Stem cell mobilization with G-CSF analogs: a rational approach to separate GVHD and GVL?

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The separation of graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) remains the "holy grail" of allogeneic stem cell transplantation, and improvements are urgently needed to allow more effective therapy of malignant disease. The use of G-CSF–mobilized peripheral blood as a clinical stem cell source is associated with enhanced GVL effects without amplification of significant acute GVHD. Preclinical studies have demonstrated that G-CSF modulates donor T cell function before transplantation, promoting T_H2 differentiation and regulatory T cell function. In addition, the expansion of immature antigen-presenting cells (APCs) and plasmacytoid dendritic cells (DCs) favors the maintenance of this pattern of T cell differentiation after transplantation. Although these patterns of T cell differentiation attenuate acute GVHD, they do not have an impact on the cytolytic pathways of the CD8$^+$ T cells that are critical for effective GVL. Recently, it has been demonstrated that modification of G-CSF, either by pegylation of the native cytokine or conjugation to Flt-3L, results in the expansion and activation of donor iNKT cells, which significantly augment CD8$^+$ T cell–mediated cytotoxicity and GVL effects after transplantation. Given that these cytokines also enhance the expansion of regulatory T cells and APCs, they further separate GVHD and GVL, offering potential clinical advantages for the transplant recipient. (Blood. 2006; 107:3430-3435)

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Introduction

Despite a wide variety of experimental approaches to tumor immunotherapy, allogeneic hemopoietic stem cell transplantation (SCT) remains the only clinically relevant platform effectively exploiting immunologic tumor clearance. Graft-versus-leukemia (GVL) effects were recognized in early murine studies[1] and subsequent clinical practice[2] and have been demonstrated against a variety of malignancies. Chronic myeloid leukemia remains the most sensitive disease reported to date, although effective responses have been demonstrated against acute myeloid leukemia, multiple myeloma, Hodgkin lymphoma, non–Hodgkin lymphoma, and a variety of nonhematologic malignancies, including renal cell carcinoma and breast cancer. Unfortunately, the pathologic reciprocal immune reaction resulting in graft-versus-host disease (GVHD) remains a major barrier to the therapeutic potential and applicability of allogeneic SCT. Although GVHD and GVL effects are closely related, more effective GVL reactivity is observed in recipients of allogeneic grafts in whom GVHD does not develop than in recipients of syngeneic or T cell–depleted allogeneic grafts.[3] Furthermore, effective adoptive immunotherapy with donor lymphocyte infusion in patients who have relapses after SCT is not uniformly associated with the development of GVHD.[4] The deliberate separation of GVHD and GVL remains the "holy grail" of allogeneic SCT, with further improvements urgently needed to maximize the immunotherapeutic potential against malignant diseases.

G-CSF is widely used during transplantation to mobilize hemopoietic stem cells. The administration of G-CSF to donors results in a complex series of events, initiated by a marked expansion of neutrophils in the bone marrow. The neutrophils secrete proteases that disrupt adhesion molecules (including VCAM-1) and chemokine receptors (including CXCL12), releasing stem cells from their bone marrow niche to be collected from the blood by apheresis.[5] G-CSF–stimulated peripheral blood has largely replaced bone marrow as a stem cell source.[6] A recent meta-analysis of randomized trials of SCT compared with bone marrow transplantation (BMT) demonstrated enhanced GVL effects in early and advanced disease after SCT.[7] Importantly, although the incidence of grades II to IV acute GVHD was not increased, increases in severe acute and chronic GVHD were noted. Thus, after stem cell mobilization with G-CSF, enhanced GVL effects are seen in conjunction with increased allogeneic responses. This does not, however, result in increased mortality; indeed, it improves overall survival in patients with advanced disease, due to reduced leukemic relapse.[7] Building on this progress represents a logical starting point from which to enhance GVL effects and further separate GVHD after SCT.

