GAINING ACCESS TO THE HEMATOPOIETIC STEM CELL NICHE: NOVEL NON-MYELOABLATIVE CONDITIONING APPROACHES

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ABSTRACT

Sickle Cell Anemia, Beta Thalassemia, Severe Combined Immunodeficiency (SCID), and Type I Diabetes share one commonality: these diverse disorders can all be attributed to faulty hematolymphoid effector cells which are largely caused by genetic mutations that alter hematopoietic cell-intrinsic function. These defective blood cells inherit their genetic deficiencies from hematopoietic stem cells (HSC) as they differentiate. Thus, each of these unique diseases should be theoretically curable through the same strategy: replacement of patients’ HSCs carrying the problematic mutation with normal HSCs from disease-free donors, thereby generating entire new, healthy hematolymphoid systems.

Replacement of disease-causing stem cells with healthy ones has been achieved clinically via hematopoietic cell transplantation (HCT) for the last 50 years, as a treatment modality for a variety of cancers and immunodeficiencies with moderate, but increasing success. However this technique bears high morbidity and mortality severely limiting its utilization. Purified hematopoietic stem cell transplantation is associated with high toxicity primarily attributed to the myeloablative pre-conditioning necessary to facilitate engraftment of the donor HSCs. However the function and mechanistic role of these reagents has been up until now poorly understood.

This thesis deconstructs the current barriers in HCT and pure HSC transplantation, and suggests that in addition to the immune barrier, hematopoietic stem cell niche availability is another critical obstacle that needs to be overcome to
allow for efficient hematolymphoid replacement. In this thesis I show that in the homeostatic state, most hematopoietic stem cell niches are occupied by host HSCs limiting the ability of incoming HSC engraftment. However, a limited number of HSC niches are vacated by HSC that are egressing from the peripheral blood into the bone marrow in a division independent manner, allowing for true but minimal HSC engraftment without myeloablative pre-conditioning.

This thesis illustrates new, up-and-coming strategies to combat the obstacle of HSC niche availability. Here I prove that one non-myeloablative conditioning approach that capitalizes upon physiological HSC egress is repetitive transplantation of purified HSCs. However, this approach is rather laborious and inefficient, and I show that another more efficient novel non-myeloablative conditioning approach to vacate the HSC niche is antibody-mediated depletion of host HSCs. Furthermore, in this thesis I demonstrate that antibody-mediated inhibition of c-kit effectively depletes host HSCs and allows for efficient, non-toxic HSC transplantation. These novel non-myeloablative conditioning methods illustrate new means of overcoming the current barriers to hematolymphoid replacement thereby allowing for the potential expansion of this technique to treat a variety of inherited diseases of hematopoietic and immune function.
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CHAPTER 1

Introduction to

Purified Hematopoietic Stem Cell Transplantation:
The Next Generation of Blood and Immune Replacement

Agnieszka Czechowicz and Irving L. Weissman

Portions of this chapter were published in the following article:

Advantages of Purified Allogeneic Hematopoietic Stem Cell Transplantation

Hematopoietic stem cells (HSCs) are the only cells within the body that at a clonal level have the ability to both self-renew for life, as well as give rise to all the different distinct mature effectors cells that comprise the blood and immune system (Spangrude et al., 1988). These two properties give HSCs the sole responsibility for the proper lifelong maintenance of hematopoietic homeostasis. However genetic abnormalities within HSCs can result in diseases such as immunodeficiency, autoimmunity, hemoglobinopathies, or hematologic malignancies, as these defects are passed down from the HSC to their mature cell progeny, which then generate the diseased blood or immune system.

The first successful hematopoietic cell transplant involving reconstitution of an infant immunological deficiency was accomplished by Good and colleagues in 1968 (Gatti et al., 1968). Since then, hematopoietic cell transplantation (HCT) has been employed as an effective strategy to treat a multitude of hematolymphoid diseases. This procedure, more commonly known as allogeneic bone marrow transplantation, replaces mutant HSCs with functional ones from donor bone marrow grafts which thereafter give rise to a complete normal hematolymphoid system that if stably engrafted persists for life (McCulloch and Till, 1960). Although allogeneic HCT can be an effective cure for most hematopoietic-intrinsic blood or immune diseases, it is rarely performed clinically except for life-threatening diseases and in near-death scenarios due to the toxicity of the procedure. Under current practices, allogeneic HCT has a transplant mortality rate of approximately ~10-20%, far too high to justify its routine use in most non-malignant settings (Michlitsch and Walters, 2008).
One of the most frequent and dangerous complications associated with allogeneic hematopoietic cell transplantation is Graft vs. Host Disease (GvHD) (Barnes et al., 1962). GvHD is a complex, immunologically mediated, host-directed, inflammatory response which is attributed to transplanted donor cells genetically disparate to their host. During GvHD, grafted mature T-cells having undergone tolerization on donor rather than host thymic epithelium, upon infusion into the host result in a violent immunologic response and particularly react against host lymphoid organs, skin, liver and gut (Keever et al., 1988) (Ferrara and Deeg, 1991). While the likelihood and severity of GvHD can be minimized by transplantation from donors that are a close histocompatible match (Schierman and Nordskog, 1963), the risks and effects of GvHD remain unacceptably high and dramatically limit hematopoietic cell transplantation.

Historically, based upon presentation of symptoms, GvHD has been classified into two distinct classes: acute and chronic. Acute GvHD is rapid, occurring within 100 days of HCT and presenting as a syndrome of dermatitis, enteritis, and/or hepatitis (Ferrara and Deeg, 1991). Chronic GvHD occurs at later time points and differs drastically from acute GvHD, often consisting of an autoimmune-like syndrome combining impairment of multiple organs or organ systems (Ferrara and Deeg, 1991). To these two commonly studied subsets of GvHD, is added a third important sub-type, which is sub-clinical but immunosuppressive GVHD (see below) (Tsao et al., 2009). Although T-cells have been shown to play a dominant role in these severe
complications of HCT, the exact molecular and cellular mechanisms underlying each sub-type remain largely unknown (Sprent and Miller, 1976).

Despite a lack of complete understanding of the pathogenesis of GvHD, one potential solution to prevent its occurrence is to transplant purified HSCs. Often the terms Hematopoietic Stem Cell Transplantation (HSCT) and Hematopoietic Cell Transplantation (HCT) / Bone Marrow Transplantation (BMT) are used interchangeably in the literature, but in reality, the clinical methodology differ dramatically. Although the efficacy of BMT relies on the activity of HSC, bone marrow is composed of a heterogeneous mixture of cells, including stem, multi-potent progenitors and mature blood cells, all of which are transferred to the patient in BMT. In contrast, HSCT refers to transfer of a highly purified population of strictly HSC obtained from the donor bone marrow. The inclusion of cell populations other than HSC and their resulting effects are what differentiate HCT/BMT from HSCT.

HSCs are defined as cells which can give rise to long-term multi-lineage reconstitution, as demonstrated when they are transferred into a hematolymphoid depleted, irradiated host. Separation based upon expression of discrete phenotypic cell surface markers and verification of their functionality in this manner, led to identification and isolation of human (Baum et al., 1992) and murine HSC (Spangrude et al., 1988). HSC are exceedingly rare cells, making up <0.1% of a bone marrow graft. Based upon the efforts of multiple scientific groups, the HSC population has been prospectively isolated and refined to purify. All long-term HSC activity in adult mouse bone marrow is believed to be contained within a population marked by the composite phenotype of c-Kit+, Thy-1.1lo, lineage marker−lo, Sca-1+, Slamfl+, Flk2−,
and CD34⁻ (Spangrude et al., 1988) (Kiel et al., 2005b) (Whitlock et al., 1987) (Christensen and Weissman, 2001) (Matsuoka et al., 2001) (Adolfsson et al., 2001). Similarly, the phenotypic profile of human HSC was validated to consist of CD34⁺ and Thy-1⁺, in addition to lacking CD38⁻, CD45RA⁻, and mature lineage markers (Baum et al., 1992) (Osawa et al., 1996) (Majeti et al., 2007). Cells with these specific phenotypes are capable of giving rise to lifelong hematopoiesis upon transplantation at the single mouse-cell level into congenic myeloablated mice (Osawa et al., 1996) (Matsuzaki et al., 2004; Wagers et al., 2002b) (Camargo et al., 2006), and at the ten human-cell level in xenogenic models with myeloablated immunodeficient mice (Majeti et al., 2007). Validation of in vivo human HSC activity with cells of this phenotype was confirmed in several Phase I clinical trials, which showed autologous HSC rescued blood formation in myeloablated recipients and provided sustained, prolonged hematopoiesis (Negrin et al., 2000) (Vose et al., 2001) (Michallet et al., 2000).

Isolation of HSC based upon the cell surface markers indicated above can be accomplished by combining magnetic bead selection and fluorescence activated cells sorting (FACS) methods, yielding purified HSC that are depleted of other polluting hematopoietic populations such as T-cells (Spangrude et al., 1988). Prospective isolation of HSC in this manner is the only effective way to completely purge grafts of contaminating, unwanted populations from clinically transplantable HSC populations. In the case of autologous transplantation to treat malignancy, human HSCs purified in this manner provide long-term hematolymphoid repopulating activity and are free of contaminating resident or metastasized cancer cells (Negrin et al., 2000). However in
allogeneic transplantation for malignancies, HSC purification eliminates T-cells that may function against the cancer and be responsible for the beneficial Graft vs. Tumor (GvT) effect (Ito and Shizuru, 1999).

In allogeneic hematopoietic cell transplantation for non-malignant diseases, purification of HSC can be profoundly beneficial and lead to significantly diminished procedure-related toxicity. Purified HSCT decreases the adverse outcomes of HCT/BMT; since removal of T-cells from allografts completely eliminates GvHD (Shizuru et al., 1996). Purification of HSC from a graft eliminates the possibility of co-transplantation of host-reactive mature donor T-cells, which are often contained within a graft and primarily responsible for both acute and chronic GvHD (Sprent and Miller, 1976). In addition to the gross lesions associated with transplantation of T-cells, low dose of T-cells within a graft also contribute to under-appreciated sub-clinical GvHD. In HCT, delays in immune reconstitution can be observed even in the setting where GvHD is not readily recognized, attributable to sub-clinical GvHD. Even post transplantation of grafts containing minimal contaminating T-cells, donor T-cells attack host lymphoid tissue and destroy tissue architecture leaving the recipient vulnerable to opportunistic infections. Transplantation of purified HSCs eliminates sub-clinical GvHD and results in significantly accelerated immune reconstitution (Tsao et al., 2009), further increasing transplantation safety. As such, the complications and toxicities of BMT and HSCT are quite distinct, and further advocate for the transplantation of purified hematopoietic stem cells especially in non-malignant settings.
Application of HSCT: Curing A Variety of Non-Malignant Hematolymphoid Diseases

Toxicity associated with HCT has dramatically restricted its current practice to life-threatening disorders such as hematologic malignancies and bone marrow failure states, where few other therapeutic options exist. However, HCT has other important potential applications beyond its current uses if HCT-associated toxicity could be eliminated. HCT has been shown to effectively reverse non-malignant genetic hematologic disorders such as sickle cell anemia and beta thalassemia as well as primary immune deficiencies (Barth et al., 2000), if sufficient hematopoietic chimerism is achieved. Additionally, early experimentation in rodents revealed that marrow transplantation could not only protect from irradiation death and prevent hematopoietic failure, but in the process induced immune tolerance and result in the creation of hematopoietic chimeras that would accept skin grafts from the donor or host strain (Main and Prehn, 1955). These and subsequent studies opened the opportunity to expand this technique as a therapeutic modality for a variety of immunological diseases and provided a potential alternative to lifelong administration of immunosuppressive drugs following organ transplantation, aims of transplant biologists and clinicians for now over half a century (Starzl et al., 1992) (Scandling et al., 2008) (Millan et al., 2002) (Alexander et al., 2008).

This phenomenon of permanent transplant tolerance is attributable to the elimination of donor-reactive T-cells, primarily through negative selection in the thymus of developing T-cells with donor-reactive antigen receptors. Transplantation of donor HSC results in new immune cell generation on a chimeric microenvironment, leading to deletion of reactive immune effector cells against both host (via the thymic
medullary epithelium) and donor (via donor derived thymic dendritic cells) (Keever et al., 1988) (Shizuru et al., 2000). Recent studies illustrate that allotransplantation of purified HSC either prior or concurrent with transplantation of matched donor heart tissue precludes injury and subsequent rejection of donor organs (Gandy and Weissman, 1998). Due to co-transplantation of either tissue organs and/or tissue stem cells with HSC, long-term immune tolerance to donor tissues by the host can be achieved and the need for hazardous life-long immuno-suppression eliminated, as best illustrated in recent trials of kidney/BM transplant patients (Kawai et al., 2008) (Scandling et al., 2008). The use of HSCT in this manner may significantly abrogate complications of solid organ transplantation, extending organ longevity and decreasing infection susceptibility. Future co-transplantation of HSC and solid organ tissue generated in vitro from the same embryonic or induced pluripotent stem cell may be possible, expanding the pool of transplant candidates.

The concept of induced immune tolerance by HSCT can additionally be extended to the treatment of autoimmune diseases. HCT and HSC transplantation have been demonstrated to have utility in blocking disease pathogenesis of a wide variety of autoimmune disorders such as diabetes mellitus type 1 (DM1) (Nikolic et al., 2004), multiple sclerosis (MS) (van Gelder et al., 1993), and systemic lupus erythematosus (SLE) (Traynor et al., 2000) (Smith-Berdan et al., 2007). These autoimmune diseases are complex, multi-factorial diseases often containing an environmental component, however they also bear a genetic element and involve HSC predisposed to generating self-reactive T-cell and/or B-cell clones that can react against and attack host tissues (Todd et al., 1987). Transplantation of the disease can
be achieved by transplantation of HSC from donors predisposed to or bearing the disorder into otherwise healthy recipients (Beilhack et al., 2003). Conversely allogeneic transplantation of normal donor HSC into diseased recipients generates tolerance and prevents attack of otherwise reactive tissues.

Cure of these diseases can be achieved by elimination of the host’s reactive T-cells, and subsequent generation of a new non-self-reactive T-cell compartment from the disease resistant donor. Current transplantation procedures eliminate host immune cells and thus at least initially suppress the autoimmune disease regardless of whether autologous or allogeneic HCT is performed. However, in these autoimmune disorders the HSC are defective and predisposed to generating self-reactive immune cells, thus autologous transplantation as illustrated in mouse models of type 1 diabetes, allergic encephalomyelitis, and systemic lupus erythematosus is not curative. As such, syngeneic transplantation of purified HSC in a mouse model of spontaneous autoimmune diabetes mellitus provides no long-term survival benefit (Beilhack et al., 2003). Conversely, transplanted allogeneic HSC are posed to generating non-self-reacting immune cells, and indeed in the same model completely prevent diabetes development throughout life (Beilhack et al., 2003). Clinical data regarding autologous transplantation for autoimmune diseases is variable. In such settings, a naïve immune system is transplanted and depending on environmental factors may not always result in rapid re-creation of the diseased state. Some patients show excellent and long-lived clinical remission of disease, whereas others enjoy initial symptomatic benefit with subsequent relapse (Krauss and Kamani, 2009).
Autologous transplantation reintroduces the hosts defective HSC, and therefore may not result in long-term cure. As the molecular basis for various monogenic hematolymphoid diseases is determined, gene therapy may become a realistic strategy to correct autologous HSC prior to transplantation. Upon transplantation, these few modified HSC could reconstitute a complete, corrected hematolymphoid system that persists for life. This strategy would be instrumental in the treatment of immune diseases, in addition to genetic and acquired nonmalignant blood diseases, such as sickle cell anemia where currently allogeneic HCT is occasionally performed. Additionally, gene therapy of HSC may play a pivotal role in generating HSC that produce immune cells that are predisposed to attacking tumors. However, to date gene therapy is individual-specific, and is limited by the current inability to achieve reliable and rapid gene transduction with vectors that do not by insertional mutagenesis induce diseases such LMO2-activated acute lymphocytic leukemia (McCormack and Rabbitts, 2004).

