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Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation

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Abstract

Purpose—The graft-versus-tumor (GVT) effect is a potent form of immunotherapy against many hematological malignancies and some solid tumors. The beneficial GVT effect after allogeneic bone marrow transplantation (BMT) is tightly linked to its most significant complication, graft-versus-host disease (GVHD). The role of interleukin-6 (IL-6) after allogeneic BMT is not well-understood. This study used a series of complementary knock-out and antibody blockade strategies to analyze the impact of IL-6 in multiple clinically relevant murine models of GVHD and GVT.

Experimental Design—We examined the effect of the source of IL-6 by analyzing the role IL-6 deficiency in donor T cells, donor bone marrow or in host tissues. We confirmed and

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extended the relevance of IL-6 deficiency on GVHD and GVT by treating BMT recipients with anti-mouse IL-6 receptor (IL-6R), MR16-1.

Results—Deficiency of IL-6 in donor T cells led to prolongation of survival. Total inhibition of IL-6 with MR16-1 caused an even greater reduction in GVHD-induced mortality. The reduction in GVHD was independent of the direct effects on T effector cell expansion or donor regulatory T cells. GVT responses were preserved after treatment with MR16-1.

Conclusion—MR16-1 treatment reduced GVHD and preserved sufficient GVT. Tocilizumab, a humanized anti-IL-6R mAb, is approved in several countries including the United States and European Union for the treatment of rheumatoid arthritis and other inflammatory diseases. Blockade of IL-6 with anti-IL-6R mAb therapy may be testable in clinical trials as an adjunct to prevent GVHD in BMT patients without a significant loss of GVT.

Keywords

cytokines; IL-6; BMT; GVHD; GVT

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HSCT) is one of the curative therapeutic options for hematological malignancies and solid tumors(1). The mechanism of tumor eradication in allogeneic HSCT recipients is the immune-mediated graft-versus-tumor (GVT) effect. The host hematopoietic system is reconstituted with donor hematopoietic system and donor lymphocytes, especially T cells that have the potential to exert GVT effects concurrently with graft-versus-host disease (GVHD) (2,3). Clinical data have demonstrated that the severity of GVHD is correlated with chance of relapse (1,4). Acute GVHD is a major side effect of allogeneic HSCT including bone marrow transplantation (BMT)(1,5).

A large number of pro-inflammatory cytokines are involved in the pathophysiology of GVHD. The presence of a pro-inflammatory milieu is attributable to the (a) conditioning regimen, (b) the allo-reaction, and (c) the damage induced in the host tissues as a consequence of them both (5,6). Several inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) are elevated after allogeneic BMT and known to perpetuate GVHD through either direct cytotoxic effects on host tissues (6) or by activation and/or priming of immune effector cells (7). Blockade of these cytokines modulated the severity of GVHD in mice and humans while several lines of experimental evidence suggest that appropriate intervention with cytokine blockade GVT {Hill, 1999 #27401, 8).

IL-6 is known to have broad effects, including maturation and activation of B cells, T cells, and macrophages (9). IL-6 has been directly implicated in many experimental and human inflammatory diseases (9). Blockade of IL-6 signaling with anti-IL-6R mAb demonstrated salutary effects in mouse models of several inflammatory diseases (9,10). More interestingly, blockade of IL-6 with tocilizumab, a humanized monoclonal antibody (mAb) specific for IL-6 receptor (IL-6R), has been shown to have therapeutic effects in many human diseases, such as rheumatoid arthritis, Castleman's disease, and juvenile idiopathic arthritis (9,11–13). Tocilizumab is now approved for clinical use in the United States, the European Union, and other countries (14).

Several lines of correlative data and indirect evidence suggest that IL-6 may be relevant in the induction and severity of GVHD. For example, IL-6 polymorphisms in the recipients of HLA-matched sibling transplants have been found to be associated with acute and chronic

GVHD (15), while serum levels of IL-6 have been found to be elevated in patients with acute GVHD (16). Infusion of exogenous IL-6 has been shown to exacerbate (17), while against the IL-6 receptor (IL-6R) reduced experimental GVHD (18). However, the source of IL-6, whether it plays a direct pathogenic role in GVHD and the impact of its blockade on the critical GVT effects after allogeneic BMT are not known (18–21). In independent and parallel experiments performed in different laboratories across two countries, we utilized a combination of genetic and antibody blockade approaches in multiple strain models to demonstrate that IL-6 plays an important role in the severity of GVHD and that its blockade can ameliorate GVHD without leading to a complete loss of GVT responses. Given the safety and efficacy of IL-6 blockade in the treatment of several immunological diseases in humans (22), our data suggest that targeting IL-6 with tocilizumab might be an immediately testable strategy in humans to mitigate acute GVHD without losing the significant anti-tumor benefits of allogeneic BMT.

