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Author
Gschweng, Eric Hans

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Cancer Immunotherapy Using Engineered Hematopoietic Stem Cells

A dissertation submitted in partial satisfaction

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Microbiology, Immunology & Molecular Genetics

by

Eric Hans Gschweng

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ABSTRACT OF THE DISSERTATION

Cancer Immunotherapy Using Engineered Hematopoietic Stem Cells

By

Eric Hans Gschweng

Doctor of Philosophy in Microbiology,

Immunology & Molecular Genetics

University of California, Los Angeles, 2015

Professor Donald Barry Kohn, Chair

Engineering the immune system against cancer ideally provides surgical precision against the antigen bearing target cell while avoiding the systemic, off-target toxicity of chemotherapy. Successful treatment of patients in the clinic has been achieved by the expression of anti-cancer T-cell receptors (TCR) and chimeric antigen receptors (CAR) in T cells followed by infusion of these cells into cancer patients. Unfortunately, while many patients initially respond showing anti-tumor efficacy, the effects are by and large transient with the majority of patients succumbing to disease. This observation speaks to the power of the engineered antigen receptor, but also indicates a need for a persistent source of cells to mount a continuous response.

This thesis work sought to investigate the feasibility of engineering immunity using hematopoietic stem cells (HSC). HSC are at the top of the hematopoietic hierarchy, and would theoretically provide a continuous supply of antigen receptor bearing T cells capable of eliminating disease in a patient. Important considerations for using HSCs in gene therapy include: efficient gene delivery to HSCs which are refractory to gene modification by nature, robust expression of the transgene within gene modified cells to provide efficacy, evaluation of successful gene modification and engraftment following transplant, the ability of gene modified T cells to mount an immune response against their intended targets, and the ability to eliminate the gene modified cells in the event of undesired outcomes.

Current systems to study human HSC biology and gene therapy are limited, and the best available are human / mouse chimeric transplant systems. Using these models, we evaluated the utility of positron emission tomography (PET) in the humanized mouse following transplant of gene modified cells, and found it superior to peripheral blood flow cytometry in being able to temporally establish successful engraftment. Using a lentiviral vector expressing both a TCR against a common cancer / testes antigen, NY-ESO-1, as well as a PET imaging / suicide gene, we demonstrated the ability to monitor engraftment, successful generation of effector cells capable of killing patient derived cancer cell lines, and the complete elimination of gene modified cells. This final work provides support for a clinical trial soon to enroll patients at UCLA.

The use of HSCs for engineered cancer immunotherapy could overcome current limitations associated with the lack of persistence of engineered terminally differentiated immune cells. Further, the rich clinical history of gene therapy using HSCs provides a broad platform for future studies aimed at the broad spectrum of cancer disease.

The dissertation for Eric Hans Gschweng is approved.

David G. Brooks

Antoni Ribas

Owen N. Witte

Donald Barry Kohn, Committee Chair

University of California, Los Angeles

2015

DEDICATION

This dissertation is dedicated to my family:

Fritz and Emily Gschweng, Bob and Fran Shoemaker,

Paul Gschweng, Robbie Shoemaker, and Nic Gschweng

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VITA

Graduate Student Researcher, UCLA, Los Angeles, CA	Sept 2009 – Mar 2015
Laboratory of Donald B. Kohn, MD	
Clinical Specialist, University of California, Los Angeles, CA	Sept 2008 - Aug 2009
Laboratory of Owen N. Witte, MD	
Research Technician II, HHMI, UCLA, CA	Sept 2006 - Sept 2008
Laboratory of Owen N. Witte, MD	
Research Associate II, UCSF, San Francisco, CA Laboratory of Randall H. Kramer, PhD	May 2004 - Sept 2006
Undergraduate, SFSU, San Francisco, CA Bachelor of Science – Biology, Emphasis – Physiology	Jan 2001 – Jan 2004
Undergraduate, SJDC, Stockton, CA	Sept 1998 – Dec 2000

Selected Publications

Cooper AR, Lill GR, **Gschweng EH**, Kohn DB. Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter. Nucleic Acids Res. 2015 Jan 9;43(1):682-90. doi: 10.1093/nar/gku1312. Epub 2014 Dec 17.

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Presentations

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UCLA Stem Cell Club, Los Angeles, Ca, 2013. "*In vivo* sr39TK PET Reporter / Suicide Gene Functionality in Human Hematopoietic Stem Cells"

3rd Annual ImmunologyLA, Skirball Center, Los Angeles, Ca, 2013. "Hematopoietic Stem Cell Based Immunotherapy for Cancer"

Awards

ASH Abstract Achievement Award 55th Annual American Society of Hematology Meeting

Dec 2013, Ernest N. Morial Convention Center, New Orleans, La

Poster Award – Best in 5th Year Group, UCLA Pharmacology Retreat

Nov 2013, Hyatt Regency Hotel, Newport Beach, Ca

Ray Owen Poster Award, 52nd Midwinter Conference of Immunologists at Asilomar

Jan 2013, Asilomar Conference Center, Pacific Grove, Ca

Other

President – UCLA Biological Sciences Council	July 2013 – June 2014
Secretary – UCLA Biological Sciences Council	July 2012 – June 2013
MIMG Representative – UCLA Biological Sciences Council	Sept 2009 – Mar 2015
Organizer – BSCRC Stem Cell Club	Sept 2013 – Mar 2015
Member – American Society of Hematology	Oct 2013 – Present
Member – Society for the Immunotherapy of Cancer	Aug 2013 – Present
Member – American Society for Gene and Cell Therapy	May 2012 – Present

CHAPTER 1:

Hematopoietic stem cells for cancer immunotherapy

T cells for TCR- and CAR-mediated immunotherapy: progress and limitations

Studies of immunotherapy using peripheral blood T lymphocytes modified with genes encoding T-cell receptors (TCRs) or chimeric antigen receptors (CARs) recognizing tumor-associated antigens have made great strides in our understanding of basic immunology and yielded excellent clinical responses in some settings. Pioneering work by Steven Rosenberg at the Surgery Branch of the National Cancer Institute paved the way for a plethora of studies that have improved the efficacy of this therapeutic approach utilizing *ex vivo* expanded antigen-specific tumor infiltrating lymphocytes reinfused into patients (1), as well as the engineering of peripheral blood T cells via viral gene transfer (2). Further work using CARs has recently shown great promise in several trials: engineering T lymphocytes with CARs directed against the B-lymphocyte antigen CD19 has led to complete remissions in a number of patients with advanced B-lineage malignancies (3–6). The potential of these therapies is indisputable, although limitations remain. Objective responses using TCR engineered T cells have been achieved in the majority of patients enrolled in trials: a measurable decrease in bulk tumor burden is often observed. However, the complete response rates have been quite low and the majority of clinical responses are short-lived with ultimate tumor relapse. This has been observed particularly with TCR-based approaches, although CAR-based approaches may also have some sub-optimal aspects as further studies are reported.

A major explanation for this sub-optimal outcome is the relatively limited in vivo survival, suppression, or exhaustion of infused engineered T cells (7, 8). Expansion of T cells to an effective therapeutic bolus is achieved by *ex vivo* culture in supraphysiologic concentrations of IL-2, increasing the cell numbers by several orders of magnitude. It has been appreciated that driving cells to expand under these conditions ages the cells from a more naive and replicative phenotype

to late-stage effectors. Characterized by the loss of markers CD45RA, CD62L and CCR7, and the acquisition of effector function such as the secretion of γ-interferon, these cells have great cytotoxic capacity but greatly diminished regenerative potential. Experimental work in murine models (9) as well as non-human primates (10) has shown the improved antitumor efficacy of central memory phenotype cells (Tcm) versus late stage effectors (9). There are promising recent reports showing that including small molecule inhibitors or modulation of the cytokine milieu in which cells are expanded *ex vivo* makes possible the maintenance and generation of the more stem like T cell populations known as stem cell memory (T_{SCM}) cells, and that these cells are capable of a more sustained response by replenishing effectors (11) similar to the previous studies with Tcm cells. A clear benefit to the transfer of less mature, more stem-like cells is evident, likely due to the increased persistence and replenishing capability of these cells *in vivo*.

In accordance with this hypothesis, studies investigating T cells transduced with CARs seem to have greater persistence and activity than those modified with TCRs, potentially demonstrating improved efficacy. Scholler et al. (12) reported long-term follow-up of subjects who received T cells modified to express a CAR-like molecule targeted against HIV-infected cells, and even 10–15 years after a single infusion of modified T cells, these cells could still be detected in the peripheral blood of many subjects. Recent trials using CARs against CD19 have shown significant clinical efficacy and also observed persistence of the engineered T cells, although with a shorter follow-up time (3–6). The inclusion of costimulatory domains derived from CD28 or 4-1BB may lead to low level tonic signaling that promotes T cell survival *in vivo*; alternatively, chronic exposure to the CD19 antigen as new B-cell precursors are produced in the marrow may induce survival of the CAR-modified cells (13).

Aside from the reduced persistence and activity, another theoretical concern for the use of mature T cells as targets for anti-cancer TCRs is the potential for mispairing: that is, the pairing of endogenous cellular TCR chains with introduced transgenic chains. In this situation, T cells can be redirected, miss the intended cancer-antigen target, and instead react against unpredictable moieties expressed on normal tissue. This has been demonstrated in a C57/Bl6 murine based study of immunotherapy using TCRa- and b-chains to engineer T cells via adoptive transfer (14). This system mimicked the clinical setting by including total body irradiation (TBI) and IL-2, and examined a variety of commonly studied TCR including OT-I, gp100, MART-1, and pmel-1. It was clearly demonstrated that mice developed symptoms of graft versus host disease (GvHD), and that the introduced T cell chains, as a pair or individually, were necessary and sufficient to cause this outcome independent of IL-2 administration. It is important to note that the development of GvHD as a result of mis-pairing of an exogenous TCR introduced into mature T cells with an endogenous TCR chain has not been an observed problem in any clinical trial to date (15), though it remains a theoretical possibility.

The real or artifactual potential for induction of frank autoimmunity aside, the reduction of desired antigen-specific TCR surface expression due to mis-pairing poses another hurdle. A fraction of the transgenic TCR chains may be tied-up in pairs with endogenous TCR chains that do not possess the desired specificity, blunting their effectiveness for anti-tumor-specific activity by reducing the surface density of engineered TCR expression. As the surface density of TCR is correlated with activation (16, 17), the reduction of cancer antigen-specific TCR may render engineered cells incapable of mounting an effective immune response, nullifying therapeutic benefit. A number of approaches to reduce or eliminate the possibility of TCR mis-pairing have been described (18).