Furthermore transplantation of HSC generating mature cells resistant to infectious agents may prove an effective strategy to combat a magnitude of viral agents. Case reports of HIV-infected patients, transplanted with HSC from donors resistant to the disease, resulted in at least preliminary cure of these patients. In these select scenarios, transplanted donor HSC generated donor T-cells bearing CCR5 defects making them impenetrable to HIV (Hutter et al., 2009). Long-term outcome of these studies is unknown and the feasibility of such treatments utilizing currently available transplantation strategies is questionable. However, these studies illustrate a
potential new therapeutic use of HCT if other hurdles such as supply of resistant-matched donor cells are overcome (van Griensven et al., 2005).

HCT has been repeatedly confirmed to be the singular curative therapy for this plethora of blood and immune diseases. To date, however, HCT has not been routinely applied in these manners to treat the hundreds of thousands of patients that suffer from these ailments primarily subsequent to concerns regarding the morbidity and mortality of allografting procedures. With elimination of GvHD by transplantation of purified HSC, that are debulked of reactive T-cells, therapy of this nature maybe become a mainstream reality.

**Barriers to Expansion of Hematopoietic Stem Cell Transplantation**

Continued improvements in the control of regimen-related toxicities are necessary to expand the applications of HCT. Current HCT methods hold exorbitant risk to the patient in terms of the transplant procedure related morbidity and mortality providing a major impediment to extrapolation of these practices to a multitude of conditions.

Although GvHD may be eliminated by transplantation of purified HSC, much toxicity of HCT is also attributable to the conditioning regimens necessary to enable HSC engraftment. Current conditioning methods include irradiation and cytotoxic drugs such as high dose chemotherapy, which can cause infertility, secondary malignancies, endocrine dysfunction, and organ damage (Ferry and Socie, 2003). Whereas in the malignant settings this conditioning serves the dual purpose of tumor eradication as well as preparation of the host, in the non-malignant disease setting
these regimens lead to inexcusable, non-beneficial toxicity. Despite the ability of 
BMT/HSCT to cure many non-malignant diseases, they have seldom been employed 
in the treatment of non-life threatening yet debilitating diseases largely due to these 
associated risks. This necessitates the need for more specific and less toxic methods to 
allow efficient HSC engraftment.

Stable, robust chimerism is necessary in the treatment of these diseases, with 
disorders such as sickle cell anemia requiring ~20% chimerism to ameliorate the side 
effects of the disease (Walters et al., 2001) (Iannone et al., 2001). In the absence of 
myeloablative therapy, this can be difficult to achieve (Storb et al., 1999). 
Additionally, engraftment of purified hematopoietic stem cells in the absence of other 
facilitator populations in the bone marrow poses an even larger engraftment challenge. 
Various facilitator populations, including in mice CD8 T-cells or CD8+ TCR- dendritic 
cells, have been indentified in bone marrow that augment HSC engraftment, however 
many of these cells may also contribute to GvHD and therefore their transplantation 
should be avoided (Gandy et al., 1999). Moreover, the identification and subsequent 
purification of non-T-cell facilitator populations in humans has not been executed, 
further limiting our ability to enhance the engraftment of HSC.

Historical clinical data has showed that T-cell depletion results in increased 
graft failures (Keman et al., 1989) (Marmont et al., 1991). This is a major impediment 
of transplantation of purified HSCs, preventing the current practice and 
consequentially exposing patients to GvHD. Various “non-myeloablative” protocols 
have been developed to permit engraftment of donor cells with attenuated conditioning 
regimens (Maloney et al., 2002), however while these protocols are not completely
myeloablative, they are still non-specific, ablate the bone marrow, and have severe regimen related toxicities instigating the need for better preparative regimens.

However, transplanting HSCs without traditional conditioning has been difficult (Tomita et al., 1994). Traditional myeloablative conditioning is thought to play a role in immune suppression as well as creating space for transplanted donor HSCs (Schofield, 1978). HSCs are thought to reside in specialized microenvironments in the bone marrow that can serve as fixed tissue niches for HSCs, thereby regulating HSC numbers and behavior. Although the precise identities of the niche cells are still largely unknown and controversial, there is a large amount of data indicating that HSC niches exist and are critical to HSC maintenance (Morrison and Spradling, 2008).

HSC require specific and special growth factors and cytokines to preserve their unique state. How they receive these signals has been a growing field of research and controversy. In 1978 Schofield proposed that a HSC site-specific niche must exist to provide these signals and in this way oversee HSC numbers, by regulating an HSC’s decisions to undergo self-renewal, differentiation, or apoptosis (Schofield, 1978). In a setting of finite numbers of such niches, transplantation of HSC in excess of these spaces would be predicted to be futile; initial experimentation carried out by Micklem and colleagues supports this hypothesis (Micklem et al., 1968). Others have since argued that space is not an important factor to donor HSC engraftment, and show in unirradiated recipients, transplantation of whole donor bone marrow readily displaces endogenous host marrow. Rather than by specialized sites, the argument can be made that HSC number is regulated by availability of diffusible factors and thus
conditioning need not be done to ensure HSC engraftment. However, these experiments were carried out with whole bone marrow, and the conclusions about broad HSC behavior and purified HSC engraftment ability must be taken into consideration in this context (Saxe et al., 1984) (Stewart et al., 1993) (Wu and Keating, 1993).

Understanding true HSC behavior is vital to optimizing hematopoietic cell transplantation, and may provide clues into strategies to allowing purified HSC transplantation without necessitating myeloablative conditioning. In the work that follows, we have studied HSC activity in the homeostatic setting and examined purified HSC engraftment in the unconditioned setting. This has enhanced our understanding of the hematopoietic stem cell niche, and illuminated approaches to gain access to the hematopoietic stem cell niche through novel non-myeloablative conditioning strategies.
CHAPTER 2

Niche Recycling Through Division-Independent Egress of Hematopoietic Stem Cells

Agnieszka Czechowicz, Deepta Bhattacharya, A.G. Lisa Ooi, Derrick Rossi, David Bryder and Irving L. Weissman

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ABSTRACT

Hematopoietic stem cells (HSCs) are thought to reside in discrete niches through stable adhesion, yet previous studies have suggested that host HSCs can be replaced by transplanted donor HSCs, even in the absence of cytoreductive conditioning. To explain this apparent paradox, we calculated, through cell surface phenotyping and transplantation of unfractionated blood, that approximately 1-5% of the total pool of HSCs enters into the circulation each day. Bromodeoxyuridine (BrdU) feeding experiments demonstrated that HSCs in the peripheral blood incorporate BrdU at the same rate as do HSCs in the bone marrow, suggesting that egress from the bone marrow to the blood can occur without cell division and can leave behind vacant HSC niches. Consistent with this, repetitive daily transplantations of small numbers of HSCs administered as new niches became available over the course of 7 days led to significantly higher levels of engraftment than did large single bolus transplantations of the same total number of HSCs. These data provide insight as to how HSC replacement can occur despite the residence of endogenous HSCs in niches, and suggest therapeutic interventions that capitalize upon physiological HSC egress.
INTRODUCTION

The concept that hematopoietic stem cell (HSC) numbers and behavior are regulated by physically discrete locations or niches within the bone marrow was first hypothesized in detail 30 years ago (Schofield, 1978). In recent years, a number of groups have begun to reveal the identity of the HSC niche, either through *in situ* identification of populations enriched for HSCs in mouse bone marrow or through genetic approaches (Arai et al., 2004; Calvi et al., 2003; Kiel et al., 2005a; Nilsson et al., 1997; Sugiyama et al., 2006; Visnjic et al., 2004; Zhang et al., 2003). Although the precise identities of the niche cells are still largely unknown and controversial (Haug et al., 2008; Kiel et al., 2007a), a large amount of data indicate that HSCs are retained within the niche through the use of specific adhesion molecules and chemokine gradients (Papayannopoulou and Scadden, 2008). Through these interactions, HSCs can be assured of receiving the appropriate supportive signals that allow them to retain their stem cell identity.

Counterbalanced against these studies, however, are data suggesting that recipient bone marrow can be readily displaced by transplanted marrow in an efficient and linear dose-dependent manner, even in the absence of conditioning (Brecher et al., 1982; Colvin et al., 2004; Rao et al., 1997; Saxe et al., 1984; Stewart et al., 1993; Wu and Keating, 1993). These studies did not directly assess HSC replacement; however the data would appear to be more consistent with a model where HSCs do not reside locked into fixed locations in the marrow, but instead receive their regulatory signals through limiting quantities of freely diffusible factors. Although more recent data have shown that actual host HSC replacement by purified HSCs, rather than simply
total marrow replacement, is less efficient than these earlier studies suggested (Bhattacharya et al., 2006b; Czechowicz et al., 2007; Prockop and Petrie, 2004), there is clearly a certain degree of HSC replacement that does occur in normal mice, even in the absence of cytoreductive conditioning. Thus, there is a need for a model that accounts for both the physically discrete bone marrow locations of HSCs that many studies have suggested, and the replacement of HSCs that occurs when transplants are performed in the absence of conditioning.

Recent studies have shown that pharmacologically-induced egress of HSCs using AMD3100, a CXCR4 inhibitor, can be used to free niches in recipient animals and allows for improved levels of donor HSC engraftment relative to untreated recipients (Chen et al., 2006). Because a number of studies have shown that HSCs and/or progenitors also circulate under physiological conditions (Abkowitz et al., 2003; Goodman and Hodgson, 1962; Massberg et al., 2007; McCredie et al., 1971; McKinney-Freeman and Goodell, 2004; Mendez-Ferrer et al., 2008; Wright et al., 2001), we hypothesized that steady-state egress of HSCs from their niches may also allow for engraftment of donor HSCs. In this model, transplanted HSCs would not directly displace host HSCs that are stably residing within a niche, but would engraft into niches that had been vacated through the physiological egress of host HSCs. In this study, we provide evidence consistent with this model, demonstrating that HSCs can enter into the bloodstream in the absence of cellular division, and that repetitive HSC transplantations can capitalize on this process of HSC niche recycling to generate higher levels of engraftment than large single-bolus transplantation of HSCs. Moreover, in our study we specifically examined in an unconditioned setting the
intrinsic behavior and replacement properties of HSCs rather than that of unfractionated bone marrow, which contains a number of different cell types that have been reported to influence engraftment and replacement, such as host-reactive T cells and stromal cells (Almeida-Porada et al., 1999; Lazarus et al., 2005; Slavin et al., 1998). To our knowledge, ours is the first such study to examine the physiological kinetics of HSC niche emptying and engraftment behavior in the absence of these variables.
RESULTS

**Numerical and functional quantification of HSCs in blood**

Two theoretical mechanisms exist that could describe the source of HSCs in the blood. The first involves an asymmetric division in which following mitosis, one daughter HSC remains positioned within the supportive niche, while the other daughter cell is displaced away (Fig. 2-1A). The daughter cell that is displaced can then intravasate into the bloodstream. The second mechanism involves division-independent egress in which an HSC exits its supportive niche in the absence of mitosis, thus leaving behind a vacant HSC niche (Fig. 2-1B).

To determine which of these two models most accurately describes the source of HSCs in the peripheral blood, we first determined whether HSCs in the bloodstream are phenotypically and functionally similar to HSCs in the bone marrow. C-Kit$^+$ lineage$^-$ Sca-1$^+$ (KLS) CD27$^+$ IL-7Ra$^-$ CD150$^+$ CD34$^-$ cells could be identified by flow cytometry in the blood that appeared to be virtually identical to phenotypic HSCs in the bone marrow (Fig. 2-2A). In agreement with some previous reports (Allman et al., 2003) but in contrast to others (Wiesmann et al., 2000), we were unable to identify KLS CD34$^-$ cells in the bone marrow that did not express CD27, although it is worth noting that CD34$^+$ HSCs express slightly lower relative levels of CD27 than do their CD34$^+$ MPP counterparts (Fig. 2-2B). We believe these discrepancies are related to improved antibody conjugates that yield brighter fluorescent signals. This marker thus aided in the specific identification of rare HSCs in the blood and distinguishing these HSCs from background CD27$^-$ events (Fig. 2-2B). Transplantation of CD27$^-$ bone
marrow did not lead to multilineage reconstitution beyond 8 weeks, confirming that all of the HSC activity is contained within the CD27+ fraction (Fig. 2-S1A).

Previous studies have shown that functional HSC activity is contained within the KLS fraction of peripheral blood (Schwarz and Bhandoola, 2004). We further confirmed through transplantation assays that only CD27+ KLS cells within the peripheral blood have multilineage reconstitution potential (Fig. 2-S1B). To verify that HSC activity was specifically contained within the KLS CD27+ IL-7Ra- CD150+ CD34- fraction, we transplanted both KLS CD27+ IL-7Ra- CD150+ CD34- and all remaining KLS CD27+ IL-7Ra- (CD34+ and/or CD150-) cells from peripheral blood into lethally irradiated recipients along with 200,000 competitor bone marrow cells from GFP+ wild type donor mice. Transplantation of as few as 8 KLS CD27+ IL-7Ra- CD150+ CD34- cells from the peripheral blood into lethally irradiated mice led to multilineage reconstitution for at least 16 weeks, while transplantation of as many as 200 of the remaining KLS CD27+ IL-7Ra- cells (encompassing both CD34+ and/or CD150- cells) from the peripheral blood failed to give detectable reconstitution at late timepoints in any recipient (Fig. 2-3A), similar to previous reports on bone marrow HSCs (Osawa et al., 1996). These data demonstrate that HSC activity in the peripheral blood is contained within the KLS CD27+ IL-7Ra- CD150+ CD34- population, just as in the bone marrow. To our knowledge, this is the first time that peripheral blood HSCs have been purified to this level, affording us the ability to specifically study their behavior.

When 8 KLS CD27+ IL-7Ra- CD150+ CD34- (PB HSCs) were transplanted alongside 200,000 competitor bone marrow cells into lethally irradiated recipients, the
mean granulocyte chimerism arising from the peripheral blood HSCs was approximately 23% (Fig. 2-3A). This is similar to the expected chimerism value of 28% if these HSCs were functionally identical to the 20 estimated bone marrow HSCs that were co-transplanted within the competitor bone marrow, assuming a bone marrow HSC frequency of 0.01% (Bryder et al., 2006). However, the lymphoid chimerism derived from the peripheral blood HSCs was slightly lower, perhaps due to the persistence and homeostatic proliferation of mature lymphocytes included in the competitor bone marrow (Ernst et al., 1999; Forster and Rajewsky, 1990; Goldrath and Bevan, 1999; Ron and Sprent, 1985). Clonal in vitro assays in which 20 single peripheral blood HSCs and 20 single bone marrow HSCs isolated from 10 mice were analyzed for colony formation demonstrated that the plating efficiency and lineage potential of peripheral blood HSCs are similar to those of bone marrow HSCs (Fig. 2-3B). Taken together, these data demonstrate that peripheral blood HSCs are phenotypically and functionally similar to bone marrow HSCs.