Pathophysiology of acute GVHD and GVL effects

During GVHD, the presentation of histocompatibility antigens by residual host antigen-presenting cells (APCs) stimulates naive donor CD8^{+} and CD4^{+} T cells,[8] whereas the presentation of host antigens by repopulating donor APCs to donor CD4^{+} T cells amplifies alloreactivity.[9] Critical to this process is the effective trafficking of donor T cells to the appropriate lymphoid environment, where they encounter professional APCs. Subsequently, donor T cells undergo type 1–biased differentiation and produce a range of proinflammatory cytokines, leading to a cascade of pathologic events. Monocytes/macrophages, primed by T_H1 cells and stimulated by LPS, release cytopathic quantities of inflammatory cytokines characteristic of the "cytokine storm."[10] Target tissue apoptosis is thus mediated in a major histocompatibility complex (MHC)–independent and –dependent fashion through inflammatory cytokines (TNF-α, IL-1, and nitric oxide)[11] and CD8^{+} T cell cellular cytotoxicity (Figure 1).[12]

Initiation of GVL reactions is dependent on a complex series of bidirectional interactions between DCs and cells of the innate and adaptive immune systems, including natural killer (NK) cells, natural killer T (NKT) cells, and CD4^{+} and CD8^{+} T cells. The result is enhancement of innate antitumor effects and licensing of DCs to allow the presentation of antigen to effector cells of the adaptive immune system. After MHC-matched allogeneic SCT, GVL effects are directed against a variety of targets, depending on the characteristics of the underlying malignancy. Targets may include ubiquitously expressed minor histocompatibility antigen (mHA), unique leukemia-associated proteins (such as BCR-ABL) or nonpolymorphic self-proteins (such as proteinase 3 or Wilms tumor 1) overexpressed by leukemic cells and presented directly by host APCs within MHC class I to donor CD8^{+} T cells.[13] Alternatively, these antigens may be presented by host or donor APCs within MHC class II to donor CD4^{+} T cells. In addition, the loss or mismatch of inhibitory KIR ligands (particularly MHC class 1) may be recognized by donor NK cells and trigger MHC-independent cytoxicity.[14] Thus, depending on the nature of the leukemia, donor CD8^{+} T cells, CD4^{+} T cells, or NK cells may be activated and may contribute to the effector phase of GVL, using perforin,[15] TNF-related apoptosis inducing ligand (TRAIL),[16] and TNF-α[17] cytolytic pathways (Figure 2).

Chronic GVHD

The incidence of extensive chronic GVHD is increased after G-CSF–mobilized SCT.[7] The pathophysiology of chronic GVHD is poorly understood, but it is becoming increasingly clear that the process is fundamentally distinct from that of acute GVHD. Although donor T cells are critical in the initiation of chronic GVHD, it is now established that the effector pathways involve cells of the myeloid lineage and fibrogenic cytokines such as TGF-β, all of which are amplified by G-CSF.[18,19] Leukemia relapse rates are markedly reduced in patients in whom chronic GVHD develops,[20] reflecting ongoing effective CTL responses and, perhaps most important, the chronicity of the process, which is in contrast to that in patients in whom severe acute GVHD develops.

Separation of GVHD and GVL after G-CSF mobilization and allogeneic SCT

The mobilization of stem cells with G-CSF may have a number of immunomodulatory effects. First, the disruption of adhesion molecules by proteases released from myeloid precursors may influence the ability of T cells to traffic to lymphoid tissue and induce GVHD. Consistent with this, CD62L expression on donor T cells is profoundly reduced after G-CSF administration.[21] Second, the distortion of the lymphoid environment by expanded myeloid precursors will alter the anatomic ability of T cells to interact with professional APCs and increase the frequency with which T cells encounter cytokine-expanded immature APCs. Third, hemopoietic tissue expanded by G-CSF and stromal tissue stimulated by G-CSF may release soluble immunomodulatory proteins, such as IL-10, TGF-β, and IFN-α, which may subsequently modulate donor T cell function.

