

# Cellular and Cytokine Effectors of Acute Graft Versus Host Disease

James L.M. Ferrara

*Blood and Marrow Transplant Program, Division of Hematology/Oncology,  
Departments of Internal Medicine and Pediatrics, University of Michigan Cancer Center,  
Ann Arbor, MI, USA*

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## Abstract

Graft-versus-host-disease (GVHD) is the major complication of allogeneic Bone Marrow Transplant (BMT) and Older BMT recipients are at greater risk for acute graft-versus-host-disease. Using well-characterized murine BMT models we have explored the mechanisms of increased GVHD in older recipients. GVHD mortality and morbidity, as well as pathologic and biochemical indices were all worse in old recipients. Donor T cell responses were significantly increased in old recipients both *in vivo* and *in vitro* when stimulated by antigen-presenting cells (APCs) from old mice. In a haploidentical GVHD model, CD4<sup>+</sup> donor T cells mediated more severe GVHD in old mice. We confirmed the role of aged APCs in GVHD using B6D2F1 BM chimeras created with either old or young BM. APCs from these mice also stimulated greater responses from allogeneic cells *in vitro*. We also evaluated whether alloantigen expression on host target epithelium is essential for tissue damage induced by GVHD in mouse models. In bone marrow chimeras recipients in which either MHC II or MHC I alloantigen was expressed only on APCs, we found that acute GVHD does not require alloantigen expression on host target epithelium and that neutralization of tumor necrosis factor-alpha and interleukin-1 prevents acute GVHD. These results suggest new strategies for the prevention and treatment of this toxic complication of BMT.

*Key words:* Bone marrow transplant;Cytokine;T lymphocyte;Gastrointestinal tract;Antigen presenting cells

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Graft Versus Host Diseases (GVHD) is a life threatening complication of allogeneic bone marrow transplantation (BMT), an important therapy for a number of hematologic diseases. In its acute phase, GVHD involves activation of proinflammatory cytokine cascades and immune effector cells that result in target tissue damage [1,2]. Multiple cellular populations and cytokines interact in a complex process that ultimately results in apoptotic injury in target organs (skin, gut, liver) and systemic disease. Shlomchik and colleagues have shown that host-derived antigen presenting cells (APCs) play a key role in the initiation of acute GVHD [3].

Advanced age of the BMT recipient is an important determinant of GVHD severity [4], and the increased risk of GVHD is one factor that often excludes older patients from consideration for allogeneic BMT. We have investigated the mechanisms by which advanced

recipient age increases GVHD in murine BMT models of acute GVHD studies.

## 1. Results

We evaluated the effect of recipient age in well-established murine models of GVHD elicited by both major and minor histocompatibility antigen differences between donors and recipients. Young (2 months), old (14-16 months) and very old (22 months) B6D2F1 (H-2<sup>b/d</sup>) mice were irradiated with 11 Gy TBI and transplanted with BM and T cells as published [5]. We found that recipient age was a continuous variable with respect to GVHD severity. Severe GVHD developed in very old recipients with 100% mortality by day 11. In old mice mortality was slightly but significantly delayed ( $P=0.03$ ), and it was further delayed in young recipients

of allogeneic BMT, 50% of which were alive on day 50 ( $P < 0.01$ ). Similar results were observed in two other donor/recipient strain combinations.

Increased GVHD was also observed in old compared to young recipients after a variety of conditioning regimens, including cyclophosphamide alone. B6D2F1 mice were treated with 100 mg/kg Cytoxan at day -2 and -1 and transplanted with  $5 \times 10^6$  B6 BM cells and  $20 \times 10^6$  B6 spleen cells. Day 40 survival in 16 months old BMT recipients was significantly decreased compared to young mice (0% vs. 83%,  $P = 0.01$ ). Thus, increased GVHD mortality and morbidity in aged recipients was neither strain nor conditioning dependent.

Accelerated GVHD in old mice was confirmed by histopathologic analysis of the small and large intestine in the B6→B6D2F1 model. Acute GVHD histologic features (diffuse apoptosis, lymphocytic infiltrate, brush border loss, mucosal sloughing into the lumen, epithelial degeneration and crypt regeneration) were summed to produce a semiquantitative pathology index as previously described [6]. Damage to the GI tract was twice as severe in older recipients. Serum levels of LPS and TNF were also significantly increased in older recipients, consistent with the pathologic data [5]. Thus by all clinical, pathologic and biochemical indices, acute GVHD was worse in old recipients.

