ALLOGENEIC BONE MARROW TRANSPLANTATION

Internal Medicine Grand Rounds
December 7, 2000

Robert Collins, Jr., M.D.
Department of Internal Medicine
Harold C. Simmons Cancer Center

This is to acknowledge that Robert Collins, Jr. M.D. has disclosed no financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Collins will not be discussing "off-label" uses in his presentation.

Robert Collins, Jr., M.D.

Associate Professor of Internal Medicine and Director of Bone Marrow Transplantation in the Division of Hematology / Oncology and the Harold C. Simmons Cancer Center.

He holds the R. Lloyd and Willye V. Skaggs Professorship in Medical Research

His research has focused on donor leukocyte infusions, nonmyeloablative stem cell transplants, and application of new assays to the study of immunologic mechanisms in bone marrow transplantation.

INTRODUCTION

Allogeneic bone marrow transplantation is an effective therapy for many otherwise incurable diseases. Unfortunately, as currently practiced, the procedure continues to be associated with significant morbidity and mortality. This is because the procedure has consisted largely of variations based on a framework devised in the 1960s when comparatively little was known about immunology and stem cell biology. However, recent advances in the understanding of the basic biology underpinning bone marrow transplantation suggest new approaches that may significantly lessen toxicity of the procedure and broaden its usage in the management of malignancies and genetic disorders--involving both hematopoietic and non-hematopoietic tissues.

BRIEF HISTORY OF THE FIELD

E. Donnall Thomas, the father of clinical bone marrow transplantation, who hails from Mart, Texas, began research in the area in the early 1950s [1-3]. Clinical studies in the late 1950s were halted because of severe problems with graft-versus-host disease (GVHD). Re-institution of clinical investigation awaited the delineation of major histocompatibility determinants in the dog and in man [4]. With the ability to serotype sibling pairs for HLA determinants, allogeneic BMT seemed feasible and clinical studies began in earnest. The first successful allogeneic transplants were carried out in 1968, in 3 children with immunodeficiency disorders (Wiskott Aldrich syndrome in one and severe combined immunodeficiency in the other two) [5]. All 3 were alive and well when reported at the 25th anniversaries of their transplants. Early studies in end-stage refractory leukemia patients were marked mostly by abject failure, but the 6-7% of the patients who were cured by the procedure (and were still alive when reported on 11-14 years later [6]) encouraged a relatively small band of like-minded to carry on. Over the years, various clinical investigators—some methodical and patient, others perhaps not as methodical—gradually improved the field to where it is now. Approximately 15,000 allogeneic transplants are carried out each year worldwide [7] (and International Bone Marrow Transplant Registry statistics, 2000).

BRIEF OVERVIEW OF CLINICAL ALLOGENEIC BMT

In the basic schema of allogeneic bone marrow transplantation, the patient first receives high doses of chemotherapy and often radiation. After completion of the therapy the hematopoietic stem cells are collected from the stem cell donor and infused into the recipient; donor stem cells engraft and hematopoiesis is evident within 2 to 3 weeks. (Note: the terms bone marrow transplantation (BMT) and stem cell transplantation (SCT) are used interchangeably in this protocol.)

Indications

Allogeneic stem cells are transplanted for one of 4 basic indications:

1) To provide hematopoiesis in cases in which hematopoietic stem cell function is impaired (e.g. aplastic anemia);

2) To provide lymphopoiesis in diseases of impaired lymphocyte function (e.g. severe combined immunodeficiency disease). In these diseases, production of non-lymphoid blood cells (e.g. red cells, platelets, granulocytes, and macrophages) is often normal; the infused hematopoietic stem cells cure the disease by differentiating into normal lymphocytes;

3) To rescue the patient from the effects of high dose anticancer therapy (e.g. leukemia);

4) As gene therapy; in this instance, the infused stem cells differentiate into normal blood cells containing genes which are defective in the patient (examples include Sickle Cell Disease and Gaucher's Disease).

Finding a Donor

Successful transplantation requires a donor who is closely matched at the human leukocyte antigen (HLA) complex located on Chromosome 6 (Figure 1)[8, 9]. The HLA complex encodes 2 classes of HLA molecules, called HLA Class I and HLA Class II molecules, that bind peptide fragments from pathogens and endogenous proteins and display them on the cell's surface for recognition by appropriate T-cells. The HLA genes are highly polymorphic, that is, there are multiple alleles of each gene, and differences in HLA molecule structure between members of a donor-recipient pair lead to activation of immune cells from the differing individuals (this process is termed alloreactivity). HLA genes are inherited in an

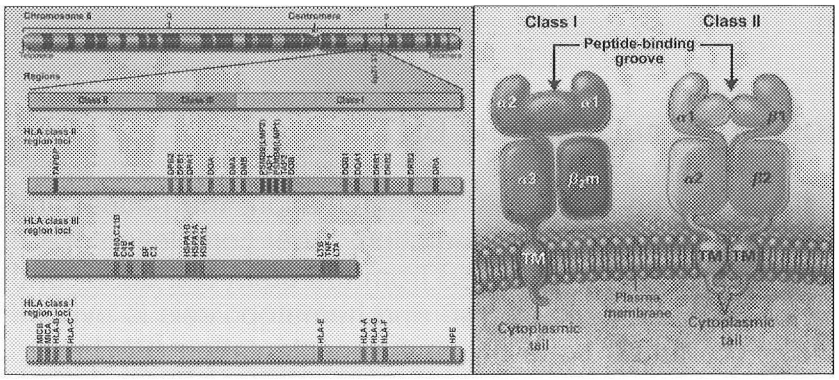


Figure 1. The HLA complex encodes HLA class I and II molecules which are expressed on the cell surface. From reference 8.

autosomal codominant fashion. Thus, a given sibling pair has a one-in-four chance of being HLA matched; the likelihood of an individual having a matched sibling is thus determined by the number of siblings (calculated by the formula $1-(0.75)^n$ where n is the number of siblings).

The HLA type may be determined by a variety of methods. In the past, serotypes were determined by allosera specific for particular HLA molecules. Increasingly, however, the HLA type is being determined at the genetic level at varying levels of resolution, up to direct sequencing of the gene [10]. Three genes are generally typed on each chromosome (two Class I molecules, HLA-A and HLA-B, and one Class II molecule, HLA-DRβ1); the optimal donor is matched at all 6 loci and is thus termed a 6-of-6 match. If an HLA matched sibling is not identified, then the parents and children are HLA typed. Parents and children share the HLA genes from one chromosome due to autosomal codominant inheritance; occasionally, by chance, they share genes on the other chromosome as well. Clinical experience suggests that a mismatch at only one antigen is often compatible with successful transplantation; thus, 5-of-6 antigen matched family members may donate [11]. Transplants with greater degrees of mismatch, i.e. two or three antigen mismatches, have been attempted and are sometimes successful; however, the risk of fatal alloreactions is so high that only a few centers routinely attempt these so-called haploidentical transplants [12, 13].

If a suitably HLA matched family member is not identified, then a search for a suitably matched unrelated donor is carried out [14-16]. Various registries around the world contain the HLA types of roughly 5 million individuals who have agreed to donate bone marrow if asked. These registries are accessed in the United States through the National Marrow Donor Program. Overall, the likelihood of finding a suitably matched unrelated donor is approximately 50%.

Umbilical cord blood is relatively rich in hematopoietic stem cells and has been used as a stem cell source in several hundred successful transplants, from both related and unrelated cord blood donors [17-19]. For still not completely defined reasons, cord blood is less likely to cause severe alloreactivity and thus greater degrees of HLA mismatch are compatible with a successful transplant. The actual number of stem cells in a cord blood collection is fairly low, thus limiting this approach for the most part to children; however, successful cord blood transplants in adults have been reported [20].

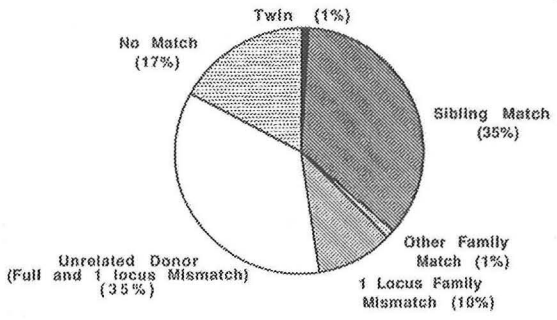


Figure 2. The likelihood of finding a suitably HLA matched donor

Pretransplant Eligibility Determination

To be eligible then for transplantation, the patient must have an appropriate disease and disease status as well as a suitable source of stem cells. In addition, the patient must have good organ function and no serious co-morbid illnesses. The upper age limit is 55 to 65, depending on the center's policies and the individual's physiologic age; less toxic preparative regimens (discussed below) are allowing exploration of transplantation in older patients.

Preparative Regimen

Before transplantation the patient receives a preparative regimen consisting of high doses of chemotherapy, often in combination with total body radiation (TBI)[21]. The purpose of the preparative regimen is two-fold: 1) to immunosuppress the patient enough to prevent rejection of stem cells. Bone marrow is highly immunogenic and without intensive immunosuppression, the recipient will routinely reject the marrow; 2) to kill cancer cells in patients with malignancy. Traditionally, an additional reason for the preparative regimen is to create space for donor cells to graft. For example, one could imagine that there would be no room for donor cells to take root in a thalassemic patient with a greatly expanded marrow, full of abnormal hematopoiesis. Thus, common sense suggests that the idea of creating "hematopoietic space" is a viable concept, but it should be stressed that, based on laboratory investigation, the concept of hematopoietic space is uncertain ("hematopoietic space" is one of those medical terms invariably written or spoken surrounded by quotation marks).

Preparative regimens were adapted from animal studies and deliver high doses of drugs or radiation that are both myeloablative and immunosuppressive. The two most commonly used regimens are high dose cyclophosphamide (120 mg/kg) with total body radiation (1200 cGy) and high dose busulfan (16 mg/kg) with cyclophosphamide (120 mg/kg). Many other regimens exist, most of them variations on these two regimens; there is little evidence that any one regimen is superior to another in terms of survival, although some may be less toxic. Significantly less toxic regimens are currently under development (discussed below).

Lastly, it should be pointed out that some patients with immunodeficiencies do not require a preparative regimen, as they are already immunosuppressed by their disease.

Stem Cell Harvesting and Transplantation

Hematopoietic cells can be harvested from the bone marrow or peripheral blood. In the bone marrow harvest procedure, marrow cells are aspirated under general anesthesia through multiple needle punctures in the posterior iliac crest and sometimes the anterior iliac crest and sternum [22]. Approximately 10 ml/kg of marrow (mixed with blood) yields an adequate number of stem cells in most cases. Donors have residual discomfort for a few days after the procedure, but are generally well within a week; serious complications have been reported, but are rare. In the peripheral blood stem cell harvest procedure [23], donors receive subcutaneous injections of granulocyte-colony stimulating factor (G-CSF) for several days. G-CSF mobilizes stem cells to the peripheral blood, apparently by altering the expression of certain adhesion molecules [24, 25]. Cells can then be collected by 1 or 2 3-4 hour apheresis procedures using peripheral veins (occasionally a central line is required). Donors occasionally have constitutional symptoms related to G-CSF administration; serious complications, generally related to central line placement, are unusual. Peripheral stem cell harvesting generally yields approximately 3 times as many stem cells and 10 times as many T-cells as bone marrow harvesting. Stem cells harvested from blood engraft sooner and more robustly; despite the increased number of T-cells, the incidence of acute graft-vs.-host disease (GVHD) is not increased although chronic GVHD probably is [26].

Stem Cell Processing

Stem cells collected by bone marrow harvesting are first filtered to remove particulate matter and achieve a single cellular suspension. In cases of ABO incompatibility, red cells are removed by centrifugation if there is a major mismatch (e.g. donor blood type A and recipient blood type O) and plasma is removed if there is a minor mismatch (e.g. donor blood type O and recipient blood type A). (Thus, red cell ABO incompatibility is not an impediment to bone marrow transplantation.) Stem cells collected by apheresis generally do not require additional processing to remove red cells.

As discussed below, T-cell depletion lessens the incidence of GVHD and is a common practice at many centers [27]. T-cells may be depleted by a variety of physical or immunologic methods. T-cells are most commonly depleted immunologically, either by "negative depletion," i.e. using antibodies directed to T-cells or by "positive stem cell selection" using monoclonal antibodies to CD34, an antigen expressed by most stem cells but not by T-cells. T-cell depletion methods lead to depletion of anywhere from 1.5 to 3.5 logs of T-cells.

