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Bone marrow transplantation and approaches to avoid graft-versus-host disease (GVHD)

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Haematopoietic stem cell transplantation (HSCT) offers promise for the treatment of haematological and immune disorders, solid tumours, and as a tolerance inducing regimen for organ transplantation. Allogeneic HSCTs engraftment requires immunosuppression and the anti-tumour effects are dependent upon the immune effector cells that are contained within or generated from the donor graft. However, significant toxicities currently limit its efficacy. These problems include: (i) graft-versus-host disease (GVHD) in which donor T cells attack the recipient resulting in multi-organ attack and morbidity, (ii) a profound period of immune deficiency following HSCT, and (iii) donor graft rejection. Currently available methods to prevent or treat GVHD with systemic immunosuppression can lead to impaired immune recovery, increased opportunistic infections, and higher relapse rates. This review will provide an overview of GVHD pathophysiology and discuss the roles of various cells, pathways, and factors in the GVHD generation process and in the preservation of graft-versus-tumour effects. Variables that need to be taken into consideration in attempting to extrapolate preclinical results to the clinical paradigm will be highlighted.

Keywords: graft-versus-host disease; bone marrow transplantation; graft versus leukaemia; cytokines; allogeneic

Bone marrow transplantation (BMT), or perhaps more accurately, haematopoietic stem cell transplantation (HSCT), offers great promise for the treatment of a variety of diseases ranging from cancer, autoimmunity, aplastic anaemia and other diseases of haematopoietic origin. BMT originated as a means to repopulate the haematopoietic stem cell compartment after myeloablative exposure to radiation as a means of 'rescue'. It has evolved into a means to induce anti-tumour responses when used in cancer. HSCT can be either autologous (the transferred haematopoietic stem cells (HSCs) are obtained from the patient) or allogeneic (HSCs are from a donor that is, optimally, totally matched at the major histocompatibility complex (MHC) loci and is related to the patient). Classically, HSCT involves some means of cytoreductive conditioning the recipient either by irradiation and/or chemotherapy. Initially HSCs were provided exclusively by bone marrow cells (BMC). Subsequently, other sources that contain HSCs have shown efficacy for haematopoietic reconstitution including mobilized peripheral blood, and more recently, cord blood. The advent of non-myeloablative or reduced intensity conditioning has allowed HSCT3 to be performed in patients with advanced age due to lesser toxicities from the use of lower cytoreductive conditioning. Allogeneic HSCTs engraftment requires immunosuppression and the anti-tumour effects are dependent upon the immune effector cells that are contained within or generated from the donor graft. The latter is supported by earlier observations that the relapse rates for allogeneic BMT were markedly lower than autologous BMT due to the occurrence of graft-versus-tumour (GVT) effects after the transplant. However, allogeneic HSCT is hampered by significant toxicities, which currently limit its efficacy. These problems include: (i) graft-versus-host disease (GVHD) in which donor T cells attack the recipient resulting in multi-organ attack and morbidity, (ii) a profound period of immune deficiency following BMT leaving the patient susceptible to opportunistic infections, and (iii) donor graft rejection. Unfortunately, common means to prevent or treat GVHD with systemic immunosuppression (i.e. by corticosteroids and use of cyclosporin A) can lead to impaired immune recovery, increased opportunistic infections, and higher relapse rates. This review will discuss current strategies in the prevention and treatment of GVHD. The review will begin with an overview on the biology of GVHD, then discuss the roles of various cells, pathways, and factors in the GVHD generation process along with providing an overview on the use of different animal models used to study GVHD and variables that need to be taken into consideration in attempting to extrapolate preclinical results to the clinical paradigm. The review will conclude with discussions on developing means to prevent and treat GVHD without hampering GVT.

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One contribution of 16 to a Theme Issue 'Immunoregulation: harnessing T cell biology for therapeutic benefit'.

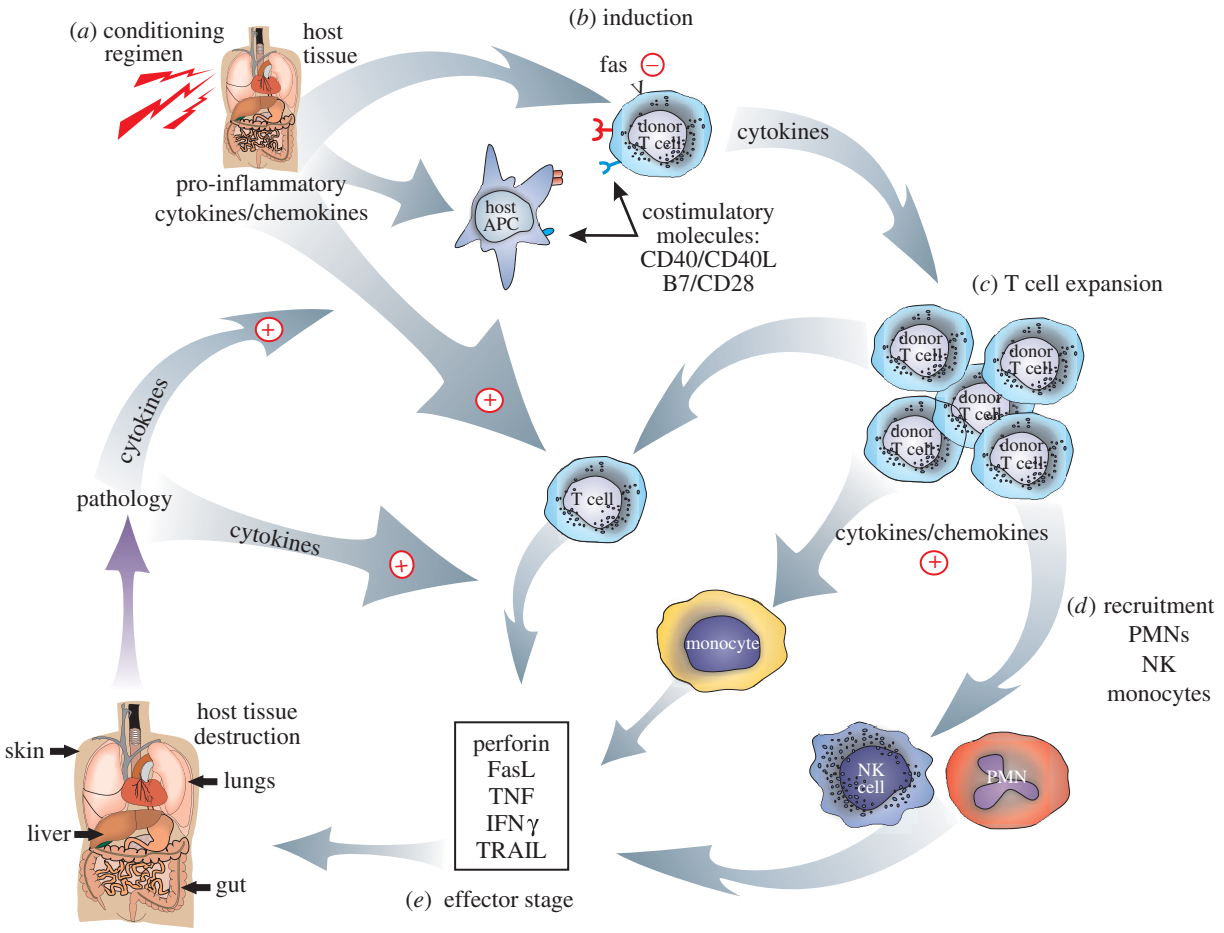

Figure 1. Overview of GVHD pathophysiology.

1. GVHD

(a) *GVHD pathophysiology*

GVHD has a complex aetiology but ultimately is the result of donor T cell attack of an immunocompromised and genetically disparate recipient and is a significant cause of morbidity following allogeneic BMT. Unfortunately, these same donor T cells also mediate GVT effects following HSCT as evidenced by studies demonstrating that prior removal of T cells in the donor graft prevents GVHD occurrence but also can increase tumour relapse rates especially for patients with certain types of haematological malignancies.

GVHD has been postulated to occur in several stages. The first stage in GVHD induction revolves around the cytoreductive conditioning or immunosuppression used to allow for donor cell engraftment (figure 1). Particularly for conventional (fully myeloablative) BMT, the extensive cytoreductive conditioning results in the release of proinflammatory cytokines which affects host antigen presenting cells (APCs; by increasing their maturation and upregulation of costimulatory molecules/cytokines) and helps fuel the alloreactive donor T cell response. It has been shown in murine models that host APCs can play a particularly important role in acute GVHD induction (Shlomchik *et al.* 1999). This is followed by the 'induction phase' which involves classical T cell recognition involving cell surface events that result in T cell receptor (TCR) ligation (providing a first signal to the T cell) and engagement of one or more costimulatory molecules (providing a second signal to the T cell); both TCR ligation and costimulation must be delivered to achieve full T cell activation and subsequent expansion. Costimulation of donor T cells is typically provided by ligands expressed on the surface of host APCs. As the alloreactive T cells expand (expansion phase) they home to various GVHD target organs (skin, gut, liver, lung), a process controlled by adhesion molecules, integrins and chemokines. The production of chemokines in inflamed and injured tissue also results in the recruitment of other cell-types (neutrophils, natural killer (NK) cells, monocytes) to the GVHD target organ sites further contributing to GVHD pathology. The 'effector phase' is noted for the destruction of host tissue by immune effector molecules (e.g. fas ligand, perforin, granzymes, interferon-gamma (IFNγ), tumour necrosis factor (TNF)) and results in the continued production of proinflammatory cytokines which then continues to fuel the entire GVHD process (figure 1). This 'cytokine storm' during the recruitment and effector phases is particularly difficult to control. Thus, many attempts to arrest GVHD have focused on targeting early during the induction phase before the massive T cell expansion, homing/migration and cytolytic process has been fully developed.