To re-examine the frequency of functional HSCs within the blood of adult mice, we transplanted 200 ml of whole peripheral blood (approximately 10% of the total blood volume of a mouse) from unmanipulated animals into lethally irradiated recipients alongside 200,000 competitor bone marrow cells. These experiments showed that whereas almost all recipients (30/34) showed clear lymphoid lineage chimerism, only 4/34 recipients showed detectable donor granulocyte reconstitution at 16 weeks post-transplant (Fig. 2-S2A), likely indicating the persistence and/or homeostatic proliferation of mature donor lymphocytes, but relatively infrequent HSC activity. Assuming single-cell reconstitution frequency ranges of 20-100% (Camargo
et al., 2006; Ema et al., 2005; Matsuzaki et al., 2004; Osawa et al., 1996; Wagers et al., 2002c), these data suggest that there are not more than 1-5 circulating HSCs in the peripheral blood of 10-12 week-old animals at any given point. Consistent with this, we were only able to sort on average 2 HSCs per adult animal from the entirety of the peripheral blood (Fig. 2-2A). These numbers are lower than our previous estimates for reasons that are not entirely clear (Wright et al., 2001). The difference does not appear to be due to the use of younger animals in our previous studies, as peripheral blood from 4-week old mice reconstituted granulocytes at an identical efficiency (2/14 recipients) as did peripheral blood from 12-week old mice (2/14 recipients) (Fig. 2-S2B). Recent studies have found that diverse stimuli, such as circadian rhythms, can influence the frequency of HSCs in the blood (Mendez-Ferrer et al., 2008). While this particular stimulus is unlikely to account for all of the difference between our current study and the previous work, it is clear that environmental factors, many of which likely remain to be discovered, can influence the numbers of HSCs in the blood. It is entirely possible that some of these environmental factors were different between the current study and our previous work, performed 8-9 years ago. Regardless, these current revised estimates are more consistent with data presented by other studies and with the observation that cross-engraftment of the bone marrow HSC compartment in parabiotic partners does not reach 50%, even after long periods of conjoinment (Abkowitz et al., 2003; McKinney-Freeman and Goodell, 2004; Nygren et al., 2004; Wright et al., 2001). Given that there are 1440 minutes per day and assuming an intravascular residence time of 5 minutes (Wright et al., 2001), we now estimate that 300-1500 HSCs (and likely many more multipotent and oligopotent progenitors),
representing up to 1-5% of the total pool, can briefly enter into the circulation each day in adult mice.

**Division-independent egress of HSCs**

If circulating HSCs egress from the bone marrow in a division-dependent manner (Fig. 2-1A), after an appropriate period of BrdU feeding, this model of HSC egress would predict that all peripheral blood HSCs would have incorporated BrdU while only a fraction of the bone marrow HSCs would have incorporated BrdU. More specifically, HSCs destined for peripheral blood would incorporate BrdU at more rapid rates than would the total pool of bone marrow HSCs. In contrast, the division-independent model (Fig. 2-1B) would predict that after an appropriate labeling period, not all blood HSCs would have incorporated BrdU, and instead the extent of BrdU incorporation would be similar to that of bone marrow HSCs. To test these models, 20 mice were fed BrdU in their drinking water for 3 days and the levels of BrdU incorporation in pooled peripheral blood and bone marrow HSCs were quantified. Only ~9% of both blood and bone marrow HSCs had incorporated BrdU, suggesting that cellular division is not a requirement for HSC egress into the bloodstream (Fig. 2-4, experiment 1). Even after a longer BrdU feeding period of 6 days in which HSCs from 20 mice were pooled and analyzed, only 18% of peripheral blood and bone marrow HSCs had incorporated BrdU (Fig. 2-4, experiment 1). These experiments were repeated with similar results. After 9 and 12 days of BrdU feeding, the frequency of BrdU+ HSCs found in the peripheral blood had still not exceeded that in the bone marrow (Fig. 2-4, experiments 2 and 3). Interestingly, the percentage of
HSCs in the bone marrow that had incorporated BrdU did not change between 9 and 12 days of feeding, consistent with the proposal that distinct proliferative and relatively non-proliferative HSC populations exist (Nygren and Bryder, 2008; Wilson et al., 2008). It is possible that the actual levels of proliferation may be even less than these values indicate, as BrdU itself has been suggested to induce the proliferation of HSCs (Nygren and Bryder, 2008; Wilson et al., 2008). Our levels of BrdU incorporation in bone marrow HSCs are likely lower than that reported by two recent studies due to the lower dose of BrdU administered in this study (Kiel et al., 2007b; Wilson et al., 2008). This lower BrdU dose was still sufficient for distinguishing differences in proliferative rates, as highly proliferative myeloid progenitors had incorporated more BrdU than had HSCs at all timepoints (Fig. 2-4). Thus, given the purity of these peripheral blood HSCs as shown by the clonal assays and in vivo reconstitutions (Fig. 2-3), it is clear from both experiments that not all peripheral blood HSCs incorporated BrdU, strengthening the interpretation that HSC intravasation need not necessarily be accompanied by cellular division.

Recent studies have shown that HSCs can circulate not only through the peripheral blood, but also through the lymph and other organs (Massberg et al., 2007). Thus, it remained possible that the HSCs in the blood that had not divided were cells that had in fact undergone egress from the bone marrow in a division-dependent manner prior to the labeling period, and had been circulating continuously for the duration of the BrdU feeding. To determine the maximum duration that HSCs can circulate and yet still retain their phenotypic identity, 10,000 single-sorted HSCs were transplanted into unconditioned wild type animals and bone marrow HSC chimerism
was measured between 1-7 days post-transplant. Strikingly, no increase was observed in HSC chimerism between 1 and 3 days post-transplantation, indicating that HSC homing to the marrow occurs rapidly after intravenous transplantation (Fig. 2-5, top panel), consistent with previous studies on partially purified HSCs (Nilsson et al., 1997; Plett et al., 2002). At both 1 and 3 days post-transplant, most donor cells retained their phenotypic HSC identity (Fig. 2-5, bottom panel). A 2-fold increase in HSC chimerism did occur between 3 and 5 days post-transplant (Fig. 2-5, top panel), but this was also accompanied by a reduction in the percentage of donor cells that retained their HSC surface phenotype, indicating differentiation (Fig. 2-5B, bottom panel; Fig. 2-S3). These data suggest that transplanted HSCs must begin to divide and differentiate by 5 days. Thus, the observed jump in HSC chimerism between 3 and 5 days may be partially due to local proliferation rather than additional HSC homing from peripheral sites. Regardless, no further increases were observed between 5 and 7 days post-transplant (Fig. 2-5, top panel), and the HSC chimerism values at these timepoints were similar to the values at 16 weeks post-transplant when similar numbers of HSCs were infused in a single bolus (see Fig. 2-6A, top panel below). These data imply that few HSCs identified in the peripheral blood could have been circulating continuously for the duration of the BrdU labeling experiment in Fig. 2-4. The total number of HSCs found per spleen remained between 20-50 cells throughout the course of the experiment with no statistically significant differences observed, and we were unable to detect more than 1 donor HSC in the blood in any recipient at any timepoint (data not shown). Taken together, these data confirm that HSCs can exit their supportive niches without dividing under physiological conditions.
Niche vacancy under homeostatic conditions

Division-independent HSC egress would be predicted to leave behind a small number of available niches for donor HSC transplantation, even in the absence of conditioning (Fig. 2-1B). Moreover, because physiological HSC migration is thought to be a continuous process (Wright et al., 2001), division-independent egress would be predicted to lead to only a finite period of niche saturation following transplantation of an excess of donor HSCs. Previous studies have shown that repetitive injections of unfractionated bone marrow lead to more hematopoietic replacement than single bolus injections of unfractionated marrow (Colvin et al., 2004; Quesenberry et al., 1994), although whether these findings apply to HSC replacement is currently unknown. Indeed, as HSCs comprise less than 0.01% of all bone marrow cells, and because transplanted accessory cells can potentially clear endogenous HSCs and/or create additional niches (Almeida-Porada et al., 1999; El-Badri et al., 1998; Slavin et al., 1998), it is difficult to assess the intrinsic ability of donor HSCs to replace host HSCs in the absence of conditioning through unfractionated marrow transplants. To define the interval of HSC niche saturation, wild type CD45.1 x CD45.2 animals were transplanted with a large single bolus of either 12,700 single-sorted (Fig. 2-6A, top panel) or 11,070 double-sorted HSCs (Fig. 2-6A, bottom panel) or the same total number of HSCs administered over the course of 7 daily injections. The animals receiving the single bolus of HSCs were injected six additional times daily (three times before HSC transplantation and three times afterwards) with saline to control for mobilizing effects of the injections themselves. The group of mice that received daily HSC injections showed approximately 2-2.5-fold increases in bone marrow HSC
chimerism, representing value ranges of 5-10%, over the group that received a single bolus of HSCs, which displayed HSC chimerism values of 2-5%, when sacrificed 16 weeks post-transplant (Fig. 2-6A). These data demonstrate that niche saturation following transplantation is transient.

Unlike in unconditioned immunodeficient recipients (Bhattacharya et al., 2006b; Czechowicz et al., 2007), granulocyte chimerism correlated poorly with HSC chimerism in this wild type setting, as has been observed before in other studies (Wu and Keating, 1993). Peripheral blood granulocyte chimerism at the time of sacrifice underestimated the absolute HSC chimerism in individual mice, and did not show a linear relationship with HSC chimerism when examined in all recipients (Fig. 2-6B). Interestingly, when we transplanted CD45.1 x CD45.2 mice with 6.5 x 10⁷ unfractionated marrow cells from a CD45.1 donor, granulocyte chimerism following transplantation overestimated bone marrow HSC chimerism, but not overall KLS chimerism, demonstrating the unique and specific difficulty of replacing HSCs with transplanted cells (Fig. 2-6C) (Czechowicz et al., 2007). Thus, in this experimental system of unconditioned wild type recipients, it appears that the most reliable method to measure bone marrow HSC chimerism is to quantify donor HSCs directly using stringent proper HSC markers. The biological basis for this difference between HSC chimerism and mature cell output is currently unclear, but has been observed before in other systems (Rossi et al., 2007; Wu and Keating, 1993). It is possible that the absences of certain cellular lineages and/or effects induced by different experimental manipulations can affect the extent to which HSC chimerism correlates with mature cell chimerism. Thus, different experimental conditions require independent
verifications of these correlations. These differences may explain the discordance between our conclusions regarding transplanted HSC dose-dependent decreases in transplantation efficiencies (Fig. 2-7A) and other studies that inferred efficient HSC replacement based upon linear dose-dependent replacement of total marrow (Bhattacharya et al., 2006b; Brecher et al., 1982; Colvin et al., 2004; Czechowicz et al., 2007; Saxe et al., 1984; Stewart et al., 1993; Wu and Keating, 1993).

Alternatively, it is possible that unfractonated marrow contains cells that can empty HSC niches through immunological clearance or through mobilization (Slavin et al., 1998). Finally, it is also possible that HSCs transplanted in the context of unfractonated marrow survive for longer durations than do purified HSCs (Benveniste et al., 2003), thus allowing these cells to engraft into empty niches as they become available over a longer period of time.

The existence of finite numbers of empty niches predicts that the likelihood of engraftment for any given cell within the transplant should decrease as the number of cells transplanted in a single bolus increases. Previous studies using unfractonated marrow transplants have given mixed answers to this prediction, with some studies suggesting an absolute linear dose-dependent replacement of host hematopoietic cells (Brecher et al., 1982), others demonstrating no dose-dependent increases in chimerism in certain mouse strains (Saxe et al., 1984), others finding no detectable engraftment in the absence of conditioning (Gambel et al., 1984), and still others finding dose-dependent curves intermediate to these findings (Rao et al., 1997). To experimentally determine this dose-dependent efficiency of engraftment specifically for HSCs in the absence of immune barriers, we transplanted various numbers of purified CD45.2
HSCs into unconditioned wild type CD45.1 x CD45.2 animals. At 4 weeks post-transplant, animals were sacrificed and bone marrow HSC chimerism was determined directly as above. The efficiency of engraftment of the lowest transplanted dose (250 HSCs) was approximately 2-fold higher than the efficiency of the 15,000 HSC dose (Fig. 2-7A). Importantly, the efficiency of transplantation never approached zero, even at the highest transplanted HSC doses. These data are consistent with the concept that HSC niches are continuously being emptied and refilled, making complete saturation difficult to achieve.

Given that the efficiency of engraftment of low numbers of transplanted HSCs is approximately 2-fold higher than the efficiency of high doses of transplanted HSCs (Fig. 2-7A), we estimated that multiple injections of low doses of HSCs would lead to 2-fold higher levels of HSC engraftment than single bolus transplants (as in Fig. 2-6A) only if the number of empty HSC niches were being reset within the duration between the injections. In contrast, if niche availability were reset after longer periods of time, HSCs transplanted during later injections would encounter fewer available niches than those transplanted at the first injection, thereby reducing the overall efficiency of engraftment. Because the HSC engraftment from daily injections was more than 2-fold greater than that achieved by the single bolus transplant (Fig. 2-6A), we estimate that HSC niche availability must be reset within at least 24 hours. Thus we, for the first time, have specifically assessed the number of available HSC niches in normal wild type animals and the rate of their emptying under steady-state conditions.

To verify that our surface phenotypic characterization accurately identified functional donor HSCs, we transplanted 12-14 single-sorted phenotypic donor HSCs
from unirradiated primary transplanted recipients into lethally irradiated secondary recipients. At 16 weeks post-secondary transplant, multilineage engraftment derived from these re-isolated donor HSCs was observed in 5/7 recipients (Fig. 2-7B). These data confirm that we are accurately quantifying donor HSC chimerism in the primary unirradiated transplanted recipients.

Apoptosis does not play a role in HSC niche clearance

Aside from the division-independent egress of HSCs, apoptosis of HSCs might also lead to the emptying of appropriate niches. To test this hypothesis, approximately 2500 single-sorted Bcl2-transgenic CD45.2 HSCs were transplanted into unconditioned CD45.1 x CD45.2 Bcl2-transgenic mice, which show increased numbers of HSCs through the prevention of apoptosis (Domen and Weissman, 2000), or into wild type CD45.1 x CD45.2 animals. Bone marrow HSC chimerism levels were similar between the 2 groups of recipients at 16 weeks post-transplantation (Fig. 2-8). These data suggest that host HSC apoptosis is not a major mechanism of niche clearance within the timeframe that transplanted HSCs engraft.
DISCUSSION

The ability of transplanted HSCs to home to the bone marrow and reconstitute the recipient's blood supply is the basis for the routine use of bone marrow transplants to correct both inherited and acquired hematopoietic disorders. The success of these treatments is contingent upon the replacement of the malfunctioning endogenous HSCs with normal donor HSCs. The need for ablating host HSCs prior to transplantation to achieve high levels of donor HSC engraftment has been a hotly debated issue over the years, with a number of groups claiming efficient HSC replacement in the absence of prior cytoreductive conditioning of the host (Brecher et al., 1982; Colvin et al., 2004; Rao et al., 1997; Saxe et al., 1984; Stewart et al., 1993; Wu and Keating, 1993), while experimental and clinical studies from our group and others found little evidence for extensive HSC replacement in unconditioned recipients (Bhattacharya et al., 2006b; Cavazzana-Calvo et al., 2007; Czechowicz et al., 2007; Gambel et al., 1984; Muller et al., 2000; Prockop and Petrie, 2004; van Os et al., 2001; Xu et al., 2004). Our studies specifically examined the behavior of purified HSCs and HSC replacement rather than that of unfractionated marrow; thus we believe our results are more reliable and meaningful than previous and often conflicting estimates based on whole marrow transplants (Brecher et al., 1982; Colvin et al., 2004; Gambel et al., 1984; Quesenberry et al., 1994; Rao et al., 1997; Saxe et al., 1984; Stewart et al., 1993; Stewart et al., 1998; Wu and Keating, 1993). This is particularly important because clinical unfractionated bone marrow transplants are associated with high rates of acute graft versus host disease, and as such most transplants are performed with HSC-enriched populations.
While the exact amount of HSC replacement that occurs in the absence of conditioning is still controversial, it is clear that a certain fraction of transplanted HSCs do engraft in unconditioned recipients, a finding that appears to be inconsistent with the assumptions that the number of HSC niches is equivalent to the number of HSCs (Moore et al., 1997) and that HSCs remain fixed into place within these physically discrete niches (Schofield, 1978). In this study, we provide evidence for a model that is consistent with both the existence of physically discrete HSC niches and HSC replacement by transplanted cells in the absence of conditioning. In this model, the division-independent egress of HSCs leaves behind a certain fraction of empty niches that can be filled by transplanted HSCs, thus leading to the functional replacement of a small fraction of endogenous HSCs.

