Advances in the understanding of acute graft-versus-host disease

Edward S. Morris1,2 and Geoffrey R. Hill2

1Department of Haematology, Royal Hallamshire Hospital, Sheffield, UK, and 2Bone Marrow Transplantation Laboratory, Queensland Institute of Medical Research, Brisbane, Qld, Australia

Summary

Allogeneic stem cell transplantation (SCT) remains the definitive immunotherapy for malignancy. However, morbidity and mortality due to graft-vs-host disease (GVHD) remains the major barrier to its advancement. Emerging experimental data highlights the immuno-modulatory roles of diverse cell populations in GVHD, including regulatory T cells, natural killer (NK) cells, NK T cells, γδ T cells, and antigen presenting cells (APC). Knowledge of the pathophysiology of GVHD has driven the investigation of new rational strategies to both prevent severe GVHD and treat steroid-refractory GVHD. Novel cytokine inhibitors, immune-suppressant agents known to preserve or even promote regulatory T-cell function and the depletion of specific alloreactive T-cell sub-populations all promise significant advances in the near future. As our knowledge and therapeutic options expand, the ability to limit GVHD whilst preserving anti-microbial and tumour responses becomes a realistic prospect.

Keywords: graft-vs.-host disease, cytokines, regulatory T cells, natural killer T cells, antigen presenting cells.

The pathophysiology of acute graft-vs.-host disease

Morbidity and mortality due to graft-vs.-host disease (GVHD) remain major barriers to the advancement of allogeneic haemopoietic stem cell transplantation (SCT). Advances in our understanding of the pathophysiology of GVHD and peripheral regulatory mechanisms, along with novel approaches to the prophylaxis and treatment of GVHD, have significantly advanced the field. Translation of basic mechanistic knowledge from animal models into clinical practice is crucial if the rapid advancement of allogeneic transplantation is to continue.

The pathophysiology of GVHD may be divided into three distinct but collaborative phases (Fig 1): 1 Tissue damage attributable to previous therapy and conditioning regimens; 2 Donor T-cell activation following interaction with antigen presenting cells (APC); 3 Cytokine- and cell-mediated target tissue damage.

Conditioning related tissue damage

Tissue damage is the catalyst for the development of rapid GVHD, and often occurs well before the transfer of the allogeneic stem cell graft. Underlying malignancy, effects of previous induction therapies and new cellular damage induced by transplant conditioning regimens lead to generation of large amounts of inflammatory cytokines that enhance early host APC activation (Zhang et al, 2002; Ferrara et al, 2003). Crucially, total body irradiation (TBI) induces secretion of pro-inflammatory cytokines (including tumour necrosis factor [TNF], interleukin [IL]-1 and IL-6) (Xun et al, 1994) and direct epithelial damage to the gastrointestinal (GI) tract (Paris et al, 2001). Translocation of lipopolysaccharide and other putative bacterial-derived glycolipids across damaged intestinal mucosa activates the innate immune system and promotes the inflammatory cytokine cascade (Hill et al, 1997; Hill & Ferrara, 2000).

Donor T-cell activation

Recent data have demonstrated that naïve but not memory donor T cells are capable of inducing acute GVHD (Anderson et al, 2003; Zhang et al, 2004). This finding has reignited the concept of specific (naïve) donor T-cell depletion with the concept of transferring only memory T cells that contain responses to nominal infectious antigens. The reasons for the inability of memory T cells to induce GVHD are not yet clear but may relate to ineffective trafficking to lymphoid organs (due to the absence of adhesion molecules like CD62L), restricted T-cell receptor (TCR) repertoire (i.e. a very low precursor frequency of host reactive T cells), or combinations of both.

Graft-vs.-host disease requires the stimulation of naïve donor T cells by APC, and a large body of recent work has focused on the requirement for alloantigen expression by specific cellular compartments. Induction of CD4-dependent
GVHD requires the expression of alloantigen on APC, but not target tissue epithelium, whereas maximal CD8-dependent GVHD requires alloantigen to be expressed on both APC and target tissue (Teshima et al., 2002). Although residual host APC alone are absolutely required and sufficient for the induction of CD8-dependent GVHD (Shlomchik et al., 1999), once initiated GVHD may be amplified by donor-derived APC, thought to be attributable to donor APC cross-priming alloreactive CD8+ T cells (Matte et al., 2004). In contrast, the efficient presentation of exogenous host antigens through the classical major histocompatibility complex (MHC) class II-dependent pathway means that expression of alloantigen on donor APC alone is sufficient to induce CD4-dependent GVHD (Anderson et al., 2005). While differences in the class I and class II major histocompatibility antigens stimulate CD8+ and CD4+ T-cell responses (Sprent et al., 1988), the relative efficacy of either T-cell subset in inducing GVHD following human leucocyte antigen (HLA)-matched SCT remains unclear, although both cell subsets are important in animal models of GVHD directed to minor antigens (Sprent et al., 1988). Minor histocompatibility antigens (miHA) are peptides from polymorphic cellular proteins, encoded by regions outside the MHC and presented within class I or class II MHC (Chao, 2004). The precise nature and importance of individual miHA, and the potential to exclude miHA-reactive T cells specifically, is a major focus for strategies to prevent GVHD. Furthermore, tissue distributions of miHA may differ and, in particular, miHA expressed only by haemopoietic tissues represent a very attractive target for the induction of graft-vs.-leukaemia (GVL) effects without promoting GVHD.

