Immunobiology of Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract
Allogeneic hematopoietic stem cell transplantation (HSCT) has evolved into an effective adoptive cellular immunotherapy for the treatment of a number of cancers. The immunobiology of allogeneic HSCT is unique in transplantation in that it involves potential immune recognition and attack between both donor and host. Much of the immunobiology of allogeneic HSCT has been gleaned from preclinical models and correlation with clinical observations. We review our current understanding of some of the issues that affect the success of this therapy, including host-versus-graft (HVG) reactions, graft-versus-host disease (GVHD), graft-versus-tumor (GVT) activity, and restoration of functional immunity to prevent transplant-related opportunistic infections. We also review new strategies to optimize the GVT and improve overall immune function while reducing GVHD and graft rejection.

Key Words
graft-versus-host disease, graft rejection, immune reconstitution
INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) developed from research to treat the sequelae of radiation exposure, which became a great concern with the start of the nuclear age and the Cold War. Lorenz and colleagues (1, 2) demonstrated that transfusion of bone marrow (BM) after lethal radiation exposure could cure mice of radiation sickness. Using myeloablative doses of radiation and BM rescue, it became possible to treat experimental tumors, resulting in prolonged tumor-free survival. In these early studies, researchers showed that BM from either genetically identical or disparate donors can rescue mice from radiation aplasia; however, transplantation of allogeneic BM was also found to cause a lethal secondary or runting disease, defined by wasting, diarrhea, and skin lesions (3). This disease is now commonly known as graft-versus-host disease (GVHD) and develops when immunocompetent cells of the graft recognize histocompatibility antigens and mount an immune attack against the cells in the recipient. In contrast, immunocompetent cells remaining in the recipient following irradiation can mount an immune attack against the graft, resulting in graft rejection or failure, known as host-versus-graft (HVG) reaction.

The discovery of human histocompatibility antigens in 1958, followed by the development of immunosuppressive drugs for the prevention of GVHD, paved the way for the successful use of allogeneic HSCT as a curative modality for both neoplastic and non-neoplastic disease states. Despite this accomplishment, cancer relapse, GVHD, graft rejection, and opportunistic infections due to prolonged immunodeficiency following the transplant remain the major complications limiting the efficacy of allogeneic HSCT.

CLINICAL CONSIDERATIONS OF ALLOGENEIC HSCT

Hematopoietic Stem Cell Sources for Allogeneic HSCT

BM is a rich source of hematopoietic stem cells (HSCs) and was virtually the sole source of donor cells for HSCT until the 1990s. The successful use of peripheral blood (PB) as a source of HSCs and hematopoietic reconstitution in dogs occurred in 1979 (4), but not until the mid-1990s, in part because of the success of mobilization protocols for hematopoietic precursors into the PB (using the cytokines G-CSF or GM-CSF) for autologous HSCT, was mobilized PB used as a source of allogeneic HSCs (5). Since the Schmitz et al. (5) report, the use of PB cell products for allogeneic HSCT has increased dramatically (6). Advantages of mobilized PB for HSCT include faster neutrophil and platelet recovery.

Umbilical cord blood (UCB), which is also a rich source of HSCs, has also been investigated as an attractive alternate source of HSCs for transplantation. The first report for the use of UCB as a source of HSCs for pediatric allogeneic HSCT for leukemia was reported in 1995 (7). Because of the limited size of the graft product, the first transplants were restricted to children. However, Laughlin et al. (8) reported in 2004 the successful transplants of a large series of adults with UCB grafts from unrelated donors. To increase the utility of UCB as a source of HSC grafts for adults, the use of two unrelated, partially matched UCB products has been examined and has been shown to provide a sufficient number of hematopoietic progenitor cells to engraft adults (9).

Mobilized PB and UCB products offer unique immunotherapeutic benefits. Despite the presence of up to a log more CD3+ T cells in a mobilized PB graft than in a BM graft, the incidence and severity of acute GVHD are not increased (10). The difference in the
ability of T cells in BM and PB grafts to induce GVHD appears to be due, at least in part, to the mobilization methods that also polarize the cells in the graft to a Th2/DC2 phenotype. The immunologically naive status of UCB T cells appears also to provide unique properties to this graft product (11), and, indeed, the incidence and severity of GVHD associated with this source of donor cells in transplants of HLA-matched sibling are reduced compared with historically matched, HLA-matched sibling BM transplants (12). However, the delayed immune reconstitution with UCB grafts compared with other HSC sources remains a significant barrier. Thus, HSCT has evolved such that BM, PB, and UCB can each be used as HSC sources but appear to have significant biological differences affecting outcome. Importantly, the vast majority of preclinical studies in mice solely use BM as a source of HSCs, leading to a greater reliance on clinical studies to determine the biology of PB and UCB transplants.

**Conditioning, Graft Manipulation, and Post-Transplant Protocols for Allogeneic HSCT**

The conditioning or preparative regimen to the recipient is essential for the success of the transplant. The principal aim of an effective protocol is (a) to eliminate or suppress host immunity to prevent rejection of the graft, (b) to eliminate malignant cells, (c) to minimize GVHD without jeopardizing engraftment or graft-versus-tumor (GVT) effects, and (d) to minimize toxicity to other tissues. The strategies employed to obtain these goals fall into three categories:

1. Preparative (conditioning) treatment of the recipient prior to the infusion of the graft cells to permit engraftment,
2. manipulation of the graft to minimize GVHD, and
3. post-transplant immunosuppressive treatment of the recipient to prevent graft rejection and GVHD.

High-dose myeloablative conditioning typically includes one or more cytotoxic drugs and may contain whole-body irradiation. Increasing the intensity of host conditioning reduces the immunocompetent cells in the recipient, permitting transplantation of even unrelated, mismatched BM (13), but it can also lead to markedly increased toxicities (14). Reduced intensity conditioning (RIC) uses lower doses of the cytoreductive treatments and incorporates more directed immunosuppressive agents, such as the purine analog fludarabine, to provide antitumor activity (15) and to suppress host immune attack (16). RIC regimens may incorporate the use of total lymphoid irradiation and/or administration of T cell–depleting antibodies and are designed specifically to delete (17) and/or suppress immunocompetent cells in the host (reviewed in 18). Importantly, by reducing toxicities with RIC, older patients who cannot as readily tolerate the toxicities associated with conventional cytoreductive conditioning regimen side effects can be more safely transplanted (19) without a compromise in overall survival (20).

