Immune Responses to Major Histocompatibility Complex Homozygous Lymphoid Cells in Murine F1, Hybrid Recipients: Implications for Transfusion-Associated Graft-Versus-Host Disease

By L.D. Fast, C.R. Valeri, and J.P. Crowley

Graft-versus-host disease (GVHD) is currently encountered after bone marrow transplantation (BMT), solid organ transplantation, or transfusion of leukocyte-containing blood. The GVHD that follows BMT is clinically similar to GVHD that is associated with blood transfusion (TA-GVHD), except that the TA-GVHD is almost invariably fatal within 30 days, whereas BMT-GVHD can often be controlled. Possible factors that may explain the greater intensity of the TA-GVHD response include the immune status of the recipient, the number of lymphoid cells transfused, and the viability of the lymphocytes that depends on the length of time that the blood has been stored. Particular emphasis has been placed on the seeming lack of recipient immune responses in the development of TA-GVHD. All of the early reports of TA-GVHD were in patients who received blood from a donor that was homozygous for one of the recipient's HLA haplotypes. In this situation, the recipient lymphocytes would perceive the donor cells as self and thus would not respond to donor cells, whereas the donor cells would recognize the recipient cells as foreign and generate a response. Therefore, it would be predicted that the only immune response possible in this situation would be a donor-antirecipient response. These findings would indicate that a lack of recipient antidonor response is a critical element in the development of TA-GVHD and may contribute to the severity of TA-GVHD. However, if this is true, why have there been no reported TA-GVHD cases in severely immunodeficient patients with acquired immunodeficiency syndrome? Moreover, theoretical calculations of the frequency of an HLA heterozygous recipient receiving blood from a homozygous donor would indicate that the incidence of TA-GVHD in immunocompetent individuals is much lower than its predicted frequency, implying that a protective host response to donor lymphocytes must exist.

Determination of an explanation for these observations is difficult to study in human patients because of (1) the lack of routine HLA typing for donors and recipients, (2) the variables introduced by the underlying conditions that require a blood transfusion, and (3) the limited ability to manipulate the immune system. A mouse model of GVHD enables the investigator to overcome many of these shortcomings with the expectation that the findings would be applicable to the human patients because of the many similarities of the murine and human immune system. A mouse model of GVHD that mimics the immune conditions in TA-GVHD has been extensively studied in this laboratory.

GVHD is induced by the intravenous injection of homozygous parental spleen cells into unirradiated immunocompetent F1 hybrid recipients. This is analogous to the HLA combination in humans, which appears to be conducive to the development of TA-GVHD. The resulting murine GVHD is similar to TA-GVHD because it induces severe hypoplasia of the lymphohemopoietic system, leading to severe pancytopenia. This murine model was used to assess the role that the recipient immune system plays in the regulating GVHD responses in this donor/recipient combination, and the results indicated that functional recipient CD8+ and NK cells play an important role in preventing GVHD in this donor/recipient combination.
MATERIALS AND METHODS

Mice. C57BL/6, CBA, A, B6AF1, and B6D2F1 mice were obtained from Jackson Laboratory (Bar Harbor, ME). All protocols using mice have been approved by the Rhode Island Hospital Animal Welfare Committee.

Antibodies. The antibodies used in these experiments include 16-13.1 (anti-Ly 2.1; American Type Culture Collection [ATCC], Rockville, MD), 2.43 (rat IgG2b, anti-Ly 2.2; ATCC), 17 GK1.5 (rat IgG2b, anti-L3T4; ATCC), 18 and YTH 89.1.8 (rat IgG2b, antihuman glycophorin; obtained from Dr H. Waldmann; Addenbrooke’s Hospital, Cambridge, UK). Commerically obtained antibodies included rabbit anti-asialo GM1 (986-1001; Wako, Richmond, VA), which is used for in vivo depletion of NK cells. 20,21

