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The allure and peril of hematopoietic stem cell transplantation: overcoming immune challenges to improve success

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Abstract

Since its inception in the mid-twentieth century, the complication limiting the application and utility of allogeneic hematopoietic stem cell transplantation (allo-HSCT) to treat patients with hematopoietic cancer is the development of graft-versus-host disease (GVHD). Ironically, GVHD is induced by the cells (T lymphocytes) transplanted for the purpose of eliminating the malignancy. Damage ensuing to multiple tissues, e.g., skin, GI, liver, and others including the eye, provides the challenge of regulating systemic and organ-specific GVH responses. Because the immune system is also targeted by GVHD, this both: (a) impairs reconstitution of immunity post-transplant resulting in patient susceptibility to lethal infection and (b) markedly diminishes the individual's capacity to generate anti-cancer immunity—the raison d'etre for undergoing allo-

HSCT. We hypothesize that deleting alloreactive T cells ex vivo using a new strategy involving antigen stimulation and alkylation will prevent systemic GVHD thereby providing a platform for the generation of anti-tumor immunity. Relapse also remains the major complication following autologous HSCT (auto-HSCT). While GVHD does not complicate auto-HSCT, its absence removes significant grant anti-tumor responses (GVL) and raises the challenge of generating rapid and effective anti-tumor immunity early post-transplant prior to immune reconstitution. We hypothesize that effective vaccine usage to stimulate tumor-specific T cells followed by their amplification using targeted IL-2 can be effective in both the autologous and allogeneic HSCT setting. Lastly, our findings support the notion that the ocular compartment can be locally targeted to regulate visual complications of GVHD which may involve both alloreactive and self-reactive (i.e., autoimmune) responses.

Keywords

Allogeneic HSCT; Autologous HSCT; GVHD; Cyclophosphamide; gp96; IL-2 complex

Introduction

According to the Center for International Blood and Marrow Research (CIBMTR), >18,000 hematopoietic stem cell transplants (HSCT) comprised of allogeneic (~40 %) and autologous (~60 %) were performed in the USA alone in 2011 [1]. Because the majority of these cellular transplants are performed in patients with hematopoietic malignancy, one goal common to both allogeneic and autologous HSCT from the immunology vantage point is to improve anti-tumor (GVL) responses in patients post-transplant (Table 1). For almost 50 years, the major complication limiting the application and utility of allogeneic hematopoietic stem cell transplantation (allo-HSCT) to treat such patients has been the development of graft-versus-host disease (GVHD). The CIBMTR recently reported results from the registry of almost 1100 patients transplanted for acute myelogenous leukemia (AML) using matched unrelated donors (MUD). Clinical and life-threatening GVHD was reported in 51 and 25 % of these patients, respectively [2]. Ironically, GVHD is induced by the same cells (T lymphocytes) transplanted for the purpose of eliminating the malignancy. GVHD damage ensuing to multiple tissues including the immune system both (a) impairs reconstitution of immunity post-HSCT resulting in the patient's susceptibility to lethal infection and (b) markedly diminishes the individual's capacity to generate anti-cancer immunity—the raison d'etre for undergoing allo-HSCT. Unfortunately, despite transplant advances and the promise of immunotherapy, relapse continues to be the primary cause of lethality (40–50 %) in allo-HSCT [1]. Notably, although GVHD and HVG (host-versus-graft responses) do not complicate auto-HSCT, tumor relapse also remains the primary cause of lethality (>70 %) following these transplants [1]. In summary, although these types of transplants typically occur following chemotherapy and conditioning to make room for donor stem cells, despite a state of minimal "residual" disease (MRD) as well as lymphopenia—two conditions of potential benefit for therapeutic immune manipulation—strategies still need to be developed for both allo- and auto-HSCT, which can circumvent immune complications and provide a cure for the malignant condition present.

Strategies to control GVHD and maintain engraftment and anti-tumor responses in allo-HSCT

Immunologists continue to grapple with the enigma of how best to harvest the anti-tumor activity associated with allogeneic HSCT. Conceptually, the two most broad approaches which have been considered (Table 2) are as follows: (a) enabling GVHD to “unleash” the power of anti-host alloreactive T-cell responses against the tumor followed by regulation/abrogation of GVHD and (b) preventing development of GVHD concomitant with the generation of anti-tumor-specific T cells. Because the latter is not yet readily feasible in many malignant conditions, approaches based on controlling GVHD have remained the major thrust clinically to improve the outcome for patients receiving allo-HSCT. Following transplant of donor T cells with anti-host reactivity, suppressing function with steroids and more directed drugs (e.g., calcineurin inhibitors, ex. CsA) has clearly advanced control of GVHD (Table 1). Subsequently, strategies transplanting T cells with less broad (i.e., tissue specific) reactivity and most recently the application of regulatory T cells (Tregs) to diminish alloreactivity may represent important advances on the horizon (Table 1). For more than 30 years, a number of approaches have evolved to become highly effective at depleting donor T cells prior to transplant in the order of 1–2 logs. These strategies have employed a variety of techniques including gradient separation, lectin-based fractionation, cytotoxic drugs, and the use of anti-sera or monoclonal antibodies alone, with complement or conjugated to toxins to diminish overall T cell numbers prior to transplant (Table 2; [3, 4]). While diminishing GVHD, the unwanted consequences of these deletions include overall diminution of T-cell immunity which includes its ability to promote engraftment and mediate rapid anti-tumor responses following transplant. Attempts to more selectively diminish donor T-cell responses, that is, those T cells with reactivity against the recipient histocompatibility (“allo”) antigens provide an opportunity to transplant non-anti-host-reactive T cells while concomitantly diminishing or preventing GVHD. Several examples included here (Table 2) have involved ex vivo methodologies, for example, coupling cytolytic molecules to antibodies or cytokines which can then target donor T cells that have been activated against the recipient’s alloantigens. One effective approach has taken advantage of activated T-cell expression of the alpha chain of the IL-2 receptor, i.e., CD25. Ricin conjugated to anti-CD25 antibody was then capable of effectively diminishing the anti-host alloreactive response in the transplanted inoculum [5, 6]. The latter more “selective deletion” in vitro strategies possess some important benefits compared with more broad, overall T-cell depletion—most notably the transplant of immune-competent donor T cells that have the potential to provide anti-pathogen and anti-tumor antigen immunity. Nonetheless, all ex vivo strategies predicated on deleting donor T cells prior to transplant share the common limitation that removal of anti-recipient alloreactive T cells—while diminishing or abrogating GVHD—will by definition be accompanied by weakening hematopoietic engraftment as well as initial anti-tumor responses immediately post-transplant (Fig. 1). Thus, during the period of minimal residual disease and the presence of lymphopenia, the latter which provides opportunity for enhancing desired immune responses—what is arguably the “most timely opportunity” to cure the malignant state—is dramatically diminished or lost when such strategies are employed.

Although potentially more “risky,” strategies aimed at deleting T cells after transplant offer the opportunity to harvest beneficial effects of donor cells “prior to their demise.” A sophisticated and potentially powerful strategy rooted in basic research and then performed clinically to capture the “good” of donor anti-host alloreactivity employed insertion of a “suicide gene” (i.e., tk, thymidine kinase) into the donor’s T cells prior to transplant. This was demonstrated to successfully enable their subsequent elimination following anti-tumor activity resulting in remission without GVHD (Table 2; [16]). Unfortunately, such an elegant approach requires individualized patient molecular methodology involving cell culture, gene transfection, selection, and other techniques, which presents practical limitations preventing large-scale clinical implementation.