Several groups are investigating methods to expand stem cell numbers *in vitro* [28, 29]. Most methods investigated seem to cause stem cells to differentiate, losing their self-replicating and totipotent ability. However, some

investigators have reported preliminary findings suggesting that it may be possible to increase stem cell numbers without causing them to lose their "stemness". Such a procedure would be of special usefulness in cases where low stem cell numbers limit the outcome of the procedure, such as cord blood transplants in adults.

Stem cells can be frozen and remain viable for years. However, only rarely is cryopreservation necessary in allogeneic transplantation as the cells are harvested and infused on the same day. (When a donor seems particularly unreliable, it may be prudent to collect and cryopreserve cells before the regimen begins.)

Stem Cell Transplant

Stem cells are transplanted simply by an intravenous infusion; the cells then home to niches in the recipient marrow space where they begin the process of donor-derived hematopoiesis. The molecular determinants of stem cell homing have begun to be described recently [30, 31]. Stem cell recipients are commonly given hematopoietic growth factors to hasten maturation of donor hematopoietic stem cells [32].

The Early Post-Transplant Period

Patients typically have severe pancytopenia for at least 2 weeks after the transplant. During this time patients require careful medical management with close attention to various issues including infectious diseases, need for red cell or platelet transfusion, management of mucositis and nutrition, and detection and management of cardiac, pulmonary, hepatic, or renal dysfunction. If all goes well, donor cell engraftment is manifested within 2 to 3 weeks and after a few more days of recovery, the patient is discharged; even under the best of circumstances the total hospital stay is frequently 4 to 6 weeks.

The Late Post-Transplant Course

Studies are generally done 1 month after transplantation to assess donor cell engraftment and disease status. Donor cell engraftment is confirmed by molecular tests (restriction fragment length polymorphisms [RFLPs] or variable number of tandem repeats [VNTRs]) or demonstration of donor sex chromosomes in sex-mismatched transplants using fluorescence *in situ* hybridization [33]. Disease status is assessed by routine staging studies such as bone marrow examination or CT scans, depending on the disease.

Even after establishment of donor cell engraftment, patients require very close follow-up, mainly to assess for evidence of immunologic dysfunction, i.e. GVHD or infection. If all goes (perfectly) well, patients are close to normal within 6 to 12 months post-transplant. A successfully transplanted patient is free of underlying disease, has solid, donor-derived hematopoiesis, has no evidence of GVHD, and has an intact immune system. Last, but not least, the patient is off immunosuppression and the donor-derived immune system is tolerant of the patient.

Complications

Complications are very common after allogeneic BMT (see Table 1). In the early period of severe pancytopenia, hemorrhage or infection may occur. The most common infections at this stage are caused by gram positive or gram-negative bacteria and occasionally by yeast species [34]. Early empiric treatment with broad-spectrum antibiotics usually manages infections successfully, but fatal sepsis occurs in a small percentage of patients. Five percent or fewer patients die of infection or hemorrhage early post transplant.

Table 1. Complications of Allogeneic BMT

Complication	Cause of Death
Toxicity of high-dose chemoradiotherapy • Infections/bleeding during period of marrow hypoplasia • Veno-occlusive disease of the liver • Myopericarditis	5–10%
Rejection of graft	<1%
Acute GVHD (and associated infections including CMV IP)	15–20%
Chronic GVHD	5%
Interstitial pneumonitis—idiopathic (not due to CMV)	5%

High dose therapy regimens are designed using drugs or radiation whose main side effects relate to myelosuppression (which is ameliorated by the stem cell infusion); however, high-dose therapy damages normal tissues, subclinically probably in most patients and severely enough to be life threatening in some. Toxicity of high-dose therapy may involve the heart (hemorrhagic myopericarditis [35]), the liver (venoocclusive disease [36]), the lungs (idiopathic pneumonitis [37]), and, less commonly, other organs. Heavily pretreated patients are more likely to have regimen-related organ toxicity and certain regimens are

more or less likely than others to cause damage to particular organs; overall, 5% of patients die of organ toxicity.

Graft rejection and resulting marrow failure is uncommon because the preparative regimen suppresses the recipient's immune system so profoundly [38]. In the standard setting of a non T-cell depleted HLA matched sibling transplant, the likelihood of graft rejection is 2 % or less. However, certain features increase the risk of rejection, including T-cell depletion of the graft, greater degrees of histoincompatibility and less intensive preparative regimens. Most patients with graft failure die, but it is interesting to note that occasional patients who are able to survive with antibiotics and transfusion support after graft failure have recovery of autologous hematopoiesis, thus demonstrating that transplant preparative regimens, despite their high doses, are not truly myeloablative in all patients.

GVHD is the most common and serious complication of allogeneic BMT [39-42]. The pathogenesis of this syndrome is discussed in more detail below. Briefly, acute GVHD is caused by donor T-cells which recognize recipient alloantigens. The donor T-cells and secondary effector cells and cytokines cause damage to host tissues, particularly the skin, liver and intestines. The syndrome occurs a median of 21 days after transplantation and ranges in severity from mild to severe. A fairly reproducible grading system is used to assess severity of the syndrome with a score assessed from I to IV. Without prophylactic measures, the incidence of GVHD is greater than 90%, but even with prophylactic immunosuppression, the incidence of moderate to severe (grade II to IV) acute GVHD is approximately 35%; 20% develop grade III to IV disease, which is considered life threatening. Risk factors for GVHD include degree of histoincompatibility, older age, multiparous female donor for male recipient, lower intensity of post-transplant immunosuppression, and concomitant viral infection. GVHD is, by itself, profoundly immunosuppressive and the agents used to treat it compound the situation even further; opportunistic infections—especially CMV and invasive fungal infections—are the most common cause of death.

Chronic GVHD occurs, by definition, 100 or more days after BMT and is different from acute GVHD in terms of both pathogenesis and clinical manifestations. Animal data suggest that the syndrome may be an autoimmune phenomenon, mediated by T-cells that are reactive with HLA determinants shared by the donor and host. Clinically the syndrome resembles an autoimmune disease, with production of autoantibodies and manifestations similar to those of discoid and systemic lupus erythematosis, Sjogren's syndrome, systemic sclerosis, polymyositis, and primary biliary cirrhosis. Roughly 50% of transplant recipients develop the syndrome, which may range significantly in severity. Risk factors for chronic GVHD include prior acute GVHD and older age. Like acute GVHD, chronic GVHD and its treatments are associated with pronounced immunosuppression and frequent opportunistic infections.

Late infections are a common problem in allograft recipients due to the combined effects of prophylactic and therapeutic immunosuppressive drugs, the immunosuppressive effects of GVHD, and the long time required for full maturation of a new donor-derived immune system [34, 43, 44]. The most common opportunistic infections include invasive fungal organisms, CMV, varicella-zoster, and encapsulated bacteria, although just about any opportunistic infection conceivable has been reported. CMV in particular, has been a major cause of mortality throughout the history of BMT [43]. The pathogenesis of this disease in BMT patients is clearly different from that in other immunocompromised patients, being closely intertwined with GVHD. Avoidance of CMV positive blood products (by choosing CMV seronegative donors or by depleting transfusions of white cells) prevents CMV infection in the occasional instance in which both donor and recipient are CMV seronegative. In donor recipient pairs where at least one is CMV seropositive prior to transplant, close monitoring for early reactivation (using the PP65 assay or PCR) with early ganciclovir treatment for patients who reactivate has significantly lessened mortality due to CMV.

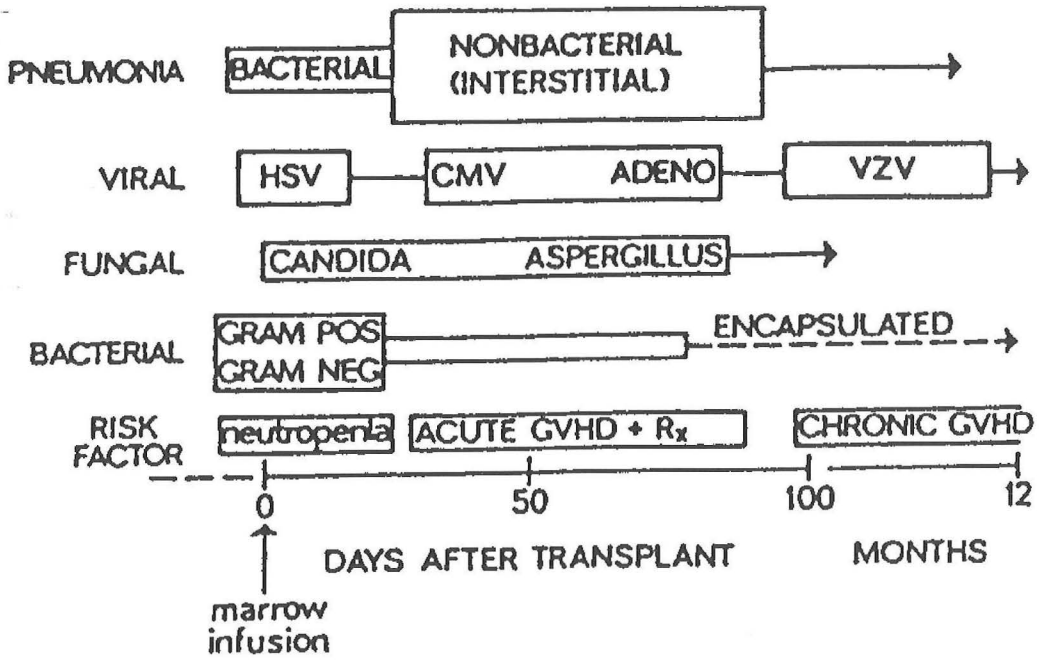


Figure 3. Infectious Syndromes at Various Times After Bone Marrow Transplantation

Late complications, generally attributable to effects of the chemotherapy and preparative regimen, are common in long-term survivors [45] (see Table 2). An especially important late complication is the development of secondary malignancy, [46] emphasizing the importance of careful surveillance of these patients.

Table 2. Delayed Complications of Allogeneic BMT

Endocrine	Infertility Hypothyroidism Growth dysfunction (children) Adrenal insufficiency
Skeletal	Osteoporosis AVN
Ophthalmological	Cataracts Sicca syndrome
Other organs: lungs, liver, kidney, CNS	Less common. Refer to Thomas, et al. (eds.) Hematopoietic Cell Transplantation. 1999
Second malignancies	Leukemia/MDS, esp. auto BMT patients Solid tumors-prior RT

Disease Free Survival

Despite its toxicity, allogeneic BMT has curative potential in a wide variety of malignant and non-malignant diseases[47-61] (see Table 3), with patients remaining in complete molecular remissions after long periods of follow-up. Survival is better in patients with earlier disease such as acute leukemia in first remission as opposed to relapse, in younger patients, and in patients who received transplants from matched siblings as opposed to unrelated donors.

Table 3. Allogeneic BMT: Disease-free Survival*

Disease	Survival (%)
AA[1]	
Untransfused	80–90
Previously transfused	65–70
CML[2]	
Chronic phase	55–75
Accelerated phase	30–40
Blastic phase	5–15
AML[3]	
First CR[4]	45–70
Second CR or early first relapse	20–45
Refractory	10
ALL[5]	
First CR	45–65
Second CR	30–45
Refractory	5–10
Myelodysplastic Syndrome	45
Myelodysplastic Syndrome	45
Hodgkin's Disease	
failed standard approaches	40
Non-Hodgkin's Lymphoma	
failed standard approaches	30
SCID[6]	80
Wiskott-Aldrich Syndrome	80
Severe Hemoglobinopathies	80
Multiple Myeloma	
Fanconi Anemia	
Congenital Pure Red Cell Aplasia	
Paroxysmal Nocturnal Hemoglobinuria	
Hereditary Storage Diseases	
Osteopetrosis	
Congenital Leukocyte Dysfunction Syndrome	

*Percentages denoted are approximations derived from the literature and represent results in matched sibling transplants. Diseases without percentages listed have been successfully treated but experience is relatively limited.

EMPHASISING THE DOWNSIDES OF CURRENT ALLOGENEIC BMT METHODS

Inspection of any bone marrow survival curve will allow an individual to take away two lessons. First, the flat portion of the survival curve indicates the curative potential of the procedure, but the second point is often under appreciated: the earlier part of the survival curve, always showing a steep decline to the plateau, indicates the pronounced treatment-related mortality of allogeneic transplantation under all circumstances.