Hematopoietic stem cells for TCR- and CAR-mediated immunotherapy

Approaching T-cell immunotherapy by engineering of autologous hematopoietic stem cells (HSCs) with a prearranged anti-cancer-antigen TCR or CAR may offer a one-two punch, solving both the problem of effector cell persistence and TCR mis-pairing at the same time. HSCs are the most primitive of blood lineage cells. Residing predominantly in the bone marrow, HSCs produce all mature blood cells by proliferation and differentiation through a series of increasingly lineage restricted progenitors. HSC transplantation has been a clinical practice for more than four decades to promote life-long hematological recovery after high doses of chemotherapy or radiation to treat malignancies, for marrow reconstitution in marrow failure states such as aplastic anemia, and to treat genetic diseases of blood cells, by providing a source of normal stem cells that can produce the needed normal blood cell type. HSCs can be obtained for transplant from the bone marrow of a suitably matched donor, by leukopheresis of peripheral blood after mobilization by administration of pharmacological doses of cytokines such as G-CSF [peripheral blood stem cells (PBSCs)], or from umbilical cord blood (UCB) collected from the placenta after delivery. The marrow, PBSCs, or UCB may be transplanted without processing, or the HSCs may be enriched by immune-selection with a monoclonal antibody to the CD34 surface antigen, especially if the HSC are to be modified by *ex vivo* gene transfer. Further enrichment of HSCs is an active area of research (19, 20) that will no doubt improve subsequent gene therapeutic approaches to immunotherapy.

Gene transfer and expression in HSCs has been under study for more than three decades (21). Vectors derived from viruses of the Retroviridae family, either γ-retroviral, such as murine leukemia viruses, lentiviral, such as the human immunodeficiency virus (HIV), or spumaviral, such as the human foamy virus, have been most effective for permanent gene insertion into the

chromosomes of HSCs. This results in stable transmission to all progeny progenitors and mature blood cells. Typical clinical approaches to gene transduction of HSCs involves enrichment for the CD34+ T-cell fraction, culture in medium containing a cocktail of recombinant human cytokines including c-kit ligand, flt-3 ligand, and thrombopoietin to activate or prestimulate the HSC from quiescence for 1–2 days, and then exposure to the gene delivery vector for 1–2 days by its addition to the culture. At the completion of transduction, the cells are washed and formulated for either direct intravenous infusion or cryopreservation for transplantation at a later date. Using these current optimized methods, insertion of 1–3 copies of the delivered transgene may be achieved into the majority of the HSCs, with preservation of their engraftment and multipotent blood cell production capacity.

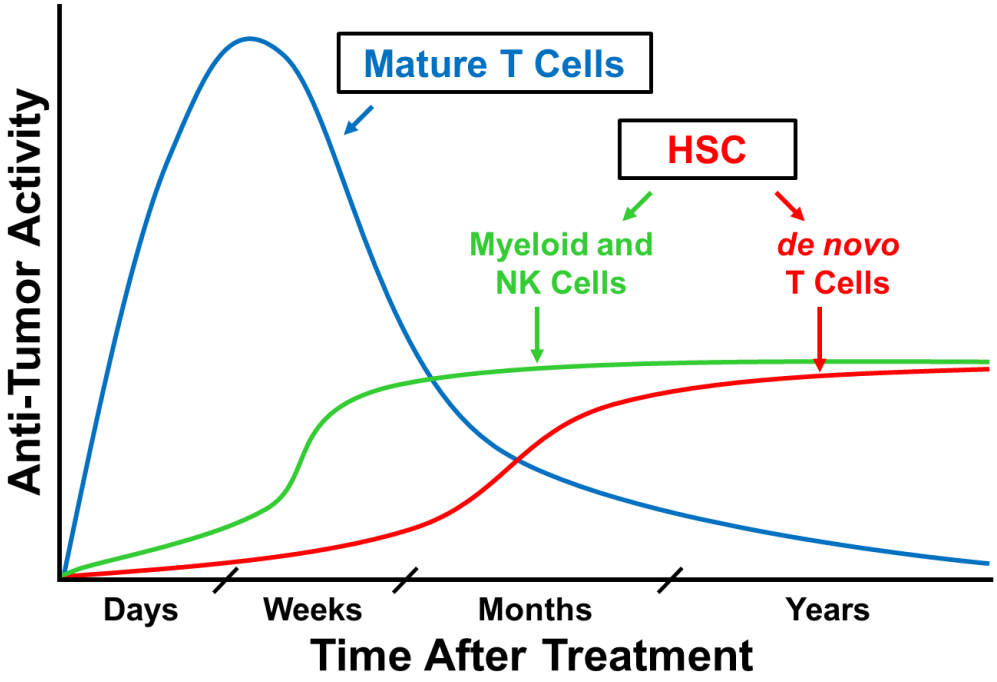
Expression of the delivered transgene is typically achieved by the use of a strong constitutive transcriptional control element such as a viral or cellular enhancer/promoter fragment. The transgene product may be transcribed in blood cells of all lineages produced from the HSCs, although other factors may modulate their expression. For example, the requirement for co-expression of the CD3 complex of proteins limits surface display of a transgenic TCR to T lymphocytes, even though the vector may be present and transcribed in all blood cells derived from a transduced HSC (notably, CARs are not limited by CD3 expression). Alternatively, some vectors are designed to use lineage-specific transcriptional control elements to restrict expression to specific cell types, such as erythroid, myeloid, etc.

The approach of using HSCs as the targets for TCRs or CARs is attractive for at least three reasons. First, as with approaches that use Tcm or Tscm as targets for gene modification, the regenerative nature of HSCs may provide a long lasting (potentially life-long) supply of effector T cells engineered against the antigen of interest. TCR- or CAR modified HSCs will continuously

produce T-lymphocyte progenitors that will undergo normal thymopoiesis and development, increasing the potential for development of immunological memory (Fig. 1). This is in contrast with mature T cells massively expanded *ex vivo* before infusion, with most having a finite lifespan. Second, the off-target cytotoxicity or blunted surface expression of the engineered TCRs due to mis-pairing described above as a potential complication from transducing mature T cells may be eliminated by tackling the approach from HSCs that have not yet rearranged their germline TCR loci (22) if the transgenic TCR suppresses rearrangement of the endogenous TCR loci. This allelic exclusion would ensure the robust surface expression of an exclusive, antigen-targeted TCR. Third, it has been demonstrated that increasing the intensity of the preconditioning regimens used when administering tumor-infiltrating lymphocytes correlates with increasing clinical efficacy (23). Very high doses of conditioning (essentially cytoablative) mandate HSCs rescue of the patient, which provides an ideal setting for engineering a portion of the HSC graft for immunotherapy.

In the following text, we discuss the known benefits and potential pitfalls associated with using HSCs for engineered immunotherapy and recent work focused on moving this technology to a clinical setting.

Figure 1. Theoretical time course of anti-tumor activity.



The theoretical anti-tumor activity produced in patients is depicted following a single infusion of T-cell receptor (TCR)- or chimeric antigen receptor (CAR)-transduced mature T cells (blue), and following stable engraftment of TCR- or CAR-transduced hematopoietic stem cells, yielding myeloid and natural killer cells expressing CARs (green) within weeks of transplant and de novo T cells expressing TCRs or CARs (red) within months.

TCRs into HSCs: Imperium sine fine dedi

Stable retroviral or lentiviral introduction of a prearranged cancer antigen-specific TCR or CAR to HSCs can provide an ongoing source of targeted T cells and, perhaps, other effectors. While infusions of TCR- or CAR-modified mature T cells have an anti-neoplastic impact within days to weeks, production of anti-tumor effector cells from engineered, transplanted HSCs may take longer, with myeloid and NK cells emerging within 1–3 weeks after transplantation and *de novo* T lymphopoiesis occurring only after several months. Although delayed, it is hypothesized that the stable engraftment of TCR or CAR-modified HSCs will lead to sustained production of targeted effector cells, potentially providing continuous anti-tumor activity.

It is well established that the transduction and transplant of HSC with a prearranged TCR can give rise to antigen-specific T cells in murine models. Early reports (24, 25) used retroviral vectors to deliver TCRα and TCRβ chains to mouse bone marrow followed by transplantation into lethally irradiated recipients. Long term development of T cells expressing the introduced TCR was demonstrated, including in secondary recipients, providing evidence of gene transfer to long-term HSCs. Later work showed that engineered T cells produced from TCR gene-transduced murine HSCs that were transplanted into lethally irradiated hosts were responsive to antigen stimulation by dendritic cells, developed a memory cell phenotype, and provided protection against in vivo tumor challenge (26, 27). Alajez et al. (28) used a novel fusion protein consisting of a single chain TCR that recognized a segment of the MUC1 tumor-associated protein in a non-MHC-restricted manner with the CD3ζ chain intracellular signaling domain (in effect a chimeric antigen receptor) to transduce murine bone marrow which was transplanted into SCID mice. The recipients of the fusion protein-transduced marrow rejected MUC1 positive tumors more effectively than did controls.

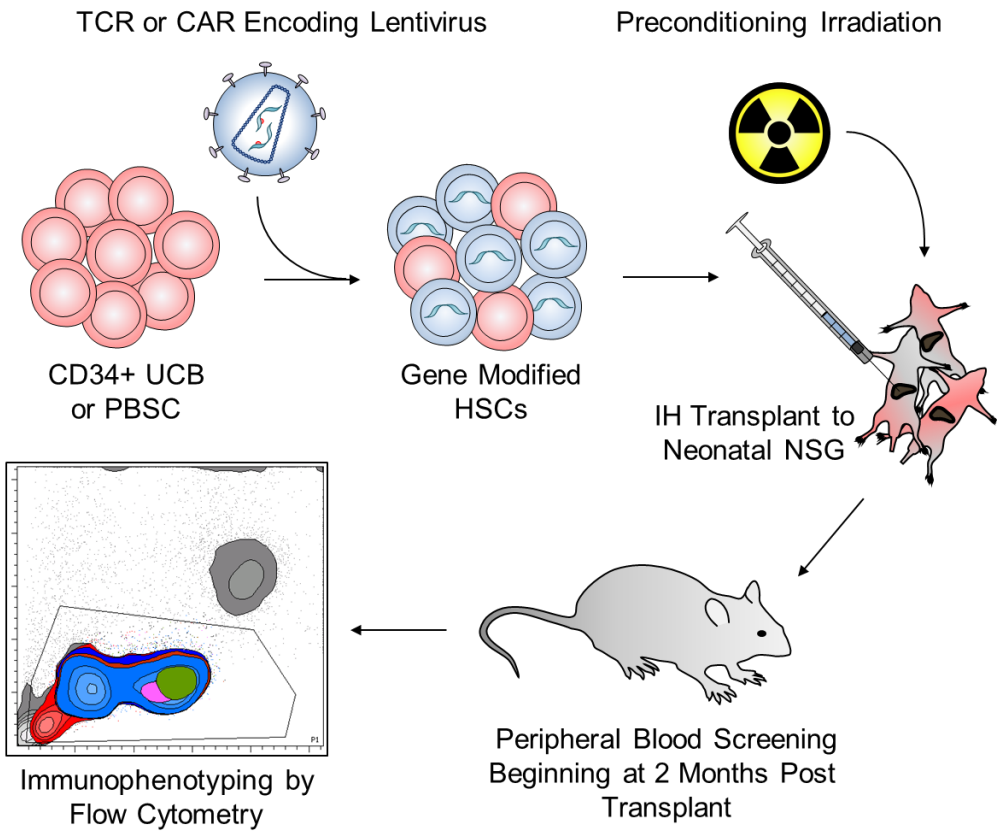
Two studies examined the potential to produce T cells from transduced human HSCs using in vitro differentiation on the murine OP9-DL1 stromal cell line. Zhao et al. (29) transduced human UCB with retroviral vectors encoding a TCR against relevant human tumor associated antigen peptides (NY-ESO-1 and p53) and directed their differentiation to T lymphocytes by coculture on OP9-DL1 stromal cells. The produced T cells displayed antigen-specific, HLA-restricted cytolytic activity. Van Lent et al. (30) used retroviral vectors to introduce the cDNA for human TCRa and TCRb chains recognizing peptides from either HA-2, CMV, or MART-1 into either human thymic T-lymphoid progenitor cells (CD34+CD1a-) or human UCB (CD34+CD38-) progenitor/stem cells. Mature T cells were produced in vitro on OP9-DL1 stroma that possessed cytolytic activity with the antigen specificity of the introduced TCR. Interestingly, the introduction of only a TCRb chain greatly accelerated T lymphopoiesis, postulated to result from prolonged signaling from a pre-TCR complex formed with a pTa chain, whereas introduction of both the TCRβ and a chains only moderately increased T-cell production, compared with cultures receiving only the TCRα chain or control non-transduced cultures.

