Combinatorial Therapeutic Vaccination Strategies for Hematologic Malignancies in the Early Period following Experimental Autologous Hematopoietic Stem Cell Transplantation

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COMBINATORIAL THERAPEUTIC VACCINATION STRATEGIES FOR HEMATOLOGIC MALIGNANCIES IN THE EARLY PERIOD FOLLOWING EXPERIMENTAL AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

By

Robert G. Newman

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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COMBINATORIAL THERAPEUTIC VACCINATION STRATEGIES FOR HEMATOLOGIC MALIGNANCIES IN THE EARLY PERIOD FOLLOWING EXPERIMENTAL AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Tumor relapse is the primary cause of mortality in patients with hematologic cancers following autologous hematopoietic stem cell transplantation (HSCT). Vaccination early post-HSCT can exploit both the state of lymphopenia and minimal residual disease for the generation of anti-tumor immunity. Here, multiple vaccinations utilizing lymphoma cells engineered to secrete the heat shock protein fusion gp96-Ig within two weeks of T cell replete syngeneic HSCT led to antigen cross-presentation and increased survival of lymphoma bearing mice. To enhance vaccine efficacy, IL-2 was directed to predominantly memory phenotype CD8^+ T lymphocytes and natural killer cells via administration bound to anti-IL-2 monoclonal antibody clone S4B6 (IL-2_S4B6). Combination therapy with gp96-Ig vaccination and coordinated infusions of IL-2_S4B6 resulted in a marked prolongation of median survival time and overall survival, which directly correlated with a ~500% increase in effector CD8^+ T cell numbers. Notably, this dual regimen elicited large increases in both donor CD8^+ T lymphocytes and natural killer cells, but not CD4^+ T lymphocytes, the former two populations essential for both vaccine efficacy and protection against opportunistic infections post-HSCT. Indeed, IL-2_S4B6 treated HSCT recipients infected with *Listeria monocytogenes* exhibited decreased bacterial levels. These pre-clinical studies validate a new strategy particularly well suited to the post-HSCT environment, which may rapidly augment adaptive and innate immune function in patients with malignant disease following autologous HSCT.
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CHAPTER I: Introduction

Section 1: Hematologic Malignancies and Current Treatment

Cancer is the second leading cause of death in the United States, exceeded only by heart disease, and will account for nearly 25% of all mortality in 2014 [1]. Even though nearly 1.7 million people will be diagnosed with some form of cancer in 2014 [1], the overall incidence has been declining yearly since the early 1990s, on average by about 1.5% per year in the last decade [2]. Malignancies of hematopoietic origin (leukemia, lymphoma, and myeloma) account for 9% of all new cases of cancer and over 9% of all cancer related mortalities [3]. Cancer is a disease of aging, but hematologic malignancies, especially leukemia, account for over 40% of all childhood cancer [3]. The maximal incidence for hematologic malignancies occurs between the ages of 80 and 84, except for Hodgkin lymphoma, which has a bimodal incidence, with the first maximal incidence occurring between the ages of 20 and 24 [3]. B cell lineage tumors (e.g., B cell chronic lymphocytic leukemia (B-CLL), diffuse large B cell lymphoma (DLBCL), and multiple myeloma) comprise the majority of hematologic malignancies [3], and arise at different stages of B cell development (Figure A,top) [4]. Similarly, malignancies of T cell origin (e.g., T cell acute lymphoblastic leukemia (T-ALL), T cell lymphoblastic lymphoma (T-LL), and T cell CLL) arise at different stages of T cell development and are associated with certain genetic mutations (Figure A,bottom) [5]. It is estimated that over 1 million Americans are currently living with or in remission from hematologic malignancies [3].

Even though five year relative survival rates for patients with hematologic malignancies are slowly rising due to accurate diagnosis of subtype and improved
treatment [3], there is still a need for more effective therapies. The first class of
chemotherapeutic agents (i.e., nitrogen mustard, e.g., cyclophosphamide) was
administered to a patient with non-Hodgkin lymphoma over 70 years ago, after
physicians noted lymphoid hypoplasia and myelosuppression in deceased soldiers
exposed to mustard gas during World War I [6]. A few years later, the second class of
chemotherapeutic drugs (i.e., folate analogues, e.g., methotrexate) was used in children
with acute lymphoblastic leukemia, after the observation that folic acid stimulated the
proliferation of these cells in patients [7]. In addition to conventional chemotherapy and
radiation, hematologic malignancies are now being treated with a wide array of targeted
drugs, including kinase inhibitors, histone deacetylase inhibitors, hypomethylating or
demethylating agents, immunomodulators, monoclonal antibodies, antibody-drug
conjugates, and proteasome inhibitors [3]. Recently, June and colleagues have pioneered
the use of adoptive cellular therapy with CD8+ T cells expressing chimeric antigen
receptors targeting CD19 or CD20 [8], which have shown efficacy in patients with B cell
leukemias and lymphomas [9-16]. Patients who are nonresponsive to this arsenal of
therapies might be candidates for hematopoietic stem cell transplantation (HSCT) [3].

In 2014, it is estimated that myeloma will be diagnosed in over 24,000 people and
cause over 11,000 deaths in the United States [1]. There are almost 90,000 people in the
United States living with or in remission from myeloma [3], and the five year relative
survival rate has significantly increased from 25% in 1975-1977 to 45% in 2003-2009
[1], a time period during which incidence has increased almost 30% [3]. The median age
at diagnosis is 69 years, and interestingly, myeloma rarely occurs under age 45 [3]. Ever
since a landmark prospective randomized study showed that high dose chemotherapy
followed by autologous HSCT was superior to conventional chemotherapy [17], symptomatic myeloma is now treated with induction therapy composed of dexamethasone (i.e., glucocorticoid, e.g., cortisol) and bortezomib (proteasome inhibitor) with thalidomide or lenalidomide, followed by autologous HSCT [18]. Individuals not eligible for HSCT because of advanced age or coexisting conditions receive chemotherapy typically composed of melphalan (nitrogen mustard), prednisone (glucocorticoid), thalidomide, and bortezomib [18]. Subsequently, all patients receive consolidation or maintenance therapy composed of a single agent or combinations of the aforementioned drugs [18].

Section 2: Hematopoietic Stem Cell Transplantation for Hematologic Malignancies

HSCT was first used to treat inherited anemias or immune deficiencies, but has now also been applied to benign hematologic disorders, autoimmune diseases, and tolerance induction for solid organ transplant [19]. Initially, HSCT served as a means of hematopoietic rescue following high dose chemotherapy and radiation to enable aggressive tumor cell killing. The first successful HSCT for a cancer patient was performed by E. Donald Thomas and colleagues at the Fred Hutchinson Cancer Research Center (FHCRC) in Seattle, WA in 1959, an accomplishment later recognized by his award of the Nobel Prize in Medicine in 1990. Earlier in the 1950s, several groups performed pivotal animal studies demonstrating survival of mice exposed to lethal total body irradiation (TBI) following shielding of the spleen [20] or intravenous infusion of unfractionated bone marrow cells [21]. The latter was eventually shown to be dependent on the colonization of recipient bone marrow by transplanted donor cells [22-26]. This groundbreaking pre-clinical research provided the foundation for subsequent translational
studies that first demonstrated the safe infusion of bone marrow cells into cancer patients receiving radiation and/or chemotherapy with evidence of donor cells in a subset of recipients suggesting transient engraftment [27].

Autologous HSCT has been primarily used for patients with lymphoma or myeloma (Figure B) [28]. However, for a brief period from the 1980s to 1990s, autologous HSCT was assessed in breast cancer patients, and at one point was the malignancy receiving the most transplants (Figure C) [28]. Stem cell rescue with autologous HSCT permitted the use of highly aggressive therapy up to twice the standard non-transplant dose in breast cancer patients [29,30]. Since 1999, 15 randomized clinical trials compared high dose chemotherapy paired with autologous HSCT to standard of care regimens for breast cancer patients [29,30]. Unfortunately, meta-analysis of these breast cancer trials, which included over 6000 patients, found no benefit for autologous HSCT with regard to overall survival [29,30]. Notably, recipients of autologous HSCT had increased relapse free survival suggesting anti-tumor activity in breast cancer [29,30].

Initially, conditioning regimens for HSCT consisted of a single high dose of total body irradiation (TBI). Conditioning is required to make space for the transplanted stem cells and promote long term engraftment by deleting T cells that would attack the transplanted cells, in addition to the tumoricidal effects of the conditioning. Current conditioning protocols employ fractionated low doses of targeted irradiation and/or drugs that target tumor cells preferentially over hematopoietic stem cells. Allogeneic HSCT inherently possesses anti-tumor immunity by the attack of T cells on the tumor, but carries the serious complication of graft vs host disease and susceptibility to pathogens by
the attack of T cells on the non-tumor hematopoietic and non-hematopoietic compartments. On the contrary, autologous HSCT does not possess the risk of graft vs host disease because the self T cells do not attack the recipient and has a lower incidence of infection because of the sparing of some of the hematopoietic compartment, but superficially lacks anti-tumor immunity because of the self T cells that have been tolerant to the tumor. Leukemias are generally not treated with autologous HSCT because lower relapse rates can be achieved with allogeneic HSCT. Similarly, lymphoma and myeloma are generally not treated with allogeneic HSCT because less graft vs tumor effects have been observed. Regardless of autologous or allogeneic HSCT, relapse of the primary disease remains the major cause of mortality in this patient population [28]. Thus, there is a critical need for more effective therapies in patients receiving HSCT.

Section 3: Vaccination for Hematologic Malignancies following Autologous HSCT

William Coley is credited as the father of immunotherapy for his use of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (Coley toxin) in the 1890s that he injected directly into tumors. Coley noted regressions following development of fevers in patients. The first therapeutic cancer vaccine was not approved by the Food and Drug Administration in the United States until 2010. Sipuleucel-T (Provenge®) is an antigen presenting cell therapy for metastatic hormone-refractory prostate cancer. The vaccine consists of autologous dendritic cells obtained by leukapheresis exposed to a fusion protein of prostatic acid phosphatase, which is expressed by 95% of prostate cancer cells, and granulocyte-macrophage colony stimulating factor, to mature and activate the dendritic cells [31]. Meta-analysis of 3 clinical trials comparing Sipuleucel-T to placebo
that included over 700 patients revealed a reproducible increase in overall survival for Sipuleucel-T treated patients [32].