Clinical GVHD occurs in 'acute' and 'chronic' forms. The designation was initially applied based on differences in time of onset clinically with acute GVHD occurring shortly after BMT and chronic GVHD being more delayed (after 100 days). It now is clear that these are two separate pathophysiological entities with different aetiologies, target organs, therapeutic

Table 1. Variables in the study of experimental murine BMT.

parameters	examples
intensity of conditioning regimen	no conditioning
	sublethal conditioning without stem cell rescue
	myeloablative conditioning with stem cell rescue
strain disparity	full major and minor MHC disparity
	full or partial major MHC disparity
	minor MHC disparity
	parent into F1
donor graft composition	unfractionated splenocytes and/or lymph node cells
	purified T cells
	purified CD4+ or CD8+ T cells
endogenous microbiota	*Helicobacter*
	orphan parvoviruses

responses and sequelae. However, there are incidences where they can overlap in time after transplantation and GVHD target organ involvement. Acute GVHD targets skin, gut, liver, and lung whereas chronic GVHD appears with primarily skin and mucosal manifestations, immune perturbations, and has many autoimmune characteristics. Predominant means of controlling GVHD, both acute and chronic, is through the prophylactic use of immunosuppression (i.e. corticosteroids and other agents) as GVHD is difficult to treat once ongoing.

(b) *Animals models in the study of GVHD*

Murine and canine models are the two predominant animal models used to study GVHD pathophysiology. Within the animal model studies for GVHD there are key variables to take into consideration prior to drawing conclusions regarding the results and attempting to correlate them to the clinical GVHD scenario (table 1). These factors include (but are not limited to): (i) the presence or absence of, and type, of cytoreductive conditioning used on the recipient, which can range from none to lethal. Lethal cytoreductive conditioning must then be followed by some form of HSC rescue. The conditioning regimen intensity is perhaps one of the most important variables in the different GVHD models. (ii) The strain combinations used is a key variable which can affect the kinetics and extent of GVHD lethality as well as the particular target organs involved. For example, in some strain combinations, $CD4^+$ T cells are the only, major, or are not involved at all in GVHD pathophysiology, whereas in others, $CD8^+$ T cells may be the dominant effectors. The MHC and/or minor histocompatibility (miH) antigens recognized could determine the frequency and quality of the immune response of donor T cells that respond to the host. Variations include donor–recipient pairs with semi-allogeneic (parent-into-F1), full mismatch MHC, multiple miH antigens, isolated MHC antigens, or combinations thereof. (iii) The type and number of the donor immune cells transferred. Many models use donor splenocytes to induce GVHD as the extent of contaminating T cells present in BMC preparations are usually inadequate to induce acute GVHD except in certain strain combinations. T cells from lymph node (LN), bone marrow (BM), or peripheral blood can also be used to supplement BMC preparations. The homing and chemokine receptors expressed on T cells in these various locations are distinct and therefore the adoptive transfer of T cells from these sites may differentially affect GVHD. The donor T cells transferred can be purified based on CD3, CD4 or CD8 antigen expression. Contingent on these variables, different organ pathologies (sometimes selective attack occurs in which only the liver or gut is affected versus other models where haematopoietic attack or autoantibody production results) and kinetics of progression (ranging from days to months) results. The number of donor T cells infused and constituency of T cell subpopulations (TCR γ/δ cells; natural killer T (NK/T) cells; naïve versus memory cells; Th1 versus Th2 cells; regulatory T cells) may also influence the kinetics and pathophysiology of GVHD. (iv) The presence of endogenous microenvironmental pathogens. Another variable is the potential presence of environmental pathogens including those that might cause subclinical infections in the mouse colony where the studies are performed and conversely the use of non-absorbable or systemically administered prophylactic antibiotics to decrease intestinal pathogens. This may affect radiosensitivity, particularly in the gut. All of these issues are important, particularly when assessing potential roles of proinflammatory cytokines and chemokines in GVHD pathobiology. Therefore, care must be taken when correlating different murine studies with clinical GVHD paradigms.

(c) *Donor alloreactive T cell homing to GVHD target organs*

There has been much interest in altering GVHD progression by affecting T cell trafficking and preventing T cells from arriving at the GVHD target organs. T cell homing involves a complex and coordinated response mediated by chemokines and the appropriate adhesion molecules (Wysocki *et al*. 2005). Chemokines are a large family of small proteins which function primarily as chemoattractants (Rollins 1997; Moser *et al*. 2004). The family is subdivided based on the number and position of NH2-terminal cysteine (C) residues. The majority of chemokines fall into the CC (CCL1-28) and CXC (CXCL1-16) subfamilies, while the C family contains only two (XCL1 and XCL2), and CX3 C only one member (CX3CL1; Murphy *et al*. 2000; Murphy 2002). It has been hypothesized that by

selectively targeting certain chemokines involved in T cell trafficking to particular GVHD organs (i.e. skin, gut, liver and lung), GVHD pathology could be blunted without significant impairment of GVT effects, particularly with leukaemia/lymphoma. Since intestinal LNs and specifically Peyer's patches (PP) have been proposed to be critical for the induction of acute GVHD, blocking cellular migration into these organs represents a potential target for GVHD control (Murai *et al.* 2003). Studies have indicated that alloreactive T cells first home to secondary lymphoid organs such as PP after BMT but the role of these organs in the induction of GVHD was unclear (reviewed in Wysocki *et al.* 2005). Studies performed by Murai *et al.* which analysed recipients that received either no conditioning or non-myeloablative conditioning without HSC rescue found that the absence of PP resulted in significant blunting of acute GVHD (Murai *et al.* 2003). This murine model involved the transfer of parental lymphocytes into F1 hybrid recipients and produced only donor-anti-host T cell reactions under non-inflammatory or low inflammatory conditions. However, recently we have found that intensively conditioned tumour necrosis factor (TNF) alpha knockout (KO) recipients, which totally lack PP, developed acute GVHD affecting both gut and liver pathology that was unimpaired in comparison to wild-type mice (Welniak *et al.* submitted). These seemingly contradictory data suggest that the induction of proinflammatory cytokines/chemokines after extensive cytoreductive conditioning can have a dramatic impact on the necessity of secondary organs and chemokines in GVHD induction/progression. This was further illustrated by GVHD studies assessing the role of CCR5, a chemokine receptor that was found in the Murai *et al.* report to be essential for recruitment of donor T cells to PP. Using a similar GVHD model in which no cytoreductive conditioning was applied to examine the role of CCR5 in GVHD, it was reported that use of blocking antibodies to CCR5 resulted in significant abrogation of liver GVHD (Murai *et al.* 1999). In striking contrast, using models in which lethal cytoreductive conditioning was applied, donor T cells from CCR5KO mice resulted in GVHD that was markedly increased as compared to wild-type donor mice (Welniak *et al.* 2004). In another study also reporting increased GVHD using cells from CCR5 KO mice, it was found that extensive cytoreductive conditioning could affect the homing of immune suppressor $CD4^{+}25^{+}$ regulatory T cells (Tregs) which could be correlated with the increased GVHD when CCR5 KO donor T cells were used (Wysocki *et al.* 2004). Thus, caution must be exercised in interpreting the various GVHD models, and factors such as cytoreductive conditioning, strain combinations used, and donor cells transferred all appear to dramatically affect GVHD induction and progression resulting in apparently contradictory results being reported.

Other chemokines have been examined for roles in GVHD pathophysiology. Fully allogeneic donor CXCR3 KO T cells infused into lethally irradiated recipients resulted in fewer donor T cells in the gut and liver, albeit more in the spleen, resulting in less overall GVHD lethality (Duffner *et al.* 2003). Chemokines also regulate the homing and migration into the lung which is a target of immune mediated injury during GVHD responses. Donor T cells from RANTES (CCL5) KO mice given to lethally irradiated semi-allogeneic recipients resulted in less severe GVHD-induced lung injury as compared to wild-type donor T cells (Hildebrandt *et al.* 2004). In another GVHD-induced lung injury model system, the transfer of donor T cells from MIP-1α (CCL3) KO mice infused into mice conditioned with both chemotherapy (cyclophosphamide) and lethal irradiation resulted in accelerated lung pathology (Panoskaltsis-Mortari *et al.* 2003). In a GVHD model involving CD4-only mediated responses where the recipient mice succumb to bone marrow aplasia, T cells from CCR2 KO donors resulted in increased GVHD suggesting that this pathway may suppress GVHD, much like CCR5 (Rao *et al.* 2003).

