

Enhancing graft-versus-leukemia after transplant: the rise of anti-cancer vaccines

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1. ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the only truly effective curative treatment for refractory hematological malignancies. Unfortunately, relapse and transplant rejection continue to be of major concern. In order to enhance the effectiveness of the HSCT, various strategies have been explored to amplify the graft versus leukemia (GvL) effect. Cancer vaccines have emerged in recent years as a promising strategy for the immunotherapeutic treatment of cancer. Evidence shows that they are most likely to have the greatest effect in the setting of minimal residual disease and as adjuvant agents. With this in mind, researchers have begun to explore the use of cancer vaccines in conjunction with HSCT, with exciting results. There has also been recent work examining the effect of novel adjuvants or blockers of negative immune regulation to augment the effect of cancer vaccines in both the transplant and non-transplant settings. The addition of these agents may prove vital to developing effective vaccine based strategies.

2. RECONSTITUTION OF DONOR IMMUNITY FOLLOWING HSCT OFFERS A UNIQUE OPPORTUNITY TO APPLY CANCER VACCINES TO EXPAND ANTI-TUMOR IMMUNITY

Allogeneic hematopoietic stem cell transplant (HSCT) is a well-established curative therapy for many hematologic malignancies. It was first successfully used in the treatment of leukemia over 50 years ago (1). Since then, numerous modifications to this treatment approach have been developed to enhance the safety and efficacy profile of HSCT. These improvements include the development of reduced ablative conditioning regimens, refinements in HLA matching, expansion of available stem cell sources, and development of adjunct chemo- and radio-therapy. The clinical effectiveness of HSCT critically relies on recognition and elimination of recipient hematological cells (including malignant cells) by donor-derived immune cells. Existence of the graft versus leukemia (GvL) effect and the critical role played by donor T cells in this response has been long appreciated, based on clinical and laboratory studies demonstrating the improved relapse-free survival following allogeneic compared to autologous HSCT,

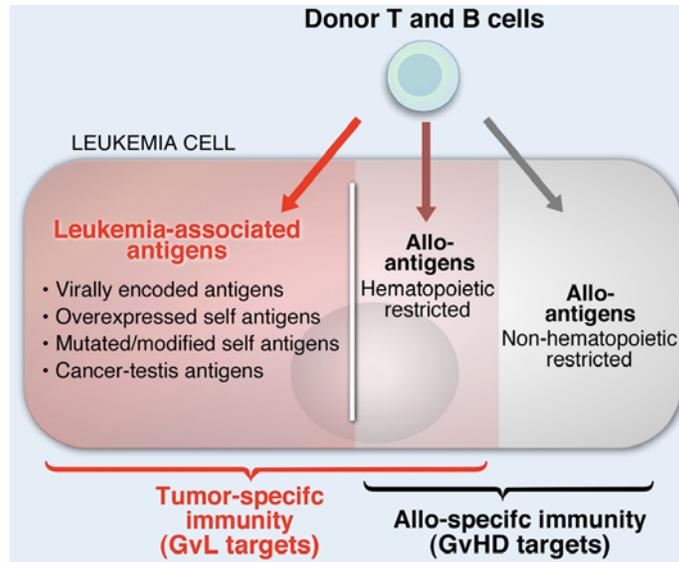


Figure 1. The separation of donor lymphocyte antigenic targets is important for tumor specific responses. Donor lymphocytes target antigens on recipient cells. These include both tumor- and allo- antigens. Targeting of allo-antigens by donor lymphocytes can result in GvHD, as well as GvL. By targeting tumor-specific antigens, it is possible to augment the GvL effect while minimizing GvHD.

increased disease relapse following HSCT using T cell-depleted grafts, and examples of leukemia regression observed following infusion of donor T cells (2-4).

Donor-derived T cells can mediate GvL effects through two general mechanisms. One, engraftment of donor cells restores normal immune function, thus overcoming tumor- or treatment-induced host immune defects and restoring immunosurveillance of malignant cells (5). After HSCT using T cell-replete grafts, early immune recovery (up to the first ~3 months) occurs through a thymic-independent process of peripherally-driven clonal expansion of mature graft-derived T cells. Mounting evidence suggests that a lymphopenic milieu—observed during the immediate weeks following stem cell infusion—supports the rapid expansion of lymphoid cells (especially CD8⁺ mature effector cells), as there is an excess of cytokines (i.e. IL-15, IL-21 and IL-7) that control lymphoid homeostasis (6). Moreover, regulatory T cells appear to be preferentially depleted compared to conventional CD4⁺ T cells (7). Recovery of broad polyclonal T cell immunity, however, requires the development and differentiation of T precursors derived from engrafted hematopoietic stem cell progenitors that differentiate in the thymus to generate mature naïve T cells. This thymic-dependent process of T cell neogenesis can occur as early as 3 months following HSCT, but more typically begins between 3-6 months after HSCT. Although thymic activity is age-dependent, with adults having largely involuted thymuses, multiple adult studies have demonstrated that thymic-dependent T cell neogenesis occurs in both myeloablative and nonmyeloablative settings (8-10). Impaired T cell reconstitution has been associated with graft versus host disease (GvHD) and susceptibility to fatal infections, the two most common and potentially fatal toxicities of HSCT. On the other hand, timely recovery of

a broad T cell repertoire is associated with complete donor chimerism and long-lasting freedom from leukemia (11, 12).

An alternate important mechanism by which donor-derived T cells effect GvL is through recognition of host antigens and through elimination of cells bearing these antigens. One conceptual framework to understand how GvL (and GvHD) responses are targeted is through understanding the classes of antigens targeted by reconstituting T cells. As shown in Figure 1, one class of target antigens is allo-antigens, which are derived from genetic polymorphisms that exist throughout the human genome, differing between the donor and the recipient (13). Genetic polymorphisms can give rise to allo-antigens through various mechanisms, including amino acid substitutions that create antigenic peptides, creation of alternate transcripts, modification of proteasomal processing, post-translational modifications, or gene deletion (14). Targeting allo-antigens that are broadly expressed in normal recipient tissues (hematopoietic and non-hematopoietic) can result in GvHD. When these allo-antigens are also expressed on leukemia cells, targeting these antigens contributes to GvL. While allo-antigens are known to be important targets after allogeneic HSCT, relatively little is known about donor immune responses directed against antigens solely expressed on leukemia cells. Some potentially important classes of antigens with leukemia-restricted expression are already known. These include virally encoded antigens (latent EBV epitopes), over-expressed self-antigens (proteinase-3, WT-1), or cancer-testis antigens (NY-ESO-1). In addition, the same genetic mechanisms that generate allo-antigens can also generate leukemia neo-antigens through acquired genetic changes that distinguish the leukemia cell from normal donor or host cells.

