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REPORT

NCI First International Workshop on The Biology, Prevention, and Treatment of Relapse After Allogeneic Hematopoietic Stem Cell Transplantation: Report from the Committee on the Biology Underlying Recurrence of Malignant Disease following Allogeneic HSCT: Graft-versus-Tumor/Leukemia Reaction

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The success of allogeneic hematopoietic stem cell transplantation (HSCT) depends on the infusion of benign stem cells as well as lymphocytes capable of participating in a graft-versus-tumor/leukemia (GVL) reaction. Clinical proof of concept is derived from studies showing increased relapse after the infusion of lymphocyte depleted hematopoietic grafts as well as the therapeutic efficacy of donor lymphocyte infusions without chemotherapy to treat relapse in some diseases. Despite this knowledge, relapse after allogeneic HSCT is common with rates approaching 40% in those with high-risk disease. In this review, we cover the basic biology and potential application to exploit adaptive T cell responses, minor histocompatibility antigens, contraction and suppression mechanisms that hinder immune responses, adaptive B cell responses and innate NK cell responses, all orchestrated in a GVL reaction. Optimal strategies to precisely balance immune responses to favor GVL without harmful graft-versus-host disease (GVHD) are needed to protect against relapse, treat persistent disease and improve disease-free survival after HSCT.

Biol Blood Marrow Transplant 16: 565-586 (2010) © 2010 American Society for Blood and Marrow Transplantation

KEY WORDS: HCT, relapse, Graft-versus-host disease, Immune modulation, Graft-versus-leukemia reaction

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has been successfully used in patients receiving myeloablative (MA) chemotherapy and irradiation for the treatment of a variety of hematologic malignancies. Initially, dose intensification to induce a maximal antitumor effect was considered essential. Because allogeneic HSCT can be accompanied by harmful immunologic complications including graft-versus-host disease (GVHD), early speculation favored the use of autologous hematopoietic stem cells (HSCs) or stem cells derived from a homozygous twin as the best source of cells for transplantation. However, subsequent clinical studies illustrated that the positive counterbalance of GVHD was a decreased likelihood of relapse after transplantation, compensating for the immune complications of allogeneic HSCT, leading to better

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Financial disclosure: See Acknowledgments on page 581.

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Received February 3, 2010; accepted February 5, 2010

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1083-8791/10/165-0001$36.00/0
doi:10.1016/j.bbmt.2010.02.005

disease-free survival (DFS). Apparently, donor-derived immune effector cells, primarily T cells, were not only responsible for the development of GVHD, but also for the reduced risk of relapse. As such, depletion of T cells from the graft to prevent GVHD resulted in an increased incidence of relapse after transplantation indicating that donor T cells were capable of mediating a graft-versus-leukemia/lymphoma (GVL) effect. Because transplantation with a T cell-replete graft from a homozygous twin did not result in GVL reactivity, it was concluded that the mere presence of T cells in the graft expressing a broad repertoire of specificities was not sufficient to mediate GVL reactivity, but that alloreactive T cells per se were responsible for the antitumor effect. Further proof of the capacity of donor-derived T cells to mediate a curative GVL effect came from the administration of donor lymphocytes to patients with recurrence of their malignancy after allogeneic HSCT. Donor lymphocyte infusion (DLI) as a treatment for recurrence of the malignant disease has resulted in 20% to 90% complete remissions depending on the malignancy. Chronic myelogenous leukemia (CML) in chronic phase (CP) was found to be most susceptible to DLI, but also patients treated for relapsed acute myelogenous leukemia (AML), multiple myeloma (MM), chronic lymphocytic leukemia (CLL), or non-Hodgkin lymphoma (NHL), and a few patients with acute lymphoblastic leukemia (ALL), showed clinically evident responses.

Although T cells mediate an antitumor effect following allogeneic HSCT, in HLA mismatched transplantations, complete removal of T cells of donor origin did not result in full abrogation of the GVL effect. Alloreactive natural killer (NK) cells of donor origin present in the graft or developing from donor stem cells following transplantation were also found to be capable of mediating an antitumor effect. Thus, T cells and NK cells make use of the genetic disparity between donor and recipient, resulting in better DFS in patients with high-risk hematologic malignancies. In addition, the role of donor antihost antibodies developing after allogeneic HSCT may also play a role in antitumor effects and GVHD.

From these observations, it can be concluded that the main advantage of allogeneic HSCT over autologous HSCT is the exploitation of the immune response to further eradicate the malignancy. This insight into the immunobiology of allogeneic HSCT has led to the development of strategies to reduce the intensity of the conditioning regimens to limit the toxicity of the transplant procedure. Reduced-intensity conditioning (RIC) using profound immunodepletion of the recipient instead of full myeloablation has greatly increased the applicability of allogeneic HSCT, allowing treatment of elderly patients with limited transplant-related morbidity and mortality. RIC has also changed the balance between donor and recipient cells following transplantation. This may change the balance between GVHD and GVL in favor of the antitumor response, which can improve the outcome of allogeneic HSCT with a better quality of life.

To allow appropriate execution of the immune-mediated antitumor effects, several immunologic phenomena have to take place. First, immune cells have to be activated in vivo or in vitro leading to the appropriate production and expansion of T cells, NK cells, or antibody-producing cells. Cells and alloantibodies have to localize to the tumor sites and mediate effector mechanisms, resulting in tumor destruction. Preferentially, following a contraction phase of the immune response, a memory response should develop capable of sustained antitumor control. In this report, we will discuss several aspects known to play a role in the development and execution of the immune response, and also try to identify missing links in understanding the biology of the immune reaction. Insight in these reactivities will lead to more specifically directed immune responses following transplantation, leading to increased antitumor reactivity with decreased immunologic complications following allogeneic HSCT.

ADAPTIVE T CELL-IMMUNE RESPONSES

T Cell Phenotype: Naïve/Effector/Memory

Naïve T cells develop in vivo following an education and selection process in the thymus, and circulate and migrate to lymphatic tissues to be activated upon encountering their specific target antigen. Following activation, they expand and execute their function, and following a contraction phase, a subset of T cells becomes memory cells, ready to be activated upon renewed antigen exposure. During life, an expanding repertoire of antigen-experienced T cells develops, depending on the exposure to foreign antigens. Thus, the donor T cell repertoire infused into the patient will be heterogeneous and will depend on the immunologic history of the donor. The donor T cells can be divided into naïve (T_N; never antigen-activated), effector (T_E; recently activated), and memory T cells (T_M), which can be classified further into effector memory (T_{EM}) and central memory cells (T_{CM}) [1,2]. Because of the fact that the T_M subset only contains T cells that have been activated in the past, the T cell receptor (TCR) repertoire of T_M is more narrow than T_N. However, because T_M have a lower threshold for activation than T_N , if the donor T cell repertoire contains T_M that can recognize recipient tissues, these cells may be readily activated after infusion, leading to an in vivo immune response. Donor T cells have diverse activation histories, which can impact their ability to mediate GVL and GVHD. The T cell subsets differ in various ways, including how they traffic, become activated, expand, and function after infusion. These

repertoire-dependent and -independent features of T cell subsets have an impact on GVL mediated by polyclonal unmanipulated donor T cell infusions. T_N, T_{EM}, and T_{CM} cells can all potentially mediate GVL [3-5], although it is likely that T_{EM} are the least potent in curing tumors because of their reduced ability to expand after transfer [4]. It is not likely that simple separation of the T cell subsets before infusion will lead to induction of GVL without GVHD.

Separation of GVHD from GVL and improvement of effectiveness and specificity of GVL may be obtained by in vitro selection or expansion of antigen specific T cells, or with genetically modified T cells with redirected specificities. For adoptive transfer of in vitro selected and expanded T cells with defined specificities, T_{CM} cells may be ideal [6,7]. Yet, some studies indicate that T_N rather than T_{CM} cells are superior after gene modification and adoptive transfer [8]. Despite this progress in understanding T cell biology, the relative contributions of T_N or T_M alloreactive T cells to controlling relapse after transplantation is unknown, and may depend on the repertoire of the donor, and the genetic disparity between donor and recipient.

Antigen Presentation and Antigen-Presenting Cells

In allogeneic HSCT, $CD4^+$ and $CD8^+$ T cells that mediate GVL must interact with major histocompatibility complex (MHC)/peptide complexes present on leukemia target cells [9-11]. In the context of HLA matched transplantation most leukemia-reactive, minor histocompatibility antigen (mHA) specific T cells are T_N. These T cells must likely be primed by professional antigen presenting cells (APCs) to be appropriately activated in vivo, although the precise roles of APC subsets (dendritic cells [DC], macrophages, B cells) are not well defined. Therefore, an effective GVL response may require that professional APCs presenting the antigens expressed by leukemia cells or that the leukemic cells themselves acting as professional APCs, as may be the case in CML, are present to provoke a T cell response of a sufficient magnitude. Early after transplant, when there are residual conditioning regimen-resistant host APCs, direct presentation of antigens derived from genes endogenously coexpressed by APCs and the malignant cells likely provides a sufficient source of antigen to drive effective GVL; and data in mouse models support this hypothesis [9,12]. The kinetics of an appropriate mHA-reactive T cell response show early activation, expansion, and then a decline, typically mimicking T cell responses in pathogen systems [13,14]. Because a maximal GVL effect may require a sustained response, this may be achieved by repeated stimulation of initially activated cells, new recruitment of T cells directed against these specificities, or subsequent activation of T cells directed against other antigens.