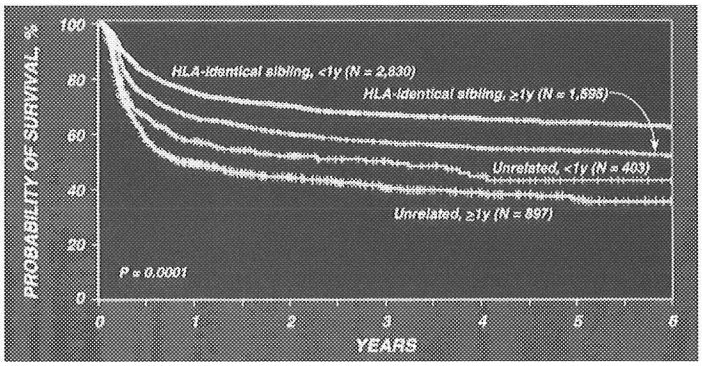


Figure 4. The good and the bad of allogeneic BMT. The flat portion of the curve indicates curative potential in CML. The early steep downward-going slope indicates early treatment-related mortality.

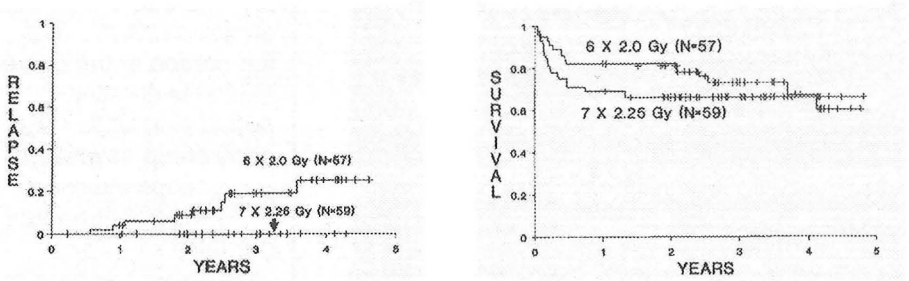
Transplant related mortality varies depending on the age and underlying physical condition of the recipient, and the degree of histocompatability between the donor and recipient. In a child with early leukemia and thus not heavily pretreated, with an HLA matched sibling donor, the risk of mortality is 15-20%. In a 30-year-old adult with early leukemia and an HLA-matched matched sibling, the risk is 20-25%; in a 50 year old with early leukemia and an unrelated donor, the risk is 45%; in the worse case, an older patient with advanced leukemia and an unrelated donor the risk of death is 50% or more (International Bone Marrow Transplant Registry statistics, 2000).

The main reason for the excessive toxicity of allogeneic BMT stems from the fact that the basic framework describing how to do the transplant was constructed in the 1960s when we knew comparatively little about immunology, and the basic framework hasn't changed since. The basic framework requires: that engraftment be achieved through very high doses of chemotherapy and radiation therapy; that cancer cells be killed, also, by very high doses of chemotherapy and radiation therapy; and that GVHD be prevented by very broadly immunosuppressive methods. Achieving adequate immune reconstitution really isn't assessed in this framework; we just hope. As illustrated in Table 4 and Figure 5, essentially all clinical research over the past 30 years addressing the

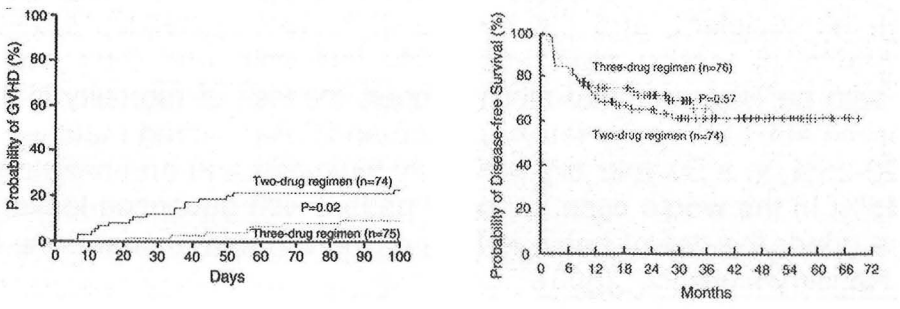
major issues of allogeneic transplantation has worked off of this framework, all variations on the same theme.

Table 4. The Basic Framework of Allogeneic BMT

PROBLEM	APPROACH	EXAMPLES
Engraftment	Very high doses of chemo/RT	CTX/TBI, VP16/CTX/TBI, VP/TBI, Bus/Cy
Kill cancer cells	Very high doses of chemo/RT	CTX/TBI, VP16/CTX/TBI, VP16/TBI, Bus/Cy
Prevent GVHD	Broad immunosuppression	CSA/MTX, CSA/MP, CSA/MTX/MP, FK/MTX, FK/MKP, FK/MTX/MP, CSA/MMF, T cell depletion
Immune reconstitution	Just hope	



A.



B.

Figure 5. Examples of Research Studies Confined by the Prescribed Framework

Panel A: The problem addressed by the study illustrated here is the fact that relapses still occur after bone marrow transplantation. To address the problem within the framework, the dose of radiation in the preparative regimen was increased by 30% (62). This resulted in a significant reduction in relapse rate which, however, was offset by an increase in treatment-related mortality; overall survival was the same.

Panel B: The problem addressed by the study illustrated here is the continuing high incidence of GVHD despite immunoprophylaxis with two drugs. To address the problem within the framework a third drug was added to the immunoprophylaxis regimen, resulting in even broader

immunosuppression [63]. This resulted in a reduction in GVHD which, however, did not result in any improvement in overall survival.

Thus, in many ways bone marrow transplantation has been stuck in a rut and one might reasonably charge the somewhat insular community of transplanters as being too blithe in its acceptance of the high rate of transplant-related mortality. It seems clear that meaningful advances in the field will require significant changes in the framework. Insights both from basic research and from the clinic have begun to suggest new approaches and recently developed assays may allow several important remaining questions to be addressed. Thus, one can begin to imagine new ways of doing transplants that are more firmly rooted in modern immunology and stem cell biology.

ALTERING THE BMT FRAMEWORK BASED ON CLINICAL INSIGHTS—HARNESSING GRAFT VERSUS LEUKEMIA

The bone marrow transplant field has relatively recently come to an appreciation that much of the curative potential of allogeneic BMT derives from an anti-tumor effect of allogeneic lymphocytes, termed graft-versus-leukemia (GVL), rather than from high doses of chemotherapy and radiotherapy [64-66]. This appreciation is leading to a significant departure from the old BMT framework, as investigators attempt new strategies which emphasize this GVL effect and de-emphasize the reliance on high dose chemo-radiotherapy.

Graft-Versus-Leukemia Background

Murine studies in the 1950s first suggested the existence of a GVL effect [67]. In these studies, leukemic mice exposed to high dose total body radiation were rescued with either syngeneic or allogeneic marrow cells. The animals receiving syngeneic cells died of leukemia, whereas the animals receiving allogeneic cells died of GVHD; importantly, the animals that died of GVHD did not have evidence of leukemia. Thus, allogeneic cell infusion had a pronounced anti-tumor effect. Over subsequent years, the GVL effect has been well documented and characterized in many animal studies [68]. These studies concur in showing a significant GVL effect, but differ in many other respects according to the animal model—in some, GVHD is closely correlated with GVL, while in others, the two are separate; in some, GVL is mediated by T-cells, in others by NK cells; in some by CD4+ T-cells, in others by CD8+ T cells, and so on.

Despite the abundant evidence in animal models, a human GVL effect wasn't appreciated for many years. It was only in the late 1970s and especially in the 1980s that a series of clinical observations began to suggest the likelihood of a human GVL effect. These observations included the human equivalent of the earliest animal studies, that is, recipients of syngeneic transplants were much more likely to relapse than recipients of allogeneic transplants [65]. In addition, it

was observed that among allogeneic transplants, those who developed GVHD were much less likely to relapse than those who did not develop GVHD [64]. Lastly, depletion of T-cells from the donor bone marrow was very effective in preventing GVHD, but was associated with a markedly increased relapse rate [69]. Observations from case reports and relatively small series led to a very large retrospective analysis by the International Bone Marrow Transplant Registry; this report confirmed, and to some extent extended, previous conclusions about human GVL [66]. Analysis of such data allowed one to get an idea of just how powerful the GVL effect is. For example, the relapse rate in syngeneic transplant recipients was 60% and in allogeneic transplant recipients was 20%. All of the cures in the syngeneic transplants (40% of the total number of patients) and half of the cures in the allogeneic patients (the same 40% of the total) would be attributable to high dose chemo-radiotherapy. However, the additional cures in the allogeneic patients, another 40% of the total, would have to be attributable to the allogeneic cells, the only difference between the two types of transplant. Thus, through this fairly reasonable, if not precise, variety of inference, it appeared that GVL might account for half the curative potential of allogeneic BMT.

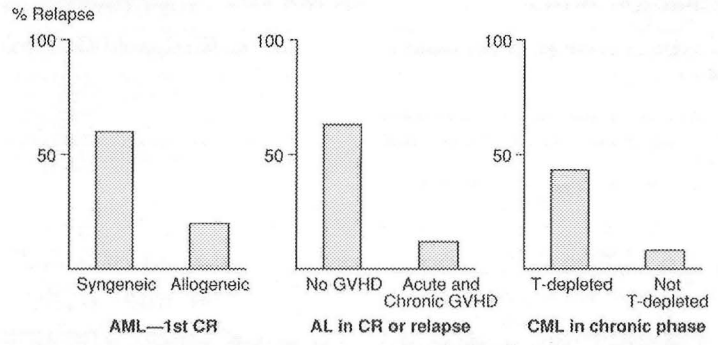


Figure 6. Clinical Evidence for Graft-vs.-Leukemia

Attempts to Purposefully Harness GVL

Based on an appreciation of the power of the GVL effect and the understanding that it was mediated by donor immune cells, most likely T-cells, numerous investigators attempted to purposefully harness the effect. In a few patients, in relapse after bone marrow transplantation, discontinuation of immunosuppression associated with a flare of GVHD resulted in complete remission [70, 71]. The field really took off when relapsed patients were treated with an infusion of additional donor lymphocytes, obtained by apheresis from the original bone marrow donor, and then given to the recipient without the cover of immunosuppression [72-76]. This procedure, termed donor leukocyte infusion (DLI) resulted in a high rate of complete remission in chronic myelogenous leukemia (CML) patients; many of these patients had no evidence of disease

even when tested by sensitive molecular techniques and the majority of these patients remain in remission, in many instances years after the DLI [77]. Thus a single infusion of the immune cells was capable of putting a high percentage of otherwise incurable CML patients into remission, quite possibly cured of their disease. DLIs have activity in many hematologic malignancies, including acute myelogenous leukemia (AML), acute lymphocytic leukemia (ALL), myelodysplastic syndrome (MDS), multiple myeloma and non-Hodgkin's lymphoma (NHL) [73, 75, 78, 79]. However, the likelihood of response varies by disease ranging from highest in CML (approximately 75%) to lowest in ALL (approximately 10%). Responses are usually, but not always, associated with GVHD. Certain variations on the procedure, such as giving lower T-cell doses or certain T-cell subsets, may lessen the likelihood of GVHD while maintaining the GVL effect [80, 81].

Changing the Framework of Allogeneic of BMT by Emphasizing GVL and De-emphasizing High Doses of Chemotherapy and Radiation

Because GVL is clearly such a powerful phenomenon, several investigators recently have begun to change the framework of allogeneic BMT from one which emphasizes high doses of chemotherapy to one which emphasizes the anti-tumor effect of allogeneic immune cells [82-88]. In this new type of transplant, termed non-myeloablative stem cell transplantation, the patient receives much lower doses of chemotherapy (and sometimes radiotherapy) than is given in a standard transplant. However, in the non-myeloablative transplant, the drugs in the preparative regimen are chosen for their potent immunosuppressive effect. Thus, much more tolerable doses of the drug are given with the aim of simply immunosuppressing the patient enough to allow the patient to accept the donor stem cell and lymphocyte graft; it is hoped then that the engrafted donor lymphocytes will mediate an anti-tumor effect.

A case report illustrates this approach:
A 60-year-old woman with low-grade non-Hodgkin's lymphoma, after several years of disease control, had become refractory to treatment and now had progressive disease, minimally responsive to any standard agents. The patient had deep-seated chronic sinusitis and a moderate reduction in her cardiac ejection fraction due to prior anthracycline therapy. Although prior studies have shown that allogeneic BMT has curative potential in this disease, this patient clearly was not a candidate because of her age, decreased ejection fraction, and overall poor condition. She was treated with a non-myeloablative transplant regimen, consisting of cyclophosphamide 2g/m^2 and fludarabine 90 mg/m^2. This is an easy regimen to give—doses are comparable to those given in a general oncologist's office. She then received G-CSF mobilized peripheral blood stem cells from her HLA matched sibling and standard immunoprophylaxis for GVHD after the transplant. She engrafted, had mild to moderate acute GVHD and

gradually evolved into complete remission; she remains in remission more than two years after the transplant.