Unfractionated BM, PB, or UCB cells are typically given in HLA-matched related transplants. Donor T cells may be removed to reduce the frequency and/or severity of GVHD in HLA-disparate or in matched, unrelated BM or PB transplants, although the risk of graft rejection, viral infection, and tumor relapse increases in certain situations compared with the infusion of T-replete grafts (21). Escalation of the number of HSCs (megadose) infused decreases rejection despite the use of vigorously T cell–depleted (22) or CD34+–selected HSC grafts (23, 24), which is thought to be effective, at least in part, by tolerizing the recipient to the engrafting cells (25).

Post-transplant prophylaxis for GVHD is given in most transplant protocols using T-replete grafts. Commonly used pharmacological agents include (a) the calcineurin inhibitors cyclosporin A (CsA) and tacrolimus (FK506), which block interleukin (IL)-2

**HLA:** human leukocyte antigen  
**GVT:** graft-versus-tumor  
**RIC:** reduced intensity conditioning
Treg cell: T regulatory cell
MHC: major histocompatibility complex
MiHA: minor histocompatibility antigen
MUD: matched unrelated donor

production and T cell expansion (26); (b) rapamycin, an inhibitor of cytokine responsiveness that may act in part by expanding T regulatory (Treg) cells (27, 28) and by inducing conventional T cells to acquire regulatory cell function (29); and (c) a lympholytic agent such as corticosteroids, alone or in combination. Other methods of GVHD prophylaxis include in vivo depletion of T cells with antithymocyte globulin (ATG), which has been in clinical use for decades, and the humanized antibody against human CD52 (CAMPATH-1H) to control GVHD and graft rejection (30, 31).

Histocompatibility Antigen Testing in HSCT

Siblings have a 25% chance of being matched at all human major histocompatibility complex (MHC, or HLA) loci, whereas all family members (parent and sibling) usually share one HLA haplotype with the patient but can differ in 0 to 3 loci at HLA-A, -B, and -DR on the second haplotype. Owing to the contribution of minor histocompatibility antigens (MiHAs), encoded outside of the MHC locus, to GVHD generation, the risk of GVHD is increased in recipients of matched unrelated donors (MUD) compared with related HLA-identical donor grafts. The noted exception is in transplants of related HLA-identical donor BM and UCB grafts in which the risk for severe GVHD was lower for recipients of UCB despite the greater number of MiHA disparities with the recipient than would be the case using HLA-identical related BM grafts (12, 32). MiHAs have also been identified as targets for GVT activity (33, 34).

There has recently been much interest in human killer cell immunoglobulin-like receptors (KIR), which are expressed on natural killer (NK) cells as well as on subpopulations of T cells. The gene complex is located on human chromosome 19q13.4 and thus is inherited independently of HLA on chromosome 6. KIRs are a family of inhibitory and activating receptors that specifically recognize HLA-A, -B, and -C alleles (reviewed in 35). The balance of activating and inhibitory signals derived from KIRs and other NK cell receptors together regulates NK cell activation and can therefore affect GVHD/GVT (36). The absence of a KIR ligand on target cells can result in the recognition and subsequent attack by cells bearing the corresponding KIR. Similar to HLA mismatches, the direction of KIR/KIR ligand incompatibility appears to impact outcomes in graft rejection, GVHD, and GVT in some (36) but not all (37, 38) clinical studies; the reasons for the differences are being intensively investigated. Not all HLA typing protocols include HLA-C; thus, KIR/KIR ligand mismatches may play an underappreciated role in transplantation, even in transplants designated as HLA-identical on the basis of HLA-A and -B typing (39).

PRECLINICAL MODELS FOR THE STUDY OF ALLOGENEIC HSCT

Much of our understanding of the biology of GVHD has developed from work largely using two preclinical animal models, the mouse and the dog. As an outbred large animal model, the dog has been invaluable in the development of transplant protocols, including preclinical testing of conditioning regimens (13, 40), prophylaxis and treatment of GVHD (41–43), and understanding of the pathology of GVHD (44, 45). The inbred mouse model has been used as the basis for much of our knowledge of the immunological mechanisms of GVHD and GVT. However, there are significant species differences between humans and mice to consider. The differences include, but are not limited to, significant variation in the ratios between leukocyte subsets, NK cell receptors used, expression of Toll-like receptors, costimulatory molecules, cytokines, and chemokine production/function (46). In addition, there are important caveats to consider when drawing conclusions from studies with animal models before correlating them to the
Clinical allogeneic HSCT scenario. Some of these issues also apply when attempting to draw broad conclusions from clinical studies. These variables may include the following:

1. The presence, intensity, and type of conditioning regimen used to prepare the recipient: In murine GVHD studies, irradiation alone is typically used, but it can range from no conditioning for the adoptive transfer of large numbers of allogeneic T cells to myeloablative doses of whole-body irradiation followed by HSC rescue. Compared with immunosuppressive/nonmyeloablative conditioning, the use of high-dose cytoreductive conditioning can lower host resistance (47), induce potent proinflammatory mediators necessary for T cell recruitment (48), and induce tissue damage, and can be one of the most critical factors to consider when drawing conclusions from allogeneic HSCT studies performed on mice or humans.

2. The immunological disparity between donor and recipient: This disparity can affect the kinetics and pathophysiology of GVHD and GVT. A diverse array of mouse strain combinations is used, resulting in a variety of MHC- and/or MiHA-disparate models. Disparity can range from full MHC and MiHA mismatch, to only multiple MiHA mismatch, to single MHC allele mismatch and semiallogeneic (parent into F1). These different strain combinations have different Th1/Th2 as well as Treg content and can sway the dominance of CD4+ or CD8+ T cell effectors in GVHD. In addition, other genetic differences outside of MHC and MiHA differences in the strains can strongly influence GVHD. In a striking example, the infusion of donor spleen cells from the other parent, C37BL/6, induces acute GVHD (49).