MATERIALS and METHODS

Mice

Female C57BL/6 (B6, H-2^b, CD45.2⁺), BALB/c (H-2^d, CD45.2⁺), B6-Ly5.2/Cr (B6-CD45.1, H-2^b, CD45.1⁺), and B6D2F1 (F1, H-2^{b/d}, CD45.2⁺) mice were purchased from Charles River Laboratories (Wilmington, MA) and B6.129S2-*Il6^{tm1Kopf}/J* (IL-6^{-/-}, H-2^b, CD45.2⁺), C3H.SW (H-2^b, CD45.2⁺), and B6(C)-*H2^{bm12}/KhEgJ* (B6-H-2^{bm12}, CD45.2⁺) mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6-background GFP-FoxP3 knock-in (GFP-Foxp3, H-2^b, CD45.2⁺) mice were provided by Dr. Rudensky (University of Washington, Seattle) (23). All animal experiments were performed under the regulations approved by the University Committee on Use and Care of Animals of the University of Michigan and the Queensland Institute of Medical Research.

Tumor

DBA/2 (H-2^d) derived mastocytoma P815 tumor cells and BALB/c (H-2^d) derived plasmacytoma J558 cells were obtained from ATCC and maintained in vitro within 2 weeks for GVT experiments (24,25).

Bone marrow transplantation and antibody treatment

T cells from B6, C3H.SW, BALB/c or GFP-FoxP3 donors were enriched by AutoMACS using CD90-microbeads, Pan T cell isolation kit or CD8 isolation kit (Miltenyi, Auburn, CA). Recipient mice were irradiated (¹³⁷Cs source) with 8–10 Gy total body irradiation (TBI) 1 day before or on the same day as the bone marrow transplant (BMT). Syngeneic or allogeneic T cell-depleted bone marrow (5×10⁶) and isolated T cells were infused through the tail vein. Anti-mouse IL-6R (MR16-1) or control rat IgG (Sigma, St Louis, MO) (0.5 mg/dose) were given to BMT hosts intravenously on day –1 and day 3 of BMT time course (26). Mice were housed in sterilized microisolator cages and maintained on acidified water (pH < 3) for 3 weeks as described previously (25,27). Survival was monitored daily. Clinical GVHD was assessed weekly as described previously (25,27). For GVT experiments, P815 tumor cells (2×10³ cells) (B6D2F1 recipients) or J558 tumor cells (BALB/c recipients, 1×10⁵ cells) were inoculated with bone marrow cells and T cells through the tail vein. Tumor burden was examined by necropsy of tumor-inoculated recipients (28).

In vitro restimulation of donor T cells

Splenocytes from individual animals were harvested 7 days after BMT and plated such that 4 × 10⁵ T cell were present in each well and stimulated with CD3 (145-2C11) at 2 µg/mL. Culture supernatants were collected 18–24 hours later for cytokine analysis.

Flow cytometric analysis

Flow cytometric analysis was performed using FITC-, PE, or APC-conjugated monoclonal antibodies (mAbs) to mouse CD3e (clone:145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD90.2 (53-2.1), CD229.1 (30C7), CD45.1 (A20), CD45.2 (104), and H-2K^b (AF6-88.5) (BD Pharmingen, San Jose, CA). PE-conjugated anti-FoxP3 mAb (clone FJK-16s) was purchased from eBioscience (San Diego, CA). Spleen cells or peripheral blood cells were collected, stained, and analyzed on FACS Vantage SE (Becton Dickinson, San Jose, CA) or C6 (Accuri Cytometers, Ann Arbor, MI) (28,29).

Cytokine analyses with intracytoplasmic staining and ELISA

Cytokine levels of IL-6, IFN- γ , TNF- α , IL-4, IL-10, and IL-5 in sera or culture supernatant cytokines were determined by either cytokine bead array (BD Biosciences, CA) or by ELISA (BD Pharmingen) per manufacturer's instructions (30,31). Intracellular cytokines were determined in the donor CD4 T cell fractions by flow cytometry. Splenocytes from day 7 post-BMT recipients were stimulated with soluble anti-CD3e mAb (2 μ g/ml) and brefeldin A (Biolegend, San Diego, CA) at 1/1000 dilution for 4 hours. Cells were processed for intracellular cytokine staining according to manufacturer's protocol (BD Cytofix/Cytoperm Kit; BD Pharmingen).

Histology

Formalin-preserved gut and liver were embedded in paraffin, cut into 5- μ m thick sections, and stained with haematoxylin and eosin for histologic examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD (10).

Apoptotic cell detection

Irradiated (10 Gy) B6-CD45.1 mouse large intestine was removed, cut and incubated in the K-SFM media (Invitrogen, Carlsbad, CA) with or without 20 ng/ml of recombinant murine IL-6 (Peprotech, Rocky Hill, NJ) for 4 hours (32). Tissue was fixed with 10% formalin and embedded in paraffin. Apoptotic cells were detected using ApopTag, Apoptosis Detection Kit (Millipore, Billerica, MA) according to manufacturer's protocol. Samples were scored using the following criteria, if the apotag positive nuclei were confined to the surface epithelium, or was less than 33% of the total number of nuclei, it was scored as 1+, greater than 33%, but less than 67% immuno-positive nuclei was recorded as 2+. Positive immuno-reactivity in excess of 67% nuclei was scored as 3+.