Genetic deficiencies in genes such as Egr1 and certain mobilizing regimens such as cyclophosphamide + G-CSF appear to trigger a large amount of proliferation of bone marrow HSCs along with the egress of HSCs into the blood, spleen, and liver (Min et al., 2008; Morrison et al., 1997), perhaps causing an excess of HSCs relative to the number of available niches available in the marrow. These mobilizing regimens have been shown to not be effective as transplantation conditioning regimens (Robinson et al., 2000). On the other hand, the CXCR4 antagonist AMD3100, interleukin-8, and blocking antibodies or antagonistic small molecules against VLA-4 all cause HSC release into the bloodstream in the apparent absence of cellular division, suggesting settings in which there is no absolute requirement for proliferation prior to HSC egress (Broxmeyer et al., 2005; Chen et al., 2006; Craddock et al., 1997; Laterveer et al., 1995; Ramirez et al., 2009). These types of mobilizing agents have
been shown to be effective as transplantation conditioners (Chen et al., 2006). Consistent with these latter findings, our results using BrdU incorporation indicate that under steady-state conditions, HSCs need not proliferate in order to egress into circulation from their supportive niches. This process allows a certain degree of HSC replacement to occur following transplantation, even in the absence of cytoreduction.

Given that the physiological circulation of HSCs is continuous, the division-independent egress of HSCs would be expected to generate a process by which HSC niches are continuously being emptied, refilled, and recycled. Consistent with such a mechanism, we demonstrate that transplantation of an excess of donor HSCs reveals these transiently vacant niches and leads to the replacement of a certain fraction of host HSCs. Taken together, our data suggest that engraftment of transplanted HSCs is limited by the number of empty niches that arise while the transplanted cells survive. We thus propose a two-phase model of donor HSC engraftment: in the first phase, the niches that are already available at the time of transplant are efficiently engrafted by a portion of the transplanted HSCs through non-stochastic chemotaxis and adhesion. In the second phase, the remaining transplanted HSCs survey for unoccupied niches until they differentiate or die; the likelihood of encountering a niche that becomes vacated by host HSC egress is dependent on the number of cells transplanted and the subsequent concentration of donor HSCs in the recipient animal.

While HSCs are known to migrate between HSC-supportive niches at different sites during embryonic development (Gekas et al., 2005; Johnson and Moore, 1975; Moore and Metcalf, 1970; Samokhvalov et al., 2007), we do not know or test here
what fraction of endogenously migrating HSC in the adult are destined to re-enter HSC niches; some could be fated for non self-renewing multipotent progenitor (MPP) niches, or even specialized niches, perhaps for the production of selected lineages such as megakaryocytes and erythrocytes. Indeed, the ability of circulating HSCs to re-enter appropriate bone marrow HSC niches under homeostatic conditions has been suggested to be low by several studies (Abkowitz et al., 2003; McKinney-Freeman and Goodell, 2004). Therefore it is still a question whether MPP niches are physically proximal to HSC niches or positioned at entry sites from the vasculature that ends in sinusoids.

While apoptosis of endogenous HSCs does not appear to be a major mechanism by which niches are emptied, it is important to note that our data do not exclude other potential mechanisms by which niches might be emptied under physiological conditions. For example, the number of HSCs expands dramatically with age, with 5-10 fold increases in HSC numbers in 24-month animals vs. 12-week old animals (de Haan and Van Zant, 1999; Harrison et al., 1989; Morrison et al., 1996; Rossi et al., 2005; Sudo et al., 2000). It is possible that the number of HSC niches also expands and precedes the HSC expansion slightly, leading to transient periods of niche vacancy. This process would be predicted to generate approximately a ~0.2% increase in the number of niches every day. While these numbers are insufficient to explain the levels of engraftment achieved in unconditioned animals, such processes may contribute to HSC replacement following transplantation. Formal testing of this hypothesis will require the precise identification of the cellular components of the niche. Additional processes such as bone remodeling may also play a role in the
creation of new niches and/or HSC egress under steady-state conditions (Kollet et al., 2006).

Our data suggest that in clinical HSC transplantation settings where cytoreductive conditioning is not applied, such as for the treatment of severe combined immunodeficiency (Buckley et al., 1999; Gatti et al., 1968), the levels of HSC engraftment, which are generally very poor (Cavazzana-Calvo et al., 2007; Muller et al., 2000), might be improved by capitalizing on the physiological egress of HSCs through the repetitive transplantation of smaller doses of donor HSCs. Moreover, our study provides a model that is consistent with host HSC replacement following donor HSC transplantation in unconditioned recipients, yet is also consistent with data suggesting the existence of a physically discrete niche which effectively retains and regulates HSCs.
MATERIALS AND METHODS

Mice

All animal procedures were approved by the International Animal Care and Use Committee and Stanford University's Administrative Panel on Laboratory Animal Care. Recipient mice used in these studies were 8-12-week old wild type C57Bl/Ka CD45.1, CD45.2, or CD45.1 x CD45.2 wild type or H-2k Bcl2-transgenic mice (Domen and Weissman, 2000). Donor mice used were 10-12 weeks congenically distinguishable CD45.1 or CD45.2 C57Bl/Ka wild type or H-2k Bcl2-transgenic mice (Domen and Weissman, 2000). All mouse strains were bred and maintained at Stanford University's Research Animal Facility.

HSC Transplantation

Bone marrow was harvested from donor mice by crushing bones and removing debris on a density gradient using Histopaque 1119 (Sigma, St. Louis, MO). Bone marrow was then c-kit+ enriched using CD117+ microbeads (AutoMACS, Miltenyi Biotec, Auburn, CA). Peripheral blood was isolated from the tail vein where indicated, or by puncture of the right atrium and perfusion of PBS + 10mM EDTA through the left ventricle of tribromoethanol-anesthetized mice. Erythrocytes were removed using a 1:1 gradient mixture of Histopaque 1119 and 1077. Cells were stained with antibodies described below and HSCs were isolated by single or double FACS based on previously defined reactivity for particular cell surface markers (Lineage− c-kit+ Sca-1+ IL7ra− CD27+ CD150+ CD34−) on a BD FACS-Aria (BD Biosciences, San Jose, CA). Cells were transplanted by retro-orbital injection. In some cases 200 ml peripheral
blood was collected from the tail vein of unmanipulated wild type mice in heparinized tubes, washed once with PBS + 10mM EDTA, and injected into lethally irradiated recipients alongside $2 \times 10^5$ competitor bone marrow from congenically distinguishable wild type mice.

Antibodies

The following monoclonal antibodies were purified and conjugated using hybridomas maintained in our laboratory: 2C11 (anti-CD3), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), 6B2 (anti-B220), 8C5 (anti–Gr-1), M1/70 (anti–Mac-1), TER119 (anti-Ter119), A20.1.7 (anti-CD45.1), AL1-4A2 (anti-CD45.2), 2B8 (anti–c-kit), E13-161-7 (anti–Sca-1). Antibodies were conjugated to biotin, Pacific Blue, PE, allophycocyanin (APC), Alexa 488, Alexa 647 or Alexa 680 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The following were purchased from eBiosciences (San Diego, CA): antibodies against CD3, CD4, CD8, B220, Mac-1, Ter119, and Gr-1 conjugated to PE-Cy5; anti–c-kit and anti–Mac-1 conjugated to PE-Cy7; anti-CD135 (A2F10) conjugated to PE, PE-Cy5, and biotin; anti-CD34 conjugated to FITC, Alexa 647, Alexa 700, and biotin; anti-CD27 conjugated to APC (LG.7F9); and anti-B220 conjugated to APC-Cy7. Anti-CD150 conjugated to PE and to Alexa 647 was purchased from Biolegend (San Diego, CA). Streptavidin conjugated to Alexa 488 and Alexa 680, and goat-anti-rat conjugated to Alexa 488 was purchased from Invitrogen. Streptavidin conjugated to Quantum Dot 605 was purchased from Invitrogen. Anti-BrdU conjugated to FITC was purchased from BD Pharmingen.
**Clonal in vitro assays**

Single cells were deposited using the ACDU unit of the BD FACS Aria into individual wells of a 96-well round bottom plate containing 150 ml DMEM/F12 (Invitrogen) with 10ng/ml each of stem cell factor (SCF, Peprotech, Rocky Hill, NJ), thrombopoietin (TPO, Peprotech), Flt3 ligand (eBiosciences), IL-3 (Peprotech), granulocyte/monocyte-colony stimulating factor (GM-CSF, Peprotech), erythropoietin (EPO, R&D systems, Minneapolis, MN), and 10% fetal bovine serum (Hyclone, Logan, UT). Wells were harvested after 12 days, cytospins were prepared and stained with May-Giemsa, and colonies were visually scored for lineage output.

**BrdU Analysis**

C57Bl/6 mice were maintained on 0.8 mg/ml BrdU in their drinking water for 3-12 days. The bottles containing BrdU water were protected from light and changed daily. To obtain peripheral blood, the mice were perfused with PBS containing 10mM EDTA and erythrocytes were removed using Histopaque gradients as before. The bone marrow was harvested from the mice by crushing bones and removing debris on density gradient using Histopaque 1119 (Sigma, St. Louis, MO). Cells from peripheral blood and bone marrow were stained with antibodies as described above. The cells were fixed and permeabilized using the Fix and Perm kit (Invitrogen, Carlsbad, CA). The cells were subjected to DNaseI treatment for 45 minutes at room temperature and BrdU incorporation was visualized using Anti-BrdU antibody conjugated to FITC.
**Engraftment Analysis**

Blood was obtained from the tail vein of transplanted mice at various time points and erythrocytes were sedimented using 2% dextran in PBS at 37ºC for 30 min, and supernatants were subsequently lysed using ACK lysis buffer (150mM NH₄Cl, 1mM KHCO₃, and 0.1mM EDTA) for 5 minutes. Cells were stained with antibodies described above and analyzed on the BD FACS-Aria. Donor granulocyte chimerism was determined by analyzing the percentage of Ter119°C3’ B220’ Gr-1<sup>high</sup> side scatter<sup>high</sup> cells that were also donor<sup>+</sup>.
ACKNOWLEDGEMENTS

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**Figure 2-1:** Hematopoietic stem cell egress is either division dependent or independent.

Hematopoietic stem cells can either undergo an extrinsically asymmetric division, in which one daughter cell is positioned away from a supportive niche and can thus intravasate to the blood (A) or can exit the supportive niche in the absence of cellular division (B). In the former model, all HSCs in the blood would be expected to have incorporated BrdU (gray shaded cells) after an appropriate feeding period, while the latter model would predict similar low BrdU incorporation rates between bone marrow and blood HSCs.
FIGURE 2-1:
**Figure 2-2:** Phenotypic HSCs can be identified and purely isolated from peripheral blood.

* A. Bone marrow and peripheral blood from 12 week-old mice were stained with the combination of markers indicated. The plots from the peripheral blood represent pooled events collected from 25 animals, in which approximately 50 HSCs were analyzed. Lineage cocktail antibodies were divided into different channels to minimize the chance of contamination of mature cells in the HSC gate.

* B. **CD27 is expressed on HSCs.** Lineage- c-kit+ Sca-1+ IL7ra- cells from the bone marrow and blood were examined for CD34 and CD27 expression. CD34- cells segregated almost exclusively to the CD27+ population in the bone marrow, and thus the CD27 marker was useful for distinguishing HSCs from background events in the blood. All stains were repeated in 8 independent experiments.
FIGURE 2-2:
**Figure 2-3:** *Functional HSCs can be found in the peripheral blood under physiological conditions.*

**A. Transplantable peripheral blood HSCs are contained within the CD34- fraction of CD27+c-kit+Sca-1+lineage- cells.* Either 8 KLS CD27+CD150+IL-7ra-CD34- or 200 of the remaining KLS CD27+IL-7ra- cells were transplanted into lethally irradiated recipients along with 200,000 competitor bone marrow cells and chimerism in various blood lineages was determined 16 weeks post-transplant. Means +/- S.E.M. are shown (n=5 for each group; experiment was repeated 3 times).

**B. Clonal lineage potential of bone marrow and peripheral blood HSCs is similar.** Single bone marrow or peripheral blood HSCs were cultured in the presence of SCF, Flt3L, IL-3, M-CSF, GM-CSF, TPO, and EPO for 12 days, and the lineage composition of the colonies was determined through cytospins and May-Giemsa stains. Twenty wells were analyzed for both peripheral blood and bone marrow HSCs. Two independent experiments were performed.
Figure 2-4: HSCs can egress into the peripheral blood without dividing.

Mice were fed BrdU in the drinking water for 3 or 6 days (experiment 1), 9 days (experiment 2), or 12 days (experiment 3) and the percentage of peripheral blood HSCs that had incorporated BrdU was quantified. Control mice were not fed BrdU, but bone marrow HSCs were isolated identically as from experimental groups. HSCs were identified as described in Figure 3A, and myeloid progenitors (MP) were identified as lineage- c-kit+ Sca-1- cells. Peripheral blood was pooled from 20 mice for each experiment and 2 independent experiments were performed for each timepoint.
FIGURE 2-4:

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<th>Expt. 3</th>
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Figure 2-5: HSCs home to the bone marrow rapidly after transplantation.

10,000 purified CD45.1 HSCs were transplanted into unconditioned CD45.1 x CD45.2 wild type recipients and donor bone marrow HSC chimerism (top panel) as well as the percentage of donor cells that retained HSC phenotypes (bottom panel) was determined at the indicated timepoints (n=4-5 mice for each timepoint). Mean chimerism values +/- S.E.M and p-values from the students’ unpaired two tailed t-test are shown. Two independent experiments were performed.
FIGURE 2-5:

![Graph showing percentage of donor HSC chimerism and percentage of HSC (of all donor cells) over days post transplantation.](image)

- **Top Graph:**
  - X-axis: Days post transplantation (1, 3, 5, 7)
  - Y-axis: % donor HSC chimerism
  - Data points and error bars are shown for each day.
  - Significant difference indicated with p=0.006.

- **Bottom Graph:**
  - X-axis: Days post transplantation (1, 3, 5, 7)
  - Y-axis: % HSC (of all donor cells)
  - Data points and error bars are shown for each day.
  - Significant difference indicated with p=0.03.
Figure 2-6: Niche availability is reset within 1 day in wild type mice.

A. Daily transplantation yields higher levels of engraftment than single bolus transplants. Wild type CD45.1 x CD45.2 mice were transplanted with a sum total of 12,700 single-sorted bone marrow HSCs (experiment 1) or with 11,060 double-sorted bone marrow HSCs (experiment 2) given as a single bolus or over the course of seven daily injections. At 16 weeks post-transplant, bone marrow was harvested and donor HSC (lineage- c-kit+ Sca-1+ CD150+ CD34-) chimerism was measured. Mean values +/- S.E.M are shown. n=10 for each group; repeat transplant experiments were performed 3 independent times, 2 of these are shown.

B. Peripheral blood granulocyte chimerism correlates poorly with donor HSC chimerism in unconditioned wild type recipients. Granulocyte chimerism was correlated with HSC chimerism in each individual mouse 16 weeks post-transplantation. R² value from linear regression is shown. Repeat transplant experiments were performed 3 independent times, 2 of these are shown. C. Mature cell chimerism correlates poorly with HSC chimerism following whole bone marrow transplantation. 6.5 x 10⁷ unfractionated nucleated bone marrow cells from wild type female CD45.2 mice were transplanted into unirradiated wild type female CD45.1 x CD45.2 F1 recipients (n=5); 2 independent experiments were performed. Total peripheral blood, BM HSC (defined as KLS CD135CD34⁻), BM total KLS, and granulocyte, B cell, T cell, natural killer cell (NK), and dendritic cell (DC) chimerism in the peripheral blood was measured 16 weeks after transplantation.
FIGURE 2-6:

A

Experiment 1

% donor HSC chimism

B

p=0.000011

Experiment 2

% donor granulocyte chimism

C

** p<0.05

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**Figure 2-7:** *Functional HSC engraftment efficiency decreases with transplanted cell dose.*

**A. Engraftment efficiency decreases with transplanted cell dose.** Various numbers of purified CD45.1 HSCs were transplanted into unconditioned CD45.1 x CD45.2 mice, and bone marrow HSC chimerism was determined 4 weeks post-transplant. Efficiency was calculated using the measured chimerism and with the approximation that 12-16 week-old mice have 25,000 endogenous HSCs as follows: 

$$\text{Efficiency} = \frac{\% \text{ chimerism} \times \text{cell dose}}{25,000}$$

Mean values +/- S.E.M are shown. n=5 for each condition; two independent experiments were performed.