In vivo administration of lymphoid cells and other reagents. Lymphoid cells were injected intravenously in the lateral tail vein of recipient mice by injecting the indicated number of donor cells in 0.2 mL phosphate-buffered saline (PBS). Spleen cells were obtained from the recipient mice on day 14 after injection of donor cells for functional testing unless indicated otherwise. In some experiments fluorescent isothiocyanate (FITC)-labeled donor cells were injected. The FITC-labeled cells were prepared by incubating spleen cell suspension in 30 μg FITC (F-7250; Sigma, St Louis, MO)/mL PBS (2 mL per spleen) for 30 minutes at room temperature on a rotating platform. After the incubation, an equal volume of cold mixed lymphocyte culture (MLC) medium was added, and then the cells were centrifuged through a layer of PBS containing 6% bovine serum albumin. The pellet was washed twice in PBS, and then the cells were resuspended in PBS to the appropriate concentration for injection. Some of the recipients had also been injected intraperitoneally (IP) with 1 mL of monoclonal antibody culture supernatant 2 to 5 days or with 100 μL of anti-asialo GM1 (diluted 1:5 with PBS) 1 day before use as recipients. Some of the recipients were injected IP with 200 μg of poly I:C (P-5764; Sigma) in PBS 1 day before injection of donor cells. Poly I:C induces the production of interferon, which activates NK cell activity. 23

Functional assays. Spleen cells obtained from recipient B6D2F1(H-2k) or B6AF1(H-2k) mice previously injected with C57BL/6(H-2b) or A(H-2d) spleen cells were tested for their functional capacity as previously described. 11 The ability of the spleen cells to lyse 105 Cr-labeled P815(H-2b), EL4(H-2d), or YAC (natural killer cell [NK]-sensitive) target cells at the effector-to-target ratios of 150:1, 75:1, 37.5:1, and 19:1 was measured in a 4-hour lysis assay. In some experiments, the number of FITC-labeled donor cells was determined by analyzing lymphocyte samples of recipient spleen, lymph node (cervical, inguinal, and mesenteric) and blood using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA). The gates were set by size on the flow cytometer so that only lymphocytes were analyzed. The number of donor CD8+ cells was determined by staining these samples with phycoerythrin-anti-CD8 (01045A; Pharmingen, San Diego, CA). The gates were set by size on the flow cytometer so that only lymphocytes were analyzed. The number of donor CD8+ cells was determined by staining these samples with phycoerythrin-anti-CD8 (01045A; Pharmingen, San Diego, CA). Because of the potentially low number of donor cells, 75,000 cells were analyzed per sample. In all or some of the experiments described, the levels of Ig production and mitogenic responses were obtained. In all experiments, concordant decrease in Ig production and mitogenic responses was observed when donor-antirecipient cytolytic T lymphocyte (CTL) was detected; thus, only the lysis results have been presented.

Cytokine production by the spleen cells from GVHD mice was measured by culturing 1.25 × 106 cells/mL of MLC medium alone or by including concanavalin A (Con A; 5 μg/mL; C-2010; Sigma) or lipopolysaccharide (LPS; 10 μg/mL; 3923-25; Difco, Detroit, MI) to stimulate cytokine production. Con A stimulates the production of interferon-γ (IFN-γ), and LPS stimulates the production of tumor necrosis factor-α (TNF-α). 24 The supernatants were collected and stored frozen at -20°C until levels of IFN-γ and TNF-α were measured using enzyme-linked immunosorbent assays (ELISAs; 80-2802-00 and 1557-00; Genzyme, Cambridge, MA).

RESULTS

Injection of C57BL/6 spleen cells into B6D2F1 recipients results in an acute form of GVHD. One of the hallmarks of acute GVHD is the presence of CD8+ CTLs of donor origin that specifically lyse target cells expressing recipient major histocompatibility complex (MHC) antigens. 11 In these experiments, the effect of manipulating the recipient was evaluated by determining the number of C57BL/6 splenic donor cells required to generate detectable donor-antirecipient CTLs as a measure of acute GVHD. The first set of experiments compared the effects of depleting recipient CD4+ or CD8+ cells on the development of GVHD. The results indicate that depletion of recipient CD8+ cells permitted fourfold fewer donor cells to generate an acute GVHD response (Fig 1). In contrast, depletion of CD4+ cells resulted in additional donor cells being required to induce an acute GVHD response. NK cells have also been implicated in removal of allogeneic cells. 22 Depletion of NK cells was accomplished by administration of anti-asialo GM1 antisera and was found to result in a twofold reduction of the number of donor cells required to induce GVHD (Fig 2). Consistent with this finding, the presence of increased NK cell activity as a result of poly I:C injection required at least twofold more donor cells to induce a GVHD response (Fig 3).

![Fig 1](https://www.bloodjournal.org/...). The effect of T-cell subset depletion on the number of donor splenocytes required to induce a GVHD response. B6D2F1, recipient mice were injected with anti-CD4 (GK 1.5; 11), anti-CD8 (116-13.1; 11), or control antibody (YTH 89.1.8; 11) and then were injected with the indicated number of C57BL/6 donor spleen cells. The presence of donor anti-recipient CTL was assessed by testing the ability of spleen cells obtained from the recipient mice 14 days after injection to lyse P815 target cells (150:1 effector to target ratio). This data is from a representative experiment of 3 such experiments.