3. The tissue source, purity, and number of donor immune cells infused: Typically spleen cells and/or lymph node cells are added to the BM graft to provide a sufficient dose of T cells for the induction of GVHD in most murine strain combinations. T cells taken from different tissues can have distinct homing characteristics, and the ratio of T cell subsets, NKT subsets, and NK cells can differ between the tissues, all of which can affect the induction of GVHD or GVT. The presence of nonlymphoid cells, such as myeloid precursors (50) or myeloid suppressors (51, 52), in the graft also influences the outcome of GVHD, GVT, and graft rejection.

4. The endogenous microflora and presence or absence of opportunistic pathogens in the animal colony: The microbial baseline of the animal facility and the use of prophylactic antibiotics can alter the immunological responses in marrow rejection or GVHD as well as markedly affect the sensitivity and response to cytoreductive conditioning. This is perhaps best illustrated in a study examining the role of effector molecules perforin and Fas ligand (FasL) in murine BM rejection (53). In some situations, the use of gastric decontamination can also influence the outcome of clinical studies (54).

5. Age of the donors and recipients: The majority of murine HSCT studies use primarily young adult mice (8- to 12-weeks old). On rare occasions, a study will also examine the influence of age by using middle-aged or old mice (>16 months old) that tend to be much more sensitive to cytoreductive conditioning and that may have differences in immune function compared with young recipients (55). Clinically, the age of donor and recipient can be important.
prognostic factors for the development and severity of GVHD.

Conflicting reported results from different laboratories can often be attributed to variables such as those described here. Therefore, researchers must take care when extrapolating the findings of animal studies from various laboratories as well as when applying them to clinical allogeneic HSCT paradigms.

BARRIERS IN ALLOGENEIC HSCT

Host-Versus-Graft Reaction (Graft Rejection)

Preclinical studies show that allogeneic HSC graft rejection can be mediated by host NK cells (56, 57), NKT cells (58), γδ TCR T cells (59, 60), and/or CD4⁺ and CD8⁺ T cells (61, 62) that recognize histocompatibility antigens on the donor cells. In clinical practice, graft rejection of related HLA-identical BM or mobilized PB after myeloablative conditioning is rare. Graft rejection or graft failure occurs primarily following transplant of cells from related HLA-mismatched or MUD donors and/or use of T cell–depleted grafts. BM graft rejection in mice has historically been assessed by quantitating the presence of hematopoietic progenitors (CFU-c) or spleen cell proliferation with incorporation of a radiolabeled thymidine analog, iododeoxyuridine, at 5–8 days post-HSCT. Alternatively, rejection or graft failure can be assessed by leukocyte numbers, bone marrow cellularity, hematocrits, or the extent of donor chimerism at one and three months post-HSCT and by the proportion of the recipients with long-term survival following myeloablative conditioning. However, early elimination of myeloid precursors does not always correlate with long-term engraftment (63), and researchers should take care to avoid over-interpretation of results.

NK cells are defined by presence of NK-specific markers (NK1.1 and DX5 in mice, CD56 and CD16 in humans) and the absence of T cell markers such as CD3 and TCR. Early studies to characterize NK cells suggested that host NK cells could reject donor BM in a non-MHC restricted manner (56, 57), as evidenced by a phenomena called hybrid resistance in which parental BM cells are rejected by F1 hybrid recipients. Investigators now recognize that NK cells bear inhibitory and activating receptors directed to MHC and other cellular determinants that are critical to target cell identification and subsequent NK cell–mediated killing. KIR family members (in humans) or the structurally unrelated family of molecules, Ly49 (in mice), recognize MHC class I–specific determinants. NKG2 family members, which associate with CD94 on the cell surface, are another class of molecules that can exert inhibitory or activating signals upon binding of the cognate ligand (inhibitory receptors, NKG2A or B, and activating receptor, NKG2C, with human HLA-E or mouse Qa-1b and activating receptor NKG2D with retinoic acid early inducible-1 proteins). The importance of KIR and Ly49 family members in BM graft rejection and GVHD is reviewed in Reference 35. NKG2D also functions in mouse BM rejection upon recognition of its ligand (64). Using a murine model, Barao et al. (65) showed that NK cell–mediated rejection of BM is suppressed by host-derived CD4⁺CD25⁺ Treg cells, and they postulated that this is dependent on transforming growth factor (TGF)–β.

During graft rejection, the effector pathways used by recipient T cells differ on the basis of prior sensitization of the host to alloantigen. In naive, unsensitized recipient mice, perforin, granzyme B, and Fas/FasL can mediate rejection of MHC- and/or MiHA-mismatched BM by CD8⁺ T cells (53, 66, 67). CD4⁺ T cells mediate allogeneic BM destruction (68). However, CD8⁺ T cells from sensitized recipients with alloantigen can reject BM by an unknown mechanism that appears independent of the numerous pathways, as determined using gene knockout mice and neutralizing antibodies (69). These studies demonstrate that naive and memory CD8⁺ T cells can use different, and perhaps
overlapping, effector mechanisms to eliminate BM precursors.

Prior exposure to histocompatibility antigens, which can occur by blood product transfusions, pregnancies, or immunization in experimental models, is attributed to cytotoxic T cells, which can be identified in alloantigen-sensitized recipients. However, antibodies capable of recognizing MHC or MiHA on donor cells can induce graft rejection or lineage-specific aplasia (70–72).

**Immune Reconstitution**

A major problem limiting the efficacy of allogeneic HSCT is the issue of promoting immune reconstitution without increasing GVHD. Patients are profoundly immunosuppressed following transplant as a result of the cytoreductive conditioning, immunosuppressive drugs to prevent GVHD, and the paucity of transplanted T cells compared with the size of the T cell compartment in an immunocompetent person. In addition, acute GVHD induces lymphoid hypoplasia, thus tying GVHD to immune impairment. This leaves the patient susceptible to a number of opportunistic infections. Although GVHD accounts for approximately 15% of deaths after allogeneic transplants, infections account for 17% of deaths after HLA-identical sibling transplants and 21% after MUD transplants (6). Infectious complications associated with neutropenia early post-transplant are no longer as prominent in clinical practice because of the use of GM-CSF and G-CSF. However, cytomegalovirus, Epstein Barr virus (EBV), and fungal infections, predominantly *Candida* species and *Aspergillus fumigatus* that arise after neutrophil recovery has occurred usually between 50 and 100 days or more following transplant, are now major contributors to the morbidity and mortality following allogeneic HSCT (74, 75). Unfortunately, there are very few preclinical models that have been developed to study these opportunistic infections and the complicating effects of GVHD on their occurrence.