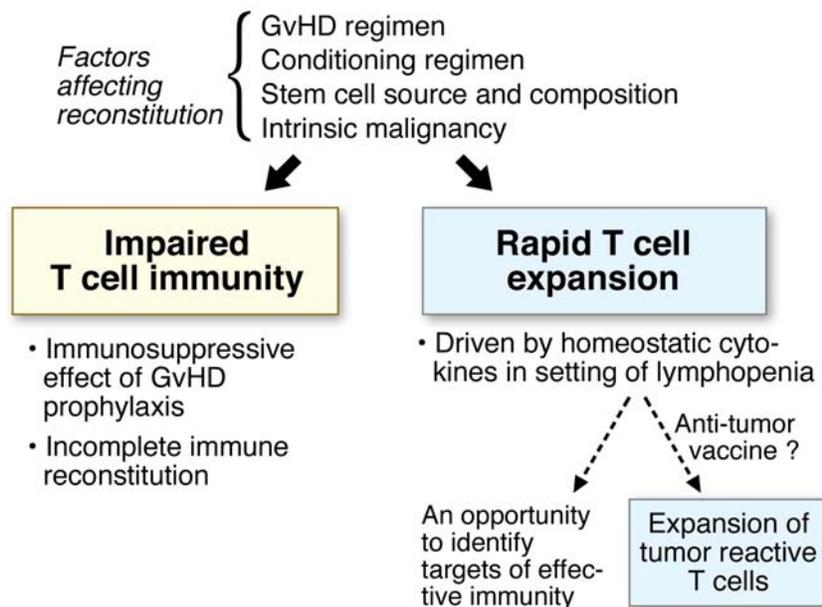


Figure 2. The early post-transplant setting may be an ideal time to discover targets for effective immunity as well as afford the opportunity to drive the rapidly expanding lymphocytes in an anti-tumor direction. Early after transplant, T cell immunity is impaired due to incomplete immune reconstitution and exogenous immunosuppression due to GvHD prophylaxis. At the same time, this is a unique environment in which there is homeostatic cytokine driven rapid T cell expansion in the setting of lymphopenia. This expansion may be potentially directed towards tumor antigens with the help of strategies such as cancer vaccines. This period also provides an opportunity to discover novel antigen targets of effective immunity.

Understanding the mechanisms underlying effective GvL now suggests a number of ways to improve how we approach developing efficacious tumor immunotherapy. First, the ability of immune-competent donor-derived cells to mount effective anti-leukemia responses suggests that the post-allogeneic stem cell transplant setting is advantageous for discovering immunogenic tumor-specific targets against which tolerance or anergy have not developed (Figure 2). Since specificity of the immune response is critically dependent on the antigens targeted, identifying appropriate immunogens is a high priority (15). In the post-transplant setting, many investigators have already identified allo-antigens as an important class of antigens targeted by donor immunity, and much focus has developed to use this information to risk stratify (13) or treat patients therapeutically (16). If tumor-specific antigen targets could likewise be identified, then immune targeting of these antigens with exclusive expression in leukemia cells would be predicted to elicit maximal anti-leukemia activity without toxicity. Section II describes approaches to discovering tumor-associated antigens in the post-transplant setting, and how this information can inform us about the components of an effective immune response.

Second, the post-transplant setting offers a unique environment for implementing vaccination approaches to enhance tumor immunity. As shown in Figure 2, the early post-transplant setting has not been conventionally considered optimal for anti-tumor vaccination because of the delayed T cell reconstitution (up

to 12-18 months following HSCT) that is typical of allogeneic HSCT. The pace of post-transplant immune reconstitution is affected by many factors and include the intensity of the transplant preparative regimen, graft composition, and the types of immunosuppressive medications selected to prevent GvHD (12, 17). Furthermore, reconstitution is also affected by host factors, such as the duration and intensity of therapy for an individual's particular malignancy. For these many reasons, adoptive cellular therapy, in which mature and immune-competent donor T cells can be directly infused into the host, has been a generally preferred therapeutic modality in the setting of post-transplant relapse. Recently, however, the lymphocyte expanding effects of the specialized cytokine milieu of a post-transplant lymphopenic environment has led to greater appreciation of the potential opportunity afforded by anti-tumor vaccines. These cytokines (IL2, IL15, IL21) appear to be instrumental in promoting lymphoid expansion and in improving both the efficacy of effector T cells and enhancing the ratio of effector to regulatory T cells (T-regs) (18-20). The balance between these two cell subsets has been thought to be vital for determining the level of immune mediated tumor eradication. Thus, the high T-effector to T-reg ratio observed in the post-transplant period suggests the potential to increase the potency of adjuvant immune therapy (21). Section III reviews the growing field of post-transplant vaccination, which has generated increasing interest, especially as the frequency of immediate toxicities of HSCT have improved, and yet disease relapse rates are still not insignificant (22). These efforts have been enhanced by

the growing body of work developing more effective vaccine adjuvants (Section IV) and agents that provide inhibition of checkpoint blockade (Section V).

3. USING RESPONSES IN THE POST-TRANSPLANT SETTING TO DISCOVER IMMUNOGENIC TUMOR ANTIGENS

Over the past 15 years, hundreds of animal studies and clinical trials of cancer vaccination have been initiated. The vast majority have used over- or aberrantly expressed tumor-associated self-antigens as vaccine immunogens, as their expression is commonly elevated in a particular cancer, and traditional antigen discovery methods have favored their identification (23) (see below). As of 2004, only ~3% of >1000 patients enrolled in cancer vaccine trials in North America had objective clinical responses to treatment across all antigens and delivery approaches, with most trials finding no responses at all (24). Thus, despite the theoretical promise of immunotherapy, vast improvements to vaccine design are clearly required, ranging from developing more potent adjuvants and vaccine delivery systems to generating approaches to circumvent tumor-mediated immunosuppression. Moreover, since vaccine specificity is determined only by the immunogen, its selection remains of central importance (15).

Recent evidence suggests that T cell responses to these well-characterized overexpressed antigens are deleted or tolerized, since the majority of tumor-associated antigens (TAAs) have been discovered in tumor-bearing hosts without evidence of clinical control of their disease. Gannage *et al* demonstrated that patients with chronic myeloid leukemia have T cells with specificity for common CML-associated leukemia antigens, but without evidence of functional responses (25). Other investigators have shown that the tumor itself can shape the T cell repertoire, such that tumor-reactive T cells are actively deleted in association with disease progression (26). In a study of therapeutic vaccination for CML using whole allogeneic tumor cells, molecularly evident tumor regression was observed, without T cell responses detected to any of a panel of common well-characterized myeloid leukemia antigens. Since immune-induced clinical responses were present, these results suggest that the panel of defined (shared) antigens selected were probably insufficient for detecting responses (27). Similar results demonstrating that well-characterized tumor antigens discovered outside of the context of therapeutic response have only limited utility as targets of an effective response have been observed for other cancers (28, 29). In fact, it was recently described that T cells with specificity to common leukemia antigens not only lack function following HSCT, but undergo replicative senescence and thereby lack the potential to expand *in vivo*.