The identity and location of APC responsible for inducing GVHD has also been the focus of intense investigation. The APC family includes dendritic cells (DC), monocytes/macrophages and B cells, and relative roles in the induction of GVHD have been difficult to dissect using available reagents. Much circumstantial evidence suggests that residual host DC are the critical APC subset that initiate GVHD (Zhang et al., 2002; Duffner et al., 2004). Tissue-specific DC populations, such as Langerhans cells (LC), may be required for the induction of organ-specific GVHD. In murine studies, depletion of host LC before transfer of donor T cells effectively prevents the development of skin GVHD (Merad et al., 2004). Interestingly, turnover of host LC appears to be dependent on donor T cells through a Fas-dependent pathway (Merad et al., 2004), and clinical studies have demonstrated that development of complete donor LC chimerism is associated with prior cutaneous GVHD (Collin et al., 2006). Conversely, host B cells seem to play little role and can even inhibit GVHD by virtue of their ability to generate IL-10 following TBI (Rowe et al., 2006). Further advances will require the ability to deplete APC subsets specifically and can be expected to move the field forward rapidly.

Although Peyer’s Patches (PP) have been shown to be a central early site of donor T-cell activation during GVHD in the absence of recipient conditioning (Murai et al., 2003), recent studies have confirmed that lethal GVHD is still induced following myeloablative SCT even in their absence (Welniak...
et al, 2006). Thus, although PP are an early site of donor T-cell activation (Beilhack et al, 2005), they, along with lymph nodes and secondary lymphoid organs, may be redundant during SCT, at least following myeloablative SCT across MHC barriers (Welniak et al, 2006). Whether this is also true following HLA-matched SCT will have important implications for the success of strategies aimed at preventing T-cell trafficking to primary lymphoid organs.

Following cognate interaction with activated APC, CD4+ T cells are driven towards T-helper cell type 1 (Th1)-biased cytokine production (Antin & Ferrara, 1992), promoting T-cell proliferation (IL-2) and further differentiation, so that very large amounts of pro-inflammatory cytokines are generated [particularly interferon γ (IFN-γ), TNF], which induce tissue damage in a MHC-independent fashion (Teshima et al, 2002). In contrast, donor CD8+ T cells differentiate into efficient cytotoxic T lymphocytes (CTL), capable of causing host tissue damage in an MHC-I dependent fashion via their perforin, granzyme and FasL cytotoxic pathways (Kagi et al, 1994; Shresta et al, 1998).

Cytokine- and cell-mediated target tissue damage

The final phase of GVHD is characterised by targeted tissue apoptosis induced by direct cytotoxic effector populations and indirect cytokine-mediated damage (Fig 1). Donor effector populations, including CD4+ T helper (Th) cells, CTL and natural killer (NK) cells, mediate cell-dependent cytotoxicity via the secretion of pre-stored perforin and granzyme or through Fas-FasL, TNF or TNF-related apoptosis-inducing ligand interactions (Kagi et al, 1994; Shresta et al, 1998; Kayagaki et al, 1999a,b). Following lethal irradiation, however, transfer of donor T cells deficient in both perforin/granzyme and FasL still results in severe GVHD (Jiang et al, 2001). Indeed, severe target organ GVHD is still seen following MHC mismatched BMT when recipient mice were bone marrow chimeras in which alloantigen were expressed on host APC but not target epithelium (Teshima et al, 2002). In these studies, target organ damage was prevented by the neutralisation of IL-1 and TNF. IL-1 is a critical promoter of pro-inflammatory immune responses, and polymorphisms in the genes encoding the 10 members of the IL-1 family have important effects on the incidence and severity of experimental GVHD (Cullup & Stark, 2005; Dickinson & Charron, 2005). TNF has central roles at several stages of GVHD, including the enhancement of APC maturation, promotion of cellular trafficking, enhancement of T-cell responses (Hill et al, 2000) and the direct induction of tissue injury (Laster et al, 1988). Tissue injury is further enhanced by macrophage-derived nitric oxide (Garside et al, 1992; Hill & Ferrara, 2000), which, importantly, significantly contributes to GVHD-induced immune suppression (Falzarano et al, 1996; Hongo et al, 2004).

Interferon γ has important effects at a variety of stages during the pathogenesis of acute GVHD, although at the present time, its roles are incompletely understood. Early non-irradiation based studies using IFN-γ deficient (IFN-γ−/−) mice as donors demonstrated a delay in GVHD mortality in the absence of IFN-γ (Ellison et al, 1998). This was associated with a T-helper cell type 2 (Th2)-biased cytokine profile (Ellison et al, 2002) and impairment of CTL function (Puliaev et al, 2004). Surprisingly, however, following TBI-based experimental transplantation, administration of exogenous IFN-γ was associated with reduced experimental GVHD (Brok et al, 1993, 1997) and GVHD was increased in recipients of IFN-γ−/−/grafts (Murphy et al, 1998; Yang et al, 1998). The mechanism responsible for the apparently contradictory effects of IFN-γ remains unclear at this time.

Although the central role of HLA matching in haemopoietic SCT is well established, many recipients of fully-matched sibling allografts still develop GVHD. It has become apparent that other genetic systems affect the development of GVHD. Although miHA contribute to alloreactivity and GVHD, genes controlling inflammatory processes, such as cytokines, chemokines and their receptors, can modulate GVHD. Gene polymorphisms affecting IL-1 (Cullup & Stark, 2005), IL-6 (Fishman et al, 1998), IL-10 (Cooke & Ferrara, 2003; Lin et al, 2003), TNF (Cavet et al, 1999), transforming growth factor β (TGF-β) (Hattori et al, 2002) and IFN-γ (Cayabyab et al, 1994) have all been implicated in the incidence and severity of GVHD, both in experimental models and immuno-genetic analysis of retrospective clinical data [recently reviewed (Dickinson & Charron, 2005)].