Administration of cyclophosphamide post-transplant (PTC): a promising experimental and clinical approach for protection against GVHD following allo-HSCT

It is well appreciated that anti-host alloreactivity by donor T cells provides both important benefits and perilous complications to patients. Thus, there has remained both the need and desire to develop advances to enable the “release and control of this genie.” A successful advance must not only be scientifically sound, i.e., capable of preventing development of GVHD while enabling the generation of anti-tumor (to eradicate disease) and anti-pathogen (to protect against infection) responses early post-transplant, but also possess the practicality for straightforward implementation by a bone marrow transplant unit in the clinic. Recently, cyclophosphamide (cyc) has been “reinvented” within the context of regulating alloreactivity not as part of pre-transplant conditioning protocols, but as a promising strategy to diminish acute GVHD following allogeneic HSCT.

Initially used as an anti-cancer drug, early studies reported PTC could (a) promote tolerance induction to skin grafts and (b) delete specific T-cell receptor families after allogeneic skin grafts were applied [20, 21]. These findings suggested that anti-alloantigen-reactive T cells could be deleted by cyc when administered at appropriate times following antigen [22, 23]. Later studies by a group at Johns Hopkins in the early 2000s demonstrated that administration of cyc after transplant cold block rejection of hematopoietic stem cell transplants leading to engraftment [24]. Subsequently, clinical trials initially by Fuchs, Luznik and colleagues and then by several centers including Thomas Jefferson and MD Anderson found that high-dose PTC injection early post-transplant—critically at days 3 and 4—could markedly diminish GVHD in MHC-mismatched and MHC-matched allogeneic (Fig. 2) transplant recipients [17–19, 25]. Indeed, we employed a lower concentration of cyclophosphamide in a pre-clinical model of MHC-matched allogeneic HSCT using the same kinetics and observed marked inhibition of GVHD [26]. Based on the DNA alkylating properties of cyc, the initial hypothesis to account for the observed effect was the elimination of alloreactive T cells post-transplant [27, 28]. Exposure of rapidly proliferating anti-host-reactive T cells to alkylation by cyc would not enable sufficient time for DNA repair to “save these cells.” Using CFSE and other labeling dyes, we observed that a large number of rapidly dividing transplanted donor T cells did not survive under the PTC protocol employed. Hence, elimination of both donor anti-host alloreactive and host anti-

donor alloreactive T cells would be consistent with the inhibition of GVHD and presence of donor engraftment post-transplant, respectively.

One interpretation of the above findings is that the mechanism for PTC protection against GVHD singularly can be accounted for by taking advantage of the "initial burst" of alloreactive T-cell responsiveness to delete GVH and HVG reactive T cells. It was therefore interesting that several years ago, Luznik and colleagues and work in my own laboratory observed that depletion of donor Treg cells prior to transplant appeared to abrogate the ability of PTC to diminish GVHD [29, 30]. Subsequent studies in our laboratory and together with our collaborators at Johns Hopkins employing rigorous depletion strategies (a) removing $CD4^+FoxP3^+$ donor cells ex vivo using FoxP3 promoter-driven RFP and GFP knock-in donor mice followed by flow cytometric cell sorting and (b) removing $CD4^+FoxP3^+$ donor cells in vivo using FoxP3 promoter-driven diphtheria toxin receptor knock-in mice, clearly demonstrated that depletion of Treg cells abolished the capacity of PTC to ameliorate GVHD. (Donor $CD4^+FoxP3^+$ regulatory T cells are required for post-transplant cyclophosphamide-mediated protection against GVHD in mice. Ganguly, S., Ross, D.B., et al. manuscript submitted, 2013) We interpret these results to indicate that deletion of alloreactive T cells is not the singular mechanism responsible for the inhibition of GVHD by PTC and that donor Treg cells are required for cyc-mediated regulation. Our working hypothesis is that not all donor anti-host alloreactive T cells are deleted by PTC, and therefore, the presence of donor Treg cells after administration of Cyc is required to maintain suppression of emergent anti-host alloreactive T cells to suppress GVHD. This hypothesis is consistent with the recent observations that Treg cells possess aldehyde dehydrogenase levels higher than levels in conventional T cells which make the former less susceptible to cyc-induced alkylation, a finding recently reported by our collaborators examining human Treg cells [31] and also observed in our mouse models (Ganguly, S., Ross, D.B., et. al. manuscript submitted, 2013). Thus, following PTC administration, a favorable Treg:Teff (effector) ratio would be capable of preventing emergence of GVHD.

How does PTC affect non-anti-host alloreactive donor T cells?

While most efforts regarding PTC have focused on the regulation of alloreactive T cells, we have been interested in asking questions regarding the impact of PTC on non-alloreactive T cells. The significance of this T-cell component in allo, as well as auto-HSCT, in providing immune reconstitution is now understood to be critical post-transplant [32]. Dependent on the nature of the transplant, including the level of conditioning and the age of the recipient, which influences potential de novo thymopoiesis contributions, peripheral expansion of transplanted donor T cells may play the major role or contribute to providing immunity post-transplant [33]. Since we observed high susceptibility to PTC by rapidly dividing alloreactive T cells, we were interested in testing the hypothesis that donor T cells undergoing slower division post-transplant—primarily as a result of lymphopenia-induced proliferation (LIP)—would preferentially survive exposure to an alkylating agent (Fig. 3). To assess donor T cells expressing TCR to antigens other than recipient histocompatibility epitopes, we examined proliferation by several TCR transgenic CD8 populations, i.e., OT-I and Pmel-1 reactive to ova-peptide (SIINFEKL, aa 257–264 chicken OVA, K^b binding) and melanin peptide (KVPRNQDWL, aa 25–33 human melanin gp100, D^b binding),

respectively. Interestingly, donor T cells undergoing slower division (i.e., non-anti-host alloreactive) as evidenced by dye dilution clearly survived more effectively than those cells undergoing rapid proliferation (i.e., anti-host alloantigen reactive). Notably, CD8 OT-I cells undergo somewhat faster lymphopenic-driven division (~1×/24 h.) than Pmel-1 cells (~1×/36 h.) and the former were found to exhibit somewhat less survival compared to the latter [26, 34]. This work is important for a number of reasons: (a) the findings raise the possibility that the sensitivity of T cells to being eliminated by exposure to cyc is determined by its rate of proliferation and (b) LIP signals do not necessarily "render" a T cell susceptible to cyc-induced death [35]. We posit that by further diminishing the rate of LIP, for example, by blocking IL-7R-mediated signaling, the extent of survival of non-anti-host alloreactive T cells can be further improved with little impact on antigen + co-stimulation-driven proliferation by donor anti-host alloreactive population.

The application of recipient alloantigen and alkylation ex vivo as a new pre-transplant strategy to harvest the "good" and control the "bad" by anti-host alloreactive T cells

Because administration of high-dose cyc on days 3 and 4 post-transplant has been shown to be effective for the prevention of GVHD, we asked would it be possible to obviate in vivo exposure to this alkylating drug and harvest the beneficial effects of alloreactive T cells by developing an ex vivo strategy to "pre-program" donor anti-host effector cells to die after their transplant? We reasoned that a strategy (Fig. 4) which "programs" only anti-recipient alloantigen-specific T cells to die following their transplant could also result in (a) a rapid and transient period of anti-tumor/pathogen responses, (b) the absence of GVHD, and (c) the reconstitution of immune function via non-anti-host TCR-bearing T cells to enable vaccination strategies for eliciting effective anti-tumor immunity in HSCT recipients. The advantages of such a strategy over in vivo PTC administration include the following: (1) the absence of large systemic doses of cyc to patients and (2) diminished likelihood of eliminating donor anti-tumor antigen-reactive cells which might if present be undergoing sufficient proliferation thereby making them susceptible to alkylation-induced death.