Adhesion molecules which play a role in T cell trafficking have been studied as a potential target in GVHD prevention (i.e. ICAM-1/LFA-1; Blazar *et al.* 1995*a*). Blocking L-selectin has been demonstrated to contribute to the inhibition of acute GVHD (Li *et al.* 2001, 2004), which is perhaps not surprising considering the critical role this molecule plays in T cell trafficking to lymphoid organs. In this study, fully MHC-mismatched splenocytes were incubated with monoclonal antibodies (mAbs) to L-selectin and alpha4-integrin and were then transferred into mice with severe combined immune deficiency that received no conditioning. This resulted in delayed GVHD induction by reducing homing to LNs (Li *et al.* 2001). This is supported by data using irradiated F1 hybrid recipients of parental donor grafts in which the *in vivo* administration of anti-alpha4-integrin mAbs resulted in protection from gastrointestinal GVHD pathology (Tanaka *et al.* 1995). Recently, intriguing data looking at lymphocyte PP adhesion molecule (LPAM or alpha_4 beta_7 integrin) has suggested that organ-specific effects may be achieved. LPAM is responsible for T cell homing in gut-associated lymphoid tissues through binding the mucosal addressin cellular adhesion molecule (MAdCAM). Using donor T cells which were selected based on LPAM expression, the transfer of LPAM^{-} cells resulted in significantly less gut GVHD compared to recipients receiving LPAM^{+} T cells (Petrovic *et al.* 2004). Importantly, GVT responses were maintained with the use of the LPAM^{-} T cells. Thus, the potential of blocking T cell homing to particular GVHD target sites or adoptive transfer of T cells selected based on chemokine receptor or adhesion molecule expression offers great promise in the selective attack of this disease.

A pharmacological agent under investigation is FTY720, a sphingosine-1-phosphate receptor agonist which has been shown to be capable of trapping T cells in secondary lymphoid organs (Chiba *et al.* 1998). It has been recently shown that FTY720 administration can inhibit GVHD without compromising GVT in a murine BMT model. While GVHD organ pathology was blunted, anti-leukaemia effects were maintained (Kim *et al.* 2003). However, using a canine model, administration of FTY720, while offering some protection from GVHD, ultimately did not protect survival of

recipients (Lee *et al.* 2003). Thus, while promising, more work on the dosing, timing, and pharmacokineticss of FTY720 in conditioned allogeneic recipients must be performed to optimize its ability to inhibit GVHD and there may be significant species differences of the particular role of chemokines and cytokines in GVHD. Taken together, these studies would suggest that altering the homing of the donor T cells via targeting chemokines/adhesion molecules may provide a means to selectively affect GVHD reactions, in particular organs, thus allowing for preservation of GVT depending on the location of the cancer.

2. PREVENTION AND TREATMENT OF GVHD

(a) *Targeting of intracellular signalling pathways as a means of preventing GVHD*

Recently, through our increased understanding of the intracellular signalling pathways that regulate T cell responses and survival, targeting of such signalling pathways has been applied to inhibit T cell alloresponses, T cell survival and hence, target GVHD. Interestingly, several of the reagents were first evaluated for their ability to directly mediate anti-tumour effects, suggesting that not only preservation of GVT but also a direction induction of anti-tumour effects may be observed when this approach is applied with BMT. For example, histone deacetylase inhibitors (HDACs) have shown promise as anti-tumour agents. HDAC inhibitors increase the acetylation of histone lysine tails in chromatin and affect gene expression, both positively and negatively. One HDAC inhibitor, suberoylanide hydroxamic acid (SAHA), has been demonstrated to: (i) inhibit tumour cell growth (Butler *et al.* 2000; Almenara *et al.* 2002; Cohen *et al.* 2002*b*) and (ii) inhibit the production of proinflammatory cytokines (interleukin 1 (IL-1), TNF-α and interleukin 12 (IL-12)) *in vivo* (Leoni *et al.* 2002). Based upon these biological functions, Ferrara and colleagues evaluated the effects of SAHA on GVHD/GVT in a murine model. The administration of SAHA resulted in significant protection from GVHD in two different murine BMT models involving lethal conditioning. While a reduction of proinflammatory cytokines was observed, no direct inhibition of T cell responses was detected and GVT was spared (Reddy *et al.* 2004).

Another means to affect GVHD is by targeting the transcription factor nuclear factor kappa B (NFκB). As NFκB has long been known to play a critical role in T cell biology, particularly with respect to cytokine responses, it has been an attractive target but been hampered in finding reagents that have favourable pharmacokinetics resulting in systemic NFκB inhibition without toxicity. Recently, a new class of proteasome inhibitors have been developed which can be administered systemically without excessive toxicity. The proteasome plays a critical role in the degradation of inhibitory kappa B (IκB) which binds and inhibits NFκB activity. Thus proteasome inhibition results in suppression of NFkB although other cellular pathways are also affected. One inhibitor, bortezomib (Velcade; formerly PS-341), has been shown to have direct anti-tumour effects and has been recently approved in the treatment of multiple myeloma (Kane *et al.* 2003; Bross *et al.* 2004). We and others have found that bortezomib could also sensitize tumour cells to immune mediated killing (i.e. tumour necrosis factor related apoptosis-inducing ligand (TRAIL); Sayers *et al.* 2003). We have recently demonstrated that bortezomib given immediately after allogeneic BMT in mice could prevent GVHD (Sun *et al.* 2004). Bortezomib could inhibit alloreactive T cell proliferation and directly induce alloreactive T cell apoptosis *in vitro* and *in vivo* (Sun *et al.* 2004), which was associated with protection from GVHD and decreased proinflammatory cytokine production. Importantly, GVT effects were still observed in advanced tumour-bearing recipients. Direct cytotoxic T lymphocyte (CTL)-mediated killing was also enhanced with leukaemia cells exposed to bortezomib indicating that the anti-tumour effects could be both direct (i.e. induction of apoptosis) and indirect (increasing tumour cell susceptibility to immune effector cell mediated death pathways). Clinical studies assessing efficacy of bortezomib with allogeneic BMT in multiple myeloma patients are currently underway.

With both of these intracellular signalling pathway targeted approaches, several issues must be taken into consideration. First, both agents have demonstrated direct anti-tumour effects, potentially adding to the anti-tumour effects of cytoreductive conditioning given prior to BMT. In addition, via effects on immune effector cells, these agents may aid in immune-mediated GVT responses. These anti-tumour effects could therefore be obtained at doses much less than if the agent was administered in the absence of BMT. GVHD was reduced but not eliminated; however, such reduction did not preclude GVT responses. While the mechanism(s) responsible for GVHD inhibition have not been fully elucidated, both agents affect multiple cell-types, including immune and parenchymal cells, and this may affect the overall effect on GVHD (figure 1). Finally, as with many studies on GVHD protection, the timing of administration may be critical. In the case of bortezomib, the timing of administration is particularly critical as it appears extremely effective in preventing GVHD early, during the induction phase. Markedly opposing effects were observed when it was administered later during active GVHD in which a lethal GVHD-dependent gut toxicity resulted (Sun *et al.* 2005). Thus, an agent that can increase death receptor expression on the tumour cell can also increase it on target tissue and subsequently, different effects may be observed contingent on the timing of administration. A recent clinical trial in which multiple myeloma patients who have relapsed after allogeneic BMT were given bortezomib demonstrated no adverse effects but some anti-tumour responses were observed (Giralt *et al.* 2004). What makes these agents attractive is that prolonged administration may not be required and augmented anti-tumour effects may still be observed.

Another means of suppressing T cell responses is through targeting intracellular signalling pathways. Janus kinase 3 (JAK3) has been demonstrated to play a pivotal role in cytokine signalling of T cells. An inhibitor of JAK3 (by the WHI-P131/JANEX-1 compound) was administered to mice after allogeneic BMT and significant increases in survival were

Table 2. Summary of cytokines and GVHD.

cytokine/growth factor	effects on GVHD	mechanism
IL-2, IL-12, IFN-γ	inhibits GVHD early promotes GVHD late	inhibits by activation-induced cell death of alloreactive T cells promotes by augmenting Th1 responses
IL4, IL10	predominantly inhibits GVHD	promotes Th2 responses and inhibits Th1
IL7	variable: no effect on or worsening of GVHD	augments T cell responses/thymopoiesis
KGF	protects from GVHD	promotes tissue repair
TNF	promotes GVHD	increases tissue destruction and inflammatory responses

observed (Uckun *et al.* 2002). Importantly, GVT effects were maintained even though the compound itself had no intrinsic anti-tumour properties. We have also observed that targeting JAK3 on T cells *ex vivo* using specific inhibitors can result in significant inhibition of allo-responses and subsequent GVHD induction (O'Shaughnessy *et al.* submitted).