Effects on donor T cells

Stem cell mobilization with G-CSF polarizes conventional $\alpha\beta$ T cells toward a T_H2 pattern of cytokine production, but this polarization is not associated with a reduction in CTL activity or GVL.[15] Similarly, other interventions to induce T_H2/Tc2 bias, including mobilization with IL-18[22] or posttransplantation administration of IL-11,[23] also prevent GVHD while maintaining GVL effects. Tayebi et al[24] reported a reduction in the production of type 1 cytokines after mobilization of healthy donors with G-CSF. Consistent with this, Franzke et al[25] reported that the expression of GATA-3, a key T_H2 transcription factor, was increased in CD4^{+} T cells after the administration of G-CSF.

Regulatory T cells (T_{regs}) are important in the attenuation of GVHD,[26] and clinical studies have shown that CD4^{+} T cells from G-CSF–mobilized donors secrete more IL-10 but less IL-4, IL-2, and IFN-γ than the same populations before G-CSF administration.[27] Purified CD4^{+} T cells were able to suppress alloproliferative responses of autologous T cells in an antigen-nonspecific manner, consistent with a regulatory Tr1-like phenotype. These effects have now been confirmed in vivo, where protection from GVHD after mobilization with G-CSF is dependent on donor T cells and both IL-10 and TGF-β.[19,28] Edinger et al[29] demonstrated that despite reducing GVHD, T_{regs} do not impair the ability of CD8^{+} CTLs to lyse tumor cells in vitro and that, at a 1:1 ratio, in vivo GVL effects are retained when sufficient numbers of effector T cells are transferred. When low numbers of effectors are transferred, however, GVL effects are impaired,[29,30] giving rise to otherwise apparently contradictory data regarding the effects of T_{regs} on GVL.