From the 1940s to the 1970s, reports started emerging suggesting that immunization with viable tumor cells could provide immunity in mice against subsequent challenge with the same cells [33-37]. For hematologic malignancies, B cell lymphoma derived Ig idiotype immunization in mice [38] led to clinical trials in lymphoma and myeloma patients [39-42]. In the 1980s, Srivastava et al. demonstrated that a 96 kDa protein could complement the immunity induced by immunization with tumor cells [43]. Glycoprotein (gp)96 is the resident endoplasmic reticulum (ER) member of the heat shock protein (hsp)90 superfamily and is intimately involved in major histocompatibility (MHC) class I restricted antigen presentation. 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 presented. Following necrosis, gp96-antigen complexes are released from dying cells and can be taken up by antigen presenting cells (APC) in tissues, which cross-present the chaperoned peptides on MHC-I to specific CD8+ T cells. APC uptake of gp96 also induces activation and expression of a multitude of cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-12, GM-CSF, MIP-1α, MCP-1, RANTES, and NO). Subsequently, Srivastava and colleagues introduced autologous tumor-derived gp96 preparations into patients with cancer, including lymphoma [44,45]. Although immune correlates were elicited, overall patient survival was relatively unchanged compared with standard of care regimens [46].
In the late 1990s, Podack and colleagues developed a strategy to have cells secrete gp96 by replacing the ER retention signal (KDEL) with the Fc portion of IgG [47]. Vaccination with tumor cell lines transfected to secrete this gp96-Ig molecule induced CD8+ T lymphocyte and natural killer (NK) cell activation and expansion, leading to enhancement of anti-tumor (mouse) and anti-viral (monkey) immunity [48-55]. Notably, the first phase I trial employing a human allogeneic lung cancer cell line engineered to secrete gp96-Ig was recently completed [56]. Interestingly, ~75% of evaluable patients displayed CD8+ T cells that secreted IFN-γ when stimulated ex vivo with the vaccine cells after one, two, or three courses of treatment, and these subjects had a median survival time (MST) of 16.5 months compared to 4.5 months for non-responders. In summary, potent vaccination of cancer patients with tumor cells secreting gp96-Ig, purified gp96, or other heat shock protein preparations is unable to provide generalized cures as single agent therapies and therefore combining vaccination, which provides the initial antigenic stimulation, with an adjuvant to enhance this antigen-specific response, might be necessary to provide sustainable cures to cancer patients.

Section 4: Interleukin-2

T cell growth factor [57], now known as interleukin (IL)-2, was originally cloned by a group in Japan in 1983 [58], and FDA approved for the treatment of metastatic renal cell carcinoma and metastatic melanoma in 1992 and 1998, respectively. In mouse models of melanoma (B16) and fibrosarcoma (MCA-207), administration of IL-2 has been combined with vaccination [59-64], including following HSCT [65]. The initial report of IL-2 complexed with anti-IL-2 mAb was published over two decades ago [66]. Complexing IL-2 to anti-IL-2 mAb greatly increases the serum half-life of the protein
Boymann et al. demonstrated that a particular anti-mouse IL-2 mAb (clone S4B6) targeted IL-2 to memory phenotype CD8+ T lymphocytes and NK cells [71], which express the β (CD122) and γ (CD132) chains of the IL-2 receptor, in the absence of the α (CD25) chain [72,73]. This ‘targeting’ of IL-2 to principally exclude cells expressing CD25 [70], reduces a side effect that greatly diminishes the clinical translatability of this therapy. In high doses, IL-2 administration can cause vascular leak syndrome (VLS, capillary leak syndrome) with symptoms including increased vascular permeability, hypotension, pulmonary edema, liver cell damage, and renal failure [74]. Initially, VLS was thought to be caused by stimulation of NK cells, which can lead to the production of vasoactive mediators, but a recent report suggests that VLS might be triggered by direct binding of IL-2 to CD25+ endothelial cells [75]. Various groups have successfully used IL-2 complexes to enhance vaccination [76,77]. Recently, Levin et al. have engineered an IL-2 protein with similar binding properties as the IL-2 complex [78]. Others have shown that IL-2 can enhance endogenous anti-pathogen responses, in a CD8+ T lymphocyte and NK cell dependent fashion [79-82]. Since infection is the second leading cause of mortality in HSCT recipients [28], a strategy that combines tumor vaccination and targeted IL-2 lend merit to use in the early period following HSCT, since it theoretically will enhance both anti-tumor and anti-pathogen responses.

The FHCRC pioneered the use of IL-2 following autologous HSCT in patients with hematologic malignancies [83] and early following HSCT for acute myeloid leukemia (AML) [84] and malignant lymphoma [85]. Similar trials were also being performed in Minneapolis, MN for acute lymphoid leukemia (ALL) [86] and malignant lymphoma [87]. The first randomized trial employing recombinant soluble IL-2
following HSCT concluded that it did not affect survival of ALL patients using the selected regimen [88], which was corroborated in a subsequent phase II trial for ALL and AML [89]. Before moving to a phase III, additional phase I/II trials were necessary since the source of the IL-2 changed [90,91]; however, subsequent phase III for trials for non-Hodgkin lymphoma (NHL) [92] and AML [93] found no effect of IL-2 on patient survival.

Section 5: Overall Rationale and Hypothesis

Given that heat shock protein vaccination alone generally results in suboptimal immune stimulation paired with minimal effects on overall survival, and the state of MRD present in the early post-HSCT period, we reasoned that transplantation with hematopoietic stem cells supplemented with clinically achievable doses of T cells would provide the foundation for effective vaccination with tumor cells secreting gp96-Ig and subsequent augmentation with an adjuvant in the form of IL-2/anti-IL-2 complexes. We sought to test this hypothesis by developing a model of minimal residual lymphoma simulating relapse by inoculating mice with tumor immediately following high dose conditioning, in the form of myeloablative lethal TBI, and autologous HSCT. Subsequently, we employed a treatment protocol established in non-tumor bearing mice to investigate if the aforementioned strategy holds merit in lethal pre-clinical models. Further we established the cellular and molecular requirements of optimal immunity.
Figure A. Cellular origin of human B-cell and T-cell malignancies.

R. Küppers

Nature Reviews Cancer 2005

I. Aifantis, E. Raetz, and S. Buonamici

Nature Reviews Immunology 2008
CHAPTER II: Heat shock protein vaccination and directed IL-2 therapy amplify tumor immunity rapidly following bone marrow transplantation in mice

Section 1: Summary

Tumor relapse is the primary cause of mortality in patients with hematologic cancers following autologous HSCT. Vaccination early post-HSCT can exploit both the state of lymphopenia and minimal residual disease for generation of anti-tumor immunity [94-100]. Here, multiple vaccinations utilizing lymphoma cells engineered to secrete the heat shock protein fusion gp96-Ig within two weeks of T cell replete syngeneic HSCT led to antigen cross-presentation and increased survival of lymphoma bearing mice. To enhance vaccine efficacy, interleukin (IL)-2 was directed to predominantly memory phenotype CD8$^+$ T lymphocytes and natural killer cells via administration bound to anti-IL-2 monoclonal antibody clone S4B6 (IL-2S4B6). Combination therapy with gp96-Ig vaccination and coordinated infusions of IL-2S4B6 resulted in marked prolongation of median survival time and overall survival, which directly correlated with a ~500% increase in effector CD8$^+$ T cell numbers. Notably, this dual regimen elicited large increases in both donor CD8$^+$ T lymphocytes and natural killer cells, but not CD4$^+$ T lymphocytes, the former two populations essential for both vaccine efficacy and protection against opportunistic infections post-HSCT. Indeed, IL-2S4B6 treated HSCT recipients infected with *Listeria monocytogenes* exhibited decreased bacterial levels. These pre-clinical studies validate a new strategy particularly well suited to the post-HSCT environment, which may augment adaptive and innate immune function in patients with malignant disease receiving autologous HSCT.
Section 2: Background

According to CIBMTR, ~80% of mortality after autologous HSCT (2010-2011) resulted from relapse of primary disease or infection in patients with myeloma, lymphoma, and leukemia [28]. Multifaceted immunotherapeutic approaches combined with HSCT for patients with hematopoietic malignancy continue to hold large, but as yet unfulfilled promise [19]. Such enthusiasm for immune-based strategies 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 state of lymphopenia present [94-100]. 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.

Heat shock protein gp96 is the resident endoplasmic reticulum protein chaperone and is intimately involved in MHC-I restricted antigen presentation [43,101-106]. Following necrosis, gp96-peptide complexes are released and can be taken up by local antigen presenting cells (APC) through CD91 receptor mediated endocytosis leading to peptide delivery and their efficient activation [107-109]. These APC can therefore cross-present gp96-chaperoned peptides to CD8+ T lymphocytes inducing their activation, expansion, and development of effector function [110,111].

The vaccine used in the present studies consisted of tumor cells engineered to secrete a modified gp96 molecule lacking the ER KDEL retention signal fused to the Fc portion of murine IgG1 (gp96-Ig) [47]. A single vaccination with OVA-expressing cells secreting gp96-Ig resulted in expansion of endogenous and transgenic OVA257-264-
specific CD8+ T cells, that expressed IFN-γ upon stimulation ex vivo and responded more strongly to a second vaccination in vivo [48]. Notably, gp96-Ig vaccination also stimulated NK cells and DC, populations hypothesized to contribute to CD8+ T cell expansion, and required peroxin-1 and IFN-γ, but were independent of FasL [49]. Subsequently, Oizumi and colleagues demonstrated that the system was independent of CD4+ T cells, CD40L expression, NKT cells, and LN, but required CD80/CD86, recruited F4/80lo cells, and expanded NK cells and DC at the site of vaccination [50].

Furthermore, it was demonstrated that vaccine-specific CD8+ T cell expansion following gp96-Ig vaccination was diminished in tumor bearing mice in a tumor antigen independent manner; however, this could be overcome following multiple vaccinations or vaccination in the absence of B cells [51]. Schreiber and colleagues then partitioned responders and non-responders, and determined that enhanced anti-tumor immunity resulted in increased Th1 and Th17 factors and decreased Th2 factors [52]. Strbo and colleagues then determined that gp96-Ig vaccination elicited responses in the gut associated lymphoid tissue and was dependent on the site of vaccination [53]. Moreover, Strbo and colleagues demonstrated that gp96-Ig vaccines expressing simian immunodeficiency virus (SIV) proteins were able to elicit polyfunctional CD8+ T cells specific for multiple SIV antigens in rhesus macaques [54], which resulted in prolonged survival following challenge with acute SIV infection [55]. Furthermore, the first clinical trial employing tumor cells secreting gp96-Ig was recently completed in lung cancer patients, and demonstrated that 75% of vaccinated patients contained CD8+ T cells expressing IFN-γ following ex vivo stimulation with the vaccine cells, who interestingly had over 3 fold enhanced survival compared to non-responders [56].
IL-2 therapy has demonstrated significant anti-tumor activity in experimental models of leukemia and has diverse affects following HSCT in part dependent on dose and time of infusion [112,113]. However, since IL-2 induced expansion of T regulatory cells (Treg) could inhibit anti-tumor immunity [114], an important advance for use of this cytokine would be to direct its activity primarily to anti-tumor effector vs Treg cells [70,71,75]. Notably, several groups have reported that IL-2 conjugated to a particular anti-IL-2 mAb can augment anti-tumor responses [76,77]. One cytokine-antibody complex utilizing mAb clone S4B6 (IL-2S4B6), which activates the intermediate affinity IL-2 receptor (β and γ), was found to stimulate the proliferation of predominately memory phenotype CD8+ T lymphocytes and NK cells—two populations essential for optimal gp96-Ig induced anti-tumor responses [49].