TCR engineering of human HSCs to produce modified T cells *in vivo* was first shown using a model in which human thymic tissue is implanted into NOD/SCID/IL2Rg$^{-/-}$ (NSG) immune-deficient mice, followed by injection of genemodified human CD34+ cells (the BLT model). This study demonstrated the development of anti-HIV T cells after transfer of the genes encoding a TCR against an HIV-1 gag peptide. Functional, antigen-specific T effector cells were produced that were capable of killing HIV-infected cells (31). This same model was then used to generate MART-1 antigen-reactive human T-cell effectors capable of killing melanoma tumors in an antigen- and HLA-specific context after transplantation of human fetal liver-derived CD34+ HSCs transduced with the genes encoding a TCR to the melanocyte antigen MART-1 (32). One caveat

to the BLT model is that the education of T cells in a human thymic organoid does not induce tolerance to the mouse host, generating self-reactive T cells that ultimately kill the humanized mouse via GvHD, limiting the duration of studies (33). Although efficacy can be demonstrated in this model in an antigen- and HLA-specific context, the examination of longterm provision of cells is hampered. To address this issue, we used a transplant model termed Hu-SRC-SCID the clinically relevant human HSC sources of CD34-enriched UCB or PBSC are transplanted into sub-lethally irradiated NSG immune-deficient neonatal mice (Fig. 2). In a direct human HSC transplant to NSG neonates, also known as the SCID repopulating cell or src-SCID model, developing human thymocytes are educated on the murine thymus. While this likely results in a reduced TCR repertoire compared with the BLT model, the mature T cells are host-tolerant, allowing long-term studies of these chimeric animals. Furthermore, using NSG hosts engineered to express human MHC molecules can allow the development of an HLA restricted T-cell repertoire (34).

In NSG and NSG-HLA*A0201 mouse strains, we studied the development of human HSCs transduced with a TCR recognizing the $_{27\text{-}35}$MART-1 nonamer. We demonstrated that both CD8+ and CD4+ T cells were produced that expressed the introduced TCR (22). The presence of the prearranged TCR in the developing thymocytes appeared to accelerate and boost CD8+ T-cell production, as had been observed *in vitro* by van Lent et al. (30). The T cells produced showed antigen-specific release of γ-interferon when tested *ex vivo* (22). Although the body of literature describing engineered immunity via HSCs is relatively young, proof of principle has been demonstrated showing the promise of this approach. Further work to classify the T-cell phenotypes developing in this model, and exploration of mouse models that mimic the human

Figure 2. *In vivo* xenograft model to study the use of hematopoietic stem cells for cancer immunotherapy.



Umbilical cord blood (UCB) or peripheral blood stem cells (PBSCs) are processed to purify the CD34+ population enriched for hematopoietic stem cells (HSCs). Cells are prestimulated and transduced with a concentrated lentiviral vector encoding a TCR or chimeric antigen receptor. Gene-modified cells are transplanted via intra-hepatic (IH) injection to preconditioned (100 cGy) NSG neonatal mice. Beginning at 2 months post-transplantation, immunophenotypic analysis of peripheral blood is performed by polychromatic flow cytometry.

cytokine milieu and how they affect T cell development and homeostasis in the context of engineered immunity via HSCs remain interesting arenas for exploration.

In addition to the potential life-long provision of effector cells, the engineering of HSCs with exogenous TCRs is an attractive solution to the issue of TCR mis-pairing. While inclusion of a number of clever designs to the TCR construct can minimize this undesired consequence, the HSC approach is capable of sidestepping this pitfall entirely due to the mechanism of allelic exclusion. Early in T-cell development, the endogenous TCRb and TCRa genes undergo V(D)J rearrangement via the VDJ recombination complex of DNA modifying enzymes. Upon successful rearrangement of one allele, the recombination of the remaining germ-line allele is inhibited (at least for TCRb). This ensures that only a single a and single b chain will be expressed in a given T cell, conferring a single MHC-specific antigenic reactivity (the same allelic exclusion mechanism is present in B cells, where it was discovered) (35).

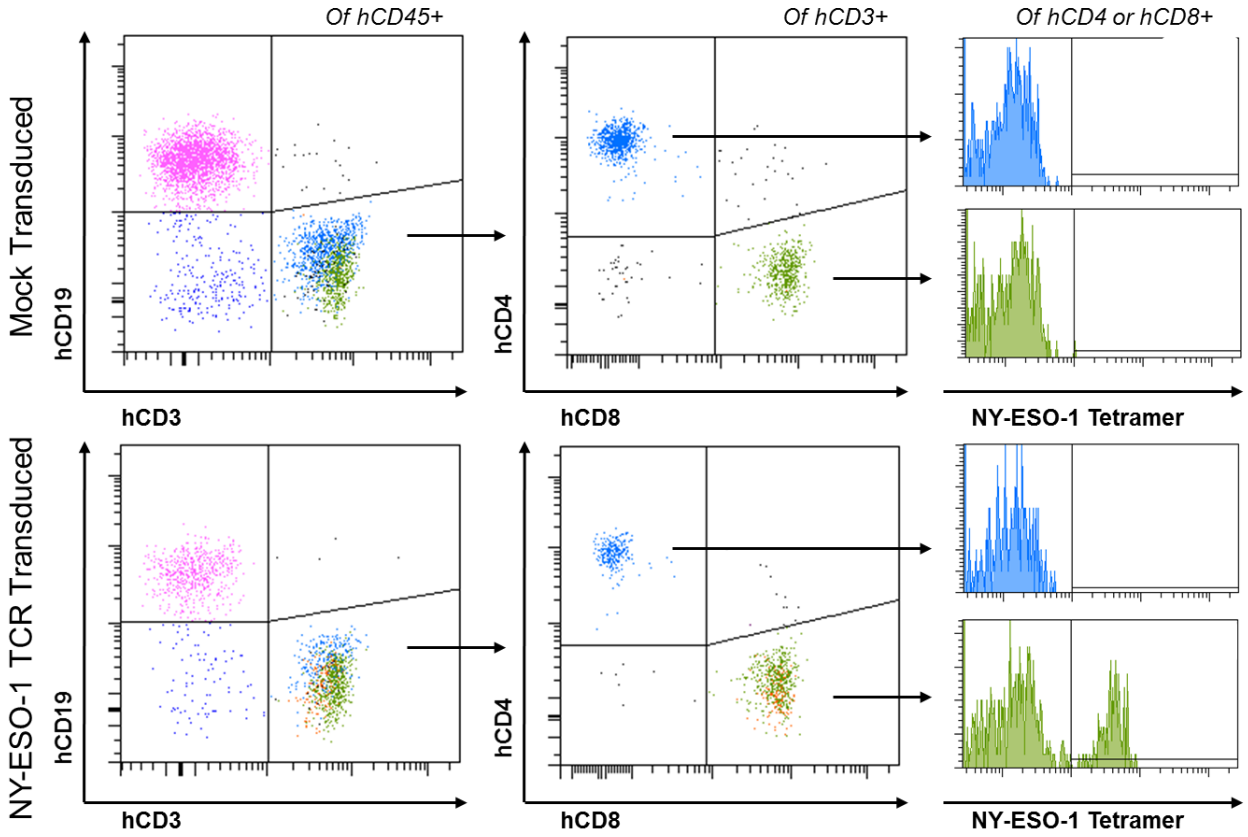
Work by our group at UCLA has demonstrated genetically (22) and molecularly (36) that the expression of a prearranged TCR upstream of T-cell development by the engineering of human HSCs does indeed induce allelic exclusion as in normal development. Deep sequencing of the TCRVb CDR3 region in T cells derived from HSCs engineered to express a transgenic TCR specific for the $_{27\text{-}35}$MART-1 peptide and differentiated into T cells in the NSG mouse showed only the introduced T-cell rearrangement, whereas T cells lacking expression of the introduced TCR contained a broad array of TCRVb gene rearrangements (22). In line with this finding, quantitative analysis of TREC, RNA spectratyping, and TCRVb chain expression by flow cytometry demonstrated allelic exclusion in MART-1+ T cells when compared with MART-1 T cells in a BLT model (36). In the latter study, while Vb family member surface staining clearly demonstrated exclusion, spectratyping of RNA showed enrichment, but not exclusive expression,

of the transgenic TCR chain. This result may suggest leakiness of allelic exclusion in this model or perhaps post-transcriptional silencing of only one rearranged TCR loci resulting in surface expression an exclusive Vb chain. These somewhat contradictory findings may highlight differences in HSC progenitors from fetal liver versus UCB, or of the thymopoietic processes in BLT versus the NSG models; a comprehensive study examining engineered immunity induced allelic exclusion at the DNA, RNA, and protein levels would be informative.

More recently, we have performed studies using a lentiviral vector carrying the cDNA for a human TCR recognizing the 157–165 peptide from the NY-ESO-1 tumor-associated antigen (37). This work has focused on the use of human PBSC as a clinically relevant HSC source compared with fetal liver or UCB, as PBSCs will be the cell source used for an upcoming trial of HSC based immunotherapy at UCLA. While higher doses of CD34+ PBSCs are needed to effectively engraft the NSG mice compared with what was used with UCB (1.0×10^6 versus 1–3 $\times 10^5$, respectively), we have nonetheless observed the production of T cells expressing the anti-NY-ESO-1 TCR in mice transplanted with transduced PBSCs. Extensive immunophenotypic characterization of T cells produced *in vivo* and expressing the TCR transgene is in progress. In contrast with the TCR against MART-1 that appeared on both CD4+ and CD8+ T cells, expression of the NY-ESO-1 TCR has been restricted to CD8+ T cells, indicating that the specific TCR plays an instructive role in T cell development (Fig. 3).

The NY-ESO-1 TCR lentiviral vector also carries the modified herpes simplex thymidine kinase gene (HSV-sr39TK) that functions as both a PET reporter as well as a potential suicide gene (see below) (38). The transferred TCR proteins will only be displayed on the surface of T cells produced from the transduced HSCs due to the requirement for co-expression of CD3,

Figure 3. Immunophenotypic analysis of humanized mice.