Because purified T cells from G-CSF–treated donors have altered cytokine and transcription profiles before transplantation, the alteration of donor T cell function occurs in the absence of alloreactivity and is likely to reflect modulation by cytokines, expanded myeloid cells, or both. G-CSF receptor (G-CSFR) mRNA is ubiquitously expressed in hemopoietic and nonhemopoietic tissue. Surprisingly, G-CSFR mRNA was detected in a small subset of purified CD4^{+} and CD8^{+} T cells from healthy donors before the administration of G-CSF.[31] Culture of purified CD4^{+} T cells in the presence of G-CSF before stimulation with mitogen resulted in increased expression of IL-4 and reduced IFN-γ mRNA.[31] Subsequent clinical studies have detected G-CSFR in T cells only after G-CSF administration.[25] Although the contribution
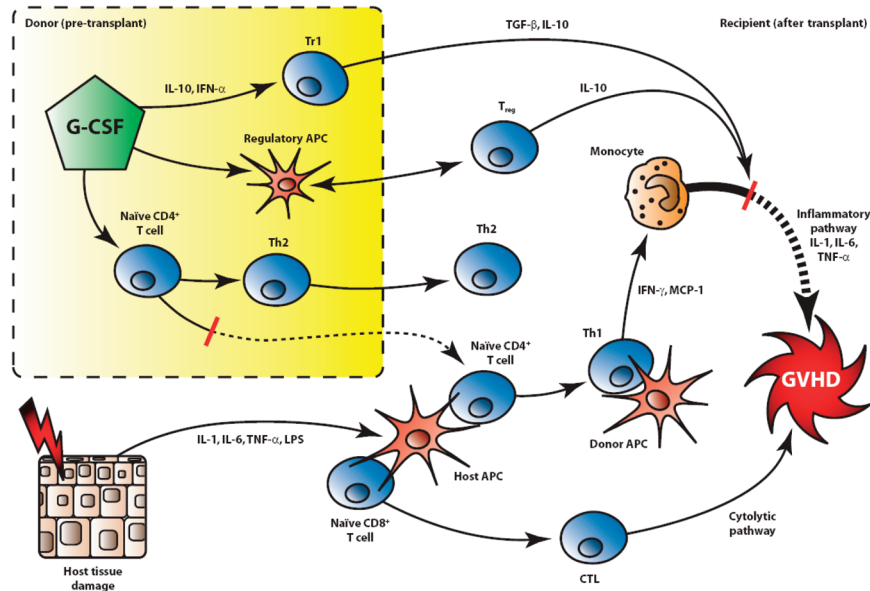
Figure 1. Stem cell mobilization with G-CSF attenuates acute GVHD through effects on T cells and APCs. After SCT, tissue injury and local inflammation (including IL-1, IL-6, and TNF-α release) are initiated by the conditioning regimen and promote the activation of host APCs. The interaction between activated host APCs (displaying disparate histocompatibility antigens) and naive donor CD4$^+$ and CD8$^+$ T cells preferentially drives type 1 differentiation, generating large amounts of IFN-γ that primes mononuclear phagocytes of donor and host origin. Donor CD4$^+$ T cell responses are subsequently perpetuated by donor APCs presenting host antigens. After activation by LPS and other gut-derived immunostimulants, monocytes primed by T_H1 cytokines secrete cytopathic quantities of proinflammatory cytokines (TNF-α, IL-1) and mediate tissue injury in the inflammatory effector pathway of GVHD. Concurrently, effector donor CD8$^+$ T cells are expanded, gain cytolytic function, and mediate target tissue GVHD through their cellular cytolytic machinery (eg, perforin, granzyme, TRAIL) in the cytolytic effector pathway. This leads to the "cytokine storm" characteristic of acute GVHD, whereby target tissues are damaged in MHC-independent and -dependent fashion. After G-CSF mobilization of stem cell donors, however, 3 key immunomodulatory effects before transplantation lead to the attenuation of GVHD. First, donor T cells up-regulate GATA-3 expression and are biased toward T_H2 differentiation, limiting T_H1-dependent monocyte activation after SCT. Second, G-CSF induces the generation of Tr1 regulatory cells (distinct from classical CD4$^+$CD25$^+$ T_{reg}) through IL-10 production. Third, G-CSF expands regulatory APCs within the donor (immature myeloid precursors and plasmacytoid DCs) which, after transplantation, promote the generation of classical CD4$^+$CD25$^+$ IL-10–producing T_{regs}. The generation of IL-10 and TGF-β from Tr1 and T_{reg} serve to further inhibit the inflammatory effector phase of GVHD, limiting target tissue damage.

of contaminating non–T cells cannot be excluded in these studies, it is possible that G-CSF may also modulate donor T cell function directly. The definitive mechanism of T cell modulation by G-CSF thus remains to be defined.

Effects on antigen-presenting cells

The presentation of alloantigen by host APCs is central to the initiation of GVHD and GVL.[32] It is becoming clear, however, that APCs are also involved in the induction and maintenance of tolerance, particularly when the predominant APCs are immature or phenotypically distinct (eg, plasmacytoid DCs).[33] APCs, therefore, represent an attractive explanation for the immunomodulatory effects of G-CSF on T cells.

G-CSF–mobilized peripheral blood stem cell grafts contain a 50-fold increase in CD14$^+$ monocytes, which suppress alloantigen-induced T cell proliferation and CD28-signaling of CD4$^+$ T cells in
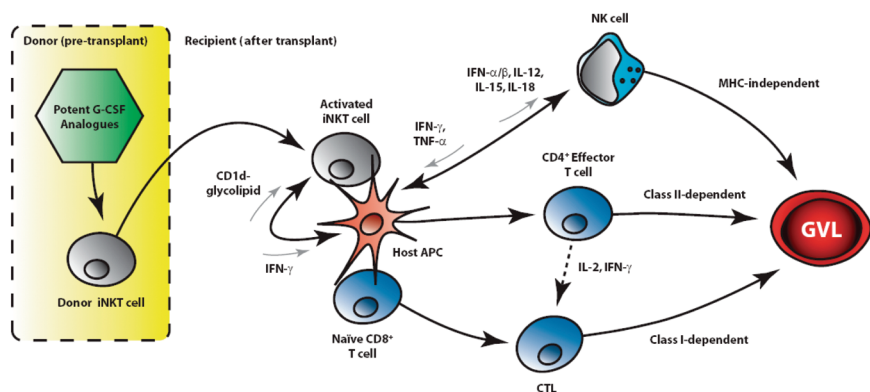