On the other hand, clinically evident anti-tumor responses can be observed after HSCT, at times in the absence of GvHD. These observations suggest that antigens with restricted tumor expression are in fact targeted by donor immune cells (14). In support of the existence of tumor-specific antigens, bulk T cell cultures

and clones with reactivity specific for tumor cells but not recipient allo-antigen bearing cells have been identified following allo-HSCT for chronic lymphocytic leukemia (30). Other investigators have identified T cells with specificity for malignant but not normal hematopoietic progenitor cells, consistent with the idea that immunologic targeting of malignant stem cells are required for curative responses (31-33). These and other studies thus support the notion that antigen discovery in the setting of successful therapeutic response may reveal immunogenic and relevant tumor-specific targets. Furthermore, their characterization may provide insights for how meaningful immune responses are mounted. Figure 3 depicts the several strategies have been employed over the years for this effort, described below.

3.1. T cell-based tumor antigen discovery

A direct method for systematic identification of T cell targets involves labor-intensive cloning of leukemia-targeted T cells followed by identification of target antigens by either biochemical methods or by expression cloning in matched MHC-expressing mammalian cells (24, 34, 35) (Figure 3). This approach has been used to discover several important minor histocompatibility targets (36). However, consistent with the technical challenges of this approach, over a period of 20 years, only a handful of T cell-defined tumor antigens have been discovered in this manner (23, 24). In the few instances that this approach was applied, in which tumor-reactive T cells were screened against autologous tumor cDNA libraries in patients with spontaneous and therapy-induced tumor regression, the dominant T cell clones recognized a variety of antigens ranging from shared and over-expressed to mutated antigens specific to the tumor (37-43).

3.2. B cell expression library cloning

A highly popular method that many groups, including our own, have used to identify target antigens is SEREX (44-47). In this approach, patient plasma is used to screen expression libraries for antibody binding. While this method does not directly identify T cell targets, some of the identified B cell targets are also targeted by T cells (48) (Figure 3).

We initiated this approach based on an unexpected finding of peripheral B cell lymphocytosis and plasma cell marrow infiltration developing at the time of clinical response to donor lymphocyte infusion (DLI) (that was not complicated by concurrent GvHD) (49) in a series of patients with CML enrolled on trials of DLI at our center (49, 50). For these studies we used post-therapy plasma from these patients as a source of antibody to discover targets of GvL immune responses. These screening studies were initially conducted using bacteriophage cDNA expression libraries, and led to the detection of potent humoral immunity -- at levels comparable to anti-viral responses -- developing in close temporal correlation with clinical tumor regression (44). We have subsequently confirmed and extended these results using recently available high-density protein microarrays, with >5000 protein open reading frames (Proto-array; Invitrogen). Responders of DLI consistently developed high-titer

Discovering tumor specific targets

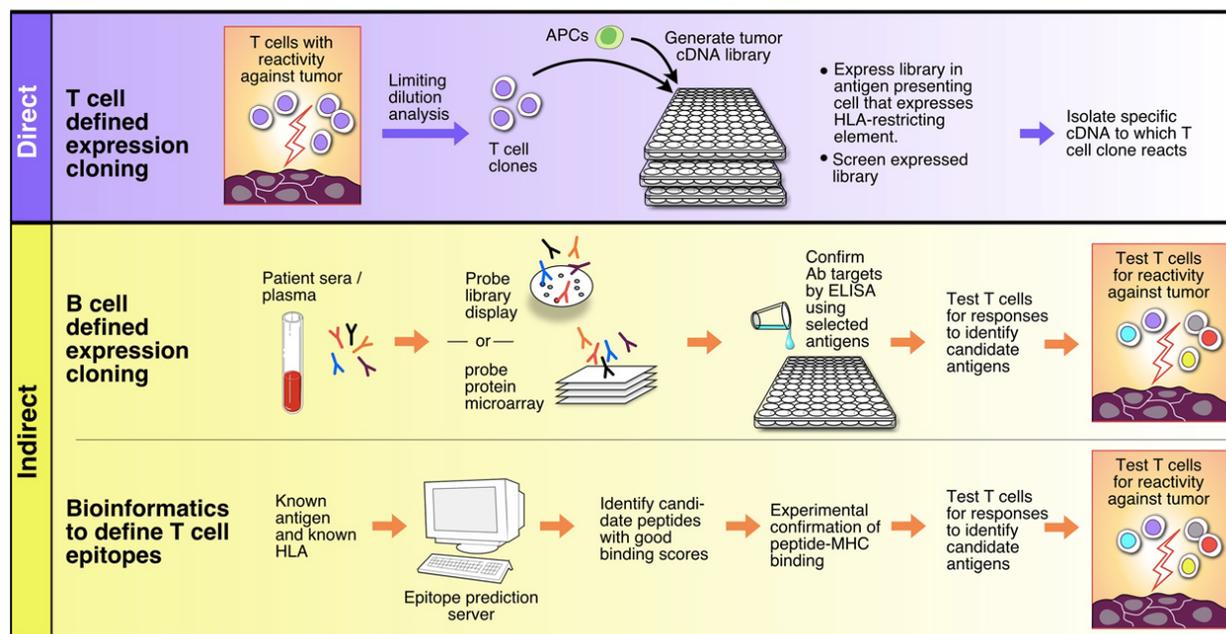


Figure 3. Discovery of tumor specific targets may be accomplished through direct or indirect means. In the direct approach, tumor-reactive T cells are isolated through limited dilution assay and cloned. T cell clones are then screened against a cDNA library generated from tumor cells to identify the precise cDNA eliciting reactivity. B cell defined expression cloning is an indirect method in which patient sera-derived antibodies are probed against protein microarray or tumor cDNA expression libraries. The antigen specificity of these antibodies are then confirmed using ELISA. Candidate B cell defined antigens can then be tested against T cells to determine if they elicit reactivity. Finally, the bioinformatics approach uses epitope prediction to identify peptides that are likely to be good binders, based on known antigens and HLA. Peptide thus identified are then confirmed to have MHC binding and subsequently tested experimentally for their ability to elicit T cell reactivity.

antibody responses to several target antigens, an outcome not observed in non-responders (Figure 4A) (51). Although occasional targets elicited antibody reactivity from more than one individual, the vast majority of targets were discovered to be unique to each individual (Figure 4B). We have made analogous findings for patients with CLL and myeloma that were treated with HSCT and DLI (52, 53). These results clearly illustrate the heterogeneity of anti-leukemia immune responses of individuals, due either to individual differences in the immune system (e.g. HLA) or in tumor antigen composition.