The pre-clinical studies described here investigated the efficacy of vaccination with tumor cells secreting gp96-Ig together with IL-2S4B6 complex in experimental mouse models of minimal residual lymphoma following syngeneic HSCT. The results obtained support the notion that the primary effect of gp96-Ig vaccination via antigen cross-presentation early following autologous HSCT was to elicit tumor-reactive CD8+ T cells, and together with directed IL-2 treatment, markedly augmented effector CD8+ T cell levels. Global expansion of donor CD8+ T lymphocytes and NK cells, but not CD4+ T lymphocytes, following administration of this IL-2S4B6 complex contributed to prolonged survival of lymphoma bearing HSCT recipients as well as augmented anti-pathogen responsiveness early post-HSCT.
Section 3: Methods

Mice. C57BL/6 wild type mice (B6, CD45.2+ CD90.2+), B6-CD45.1+ and B6-CD90.1+ congenic strains, and B6-CD80−/−CD86−/− double deficient mice (B7KO) [115] were obtained from Charles River Laboratories, Taconic Farms, The Jackson Laboratory, or the National Cancer Institute. B6-OT-I mice [116], provided by M. Bevan, were backcrossed onto B6-RAG1−/− background [117]. Batf3 deficient (B6-Batf3−/−, Batf3KO) [118,119] BM was provided by the laboratory of P. Reddy who received animals from K. Murphy. Perforin-1 deficient mice (B6-Pfp−/−, PrfKO) were generated as described [120]. B6-FoxP3mRFP mice [121] were provided by R. Flavell. Mice were subsequently bred and maintained under DVR supervision and IACUC approved protocols.

Antibodies and staining. Fluorescent antibodies were purchased from BD-Biosciences, eBioscience, BioLegend, or Life Technologies, and used for flow cytometric analysis: anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (6D5), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD62L (MEL-14), anti-CD90.1 (OX-7), anti-CD122 (TM-β1), anti-CD127 (A7R34), anti-F4/80 (BM8), anti-Gr-1 (RB6-8C5), anti-IFN-γ (XMG1.2), anti-KLRG-1 (2F1), anti-NK1.1 (PK136), anti-TNF-α (MP6-XT22), anti-Vα2 (B20.1), anti-Vβ5.1/5.2 (MR9-4). To enumerate TCR OVA257-264 (SIINFEKL) reactive CD8+ T cells, 0.5 μg DimerX−Kb (BD Biosciences) was incubated with 0.33 μg OVA257-264 overnight. Intracellular cytokine staining was performed using single cell suspensions prepared from tissues followed by incubation (1.0 x 10^6 cells/mL) in 10% complete medium with 0.1 nM OVA257-284 for 4-6 h at 37°C, with monensin (GolgiStop, BD-Biosciences) or brefeldin A (GolgiPlug, BD-Biosciences). Cells were then surface stained, fixed and
permeabilized with the FoxP3 staining kit (eBioscience) overnight, followed by intracellular staining and analysis. Data was acquired on a BD-LSRFortessa, LSRII, or Accuri-C6 flow cytometer and results analyzed using BD-FACSDiva or CFlow software.

**Tumor cell lines.** EL-4 lymphoma cells [122] expressing OVA (E.G7) [123], provided by M. Bevan, were engineered to secrete gp96-Ig (E.G7-gp96-Ig as well as EL4-gp96-Ig) as described [47]. Cell lines were maintained in Iscove's modified Dulbecco's medium containing 10% FBS, 1 μg/mL gentamicin, 0.05 mmol/L β-mercaptoethanol, and the appropriate antibiotics: G418 (0.4 mg/mL) or L-histidinol (2 mmol/L).

**Preparation of donor T cell populations.** CD8+ T cells specific for OVA257-264 (OT-I) were purified by negative selection from spleen and LN of B6-OT-I (RAG1−/−) mice with the CD8+ T Cell Isolation Kit (Miltenyi Biotec) using magnetic separation (≥95% by flow cytometry). Polyclonal CD4+ and CD8+ T cells were enriched (≥75%) following B cell depletion (≥90%) from spleen and LN of donor mice using plate bound goat anti-mouse IgG/M (Millipore) as described [124,125]. Polyclonal CD4+ (≥88%) and CD8+ (≥90%) T cells were purified by negative selection following B cell depletion.

**BM transplantation.** BM was isolated from femurs, tibias, and vertebral columns as described [124,125]. Briefly, T cells were removed by incubation with anti-CD90.2 (HO134), anti-CD4 (RL174), and anti-CD8 (H022) ascites (1:5) with 12% vol/vol Low-Tox M rabbit complement (Cedarlane Labs) at a final concentration of 25.0 x 10^6 cells/mL on ice for 15 min followed by 37 °C incubation for 30 min. B6 mice were conditioned with 9.5 Gy total body irradiation. The following day, 5.0 x 10^6 congenic B6-CD45.1+ T cell depleted (TCD)-BM cells were infused intravenously alone or together
with the selected donor T cell populations (0.2 mL RPMI-1640). Mice were maintained briefly on gentamicin supplemented water.

Tumor cell inoculation into mice used as BM recipients or T cell donors. E.G7 and EL-4 lymphoma cells were isolated from log-phase cultures. One day following BM transplants, 1.0 x 10^5 or 5000 cells, respectively, were injected intraperitoneally (0.5 mL RPMI-1640). Tumor bearing T cell donor mice were inoculated intraperitoneally with 4.0 x 10^6 E.G7 cells 3 wk before T cell harvest. Some donor mice were injected intravenously with 5.0 x 10^5 negatively selected CD8^+ OT-I T cells 1 d preceding tumor inoculation. For subcutaneous tumor challenge experiments, 1 x 10^6 E.G7 cells were inoculated (0.1 mL RPMI-1640).

Vaccination. E.G7-gp96-Ig and EL4-gp96-Ig cells were isolated from log-phase cultures. Two or three days after adoptive transfer of T cells, 1.0 x 10^7 irradiated (40-120 Gy) cells were injected intraperitoneally (0.5 mL RPMI-1640). Vaccinations were repeated on subsequent days as indicated.

IL-2 cytokine-antibody complex. 1.5 μg recombinant mouse IL-2 (eBioscience) was incubated with 8 μg anti-mouse IL-2 mAb clone S4B6 (BD-Biosciences) for 15 min at room temperature (IL-2S4B6) as described [71]. IL-2 complexes were injected intraperitoneally as indicated (0.5 mL PBS).

NK cell depletion. Anti-NK1.1 mAb clone PK136 was administered intraperitoneally (0.5mL PBS) 1 d before total body irradiation (200μg) and 3d (100μg), 9d (50μg), and 15d (50μg) post-HSCT.

Bacterial infection and determination of CFU. Recombinant *Listeria monocytogenes* expressing OVA_{134-387} [79,126], provided by H. Chen, was grown in
brain/heart infusion broth. Log-phase growing bacteria (OD$_{560}$ 0.2) were diluted (PBS) to 1.0 x $10^4$ CFU and injected intravenously (0.2 mL HBSS). The number of bacteria injected was calculated and confirmed by subsequent growth on brain/heart infusion agar plates. Seven days post-infection, single cell suspensions of spleens were prepared by isolation and disruption using a glass stopper on 0.2 μm screens in 0.05% Triton X-100. CFU were determined by serial dilutions after incubation for 18 h at 37 °C on brain/heart infusion agar plates.

**Statistical analysis.** Paired comparisons performed using t test, multiple analyses performed using one-way or two-way ANOVA as applicable, and survival analyses performed using log-rank test: *p ≤ 0.05, **p ≤ 0.01, ***p≤0.001, ****p≤0.0001.

Section 4: Results

**CD8$^+$ T cell response elicited following vaccination with tumor cells secreting gp96-Ig in syngeneic HSCT recipients.** To assess the immune consequences of administering gp96-Ig secreting tumor cells early following autologous HSCT, B6 mice were conditioned (9.5 Gy) and transplanted with B6-CD45.1$^+$ TCD-BM cells. To monitor a tumor-reactive CD8$^+$ T cell population, TCD-BM was supplemented with 1.0 x $10^6$ CD8$^+$ T cells specific for OVA$_{257-264}$ (OT-I). Following a single intraperitoneal vaccination with 1.0 x $10^7$ EL-4 lymphoma cells (120 Gy) expressing OVA (E.G7) secreting gp96-Ig (E.G7-gp96-Ig), large numbers of macrophages (F4/80$^{hi}$ CD11b$^{hi}$ Gr-1$^{lo}$) and inflammatory monocytes (Gr-1$^{hi}$ CD11b$^{mid}$ F4/80$^{lo}$) [127,128] rapidly accumulated at the vaccine site (2 d post-vaccination), comprising ~90% of its entire cellularity ([Figure 1A](#)). Initially, these myeloid cells were of recipient origin, but were rapidly replaced by their donor counterparts over the course of the immune response.
(within 1 wk, data not shown). Interestingly, at 2 d post-vaccination, tumor-reactive CD8+ T lymphocytes could not be detected at the vaccine site (Figure 1A); however, within 24 h, elevated levels were observed in the blood (data not shown) and maximum numbers were reached in the peritoneal cavity 5 d following a single vaccination (Figure 1B). At this time, large numbers of macrophages could still be enumerated in this compartment, together with lower numbers of inflammatory monocytes (Figure 1B).

Notably, vaccination with parental E.G7 cells containing endogenous gp96 did not induce these cellular responses (Figure 1A-B).

Syngeneic HSCT recipients vaccinated a second time (6 d following the first vaccination) with tumor cells secreting gp96-Ig contained similar numbers of tumor-reactive CD8+ T cells at the vaccine site as observed after a single vaccination, and elevated numbers of these cells were now detected in the spleen (Figure 1C). Proliferative signals engendered by lymphopenia diminish over time in myeloablative conditioned mice [99,129]. We found that vaccination could be delayed 1-2 wk post-HSCT and similar levels of tumor-reactive CD8+ T lymphocytes were observed at the vaccine site 5 d following vaccination (Figure 1D). CD8+ T cells introduced into a lymphopenic environment assume a central-memory phenotype (CD62L+ CD44+, Figure 1E) [72,73,130-132]. Following a single vaccination with gp96-Ig secreting tumor cells, ≥80% of tumor-reactive CD8+ T cells transitioned to effector cells (CD62L- CD44+, Figure 1E). Peptide stimulation of these cells ex vivo corroborated this effector phenotype since ≥60% expressed IFN-γ (Figure 1F). In non-transplanted mice, gp96 has been reported not to induce stimulation of CD4+ T cells [47,101], and we detected no appreciable tumor-reactive (OVA323-339) CD4+ T cell (OT-II) expansion following
transfer of $1.0 \times 10^6$ CD4$^+$ OT-II T cells and a single vaccination with tumor cells secreting gp96-Ig in syngeneic HSCT recipients (data not shown).

To determine if B7 costimulation was required for antigen-specific CD8$^+$ T cell expansion induced by gp96-Ig vaccination in HSCT recipients as well as the source of this signal regarding APC, transplants were performed utilizing donor and/or recipient mice deficient in CD80/CD86 (B7KO). When vaccine was administered 2 d post-HSCT, donor and recipient B7 expressing cells were both found to contribute to overall tumor-reactive CD8$^+$ T lymphocyte expansion (Figure 2A). Strikingly, when vaccination was delayed 1 wk post-HSCT, only B7 expression by donor cells contributed to the response, since such expansion was not observed when B7 deficient BM cells were transplanted into WT hosts (Figure 2B).