Figure 2. Stem cell mobilization with potent G-CSF analogs activates iNKT cells with subsequent promotion of donor CTL function and GVL effects. After stem cell mobilization with potent G-CSF analogs, donor iNKT cells are expanded and functionally activated. These iNKT cells interact with residual host APCs and may be activated directly through CD1d-presented glycolipid or indirectly through cytokines (including IL-12 and IL-18). After activation, iNKT cells secrete large amounts of cytokine, including IFN-γ, which further primes host APCs and activates cellular effectors of the innate (NK cell) and adaptive (CD4$^+$ and CD8$^+$ T cell) immune systems. NK cells are activated by host APCs through activating receptor interactions (including NKG2D-NKG2DI and CD70-CD27) and cytokines (including IFN-α/β, IL-12, IL-15, and IL-18). NK cells reciprocally enhance APC activation through the secretion of IFN-γ and TNF-α and directly mediate MHC-independent GVL through interactions with activating ligands, KIR mismatch, or the recognition of leukemic targets lacking MHC class 1. Donor CD4$^+$ T cells, activated by host hemopoietic or leukemia-specific antigens presented by host (or donor) APCs, mediate GVL effects against MHC class 2$^+$ leukemic targets expressing the relevant antigens. Donor CD8$^+$ T cells activated by a similar range of antigens, presented by host APCs only, mediate GVL against leukemic targets expressing the relevant antigens within MHC class 1.

vitro.[34,35] T-cell proliferation and IFN-γ production are reduced after the addition of G-CSF–mobilized monocytes to mixed lymphocyte cultures, in part through IL-10–mediated effects.[36] The expansion of granulocyte-monocyte precursors by G-CSF is associated with the modulation of GVHD, and these cells differentiate into MHC class 2+, CD80/86+, CD40− APCs after allogeneic SCT.[37] Adoptive transfer of these immature myeloid APCs promotes transplantation tolerance by the MHC class II–restricted generation of IL-10–secreting, antigen-specific regulatory T cells, predominantly in the classical CD4+CD25+ fraction. Despite potently inhibiting GVHD, these immature myeloid cells do not prevent GVL.[37]

Serum IL-10 and IFN-α have been shown to be increased after the administration of G-CSF to healthy donors, and culture of monocytes in the presence of serum from G-CSF–mobilized donors resulted in maturation to a DC-like population with low IL-12 secretion and poor allostimulatory capacity.[38] Furthermore, coculture of naive CD4+ T cells with this DC-like population led to IFN-α– and IL-10–dependent generation of IL-10– and TGF-β–producing antigen-specific regulatory T cells.[38] Human peripheral blood contains CD11c^hi (myeloid) and plasmacytoid DCs.[39] Myeloid DCs express high levels of costimulatory molecules and initiate primary T cell–dependent immune responses that are T_H1 biased.[40] Plasmacytoid DCs preferentially express a distinct profile of TLRs (TLR7 and TLR9) that respond to single-stranded RNA and DNA viruses with rapid production of type I interferons (IFN-α and IFN-β).[41] Plasmacytoid DCs promote T_H2-biased T cell responses[42] and regulatory T cell function.[43] After G-CSF administration, the number of plasmacytoid DCs in peripheral blood is increased approximately 5-fold,[44] which may reflect altered trafficking caused by the down-regulation of CD62L and the up-regulation of CCR7.[45] It has been postulated that plasmacytoid DCs may contribute indirectly to T_H2 polarization of donor T cells by G-CSF,[44] and they appear to contribute to the inhibition of disease development in autoimmune models of diabetes.[46] However, a causal relationship between the effects of G-CSF on plasmacytoid DCs and the inhibition of GVHD has yet to be confirmed in animal models because their adoptive transfer during transplantation has failed to confer protection from GVHD.[37]