There are two sources for T cells in the recovering recipient: peripheral expansion of mature T cells and de novo production of naive T cells derived from transplanted stem cells and produced in the recipient thymus. However, the thymus begins to involute at puberty, and the capacity for thymic-derived T cell production is greatly diminished in adulthood. In addition, the cytoreductive conditioning can induce tissue damage to the epithelial cells of the thymus and a decreased ability to produce IL-7 (76), a cytokine necessary for thymocyte survival and development. Thus, a reduced ability to generate new T cells is a function both of increasing age and of conditioning dose intensity (77, 78). An older HSCT recipient is especially prone to limited recovery of the CD4+ T cell repertoire following allogeneic HSCT. A slow recovery is associated with an increased risk of opportunistic infections (79) and a decreased ability to generate a response to vaccination (80). The benefit of de novo generation of T cells post-transplant is the production of donor-derived T cells that are tolerant of both the graft and the recipient and generation of a broad TCR repertoire. Administration of fibroblast growth factor-7 [also termed keratinocyte growth factor (KGF)] prior to transplant protects cells and/or speeds the repair of epithelial cell–rich tissues (81), including cells in the thymic microenvironment, from conditioning regimen injury, resulting in increased immune reconstitution post-transplant (82). Administration of IL-7 also increases thymopoiesis and peripheral T cell expansion (reviewed in 83). However, the experimental data with IL-7 administration have been conflicting (84, 85), with one study showing no promotion of GVHD and another demonstrating exacerbation of disease. Likewise, administration of IL-15 and FLT3L improves reconstitution by promoting expansion of T cell and dendritic cell (DC) populations, respectively (86, 87). As reported with IL-7 administration, these cytokines under appropriate conditions can also promote GVHD (88, 89).
Sex steroids exert inhibitory effects on thymopoiesis, and chemical or surgical castration can improve thymic function in aged rodents (90, 91). Chemical castration of young recipient mice prior to allogeneic HSCT can enhance thymic recovery through expansion of HSC and thymic precursor populations and via effects on the thymic microenvironment (92). Most of these studies use T cell recovery as an endpoint. Currently, there are very few studies looking at infectious disease resistance as a readout post-HSCT.

Newer strategies under investigation for the promotion of immune reconstitution without GVHD include both the transplantation of T cell subsets that induce GVT but not GVHD in experimental models and the transplantation of grafts that are depleted of cells that proliferate in response to alloantigen (93–95). These and other treatment strategies in preclinical development offer promise for improving overall outcome following allogeneic HSCT by reducing infectious complications without worsening GVHD.

GVHD AND GVT

Early rodent experimentation with BM transplants led to the recognition that infusion of BM between different strains of mice, but not syngeneic animals, resulted in the development of acute GVHD following the recovery of both syngeneic and allogeneic recipients from radiation toxicity (3). Interestingly, GVT activity was also recognized in these early studies (96, 97). The principles necessary for the development of GVHD were described by Billingham in 1966 (98). GVHD requires that the host must be incapable of adequately rejecting the graft, the graft must contain immunocompetent cells, and there must be incompatibilities in transplantation antigens between the host and donor (98). Investigators thus recognized that GVHD is a T cell–mediated inflammatory disease that can be classified into acute or chronic disease. Unfortunately, it is often closely associated with the beneficial GVT activity, and much effort has been put forth to reduce GVHD while maintaining GVT.

Clinical GVHD occurs in acute and chronic forms. Acute GVHD was originally defined as disease appearing within the first 100 days post-transplant, whereas chronic GVHD was more delayed. These two forms are clearly separate entities, with different etiologies and pathophysiology as well as responses to therapy. In addition, they can overlap in time after transplant, especially in recent experiences following treatment with calcineurin inhibitors and the introduction of RIC regimens. Acute GVHD targets the skin, intestine, liver, lung, thymus, and secondary lymphoid tissues and is most often characterized by a Th1-type cellular response and associated B cell lymphopenia. The lung can also be targeted in a related syndrome known as idiopathic pneumonia. Chronic GVHD can target skin and mucosa, but it also involves serous membranes and exocrine glands. It resembles collagen vascular diseases (99) and is characterized in experimental models by Th2-type responses and the presence of autoimmune characteristics, including autoantibody formation. The pathophysiology of chronic GVHD is less well understood than is acute GVHD, in part because of the lack of good animal models that represent the full pathological spectrum for this disease.

Pathophysiology of GVHD

GVHD is a complex disease resulting from donor T cell recognition of a genetically disparate recipient that is unable to reject the foreign (donor) cells following allogeneic HSCT. Investigators have proposed that GVHD develops over multiple stages (100) (see Figure 1). The first stage is characterized by priming of the immune response. Cytoreductive conditioning induces tissue damage and the release of a storm of proinflammatory cytokines that promote the activation and maturation of
antigen-presenting cells (APCs) and the rapid amplification of donor T cells. Conditioning may also change the repertoire of antigenic peptides presented on host APCs. (101). As mentioned above, the importance of conditioning to fuel acute GVHD is demonstrated in the delay of onset following RIC or after delayed donor lymphocyte infusions (DLI) and in mouse studies in which escalation of total body radiation of recipients is associated with more severe GVHD (48, 102). Strategies that reduce tissue damage through the use of cytoprotective agents [e.g., fibroblast growth factor-7 (103, 104)] have proven beneficial in
preventing GVHD in preclinical models and are under clinical investigation.

The second stage, induction of T cell activation, begins with the recognition and interaction of cell surface molecules on the T cell (TCR and costimulatory molecules), with their cognate ligands expressed on the surface of the APC in secondary lymphoid tissues. In murine models, host hematopoietic APCs are a critical component in the induction of GVHD (105), although both donor and host APCs can drive further T cell activation during GVHD (106, 107). In the third stage, alloreactive T cells undergo expansion and differentiation into Th1/Th1 or Th2/Th2 cells (as defined by cytokine production) that have been associated with differences in the manifestations of GVHD (108–110). The fourth stage is characterized by migration of the activated cells to GVHD target tissues (gut, liver, skin, and lung), which is followed by the recruitment of other effector leukocytes, resulting in subsequent tissue injury. Homing of T cells and other cell types to the target tissues is regulated by adhesion, addressin, and chemokine receptor molecules and is enhanced by the production of chemokines in the injured tissue. Chemokine production is initially due to the conditioning treatment and then amplified by the disease process (111–114).