This approach differs from other deletion strategies that eliminate anti-host-reactive cells prior to transplant removing donor T-cell capacity to affect graft-promoting and anti-tumor activity in recipients. Nonetheless, continued exposure to alloantigen in recipients is hypothesized to drive rapid proliferation and thereby prevent sufficient time for DNA repair by this population. In contrast, we hypothesize that non-activated donor T cells in the ex vivo culture will undergo slow, lymphopenia-induced division and will be spared from death as a consequence of DNA repair activity. These cells can therefore persist in recipients and provide immunity during the early post-HSCT period. To bypass cyclophosphamide's need for hepatic activation to generate its active metabolic product, we selected mafosfamide to initiate DNA damage since this compound spontaneously hydrolyzes to the active metabolite 4-hydroxy-cyclophosphamide. Our initial findings monitored the death of transgenic CD8 T-cell populations in vitro following exposure to varying concentrations of mafosfamide for varying time periods (data not shown). We identified an effective concentration that was added after 24 h of co-culture between donor CD8 T cells and syngeneic spleen cells pulsed

with appropriate peptide. At concentrations >2.0 ug/ml, almost 100 % of CD8 T cells had died by 120 h of culture (data not shown). Notably, assessment for intracellular cytokine (Fig. 5) demonstrated that this protocol resulted in CD8 T-cell production of IFNc and GzmB by mafosfamide-treated cells between 29 and 52 h post-antigen stimulation. These results demonstrate that such cells transferred into recipients immediately following mafosfamide washing at 29 h would be producing effector molecules.

We have recently performed several preliminary experiments co-culturing B6 T cells with BALB/c-stimulating cells ex vivo followed by brief exposure to mafosfamide immediately prior to transplant (Fig. 6). The findings observed indicated that although recipients die in each of the three “control”-treated donor T-cell groups, donor T cells treated with BALB/c antigen and mafosfamide did not induce any lethality in this complete MHC-mismatched allo-HSCT. Our recent findings have identified donor $CD19^+$ B cells > 1 month post-transplant demonstrating donor bone marrow engraftment occurred in these recipients (data not shown). Experiments are underway to assess the fate of mafosfamide-treated T-cell populations post-transplant including non-recipient-reactive T cells. These experiments will test the hypothesis that such cells survive mafosfamide treatment and can affect immune function to tumor cells (GVL) and pathogens, i.e., viral infection (GVP). Our working hypothesis is that slowly dividing non-host alloantigen-reactive T cells will persist in these mice to provide a platform for vaccination against tumor and pathogen antigens.

Ocular graft-versus-host disease: a model to visualize and dissect immune mechanisms that cause allo-HSCT-induced tissue-specific damage

As described above, GVHD is a complex, multi-organ disorder arising from an immunologic attack by donor alloreactive T cells that result in damage caused by inflammatory responses in specific targeted organs. These include the skin, gastrointestinal tract, liver, mucosal surfaces, and the ocular adnexa [36]. Even though major advances have been made in the understanding of immune dysregulation in systemic GVHD, a critical question in the field is to understand the relationship between systemic and organ-specific GVHD. More specifically, a central unanswered question regarding systemic GVHD and subsequent damage in various tissues is the involvement of not only alloreactive, but self-reactive (i.e., autoimmune) responses, which have also been demonstrated post-HSCT [37]. A major limitation for this deficiency of knowledge regarding tissue-specific immune dysregulation is due in part, to the technical difficulties in having direct access to these tissues to study and characterize infiltrating immune cells.

Our group has utilized the eye to study in vivo and in real time the development of immune responses after inflammatory insults and transplantation [38–40]. The direct access to this organ and its transparency provides a unique opportunity to perform intravital microscopy of fluorescently labeled immune cells to monitor their recruitment and allow isolation for characterization of their phenotype and effector/regulatory functions. Moreover, ocular GVHD occurs in >60 % of patients with GVHD and is characterized by dry eye, conjunctiva damage, punctate keratopathy, corneal ulceration, and perforation [41, 42]. Patients with ocular GVHD suffer and are incapacitated because of severe ocular discomfort, pain, and

poor vision. Therefore, understanding the roles of alloreactive and auto-reactive T cells in the pathogenesis of eye damage can lead to the development of novel therapies for not only the eye but also other affected organs.

The role of alloreactive T cells in the development of ocular GVHD has been well known. However, even though there has been no direct evidence of self-reactive T-cell involvement, the chronic form of GVHD is characterized by “autoimmune” features that often resemble clinical findings in scleroderma and Sjögren syndrome, which cause dry eye and ocular surface damage similar to ocular GVHD [43–47]. Our group has developed a pre-clinical animal model of systemic GVHD that develops ocular manifestations similar to the ones observed in patients receiving an allogeneic HSCT. To understand the immune mechanisms responsible for the development of ocular GVHD, an MHC-matched minor histocompatibility-mismatched HSCT model developed in our laboratory was utilized [48, 49]. C3H.SW (H-2b, Ly9.1^{+}) mice transplanted with B6 (H-2b, Ly9.1^{-}) T-cell depleted bone marrow (TCD-BM) and T cells (a) underwent weight loss and began exhibiting clinical signs of GVHD ~ 3 weeks post-HSCT, (b) contained damaged thymuses, and (c) expressed an inverted CD4/CD8 ratio in the peripheral lymphoid compartments (data not shown). Importantly, these mice also developed ocular surface disease evidenced by the progression of ocular surface damage characterized by increased corneal staining and ulceration by week 6. Excitingly, histological analyses demonstrated that affected mice exhibited corneal thickening and epithelial irregularity, as well as dense inflammatory cell infiltrates that were associated with conjunctiva involvement and significant goblet cell destruction known to be a cause of sicca-mediated ocular damage (data not shown). When fluorescently labeled donor T cells were transplanted, in vivo and ex vivo fluorescent imaging studies confirmed the recruitment of donor T cells into the eye preceding the time that ocular damage was observed (Fig. 7). Importantly, our studies also demonstrate that the recruitment of macrophages was significantly increased in eye with ocular GVHD and in situ cytokine analysis confirmed the existence of IFNγ, TNFα, and IL-6 mediators of inflammation that could lead to tissue damage.

GVHD is a complex systemic disorder involving multiple tissues including the eye. The easy access to study this involved organ can provide new insights into the immune pathogenesis of “local” GVHD. Utilizing this model, we have confirmed the normal dogma that establish that donor anti-host alloreactive T cells are responsible to initiate GVHD, which also leads to pathogenesis in some tissues—including the ocular adnexa. However, self-reactive T cells arising from both thymic and peripheral pathways have been identified during GVHD, and these are believed to cause auto-immune like symptoms [43–46, 50–53]. Therefore, we posit that self-reactive T cells also play a role in ocular GVHD through similar pathways of differentiation, and in addition, the recruitment of both allo- and self-reactive T cells orchestrates the recruitment of inflammatory macrophages that eventually will cause tissue damage responsible for the clinical manifestations of ocular GVHD (Fig. 8).