The field of non-myeloablative stem cell transplantation is still early with pilot studies going on at several institutions around the world. Two studies of particular interest have been reported by Khouri et al. [85, 89] and by Childs et al [84]. Khouri et al. recently reported 2 year follow-up of 14 patients with advanced follicular lymphoma treated with a non-myeloablative transplant;[89] results in these patients were compared to a historical control group of 44 patients treated previously at the same institution with standard transplants. Patients treated with the nonmyeloablative approach received fludarabine and cyclophosphamide followed by allogeneic peripheral blood stem cell transplantation from HLA-matched siblings. Results in the patients receiving nonmyeloablative transplants were significantly better than in the historical controls, with day 100 mortality of 0% (vs. 34%), grade II-IV acute GVHD of 7% (vs. 43%), overall 2-year survival of 73% (vs. 45%), and 2-year disease-free survival of 73% (vs. 43%).

Childs et al. recently reported an investigation of this approach in patients with renal cell cancer [84]. Nineteen patients with refractory metastatic renal cell cancer received moderate doses of cyclosporine and fludarabine followed by infusion of allogeneic peripheral blood stem cells from HLA-matched siblings. Cyclosporine alone was used as immunoprophylaxis and patients were monitored carefully for engraftment of donor T cells. Cyclosporine was often tapered off to foster the establishment of complete donor T cell chimerism, and to allow donor T cells to mediate anti-tumor effects. Three patients had complete remissions, which were ongoing at 27,25, and 16 months. Partial remissions were seen in 7 patients. It was noted that responses commonly were delayed, occurring several months after the transplant, often following withdrawal of cyclosporine and often associated with GVHD; it was noted by the investigators that several of the partial responders seemed to be continuing to gradually evolve into remission. Ten patients developed GVHD; 1 died of GVHD and another died of bacterial sepsis.

At this point the field of nonmyeloablative stem cell transplantation is early, but it is possible to begin to draw some conclusions. Although nonmyeloablative regimens vary greatly in intensity, all of them are less intensive than standard transplant regimens. Thus, the early phase of the nonmyeloablative transplant process is significantly less toxic that that of the standard transplant. Nonmyeloablative regimens are commonly given in the outpatient setting, post-transplant pancytopenia is less severe (in fact, with some regimens, patients commonly do not develop severe neutropenia and do not require transfusion), and regimen-related mucositis and organ toxicity is much less common. The generally smooth early course of the nonmyeloablative transplant has inspired such monikers for the procedure as "mini-transplant," "transplant-lite," and "drive-thru transplant." However, since fully capable allogeneic T cells are

infused in the process and immunoprophylaxis regimens are similar to those used in standard transplants, it should come as no surprise that GVHD remains a significant problem. This is especially true since part of the treatment strategy often involves a purposeful attempt to induce GVHD and associated graft-vs.-tumor by decreasing cyclosporine doses and even infusing additional lymphocytes from the donor. Thus, GVHD is a common problem and treatment-related mortality, occurring late and due to GVHD and associated infections is not inconsequential, ranging from 10% to nearly 40% in some series. It shouldn't be surprising that mortality would continue to be a problem with nonmyeloablative transplants despite less early toxicity; only 5% or so of *standard* allograft recipients die of early toxicity, with the majority of deaths being due to GVHD and infection.

With regards to disease activity, studies thus far are too preliminary to allow any definitive conclusions about likelihood of response or duration of response in a given disease. However, response rates appear promising in CML, CLL, low-grade NHL and renal cell cancer. How responsive other hematologic malignancies, e.g. AML, ALL, MDS, and multiple myeloma, will be remains uncertain, but it should be kept in mind that previous experience with donor leukocyte infusion doesn't bode well for nonmyeloablative transplants in certain diseases. For example, since ALL rarely responds to DLI, then one wouldn't expect it to respond to nonmyeloablative stem cell transplantation. Whether nonmyeloablative stem cell transplantation will be useful in solid tumors besides renal cell cancer is unknown. Renal cell cancer was chosen for study because of prior clinical observations that had suggested it might be an "immune-responsive disease." However, malignant melanoma, despite also being sensitive to immunologic maneuvers, hasn't appeared responsive to nonmyeloablative stem cell transplantation, at least in early studies (R. Childs, personal communication). The approach is being tested in other solid tumors but results have not yet been reported.

Thus, nonmyeloablative stem cell transplantation represents a real shift from the old framework of BMT; high dose chemoradiotherapy is substituted for by less toxic doses to achieve engraftment, and by donor immune cells to attack cancer cells. However, GVHD remains a problem and it remains uncertain if graft-vs.-tumor can be delivered without having to be associated with GVHD. Improvement in the situation will require a stronger foundation in terms of basic immunology and stem cell biology; this will involve application of recent basic studies, and addressing remaining questions by recently developed assays.

ALTERING THE BMT FRAMEWORK BASED ON INSIGHTS FROM LABORATORY RESEARCH

Basic and translational research over the past several years has led to major advances in our understanding of the basic immunologic issues of allogeneic

transplantation—alloreactivity, graft-vs.-host disease, graft-vs.-leukemia, and immune reconstitution [8, 9, 40, 42, 68, 90-97]. In addition, although several important questions remain to be answered, new assays show promise in being able to address them. This enhanced understanding of fundamental issues suggests additional ways of altering the old framework of allogeneic BMT, which may result in less toxic and more effective methods of the procedure.

Molecular and Cellular Basis of Alloreactivity

Alloreactivity is the most serious impediment to successful transplantation. This process involves the recognition of non-self peptides by donor or recipient immune cells, resulting in graft rejection or GVHD. Although rejection is not common in clinical transplantation this is because the recipient is immunosuppressed by often very toxic methods. GVHD, of course, remains a major problem despite administration of standard immunoprophylactic medications.

A review article written 10 years ago about GVHD was able to paint GVHD pathogenesis with only broad brushstrokes [42]. In this model, GVHD is initiated by an antigen-presenting cell presenting antigen to a CD4+ T cell. Upon activation, the CD4+ cell secretes IL-2, recruiting other cellular effectors, which, along with various cytokines, mediate tissue damage (see Figure 7). This model is essentially correct, but 10 years ago the details were very sketchy, with limited understanding of the nature of minor histocompatibility antigens, optimal antigen presentation, costimulation, signaling pathways, lymphocyte homing, pathways of apoptosis, etc. Great strides in basic immunology over the past decade, both in terms of a better understanding of various molecular interactions from a reductionistic point-of-view, as well as a better "big picture" understanding of the highly organized integration of the immune system's various components [98, 99], have allowed the basic model of GVHD to be fleshed out significantly.

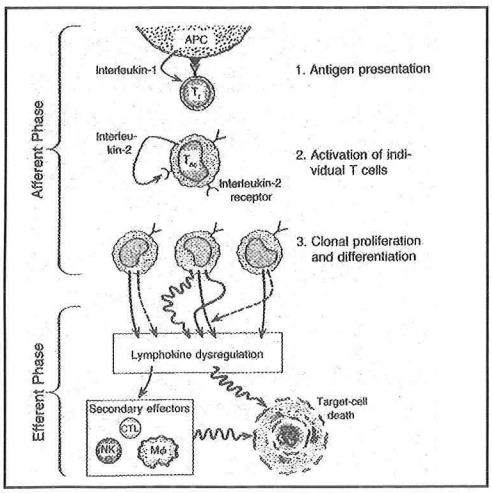


Figure 7. Model for acute GVHD. From reference 42.

The first major requirement of alloreactivity is that an antigen exist that will be recognized as foreign. The most potent alloantigens are the major histocompatibility antigens encoded by the Major Histocompatibility Complex (MHC) (termed the HLA Complex in man) [8, 9]. However, most bone marrow transplants are carried out in the HLA-identical setting; antigens inducing alloreactivity in this setting are termed minor histocompatibility antigens [95, 100-103]. It is now understood that minor histocompatibility antigens are peptides derived from polymorphic proteins, which may be encoded by autosomal chromosomes, the Y chromosome, or mitochondrial DNA. These proteins are degraded as part of the normal turnover of intracellular proteins, and peptides resulting from this process, a few amino acids in length, are incorporated into the peptide binding cleft of HLA molecules as they are assembled in the endoplasmic reticulum [104]. The HLA molecule/peptide is transported to the surface of the cell where the tertiary structure of HLA molecule/bound peptide is presented to the T cell receptor of T cells passing by. For example, the protein encoded by the KIAA0223 gene has 2 alleles, one containing an arginine amino acid in a particular position and the other a histidine amino acid in this position [103]. A peptide containing one amino acid or the other is transported to the cell surface along with a particular restricting HLA molecule (HLA-A2 in this case), where it serves as an alloantigen for an individual who has inherited the opposite allele. Several minor histocompatibility antigens have been characterized although many more remain to be. Although a great many proteins likely have polymorphisms, not all of these polymorphisms will translate into minor histocompatiblility antigens. For example, the polymorphic peptide may be produced in tiny amounts, or may not bind to the peptide-binding site of a given HLA molecule or may not lead to significant enough changes in tertiary structure to be recognized. The total number of relevant minor histocompatibility antigens in humans may be only a few dozen, and it is likely that in an individual donor-recipient pair only one or a very few minor antigens are immunodominant, meaning that they can elicit an alloreactive response. In one clinical study, disparity in a single minor histocompatibility antigen was able to account for most of the clinically significant GVHD observed [105].

The second major requirement of alloreactivity is that a T cell with T cell receptor structure exists that is capable of recognizing as foreign the histocompatibility antigen. The T cell repertoire is formed in the thymus [106-108]. T cell precursors rearrange their T cell receptor genes in a process of recombination analogous to that observed in immunoglobulin genes of B cells. Millions of different T cell receptors result [109]. Many of these are non-functional, leading to apoptosis of the cell. Many others do not adequately interact with self-MHC molecules expressed by thymic epithelial cells; T cells containing such receptors would not be able to interact with self tissue to carry out surveillance for foreign peptides

and thus die by apoptosis also [110, 111]. Some T cells contain T cell receptors that bind with excessive affinity to self-peptide/self MHC expressed by dendritic cells within the thymic medulla; these autoreactive T cells are deleted [112]. Thus, there is a great amount of T cell death in the thymus and the only T cells that make in into the circulation are the ones that can interact with self MHC but not too tightly, those reactive with self peptides having been deleted. Thus, in the example above [105], an individual who had the arginine-coding allele of KIAA0223 would have T cells reactive to the histidine-containing peptide but would lack T cells (self)-reactive to the arginine-containing peptide; the reverse would be true in an individual who had the histidine-coding allele.

Graft-vs.-host disease requires, in addition to histoincompatibility and immunocompetent alloreactive T cells, that the host be unable to reject donor T cells. After high dose therapy, donor alloreactive cells have a numerical and perhaps functional superiority over host alloreactive cells. With these three requirements of Billingham's classic triad met [113], the stage is set for GVHD to occur.

Infused donor T cells circulate in the secondary lymphoid tissues (lymph nodes, Peyer's patches and spleen) and percolate through the T cell regions of these tissues until they encounter their cognate antigen [98]. The antigen is presented by highly specialized antigen presenting cells which have become activated in the peripheral tissues. The main antigen presenting cells involved are the dendritic cells [114, 115], which reside in peripheral tissues, serving as sentries to alert the immune system to the presence of foreign antigens that are causing tissue damage [116]. The high dose preparative regimen directly causes tissue damage and allows infectious agents to breach epithelial borders in the gut, skin and liver; the tissue damage from the regimen is thus compounded by infection, leading to an "activation" of the tissue [40]. Activated tissue releases various cytokines, especially IL-1, TNF-alpha, and GM-CSF, which, along with still undefined factors, activate dendritic cells. The activated dendritic cells, which may have engulfed antigen from microorganisms but also contain antigen that will be seen as foreign by donor T cells, migrate to the lymph node. In the lymph node the activated dendritic cell serves to present its complement of antigens (including allo-peptides) to donor T cells [99, 114]. Donor CD4+ T cells interact with peptide presented in the context of HLA class II molecules and donor CD8+ T cells interact with peptide presented in the context of HLA class I molecules.