Diagnostic difficulties

A major recurring difficulty for transplant physicians is the ability to differentiate immune-mediated GVHD damage from tissue injury induced by other processes (Atkinson et al, 1988). The definitive diagnostic procedure remains the demonstration of GVHD in biopsy specimens from affected organs, although this is associated with inherent delays due to difficulties in obtaining specimens, processing of samples and examination by an experienced histopathologist. Even when appropriate specimens are available histological diagnosis may still be difficult, and there may be significant overlap in the pathological appearances of acute GVHD and damage attributable to direct radiation damage, drugs, infectious agents and microangiopathy. Although novel diagnostic techniques, such as serum proteomic analysis (Kaiser et al, 2004; Srinivasan et al, 2006), may allow accurate and rapid diagnosis of GVHD in the future, at the present time differentiation of GVHD from other pathological processes frequently still rests on the input of experienced transplant clinicians (Deeg & Antin, 2006).

The emerging roles of novel cell populations in GVHD

The fine control of alloreactivity following SCT reflects a complex set of interactions between the innate and adaptive...
immune systems including NK T cells (NKT cells), NK cells and γδ T cells. Alloreactivity may be further modulated by regulatory cell populations including regulatory T cells (T_{reg}), regulatory APC populations and perhaps, mesenchymal stem cells.

NKT cells

In the late 1980s, a number of groups independently reported the existence of a distinct subset of γδ T cells in mice which secreted large amounts of potentially immunoregulatory cytokines, including IFN-γ, IL-4 and TNF (Budd et al., 1987; Ceredig et al., 1987; Fowlkes et al., 1987). The population was subsequently shown to express the C-type lectin, NK1.1, previously thought to be restricted to NK cells (Sykes, 1990; Arase et al., 1993), and is now known to include a diverse variety of functionally distinct cell types, collectively referred to as NKT cells. NKT cells are dependent on the presentation of hydrophobic glycolipid molecules by the MHC class-I like molecule, CD1d, for positive selection within the thymus and subsequent activation in the periphery. Type 1 NKT cells (also referred to as invariant or iNKT cells) are further characterised by their specific recognition of the synthetic glycolipid, α-galactosylceramide (α-GalCer), and may therefore be identified via their specificity for α-GalCer-loaded CD1d-tetramer. Type 1 NKT cells express highly conserved TCRs consisting of an invariant TCR α chain and a limited selection of TCR β chains due to marked skewing of Vβ gene usage. In mice, both CD4^+ and double negative (DN; CD4^-CD8^-) subsets are recognised, existing in different proportions in different tissues (Eberl et al., 1999). Type 1 NKT cell recognition of glycolipid antigens characteristically leads to rapid production of immuno-modulatory cytokines, particularly IFN-γ and IL-4 (Crowe et al., 2003). The functional significance of different subsets is becoming clear, and it appears that CD4^+ type 1 NKT cells produce both Th1 and Th2 cytokines, whereas DN NKT cells produce predominantly Th1 cytokines (Lee et al., 2002) and may have more important roles in tumour surveillance (Crowe et al., 2005; Morris et al., 2005). Type 2 NKT cells have been less well characterised, largely because they cannot be specifically identified using conventional technologies (Godfrey et al., 2004a).

The immuno-modulatory effects of NKT cells following allogeneic transplantation are critically dependent on whether donor or residual host NKT cells predominate. The Strober group has demonstrated that specific enrichment of host NKT cells by conditioning with total lymphoid irradiation and anti-lymphocyte globulin significantly abrogates GVHD in both preclinical (Lan et al., 2001, 2003) and clinical studies (Lowsky et al., 2005) and this appears dependent on the ability of NKT cells to generate IL-4 (Lan et al., 2001; Higuchi et al., 2002). Consistent with this, the administration of α-GalCer to recipients in a mouse model of allogeneic SCT following sublethal irradiation significantly reduced GVHD (Morecki et al., 2004), due to residual host NKT cell-dependent Th2 polarisation of donor T cells (Hashimoto et al., 2005). The host NKT cell subset responsible for this effect has not been characterised. Conversely, donor DN NKT cells within the stem cell graft are associated with increased GVHD severity but significantly augment GVL activity (Morris et al., 2005). We recently reported that stem cell mobilisation with potent granulocyte colony-stimulating factor (G-CSF) analogues can modulate the NKT cell compartment, with dramatic effects on both GVHD and GVL responses (discussed below). Thus NKT cells represent an important new cell subset that may be modulated to improve GVHD and GVL outcomes.

NK cells

Natural killer cells are a unique lymphocyte population that participate in complex bi-directional interactions with APC and are central to the co-ordinated induction of innate and primary adaptive immunity (Degli-Esposti & Smyth, 2005). Although heterogeneous, two broad functional subsets can be determined in humans: CD56^bright NK cells, which produce large amounts of immuno-modulatory cytokines (including IL-2, IL-12 and IL-18), and CD56^dim NK cells, which are highly cytotoxic (mediated predominantly via the perforin pathway) (Cooper et al., 2001). NK-cell reactivity is tightly controlled through a balance between activating and inhibitory receptors (Lanier, 2005). Lysis of autologous targets is physiologically prevented through NK-cell expression of inhibitory killer cell immunoglobulin-like receptors (KIR) that recognise self class I MHC molecules. Normal NK-cell development dictates that NK cells tolerate healthy autologous cells due to the interaction of the particular inhibitory KIR receptor with autologous HLA class I ligands (Valiante et al., 1997).