NSG mice transplanted with either mock-transduced and NY-ESO-1-TCR vector transduced PBSCs reconstitute a human immune system in vivo, as evidenced by human CD19+ B cells and both CD4+ and CD8+ human CD3+ T cells. CD8+, NY-ESO-1 tetramer-binding effector T cells that developed from gene-modified HSCs are detectable (bottom right).

limiting detection of total gene modified cells using a TCR assay based approach, such as binding to MHC-tetramer. However, the HSV-sr39TK reporter gene will be expressed at high levels from the vector's MSCV LTR promoter in cells of all hematopoietic lineages. This reporter allows in vivo imaging to be performed to examine sites of hematopoiesis, visualizing the gene-modified progeny of the transduced CD34+ cells *in vivo* in the NSG recipients. Following administration of an [^{18}F]-FHBG PET probe (250μCi) to the mice, we have observed hematopoietic niches including the long bones, sternum, spleen, vertebrae, ribs, as well as the thymus as hot spots of reporter signal, indicative of engrafted, transduced human HSCs and their cell progeny in NSG hosts (see Chapter 4).

As central tolerance limits the development of self-reactive T cells, and cancer antigens targeted in T-cell therapy are frequently self-expressed antigens (39). As such, the isolation of self-reactive TCRs is quite rare and, when present, the TCR affinity is accordingly low (40). This principle potentially poses a challenge for the TCR-modified HSC approach. Mature T cells have already passed the stage of development where auto-reactive cells have been deleted, and the introduction of a self-reactive TCR into these cells is possible. However, in a prethymic stem/progenitor cell destined for thymic education and surveillance, auto-reactivity may result in deletion of thymocytes expressing self-reactive TCR.

An elegant approach to circumvent this limitation has been demonstrated in the mouse (41). In this study, the investigators determined that microRNA miR-181 family members were expressed transiently at high levels during thymopoiesis, with peak expression occurring at the double positive thymocyte stage, and falling upon maturation and migration of the T cells to the periphery. They designed a vector carrying a conalbumin-specific TCR in which the vector transcript was made sensitive to suppression by endogenous miR-181a by inclusion of repetitive

miR-181a target sequences following the transgene. In a murine model of HSC transplant to B10.D2 mice, known to negatively select this specific TCR, only transplants containing the TCR vector with the miR-181a target sequences resulted in the development of engineered mature TCR-expressing T cells that survived through thymic selection. Importantly, peripheral T cells expressing the conalbumin-specific TCR expanded when the animals were vaccinated with antigen-pulsed dendritic cells, and *ex vivo* functional assays showed responsiveness in an antigen dose-dependent fashion. This latter point demonstrates the feasibility for use of this approach for other potentially self-reactive TCRs (or CARs) in the clinical setting, as modulation of the TCR through development allowed self-reactive T-cell development yet did not ablate mature cell function. It would be of interest to know if the downregulation of TCR expression coincident with high levels of miR-181a during development will abrogate allelic exclusion and allow endogenous TCR rearrangement to occur, thereby permitting potential TCR mis-pairing in T cells expressing endogenous and exogenous TCR genes.

TCRs have the advantage over CARs of being able to recognize a wider variety of tumor-associated antigens, not limited to those that are expressed on the cell surface. Thus, the use of HSCs as a vehicle to continuously produce fresh TCR-modified T cells may provide an additional means to extend the activity of this approach.

Driving CARs into HSCs

As with gene modification of HSCs with TCRs, modification of HSCs with genes encoding CARs brings the prospect of long-term production of immune effector cells targeted to tumor-associated antigens. One major potential advantage with CARs is that their expression will not be limited to T cells, as is the case with TCR genes introduced into HSCs, since the surface display of CARs does not require CD3 co-expression. Thus, CARs may be expressed on multiple

hematopoietic lineages (T, B, NK, myeloid), amplifying the potential graft-versus-cancer activity (21, 42–45). While *de novo* production of T cells from transplanted HSCs may be slow and limited in adult recipients, myeloid and NK cells should be produced rapidly after transplant, yielding an early source of anti-tumor immunity (42–44). It is expected that CAR-bearing T cells would be the most efficient cytotoxic effectors cells against tumor cells produced by CAR-modified HSCs due to their longer lifespan, greater activation and proliferation properties, and potential for generation of immunological memory. Results from studies assessing CAR-modified NK and myeloid cells indicate that NK cells have similar antigen-specific cytotoxicity to T cells (42, 43, 46). However, the continued generation of large numbers of CAR-bearing neutrophils, monocytes and NK cells may constitute a powerful anti-cancer barrier, thanks to their tissue homing, large circulating pool in peripheral blood, and vascular diapedesis. Myeloid and NK cells do not depend on thymopoiesis to become active cytotoxic effectors and could exponentially amplify the anti-tumor immune response directed by CAR. The multi-lineage expression of CARs, associated with potent engineered antigen-specific cytotoxicity, makes the CAR modification of HSC a very promising cancer immunotherapy approach to be explored.

CAR-modified HSCs can be infused in the context of the standard medical procedure of HSC transplantation, when the chemotherapy conditioning regimen would create optimal conditions for the efficacy of this approach, favoring engraftment of gene modified cells, as well as decreasing tumor burden and decreasing the risks of immunogenicity of the CAR molecules. Recently, a clinical trial has been opened infusing CD19-specific CAR-modified T cells in patients with high-risk B-lineage malignancies undergoing autologous HSC transplant using UCB, to add potential graft-versus-leukemia effects to the relatively naive T cells contained within the graft

(47). In this setting, CAR modification of a portion of the donor HSC may add additional anti-leukemic effects.

Studies performed by the research group at Cell Genesys, Inc. in the mid-1990s were the first efforts to explore the potential for using HSCs as the cell platform for expressing a CAR-like molecule (42, 43). They produced a CAR-like fusion protein between the extracellular domain of human CD4 and the intracellular CD3ζ chain, termed a universal receptor (UR), to direct cytolytic effector cells to kill cells infected with HIV-1 through binding of the CD4 domain with HIV-1 gp120 displayed on the surface of the HIV infected cell. This UR had been shown to arm T cells to kill HIV-infected cells and was advanced to several clinical trials into peripheral blood T cells, where it showed some evidence of *in vivo* anti-HIV effects (42, 48, 49).

Moving toward a stem cell approach, this same investigative group studied the activity of the UR introduced into murine bone marrow HSCs, which was then transplanted into recipient mice. They demonstrated that it led to production of effective cytolytic cells, using a leukemia cell line transduced to express HIV-1 gp120 as a surrogate target for the CD4/CD3ζ fusion construct. Expression of the UR was observed on leukocytes of multiple lineages derived from the transduced HSCs, including granulocytes, monocytes, and NK cells. A direct demonstration that non-T-cell effectors (myeloid and NK cells) were responsible for the cytolytic activity was obtained by transducing the UR gene into bone marrow from SCID mice (that would be genetically incapable of producing T lymphocytes), which still led to elimination of the gp120-expressing target cells *in vivo* after transplant (42). They also reported introduction of the UR into human blood NK cells and into human CD34+ cells that produced CAR-targeted myeloid cells upon *in vitro* differentiation and again demonstrated specific cytolytic activity (43).

In a later article, these authors showed the insertion of the same CD4/CD3ζ fusion gene into marrow HSCs from normal mice led to production of UR-expressing myeloid, NK, and B cells, but a complete absence of transgenic T cells, with a blockade to production of UR-expressing T cells occurring during the T1-T2 transition of thymopoiesis. This observation implies that intrathymic negative selection of cells expressing a self-reactive CAR or TCR may prevent their maturation to mature T cells. However, the finding may be specific to this UR with a CD4 extracellular domain, because the UR-expressing T cells were not eliminated when the transduced HSC were transplanted into MHCII gene knockout mice, suggesting that it was a specific interaction between murine MHCII and human CD4 that triggered their elimination (50).

In exploring the role of the intracellular signaling domain, they also compared the CD3ζ domain, used for TCR signaling in T cells, to the analogous domain from the Fc receptor-c (FccR1), which would normally be used in myeloid and NK cells (43). CD3ζ and FcRγ chains share the immunoreceptor tyrosine-based activation motif (ITAM), and both trigger activation pathways leading to target cytolysis, phagocytosis, and effector cell degranulation. In both murine and human T, myeloid, and NK cells, the UR with the CD3ζ domain consistently displayed greater activity than one with the FcRγ domain, which may be attributed to the presence of three ITAMs in the CD3ζ domain but only one in the FcRγ domain (42, 43). Other studies have reported similar findings on the greater activity of the CD3ζ domain over the FcRγ domain in T cells (42, 43, 51–54).

The optimal intracellular signaling domains for CAR to be used via HSCs are not fully known. The development of CAR activating moieties have mostly focused on the activation of T cells, with CD3ζ being the most commonly used moiety (51, 55), as the terminal portion of the CAR construct, by itself (first generation CAR) or following the inclusion of different co-

stimulatory domains (second and third generation CARs) (56, 57). Some knowledge has been obtained on the molecular events of the T cell activation triggered by CARs, generally observing that the same activation pathways activated by TCR stimulation are involved. It has been demonstrated that CARs in T cells leads to immunological synapse formation (58, 59), calcium influx, and activation of the phosphatidylinositol and tyrosine kinase pathways (60, 61).

Although it is generally agreed that costimulation is required for efficacy in cancer immunotherapy approaches (4, 47, 56, 62, 63), at this moment it is still not fully resolved which costimulatory molecule produces the best results in CAR-mediated T-cell activation (nor for myeloid and NK cells). It has been shown that CAR signaling mediates cell activation in neutrophils, monocytes, and NK cells, besides T cells, with either the FcRγ or CD3ζ activation moieties (42, 43, 46, 64). Activation of NK cells has been shown to require costimulation, with successful antigen specific activation only achieved with second-generation CAR (46, 64). It was found that the 4-1BB costimulatory domain was most effective when CARs were introduced into mature human NK cells (64–66). Our group and others have shown that first generation CARs successfully activated neutrophils and monocytes (42, 43), and their antigen-specific cytotoxicity did not differ from that triggered by second generation CARs (67).

Gene modification of HSCs requires stable transgene integration, ideally delivering one transgene copy per HSC (21). We have consistently achieved 40–50% of transduction efficiency of human HSCs with CARs at an average of 1–3 copies/cell using lentiviral vectors with internal enhancer/ promoters from the MND LTR. Higher transduction rates may not be warranted, as it would not be desired to completely devote the immune system to mono-specific antitumor effector cells; fortunately, for the immunotherapeutic applications, such high level engraftment of gene-

modified human HSCs has not been achieved in clinical trials for genetic diseases where complete gene correction of all engrafted HSCs may be clinically useful.