If host hematopoiesis after transplantation is completely replaced by cells of donor origin, including donor-derived APCs, and the immune response cannot be directly triggered by leukemic APCs, the recipient (malignancy) associated antigens cannot be endogenously presented by professional APC. Under those circumstances, donor T cell activation has to be triggered by host antigens that are crosspresented by donor APCs [15]. Mouse models have demonstrated that indirect presentation occurs, although how effective this process is after transplant is less well studied [10,15-17]. Late-onset GVHD in clinical allogeneic HSCT, when full donor chimerism has developed, may indicate that this also occurs in humans. If at this time indirectly presented host antigens are derived from nonhematopoietic cells, it is possible that the host mHAs targeted may not be expressed on leukemia cells, leading to GVHD, but not GVL. If allo-immunity is directed against ubiquitously expressed host antigens, both GVHD and GVL can be expected. This type of immune response may be sustained because of continuous antigen access for donor-derived APCs; although the continuous host antigen/peptide exposure to donor T cells can also lead to trapping or consumption of alloreactive and potentially GVL-inducing T cells in tissues [18] and T cell exhaustion [19,20]. To induce crosspresentation, activation signals are likely necessary. In mouse models, CD4 "help" by CD40L-expressing T cells, type I interferons (IFN), and Toll-like receptor (TLR) ligation all promote crosspresentation [21-26]. How to exploit these mechanisms in patients who relapse after transplantation is less clear. In a patient who has relapsed, the standard approach is to first withdraw immunosuppressive drugs, and, if this is ineffective, to administer DLI. These are both T cell-directed interventions, and little attention is paid to the APC component of the response. Possible strategies that would target the APC component could include adding vaccination against hematopoietically expressed host mHAs or stimuli that promote crosspresentation of host antigens by donor APCs.

T Cell Allo-Activation (Signal 1)

If allogeneic HSCT is performed over HLA barriers, a profound immune response is likely to occur. Allogeneic T cell responses against HLA antigens are comprised of multiple responses directed against a variety of peptides presented in the context of nonself HLA molecules. These T cell responses are caused by normal T cells educated to recognize foreign peptides in the context of self MHC, but are crossreactive with unrelated antigens when presented in the context of nonself MHC molecules. As a consequence, these T cell responses are present in both the T_N and the T_M

compartments of the donors. Therefore, following HLA-mismatched HSCT, T_M cells, capable of recognizing a variety of antigens presented in various tissues of the recipient, will be infused. Because T_M cells can be activated in the absence of professional APCs, severe GVHD is more likely to occur if patients are transplanted with HLA-mismatched donor grafts. On the other hand, if patients are transplanted with a donor graft from a very young individual or umbilical cord blood, the likelihood of large numbers of donor T_M cells capable of recognizing allo-HLA antigens is more limited, and therefore, grafts from umbilical cord blood or very young donors are less likely to cause this severe type of GVHD because the allo-HLA-directed T_N cells must be activated by professional APCs. If allo-HLA responses develop, they will be directed against both normal and malignant target cells, leading to both GVL and GVHD. After HLA-matched transplantation, the mHA presented and recognized in the context of self MHC are less likely to be previously encountered by donor T cells, and therefore, professional APCs with additional costimulatory molecules play a more crucial role in the initiation of the allo-immune response. If primary immune responses against hematopoiesis-associated host mHA (discussed in detail below) expressed by the APCs develop, these T cell responses may lead to separation of GVL and GVHD, whereas transplantation over HLA barriers are likely to cause immune responses leading to both GVHD and GVL reactivity.

T Cell Allo-Activation (Signals 2 and 3)

Costimulatory molecules that deliver a second signal to donor T cells can play pivotal roles in the donor T_N cell activation required for GVHD induction. The most well-studied pathway, CD28/CTLA-4:B7, involves homologs that can deliver positive (CD28/B7) or negative (CTLA-4/B7) interactions. Specific blockade of the positive pathway reduces GVHD, whereas blockade of the inhibitory pathway increases GVHD lethality [27]. Other CD28 family members include inducible costimulator (ICOS) and the programmed death-1 (PD-1) pathways. ICOS is present on activated and T_M cells, binds ICOS-ligand (a.k.a. B7h), and promotes T_E cell responses [28,29]. Precluding ICOS signals on donor T cells diminishes donor T cell-mediated alloresponses, especially of the gastrointestinal tract and liver [30,31], which proved to be $CD4^+$ T cell-dependent disease in some [32], but not in other studies [33]. Loss of ICOS signaling worsens $CD8^+$ T cell-mediated GVHD, as a consequence of increased expansion of donor $CD8^+$ T cells [33]. PD-1, an inhibitor of activated T cells, also is expressed in the cytoplasm of $CD4^+CD25^+$ T regulatory (Treg) cells [32]. Blockade or absence of PD-1 on donor cells accelerates both $CD4^+$- and $CD8^+$-mediated GVHD [34].

Members of the TNF receptor (TNFR) family function as costimulatory molecules and modulate GVHD and GVL. OX40 is present on both activated $CD4^+$ and $CD8^+$ T cells as well as on activated APCs. Despite the presence of the receptor on both T cell populations, activation of OX40 has been reported to promote $CD4^+$ but not $CD8^+$ T cell-mediated GVHD [35]. CD40 ligand (CD40L) is expressed on activated $CD4^+$ T cells, and CD40:CD40L interaction increases acute GVHD[36] by promoting $CD4^+$ T cell-mediated tissue destruction and $CD8^+$ T cell expansion [37]. All 4 of the aforementioned costimulatory pathway members (CD28, ICOS, OX40, and CD40L) act independently, as inhibition of any single pathway does not eliminate GVHD, and co-blockade results in greater protection [38,39]. Two other TNFR members include 4-1BB and glucocorticoid-induced tumor necrosis factor receptor (GITR). 4-1BB is expressed on activated $CD4^+$ and $CD8^+$ T cells and on NK cells [40,41]. Inhibition of 4-1BB binding to 4-1BB ligand reduces $CD8^+$ T cell-mediated GVHD lethality and $CD4^+$ Th1 generation in GVHD [42,43]. GITR is expressed constitutively on $CD4^+CD25^+$ Treg cells and activated $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells [44]. Stimulating GITR on Treg cells or removal of $GITR^+$ cells reverses suppression, leading to the development of autoimmune disease [44]. GITR stimulation on $CD4^+CD25^-$ T cells reduced GVHD in MHC II-disparate recipients, whereas stimulation of GITR of $CD8^+CD25^-$ T cells increased proliferation and GVHD in a MHC I-disparate murine model [45].

In addition to signals 1 and 2, activation of naïve $CD8^+$ T cells to undergo clonal expansion and develop effector function requires a third signal that can be delivered by IL-12, type I interferon (IFN), or adjuvant. The third signal, most important when antigen levels are low [46], results in the upregulation of bcl3 [47] and granzyme B expression [48]. Even at high antigen levels, the third signal, although not needed for proliferation, is necessary for the full development of cytolytic effector function [46]. The third signal is critical in determining whether stimulation by antigen results in tolerance versus development of effector function and establishment of a responsive memory population. Prolonged exposure to antigen and costimulation, along with a third signal are required for $CD8^+$ T cell clonal expansion. Importantly, signal 3 availability at the tumor site can influence $CD8^+$ T cell responses to a solid tumor [49].

T Cell Trafficking

Manipulation of T cell trafficking may be an attractive strategy to separate GVL and GVHD. Trafficking and migration involve a complex series of events mediated by integrins, chemokine receptors,

and selectins on the surface of lymphocytes, and the interaction of these molecules with cognate extracellular ligands. Selectins (addressins) affecting lymphocyte migration may also be expressed on endothelial cells of lymphoid and target tissues [50]. The biologic roles of homing molecules can be pleiotropic, for example, contributing to the binding of T cells and APCs at the immunologic synapse [51].

The CC-chemokine receptor-7 (CCR7) is thought to play an important role in the entry of naïve lymphocytes into secondary lymphoid organs prior to their activation by APCs, after which it is downregulated as activated T cells migrate to target tissues to execute effector functions [52,53]. Individual selectins, integrins, and chemokines/chemokine receptors have all been implicated in the pathogenesis of GVHD [50].

Chemokine receptor CCR2 knockout donor T cells and β7 integrin knockout donor T cells have both demonstrated decreased homing to the liver and GI tract and decreased GVHD, but intact GVL responses [54-56]. A humanized antibody to the α4 subunit of certain integrin heterodimers, natalizumab, has been tested for use in inflammatory bowel disease and multiple sclerosis [57-61]. Heterodimers with the α4 subunit are important for homing to Peyer's patches and gut mucosa, and may thus be useful for blocking migration of activated T cells to GVHD target sites, although still permitting activation of GVL effectors in lymphoid tissue [62]. This hypothesis remains to be tested, but it is supported by the knowledge that the α4 subunit forms heterodimers with the β7 subunit described earlier. Broad questions remain regarding the function of homing molecules and T cell trafficking during GVL, but an assessment of the homing molecules implicated in GVHD presents at least an initial road map [50]. Such studies are necessary to distinguish molecules critical for GVL from those that may be blocked to prevent GVHD without impairing GVL.

T Cell Cytolysis

Cytotoxic T cell lymphocytes (CTLss) execute their function via use of the perforin/granzyme system and death receptor ligands (FasL, tumor necrosis factor [TNF], TNF-related apoptosis inducing ligand [TRAIL], tumor necrosis factor-like weak inducer of apoptosis [TWEAK]), which utilize the target cell's own machinery to initiate apoptosis [63]. As with mechanisms of lymphocyte homing, the role of cytotoxicity has been studied in greater detail in GVHD than in GVL. Most studies have emphasized preservation of GVL cytotoxicity and suppression of GVHD. Studies with patient samples have demonstrated varying levels of leukemic sensitivity to FasL and perforin, the 2 classic cytotoxic effector molecules [64].