Through these screening efforts, we have identified CML66, CML28, TBCE, RAB38 and DUSP12 as TAAs, that all are highly expressed in malignant myeloid progenitor cells and a broad array of tumors, but in only a narrow range of normal tissues (51, 54, 55) (Figure 5C). These over-expressed proteins are not allo-antigens as no sequence differences between donor and host cells were found (54). These DLI-associated antigen-specific B cell responses appear to be coordinated with T cell immunity. For example, CD8+ memory T cell responses against CML66 can be detected in blood and marrow starting ~1 month after DLI—immediately preceding achievement of cytogenetic remission—while antigen-specific antibody responses begin ~2-3 months after DLI (Figure 5A, 5B). Donor-derived T cells against the DLI-associated antigen

CML66 were present in leukemic marrow prior to DLI, leading us to speculate that DLI-induced GvL is associated with pre-existing immunity to leukemia antigens. In support of this notion, we recently discovered that presence of >5% infiltrating CD8+T cells detected on pre-treatment marrow biopsies of 30 CML patients who received DLI was significantly associated with ensuing effective clinical response (56). Together, these studies imply that DLI activates a pre-existing pool of leukemia-reactive T cells to expand and reject CML.

3.3. The bioinformatics approach to antigen discovery

During the past 10 years, the performance of multiple peptide-binding prediction programs has increasingly improved such that a subset of computational tools have attained useful levels of sensitivity and specificity for predicting peptide binding to common HLA alleles. Recently, the performance of >30 prediction algorithms was assessed using a validated test set of peptides from a tumor antigen, survivin, and from a CMV matrix protein (57). Several algorithms, including NetMHC, which applies artificial neural networks, and IEDB, were assessed to demonstrate high accuracy over a range of HLA class I alleles compared to other prediction algorithms, with close correlation between peptide prediction and experimentally determined binding affinities ($p < 0.001$). Thus, these bioinformatic tools may now be

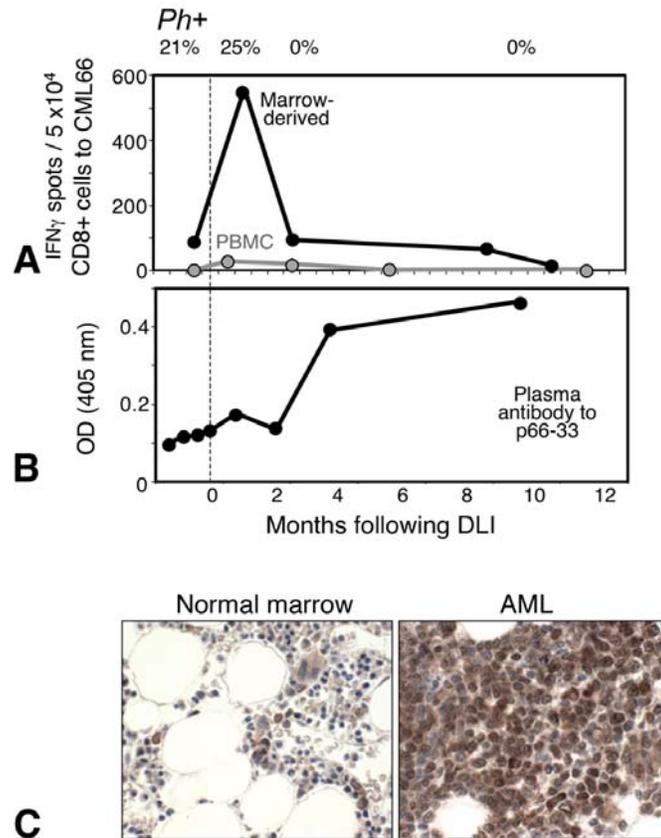


Figure 5. Post-DLI, there is a coordinated B and T cell response to CML66. A, In the first 3 months after DLI, T cells reactive against the CML-associated antigen CML expand from peripheral blood and marrow, as measured on ELISPOT. B. Plasma reactivity (diluted 1:200) against peptide 66-33 following DLI increases over the first 10 months, with the first major increase coinciding with the end of T cell expansion (as measured by ELISA assay). C. Immunohistochemical staining of AML marrow with anti-CML66 specific monoclonal antibody reveals high CML66 expression among CD34+ malignant cells. In normal marrow, CML66 antibody stains rare normal myeloid progenitor cells..

3.4. Summary

Our prior analyses of a highly effective cellular immunotherapy for the treatment of CML and CLL, namely DLI, has revealed that the durable anti-leukemia immune responses are comprised of coordinated B (antibody) and T cell (cytotoxic) responses against multiple antigens that are expressed in leukemia cells (5, 44, 51, 61, 62). Moreover, the targeting of individual antigens was typically specific to each individual (51). In general, these insights support vaccination approaches that will stimulate multivalent rather than univalent responses. Furthermore, as discussed in Section IV, approaches using autologous tumor (and hence, are personalized) as immunogen are promising for enhancing GvL effects.

4. NOVEL POST-TRANSPLANT INTERVENTIONS TO ENHANCE ANTI-LEUKEMIA RESPONSES: RECONSTITUTION-ASSOCIATED THERAPIES

Based on the mechanistic insights gained from studies of immune reconstitution and clinical responses following HSCT, enhancement of GvL responses is now possible through a number of approaches. As shown in

Figure 6, because of the central importance of reconstitution of normal donor immunocompetence in anti-tumor responses, one line of investigation has focused on enhancement of T cell reconstitution and thymic regeneration (63-65). Several ongoing investigations have suggested cytokines as the most promising potential agents to enhance T cell reconstitution. Of these, IL-7 appears to be amongst the more promising of these, and clinical trials evaluating IL-7 are currently being carried out (66-68).

Because T cell immune reconstitution is typically delayed following HSCT, another productive therapeutic approach that essentially provides replacement immunocompetent T cells is through administration of post-transplant adoptive cellular therapy. For example, infusion of donor T cells with defined antigen specificity can effectively treat viral infections (69-71). For treatment of malignancy, the effectiveness of this approach is best exemplified by the successes of DLI, routinely used in clinical practice for over 15 years now. In particular, 75-80% of patients with CML who are treated with DLI demonstrate durable clinical remissions. The mechanism by which DLI effects its response is still unknown, but

