
Immunologic targeting of the cancer stem cell

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Table of Contents

1. Introduction	2
2. Lessons from CML, a disorder of the hematopoietic stem cell	2
3. Allogeneic hematopoietic stem cell transplantation: a curative immune-based therapy resulting in eradication of malignant progenitor cells	3
3.1. Evidence supporting the existence of the Graft versus Leukemia effect	4
3.2. Dissection of the immunologic basis of GvL	5
3.2.1. Donor T cells as mediators of GvL and their target antigens	5
3.2.2. Donor B cells as mediators of GvL and their target antigens	6
3.2.3. Donor natural killer (NK) cells as mediators of GvL	8
3.3. Evidence that malignant progenitor cells are targeted by GvL	8
3.3.1. Effective GvL is associated with molecularly undetectable disease	8
3.3.2. GvL-associated T cell responses recognize leukemic progenitors	9
3.3.3. GvL-associated B cell responses target hematopoietic progenitor cells	10
4. Strategies for immunologic targeting of the stem cell population	10
4.1. Defining the tumor-initiating cell	11
4.2. Immunologic targeting of tumor-initiating cells	13
5. Acknowledgements	15
6. References	15

Abstract

Growing evidence has suggested that lack of eradication of the malignant stem cell forms the basis for cancer relapse and progression. In this regard, the clinical experiences of treating chronic myelogenous leukemia (CML), a prototypical stem cell disease, have been instructive, and are illustrative of the challenges facing the treatment of cancer when using potent cytoreductive agents that incompletely eradicate minimal residual disease. On the other hand, several decades of clinical and laboratory experience have demonstrated the curative potential of allogeneic stem cell transplantation for CML and other hematologic malignancies. As reviewed in this chapter, these studies have clearly demonstrated the curative potential of immune-based recognition of tumor cells, including the malignant progenitor cell population. These data set the stage for newer approaches that focus on immune targeting of antigens that are present on the cancer stem cell. Rational immune targeting

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of the tumor-initiating population critically depends on (1) identifying the unique surface markers of these cells so that they may be isolated, and on (2) defining antigens that are uniquely or preferentially expressed within the malignant cells with stem-cell like functions compared to normal cells. While debate continues as to the exact nature and defining characteristics of the cell population that is capable of propagating tumor, and hence the critical tumor cell sub-population that is required for immune targeting, several promising approaches for cancer immunotherapy are under investigation. Ultimately, combination therapy that includes both pharmacologic cytoreductive agents together with immunologic targeting of malignant stem cell populations may provide an effective curative approach with acceptable toxicity for the treatment of malignant diseases.

1. Introduction

By now, the existence of cancer initiating cells has been demonstrated in many malignancies (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Jamieson et al., 2004; Singh et al., 2003) (Schatten et al., 2008). These are rare quiescent cell populations that can be serially transplanted, have self-renewal capacity, and are frequently resistant to the cytotoxic effects of standard chemotherapy (Clarke et al., 2006). These characteristics have clear implications on disease progression and approaches to therapeutic intervention. Growing evidence has suggested that lack of eradication of the malignant stem cell forms the basis for disease relapse and progression. In this regard, the clinical experiences of treating chronic myelogenous leukemia (CML), a disease derived from a transformed stem cell population, have been instructive (Section 2), and are illustrative of the challenges facing the treatment of solid tumor malignancies as well. These studies have demonstrated the potent cytoreductive capability of recently developed pharmacologic therapies, but also their general inability to completely eradicate residual disease. On the other hand, a large body of clinical and laboratory experience over the past three decades has demonstrated the curative potential of allogeneic stem cell transplantation for CML and other hematologic malignancies. As reviewed in Section 3, these studies have clearly demonstrated that donor engraftment results in immunologic recognition of tumor cells, including the malignant progenitor cell population. These data set the stage for newer approaches that focus on immune targeting of antigens that are present on the cancer stem cell. While debate continues as to the exact nature and defining characteristics of the cell population that is capable of propagating tumor, and hence the extent of the total tumor cell population that is required for immune targeting, several promising approaches for cancer immunotherapy are under investigation. These approaches could include immunization and targeting of antigens with selective expression on malignant stem cells, coupled with reagents that can regulate the potency of the immune system (Section 4). Ultimately, combination therapy that includes both pharmacologic cytoreductive agents together with immunologic targeting of malignant stem cell populations may provide an effective curative approach with acceptable toxicity for the treatment of malignant diseases.

2. Lessons from CML, a disorder of the hematopoietic stem cell

Chronic myelogenous leukemia (CML) is a myeloproliferative disease associated with the *t(9; 22)* chromosomal translocation which encodes the oncogenic *BCR-ABL* fusion protein. This chimeric protein has tyrosine kinase activity and is essential for malignant transformation and the excessive myeloid cell expansion that characterizes CML. The natural history of CML has been well characterized; patients typically remain in chronic phase (CP) for several years, but inevitably transform to a more aggressive clinical syndrome (accelerated phase), and eventually to fatal blast crisis (BC) (Faderl et al., 1999).

Genetic studies performed over 20 years ago demonstrated that the malignant clone in CML is a pluripotent hematopoietic stem cell, capable of differentiating into myeloid cells, monocytes, erythrocytes, and platelets (Bernstein et al., 1992; Douer et al., 1981; Fialkow et al., 1977; Koeffler et al., 1980; Martin et al., 1980; Singer et al., 1980; Singer et al., 1979). These studies identified that only single enzyme phenotypes of the X-linked G6PD isoenzyme were present in cells across the spectrum of hematopoietic lineages in women with CML who were heterozygous for this gene. Thus, the malignant clone was determined to originate from the earliest hematopoietic progenitor cell. Phenotypic characteristics of human hematopoietic stem cells, precursors, and progenitor populations have been identified (Kondo et al., 2003), and in general, these subpopulations can be identified based on CD34+ expression in combination with present or absent expression of additional immunophenotypic markers. More recently, Jamieson et al. have demonstrated the property of self-renewal within the stem cell population of CML patients with chronic-phase disease and within the stem cell and myeloid progenitor populations in patients with blast crisis, that are associated with increased activity of the β -catenin pathway (Jamieson et al., 2004).

Inhibition of the *ABL* kinase, which is constitutively activated in CML as a result of the *BCR-ABL* translocation, has been a successful recently developed strategy for the treatment of CML. The specific tyrosine kinase inhibitor, imatinib mesylate, competitively inhibits the binding of ATP to the *ABL* kinase domain (Buchdunger et al., 1996), and thus has specific inhibitory activity for the tyrosine kinase encoded by the *BCR-ABL* fusion transcript (Buchdunger et al., 1996; Deininger et al., 1997; Druker et al., 1996). Phase I and II studies have demonstrated a high frequency of hematologic and cytogenetic responses in patients with accelerated phase or BC CML, as well as with newly diagnosed CP (Druker et al., 2001a; Druker et al., 2001b; Kantarjian et al., 2002). (Kantarjian et al., 2002; Sawyers et al., 2002). Moreover, a phase III randomized controlled trial has shown the superiority of imatinib to the combination of IFN α and cytarabine in newly diagnosed patients with CP-CML (IRIS trial) (O'Brien et al., 2003). Hematologic and major cytogenetic responses were observed in 98% and 84% of patients, respectively, on the imatinib arm, and these results were achieved with low toxicity. These impressive results led to its rapid FDA approval in 2001, and imatinib has emerged as standard front-line therapy for CML. Over the recent years, *ABL* kinase inhibitors of increased potency have been developed for the treatment of imatinib-refractory disease, and include dasatinib (FDA-approved in June, 2006) and nilotinib (FDA-approved in October, 2007) (Brave et al., 2008; Cortes et al., 2007; Guilhot et al., 2007; Hochhaus et al., 2008; Hochhaus et al., 2007; Kantarjian et al., 2007a; Kantarjian et al., 2007b; le Coutre et al., 2008). Taken together, the treatment of CML with *ABL* kinase inhibitors serves as a model for the successes of molecular targeting of signaling pathways that are vital for the survival of the malignant cell. This model has inspired the development of strategies that disrupt essential signaling pathways in other tumors, including in gastrointestinal stromal cell tumors (Blanke et al., 2008), and in lung cancer (Giaccone, 2005; Sequist et al., 2007).