Optimal activation of an alloreactive T cell by an antigen presenting cell requires 3 components [117]: 1) engagement of the T cell receptor by its cognate peptide/MHC complex; 2) tight adhesion between antigen presenting cell and T cell; and 3) importantly, delivery by the antigen presenting cell of a so-called costimulus [118]. Dendritic cells abundantly express molecules important for all these components: HLA class I and II molecules, and several adhesion and costimulatory molecules. Engagement of the T cell receptor and engagement of receptors for costimulatory molecules (the best characterized costimulatory

interaction is between B7 expressed by the antigen presenting cell and CD28 expressed by the T cell) activates the T cell through increasingly well-defined signaling pathways [119]. Particular dendritic cells, by expressing certain profiles of cytokines and other molecules, tend to polarize responding T cells toward expressing certain cytokine profiles themselves [114]. These cytokine profiles will favor either a cellular response to antigen (TH1 cells) or an antibody response to antigen (TH2 cells)[120]. In the case of GVHD, T cells are polarized toward a TH1 response [40]. Under these circumstances, activation of the T cell involves expression of IL-2 and its receptor [99, 121], expression of other cytokines, especially gamma interferon [40], and, in the case of cytotoxic cells, protein esterases required for killing activity. In addition, a new array of homing molecules is expressed that allows the T cells to home to peripheral sites which have been activated by inflammation [98]. Thus, the 2 signals delivered by the antigen-presenting cell to the T cell lead to profound changes in the phenotype and function of the alloreactive T cell. Again, the first signal is delivered by the interaction of the MHC-peptide/T cell receptor interaction, and the second is delivered by the costimulus. If, however, the first signal is delivered but the second is not, then the T cell actually becomes either anergized or deleted and can no longer respond to its cognate peptide/MHC [117, 118] (this has important therapeutic implications as discussed below). A last cellular component of this phase of GVHD (again of potential therapeutic importance) is a cell type which plays the role of a negative regulator and can suppress alloreactive cells [122]; the balance between these cells and alloreactive cells conceivably may control the intensity of GVHD.

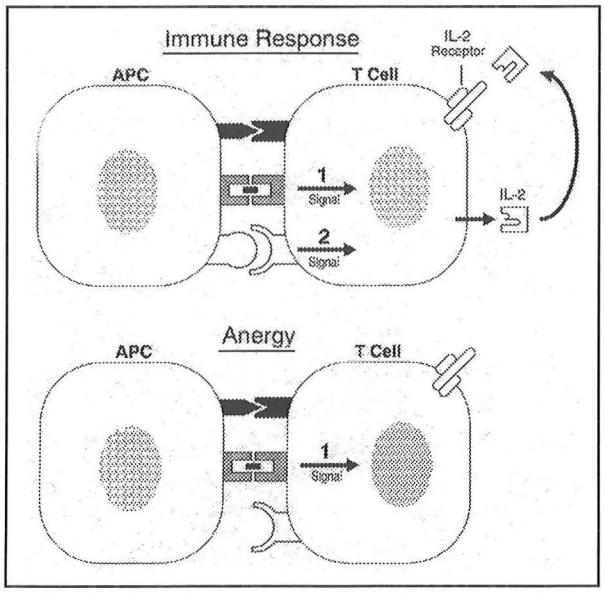


Figure 8. Effective interactions between antigen presenting cell and alloreactive T cell involve 3 major components: adhesion, engagement of the T cell receptor (Signal 1), and a costimulus (Signal 2). Delivery of Signal 1 without signal 2 results in anergy or deletion of the T cell. From reference 117.

The effector phase of the GVH reaction involves the homing of activated T cells (CD4+ and CD8+) to peripheral sites [98], along with the recruitment of other cell

types and release of various cytokines [40]. Earlier concepts that cytotoxic T cells mediated the bulk of tissue destruction in GVHD were too simple. Clearly other cell types are recruited, including NK cells and macrophages. TH1 cytokines, especially gamma-interferon, prime macrophages to receive a second triggering signal, especially lipopolysaccharide which may leak through damaged intestinal mucosa. These activated macrophages release IL-1, nitrous oxide, and TNF-alpha; TNF-alpha, in particular, is responsible for much of the tissue damage associated with GVHD [123]. Tissue destruction is mediated by a number of different mechanisms [40]. Cytotoxic lymphocytes and natural killer cells kill through release of perforin and granzymes, and through Fas ligand/Fas interactions [124]. Cytokines, released by the various cells involved in the reaction kill through engaging their receptors on target tissue and engaging apoptotic pathways. Although epithelial tissues are the targets of GVHD, exactly which cells within the tissue are targeted is unknown. The localization of lymphoid infiltrate and apoptotic bodies to the stem cell regions of these tissues--rete ridges of the skin and intestinal crypts in the intestinal mucosa--suggest that epithelial stem cells may be the target.

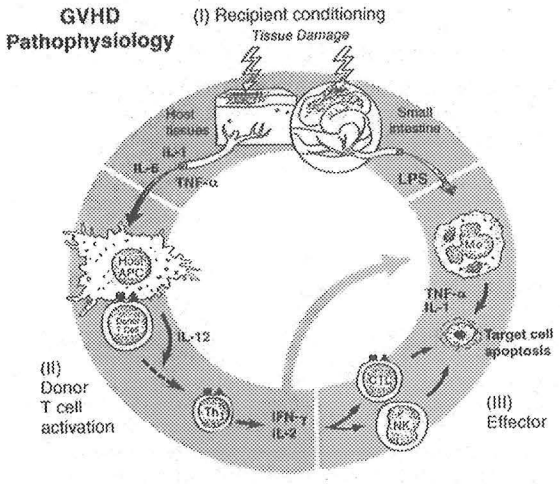


Figure 9. Model for acute GVHD which incorporates some recent concepts. From reference 40.

Chronic GVHD appears to have a different pathophysiology from acute GVHD [41]. Animal studies suggest that the effector cells of chronic GVHD are actually autoreactive rather than alloreactive. The autoreactive T cells appear to be directed against a framework antigen of MHC class II molecules which is shared between donor and host. The cause for the development of these autoreactive clones is uncertain but thymic damage has been proposed as the major instigator. The thymus may become damaged in allogeneic transplant recipients from a variety of insults, including radiation, cyclosporine, and, especially, acute GVHD. It is postulated that the damaged thymus fails to delete autoreactive T cells and fails to produce the suppressor T cells that would normally inhibit autoreactive cells that managed to make it into the periphery. Another possible

explanation for the existence of autoreactive clones is that the state of severe T lymphocytopenia after BMT fosters the survival of autoreactive clones [98]. Under normal circumstances various T cell clones compete for niches capable of supporting their survival; in a state of lymphocytopenia autoreactive clones are more likely to be able to survive this competitive struggle. Another feature of chronic GVHD is that it is especially characterized by excessive production of cytokines, some of which stimulate fibroblasts to produce collagen. Clinically, chronic GVHD is notable for its similarity to a variety of autoimmune diseases and for severe cutaneous fibrosis.

Important issues about alloreactivity remain to be completely addressed, in particular the identity of the majority of the minor histocompatibility antigens and the relative importance of various cytokines. But the deep understanding of many of the immunologic details of GVHD has suggested many new approaches which may lead to a more precise approach to the problem [125, 126] (see Table 5). I will limit my discussion below to two promising approaches.

Table 5. Potential Sites for GVHD Intervention

SITE FOR INTERVENTION	METHOD OF INTERVENTION
Activated host dendritic cells	Deplete host dendritic cells Minimize host tissue damage - germ-free environment - less toxic regimen
Alloreactive donor T cells	T cell deplete BM Selectively T cell deplete BM Target T cells *in vivo*
Interaction between DC/T cells	MoAb to adhesion molecules Dummy peptides inhibiting TcR/MHC interaction Co-stimulation blockade
Inhibit T cell signaling	TcR signaling IL-2R signaling Co-stimulatory pathway signaling
Target cytokines	Shift response to TH2 (IL-10, IL4) Receptor antagonists, soluble receptors, MoAbs to cytokines IL-2, IL-1, γ-INF, TNF
Block homing to inflamed sites	MoAbs, antagonists to homing molecules

Methods to selectively deplete or inhibit alloreactive T cells

Since GVHD is initiated by donor T cells, it seems reasonable to assume that an effective method for preventing GVHD would be simply to deplete the bone marrow cells of T cells. Indeed, this approach has been intensively investigated and shown to prevent GVHD, depending on completeness of T cell depletion [27]. If T cells are nearly completely removed, then the incidence of acute GVHD approaches zero, even without the use of prophylactic immunosuppressive

medications. Importantly, the incidence of chronic GVHD approaches zero as well. Unfortunately these beneficial effects are balanced by a significant increase in the likelihood of graft rejection, disease relapse, and poor immune reconstitution. In most instances these downsides are so prominent as to cancel out the beneficial effect of GVHD prevention. Thus a goal in T cell depletion would be to selectively deplete alloreactive T cells, leaving the others intact.

We are currently investigating an approach which takes advantage of the expression by alloreactive T cells of various cell surface antigens, in particular the IL-2 receptor. In this approach a mixed lymphocyte reaction is set up in which irradiated recipient peripheral blood mononuclear cells (containing antigen presenting cells) are cocultured with donor T cells. The alloreactive T cells are activated and express CD25, a subunit of the IL-2 receptor. CD25-expressing cells are targeted with an anti-CD25 monoclonal antibody linked to the ricin A chain, a potent compound which poisons cells by inhibiting ribosomal activity. Preclinical studies have shown that the procedure depletes alloreactive T cells while leaving responses to third party antigens intact [127] (see Figure 10). Additional optimization of the procedure is ongoing and preliminary work has begun to upscale the procedure for clinical use.

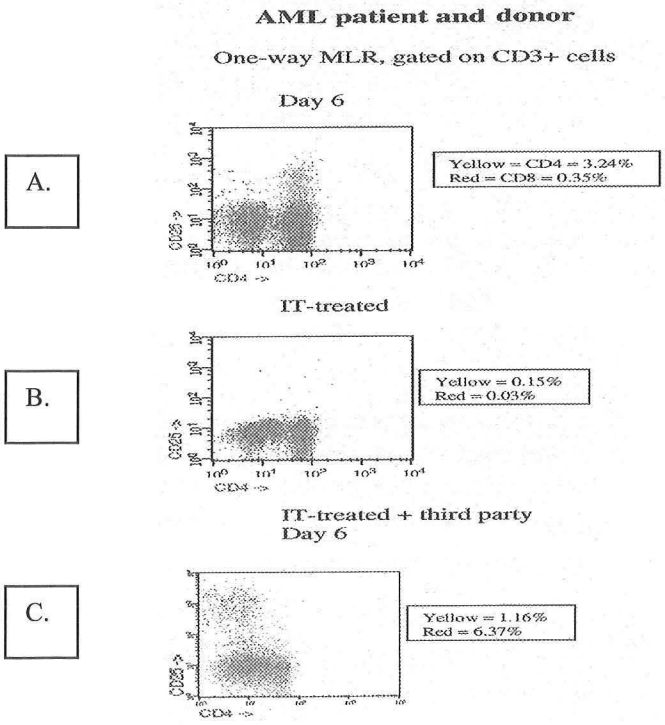


Figure 10. Flow cytometric analysis showing selective depletion of alloreactive T cells by targeting IL-2 receptor with immunotoxin. Panel A. Alloreactive T cells expressing CD25 after stimulation by recipient cells in MLR (red dots are CD8 cells and yellow dots are CD4 cells). Panel B. Depletion of alloreactive T cells by immunotoxin to CD25 Panel C. T cell responses to third-party antigens are preserved.

Another approach aimed at specifically inhibiting alloreactive responses is based on appreciation of the importance of costimulation in T cell responses to antigen.

As outlined above, inhibition of costimulation during the engagement of a T cell receptor with its cognate antigen will either anergize or delete the particular T cell. Numerous agents inhibit costimulation. Guinan et al. have reported a series of 12 patients who were undergoing haploidentical transplantation (i.e. mismatch for an entire HLA haplotype)(108). Before infusion, donor bone marrow was cocultured with irradiated recipient cells in the presence of CTLA4Ig, an inhibitor of B7:CD28 mediated costimulation. After coculture the frequency of recipient-specific donor T cells was markedly reduced, while third party responses remained intact. The incidence and severity of GVHD after infusion of the "anergized" marrow was significantly less than that usually seen in haploidentical transplant patients.

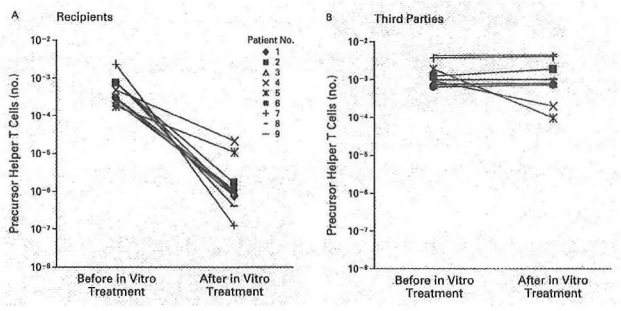


Figure 11. Donor precursor helper T cell frequencies after coculture of donor bone marrow and irradiated haploidentical recipient cells in the presence of CTLA4Ig to block costimulation. Panel A: Activity specific for recipients is markedly reduced. Panel B: Activity for 3rd-party antigens is maintained. From reference 128.