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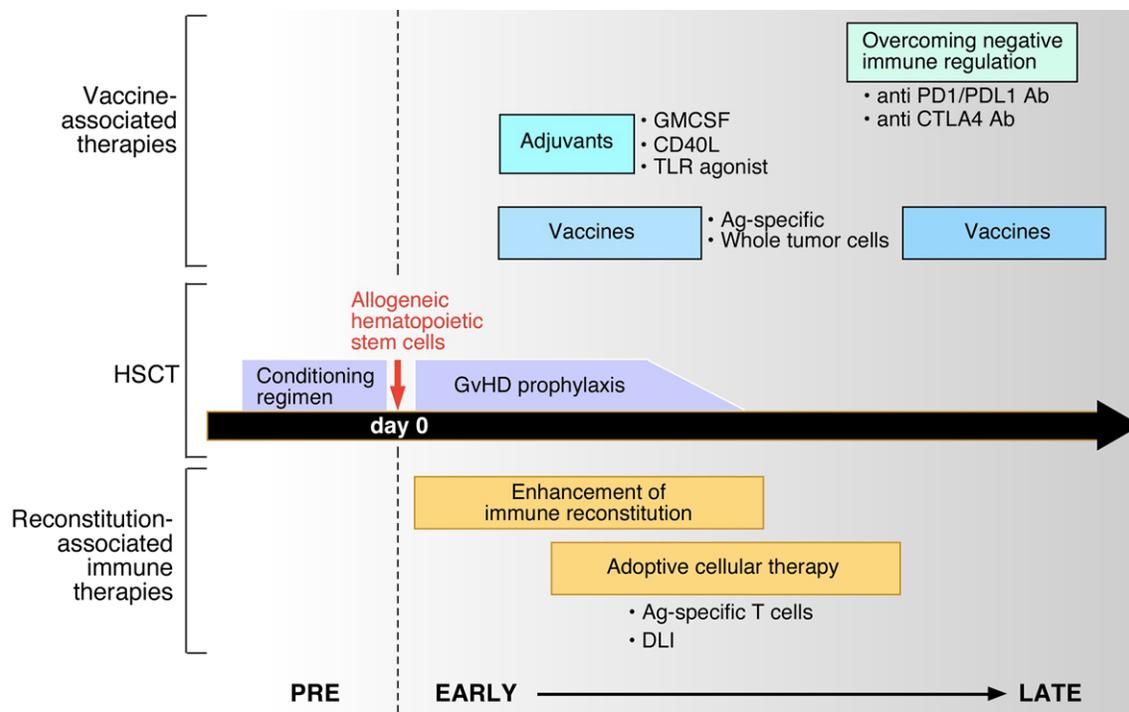


Figure 6. Many interventions are possible to enhance GvL in the post-transplant period. Options include early or late post-HSCT vaccines and associated adjuvant therapies, as well as additional agents to block negative immune regulation. Other strategies such as adoptive cellular therapy and infusions of homeostatic cytokines aim to enhance immune reconstitution.

much evidence suggests that it provides immunologic help to surmount ineffective immunity. Many studies have demonstrated that administration of DLI is associated with broadening of T cell repertoire diversity and enhancement of T cell neogenesis (72-74). Various schedules of administration of DLI exist, with no uniform standard regimen, but the beneficial effects of DLI on immune reconstitution have been observed whether administered in the early prophylactic setting (49) or much later following transplant in the setting of treatment of relapse (72, 75). Despite the potentially beneficial effects of DLI, its most common and potentially fatal toxicity is GvHD. Hence, new investigational directions now focus on expansion or isolation and infusion of particular immune cell subsets (76, 77) that preferentially target tumor cells rather than non-tumor host cells that express alloantigen. Some centers have favored nonspecific expansion of activated cells (78), while other groups have isolated and infused cells that target particular antigen specificities. For example, relapsed leukemia has been treated with post-transplant infusion of donor T cells specific for single or a few recipient alloantigens. However, while alloantigen-based adoptive cellular therapy appears to have clinical anti-tumor activity, this resulted in either short-lived efficacy or significant toxicity due to non-tumor specific alloantigen expression (79, 80).

5. NOVEL POST-TRANSPLANT INTERVENTIONS TO ENHANCE ANTI-LEUKEMIA RESPONSES: ANTI-CANCER VACCINES

A cancer vaccine consists of three basic components (81): an antigen, to provoke an immune

response; an adjuvant to enhance the immune response; and a delivery system, to ensure that the antigen is made available to the immune system for recognition. Compared to adoptive cellular therapy (Section III), cancer vaccines offer several advantages. First, vaccines are a treatment option with low a priori toxicity. In the many clinical trials that have been carried out to date, the vast majority of reported side effects have been insignificant: mild flu-like symptoms and injection site discomfort (24, 82-84). Second, vaccines are relatively simpler to manufacture, and lack the technical and regulatory requirements for *ex vivo* cell expansion and isolation. Third, for the very same reasons, vaccines are also consequently relatively less expensive than cellular therapies.

Typically, therapeutic vaccination has been thought to be effective only in settings of immune competence. For these reasons, the post-HSCT setting, with its delayed immune reconstitution and the typical use of immunosuppressant medications to prevent or treat GvHD, has not been considered an optimal setting for vaccination. Recently, however, evidence has not supported this premise, and in fact has suggested that HSCT provides a beneficial platform for stimulating anti-tumor immunity. For example, vaccination for polio and hepatitis B have been effective in the post-transplant period, indicating that the immune system is still very able to respond to antigen in the form of vaccine at this time (85, 86). A number of new insights and the availability of novel immunotherapeutic reagents have challenged the view that early post-HSCT vaccination is ineffective, and particularly

Enhancing gvL after transplant with anti-cancer vaccines

invite revisitation of the concept of anti-tumor vaccination following HSCT (see Figure 6). First, a number of studies have supported the idea that T cells can be rapidly expanded in the early post-transplant period, when homeostatic cytokines that support lymphoid expansion are in high abundance (87, 88). Secondly, newer adjuvants are now available that can potently stimulate the immune response (Section V). Finally, an exciting new class of reagents, the checkpoint blockade inhibitors have shown great clinical promise and appear to be effective in overcoming negative immunoregulation, and hence serve to amplify and render effective primed immune responses (Section VI).

5.1. Defined antigen vaccines

Antigen-specific vaccines have been the focus of the vast majority of tumor vaccine studies. A major advantage of this approach is the ability to mount a highly focused immune response, with specificity of the response dependent on the expression of the antigen on the target tumor tissue (and not normal tissue). Possibly formulations of defined antigen vaccines include peptides, proteins or DNA that encode specific tumor epitopes. Examples of well-characterized antigens that have been the focus of antigen-specific vaccines for the hematologic malignancies include BCR-ABL, Wilm's tumor-1 (WT-1), proteinase-3 (PR-3), receptor for hyaluronic acid-mediated motility (RHAMM) and NY-ESO-1. In the transplant setting, it has been observed that T cells specific for known tumor antigens may exist in some healthy controls as well as in leukemic patients both before and after transplant (89, 90). In leukemic patients, these antigen-specific T cells may well aid in the GvL response, with HSCT creating an environment amenable for their expansion and action (91).

Only few antigen-specific vaccines have been tested in the post-HSCT setting. The majority have utilized a dendritic cell (DC) based approach, whereby DCs are loaded with antigen. In a case report (92), a patient with relapsed AML after allogeneic HSCT was treated with a keyhole limpet hemocyanin (KLH) and WT-1 pulsed DC vaccine, starting 6 months post-transplant. Although DTH reactions to WT-1 pulsed DCs were detected, no immune responses were detectable in either peripheral blood or bone marrow. It was not reported if the patient displayed a clinical response to the vaccine. Two separate groups have also explored idiosyncrasy-loaded DC vaccines, augmented with KLH, administered months after combination treatment with high-dose therapy and autologous transplant in multiple myeloma (93, 94). The idea explored in this setting is that there may be improved immunocompetence due to both minimal disease and absence of exogenous immunosuppression (95). Both groups observed that a small proportion of patients developed idiosyncrasy specific cytotoxic T cell responses (2/12 and 4/26 respectively), but that more than 90% of patients produced KLH specific cellular proliferative immune responses, indicating that patients were generally immunocompetent at the time of vaccination.