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
The fate of the injected donor cells was studied to determine the mechanisms involved in the regulation of GVHD responses in unirradiated F1 recipients. B6D2F1 recipients that had or had not been previously injected with anti-CD8 antibodies were injected with $25 \times 10^6$ FITC-labeled C57BL/6 cells. This dose of donor cells was used because it had been previously shown to be unable to induce donor-antirecipient CTLs in control recipient mice but could do so in CD8-cell-depleted mice. When the presence of donor cells was examined on day 3 after injection of cells, the results indicated that donor cells and specifically CD8+ donor cells could still be detected in both the control and CD8-cell-depleted mice (Table 1). The inability to deplete donor cells was not caused by a lack of NK cell activity, because these mice showed elevated levels of NK activity (Table 1).

Because production of cytokines such as IFN-γ and TNF-α and IL-4 are critical factors in the development of GVHD, experiments were performed to determine if the disease could be regulated by these cytokines. Treatment of the recipient with anti-CD4 antibody was shown to be ineffective in preventing acute GVHD, indicating that CD4 cells are not the primary producers of these cytokines in the F1 model system.

One possible explanation for the minimum number of donor cells that are required to induce an acute GVHD response is that a certain number of donor cells are lost as a result of nonspecific trapping. If this were the case, addition of carrier cells syngeneic with the recipient should permit fewer number of C57BL/6 donor cells to induce a GVHD response. Addition of $50 \times 10^6$ B6D2F1 cells to varying numbers (10 to $100 \times 10^6$) of C57BL/6 donor cells did not effect the GVHD responses of the C57BL/6 cells (data not shown). Induction of TA-GVHD is often associated with the receipt of a blood transfusion from a donor homozygous for one of the recipient’s MHC haplotypes. One possibility is that the appearance of TA-GVHD in this situation could be facilitated by the immune responses that result from a blood transfusion received simultaneously from a donor that is allogeneic to both the recipient and the homozygous donor. This possibility was assessed by adding from $10$ to $75 \times 10^6$ allogeneic CBA(H-2k) spleen cells to varying numbers of C57BL/6 (H-2b) donor spleen cells and measuring the GVHD response that occurred when these cells were injected into B6D2F1(H-2nd) recipients. The results of seven experiments indicated that only minimal and inconsistent changes were detected in the ability of the C57BL/6 cells to induce a GVHD response in this model system as a result of coinjecting allogeneic donor cells (data not shown).

Hybrid resistance is a phenomenon in which parental donor BM cells are rejected by NK cells found in irradiated F1 recipients. As an approach to determine if hybrid resistance played a role in unirradiated F1 recipients, the ability of varying number of parental donor spleen cells to induce acute GVHD was studied in B6AF1 recipients. This recipient strain was chosen because donor spleen cells from both the C57BL/6 and A parents induce acute GVHD, and because C57BL/6 BM cells are susceptible to hybrid resistance, whereas AJ BM cells are not affected by hybrid resistance. Induction of GVHD with varying numbers of spleen cells from both strains resulted in similar dose curves (Fig 4), indicating that hybrid resistance was not playing a major role in controlling the GVHD responses of donor spleen cells in naive unirradiated F1 recipients.
have been implicated in the pathogenesis of GVHD, the production of TNF and IFN-γ was compared with the development of donor-antirecipient CTLs after the injection of varying numbers of donor lymphoid cells into unirradiated B6D2F1 recipients. TNF-α production and CTL generation were found to be inversely correlated in that there was no TNF-α production in those combinations which generated donor-antirecipient CTLs (Fig 4). In contrast, increased IFN-γ production correlated with increased CTL activity and could still be detected at a donor cell dose that did not induce donor-antirecipient CTLs (25 × 10⁶ cells; see Fig 5).