(b) *Targeting cytokine responses as a means of preventing GVHD*

Given the pivotal role of cytokines in GVHD pathobiology, it is perhaps no surprise that approaches focused upon regulating cytokine production and function have also been studied as a strategy to prevent GVHD (table 2). Blocking cytokines that may participate in GVHD has been a subject of intense investigation. The historical dogma on the role of cytokines in GVHD pathophysiology is that Th1-type cytokines (IFNγ, IL-12) fuel GVHD generation. Interestingly, in several recent studies, it has been shown that Th1 cytokines can be protective if present in high amounts in the very early stage after allogeneic BMT. For example, recipients of donor T cells from IFNγ KO mice were found to exhibit increased GVHD (Murphy *et al.* 1998). Interleukin 18 (IL-18) has also been shown to inhibit GVHD (Reddy *et al.* 2001, 2003) but recent studies suggest that this may be dependent on CD4 versus CD8-mediated responses (Min *et al.* 2004). One mechanism responsible for Th1 cytokine mediated GVHD protection has been the induction of fas-mediated activation-induced death of the donor alloreactive T cells (Sykes *et al.* 1990, 1995). At the other end of the spectrum, skewing donor T cells to Th2-type cytokine profile (interleukin-4 (IL4), interleukin-10 (IL-10)) responses has also been shown to be protective (Jung *et al.* 2003). However, the amount and timing of cytokines is likely to be critical to regulating GVHD; high doses of IL-10 can accelerate GVHD (Blazar *et al.* 1995*b*). Timing also is critical in influencing the outcome of Th1 cytokine effects on GVHD and long-term survival. For example, the timing of exogenous IL-12 administration in relationship to irradiation can either prevent GVHD or conversely result in rapid peri-BMT lethality (Sykes *et al.* 1999). Together, these data would suggest that cytokines may have effects, sometimes opposing, contingent on the phase of GVHD where they are applied and that caution must be exercised when attempting systemic administration or global blockade clinically.

Blocking TNF has shown to have efficacy in preclinical models (Wall & Sheehan 1994; Speiser *et al.* 1997; Brown *et al.* 2002; Korngold *et al.* 2003), although the effects may be contingent on the conditioning, strain combination, and donor cell transferred. It has been demonstrated that TNF can play a role in GVHD progression, particularly with the gut (Brown *et al.* 2002). Clinical trials evaluating efficacy of TNF blockade in acute GVHD progression have been pursued. Given the complex and dual role of TNF as an effector molecule in many protective immune responses to pathogens, resistance to opportunistic infections and effects on relapse are being scrutinized in these studies.

There has been much attention to not only controlling or modulating the T cell response but also tissue damage resulting from GVHD attack. One of the most promising cytokines, keratinocyte growth factor (KGF; a.k.a. fibroblast growth factor-7), has been demonstrated to affect GVHD by multiple pathways. The KGF receptor, fibroblast growth factor receptor II–IIIb is expressed on epithelial cells, which are targets of GVHD. KGF stimulates epithelial cell proliferation increasing the thickness of the epithelial cell layers especially of the gastrointestinal tract, which may permit epithelial cell rich target organs to better withstand GVHD mediated destruction. In rodents, KGF given prior to chemoradiotherapy conditioning clearly can blunt the tissue injury from GVHD attack (particularly in the gut and the lung) but can modulate T cell responses as well (Krijanovski *et al.* 1999; Panoskaltsis-Mortari *et al.* 2000). Graft versus leukaemia (GVL) responses were not impaired after KGF administration (Krijanovski *et al.* 1999). KGF administration has been shown to also promote thymic recovery due to the effect of KGF on thymic epithelial cells which are damaged by chemoradiotherapy conditioning regimens and are targets of GVHD (Min *et al.* 2002; Rossi *et al.* 2002). KGF was recently approved as a means of decreasing oral mucositis after HSCT (Spielberger *et al.* 2004), an effect likely to be the result of the initial induction of epithelial cell proliferation in the oral mucosa. Clinical studies assessing the effects of KGF in allogeneic BMT recipients are underway. Hepatocyte growth factor administration has also been recently shown to protect in liver GVHD, presumably by similar mechanisms as KGF (Kuroiwa *et al.* 2001; Imado *et al.* 2004).

Other cytokine infusions specifically are being investigated as means to diminish the period of immune suppression that occurs post-BMT. The potential impact on GVHD remains a paramount concern though. Similar to KGF, interleukin 7 (IL-7) has been shown to promote T cell recovery after BMT.

Table 3. CD28 costimulatory receptor family.

Receptor: CD28 family	other names	function	expression	ligand: by family	other names	expression
CD28	T44	costimulation	T and NK cells	B7.1	CD80	activated APC
				B7.2	CD86	APC (upregulated) activated T cells
CTLA-4	CD152	inhibition	activated T cells	B7.2	CD86	APC (upregulated) activated T cells
ICOS	H4, CRP-1, AILIM	costimulation	activated T cells	B7RP-1	ICOSL, GL50, B7-H2, B7h	APC
?				B7-H3		B, T and NK cells, macrophages, DCs

IL-7 has been demonstrated to play a critical role in both thymopoiesis as well as peripheral T cell homeostasis (Alpdogan *et al*. 2001). Conversely, KGF administration has been shown to induce IL-7 mRNA expression in the thymus (Min *et al*. 2002). However, the role of IL-7 in GVHD has been conflicting with some reports demonstrating no effects and other showing increased GVHD in murine models (Alpdogan *et al*. 2001; Sinha *et al*. 2002; Gendelman *et al*. 2004).

(c) *Targeting costimulatory pathway responses as a means of preventing GVHD*

Allogeneic HCT provides a unique situation in which large numbers of donor immune cells are purposefully infused and exposed to allogeneic host MHC and miH antigens typically in the context of a conditioning-regimen induced tissue injury and inflammation which occurs throughout the body and requires several weeks to subside. Such inflammation results in the cell surface upregulation as well as the systemic release of alloantigens that are available for uptake by cells capable of supporting a T cell immune response such as DCs and macrophages. The net result of conditioning regimen injury is a rich environment in which donor T cells need not search far for alloantigen, cytokines or costimulation. Although pan-T cell depletion, if sufficiently rigorous, can prevent GVHD, the lack of mature T cells infused and the slow time to recovery of thymic-derived mature T cells renders the recipient susceptible to infections, tumour recurrence, and host anti-donor BM graft rejection responses. This section will focus on only those approaches that have been shown to affect acute GVHD mortality in lethally irradiated mice to best simulate the human myeloablative allogeneic BMT setting.

Given the important role of host DCs to acute GVHD induction (Teshima *et al*. 2002*a*; Zhang *et al*. 2002; Matte *et al*. 2004), one approach to GVHD prevention could target DCs for depletion. Since permanent donor and host DC depletion is not feasible yet, even in rodents, a preferred approach would be to block T cell–APC interaction or functions as can be accomplished via costimulatory pathway blockade. In solid organ graft rejection, costimulatory pathway blockade can be highly effective in preventing cardiac and to a lesser extent skin allografts rejection. In allogeneic BMT, positive results may be more difficult to achieve due to the extensive and widely distributed tissue injury caused by conditioning regimen injury. Perhaps the most substantial effects on reducing GVHD lethality have been seen by blocking the CD28/B7 pathway (table 3), which is critically involved in the initiation of T cell responses. Studies using donor $CD28^{-/-}$ mice or fusion proteins that bind B7 ligands, have shown that GVHD lethality is markedly inhibited but not uniformly eliminated in heavily conditioned mice (Blazar *et al*. 1994, 1996; Hakim *et al*. 1995; Saito *et al*. 1998; Yu *et al*. 1998). These reagents also block the binding of the B7 ligands to cytotoxic lymphocyte antigen-4 (CTLA-4; CD152), a molecule that is homologous to CD28, is expressed predominantly intracellularly, and is upregulated on activated T cells. CTLA-4 binds B7 ligands with higher affinity than CD28. In contrast to CD28/B7 interactions, CTLA-4 ligation confers a negative signal to the T cell to cause cell cycle arrest, terminating proliferation. Interruption of CTLA-4/B7 binding accelerates GVHD lethality (Blazar *et al*. 1999). In addition to the dual block of CD28/B7 and CTLA-4/B7 interactions, anti-B7 mAbs also may deplete the $CD4^{+}25^{+}$ Tregs. Since depletion of donor or host Tregs increases acute GVHD lethality (Salomon *et al*. 2000; Taylor *et al*. 2001*a*; Cohen *et al*. 2002*a*), the inhibitory effects of anti-B7 mAbs on GVHD initiation are partially offset by the blockade of CTLA-4/B7 pathway and depletion of Tregs. Because CD28/B7 interactions facilitates Treg generation in the thymus, $CD28^{-/-}$ mice have a paucity of Treg cells (Salomon *et al*. 2000). In a different approach, Yu and Anasetti have used anti-CD28 mAbs to inhibit GVHD generation and have made the surprising observation that alloreactive T cells were specifically depleted via an IFNγ dependent mechanism, opening up new possibilities for selectively targeting the CD28 pathway (Yu *et al*. 1999, 2000). Moreover, these investigators recently have demonstrated that the combination of CD28 blockade with the pharmacological agent, rapamycin (sirolimus), which binds to the molecular target of rapamycin (mTOR), produced superior responses in inhibiting GVHD (Albert *et al*. 2005) as compared to either agent alone.

In contrast to the CD28 pathway, CD40 ligand (CD154) is expressed on alloactivated $CD4^{+}$ and not $CD8^{+}$ T cells. The CD154–CD40 pathway (table 4) directly regulates $CD4^{+}$ T cell and typically only indirectly affects $CD8^{+}$ T cell alloresponses. Although anti-CD154 mAbs or the use of $CD154^{-/-}$ $CD4^{+}$ T cells reduces GVHD lethality, typically about one-half of mice will survive under heavy irradiation

Table 4. Members of the TNF receptor superfamily associated with GVHD.