The effector stage of the disease process is defined by the destruction of the target tissues by these cells via cell surface and soluble immune effector molecules [i.e., FasL, tumor necrosis factor (TNF)-α, TNF-related apoptosis inducing ligand (TRAIL), perforin, granzymes, interferon (IFN)-γ]. Tissue damage then leads to increased inflammatory signals, perpetuating and augmenting the disease process by contributing to the cytokine storm that fuels GVHD, as detailed below. The incidence and severity of GVHD in preclinical studies with mice can be assessed by overall survival, measurement of weight loss, scoring of clinical symptoms, and/or histopathological evaluation of affected tissues (115).

Presentation of Alloantigen

Murine studies with MhA-disparate models have demonstrated that the initiation of CD8+ T cell–mediated GVHD and GVT requires donor T cell recognition of host antigen in the context of host APCs (105). This critical interaction occurs despite the early disappearance of the host APCs post-HSCT (106, 107), although alloreactive T cells do undergo several rounds of proliferation prior to the loss of host APCs (106). Donor-derived APCs are then able to augment CD8+ T cell–mediated GVHD, presumably by acquiring and presenting host antigens (cross-priming) (107). In addition, there is evidence that tolerance to self (donor) antigens may be lost during GVHD and that donor-derived antigens can contribute to ongoing GVHD (116).

In full MHC or single MHC-disparate murine BM transplants, antigen presentation by host APCs is critical for the induction of GVT (107, 117, 118). In contrast, there are conflicting data regarding the ability of donor APCs to participate in antitumor responses, which may be related to the differences in the experimental protocols or tumor models tested. Mice were unable to mount recall responses to the leukemia upon rechallenge following full conversion to donor-derived BM following delayed DLI into hematopoietically mixed chimeras (119). In another study, the use of myeloablative conditioning and an aggressive tumor model did not find a critical role of donor APCs for GVT (107). In a third study, Reddy et al. (118) used an allogeneic BM chimera to demonstrate that cross-presentation of alloantigens by donor APCs can occur and may sustain GVT activity.

T Cell Subsets in GVHD and GVT

In mice, naive CD4+CD62L+ CD8+ T cells generate and sustain allogeneic CD8+ T cell subsets in GVHD reactions (120). T cells with a memory phenotype (CD62L− T cells (94, 121) and CD4+ memory (CD45R+CD62L−) T cells (93)) from donors that have not been
previously sensitized to recipient-specific antigens fail to induce GVHD in experimental models. Alloantigen-sensitized effector memory CD44hiCD62Llo as well as naive phenotype CD44hiCD62Lhi, but not central memory CD44hiCD62Lhi CD8+ T cells, can maintain GVHD reactions in secondary recipients (120). Indeed, both alloantigen-sensitized memory CD4+ and CD8+ T cells are involved in the persistence of disease (120, 122). Comparison of T cell subsets in patients following allogeneic HSCT has corroborated and extended these findings to show that CD4+ and CD8+ effector memory cells predominate in chronic GVHD (123, 124).

**T Cell Costimulation and GVHD**

Costimulatory molecules from two major families play pivotal roles in GVHD. CD28:B7 interactions confer an activating signal to the T cell, whereas CTLA-4:B7 interactions confer a negative signal that inhibits T cell proliferation. Reduction in GVHD lethality has been seen in mice treated with B7 antagonists, including CTLA-4-Ig (125, 126). Not surprisingly, use of a specific blockade of CD28 confers greater protection from GVHD lethality than the blockade of both CD28:B7 and CTLA-4:B7 interactions (127).

Other CD28 family members include ICOS and programmed death-1 (PD-1). ICOS is present on activated and memory T cells, binds the ligand B7hi, and promotes effector responses (128, 129). The blockade or absence of ICOS on donor T cells diminishes GVHD associated with the gut and liver (130, 131). The reduction in intestinal and hepatic GVHD was related to CD4+ T cell–dependent disease in some (132) but not other (131) studies. Loss of ICOS signaling worsens CD8+ T cell–mediated GVHD and is associated with increased expansion of the donor CD8+ T cells (132). PD-1 (CD279) is an inhibitor of activated T cells. PD-1 is primarily expressed in the cytoplasm of CD4+ CD25+ Treg cells (133). Blockade or absence of PD-1 on donor cells accelerates both CD4+- and CD8+-mediated GVHD and is associated with increased IFN-γ production (134). This observation, taken together with the findings that IFN-γ upregulates PD-L1 expression (135), suggests that IFN-γ may inhibit GVHD (136), at least in part through the PD-1 pathway.

Members of the TNF receptor (TNFR) family also function as costimulatory molecules and modulate GVHD. OX40 (CD134) is present on both activated CD4+ and CD8+ T cells, and its cognate ligand, OX40L, is present on activated APCs. Despite the presence of the receptor on both T cell populations, the absence of the receptor or ligand or the use of blocking antibody demonstrates that activation of OX40 promotes CD4+ but not CD8+ T cell–mediated GVHD (137). In contrast, CD40L (CD154) is expressed only on activated CD4+ T cells. Endogenous CD40:CD40L interaction increases acute GVHD lethality (138) by promoting both direct CD4+ T cell–mediated tissue destruction and CD8+ T cell expansion (139). In addition, CD40L blockade can inhibit CD4+ T cell–mediated allogeneic BM rejection (140) even under conditions in which no other conditioning is applied (141). Four of these costimulatory pathways (CD28, ICOS, OX40, and CD40L) act independently, as inhibition of any single pathway does not eliminate GVHD, and coblockade results in greater protection (142, 143).