**DISCUSSION**

TA-GVHD and the acute form of BMT-GVHD manifest similar symptoms except for the more severe pancytopenia that is found in TA-GVHD. Why is TA-GVHD so much more severe, with death occurring in almost all cases within 30 days? One distinction is that the BM cells in the patient with TA-GVHD are of recipient origin, whereas the BM cells in a transplant patient are of donor origin. Thus, the conditions that permit TA-GVHD result in a strong donor-antirecipient response which could also potentially lyse or inhibit BM stem cells in the patient with TA-GVHD but not in the BM transplant patient. The aplasia that results from this response could be a major contributor to the severity of the TA-GVHD. The strong donor-antirecipient responses observed in TA-GVHD could be caused in part by a lack of recipient antidonor responses. Although BMT recipients undergo intensive conditioning before receiving the BM inoculum, there are recipient antidonor responses that can still function. This has been shown by the finding of an increased incidence of graft rejection after transplant of T-cell-depleted BM cells. In this regard, it is interesting that, in most of the cases of TA-GVHD that have been analyzed so far, the HLA heterozygous recipient had received blood from a donor that is homozygous for one of the recipient’s HLA haplotypes. In this combination, a lack of a specific recipient antidonor response would be predicted. This is because T lymphocytes that undergo a negative selection step have been selected not to respond to self MHC molecules. In this combination, T cells would see leukocytes from the donor as self, whereas the donor cells recognize the alloge neic HLA antigens expressed on recipient cells as foreign. Although this combination would be predicted to limit the response to a donor-antirecipient response, it is clear that the frequency of TA-GVHD is much lower than the calculated frequency of which an HLA heterozygous recipient receives blood from a donor homoyzgous for one of the recipient’s HLA haplotypes.

What is the explanation for the discrepancy between the frequency of the donor/recipient combination and the incidence of TA-GVHD? One possible explanation is that, although the recipient lymphocytes are unable to mediate specific immune responses in this donor/recipient combination, they may generate nonspecific responses that are able to regulate the donor cells in this situation. In this study, we have shown that recipient CD8+ cells and NK cells are capable of preventing GVHD responses of donor cells. How can these cells nonspecifically mediate this effect? A recent study has shown that lethally irradiated mice rapidly reject alloge neic or parental BM cells and lymph node T cells. The genetic regulation of rejection and mechanisms involved are quite different for donor BM cells and donor T cells. NK

---

**Table 1. Detection of Donor Lymphoid Cells in B6D2F1 Recipients**

<table>
<thead>
<tr>
<th>Antibody Injected</th>
<th>B6D2F1, Recipients</th>
<th>% Donor Cells in Spleen</th>
<th>NK Cell Activity (LU/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells Injected</td>
<td>All Donor Cells</td>
<td>CD8+ Cells</td>
</tr>
<tr>
<td>YTH 89.1.8 (control antibody)</td>
<td>25 × 10⁶ FITC-C57BL/6 cells</td>
<td>0.34, 0.47</td>
<td>0.03, 0.04</td>
</tr>
<tr>
<td>116-13.1 (anti-Ly2.1)</td>
<td>25 × 10⁶ FITC-C57BL/6 cells</td>
<td>0.30, 0.35</td>
<td>0.03, 0.03</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>&lt;0.01, &lt;0.01</td>
<td>&lt;0.01, &lt;0.01</td>
</tr>
</tbody>
</table>

B6D2F1 recipient mice (2 per group) were injected intraperitoneally with the indicated antibody 2 days before being injected with the indicated cells. Three days after injection of cells, the spleen cells were obtained from the recipient and analyzed on the flow cytometer (75,000 cells per sample) or were tested for NK lysis of yeast artificial chromosome targets. One lytic unit (LU) is the number of effector cells required to achieve 30% lysis. Data shown are from one representative experiment of four experiments.
cells reject BM cells, whereas CD8+ cells are important for rejection of T-cell grafts in most donor/recipient combinations. However, the rejection of C57BL/6 T-cell grafts by irradiated B6D2F1 recipients could be inhibited by depletion of NK1.1+ or CD8+ cells, indicating that both NK and CD8+ cells can play a role in this combination. This is similar to the findings of these studies using unirradiated recipients. The CD8+ cells that play a role in rejection of parental grafts appear to be classical CD8+ cells, which can mediate rejection without requiring a priming period. Thus, the effector cells responsible for the inhibition of donor cell responses depend on the type of donor cells, the strain combinations studied, and whether the recipient mice are irradiated or unirradiated.