Murine models have suggested the perforin pathway is important for GVL, whereas FasL appears to have a greater role in GVHD [63]. However, both molecules have been implicated in both GVHD and GVL [63,65,66]. TNF-α, which plays a central role in GVHD pathogenesis, has also been implicated in GVL, although it is possible that the GVL effect of TNF-α may be related to an effect on donor T cells rather than on host neoplastic cells [10,63,67]. The role of TWEAK in GVL is unknown, however TRAIL may have a role limited to GVL without potential for induction of GVHD[63].

THE ROLE OF MINOR HISTOCOMPATIBILITY ANTIGENS IN THE GVL EFFECT

Minor Histocompatibility Antigens

GVHD and GVL activity occur in the majority of allogeneic HSCT recipients who receive T cell-replete grafts and/or DLI from genotypically MHC-matched donors. Thus, GVHD and GVL in this setting are triggered by donor-recipient genetic disparity at polymorphic loci outside the MHC, which are consequently referred to as minor histocompatibility loci. Studies in both mice and humans have demonstrated that most minor histocompatibility loci encode short peptides, termed mHAs, which are encoded by polymorphic genes. These are presented on the cell surface by MHC class I and II molecules, where they can be recognized by donor $CD8^+$ and $CD4^+$ T cells, respectively. $CD8^+$ and $CD4^+$ T cell clones specific for recipient mHAs can be isolated from most HSCT recipients of T-replete grafts or after DLI.

Molecular characterization of mHAs recognized by T cells after MHC-matched HSCT has demonstrated that the genetic loci encoding mHAs can be divided into 2 broad categories based on their chromosomal location (Figure 1). The first category comprises a small class of Y chromosome genes that encode male-specific mHAs (H-Y antigens), which are targets for female T cells in sex-mismatched transplants. The genes encoding all of the known H-Y antigens have X chromosome homologs that encode proteins that are 81% to 99% identical in sequence with the Y chromosome-encoded isoforms, and are expressed both in and outside the testis. The extensive sequence disparity between the Y and X chromosome-encoded isoforms creates a large number of male-specific peptides that could potentially serve as targets for female T cells in MHC-matched transplants. The immunologic significance of H-Y antigens is enhanced by the fact that the Y chromosome is inherited as a single functional genetic element, and, therefore, all H-Y antigens are in complete linkage disequilibrium. At least 12 H-Y antigens encoded by 6 different Y chromosome genes have been identified to date, and many more remain to be identified.

A heterogeneous group of polymorphic autosomal loci widely distributed throughout the genome

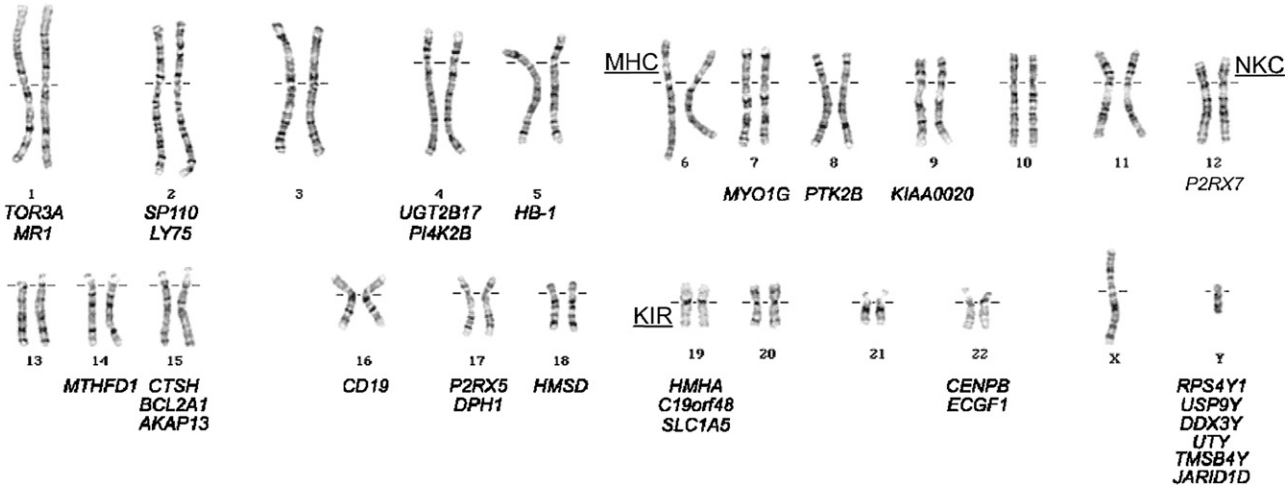

Figure 1. Chromosomal distribution of the 30 genes known to encode mHAs. The chromosomal locations of the Major Histocompatibility Complex (MHC), Natural Killer Complex (NKC), and Killer-cell Immunoglobulin-like Receptor (KIR) locus are also indicated.

comprise the second major class of mHA-encoding genes (Figure 1). Although some of these loci encode well-characterized proteins with known functions, there are several whose protein products are as yet completely uncharacterized. The polymorphism in autosomal minor histocompatibility loci is generated by variation in human genome sequences, which creates most of the autosomal mHAs identified to date. Variation in genome structure, which creates at least 4 known mHAs, likely creates other mHAs that have yet to be characterized. Nonsynonymous single nucleotide polymorphisms (SNPs) in the coding sequence of normal self proteins that create amino acid polymorphisms in the sequence of these proteins create most of the known autosomal mHAs. Such polymorphisms can alter how peptides are generated from self-proteins inside the proteasome. Peptides are then transported into the endoplasmic reticulum by the transporter associated with antigen processing, bind to MHC molecules, or are recognized by T cells when presented on the cell surface in a complex with MHCs. Both mHAs encoded within normal, as well as alternative open reading frames are frequently found [68,69]. Alternative mRNA splicing because of SNPs in the introns of protein-coding genes [70] and single nucleotide deletions leading to frameshift polymorphisms [71] can also create mHAs. Larger scale variation in the human genome structure creates mHAs through deletion of entire protein-coding sequences. A common deletion polymorphism on chromosome 4q that spans 117 kb and includes the entire UGT2B17 locus creates at least 3 different mHAs [72-74].

Expression of mHAs in Normal and Malignant Tissues

Evaluation of the expression of mHAs in normal and malignant tissues has provided insight into the potential contributions of mHA-specific T cell responses to GVL activity. Many mHAs are expressed in both hematopoietic and nonhematopoietic cells in vitro, suggesting that T cell responses to these antigens could contribute to both GVL and GVHD. However, a significant fraction of mHAs show expression that is limited to cells of hematopoietic origin, raising the prospect that T cell responses against these mHAs might contribute selectively to GVL activity. Because HLA class II molecules under most conditions are preferentially expressed on HSCs compared with non-HSCs HLA-class II restricted mHAs have a higher likelihood of being selectively expressed by malignant hematopoietic cells [75-77]. $CD4^+$ and $CD8^+$ T cell clones specific for mHAs that are selectively expressed in HSCs recognize lymphogenous and myelogenous leukemic cells in vitro and inhibit the growth of clonogenic leukemic cells in in vitro culture [78,79]. $CD8^+$ mHA-specific CTL clones also inhibit the engraftment of human acute leukemia into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, demonstrating that leukemic stem cells [80,81] express mHAs and can be targeted by mHA-specific CTLs [82,83], which can be active against established hematopoietic tumors in vivo [84].

Contributions of mHA-Specific T Cell Responses to GVL

Cellular and molecular dissection of immune responses occurring in recipients after HSCT or DLI has provided compelling evidence that both autosomal and H-Y mHAs expressed on recipient tumor cells are targets of GVL responses in MHC-matched HSCT. Flow cytometric analysis with mHA peptide/MHC tetramers has shown that mHA-specific T cells with in vitro reactivity against recipient leukemic cells increase in frequency in peripheral blood before and

during clinical regression of malignancy [68,69,71,85,86]. Analysis of the T cell repertoire in patients with persistent or recurrent disease who responded to DLI showed that the T cell response associated with GVL is polyclonal and likely directed at a significant number of distinct antigens [87]. Comprehensive application of single-cell cloning has directly confirmed this hypothesis, and demonstrated that GVL reactions involving T cells with diverse antigen specificity occur in patients with CML [88,89] and CLL [90].

Retrospective analyses of transplant outcome have attempted to evaluate the contribution of mHA-specific T cells to GVL activity by correlating donor-recipient disparity at specific minor histocompatibility loci with posttransplant relapse or remission of disease. Studies that focused on disparity at autosomal mHAs have had inconsistent results, but the largest of such studies showed no significant correlation between donor-recipient disparity at specific loci with tumor control after HSCT. In contrast, several large retrospective analyses have identified a significantly lower rate of relapse in male recipients of female hematopoietic cell grafts (F → M HSCT) than in any other donor/recipient sex combination, suggesting that T cell responses to H-Y antigens have a clinically measurable antileukemic effect [91,92]. Thus, T cell responses against multiple H-Y antigens are thus likely to be elicited in any F → M HSCT recipient.

Human Genomic Diversity and the Number of mHAs

At least 30 different autosomal and Y chromosome genes have been shown to encode mHAs (Figure 1), but it is likely that many more mHAs remain to be identified. The human genome is characterized by an enormous quantity of sequence and structural variation. To date, more than 150,000 SNPs that cause amino acid polymorphisms in the sequence of known or predicted proteins (www.hapmap.org), and common deletion polymorphisms that span several dozen known and predicted genes have been identified. Thus, there are a very large number of polymorphic protein and peptide sequences that, in the setting of MHC-matched allogeneic HSCT, could potentially encode mHAs. Recognition of the enormous diversity in the human genome demonstrates that there is abundant polymorphism that could potentially be exploited to enhance GVL activity after MHC-matched HSCT, but also suggests that whole-genome approaches to understanding GVL will likely provide the key to its successful exploitation.