We next wanted to formally address if cross-presentation was taking place early post-HSCT since gp96 is thought to function by shuttling antigen into the MHC-I pathway inside the APC. To address this, TCD-BM from Batf3 deficient mice (Batf3KO), which lack CD11c$^+$ CD8α$^+$ cross-presenting dendritic cells [118,119], was used for transplantation (Figure 2C,top). These recipients were unable to expand tumor-reactive CD8$^+$ T cells following gp96-Ig vaccination (Figure 2C,bottom). Our group reported that perforin-1 was required for tumor-reactive CD8$^+$ T lymphocyte expansion induced by vaccination with tumor cells secreting gp96-Ig in non-transplanted mice [49]. Using either donors or recipients deficient in perforin-1 (PrfKO), transplants determined that perforin-1 was also required following HSCT, and interestingly, donor or recipient derived perforin-1 was sufficient to support this expansion (Figure 2D).
Effectiveness of gp96-Ig secreting tumor cell vaccination in a pre-clinical minimal residual lymphoma syngeneic HSCT model. Similar to what occurs in autologous clinical transplants, T cells were obtained for these experiments from syngeneic donor mice bearing tumor. Accordingly, TCD-BM (as in Figure 1) was supplemented with $2.0 \times 10^6$ enriched CD4$^+$ and CD8$^+$ T lymphocytes from tumor bearing B6-CD90.1$^+$ donors. To enable CD8$^+$ tumor-reactive T cell monitoring, some tumor bearing T cell donors were infused intravenously with $5.0 \times 10^5$ CD8$^+$ OT-I T cells 1 d preceding tumor inoculation. Three weeks later, based upon TCR V$\alpha$V$\beta$ analysis, we calculated that ~1000 transgenic tumor-reactive CD8$^+$ T lymphocytes were present with the $2.0 \times 10^6$ donor T cell inoculum when such tumor bearing animals were employed (Figure 3A). Two days post-HSCT, B6 recipients were vaccinated intraperitoneally with $1.0 \times 10^7$ gp96-Ig secreting tumor cells (40 Gy) and repeated every 3 d (for a total of 5 vaccinations). Rapid and potent tumor-reactive CD8$^+$ T lymphocyte expansion was identified within the first 3 wk post-HSCT (Figure 3B). Five days following the third vaccination (at the peak of the response, Figure 3B), tumor-reactive CD8$^+$ T cells were identified at the vaccine site (>1000x the total input number), spleen (>5000x), and draining LN (>500x, Figure 3C). Following peptide stimulation ex vivo, a majority of these lymphocytes from the vaccine site exhibited polyfunctional activity [133], co-producing IFN-$\gamma$ and TNF-$\alpha$ (Figure 3D). Repeated vaccinations with parental E.G7 cells again (Figure 1B-C,E-F) failed to induce appreciable expansion or responsiveness greater than that resulting from their presence in lymphopenic conditions alone (Figure 3B-D).

Next, to simulate tumor relapse post-transplant, we developed a minimal residual lymphoma model based off the work of Korngold and colleagues [134] in which
recipients were inoculated intraperitoneally with $1.0 \times 10^5$ viable E.G7 lymphoma cells 1 d following HSCT. This resulted in 100% lethality with a median survival time (MST) of 1 mo, regardless of donor T cell source (tumor naive or tumor bearing donors, Figure 4A, left). Higher doses of T cells ($>2.0 \times 10^6$) resulted in <100% lethality (Figure 4A, right). In this minimal residual lymphoma model, similar (Figure 3B) tumor-reactive CD8$^+$ T cell expansion was observed following multiple gp96-Ig vaccinations (Figure 4B). These responses correlated with a significant MST extension and increased overall survival (20% survived >100 d post-HSCT, Figure 4C). Notably, enhanced MST was dependent on transplant of donor T cells since vaccination failed to prolong survival in recipients of BM only lacking donor T cells (Figure 4C). Vaccination with parental E.G7 cells again (Figure 3B) failed to affect expansion or survival (Figure 4B-C) and the source of donor T cells (tumor naive or tumor bearing donors) again (Figure 4A) did not alter the survival advantage engendered by gp96-Ig vaccination (Figure 4D).

Directed IL-2 augmented CD8$^+$ T lymphocyte expansion and markedly enhanced survival of lymphoma bearing HSCT recipients. The initial studies demonstrated that vaccination with gp96-Ig secreting tumor cells could stimulate tumor-reactive CD8$^+$ T cells and extend survival. To attempt to expand tumor-reactive CD8$^+$ T cells IL-2 was introduced following vaccination. Recipient mice were transplanted (as in Figure 4) and IL-2S4B6 was injected i.p. 1 d following each vaccination. We first observed an increase in total CD8$^+$ T lymphocyte expansion in the peripheral blood and in tissues of HSCT recipients (Figure 5A, left and data not shown). However, IL-2S4B6 administration only marginally affected overall CD4$^+$ T lymphocyte (Figure 5A, right) and notably CD4$^+$ FoxP3$^+$ Treg levels (Figure 5A, bottom). Analysis of the vaccine site, spleen, and LN
compartments (2 wk post-HSCT at the peak of the response, Figure 5B,left) following vaccination in combination with IL-2S4B6 demonstrated >30,000x expansion of CD8+ OT-I T lymphocytes had occurred over the input number of ~1000, reaching greater than >30.0 x 10^6 cells (Figure 5B,right). Consistent with such expansion, this combinatorial regimen (gp96-Ig vaccination and IL-2S4B6) also resulted in a striking increase in MST (>100 d post-HSCT) and overall survival (60%, Figure 5C,bottom). In contrast, administration of the same dose and schedule of unbound IL-2 (not complexed to mAb) together with vaccination exhibited no effect on overall CD8+ T lymphocyte expansion (Figure 5C,left) or CD8+ OT-I T cell levels (Figure 5C,right). Importantly, this did not result in an increase in MST or overall survival compared to vaccination alone (Figure 5C,bottom). Furthermore, to elicit marked expansion of CD8+ OT-I T cells, IL-2S4B6 must be combined with vaccination since although monotherapy with IL-2S4B6 induced strong total CD8+ T cell expansion (Figure 5D,left), IL-2S4B6 alone only elicited a small response by CD8+ OT-I T lymphocytes present (Figure 5D,right). Not surprisingly, this resulted in similar MST and overall survival compared to vaccination alone (Figure 5D,bottom). Notably, IL-2S4B6 treatment in combination with parental E.G7 cells not secreting gp96-Ig (“weak” vaccine) elicited only a modest response by CD8+ OT-I T cells above that of IL-2S4B6 alone (Figure 5D,right).

Importantly, MST and overall survival, as well as total CD8+ T cell expansion in response to IL-2S4B6, were independent of transgenic OT-I presence (Figure 5E,right and 5E,left). Notably, in vaccinated recipients not receiving OT-I cells, IL-2S4B6 elicited enhanced levels of CD8+ T effector cells (Figure 5F,left), which has been described with other vaccination strategies [135]. To determine if tumor-reactive non-TCR transgenic
CD8+ T cells were elicited following gp96-Ig vaccination, DimerX–Kb (mAb with MHC-I fused to heavy chain after variable region) loaded with peptide was utilized to detect OVA257-264 specific cells. Importantly, endogenous CD8+ T lymphocytes specific for OVA257-264 were readily apparent only in recipients of gp96-Ig vaccination, while a markedly increased CD8+ effector T cell response was only elicited following vaccination together with IL-2S4B6 treatment (Figure 5F,right).

To test the vaccine strategy using a system without OVA, a highly lethal minimal residual lymphoma model employing intraperitoneal inoculation of 5000 EL-4 lymphoma cells 1 d post-HSCT was employed [136,137]. The results illustrated that EL4-gp96-Ig vaccination in combination with IL-2S4B6 significantly increased CD8+ effector T cells levels (Figure 5G,left) and extended MST compared to monotherapy with either reagent (Figure 5G,right). Moreover, the MST of the individual treatments were indistinguishable from non-vaccinated mice (Figure 5G,right).

To identify which donor leukocyte populations were required for augmented anti-tumor immunity following vaccine and IL-2 therapy, donor TCD-BM was supplemented with purified CD8+ or CD4+ T cells in the absence of OT-I (Figure 6A). Notably, donor CD8+ T cells expanded following vaccine and IL-2S4B6 treatment in the absence of donor CD4+ T cells (Figure 6B,left and 6B,right). However, a significantly smaller portion of these expanded donor CD8+ T cells exhibited an effector cell phenotype (CD62L− CD44+, Figure 6C). These recipients also trended to shortened MST and overall survival compared to recipients of CD4+ and CD8+ T cells (Figure 6D). Importantly, recipients of purified CD4+ T cells treated with gp96-Ig vaccination and IL-2S4B6 displayed no survival benefit compared to non-vaccinated recipients (Figure 6D).
To assess for a memory response in previously vaccinated HSCT recipients, surviving mice (>100 d post-HSCT, Figure 4-6) were challenged with a lethal number (1.0 x 10^6) of E.G7 lymphoma cells subcutaneously to determine if tumor-specific memory had been generated. Surviving HSCT recipients (n = 20) previously vaccinated with tumor cells secreting gp96-Ig alone (n = 6) or in combination with IL-2S4B6 (n = 14) exhibited a rejection rate of 90% (Table 1). The animals that were not protected following re-challenge (2/20) were greater than 1 y of age and as expected, untreated, non-transplanted mice were also not protected (9/9, see Table 1 Figure,left for kinetics of tumor growth and Table 1 Figure, right for recipient survival). Interestingly, recipients transplanted with purified CD8^+ T cells (n = 2) also rejected this subcutaneous tumor challenge (data not shown).

Administration of directed IL-2 enhanced innate and adaptive immunity in the early post-HSCT period. The vaccine site was analyzed following 3 treatments with IL-2S4B6 (2 wk post-HSCT at the peak of the CD8^+ T cell response, Figure 5A) to address the effect of directed IL-2 on NK cells post-transplant [138]. Notably, NK cell numbers were significantly increased at the site of vaccination (>20x) compared with non-IL-2 treated recipients (Figure 7A). To determine if the IL-2 induced increases in NK cells and CD8^+ T lymphocytes altered anti-pathogen immunity early following HSCT, recombinant Listeria monocytogenes was inoculated intravenously into mice 2 wk post-HSCT. One wk post-inoculation, untreated non-transplanted mice and HSCT recipients contained a similar number of CFU in the spleen (Figure 7B,right). IL-2S4B6 treated HSCT recipients exhibited elevated CD8^+ T cell levels following 4 IL-2S4B6 infusions (Figure 7B,left) and significantly fewer CFU numbers 1 wk later (Figure 7B,right).
Section 5: Discussion

Enhancing diminished immune function is a major challenge following regimens involving aggressive therapy and autologous HSCT particularly in patients with hematologic malignancies. An ideal approach would simultaneously elicit rapid and potent tumor-specific responses, while successfully augmenting overall anti-pathogen immunity. The principal objective of these experiments was to evaluate the efficacy of a tumor cell vaccine combined with IL-2 in experimental models of minimal residual lymphoma during the early period following myeloablative conditioning and syngeneic HSCT. 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-reactive CD8^+ OT-I T lymphocytes. Notably, administration of IL-2 pre-bound to an anti-IL-2 mAb clone S4B6 alone or together with vaccination, led to rapid and extensive expansion of total CD8^+ T lymphocytes and NK cells, and augmented the generation of anti-pathogen (*Listeria monocytogenes*) responses. Overall, this strategy of coupling directed IL-2 to heat shock protein vaccination induced remarkable expansion of effector CD8^+ T lymphocytes and led to dramatic increases in MST and overall survival in HSCT recipients as well as the generation of long lived anti-tumor memory.