5.2. Whole tumor cell vaccines

Within the many approaches to cancer vaccine

design, whole tumor cell-based vaccines have so far proved to be the most promising (24, 95). This format has the advantage that multiple antigenic epitopes are available to stimulate immune responses. On the other hand, since tumor cells also contain many self-antigens, this approach risks induction of autoimmunity. Whole cell-based vaccines can be autologous or allogeneic, and may be administered unmodified, (i.e. as cell lysates) or modified whole cells (i.e. cells modified to express cytokines). In all of these cases, tumor cells are rendered incapable of replication prior to administration, usually by ionizing irradiation.

Autologous tumor cell vaccines are generated from a patient's own tumor cells. The main advantage of an autologous tumor cell vaccine approach is the personalized nature of the vaccine – the tumor-associated antigens within the vaccine are unique to the individual patient. However, the creation of autologous tumors requires harvestable tumor, which is not always possible; for example, in cases of minimal residual disease. Allogeneic vaccines are created from pre-existing tumor cell lines, and therefore consist of a mixture of common tumor antigens and alloantigens. Allogeneic vaccines overcome many of the disadvantages of the autologous approach, eliminating the need for harvestable tumor and providing an easily manufactured, off-the-shelf product. Their main disadvantage compared to the autologous tumor cell vaccines is that they do not ensure good coverage of patient-specific tumor associated antigens, and that moreover, they contain many 'self' peptides. Preclinical studies in mice have shown that whole tumor cell vaccines are capable of inducing autoimmunity (96, 97), but in human studies this has been largely limited to reports of vitiligo at vaccination sites in melanoma patients (98-100).

The clinical use of whole tumor cell vaccines has been well-supported by animal studies. Mundhada *et al.* (101) studied healthy mice undergoing allogeneic, myeloablative BMT. Immune reconstitution was assessed by flow cytometry of peripheral blood. When judged complete, mice were immunized with irradiated whole cells from an acute leukemia line (C1498) weekly for up to four weeks. When challenged with unirradiated tumor cells 8-10 days post final vaccine, vaccinated mice had a statistically significant survival benefit compared to the controls. Furthermore, they also showed a significantly reduced tumor burden (as assessed by PCR), reduced numbers of lung nodules, and consistently higher numbers of activated interferon gamma A positive T cells. These results serve to illustrate that whole cell vaccines are capable of inducing significant anti-tumor responses *in vivo*. Similarly, in a melanoma model (102), healthy mice vaccinated with irradiated GM-CSF secreting melanoma B-16 cells post myeloablative BMT showed a strong survival advantage when challenged with tumor one week later.

Two studies have evaluated the anti-tumor effects of post-HSCT vaccines in the more clinically relevant setting of established tumors. In the first, Moyer *et al.* (103) used mice with established 6-day melanoma tumors. The mice were then treated with allogeneic BMT

followed by, on day 3 post-transplant, 3 weekly vaccines with B16 melanoma lysate-pulsed DCs. GvHD was not exacerbated in the treatment group compared to the control groups. Compared to control mice (BMT alone without vaccine), vaccinated mice showed significantly decreased tumor area, and significantly increased numbers of interferon-gamma secreting B16-reactive lymphocytes, that were present in lymph nodes draining the DC vaccination site. This occurred in the absence of complete immune reconstitution in the peripheral blood, indicating that the reconstituting immune system is indeed capable of generating significant and adequate immune responses to vaccine. In the second study, Luznik *et al.* (104) inoculated healthy mice with a mouse mammary carcinoma. On day 13 after tumor establishment, mice underwent tumor resection, followed the day after with non-myeloablative BMT. DLI was administered on day 28 and finally, a vaccine was given on day 31. The vaccine consisted of irradiated whole cells from the mammary carcinoma cell line used for tumor establishment, combined with a GM-CSF secreting melanoma bystander cell line. For mice that received all four treatments, survival was significantly longer ($p < 0.01$) than for any animals receiving three or less of the four components. Animals that received all four treatments retained increased tumor protection when a subsequent tumor challenge was administered at day 120.

The clinical studies adopting the approach of early-post transplant whole tumor cell vaccination appear promising. Ho *et al.* (105) developed an autologous GM-CSF secreting vaccine, which was administered to 15 patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) within 45 days of non-myeloablative allogeneic HSCT, to a total of six vaccines. Of the ten patients who received all six vaccines, nine achieved durable complete remission (median follow-up 26 months), with 7 long-term responders. Vaccine subjects demonstrated positive DTH responses (7 of 8), and were also noted to have decreases in the levels of a protein (MICA) which has been shown to be linked with tumor destruction (13 of 15). The results for this study show significant 2-year survival advantage for vaccinated subjects compared to historical controls, a highly promising result that bears further investigation with additional trials. This approach has now been extended to patients with advanced chronic lymphocytic leukemia undergoing reduced-intensity transplant in an ongoing study (106).

6. NOVEL ADJUVANTS TO ENHANCE POST-TRANSPLANT VACCINES

Adjuvants are substances that enhance the effectiveness of vaccines. First used in prophylactic vaccines for infectious diseases, adjuvants have been increasingly recognized as playing a critical role in therapeutic cancer vaccines as well. While the mechanism by which each adjuvant exerts its immune-stimulatory effect is different, their general mode of action is to aid in the priming of the immune cells. In recent years, the use of granulocyte-macrophage colony stimulating factor (GM-CSF), toll-like receptor (TLR) agonists and CD40-ligand (CD40L), described below, have become increasingly common.

6.1. GM-CSF

GM-CSF is a cytokine secreted from macrophages and T cells that stimulates the production of granulocytes and monocytes from stem cells. It has been shown that GM-CSF is capable of inducing potent immune responses that augment the effect of cancer vaccines (107). It is believed that the reason for this is due to GM-CSF's role in the recruitment and maturation of DCs. With more effective DCs, tumor antigen presentation is improved, as is the activation of macrophages, granulocytes and NK cells (108).

GM-CSF can be administered in a number of different ways as adjuvant. In its simplest form, recombinant GM-CSF can simply be injected subcutaneously or intravenously. Alternatively, if a cellular vaccine is used, GM-CSF can be incorporated into the vaccine itself. This can be accomplished through viral transduction into either the antigenic cell or a bystander cell line. It is also possible to use these transduced bystander cell lines in non-cellular vaccines as adjuvant (109, 110). As an adjuvant, GM-CSF has been used extensively for a wide range of cellular based vaccines, including melanoma, renal cell carcinoma, pancreatic cancer, prostate cancer, lung cancer and leukemia, amongst others (105, 111-117), with moderate success. Phase III trials are currently being carried out in melanoma and leukemia (<http://clinicaltrials.gov/ct2/show/NCT00769704>; <http://clinicaltrials.gov/ct2/show/NCT00454168>).