T Cell Responses to Nonpolymorphic Antigens

In addition to its direct antitumor action, the mHA-specific T cell response that occurs in recipients of allogeneic HSCT can enable and recruit antitumor T cell responses to nonpolymorphic antigens that are derived from normal self proteins and are over or aberrantly expressed in recipient tumor cells. $CD8^+$ T cells specific for the HLA-A*0201-restricted PR1 peptide that is contained within both proteinase-3 and elastase, the major constituents of the primary azurophil granules of normal promyelocytes as well as AML and CML blasts, can be detected in the blood of many HLA-A*0201^+ CML patients who achieve remission after MHC-matched HSCT [93,94]. PR1-specific CTLs recognize myelogenous leukemia cells in vitro [95] and inhibit the growth of CML colony-forming units [96]. The protein product of the WT1 gene is also a target of spontaneous posttransplant T cell responses that could contribute to GVL activity against myelogenous and lymphogenous leukemias. $CD8^+$ HLA-A*0201-restricted responses to WT1 have been observed in HLA-A*0201^+ ALL patients after HSCT and their appearance was correlated with clearance of molecular markers of residual disease [97].

Increasing evidence also suggests that donor T cell responses against recipient tumor-specific antigens frequently occur in allogeneic HSCT recipients and could make significant contributions to GVL activity. Regression of MM in several patients who received DLI with $CD4^+$ cells was associated with the appearance and selective expansion of myeloma-specific $CD8^+$ T cells [98]. Antigens encoded by cancer-testis (C-T) genes including NY-ESO-1 and the MAGE and SSX gene families are often expressed in advanced or poor-prognosis myeloma, and B and T cell responses to these C-T antigens are preferentially detected in patients who have undergone allogeneic HSCT [99]. CML cells express several tumor-specific antigens that can elicit T cell responses in cancer patients, including BCR-ABL junctional peptides, and BCR-ABL-specific T cells have been detected in CML patients both before and after HSCT [94]. In recent studies remission of CLL after nonmyeloablative MHC-matched HSCT was closely associated with appearance of tumor-specific $CD8^+$ T cells [90,100].

Role of HLA-DP Mismatch in Unrelated Donor (URD) Transplantation

Recent genetic and clinical studies suggest that donor/recipient mismatch at loci within the classical MHC, most notably at HLA-DP, may play a uniquely important role in preventing or treating relapse after transplantation from unrelated donors [101]. High-density SNP genotyping of 1500 HSCT donor/recipient pairs at the Fred Hutchinson Cancer Research Center in Seattle has shown that sibling donor/recipient pairs who are identical at the HLA-A, -B, and -DR loci are invariably also identical at HLA-C, HLA-DQ, and HLA-DP, and, in fact, are identical throughout

the entire ~4 Mb region encompassing the MHC. This is presumably because of inheritance of the same 2 copies of chromosome 6 from their parents (S. Li and E.H. Warren, in preparation). However, the same is not true for unrelated donor/recipient pairs, even those who are matched at the DNA sequence level at the HLA-A, HLA-B, and HLA-DR loci. Significant genetic nonidentity within the classical MHC exists within unrelated donor/recipient pairs who are otherwise identical at the DNA sequence at the HLA-A, -B, and -DR, loci, and this nonidentity is particularly prominent at the HLA-DP locus (S. Li and E.H. Warren, in preparation). Donor/recipient mismatch for HLA-DP represents an MHC mismatch that would be expected to stimulate a polyclonal HLA-DP-restricted, donor anti-recipient T cell response in most, if not all, mismatched pairs, and an HLA-DP-restricted response could potentially have a significant antitumor effect [101,102]. Two retrospective analyses, which collectively included more than 10,000 unrelated donor transplants facilitated by the National Marrow Donor Program and the Japan Marrow Donor Program, have, in fact, confirmed that donor/recipient mismatch at HLA-DP is associated with a statistically significant decrease in the rate of posttransplant relapse [103,104]. A similar effect associated with donor/recipient mismatch at HLA-C was also identified, but the clinical significance of HLA-C mismatch is not as great as that associated with HLA-DP mismatch because it is observed in far fewer unrelated donor/recipient pairs (S. Li and E.H. Warren, in preparation).

In conclusion, genetic disparity between donor and recipient is the key to the therapeutic efficacy of HSCT, but is also the root of GVHD, its primary limitation. Cellular and molecular dissection of GVL responses have demonstrated that GVL after MHC-matched HSCT is initiated by donor $CD8^+$ and $CD4^+$ T cells that recognize recipient mHAs encoded by polymorphic genes. This alloresponse likely recruits additional effector cells recognizing tumor-specific or tumor-associated antigens encoded by nonpolymorphic genes that are over or aberrantly expressed in recipient tumor cells. The GVL response in most HSCT recipients is therefore likely to comprise a broadly focused immune response directed at a large number of polymorphic and nonpolymorphic target antigens. Deeper understanding of the breadth and the antigenic specificity of the GVL response will be required to exploit it successfully. Selective expression of some GVL target antigens in recipient HSCs and recipient tumor cells, with limited expression in nonhematopoietic tissues, may allow the development of therapeutic strategies for enhancing GVL activity without inducing or aggravating GVHD. Characterization of the extensive sequence and structural variation in the human genome suggests that there are likely to be a large number of polymorphic protein and peptide sequences at which immunotherapy to augment GVL could potentially be directed.

IMMUNE CONTRACTION AND SUPPRESSION

Survival of the Organism from a Potentially Overzealous Immune Response

Analogous to our understanding of tumoriogenesis, in which the initial emphasis on oncogenes became supplanted with the characterization of tumor suppressors as critical checkpoints that need to be overridden, there has been an increasing realization that merely stimulating the immune system in cancer will not be sufficient. Immune responses to any stimuli eventually undergo a contraction phase and also induce potent suppressive pathways, as biologic responses are geared to end. Immunotherapy in cancer has shown great promise but has been lacking with sustained responses and overall survival being affected. In part, this can be attributed to contraction and suppression pathways inherent to the immune response itself and in part because of manipulation of the suppressive pathways by the tumor. It has become clear that either augmenting the immune response in cancer or providing adoptive immunotherapy will need to overcome these hurdles to maintain the response. This represents a key pathway that, despite the successful generation of antitumor effectors, will limit responses.

Naïve T Cell Expansion, Differentiation, Contraction and Memory T Cell Generation

Whereas cytoreductive antitumor therapies can reduce disease burden, residual tumor cells must be eradicated or held in check by the immune system. The most compelling evidence for a T cell-mediated immune-mediated antitumor response can be derived from the adoptive transfer of allogeneic donor lymphocytes used to treat leukemia recurrence following transplantation [105]. Assays for minimal residual disease in CML have shown a gradual reduction in tumor burden, often requiring months to years to achieve the full biologic effect. These data are consistent with a critical threshold number of antitumor reactive T cells that is needed to achieve and sustain an antileukemia effect.

Both T_N and T_M cells can contribute to antitumor responses. With age and repetitive radiation or chemotherapy courses, fewer näive T cells are produced [106]. Under those conditions, T_M cells may be the dominant antitumor reactive T cell responding population. Naïve T cells encountering immunogeneic tumor peptides or antigens can be skewed toward an antileukemia response, especially during periods of lymphopenia

induced by cytoreductive therapies. This results in cytokine accumulation (eg, IL-7, IL-15) and substantial T cell homeostatic expansion and differentiation of naïve T cells into T_E cells that reduce tumor burden [107]. Typically within the first week, a second and profound contraction phase occurs because of activation-induced cell death, cytokine consumption, and/or inhibitory mechanisms (eg, exposure to IFN-gamma or TGF-β immunosuppressive cytokines, upregulation of CTLA-4 or PD-1 inhibitory coreceptors or TRAIL or Fas pathway death receptors). Following the contraction phase, a small subset of antigen-reactive T_{EM}, T_{CM}, and putative memory stem cells survive, each of which may retain an antitumor response capacity. In the presence of $CD4^+$ T cell help, posthomeostatic proliferation-induced memory-like cells are produced, which can have similar responses to conventional $CD8^+$ T_M cells [108]. The precise mechanisms by which $CD4^+$ T cells maintain $CD8^+$ T cell memory are still poorly understood, and this may impact the efficacy of $CD8^+$ T cell transfer.

If tumor antigen levels are sufficient to trigger their TCR, memory cells may receive survival signals that promote their persistence and function, thereby permitting further reductions in tumor burden and continued immune surveillance. If antigen levels are too high, T cells may become exhausted and are known to express inhibitory molecules such as PD-1, which upon engagement by PD-1 ligand (PD-L) render antigen-reactive T cells hyporesponsive in terms of proliferation, cytokine production, and killing capacity [20]. If antigen levels are too low, these memory populations would become quiescent until they are reengaged by antigens that trigger their TCR and sufficient adjuvant signals such as cytokines or costimulatory pathway receptors that can reawaken cells to proliferate and lyse tumor cells.

The Tumor Microenvironment

A key factor that regulates the size and activation status of the memory cell pool is tissue location. Antitumor reactive T_M cells may reside in secondary lymphoid organs, parenchymal organs, bone marrow (BM), or the tumor microenvironment itself. Some but not all of these environments are conducive to an antitumor response by providing positive signals (eg, costimulation, stimulatory cytokines) and only a limited number of inhibitory signals or cell populations that restrain memory cell activation, expansion, or acquisition of lytic function. Another key factor in the naïve and memory T cell response is the capacity of antitumor reactive T cell to migrate to the tumor site. Therefore, T cell populations not residing at the tumor site, and lacking necessary addressins, selectins, or chemokine receptors for migration to the tumor site are unlikely to substantively contribute to the immune response.