In vitro T cell proliferation assay

Whole T cells from B6 mice were isolated by positive selection using CD90-microbeads (miltenyi) and used as responder. Irradiated (20 Gy) B6 or B6D2F1 spleen cells were used as stimulator. Responder and stimulator cells were incubated in 96-well round bottom plate. Incorporation of ³H-thymidine (1 μ Ci/well) by proliferating cells was measured during the last 6 hours. To test the effect of exogenous IL-6, recombinant murine IL-6 (Peprotech), was added in the culture at 20 ng/ml concentration. To test the effect of IL-6 blockade, control Rat IgG (Sigma) or MR16-1 were added in the culture at 10 μ g/ml concentration.

Statistical analysis

Survival curves were plotted and compared by log-rank analysis. $P < 0.05$ was considered statistically significant. A paired t test was used to evaluate significant differences between groups in cytokine studies. Data are expressed as mean $\pm$ SEM.

RESULTS

IL-6 levels are higher after MHC mismatched and matched allogeneic BMT

We first determined whether IL-6 production was altered after allogeneic BMT by analyzing serum levels of IL-6. Lethally irradiated B6, F1 or C3H.SW hosts were transplanted with BM and T cells from either syngeneic or MHC mismatched (BALB/c→ B6 and B6-CD45.1→B6D2F1) or matched (B6→C3H.SW) allogeneic donors as in Materials and Methods. Recipient sera were harvested on days 4 and 7 and IL-6 levels were measured by ELISA. Consistent with human observations, allogeneic BMT recipients demonstrated significantly elevated serum levels of IL-6 on days 4 and 7 after BMT (Fig. 1A), while the syngeneic recipients had no detectable levels (data not shown) (15,33–35). When serum IL-6 levels were measured at later time-points (~ 2weeks), they were significantly reduced, almost to baseline, demonstrating that the levels peaked early after BMT (Figure 1A).

Donor T cell-derived IL-6 is involved in GVHD

The elevated levels of IL-6 suggested that it may play an important role in GVHD. IL-6 can be produced by a number of different cells. We therefore first determined whether donor- or host-derived IL-6 is critical for the severity of GVHD. We first examined whether donor derived IL-6 was important. Whole T cells (1×10^6) and TCD BM (5×10^6) from either wild type (WT) B6 or IL-6 KO mice were infused into lethally-irradiated BALB/c mice. As shown in Fig. 1B, survival of the BMT recipients that received BM and T cells from IL-6 KO donors was significantly prolonged compared to WT T cell recipients ($P < 0.05$).

Because donor T cells are major effectors for induction of GVHD (7,36), we next examined whether only the donor T cell-derived IL-6 is critical for the severity of GVHD under similar experimental conditions in the same B6 into BALB/c model of allo-BMT. To this end we infused T cells (1×10^6) from wild type (WT) B6 or IL-6 KO mice with WT B6 TCD bone marrow. As shown in Fig. 1C, the absence of donor-derived IL-6 from T cells also resulted in a similar and significant delay in GVHD mortality ($P < 0.002$) although, eventually, all recipients died from GVHD. These data demonstrated that donor T cell-derived IL-6 contributes to the overall severity of GVHD.

Because IL-6 can modulate the expansion or differentiation of T effector cells (9,10,37) and Foxp3+ T regulatory cells (38), it was possible that the absence of IL-6 secretion by donor T cells could alter their responses to host antigens. We therefore measured multiple parameters of donor T cell function in the spleens of BMT recipients. The overall donor T cell counts in the allogeneic recipients of WT and IL-6^{-/-} T cells were equivalent in CD4 ($0.494 \times 10^6 \pm 0.090$, $n=5$ vs. $0.338 \times 10^6 \pm 0.0187$, $n=5$, $P=NS$) and CD8 (1.528 ± 0.198 , $n=5$ vs. 1.318 ± 0.115 , $n=5$, $P=NS$). We measured serum levels of IFN- γ , IL-17, IL-10, and IL-5 to analyze whether absence of IL-6 secretion by donor T cells skewed their differentiation into Th1, Th17 or Th2 cells. As shown in Figure 2A, when compared to allo-recipients of WT T cells, the animals that received IL-6^{-/-} donor grafts showed similar serum levels of IFN- γ , IL-17A, IL-10, and TNF- α , and a modest increase in IL-5. To further directly demonstrate the impact on T cell differentiation at the single cell level, we measured intracytoplasmic expression of the key effector cytokines in the donor T cells and found no significant differences in the T cells making either IFN γ , IL-4, IL-5, or TNF α but found a modest difference in the number of Th17 cells in the animals that received IL-6^{-/-} donor T cells (Figure 2B). Interestingly, despite the survival advantage seen its absence in the donor-derived T cells and BM, IL-6 was detected, albeit in much lower concentration, in the sera of these BMT recipients, suggesting host-derived source (Figure 2A.) Furthermore, when splenocytes were harvested and restimulated ex vivo, despite a lack of IL-6 secretion by the T cells, there was once again no difference in the secretion of IFN- γ , IL-17A, and TNF-

α (data not shown). These data collectively show that significant and consistent differences in the polarization of donor T cells were not observed.

Consistent with an equivalent increase of donor T cells, no differences were observed in the expansion of donor Foxp3+ T cells in the spleens (Supplemental Fig. 1). Collectively these data show that donor T cell-derived IL-6 plays a significant role in enhancing the severity of GVHD but does not alter donor T cell expansion or the amount of Th1 and Th17 cytokines that have been shown to play pathogenic roles in GVHD.