Despite these striking clinical results, a few observations are notes for caution. First, in patients with advanced disease, drug-induced remissions in CML patients with advanced disease are not durable (Druker et al., 2001a) (Ottmann et al., 2002; Sawyers et al., 2002). Investigations into the mechanisms of imatinib drug resistance have supported the notions that: (a) *BCR-ABL* leads to overall genomic instability that may induce secondary genetic alterations (*BCR-ABL* gene amplification, new inactivating point mutations) contributing to *BCR-ABL* independent growth and/or survival of the malignant clone (Branford et al., 2002; Gorre et al., 2001; Hochhaus et al., 2002; Roche-Lestienne et al., 2002; Roumiantsev et al., 2002; Schindler et al., 2000; Shah et al., 2002); and (b) imatinib can select for the resistant disease clone in patients with advanced disease, as supported by patient mutational analysis (Bumm et al., 2003). Second, even in patients with less advanced disease, <5% become completely PCR-negative for *BCR-ABL* despite the frequent achievement of complete cytogenetic responses (CCR) (Hughes et al., 2003). In the IRIS trial, of the 68% of patients who achieved CCR after 1 year of therapy, 30% achieved a <2 log reduction in median *BCR-ABL* transcript levels by *BCR-ABL* qPCR. This low level of decrease in *BCR-ABL* transcript was associated with a 5–15% probability of disease progression at 24 months. In contrast, patients with a > 3 log reduction remained 100% progression free at 24 months. More recent studies have demonstrated that with prolonged first-line therapy, the probability of achieving molecular remissions increase, but 50% of patients still have detectable residual disease (Branford et al., 2007).

Consistent with the persistence of molecularly detectable minimal residual disease following imatinib therapy, Bhatia et al. identified malignant hematopoietic progenitors in 15 of 15 CML patients studied, who were in CCR following imatinib (Bhatia et al., 2003). Graham et al. reported that primitive quiescent Ph⁺ progenitor cells from CML patients are insensitive to imatinib *in vitro* (Graham et al., 2002). Dasatinib and nilotinib have also been demonstrated to select for the growth of clones bearing resistance mutations (Shah et al., 2007), and to ineffectively eradicate residual disease (Copland et al., 2006; Jorgensen et al., 2007), despite their increased potency compared to imatinib. Jiang et al. recently demonstrated that freshly isolated CML stem cells from untreated patients already harbour a high frequency of *BCR-ABL* mutations. Moreover, they identified more than 70 different *BCR-ABL* mutations in progeny of cultures of CML stem cells. These and other studies show that primary CML stem cells display instability of the *BCR-ABL* fusion gene, and that a reservoir of malignant progenitor cells persists in patients treated with *ABL* kinase inhibitors, that have the potential to develop into relapsed disease (Jiang et al., 2007). Quiescent drug-resistant cell subpopulations with clonogenic potential have been also identified in other cancers (Abbott, 2003; Challen and Little, 2006; Hadnagy et al., 2006).

3. Allogeneic hematopoietic stem cell transplantation: a curative immune-based therapy resulting in eradication of malignant progenitor cells

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established curative treatment approach for many hematologic malignancies. While the intensity and composition of the conditioning regimen are important to successful HSCT, the reconstitution of donor immune cells plays a critical role in the elimination of recipient tumor cells, a process termed graft-versus-leukemia (GvL). As will be reviewed in this section, over 30 years of clinical and

Table 1. Clinical evidence for GvL activity following allogeneic HSCT

-
- Lower relapse rate after transplantation of allogeneic stem cells compared to autologous or syngeneic stem cells
 - Positive correlation between GVHD and GvL
 - Temporal association of leukemia remission with episodes of acute or chronic GVHD
 - Disease remission after stopping immunosuppressive medications
 - Decreased relapse associated with GVHD
 - T cell depletion of donor stem cell graft is associated with higher relapse rates
 - Donor lymphocyte infusion induces remission of hematologic malignancies
 - Allogeneic HSCT after nonmyeloablative conditioning induces remission of hematologic malignancies and some non-hematologic malignancies
-

laboratory experience have provided clear demonstration that immunologic targeting of malignant hematopoietic cell populations can effectively generate lasting curative responses. This approach has even been applied to the treatment of solid tumors. These studies now provide the backdrop for the development of therapies that enhance the therapeutic benefit of allogeneic transplantation while minimizing the risks and toxicities associated with treatment.

3.1. Evidence supporting the existence of the Graft versus Leukemia effect

Several lines of clinical evidence, established over the last 30 years, have convincingly demonstrated the existence of GvL and its remarkable effectiveness for providing lasting immunologic rejection of malignant cells. As summarized in Table 1, these clinical studies began in the 1970s and 1980s, in which the risk of disease relapse after HSCT was found to be highly correlated with immunologic status of the recipient. For example, anti-leukemia response (i.e. GvL) was found to be strongly correlated with the presence of an immunologic toxicity of transplantation, graft-versus-host disease (GVHD). Leukemia relapse was 2.5 times more likely in recipients of syngeneic stem cells compared to HLA-matched allogeneic recipients who developed GVHD (Weiden et al., 1979). In addition, patients who experienced GVHD were found to have decreased risk of relapse from disease, and this appeared to be related to the extent of HLA matching between recipient and donor (Fefer et al., 1987; Sullivan et al., 1989; Weiden et al., 1981); (Butturini et al., 1987; Gale et al., 1994; Jones et al., 1991; Weisdorf et al., 1987). Examples of leukemia remission have also been observed to occur in association with worsening GVHD (Odom et al., 1978; Tricot et al., 1996), or after stopping immunosuppressive medications used for prevention of GVHD (Collins et al., 1992; Libura et al., 1999). Following single-institution reports describing the positive correlation between decreased relapse and acute and/or chronic GVHD, several large confirmatory registry studies were subsequently performed in North America (Horowitz et al., 1990) and in Europe (Ringden et al., 1996). Some patients in these retrospective studies had received stem cells grafts from which T cells had been depleted *in vitro* to prevent GVHD. T cell depletion efficiently prevented severe GVHD, but higher rates of relapse in these patients were observed compared with recipients of non-T cell depleted stem cells. This effect of T cell depletion was most often noted in patients with CML. Taken together, these studies demonstrated that GVHD was associated with a highly significant GvL effect, that was mediated by donor T cells in the stem cell products (Champlin et al., 1990; Goldman et al., 1988; Horowitz et al., 1990; Martin et al., 1988).

The efficacy of GvL responses in tumor elimination was definitively demonstrated in the early 1990s through the successful experience of using infusions of donor lymphocytes (DLI) to treat relapsed CML following HSCT (Kolb et al., 1990). The observation that donor lymphocytes alone, without further chemotherapy or radiation, could induce disease remission directly demonstrated the potent anti-tumor activity of donor-derived immune effector cells. Since this initial report, multi-center experiences in Europe and North America have confirmed the efficacy of DLI for inducing GvL (Collins et al., 1997; Kolb et al., 1995). From these clinical studies, it is clear that DLI is especially effective in the treatment of stable phase CML, where durable responses occur in 75–80% of patients. In contrast, response rates of patients with multiple myeloma and CLL have ranged from 30–50% (Lokhorst et al., 1997; Mandigers et al., 2003; Rondon et al., 1996; Tricot et al., 1996); and of patients with acute leukemia, only 10–15%. Consistent with these observations, DLI is generally more effective in patients with lower disease burdens (van Rhee et al., 1994). Only 5–10% of patients with advanced CML (blast crisis/accelerated phase) respond to DLI. Interestingly, clinical responses following DLI are often delayed until 2–4 months after a single infusion. This prolonged interval may reflect the time required to mount an effective response when the frequency of naïve T cells capable of responding is low. Alternatively, delayed responses may reflect the time required to demonstrate the impact of lysing those cells that comprise the originating malignant clone. DLI responses for at least some diseases are highly durable (Mattei et al., 2001; Shimoni et al., 2001). For example, Porter et al. reported the probability of survival at 1, 2 and 3 years following DLI therapy to be 83, 76 and 73%, respectively (Porter et al., 1999).