Two other members of the TNFR family are 4–1BB (CD137) and glucocorticoid-induced tumor necrosis factor receptor (GITR). 4–1BB is expressed on activated CD4+ and CD8+ T cells and on NK cells (144, 145). Blockade of 4–1BB reduces CD8+ T cell–mediated GVHD lethality and CD4+ Th1 generation in lethally irradiated models of GVHD (146, 147). In addition, stimulation of 4–1BB with an agonistic antibody can inhibit the development of chronic GVHD in an unirradiated mouse model by depleting CD4+ T cells through activation-induced...
cell death (148). GITR is expressed constitutively on CD4⁺CD25⁺ Treg cells and activated CD4⁺CD25⁻ and CD8⁺CD25⁻ cells (149). Stimulating GITR on Treg cells with an agonist antibody or removal of GITR⁺ cells reverses suppression, leading to the development of autoimmune disease (149). In GVHD, stimulation of GITR on CD4⁺CD25⁻ T cells reduced GVHD in MHC II–disparate recipients, whereas stimulation of GITR of CD8⁺CD25⁻ T cells increased proliferation and GVHD in a MHC I–disparate murine model (150).

T Cell Trafficking and GVHD

Trafficking of naive alloreactive T cells to lymphoid tissues for activation by APCs, followed by homing to specific organ sites, is essential to the induction and pathogenesis of GVHD (Table 1). Whole animal imaging studies have been invaluable for the current understanding of the temporal and organ localization patterns of T cell and tumor trafficking postallogeneic HSCT. Several methodologies are available for such studies. T cells obtained from fluorescent reporter gene (e.g., green fluorescent protein) transgenic mice are particularly advantageous in documenting the donor cell origin and tissue localization of infused donor cells (151). Bioluminescence (e.g., firefly luciferase) imaging is highly useful in the sequential and quantitative visualization of donor cells (152, 153). Positron emission tomography using radiolabeled substrate (e.g., herpes virus thymidine kinase–expressing cells) provides a kinetic and 3-dimensional view of infused cell populations (154) but currently requires the synthesis of short-lived radiolabeled compounds. Alternatively, flow cytometry can be used to track TCR transgenic CD4⁺ and CD8⁺ T cells specifically reactive to host MHC (155) or CD8⁺ T cells reactive to host MiHA disparities (156).

Almost all tissues express transplantation antigens; however, acute GVHD pathology is primarily limited to only a few locations—gut, skin, liver, lung, secondary lymphoid organs, and thymus. One criterion for the diagnosis of acute GVHD involvement in an organ is the presence of lymphocytic infiltration. The ability of alloreactive T cells to home to specific organs is most likely due to a unique combination of signals and corresponding receptors on the tissues and T cells.

Chemokines are a family of structurally related proteins that are expressed by a wide variety of cells in response to infectious agents and following cellular damage. Their G protein–coupled receptors are expressed in unique combinations on different cells. Unlike other cytokines, many chemokine:chemokine receptor interactions are promiscuous in that chemokines can bind multiple receptors and some receptors can bind more than one chemokine. CCR7 is expressed on a subset of T cells as well as on APCs, and CCR7 is important in the migration of naive T cells, central memory T cells, and DCs into lymphoid tissue (reviewed in 157). The ligands for CCR7 are CCL21 and CCL19 (157). Blockade of CCL21 can inhibit induction of chronic GVHD in a murine model (158), whereas increased frequency of CCR7⁻CD8⁺ effector cells is associated with chronic GVHD in humans. These observations suggest that CCR7:CCL21 interaction is required for localization of naive T cells to secondary lymphoid tissue, followed by downregulation of CCR7 and subsequent migration out into target organs. CD30/CD30L (CD153) are members of the TNFR/TNF ligand families, respectively. CD30 is expressed on activated T cells, and among the many consequences of this ligation is the upregulation of CCR7 (159). Blockade of CD30L can reduce CD4⁺ T cell–mediated GVHD in both sublethal and heavily irradiated murine models of disease (160). Treatment with anti-CD30L did not change lymph node infiltration of donor cells but did block migration to the gut and skin. CCR7 expression was not assessed in this study.

Given the multiple lines of evidence, researchers believe that the requirement for
### Table 1  Summary of factors that influence GVHD I

<table>
<thead>
<tr>
<th>Species testedc</th>
<th>Comments</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Alloreactive T cell trafficking in GVHD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR7/ CCL21</td>
<td>↑</td>
<td>M, H</td>
</tr>
<tr>
<td>CD62L α4 integrin</td>
<td>↑ ↓</td>
<td>P</td>
</tr>
<tr>
<td>CCR5 (donor)</td>
<td>↑ ↓</td>
<td>M</td>
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<tr>
<td>CXCR3</td>
<td>↑ (CD8)</td>
<td>M</td>
</tr>
<tr>
<td>CCR6</td>
<td>↑ (CD4)</td>
<td>M</td>
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<tr>
<td><strong>Cytokines</strong></td>
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<tr>
<td>IFN-γ</td>
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<td>TNF-α</td>
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<td>IL-2</td>
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<td>P</td>
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<td>IL-7</td>
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<td>IL-10</td>
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<td>KGF</td>
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<td>M</td>
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<tr>
<td><strong>Cellular mediators</strong></td>
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<td>Naive T cells</td>
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<td>Effector memory T cells</td>
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<td>Treg cells</td>
<td>↓</td>
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</tr>
<tr>
<td>NK cells</td>
<td>↓ –</td>
<td>P ↑</td>
</tr>
</tbody>
</table>

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*a↑ increased or induction of GVHD, ↓ reduced GVHD, – no effect on GVHD.
bP, GVT is preserved.
cM, mouse; R, rat; D, dog; H, human.

alloreactive T cell priming in secondary lymphoid tissues is a critical step in the development of GVHD (151). For example, a pharmacological agent that acts as a sphingosine-1-phosphate receptor agonist, FTY720, traps T cells in secondary lymphoid tissues (161). In experimental models in which animals were treated with the agent beginning at the time of transplant, administration of FTY720 inhibited GVHD without loss of GVT (162). However, administration of the compound into dogs with active GVHD did not reduce lethality of the disease (163).
Less established is the requirement for target organ–associated lymphoid tissues. This may well be a result of the influence of heavy conditioning on lymphocyte trafficking in GVHD. For example, in murine models with no conditioning or nonmyeloablative conditioning with allogeneic lymphocyte infusion, Murai et al. (164) have shown that the absence of Peyer’s patches alone, blockade of the chemokine receptor CCR5, or blockade of MadCAM, the gut-associated ligand for LPAM (lymphocyte Peyer’s patch adhesion molecule, \(\alpha_4\beta_7\) integrin), were all sufficient to reduce acute GVHD (164). CCR5 expression on donor cells was also important in development of liver pathology in similar experimental models (113). However, using myeloablative conditioning and allogeneic BM transplant, we and others (165–167) have found that the absence of Peyer’s patches in recipients or CCR5\(^+\) donor cells did not affect GVHD pathology. The mechanism(s) may be twofold. Following myeloablative conditioning, priming of alloreactive T cells may be able to occur outside the lymphoid tissue or in other secondary lymphoid tissues (168). In addition, optimal inhibition of GVHD by CD4\(^+\) CD25\(^+\) T reg cells in most instances appears to require homing of these cells to secondary lymphoid tissues that can be lost in the absence of CCR5 (166) or CD62L (169, 170).