Given the overall importance of the combined (BM and T cells) donor-derived IL-6 (Fig. 1B), we next analyzed whether only the donor BM-derived IL-6, another potential donor source, is as important as the donor T cell-derived IL-6. We transplanted WT donor T cells with TCD BM from either WT or IL-6^{-/-} donors into allogeneic BALB/c recipients. In contrast to the delay in GVHD mortality when IL-6 was not secreted by either donor T cells or all donor cells, the lack of secretion by only the donor BM-derived cells alone did not alter GVHD severity and mortality (Fig. 3A). Furthermore, in additional mixing experiments the absence of donor BM-derived IL-6 did not provide additional protection when donor T cells were also unable to secrete IL-6 (data not shown).

Because IL-6 can also be secreted by the hosts, in order to examine the role of IL-6 derived from the recipients, we performed BMT utilizing IL-6^{-/-} animals as recipients. To this end we used C3H.SW as donors and IL-6^{-/-} mice as recipients and compared GVHD severity with those in wild type B6 recipients. As shown in Fig. 3B, IL-6^{-/-} recipients did not show different GVHD mortality. To rule out strain-dependent artifacts, similar experiments were performed across MHC mismatched recipients (BALB/c→B6 model) and similar observations were also noted (data not shown).

Inhibition of responses to IL-6 reduces GVHD

In light of significant delay in GVHD mortality when the donor T cells were incapable of producing IL-6 and because of the persistence of IL-6 after BMT even when the donor graft was IL-6^{-/-}, we reasoned that a complete blockade of IL-6 responses might further enhance GVHD protection. In order to test this, we treated recipients with anti-IL-6R mAb (MR16-1) in both MHC mismatched and matched models of GVHD, namely the B6-CD45.1→B6D2F1 (H-2^b→H-2^{b/d}) and B6→C3H.SW GVHD (H-2^b→H-2^b) models. B6-CD45.1 whole T cells (2×10^6) and TCDBM (5×10^6) were infused into lethally-irradiated (10 Gy) B6D2F1 recipients and B6 whole T cells (1×10^6) and TCDBM (5×10^6) were infused into lethally-irradiated (10 Gy) C3H.SW recipients. To examine the effect of IL-6 inhibition in early phase post BMT, recipients were treated with control rat-IgG or MR16-1 on day -1 and Day 3 at 0.5 mg/dose. This dose was chosen because it has been shown to effectively block IL-6 signaling in vivo (26). The schedule was chosen to obtain an adequate systemic level at the time of transplant and up to the period of elevated IL-6 levels after allo-BMT (see above, Fig. 1A). Administration of anti-IL-6R mAb significantly reduced clinical severity of GVHD and enhanced survival when compared with control IgG treated animals in both the model systems, demonstrating a strain independent benefit (Fig. 4A, B). The reduction in GVHD with the administration of anti-IL-6R mAb was also associated with a reduction in the GVHD specific histopathological severity of the target organ, GI tract after allogeneic BMT (Figure 4C).

IL-6 blockade does not alter the T effector to Treg ratio

Because data with IL-6^{-/-} grafts demonstrated equivalent expansion and differentiation of T effector cells (Fig. 2A and B), we next examined the effect of administration of MR16-1 on the expansion of T effectors. We found no statistically significant reduction of

expansion of either CD4⁺ or CD8⁺ alloreactive cytopathic T cells in multiple models (Supplementary Fig. 2A). Since GVHD severity is regulated by the balance between Tregs and allo-reactive cytopathic T cells (39–41), we analyzed the ratio of T effectors (CD4⁺ but Foxp3[−] and CD8⁺) to Tregs (CD4⁺Foxp3⁺Tregs). As shown in Fig. 5A, administration of MR16-1 did not alter the ratio between donor T effector (non-Treg) and Tregs in both MHC mismatched (B6→BALB/c) and matched (C3H.SW→B6). We confirmed similar phenomena in peripheral blood in the GFP-FoxP3→C3H.SW model (supplemental Figure 2B). We found that MR16-1 treatment did not alter the absolute number of CD4⁺FoxP3⁺ cells in recipient spleens.

We further investigated, *in vitro*, whether IL-6 has any direct effect on the expansion of allo-T cells in an MLR. As shown in Supplemental Figure 3, addition of exogenous IL-6 or blockade of IL-6 with MR16-1 did not significantly and consistently alter the expansion of T cells following stimulation with allogeneic APCs. These data collectively show that both *in vivo* and *in vitro*, IL-6 did not directly alter the expansion of alloreactive T cells

In light of these observations, we next tested whether infusion of mature Foxp3⁺ donor Tregs is necessary for reduction of GVHD. We utilized anti-IL6R mAb (MR16-1) in a MHC-matched minor antigen disparate, CD8⁺ driven model, C3H.SW (H2^b) into the B6 (H2^b), of acute GVHD (42). In this model, infusion of donor CD8 T cells alone caused lethal GVHD without co-infusion of CD4 T cells. Groups of syngeneic and allogeneic recipients received 0.5 mg/dose of control rat IgG or MR16-1 on days −1 and 3. C3H.SW CD8 T cells (>98% purity) were infused with T cell-depleted bone marrow cells (5×10⁶) into allogeneic B6 recipients. Administration of MR16-1 markedly reduced mortality after 60 days of observation as shown in Fig. 5B in allo-recipients compared with control antibody treated allo-recipients despite the lack of infusion of mature Foxp3⁺ CD4⁺ donor T cells. Collectively these data show that IL-6 blockade early after allo-BMT reduced GVHD without causing the expansion of the infused mature Tregs and regardless of their infusion at the time of BMT. However, these results do not directly evaluate the impact of either the generation or the functional relevance of donor BM-derived Tregs on GVHD after BMT. Nonetheless, the thymic dependence of this process makes this a very unlikely cause for the protection seen early after BMT following IL-6 neutralization.