The immune environment in which T_N or T_M cells reside may suppress the antitumor immune response by affecting T cell activation, proliferation, pro- or anti-inflammatory cytokine expression, cytolytic molecule expression, pro- or anti-apoptotic molecule expression, or the regulation of receptors involved in migration. The size and activation status of the T_M cell compartment is determined by the balance between positive and negative signals delivered to the memory T cell. The positive and negative immune pathways work in concert, but suppression tends to trump over stimulation. The lack of the stimulatory factors may also predispose the immune cells to the suppressive pathways. These suppressive pathways may predominate even more in the aged individual, which may significantly limit immune interventions in this population. Typically, the tumor highjacks its microenvironment to foster tumor growth and discourage a productive immune response. Tumor cells such as those seen in Hodgkin lymphoma cells can produce immunosuppressive cytokines, such as IL-10 or TGF, which suppress T cell proliferation or induce Tregs that constrain T_E-cell responses in the tumor microenvironment [109]. Tumor cells can actively attack the attackers by expressing fasL and PD-L [110].

Tumor cells may express high levels of the intracellular tryptophan catabolic pathway, indoleamine 2,3 dioxygenase (IDO), as has been demonstrated for AML cells [111]. In addition, $CD11c^+CD19^+$ plasmacytoid dendritic cells (pDC) present in draining lymph nodes or the tumor microenvironment of rodents and humans can express high levels of IDO, which may be upregulated by tumor cells (eg, melanoma, breast cancer), Toll-like receptor-9 (TLR-9) ligation from exposure to bacteria products, or the T cell response itself via the elaboration of IFN-γ or surface expression of CTLA-4 [112]. High IDO levels act in the local microenvironment to stimulate integrated stress response pathways in T cells activated by amino acid starvation resulting in cell cycle arrest or apoptosis. In rodents, IDO expression in pDC has been shown to augment mature Tregs, and, upon TLR-9 ligation, blocks the conversion of Tregs into Th17-like cells [113], while in humans, TLR-9-activated pDC can convert naïve T cells into Tregs [114].

Tumors can cause T cell dysfunction by altering TCR signaling mechanisms [115]. Depletion of the amino acid L-arginine by cytoplasmic arginase I profoundly inhibits $CD4^+$ and $CD8^+$ T cell functions [116]. L-arginine depletion is associated with the downregulation of the TCR zeta chain in T cells that infiltrate in the tumor microenvironment. Arginase I is expressed at high levels in myeloid-derived suppressor cells (MDSCs) that infiltrate the tumor microenvironment [116]. Murine MDSCs have been shown to have an increased uptake of L-arginine because of high levels of a cationic amino acid transporter, 2B

[117]. In addition, prostaglandin-E_2 is synthesized by MDSCs, which most likely contributes to immune suppression in the tumor microenvironment [118]. In humans, MDSCs are $CD34^+CD33^+CD13^+$, indicative of a myeloid lineage [119]. In mice, Gr-1^{low}-$CD11b^{high}Ly\text{-}6C^{high}SSC^{low}$ and Gr-$1^{high}CD11b^{low}$ cell fractions possessed suppressive potential [119]. The latter cells coexpressed F4/80, high levels of Ly6C, and low levels of CSF-1 receptor (CD115). Suppression was observed, albeit at as lesser potency on a cell-by-cell basis for Gr-$1^{hi}CD11b^{hi}$ cells. IFN-γ or lipopolysaccharide not only activates MDSCs, but suppresses the differentiation of DCs from BM myeloid progenitors. Therefore, an inflammatory response to tumor cells can result in the generation of MDSCs in the tumor microenvironment, offsetting the host antitumor immune response. Intriguingly, in patients with renal cell carcinoma, IL-2 therapy markedly increased MDSC percentages and arginase I levels [120]. Thus, IL-2 has a dual effect on the immune response by increasing T_E cells and NK cells as well as Tregs and MDSCs. MDSCs also can suppress T cell function via other mechanisms such as the elaboration of nitric oxide or regulation of CD80 and PD-L expression. Vascular endothelial cell growth factor (VEGF), known to be produced by almost all tumor cells, has been shown to inhibit the development of DC from the BM which was associated with an increase in immature, immune suppressive $Gr1^+$ myeloid cells [121]. In some types of cancer patients, immature DCs, in fact, are increased in the peripheral blood of patients.

A functional state of tumor-mediated suppression may occur as a result of deviation of the immune response toward a nonfavorable T_E cell phenotype (eg, Tregs type I; anti-inflammatory cytokine producing Th2 cells; TGF-β-secreting Th3 cells). Alternatively, tumor cells may inhibit antigen recognition by a variety of mechanisms. For example, some tumors downregulate major histocompatibility complex antigens (eg, MHC class I in neuroblastoma cells) or potentially immunodominant antigens (eg, EBV nuclear antigens, 3A, B, C in Hodgkin disease). Tumor cells can also secrete "decoy" molecules such as soluble MICA, which can inhibit NKG2D-mediated killing by NK cells. Defective APC function has been reported in cancer patients as a consequence of low expression of costimulatory molecules (eg, B7 ligands), exposure to high levels of IL-10 that result in tolerogenic DC, or high expression of inhibitory coreceptors (eg, PD-L).

Therapeutic Strategies to Overcome Immune Contraction and Suppression

Bypassing activation-induced cell death (AICD) is critical for sustaining activated immune cell engraftment and function. However, many cytokines and pathways can exert opposing effects. IFN-γ is a major mediator that is critical for many antitumor effector functions as well as a major mediator limiting T cell responses, particularly $CD4^+$ T cells via AICD [122]. During T cell activation, CD25 (IL-2Rα chain) is upregulated, followed by the downregulation of CD127 (IL-7Rα chain) at the time of T_E cell to effector/memory transition. As such, it is reasonable to consider the use of IL-2 or IL-7 to expand T_E cells. However, the biology of IL-7 and other cytokines such as IL-15 is complicated because of the effects of presentation of the cytokine by DC's potentially yielding opposing effects on the T cell [123]. Moreover, the consumption of cytokines that can occur during the expansion phase, particularly following lymphopenia and homeostatic expansion, may result in a relative deficiency of cytokines to support activated T_E cells. Exogenous IL-2 administration has been shown to increase T_E cells as well as NK cells and Tregs. Of note, high doses of IL-2 can cause AICD of activated T cells, and IL-2 withdrawal can cause cytokine-deprivation induced apoptosis. Therefore, the available levels of IL-2 for IL-2R engagement are critical in determining the ultimate T cell fate. Recent data suggest that the binding of IL-2 with an antibody can result in supraphysiologic effects in vivo and even bypass the need of NK cells for IL-15 [124,125]. In rodents, IL-7, the second member of the IL-2R common gamma chain family of cytokines, has been shown to be a critical growth factor for $CD8^+$ T_E and T_M cell survival. In humans, exogenous IL-7 supported a sustained increase in peripheral blood $CD4^+$ and $CD8^+$ T cells with broadening of the TCR repertoire diversity, induced T cell cycling and antiapoptotic protein (bcl2) upregulation, and expanded T_N cells, with a proportional diminution in Treg frequency [126]. Because IL-7 was well tolerated in this first in-human study, IL-7 administration may prove especially useful in situations in which IL-7 levels are limiting such as following homeostatic expansion during the resolving lymphopenia phase.

IL-15, the third member of the IL-2R common gamma chain family of cytokines, has been shown to be essential for memory cell survival. In vivo studies in rodent and nonhuman primates have demonstrated the capacity of IL-15 administration to facilitate T cell survival, especially antigen-specific central memory T cells. Studies of human T cells indicate that IL-15 favors the in vitro generation of T_{CM} versus T_E cells and also appears to be critical for NK cell survival. IL-15 is being readied for first-in-human testing in the near future. However, the "trans" presentation that IL-15 appears to require for optimal effects suggests that simple administration of the cytokine may not be sufficient [127]. IL-21, the fourth member of this family that is closely related to IL-2, also promotes the function of $CD8^+$ T_E cells [128]. In contrast to

IL-2, IL-21 inhibits the maturation of $CD8^+$ T_N cells into granzyme B- and CD44-expressing effector $CD8^+$ CTL [129]. At the same time, IL-21 increased the antitumor regressive capacity of adoptively transferred CTL. These data are consistent with the paradigm that cytokines, such as IL-21, limit both the stage of T_E cell differentiation and preserve the expression of key cell surface homing receptors such as L-selectin. Thus, IL-7, IL-15, and IL-21 are new candidate cytokines that may favor the persistence of T_M cells with improved antitumor properties, compared to IL-2. New approaches for cytokine delivery, such as cytokine-anticytokine antibody complexes may provide a superior methodology to achieve more pronounced in vivo biologic effects by increasing their otherwise short serum half-life.

Thus far, the success of bypassing negative regulators (eg, CTLA-4 or PD-1 pathway blockade for T cells, killer inhibitory receptor blockade for NK cells) may result in augmented antitumor effects but may also result in possible autoreactive toxicities. For example, antibodies directed against CTLA-4 have shown promising results in clinical trials in augmenting endogenous antitumor reactive T cells, while at the same time inducing autoimmune-mediated destruction of nontumor cells in some patients [130]. Anti-PD-1 and anti-PD-L1 antibodies are in clinical trials in cancer patients; anti-PD-L1 antibody may be particularly useful in preventing the T cell exhaustion state that chronic antigen activation may cause [20]. However, in vivo studies have suggested that targeting multiple members of the PD1/PD-L family may be needed for optimal effects. Agonistic antibodies against costimulatory pathway members (eg, CD40/CD40L, OX40/OX40L, 4-1BB/4-1BBL) alone or combined with blockade of inhibitory coreceptors may prove to be particularly efficacious in patients with both APC and T cell dysfunction.