Natural killer cell cytotoxicity contributes directly to the cellular effector stage of GVHD (Hill & Ferrara, 2000; Ferrara & Yanik, 2005), but recent data suggest that following haplo-identical SCT NK cell alloreactivity may in fact be beneficial. Bi-directional T-cell alloreactivity would clearly be predicted to represent a major barrier to haplo-mismatched transplants due to prevention of engraftment (host-vs.-graft alloreactivity) or conversely, overwhelming GVHD. The use of high-dose, vigorously T cell depleted grafts in conjunction with highly immuno-suppressive conditioning regimens may overcome these barriers (Dey & Spitzer, 2006). Adequate T-cell depletion of grafts is critical and may be achieved through negative selection and T-cell depletion or positive CD34 or CD133 stem cell selection (see below) (Bitan et al., 2005; Lang et al., 2005). The optimal method ensuring maximal T-cell depletion without excessive loss of progenitor cells remains to be determined. In clinical studies to date engraftment rates have been impressive (>90%), and the incidence of GVHD has been low with persistence of GVL effects, at least against myeloid malignancies (Aversa et al., 1998). In this specific setting, donor NK cells recognise the absence of donor class I on recipient APC and leukaemia cells and eliminate both, reducing GVHD whilst mediating GVL respectively (Ruggeri et al., 2002). Importantly, since NK cells are not dependent on
stimulation by host APC to respond to host tissues, they retain the capacity to induce GVL reactions even after host APC have been cleared (Ruggeri et al, 1999).

**Regulatory T cells**

Central tolerance is classically attributed to clonal deletion of self-reactive T cells in the thymus upon interaction with self-antigens. However, since not all self-antigens gain access to the thymus, central tolerance cannot be complete and the induction of tolerance in the periphery is critical for prevention of autoimmunity and maintenance of immune homeostasis. T_{reg} were originally identified as a CD4^{+} CD25^{+} population of γδ T cells that permitted the acceptance of allogeneic skin grafts (Sakaguchi et al, 1995), and may be characterised by their expression of FoxP3 (Fontenot et al, 2005a). In mouse models of allogeneic SCT, depletion of T_{reg} within grafts accelerates GVHD (Cohen et al, 2002; Hoffmann et al, 2002; Taylor et al, 2002), whereas adoptive transfer of large numbers of donor-derived T_{reg} effectively prevents GVHD (Cohen et al, 2002; Hoffmann et al, 2002). Although suppression of proliferation and function of conventional T cells might be predicted to impair cellular GVL effects, a number of studies have demonstrated the persistence of immune-mediated GVL effects (Edinger et al, 2003; Jones et al, 2003; Trenado et al, 2003). The mechanisms for maintenance of GVL effects remain to be clearly defined, but since GVHD is immunosuppressive in its own right (Krenger et al, 1996; Ferrara et al, 1997) it is plausible that suppression of GVHD by T_{reg} allows residual cellular effectors (including T cells and NK cells) to mount effective GVL responses. Importantly, the absolute numbers of alloreactive T cells may have critical effects, with relatively small numbers being sufficient to mount a GVL effect, but larger numbers of T cells driving robust alloreactive responses and GVHD (Edinger et al, 2003). Nguyen et al (2006) have recently demonstrated that infusion of conventional and T_{reg} subsets at almost physiological doses (10:1) prevented dramatic early proliferation of effector T cells, but allowed the persistence of sufficient numbers to mount an effective GVL response.

Stem cell mobilisation with G-CSF limits the ability of T cells to induce GVHD on a per cell basis (Morris et al, 2006) and enhanced T_{reg} activity has been demonstrated in patients following G-CSF administration (Rutella et al, 2002). Studies from our laboratory have recently demonstrated that protection from GVHD may be provided by stem cell mobilisation with the pegylated form of G-CSF (peg-G-CSF) (Morris et al, 2004). Donor T cells from peg-G-CSF treated donors had impaired proliferation in response to alloantigen stimulation and inhibited the alloreactivity of donor T cells following SCT in an IL-10 dependent manner (Morris et al, 2004). Importantly, despite reducing GVHD, GVL effects were enhanced due to expansion of donor NKT cells with enhanced functional activity following SCT (Morris et al, 2005).

Although standard GVHD prophylaxis includes IL-2 blockade by calcineurin inhibitors (see below), T_{reg} suppressor function is dependent on both IL-2 signalling (Fontenot et al, 2005b) and TCR ligation (Thornton et al, 2004). In murine studies, Zeiser et al (2006) demonstrated that culture of T_{reg} with cyclosporine (CSA) suppressed T_{reg} function in vivo and in vitro (determined by protection from GVHD). Conversely, alternative immune suppressants, such as sirolimus and mycophenolate mofetil (MMF), did not impair T_{reg} function. Clearly the nature of immune suppression may have significant effects on the functional activity of T_{reg} and future clinical trials examining combinations of agents that spare T_{reg} function are needed.