Effect of IL-6 on GI epithelium

Because of the lack of direct effect of IL-6 on either the cellular mediators, namely the infused mature donor Tregs and on the expansion or differentiation of alloreactive T cells, or on the inflammatory mediators (INF- γ , TNF α , IL-17 and IL-4) of acute GVHD, we next examined whether IL-6 has direct cytopathic effect on the GVHD target tissues. The intestinal epithelial tissues were harvested as in Materials and Methods and then exposed *ex vivo* to IL-6. The direct toxic effect of IL-6 was analyzed by staining these cells for apoptosis. As shown in Figure 5C, IL-6 caused a significant increase in the degree of apoptosis when compared to diluent treated controls. To further confirm the *in vivo* relevance of these observations, we lethally irradiated naïve B6 animals and treated them with MR16-1 as above. The intestinal tracts were then harvested 24 hours later and analyzed for amount of epithelial apoptosis. Blockade of radiation induced endogenous IL-6 by MR16-1 reduced the amount of intestinal epithelial cell apoptosis (Supplemental Figure 4). Together these data demonstrate that IL-6 has direct toxic effect of GVHD target organ, the intestinal tract.

IL-6 blockade retains sufficient GVT responses

Because donor T cells are required for GVT responses, the maintenance of donor T cell effector responses to host antigens after anti-IL6R mAb treatment suggested that this

approach might preserve at least some of the beneficial GVT activity. To directly examine the impact of IL-6 inhibition on GVT effect, we determined tumor-free survival in the allogeneic and syngeneic recipients after treatment with anti-IL-6R mAb (MR16-1). We utilized syngeneic B6D2F1→B6D2F1 and allogeneic B6-CD45.1→B6D2F1 (H-2^b→H-2^{b/d}) BMT model and infused DBA/2 (H-2^d) derived mastocytoma P815 tumor cells (thus syngeneic to the hosts) to test GVT responses. Recipients were treated with control rat-IgG or MR16-1 on day -1 and day 3 at 0.5 mg/dose. As shown in Fig. 6A, all syngeneic tumor-bearing recipients died from tumor and MR16-1 treatment did not affect their survival, thus ruling out direct anti-tumor effects. In allogeneic BMT recipients without tumor, MR16-1 treatment significantly reduced GVHD-induced mortality (Fig. 6A), as shown in Fig. 4B. Interestingly, MR16-1 treatment also reduced clinical GVHD and caused better survival even in tumor-bearing allogeneic BMT when compared with control rat-IgG treated recipients. However, 2 of the 8 animals treated with MR16-1 but none of the control treated tumor-bearing allogeneic BMT recipients showed evidence of tumor upon necropsy (P=NS). Next, to rule out any tumor or strain dependent effects on the preservation of GVT effects, we next tested the effect of IL-6 blockade with MR16-1 in the B6 into BALB/c model of BMT. We utilized syngeneic BALB/c→BALB/c and allogeneic B6→BALB/c (H-2^b→H-2^d) BMT model and infused plasmacytoma J558 tumor cells, thus syngeneic to the hosts. Recipients were once again treated with either control rat-IgG or MR16-1 as above. All syngeneic tumor-bearing recipients died from tumor regardless of treatment with control and MR16-1 (Figure 6B). By contrast, allogeneic BMT recipients, with or without tumor, that received MR16-1 demonstrated significantly prolonged survival when compared with control treated animals demonstrating that sufficient and significant GVT effects were retained.

DISCUSSION

We demonstrate an important role for IL-6 in the severity of acute GVHD. In parallel experiments performed in two separate labs from two different countries (Hill and Reddy labs) and across multiple clinically relevant murine models of GVHD, we show that (a) IL-6 levels are elevated early after allogeneic BMT, (b) mature donor T cell-derived IL-6 plays a significantly greater role than production from either donor BM or the host tissues, (c) brief inhibition of IL-6 with anti-IL6R mAb after allogeneic BMT reduced acute GVHD by clinical and histologic parameters, and (d) absence of either donor T cell-derived IL-6 or brief blockade with anti-IL6R mAb did not alter T cell activity to host antigens and thus preserved sufficient GVT effects.