**B. Donor HSCs can be secondarily transplanted.** 12-14 single-sorted donor CD45.2 HSCs from unconditioned transplanted wild type CD45.1 x CD45.2 mice were re-isolated at 16 weeks post primary transplant and secondarily transplanted into lethally irradiated CD45.1 mice along with 200,000 GFP+ unfractionated bone marrow cells. The total percent chimerism for each lineage derived from CD45.2 HSCs is shown at 16 weeks post transplant. Mean chimerism +/- S.E.M. is shown (n=7). Two independent experiments were performed.
FIGURE 2-7:
**Figure 2-8:** Apoptosis is not a major mechanism of HSC niche clearance.

2500 single-sorted bone marrow HSCs (~45% purity) from H-2\(^k\)-Bcl-2 transgenic CD45.2 mice were transplanted into unconditioned H-2\(^k\)-Bcl-2 transgenic CD45.1\times CD45.2 mice (n=4) or wild type CD45.1\times CD45.2 mice (n=3). Mean donor bone marrow HSC chimerism +/- S.E.M. was quantified 16 weeks after transplantation. Two independent experiments were performed.
FIGURE 2-8:
**Figure 2-S1:** *HSC activity segregates with CD27+ cells.*

**A. HSC activity is undetectable in CD27- bone marrow.** 5000 CD27⁺, containing ~5 KLS CD150⁺ CD34⁻ cells, or 100,000 CD27⁻ bone marrow cells from GFP⁺ animals were transplanted alongside $2 \times 10^5$ GFP⁻ CD45.2 competitor cells into lethally irradiated GFP⁻ CD45.2 mice. Peripheral blood granulocyte, B cell, and T cell GFP⁺ chimerism was determined at 4 and 8 weeks post-transplant. 5/7 recipients transplanted with CD27⁺ marrow showed multilineage reconstitution, while 0/9 recipients receiving CD27⁻ marrow showed multilineage reconstitution by 8 weeks post-transplant.

**B. All HSC activity within peripheral blood is contained within the KLS CD27⁺ fraction.** 40 single-sorted peripheral blood KLS CD27⁺ cells or $4 \times 10^5$ of all remaining non-erythrocyte blood cells from GFP⁺ animals were transplanted alongside $2 \times 10^5$ GFP⁻ CD45.2 competitor cells into lethally irradiated GFP⁻ CD45.2 mice. Peripheral blood granulocyte, B cell, and T cell GFP⁺ chimerism was determined at 4 and 8 weeks post-transplant. 0/9 mice receiving non-KLS CD27⁺ cells showed multilineage reconstitution. Chimerism values below 0.01% were considered undetectable. Both experiments were repeated once.
FIGURE 2-S1:
**Figure 2-S2:** Hematopoietic stem cells are rare in the peripheral blood of adult animals.

**A. Persistent lymphoid, but not myeloid chimerism following transplantation of whole peripheral blood.** 200 ml of unfractionated blood isolated from the tail vein of unmanipulated wild type animals was transplanted along with $2 \times 10^5$ unfractionated congenically marked competitor bone marrow cells into lethally irradiated congenically distinguishable wild type recipients. Chimerism derived from the transplanted peripheral blood was measured at 16 weeks post-transplant for the granulocyte (black filled circles), B cell (unfilled circles), and T cell (gray-filled circles) lineages. Chimerism values below 0.1% were considered undetectable. Eight independent experiments were performed; data shown are a summation of all 8 experiments.

**B. Frequency of peripheral blood HSCs is similar between young and adult mice.** 250 ml of unfractionated blood isolated from the tail vein of unmanipulated 12-week old (left panel) or 4-week old (right panel) wild type animals was transplanted along with $2 \times 10^5$ unfractionated congenically marked competitor bone marrow cells into lethally irradiated congenically distinguishable wild type recipients. Chimerism derived from the transplanted peripheral blood was measured at 16 weeks post-transplant for the granulocyte (black filled circles), B cell (unfilled circles), and T cell (gray-filled circles) lineages. Chimerism values below 0.1% were considered undetectable. Experiment was performed once.
FIGURE 2-S2:
**Figure 2-S3:** Transplanted HSCs remain undifferentiated for 3 days post-transplant into unconditioned animals.

Representative plots show the surface phenotypes of lineage- CD45.1+ CD45.2+ host cells (left panel) or all CD45.1+ CD45.2- donor cells identified in the bone marrow at various timepoints post-transplantation of 10,000 purified CD45.1+ HSCs. Donor cells uniformly lacked expression of lineage markers at these timepoints. C-kit+ Sca-1+ donor cells uniformly expressed CD150 and lacked CD34 during all timepoints. Experiment was repeated once.
FIGURE 2-S3:
CHAPTER 3

Antibody-based Depletion of Hematopoietic Stem Cells

Empties Niches for Efficient Transplantation

Agnieszka Czechowicz, Deepta Bhattacharya, Daniel Kraft and
Irving L. Weissman

Portions of this chapter were published in the following article:

ABSTRACT

Upon intravenous transplantation, hematopoietic stem cells (HSCs) can home to specialized bone marrow niches, yet engraftment levels rarely exceed 0.5% following transplantation into immunodeficient recipients without toxic conditioning. Here, we provide evidence that, aside from immune barriers, donor HSC engraftment is restricted by occupancy of appropriate niches by host HSCs. Administration of ACK2, a depleting antibody specific for c-kit, led to the transient removal of >98% of endogenous HSCs and transplantation of these animals with donor HSCs led to chimerism levels of up to 90%. Extrapolation of these methods to humans may enable efficient yet mild conditioning regimens for transplantation.
INTRODUCTION

HSCs are the only cells within the bone marrow (BM) that possess the ability to differentiate to all blood lineages and yet self-renew and maintain themselves for life. These two properties, coupled with the ability to home to highly specialized microenvironments within the bone marrow that enable them to function properly, have allowed HSCs, included within the hematopoietic preparations in BM transplants, to become the only stem cells in routine clinical use. They are widely used for the treatment of some hematological disorders, such as severe combined immunodeficiency (SCID), and hematopoietic failure following irradiation and chemotherapy. They are also used in a variety of other hematologic disorders. In animal models (Beilhack et al., 2003; Smith-Berdan et al., 2007) and anecdotally in humans, HSC from normal donors have also been implicated to be useful in the treatment of a wide range of autoimmune disorders such as diabetes, the NZB/W model of lupus, and multiple sclerosis. HSC are also capable of inducing transplantation tolerance to HSC donor tissues (Gandy and Weissman, 1998; Shizuru et al., 1990). However, the use of HSC for such disorders is not common due to the toxic conditioning regimens currently required for engraftment and the graft vs. host disease caused by T cells in the marrow or mobilized blood that supplies the HSC.
RESULTS

Available HSC Niches Can Be Saturated with Donor HSCs

Typically, efficient allogeneic transplantation requires conditioning of the recipient with toxic, cytoreductive treatments in order to prevent immunological rejection of the graft. Nevertheless, even in the absence of immune barriers, the levels of donor engraftment following HSC transplantation appear to be restricted in unconditioned recipients. Consistent with clinical data regarding BM transplantation (BMT) of SCID patients that had not been conditioned with cytoreductive drugs (Cavazzana-Calvo et al., 2007; Muller et al., 2000; Tjonnfjord et al., 1994), we found that HSC transplantation into unconditioned SCID mice led to a restoration of functional B and T lymphocytes, but donor HSC chimerism remained at <1% (Bhattacharya et al., 2006b). These low levels of chimerism could also be achieved when CD45.1 HSCs were transplanted into unconditioned wild type CD45.1 x CD45.2 mice, which are genetically incapable of rejecting the graft, but not CD45.2 recipients, which presumably can reject the graft on the basis of antigenic differences of the CD45 protein.

We hypothesized that donor HSC engraftment might be limited by the occupancy of appropriate HSC niches and that the specific removal of host HSCs from these niches might increase donor HSC engraftment. In the studies described below, we provide evidence that the specific antibody-mediated depletion of host HSCs leads to a dramatic improvement in the efficiency of donor HSC engraftment. These data confirm that niche availability regulates the efficiency of HSC transplantation. Moreover, these studies provide a framework for the use of highly specific antibodies...
to safely deplete and replace genetically abnormal HSCs for the treatment of hematological disorders.

We have shown previously that normal HSC in marrow engage in continuous recirculation from marrow to blood back to marrow (Wright et al., 2001). Cells in the blood enter the bone marrow, spleen, and liver and can establish long-term HSC self-renewal and hematopoiesis (Wagers et al., 2002a; Wright et al., 2001). We have previously demonstrated that at any instant about 0.1-0.5% of HSC are in this process, leading us to propose that there are equivalent numbers of empty HSC niches at any instant (Bhattacharya et al., 2006b; Wright et al., 2001). Consistent with this hypothesis, we found little difference in granulocyte or HSC chimerism when cell doses ranging from 500-4000 purified HSCs were transplanted in a single bolus into either unconditioned recombinase activating gene 2-deficient (RAG2-/-) or RAG2-/- interleukin-2 common gamma chain-deficient (RAG2-/-gc-/-) mice, which behave similarly to each other with respect to HSC transplantation (Bhattacharya et al., 2006b). To more rigorously address whether appropriate niches in unconditioned recipients can be saturated by transplanted HSCs in a dose-dependent manner, we transplanted unconditioned Rag2-/-gc-/- mice with varying numbers of c-kit+lineage- Sca-1+ (KLS) CD34-CD150+ HSCs from GFP-transgenic mice (Kiel et al., 2005a; Osawa et al., 1996; Spangrude et al., 1988; Wright et al., 2001). Peripheral blood granulocyte chimerism was measured at 16 weeks post-transplant, which we have previously shown to accurately reflect donor HSC chimerism in this unconditioned system (Bhattacharya et al., 2006b; Wright et al., 2001).
Donor granulocyte chimerism increased significantly in doses between 10 and 250 transplanted HSCs, but transplantation of more than 250 cells led to at most modest increases in chimerism (Fig. 3-1). Thus, increasing the dose of transplanted HSCs did not result in a linear increase in donor chimerism as would be expected in a model where endogenous HSCs can be readily replaced by transplanted HSCs. Instead, the data confirm that the HSC niche is a highly specific entity, and suggest that the number of niches available for engraftment at any given point under homeostatic conditions are similar to the number of HSCs estimated to be in their circulatory phase (Wright et al., 2001). In this model, increased doses of transplanted HSCs lead to slightly increased chimerism levels by increasing the probability that an endogenous HSC exits into circulation and vacates a niche in close spatial proximity to an as yet non-engrafted donor HSC. However, the process is clearly inefficient and non-linear, since transplantation of 1000 HSCs, equivalent to approximately 5% of the total number of HSCs in a mouse (Boggs, 1984; Colvin et al., 2004; Kiel et al., 2005a; Sudo et al., 2000), led only to 0.6% chimerism (Fig. 3-1).

To determine whether HSCs alone or other cell types as well can occupy these niches, we competitively transplanted unconditioned Rag2-/-gc-/- (CD45.2) mice with 1000 CD45.1 HSC along with 100,000 GFP KLS CD34+ progenitor cells, which are the immediate downstream progeny of HSCs (Osawa et al., 1996; Rossi et al., 2005). The inclusion of 100,000 KLS CD34+ progenitor cells in the transplant did not affect the engraftment of the HSC, as there was no significant difference in donor chimerism relative to recipients that received 1000 HSCs alone (Fig. 3-1). Despite the inclusion of a 100-fold excess of progenitor cells, the donor chimerism resulting from the 1000
HSC was significantly higher than the 10 HSC cell dose (Fig. 3-1). These data imply that KLS CD34+ progenitor cells do not effectively compete with HSCs for the same niches, and that the presence or absence of these non-self-renewing progenitor cells does not affect HSC engraftment. GFP+ donor-derived myeloid and lymphoid contribution from the transplanted KLS CD34+ cells did persist through at least 8 weeks post-transplantation (data not shown) at levels similar to those we have observed previously (Bhattacharya et al., 2006a), suggesting that HSCs and their immediate progeny use distinct niches to maintain function.

Studies in unconditioned mice have demonstrated that unfractionated donor BM transplants lead to chimerism levels that are linearly proportional to the dose of transplanted cells, suggesting that "space" is not a limiting factor in BM transplants (Stewart et al., 1993). However, these studies did not determine whether all cell types or only certain subsets of cells that are present within the heterogeneous BM compartment are readily displaced. Because HSCs represent <0.01% of all nucleated bone marrow cells (Kiel et al., 2005b; Sudo et al., 2000), we wished to determine whether the total donor chimerism measured in these studies accurately reflected HSC chimerism. To examine this issue, we transplanted 6.5 x 10^7 unfractionated bone marrow cells from female CD45.2 mice into female CD45.1 x CD45.2 F1 recipients to exclude the possibility of graft rejection. This transplanted cell dose represents 12-15% of the total recipient bone marrow cellularity by one estimate (Colvin et al., 2004), or 20-28% by another (Boggs, 1984). Sixteen weeks after transplant, we measured the total peripheral blood chimerism and the bone marrow HSC chimerism directly. The total chimerism in the peripheral blood was 11% (Fig. 3-S1), similar to
the predictions made in earlier studies (Stewart et al., 1993). However, the mean HSC chimerism was only 4.5%, approximately 2.5 fold lower than the total chimerism (Fig. 3-S1). These data demonstrate that measurement of total chimerism leads to an overestimation of BM HSC chimerism. Thus, the data confirm that even in this system, HSC replacement is inefficient in unconditioned recipients.

**ACK2 Treatment Depletes HSCs In Vivo**

Based on these data, we reasoned that the specific elimination of host HSCs that occupy these highly specific niches in unconditioned animals would allow for high levels of donor HSC engraftment. In vivo administration of antibodies specific for particular antigens, such as CD20, has been shown in many contexts to mediate specific cell depletion through opsonization, antibody-dependent cell-mediated cytotoxicity (ADCC), recruitment of complement, or the disruption of essential signaling pathways (Clark and Ledbetter, 2005). We hypothesized that antibodies might also be used to target and deplete HSCs, thereby creating more available HSC niches for donor HSC engraftment. To test this hypothesis, we compared a number of different monoclonal antibodies and selected ACK2, an antibody known to recognize c-kit, the receptor for stem cell factor (SCF) (Witte, 1990), and antagonize function in vivo (Ogawa et al., 1991).

We hypothesized that if the ACK2 antibody were capable of depleting endogenous HSCs, residual antibody in the serum of mice would also inhibit and/or deplete transplanted donor HSCs. To determine the kinetics of antibody clearance in vivo, we administered 500µg of ACK2 intravenously to Rag2-/gc-/- immunodeficient
mice and tested the serum every two days for the presence of antibody by staining c-kit+ mast cells (Nakano et al., 1985). Residual antibody was detected in the serum up to five days after injection; however all detectable ACK2 antibody was cleared from the serum by seven days after injection (Fig. 3-2A). In certain other ACK2 preparations, antibody persisted in the serum of mice for up to eight days after injection.