**γδ T cells**

Another novel T-cell population, γδ T cells, further contribute to the bridging of innate and adaptive immune responses (Hayday & Tigelaar, 2003). Although γδ T cells undergo somatic DNA rearrangement to form their TCR (Asarnow et al, 1988), they recognise both protein and non-protein moieties in the absence of MHC (and therefore do not require professional APC for activation) and react to patterns associated with tissue injury or pathogen invasion (Chien & Bonneville, 2006). Epithelial-based γδ T cells release pro-inflammatory cytokines (including IFN-γ and IL-4) and chemokines that augment mucosal immunity early in the immune response (Carding & Egan, 2002). Following an effective immune response, γδ T cells may promote its termination through the production of anti-inflammatory cytokines, such as IL-10 (Hayday & Tigelaar, 2003). Intriguingly, although γδ T cells are rare in peripheral blood and lymph nodes, they are seen in significantly larger numbers in the skin and GI tract, both of which are targets of GVHD.

Using murine models, T cells from donors transgenically modified to express high levels of γδ heterotrimerers facilitated engraftment of MHC mismatched bone marrow grafts, albeit at the expense of increased GVHD severity (Blazar et al, 1996). Drobyski et al (2000) examined the immuno-modulatory effects of transfer of ex vivo IL-2 activated γδ T cells on GVHD in a model of donor lymphocyte infusion (DLI) following fully MHC mismatched BMT. Transfer of γδ T cells and γδ T cells at day 0 significantly increased GVHD when compared with recipients of γδ T cells alone. Maeda et al (2005a) examined the role of recipient γδ T cells following fully MHC mismatched BMT. The presence of recipient γδ T cells was associated with increased GVHD severity, demonstrated to be the result of enhanced host APC activation and alloantigen presentation. In contrast, recipient γδ T cells do not appear to influence chronic GVHD directed to miHA (Anderson et al, 2004).

**Regulatory APC**

Peripheral blood contains two distinct populations of DCs: conventional DCs (cDC) and plasmacytoid DCs (pDC), both of which are myeloid in origin at steady state (MacDonald et al, 2005a). cDC are CD11c^{hi}, CD13^{+} and CD33^{+} and require
the presence of granulocyte-macrophage CSF (GM-CSF) for survival (Caux et al., 1992; Young et al., 1995). Furthermore, cDC express high surface levels of co-stimulatory molecules, secrete large amounts of cytokines (including IL-12) when stimulated with TNF or CD40L, and can initiate primary T cell-dependent immune responses biased towards Th1 cytokine profiles (Macatonia et al., 1995; Cella et al., 1996). Several recent studies, however, have suggested a role for cDC in the development of peripheral tolerance through the induction of Treg populations. The ability of cDC to induce immunity or tolerance is critically linked to maturation, RelB activity, and CD40 expression (Dhodapkar et al., 2001; Martin et al., 2003). Immature cDC generated from murine bone marrow induced T-cell unresponsiveness in vitro and prolonged cardiac allograft survival in vivo (Lutz et al., 2000). Immature cDC have also been shown to induce CD4+ Treg in vitro and CD8+ Treg in vivo, both of which produce high levels of IL-10 and low levels of IFN-γ (Jonuleit et al., 2000; Dhodapkar et al., 2001). Sato et al. (2003) described the generation of regulatory host type DC, by differentiation in the presence of IL-10, that induce transplant tolerance via the induction of regulatory CD4+CD25+ T cells. These regulatory DC were required to express host MHC to promote tolerance, demonstrating a requirement for direct antigen presentation.

Plasmacytoid DC may be defined as HLA-DR+/CD11c−, CD4− and IL-3Rα− and are dependent on IL-3 (but not GM-CSF) for survival and differentiation (Grouard et al., 1997; Kohrgruber et al., 1999). pDC preferentially express a distinct profile of toll-like receptors (TLR7 and TLR9) which respond to single stranded RNA and DNA viruses with rapid production of type 1 IFN (IFN-a and IFN-b) (Asselin-Paturel & Trinchieri, 2005). pDC may promote Th2-biased T-cell responses (Shortman & Liu, 2002) and can also induce Treg (Wakkach et al., 2003), a process that occurs predominantly within lymph nodes (Ochando et al., 2006). Haemopoietic stem cell mobilisation is known to augment pDC numbers within the blood, and this has been mooted as a major pathway of immune modulation by G-CSF (Kared et al., 2005). The adoptive transfer of splenic-derived cDC or pDC from mobilised donor mice failed to confer protection from GVHD (MacDonald et al., 2005b). However, it is possible that immature marrow- or blood-derived pDC will have more tolerogenic properties and this will require further study. We have demonstrated that stem cell mobilisation expands a novel granulocyte–monocyte precursor population (termed GM cells) (MacDonald et al., 2005b) that promote transplant tolerance and prevents GVHD by MHC class II-restricted generation of IL-10-secreting, antigen-specific Treg. These effects are all enhanced by mobilisation with newer potent G-CSF analogues and their effects in large clinical trials are awaited.