The induction of acute GVHD is a direct consequence of donor T cell responses to host alloantigens and the deregulation of pro-inflammatory cytokines (6). The pro-inflammatory cytokines such as IL-1 and TNF- α have been shown to play a direct role in enhancement of GVHD by causing direct damage to gut mucosa and by modulating donor T cell responses (6,7). IL-6, also a known pro-inflammatory cytokine, is elevated after clinical allogeneic BMT (18,33–35). Moreover, IL-6 gene polymorphism studies have shown an association with increased GVHD severity after allogeneic BMT (15). These observations, however, do not address whether IL-6 plays a direct role in causing or amplifying GVHD severity. Furthermore, it is also not known whether the source of IL-6 is critical for GVHD. In the current study, we demonstrate that reduction of IL-6 secretion or its effects by its receptor blockade reduced GVHD across multiple models. IL-6 is produced by numerous cell types and the effects of IL-6 are varied depending on the type of responding cells and whether they are activated directly or *in trans* (9,43). Our data suggest that while donor T cells are the most significant source of IL-6 production for increasing GVHD severity, global blockade of its activity induced significantly better GVHD protection. The presence of equivalent T cell expansion and serum levels of IFN- γ , IL-4, IL-5 and IL-17 suggest that

direct or indirect effects on mature donor T cell expansion and differentiation is not critical in IL-6-induced augmentation of GVHD.

A recent study has shown that blockade of IL-6 increases the numbers of donor Tregs as a direct consequence of peripheral conversion, as well as from the donor BM (18). Our data confirm and extend the observations from that study in demonstrating an important role for GVHD. However, in contrast to the earlier study, we show that a brief duration of IL-6 inhibition did not increase the absolute numbers of mature donor Tregs (18). This could be a consequence of the several key differences between the models, including the dose of radiation, the infusion of unsorted splenocytes (we used purified donor T cell subsets), and the longer duration of the IL-6 blockade (18). In addition, we also found a similar lack of increase in donor mature Tregs despite the reduction in GVHD severity when T cells from IL-6^{-/-} mice were used as donors. It is also important to note that in contrast to Chen et al, we only focused on the role of mature Tregs, and our data do not directly explore the development or the role for donor Tregs generated from peripheral conversion from donor BM (18). Furthermore, Chen et al did not evaluate the impact of IL-6 blockade on GVHD by depletion of mature Tregs from donor inoculums either before after or after BMT (18). By contrast, our data demonstrate that IL-6 inhibition reduces GVHD despite infusion of mature Treg-depleted donor CD8⁺ T cells, demonstrating that infusion of mature Tregs is not obligatory for GVHD protection after inhibition of IL-6. Thus collectively our observations suggest that the GVHD protection from reducing the effects of IL-6 is neither a direct consequence of reducing alloreactive T cell responses nor dependent on infusion of mature Tregs. Because IL-6R signaling can induce cytopathic effects in hepatic or intestinal cells, the epithelial targets of GVHD and given that our data show a direct cytopathic effect of IL-6 on intestinal epithelium, collectively these data suggest that reduction in GVHD is likely a consequence of direct reduction in IL-6 induced inflammation and cytopathic damage of the target tissues (43–47). Our findings extend previous work (17,18) and show that IL-6 blockade attenuates GVHD across multiple models under a variety of experimental BMT conditions.

The toxicity of GVHD is difficult to separate from GVT effects, a well-recognized benefit of immunotherapy for malignancies (1–4). Inflammatory cytokines significantly contribute to the toxicity of GVHD but have a more limited role in the eradication of residual leukemia, which is primarily mediated by donor cytotoxic T lymphocytes and NK cells in BMT experimental models using established tumor cell lines (48). Thus, inhibition of IL-6 leads to disruption of the direct inflammatory effects of IL-6 while maintaining donor T cell functions and thereby successfully preserving sufficient GVT responses in two different strain and tumor models. However, the GVT models have certain limitations(48). For example, the tumor cell lines, despite exhibiting the classic hallmarks (49), may demonstrate variability in subcloning efficiency and outgrowth of dominant clones that likely alter the nature, immunogenicity, and density of relevant antigens (48). Moreover, the immune repertoire of tumors that develop in these models, unlike in humans, is more homogenous and not subjected to immunoediting (50). Nonetheless, such tumor systems have been useful in elucidating the effector mechanisms of immunotherapy and GVT activity (48).

Our findings demonstrate a direct role for IL-6 in the severity of GVHD and show that its brief inhibition early after allo-BMT reduces GVHD while preserving donor T cell responses and meaningful GVT effects. Because anti-IL-6R mAb (tocilizumab) is now FDA approved, and is currently being tested in phase I/II clinical trials in human cancer patients (13,14,22,51), it may soon be testable in allogeneic BMT recipients as an anti-inflammatory adjunct to standard GVHD prophylaxis and treatment. It is likely that inhibition of IL-6 after allogeneic BMT might be particularly attractive for clinical translation and especially

relevant in cancers that are characterized by pathological IL-6 overproduction such as multiple myeloma and certain lymphomas (22,52).