To determine whether ACK2 administration could deplete HSCs in vivo, we quantified HSC numbers and frequencies in the bone marrow of treated mice at the time of ACK2 clearance. At this time point, we observed a ~99% decrease in the number of phenotypically identifiable HSCs (KLS CD135- CD150+) (Fig. 2B). Because we were uncertain that c-kit expression could reliably be used to mark HSCs following ACK2 treatment despite using a different c-kit clone (3C11), we also quantified the number of bone marrow HSCs using expression of Sca-1 and CD150, coupled with the lack of expression of CD34, CD135, CD244, CD48, and CD41 and other antigens associated with lineage commitment (Adolfsson et al., 2001; Christensen and Weissman, 2001; Kiel et al., 2005b) and observed a similar decrease in HSC numbers and frequency (data not shown). This treatment did not lead to obvious HSC mobilization, since an increase in phenotypic HSCs was not detected in the spleens or blood of ACK2-treated mice (data not shown).

In order to control for the possibility that ACK2 treatment changed the cell surface phenotype of host HSCs and to verify that ACK2 depleted functional host HSCs, unfractionated bone marrow from ACK2-conditioned or rat IgG-treated control animals were transplanted into irradiated recipients alongside competitor marrow.
Rag2-/-gc-/- mice were conditioned with 500µg ACK2, and at time of antibody clearance, 200,000 bone marrow cells from ACK2-conditioned animals were transplanted alongside 200,000 congenically marked competitor bone marrow cells from untreated wild type mice into lethally irradiated recipients. Transplantation of BM from ACK2-treated animals led to markedly reduced engraftment values relative to those obtained by transplantation of control BM (Fig. 3-2C). These data demonstrate that ACK2 depletes functional HSCs from the BM.

To determine the potential direct mobilizing effects of ACK2 treatment, the entire splenocyte population of ACK2-conditioned or control animals was transplanted alongside 200,000 congenically marked competitor bone marrow cells from untreated wild type mice into lethally irradiated recipients. The donor chimerism resulting from transplantation of splenocytes from ACK2-conditioned animals was significantly reduced relative to the chimerism resulting from transplantation of control splenocytes (Fig. 3-2D). These data indicate that ACK2-treatment depletes HSCs from the spleen as well as the BM, and does not directly induce early HSC mobilization.

In order to determine the mechanism by which ACK2 depletes HSCs, we compared the effects of ACK2 treatment to that of 2B8, another c-kit monoclonal antibody of the same IgG2b isotype. If ACK2 were to deplete HSCs through Fc-mediated functions such as opsonization, ADCC, or recruitment of complement, we hypothesized that 2B8, which stains c-kit expressing cells with equivalent intensity as ACK2 (data not shown), would also deplete HSCs in vivo. However, 2B8 treatment did not decrease functional HSC numbers in vivo as transplantation of bone marrow cells from mice treated with 2B8 resulted in normal engraftment (Fig. 3-2C). Thus,
we hypothesized that ACK2 may deplete HSCs by antagonizing SCF-mediated c-kit function, as has been previously shown in melanocytes (Nishikawa et al., 1991). To test this hypothesis, we cultured purified HSCs in the presence of ACK2 and found that it inhibited SCF-dependent proliferation (Fig. 3-2E), but not thrombopoietin (TPO)-mediated proliferation (Fig. 3-2E). Unlike ACK2, the effects of 2B8 on SCF-mediated proliferation were modest (Fig. 3-2E). Therefore, it is likely that ACK2 causes HSC depletion through the inhibition of SCF signaling while 2B8 binds to a region of the c-kit molecule that is not essential for signaling. The importance of SCF-mediated c-kit signaling for HSCs has been demonstrated in W/W mice, which lack functional c-kit expression and die in utero unless transplanted with normal HSCs (Fleischman and Mintz, 1979).

Additionally, we assayed the effect of ACK2 on a variety of hematopoietic progenitor cells in vivo at two and nine days after ACK2 administration. Two days post ACK2 administration, all myeloid progenitors in the bone marrow, which express similar levels of c-kit as do HSCs (Akashi et al., 2000), began to decline, however HSC were most dramatically impacted (Fig. 3-2F). Thus, these data are again inconsistent with a direct Fc-mediated depleting activity of c-kit+ cells by ACK2 and rather support a mechanism by which progenitor cells are gradually lost either due to the lack of replenishment by HSCs and other early progenitors within the KLS compartment, or by blockade of their c-kit receptors. Consistent with this mechanism, by nine days post ACK2 administration, HSCs (Fig. 3-2F), myeloid progenitors (Fig. 3-2F), and common lymphoid progenitors (data not shown) were severely diminished. Upon histological examination, we found a similar gradual diminishment of bone
marrow cellularity (Fig. 3-S2). Additionally, we observed a progressive increase in
the size of osteoblasts (Fig 3-S2, right column).

These data indicate that ACK2 causes a significant but transient depletion of
host HSCs and results in a short window in which ACK2-treated animals might be
receptive to donor HSC transplantation. Importantly, all mice survived the treatment
with no obvious signs of distress aside from a temporary loss of coat color, as
previously reported (Nishikawa et al., 1991; Ogawa et al., 1991). The lack of
mortality in ACK2-treated mice is likely due to the mechanism of ACK2 depletion, in
that mature effector blood cells are not directly affected by the treatment and are only
lost gradually due to attrition. This hypothesis is supported by the observation that a
number of hematologic parameters in the peripheral blood are only modestly affected
by ACK2 treatment (Fig. 3-TS1). Moreover, significant numbers of both mature
erythrocytes and regenerating erythroid colonies were observed in the bone marrow 9
days after ACK2 treatment (Fig. 3-S2, bottom left panel). Additionally, both male and
female mice treated with ACK2 remained fertile and had viable offspring. Thus, the
side effects of antibody-mediated depletion of HSCs stand in marked contrast to lethal
irradiation, which requires the early transplantation of bone marrow or hematopoietic
progenitors to prevent the death of the animal (Lorenz et al., 1951; Nakorn et al.,
2003; Salisbury et al., 1951; Uchida et al., 1994), a procedure which in humans is
accompanied by high levels of morbidity and significant mortality.

Near complete HSC depletion was observed as early as two days post ACK2
administration. However, some HSCs clearly do remain and retain the capacity to
self-renew because by two weeks post-serum clearance of antibody, HSC cell surface
profiles (Fig. 3-S3) and numbers (Fig. 3-2B) had returned to near normal levels. Although ACK2 treatment does not directly cause host HSC mobilization (Fig. 3-2D), significant splenic extramedullary hematopoiesis does occur during the recovery phase by one week after ACK2 clearance (Fig. 3-S3). By two weeks after clearance of ACK2 from the serum, the spleens of the animals appeared identical to untreated control animals (data not shown). Wild-type mice as well as B cell-deficient mMT mice (Kitamura et al., 1991) also showed decreased levels of HSCs in their bone marrow post ACK2 treatment (Fig. 3-S4). Interestingly, the recovery of HSC numbers appeared to be more rapid in B cell-sufficient or T cell-sufficient animals after ACK2 clearance (data not shown), suggesting an unexpected potential role for lymphocytes in stimulating hematopoietic recovery.

**ACK2 Treatment Enhances HSC Engraftment**

To test whether the ablation of host HSCs could improve the efficiency of donor HSC engraftment, we conditioned RAG2/- (CD45.1) mice, which behave identically to RAG2/- ge-/- with respect to antibody treatment (data not shown), with 500µg ACK2. These mice were then transplanted with 5000 wild type CD45.2 LT-HSCs seven days post antibody administration, a time point at which there was no detectable ACK2 in the serum. Peripheral blood was obtained from the recipients every four weeks post-transplantation and granulocyte chimerism was quantified. The mean donor granulocyte chimerism at 37 weeks post transplant was 16.1%, reflecting a >10-fold increase over untreated control animals that were transplanted with the
same number of HSCs (Fig. 3-3A). The engrafted HSC also gave rise to donor-derived peripheral B cells and T cells (Fig. 3-3B).

We also analyzed donor bone marrow HSC chimerism directly at this late time point to confirm the increase in donor engraftment, and indeed found that it correlated well with the donor peripheral blood granulocyte chimerism (Fig. 3-3C). Finally, to verify that we were accurately identifying functional donor HSCs with normal cell surface phenotypes, we re-isolated KLS CD34- CD150+ donor HSCs from the bone marrow of primary recipients and performed secondary transplants into irradiated recipient mice along with 200,000 wild type competitor bone marrow cells. These HSCs gave rise to multi-lineage engraftment for at least 16 weeks post-transplant (Fig. 3-3D), confirming that transplanted HSCs regain their normal cell surface phenotype by at least 7-9 months post-transplant in ACK2-treated animals. Consistent with this observation, we found that the cell cycle profiles of both host and donor HSCs in the BM of mice treated with ACK2 and transplanted 7 months prior was identical to that of untreated animals (Fig. 3-S5).

_Donor Chimerism Increases with Transplanted HSC Cell Number in ACK2-Treated Mice_

These data indicate that through specific ablation of host HSCs, we were able to significantly increase donor HSC engraftment. However, it was unclear whether ACK2 treatment increased niche space, leading to a high level of initial HSC engraftment following transplantation, or whether, through the depletion of host HSCs, small numbers of initially engrafted donor HSCs in ACK2-treated mice could
competitively expand. If niche space had truly been freed, HSC chimerism would be expected to increase with transplanted cell dose. In contrast, if the initial HSC engraftment were the same between ACK2-treated and unconditioned mice, no linear increase in donor chimerism would be expected at transplanted HSC numbers above 250 cells, as doses higher than this do not lead to linearly proportional increases in chimerism in unconditioned animals (Fig. 3-1). In order to determine the effects of cell dose in this system, we conditioned RAG2/-/-gc/-/- (CD45.2) recipient mice with 500µg ACK2 as above and transplanted them with varying doses of double-sorted CD45.1 LT-HSCs. Peripheral blood was assayed every four weeks for donor granulocyte chimerism until 24 weeks after transplantation.

Donor engraftment increased linearly with transplanted HSC dose (Fig. 3-4A), and thus proved that the dose of transplanted HSCs required to saturate available niches had been significantly increased relative to unconditioned mice (Fig. 3-1). These data demonstrate that ACK2 treatment increases the proportion of niches available for engraftment. Furthermore, the results suggest that niche availability is regulated by host HSC occupation and that overcoming this barrier can allow high levels of donor HSC engraftment. Other studies that attempted to increase the number of available niches for transplantation by mobilizing endogenous HSCs may have failed, perhaps due to the residual presence of mobilizing drugs at the time of transplantation or due to the damaging effects of these agents on the HSC-niche cells themselves (Robinson et al., 2000).

In the first experiment, both mice that received 35,000 HSCs (1.4x106 HSC/kg) had extremely high levels of donor chimerism (90.1% and 62.7%- Fig. 3-
4B). It is important to note that obtaining high numbers of HSCs has already proven to be clinically feasible, because most human HSC grafts are isolated from the peripheral blood of mobilized donors using regimens that lead to an expansion of HSCs (Morrison et al., 1997; Negrin et al., 2000). However, transplantation of such a large bolus of cells might potentially be avoidable since similarly high levels of donor chimerism were consistently obtained through three rounds of conditioning and transplantation of 5000 HSCs (for a total of 15,000 transplanted HSCs) (Fig. 3-S6). The total contribution of the third transplant was consistently higher than the first two HSC transplants combined.
DISCUSSION

Allogeneic BMT is used routinely for a number of clinical purposes, most commonly for the treatment of malignancies following high dose irradiation and chemotherapy, but also for the treatment of inherited hematopoietic deficiencies such as SCID. The minimum therapeutic levels of donor HSC chimerism vary between diseases, ranging from <1% for certain forms of SCID (Bhattacharya et al., 2006b; Muller et al., 2000) to ~20% for sickle cell anemia (Iannone et al., 2001). Nevertheless, the minimum therapeutic level of chimerism is not necessarily clinically optimal. For example, HSC chimerism levels of <1% in gc-deficient SCID patients can restore functional T cells, but B cells remain largely of host origin (Muller et al., 2000). Moreover, new T cell production correlates with myeloid chimerism and presumably HSC chimerism as well (Cavazzana-Calvo et al., 2007), suggesting that low levels of initial HSC engraftment lead to only finite periods of T cell production in SCID patients. Here, we provide evidence that donor HSC engraftment is limited by the occupancy of appropriate niches by host HSCs. These data offer a clear explanation for the poor donor HSC engraftment observed in unconditioned SCID patients (Muller et al., 2000; Tjonnfjord et al., 1994).

The challenge for BMT, particularly for the treatment of non-malignancies, is maximizing the efficiency of donor HSC engraftment while minimizing harmful side effects. One of the major side effects associated with allogeneic BMT is graft versus host disease (GVHD), a potentially life-threatening condition in which the presence of alloreactive T cells within the graft leads to the destruction of host tissues. Importantly, studies have shown that purified allogeneic HSC transplantation does not
cause GVHD (Shizuru et al., 1996). Other serious side effects of BMT are often associated with cytotoxic conditioning regimens. Even if employed in moderation through non-myeloablative conditioning, cytotoxic conditioning regimens carry significant health risks including infertility, prolonged thrombocytopenia, organ damage, immunosuppression and high rates of secondary malignancies (Ferry and Socie, 2003). Indeed, it is these very side effects that prevent the routine clinical use of BMT for the treatment of serious but not immediately life-threatening diseases such as sickle cell anemia and certain types of autoimmunity, which have been shown to be cured by HSC transplantation both in animal models and limited numbers of clinical cases (Burt and Traynor, 1999; Iannone et al., 2001; Nelson et al., 1997).

We postulated that the most specific and consequently the safest way to increase HSC niche space would be through targeted antibody-mediated depletion. A large body of clinical precedent supports this premise, including the highly successful use of α-CD20 antibodies (Rituximab) for the treatment of B cell malignancies (Maloney et al., 1997). To generate preclinical evidence to support the use of HSC-depleting antibodies, we demonstrate above that administration of ACK2 in vivo leads to the rapid but transient depletion of host HSCs and progenitors. Transplantation of highly purified HSCs into ACK2-conditioned recipients leads to donor chimerism levels of up to 90%. These levels would almost certainly be therapeutic for inherited hematopoietic deficiencies.

For SCID patients, the specific depletion of HSCs might well be the only requirement of an effective conditioning regimen to allow for permanent and fully functional immune reconstitution by donor HSCs. For other types of inherited
hematopoietic disorders, HSC-depleting antibody treatments in combination with specific immunosuppressive agents could potentially be employed in place of or in combination with lowered doses of drugs designed for myelosuppression. Thus, the use of HSC-depleting antibodies is an attractive alternative to conventional methods of conditioning for BMT and may reduce the risks currently associated with bone marrow transplantation considerably, thereby increasing its utility in the treatment of a variety of hematologic and non-hematologic disorders. The development of depleting antibodies specific for human HSCs will be an important next step.
Studies in unconditioned mice have demonstrated that unfractionated donor BM transplants lead to chimerism levels that are linearly proportional to the dose of transplanted cells, suggesting that "space" is not a limiting factor in BM transplants (Stewart et al., 1993). However, these studies did not determine whether all BM cell types or only certain subsets of cells are readily displaced. To examine this issue, we transplanted 6.5 x 10^7 unfractionated bone marrow cells from female CD45.2 mice into female CD45.1 x CD45.2 F1 recipients to exclude the possibility of graft rejection. This transplanted cell dose represents 12-15% of the total recipient bone marrow cellularity by one estimate (Colvin et al., 2004), and 20-28% by another (Boggs, 1984). The mean total chimerism in the peripheral blood at 16 weeks post-transplant was 11% (Fig. 3-S1), similar to the predictions made in earlier studies (Stewart et al., 1993). However, the mean BM HSC chimerism was only 4.5%, approximately 2.5 fold lower than the total chimerism (Fig. 3-S1). These data demonstrate that measurement of total chimerism leads to an overestimation of BM HSC chimerism, and that even when the graft includes transplantation-enhancing non-hematopoietic cells (Devine and Hoffman, 2000; El-Badri et al., 1998), the majority of HSCs did not engraft.