Clinical studies have demonstrated that tumor-specific CD8^+ T cell responses can be elicited in myeloma patients within 1-2 mo following autologous HSCT [139]. Responses in these patients were generated employing multi-epitope predicted tumor antigen vaccines against hTERT and survivin. Following re-infusion of autologous T cells 2 d post-HSCT and multiple vaccinations beginning at 2 wk, ~1/3 of patients
receiving a prime-boost regimen exhibited specific responses to hTERT and survivin. We sought to transfer T cells from tumor bearing donors, but utilized a multi-epitope vaccine in the early post-HSCT period to induce robust and specific immunity [140]. Our strategy differed as it did not employ ex vivo stimulation, but included IL-2 together with a tumor vaccine in vivo to induce massive CD8^+ T cell expansion early post-HSCT. In contrast to clinical studies in which patients apparently required vaccination prior to T cell collection and HSCT to subsequently elicit responses to vaccine antigens [141,142], the inoculum employed here was obtained from donor mice bearing tumor, but they were not vaccinated. The present study demonstrated that vaccination was absolutely required to elicit expansion of an endogenous antigen-specific T cell clone (Figure 5F,right). Nonetheless, the response of this individual non-TCR transgenic tumor-reactive CD8^+ T cell population cannot account for the markedly increased survival observed in recipients of the combinatorial strategy described here. We hypothesize that additional tumor-specific clones – which cannot be directly monitored – resulting from the multi-epitope nature of the heat shock protein vaccine, must contribute to the increased survival observed in recipients of vaccine and IL-2. Based on the enormous increase in CD8^+ T effector cell numbers following combination treatment (Figure 5F,right), we speculate this response includes low TCR affinity clones that require strong stimulating signals and can be expanded in the presence of IL-2_{S4B6}.

There have been few pre-clinical reports concerning therapeutic anti-tumor vaccination in myeloablative models of syngeneic HSCT supplemented with T cells. In contrast to the multipronged vaccination strategy employed here, those studies required tumor cell vaccination after de novo thymic T cell poiesis following transplant, higher
doses of donor T cells (>2.0 x 10^6), or presensitization of donor T cells with the tumor vaccine [143-145]. One objective of this research was to rapidly elicit T cell responses by transplanted donor cells prior to de novo production of thymic derived T cells. Importantly, expansion of donor tumor-reactive CD8^+, but not CD4^+, T cells was observed following gp96-Ig vaccination alone or together with directed IL-2 within 10 d (Figure 4B and 5B,left). However, only this combination strategy induced a marked increase in overall CD8^+ T effector cell numbers (~500%) ≤2 wk post-HSCT (Figure 5F,right). This time frame is prior to production and emigration of new thymic derived T cells, which takes place 2-3 wk post-HSCT [146]. Together with the observation that gp96-Ig vaccination alone or together with IL-2.ISB did not evoke protective immunity in recipients receiving TCD-BM grafts (Figure 4C) or purified CD4^+ T cells (Figure 6D), respectively, we conclude that the vaccination regimens here expanded transplanted donor CD8^+ T cells very early following HSCT, paralleling clinical findings requiring the addition of large numbers of T cells to provide detectable immunity early post-HSCT [141].

Few pre-clinical studies have incorporated heat shock protein preparations into vaccine strategies for treating hematologic malignancies, including myeloma [147,148]. In the context of experimental syngeneic HSCT, multiple vaccinations with purified syngeneic lymphoma derived gp96 resulted in increased survival in a model of minimal residual lymphoma [149-151]. First, 2-4x the quantity of gp96 was required perhaps because our system secreted 1.0 μg/vaccination. Second, the first vaccination was administered 2 wk post-HSCT, with subsequent vaccinations after the emergence of
thymic derived T cells perhaps because the authors could not elicit a strong enough response with vaccination alone early following HSCT.

Notably, in our immediate vaccination strategy, although recipient APC initially contributed to eliciting tumor-reactive CD8⁺ T cells (Figure 2A), the response rapidly became singularly dependent on donor B7 expressing APC (Figure 2B), which included a critical CD11c⁺ CD8α⁺ cross-presenting DC population (Figure 2C), and required perforin-1 (Figure 2D). Therefore, enriched donor T cells plus CD34⁺ progenitors may not be optimal for use with this vaccine strategy. The role of perforin-1 remains obscure, but could involve NK-APC interactions early during the response [152-154].

The initial use of IL-2 in combination with vaccination or complexed to an anti-IL-2 mAb in the 1990s reported increased CD8⁺ T lymphocyte proliferation, NK cell activity, and enhanced anti-tumor immunity in vivo [59,66,67]. The transplant system employed here was uniquely suitable for IL-2S4B6 complex usage since virtually all CD8⁺ T cells transplanted into lymphopenic hosts assume a central-memory phenotype (CD62L⁺ CD44⁺ CD122⁺ CD127⁺ KLRG-1⁻, data not shown) and express IL-2 receptor β and γ chains presumably rendering these donor CD8⁺ T cells sensitive to IL-2S4B6 stimulation [72,73]. Boyman and colleagues demonstrated rapid CD8⁺ T cell expansion following daily (1 wk) administration of IL-2S4B6 post-HSCT [71]. Similarly, in the transplant model employed here, we detected virtually no change in CD4⁺ T cell and Treg levels (Figure 5A, right and 5A, bottom), indicating that under these conditions, IL-2S4B6 complex most efficiently targeted transplanted donor CD8⁺ T cells. Notably, our data illustrated that vaccination increased the frequency and numbers of tumor-reactive
CD8⁺ OT-I T cells and subsequently IL-2S₄B₆ markedly amplified CD8⁺ effector T cell levels, which directly correlated with the observed increases in MST and overall survival.

Using an independent, highly lethal model of minimal residual lymphoma, increased MST was only observed following vaccination and IL-2 therapy (Figure 5G) further supporting the necessity for the combination. Studies have also demonstrated that human IL-2 complexed to human anti-IL-2 mAb effectively expanded murine CD8⁺ T cells and could enhance anti-tumor immunity [70,71,75]. Interestingly, Levin et al. have engineered human IL-2 to possess binding properties similar to those when natural IL-2 is complexed to anti-IL-2 mAb [78]. These human IL-2 complexes, ‘super-IL-2,’ and new IL-2 fusion products may therefore provide next generation reagents to implement and clinically test the regimen developed here. Similar to IL-2/anti-IL-2 complexes, IL-15 can be complexed with the α chain of the IL-15 receptor (IL-15Rα) leading to enhanced anti-tumor immunity [155,156].

In addition to CD8⁺ T cells, the individual capacities of gp96 and IL-2 to activate APC and NK cells, respectively, is well documented [66,107,108,138] and therefore CD4⁺ T cell presence may not be required for post-HSCT vaccination. IL-2S₄B₆ treatment following transplant with purified CD8⁺ T cells (Figure 6A and 6B,right) resulted in marked donor CD8⁺ T cell expansion (Figure 6B,left). However, there were decreased levels of effector (CD62L⁻ CD44⁺) CD8⁺ T cells (Figure 6C), which might have contributed to the reduced survival observed in these recipients (Figure 6D). These findings support the notion that donor CD4⁺ T cell licensing of APC is unnecessary for this CD8⁺ T cell expansion and purified donor CD4⁺ T cells failed to provide detectable anti-tumor immunity following vaccine and IL-2 treatment.
We observed potent NK cell expansion following vaccination and IL-2<sub>S4B6</sub> infusion (Figure 7A), a population critical for gp96-Ig based strategies [49]. Thus, we posit IL-2<sub>S4B6</sub> enhanced vaccine efficacy in both an indirect and direct fashion via NK cell and tumor-reactive CD<sup>8+</sup> T lymphocyte expansion. In addition to relapse of primary disease, infection is the second leading cause of mortality in HSCT recipients [28]. Both CD<sup>8+</sup> T lymphocytes and NK cells are known to contribute to immunity against *Listeria monocytogenes* [79-81,126,157,158]. Consistent with this, we found that IL-2<sub>S4B6</sub> treated HSCT recipients, which contain increased CD<sup>8+</sup> T lymphocyte and NK cell numbers early post-HSCT, displayed diminished splenic bacterial levels (Figure 7B, right). This is the first demonstration we are aware of reporting IL-2 enhanced anti-pathogen immunity early following myeloablative conditioning and syngeneic HSCT. In models of persistent viral infection, IL-2 has been shown to enhance CD<sup>8+</sup> T cell levels and reduce viral burden in non-transplanted mice infected with lymphocytic choriomeningitis virus (LCMV) [159] and murine γ-herpesvirus 68 (MHV-68) [160].

Srivastava and colleagues introduced autologous tumor-derived gp96 preparations into patients with cancer, including lymphoma [44,45]. Although immune correlates were elicited, overall patient survival was relatively unchanged compared with standard of care regimens [46]. Notably, the first successful phase I trial employing a human allogeneic lung cancer cell line engineered to secrete gp96-Ig was recently completed [56]. Interestingly, ~75% of evaluable patients displayed CD<sup>8+</sup> IFN-γ responses from PBMC against the vaccine cells ex vivo, and these subjects had a MST of 16.5 mo vs 4.5 mo for non-responders. The present findings support the notion that a more effective combinatorial strategy can first activate and then expand tumor-reactive CD<sup>8+</sup> T cells.
Based on the findings presented here, the tumor cell secreted heat shock protein vaccine and directed IL-2 regimen developed in the pre-clinical model here represents a promising strategy with translational applications for patients with hematologic cancers.

In addition to IL-2\textsubscript{S4B6}, IL-2 can be complexed with other anti-mouse IL-2 mAb such as clone JES6-1A12 (IL-2\textsubscript{JES6}), which has been shown to efficiently stimulate the proliferation of cells expressing IL-2 receptor α (CD25) including CD4\textsuperscript{+} FoxP3\textsuperscript{+} Treg and recently activated CD8\textsuperscript{+} T cells [69-71,75,161]. Experiments were performed to compare IL-2\textsubscript{JES6} to IL-2\textsubscript{S4B6} as monotherapies and in combination with vaccination. In contrast to IL-2\textsubscript{S4B6}, CD8\textsuperscript{+} T lymphocytes only expanded following IL-2\textsubscript{JES6} treatment in combination with vaccination, but not as a monotherapy (Figure 8A, left). IL-2\textsubscript{JES6} treated vaccinated mice also displayed elevated levels of tumor-specific CD8\textsuperscript{+} OT-I T cells (Figure 8A, right), which was consistent with an enhancement of recipient survival (Figure 8A, bottom). Another difference between IL-2\textsubscript{S4B6} and IL-2\textsubscript{JES6} was that monotherapy with the latter was able to induce tumor-specific CD8\textsuperscript{+} OT-I T cells, although only in a subset of animals (3/5), with delayed kinetics compared to vaccination alone (Figure 8A, right). Monotherapy (Figure 8B) and combination therapy (Figure 8C) are displayed in the same graphs for comparison purposes. Note that there are subtle differences in magnitude and kinetics of CD8\textsuperscript{+} T cell responses (total and tumor-specific OT-I, Table 2), but overall recipient survival is similar comparing either complex alone or in combination with vaccination.
Section 6: Future Directions

We observed reduced activation of donor CD8\(^+\) T lymphocytes and shortened MST and overall survival in tumor bearing syngeneic HSCT recipients transplanted with purified CD8\(^+\) T cells treated with gp96-Ig vaccination and IL-2 complex. To determine what non-CD8\(^+\) cell population contained in the donor T cell inoculum was required for optimal donor CD8\(^+\) T lymphocyte activation, MST, and overall survival in recipients of enriched CD4\(^+\) and CD8\(^+\) T cells, the next set of experiments could systematically remove either donor CD4\(^+\) T lymphocytes or NK cells. In a preliminary experiment, mice were transplanted with either enriched CD8\(^+\) T cells (CD4\(^+\) depleted) or purified CD4\(^+\) and CD8\(^+\) T cells (NK, etc., depleted). In both groups, MST and overall survival were similar to recipients of enriched CD4\(^+\) and CD8\(^+\) T cells, suggesting that CD4\(^+\) T cells and another non-CD8\(^+\) cell population (most likely NK cells) were both contributing to optimal tumor immunity. Interestingly, it appeared that CD4\(^+\) T cells were more important for CD8\(^+\) effector T cell generation as recipients of purified CD4\(^+\) and CD8\(^+\) T cells (NK depleted) were more similar to recipients of enriched CD4\(^+\) and CD8\(^+\) T cells, whereas recipients of enriched CD8\(^+\) T cells (CD4 depleted) were more similar to recipients of purified CD8\(^+\) T cells.