The understanding of pathways responsible for “tissue” GVHD is extremely important to develop new therapeutic approaches to this disease. In the eye, we have identified an intimate relationship between innate and adaptive immune responses. This “real-time” information has led us to investigate the application of novel small biomolecules and

compounds to target the specific pathways identified within this model. The goal of these studies is to interfere and abrogate the access of the relevant immune cells and/or their function within the ocular tissue to inhibit damage. Importantly, the rationale of developing such targeted local immunotherapy is to prevent ocular GVHD without interfering with anti-tumor (i.e. GVL) activity while maintaining vision and improving patient quality of life.

Vaccination using a heat shock protein-based vaccine coupled with targeted IL-2 can markedly augment anti-tumor immunity in the early period post-autologous HSCT

Autologous HSCT is primarily utilized for patients with lymphoma or myeloma [1]. As noted earlier, the major cause of mortality after auto-HSCT for patients with these malignancies results from relapse of primary disease or infection [1]. Enthusiasm for combining immune-based strategies with auto-HSCT rests in part from the notion that vaccination regimens can be employed early post-HSCT during “reboot” of the immune system to promote efficient anti-tumor and anti-pathogen immunity by taking advantage of minimal residual disease as well as the lymphopenia present [32, 54–60]. Nevertheless, generating successful protocols early post-HSCT must account for the relative dearth of T cells as well as the need for a vaccine with appropriate tumor or pathogen antigens to promote successful immunity.

Although research to demonstrate immunotherapy against tumors has been ongoing for decades, the first therapeutic cancer vaccine consisting of autologous dendritic (i.e., antigen presenting) cells obtained by leukapheresis exposed to prostatic acid phosphatase was not approved by the Food and Drug Administration in the USA until 2010; Sipuleucel-T (Provenge®). Heat shock protein gp96 is the resident endoplasmic reticulum protein chaperone and is intimately involved in MHC-I restricted antigen presentation [61–67]. Following necrosis, gp96–peptide complexes are released and can be taken up by antigen-presenting cells (APC) leading to peptide delivery and their efficient activation [68, 69]. In the 1980s, Srivastava et al. demonstrated that a 96-kDa protein was responsible for the immunity induced by immunization with tumor cells [61]. Once proteins are processed by the proteasome and their antigenic fragments enter the ER, peptides with appropriate amino acid sequence are transferred from gp96 to calreticulin to MHC-I before transiting to the cell surface where peptide is cross-presented to specific $CD8^+$ T cells. Notably, APC uptake of gp96 also induces activation and expression of a number of cytokines and chemokines. Subsequent to this overall understanding, Srivastava and colleagues introduced autologous tumor-derived gp96 preparations into patients with cancer, including lymphoma [70, 71]. Although immune correlates were elicited, overall patient survival was relatively unchanged compared with the standard of care regimens [72].

In the late 1990s, our colleagues developed a strategy to have cells secrete gp96 by replacing the ER retention signal (KDEL) with the F_C portion of IgG [73]. Following transfection into tumor cell lines, this secreted gp96-Ig molecule-induced $CD8^+$ T lymphocyte and natural killer (NK) cell activation and expansion, leading to enhancement of anti-tumor and anti-viral immunity [74–81]. Notably, the first phase I trial employing a human allogeneic lung

cancer cell line engineered to secrete gp96-Ig was recently completed [82]. Interestingly, ~75 % of evaluable patients displayed $CD8^+$ T cells that secreted IFN-γ when stimulated ex vivo with the vaccine cells, and these subjects had a median survival time (MST) of 16.5 months compared to 4.5 months for non-responders. While encouraging, we hypothesized that vaccination with tumor cells secreting gp96-Ig as a single agent can be improved by taking advantage of its capacity to activate multiple clones of potential anti-tumor-specific non-tolerant donor T cells early following auto-HSCT.

Interleukin (IL)-2 therapy has demonstrated significant anti-tumor activity in experimental models and has diverse effects following HSCT in part dependent on dose and time of infusion [83, 84]. However, since expansion of T regulatory cells (Treg) by IL-2 could dampen anti-tumor immunity, an important advance for this cytokine's in vivo usage would be to target its activity primarily to effector compared to Treg cells [85–87]. Notably, recent findings have reported that IL-2 conjugated to a specific anti-IL-2 monoclonal antibody (mAb) can augment anti-tumor responses [88, 89]. Boyman et al. demonstrated that a specific anti-mouse IL-2 mAb (clone S4B6) targeted IL-2 ($IL\text{-}2_{S4B6}$) predominantly to memory phenotype $CD8^+$ T lymphocytes and NK cells [85], both of which express the β (CD122) and γ (CD132) chains of the IL-2 receptor, typically in the absence of the α (CD25) chain [90, 91]. This "targeting" of IL-2 to principally exclude cells (including $CD4^+$ $FoxP3^+$ Treg cells) expressing CD25 thereby should reduce one complicating side effect, which could hinder the clinical translation of this therapy [86, 87]. In the context of our studies, it is notable that both $CD8^+$ T lymphocytes and NK cells are populations essential for optimal gp96-Ig-induced anti-tumor responses [75].

Several groups have successfully used IL-2 complexes to enhance vaccination [88, 89] and others have shown that IL-2 can also enhance endogenous anti-pathogen responses, in a $CD8^+$ T lymphocyte and NK cell-dependent fashion [92, 93]. Since infection is the second leading cause of mortality in HSCT recipients [1], a strategy that combines tumor vaccination and targeted IL-2 lends merit to its use in the early period following HSCT, since it possesses the capacity to enhance both anti-tumor and anti-pathogen responses. Since overcoming diminished immune function is a major challenge following regimens involving aggressive therapy and auto-HSCT, an "ideal" strategy would simultaneously elicit rapid and potent tumor-specific responses, while successfully augmenting overall anti-pathogen immunity. The objective of our studies was to evaluate the efficacy of a tumor cell vaccine combined with IL-2 in an experimental model of minimal residual lymphoma during the early period following myeloablative conditioning and syngeneic HSCT (Fig. 9). The results demonstrated that vaccination with tumor cells engineered to secrete gp96-Ig initiated within a few days post-HSCT induced the activation, expansion, and functional competence of tumor-specific $CD8^+$ T lymphocytes (Newman, R., et al. manuscript submitted 2013). Notably, administration of IL-2 pre-bound to an anti-IL-2 mAb led to rapid and extensive expansion of total $CD8^+$ T lymphocytes and NK cells (Newman, R., et al. manuscript submitted 2013). Importantly, one consequence of this expansion we identified was enhancement of the generation of anti-pathogen responses as assessed following *Listeria monocytogenes* infection after auto-HSCT (Newman, R., et al. manuscript submitted 2013). Overall, this combinatorial strategy of targeted IL-2 following heat shock protein vaccination induced remarkable expansion of tumor-specific $CD8^+$ T lymphocytes

and led to dramatic increases in MST and overall survival in HSCT recipients (Fig. 9) as well as the generation of long-lived anti-tumor memory (Fig. 10) (data not shown).