We next analyzed donor T cell expansion in the spleens of BMT recipients four days after transplant. Using B6.Ly5.2 congenic donors, we observed significantly increased number of donor T cells ( $CD4^+$ ,  $CD8^+$ ,  $CD45.1^+$ ) that correlated with advancing recipients age mainly due to the increase of donor  $CD4^+$  T cells. Flow cytometric analysis of intracellular IFN- $\gamma$ , an important cytokine mediator of acute GVHD, showed a twofold increase in the number of IFN- $\gamma$  expressing  $CD4^+$  donor T cells in the spleens of old recipients 5.

To evaluate the role of donor  $CD4^+$  T cells with respect to the severity of GVHD in old recipients, we depleted either  $CD4^+$  or  $CD8^+$  T cells from donor B6 splenic T cells with mAb coupled to magnetic beads. Depletion of  $CD4^+$  T cells from the donor-cell inoculum effectively eliminated GVHD mortality and both young and old recipients of  $CD8^+$  cells survived the entire observation period 5. By contrast, old recipients of  $CD4^+$  T cells experienced significantly greater mortality than young recipients ( $P < 0.001$ ).  $CD4^+$  donor T cells are therefore the principal effectors in acute GVHD severity in this haploidentical mouse model and responsible for the increased GVHD in old recipients.

We next compared the ability of APCs from freshly isolated B6D2F1 splenocytes to stimulate naive B6 responders in vitro. Donor T cells proliferated more rapidly and produced greater amounts of IFN- $\gamma$  and IL-2 to stimulator cells from old animals in two responder/stimulator combinations. We also compared the allostimulatory capacity of BM-derived DCs from young versus old mice and found that DCs from old mice induced greater proliferation of allogeneic T cells compared to DCs from young mice. TNF- $\alpha$  and IL-12, known to play an importance role in augmenting

alloreactive T cell response, were also produced in significantly greater amounts by old DCs after LPS stimulation.

To investigate whether aged APCs were sufficient to induce more severe GVHD, we generated bone marrow chimeras in which cells of hematopoietic lineages were derived from old mice and non-hematopoietic cells (including all epithelial GVHD targets) were from young mice [5]. We hypothesized that if increased age of APCs were the primary cause of increased GVHD severity, then [old→young] chimeras with old APCs should develop more intense disease than [young→young] chimeras despite the identical age of the GVHD target tissues. GVHD was significantly more severe in [old→young] chimeras than in [young→young] chimeras. Analysis of CFSE labeled donor T cell response in vivo at day 3 post BMT confirmed greater  $CD4^+$  donor T cell expansion in response to old APCs in vivo. These data indicating that old APCs are sufficient to induce an increased response from allogeneic donor T cells and cause more severe GVHD irrespective of the age of the recipient target organs.

In our next series of experiments, we evaluated the requirement for MHC alloantigen to be present on host target tissues during GVHD. We first tested the requirement for the expression of MHC class II antigens to stimulate allogeneic  $CD4^+$  T cells in vivo. B6 mice received 13 Gy total body irradiation (TBI) and were transplanted with allogeneic BM and T cells from donor bm12 mice that differ at a single MHC class II allele [7]. Normal B6 recipients experienced 100% mortality from GVHD by day 7, whereas 0% mortality was observed in  $II^{-/-}$  B6 recipients [8]. To test the requirement for the expression of MHC class II on host APCs in GVHD we generated BM chimeras expressing MHC class II on BM-derived APCs but lacking MHC class II on target cells by reconstituting lethally irradiated  $II^{-/-}$  mice with B6 BM cells ([B6→ $II^{-/-}$ ] chimeras). Four months later, flow cytometric analysis of splenic DCs isolated from these animals showed complete replacement of splenic DCs by donor BM cells with MHC class II expression on >95% of  $CD11c^+$  DCs. Identically treated [B6→B6] chimeras were created as controls. [B6→ $II^{-/-}$ ] and [B6→B6] chimeras were transplanted with BM and T cells from bm12 donors after 13 Gy TBI. As expected, GVHD was very severe in [B6→B6] recipients of bm12 T donors: all recipients died at day 5 of BMT in contrast to 90% survival of recipients of syngeneic B6 donors. GVHD was equally severe in [B6→ $II^{-/-}$ ] recipients of bm12 T cells as in [B6→wt] recipients and all died by day 7. GVHD pathology scores of the liver and small intestine in allogeneic [B6→wt] and [B6→ $II^{-/-}$ ] recipients on day 5 post-BMT were significantly greater than those in corresponding syngeneic recipients. Liver histology showed standard histological features of acute GVHD included mild mononuclear cells infiltrates in bile ducts and portal triads, acidophilic bodies, and endothelialitis, which is characteristics of severe GVHD [9]. These changes were, if anything more prominent in [B6→ $II^{-/-}$ ] recipients that

lacked MHC class II expression on target cells. Small intestine histopathology also showed significant changes of GVHD in [B6 $\rightarrow$ II $^{-/-}$ ] recipients of bm12 T cells, including villous atrophy with epithelial apoptosis. Mice receiving 13 Gy TBI and no BM survived more than 10 days (data not shown), ruling out graft failure as a cause of this early mortality as described [10].