With the demonstration that GvL plays an important role in the elimination of leukemia cells following transplant, many studies have begun to examine the feasibility of using less intensive, non-myeloablative conditioning regimens to prepare patients for allogeneic HSCT. A variety of non-myeloablative conditioning regimens have been developed and a large number of clinical studies have demonstrated the effectiveness of this approach, especially in patients who are ineligible for more intensive conditioning (Khouri et al., 1998; McSweeney et al., 2001) (Alyea et al., 2005; Morris et al., 2004). Reduced intensity conditioning regimens are associated with substantially less toxicity but nevertheless provide sufficient immune suppression of the recipient to prevent rejection of allogeneic hematopoietic stem cells. Since non-myeloablative conditioning regimens are insufficient to eliminate leukemia cells in the recipient, long-term remissions are primarily dependent on immunologic mechanisms mediated by donor cells. Similar to the myeloablative setting, GvL responses following non-myeloablative transplant are often associated with GvHD (Crawley et al., 2005). Using this approach, recent clinical trials in patients with solid tumors suggest that graft versus tumor responses can be observed in at least some of these patients (Childs et al., 2000; Demirer et al., 2008; Tykodi et al., 2004; Ueno et al., 2003)

3.2. Dissection of the immunologic basis of GvL

Many laboratory studies have sought to define the immunologic mechanisms that contribute to GvL. Although stem cell and DLI products contain a variety of mononuclear cell types, T cells have been generally presumed to comprise the predominant effector cells in these products. Recent studies, however, have also implicated a role for B and NK responses in GvL responses. In this section we consider evidence of the role of each of these cell populations in GvL.

3.2.1. Donor T cells as mediators of GvL and their target antigens

In murine models, both CD4+ and CD8+ T cell populations have been shown to contribute to GvL activity *in vivo* (Truitt and Atasoylu, 1991). Removal of either of these T cell populations results in compromise of GvL reactivity, indicating that CD4+ and CD8+ T cells are both required for generating optimal GvL. In patients who have undergone allogeneic HSCT, both CD4+ and CD8+ leukemia-reactive T cells have also been identified. In recent years, much activity has been directed towards the precise identification of the target antigens of donor T cells after allogeneic HSCT, since a better understanding of the precise peptide epitopes recognized by T cells can potentially lead to the optimization of strategies to distinguish GvL and GvHD effects *in vivo*.

Most strategies for identification of T cell epitopes have relied on examination of cytolytic activity of donor-derived T cells against host antigens, identified by either biochemical approaches or by testing against cDNA expression libraries derived from host tissue. Using these strategies, many minor histocompatibility antigens (mHAs) have been identified. mHAs are antigens that occur as a result of genetic polymorphisms that exist throughout the human genome (Mullally and Ritz, 2007), and thus are a reflection of those polymorphic self-peptides that distinguish any two individuals. Transplantation of mature T cells during allogeneic HSCT results in the transfer of large numbers of cells capable of recognizing these mHA.

As schematically represented in Figure 1, the clinical consequences of mHA appear to result entirely from their expression in different cell types and the recognition of these antigens by donor T cells (Akatsuka et al., 2003; Dickinson et al., 2002; Kloosterboer et al., 2005). Most of the human mHA identified thus far have broad tissue expression and the targeting of these varied cell types by donor T cells represents one of the initiating events of GvHD (Dickinson et al., 2002). For example, one well-characterized class of broadly expressed mHA are genes on the Y chromosome. Males are tolerant to these Y chromosome-encoded proteins (H-Y antigens) but T cells reactive with H-Y peptides are not deleted in normal females (Foote et al., 1992; Wang et al., 1995). Females are tolerant to the expressed X chromosome homologue and when exposed to H-Y antigens through pregnancy or blood transfusion can develop long-lived T cell responses to these mHA (James et al., 2003; Verdijk et al., 2004). Similarly engraftment of female T cells in male recipients can lead to the expansion of H-Y specific donor T cells (Pierce et al., 1999; Takami et al., 2004; Vogt et al., 2000a). Evidence that these are targets of donor-derived immunity include the discovery of increased incidence of GvHD in male recipients of stem cell grafts from female donors (Atkinson et al., 1986; Flowers et al., 1990; Gratwohl et al., 2001; Randolph et al., 2004). This is presumed to be due to the broad tissue and cellular expression of H-Y proteins and the immunogenicity of these antigens. Many of the H-Y mHA contain multiple disparate amino acids when compared to their X-homologues and these peptide epitopes are presented by both MHC class I and class II molecules (Spierings et al., 2003b; Torikai et al., 2004; Vogt et al., 2002). Both of these factors likely contribute to the high level of immunogenicity of these antigens. Non-Y chromosome encoded

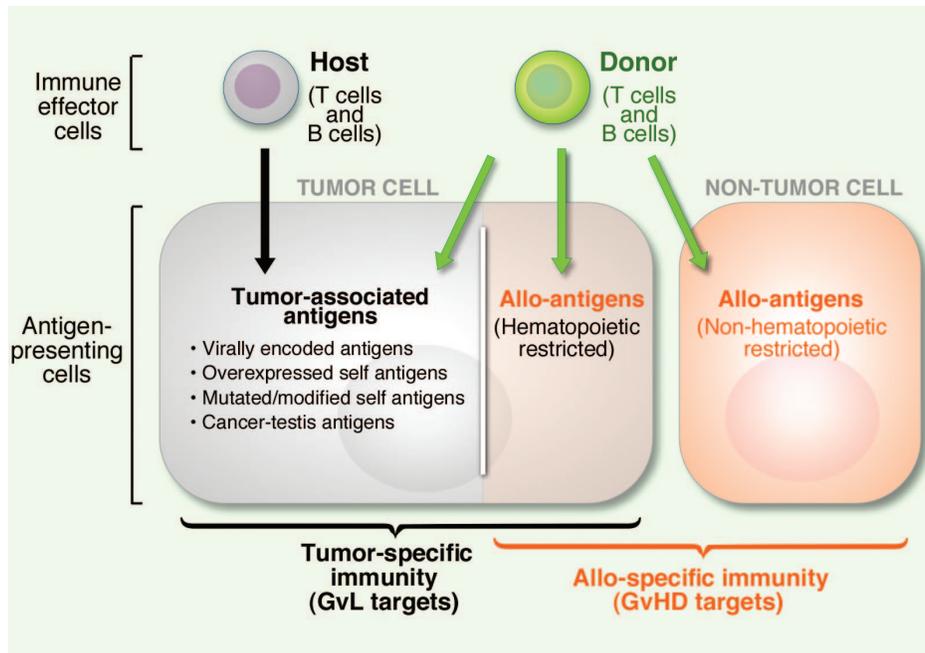


Figure 1. The spectrum of target antigens associated with tumor immunity and allo-immunity after allogeneic HSCT. Host-derived T and B cells can be induced to recognize tumor-associated antigens, whereas donor-derived B and T cells can recognize both tumor-associated antigens and alloantigens.

mHA include autosomal mHA that are generated on the basis of single nucleotide polymorphisms that differ between donor and recipient. These genetic differences can lead to creation of alternate transcripts, differences in proteasomal processing (Brickner et al., 2001; Spierings et al., 2003a) and distinct post-translational modifications (Meadows et al., 1997) as well as simple substitution of single amino acids in the antigenic peptide (den Haan et al., 1998; Mommaas et al., 2002; Pierce et al., 2001; Vogt et al., 2000b). Recently described gene deletion polymorphisms may also play an important role in the generation of mHA (Murata et al., 2003).

To the extent that mHA are expressed by both malignant and normal hematopoietic cells in the recipient but not other tissues, targeting of these antigens will contribute to GvL and conversion to full donor hematopoiesis but will not contribute to GvHD (Bleakley and Riddell, 2004). For this reason, targeting mHA with restricted hematopoietic expression has been proposed as an important mechanism for distinguishing GvL from GvHD following allogeneic HSCT and DLI (Hambach and Goulmy, 2005; Mutis and Goulmy, 2002; Riddell et al., 2003).