TNFR family	other names	function	expression	ligand: TNF family	other names	expression
CD27	T14	costimulation	T cells, B subset, NK	CD70		activated B cells
CD30	Ki-1	costimulation, apoptosis	activated T, NK and B cells	CD153	CD30L	neutrophils, activated B and T cells
CD40		activation	APC, T subset, endothelium, cardiac myocytes, fibroblasts	CD40L	CD154, gp39 TRAP	activated T cells
4-1BB	CD137	costimulation	activated T cells	4-1BBL		activated B, DC, peritoneal cells
OX40	CD134	activation, differentiation, apoptosis	activated T cells	OX40L		activated B cells, cardiac myocytes
Fas	CD95, Apo-1	activation, apoptosis	leukocytes	FasL	CD95L, CD178	activated T cells

conditions and no biological effects are notable in models in which only $CD8^+$ T cells are infused (Blazar *et al.* 1997). However, in situations in which $CD4^+$ T cells are coinfused to drive $CD8^+$ T cell expansion, signals delivered via this pathway to $CD4^+$ T cells are needed to optimally support $CD8^+$ T cell expansion and GVHD generation (Buhlmann *et al.* 1999). Recent evidence exists that the effects of CD154/CD40 pathway blockade, dependent upon the conditions, are mediated in part or in whole via Treg cell suppression of alloresponses. *In vitro* depletion of Tregs from the responding $CD4^+$ T cell population in an allogeneic mixed lymphocyte reaction (MLR) culture precludes the induction of alloantigen hyporesponsiveness by CD154/CD40 pathway (and CD28/CTLA-4–B7 pathway) blockade (Taylor *et al.* 2001*a*). Similarly, the *in vivo* induction of hyporesponsiveness to alloantigen by CD154–CD40 pathway blockade combined with donor specific transfusion also can be dependent upon the presence of Tregs (Jarvinen *et al.* 2003). The clinical application of anti-CD154 mAb in allogeneic BMT will likely await results of anti-CD154 mAb studies in patients with autoimmunity and solid organ transplants.

CD134 is expressed on activated $CD4^+$ and $CD8^+$ T cells in rodents and humans (Mallett *et al.* 1990; Calderhead *et al.* 1993; Baum *et al.* 1994*a*,*b*; Latza *et al.* 1994; Birkeland *et al.* 1995). Nonetheless, much of the literature indicates that CD134–CD134 ligand (CD134L) interactions may play a more critical role in $CD4^+$ T cell responses than in CD8 T cell responses. CD134 receptor engagement promotes $CD4^+$ T cells to proliferate, produce Th1 and Th2 cytokines and anti-apoptotic proteins, to clonally expand, survive, and develop into memory cells (Flynn *et al.* 1998; Gramaglia *et al.* 1998, 2000; Ohshima *et al.* 1998; Weinberg 1998, 2002; Chen *et al.* 1999; Kopf *et al.* 1999; Pippig *et al.* 1999; Akiba *et al.* 2000; Murata *et al.* 2000; Rogers & Croft 2000; Weinberg *et al.* 2000; Bansal-Pakala *et al.* 2001; Evans *et al.* 2001; Rogers *et al.* 2001; Weatherill *et al.* 2001; De Smedt *et al.* 2002). CD134L is expressed on APCs that have been activated by known stimuli such as CD40 or proinflammatory mediators (Stuber *et al.* 1995; Ohshima *et al.* 1997; Brocker *et al.* 1999). Anti-CD134L mAb was able to ameliorate GVHD-mediated lethality (Tsukada *et al.* 2000; Blazar *et al.* 2003). Some studies have indicated that CD134 is critical for inducing Th2 responses. However, CD134/CD134L blockade was effective in reducing GVHD mediated by Th1- or Th2-deficient splenocytes as well as when using donor $CD28^{-/-}$, splenocytes indicating non-redundancy between the CD134 and CD28 pathways. Coblocking CD154/CD40 and CD134/CD134L pathways was superior to either pathway blockade alone or combined CD154/CD40 and CD28/CD152–B7 blockade in a full MHC disparate strain combination (Patricia A. Taylor and BRB, unpublished data).

Similar to CD28 and CD134, 4-1BB (CD137) is expressed on activated $CD8^+$ and $CD4^+$ T cells, in addition to NK cells (Kwon & Weissman 1989; Pollok *et al.* 1993; Melero *et al.* 1998). Early data suggested that the CD137–CD137L pathway plays a more prominent role in $CD8^+$ T cell responses than in $CD4^+$ T cell responses (Shuford *et al.* 1997; Takahashi *et al.* 1999; Tan *et al.* 1999, 2000). CD137 signals induce T cells to produce interleukin 2 (IL-2), proliferate, and differentiate, and protect T cells from activation-induced cell death (ACID; Goodwin *et al.* 1993; Pollok *et al.* 1993; Alderson *et al.* 1994; Hurtado *et al.* 1995, 1997; Saoulli *et al.* 1998; DeBenedette *et al.* 1999; Takahashi *et al.* 1999). CD137 ligand (CD137L) is expressed on APCs (Pollok *et al.* 1994; DeBenedette *et al.* 1997). Antagonistic anti-CD137L mAbs significantly reduced GVHD-induced mortality in a lethally irradiated model of acute GVHD (Nozawa *et al.* 2001). Expansion of donor $CD8^+$ T cells, but not $CD4^+$ T cells, was reduced in anti-CD137L mAb-treated mice. Anti-CD137L mAb ameliorated lethality that was associated with impaired donor $CD8^+$ T cell expansion and IFNγ production without significant effects on $CD4^+$ T cell expansion. Although signalling via CD137 had been reported to be a relatively weak agonist for $CD4^+$ cells (Shuford *et al.* 1997), we found that the CD137–CD137L pathway was important in regulating GVHD by $CD4^+$ or $CD8^+$ T cells (Blazar *et al.* 2001*a*). Taken together these studies indicate that

Table 5. Inhibitory receptors of the B7 family.

inhibitory molecules	other names	function	expression	ligand: B7 family	other names	expression
PD-1		inhibition	activated T, B cells and macrophages	PD-L1	B7-H1	leukocytes
				PD-L2	B7-DC	monocytes, macrophages, DC
CTLA-4	CD152	inhibition	activated T cells	B7.1	CD80	activated APC
				B7.2	CD86	APC (upregulated) activated T cells

the CD137/CD137L pathway regulates acute GVHD caused by $CD8^+$ T cells and $CD4^+$ Th1 cell generation, whereas the effects of this pathway on $CD4^+$ T cell mediated GVHD lethality may be dependent upon the particular donor-recipient strains tested and conditions used for acute GVHD generation.

Inducible costimulator (ICOS) is expressed on $CD4^+$ and $CD8^+$ T cells within 24–48 h after activation (Hutloff *et al*. 1999; Coyle *et al*. 2000; Kopf *et al*. 2000; Sperling & Bluestone 2001). The ligand for ICOS (ICOS-L is constitutively expressed by B cells, monocytes and some T cells; Swallow *et al*. 1999; Yoshinaga *et al*. 1999; Aicher *et al*. 2000; Ling *et al*. 2000; Wang *et al*. 2000; Gonzalo *et al*. 2001*b*; Ling *et al*. 2001; Sperling & Bluestone 2001). Much but not all of the literature indicate that ICOS/ICOS-L ligation has a preferential effect on inducing Th2 rather than Th1 cytokines (Hutloff *et al*. 1999; Yoshinaga *et al*. 1999, 2000; Coyle *et al*. 2000; McAdam *et al*. 2000; Coyle & Gutierrez-Ramos 2001; Dong *et al*. 2001; Gonzalo *et al*. 2001*a*; McAdam *et al*. 2001; Sperling & Bluestone 2001; Tafuri *et al*. 2001; Tamura *et al*. 2001; Tesciuba *et al*. 2001; Khayyamian *et al*. 2002). The inducible expression of ICOS shortly after T cell activation suggests that ICOS may be particularly important in providing costimulatory signals to activated T cells or memory cells (Hutloff *et al*. 1999; Yoshinaga *et al*. 1999; Coyle & Gutierrez-Ramos 2001). ICOS signalling does not appear to be required for naïve cell responses with the exception of superantigen (Gonzalo *et al*. 2001*a*). ICOS-immunoglobulin (ICOS-Ig) infusion has more pronounced effects when given during induction of the secondary rather than primary immune responses in contrast to CTLA-immunoglobulin (CTLA4-Ig), a fusion protein that binds to B7 ligands thereby reducing or eliminating B7 ligand binding to their endogenous receptors (Coyle *et al*. 2000; Gonzalo *et al*. 2001*b*). ICOS/ICOS-L has been shown to reduce $CD4^+$ and $CD8^+$ T cell mediated GVHD under lethal irradiation conditions (Taylor *et al*. 2004*a*). Delaying ICOS blockade until day 5 post-BMT, a time in which T cells have upregulated ICOS expression and have dramatically expanded and gained effector function, improved outcome but did not prevent eventual GVHD-induced lethality. In contrast, anti-CD154 mAb, which is highly protective when initiated at time of BMT, had no effect when mAb infusion was given at that time. Survival was significantly improved by combining anti-ICOS and anti-CD154 mAbs. Thus, ICOS/ICOS-L blockade may be an especially attractive target for downregulating T cell responses once they have been initiated and for use in combined costimulatory pathway blockade.