To examine the role of NK cells in our minimal residual lymphoma model, transplant recipients treated with vaccine and IL-2\(_{S4B6}\) were administered anti-NK1.1 mAb clone PK136 the day before conditioning and every 5-6 d for a total of 4 injections spanning the vaccine protocol. NK cells were depleted for at least 3 wk post-HSCT (Figure 9, left). Interestingly, these mice displayed delayed kinetics of and reduced levels of endogenous tumor-reactive CD8\(^+\) T cell expansion compared to similarly treated
recipients with an intact NK cell compartment (Figure 9,right), which might have contributed to reduced MST and overall survival (Figure 9,bottom). In order to remove NK cells only from the donor T cell inoculum, T cells would be enriched from IL-15 deficient mice, which lack NK cells [162], and then CD4⁺ T cells would be depleted. This would be compared to recipients of similarly treated cells from wild type mice along with purified CD8⁺ T cells from wild type mice.

To further address how CD4⁺ T cells contributed to tumor immunity in HSCT recipients of purified CD4⁺ and CD8⁺ T cells, purified CD4⁺ T cells will be obtained from CD40L or IL-2 deficient mice and mixed with purified CD8⁺ T cells from wild type mice. This will be compared to recipients of purified CD4⁺ and CD8⁺ T cells from wild type mice along with recipients of purified CD8⁺ T cells from wild type mice.

To identify which functional molecules donor CD8⁺ T cells employed for optimal tumor immunity, purified CD8⁺ T cells will be obtained from perforin-1 or granzyme deficient mice and mixed with CD8⁺ depleted cells from wild type mice. These groups will be compared to recipients of enriched CD4⁺ and CD8⁺ T cells from wild type mice along with recipients of purified CD8⁺ T cells from wild type mice.

**Proposed model for gp96-Ig secreting tumor cell vaccination and IL-2S4B6 therapy:**

\[
\text{vaccine} \rightarrow \text{gp96-Ig} \rightarrow \text{CD11c⁺ CD8α⁺ DC} \quad \text{IL-2S4B6} \quad \text{tumor/pathogen} \\
\downarrow \quad \downarrow \quad \uparrow \\
\text{NK cell} \quad \text{CD8⁺ T cell} \quad \text{CD8⁺ T cell}
\]
FIGURES AND TABLES

Figure 1A-B. Peritoneal vaccination with tumor cells secreting gp96-Ig in syngeneic HSCT recipients led to the infiltration of myeloid cells and expansion of tumor-reactive CD8^+ T lymphocytes in the peritoneal cavity. Conditioned (9.5 Gy) B6 mice were transplanted with 5.0 x 10^6 B6-CD45.1^+ TCD-BM cells and adoptively transferred with 1.0 x 10^6 CD8^+ T cells specific for OVA_{257-264} (OT-I) 5 d later. After a 2 d resting period, recipients were vaccinated intraperitoneally with irradiated (120 Gy) EL-4 lymphoma cells expressing OVA (E.G7) engineered to secrete gp96-Ig (E.G7-gp96-Ig). (A) Macrophage (F4/80^{hi} CD11b^{hi} Gr-1^{lo}) and inflammatory monocyte (Gr-1^{hi} CD11b^{mid} F4/80^{lo}) numbers 2 d following vaccination; n = 9 from pool of 3 experiments. (B) Tumor-reactive CD8^+ T lymphocyte (OT-I: CD8^+ CD45.1^- V_{a2}^+ V_{b5}^+) numbers 5 d post-vaccination; n = 8 from pool of 3 experiments.
Figure 1C. A second vaccination with tumor cells secreting gp96-Ig led to accumulation of tumor-reactive CD8\(^+\) T lymphocytes in secondary lymphoid organs. Mice were transplanted and treated as in Figure 1A and received a 2\(^{nd}\) vaccination 13 d post-HSCT. Tumor-reactive CD8\(^+\) T cell numbers 18 d following HSCT; n = 3.
Figure 1D. Equivalent expansion of tumor-reactive CD8$^+$ T lymphocytes following vaccination up to 2 wk post-HSCT. Transplants were performed as in Figure 1A, but tumor-reactive CD8$^+$ T cells were co-transplanted with TCD-BM, and recipients were vaccinated 2, 7, or 14 d following HSCT. Tumor-reactive CD8$^+$ T cell number 5 d post-vaccination; n = 3-8 from 3 independent experiments.
Figure 1E. Tumor-reactive CD8$^+$ T lymphocytes are activated following vaccination. Mice were transplanted and treated as in Figure 1A. Tumor-reactive CD8$^+$ T lymphocytes transitioned from central-memory (T\textsubscript{CM}, CD62L$^+$ CD44$^+$) to effector cells (T\textsubscript{eff}, CD62L$^-$ CD44$^+$) at the site of vaccination; representative dot plots (left), n = 5-6 from pool of 2 experiments (right).
Figure 1F. Vaccination results in functional tumor-reactive CD8$^+$ T lymphocytes. Transplants and treatments were performed as in Figure 1A. Vaccination induced highly IFN-$\gamma^+$ tumor-reactive CD8$^+$ T cells at the site of vaccination; representative dot plots (left), n = 4 from representative of 3 experiments (right).
Figure 2A-B. B7 costimulation was required for optimal tumor-reactive CD8+ T lymphocyte expansion induced by vaccination with gp96-Ig secreting tumor cells following syngeneic HSCT. Transplants were performed as in Figure 1 utilizing mice deficient in CD80 and CD86 (B7KO) as donors and/or recipients with (A) co-infusion or (B) delayed infusion of 1.0 x 10^6 tumor-reactive CD8+ T cells (OT-I) and the peritoneal cavity was analyzed 5 d following intraperitoneal vaccination with tumor cells secreting gp96-Ig. (A) Donor and recipient APC contributed to expansion of tumor-reactive CD8+ T cells, co-transplanted with the BM, following vaccination with gp96-Ig secreting tumor cells; n = 3-9 from pool of 3 experiments. (B) Only donor APC contributed to expansion of tumor-reactive CD8+ T lymphocytes, infused 5 d post-HSCT, induced by vaccination with tumor cells secreting gp96-Ig; n = 2-4 from pool of 2 experiments.
Figure 2C. Batf3 was required for optimal tumor-reactive CD8$^+$ T lymphocyte expansion induced by vaccination with gp96-Ig secreting tumor cells following syngeneic HSCT. Conditioned (9.5 Gy) B6 mice were transplanted with $5.0 \times 10^6$ B6 wild-type (B6WT) or Batf3 deficient (Batf3KO) TCD-BM cells and adoptively transferred with $0.5 \times 10^6$ CD8$^+$ T cells specific for OVA$_{257-264}$ (OT-I) 5 d later. After a 2 d resting period, recipients were vaccinated intraperitoneally with irradiated (40 Gy) EL-4 lymphoma cells expressing OVA (E.G7) engineered to secrete gp96-Ig (E.G7-gp96-Ig).

(Top) Loss of CD11b$^{lo}$ CD11c$^{hi}$ DC at the site of vaccination in recipients of Batf3 deficient TCD-BM receiving gp96-Ig secreting tumor cells. CD11c$^{hi}$ CD11b$^{lo}$ F4/80$^{lo}$ Gr-1$^{lo}$ frequency in the peritoneal cavity; representative dot plots (left), n = 5 (right).