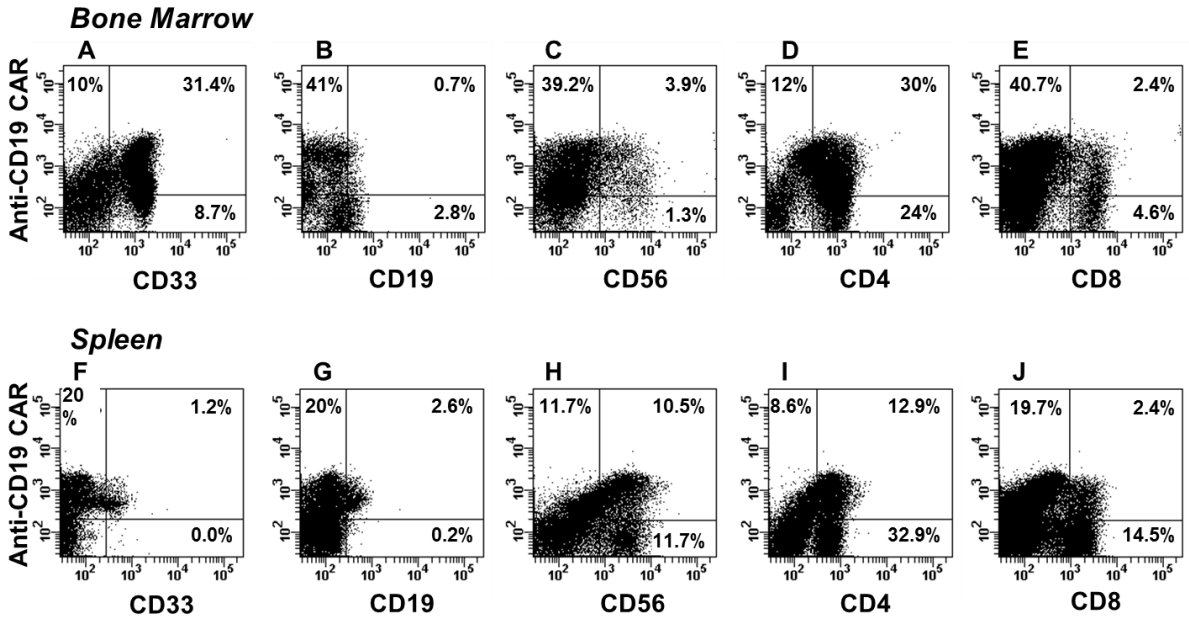
CAR-modified HSCs undergo normal differentiation and proliferation, and their cell progeny are morphologically and functionally normal (67). We have successfully engrafted anti-CD19 CAR-modified human CD34+ HSC in NSG mice, with CAR-bearing cells detected in all hematopoietic lineages in bone marrow, spleen, and peripheral blood (Fig. 4). Notably, the absence of human CD19+ B cells is indirect evidence of active cytotoxicity by the anti-CD19 CAR expressing cells, as B lymphocytes are normally the most common human cells to develop in this model.

With this initial progress toward modification of HSCs with CARs, several questions remain unresolved. The relative benefits of adding the activity of CAR-armed myeloid and NK cell effectors to the T cells' army remain to be determined. It also remains to be determined whether the presence of the CAR will have adverse effects on *de novo* T-cell production, by causing negative selection, whether the CAR will suppress endogenous TCR gene rearrangement by allelic exclusion, as has been shown with TCRs, and whether the diverse CAR expressing leukocytes will cause unique toxicities. Ongoing studies in the NSG mice may address some of these questions and initial clinical trials may provide more answers.

Suicide genes to eliminate TCR/CAR modified HSC grafts

With the engineering of HSCs comes the risk of insertional oncogenesis, as has occurred in several clinical trials in which γ-retroviral vectors were used to transduce autologous HSCs that were transplanted for patients with primary immune deficiency diseases (68). The introduction of these retroviral vectors, containing long terminal repeats with strong

Figure 4. CD19-specific chimeric antigen receptor (CAR)-bearing cells in humanized NSG mouse.



Representative flow cytometry dot plots from a humanized mouse engrafted with CAR-modified human hematopoietic stem cells are depicted. (A–E) Cells from bone marrow were analyzed after staining with a FITC-labeled anti-IgG Fc to detect CAR (y-axis) versus antibodies against the human surface markers CD33 (myeloid cell marker), CD19 (B-cell marker), CD56 (natural killer cell marker), CD4 and CD8 (T-cell markers), represented on x-axis. (F–J) Cells from spleen of the same mouse stained using same antibody mix. Bold numbers in quadrants represent percentages from total human CD45 population in each sample.

promoter / enhancer elements, into the susceptible genome of HSCs is capable of activating proximal cellular proto-oncogenes, and inducing cell proliferation that can progress to frank transformation. While the potential for this to occur in the setting of adult patients who would likely not have the high level of engraftment and proliferation seen in young immune deficiency patients, it remains a hypothetical risk. The long-term production of targeted effector cells, an attractive benefit of using HSC for immunotherapy, also poses potential problems if there is toxicity, either on-target/off-tissue toxicity, such as sustained suppression of CD19+ B cells, or the previously mentioned off-target reactivity possible by the introduction of a self-reactive TCR.

To alleviate these risks, the inclusion of a suicide gene to ablate gene-modified cells has been undertaken in a number of studies. The most widely studied suicide gene is derived from the herpes simplex virus thymidine kinase gene, which confers sensitivity to the anti-viral nucleoside analogue ganciclovir (69–74). Other suicide genes have been produced using fusion proteins that express FAS signaling moieties or caspase components under the post-transcriptional control of small molecule ligand (75–78). These latter genes are attractive in that they do not carry the immunogenicity demonstrated by the HSV-sr39TK construct, although they do not allow PET functionality to visualize modified cell tracking. The presence of a suicide gene in the TCR or CAR vector would allow cells that are over-proliferating or causing toxicity to be eliminated by administration of the pro-drug or inducing agent.

Our studies using the NY-ESO-1 TCR include HSV-sr39TK in the lentiviral vector payload: we examined the potential of the latter gene to eliminate the cell products of the TCR-transduced human HSCs in the NSG mice. Upon administration of ganciclovir (50 mg/kg IP, 5 days), the transduced cells were completely eliminated from the mice, as indicated by both repeat PET scans, FACS analysis for NY-ESO-1 tetramer staining in CD8+ T cells, and qPCR of blood,

spleen, thymus, and bone marrow for the lentiviral psi element. The ability to eliminate the engineered graft is attractive in case of a serious adverse event, and remains an important safety switch for inclusion in HSC-based approaches to engineered immunity.

Clinical considerations for use of TCR/CAR-modified HSCs

Sustained HSC engraftment requires pretransplant conditioning with agents that have myeloablative or at least marrow cytoreductive activity, such as total body irradiation or busulfan. These may be used at sub-myeloablative doses (e.g. 200 cGy TBI or 4 mg/kg busulfan) for marrow cytoreduction with minimal acute toxicity. In contrast, the lymphodepleting agents typically used prior to T cell immunotherapy (e.g. fludarabine, cyclophosphamide) are not significantly myeloablative and would not be expected to increase HSC engraftment. The need for cytoreductive conditioning as preparation for HSC immunotherapy would make application in disease settings where autologous or allogeneic HSCT is already used (e.g. leukemia and lymphoma) as most logical first choices.

The clinical application of TCR or CAR therapies via HSC transplant holds several challenges. Adult HSCs are generally more myeloid skewed than those from younger people, which may limit the numbers of T cells that can be produced from the transplanted, engineered HSCs (79). In addition, the adult thymus is certainly much less active, undergoing involution with advancing age and the potential for *de novo* thymopoiesis is diminished, although some activity remains (80, 81). Prior treatments, such as chemotherapy or radiation therapy, may also decrease thymic function. These potential limits to new T cell development emphasize the potential benefits derived from non T cell effectors that CAR-modified HSCs may produce.

Summary

The potential advantages and proof-of-principles have been provided for approaches to cancer immunotherapy using TCR- or CAR-modified HSCs. It remains to be proven in clinical trials whether these approaches will lead to additive efficacy to those realized using mature T cells as targets. Certainly the two approaches may be complementary when applied together in some settings. Clinical trials are being developed that will provide some initial assessments.

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CHAPTER 2:

Long-term *in vivo* monitoring of mouse and human hematopoietic stem cell engraftment with a human positron emission tomography reporter gene.

Introduction

Genetically modifying cells can offer novel therapeutic strategies for currently untreatable diseases (1). Standard methods for monitoring the long-term viability of transplanted cells are inadequate. Improved methods to serially detect transplanted cells in several tissues throughout the body simultaneously and noninvasively are critical to measure therapeutic efficacy (2, 3).

Hematopoietic stem cell transplants (HSCT) from both autologous and allogeneic sources have been successfully used in regenerative medicine (4). Genetic engineering through viral vector integration repairs defects in HSCs expanding clinical applications (5). Effective transplantation requires the engraftment of HSCs followed by an expansion into mature hematopoietic lineages repopulating multiple organs and peripheral blood. Measurement of mature hematopoietic cells in the peripheral blood is the primary diagnostic method for evaluating transplant efficacy. The major limitation of this approach is the lack of information about the engraftment within hematopoietic tissues.

Cells engineered to express a positron emission tomography (PET) reporter gene can be serially imaged *in vivo* with a reporter-specific probe (2). Most studies have used variants of the herpes simplex virus type 1 thymidine kinase (HSV1-TK or HSV1-sr39TK) and a radiolabeled penciclovir analog (9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)guanine, [^{18}F]-FHBG) to detect reporter-labeled cells (6, 7). However, HSV1-TK is immunogenic and cells expressing this enzyme are selectively cleared over time potentially causing therapeutic failure (8–10). This immunogenicity has prevented the routine use of PET reporter genes clinically (11, 12).

Alternative potentially nonimmunogenic PET reporter genes have been investigated (3). Human nucleoside kinases deoxycytidine kinase (dCK) and thymidine kinase 2 (TK2) have similar

substrate specificity to HSV1-TK. Several studies demonstrated the specific detection of reporter-labeled cells in mouse models with these human nucleoside kinases as PET reporters. Two studies developed xenografts expressing truncated TK2 or a mutant TK2 demonstrating reporter-specific imaging when probed with [^{18}F]-thymidine analogs (13, 14). Infiltrating tumor-specific human T cells expressing a mutant dCK (dCKDM: R104M, D133A) reporter were detected within lung lesions of mice after transplantation by 2′-[^{18}F]fluoro-5-ethyl-1-beta-D-arabinofuranosyluracil ([^{18}F]-FEAU) PET (15). These reporter-labeled T cells were tested for cytolytic activity *in vitro* against target cells demonstrating that expression of dCKDM did not alter their short-term function (15).

Further investigation of human dCK as a PET reporter was selected based on multiple factors. mRNA encoding DCK is ~800bp, smaller than HSV1-TK, causing a minimal size increase when inserted into therapeutic vectors. The biological function of dCK has been described in genetic knockout mice (16, 17). The enzyme structure and kinetics of dCK are well characterized (18) with known point mutations that shift substrate specificity (18–20). A previous study successfully demonstrated the use of an alternate mutant dCK reporter and probe (15). Endogenous dCK activity can be monitored with PET using an alternate radiolabeled nucleoside analog (21).

How human nucleoside kinase reporters affect long-term cell-based therapies remains uncertain. Specifically it is unknown if constitutive expression in reporter-labeled cells is maintained within the recipient with no perturbation on cell function. Knockout dCK mice have a significant reduction in the total quantity of T and B lymphocytes caused by cellular stress from imbalanced nucleotide pools (22). Ectopic expression of nucleoside kinases could cause similar imbalances. Potential complications may include growth defect, disadvantage, or counterselection resulting in the loss of engrafted cells over time.

Our study demonstrates that a hdCK PET reporter can successfully monitor transplanted cells long term with no toxicity or survival disadvantage in modified cells. Models of mouse and human hematopoietic reconstitution were used to compare our reporter tracking for monitoring engraftment to peripheral blood sampling. Reporter-labeled cells exhibited identical behavior to nonlabeled cells with no differences detected regarding cell cycle, lineage, or tissue location. Our data provide evidence that hdCK3mut is an optimal reporter gene for hematopoietic cell tracking with future applications in a broad range of therapeutic cell transplants.