CD30 is expressed on B cells and mitogen-stimulated T cells (Durkop *et al*. 1992; Shanebeck *et al*. 1995; Bowen *et al*. 1996). CD30 expression is induced on $CD8^+$ and to a lesser extent on $CD4^+$ T cells by alloantigen exposure (Bowen *et al*. 1996; Martinez *et al*. 1998; Chan *et al*. 2002). CD30 signalling regulates CTL generation and also renders T cells sensitive to cell death (Amakawa *et al*. 1996; Duckett & Thompson 1997; Telford *et al*. 1997; Chiarle *et al*. 1999; Kurts *et al*. 1999). CD30 ligand (CD30L, CD153) is expressed on macrophages, activated B cells and T cells, primarily $CD4^+$ T cells of both Th1 and Th2 phenotype, and can provide signals for B cell growth and differentiation (Shanebeck *et al*. 1995; Bowen *et al*. 1996; Wiley *et al*. 1996; Shimozato *et al*. 1999). The CD30/CD153 pathway is a potent regulator of $CD4^+$ but not $CD8^+$ T cell-mediated GVHD. Although blocking CD30/CD153 interactions *in vivo* did not affect alloreactive $CD4^+$ T cell proliferation or apoptosis, a substantial reduction in donor $CD4^+$ T cell migration into the gastrointestinal tract, a GVHD target organ, was readily observed with lesser effects in other GVHD organs. Thus, blockade of the CD30/CD153 pathway represents a viable approach for preventing $CD4^+$ but not $CD8^+$ T cell-mediated GVHD.

Similar to CD152, programmed death-1 (PD-1) is a negative costimulatory pathway with two known ligands: PD-L1 and PD-L2 (table 5; Ishida *et al*. 1992; Shinohara *et al*. 1994; Agata *et al*. 1996; Nishimura *et al*. 1999; Freeman *et al*. 2000; Kingsbury *et al*. 2001; Latchman *et al*. 2001; Tseng *et al*. 2001). The expression of PD-1 is induced on mature peripheral T cells, B cells and myeloid cells upon activation (Ishida *et al*. 1992; Agata *et al*. 1996; Vibhakar *et al*. 1997) PD-1 and CD152 have structural similarities and PD-1 has an immunoreceptor tyrosine based inhibitory action suggesting an inhibitory function (Ishida *et al*. 1992; Shinohara *et al*. 1994; Vivier & Daeron 1997; Coyle & Gutierrez-Ramos 2001; Sharpe & Freeman 2002). PD-1 ligand, PD-L1, also termed B7-H1, is constitutively expressed on DCs, some activated $CD3^+$ cells, and IFNγ stimulated monocytes and keratinocytes (Freeman *et al*. 2000) while PD-L2, also termed B7-DC, is constitutively expressed on resting monocytes (Latchman *et al*. 2001; Tseng *et al*. 2001). Some tissues (lung, liver, pancreas) express PD-L2 but not PD-L1, whereas others (heart, skeletal muscle) express both. Although investigators have reported that T cell proliferation is enhanced by the PD-1 pathway *in vitro* under some conditions, others have demonstrated that the dominant function of the PD-1/PD-L pathway is to inhibit T cell responses (Dong *et al*. 1999; Freeman *et al*. 2000; Latchman *et al*.

2001; Tseng *et al.* 2001). In lethally irradiated recipients of full MHC-disparate donor T cells, PD-1 infiltrating cells were found in increased frequency in GVHD target organs as compared to BMT controls (Blazar *et al.* 2002). Blockade of the PD-1/PD1-L pathway significantly augmented GVHD mortality. The combined administration of anti-CD152 and anti-PD-1 mAbs was significantly more potent than either alone in accelerating GVHD lethality, indicating that these pathways are not fully redundant. Reagents that may provide a signal to PD-1$^+$ T cells may be of benefit in inhibiting GVHD.

It is important to point out several factors that must be considered before applying these principles to humans. An important consideration for *in vivo* costimulatory pathway blockade is that such an approach is likely to be added to conventional immune suppressive pharmacological agents. In order for costimulatory pathway blockade to be effective, donor T cells must receive sufficient TCR signalling. Calcineurin inhibitors such as cyclosporin A or tacrolimus (FK506) blunt effective TCR signalling which may interfere with tolerance induction by blocking T cell apoptosis whilst blocking T cell proliferation. In contrast, sirolimus (rapamycin) as a target interferes with cytokine responsiveness and not TCR signals. Studies in rodents have shown that sirolimus does not interfere with tolerance induction *in vivo* and in fact massively increases the proportion of proliferating cells destined for apoptosis thereby limiting the size of alloreactive T cells potentially below a threshold level required to cause GVHD (Li *et al.* 1999). One note of caution in extrapolating these studies from the solid organ grafting to the GVHD arena is that the level of TCR signalling and tissue destruction may not preclude the use of calcineurin inhibitors should T cell proliferation induced by these proinflammatory events proceed, albeit at a reduced level, if such inhibitors merely serve to dampen TCR signals to levels that still exceed a threshold response. Testing in the context of GVHD in rodents and in humans will be required before conclusions can be reached as to whether calcineurin inhibitors are contraindicated in such situations, especially considering that some but not all non-myeloablated BMT rodent models have shown that calcineurin inhibitors can be additive with costimulatory pathway blockade in promoting alloengraftment.

While anti-B7 mAbs have been tested *in vivo* in solid organ patients and *in vivo* CTLA4-Ig and anti-CD154 mAb in patients with autoimmune disorders, these reagents have not been tested *in vivo* in the context of allogeneic BMT and the status of clinical testing of reagents for the other pathways in patients undergoing BMT remains uncertain. The BMT setting poses additional challenges for costimulatory pathway blockade. For example, strategies that block GVHD but have detrimental effects on alloengraftment, GVT effects, or immune recovery must be carefully considered before use in allogeneic BMT patients. Factors found in rodents that may adversely affect the efficacy of mAbs or fusion proteins to block T cell costimulation early post-BMT in fully myeloablated recipients include: (i) the recent observations that profound lymphopenia, which induces homeostatic T cell proliferation to fill up the lymphoid compartment, may be a significant barrier to tolerance induction via costimulatory pathway blockade (Kreisel *et al.* 2002); (ii) heterologous immunity, a response to an infectious pathogen that can preclude tolerance induction (Williams *et al.* 2001). Since all fully myeloablated BMT patients have a period of profound lymphopenia and viral infection can occur early post-BMT, although typically significant viral infections do not occur in the first several weeks post-BMT, should the rodent data be translatable to the clinical arena, the potency of costimulatory pathway blockade may be more limited in humans than rodents. Nonetheless, blockade of T cell costimulation may prove to be an important new approach to specifically target those T cells that are alloreactive, especially when used early post-BMT when tumour and viral antigens may be present at far lower amounts than alloantigens.

(d) *GVHD prevention by ex vivo* approaches

The prior discussions have focused on *in vivo* mAbs or fusion proteins administration. An *ex vivo* tolerization approach involves the culturing of donor T cells with host alloantigen in the presence of mAbs or proteins that are capable of blocking T cell costimulation. An *ex vivo* tolerization procedure has several theoretical advantages over *in vivo* tolerization attempts. These include: higher likelihood of tolerance induction by ensuring costimulatory pathway blockade is delivered to the site of T cell–APC interactions; achieving tolerance induction before *in vivo* infusion into an inflammatory milieu which may diminish the likelihood of tolerance induction; capacity to monitor the depth of tolerance induction *in vitro*; avoidance of potential *in vivo* toxicities of mAbs or proteins.

We have described an *ex vivo* approach in which the blockade of the CD154/CD40 or CD28/CD152–B7 pathways added to a 7 to 10-day MLR culture of CD4$^+$ T cells and irradiated MHC class II-disparate stimulators induces tolerance leading to complete GVHD prevention (Blazar *et al.* 1998; Taylor *et al.* 2000, 2001*b*, 2002*a*). Tolerant CD4$^+$ T cells were hyporesponsive to initial alloantigen challenge and to restimulation *in vitro*. Tolerance was long-lived and not readily reversible *in vivo* (Blazar *et al.* 1998). Tolerance induction resulted in the generation of potent immunoregulatory CD4$^+$25$^+$ T cells that could inhibit both naïve and primed alloresponses as assessed by *in vitro* and *in vivo* assays (Taylor *et al.* 2002*a*). With respect to the latter, the separate coinfusion of tolerized cells with an otherwise uniformly lethal dose of naïve CD4$^+$ cells prevented GVHD mortality in 75% of mice. The generation of this regulatory capacity during tolerization may be of added benefit by the provision of a fail-safe mechanism to control alloreactive T cells that may escape tolerization. In a clinical trial, donor BM cocultured with irradiated recipient in the presence of CTLA4Ig (Guinan *et al.* 1999) resulted in a reduction in the frequency of T cells capable of recognizing host alloantigens. The incidence and severity of acute GVHD appeared to be reduced from what might be expected after taking into account the degree of HLA disparity and the number of mature T cells. In a

different *ex vivo* approach, we have shown that $CD4^+$ T cells can be rendered tolerant via exposure to immunoregulatory cytokines (e.g. IL-10 and transforming growth factor-beta, TGFβ; Zeller *et al.* 1999; Boussiotis *et al.* 2001; Chen *et al.* 2003). Tolerization leads to GVHD prevention in most recipients and to the induction of suppressor cells that inhibited naïve T cell mediated GVHD (Zeller *et al.* 1999; Chen *et al.* 2003). A clinical trial in humans is underway in a haploidentical BMT setting using exogenous IL-10 to tolerize donor T cells to host alloantigens *in vitro* before infusing these cells later post-BMT (Bacchetta *et al.* 2000).