Thus, new insights into the biology of alloreactivity have allowed investigation of novel approaches that may more specifically inhibit alloreactive cells and thus prevent GVHD. Another goal of transplantation is to achieve engraftment of stem cells without the use of immunosuppressive chemotherapy or radiation. We have observed two recipients of standard liver transplants who had complete engraftment of donor hematopoietic stem cells with establishment of donor-derived hematopoiesis, demonstrating the feasibility of donor hematopoietic stem cell engraftment without standard pre-treatment of the recipient [129, 130]. Since the alloreactivity of graft rejection is in many ways the mirror image of GVHD, one can imagine applying the novel approaches for GVHD prevention discussed above for graft rejection as well. For example, one could imagine exposing host cells to a donor alloantigen in the presence of a costimulus-blocking agent. Such an approach might render the host cells non-reactive to donor cells, allowing them to engraft without any chemotherapy, radiation, or broadly immunosuppressive drugs.

Investigating the Role of the Thymus in Immune Reconstitution

Infections, often in association with GVHD, are the most common cause of death after allogeneic stem cell transplantation. The most serious infections are those

that are normally handled by intact T cell mechanisms. Thus, the mechanisms of T cell immune reconstitution are essential to understand if transplants are to be done more safely [96, 131, 132].

The thymus produces the entire T cell repertoire in childhood. After puberty the thymus involutes and the individual lives for the rest of his life off of the immune repertoire that was present when the thymus stopped functioning. Under normal circumstances this is compatible with a long lifespan but what happens when the system is stressed by bone marrow transplantation [131]? The high doses of chemoradiotherapy wipe out the recipient's immune system, which is then replaced by the relatively limited number of donor T cells contained in the donor bone marrow inoculum. In the child the thymus is thought to be intact and can thus contribute to production of a full T cell repertoire. However, in adults it has been thought that full T cell reconstitution is not possible because the thymus is involuted. Any increase in T cell number would require peripheral expansion of post-thymic T cells present in the donor bone marrow inoculum; expansion of this limited number of cells would likely lead to a restricted immune repertoire. The issue of the thymus' contribution to immune reconstitution has been difficult to address because of the lack of an assay for directly measuring thymic output.

Douek et al. have recently developed an assay that allows direct assessment of thymic function in bone marrow transplant recipients [133]. This assay is based on the T cell receptor gene rearrangement process that occurs during T cell maturation in the thymus, as described above. As recombination occurs segments of intervening DNA are looped out and then exist as episomes, which are termed T cell receptor gene rearrangement excision circles (TRECs). However, since non-chromosomal DNA is not replicated during cell division, the episomes are diluted out when T cells go through cell divisions. Thus recent emigrants from the thymus contain these TRECs while post-thymic T cells that have undergone cycles of cell division, generally (although not necessarily) after exposure to antigen, do not. Douek et al. found that a particular segment of DNA was rearranged in 70% of $\alpha\beta$ T cell receptor-containing T cells, resulting in a particular episome with a conserved DNA sequence at the joining region; this allowed construction of a quantitative PCR assay that could be used to determine TREC levels *in vivo* as a measure of thymic function. TRECs are not present in children with DiGeorge's syndrome but are present after thymic transplantation [134]. After control of HIV by antiretroviral therapy, adult HIV patients have rising TREC levels [133], indicating that adults, contrary to prior dogma, do have a functioning thymus.

We have used the TREC assay to study thymic function in both autologous and allogeneic transplant recipients. The study in autologous patients, all of whom were adults, showed evidence of thymic production of new T cells by day 100 after transplantation [135]. Although there was an inverse correlation between thymic function and age, significant thymic output was observed in many older patients, even into the sixth and seventh decades. Increased thymic output correlated with, and was predictive of, increased naïve T cell numbers and

broader T cell receptor repertoires. Likewise, analysis of TRECs in allogeneic transplant recipients has shown evidence of thymic function in adults [136]. Importantly, GVHD appears to inhibit the thymus' ability to function. This is particularly true of chronic GVHD, in line with animal studies that have demonstrated that the thymus is a major target of GVHD.

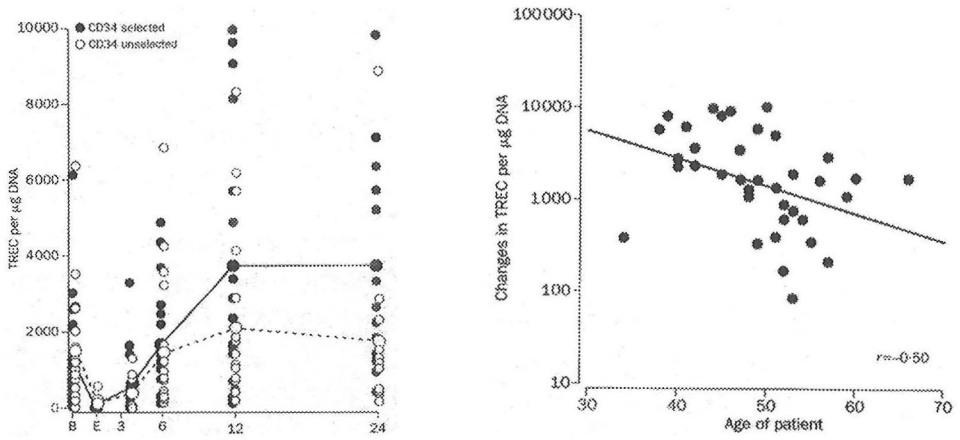


Figure 12. TREC Assay in Autologous Transplant Recipients.

Left panel: Increases in numbers of TREC in peripheral blood mononuclear cells post transplant. B=baseline. E=engraftment of neutrophils. Solid line=mean for CD34+ selected grafts, dashed line=mean for CD34-unselected grafts. Right panel: Relation between age and numbers of TRECs. From reference 135.

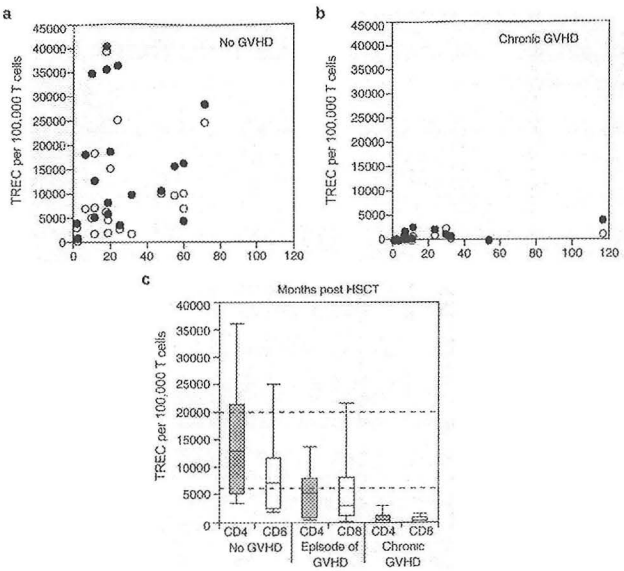


Figure 13. TREC assay in allogeneic transplant patients. Individual CD4+ (dark circles) and CD8+ (open circles) T cell TREC levels are shown for patients ≤ 25 with no history of GVHD (panel a) and with chronic GVHD (panel b). A composite box plot for TREC levels is shown in the lower panel. From reference 136.

Thus, the TREC assay has shown that the thymus may reawaken in adults after transplantation and emphasizes the importance of avoiding GVHD if full immune reconstitution is to occur. Preclinical studies have suggested that certain hormones and cytokines, in particular IL-7 [137], have significant thymopoietic activity. One can imagine that it may be possible to use such an agent to enhance thymic recovery after stem cell transplantation, thus hastening the reconstitution of a full T cell repertoire.

Investigating GVL- and GVH-specific clones using a new assay of antigen-specific T cells

As discussed above, GVL is an extremely potent anti-tumor phenomenon. However, although mechanisms have been worked out in animal models [68] (and vary from model to model), the mechanisms in humans are poorly understood [138]. In particular, it is not known if GVL and GVHD are directed against the same or different antigens. Theoretically tumor cells could express tumor-specific antigens, i.e. peptides derived from mutant proteins or over-expressed proteins. On the other hand tumor cells may express the same alloantigens expressed on normal tissues and thus be targeted by the same cells that cause GVHD. Many clinical observations suggest that the GVL phenomenon is really an alloreactive phenomenon, for example, the lower relapse rate post-transplant of patients who develop GVHD [64] and the close correlation of GVHD to response in recipients of donor leukocyte infusions [75]. However, some clinical observations suggest that GVL sometimes may be separate from GVHD; for example, allograft recipients who do *not* develop GVHD nevertheless have a lower relapse rate than recipients of syngeneic transplants [66]. In addition, many patients have been reported who had responses to donor leukocyte infusions but did not have GVHD [77, 80, 81]. Whether antileukemia activity was mediated in such patients by subclinical alloreactivity or by truly leukemia-specific mechanisms is unknown. Dissection of the relevant mechanisms has been limited by insufficient assays.

Douek and colleagues have recently developed a quantitative PCR assay for monitoring antigen-specific T cells [139, 140] (and unpublished observations). This assay was initially developed for responses to viral antigens. Briefly, a T cell population is exposed to a given antigen presented by antigen presenting cells. The antigen-specific T cells are activated and express activation antigens, allowing them to be sorted according to cell surface molecule expression. cDNA is then prepared from the responding T cells and the complementarily determining region 3 (CDR3) region of the T cell receptor is expanded using anchored PCR and a primer for the constant region of the gene. The PCR product is then ligated into E coli; colonies are plucked and the inserts are sequenced. Roughly 10-15 separate clones are thus isolated in the typical T cell response to a virus, with 2 or 3 clones being dominant. Knowledge of the

specific clonal CDR3 region sequence allows clone-specific primers to be constructed for a quantitative PCR reaction. This assay has been used to monitor viral-antigen specific T cell clones and has recently been adapted to

allow study of responses to tissue antigens, i.e. minor histocompatibility antigens or leukemia-specific antigens. Isolated populations of host leukemia cells and normal cells can be used to separately stimulate donor T cells. Putative GVH-specific and GVL-specific clones can then be followed *in vivo* and correlated with clinical GVHD and clinical GVL (as measured by quantitative PCR for leukemia-specific chromosomal translocations). T cell receptors that appear to be associated with GVH or GVL can be used to probe cDNA libraries to determine the antigens to which they are directed [141]. We are just beginning these studies in our program and thus have no news to report, but it should be clear that sophisticated immunologic assays might shed light on GVH and GVL mechanisms. If GVL antigens were found to be different from GVH antigens then one could imagine using this knowledge to enhance GVL, such as by adoptively transferring antigen-specific T cell clones expanded *in vitro* [142], or by vaccinating either the donor pre-transplant [143], or the recipient post-transplant, with the leukemia-specific antigen.

An Entirely New Framework for BMT

Thus, advances in basic immunology allow one to imagine completely changing the framework of allogeneic BMT, from the toxic approach mandated by the state of knowledge in the 1960s, to a more sophisticated approach incorporating our current, deeper understanding of the essential immunologic issues (Table 6). Based on safer and more precise methods, allogeneic transplantation is likely to evolve significantly over the next decade. It is likely that genetic disorders involving hematopoiesis, especially sickle cell disease and thalassemia, will be transplanted more safely and frequently. Transplantation for malignancies will continue, with an emphasis on allogeneic immunotherapy in immune-sensitive malignancies. However, as more novel therapeutics become available that specifically target the molecular abnormalities of particular cancers, transplantation for certain malignancies may wane; this phenomenon is already being observed in the case of CML, which appears to be especially sensitive to a new agent, STI571, which selectively inhibits the overactive abl tyrosine kinase that drives the disease [144].

Table 6. Reasonably Conceivable Changes to the Framework of Allogeneic BMT

PROBLEM	OLD	NEW
Engraftment	High-dose chemo/RT	Donor-specific tolerance through blockade of costimulation
GVHD	Broad immunosuppression	Recipient-specific tolerance or selective depletion of alloreactive T cells
Immune reconstitution	Just hope	Stimulation of thymus by certain cytokines, hormones
Anti-leukemia	High-dose chemo/RT	Tumor-specific T cells - adoptively transfered - vaccination with tumor-specific antigen

NEW INDICATIONS: HEMATOPOIETIC STEM CELLS FOR NON-HEMATOLOGIC DISEASES

A remarkable series of experiments has recently demonstrated the extraordinary plasticity of tissue stem cells [145]. A long-held dogma has described development in terms of embryonic stem cells giving rise through intermediaries to increasingly differentiated tissues. Differentiated skin cells were skin cells-only and differentiated muscle cells were muscle cells-only, but even stem cells of given tissues—i.e. hematopoietic stem cells, neural stem cells, etc. were felt to be differentiated such that they could only give rise to cells of a particular lineage, i.e. hematopoietic stem cells could only make blood cells.