The chemokine receptor CCR6, which is preferentially expressed on donor CD4\(^+\) T cells, targets these cells to the gut and skin in both sublethal and heavily irradiated recipient murine GVHD models (171). Expression of the CXCR3 or CCR2 on donor CD8\(^+\) T cells targets these cells to the gut and liver but not lung (115, 172, 173) in recipients prepared with myeloablative conditioning. However, the absence of CCR2 on donor CD4\(^+\) T cells results in increased GVHD lethality in a BM aplasia model that was associated with increased IFN-\(\gamma\) production (174). These results suggest that affecting T cell migration, particularly to GVHD target organs, offers great promise, but the redundancy of chemokines and their receptors may make this difficult to apply.

**Effector Functions of T Cells and Their Role in GVHD**

After migration of alloreactive effector T cells to the target tissues of GVHD, these cells can mediate tissue destruction through both direct cytotoxic activity and the recruitment of other leukocytes (Table 1). Targeting these effector pathways has been studied as potential strategies to prevent or reduce the severity of GVHD.

Researchers have considered acute GVHD, on the one hand, to be a Th1/Tc1-type (IL-12, IL-2, IFN-\(\gamma\)) disease on the basis of the predominance of cytotoxic T cell–mediated pathology and of increased production of Th1-type cytokines, including IFN-\(\gamma\). Chronic GVHD, on the other hand, has been described as a Th2/Tc2-type (IL-4, IL-10) disease on the basis of its autoimmune-like features and of the predominance of Th2-type cytokines. Infusion of ex vivo alloantigen-activated cells polarized to a Th2 phenotype resulted in amelioration of acute GVHD (175). Development of acute or chronic GVHD in an unirradiated GVHD murine model is associated with Th1 and Th2 cytokine production, respectively (110). T cells and DCs from G-CSF-mobilized grafts are skewed to a Th2 cytokine phenotype (176, 177) and are associated with reduced acute GVHD lethality in mice (176). However, several recent studies have suggested that the influence of Th1 and Th2 cytokines in acute and chronic GVHD is not so simply explained. The production of IFN-\(\gamma\) by both CD4\(^+\) and CD8\(^+\) donor T cells limits the severity of acute GVHD in recipient mice after myeloablative conditioning (136, 178, 179), but the cytokine is needed for the retention of GVT activity in a murine leukemia model (178) and for CD4\(^+\) T cell–mediated graft-versus-host BM aplasia (136). These results demonstrate the dual nature of IFN-\(\gamma\) in GVHD. Likewise, IL-18, which
promotes IFN-γ production, can inhibit CD4+−dependent GVHD (180). Neutralization of IL-4 with monoclonal antibodies after HSCT (181) or the use of donor cells that lack the IL-4 gene in a murine model attenuates disease (136). The use of donor T cells from mice lacking STAT4 or STAT6, transcription factors required for development to Th1 or Th2 phenotypes, respectively (108), or the ablation of either IL-2-producing (Th1-type) or IL-4-producing (Th2-type) donor T cells following the onset of clinical symptoms of GVHD (109) demonstrate that both Th1- and Th2-type donor T cells can induce acute GVHD.

Dose and timing of cytokine production appear to be critical factors with regard to their role in GVHD. IL-10 is produced by APCs and Th2 cells, promotes Th2 responses, and is important in the tolerance of Treg cells to allografts (182, 183). Higher production of IL-10, as demonstrated in spontaneous release in culture of recipient PB mononuclear cells (184), or the presence in recipients of a polymorphism linked with increased IL-10 production (185) is associated with reduced occurrence and severity of GVHD in patients. Paradoxically, high-dose IL-10 can accelerate GVHD in a murine model (186), and high-serum IL-10 levels in patients after HSCT are associated with a fatal outcome (187), whereas administration of low doses of IL-10 is protective in murine acute GVHD (188). Interestingly, administration of IL-2 peri-transplant can inhibit GVHD (189), and depletion of IL-2 responsive cells can exacerbate GVHD (190), yet depletion of IL-2-responsive cells following the onset of clinical GVHD symptoms reduces the severity of disease (191). These findings highlight the pleiotropic, sometimes opposing, nature of cytokines during the different phases of GVHD pathogenesis and on various effector and regulatory cell populations.

T cells mediate the final effector pathway in both GVHD and GVT by multiple pathways. Murine studies have shown that the cytolytic molecules perforin and Fas/FasL together are the predominant mediators of lethal acute GVHD (192), whereas TNF/TNFR (193, 194) can contribute to GVHD mortality and histopathology. Fas-mediated cytotoxicity can be upregulated by IFN-γ production (195) and is associated with intestinal and hepatic GVHD, lymphoid hypoplasia, and GVT (196–198). Both CD4+ and CD8+ T cells can use perforin to mediate lethal GVHD (199). The perforin and TRAIL cytotoxic pathways are associated with CD8+ T cell–mediated GVT (198, 200). Of note, the available experimental data are strongly skewed toward CD8+ T cell–mediated GVT based on the dominant role of this effector population in most murine GVT models; however, CD4+ T cells can mediate GVT (201–203). Taken together, there is some distinction between the use of different lytic pathways in GVHD and GVT.

In studies of transplant patients, polymorphisms in the TNF gene of HSCT recipients are associated with higher levels of production of the cytokine and are correlated with a higher incidence of severe GVHD (204, 205), which suggests that, in humans, induction of TNF from recipient cells may make an important contribution to disease. The role of recipient-derived TNF was not observed in a murine model of GVHD (167). Regardless of the source of TNF, its importance in GVHD is borne out with the demonstration that treatment of steroid-resistant GVHD with a TNF-α blocker has shown efficacy, especially against gastrointestinal disease (206).