There have been few pre-clinical studies employing therapeutic anti-tumor vaccination in myeloablative models of syngeneic HSCT supplemented with T cells. In contrast to the multipronged vaccination strategy employed by our laboratory, those studies required tumor cell vaccination *after* de novo T cell genesis following transplant, higher doses of donor T cells ($> 2.0\times10^6$), or presensitization of donor T cells with the tumor vaccine [94–96]. One objective of our work was to rapidly elicit T-cell responses by transplanted donor cells, which would necessitate kinetics prior to *de novo* production of thymic-derived T cells. Expansion of donor tumor-specific $CD8^+$ T cells was observed following gp96-Ig vaccination alone or together with targeted IL-2 within 10 days, and this combination strategy induced >30,000 × expansion <2 weeks post-HSCT (Newman, R., et al. manuscript submitted 2013), a time frame prior to production and emigration of new thymic-derived T cells which takes place 2–3 weeks post-HSCT [97]. Together with the observation that gp96-Ig vaccination did not evoke protective immunity in recipients receiving TCD-BM grafts (Fig. 9), we conclude that the vaccination regimens here expanded transplanted donor T cells very early following HSCT, paralleling clinical findings requiring the addition of large numbers of T cells to provide detectable immunity [98].

Our working model emphasizes the rapid and multiple interactions proposed early post-HSCT in mice vaccinated with gp96-Ig-transfected tumor cells and coordinated infusions of IL-2/αIL-2mAb complex (Fig. 10). Following administration of vaccine cells and $IL\text{-}2_{S4B6}$ into the peritoneal cavity, NK and CD8 T cells rapidly infiltrate this compartment leading to activation ($CD44^{hi}CD62L^{lo}$, data not shown) followed by expansion of antigen-specific CD8 T cells. This treatment leads to global expansion of memory phenotype CD8 T cells as well as NK cells. Following CD4 T-cell transplant, vaccine + $IL\text{-}2_{S4B6}$ failed to demonstrate any prolonged survival compared to non-vaccinated recipients transplanted with CD4 and CD8 T cells. Moreover, vaccine failed to expand antigen-specific CD4 T cells further emphasizing the crucial role of donor CD8 T cells in this protocol.

Future directions

We have found that a large component of the donor T-cell pool in recipients after PTC is derived from mature T cells contained in the donor inoculum (Ross, Levy, unpublished observations) consistent with our findings that non-anti-host-reactive T cells preferentially survive alkylation due to their slower rates of proliferation early post-transplant [26]. Although presently it is not understood how PTC affects the donor inoculum with respect, for example, to naïve and memory populations, understanding how PTC impacts transplanted donor T-cell populations is critical for the design of more effective strategies to augment tumor (GVL) and overall immunity in allo-HSCT recipients. In some respects, the ability of PTC treatment to abrogate GVHD may be considered as “converting” allo-HSCT toward an auto-like HSCT environment, i.e., a result of the absence of GVHD, sparing damage to the marrow and thymic compartment and consequent improved kinetics of immune reconstitution. Accordingly, based on our findings in the autologous HSCT model to date, we propose to take advantage of the powerful strategy utilized to expand anti-tumor

reactive T cells by implementing a similar vaccine strategy coupled with directed IL-2. This regimen will itself result in a rigorous assessment of the ability of alkylation both in vivo (post-transplant cyclophosphamide) and ex vivo (pre-transplant mafosfamide) to prevent GVHD since IL-2 infusion will likely provide additional stimulation to drive surviving transplanted donor anti-recipient alloreactive T cells. Such types of experiments will begin testing the possibility that deletion strategies being developed can convert the post-transplant allo-HSCT environment toward that present in an auto-HSCT recipient, which may thereby enable the elicitation of truly effective responses against pathogens and tumor by transplanted, mature non-tolerant donor T cells.

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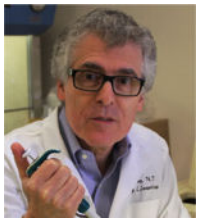
Biography



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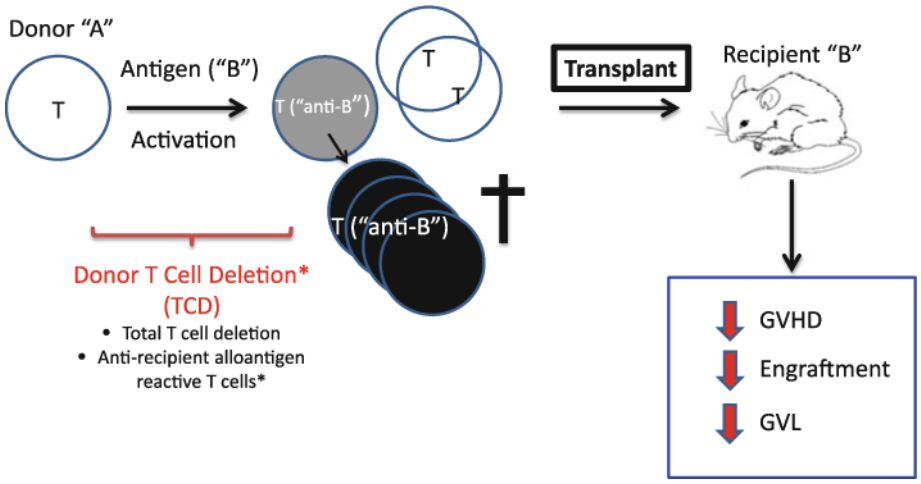

Fig. 1.

Deletion of anti-recipient-reactive T cells prior to allogeneic HSCT. Historically, there have been a number of approaches employed to reduce/delete total T cells or specifically, anti-recipient alloantigen-reactive T cells ex vivo. These include antibodies specific to T-cell determinants + complement, antibodies targeting activated T cells (ex. CD25) conjugated with toxins (ex. ricin) and cytokine conjugated to toxins (ex. IL-2-diptheria toxin). Not unexpectedly, these strategies have resulted in reducing GVHD accompanied by diminished engraftment and anti-tumor responses (GVL)

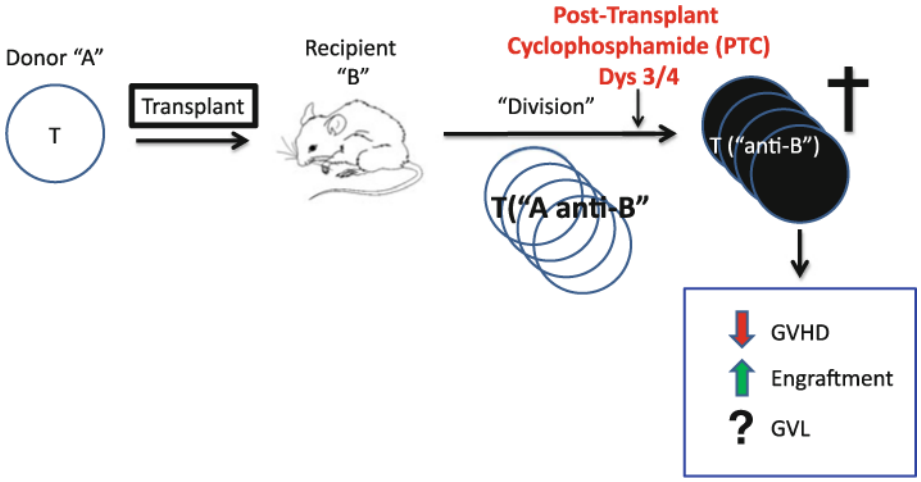

Fig. 2.