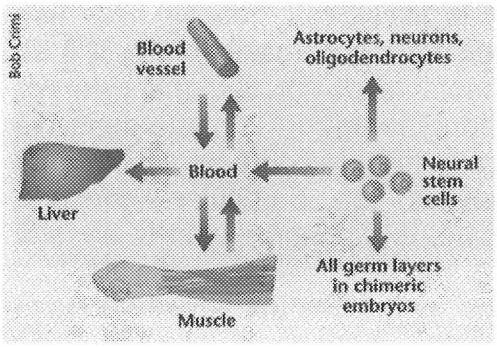


Figure 14. Hematopoietic stem cells can differentiate into many different tissues. From reference 145.

However, even in the 1950s, experiments had shown that nuclei from specialized cells when placed into oocytes could redirect cell fate[146]. Dolly affirmed this when differentiated mammary gland cells were redirected into a fully developed animal [147]. It has recently become clear that this developmental plasticity is not restricted to an embryonic environment. Stem cells from various tissues, under the right circumstances, can differentiate into other tissues. Neural stem cells

are especially multipotent, giving rise to blood cells, nerve cells, and all germ layers in chimeric embryos [148, 149]. Hematopoietic stem cells can give rise to blood vessels [145], liver tissues [150-152], and muscle cells[153, 154]. In early studies, adult hematopoietic stem cells, fairly easily isolated by immunologic techniques, have been used to successfully treat animal models of muscular dystrophy [154], a hepatic enzymatic defect [150], and myocardial infarction [155]. Although much work remains to be done, it seems quite likely that hematopoietic stem cells will be used to treat a variety of non-hematologic diseases in the future. Autologous stem cells will probably be used in instances where repair of otherwise normal tissue is desired, but allogeneic stem cells may be used in diseases characterized by lack of a normal gene (e.g. muscular dystrophy). In this instance all of the same immunologic issues discussed above would apply and is encouraging to think that the immunology is coming well enough along that it may be possible to carry out such transplants safely.

1. Thomas ED, Storb R. The development of the scientific foundation of hematopoietic cell transplantation based on animal and human studies. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 1-11.
2. Thomas ED, Lochte Jr. HLC, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med* 257:491-496, 1957
3. Thomas ED, Lochte HLJ, Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. *J Clin Invest* 38:1709-1716, 1959
4. van Rood JJ, van Loeuwen A. Leukocyte grouping: a method and its application. *J Clin Invest* 42:1382-1390, 1963
5. Bortin MM, Bach FH, van Bekkum DW, Good RA, van Rood JJ. 25th Anniversary of the first successful allogeneic bone marrow transplants. *Bone Marrow Transplantation* 14:211-212, 1994
6. Fefer A, Thomas ED. Marrow transplantation in the treatment of acute leukemia. In: Henderson ES, Lister TA, editors. Leukemia. Philadelphia: W. B. Saunders Company; 1990. p. 431-441.
7. Horowitz MM. Uses and growth of hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 12-18.
8. Klein J, Sato A. The HLA system: First of two parts. *N Engl J Med* 343(10):702-709, 2000
9. Klein J, Sato A. The HLA system: Second of two parts. *N Engl J Med* 343(11):782-786, 2000
10. Mickelson E, Petersdorf EW. Histocompatibility. In: Thomas ED, Blume K, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 28-37.
11. Beatty PG, Clift RA, Mickelson EM, et al. Marrow transplantation from related donors other than HLA-identical siblings. *N Engl J Med* 313:765-771, 1985
12. Henslee-Downey PJ, Parrish RS, MacDonald JS, et al. Combined *in vitro* and *in vivo* T lymphocyte depletion for the control of graft-verses-host disease following haploidentical marrow transplant. *Transplantation* 61:738-745, 1996
13. Aversa F, Tabilio A, Terenzi A, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. *Blood* 84:3948-3955, 1994
14. Hansen JA, Petersdorf EW. Unrelated donor hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 915-928.

15. Mori M, Beatty PG, Graves M, Boucher KM, Milford EL. HLA gene and haplo-type frequencies in the North American population: The National marrow Donor Program Donor Registry. *Transplantation* 64:1017-1027, 1997
16. Kernan NA, Bartsch G, Ash RC. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Program. *N Engl J Med* 328:593-602, 1993
17. Broxmeyer HE, Smith FO. Cord blood stem cell transplantation. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 431-443.
18. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. *N Engl J Med* 337:373-381, 1997
19. Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med* 339(22):1565-1577, 1998
20. LaPorte JP, Gornin NC. Cord-blood transplantation from an unrelated donor in an adult with chronic myelogenous leukemia. *N Engl J Med* 335:167-170, 1996
21. Bensinger WI, Buckner CD. Preparative Regimens. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 123-134.
22. Confer DL, Stroncek DE. Bone Marrow and Peripheral Blood Stem Cell Donors. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. Second ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 421-430.
23. Korbling M. Peripheral blood stem cells for allogeneic transplantation. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA.: Blackwell Science, Inc.; 1999. p. 469-480.
24. Mohle R, Murea S, Kirsch M, Haas R. Differential expression of L-selectin, VLA-4, and LFA-1 on $CD34^+$ progenitor cells from bone marrow and peripheral blood during G-CSF enhanced recovery. *Exp Hematol* 23:1535, 1995
25. Vermeulen M, Le Pesteur F, Gagnerault MC, Mary JY. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. *Blood* 92(3):894-900, 1998
26. Champlin RE, Schmitz N, Horowitz MM, Chapuis B, et al. Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). *Blood* 12(95):3207-3209, 2000

27. Kernan NA. T-cell depletion for the prevention of graft-versus-host disease. In: Thomas ED, Bloom KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 186-196.
28. Srour EF. Proliferative history and hematopoietic function of *ex vivo* expanded human CD34(+) cells. *Blood* 96(4):1609-1612, 2000
29. Paquette RL, Dergham ST, Karpf E, Wang HJ, Slamon DJ, Souza L, Glaspy JA. *Ex vivo* expanded unselected peripheral blood: progenitor cells reduce posttransplantation neutropenia, thrombocytopenia, and anemia in patients with breast cancer. *Blood* 96(7):2385-2390, 2000
30. Whetton AD, Graham GJ. Homing and mobilization in the stem cell niche. *Trends Cell Biol* 9(6):233-238, 1999
31. Quesenberry PJ, Becker PS. Stem cell homing: rolling, crawling, and nesting. *Proc Natl Acad Sci U S A* 95(26):15155-15157, 1998
32. Bishop MR, Tarantolo SR, Geller RB, Lynch JC, Bierman PJ, Pavletic S, et al. A randomized, double-blind trial of filgrastim (granulocyte colony-stimulating factor) versus placebo following allogeneic blood stem cell transplantation. *Blood* 96(1):80, 2000
33. Bryant E, Martin PJ. Documentation of engraftment and characterization of chimerism following hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 197-206.
34. van Burik JA, Weisdorf DJ. Infections in recipients of blood and marrow transplantation. *Hematol Oncol Clin North Am* 13(5):1065-89, viii.,1999
35. Braverman AC, Antin JH, Plappert MT, Cook EF, Lee RT. Cyclophosphamide cardiotoxicity in bone marrow transplantation: a prospective evaluation of new dosing regimens. *J Clin Oncol* 7(9):1215-1223, 1991
36. Richardson P, Guinan E. The pathology, diagnosis, and treatment of hepatic veno-occlusive disease: current status and novel approaches. *Br J Haematol* 3107:485-493, 1999
37. Crawford SW. Critical care and respiratory failure. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 712-722.
38. Anasetti C, Amos D, Beatty PG, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med* 320:197-204, 1989
39. Flowers ME, Kansu E, Sullivan KM. Pathophysiology and treatment of graft-versus-host disease. *Hematol Oncol Clin North Am* 5(13):1091-1112, 1999
40. Ferrara JL, Levy R, Chao NJ. Pathophysiologic mechanisms of acute graft-vs.-host disease. *Biol Blood Marrow Transplant* 6(5):347-356, 1999
41. Parkman R. Chronic graft-versus-host disease. *Curr Opin Hemato* 5(1):22-25, 1998
42. Ferrara JL, Deeg HJ. Graft-versus-host disease. *N Engl J Med* 324(10):667-674, 1991

43. Zaia JA, Forman SJ. Cytomegalovirus infection in the bone marrow transplant recipient. *Infect Dis Clin North Am* 9(4):879-900, 1995
44. Ochs L, Shu XO, Miller J, Enright H, Wagner JW, et al. Late infections after allogeneic bone marrow transplantations: comparison of incidence in related and unrelated donor transplant recipients. *Blood* 86(10):3979-86, 1995
45. Mauch P, Constine L, Greenberger J, Knospe W. Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. *Int J Radiat Oncol Biol Phys* 5(31):319-339, 1995
46. Deeg HJ, Socie G. Malignancies after hematopoietic stem cell transplantation: many questions,some answers. *Blood* 91(6):1833-1844, 1998
47. Young NS, Barrett AJ. The treatment of severe acquired aplastic anemia. *Blood* 85:3367-3377, 1995
48. Clift RA, Anasette C. Allografting for chronic myeloid leukemia. *Baileres Clin Haematol* 10:319-336, 1997
49. Gale RP, Hehlmann R, Zhang MJ, et al. Survival with bone marrow transplantation versus hydroxyurea or interferon for chronic myelogenous leukemia. The German CML Study Group. *Blood* 91:1810-9, 1998
50. Lee SJ, Kuntz KM, Horowitz MM, et al. Unrelated donor bone marrow transplantation for chronic myelogenous leukemia: a decision analysis. *Annal Intern Med* 127:1080-8, 1997
51. Clift RA, Buckner CD. Marrow transplantation for acute myeloid leukemia. *Cancer Invest* 16:53-61, 1998
52. Barrett AJ. Bone marrow transplantation for acute lymphoblastic leukemia. *Baillieres Clin Haematol* 7:377-401, 1994
53. Anderson JE, Appelbaum FR, Schoch G, et al. Allogeneic marrow transplantation for refractory anemia: a comparison of two preparative regimens and analysis of prognostic factors. *Blood* 87:51058, 1996
54. Bensinger WI, Buckner D, Gahrton G. Allogeneic stem cell transplantation for multiple myeloma. *Hematol Oncol Clin North Am* 11(1), 1997
55. Anderson JE, Litzow MR, et al. Allogeneic, syngeneic, and autologous marrow transplantation for Hodgkin's disease: the 21-year Seattle experience. *J Clin Oncol* 11:2342-2350, 1993
56. van Besien KW, Khouri IF, Giralt SA, et al. Allogeneic bone marrow transplantation for refractory and recurrent low-grade lymphoma: the case for aggressive management. *J Clin Oncol* 13:1096-1102, 1995
57. Khouri IF, Przepiorka D, van Besien K, et al. Allogeneic blood or marrow transplantation for chronic lymphocytic leukaemia: timing of transplantation and potential effect of fludarabine on acute graft-versus-blood-host disease. *Br J Haematol* 97:466-473, 1997
58. Ratanatharathorn V, Uberti J, Karanes C, et al. Prospective comparative trial of autologous versus allogeneic bone marrow transplantation in patients with non-Hodgkin's lymphoma. *Blood* 84:1050-1055, 1994