**Mesenchymal stem cells**

Mesenchymal stem cells (MSC) are multipotential non-haemopoietic progenitor cells, resident within the bone marrow, that are able to differentiate into the various lineages of the mesenchyme (including chondrocytes, myocytes and neurons) (Pittenger et al., 2002). MSC are characterised by the absence of haemopoietic markers (CD45− and CD34−) and the expression of specific patterns of adhesion molecules (including CD106 and CD105+). MSC have been shown to suppress primary and secondary in vitro mixed lymphocyte reactions (MLR) in an indoleamine 2,3-dioxygenase (IDO)-dependent fashion (Meisel et al., 2004) and, in murine models, delay skin graft rejection across MHC barriers (Bartholomew et al., 2002), possibly via contact-dependent induction of regulatory APC populations (Beyth et al., 2005). Lazarus et al. (2005) demonstrated the feasibility of administering bone marrow-derived, ex vivo expanded MSC to 56 recipients of HLA-identical sibling allografts following myeloablative conditioning. Grade II to IV GVHD was seen in 28% of recipients and chronic GVHD in 61% of patients surviving beyond day 100. Further randomised clinical studies are needed to confirm the efficacy of MSC in modulating GVHD.

**Therapeutic strategies against acute GVHD**

Prophylactic immune-suppression remains central to the management of GVHD in clinical practice. The cornerstones of GVHD prophylaxis remain the combination of methotrexate (MTX) and calcineurin inhibitors (Chao & Chen, 2006), although the optimal calcineurin inhibitor (CSA or tacrolimus) remains the focus of debate (Ratanatharathorn et al., 1998; Nash et al., 2000; Hiraoka et al., 2001; Yanada et al., 2004). Novel strategies will ultimately aim to potently inhibit GVHD with relative preservation of anti-microbial immunity and immunological anti-tumour effects.

**Preventative strategies**

**IL-2 inhibition: novel calcineurin inhibitors**

Sirolimus (also known as Rapamycin) is a novel macrolide which has received recent attention because of its combination of immunosuppressive, anti-fungal, anti-viral and anti-neoplastic properties (Cutler & Antin, 2004). Excitingly, this agent does not appear to inhibit Treg function in vivo (Zeiser et al., 2006) and may even augment activity due to TGF-β dependent induction of Treg (Battaglia et al., 2005). Although structurally related to other calcineurin inhibitors, it has a novel mode of action and appears to exert some of its immunosuppressive properties via inhibition of DC activity through interference with antigen uptake (Monti et al., 2003), cellular maturation (Hackstein et al., 2003), intracellular signalling (Chiang et al., 2002) and apoptosis induction (Woltman et al., 2003). Cutler and Antin (2004) described the outcomes of three clinical trials using Sirolimus as prophylaxis against GVHD after allogeneic SCT. They reported excellent GVHD control even when mismatched and unrelated donors were used, and reduced mortality associated with transplantation due to a reduction in the
necessity to omit or reduce doses of prophylactic MTX. Although rates of cytomegalovirus reactivation and invasive fungal infection were low, concern was noted regarding increased rates of thrombotic microangiopathy. Randomised clinical studies examining the combination of Sirolimus with a variety of immunosuppressive reagents are eagerly awaited.

**IL-2 inhibition: monoclonal antibodies**

Dacluzimab (Zenapax), basiliximab (Simulect) and inolimomab are monoclonal antibodies that bind to the IL-2Rα receptor (CD25) and inhibit IL-2 signalling. These molecules, as expected, all have activity in the SCT setting although they are limited by their high cost. Lee et al (2004) examined the utility of first-line therapy of acute GVHD with steroids and dacluzimab in a randomised study. The study was stopped early due to increased relapse and GVHD mortality in the dacluzimab arm. Other studies in the setting of steroid refractory GVHD have yielded conflicting results (Massenkeil et al, 2002; Bay et al, 2005; Wolff et al, 2005; Teachey et al, 2006), but have generally not been superior to other approaches. Perhaps the real advantage of these agents is their lack of toxicity (save immune suppression), which potentially makes them ideal agents in the setting of calcineurin-related toxicity [e.g. renal failure, thrombotic thrombocytopenic purpura (TTP)] to provide effective IL-2 inhibition until such time as calcineurin inhibitors can be re instituted (Wolff et al, 2006).

**T-cell depletion.**

CD52 is expressed on human T and B cells, monocytes/macrophages and DC. Campath-1H (alemzumab) is a humanised rat monoclonal anti-CD52 antibody that can be used intravenously for in vivo T-cell depletion or added directly to the stem cell graft for in vitro purging (Chakrabarti et al, 2004). The use of Campath in vivo following unrelated donor myeloablative transplantation or as part of non-myeloablative regimens is associated with durable engraftment and a low incidence of GVHD, but at the expense of a significant delay in immune reconstitution (Kottaridis et al, 2001; Chakrabarti et al, 2002) and the approach of general T-cell depletion significantly impairs the GVL effect (Gale & Horowitz, 1990; Horowitz et al, 1990).

Pan T-cell depletion can be refined with highly specific depletion of alloreactive T-cell subpopulations. Following in vitro stimulation in MLR, alloreactive T cells can be identified by the expression of surface activation markers (including CD25 and CD69), proliferation or preferential retention of photoactive dyes. Alloreactive cells can be eliminated using immunotoxins (Montagna et al, 1999), immuno-magnetic separation (van Dijk et al, 1999; Koh et al, 1999; Davies et al, 2004), flow cytometric cell sorting (Godfrey et al, 2004b) or photodynamic purging (Martins et al, 2004). These techniques aim to eliminate host reactive T cells and allow transfer of the remainder of T cells to provide immunity to microbial antigens, and early clinical results are encouraging (Amrolia et al, 2006).