Deletion targeting anti-recipient reactive donor T cells after allogeneic HSCT. The experimental administration of cyclophosphamide post-transplant (PTC) and recently following clinical allo-HSCT has been hypothesized to eliminate rapidly dividing cells including host-versus-graft (HVG)- and graft-versus-host (GVH)-reactive T cells. This dampening of alloreactive T cells is proposed to ameliorate HVG and GVHD. The impact on the beneficial anti-tumor response (GVL) is not yet understood

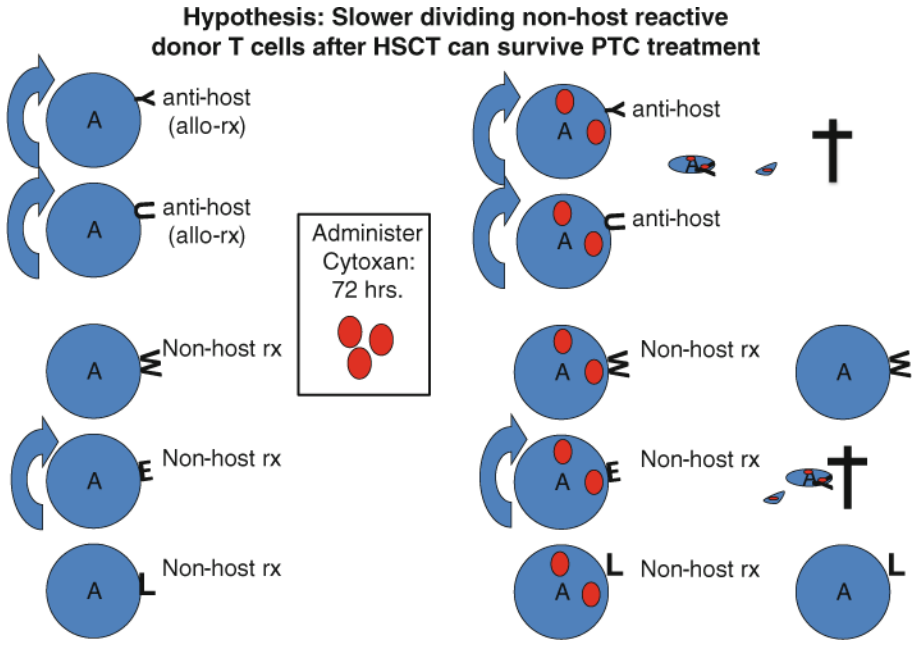

Fig. 3.

Deletion of rapidly but not slowly dividing donor T cells exposed to post-transplant cyclophosphamide. We hypothesize that alloantigen stimulated donor T cells by recipient APC providing transplantation antigen plus co-stimulation leads to rapid division and insufficient time for DNA repair following alkylation. In contrast, more slowly dividing donor T cells are spared following alkylation due to slower division as a result of stimulation by lymphopenic signals enabling sufficient time for such cells to repair alkylated DNA

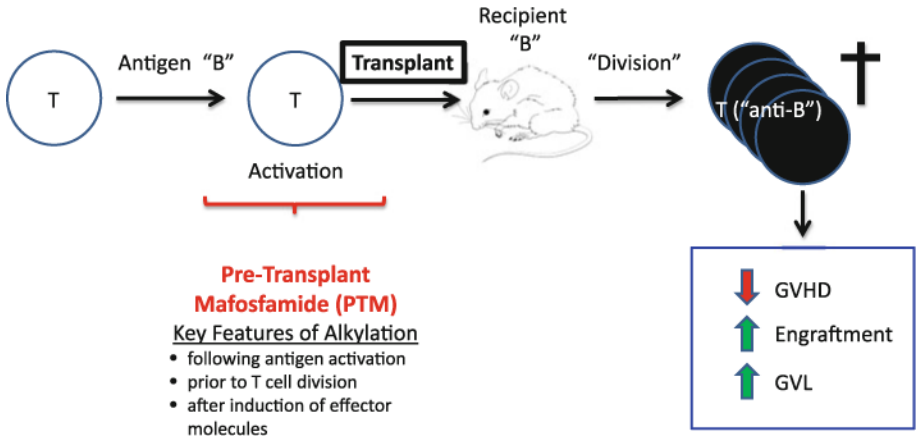

Fig. 4.

Ex vivo programming of donor anti-recipient-reactive T cells to die following transplant into antigen-expressing allogeneic HSCT recipients. The hypothesis presented is that donor anti-recipient alloantigen-specific T cells can be programmed ex vivo to undergo death in the recipient early post-transplant. The strategy is to induce donor T-cell activation specifically against recipient alloantigens combined with alkylation using the active hepatic metabolic product of cyclophosphamide, i.e., mafosfamide

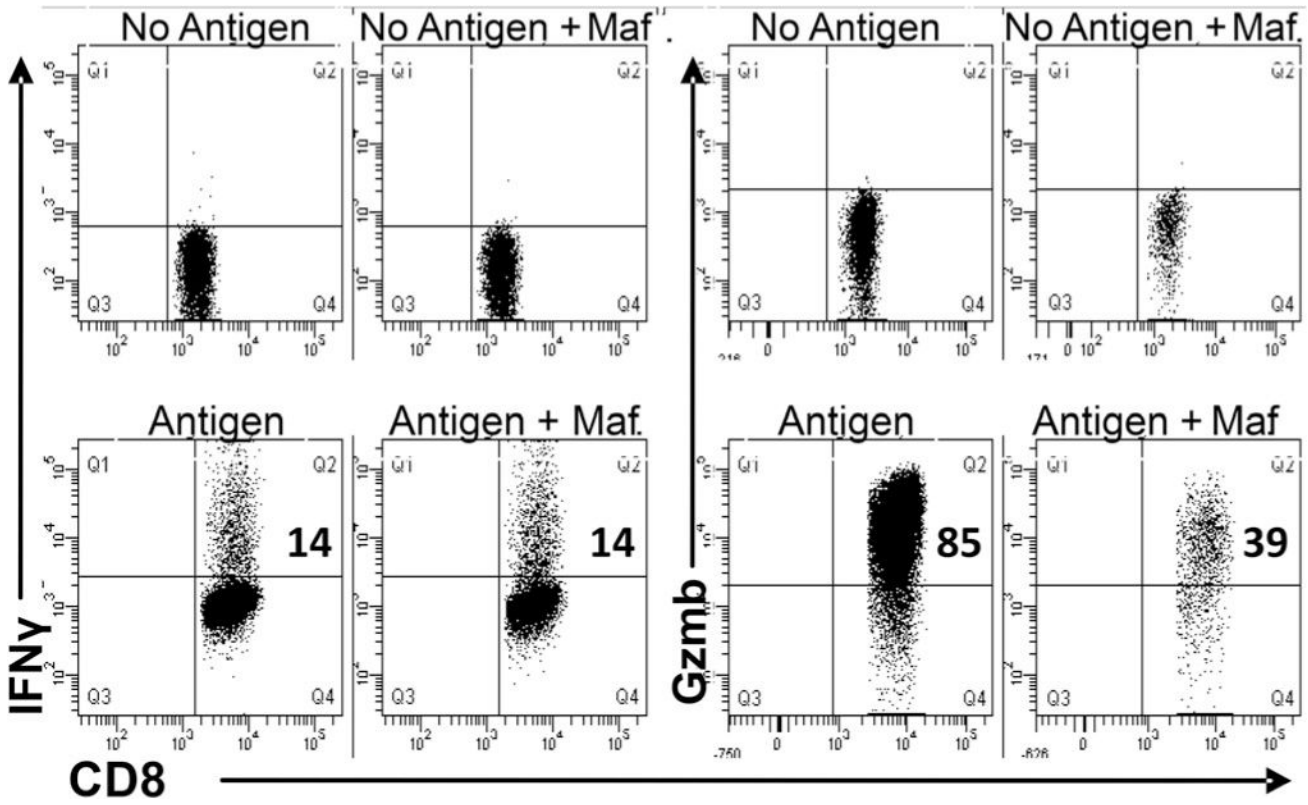

Fig. 5.