Importantly, all mice survived the treatment with no obvious signs of distress aside from a temporary loss of coat color, as previously reported (Nishikawa et al., 1991; Ogawa et al., 1991). The lack of mortality in ACK2-treated mice is likely due to the mechanism of ACK2 depletion, in that mature effector blood cells are not directly affected by the treatment and are only lost gradually due to attrition. This is
supported by the observation that a number of hematologic parameters in the peripheral blood are only modestly affected by ACK2 treatment (Fig. 3-TS1). Moreover, significant numbers of mature erythrocytes and regenerating erythroid colonies were observed in the bone marrow 9 days after ACK2 treatment (Fig. 3-S2, bottom left panel). Upon histological examination, we found only a gradual diminishment of bone marrow cellularity (Fig. 3-S2), accompanied by a progressive increase in the size of osteoblasts (Fig 3-S2, right column). Additionally, both male and female mice treated with ACK2 remained fertile and had viable offspring. Thus, the side effects of antibody-mediated depletion of HSCs stand in marked contrast to lethal irradiation, which requires the early transplantation of bone marrow or hematopoietic progenitors to prevent the death of the animal (Lorenz et al., 1951; Nakorn et al., 2003; Salisbury et al., 1951; Uchida et al., 1994), a procedure which in humans is accompanied by high levels of morbidity and significant mortality. Indeed, conditioning regimens carry significant health risks including infertility, prolonged thrombocytopenia, organ damage, immunosuppression and high rates of secondary malignancies (Ferry and Socie, 2003). These risks are precisely the reason why many clinicians have chosen to treat SCID patients with BM transplants without cytotoxic conditioning.

Wild-type mice as well as B cell-deficient mMT mice (Kitamura et al., 1991) also showed decreased levels of HSCs in their bone marrow post ACK2 treatment (Fig. 3-S3). Interestingly, the recovery of HSC numbers appeared to be more rapid in B cell-sufficient or T cell-sufficient animals after ACK2 clearance (data not shown),
suggesting an unexpected potential role for lymphocytes in stimulating hematopoietic recovery.

The importance of SCF-mediated c-kit signaling for HSCs has been demonstrated in W/W mice, which completely lack functional c-kit expression and die in utero unless transplanted with normal HSCs (Fleischman and Mintz, 1979). The inhibition of c-kit signaling apparently must be complete to deplete HSCs, since neither the partial inhibition of c-kit signaling caused by 2B8 (Fig. 3-2E) nor genetic mutations that lead to a partial abrogation of c-kit function lead to a substantial diminishment of HSC numbers in vivo (Miller et al., 1996).

Additionally, we assayed the effect of ACK2 on a variety of hematopoietic progenitor cells in vivo after ACK2 administration. At two days post-treatment, all myeloid progenitors in the bone marrow, which express similar levels of c-kit as do HSCs (Akashi et al., 2000), began to decline; however HSCs were most dramatically impacted (Fig. 3-S6). By nine days post-treatment, HSCs (Fig. 3-S6), myeloid progenitors (Fig. 3-S6), and common lymphoid progenitors (Kondo et al., 1997) (data not shown) were severely diminished. These data support a mechanism by which progenitor cells are gradually lost due to the lack of replenishment by HSCs.

It is important to note that obtaining high numbers of HSCs has already proven to be clinically feasible, because most human HSC grafts are isolated from the peripheral blood of mobilized donors using regimens that lead to an expansion of HSCs (Morrison et al., 1997; Negrin et al., 2000). However, transplantation of such a large bolus of cells might potentially be avoidable since similarly high levels of donor chimerism were consistently obtained through three rounds of conditioning and
transplantation of 5000 HSCs (for a total of 15,000 transplanted HSCs) (Fig. 3-S8). The total contribution of the third transplant was consistently higher than the first two HSC transplants combined.
MATERIALS AND METHODS

Mice
All animal procedures were approved by the International Animal Care and Use Committee. Recipient mice used in these studies were 4-8 weeks old recombinase activating gene 2-deficient (RAG2/-) (Shinkai et al., 1992), RAG2/- interleukin-2 common gamma chain-deficient (RAG2/-gc/-) (Goldman et al., 1998), or mMT mice (Kitamura et al., 1991). Donor mice used were 8-12 weeks old GFP transgenic mice expressing GFP from the chicken b-actin promoter (Wright et al., 2001), or congenically distinguishable CD45.1 or CD45.2 C57Bl/Ka mice. All mouse strains were bred and maintained at Stanford University's Research Animal Facility.

HSC Transplantation
Bone marrow was harvested from donor mice by crushing bones, lysing red blood cells with ACK lysis buffer (150mM NH₄Cl, 1mM KHCO₃, and 0.1mM EDTA), and removing debris on density gradient using Histopaque 1119 (Sigma, St. Louis, MO). Bone marrow was then c-kit⁺ enriched using CD117⁺ microbeads (AutoMACS, Miltenyi Biotec, Auburn, CA). Cells were stained with antibodies described below and HSC were isolated by single or double FACS based on previously defined reactivity for particular cell surface markers (c-kit⁺lineage⁻Sca-1⁻CD34⁻CD150⁺) on the BD FACS-Aria (BD Biosciences, San Jose, CA). Cells were transplanted by retro-orbital injection.
**Antibodies**

The following monoclonal antibodies were purified and conjugated using hybridomas maintained in our laboratory: 2C11 (anti-CD3), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), 6B2 (anti-B220), 8C5 (anti–Gr-1), M1/70 (anti–Mac-1), TER119 (anti-Ter119), A20.1.7 (anti-CD45.1), AL1-4A2 (anti-CD45.2), 2B8 (anti–c-kit), 3C11 (anti-c-kit), E13-161-7 (anti–Sca-1). Antibodies were conjugated to biotin, Pacific Blue, Pacific Orange, PE, allophycocyanin (APC), Alexa 488, Alexa 647 or Alexa 680 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The following were purchased from eBiosciences (San Diego, CA): antibodies against CD3, CD4, CD8, B220, Mac-1, Ter119, and Gr-1 conjugated to PE-Cy5; anti–c-kit and anti–Mac-1 conjugated to PE-Cy7; anti-CD135 (A2F10) conjugated to PE; anti-CD34 conjugated to FITC or biotin; and anti-B220 conjugated to APC-Cy7. Anti-CD41 conjugated to FITC, anti-CD48 conjugated to FITC, goat-anti-rat conjugated to APC, and anti-TCRβ (H57-597) conjugated to APC were purchased from BD Biosciences. Anti-CD150 conjugated to Alexa 647 was purchased from Biolegend (San Diego, CA). Streptavidin conjugated to Alexa 488 and Alexa 680, and goat-anti-rat conjugated to Alexa 488 was purchased from Invitrogen. Streptavidin conjugated to Quantum Dot 605 was purchased from Invitrogen.

**ACK2 Production and Purification**

The ACK2 hybridoma was a generous gift of S. Nishikawa (Kyoto, Japan). The cell line was expanded and subcloned to establish an ACK2 high producing hybridoma cell line. Cells were grown in the Integra flask system (Integra Biosciences, Chur,
Switzerland) and media containing antibody was collected. ACK2 was purified on an IgG purification column by binding the ACK2 to the column and eluting with 100mM Glycine and 5mM NaN₃. The eluted positive fractions (OD₂₈₀ >0.2) were combined, dialyzed for 12 hours in PBS, and concentrated using a Vivaspin concentrator (Sartorius AG, Goettingen, Germany). Subsequent ACK2 preparations were prepared by Bio Express (W. Lebanon, New Hampshire).

ACK2 Administration and Clearance
500µg of ACK2 was administered through retro-orbital injection to 4-8 week RAG2-/- gc-/- mice. Peripheral blood was isolated from the tail vein of these mice every other day and allowed to clot for 1 hour. Samples were centrifuged for several minutes and serum was isolated. 10,000 mast cells (Nakano et al., 1985), a generous gift of A. Piliponsky and S. Galli, were subsequently stained with 50µl of serum, followed by goat-anti-rat IgG APC or Alexa 488, to test for ACK2 antibody presence. In addition, mast cells were stained with a known anti-c-kit antibody, 2B8 conjugated to APC as a control. These cells were analyzed on the BD FACS-Aria. To determine HSC depletion, both femurs and tibia were obtained from conditioned mice and prepared as above. Cells were counted and HSC frequency was determined on the BD FACS-Aria by gating on KLS CD135⁺CD150⁺ cells.

ACK2 Conditioning and Transplantation
500µg of ACK2 was administered intravenously to 4-8 week Rag2-/- or Rag2-/-gc-/- mice. Mice were transplanted at the time point that ACK2 was shown to no longer be
present in the serum (D7 or D9 depending on preparation). HSC for transplantation were obtained from bone marrow of donor mice, which were isolated on the BD FACS-Aria by gating on c-kit^+lineage^-Sca-1^+CD34^-CD150^+ cells.

**Engraftment Analysis**

Blood was obtained from the tail vein of transplanted mice at various time points. It was separated using 2% dextran at 37°C for 30 min, and subsequently lysed using ACK lysis buffer (150mM NH₄Cl, 1mM KHCO₃, and 0.1mM EDTA) for 5 minutes. Cells were stained with antibodies described above and analyzed on the BD FACS-Aria. Donor granulocyte chimerism was determined by analyzing the percentage of Ter119^-CD3^-B220^-Mac1^{high} side scatter^{high} cells that were also donor^+^+. Several animals were sacrificed and HSCs were isolated similarly to donor mice in order to confirm that the HSC chimerism mimicked the granulocyte chimerism. To determine the effects of ACK2 on hematopoietic progenitors, bone marrow was isolated from mice treated with 500µg ACK2 as above. Cells were counted and progenitor frequency was determined on the BD FACS-Aria. Cell numbers were compared to those of untreated animals. HSC were gated as lin^-c-kit^-Sca-1^-CD135^-CD150^+, MEP were gated as lin^-c-kit^-Sca-1^-CD34^-FcgR^-, CMP were gated as lin^-c-kit^-Sca-1^-CD34^{low} FcgR^{low}, and GMP were gated as lin^-c-kit^-Sca-1^-CD34^{high} FcgR^{high}.

**Transplantation into Irradiated Recipients**

Recipient mice were treated with 950cGy prior to transplantation. Each mouse was transplanted with the entire splenocyte population from an ACK2 mouse treated 9
days prior, 200,000 unfractionated bone marrow cells from an ACK2 mouse treated 9
days prior, or 100 donor+ KLS CD34-CD150+ HSC from a primary transplanted
mouse (ACK2 conditioned and transplanted with 5000 donor HSC 39 weeks prior).

In Vitro Culture

Exactly 10 HSCs (KLS CD34-CD150+) were clone sorted on the BD-Aria into 96-well
round-bottom plates and cultured in the presence of 50 ng/ml SCF (R&D Systems) or
50 ng/ml TPO (R&D Systems) in Iscove's Modified Dulbecco's Medium (Invitrogen)
with 10% fetal calf serum (Omega), 1 mM sodium pyruvate (Invitrogen), 100 mM
non-essential amino acids (Invitrogen), and 50 mM b-mercaptoethanol. HSC treated
with antibody received 10µg/ml of ACK2 or 2B8. Viable cells were counted each day
under the microscope.

Cell Cycle Analysis

Bone marrow cells were c-kit-enriched as before and stained with anti-CD34 FITC,
anti-c-kit (2B8) PE-Cy7, anti-CD150 Alexa 647, anti-Sca-1 Alexa 680, and anti-
lineage PE-Cy5. Cells were then fixed with 2% paraformaldehyde in PBS for 20
minutes at room temperature, washed twice with PBS, and resuspended in PBS/ 0.2%
saponin + 2mg/ml 4',6-diamidino-2-phenylindole (DAPI) prior to analysis.

Histology

Mice were treated with 500µg ACK2 and at 2 days and 9 days post treatment, the
humerus was removed and placed in Bouin's fixative for >1 day. Bones were
decalcified in formic acid, paraffin-embedded, sectioned, stained with hematoxylin and eosin, and mounted using xylene-based media.

Analysis of peripheral blood

Peripheral blood was collected from the tail vein and deposited directly into heparin-coated tubes. Analysis was performed by the Department of Comparative Medicine's Diagnostic Laboratory at Stanford University.
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Affiliations that might be perceived to have biased this work are as follows: I.L.W. owns significant Amgen stock, cofounded and consulted for Systemix, is a cofounder and director of Stem Cells, Inc., and cofounded and is a director of Cellerant, Inc. All other authors have no conflicting financial interests.
**Figure 3-1:** *Available HSC niches can be saturated with donor HSCs.*

Peripheral blood of transplanted unconditioned RAG2-/-gc-/- mice was analyzed 16 weeks after HSC transplantation for GFP⁺ donor-derived Ter119⁺CD3⁻B220⁻Mac-1<sup>high</sup> side scatter<sup>high</sup> granulocytes. In the right panel, mice were co-transplanted with CD45.1 1000 HSCs and 100,000 GFP⁺ KLS CD34⁺ cells. Mean values +/- SEM are shown (n=4-5 for each dose); ** indicates p-value<0.05 relative to the chimerism arising from the 10 HSC-transplanted group. The dashed line represents the theoretical HSC chimerism if engraftment were to increase linearly with transplanted cell dose.
FIGURE 3-1:
**Figure 3-2:** ACK2 treatment depletes HSCs in vivo.

**A. ACK2 is cleared from serum of RAG2-/-gc-/- mice seven days after injection.**
Serum of mice receiving 500µg ACK2 was analyzed every two days for persistence of the ACK2 antibody by staining c-kit⁺ mast cells.

**B. ACK2 administration leads to depletion of BM HSCs.** The number of KLS CD135⁻ CD150⁺ HSCs in both femurs and tibia of ACK2-treated and control mice was determined by flow cytometry at several timepoints after treatment. Mean values +/- SEM are shown (n=3 for each time point); ** indicates p-value<0.001.

**C. ACK2, but not 2B8 treatment, depletes functional HSCs from BM.** Transplantation of 200,000 unfractionated bone marrow cells from mice treated with 500µg ACK2 nine days earlier into irradiated recipients leads to decreased chimerism as compared to controls and another c-kit antibody, 2B8. Mean values +/- SEM are shown (n=5-8); ** indicates p-value<0.01.

**D. ACK2 treatment does not directly cause HSC mobilization to the spleen.** Single-cell suspensions of the spleens were generated from mice treated with 500µg ACK2 nine days earlier and the entire suspensions were transplanted alongside 200,000 competitor bone marrow cells from wild type mice. Mean values +/- SEM are shown (n=3-9); ** indicates p-value<0.001.
E. **ACK2 inhibits SCF mediated HSC proliferation.** HSC were isolated from wild type mice and cultured in the presence of SCF or TPO and ACK2 or 2B8. Proliferation was observed by light microscopy. ** indicates p-value<0.05 as compared to ACK2 treated samples.

F. **ACK2 treatment selectively depletes HSCs at early timepoints.** Total numbers of HSCs (lin⁻c-kit⁺Sca-1⁺CD135⁺CD150⁺), MEPs (lin⁻c-kit⁺Sca-1⁻CD34⁻FcgR⁻), CMPs (lin⁻c-kit⁺Sca-1⁻CD34⁻lowFcgR⁻low), and GMPs (lin⁻c-kit⁺Sca-1⁺CD34⁺highFcgR⁺high) were quantified and compared to untreated control mice. Mean values +/- SEM are shown (n=3); ** indicates p-value<0.001 as compared to the relative number of HSCs at the same time point.
FIGURE 3-2:
Figure 3-3: **ACK2 treatment enhances HSC engraftment.**

**A. ACK2 conditioning leads to higher donor myeloid chimerism.** Donor granulocyte chimerism was measured following transplantation of 5000 HSCs in RAG2-/- mice conditioned with ACK2 seven days prior to transplant and compared to that of unconditioned mice. Mean values +/- SEM are shown (n=4); ** indicates p-value<0.01.