Other categories of GvL targets include tumor-specific antigens, virally encoded tumor antigens, overexpressed self-antigens, mutated or modified self-antigens, and cancer-testis antigens. For hematopoietic malignancies, there are many potentially important antigens in these categories, including chimeric *BCR-ABL* (Bocchia et al., 1995; Cathcart et al., 2004) and *PML/RAR α* (Bocchia et al., 1995) proteins, latent EBV antigens, proteinase-3 (Molldrem et al., 2000), WT-1 (Azuma et al., 2002), survivin (Reker et al., 2004), ML-IAP (Schmollinger et al., 2003) and cancer-testes antigens such as SLLP1 (Wang et al., 2004). In these instances, the immunogenicity of these targets is not dependent on genetic disparity between recipient and donor. Since these are not targets of allo-reactivity, these represent possible targets of the host immune effectors (Symons et al., 2008). However recipients with leukemia may have become tolerant to these antigens, whereas normal donors may not be tolerant to the antigens and remain capable of developing effective immune responses after transplantation.

3.2.2. Donor B cells as mediators of GvL and their target antigens

Several recent studies have suggested that B cells are also likely to play an important role in GvL. As part of the adaptive immune response, B cells can enhance immunogenicity of tumors by secretion of cytokines and chemokines and antigen-antibody immune complexes facilitate antigen delivery to antigen presenting cells and can thereby enhance antigen-specific T cell activation. When directed against cell surface molecules, antigen-specific antibodies that can directly lyse tumor cells through antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated lysis.

As for T cells, mHA and tumor associated antigens are both potential targets of donor B cells that can contribute to GvL (and GvHD) following allogeneic HSCT (Figure 1). Miklos et al. reported that the Y encoded mHA DBY is frequently targeted by antibody responses following female → male HSCT (Miklos et al., 2004). Of note, 50% of male patients who received stem cell grafts from female donors developed humoral immunity to recombinant DBY protein compared to 5% of male patients with male donors. Very few of the patients developed antibodies against the X-encoded homologue, DBX, and antibody responses were directed primarily against areas of amino acid disparity between DBY and DBX. When this analysis was extended to a panel of 5 recombinant H-Y encoded proteins (DBY, UTY, ZFY, RPS4Y, and EIF1AY) and their X chromosome homologues, 89% of patients with at least one H-Y antibody developed chronic GVHD compared to only 31% of patients without antibodies to this panel ($p < .0001$). Moreover, 48% of patients without H-Y antibodies relapsed compared to 0% of patients with H-Y antibodies ($p < .0001$) (Miklos et al., 2005). Clinical studies at our center and others have recently suggested that rituximab, a B cell directed therapy, may improve some of the clinical manifestations of chronic GvHD and larger trials evaluating this approach have been initiated (Ratanatharathorn et al., 2003) (Cutler et al., 2006). However, inhibition of B cell responses may also reduce GvL activity and this should be closely monitored in these patients.

Antibody responses against tumor-associated antigens have also been identified in association with GvL activity after allogeneic HSCT (Bellucci et al., 2004; Hishizawa et al., 2005; Wu et al., 2000). In a series of previous studies, we were motivated to discover the target antigens of the DLI associated B cell immune response, based on unexpected finding of clear peripheral B cell lymphocytosis and plasma cell marrow infiltration developing at the time of clinical response (that was not complicated by concurrent GvHD) (Bellucci et al., 2002) in a series of patients enrolled on trials of DLI at DFCI (Alyea et al., 1998; Bellucci et al., 2002). These studies have led to the detection of the presence of potent humoral immunity – at levels comparable to anti-viral responses – that was temporally correlated with clinical tumor regression in a series of patients with CML (Wu et al., 2000). We first detected the presence of anti-tumor humoral immunity in association with DLI when we immunoblotted lysates generated from a CML cell line, K562 cells against sera derived from a DLI-treated patient, and we visualized new bands detected by post DLI but not pre-DLI sera, signifying the detection of new antibody responses to CML target antigens following DLI. Subsequently, by antibody-based expression cloning, in which plasma from responders of DLI therapy was used as a source of antibody to screen a CML cDNA expression library that we generated from patient samples, a series of candidate target antigens against which post-DLI but not pre-DLI nor pre-BMT sera was reactive were identified (Sahin et al., 1997; Wu et al., 2000). These encoded a variety of known and novel genes involved in various cellular functions, including gene transcription, cell cycle, and cell signaling. A subset of antigens was found to be highly expressed in a broad array of tumors, but in only a narrow range of normal tissues. Two of the novel CML-associated antigens in this group, CML66 and CML28, have been characterized in depth (Yang et al., 2002; Yang et al., 2001). Antibody responses to both of these targets were not present before transplant or before DLI. High titer antibodies developed 2–3 months after DLI coincident with the achievement of cytogenetic and molecular remission of CML. Unlike H-Y genes, neither of these genes appears to encode polymorphisms that distinguish the alleles expressed in the transplant donor and recipient. Moreover, antibodies to CML66 and CML28 were not present in patients with chronic GvHD, patients with CML who underwent T cell depleted allogeneic HSCT or normal donors. Remarkably, antibodies to these 2 antigens were also present in patients with CML who had responded to treatment with interferon- α , but not in patients treated with hydroxyurea or imatinib. These studies suggest that the immunogenicity of both of these proteins derives from their high-level expression in leukemia cells and not because they represent novel allo-antigens.

Some targets of immune treatment induced humoral immunity have been identified to be surface expressed antigens. For example, Bellucci et al. identified BCMA, a member of the TNF receptor superfamily that is selectively expressed on the surface of mature B cells and myeloma cells, as a DLI-associated target in patients with myeloma (Bellucci et al., 2005). Antibodies that developed *in vivo* after DLI were found to be reactive with cell surface domain of BCMA. When tested in functional assays, these IgG antibodies in patient serum were able to mediate complement-induced lysis and ADCC of stably transfected BCMA expressing cells or primary BCMA expressing myeloma cells. These antibodies were detected in 2 of 9 DLI responders and persisted for long periods after DLI. Other recent studies have demonstrated that antibodies against surface expressed antigens can generate specific receptor-ligand effects that enhance tumor cytotoxicity (Jinushi et al., 2006) (Jinushi et al., 2008). On the other hand, the great majority of antigens identified by serological screening are intracellular proteins. While these may merely represent the development of an immune response to extruded contents of lysed tumor cells, antibodies to intracellular antigens are able to facilitate cross-presentation of target antigens through an Fc γ R mediated pathway in dendritic cells, and can result in the stimulation of CD8+ T cell responses to peptide epitopes within the target protein (Amigorena, 2002; Dhodapkar et al., 2002; Kita et al., 2002) (Valmori et al., 2007) Taken together, these observations suggest that B cell responses to leukemia-associated likely contribute to GvL activity *in vivo* through a variety of mechanisms.

3.2.3. Donor natural killer (NK) cells as mediators of GvL

Natural killer (NK) cells are the first of lymphoid lineage cells to reconstitute following allogeneic transplantation, and adequate recovery of NK cell number in the early post-transplant period has been associated with improved relapse-free outcome (Jiang et al., 1997). Engagement of NK cell receptors results in stimulation or inhibition of NK cell effector function, depending on intracellular signaling mediated through the cytoplasmic tail or adaptor molecules associated with each receptor (Chiesa et al., 2005; Moretta and Moretta, 2004). Although the NK cell response to a target depends on the net effect of activating and inhibitory receptors, it is predominantly negatively regulated by KIRs, or MHC class I specific killer inhibitory receptors (KIR) or receptors. Adequate expression of appropriate inhibitory NK receptor ligands protects healthy “self” cells against NK cell lysis. However, in the absence of this inhibitory pathway, targets become susceptible to NK mediated lysis. In the setting of allogeneic HSCT, the results of NK activity depend on the directionality of lysis (Hsu and Dupont, 2005; Ruggeri et al., 2005a). When the NK cells are donor-derived and recipient cells lack expression of the cognate KIR ligand, the donor NK cell lysis of recipient target cells can result in GvL and/or GvHD, depending on the tissue origin of the NK target. However, if the target cell is of donor origin, and the NK effector cell is of recipient origin, NK cell lysis can result in graft rejection. Recent studies have demonstrated that NK cell effector capacity is influenced by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. (Pfeiffer et al., 2007; Yu et al., 2007) It has been estimated that NK cell alloreactivity can be expected to occur in about 50% of unrelated donor transplants with one or more HLA allele level mismatches.