Translational Relevance

The graft-versus-tumor (GVT) effect after allogeneic bone marrow transplantation (BMT) can potentially cure many hematopoietic malignancies. Its applicability has been limited by its major complication, graft-versus-host disease (GVHD). In this study, we show the relevance of cytokine IL-6 and its blockade with anti-IL-6R monoclonal antibody (mAb) on experimental GVT and GVHD in multiple clinically relevant models of BMT. Our data, consistent with clinical observations, demonstrate elevation of serum levels of IL-6 in the hosts after allogeneic bone marrow transplantation (BMT). Donor T cell-derived IL-6 contributed to GVHD severity. Inhibition of IL-6 with anti-IL-6R mAb caused reduction of GVHD but preserved sufficient GVT effects. Importantly, tocilizumab, a humanized anti-IL-6R mAb, is now approved for use in the United States, European Union, and some other countries. Our data indicate that inhibition of IL-6 with anti-IL-6R mAb may soon be testable in allogeneic BMT recipients as an adjunct to standard GVHD prophylaxis and treatment, which is an important step toward more effective treatment for these patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IL-6 elevation after BMT and involvement of donor IL-6 in GVHD

(A) B6 (H-2^b) recipients were irradiated (10 Gy) on day -1 and transplanted with BALB/c(H-2^d) donor 5×10^6 T cell-depleted bone marrow (TCDBM) and 2×10^6 whole T cells (top left). B6D2F1 (H-2^{b/d}) recipients were irradiated (10 Gy) on day -1 and transplanted with B6 5×10^6 TCDBM and 2×10^6 whole T cells (top, middle). C3H.SW recipients were irradiated (10 Gy) on day -1 and infused with B6 5×10^6 TCDBM and 1×10^6 whole T cells (top, right). Sera were collected from recipients (4-5 recipients/group) on day 4, 7 and 16. IL-6 levels of each sample were measured by ELISA.

(B) BALB/c recipients were irradiated (9 Gy) on day 0 and received 10×10^6 B6 TCDBM (◆, $n=8$), 10×10^6 wild type (WT) B6 TCDBM and 5×10^5 WT B6 whole T cells (■, $n=17$) or 10×10^6 IL-6^{-/-} B6 TCDBM and 5×10^5 IL-6^{-/-} whole T cells (○, $n=17$). Survival of the recipients was monitored daily. ■ vs. ○ $P < 0.05$ (C) BALB/c recipients were irradiated (8 Gy) on day -1 and received 5×10^6 syngeneic TCDBM (●, $n=15$), 5×10^6 wild type (WT) B6 TCDBM and 1×10^6 WT B6 whole T cells (■, $n=23$) or 5×10^6 WT B6 TCDBM and 1×10^6 IL-6^{-/-} whole T cells (○, $n=23$). Survival of the recipients was monitored daily. ■ vs. ○ $P=0.0013$.

Figure 2. Serum cytokine levels in BMT recipients

(A). BALB/c recipients were irradiated (9 Gy) on day 0 and received 10×10^6 B6 TCDBM (■, $n=6$), 10×10^6 wild type (WT) B6 TCDBM and 5×10^5 WT B6 whole T cells (■, $n=12-20$) or 10×10^6 IL-6 $^{-/-}$ B6 TCDBM and 5×10^5 IL-6 $^{-/-}$ whole T cells (□, $n=12-20$). Sera were collected from recipients on day 7. Cytokines were determined by cytokine bead array. **(B).** Splenocytes from day 7 recipient (□, WT B6 donor, ■ IL-6 $^{-/-}$ B6 donor, $n=20$ respectively) were stimulated with soluble anti-CD3 mAb (2 μ g/ml) in the presence of brefeldin A (1/1000 dilution; BioLegend) for 4 hours. Cells were processed for intracellular cytokine staining (ICC), per the manufacturer's protocol (BD Cytotfix/Cytoperm Kit; BD Pharmingen). Representative plots of cytokine expression gated on donor (H-2K b -positive) CD4 T cells were shown.

A **B**

Figure 3. Donor bone marrow or host-derived IL-6 is not involved in GVHD

(A) BALB/c recipients were irradiated (9 Gy) on day 0 and received 10×10^6 B6 TCDBM (◆, $n=6$), 10×10^6 wild type (WT) B6 TCDBM and 5×10^5 WT B6 whole T cells (■, $n=12$) or 10×10^6 IL-6^{-/-} B6 TCDBM and 5×10^5 WT B6 whole T cells (◇, $n=12$). Survival of the recipients was monitored daily.

(B) B6 recipients were irradiated (10 Gy) on day -1 and received syngeneic donor B6 5×10^6 TCDBM (●, $n=12$), or allogeneic donor C3H.SW 5×10^6 TCDBM and 2×10^5 CD8 T cells (■, $n=12$). IL-6^{-/-} recipients were irradiated (10 Gy) on day -1 and received donor C3H.SW 5×10^6 TCDBM and 2×10^5 CD8 T cells (○, $n=14$). Survival of the recipients was monitored daily.