Perhaps most exciting is the potential to specifically deplete native T cells from the stem cell graft (on the basis of CD45RA or CD62L) in order to retain immunity to microbial antigens within the memory T-cell compartment. This approach lends itself to available large scale separation techniques and thus clinical trials of this approach are eagerly awaited. The approach is likely, however, to eliminate effective GVL and preclinical studies are awaited to document the potential GVL effects retained in central and effector memory T-cell populations. If, as might be expected, these memory T cells retain minimal GVL effects, subsequent DLI-based approaches will be required in the clinic. Nevertheless, this remains the most promising new approach for improving transplant outcome for some time and was born from innovative preclinical studies (Anderson et al, 2003; Zhang et al, 2004).

**Stem cell selection.**

An alternative approach to T-cell depletion is the implicit exclusion of alloreactive populations via the specific positive selection of haemopoietic stem cells. A number of groups have demonstrated excellent rates of engraftment and low incidence of severe GVHD following transplantation with CD34⁺ selected grafts, typically using immune-adsorption techniques (Finke et al, 1996; Matsuda et al, 1998; Lang et al, 2003, 2004), although high rates of GVHD were observed in some early studies (Bensinger et al, 1996). Furthermore, Cao et al (2001) reported the effects of CD34-enrichment via density-gradient separation. Although the incidence of acute GVHD was low (26%), there was a surprisingly high rate of non-relapse related mortality (62% by day 180) including infection (fungal and viral), venoocclusive disease, TTP and idiopathic pneumonia syndrome. Further studies examining immune reconstitution and antitumour effects are awaited, both of which would be predicted to be compromised by this approach.

CD133 is a recently described glycoprotein (Yin et al, 1997) reported to identify a CD34⁺ stem cell population with repopulating capacity, likely to be the precursor population of CD34⁺ cells (Bhatia et al, 1998). Lang et al (2004b) recently reported the feasibility of transplantation using magnetically selected CD34⁺ and CD133⁺ grafts in a paediatric population. All patients engrafted and although CD133-selection was associated with a lower depletion of T cells when compared with CD34⁺ selection, no GVHD greater than grade 2 was seen. Bitan et al (2005) reported the successful use of CD133-selected stem cell grafts in patients undergoing haplotype mismatched transplantation. Again, all patients engrafted and no patients developed acute GVHD although overall mortality was high.

An alternative strategy involves a combined approach utilising CD34⁺ selection in combination with addition of defined doses of purified CD3⁺ T cells at the time of transplant (Bunin et al, 2006) or planned DLI (Goerner et al, 2003). Although initial reports are encouraging, with acceptable rates of GVHD and apparent persistence of GVL effects, further data is required.
FTY720. FTY720 is a novel class of immuno-modulatory agent that is agonistic for the sphingosine 1 phosphate receptor and thereby inhibits lymphocyte migration from secondary lymphoid tissues (Chiba et al., 2006). Synergyism with other agents, including CSA and tacrolimus (Furukawa et al., 2000), makes it a very attractive agent for the therapy of GVHD. Preclinical studies have demonstrated that FTY720 potently inhibits the initiation of epithelial GVHD, but importantly GVL effects within the lympho-haemopoietic system were preserved (Kim et al., 2003). Further investigation and clinical translation is eagerly awaited.

Keratinocyte growth factor. Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family with specificity for epithelial tissues expressing its receptors, including the skin, GI tract and liver (Housley et al., 1994; Pierce et al., 1994). Administration of KGF reduced GVHD mortality, whilst retaining GVL effects, in preclinical models (Panoskaltsis-Mortari et al., 1998; Krijanovski et al., 1999; Ellison et al., 2004). Interestingly, the prevention of GVHD by KGF is not attributable to facilitation of repair of conditioning-induced tissue injury (Panoskaltsis-Mortari et al., 2000), but is associated with preservation of thymopoiesis (Rossi et al., 2002), prevention of gut injury (Panoskaltsis-Mortari et al., 1998; Krijanovski et al., 1999; Ellison et al., 2004) and relative preservation of peripheral regulatory mechanisms (Min et al., 2002). KGF has significant utility for the prevention of chemo- and radio-therapy induced mucositis (Snyder et al., 2006), and the effects of KGF on the incidence and severity of GVHD following allogeneic transplantation are currently under investigation. Although other interventions, such as the administration of IL-7 may also promote thymic T-cell generation (Snyder et al., 2006), reports of increased GVHD severity in preclinical models (Sinha et al., 2002) suggests that caution must be exercised.

Treatment strategies

Therapy with high dose methylprednisone remains the gold-standard therapy for the treatment of acute GVHD developing despite prophylaxis. One of the largest studies examining primary therapy of acute GVHD prior to 2000 reported a disappointing complete response rate of 35% and overall responses of 53% (MacMillan et al., 2002). Additional therapeutic strategies for incomplete responders have included the addition of pharmacological immune suppressants, such as MMF, antibody therapies, such as polyclonal antithymocyte globulin (ATG) or monoclonal TNF inhibitors, and novel procedures, such as extracorporeal photochemotherapy (ECP).

