F. Carroll, and D.A. Vallera. 1991. In vivo administration of anti-CD3 monoclonal antibodies or immunotoxins in murine recipients of allogeneic T cell-depleted marrow for the promotion of engrafment. J. Immunol. 147:1492.

32. Hiruma, K., R. Hirsch, M. Patchen, J.A. Bluestone, and R.E. Gress. 1992. Effects of anti-CD3 monoclonal antibody on engraftment of T-cell-depleted bone marrow allografts in mice: host T-cell suppression, growth factors, and space. Blood. 79:3050.

33. Dannert, D., C.G. Anderson, and J. Warner. 1985. T killer cells play a role in allogeneic bone marrow graft rejection but not in hybrid resistance. J. Immunol. 135:3729.

34. Murphy, W.J., V. Kumar, and M. Bennett. 1987. Acute rejection of murine bone marrow allografts by natural killer cells and T cells. Differences in kinetics and target antigens recognized. J. Exp. Med. 166:1499.

35. Nakamura, H., and R.E. Gress. 1990. Graft rejection by cytolytic T cells. Transplantation (Baltimore). 49:453.

36. Cobbold, S.P., G. Martin, S. Qin, and H. Waldmann. 1986. Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance. Nature (Lond.). 323:164.

37. Donohue, J., and N.A. Kernan. 1991. Characterization of cells emerging at the time of graft failure following BMT from an unrelated marrow donor. Blood. 78:402a. (Abstr.)

38. Nakamura, N., Y. Kusunoki, and M. Akiyama. 1990. Radio-sensitivity of CD4 or CD8 positive human T lymphocytes by an in vitro colony formation assay. Radiat. Res. 123:224.

39. McCarthy, S.A., and A. Singer. 1986. Recognition of MHC class I alldeterminants regulates the generation of MHC class II-specific CTL. J. Immunol. 137:3087.

40. Murphy, W.J., V. Kumar, J.C. Cope, and M. Bennett. 1990. An absence of T cells in murine bone marrow allografts leads to an increased susceptibility to rejection by natural killer cells and T cells. J. Immunol. 144:3305.

41. Lapidot, T., I. Lubin, A. Terenzi, Y. Faktorowich, P. Erlich, and Y. Reisner. 1990. Enhancement of bone marrow allografts from nude mice into mismatched recipients by T cells void of graft-versus-host activity. Proc. Natl. Acad. Sci. USA. 87:4595.

42. Goldberg, H., T. Mizuochi, S.A. McCarthy, C.A. Cleveland, and A. Singer. 1987. Relationship among function, phenotype, and specificity in primary allospecific T cell populations: identification of phenotype-identical but functionally distinct primary T cell subsets that differ in their recognition of MHC class I and class II alldeterminants. J. Immunol. 138:10.

43. Widmer, M.B., and F.H. Bach. 1981. Antigen driven helper cell independent clonal cytotoxic T lymphocytes. Nature (Lond.). 294:750.

44. Klein, J. 1986. Natural History of the Major Histocompatibility Complex. Wiley-Liss, Inc., New York.

45. Nakamura, H., and R.E. Gress. 1990. Interleukin 2 enhancement of veto suppressor cell function in T-cell-depleted bone marrow in vitro and in vivo. Transplantation (Baltimore). 49:931.

46. Lapidot, T., Y. Faktorowich, I. Lubin, and Y. Reisner. 1992. Enhancement of T-cell-depleted bone marrow allografts in the absence of graft-versus-host disease is mediated by CD8+ CD4− and not by CD8− CD4+ thymocytes. Blood. 80:2406.

47. Lapidot, T., A. Terenzi, T.S. Singer, O. Salomon, and Y. Reisner. 1989. Enhancement by dimethylmyleran of donor type chimerism in murine recipients of bone marrow allografts. Blood. 73:2025.

48. Fu, S.M., N. Chiorazzi, C.Y. Wang, G. Montazeri, H.G. Kunkel, H.S. Ko, and A.B. Gottlieb. 1978. la-bearing T lymphocytes in man. Their identification and role in the generation of allogeneic helper activity. J. Exp. Med. 148:1423.

49. Champlin, R., W. Ho, and J. Gajewski. 1990. Selective depletion of CD8+ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Blood. 76:418.