Studies performed two decades ago demonstrated the presence of lytic NK cell activity against host-derived leukemia cells following HSCT (Hauch et al., 1990; Hercend et al., 1986). Compelling studies performed more recently by Ruggeri et al. have examined the role of NK cell activity in patients who received T cell depleted stem cell transplants from HLA-mismatched donors (Ruggeri et al., 1999; Ruggeri et al., 2002; Ruggeri et al., 2005b). In this setting, KIR on donor NK cells are potentially mismatched with their inhibiting HLA-ligands and are capable of recognizing and killing recipient leukemia cells. Moreover, NK alloreactivity was predicted on the basis of HLA-B and HLA-C typing, and donor-recipient pairs were divided into two groups: those with KIR ligand incompatibility in the donor versus host direction, and those without. In a clinical trial that included 112 patients with high-risk acute leukemia, predicted NK alloreactivity was highly correlated with transplant outcome in patients with AML. Notably, event-free survival for AML patients in the KIR ligand incompatible group was 60%, compared to only 5% in the compatible group. Moreover, screening donor-derived NK clones for lysis against recipient cells confirmed that KIR ligand incompatibility correlated closely with the detection of donor NK clones killing recipient targets. These and other studies have demonstrated that KIR ligand incompatibility in the donor versus host direction appeared to protect patients with AML against graft rejection, GVHD and leukemia relapse (Hsu et al., 2005; Savani et al., 2007). The feasibility and safety of adoptive transfer of expanded NK cells for the treatment of relapsed leukemia has been demonstrated, and ongoing efforts are designed to evaluate the effectiveness of this approach (Miller et al., 2005).

3.3. Evidence that malignant progenitor cells are targeted by GvL

The curative potential of stem cell transplantation and DLI implies that malignant progenitor cells are included within the scope of recipient cells targeted by GvL. In this section, we summarize evidence from studies of molecular monitoring of disease response to therapy, and of characterizing the target antigens of T and B cells responses following HSCT that support this concept. Representative examples of these studies are shown in Figure 2.

3.3.1. Effective GvL is associated with molecularly undetectable disease

The presence of the disease-specific translocation *BCR-ABL* has facilitated the development of sensitive molecular assays to detect transcript expression of this fusion gene product as a measure of disease burden. The achievement of molecularly undetectable status consistent with curative responses and elimination of malignant self-renewing populations has been consistently demonstrated in CML patients following treatment with HSCT. Figure 2A depicts the results of molecular monitoring of a group of 50 patients treated at the Dana-Farber Cancer Institute/Brigham and Women’s Hospital, Boston (unpublished data). Our laboratory uses the *GUS* (β -glucuronidase) as a control transcript, which has been previously validated (Muller et al., 2008). As shown in this figure, untreated CML patients express a %*BCR-ABL/GUS* ratio between 10–100% (our standard reference baseline), whereas patients without the *BCR-ABL* transcript (i.e. patients without CML, such as patients with chronic lymphocytic leukemia (CLL)) have undetectable transcript levels (%*BCR-ABL/GUS* = <0.001–0.0001%). Consistent with results reported by other investigators, samples received from patients treated with imatinib for > 1 year, *BCR-ABL* transcript levels decreased variably over a 5 log range, with many achieving a < 2 log transcript reduction from the standard reference baseline following imatinib monotherapy. In contrast, our assay measures low to undetectable amounts of disease in patients who have achieved

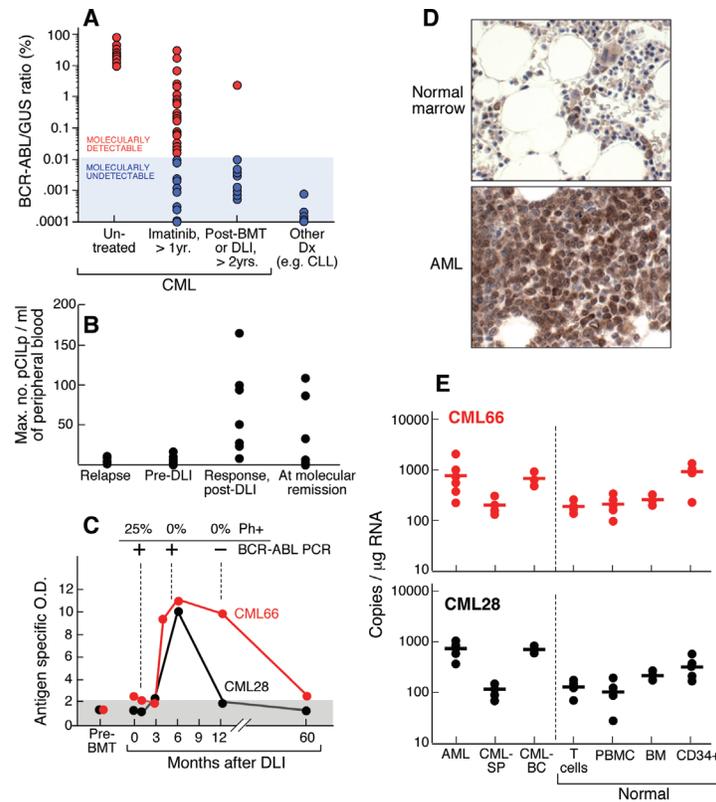


Figure 2. Representative studies providing evidence that malignant progenitor cells are targeted by GvL responses. (A) Effective GvL is associated with molecularly undetectable disease. Results of molecular monitoring of *BCR-ABL* transcript (relative to control *GUS* transcript) of 50 patients treated at BWH/DFCI with untreated disease, following imatinib therapy, following allogeneic HSCT, and of patients without CML. (B) Effective GvL is associated with T cell responses that recognize leukemic progenitors (adapted from Smit et al. 1998). The pCILp assay measures the frequency of T cell precursors in peripheral blood that are capable of suppressing the growth of CD34+ CML progenitor cells. (C) Effective GvL is associated with B cell responses against antigens expressed on leukemic progenitors. Clinical response to DLI in a CML patient responses is associated with high titer antibody responses against CML66 and CML28. Dashed line represents 250D above mean of 10 normals. (D) CML66 is highly expressed in myeloid progenitor cells based on marrow immunohistochemical staining of marrows from patients with AML compound to normal bone marrow. (E) CML28 and CML66 are highly expressed in myeloid progenitor cells based on studies of quantitation of RNA transcripts. Relative copy numbers of CML66 and CML28 transcripts was measured in total RNA derived from differentiated and undifferentiated normal and malignant hematopoietic cells, measured by gene-specific RT-PCR. CML-SP = stable phase CML; CML-BC = blast crisis CML; AML = acute myeloid leukemia.

immune-therapy (BMT- or DLI-) induced remission. Moreover, these patients remain molecularly undetectable for years.

Successful allotransplant for the treatment of chronic lymphocytic leukemia and multiple myeloma has also resulted in molecularly undetectable disease remissions (Corradini et al., 2003) (Esteve et al., 2001). The durability of clinical responses following DLI appears to be linked to achievement of molecular remission, suggesting that the effectiveness of DLI similarly results from immunologic elimination of the malignant stem cell clone (Dazzi et al., 2000).

3.3.2. GvL-associated T cell responses recognize leukemic progenitors

Pichert et al. examined the relative contributions of the transplant preparative regimen and immunologic mechanisms post-transplant on the elimination of leukemia cells in the recipient (Pichert et al., 1995). Transplants were performed using T cell depleted or unmanipulated marrow products. Of 92 patients treated with TCD myeloablative transplant for CML, who demonstrated disease suppression without clinically apparent GvHD, laboratory studies indicated that the *BCR-ABL* positive cells detected in these individuals represented early progenitor cells derived from the CML clone (Pichert et al., 1994). The persistence of these CML cells in the vast majority of patients who received myeloablative therapy therefore suggested that high-dose conditioning regimens, by themselves, did not effectively eliminate leukemia cells. In contrast, immunologic mechanisms mediated by donor T cells appeared to be more important for suppressing leukemia cells after transplant and preventing leukemia relapse.