Despite the promise of GM-CSF as a cytokine adjuvant, questions regarding the extent that the action of GM-CSF on the immune system is stimulatory or suppressive have been raised. For instance, many tumor cell lines have been shown to secrete GM-CSF, and that tumor-secreted GM-CSF recruits myeloid suppressor cells (108). This dichotomous effect of GM-CSF on anti-tumor immunity is likely dependant on the dose administered or secreted by cellular vaccines. Serafini *et al.* (118) demonstrated that GM-CSF, above a certain concentration, caused a vaccine to become ineffective and actually promoted immunosuppression. Thus, accurate quantification of GM-CSF levels in immunotherapeutic formulations may be important for ensuring treatment within immunostimulatory ranges.

6.2. TLR agonists

Toll like receptors (TLRs) are a group of innate immune system membrane-spanning proteins involved in pathogen recognition and immune activation. TLR agonists are substances, often microbial, which bind TLRs and activate the immune system through a complex inflammatory response. When TLRs become activated through pathogen binding, inflammatory responses are initiated through cytokine cascade, as are gene expression patterns that initiate an adaptive immune response. TAA non-specific activation of the innate immune system, such as that which occurs through the activation of TLRs, has long been known to positively correlate with tumor regression, with spontaneous remissions associated with severe infections going back more than a hundred years (119).

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This activation of the innate immune response is particularly useful in cancer therapy, where it can result in breaking immune tolerance to self-antigens present on tumor cells. TLR agonists can convert tumor cells into a state where they are sensitive to both immune mediated and chemotherapeutic cell destruction (120). Imiquimod, used in the treatment of basal and squamous cell carcinomas, is perhaps the most successfully used TLR to date; it is now part of the standard management of such cancers. Additionally, it has been shown that the stimulation of TLR through the use of agonists causes the suppression of regulatory T cells, activation of DCs, and activation of effector T cells in the absence of antigen. TLR agonist stimulated T cells have superior expansion and activation *in vivo* when compared to non-conditioned T cells (121, 122). Accordingly, TLR agonists are increasingly being investigated for their potential in cancer immunotherapy, either alone or as adjuvants with vaccines.

Data from murine models have illustrated the promise of using TLR agonists as primary anti-tumor intervention or as an adjuvant to vaccination. For example, in a lymphoma mouse model, immunocompetent mice injected intratumorally with the TLR agonist CpG-ODN were found to develop a tumor-specific response in CD4 and CD8 T cells (123). The activity of these activated cells was preserved when adoptively transferred to knockout mice, eradicating tumor after tumor challenge.

In a separate clinical study, melanoma patients were vaccinated in a series of four monthly treatments using a TLR agonist, CpG 7909. Patients who received the CpG 7909 augmented vaccine displayed a significantly higher proportion of melanoma antigen specific T cells compared with patients who received the melanoma antigen vaccine only. When tested *in vitro*, this expanded cell population targeted melanoma cells in an antigen-specific manner (124).

TLRs have also been studied as vaccine adjuvants. In a renal cell carcinoma vaccine study, patients with metastatic renal cell carcinoma were vaccinated post-nephrectomy with autologous tumor cells combined with a CpG (a TLR) and GM-CSF (125). Three vaccine injections were administered followed by subcutaneous injection of interferon and CpG. Patients with remission or stable disease went on to receive three further vaccines. Post-vaccination DTH responses were only positive for autologous tumor cells, suggesting a tumor specific response. In this study, it is difficult to differentiate the immune effects contributed by each of the three different components of the vaccine. Clinically, 3 of 12 vaccinated patients achieved a partial response of up to 6 months duration, and a further two patients had stable disease.

TLR agonists have been shown to be effective in enhancing the post-transplant GvL effect of DLI. Durakovic *et al.* (126) showed that in chimeric mice, the addition of TLR agonists amplified the GvL activity of DLI, with conversion to full donor chimerism when TLR agonists were applied topically or intraperitoneally. Additionally, Blazar *et al.* (127) demonstrated that TLR

agonists administered as early as two weeks after transplant are capable of inducing anti-tumor responses in mice, a period where immune reconstitution is incomplete. They also showed that when given with DLI, TLR agonists were capable of preventing leukemia related mortality.

Recently, Lin & Zhang *et al.* have demonstrated that effective anti-tumor responses in patients receiving DLI for treatment of relapsed CML are associated with the production of endogenous adjuvants, present in the plasma of reactive patients, in the form of nucleic acid-antibody complexes. Specifically, they detected circulating antibody-antigen complexes that carry nucleic acids that could engage the RNA and DNA sensors TLR-8 and TLR-9. These TLR activating activities were present as early as 2 weeks following DLI, preceding the development of high titer antibody, that typically start at 2-3 months after DLI (44). Furthermore, cytokine signatures associated with TLR activation could be detected directly *in vivo* from patients with productive immune responses. These studies demonstrate that effective immune responses are associated with presence of endogenous immunostimulatory complexes that are able to induce potent antigen-specific immunity against malignant cells, and represent a novel mechanism by which effective anti-leukemia immunity is initiated and propagated *in vivo* (128).

6.3. CD40L

CD40 ligand (CD40L), a member of the tumor necrosis factor (TNF) family, is a co-stimulatory molecule expressed on the surface of activated lymphocytes, monocytes and DCs. It binds to the CD40 receptor found on antigen presenting cells, including DC, B-cells and macrophages, and induces activation of these cells. CD40L can also be used to bind to the CD40 receptor on leukemia and lymphoma cells; this activates the antigen presenting capabilities of the cells and effectively allows the malignant cells to present themselves as antigen to generate an anti-tumor response.

In the non-transplant setting, CD40L administration has been successfully used to augment anti-tumor responses when combined with other adjuvants or vaccine. In an ex-vivo study using human tumor and lymph node tissue from various different cancers, T cell responses in response to a vaccine derived from K562/GM-CSF and CD40L expressing cells were tested (129). When autologous tumor cells were mixed with the GM-CSF and CD40L expressing cells, significant anti-tumor T cell responses were induced. The study concluded that the anti-tumor activity induced by the combined GM-CSF/CD40L adjuvant was significant enough to warrant further investigation in clinical trials.

Several methods of expressing CD40L in vaccines have been explored. Dotti *et al.* (130) developed a murine model using CD40L expressing fibroblasts, in which animals were co-injected subcutaneously along with either CD40-positive or CD40-negative myeloma tumor cells, to induce tumor. Mice who had been treated with the CD40L fibroblasts demonstrated inhibition of tumor growth compared to controls, as well as resistance to

further tumor challenge. This effect was abrogated when lymphocytes were depleted. As the results were similar in both CD40-positive or CD40-negative tumor, the actions of the CD40L appear to be primarily on the activation of antigen presenting cells rather than CD40 activation of the malignant cells.