Amongst other approaches that have been explored that show promise include the depletion of alloreactive T cells by affinity columns, magnetic beads, or toxins that bind to T cells expressing activation antigens (CD69; CD25; IFNγ; Montagna *et al.* 1999; Koh *et al.* 2002; Solomon *et al.* 2002; Amrolia *et al.* 2003). In a different approach, Chen *et al.* describe the application of a photoactive rhodamine derivative [4,5-dibromorhodamine 123 (TH9402)], that is selectively retained in the mitochondria of activated T cells added to MLR cultures (Chen *et al.* 2002). Upon exposure to visible light this depletes the TH9402-enriched activated host-reactive cells in the MLR culture. Treatment with photodynamic cell purging process inhibited anti-host CTL and IFNγ responses and importantly prevented GVHD lethality.

Recent data show that memory T cells in rodents have a markedly reduced capacity to cause GVHD lethality (Anderson *et al.* 2003). These studies have demonstrated that effector memory CD4 T cells ($CD62L^-$, $CD44^+$) induced less GVHD when compared with unfractionated or naïve $CD4^+$ T cells (Anderson *et al.* 2003). Importantly, engraftment occurred and generation of antigen-specific responses resulted. The observations that the $CD62L^-$ T cell infusion can result in markedly reduced GVHD lethality capacity in rodents has been confirmed by others (Chen *et al.* 2004), and extended into the human preclinical setting with data showing that human $CD62L^-$ T cells are less potent in producing MLR responses *in vitro* when compared to $CD62L^+$ T cells (Foster *et al.* 2004). These data are intriguing in that assessment of TCR usage showed limited diversity in the memory population. These data also would suggest that isolation and transfer of these cells could prevent GVHD and allow for GVT to be maintained should these data in human *in vitro* cultures be as reproducible as initial studies have been in rodents.

(e) *Cellular therapies to prevent GVHD (table 6)*

(i) *T regulatory cells (Tregs)*

A variety of murine cells that display regulatory function *in vitro* or *in vivo* have been described and a review of all types is outside the scope of this manuscript. Instead, we will focus upon $CD4^+25^+$ Treg cells shown to have potent suppressor activity (Sakaguchi *et al.* 1995, 2001; Suri-Payer *et al.* 1998; Thornton & Shevach 1998; Baecher-Allan *et al.* 2001; Dieckmann *et al.* 2001; Jonuleit *et al.* 2001; Levings *et al.* 2001; Shevach 2001, 2002; Shevach *et al.* 2001; Jonuleit *et al.* 2002). In rodents, this population comprises 8–12% of lymph node $CD4^+$ T cells in most strains, whereas in humans, $CD4^+CD25^{bright}$ cells are present in far lower frequencies (1–3% of peripheral blood). There has been agreement that donor or host Treg cell depletion accelerates GVHD and Treg cell add-back inhibits acute GVHD lethality (Cohen *et al.* 2002*a*; Hoffmann *et al.* 2002*a*,*b*; Taylor *et al.* 2002*b*, 2004*b*; Jones *et al.* 2003; Trenado *et al.* 2003). Whereas fresh Treg cell add-back has had biological effects in reducing GVHD, some studies have shown more striking anti-GVHD effects than others. In direct comparative studies, *ex vivo* expanded and hence activated Tregs are more effective on a cell-to-cell basis than fresh cells whether Tregs are expanded using polyclonal stimulators (anti-CD3 mAb) or recipient APCs to cross-link the TCR (Taylor *et al.* 2002*b*; Jones *et al.* 2003). These data are consistent with the findings that activated Tregs suppress more potently than resting Tregs (Thornton & Shevach 2000). The additional advantage of *ex vivo* expansion prior to infusion is to increase the number of Treg in human blood that would be available for infusion. While Tregs can be generated against specific antigens including alloantigens, once activated Tregs can suppress alloresponses in an antigen-non-specific fashion (Godfrey *et al.* 2005). Nonetheless, it is possible that recipient-specific Tregs may be advantageous as compared to polyclonal Tregs, at least on a per cell basis, if TCR ligation *in vivo* is required to sustain Treg persistence or increase suppressor cell function. In that event, anti-recipient specific Tregs may be reactivated by host cells *in vivo* although Treg cell persistence may not necessarily be desirable in patients if such persistence results in global immune suppression during the time of infectious challenge or early tumour recurrence eliminating GVT. Studies in rodents will be needed to address these issues first. In addition, acquiring non-malignant, non-infected host APCs will be needed and, in some instances, the potency of *in vitro* stimulation of Tregs against host alloantigens (e.g. in miH antigen alone) may be insufficient to permit full expansion or suppressor cell function (table 6).

Strategies have been developed to expand human peripheral blood or cord blood Tregs using a bead-based approach (Godfrey *et al.* 2004, 2005; Hoffmann *et al.* 2004). In the majority of these protocols, such purified Tregs will be polyclonally expanded and infused into recipients to prevent GVHD. It will be important to ensure that Treg cells are suppressive after expansion and to utilize available expansion approaches or develop new approaches that lead to the retention of appropriate homing receptors such as CD62L (L-selectin) that in rodents have been shown to improve GVHD prevention via trafficking of Treg cells to putative sites of GVHD initiation (Taylor *et al.* 2004*b*). Such trials should commence this year. Lastly, it is worth noting that in contrast to some other forms of GVHD prevention, Treg cell infusions have increased alloengraftment by suppressing host anti-donor interactions and, in most instances, have not eliminated the GVL effect of allogeneic BMT against haematopoietic tumours (Edinger *et al.* 2003; Jones *et al.* 2003; Trenado *et al.* 2003; Joffre *et al.* 2004; Taylor *et al.*

Table 6. Cellular therapy in GVHD.

cells	mechanism of suppression
Tregs	suppression of alloreactive T cells
NK cells	elimination of host DCs, production of TGF-β and IFN-γ
NK/T cells	IL4 production
myeloid suppressor cells	inhibition of alloreactive T cell responsiveness
mesenchymal stem cells	suppression of alloreactive T cells promotion of tissue repair?

2004*a*,*b*; Hanash & Levy 2005). GVHD-induced immune suppression has been diminished by Treg cell infusion (Trenado *et al.* 2003), an approach that might also reduce the need for post-BMT immune suppressive drugs or T cell depletion.

(ii) *NK and NK/T cells*

Multiple other immunomodulating cell-types are being assessed for their ability to impede GVHD yet still allow for GVT effects. NK and NK/T cells have both been demonstrated to exert such effects. Donor-type NK cells have been demonstrated in preclinical models to inhibit GVHD and promote GVT (Asai *et al.* 1998). The mechanism underlying this protection is not definitively known. Studies have demonstrated that transforming growth factor-beta (TGF-β), an immunosuppressive cytokine, may be at least partially responsible for the suppression of alloreactivity in this model. Recent studies have also suggested that donor NK cells can attack host DCs which have been shown to play an important role in GVHD (Shlomchik *et al.* 1999; Zhang *et al.* 2002; Matte *et al.* 2004; Anderson *et al.* 2005). It is possible that all of these pathways may play a role. Interestingly, studies have suggested that the timing of NK cell transfer is also important, paralleling the studies with proteasome inhibition in that NK cell administration needs to be early after BMT. When donor-type NK cells were administered later during ongoing GVHD, increased GVHD mortality was observed (Asai *et al.* 1998). This is similar to reported results in earlier studies administering IL-2 and IL-12 which showed protection only when given early after BMT (Sykes *et al.* 1990, 1995).

The characterization of NK cell subsets bearing receptors for MHC and other determinants has revolutionized our understanding of their biology and also how to potentially exploit their clinical potential. NK cells bear inhibitory and activating receptors directed to MHC and other determinants (killing immunoglobin-like receptors (KIR) in humans, Ly49 in mouse, and NKG2D in both), which significantly affects their responses. Clinical BMT studies using KIR ligand mismatch combinations demonstrated heightened anti-tumour effects without increased GVHD in acute myelocytic leukaemia (AML; Ruggeri *et al.* 2002). Some clinical BMT studies failed to confirm these results but significant differences in the conditioning and BMT protocols may have affected NK cell recovery and ultimate outcome (Davies *et al.* 2002). Recently, a clinical study examining adoptive transfer of donor-type NK cells after allogeneic BMT yielded encouraging results in which increased anti-tumour effects without increased GVHD (Koehl *et al.* 2004). These studies suggest that NK cells, either as adoptive immunotherapy or using means to augment their recovery after BMT, may be of use to prevent GVHD and promote GVT responses. Further characterization and exploitation of NK cell subsets may allow for augmentation of these protective effects.