(Bottom) Donor cross-presenting CD11c$^+$ CD8$\alpha^+$ DC were required for optimal expansion of tumor-reactive CD8$^+$ T cells following vaccination with gp96-Ig secreting tumor cells; n = 5.
Figure 2D. Perforin-1 was required for optimal tumor-reactive CD8\(^+\) T lymphocyte expansion induced by vaccination with gp96-Ig secreting tumor cells following syngeneic HSCT. Transplants and treatments were performed as in Figure 1 utilizing mice deficient in perforin-1 (PrfKO) as donors and/or recipients with delayed infusion of 1.0 x 10\(^6\) tumor-reactive CD8\(^+\) T cells (OT-I) and the peritoneal cavity was analyzed 5 d following intraperitoneal vaccination with tumor cells secreting gp96-Ig. (D) Perforin-1 could be supplied by either donor or recipient cells for optimal expansion of tumor-reactive CD8\(^+\) T lymphocytes following vaccination with tumor cells secreting gp96-Ig; n = 2-4 from pool of 2 experiments.
Figure 3A. CD4+ and CD8+ splenic and LN T cells from a typical tumor bearing donor mouse. Donor mice were injected 3 wk pre-HSCT with 5.0 x 10^5 CD8+ OT-I T lymphocytes intravenously and 4.0 x 10^6 E.G7 lymphoma cells intraperitoneally and bear progressively growing tumors. Tumor was only detectable at the site of injection (peritoneal cavity) at this time. Total events analyzed represented 2.5 x 10^6 viable cells and CD8+ OT-I T lymphocytes were clearly identified (1000 CD8+ CD90.1- Vα2+ Vβ5+ / 1.0 x 10^6 CD8+ CD90.1+). 2.0 x 10^6 CD4+ and CD8+ T lymphocytes obtained from E.G7 lymphoma bearing B6-CD90.1+ donors contained ~1000 tumor-reactive CD8+ T cells (OT-I).
Figure 3B-C. Tumor-reactive CD8$^+$ T lymphocytes obtained from tumor bearing donors were expanded following transplantation into syngeneic HSCT recipients and vaccination with tumor cells secreting gp96-Ig. Conditioned (9.5 Gy) B6 recipients received B6-CD45.1$^+$ TCD-BM cells supplemented with $2.0 \times 10^6$ B6-CD90.1$^+$ CD4$^+$ and CD8$^+$ T lymphocytes from Figure 3A. Recipients were vaccinated intraperitoneally with irradiated (40 Gy) E.G7 cells secreting gp96-Ig 2 d post-HSCT and repeated every 3 d for a total of 5 vaccinations. (B) Multiple vaccinations with gp96-Ig secreting tumor cells induced expansion of tumor-reactive CD8$^+$ T cells obtained from tumor bearing donors; n = 4; ♦: E.G7, ●: E.G7-gp96-Ig. (C) Tumor-reactive CD8$^+$ T lymphocytes expanded at the vaccine site and other lymphoid tissues 5 d following 3 vaccinations with tumor cells secreting gp96-Ig; n = 2.
Figure 3D. Tumor-reactive CD8⁺ T lymphocytes obtained from tumor bearing donors were functional following transplantation into syngeneic HSCT recipients and vaccination with tumor cells secreting gp96-Ig. Mice were transplanted and treated as in Figure 3B. Vaccination with gp96-Ig secreting tumor cells induced highly IFN-γ⁺ and TNF-α⁺ tumor-reactive CD8⁺ T cells 5 d following 3 vaccinations; representative dot plots from n = 2.
Figure 4A. Development of minimal residual lymphoma model following syngeneic HSCT. Transplants were performed as in Figure 3 and recipients were inoculated intraperitoneally with $1.0 \times 10^5$ E.G7 lymphoma cells the following day to simulate tumor relapse post-HSCT. (Left) Equivalent survival of HSCT recipients with lymphoma receiving T cells from tumor bearing or tumor naive donors or no T cells; n = 4-5 from 3 independent experiments; x: no vaccine (tumor), +: no vaccine (naive), -: no T cells. (Right) Increased numbers of T cells ($>2.0 \times 10^6$) results in <100% lethality; n = 4-5 from 2 independent experiments; x: 2.0 $\times 10^6$ T cells, +: 5.0 $\times 10^6$ T cells, -: 10.0 $\times 10^6$ T cells.
Figure 4B. Multiple vaccinations with tumor cells secreting gp96-Ig expanded tumor-reactive CD8+ T lymphocytes in syngeneic HSCT recipients with lymphoma. Transplants and tumor inoculation were performed as in Figure 4A. Two days following tumor inoculation, recipients were vaccinated intraperitoneally with irradiated (40 Gy) E.G7 lymphoma cells secreting gp96-Ig and repeated every 3 d for a total of 5 vaccinations; n = 20 from pool of 4 experiments; x: no vaccine, ♦: E.G7, ●: E.G7-gp96-Ig.
Figure 4C-D. Multiple vaccinations with tumor cells secreting gp96-Ig increased survival in T cell replete syngeneic HSCT recipients with lymphoma. Transplants, tumor inoculation, and treatments were performed as in Figure 4B. (C) Vaccination with tumor cells secreting gp96-Ig led to increased MST and overall survival of lymphoma bearing HSCT recipients; n = 4-5; x: no vaccine, ◆: E.G7, ●: E.G7-gp96-Ig, ○: E.G7-gp96-Ig (no T cells). The ‘no vaccine’ group illustrated here was shown in Figure 4A with other non-vaccinated groups. (D) Equivalent survival of vaccinated HSCT recipients receiving T cells from tumor bearing or tumor naive donors; n = 6; ●: E.G7-gp96-Ig (tumor), ○: E.G7-gp96-Ig (naive).
Figure 5A. IL-2 administered in vivo after complex with anti-IL-2 mAb effectively expanded CD8+, but not CD4+, T lymphocytes induced by vaccination with tumor cells secreting gp96-Ig in lymphoma bearing syngeneic HSCT recipients. Transplants, tumor inoculation, and treatments were performed as in Figure 4 and mice received IL-2S4B6 (1.5 μg IL-2 + 8 μg anti-IL-2 mAb clone S4B6) 1 d following each vaccination. (Left) IL-2S4B6 treatment induced robust expansion of CD8+ T cells in vaccinated HSCT recipients with lymphoma during the first 3 wk post-HSCT. CD8+ T cell frequency in the peripheral blood; n = 20 from pool of 4 experiments; ✱: no vaccine, ♦: E.G7, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2S4B6. (Right) Systemic CD4+ T cell levels were unchanged following IL-2S4B6 treatment in syngeneic HSCT recipients. CD4+ T cell frequency in the peripheral blood; n = 10 from pool of 2 experiments; ✱: no vaccine, ♦: E.G7, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2S4B6. (Bottom) Systemic CD4+ FoxP3+ Treg levels were marginally changed following IL-2S4B6 treatment in syngeneic HSCT recipients. FoxP3+ frequency within CD4+ T cells in the peripheral blood utilizing mice expressing red fluorescent protein under control of the FoxP3 promoter as T cell donors; n = pool of 5; ✱: no vaccine, ♦: E.G7, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2S4B6.
Figure 5B. IL-2 administered in vivo after complex with anti-IL-2 mAb effectively expanded tumor-reactive CD8\(^+\) T lymphocytes induced by vaccination with tumor cells secreting gp96-Ig in lymphoma bearing syngeneic HSCT recipients. Transplants, tumor inoculation, and treatments were performed as in Figure 5A. (Left) Tumor-reactive CD8\(^+\) T cells reached maximal levels in the peripheral blood 2 wk post-HSCT following vaccination and IL-2 therapy; n = 20 from pool of 4 experiments; x: no vaccine, ♦: E.G7, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2\(_{S4B6}\). The ‘no vaccine,’ ‘E.G7,’ and ‘E.G7-gp9-Ig’ groups from Figure 4B were included as a reference to illustrate the kinetics of expansion with vaccination and IL-2\(_{S4B6}\). (Right) Tumor-reactive CD8\(^+\) T lymphocytes markedly expanded at the vaccine site and other lymphoid tissues of lymphoma bearing HSCT recipients 5 d following 3 treatments with IL-2\(_{S4B6}\) and gp96-Ig vaccine. Tumor-reactive CD8\(^+\) T cell number in the peritoneal cavity, spleen, and draining LN; n = 2.
Figure 5C. IL-2 administered in vivo after complex with anti-IL-2 mAb augmented anti-tumor immunity induced by vaccination with tumor cells secreting gp96-Ig in lymphoma bearing syngeneic HSCT recipients. Transplants, tumor inoculation, and treatments were performed as in Figure 5A. (Left) Non-complexed, unbound IL-2 in combination with vaccination failed to expand CD8⁺ T lymphocytes. CD8⁺ T cell frequency in the peripheral blood; n = 5; ●: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2, ■: E.G7-gp96-Ig + IL-2S4B6. (Right) Non-complexed, unbound IL-2 in combination with vaccination failed to expand tumor-reactive CD8⁺ T lymphocytes. Tumor-reactive CD8⁺ T cell frequency in the peripheral blood; n = 5. ●: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2, ■: E.G7-gp96-Ig + IL-2S4B6. Extended analyses of these CD8⁺ T lymphocyte frequencies in mice treated with vaccination and IL-2S4B6 suggested that slightly increased levels were maintained for at least 6 wk post-HSCT, or 1 mo following cessation of treatment. (C) Combination therapy with vaccination and IL-2S4B6 increased MST and overall survival of syngeneic HSCT recipients with lymphoma. Non-complexed, unbound IL-2 in combination with vaccination failed to enhance survival of lymphoma bearing HSCT recipients; n = 5; ●: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2, ■: E.G7-gp96-Ig + IL-2S4B6.
Figure 5D. IL-2_{S4B6} in the absence of vaccination markedly expanded CD8^{+} T cells, but only elicited a small response by tumor-reactive CD8^{+} T lymphocytes. Transplants, tumor inoculation, and treatments were performed as in Figure 5A. (Left) IL-2_{S4B6} in the absence of vaccination markedly expanded CD8^{+} T cells. CD8^{+} T cell frequency in the peripheral blood; n = pool of 5; x: IL-2_{S4B6}, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_{S4B6}. (Right) IL-2_{S4B6} in the absence of vaccination only elicited a small response by tumor-reactive CD8^{+} T lymphocytes. Tumor-reactive CD8^{+} T cell frequency in the peripheral blood; n = pool of 5; x: IL-2_{S4B6}, ●: E.G7 + IL-2_{S4B6}, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_{S4B6}. (Bottom) IL-2_{S4B6} in the absence of vaccination marginally enhanced survival of lymphoma bearing HSCT recipients; n = 5; x: IL-2_{S4B6}, ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_{S4B6}.
Figure 5E. CD8+ T cells markedly expanded following vaccination and IL-2S4B6 in the absence of transgenic CD8+ OT-I T lymphocytes resulting in enhanced survival. Transplants, tumor inoculation, and treatments were performed as in Figure 5A. (Left) CD8+ T cell frequency in the peripheral blood; n = pool of 5; ●: E.G7-gp96-Ig, ○: E.G7-gp96-Ig (no OT-I), ■: E.G7-gp96-Ig + IL-2S4B6, □: E.G7-gp96-Ig + IL-2S4B6 (no OT-I). The small increase observed in CD8+ T lymphocyte frequency in both groups containing CD8+ OT-I T cells likely represents the presence of the transgenic CD8+ T cell population. (Right) MST and overall survival of T cell replete syngeneic HSCT recipients with lymphoma following vaccination and IL-2S4B6 were independent of transgenic antigen-specific CD8+ T cell presence; n = 5; ●: E.G7-gp96-Ig, ○: E.G7-gp96-Ig (no OT-I), ■: E.G7-gp96-Ig + IL-2S4B6, □: E.G7-gp96-Ig + IL-2S4B6 (no OT-I).
Figure 5F. IL-2_{S4B6} in combination with vaccination elicits potent effector CD8^+ T cell response. Transplants, tumor inoculation, and treatments were performed as in Figure 5A. (Left) CD8^+ T_{eff} (CD62L^- CD44^+) cell frequency in the peripheral blood; n = 21 from pool of 4 experiments; ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_{S4B6}. (Right) CD8^+ effector T cell numbers at the vaccine site were markedly increased only following vaccine and IL-2_{S4B6} treatment. CD8^+ T_{eff} (CD62L^- CD44^+), including endogenous non-TCR transgenic DimerX–K^b–OVA_{257-264}^+, cell numbers in the peritoneal cavity 5 d following 3 vaccinations; n = 5.
Figure 5G. Combined IL-2_{S4B6} and gp96-Ig vaccination elicited increased CD8$^+$ effector T cells and enhanced survival in a highly aggressive minimal residual lymphoma model post-HSCT. Transplants were performed as in Figure 5A and recipients were inoculated with 5000 EL-4 lymphoma cells 1 d post-HSCT. Mice were vaccinated with EL4-gp96-Ig and received IL-2_{S4B6} 1 d following each vaccination for a total of 5 treatments. (Left) IL-2_{S4B6} enhanced CD8$^+$ effector T cell levels induced by gp96-Ig vaccination. CD8$^+$ T_{eff} (CD62L$^-$ CD44$^+$) cell frequency in peripheral blood; n = 5; ○: EL4-gp96-Ig, ■: EL4-gp96-Ig + IL-2_{S4B6}. (Right) Only combined vaccination and IL-2_{S4B6} increased survival post-HSCT; n = 5; □: no vaccine, ♦: IL-2_{S4B6}, ●: EL4-gp96-Ig, ■: EL4-gp96-Ig + IL-2_{S4B6}. 
Figure 6A. CD4$^+$ and CD8$^+$ T cell content of purified CD4$^+$ or CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cells were purified following B cell depletion using negative selection.
Figure 6B. Donor CD8⁺ T cells efficiently expanded following vaccination and IL-2S4B6 in the absence of donor CD4⁺ T cells. Transplants, tumor inoculation, and treatments were performed as in Figure 5, however some recipients received BM supplemented with purified CD4⁺ (≥88%) or CD8⁺ (≥90%) T cells. (Left) Donor CD8⁺ T cell frequency in the peripheral blood; n = 5; ●: E.G7-gp96-Ig, ○: E.G7-gp96-Ig (Purified CD8), ■: E.G7-gp96-Ig + IL-2S4B6, □: E.G7-gp96-Ig + IL-2S4B6 (Purified CD8). The results for ‘E.G7-gp96-Ig + IL-2S4B6’ and ‘E.G7-gp96-Ig + IL-2S4B6 (Purified CD8)’ were repeated in an independent experiment. (Right) Donor CD4⁺ T cells were minimally detectable in recipients of purified CD8⁺ T cells. CD4⁺ T cell frequency in the peripheral blood; n = 5; ●: E.G7-gp96-Ig, ○: E.G7-gp96-Ig (Purified CD8), ■: E.G7-gp96-Ig + IL-2S4B6, □: E.G7-gp96-Ig + IL-2S4B6 (Purified CD8). The results for ‘E.G7-gp96-Ig + IL-2S4B6’ and ‘E.G7-gp96-Ig + IL-2S4B6 (Purified CD8)’ were repeated in an independent experiment.
Figure 6C. In the absence of donor CD4$^+$ T cells, donor CD8$^+$ T effector cells were not efficiently generated following vaccine and IL-2S4B6. Transplants, tumor inoculation, and treatments were performed as in Figure 6B. Donor CD8$^+$ Teff (CD62L$^-$ CD44$^+$) cell frequency in the peripheral blood; n = 5 from representative of 2 experiments; ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2S4B6, □: E.G7-gp96-Ig + IL-2S4B6 (Purified CD8). The results for ‘E.G7-gp96-Ig + IL-2S4B6’ and ‘E.G7-gp96-Ig + IL-2S4B6 (Purified CD8)’ were repeated in an independent experiment.
Figure 6D. Donor non-CD8\(^+\) cells were required for optimal anti-tumor immunity induced by combination therapy with gp96-Ig secreting tumor cell vaccination and IL-2 cytokine-antibody complexes in syngeneic HSCT recipients with lymphoma. Transplants, tumor inoculation, and treatments were performed as in Figure 6B. Survival benefit of vaccination and IL-2\(_{S4B6}\) therapy is reduced in lymphoma bearing HSCT recipients receiving TCD-BM supplemented with purified CD8\(^+\) T cells and abolished in recipients of purified CD4\(^+\) T cells; \(n = 5-10\) from pool of 3 experiments; \(\blacklozenge\): no vaccine, \(\bullet\): E.G7-gp96-Ig, \(\blacksquare\): E.G7-gp96-Ig + IL-2\(_{S4B6}\), \(\square\): E.G7-gp96-Ig + IL-2\(_{S4B6}\) (Purified CD8), \(\blacklozenge\): E.G7-gp96-Ig + IL-2\(_{S4B6}\) (Purified CD4).
Table 1. Anti-tumor memory response in previously vaccinated HSCT recipients.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. with tumor(c/)</th>
<th>No. injected(d)</th>
<th>% with tumor(c)</th>
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<td>Untreated</td>
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<td>100</td>
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<tr>
<td>Non-transplanted</td>
<td></td>
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</tr>
<tr>
<td>E.G7-gp96-Ig(a)</td>
<td></td>
<td>0 / 6</td>
<td>0</td>
</tr>
<tr>
<td>BM + T cells(b)</td>
<td></td>
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<tr>
<td>E.G7-gp96-Ig + IL-2(b)</td>
<td>2 / 14</td>
<td></td>
<td>14.3</td>
</tr>
<tr>
<td>BM + T cells(b)</td>
<td></td>
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</tbody>
</table>