Other investigators have examined the antigenic specificities of bulk T cell lines and T cell clones expanded from patients with clinical GvL responses, and have demonstrated targeting of the CD34+ cell population. As shown in Figure 2B, Smit et al. detected elevated percentages of CTLs that were inhibitory to CML CD34+ cells in patients who demonstrated clinically evident GvL following DLI therapy (Smit et al., 1998). Falkenburg et al. isolated CTL clones directed against mHA that are capable of antigen-specific lysis of freshly obtained leukemic cells and inhibition of leukemic precursor cells *in vitro* (Falkenburg et al., 1999). Bonnet et al. demonstrated that human CTL clones specific for mHA can preferentially target leukemia stem cells and that T cells clones with this type of specificity could effectively eliminate transplanted leukemia cells in NOD/SCID mice (Bonnet et al., 1999). Rezvani et al. have detected an association between post-transplant GvL effects and detection of cytotoxic CD8+ T cells against WT-1, which is highly expressed in malignant hematopoietic progenitor cells (Rezvani et al., 2007).

3.3.3. GvL-associated B cell responses target hematopoietic progenitor cells

As shown in Figure 2C–E, CML66 and CML28, B cell targets associated with GvL responses following DLI, have been found to be highly expressed in myeloid progenitor cells but not in more mature myeloid cells (Wu et al., 2005). We propose that the combination of serologic immunoscreening with studies of gene expression can efficiently identify promising antigens for the immunologic targeting of stem cells. Recently, with expanded serology-based antigen discovery of targets of GvL, we have discovered that the vast majority of DLI-associated antigens are expressed on CD34+ cells. We probed two complementary immunoproteomic platforms, a bacteriophage expression library and a high-density protein microarray, using post-therapy plasma immunoglobulin from seven CML patients who each demonstrated clinically apparent GvL without graft-versus-host disease (GvHD) after DLI. In total, 62 antigens elicited increased reactivity in post-DLI compared to pre-DLI plasma. Analysis of gene expression in normal and malignant myeloid cells using HG-FOCUS and HG-U133A Affymetrix microarrays confirmed that >70% of the antigens have detectable gene expression in CML CD34+ cells. Four antigens (RAB38, TBCE, DUSP12, and VPS4B) were expressed at higher levels in CML compared to normal CD34+ cells ($p < 0.002$). As a collection, the identified antigens represent potential immunogens or reagents for the monitoring of immunotherapeutic strategies designed to eliminate myeloid leukemia stem cells (Biernacki et al., 2007).

In an analogous fashion, Spisek et al. recently described the detection of antibody responses in patients with the myeloma precursor disorder, monoclonal gammopathy of unknown significance (MGUS) but not with myeloma against SOX2, a gene required for self-renewal in embryonal stem cells. SOX2 was found to be highly expressed in the progenitor cells that mark the clonogenic compartment of MGUS, and to elicit frequent cellular immunity in patients with MGUS. Of note, CTLs generated against this antigen inhibited clonogenic growth of MGUS *in vitro*. Furthermore, detection of antigen-specific CTLs in MGUS patients was associated with diminished progression to malignant disease and improved clinical outcome (Spisek et al., 2007).

4. Strategies for immunologic targeting of the stem cell population

The previous sections of this review have summarized a large body of data demonstrating the potency of the donor immune system for the elimination and long-term control of malignant cells. As schematically shown in Figures 3A and 3B, while myeloablative transplant preparative regimens by themselves can reduce disease burden, the studies described in the previous sections demonstrate that immune responses generated from engrafted donor-derived immune cells are instrumental to the final elimination of residual disease, leading to cure. Although HSCT and DLI continue to be associated with significant toxicity and disease control is not achieved in all patients, allogeneic transplant represents an excellent example of the clinical results that can be achieved through a therapy based on immunologic mechanisms.

Many aspects of GvL after allogeneic HSCT are not well understood. However, steady progress in the characterization of the GvL targets of T and B cell responses has revealed that clinically effective immune responses appear to be polyclonal, are directed at a wide variety of both allo- and tumor-associated antigens, including those with expression on progenitor cells, and frequently involve a coordinated cellular, humoral and innate immunity. Moreover, clinical responses are more easily generated in the setting of low disease burden and slowly proliferating tumor cells, especially when less intensive chemotherapy and radiation therapy are used to prepare patients for allogeneic HSCT. As shown in Figure 3C, GvL effects can be potentially enhanced by incorporating active vaccination of the recipient after engraftment of donor cells to either induce or enhance responses to mHA or tumor-associated antigens. Model systems have suggested that this approach is feasible and potentially effective (Anderson et al., 2000; Durakovic et al., 2007; Luznik et al., 2003; Teshima et al., 2002) but the selection of methods for vaccination as well as timing and dosing of vaccines must take into consideration the ability of the reconstituting donor immune system to respond to antigenic challenge and a variety of patient factors.

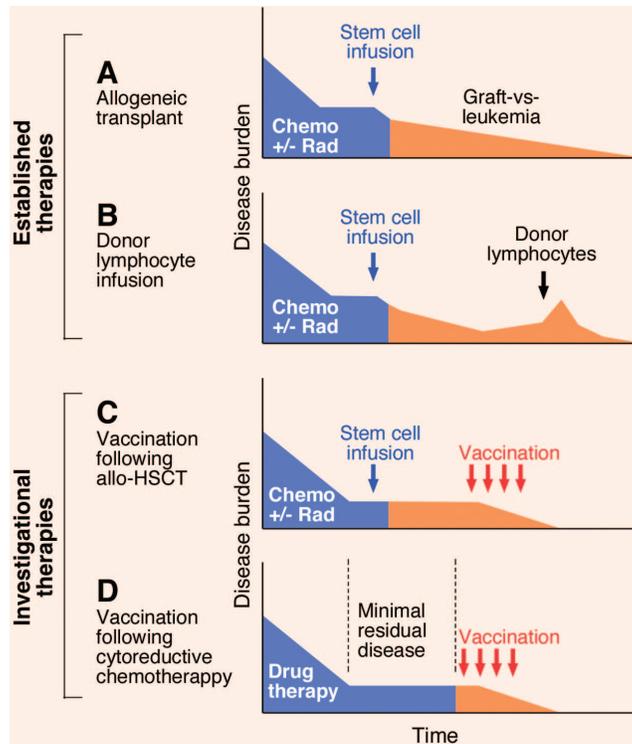


Figure 3. Schematic representation of current (A and B) and promising future strategies (C and D) to stimulate anti tumor immune responses (in orange).

Or, as suggested in Figure 3D, can the principles of the graft-versus-leukemia response be applied to the development of effective tumor-specific immunotherapy in the autologous setting? More specifically, can polyvalent cellular and humoral immune responses be targeted against the malignant stem or progenitor cell population? Optimally, this would require achievement of a state of minimal residual disease, and would spare nonmalignant cells, and hence minimize the risk for autoimmunity. As discussed in this section, rational immune targeting of the tumor-initiating population critically depends on (1) identifying the unique surface markers of these cells so that they may be isolated, and on (2) defining antigens that are uniquely or preferentially expressed within the malignant cells with stem-cell like functions compared to normal cells. Achieving this goal, however, is complicated that the fact that the exact immunophenotype of the initiating cells of many tumor types (especially solid tumors) is presently highly controversial (LaBarge and Bissell, 2008). Clarification of the identity of the tumor-initiating cell has important implications on the extent and feasibility of using immune responses to therapeutically target cancer: e.g. whether targeting is required against the entire tumor cell population, or only single or a collection of distinct cell subpopulations. Strategies for immune targeting are discussed.