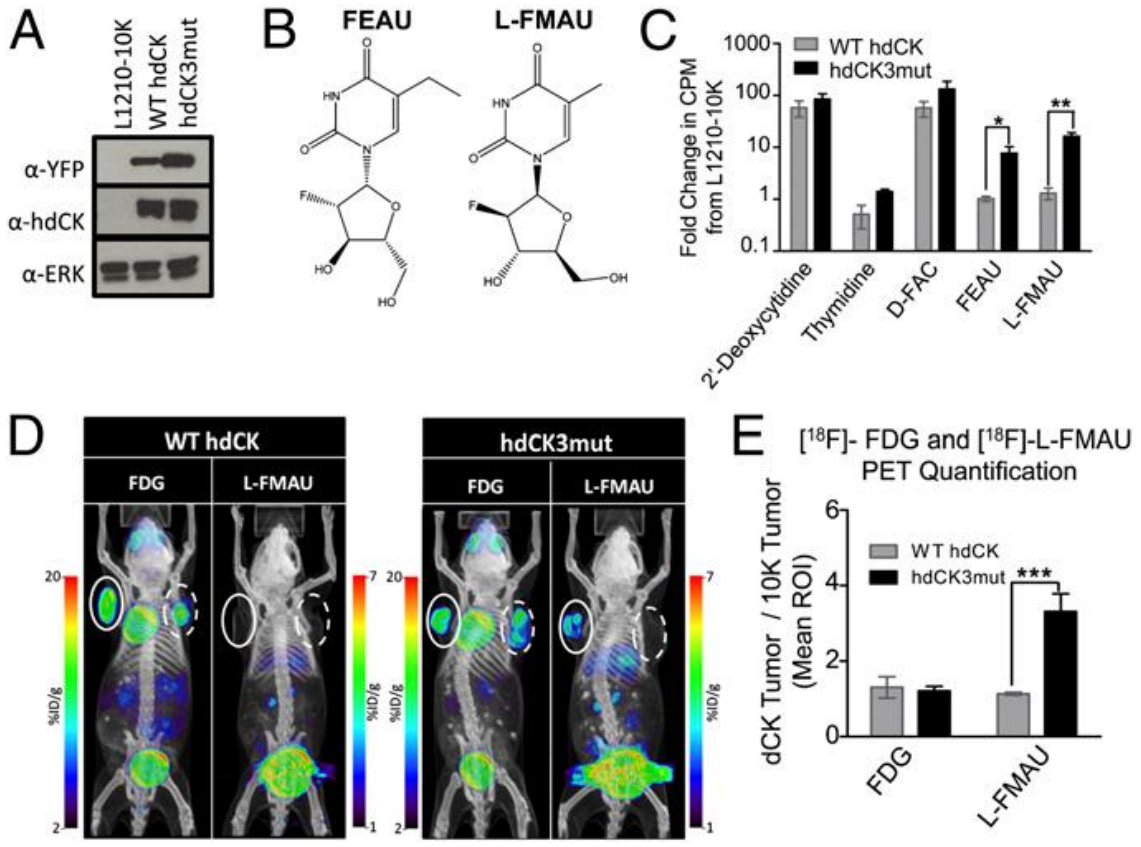
Results

Mutant Human dCK Functions as a PET Reporter When Probed with Thymidine Analogs.

hdCK3mut contained three point mutations (A100V, R104M, and D133A) that were chosen based on a previous study that demonstrated a 1,100-fold increase in thymidine activity compared with wild-type dCK in enzyme kinetic assays (20). L1210-10K, a mouse leukemic cell line with no endogenous dCK was selected as a model cell line for *in vitro* studies (23). Stable expression of wild-type (WT) hdCK or hdCK3mut coexpressed with yellow fluorescent protein (YFP) through an internal ribosome entry site (IRES) in L1210-10K cells were generated to test probes for specific retention in hdCK3mut cells (Fig. 1A and vector maps in Fig. S1). Two thymidine analogs, 2′-fluoro-2′-deoxyarabinofuranosyl-5-ethyluracil (FEAU) and 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU), showed significant accumulation in reporter cell lines compared with wild type. Retention of the probe L-FMAU was 18-fold higher in hdCK3mut cells compared with WT hdCK (Fig. 1B and C).

Enzyme kinetic analysis further demonstrated high substrate affinity of hdCK3mut to L-FMAU with a measured Km of ∼13μM. hdCK3mut had a fourfold lower Km for L-FMAU compared with the previously published dCKDM reporter (13μM versus 56μM). A high affinity PET reporter and probe combination is optimal because probes are administered at high specific activities with low concentrations of substrate. The decreased Km of hdCK3mut for L-FMAU demonstrates that it will achieve a higher velocity at a lower substrate concentration (Table S1). Immune compromised NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were implanted with two s.c. grafts. The right side contained L1210-10K cells and the left side contained L1210-10k cell lines engineered to express WT hdCK or hdCK3mut. To determine tumor viability,

Figure 1. Development of a human thymidine selective PET reporter gene hdCK3mut.



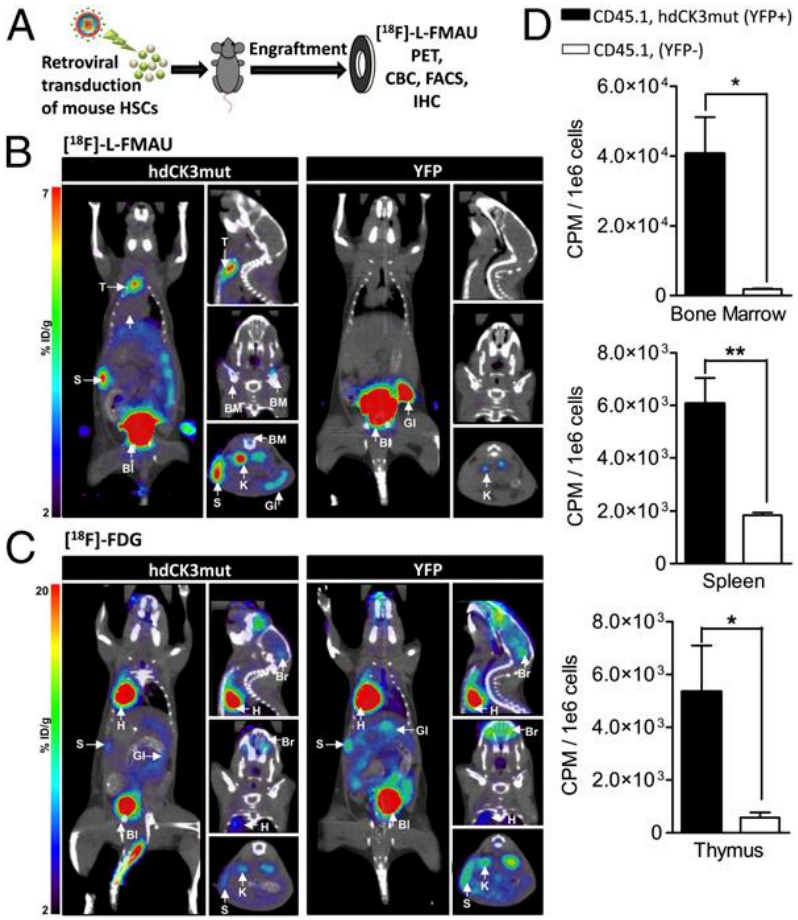
(A) Western blot analysis for equal expression of dCK and the linked fluorescent marker YFP in stable cell lines. (B) Chemical structure of two thymidine analogs FEAU and L-FMAU. (C) *In vitro* [^{3}H]-nucleoside uptake assay. Results are displayed on a log10 scale as a fold change in counts per minute (cpm) from L1210-10K, a dCK-deficient cell line. (FEAU P = 0.027, L-FMAU P = 0.0052) (D) [^{18}F]-FDG and [^{18}F]-L-FMAU MicroPET scans of NSG mice with s.c. grafts. Right side is control L1210-10K (dotted line). Left side is L1210-10K cells with stable expression of WT dCK or hdCK3mut (solid line). (E) Region-of-interest quantification for [^{18}F]-FDG and [^{18}F]-L-FMAU (P = 0.0006).

animals were imaged by PET/CT with 2-deoxy-2-18fluoro-D-glucose ([^{18}F]-FDG), a glucose analog used to measure glycolytic consumption. F-18 has a half-life of ∼110 min with probes decayed to undetectable levels within 24 h allowing for sequential scans with alternate probes. The following day [^{18}F]-L-FMAU PET/CT scans detected hdCK3mut reporter expression with signal observed within hdCK3mut grafts (Fig. 1D). PET images were then quantified for total probe accumulation. Images were dose corrected to total radioactivity at the scan start time. Tumors were then selected in a region of interest (ROI) and the mean percent injected dose per gram (%ID/g) over the entire tumor was calculated. Tumor signal of the dCK transduced graft is compared as the fold change in probe retention to the nontransduced L1210-10K tumors of each animal. Tumors expressing hdCK3mut had a 3.3-fold increase in [^{18}F]-L-FMAU retention ($P = 0.0006$) compared with WT hdCK grafts (Fig. 1E and Fig. S2). These results determined that hdCK3mut and L-FMAU make a suitable PET reporter gene and probe combination for *in vivo* studies.

Expression of hdCK3mut in Mouse HSCs Allow Noninvasive Detection of Reporter Cell Transplantation Before Normalization of Peripheral Blood Counts.

A competitive mouse bone marrow transplantation (BMT) study was chosen to test whether hdCK3mut can detect transplanted cells during early hematopoietic reconstitution (24–28). Donor cells were generated by treating mice with 5-flourouracil 5d pre-harvest for HSC enrichment. Collected bone marrow was retrovirally infected with ∼40–60% transduction efficiency to express hdCK3mut (coexpressed with YFP through an IRES) or the control of IRES-YFP only (Fig. S1). Recipient mice then received a lethal irradiation dose of 900rad to eliminate host bone marrow. Mice were transplanted with the mixed population of reporter / nonreporter HSC-enriched donor bone marrow (Fig. 2A).

Figure 2. hdCK3mut and [^{18}F]-L-FMAU PET can track reporter-labeled mouse hematopoietic cells during early engraftment and expansion in bone marrow chimera mice.



(A) Lethally irradiated C57BL/6 (CD45.2) mice were transplanted with retro-transduced 5-FU–enriched HSCs (CD45.1). MicroPET scans shown from Left,coronal; Right Upper, sagittal; Center, coronal; and Lower, transverse. (B) [^{18}F]-L-FMAU at 4wk post-transplant. Reporter signal observed in hdCK3mut animals in spleen (S), thymus (T), and bone marrow (BM). Probe metabolism in both cohorts seen in gastrointestinal (GI), bladder (Bl), and kidney (K). (C) [^{18}F]-FDG MicroPET scan at 4wk post-transplant. Background signal observed in both cohorts in heart (H), spleen (S), gastrointestinal (GI), brain (Br), with metabolism in kidneys (K) and bladder (Bl). (D) *In vivo* accumulation of [^{18}F]-L-FMAU in sorted hematopoietic cells from hdCK3mut animals. Reporter positive: CD45.1+, YFP+ and reporter negative: CD45.1+, YFP−. ($P < 0.05$).