Antigen-stimulated effector cells exposed to mafosfamide produce effector cytokines until they die. No difference was detected following 24 h stimulation with antigen followed by exposure to mafosfamide (4.0 lg/ml, 5 h.) for IFNγ production (*left*). At 52 h post-culture initiation (day 2–3), when some mafosmfamide cells are dying (not shown), these CD8 T cells are still producing Gzmb (*right*) important for effector function

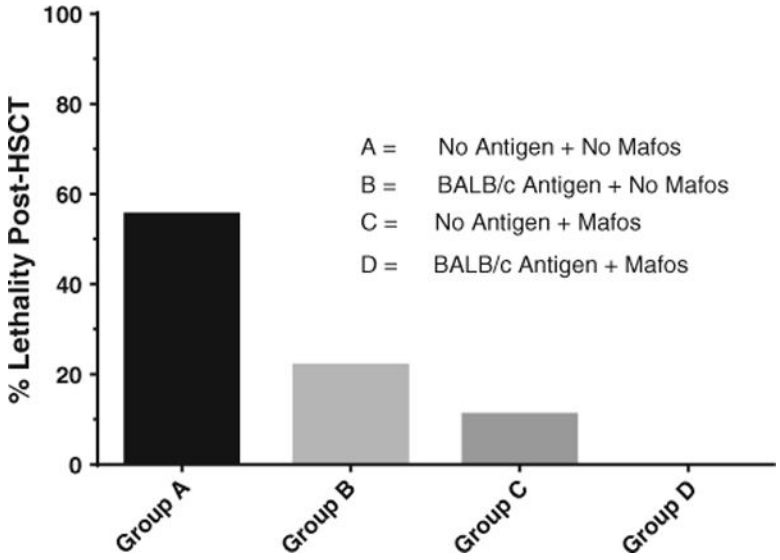

Fig. 6.

Donor B6 T-cell cultures stimulated with recipient antigen and pulsed with mafosfamide do not induce lethality following transplantation. B6 lymph node cells were cultured for 24 h alone or together with BALB/c antigen and/or brief exposure to mafosfamide immediately prior to transplant. BALB/c recipients were lethally irradiated (8.5 Gy TBI) 1 day prior to receiving cultured T cells and T-cell-depleted B6-bone marrow cells. Summary of four experiments is shown (n = 18/group)

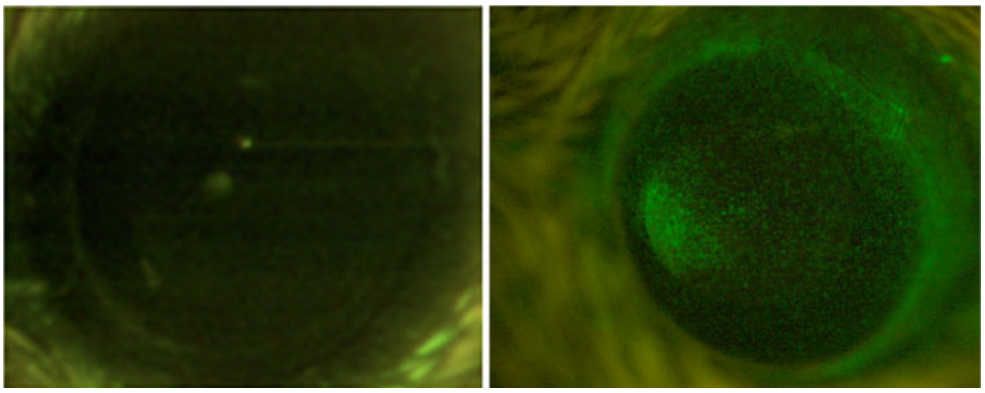

Fig. 7.

In vivo visualization of B6-EGFP-labeled donor T cells in the cornea of animals with systemic and ocular GVHD. C3H.SW H2b mice were transplanted with either bone marrow cells only (*left panel*) or BM cells and EGFP-labeled T cells (*right panel*) from EGFP-C57BL/6 H2b. In vivo fluorescent intravital microscopy of the cornea was performed at different time points after transplantation. In contrast to control bone marrow only transplanted animals (*left panel*), EGFP donor T cells were readily detectable in the cornea (*green dots*) of animals undergoing systemic and ocular GVHD by week four after transplantation

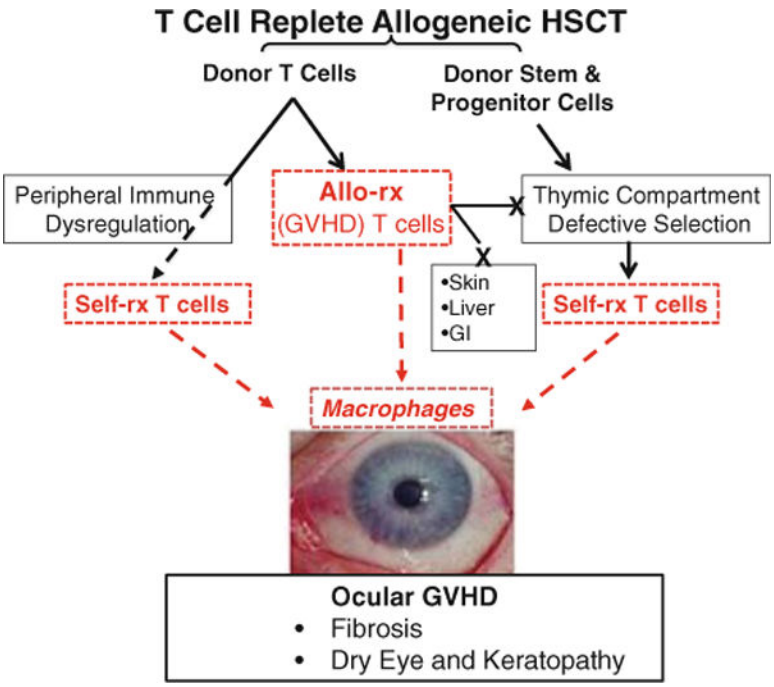

Fig. 8.