\(a\) Transplant recipients were \(\geq 100\) d post-HSCT (Figure 4-6).

\(b\) \(\geq 75\%\) CD4\(^+\) and CD8\(^+\); \(\leq 3\%\) CD19\(^+\) (Methods).

\(c\) Mice with progressively growing tumors (sacrificed \(\geq 225\) mm\(^2\)).

\(d\) Mice were challenged with E.G7 lymphoma cells subcutaneously \((1.0 \times 10^6)\).

\(e\) Total rejection rate of HSCT recipients was 90\% (18/20).

Table 1 Figure. Kinetics of tumor growth and survival in previously vaccinated HSCT recipients challenged with a lethal number of tumor cells. Previously vaccinated transplant recipients (>100 d post-HSCT; Figures 4-6) were challenged with a lethal number \((1.0 \times 10^6)\) of E.G7 tumor cells subcutaneously to determine if anti-tumor memory had been generated by either vaccine strategy. **(Left)** Kinetics of tumor growth; \(\times\): Untreated and Non-transplanted (pooled), \(\bullet\): E.G7-gp96-Ig (individual), \(\blacksquare\): E.G7-gp96-Ig + IL-2\(b\) (individual). **(Right)** Survival post-challenge; \(n = 6-14\) from pool of 2 subcutaneous challenge experiments; \(\times\): Untreated and Non-transplanted, \(\bullet\): E.G7-gp96-Ig, \(\blacksquare\): E.G7-gp96-Ig + IL-2\(b\).
Figure 7A. IL-2 complex therapy enhanced NK cell numbers in HSCT recipients. Transplants and treatments were performed as in Figure 5. IL-2s486 therapy elicited large numbers of NK cells in the peritoneal cavity of intraperitoneally vaccinated HSCT recipients 5 d following the 3rd treatment. NK1.1+ cell numbers in the peritoneal cavity; n = 2.
Figure 7B. IL-2 complex therapy enhanced and anti-pathogen immunity in HSCT recipients. Transplants were performed as in Figure 5 and HSCT recipients were infused with IL-2S_{4B6} 3 d post-HSCT and every 3 d for a total of 4 infusions. (Left) IL-2S_{4B6} induced expansion of CD8^+ T cells in HSCT recipients immediately prior to *Listeria monocytogenes* challenge. CD8^+ T cell frequency in the peripheral blood 2 wk post-HSCT; n = 5 from representative of 2 experiments; x: no HSCT, ●: HSCT, ■: HSCT + IL-2S_{4B6}. (Right) IL-2S_{4B6} treated HSCT recipients displayed fewer splenic bacterial CFU following intravenous inoculation with 1.0 x 10^4 CFU *Listeria monocytogenes* 14 d post-HSCT and 4 infusions of IL-2S_{4B6} starting 3 d post-HSCT and repeated every 3 d. CFU numbers in the spleen 7 d post-infection; n = 5 from representative of 2 experiments; x: no HSCT, ●: HSCT, ■: HSCT + IL-2S_{4B6}.
Figure 8A: Effects of IL-2JES6. Transplants were performed as in Figure 5 and mice received IL-2 pre-bound to anti-IL-2 mAb clone JES6 (IL-2JES6) 1 d following each vaccination or in the absence of vaccination. (Left) IL-2JES6 only expanded CD8⁺ T cells in combination with vaccination. CD8⁺ T cell frequency in the peripheral blood; n = 5; †: IL-2JES6, •: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2JES6. (Right) IL-2JES6 augmented vaccine induced tumor-reactive CD8⁺ T cell expansion. Tumor-reactive CD8⁺ T cell frequency in the peripheral blood; n = 5; †: IL-2JES6, •: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2JES6. (Bottom) Combination therapy with vaccine and IL-2JES6 enhanced MST and overall survival; n = 5; †: IL-2JES6, •: E.G7-gp96-Ig, ♦: E.G7-gp96-Ig + IL-2JES6.
Figure 8B. Comparison of IL-2_mAb monotherapy. Composite data from Figures 5D and 8A. **(Left)** Monotherapy with IL-2_JES6 did not expand CD8⁺ T cells as compared to IL-2_S4B6. CD8⁺ T cell frequency in the peripheral blood; n = 5-10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ×: IL-2_S4B6, +: IL-2_JES6. **(Right)** IL-2_JES6 expanded tumor-reactive CD8⁺ T cells in a subset of mice compared to minimal expansion by IL-2_S4B6. Tumor-reactive CD8⁺ T cell frequency in the peripheral blood; n = 5-10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ×: IL-2_S4B6, +: IL-2_JES6. **(Bottom)** Monotherapy with either IL-2_mAb reagent results in similar MST; n = 5-10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ×: IL-2_S4B6, +: IL-2_JES6.
Figure 8C. Comparison of IL-2mAb in combination with vaccination. Composite data from Figures 5C-D and 8A. (Left) Delayed expansion and increased contraction of CD8⁺ T cells following vaccination in combination with IL-2_JES6 compared to IL-2_S4B6. CD8⁺ T cell frequency in the peripheral blood; n = 10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_S4B6, ♦: E.G7-gp96-Ig + IL-2_JES6. (Right) Augmented expansion and contraction of tumor-reactive CD8⁺ T cells following vaccination in combination with IL-2_JES6 compared to IL-2_S4B6. Tumor-reactive CD8⁺ T cell frequency in the peripheral blood; n = 10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_S4B6, ♦: E.G7-gp96-Ig + IL-2_JES6. (Bottom) IL-2_JES6 and IL-2_S4B6 in combination with vaccination enhanced MST and overall survival; n = 10 from pool of 2 experiments; ●: E.G7-gp96-Ig, ■: E.G7-gp96-Ig + IL-2_S4B6, ♦: E.G7-gp96-Ig + IL-2_JES6.
Table 2: Summary of IL-2/anti-IL-2 complex induced CD8^{+} T cell responses in the presence and absence of vaccination with gp96-Ig secreting tumor cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor CD8^{+} T cells</th>
<th>Tumor reactive CD8^{+} OT-1 T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>no vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2_{S4B6}</td>
<td>+</td>
<td>+/−^{a}</td>
</tr>
<tr>
<td>IL-2_{JES6}</td>
<td>−</td>
<td>+^{b}</td>
</tr>
<tr>
<td>gp96-Ig vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2_{S4B6}</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IL-2_{JES6}</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

^{a}below level of detection
^{b}3/5
Figure 9. Depletion of NK cells in HSCT recipients resulted in diminished tumor-reactive CD8\(^+\) T cell expansion and survival following vaccine and IL-2. Transplants, treatments, and tumor inoculation were performed as in Figure 6. Anti-NK1.1 mAb clone PK136 was administered 1 d before total body irradiation (200 \(\mu\)g) and 3 d (100 \(\mu\)g), 9 d (50 \(\mu\)g), and 15 d (50 \(\mu\)g) post-HSCT. **(Left)** Depletion of NK cells in HSCT recipients administered anti-NK1.1 mAb clone PK136 treated with vaccine and IL-2. Donor NK cell frequency in the peripheral blood; \(n = 5\); ■: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\), □: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\) (αNK1.1 mAb). **(Right)** Depletion of NK cells in HSCT recipients resulted in delayed and diminished tumor-reactive CD8\(^+\) T cell expansion following with vaccine and IL-2. Donor CD8\(^+\) OVA\(_{257-264}\) \(^+\) T cell frequency in the peripheral blood; \(n = 5\); ■: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\), □: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\) (αNK1.1 mAb). **(Bottom)** Diminished survival of HSCT recipients treated with vaccine and IL-2 depleted of NK cells; \(n = 5\); ✖: IL-2\(_{S_{4B6}}\), ■: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\), □: E.G7-gp96-Ig + IL-2\(_{S_{4B6}}\) (αNK1.1 mAb)
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