4.1. Defining the tumor-initiating cell

Achieving the goal of immunologically targetting the “cancer stem cell” requires knowledge of precise immunophenotype of this cell population, so that the antigens unique to this cell population can be identified. While this notion is conceptually straight-forward, and while there is general agreement that tumors inevitably arise from a cell population that can self-propagate and renew, exact origins of this cell are the subject of great debate. As shown in Figure 4, at least two different models have been proposed for tumor propagation, based on experimental evidence (Adams and Strasser, 2008; Shipitsin and Polyak, 2008). On the one hand, the “hierarchical model” or “cancer stem cell hypothesis”, is based on the concept that only a rare cell population is responsible for the self-renewing and self-propagating properties of cancer (see Figure 4A). These specialized cell populations were originally identified in myeloid leukemia as cells with primitive hematopoietic progenitor phenotype ($CD34^+ CD38^-$) that, when injected into immunodeficient mice, could engraft and regenerate the same human leukemia. Moreover, it was established that the resulting leukemia could be serially transplanted into secondary recipients, whereas injection of more differentiated leukemic cells could not (Bonnet and Dick, 1997; Lapidot et al., 1994). Using this same methodologic approach, cell populations with these characteristics have been defined for a number of solid tumors. For example, Al-Hajj et al. reported that $CD24^{-/low}/CD44^+$ fractions from metastatic pleural effusions and a primary invasive breast

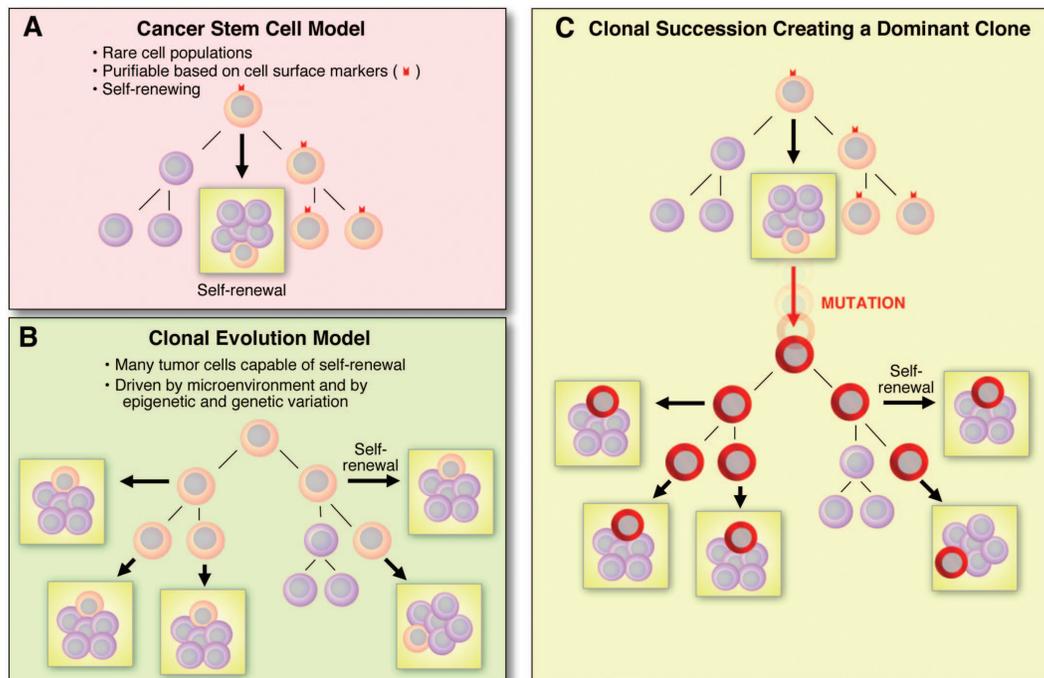


Figure 4. Models for the nature of sustained tumor growth (adapted from Adams and Strasser 2008 with permission).

tumor had higher tumorigenic potential when injected into the mammary fat pad of female NOD/SCID mice than CD24⁺/CD44^{+/−} cell fractions (Al-Hajj et al., 2003). In support of the cancer stem cell model in breast cancer, Liu et al. identified a distinct gene signature associated with tumorigenic CD24^{−/low}/CD44⁺ cell populations compared to normal breast epithelium and observed correlation between this “invasive” gene signature with shorter distant metastasis-free and overall survival. These studies suggest that presence and frequency of this cell population has prognostic relevance (Liu et al., 2007). Other investigators have used the stem cell markers CD133 or CD44 to purify putative cancer stem cells in several tumor types (Collins et al., 2005; Li et al., 2007; Zhang et al., 2008). Of note, treatment of mice transplanted with human AML cells with activating anti-CD44 antibody markedly reduced leukemic repopulation, presumably due to interference with AML stem cell engraftment and repopulation capabilities (Jin et al., 2006).

Alternatively, proponents of the “stochastic” or “clonal evolution” model propose that not a rare population, but most tumor cells are capable of self-renewal and can contribute substantially to tumor maintenance. As shown in Figure 4B, they suggest that acquisition of genetic changes, mutations or certain responses to environmental cues can result in cells that acquire stem-like programs and functions (Kelly et al., 2007). First and foremost, they question whether successful xenotransplantation – the cornerstone of the former model – accurately reflects *in vivo* human stem cell biology, or simply defines a human cancer cell population that is adaptable to the mouse microenvironment. Thus, they argue that using this methodology to define “stem-ness” underestimates the number of tumor-initiating cells. Several lines of argument support this alternate hypothesis. First, Kelly et al. has shown that in the absence of the barriers of xenotransplantation, such as in murine lymphoma models and a PU.1 knockout AML mouse model, both cells with or without stem cell-like phenotype can cause tumors in recipient mice (Kelly et al., 2007). Second, some investigators have pointed out that cell markers utilized for defining putative stem cells, such as CD133+, can account for up to 20% of the tumor. Similarly, Shipitsin et al. found that the putative breast cancer stem cell population, based on the phenotype of CD24^{−/low}/CD44⁺, was rather large – 12–60% of tumor cells (Shipitsin et al., 2007). Thus, these and other studies suggest that in poorly differentiated tumors, cells with stem cell functions may constitute the majority of tumor cells. Finally, confusion persists regarding the exact cell surface markers that define the stem cell population. Beier et al. demonstrated that depending on the tumor analyzed, glioblastoma cancer stem cells can be either CD133+ or CD133−, suggesting that markers for cancer stem cells are not well-established, or that all tumor cells are tumorigenic, but to a varying degree (Beier et al., 2007). Similar confusion of markers has been recently reported in the colon cancer field (LaBarge and Bissell, 2008).

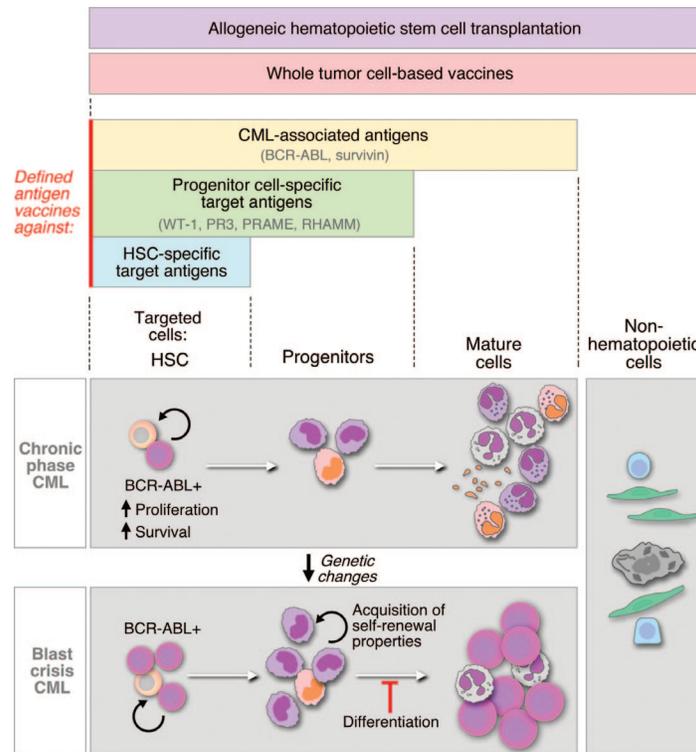


Figure 5. Specificity of immune responses against the malignant cancer-initiating cell depends on where antigen targets are expressed in the cell differentiation hierarchy. In the example of CML, increased capacity for malignant self-renewal can arise from transformation of the hematopoietic stem cell (HSC) to give rise to chronic phase CML or from more differentiated progenitor cells, to give rise to blast crisis CML. Leukemic stem cell activity can result from impaired differentiation, increased cell survival, increased proliferation, increased genomic instability and/or increased self-renewal capacity. Examples of vaccination strategies are provided.

In attempt to reconcile these two models, Adams et al. suggest that the behaviour of individual cancers may follow one or the other model more closely (Adams and Strasser, 2008). Thus, the growth of hematopoietic cancers, whose the cell differentiation pathways have been well-characterized, may more often demonstrate a hierarchical pattern, while that of solid tumors – typically heterogeneous diseases and more reliant on the supporting infrastructure of endothelial cells and fibroblasts that form intratumoral blood vessels, and that provide paracrine factors (that would be absent in an immunodeficient mouse) – may be more consistent with a stochastic pattern. Still another model, also shown in Figure 4C, fuses the two models and proposes that there may exist an originating cancer stem cell, but that acquisition of genetic changes by a secondary cancer cell might render it stem-like, resulting in it becoming the predominant clone (Adams and Strasser, 2008).