A chemical inhibitor of the IDO pathway (1-methyl-tryptophan) is being studied in phase I trials in cancer patients. COX-2 inhibitors have been used successfully, especially in patients with solid tumors, to reverse PGE2-mediated suppression and the frequency of inducible Tregs that can occur as a result of the tumor microenvironment [131]. MDSC-mediated inhibition of antitumor T cell or NK cell immune responses may be overcome by sunitinib malate [132], a receptor kinase inhibitor, TGF-β neutralization [133], modulators of arginine metabolism (see above) or nitric-oxide synthase inhibitor (eg, N[G] nitro-L-arginine methyl ester [L-NAME]) [134]. Anti-VEGF antibody in rodent but not human studies decreases suppressive DC numbers [120,121].

Cellular depletion approaches such as those targeting Tregs (eg, IL-2 diphtheria toxin, a.k.a. denileukin diftitox or Ontak®) have been variably efficacious in augmenting endogenous T cell responses by depleting Tregs. The transfer of ex vivo expanded and activated antitumor reactive T cells, including those that are forced to express an antitumor reactive TCR by gene transfer, have been reported to be efficacious in patients with melanoma, generally an immune responsive disease [135]. Rodent studies have indicated that combined in vivo Treg depletion with the adoptive transfer of syngeneic CTL [136] may be a useful strategy that circumvents the in vivo dysfunction of antitumor reactive T cells (eg, TCR zeta chain downmodulation) and inhibits the suppression conferred by Tregs.

The role of $CD4^+$ T cells (and the ratio of their subsets may be critical) in maintaining $CD8^+$ T cell responses (survival and function) also needs to be explored. More attempts at combination approaches need to be undertaken. Recent data also suggests that activated DCs may do more than simply initiate the adaptive response but may also play a role in maintaining antigen-specific $CD8^+$ T cell function and survival [137]. These data indicate that cytoreductive conditioning in HSCT may do much more than simply promote T cell expansion via homeostasis, but instead activates DCs via TLRs. Understanding the mechanisms involved may have a profound impact on the use of HSCT and adoptive immunotherapy. Finally, it has been shown in numerous models that peripheral immune readouts may have no bearing on antitumor responses. Studies are needed that understand how normal contraction processes occur within any attempt to stimulate or provide immune responses. AICD coupled with immune suppression and deprivation of supportive cells and cytokines may prematurely diminish responses and make the host resistant to further to attempts at stimulation. Targeting immune responses to the tumor site and, importantly, assessing immune effects within the tumor itself, may offer the best indicators of success as well as limit systemic toxicities. Understanding normal immune contractions/suppression and how the tumor can further subvert it is critical if the immune system is to be applied in cancer therapies.

ADAPTIVE B CELL-IMMUNE RESPONSES IN GVL

The Role of B Cells and Antibodies in GVL

Evidence that B cells contribute to tumor immunity after allogeneic HSCT comes primarily from studies that have attempted to characterize immunologic events in patients with documented effective GVL responses in vivo. For example, Wu et al. [138] studied patients with relapsed CML who responded to DLI. Antibodies reactive with CML cells were identified after DLI response, and specific protein targets were identified using serologic analysis of recombinant cDNA expression libraries (SEREX). Several target proteins were characterized in greater detail, and it was further shown that the

generation of antibodies specific for these targets coincided with the disappearance of CML cells in vivo. Similar antibodies were not present before transplant, before DLI, or in patients who developed chronic GVHD (cGVHD) after transplant. Antibodies to these CML-associated antigens were also not present in healthy donors or in patients who did not respond to DLI. Further studies demonstrated that several of these target antigens were preferentially expressed in both normal and CML progenitor cells [139].

Antibody responses to tumor-associated antigens have also been identified in patients with myeloma after allogeneic HSCT. Compared with CML, graft-versus-myeloma (GVM) responses after allogeneic HSCT occur less frequently and often only result in partial responses. Patients with myeloma who received prophylactic DLI early after transplant were found to have increased numbers of normal polyclonal $CD20^+$ B cells in peripheral blood [140]. Using a similar SEREX approach Bellucci et al. [141] identified a panel of myeloma-associated antigens that were targets of antibody responses in patients with myeloma who responded to DLI. Antibody responses were not detected before DLI or in patients who did not receive DLI after transplant. These target proteins were highly expressed in myeloma cells, and antibodies to some of these antigens were detected in different DLI responders. Taken together, these studies in different hematologic malignancies suggest that polyclonal antibody responses to a variety of tumor-associated antigens frequently occur in patients with effective graft-versus-tumor responses in vivo. Nevertheless, the mechanisms whereby these antibody responses contribute to elimination of tumor cells in vivo have not been established.

Target Antigens and Potential Mechanisms of Action of Antibodies

Several studies have reported that posttransplant patient antibodies can recognize cell membrane proteins expressed on tumor cell targets. This was recently shown for a polymorphic protein, ILT5, which is expressed on the cell membrane of normal DC [142]. This antigen is also expressed on myelogenous leukemia cells, and antibodies in patient serum were able to induce both complement-mediated cytotoxicity of AML cells as well as antibody-dependent cellular cytotoxicity (ADCC). In this instance, immunogenicity was because of a polymorphism in the ILT5 gene. As a result of this polymorphism, donor B cells were able to recognize a variant of this protein in the transplant recipient that was not present in the HLA identical transplant donor. Overall, ILT5 reactive antibodies were found in 5.4% of HSCT patients, but not in solid organ transplant recipients, patients with autoimmune diseases, multiparous women, or healthy individuals.

Antibodies to a B cell membrane antigen, BCMA, have also been found in patients with myeloma [143]. BCMA is a transmembrane receptor of the TNF superfamily that is selectively expressed by mature B cells. In this setting anti-BCMA antibodies were also able to induce both complement-mediated cytotoxicity of myeloma cells, as well as ADCC. BCMA antibodies were only detected in patients with myeloma who received DLI. However, in this case, no genetic polymorphism in the BCMA gene was detected to account for the immunogenicity of this specific protein. Although few antibodies to cell membrane proteins have been described, few studies have specifically attempted to identify these types of antibodies as current methods that have been used to determine the specificity of antibodies in patient sera such as SEREX are not well suited to detect membrane proteins. It is therefore possible that antibodies to cell membrane proteins are a more common phenomenon in patients with GVL.

Most antigens that have been identified as targets of posttransplant antibodies are intracellular proteins. As with cell membrane proteins described above, the immunogenicity of some of these proteins is because of genetic polymorphisms that distinguish recipient and donor. For example, Y chromosome encoded proteins are immunogenic in male patients who receive stem cell grafts from female donors. Males are tolerant to these "self" antigens, but H-Y proteins are highly immunogenic in females and elicit both B and T cell responses after HSCT. H-Y proteins are widely expressed in normal tissues as well as tumor cells. B cell responses to H-Y proteins have been associated with cGVHD, but patients with antibodies to HY proteins also have a significantly lower risk of relapse after transplant than patients without H-Y antibodies [144]. In addition to H-Y proteins, a number of other intracellular proteins have been identified as targets of antibody responses in patients with GVL [138,141]. In most of these cases, polymorphisms that distinguish recipient from donor have not been identified, and the immunogenicity of these proteins has not been explained. Nevertheless, the persistence of high titer IgG antibodies for long periods after transplant suggests that these proteins are highly immunogenic. It is also possible that specific T cell responses directed against peptide epitopes derived from the same proteins are also present in these patients. Coordinated B and T cell responses have been reported for H-Y proteins, and similarly coordinated responses may also exist for many of these other proteins [145]. In these cases, the presence of specific antibodies reactive with soluble proteins or protein fragments may facilitate antigen presentation and the development of specific $CD4^+$ and $CD8^+$ T cell responses to distinct peptide epitopes contained within these larger fragments.

Although these have not been studied as extensively, antibodies directed against soluble or secreted proteins have also been detected after allogeneic HSCT. Antibodies specific for secreted proteins may significantly deplete these proteins in vivo, resulting in profound functional effects. For example, some solid tumor patients who respond to tumor vaccines and infusions of anti-CTLA4 antibody have been found to develop high titer antibodies specific for soluble MICA [146]. MICA is expressed on tumor cell membranes in response to DNA damage and is a known ligand for NKG2D expressed on NK cells and $CD8^+$ T_E cells. However, soluble MICA secreted by tumor cells results in the downregulation of NKG2D on NK cells and T_E cells with subsequent loss of cytolytic function. Antibodies to MICA induce the clearance of this soluble protein, resulting in the reexpression of NKG2D and restoration of cytolytic effector cell function. In patients with AML after allogeneic HSCT, Ho et al. [147] recently reported a decrease in soluble NKG2D ligands following vaccination with autologous leukemia cells genetically engineered to secrete granulocyte macrophage colony stimulating factor (GM-CSF). Elevated levels of soluble MICA have also been found after allogeneic HSCT [148]. In this study, elevated levels of anti-MICA were associated with decreased cGVHD and increased risk of relapse after allogeneic HSCT. In many patients, the effectiveness of the GVL response is limited by immune suppressive factors secreted by the tumor cells themselves. The generation of specific antibodies directed against immune suppressive molecules may therefore be an indirect mechanism whereby donor B cells can promote GVL responses mediated by other effector cells. Further studies are needed to explore this novel mechanism whereby antibodies can potentially contribute to the GVL response in vivo.

Although the development of B cell responses after allogeneic HSCT appears to be associated with effective GVL responses, it has also been noted that antibodies to tumor cell antigens are frequently found in patients with solid tumors. In contrast to the posttransplant setting, the presence of antibodies is not clearly associated with effective autologous tumor immunity in patients with solid tumors [149]. A very large database of antigens identified by SEREX is now available in the Cancer Immunome Database (http://ludwig-sun5.unil.ch/CancerImmunomeDB). In some cases such as NY-ESO-1, T cell responses to distinct epitopes within these proteins have also been identified, but the clinical relevance of these endogenous antibody responses has not been established [150] . In mouse models that have examined the role of B cells in tumor immunity, it has been found that the presence of B cells can actually inhibit effective tumor immunity [151].