In the post-HSCT setting, Rousseau *et al.* (131) evaluated ten patients with acute myeloid or lymphoid leukemia who were in cytologic remission following HSCT (n=9) or chemotherapy (n=1), and who were vaccinated with an IL-2- and CD40L-expressing autologous tumor vaccine consisting of leukemic blasts mixed with skin fibroblasts which had been adenovirally transduced to express human IL-2 (hIL-2) and hCD40L. Patients received up to 6 vaccines subcutaneously without any severe adverse effects. Following immunization, a consistent rise in circulating CD4+ and CD8+ as well as activated (HLA-DR+) lymphocytes was observed. Importantly, large increases in anti-leukemia cytotoxic cells as well as helper T cells were observed, persisting for up to three months post vaccination. Two patients also generated antibodies to their own leukemia blasts. Although these results are promising, further testing is required to adequately assess the true value of this immunologically active vaccine.

7. AGENTS FOR CHECKPOINT BLOCKADE TO ENHANCE POST-TRANSPLANT ANTI-TUMOR RESPONSES

A novel approach to enhancing anti-tumor activity centers on the blockade of immune checkpoints, molecules found on the surface of lymphocytes that normally act to down-regulate or suppress anti-tumor activity. A number of these negative regulators have been identified, chief amongst them CTLA-4 and PD-1. The use of antibodies to block these molecules may therefore be helpful in T-cell expansion and anti-tumor activity.

7.1. Anti-CTLA4 blocking antibody

Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) is a protein expressed on the surface of CD4 cells and intracellularly in regulatory T cells that transmits inhibitory signals to T cells. CD28 binds the same ligands as CTLA4, but ligand-binding to CD28 leads to T cell activation and proliferation. CD28 and CTLA4 thus compete for binding ligands to CD80 and CD86 ligands on antigen presenting cells and have opposing effects on T cell activity. CTLA4 has a higher binding affinity, which, while important in maintaining self-tolerance, is probably also at least partly the reason for suboptimal responses to tumor antigen and therapeutic cancer vaccines (132, 133). For this reason, anti-CTLA4 treatments are being explored as a means to augment cancer vaccines.

CTLA4 blockade has been investigated as a treatment both alone and as adjuvant in a number of solid and hematological malignancies. Definitive phase III studies have recently been completed that describe the improved survival of patients with metastatic melanoma receiving ipilimumab (anti-CTLA4 blocking antibody) with or without a gp100 antigen vaccine (134). CTLA4 blockade

may also have a role in the treatment of cancer relapse following HSCT. Bashey *et al.* evaluated the effects of a single dose of ipilimumab for treatment of post-HSCT relapse in 29 patients with various hematological and solid malignancies. (135) Three patients developed clinical responses following treatment, including two durable remissions. Four of 29 patients did develop organ-specific immune events that were thought to be related to ipilimumab; however, GvHD was not unduly exacerbated. The authors concluded that ipilimumab dosing in the post-transplant was safe and potentially capable of inducing tumor regression post-transplant.

The timing of administration of anti-CTLA4, a well as the dosing, may be important to the development of autoimmunity with this treatment. For instance, Phan *et al.* (136) used a bivalent peptide vaccine augmented with anti-CTLA4 to treat patients with metastatic melanoma, and observed that a large proportion of patients (including those with objective responses) developed grade III/IV autoimmunity. On the other hand, Hodi *et al.* (21), administered anti-CTLA4 blocking antibody 1 and 4 months after treatment with an autologous GM-CSF-secreting irradiated tumor cell vaccine to treat 11 melanoma patients with different results. Eight of the 11 patients in this trial showed tumor regression, with 3 patients demonstrating partial responses. No grade III/IV autoimmunity was observed in any patients. Histological analysis of tumor showed an increase in the CD8/T-reg ratio, as observed by other investigators (137). These experiences suggest that initial priming of T cell responses to tumor through vaccination can polarize subsequent immune responses induced by anti-CTLA4 blockade away from auto-immunity.

7.2. Anti-PD1/PDL1 antibody

Programmed Death 1 (PD1) is a cellular protein in the CD28/CTLA4 family which is expressed on activated lymphoid cells and involved in negative regulation of T cells. Overexpression of PD1 on effector cells is correlated with an immunosuppressive microenvironment and is associated with development of tumor, including melanoma and CML (138, 139). Binding of PD1 with its ligands causes inhibition of both proliferation and cytokine secretion in CD4 and CD8 T cells (140). Blockade of PD1 ligand-binding with anti-PD1 can reverse this “exhausted” phenotype, creating a more dynamic environment for antigen presentation and cytotoxic action (141).

Anti-PD1 has been explored extensively in murine studies in conjunction with vaccine and adoptive transfer. For example, two recent mouse melanoma studies, one with peptide-pulsed DC vaccine (142) and one with a GM-CSF-expressing B16 melanoma vaccine (143), both successfully used anti-PD1 monoclonal antibody (MoAb) as adjuvant. In both studies, addition of anti-PD1 MoAb led to greater numbers of antigen specific CD8 cells. In the melanoma peptide-pulsed DC vaccine, when adoptive transfer of antigen specific CD8 T cells were added as additional treatment, mice treated with anti-PD1 MoAb in combination with vaccine showed increased activation and

persistence of these transferred T cells as well as a higher number of tumor infiltrating CD8 T cells. Zhou *et al.* (144) examined the effect of anti-PD1 MoAb and the adoptive transfer of cells (without vaccine) for the treatment of acute myeloid leukemia (AML). Mice which were intravenously injected with a CML cell line received anti-PD1 MoAb before and after adoptive transfer of AML-specific CD8 T cells (administered on day 14). Either therapy alone was ineffective, but significant survival advantage was observed when using both therapies in combination. Anti-PD1 MoAb treatment additionally increased both the proliferation and activation of cytotoxic T cells.

Clinical use of anti-PD1 therapy in patients is still under investigation, but the early results are promising. Berger *et al.* (145) executed a phase I study examining the effects of a single dose of anti-PD1 therapy in patients with a range of advanced hematologic malignancies, including 5 with previous HSCT. A clinical response was observed in 6 of 17 patients, with one complete remission. On immunological examination, patients were found to have an increased percentage of peripheral CD4 cells compared to pre-treatment, and this effect was increased with higher doses of anti-PD1 MoAb.

7.3. Summary

In order for immunotherapy to be as effective as possible, it is vital that all aspects of immune dysfunction induced by tumor be addressed. As such, in addition to up-regulating anti-tumor activity with the use of adjuvants, the use of checkpoint blockade inhibitors such as PD-1 and CTLA-4 to block negative immunoregulation is increasingly being explored, with promising results.

8. PERSPECTIVES

We are developing an increasingly large toolbox from which to manipulate immune responses. Increasing insights into the components of effective immunity – the need to target the immunologic heterogeneity of cancer, in a manner that utilizes the various arms of the immune responses in a coordinated fashion – are now providing us with clues on how to develop truly effective immune-based therapy with an acceptable toxicity profile. Most promisingly, many individual immunotherapy components now appear to be have clinically apparent anti-tumor activity, as we have reviewed in this chapter. In the future, rational combinations of these approaches (i.e. HSCT together with DLI and vaccine, +/- checkpoint blockade inhibitor) may provide consistent, enduring and well-tolerated responses.

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