Studies on the fate of the donor C57BL/6 cells injected into B6D2F1 recipients found that donor C57BL/6 cells or donor CD8+ cells could be detected for at least 12 days after injection into naive or CD8-cell-depleted recipient mice. The continued appearance of donor cells did not correlate with the appearance of donor-antirecipient CTLs. These results would indicate that recipient CD8+ or NK cells are not effecting donor cell responses by eliminating all donor cells but by possibly selectively eliminating or anergizing the donor cells that react with recipient antigens. B6D2F1 recipient cells acting as veto cells are one possible explanation for these findings. Veto cells are cells that inactivate CTL precursor cells that recognize and bind to the veto cell and have been implicated in the development of transplantation tolerance. The specificity is supplied by the CTL precursor cells, not the veto cells. In the same murine GVHD model used for these experiments, recipient F1 CD8+ cells have been shown to inhibit the donor cytolytic response by acting as veto cells. These B6D2F1 veto cells were found to be radiosensitive, inhibited by anti-CD8, and potentiated by incubation with interleukin-2 (IL-2). The potential role of veto cells will be tested by determining the immune responsiveness of the remaining donor cells. Human CD8+ cells that inhibited donor antihost responses have been identified in posttransfusion responses. These findings also provide an explanation for the lack of reported TA-GVHD after transfusion in patients with acquired immunodeficiency syndrome. Although these patients are immunosuppressed because of a lack of CD4+ cell function, CD8+ cells are still functional in these patients until the advanced stages of the disease.

These results would suggest that procedures or conditions

![Figure 5](https://www.bloodjournal.org/content/112/6/2076/F5)

**Figure 5.** The effect of donor cell dose on donor-antirecipient CTL responses and the production of TNF-α and IFN-γ. B6D2F1 mice were injected with the indicated dose of C57BL/6 spleen cells. Fourteen days later, the spleen cells were obtained from these recipient mice, and the presence of donor-antirecipient CTLs was assessed by testing the ability of spleen cells obtained from the recipient mice 14 days later to lyse P815 target cells (150:1 effector-to-target ratio; C), for the cells ability to produce TNF-α (A) when cultured overnight in medium (■) or with LPS (□), or for IFN-γ production (B) in overnight culture with medium (■) or with Con A (□). This is a representative experiment of three such experiments.
that result in the lack of recipient CD8 and NK cell function would be conducive to the development of TA-GVHD. For example, anesthesia and surgery have been shown to be associated with a depression in cell-mediated immunity. This is manifested by a decrease in T- and NK-cell number, and by depressed delayed-type hypersensitivity responses in vivo. The magnitude of the depression in immune responses is increased as the extent of the surgery increases. The development of TA-GVHD has often been found to be associated with the recipient receiving blood when undergoing major surgery, thus correlating the loss of recipient immune function with increased incidence of TAGVHD.

It has been proposed that the symptoms associated with GVHD are the result of dysregulation of the production of inflammatory cytokines including IL-1, IL-2, and TNF-α. This hypothesis has been supported by the finding that administration of TNF-α generates GVHD lesions and inhibition of TNF reduces the severity of GVHD. Inhibition of IL-1 using IL-1-receptor antagonist also blocked the development of GVHD symptoms. These studies have been performed in murine models of BMT using irradiated recipients. Analysis of the production of cytokine mRNA 14 days after the injection of C57BL/6 spleen cells into unirradiated B6D2F1 recipients detected increased mRNA levels for IL-1α, IL-10, IFN-γ, and macrophage-inhibitory protein-1α and decreased mRNA levels for TNF-β. Unchanged mRNA levels for TNF-α were detected. Thus, our findings of increased IFN-γ correlated with increased levels of mRNA. However, despite no change in mRNA levels, there was decreased production of TNF-α. Additional studies using mice that are selectively unable to produce cytokines such as IFN-γ or TNF-α or to mediate cytolytic activity will help distinguish whether cytokine production or cytolytic activity is important for the development of GVHD.

These findings would suggest that any recipient with impaired CD8 and/or NK cells or who will be undergoing a procedure that inhibits the function of CD8 and/or NK cells would be at an increased risk for developing TA-GVHD. This would be especially true if they also received a blood transfusion from an individual that is homozygous for one of their HLA haplotypes and if the blood contains large numbers of viable lymphocytes. The threshold number of lymphocytes in blood products below which TA-GVHD would not occur is unknown. Therefore, we expect that irradiation of transfused blood in cases in which impaired recipient NK or CD8⁺ cell function has been identified would reduce the risk of developing TA-GVHD.

**ACKNOWLEDGMENT**

We would like to thank Joann Ferland, Jacqui Poore, and Gilbert DiLeone for their excellent technical assistance.

**REFERENCES**

22. Sheng-Tanner X, Miller RG: Correlation between lympho-
Immune responses to major histocompatibility complex homozygous lymphoid cells in murine F1 hybrid recipients: implications for transfusion-associated graft-versus-host disease

LD Fast, CR Valeri and JP Crowley