Methods to Identify Target Antigens

Thus far, most studies that have attempted to define the specificity of antibodies in the posttransplant setting have relied on the SEREX approach to identify and clone specific target proteins. This has been a very useful tool and has allowed the broad characterization of antibody specificity. However, the SEREX method also has limitations, and may not be able to identify all relevant target antigens. Because target genes are identified in a cDNA expression library, this method favors the identification of highly expressed genes and protein targets represented by low copy number mRNAs are difficult to detect. This method also does not favor the identification of cell membrane proteins. Protein microarrays that include very large numbers of targets are now available, and these can provide an alternative approach for identification of antibody specificity after allogeneic HSCT, especially in the setting of GVL. Protein microarrays have the advantage of providing an unbiased approach to target identification and also greatly simplify and standardize the procedure for characterizing the specificity of complex polyclonal antibody responses. Although current protein microarrays now contain several thousand proteins, this approach also has limitations. Many relevant proteins may not be included in these arrays, and this approach is not designed to detect responses specific for protein polymorphisms. The cost of these arrays may be prohibitive for large-scale studies, but it is likely that this will also be a useful approach for defining antibody specificities in the setting of GVL responses in vivo.

As methods for high throughput genotyping and whole genome sequencing continue to evolve rapidly, it is likely that these methods will also facilitate the ability to identify antibody targets. For example, the identification of genetic disparities that distinguish recipients and stem cell transplant donors has already been used to predict candidate immunogenic targets for GVHD responses. Using current bioinformatic tools, it is also possible to identify genetic disparities or mutations that predict for the creation of immunogenic epitopes. Once specific epitopes are identified in silico, the immunogenicity of these epitopes can be validated in patients who have undergone allogeneic HSCT. In the future, this approach can be used to validate both B and T cell epitopes and to examine whether they are associated with either GVL or GVHD (or both).

Mechanisms That Promote Antibody Responses and Implications for Therapy

The administration of MA or intensely immune suppressive therapy prior to allogeneic HSCT leads to profound lymphopenia that often persists for prolonged periods. The physiologic response to lymphopenia facilitates the homeostatic proliferation

of donor B and T cells and supports the recovery of these cells to normal levels. In this environment, recent studies have suggested that B cell recovery following HSCT is supported, at least in part, by high levels of B cell activating factor (BAFF) [152,153]. This homeostatic cytokine promotes B cell survival and differentiation, and thus supports the secretion of antibodies generated by donor B cells. As noted above, these antibodies may contribute to the GVL response, and the persistence of high levels of BAFF may therefore be an important mechanism to support this functional activity. Other cytokines and microenvironmental factors likely also play important roles in the reconstitution of B cell numbers and repertoire following transplant. These factors have not been well defined in the clinical setting, and further studies to identify factors that modulate B cell reconstitution are warranted. As we develop a better understanding of the role of B cells in the GVL response in vivo, the characterization of these factors may provide novel opportunities for modulating B cell reconstitution to promote GVL after allogeneic HSCT.

Although B cell responses noted above have been associated with GVL, it is important to acknowledge that B cells also likely contribute to the development of cGVHD after allogeneic HSCT [144,152,154]. This is further supported by the finding that B cell-directed therapy with anti-CD20 monoclonal antibody (rituximab) can provide effective therapy for some patients with steroid-resistant cGVHD [155-157]. Despite many attempts to distinguish GVL and GVHD, it is evident that there is substantial overlap between immune responses directed against either cancer cells or normal tissues in the transplant recipient. In this setting, new approaches to enhance B cell responses to promote GVL may also have the unwanted toxicity of increasing cGVHD. Similarly, new and more effective strategies to treat or prevent cGVHD by targeting donor B cells may also have the unwanted effect of reducing GVL and increasing the risk of relapse after allogeneic HSCT. These issues will need to be carefully considered in the design and evaluation of any new clinical strategies to modulate B cell reconstitution following transplant.

INNATE NK CELL IMMUNE RESPONSES

NK Cell Biology

The GVL effect goes beyond adaptive immune responses, and a role for NK cells has been shown in humans and the mouse. Yet there are striking species differences between the mouse and humans that need to be considered when attempting to translate results. Dominant among these differences is the expression of CD56 as a marker on human NK cells and the existence of $CD56^{bright}$ and $CD56^{dim}$ subsets. $CD56^{bright}$ NK cells are present in the lymph node and blood in humans, are highly proliferative, and produce cytokines, but are weakly lytic, suggesting they exert immunoregulatory functions. Mice lack both CD56 as a marker on their NK cells and have few NK cells in the lymph nodes. The MHC binding receptors in the mouse and humans are also fundamentally different. In humans, killer-immunoglobulin-like receptors (KIRs) are the dominant HLA-binding molecules that regulate NK cell activity, whereas mice express the lectin-binding Ly49 family. Additionally, mice are housed under specific pathogen-free (SPF) conditions and their baseline NK cell killing is relatively poor, which correlates with very low granzyme/perforin expression unless activated, whereas human NK cells readily express these lytic molecules and exhibit de novo function without activation. In addition, human NK cells survive better ex vivo, whereas murine NK cells inevitably die after 2 weeks in culture. Despite these differences, there are many similarities in biologic paradigms between human and mouse NK cells including the expression of regulatory molecules (NKG2D, CD94/NKG2A, and others), lytic pathways, and effects of cytokines (IL-15, IL-2, Flt3L) critical for their development. Attempts to use xenograft models to study human NK cell development and activity has been inconsistent, but progress has been made by the discovery that IL-15 trans-presentation by species specific IL-15Rα is required and warrants further study.

Although both human and murine inhibitory receptors (KIR and Ly49, respectively) recognize MHC molecules, the natural ligands for many KIRs are unknown, including all of the activating KIRs, even though some may bind MHC molecules at low affinity. In the homologous murine Ly49 system, the activating receptor Ly49H recognizes the murine cytomegalovirus (CMV) glycoprotein m157, providing "proof of principle" that activating receptors may recognize viral proteins. Until recently, it had been presumed that only cells of the adaptive immune system have memory. Recently, the Lanier lab has shown that $Ly49H^{+}$ (the receptor for muCMV) NK cells dramatically expand and then contract after viral control, yet amazingly these cells can be found in the recipient 3 months after infection suggesting that "NK cell memory" exists [158]. A role for human activating receptors in infection is supported by studies of patients with HIV showing an association between AIDS progression and in transplantation by association with human CMV, but memory NK cells are not well studied in humans.

Development of NK Cell Self-Tolerance

The mechanism by which NK cells acquire self-tolerance and alloreactivity has been referred to as

NK cell education or licensing. This is one of the most widely debated topics in NK cell biology over the past several years. Self-tolerance is the process by which NK cell function is suppressed after ligation of "self" through class I recognizing inhibitory receptors. Although alloreactivity is determined by a lack of inhibition and a positive balance of activating signals, how an NK cell acquires effector function is more complicated. Several models have been proposed to explain why inhibitory receptor expression correlates with the acquisition of effector function. These concepts differ in their implied mechanisms and whether the process is one of activation or loss of function. What is agreed upon between these and other models is that human NK cells lacking inhibitory receptors are hyporesponsive [159-161]. Although the exact mechanism remains unknown, self-tolerance may be the result of coordinated developmental pathways whereby mature NK cell function is synchronized with the acquisition of self-inhibitory receptors and their subsequent ligation. The complexity of these interactions is highlighted by the expression of multiple inhibitory receptors capable of recognizing self-MHC, the net summation of activating receptor signals and the variable expression of ligands resulting in a "rheostat" [162] or a continuum "tunable rheostat" model integrating a cadre of different signals on the NK cell itself and its potential targets [163]. In humans, the best evidence for NK cell education is the finding of enhanced function of self-KIR expressing NK cells (KIRs in an individual who also expresses its cognate HLA ligand) where NK cells expressing nonself-KIRs are hyporesponsive. Although KIR interactions dominate the clinical literature, receptors beyond KIR are operant in rheostat models and include class I-recognizing NKG2A (recognizes HLA-E), LIR-1 (recognizes HLA-A, B, and G), and class I MHC-independent interactions including but not limited to: CD16 (FcRIII), natural cytotoxicity receptors (NKp30, 44, 46), DNAM-1, LFA-1, NKG2D, and CD244 (2B4).

Above and beyond the control of NK cytotoxicity by MHC class I expression on tumor cells, we are beginning to appreciate tissue-specific differences in the ability of NK cells to kill targets. Most notably, transplant data suggest that myelogenous malignancies are more susceptible to NK cell control than lymphogenous malignancies [164,165]. Why myelogenous cells are more susceptible is not known. One possibility is that myelogenous leukemias share with normal myelogenous APCs the activation-induced C-type lectin (AICL) ligand for NKp80, another C-type lectin NK cell recetor, which could license the NK cell for cytotoxicity [166]. AICL is a myelogenous specific activating receptor that is upregulated by TLR stimulation, suggesting that inflammation may prime NK cells to kill targets. Other differences in receptor-ligand interaction strength have also been proposed and are presumed to be target specific. For example, in CML NKG2D interacting with MICA/B, abundantly expressed on leukemic cells, appears to be the mechanism of NK cell-target engagement [167]. A critical question in determining their clinical potential is whether NK cells exhibit cytotoxicity against leukemia stem cells. Some data suggest that, at least in CML, NK cells can engage early leukemic $CD34^+$ progenitors, although the cytotoxicity is weak. NK cells exert cytotoxicity via perforin-granzyme pathways, but recently a role for TRAIL engagement with TRAIL receptor on the NK cell has been shown to be important. Strategies to exploit this mechanism may be of therapeutic importance. Notably, the proteosomal inhibitor, bortezomib, upregulates TRAIL and can increase cytotoxicity of NK cells to renal cell cancer and CML progenitors [168]. Studies aimed at sensitizing targets to NK cell killing warrant further study.