Under standard conditions mice will display normalized engraftment and complete blood counts (CBC) within ~8wk after BMT (27). We hypothesized that early engraftment and expansion could be monitored by reporter imaging before normalization of peripheral blood measurements. At 4wk post-BMT, animals received PET/CT scans with [^{18}F]-FDG and the following day [^{18}F]-L-FMAU (Fig. 2 B and C).

Whole-body glucose consumption was measured by [^{18}F]-FDG MicroPET and was indistinguishable between the two cohorts of animals. Weak signal was detected within the spleen and bone marrow indicating a similar glycolytic rate across all animals (Fig. 2C). The following day PET/CT with [^{18}F]-L-FMAU detected only hdCK3mut cells engrafted within the spleen, thymus, and focal areas within the bone marrow of reporter-labeled animals (Fig. 2B and Fig. S3). Animals in the control YFP cohort had no hematopoietic signal observed with [^{18}F]-L-FMAU (Fig. 2B). Visualization of [^{18}F]-L-FMAU in hematopoietic tissues of hdCK3mut recipients verified that reporter imaging can monitor early engraftment after BMT.

To confirm that [^{18}F]-L-FMAU accumulation was specific for hdCK3mut cells, *in vivo* reporter and nonreporter accumulation was measured. Donor hematopoietic cells from hdCK3mut recipients were sorted for reporter positive or negative and then were counted for total radioactivity in counts per minute (cpm) normalized to cpm/1 x 10^6 cells. Cells expressing hdCK3mut had a significantly ($P < 0.05$) higher accumulation of [^{18}F]-L-FMAU compared with unlabeled cells in all hematopoietic tissues (Fig. 2D).

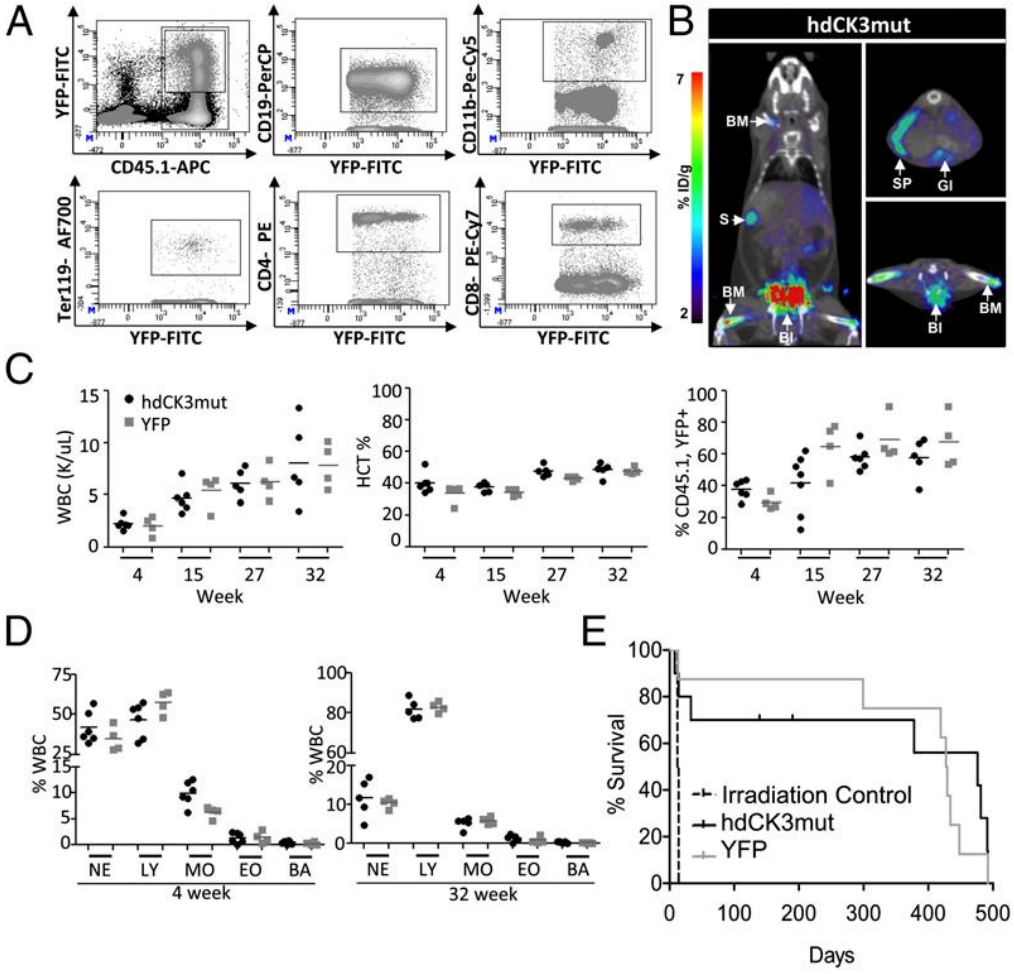
Reporter Labeled Mouse HSCs Retain Expression of hdCK3mut with Equivalent Engraftment and Differentiation Capacity.

Overexpression of enzymes or reporter genes can potentially cause cellular stress, developmental defects during differentiation, growth disadvantage, or transformation (29, 30). The long-term effects from forced expression of hdCK3mut on mouse HSC's engraftment and differentiation capacity was investigated. Reconstituted chimeric mice were evaluated to confirm that hdCK3mut expression was preserved and that mouse HSCs expressing the reporter maintained normal function after transplantation.

Reconstituted chimeric mice 6–8 wk post-BMT were analyzed by flow cytometry and immunohistochemical (IHC) analysis (Fig. 3A and Figs. S4 and S5). Flow cytometry analysis evaluated the spleen, thymus, bone marrow, and peripheral blood for total donor engraftment by lineage, reporter expression (YFP expression), and cell cycle. A representative fluorescent-activated cell sorting (FACS) plot of hdCK3mut engraftment within the spleen is displayed (Fig. 3A). No significant difference in reporter engraftment based on tissue location, lineage distribution, or cell cycle profiles from nonreporter labeled cells or in comparison with the YFP cohort was observed (Fig. S4).

Tissue architecture of the spleen and thymus was examined by hematoxylin and eosin staining (H&E) with normal morphology in both hdCK3mut and YFP mice. hdCK3mut engraftment was then detected although IHC of anti-dCK with no staining seen in YFP recipients. Anti-YFP IHC detected the linked fluorescent marker in both tissues and cohorts of animals confirming the flow cytometry data. Both dCK and YFP IHC identified the same engrafted hematopoietic cells in hdCK3mut animals, demonstrating the specificity of reporter detection using a newly developed monoclonal antibody generated in our laboratory (Fig. S5).

Figure 3. hdCK3mut mouse HSCs persist *in vivo* allowing long-term monitoring of therapeutic cell transplantation.



(A) Representative FACS plot for hdCK3mut engraftment within the spleen. Cells were monitored for CD45.1 (donor) and YFP (reporter) positive. Further gating demonstrates that reporter positive (YFP+) cells can be found in all major lineages. (B) [^{18}F]-L-FMAU MicroPET at 32wk post-BMT. (C) Serial monitoring of peripheral blood. Animals were monitored for total white blood cell (WBC), hematocrit (HCT), and reporter-labeled donor engraftment (CD45.1+, YFP+). (D) Distribution of white blood cells at early and late engraftment are indistinguishable between YFP and hdCK3mut animals. NE, neutrophils; LY, lymphocytes; MO, monocyte; EO, eosinophil; BA, basophil. (E) No survival disadvantage seen in hdCK3mut reporter animals.

Mice received HSC-enriched bone marrow that was retrovirally transduced to express hdCK3mut or YFP in ~50% of cells. This enrichment technique also contains residual committed short-term progenitor cells that can express the reporter. Transplantation of these progenitor cells is necessary for animal survival but these cells confound analysis of HSC differentiation at early time points. Measurements of mature hematopoietic cells from HSCs and progenitor cells are indistinguishable in peripheral blood analysis. A methylcellulose (MC) colony forming assay measured the expansion and differentiation capacity of reporter-labeled bone marrow 6 wk posttransplantation. Recipient animals' bone marrow was harvested, sorted, and placed in MC for 12 d (Fig. S6A) (25). Quantification of the MC assay determined that cells from hdCK3mut recipient mice were equivalent to YFP animals and nonchimeric bone marrow in colony forming capacity (CFC) as well as colony type distribution (Fig. S6 B–F). Sorted hdCK3mut or YFP positive cells were comparable in CFC and colony type, demonstrating that expression of hdCK3mut does not cause a disadvantage during *in vitro* differentiation (Fig. S6E). Sorted cells from both YFP and hdCK3mut recipients retained reporter expression during *in vitro* differentiation detected through flow cytometry, confirming the continued expression of hdCK3mut throughout cell development (Fig. S6F).

Together these experiments demonstrate that expression of the hdCK3mut reporter gene has no observable selective disadvantage on hematopoietic cell engraftment and expansion capacity in a mouse HSC transplantation model.

hdCK3mut Mouse HSCs Persist *in vivo* Allowing Long-Term Monitoring of Therapeutic Cell Transplantation.

Long-term effects from the expression of human nucleoside reporters are poorly defined. Potential concerns include selective vector silencing or counterselection of reporter-labeled cells over time.

We examined whether reporter cells in BMT-recipient mice retained the PET reporter function through serial imaging *in vivo*. Consecutive scans and peripheral blood analysis were obtained at 4, 15, 27, and 32 wk post-BMT allowing for detection of both short- and long-lived reporter HSCs (27, 28).

At 32 wk post-BMT hdCK3mut reporter-specific signal was detected within the spleen and bone marrow, demonstrating long-term engraftment capability of reporter-labeled hematopoietic cells (Fig. 3B). Previous scans of the same animals demonstrated similar signal at 15 and 27 wk (Fig. S7). Serial detection of hdCK3mut through PET/CT reveals that the reporter gene functions through hematopoiesis. It is hypothesized that each sequential scan is detecting different hematopoietic cells that have homed to the spleen.

Peripheral blood was collected at each time point and analyzed for CBC and reporter engraftment by flow cytometry. No significant difference was observed in the retention of circulating reporter positive hematopoietic cells between hdCK3mut and YFP recipients. Comparison of the total white blood cell (WBC) count and hematocrit were normal and equivalent between both hdCK3mut and YFP after BMT (Fig. 3C). Peripheral WBC differential demonstrated that at both early and late engraftment the distribution of WBC subtypes were consistent between both groups (Fig. 3D).