59. Walters MC, Storb R, Patience M, Leisenring W, et al. Impact of bone marrow transplantation for symptomatic sickle cell disease: an interim report. *Blood* 6:1918-1824, 2000
60. Giardini C, Galimberti M, Lucarelli G. Bone marrow transplantation in thalassemia. *Annu Rev Med* 46:319-330, 1995
61. O'Reilly RJ, Friedrich W, Small TN. Hematopoietic cell transplantation for immunodeficiency disease. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 1154-1172.
62. Clift R, Buckner C, Appelbaum F, et al. Allogeneic marrow transplantation in patients with chronic myeloid leukemia in the chronic phase: A randomized trial of two irradiation regimens. *Blood* 177(8):1660-1665, 1991
63. Chao NJ, Schmidt GM, Niland JC, Amylon MD, Dagis AC, Long GD, et al. Cyclosporine, methotrexate, and prednisone compared with cyclosporine and prednisone for prophylaxis of acute graft-versus-host disease. *N Engl J Med* 329(17):1225-1230, 1993
64. Weiden PL, Sullivan KM, Flournoy N, et al. The Seattle marrow transplant team: Antileukemic effect of chronic graft-versus-host disease. Contribution to improved survival after allogenic marrow transplantation. *N Engl J Med* 304:1529-1533, 1981
65. Gale RP, Champlin RE. How does bone marrow transplantion cure leukemia? *Lancet* 2:28-30, 1984
66. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringden O, Rozman C, Speck B, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 75(3):555-562, 1990
67. Barnes D, Louti J, Neal F. Treatment of murine leukemia with x-rays and homologous bone marrow. *Br Med J* 2:626-630, 1956
68. Truitt R, Johnson B. Principles of graft-verses-leukemia reactivity. *Biol Blood Marrow Transplant* 1(2):61-68, 1995
69. Mitsuyasu RT, Champlin RE, Gale RP. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of graft-verses-host disease: a prospective, randomized, double-blind trial. *Ann Intern Med* 105:20-26, 1986
70. Higano CS, Brixey M, Bryant EM, Durnam DM, Doney K, Sullivan KM, Singer JW. Durable complete remission of acute nonlymphocytic leukemia associated with discontinuation of immunosuppression following relapse after allogeneic bone marrow transplantation. A case report of a probable graft-versus-leukemia effect. *Transplantation* 50(1):175-177, 1990
71. Collins RH, Rogers ZR, Bennett M, et al. Hematologic relapse of chronic myelogenous leukemia following allogeneic bone marrow transplantation: Apparent graft-versus-leukemia effect following abrupt discontinuation of immunosuppression. *Bone Marrow Transplant* 10:391-395, 1990
72. Kolb HJ, Mittermuller J, Cemm C, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* 76:2462-2465, 1990

73. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 86:2041-2050, 1995
74. Slavin S, Naparstek E, Nagler A. Allogeneic cell therapy with donor peripheral blood cells and recombinant human interleukin-2 to treat leukemia relapse after allogeneic bone marrow transplantation. *Blood* 87:2195-2204, 1996
75. Collins RH, Shpilberg O, Drobyski WR, Porter DL, Giralt S, Champlin R, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J of Clin Oncol* 15(2):433-443, 1997
76. Porter DL, Antin JH. The graft-versus-leukemia effects of allogeneic cell therapy. *Annu Rev Med* 50:369-386, 1999
77. Dazzi F, Szydlo RM, Cross NC, Craddock C, et al. Durability of responses following donor lymphocyte infusions for patients who relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. *Blood* 96(8):2712-2716, 2000
78. Collins RHJ, Goldstein S, Giralt S, Levine J, et al. Donor leukocyte infusions in acute lymphocytic leukemia. *Bone Marrow Transplant* 26(5):511-516, 2000
79. Salama M, Neville T, Marcellus D, Collins R. Donor leukocyte infusions for multiple myeloma. *Bone Marrow Transplant*, in press
80. Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of GVL responses from GVHD. *Blood* 86:1261-1268, 1995
81. Giralt S, Hester J, Huh Y, et al. CD8-depleted lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. *Blood* 86:4337-4343, 1995
82. Slavin S. Immunotherapy of cancer with alloreactive lymphocytes. *N Engl J Med.* 343(11):802-803, 2000
83. Slavin S, Nagler A, Naparstek E, Kapelushnik Y. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 91(3):756-63, 1998
84. Childs R, Chernoff A, Contentin N, Bahceci E, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* 343(11):750-758, 2000
85. Khouri IF, Keating M, Korbling M. Transplant-lite: induction of graft-versus-malignancy using Fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol* 16(8):2817-2824, 1998

86. Champlin R, Khouri I, Komblau S, et al. Reinventing bone marrow transplantation. Nonmyeloablative preparative regimens and induction of graft-vs-malignancy effect. *Oncology (Hunting)* 13(5):621-628, 1999
87. Carella AM, Champlin R, Slavin S. Mini-allografts: ongoing trials in humans. *Bone Marrow Transplant* 25(4):345-350, 2000
88. Sandmaier BM, McSweeney P, Yu C, Storb R. Nonmyeloablative transplants: preclinical and clinical results. *Semin Oncol* 27(Suppl 2):78-81, 2000
89. Khouri I, Saliba R, Giralt S, Hagenmeister F, Korbling M, Younces A, et al. Allogeneic hematopoietic transplantation for indolent lymphomas: improved outcome with non-myeloablative versus high dose chemotherapy (HDCT) regimens. *Blood* 96(11):199a, 2000
90. Martin PJ. Overview of Marrow Transplantation Immunology. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 19-27.
91. Delves PJ, Roitt IM. The Immune System: First of two parts. *N Engl J Med* 343(1):37-49, 2000
92. Delves PJ, Roitt IM. The Immune System: Second of two parts. *N Engl J Med* 343(2):108-117, 2000
93. von Andrian UH, Mackay CR. T-cell function and migration: two sdes of the same coin. *N Engl J Med* 343(14):1020-1034, 2000
94. Sykes M, Strober S. Mechanisms of Tolerance. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. Malden, MA: Blackwell Science, Inc.; 1999. p. 264-286.
95. Goulmy E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. *Immunol Rev* 157:125-140, 1997
96. Mackall CL, Gress RE. Pathways of T-cell regeneration in mice and humans: implications for bone marrow transplantation and immunotherapy. *Immunol Rev* 157(61-72), 1997
97. George T, Yu YY, Liu J. Allorecognition by murine natural killer cells: lysis of T-lymphoblasts and rejection of bone-marrow grafts. *Immunol Rev* 155:29-40, 1997
98. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 272:60-66, 1996
99. Picker LJ, Butcher EC. Physiological and molecular mechanisms of lymphocyte homing. *Annu Rev Immunol* 10:561-591, 1993
100. Simpson E, Roopenian D, Goulmy E. Much ado about minor histocompatibility antigens. *Immunology Today* 19(3):108-112, 1998
101. Scott DM, Ehrmann IE, Elis PS, Bishop CE, Agulnik AI, Simpson E, et al. Identification of a mouse male-specific transplantation antigen, H-Y. *Nature* 376:695-698, 1995
102. den Haan JM, Sherman NE, Blokland E, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* 268:1476-1480, 1995

103. den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, Reinhardus C, Shabanowitz J, Offringa R, Hunt DF, Engelhard VH, Goulmy E. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science* 279(5353):1054-1057, 1998
104. Warrens AN, Lombardi G, Lechler RI. Presentation and recognition of major and mior histocompatibility antigens. *Transpl Immunol* 2:103-107, 1994
105. Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* 335(5):281-285, 1996
106. van Ewijk W. T-cell Differentiation is influenced by thymic microenvironments. *Annu Rev Immunol* 9:591-615, 1991
107. Anderson G, Moore NC, Owen JJT, Jenkinson EJ. Cellular interactions in thymocyte development. *Annu Rev Immunol* 14:73-99, 1996
108. Strasser A. Life and death during lymphocyte development and function. *Curr Opin Immunol* 7:228-234, 1995
109. Dudley EC, Petrie HT, Shah LM. T-cell receptor β chain gene rerrangement and selection during thymocyte development in adult mice. *Immunity* 1:83-93, 1994
110. Ignatowicz L, Kappler J, Marrack P. The repertoire of T cells shaped by a single MHC/peptifde ligand. *Cell* 84:521-529, 1996
111. Fowlkes BJ, Schweighoffer E. Positive selection of T cells. *Curr Opin Immunol* 7:188-195, 1995
112. Sprent J, R. WS. Intrathymic and extrathymic clonal deletion of T cells. *Curr Opin Immunol* 7:196-205, 1995
113. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect* 62:21-78, 1866-67
114. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 392:345-352, 1998
115. Shlomchik WD, Couzens MS, Tang CB. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 285:412-415, 1999
116. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991-1045, 1994
117. Guinan EC, Gribben JG, Boussiotis VA, et al. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood* 84(10):3261-3282, 1994
118. Lenschow DJ, Bluestone JA. T cell co-stimulation and in vivo tolerance. *Curr Opin Immunol* 5:747-752, 1993
119. Healy JL, Goodnow CC. Positive versus negative signaling by lymphocyte antigen receptors. *Annu Rev Immunol* 16:645-670, 1998
120. Coffman RF, Mocci S, O'Garra A. The stability and reversibility of Th1 and Th2 populations. *Curr Top Microbiol Immunol* 238:1-12, 1999
121. Jain J, Loh C, Rao A. Transcriptional regulation of the IL-2 gene. *Curr Opin Immunol.* 7:333-342, 1995

122. Martin PJ. Winning the battle of graft versus host. *Nature Med* 6(1):18-19, 2000
123. Armitage RJ. Tumor necrosis factor receptor superfamily members and their ligands. *Curr Opin Immunol* 6:407-413, 1994
124. Griffiths GM. The cell biology of CTL killing. *Curr Opin Immunol* 7:343-348, 1995
125. Murphy WJ, Blazar BR. New strategies for preventing graft-versus-host disease. *Curr Opin Immunol* 11(5):509-515, 1999
126. Blazar BR, Korngold R, Vallera DA. Recent advances in graft-versus-host disease (GVHD) prevention. *Immunol Rev* 157:79-109, 1997
127. Michalek J, Vitetta ES, Collins RH. The effect of different enhancers on the ability of an anti-CD25 ricin A chain immunotoxin to deplete cells which are activated in an MLR. *Blood* 96(11):312b, 2000
128. Guinan EC, Boussiotis VA, Neuberg D, et al. Transplantation of anergic histoincompatible bone marrow allografts. *N Engl J Med* 340(22):1704-1714, 1999
129. Collins RH, Anastasi J, Terstappen L, et al. Brief report: donor-derived long-term multilineage hematopoises in a liver-transplant recipient. *N Engl J Med* 328(11):762-765, 1993
130. Collins RHJ, Sackler M, Pitcher CJ, et al. Immune reconstitution with donor-derived memory/effector T cells after orthotopic liver transplantation. *Exp Hematol* 25(2):147-159, 1997
131. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: all repertoires are not created equal. *Immunol Today.* 18(5):245-251, 1997
132. Parkman R, Weinberg KI. Immunological reconstitution following bone marrow transplantation. *Immunol Rev* 157:73-78, 1997
133. Douek DC, McFarland RD, H. KP, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396(6712):690-695, 1998
134. Markert ML, Boeck A, Hale LP, et al. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* 341(16):1180-1189, 1999
135. Douek DC, Vescio RA, Betts MR, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet* 355(9218):1875-1881, 2000
136. Weinberg K, Blazar BR, Wagner JE, et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood* , In-press
137. Bolotin E, Smogorzewska M, Smith S, Widmer M, Weinberg K. Enhancement of thymopoiesis after bone marrow transplant by in vivo interleukin-7. *Blood* 88(5):1887-1894, 1996
138. Barrett AJ, Malkovska V. Graft-versus-leukaemia: understanding and using the alloimmunie response to treat hematological malignancies. *Br J Hematol* 93:754-761, 1996

139. Nixon DF, Douek D, Kuebler PJ, et al. Molecular tracking of an Human Immunodeficiency Virus nef specific cytotoxic T-cell clone shows persistence of clone-specific T-cell receptor DNA but not mRNA following early combination antiretroviral therapy. *Immunol Lett* 66(1-3):219-228, 1999
140. Maino VC, Picker LJ. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. *Cytometry* 34(5):207-215, 1998
141. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254(5038):1643-1647, 1991
142. Riddell SR, Greenberg PD. Adoptive immunotherapy with antigen-specific T-cells. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. 2 ed. Malden, MA: Blackwell Science, Inc.; 1999. p. 327-341.
143. Kwak LW, Taub DD, Duffey PL, et al. Transfer of myeloma idiotype-specific immunity from an actively immunised marrow donor. *Lancet* 345(8956):1016-1020, 1995
144. Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest* 105(1):3-7, 2000
145. Orkin SH. Stem cell alchemy. *Nature Medicine* 6(11):1212-1213, 2000
146. Briggs R, Kint TJ. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc Natl Acad Sci, USA* 38:455-463, 1952
147. Wilmut I, Schnieke AE, McWhir J, et al. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810-813, 1997
148. Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 283:534-537, 1999
149. Clark DL, et al. Generalized potential of adult neural stem cells. *Science* 288:1660-1663, 2000
150. Lagasse E, et al. Purified hematopoietic stem cells can differentiate to hepatocytes in vivo. *Nature Med* 6:1229-1234, 2000
151. Petersen BE, et al. Bone marrow as a potential source of hepatic oval cells. *Science* 284:1168-1170, 1999
152. Theise ND, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* 31:335-340, 2000
153. Ferrari G, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528-1530, 1998
154. Gussoni E, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401:390-394, 1999
155. Orlic D, Kajstura J, Chimenti S, Li B, Anderson S, Jakoniuk I, et al. Transplanted hematopoietic stem cells repair myocardial infarcts. *Blood* 96(11):221a, 2000