Our working model is that Th1 cells are recruited to the ocular compartment followed by mU where cytokine mediated cross-talk occurs between these cell populations effecting local pathogenesis that we propose defines ocular GVHD

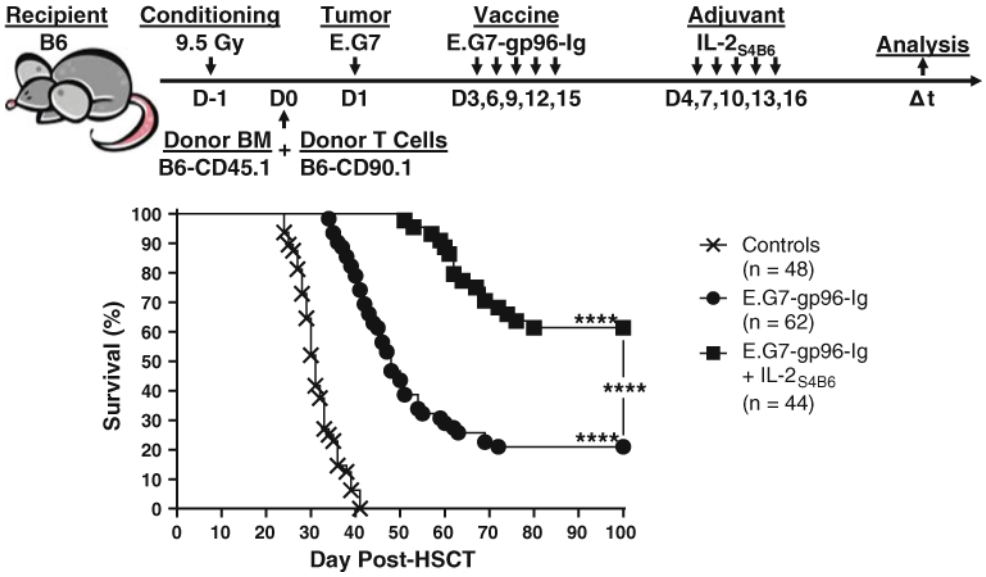

Fig. 9.

Top: Autologous (“syngeneic”) HSCT model: The time frame includes administration of gp96-Ig-transfected vaccine cells (1 × 107ip, _40 Gy) and IL-2 anti-IL-2S4B6 complex. *Bottom*: Survival data following a viable tumor (EG7) challenge on day 1 post-transplant. Control groups (*star*) include recipients without T cells and vaccine, as well as parental vaccine cells not secreting gp96-Ig. EG7–gp96-Ig (*filled circle*) groups include recipients receiving vaccine with no IL-2 or together with non-complexed (“free”) IL-2. EG7–gp96-Ig + IL-2S4B6 (*filled square*) mice. Experimental results obtained in this group exhibited identical survival with and without addition of CD8 OT-I T cells demonstrating vaccine + IL-2S4B6 efficacy in the absence of transplanting small numbers of TCR transgenic donor T cells

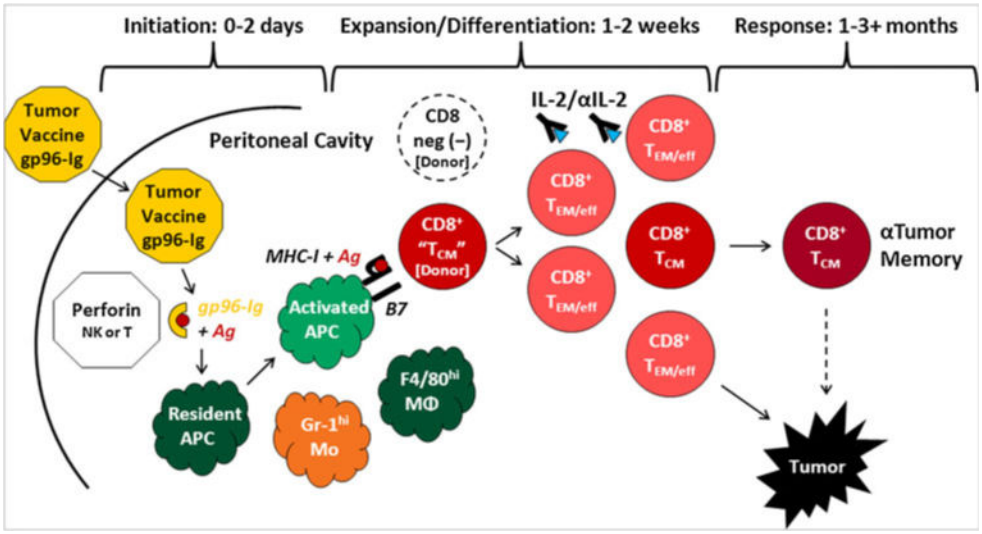

Fig. 10.

Proposed model to account for the combinatorial strategy involving gp96-Ig tumor cell vaccine + IL-2/anti-IL-2 complex (IL-2S4B6) delivery early following auto-HSCT. Intraperitoneal administration of the vaccine cells results in rapid influx of NK and T cells. NK cells interact with APC, which may involve perforin function that is known to be needed for overall vaccine efficacy post-transplant and in non-transplanted mice. Studies using purified CD8 T cells (not shown) have demonstrated that non-CD8 T cells are required for optimal vaccine + IL-2S4B6-induced CD8 T-cell effector differentiation and tumor protection

Table 1

Immune challenges in allogeneic and autologous hematopoietic stem cell transplantation

Hematopoietic stem cell transplant (HSCT)	Graft-versus-host reaction (GVHD)	Host-versus-graft rejection (HVG)	Graft versus tumor (GVL)	Immune function and reconstitution	Minimal residual disease and lymphopenia
Allogeneic (AML, ALL, CML)	Important	Important	Important	Important	Important
Autologous (Myeloma, NHL)	Not applicable	Not applicable	Important	Important	Important

Table 2

Strategies to harvest the “good” (anti-leukemia, GVL) and prevent the “bad” (anti-host, GVHD) following T-cell replete allogeneic HSCT

DONOR T CELLS TRANSPLANTED WITH HEMATOPOIETIC PROGENITOR CELLS:	
NO REDUCTION	
Following broad anti-host reactivity resulting in GVHD, administration of steroids, methotrexate and CsA control GVHD. Reduces tumor burden. *Challenge*: regulate complications of GVHD including immune deficiency, infection and relapse.	
Transplant of donor T cells with limited, tissue specific reactivity (ex. recipient hematopoietic minor Hag). “Limits” GVHD to hematopoietic compartment and tumor. *Limitation*: practical, i.e. requires cell lines.	[7, 8]
Addition of donor $CD4^+$ T regulatory (Tregs) cells. Objective is to suppress anti-recipient alloantigen-reactive T cell proliferation while permitting effector cell functions to mediate anti-tumor responses. *Challenge:* practical, i.e. generation of Tregs.	[9]
REDUCTION PRIOR TO TRANSPLANTATION	
Globally diminish (1–2 logs) CD4 and/or CD8 T cells – ex. e-rosette, lectin separation, Alemtuzumab (anti-CD52). *Limitation*: elevated tumor relapse and graft failure.	[3, 10, 11]
Specifically diminish anti-recipient alloantigen reactive T cells. Stimulate donor cells ex-vivo with recipient antigen. Delete cells via treatment with 4,5-dibromorhodaminc 123+light, CD25 conjugated toxins, etc. *Limitation:* Complete/partial deletion results in diminished anti-tumor response & GVHD, respectively.	[5, 6, 12, 13]
REDUCTION FOLLOWING TRANSPLANTATION	
Photopheresis: remove patient cells, add psoralen to sensitize to UV light induced killing and reinfusion. Used to treat ongoing GVHD, promising results. *Challenge:* How to strengthen anti-tumor response.	[14,15]
Suicide gene transfection into donor T cells. *Limitation:* practical, i.c. transfections, selection required.	[16]
Post-transplant cyclophosphamide (PTC): administer high dose of drug on days 3 and 4 post-transplant. Promising results diminishing acute GVHD from multiple *centers. Challenge:* enhance effectiveness against chronic GVHD	[17–19]