Figure 4. Anti-IL-6R mAb treatment suppresses GVHD

(A) C3H.SW (H-2^b) recipients were irradiated (10 Gy) on day -1 and injected with either syngeneic C3H.SW 5×10^6 TCDBM and 1×10^6 whole T cells were infused (●) or allogeneic B6 (H-2^b) 5×10^6 T TCDBM and 1×10^6 whole T cells were infused. Allogeneic BMT recipients were treated with control rat IgG on day -1 and 3 at 0.5 mg/dose (■, $n=12$) or with MR16-1 on day -1 and 3 at 0.5 mg/dose (○, $n=9$). GVHD clinical score was monitored weekly and survival of the recipients was monitored daily. ■ vs. ○ (survival $P=0.0235$ and clinical score $P<0.05$)

(B) B6D2F1 (H-2^{b/d}) recipients were irradiated (10 Gy) on day -1 and injected with either syngeneic B6D2F1 5×10^6 TCDBM and 2×10^6 whole T cells were infused (●, $n=6$). or allogeneic B6-CD45.1 (H-2^b) 5×10^6 T TCDBM and 2×10^6 whole T cells were infused. Allogeneic BMT recipients were treated with control rat IgG on day -1 and 3 at 0.5 mg/dose (■, $n=12$) or with MR16-1 on day -1 and 3 at 0.5 mg/dose (○, $n=12$). GVHD clinical scores were monitored weekly and survival was monitored daily. ■ vs. ○ $P=0.0139$ (survival) and <0.05 (clinical score)

(C) GVHD specific pathology on day +7: Histopathological score of GVHD severity was analyzed in from the recipients of in B6→B6D2F1 model 7 days post-BMT.

Figure 5. Effect of Anti-IL-6R mAb treatment on regulatory T cells and GVHD

(A) BALB/c mice were irradiated (8 Gy) on day -1 and received 5×10^6 B6 TCDBM 5×10^5 CD90 T cells. Recipients were treated with anti-IL-6R (MR16-1) or control rat IgG on day -1 and 3 (0.5 mg/dose) intravenously. On day 6 and 10 post-BMT, spleen cells were harvested, stained with anti-H-2K^b, CD4, CD8a and FoxP3 mAbs and analyzed by flow cytometry. H-2K^b-positive cells were defined as donor derived cells. (left). B6-CD45.1 mice were irradiated (10 Gy) on day -1 and received C3H.SW 5×10^6 TCDBM 1×10^6 whole T cells. Recipients were treated with anti-IL-6R (MR16-1) or control rat IgG on day -1 and 3 (0.5 mg/dose) intravenously. On day 5 and 29 post-BMT, spleen cells were harvested, stained with anti-CD229.1, CD4, CD8a and FoxP3 mAbs and analyzed by flow cytometry. CD229.1-positive cells were defined as donor derived cells. (right).

(B) WT B6 recipients were irradiated (10 Gy) on day -1 and received syngeneic B6 5×10^6 TCDBM (●, $n=15$). Allogeneic C3H.SW (H-2^b) 5×10^6 TCDBM and 2×10^5 CD8 T cells were infused. Allogeneic BMT recipients were treated with control rat IgG on day -1 and 3 at 0.5 mg/dose (■, $n=18$) or with MR16-1 on day -1 and 3 at 0.5 mg/dose (○, $n=18$). Survival of the recipients was monitored daily. ■ vs. ○ $P=0.0075$.

(C) B6-CD45.1 mice were irradiated (10 Gy) and large intestines were harvested. Tissue was cut and incubated in the K-SFM media with or without of recombinant murine IL-6 (20

ng/ml) for 4h. Tissue was fixed with 10% formalin, embedded in paraffin. Paraffin-sections were stained using ApopTag Apoptosis Detection Kit. Apoptosis of epithelial cells were scored as 0~3+. Typical images of apoptosis score 1+, 3+ are shown. Bar graph shows mean $\pm$ SEM (n=4)

Figure 6. Anti-IL-6R treatment preserves GVT effect

(A) B6D2F1 recipients were irradiated (10 Gy) on day -1 and transplanted with syngeneic B6D2F1 5×10^6 TCDBM and 2×10^6 whole T cells (●, $n=6$) along with or without 2×10^3 P815 cells. Syngeneic recipients were treated with control rat IgG on day -1 and 3 at 0.5 mg/dose (▲, $n=7$) or with MR16-1 on day -1 and 3 at 0.5 mg/dose (▼, $n=8$). Survival of the recipients was monitored daily (top left). B6D2F1 recipients were irradiated and treated with either control IgG-(◆ and ■, $n=7$) or MR16-1 (◇ and ○, $n=8$) and injected with or without P815 tumor cells as above. All of the animals received allogeneic B6-CD45.1 5×10^6 TCDBM and 2×10^6 whole T cells (top right). Survival of the recipients was monitored daily. ■ vs. ○ $P=0.0319$, ◆ vs. ◇ $P=0.0287$.

(B) BALB/c animals were irradiated (8 Gy, day -1) and received syngeneic BALB/c 5×10^6 TCDBM and 5×10^5 whole T cells along with or without J558 tumor cells (●, $n=13$). The animals were treated with either control rat IgG on day -1 and 3 at 0.5 mg/dose (▲, $n=7$) or with MR16-1 on day -1 and 3 at 0.5 mg/dose (▼, $n=8$). Survival was monitored daily (bottom, left). ■ vs. ○ $P=0.9816$. BALB/c animals were irradiated and treated with either rat IgG-treated (◆, day-1 and 3 at 0.5 mg/dose, $n=14$) or MR16-1-treated (◇, day-1 and 3 at 0.5 mg/dose, $n=14$) as above and were infused with allogeneic B6 5×10^6 TCDBM and 5×10^5 whole T cells with or without 1×10^5 J558 cells. Survival was monitored daily (bottom). ◆ vs. ◇ $P=0.0285$ (bottom right).