**Molecular Targeting of GVHD**

There has been intensive interest in targeting intracellular pathways to treat GVHD. Suberoylanide hydroxamic acid (SAHA) is a histone deacetylase inhibitor that inhibits tumor growth (207) and proinflammatory cytokine production in vivo (208). When SAHA was administered to mice early post allogeneic transplant, decreases in circulating TNF-α, IL-1β, and IFN-γ and attenuation of GVHD were observed without a decrease in T cell
proliferation (209). The preservation of T cell proliferative and cytotoxic responses correlated with the maintenance of GVT. Bortezomib is a proteasome inhibitor that has direct antitumor effects (210) and has been approved for the treatment of multiple myeloma. When bortezomib was given immediately after allogeneic transplant, reduction in acute GVHD mortality with preservation of GVT was observed (211). Protection was associated with reduction in alloreactive T cell expansion and proinflammatory cytokine production. However, administration of bortezomib five or more days after transplant can result in hyperacute GVHD-associated lethality in a murine model (212), demonstrating again how critical the timing of the interventions can be to the efficacy of the treatments, similar to the role of cytokines like IFN-γ in GVHD. Most murine studies demonstrate that affecting the induction phase of GVHD and not later phases results in protection.

**The Role of Other Lymphocytic Subpopulations in Allogeneic HSCT**

NK cells, CD4+ Treg cells, NKT cells (reviewed in 213), and γδ TCR T cells (59, 60) in the recipients and/or in the donor graft affect the outcome of allogeneic HSCT regarding graft rejection or tolerance, GVHD, and GVT (Table 1). Two of these populations are discussed in greater detail below.

**T regulatory cells.** CD4+CD25+Foxp3+ Treg cells have potent suppressor activity both in vitro and in vivo (reviewed in 214). Donor Treg cell infusion both blocks acute GVHD (215–217) and prevents graft rejection of MHC-disparate allografts after sublethal conditioning in mice (170, 218, 219). CD62L+ Treg cells preferentially home to secondary lymph nodes and provide protection in GVHD (170). Conversely, depletion of CD25+ cells from the graft or in the recipient immediately following allogeneic HSCT promotes acute and chronic GVHD in various mouse studies (216, 217, 220) and can maintain GVT against a lymphoid malignancy (221). Owing to the relatively low frequency of Treg cells in lymphoid organs, ex vivo expansion of Treg cells often has been used to increase the number available for in vivo infusion. Activation of Treg cells increases their suppressor cell potency, although freshly isolated Treg cells are capable of expanding in vivo and inhibiting GVHD lethality (222).

Following whole-body irradiation, CD4+CD25+ Treg cells are retained in recipient mice at a higher frequency than are other T cell populations (220). Recipient-derived CD4+CD25+ Treg cells can also reduce acute and chronic GVHD in murine models (220, 223), inhibit NK cell–mediated BM graft rejection (65), and improve immune reconstitution and GVT (222–224). Immunosuppressive drugs given to prevent or control GVHD also affect Treg cell expansion and function. Calcineurin inhibitors such as cyclosporine A decrease IL-2 production, leading to a reduction in Treg cell proliferation and function (224), whereas rapamycin can increase functional murine Treg cells (27) and increase suppressive human CD4+ T cells (29) in ex vivo culture.

Some challenges have arisen in the manipulation of human Treg cells during allogeneic HSCT when procedures are based solely on CD25 expression (225). Although the expression of the intracellular protein, Foxp3, is used to define Treg populations, permeabilization of cells to assess Foxp3 expression precludes the use of Foxp3 as a marker suitable for cell isolation procedures without impairing cell viability and function. More recently, a combination of CD4, CD25, and CD127 (IL-7Rα) resulted in a highly purified population of Treg cells that included both CD4+CD25+ and CD4+CD25− T cell subsets that were as suppressive as the classic CD4+CD25hi Treg cell subset (226, 227)

**NK cells.** Donor-derived NK cells have the potential to promote engraftment, suppress GVHD, and promote GVT, whereas host-derived NK cells can mediate graft rejection.
and affect GVHD by eliminating donor HSCs or activated T cells, respectively. Investigation into the incidence of GVT and GVHD in KIR ligand–mismatched haploidentical allogeneic HSCT has suggested increased GVT against acute myelogenous leukemia with protection from GVHD (36). However, the observation may also depend on other contributing factors in the transplant protocol, such as the extent of donor T cell depletion (228), the speed at which NK cells recover, and/or the use of post-transplant immunosuppression, as other clinical studies have not been able to identify a benefit in outcome (37).

Adoptive transfer of activated NK cells early after transplant inhibits GVHD and promotes GVT in a murine model (229). However, using the same model, administration of activated NK cells later in the course of GVHD (229) could exacerbate the disease, similar to effects seen with IL-2 and IL-12 (189, 195). Although the mechanism by which donor NK cells can inhibit GVHD is not fully understood, TGF-β may be a mediator (229). Another possible mechanism may be the accelerated depletion of host APCs (36). Indeed, NK cells promote tolerance by eliminating donor DCs in an experimental model of solid organ transplant (230). Miller et al. (231) recently demonstrated the safety and potential benefit of adoptive haploidentical–related NK cell therapy without HSCT following high-dose intensity conditioning. Twenty-six percent of a small cohort of poor prognosis patients with acute myelogenous leukemia achieved complete hematological remission of their leukemia (231). Additional studies are needed to determine how best to exploit the potential benefit of NK cells in allogeneic HSCT by promoting their recovery with cytokines such as IL-15 or by selection of specific subsets.

CONCLUDING REMARKS

Substantial progress has been made in the clinical practice of allogeneic HSCT and in our understanding of the biology underlying this therapy. In its infancy, allogeneic HSCT, when used as a treatment for cancer, was the delivery of myeloablative doses of chemotherapeutic drugs and radiation to eliminate leukemias followed by hematopoietic rescue with healthy BM. Allogeneic HSCT has now evolved into an adoptive cellular immunotherapy. Keeping in mind the numerous caveats before extrapolating results to humans, we anticipate that preclinical allogeneic HSCT models will continue to be invaluable in increasing our understanding of the immunobiology of HSCT and its application.

ACKNOWLEDGMENTS

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