**B. HSC transplantation of ACK2-treated animals leads to lymphocyte reconstitution.** Splenic donor-derived B and T-cells from ACK2-treated and unconditioned RAG2-/- mice were enumerated 39 weeks after transplantation with wild type HSCs. Mean values +/- SEM are shown (n=3-5); ** indicates p-value<0.01.

**C. Granulocyte chimerism accurately measures BM HSC chimerism.** Peripheral blood granulocyte (Ter119^CD3^-B220^-Mac-1^{high} side scatter^{high}) chimerism at 37 weeks post-transplantation was correlated with HSC (c-kit^+ lineage^- Sca-1^- CD34^- CD150^+) chimerism in the BM at 39 weeks post-transplantation upon sacrifice. Solid line illustrates linear regression with 95% confidence interval shaded in gray. Dashed line represents theoretical values if donor granulocyte chimerism were identical to donor HSC chimerism.

**D. Secondarily transplanted donor HSCs from ACK2 treated give rise to long term multilineage engraftment.** Peripheral blood chimerism of B cells (B), T cells (T), and
granulocytes (G) are shown 16 weeks post-secondary transplant for 2 independent experiments. Mean values +/- SEM are shown (n=7-8 in each experiment).
FIGURE 3-3:

A

weeks post transplant

% donor granulocyte chimerism

unconditioned

ACK2

**

B

cells in spleen ($\times 10^6$)

** unconditioned

ACK2

B

T

D

% donor chimerism

% donor HSC chimerism

unconditioned

ACK2

Exp.1

Exp.2

B

T

G

**
Figure 3-4: Donor chimerism increases with transplanted HSC cell number in ACK2-treated mice.

A. ACK2 treatment increases available HSC niche space. In two separate experiments, RAG2/-gc/- mice were treated with ACK2 and transplanted nine days later with varying doses of HSCs (CD45.1). Donor granulocyte chimerism was measured as above 24 weeks after transplantation for the first experiment, and 4 weeks for the second experiment. Mean values +/- SEM are shown.

B. Flow cytometry profiles of mice transplanted with 35,000 HSCs. Chimerism of CD3^+B220^-Mac1^high side scatter^high peripheral blood granulocytes is shown.
FIGURE 3-4:

A

24 wks- Exp. 1

4 wks- Exp. 2

% donor granulocyte chimerism

500 2000 35000

500 2500 13500

B

HSC

10^5

10^4

10^3

10^2

10^1

90.1

62.7

CD45.1 (donor)

0

10^2

10^3

10^4

10^5

0

10^2

10^3

10^4

10^5

CD45.2 (recipient)
Figure 3-S1. Total donor chimerism does not accurately reflect HSC chimerism following unfractionated bone marrow transplantation.

6.5 x 10^7 unfractionated nucleated bone marrow cells from wild type female CD45.2 mice were transplanted into unirradiated wild type female CD45.1 x CD45.2 F1 recipients. Total peripheral blood and BM HSC (defined as KLS CD135-CD34-) chimerism was measured 16 weeks after transplantation.
FIGURE 3-S1:
**Figure 3-S2:** ACK2 treatment leads to marked reduction of BM cellularity and increases in osteoblast size.

Humerus bones from untreated (Day 0) or ACK2-treated RAG2-/-gc-/- mice were isolated 2 or 9 days after antibody injection. Bones were fixed, decalcified, paraffin embedded, and hematoxylin and eosin stains were performed on sections. Ob, osteoblast; Er, immature erythroid colony.
FIGURE 3-S2:
**Figure 3-S3:** ACK2 administration results in depletion of HSC in both wild type and immunodeficient mice.

The number of KLS CD135-CD150+ HSCs in both femurs and tibia of ACK2-treated and control mice was determined by flow cytometry 2 days after treatment with 1mg of ACK2. Mean values +/- SEM are shown (n=2); ** indicates p-value<0.001.
FIGURE 3-S3:
**Figure 3-S4:** *ACK2 treatment does not result in HSC mobilization.*

The frequency of KLS CD135-CD150+ HSCs in the spleen, liver and blood of ACK2-treated and control mice was determined by flow cytometry. Spleen and liver were analyzed 4 days after treatment with 500µg of ACK2, whereas blood was analyzed in a separate experiment 9 days after treatment with 500µg of ACK2.
FIGURE 3-S4:
Figure 3-S5: ACK2 treatment results in near complete yet transient HSC depletion in vivo.

Bone marrow cellularity and HSC frequency were used to determine the number of HSCs in the bone marrow. Spleen sizes were also assessed for signs of extramedullary hematopoiesis. A) control, B) At time of ACK2 clearance (Day 9), C) One week post ACK2 clearance (Day 16). Flow cytometric profiles of HSCs in lineage- BM using 3C11, a c-kit-specific antibody that binds a distinct epitope from ACK2, as well as KLS CD135-CD150+ frequency, of which 19.5% are HSCs (Rossi et al., 2005), are shown adjacent to each corresponding time point. Mean values +/- SEM are shown (n=3 for each time point).
FIGURE 3-S5:
**Figure 3-S6:** ACK2 treatment selectively depletes HSCs at early timepoints.

Total numbers of HSCs (lin-c-kit+Sca-1+CD135-CD150+), MEPs (lin-c-kit+Sca-1-CD34-FcgR-), CMPs (lin-c-kit+Sca-1-CD34lowFcgRlow), and GMPs (lin-c-kit+Sca-1+CD34highFcgRhhigh) were quantified after administration of 500µg of ACK2 and compared to untreated control mice. Mean values +/- SEM are shown (n=3); ** indicates p-value<0.001 as compared to the relative number of HSCs at the same time point.
FIGURE 3-S6:
**Figure 3-S7:** Normal cell cycle status in recipient and donor HSCs after ACK2 treatment.

Cell cycle profiles of untreated controls and both recipient and donor HSCs from ACK2-treated RAG2-/-gc-/- mice that had been transplanted 7 months earlier with wild type HSCs were obtained. HSCs were identified as KLS CD150+CD34- cells.
FIGURE 3-S7:
Figure 3-S8: Multiple rounds of ACK2 treatment and HSC transplantation result in consistently high levels of donor chimerism.

RAG2-/-gc-/- mice were conditioned with 500µg of ACK2, and transplanted seven days later with CD45.1+ 5000 LT-HSCs. Mice were allowed to recover for seven days and were once again conditioned with ACK2 and transplanted seven days later with 5000 GFP+ LT-HSCs. A third round of ACK2 treatment and LT-HSC transplantation was performed using 5000 CD45.2+CD45.1+ HSCs. Peripheral blood was analyzed 24 weeks later for donor granulocyte chimerism as before. Chimerism values arising from each transplant in four separate animals are shown.
FIGURE 3-S8:

[Graph showing % donor granulocyte chimerism for Mouse 1 through 4, with bars color-coded for different transplant conditions: 1st Transplant (CD45.1), 2nd Transplant (GFP), 3rd Transplant (CD45.1x45.2), and Recipient (CD45.2).]
**Figure 3-TS1:** *Modest hematological effects of ACK2 treatment.*

Peripheral blood was sampled from a control mouse and an animal that had been treated with ACK2 9 days beforehand. Several hematological parameters were assessed.
FIGURE 3-TS1:

**Supplementary Table S1. Hematological effects of ACK2 treatment.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ACK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Blood Cells (x 10^6 cells/μL)</td>
<td>9.92</td>
<td>8.21</td>
</tr>
<tr>
<td>Hemoglobin (grams/dL)</td>
<td>14.5</td>
<td>11.8</td>
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<tr>
<td>Hematocrit (%)</td>
<td>45.9</td>
<td>36.9</td>
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<tr>
<td>Mean Corpuscular Volume (fL)</td>
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<tr>
<td>Mean Corpuscular Hemoglobin (pg)</td>
<td>14.6</td>
<td>14.4</td>
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<tr>
<td>Mean Corpuscular Hematocrit (g/dL)</td>
<td>31.6</td>
<td>32.0</td>
</tr>
</tbody>
</table>
CHAPTER 4

Gaining Access to the Hematopoietic Stem Cell Niche:

Novel Non-Myeloablative Conditioning Approaches

Agnieszka Czechowicz and Irving L. Weissman

Portions of this chapter were published in the following article:

Using purified HSC transplants, we have shown that in normal and immunodeficient mice, at any one point only a small number of HSC niches are readily available for transplanted donor HSCs and transplants without conditioning lead to very low donor HSC chimerism (0.5%) (Bhattacharya et al., 2006b). Regardless of the number of HSCs transplanted, once the available HSC niches are saturated additional engraftment cannot be obtained (Czechowicz et al., 2007). Importantly, only HSC can saturate these niches, and co-transplantation of 1,000 fold-excess of progenitors does not affects HSC engraftment, arguing that HSC occupy discrete niches from their downstream progeny (Czechowicz et al., 2007). These data mimic that observed by clinical transplanters who even in the absence of immune barriers, observe similarly very low levels of donor HSC chimerism upon transplantation of hematopoietic cells enriched for human HSC into immunodeficient patients not receiving conditioning (Muller et al., 2000). The low level of HSC engraftment in these patients is sufficient to restore immune function transiently through proliferation and expansion of immune progenitors, however over time these few engrafted HSC encounter exhaustion and loss of the graft is occasionally observed thereby necessitating ways to increase initial HSC engraftment even in the immunodeficiency setting (Cavazzana-Calvo et al., 2007).

Taken together, these studies suggest that in the absence of conditioning or facilitator populations, in both humans and mice donor HSC engraftment is limited by the availability of appropriate niches. Endogenous HSCs occupy appropriate, otherwise transplantable HSC niches, and therefore one strategy to enhance donor
HSC engraftment may be to deplete host HSCs. The development of reagents that specifically displace host HSCs, rather than myeloablative conditioning techniques currently in use, could lead to safer transplantation-based therapies for hematological and non-hematological disorders.

**Up-and-coming strategies to improve Hematopoietic Stem Cell Transplantation**

Hematopoietic stem cells are migratory cells (Wright et al., 2001). Under homeostatic conditions they can be found in blood circulation in addition to bone marrow, albeit at very low but physiologically relevant frequency (Bhattacharya et al., 2009). We have shown that HSC enter the blood stream via division-independent egress from the bone marrow, leaving behind empty HSC niches available for transplantation, and explaining why low levels of engraftment are observed in non-conditioned settings (Bhattacharya et al., 2009). HSC continually egress from the marrow and enter the blood, suggesting that additional HSC niches may become available over time. Concordantly, saturation of engrafted HSC niches is transient and indeed repeat rounds of HSC transplantation lead to additional donor HSC chimerism (Bhattacharya et al., 2006b) (Bhattacharya et al., 2009). This may be one important strategy through which with ease donor HSC engraftment can be increased.

Admittedly, the natural vacancy of HSC niches is very slow and therefore one proposed strategy to increase the competition between the donor and host HSC is to augment the vacancy of the HSC niches through mobilizing endogenous host HSC out of their marrow microenvironments and into circulation. This may be accomplished with reagents such as AMD3100, which cause significant mobilization without
noteworthy proliferation (Broxmeyer et al., 2005). Limited murine studies have shown such drugs to function as effective non-toxic conditioning therapeutics (Chen et al., 2006). However, even in the setting of HSC mobilization, transplanted donor HSC must still compete with displaced host HSC for HSC space. Therefore alternative strategies to enhance engraftment by eliminating endogenous competing HSC are desired.

HSC rely on a variety of signals for survival and maintenance of their stem-cell state. Specifically HSC have been shown to require continual kit-ligand (SCF) for survival, and inhibition of this signal results in apoptosis (Domen and Weissman, 2000). We have shown that ACK2, an antagonistic monoclonal antibody to the murine c-kit receptor (Ogawa et al., 1991) in immunodeficient mice eliminates murine HSC, and creates vacant HSC niches available for transplantation (Czechowicz et al., 2007). Donor HSC engraftment efficiency is significantly increased with such conditioning without any toxic side effects other than transient graying (as c-kit is additionally present on melanocytes). Transplantation of high doses of HSC or multiple rounds of ACK2 followed by HSC result in very high levels of mixed chimerism (>90%) (Czechowicz et al., 2007). Translation of such strategies, targeting human HSC, may result in non-myeloablative regimens that promote donor HSC engraftment with minimal toxicity, thereby significantly decreasing the morbidity and mortality currently experienced with present conditioning regimens.

Such novel conditioning strategies may be effective at obtaining high levels of HSC engraftment. However, conditioning methods including irradiation and cytotoxic agents not only play a role in creation of incoming space for HSC, but additionally act
as immune suppressants and play a role in immune-mediated HSC resistance. In the immunodeficient patients, such novel "space-creating" strategies in conjunction with purification of HSC may be sufficient to eliminate entirely the current toxicities associated with HCT. However, in immunocompetent settings additional reagents will need to be explored to inhibit the hosts’ immune system thereby preventing rejection of the incoming transplanted cells. Classically, T lymphocytes and natural killer (NK) cells are considered the primary immune mediators of allogeneic HSC resistance (Murphy et al., 1987). When transplant pairs are fully matched at the major histocompatibility complex (MHC) loci, T-cell immunity predominates. However, if MHC disparities exist, as in, for example, haplo-identical transplantations, NK cells also play an important role. Thus, reagents to eliminate the engraftment barrier must deplete or significantly impair the function of both types of lymphoid cells. Monoclonal antibodies may play a significant future role, as they may be used to transiently deplete host T and host NK cells prior to donor cell infusion. Multiple immunosuppressive monoclonal antibodies to human lymphocytes currently exist, including anti-CD2, CD52, CD3, CD4, and CD8, facilitating the generation of purely antibody-based non-toxic conditioning.

*Revolutionizing HCT*

Almost 60 years has passed since the early dismal but promising transplants performed by Thomas and colleagues (Thomas et al., 1957), and since we have learned much about the biology of blood and immune transplantation. Yet today we still face many of the same hurdles faced by our predecessors, namely the competing
challenges 1) complications arising from graft vs host disease syndrome and 2) toxicities associated with preparative regimens necessary for cell engraftment.

Recent data suggests we may be bordering on developing therapies that overcome these obstacles. By combining these strategies we may be at the tipping point to changing the practice and therefore application of hematopoietic cell transplantation. If the strategies outlined above, or others in their stead, are employed successfully, we may witness a new exciting wave of hematopoietic cell transplantation, and an expansion of the use of HCT from primarily for those with rapidly lethal diseases to patients with a variety of other hematolymphoid diseases for which HCT is currently unacceptable.

From the beginning of clinical hematopoietic cell transplantation, immunodeficiency has been a good initial disease target because it allows for separation of the immune transplant barrier from the other transplantation obstacles, affording scientists and clinicians the ability to sequentially optimize individual treatment components. In this manner, SCID will likely be the first disease treated with the modalities outlined above before they are extended to other applications. Moving forward, purified HSCT and novel conditioning strategies should allow for better treatment of SCID, obtaining higher donor engraftment without GvHD. Addition of antibody-based immunodepletion will subsequently allow for combating of non-malignant blood diseases. Thereafter transplant tolerance may be achievable using such strategies by co-transplantation of HSC and tissues/organs and similarly autoimmunity may be treated. The final goal is to treat patients who's organs have already been destroyed during autoimmune attacks, such as insulin-dependent type 1
diabetics lacking islet cells, and as has been shown in mice, concurrently transplant them with new organs as well as HSCs that impede rejection of the organ graft, prevent subsequent autoimmunity, and do not lead to GvHD (Beilhack et al., 2003). Such dreams may become a reality in the distant future, meanwhile the incremental successes in any of these realms will allow for the gradual expansion of hematopoietic cell transplantation as a therapeutic option for thousands of patients suffering from the diverse diseases of the blood and immune system.
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