The transplantation of peripheral blood stem cell grafts therefore results in the transfer of large numbers of immunocompetent CTLs compared with that within bone marrow and improves GVL through the cytolytic pathway. This is balanced by the transfer of large numbers of regulatory APCs and Tr1/T_H2 cells that concomitantly attenuate the inflammatory pathway of GVHD (Figure 1).

Effects of stem cell mobilization with novel G-CSF analogs

Stem cell mobilization with G-CSF modulates GVHD by promoting T_H2 and regulatory T cell function in the context of expanded regulatory or inhibitory APCs. This prompted us to question whether all G-CSF molecules behave similarly and whether differing moieties may be exploited to further separate GVHD and GVL. We have demonstrated that protection from GVHD is dependent on the G-CSF dose and can be maximized by pegylation of the native cytokine. Mobilization with pegylated G-CSF (Peg-G-CSF) results in enhanced expansion of tolerogenic APCs and augmentation of T_reg activity that in turn promotes tolerance.[28] A second family of molecules, the progenipoietins (including progenipoietin-1 [ProGP-1]) are engineered chimeric G-CSF and Flt-3L

proteins that have significantly greater ability to mobilize stem cells and APCs than either native molecule alone. Grafts mobilized by these cytokines have marked tolerogenic properties that reside in the T cell and APC compartments.[37] Surprisingly, after stem cell mobilization with pegylated G-CSF or ProGP-1, GVL and GVHD are effectively separated, and maximal GVL effects are dependent on the presence of invariant NKT (iNKT) cells (Figure 2).[47]

iNKT cells are increasingly recognized as having important roles in immunoregulation and tumor surveillance. iNKT cell recognition of glycolipid antigens, such as α-galactosylceramide (α-GalCer), characteristically leads to rapid production of immunomodulatory cytokines, particularly IFN-γ and IL-4.[48] iNKT cell activation using α-GalCer has been shown to influence disease progression in a variety of experimental models of autoimmunity and inflammation (for a review, see Van Kaer[49]). Although most studies suggest that α-GalCer prevents autoimmunity by promoting T_H2 responses, it is becoming clear that the induction of tolerogenic DCs[50] or regulatory T cells may also have a crucial role.[51] Administration of α-GalCer to recipients in a mouse model of allogeneic SCT after sublethal irradiation significantly reduced GVHD,[52] subsequently shown to be associated with T_H2 polarization of donor T cells.[53] Haraguchi et al,[54] using a nonlethal irradiation BMT model, confirmed that residual host iNKT cells provided protection from GVHD. Protection could be conferred by adoptive transfer of additional host-type iNKT cells, whereas the presence of iNKT cells in the donor graft was associated with increased GVHD severity,[47] demonstrating differential immunomodulatory effects of donor and host iNKT cells in allogeneic SCT. Stem cell mobilization with ProGP-1 expands and activates donor iNKT cells, resulting in enhanced responses to α-GalCer. After transplantation, donor iNKT cells promote the licensing of residual host DCs and enhance perforin-restricted CD8+ T cell cytotoxicity against host-type antigens. Enhanced cytotoxicity and GVL effects are not associated with Flt-3L signaling or effects on DCs but can be reproduced by prolonged G-CSF receptor engagement with pegylated G-CSF. The enhanced regulatory properties inherent in grafts mobilized using potent G-CSF analogs compensate for the increased CTL function that otherwise might be expected to result in increased GVHD.[21,28,37,47] Studies to date predict that the separation of GVHD and GVL effects after stem cell mobilization with potent G-CSF analogs will be most marked when GVHD is CD4 dependent and GVL effects are mediated by CD8+ CTL and NK cells.