## 2. Discussion

These studies demonstrate the central importance of APCs in the pathogenesis of acute GVHD. Previously, the primary hypotheses regarding increased GVHD in elderly recipients involved the altered function of the immune system of old recipients, increased load of bacterial and viral antigens with age, and reduced repair capacity of aged tissues. The process of thymic involution (decreased thymic size and function) that occurs with increasing age has long been thought to be related to increased GVHD in the elderly [11]. Decreased thymic function in aged recipients might amplify acute GVHD by reducing host resistance to donor T cell expansion. This decline in T cell function with age could be considered a type of acquired immunodeficiency that makes older patients more susceptible to a GVH reaction.

A second possibility for more severe GVHD in aged recipients is the increase in viral or bacterial antigenic exposure with age. Clinical acute GVHD is associated with viral infections, particularly CMV, and viral antigens are well known targets for T cell responses [12-15]. This phenomenon is an unlikely explanation for our observations because all recipients had been housed in specific pathogen free conditions their entire lives and all animals tested were negative for the entire panel of infectious pathogens.

A second issue related to exposure of infectious agents relates to bacterial gut flora. Bacterial lipopolysaccharide (LPS) crosses a damaged gut mucosa amplifying systemic GVHD in animal models, and blockade of LPS or selective bowel decontamination can reduce clinical GVHD and improve survival [16-19]. Some clinical studies suggest that bacterial overgrowth of the small bowel increases with age although these studies are small and may suffer from selection bias age [20,21]. Even if such bacterial loading holds possible clinical relevance, the BM chimeras experiments rule out this mechanism as an explanation for more severe GVHD in this experimental model.

Alloantigen expression on host APCs is essential for the activation phase of acute GVHD. The effector phase of GVHD is also believed to be antigen specific and to require alloantigen expression on target cells. Our results using chimeric mice challenge long held assumptions about the antigen specificity of GVHD effector mechanisms: we demonstrate for the first time that the damage to GVHD target organs does not require alloantigen expression on epithelial and nonhematopoietically derived target cells. The lack of requirement for alloantigen expression pertains to both CD4-

dependent and CD8-dependent GVHD, but is particularly true of CD4-dependent GVHD damage that was mediated primarily by cytokines. This antigen-independent effector mechanism may help to explain how CD4 $^{+}$  T cells damage host target epithelium that normally do not express MHC class II molecules. Current paradigms postulate that MHC class II aberrantly expressed in target organs during GVHD renders them vulnerable to attack by CD4 $^{+}$  effector cells [22-24]. Our results suggest that aberrant MHC class II expression is the result of tissue inflammation, probably from systemic IFN- $\gamma$  production.

Effector mechanisms of acute GVHD are thought to include both CTL-mediated and cytokine-mediated cytotoxicity. A second cytolytic pathway during GVHD is an inflammatory cascade generated when mononuclear cells are stimulated to secrete TNF- $\alpha$  and other cytokines by LPS which enters into systemic circulation from damaged gut mucosa [16]. Thus host APCs alone during GVHD can activate the inflammatory cytokine cascade, and mortality and morbidity of GVHD was dramatically suppressed when both TNF- $\alpha$  and IL-1 were neutralized. This reduction of GVHD was due to the suppression of the effector phase rather than the afferent phase of GVHD because cytokine blockade did not suppress donor T cell expansion early after BMT as previously described [25].

Surprisingly, acute GVHD mediated by CD8 $^{+}$  T cells did not require alloantigen expression on target epithelium, although acute GVHD mortality was more rapid when epithelial targets expressed the alloantigen. Neutralization of both TNF- $\alpha$  and IL-1 completely prevented GVHD target organ injury in chimeric recipients lacking alloantigens on target cells. These results demonstrate that CD8-dependent GVHD is mediated by both antigen non-specific cytokines and antigen specific cellular effectors and they confirm the delayed mortality observed in CD8-dependent GVHD models using perforin or granzyme deficient donors [26-28].

In summary, our results demonstrate that for acute GVHD; (1) host APCs are sufficient in both activation and effector phase; (2) alloantigen expression on host target cells is not required, and therefore the effector phase is not antigen specific; (3) inflammatory cytokines mediate mortality and target destruction. These conclusions are true particularly for CD4-mediated acute GVHD but also apply, at least in part, for CD8-mediated disease. Our results have important clinical implications because donor CTLs are important effectors of beneficial graft-versus-leukemia (GVL) effects [29] in patients receiving BMT for hematologic malignancies, and elective blockade of inflammatory cytokines may thus be a strategy to preserve GVL while reducing toxicity of GVHD.

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