The Role of NK Cells in the GVL Response

NK cells are attractive to exploit in the setting of hematopoietic transplantation because they are the first lymphocytes to reconstitute after transplantation at a time when the adaptive immune system is impaired. A number of groups have been interested in harnessing the activity of NK cells in the setting of autologous HSCT to prevent relapse, the biggest cause of treatment failure from this procedure. These studies employed low-dose IL-2 administration, which was capable of expanding NK cells in vivo with a modest increase in cytolytic activity. However, even higher doses of IL-2 and autologous hematopoietic cell infusions were not strong enough to result in significant clinical benefit when evaluating definitive clinical endpoints such as time to disease progression and relapse. The biology of NK cells has now explained this result to be partially a result of (1) a number of class I MHC "self"-recognizing inhibitory receptors displayed on the surface of NK cells, and (2) a consistent finding that low-dose IL-2 administration may lead to blunting of the immune response by increasing Tregs. These 2 issues underpin current strategies to exploit NK cells in the clinic. The first major advance was reported by Ruggeri et al. [164], who found a reduced risk of relapse of leukemia in AML patients who received transplants from donors who were mismatched at HLA-B or HLA-C ligands for KIRs. The advantages of KIR ligand mismatch appear to be specific to AML and dependent on potent graft T cell depletion, suggesting that the platform is critically important to see beneficial effects of early posttransplant therapy. The potential benefits include: (1)

decreased GVHD as host DC are killed by donor NK cells, (2) better antitumor activity via direct cytotoxicity, (3) improved engraftment mediated by NK cell release of hematopoietic cytokines, and (4) ultimately better survival. Additional clinical trials have either supported or failed to find relapse protection and a survival benefit [169-176], which may be explained by important differences in transplant platforms between studies including preparative regimen, extent of T cell depletion, graft source, donor, stem cell dose, disease, and disease status. Taken together, these results suggest that NK cells play a role in allogeneic HSCT and myeloid leukemia therapy; however, the complexities of the KIR system and the presence of other functional receptors on NK cells may explain some of the confusion in interpreting published studies.

KIR Genotyping: Implications for Donor Selection

The role of KIR immunogenetics is complicated by 16 genes and over 100 allelic polymorphisms, some of which define distinct functional differences. Despite these complexities, some simple associations have been made in determining clinical outcome after HSCT. Unrelated donors and recipients from 209 HLA-matched and 239 mismatched T-replete URD transplantations for AML were analyzed by KIR genotyping [177]. Based on gene content, donors were stratified as having a B/x haplotype if they contained 1 or more B-defining genes (KIR2DS1, 2, 3, 5 and KIR2DL5), whereas those lacking these genes were classified as having a KIR A/A haplotype. Three-year overall survival was significantly higher after transplantation from a KIR B/x donor {31% (95% confidence interval [CI]: 26-36) versus 20% (95% CI: 13-27); $P = .007$}. Multivariate analysis demonstrated a 30% improvement in the relative risk of relapse-free survival with B/x donors. This demonstrates that unrelated donors with KIR B haplotypes confer significant survival benefit to patients undergoing T-replete HSCT for AML. Certain B haplotype KIR groups have also been found to favorably affect outcome after T cell-depleted HLA identical sibling transplants [165]. Thus, it seems likely that the genetic characteristics of a stem cell donor could affect transplant outcome; however, data on the relative impact of specific KIR genes or groups of genes needs further study to understand why effects are not applicable to diseases beyond AML.

NK Cell Adoptive Transfer

Much of what is known about lymphocyte adoptive transfer comes from T cell infusion literature, which may extrapolate to NK cell therapy. Emerging concepts suggested that in vivo expansion of lymphocytes were dependent on a potent lymphodepleting regimen to (1) create space, (2) increase endogenous cytokines, and (3) to eliminate immune rejection of adoptively transferred cells. This has been well established in mouse models and the infusion of carboxyfluorescein diacetate, succinimidyl ester (CFSE, a fluorescence dye that gets diluted out in proliferating cells)-labeled lymphocytes that did not expand in the absence of a lymphodepleting regimen [178]. This lymphodepletion strategy was first translated into humans by Dudley et al. [179], who demonstrated in vivo expansion of melanoma specific T cells after a preparative regimen of high-dose cyclophosphamide and fludarabine. Because HSCT always uses a lymphodepleting regimen, whether fully MA or not, the same biologic concepts for lymphocyte adoptive transfer may be extrapolated.

In 2005, Miller et al. [180] were the first to show in vivo expansion of NK cells after adoptive transfer of haploidentical related donor NK cells. The success of this regimen was dependent on a lymphodepleting preparative regimen that included cyclophosphamide and fludarabine. NK cells were infused with IL-2 administration for the 2-week interval after adoptive transfer. Chimerism studies established unequivocal proof of concept that donor-derived haploidentical NK cells persisted and expanded in vivo in some patients. However, this strategy was limited by inadequate efficacy, which may correlate with failure to expand donor NK cells in vivo. This could be explained by inadequate potency of the preparative regimen, insufficient cytokines acting in vivo (endogenous IL-15 induced by lymphodepletion), or contraction of the immune response by increasing Treg cells stimulated by IL-2. In an attempt to overcome some of these barriers several groups have been exploring increased intensity of a lymphodepleting regimen with the addition of total body irradiation, approaching that used in a fully MA preparative regimen. Although these studies are ongoing, there are suggestions with both T cell and NK cell adoptive transfer that a more potent lymphodepleting regimen may be beneficial. Therefore, to maximize the effects of adoptive transfer, combination with HSCT may be warranted to avoid prolonged neutropenia.

One of the challenges in NK cell therapy is how to maximize the effector to target ratio in vivo, which likely correlates with efficacy. IL-15 has recently shown promise in primates [181] and will be made available through the National Cancer Institute for clinical testing. Although in vivo expansion of NK cells is attractive and allows little ex vivo manipulation, an alternative approach that could also avoid the potential toxicities of a potent lymphodepleting regimen is to expand NK cells ex vivo to increase numbers. One method proposed by Campana et al. [182] uses K562 stimulators transduced with 41BB-ligand and membrane-bound IL-15. Another approach is described

by Berg et al. [183], who developed GMP-compatible methods using B cell feeders to promote NK cell expansion over 500-fold. As one contemplates comparative studies between in vivo and ex vivo expansion approaches, several questions need to be answered. Does in vivo expansion lead a more efficient NK cell education and resultant antitumor function than ex vivo expansion? How do in vivo and ex vivo expansion methods affect NK cell activation, receptor expression, survival, homing, and durability of response? All of these questions warrant further study.

In conclusion, our understanding of NK cell target interactions requires more detailed exploration of the potency of specific NK molecules and their ligands on specific target cells (including malignant stem cells), the stages of NK maturation, and the effect of the recipient milieu on NK cell maturation and NK clonal selection. Also, relatively little is known about the homing of NK cells to their targets and whether NK cells are restricted in the tissues they patrol. Importantly, we do not yet have a clear idea about the ultimate potency of NK cells in their most favorable context to permanently eliminate malignancy and to best apply them to the problem of post-transplant relapse.

CONCLUSION

Immune responses following allogeneic HSCT and DLI have illustrated the robust potential of allogeneic T cell, NK cell, and/or antibody responses to treat patients with hematologic malignancies. Several issues, however, have limited the successful application of these immune responses. Key among these are GVHD, which has been difficult to separate from GVL, and either upfront resistance of tumors to GVL or late relapse. How to deliver GVL and promote immune reconstitution from donor T cells with acceptable GVHD remains a central mantra in the field. Overcoming these challenges would be facilitated by a detailed mechanistic understanding of GVL resistance and GVHD. Identification of relevant target structures, the type of effector cells necessary for optimal and specific responses, and mechanisms of action are among prerequisites for successful "next steps." We also need to understand the induction, expansion, and trafficking of immune responses, which may allow specific recognition of hematopoietic tissues from the host and not target organs of GVHD. Knowledge of the factors that limit the effectiveness of alloimmune responses to execute a specific antitumor effect is essential to increase the likelihood of success with no or limited toxicity. These factors include escape mechanisms of tumor cells to prevent recognition and elimination by alloimmune responses, which may include downregulation of the target structures to be recognized, suppression of homing or interaction at the site of the tumor, or the development of suppressive factors preventing the development of the immune responses in vivo.

Future initiatives should be explored to identify and overcome roadblocks preventing successful application of allogeneic therapy for the treatment of hematologic malignancies to exploit T cells, NK cells, or antibody mediated mechanisms. Specific strategies in mouse and in man may include (1) studies to better understand mechanisms of trafficking, target recognition, and in vivo expansion of tumor specific responses; (2) studies of mechanisms that suppress immune responses systemically or locally at the site of the tumor; (3) studies on in vitro generation, expansion, and engineering of specific immune cells for adoptive transfer; (4) studies on the susceptibility of tumor stem cells to immune attack; (5) induction of effective antitumor immunity with vaccines or inhibition of negative regulatory pathways; and (6) high throughput screening for antigens involved in antitumor responses using genome wide analysis. Support for collaborative laboratory studies and clinical trials as well as prospective banking of human samples and collection of data will facilitate translation of basic research into successful clinical research strategies. Given the wealth of talent with in the transplantation community, promoting interactive program grants between institutions will provide synergy, broaden the use of existing institution core resources, and build on individual strengths to move the field forward and systematically address strategies needed to overcome post-transplant relapse.

ACKNOWLEDGMENTS

Financial disclosure: The authors have nothing to disclose.

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