4.2. Immunologic targeting of tumor-initiating cells

The implications of the different cancer stem cell models, discussed above, is that while the hierarchical model suggests that targeting the small stem cell population is sufficient for therapeutic efficacy, the other requires that all tumor cells be targeted. This is because the latter model suggests that any tumor cell can acquire the stem cell functions. Therefore, narrowly focusing on a small phenotypically defined subset will result in tumor escape. Thus, even in CML, which can be considered the prototypical stem cell cancer, multiple pathways by which malignant cells can acquire stem cell characteristics have been identified (Passegue et al., 2003). As shown schematically in Figure 5, these include genetic changes that are acquired in the earliest cell of the differentiation program, that can lead to increased cell survival and proliferation. Alternately, acquired genetic changes downstream of the stem cell, such as the progenitor cell, can lead to increased self-renewal capacity. Therefore, developing immunity against only HSC-specific target antigens will miss elimination of progenitor cells that are self-renewing.

Given the uncertainties of what cells to target and what precise immunogens result in tumor elimination, the approach adopted by several groups has been to use whole tumor cells for vaccination (see Figure 5). This approach does not uniquely target malignant stem cells or progenitor cells, but these cell populations are included within the range of cells vulnerable to the effects of immunization. This “broader” approach has the advantage of immunizing

with patient tumor-specific antigens – including both mutated and differentially expressed/presented antigens. This has been accomplished by immunization with cytokine gene-transduced irradiated autologous tumor cells (Soiffer et al., 1998; Zhou et al., 2005), tumor lysates (Jocham et al., 2004), heat shock proteins bound to peptides derived from autologous tumors (Srivastava, 2006), or fusion hybrids between autologous tumor and dendritic cells or amplified tumor-derived RNA (Su et al., 2003). For example, at DFCI, vaccination with irradiated autologous melanoma cells engineered to secrete the powerful cytokine adjuvant GM-CSF, by adenoviral-mediated gene transfer, likely does target some melanoma-initiating cell since 29% of patients with metastatic melanoma survive at a minimum follow-up of 36 months (Soiffer et al., 2003).

The obvious limitation of autologous whole tumor vaccines is that while their use ensures that the immunogens are personalized, they are – by definition – also composed of thousands of normal self-antigens that are present in many tissues. Thus, as the potency of whole tumor vaccines in inducing immunity is enhanced, it is anticipated that strong anti-tumor immunity will be associated with concurrent and potentially fatal autoimmunity. This can be considered analogous to the induction of GvHD in the transplant setting. Early signs of this possibility have been observed in the studies of a new agent, the CTLA4-Ig blocking antibody, which can induce significant autoimmunity when co-delivered with tumor antigens (Attia et al., 2005; Beck et al., 2006; Maker et al., 2006; Phan et al., 2003; Ribas et al., 2005; Sanderson et al., 2005). On the other hand, recent modifications of CTLA4-Ig dosing schedule have led to more acceptable levels of inflammation (Hodi et al., 2008; Hodi et al., 2003). In particular, sequential dosing such that vaccination is followed by CTLA4 blockade have resulted in clinical dissociation of tumor immunity and autoimmunity, although breaches of tolerance are still seen, as evidenced by the frequent occurrence of transient skin rashes, asymptomatic bilateral hilar lymphadenopathy, and low grade colitis. Thus, a significant risk for autoimmunity remains in immunizing patients with large amounts of widely expressed antigens, especially as methods for stimulating immunity become more effective.

Given these risks, an alternate approach is to generate vaccines that more selectively target cancer-initiating cells. As an example of this approach, Pellegatta et al. used dendritic cells loaded with neurospheres, which are enriched for glioma stem cells, to vaccinate mice with tumors comprised of GL261 glioma cells. Vaccination with these loaded DCs resulted in cure of 80% of mice with these tumors, suggesting that this is a highly protective strategy (Pellegatta et al., 2006).

Other investigators have sought to develop defined antigen vaccines, using immunogens that are consistently over-expressed in malignant progenitor cells. These immunogens may be delivered as whole protein or as peptides. In general, a frequently used methodologic approach for identifying candidate immunotherapy targets has been to identify antigens that demonstrate preferential tumor-restricted expression, and then to bioinformatically predict peptides derived from the candidate antigen that can bind to common HLA alleles. Then, cytotoxic T cell reactivity is assessed against antigen-presenting cells expressing the predicted peptide. In this fashion, WT-1 has been well-characterized as an antigen with almost exclusive expression on either leukemic CD34+ stem or progenitor cells, and has been tested in clinical trials (Rezvani et al., 2008). In addition to high expression in progenitor cells, it is also known to elicit antibody responses (Elisseeva et al., 2002; Gaiger et al., 2001; Ling et al., 1998). Furthermore, WT1-specific CTLs can specifically kill *BCR-ABL* + leukemia transformed stem cells without damage to normal hematopoietic stem cells *in vitro* or in mice *in vivo* (Gao et al., 2000; Oka et al., 2000). Recently, a phase I study was conducted in which 26 patients with breast, lung, MDS or AML were vaccinated with intradermal injections of the HLA-A24 restricted natural or modified 9-mer WT1 peptides (Oka et al., 2004). Eighteen of 26 completed 3 or more injections, and 12 of 20 showed clinical responses such as reduction in leukemia blast cells or tumor sizes and markers, that was clearly correlated with an increase in frequency of WT1-specific CTLs after vaccination. No damage to normal tissues was observed. Other potential leukemia-associated antigens of this category that are each under evaluation in clinical trials include the leukemia-associated antigens RHAMM, PRAME, survivin and proteinase 3 (Greiner and Schmitt, 2008). This general approach of “reverse immunology” has been applied to identify promising candidate tumor-associated antigens for vaccination in solid tumors as well (Curigliano et al., 2006; Gnjatic et al., 2006; Purcell et al., 2007).

A potential third class of tumor antigens has rarely been used in vaccines due to technical difficulties in identifying them (Parmiani et al., 2007; Sensi and Anichini, 2006). This class consists of proteins with tumor-specific mutations that result in altered amino acid sequences. Such mutated proteins have the potential to: (a) uniquely mark a tumor (relative to non-tumor cells) for recognition and destruction by the immune system (Lennerz et al., 2005); (b) avoid central and sometimes peripheral T cell tolerance, and thus be recognized by more effective, high avidity T cells receptors (Gotter et al., 2004). Recently, large-scale traditional sequencing efforts have demonstrated that tumors contain a small number of driver and a large number of passenger mutations, and that an average tumor may have tens to hundreds of non-synonymous mutations in protein coding regions (Greenman et al., 2007; Sjoblom et al., 2006;

Thomas et al., 2007). Moreover, *in silico* analysis of sequences derived from tumor and normal cells in the same patient suggest that these somatic mutations provide several potential neoantigens for tumor vaccine development, estimated at ~10 novel epitopes per tumor that can bind HLA-A*0201 (Segal et al., 2008). Alternative splice variants of *BCR-ABL* in CML patients have been identified that are immunogenic (Volpe et al., 2007), and suggest that these are also potential immune targets for this disease. It is unknown at what stage in the CML hierarchy these variants are acquired. However, based on the analysis of *BCR-ABL* mutations following exposure to *ABL* kinase inhibitors (Section 2), these variants have a high likelihood of being detected in CML stem cells.

In closing, many of the concepts uncovered in the studies of CML are generally applicable to conceiving analogous therapeutic strategies in patients with other malignancies. Recent identification of malignant cells that possess clonogenic potential suggest the possibility that targeting specific cell subpopulations may contribute generating curative responses. The exquisite specificity of the adaptive immune response presents the opportunity to target this cell population without collateral damage to other non-malignant cell populations. The recent progress in elucidating effective adjuvants, and in dissecting the mechanisms that control negative immunoregulation excite the possibility of developing effective cancer vaccines. In the future, we can look forward to treatment strategies that leverage this information together with appropriate antigenic stimulation to target cancer-initiating cells to develop curative therapies with minimal toxicity for cancer.

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