Antithymocyte globulin. A number of studies have examined the efficacy of pan T-cell ablation with polyclonal ATG. Van Lint et al. (2006) have recently reported the results of a randomised study in which all patients with acute GVHD were initially treated with methylprednisone 2 mg/kg/d. At day +5 non-responders (n = 61) were randomised to receive either methylprednisolone 5 mg/kg/d alone or methylprednisolone 5 mg/kg/d plus ATG (1.25 mg/kg for 5 doses on alternate days). The authors concluded that although the response rate following the addition of ATG was increased, this was at the expense of increased transplant-related mortality, predominantly attributable to infection. Mollee et al. (2001) described a single centre experience in which patients developing GVHD whilst on CSA, and resistant to steroids, received therapy consisting of cessation of CSA, continuation of steroids and addition of ATG (15 mg/kg for 5 d) and tacrolimus (0.025–0.1 mg/kg/d). Within 28 d of treatment, they reported responses in 70% of patients. Within 6 weeks of therapy, however, 80% of patients developed significant infections, often with multiple organisms. Experience with alternative T-cell depletion antibodies including OKT3 (anti-CD3) and ABX-CBL (anti-CD147) have been largely disappointing (Keever-Taylor et al., 2001; Knop et al., 2005; Benekli et al., 2006; Macmillan et al., 2006).

Thus, although ATG is still the standard therapy for steroid refractory GVHD, the control of subsequent opportunistic infection remains one of the major barriers to improving outcome. It is anticipated that the introduction of new highly active anti-fungal agents and improved molecular-based viral diagnostics and anti-viral drugs may help in this regard.

Extracorporeal photochemotherapy. Extracorporeal photopheresis is based on the immuno-modulatory action of UV-A irradiation on blood mononuclear cells collected by apheresis and photosensitised by 8-methoxypsoralen (Kanold et al., 2005). The mechanism of immune suppression is poorly understood but may involve the re-infusion of irradiated APC and subsequent induction of antigen-specific Treg (Krijanovski et al., 2001; Baudard et al., 2005). ECP has been investigated as a therapeutic option for the treatment of steroid refractory acute GVHD and chronic GVHD (Greinix et al., 2006), although positive results from randomised, prospective studies are needed to definitively establish efficacy.

Mycophenolate mofetil. Mycophenolate mofetil is the prodrug of the potent immune-suppressant mycophenolic acid, a non-competitive reversible inhibitor of inosine monophosphate dehydrogenase, which blocks de novo synthesis of guanosine nucleotides (Lipsky, 1996). Lymphocytes depend on this pathway because they do not possess the salvage pathways of other cells. MMF has been examined in limited trials for the treatment of acute GVHD (Mookerjee et al., 1999; Basara et al., 2001; Baudard et al., 2002), with some efficacy. The toxicity profile of MMF, particularly its lack of renal toxicity and cross-reactivity with CSA, make MMF an attractive candidate, although bone marrow suppression and reports of high rates of infection (Baudard et al., 2002) counsel caution. Unfortunately, a recent study demonstrated that the addition of MMF to...
CSA did not offer any benefit over standard CSA and MTX (Nash et al., 2005), and the main use of MMF may therefore be in the setting of steroid-refractory GVHD (Kennedy et al., 2006).

**TNF inhibition.** Since TNF has a central role in the pathogenesis of GVHD, it represents a logical target for inhibition. Currently, there a number of clinically available anti-TNF monoclonal antibody preparations: infliximab (Remicaid – chimeric mouse/human anti-TNF antibody), adalimumab (Humira – fully humanised anti-TNF antibody) and etanercept (Embrel – recombinant human TNF receptor type II fusion protein). It must be noted that there are important differences between these molecules which may translate to differences seen in clinical trials (Ehlers, 2003). Infliximab and adalimumab bind with high affinity to both soluble and membrane-bound TNF, and do not release TNF once bound. They also bind complement and therefore have the ability to lyse cells expressing surface TNF (including tissue macrophages). In contrast, etanercept binds soluble TNF and lymphotoxin (LT) alpha homotrimer (LTα3) but has very low affinity for membrane-bound TNF (Scallon et al., 1995) and does not induce cellular lysis. Furthermore, etanercept rapidly releases bound TNF and LT, and therefore rapidly binds them in environments where they are abundant, and rapidly releases them where concentrations are low (Ehlers, 2003).

Couriel et al. (2004) reported 21 patients with steroid-refractory GVHD treated with infliximab. The CR rate was impressive at 62%, but infection rates were high. Uberti et al. (2005) examined the use of combined therapy with etanercept, methyprednisone and tacrolimus as primary therapy in 20 patients with acute GVHD. Again the response to therapy was impressive, with 75% of patients achieving CR. Kennedy et al. (2006) recently reported a retrospective analysis of 16 patients with refractory GVHD treated with a combination of ATG, tacrolimus and etanercept. Again, overall response rates were impressive at 81%, and were superior to historical data in patients receiving ATG alone.

Thus TNF appears an attractive, logical and non-overlapping target (in conjunction with T cell-directed therapy) for the treatment of steroid-refractory GVHD. While combined therapy with anti-T cell therapies (e.g. ATG or CD25 inhibition) and TNF inhibition in this setting has become routine therapy in many units, prospective randomised trials are urgently required.

**Concluding remarks**

Acute GVHD remains one of the major barriers to the advancement of allogeneic haemopoietic transplantation. Advances in our knowledge of the pathophysiology of GVHD and mechanisms of peripheral tolerance promises significant advances in the near next few years. Further investigation of combinations of pharmacological immune suppressants, particularly agents with the ability to spare beneficial regulatory populations in combination with cytokine inhibition and specific removal of alloreactive T cells, will be of great interest. The continued high rates of infectious complications following intensive immune suppression remain of great concern, but aggressive anti-fungal prophylaxis and the promotion of early reconstitution of innate and adaptive anti-microbial immune responses should begin to redress the balance.

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