NK/T cells, a subset of lymphocytes which exhibit markers present on both NK and T cells, are functionally heterogeneous. The majority of murine NK/T cells have restricted TCR expression which recognizes glycolipid antigens in the context of the MHC-like molecule, CD1d expressed by APCs (Zhou *et al.* 2004). They have previously been demonstrated to inhibit GVHD through the production of IL-4 (Hashimoto *et al.* 2005). A recent study used a means of activating them with the injection of a synthetic ligand, alpha-galactoceramide (alpha-Gal Cer) after allogeneic BMT. Significant skewing to Th2-type cytokine production (IL4) occurred and inhibition of GVHD resulted, suggesting that adoptive transfer of NK/T cells or simply their activation may suppress GVHD (Hashimoto *et al.* 2005). Interestingly, use of this same ligand in resting mice has been reported to promote Th1-type cytokine responses, suggesting that perhaps the conditioning in BMT models alters the biologic properties of the NK/T cells in response to alpha-Gal Cer. As many of the anti-tumour effects of NK/T cells appear to be due to their ability to promote Th1-type responses, it will be of interest to delineate and reconcile the immunosuppressive ability of the cells in GVHD (via production of Th2 cytokines) combined with their anti-tumour effects.

(iii) *Regulation of immune function by manipulating APCs*

APCs represent a heterogeneous population of cells with regard to various subsets (lymphoid DCs, myeloid DCs, plasmacytoid DC precursors) capable of mediating distinct effects on immune responses. Using $SHIP^{-/-}$ mice which have an increased population of myeloid suppressor cells (MySC), it was demonstrated that impaired alloreactive responses occurred and protection from GVHD resulted in the recipients (Wang *et al.* 2002; Ghansah *et al.* 2004). A similar murine MySC expressing Mac1, Ly6-G, Ly6-C was also demonstrated to protect from GVHD (Billiau *et al.* 2003). Finally, the pre-BMT depletion of host APCs in the skin and in particular Langerhan's cells has been shown, as the skin is a highly accessible site for manipulation. Langerhan cell depletion prevented GVHD in the skin of mice (Merad *et al.* 2004), suggesting that it may be possible to perform procedures that target these cells prior and during the transplant and reduce GVHD pathology in the skin.

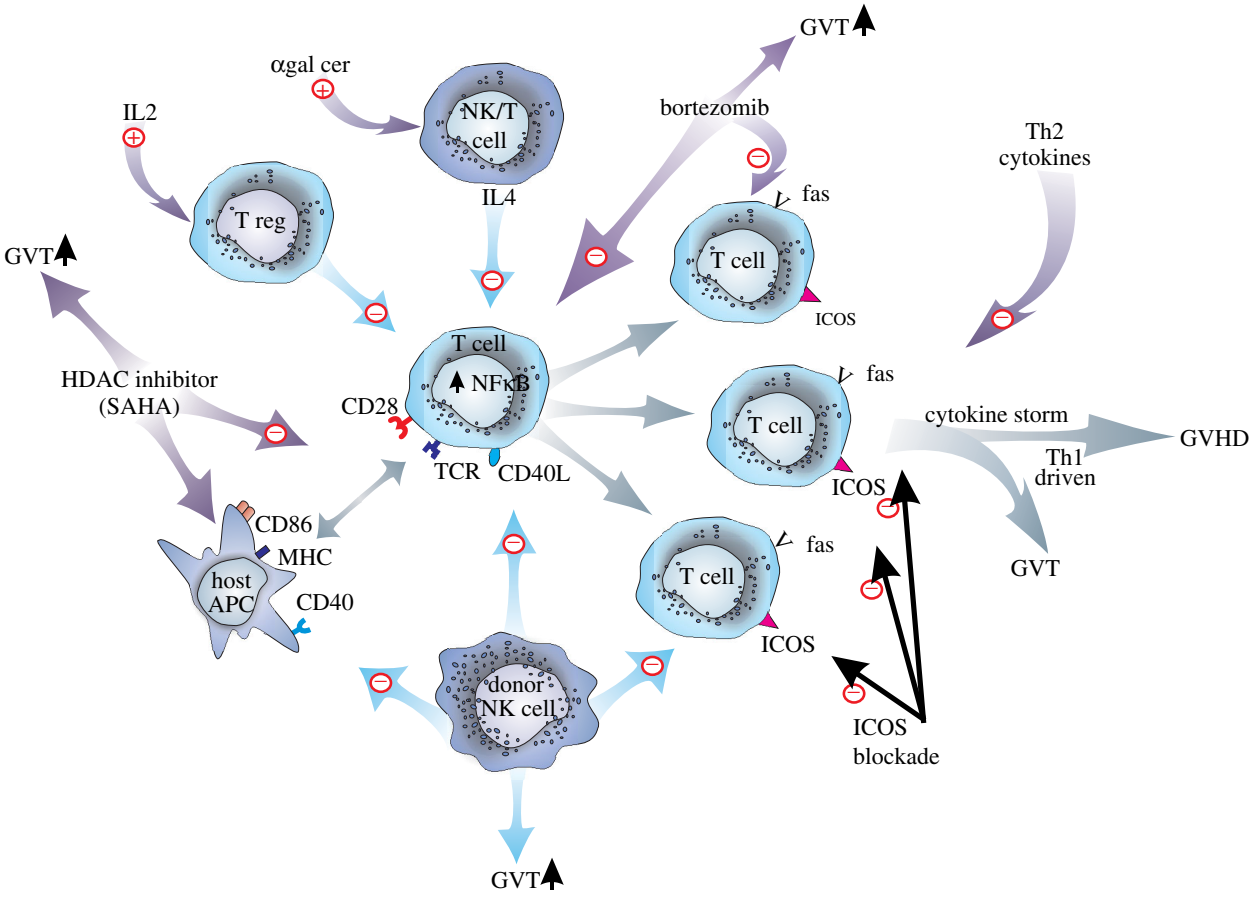

Figure 2. Current means to prevent GVHD.

Cytokines such as Flt3 ligand have also been given to recipients and shown to inhibit GVHD through the induction of CD8 alpha-positive DCs, again suggesting that exploiting the immunodulatory properties of APC subsets may be of use in GVHD (Teshima *et al.* 2002*b*), although the post-BMT administration of Flt3 ligand can paradoxically increase acute GVHD in other models (Blazar *et al.* 2001*b*). Recent data suggests, at least for chronic GVHD, that both donor and host APCs may play a role, suggesting that both may need to be targeted and that clinically both may of import in acute GVHD as well (Anderson *et al.* 2005), thus donor APC depletion may be advantageous in GVHD prevention. In all of these studies, effects on GVHD must be balanced with potential adverse effects of GVT.

(iv) *Mesenchymal stem cells (MSCs)*

Another promising cell therapy that may prevent or treat GVHD are derived from non-haematopoietic stem cells such as mesenchymal stem cells or stromal cells (MSCs) which are an adherent, $CD45^-$ BM cell population that can differentiate into a wide-spectrum of cells.

An intriguing feature of MSCs is their potency in inhibiting T cell responses. MSCs can express intermediate levels of MHC class I which is inducible to high levels with IFNγ (Le Blanc *et al.* 2003) but do not express MHC class II (Di Nicola *et al.* 2002; Tse *et al.* 2003) and express low levels of B7 ligands and CD40 (Tse *et al.* 2003). All but Potian *et al.* (2003) have shown that irradiated MSCs do not elicit vigorous T cell proliferative responses when used as stimulators (Le Blanc *et al.* 2003; Tse *et al.* 2003; Meisel *et al.* 2004). Human MSCs (hMSCs) are poor allostimulators as has been observed even after differentiation in osteogenic, chondrogenic or adipogenic media (Le Blanc *et al.* 2003). Autologous and allogeneic human BM stromal cells suppressed both $CD4^+$ and $CD8^+$ T cell proliferation in MLR and mitogen assays. A multicenter non-randomized trial of donor MSC infusion given at the time of BMT has been initiated to inhibit GVHD (Urbano-Ispizua *et al.* 2002). Acute and chronic GVHD risk appeared significantly reduced compared to historical pair-matched controls. A single case report in the literature provides intriguing evidence that MSCs can be used to treat steroid-resistant GVHD. Given the immune suppressive capacity of MSCs, it may be possible to use third-party MSCs as a universal donor source to prevent or treat GVHD.

3. FUTURE DIRECTIONS

There has been considerable progress in our understanding of GVHD and development of means to overcome this BMT complication without sacrificing GVT. Major areas under intense investigation currently have focused on exploiting Treg transfer, molecular targeting of T cell and GVHD responses, understanding the homing of T cells in part through the use of innovative imaging technologies, use of NK cells, and attempting to understand potential organ-specific responses and effects of interventions (figure 2). As more is understood on basic GVHD processes, T cell biology, and disassociating GVHD with GVT, the likelihood for increased efficacy of BMT looms larger.

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