iNKT cells are expanded in G-CSF transgenic mice,[55] and G-CSF receptor mRNA has been demonstrated in NKT-like cells.[56] In clinical studies, however, mobilization with standard G-CSF is not associated with the modulation of donor iNKT cell function, and iNKT cells are not expanded in G-CSF–mobilized stem cell grafts from healthy donors.[57,58] Consistent with this, stem cell mobilization with standard G-CSF does not alter GVL in murine models,[47] suggesting that molecular alterations in the new G-CSF moieties result in the augmentation of signaling that influences iNKT cell expansion and function. ProGP-1 binds to the G-CSF and Flt-3L receptors with an affinity similar to that for native molecules.[59] ProGP-1 is able to bind to G-CSF and Flt-3L receptors simultaneously and thus to colocalize and amplify signaling at the cell membrane. Although combined mobilization of donors with G-CSF and Flt-3L failed to reproduce the enhanced GVL effects of ProGP-1, enhanced GVL effects were observed after mobilization with pegylated G-CSF. We hypothesize, therefore, that the predominant effect is likely to be through the G-CSF receptor. Interestingly, the pegylation of G-CSF not only enhances serum half-life (and

thus receptor occupancy), it also alters the cellular trafficking of G-CSF to enhance G-CSF receptor stimulation at a cellular level.[60]

Administration of G-CSF after BMT

G-CSF is often also administered to recipients after transplantation to hasten engraftment,[61] though potential immunologic effects have recently become the source of considerable controversy. Analysis of data reported to the European Group for Blood and Marrow Transplantation (EBMT) has demonstrated that the administration of G-CSF after BMT increases the incidence of acute and chronic GVHD, with consequent reductions in overall survival.[62] Although the underlying mechanism remains unclear, it is intriguing that detrimental effects were not seen in recipients of G-CSF–mobilized PBSC grafts. It is attractive to postulate that the administration of G-CSF after BMT may activate donor iNKT cells and augment alloresponses in an environment that it is not balanced by the inhibitory effects of G-CSF on donor T cells and APCs during stem cell mobilization. We are studying this hypothesis in preclinical models; the literature cautions against the routine use of G-CSF after allogeneic BMT until further data are available.

Future directions and alternative strategies

The mobilization of stem cells with pegylated G-CSF in healthy donors will have to be closely monitored in well-designed clinical trials that focus on GVHD severity and relapse in the context of iNKT cell function. At this point, it is difficult to envisage the exposure of healthy donors to new hybrid cytokines or combinations of cytokines. The augmentation of donor iNKT cell function and GVL effects after SCT through the administration of α-GalCer (or variants thereof) or α-GalCer–pulsed DCs will have to be studied in preclinical models before clinical translation because they would be predicted to augment GVHD.

Although T-cell depletion in clinical allogeneic SCT is a concept that has largely come and gone, the ability of naive, but not memory, T cells to induce GVHD has ignited the idea of depleting T-cell subsets. In this regard, the relative ability of effector and central memory T cells to mediate GVL will have to be addressed in preclinical models before clinical trials that deplete naive T cells from the stem cell graft. Understanding the mechanism by which memory T cells fail to induce GVHD will provide important insights and may result in therapeutic approaches that block T cell adhesion molecules and subsequent trafficking of donor T cells to primary lymphoid organs.

Although the depletion of APCs to prevent GVHD initially appeared an attractive concept, it now seems clear that this approach will prevent the induction of effective GVL.[32] However, the relative role of APC subsets in the induction of GVHD remains unknown. Although this is difficult to dissect with currently available reagents, it will be the next critical step in moving the field forward. Finally, it now seems important to focus on the modification of DC function in vivo by preventing activation and maturation because this approach may promote the induction of regulatory T cells and, in turn, may retain GVL effects early after transplantation.

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