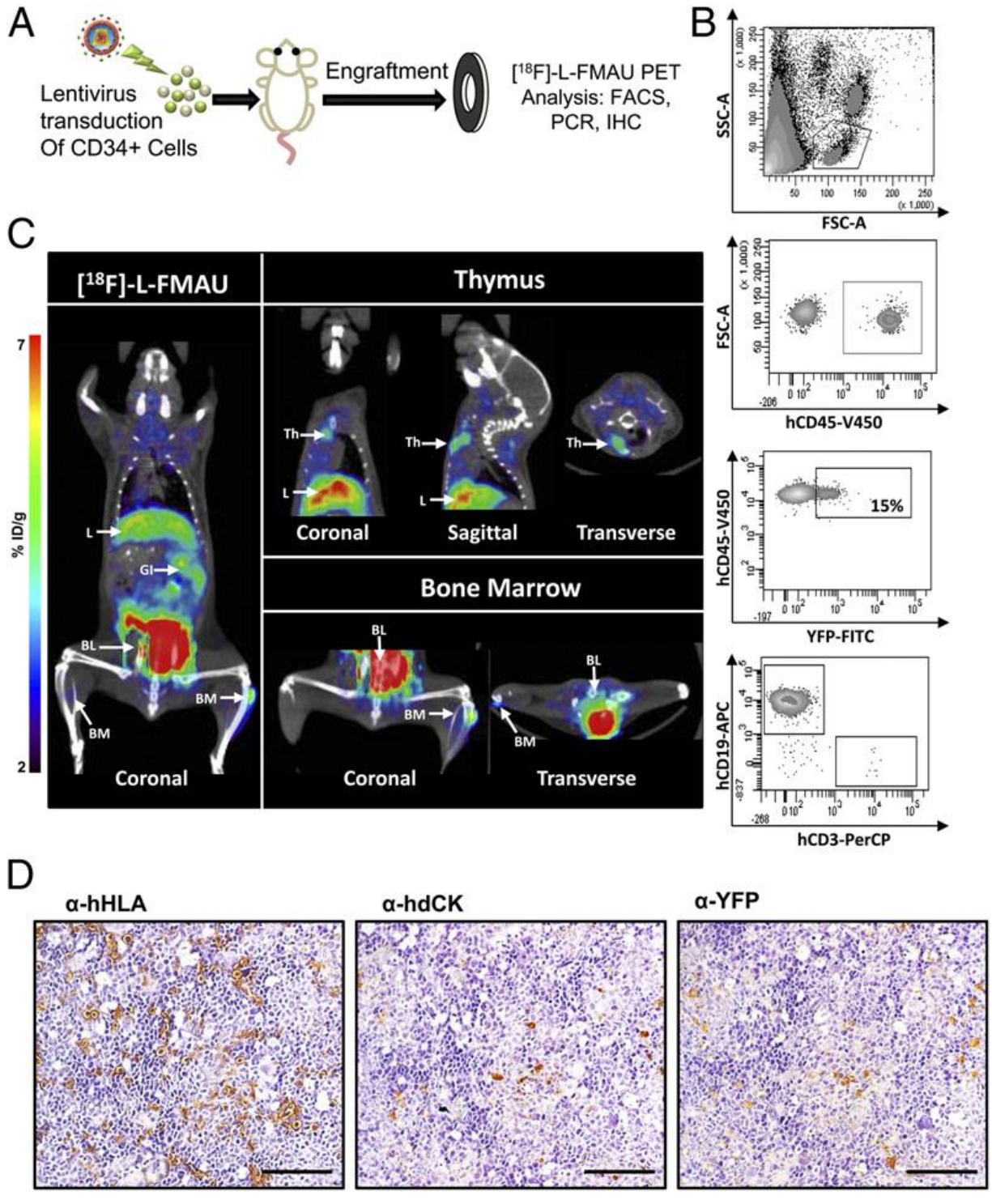
Reporter-labeled bone marrow transduced with YFP or hdCK3mut was able to successfully rescue lethally irradiated recipient animals. Long-term monitoring determined there was no survival disadvantage for hdCK3mut recipients over YFP as indicated in a Kaplan–Meier survival curve (Fig. 3E). Collectively these experiments demonstrate that expression of hdCK3mut is an inert reporter gene capable of long-term noninvasive tracking method throughout the recipients' lifespan.

hdCK3mut Allows for Noninvasive Detection of Human HSC Engraftment.

Probe retention in reporter-labeled cells is dependent upon transport of the radio-labeled probe intracellularly with sequential phosphorylation by hdCK3mut. Although mice and humans have similar nucleoside transporters, total expression or variation based on cell lineage may differ between species (31, 32). A humanized xenotransplantation model was used to validate that hdCK3mut would function as a reporter in human HSCs. Human hematopoiesis occurs within the spleen, bone marrow, and thymus of NSG mice when transplanted as neonates providing a tool to study *in vivo* human HSC differentiation (33). Isolated CD34+ cord blood HSCs were transduced with hdCK3mut or control lentiviral vectors (Fig. S1B) and transplanted into sublethally irradiated neonate NSG recipient animals (Fig. 4A). When transplanted as neonates CD34+ cells engraft and expand within multiple tissues and can home to the empty NSG thymus. These mice develop partial human hematopoietic systems with mature human myeloid, T-, and B cells.

At 8wk post human HSC transplantation peripheral blood analysis detected hdCK3mut reporter human engraftment by flow cytometry (Fig. 4B). Peripheral blood mononuclear cells (PBMCs) were stained for human CD45 to detect total human engraftment. Additional markers were used to detect human myeloid, B cell, T cell, and YFP for reporter-labeled cells. MicroPET

Figure 4. hdCK3mut allows for noninvasive detection of human HSC engraftment.



(A) Schematic of human HSC xenotransplantation. CD34+ cells from cord blood donors are transduced with lentivirus. Sublethally irradiated neonatal NSG recipients are intrahepatically transplanted. (B) FACS plots for total human engraftment at 8wk post-transplantation. hCD45 denotes total human cells, reporter cells are detected by YFP+. (C) [^{18}F]-L-FMAU MicroPET detects human hematopoietic cells expressing hdCK3mut within the bone marrow (BM) and thymus (Th). Background signal from probe metabolism is seen in liver (L), gastrointestinal (GI) with probe clearance through the bladder (BL). NSG mice displayed higher probe background compared with C57Bl6 animals seen by increased nonspecific liver signal. (D) IHC detects hdCK3mut-labeled human hematopoietic cells within the spleen. Total engraftment detected with α-hHLA, reporter cells detected by α-hdCK and α-YFP. (Scale bar, 100μm).

scans with [^{18}F]-L-FMAU detected hdCK3mut cells within the thymus and bone marrow of chimeric recipient mice (Fig. 4C). This demonstrates that human cells labeled with hdCK3mut retain expression of the reporter and are capable of engrafting after transplantation.

IHC analysis of human HSC engraftment in the spleen and thymus was performed at the experimental endpoint (Fig. 4D and Fig. S8). Total human engraftment was detected with human-specific HLA staining. Sequential sections verified reporter positive cells by anti-dCK and anti-YFP staining. Anti-dCK IHC in the spleen stained a fraction of the total engrafted human cells, consistent with the peripheral blood FACS (Fig. 4B), which revealed ~15% of human cells that were reporter positive based on YFP expression (Fig. 4D). This supports the hypothesis that human hematopoietic cell maturation and homing is retained in cells expressing hdCK3mut.

Overlapping Integration Sites in hdCK3mut-Labeled Human Hematopoietic Cells Defines a Common Cell of Origin with Multilineage Differentiation Capacity *in vivo*.

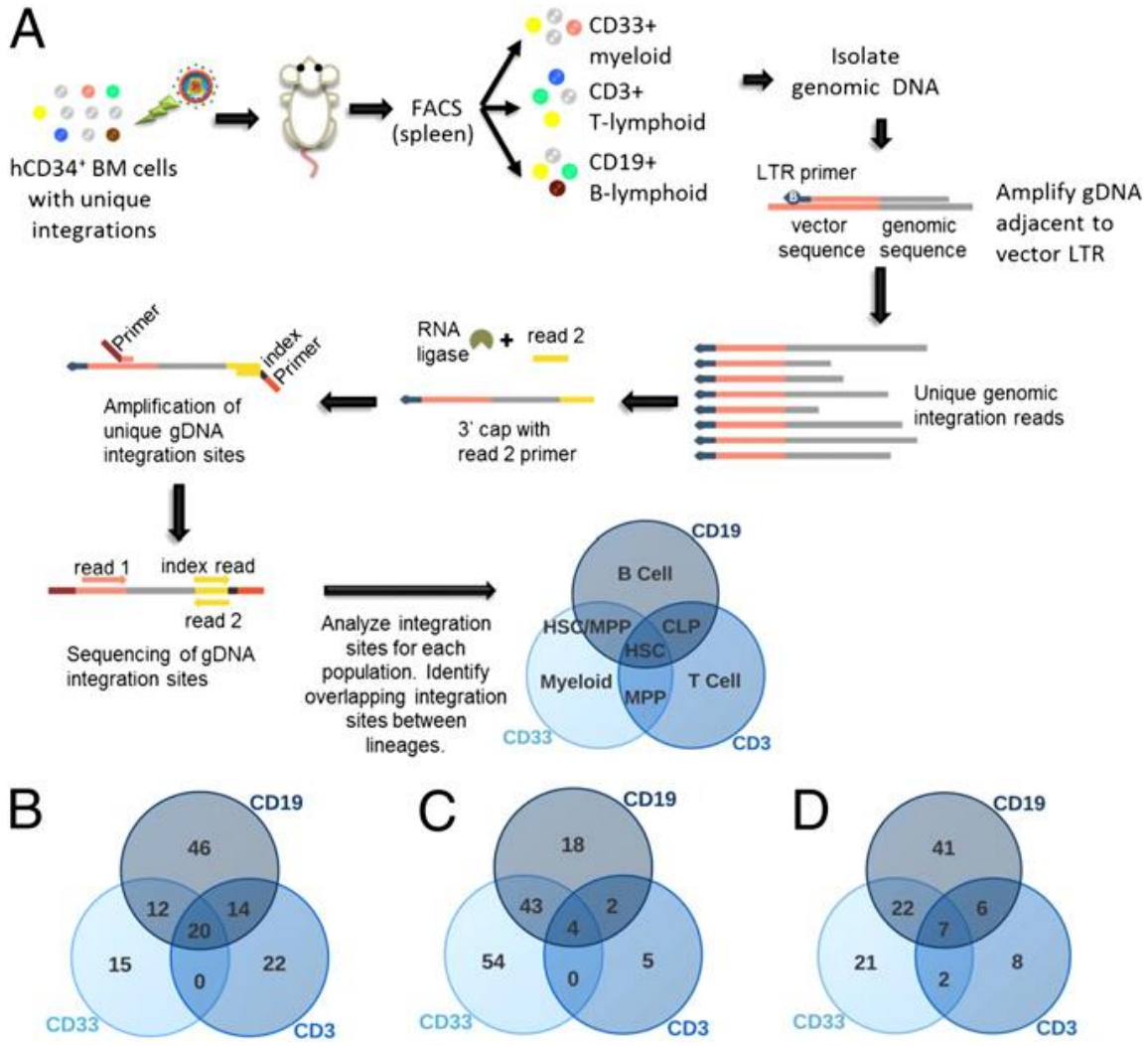
A concern of gene therapy trials for the correction of inherited diseases is the potential for insertional mutagenesis that has been observed in rare cases (34). Vector integration within tumor suppressors, near the transcriptional start site of oncogenes, or at sites that alter cell function are potential complications when using viral integration methods. Integration of lentiviral vectors is less likely to cause oncogenic transformation that was previously seen with other retroviral vectors (35). Recent studies have focused on identifying integration sites of modified hHSCs to detect potential problems such as dominant clonal expansion or lineage restriction (36, 37). Integration site analysis on long-term engrafted human chimeric mice was used to determine if expression and integration of hdCK3mut resulted in an abnormal event.

Cells were sorted from the spleens of engrafted animals into three lineages based on human CD33, CD3, or CD19 expression. Total genomic DNA was isolated and sequences flanking the vector integration sites were amplified by using common primers within the LTRs. Short primers were then ligated to the 3′ end of all amplified DNA allowing uniform ends of all fragments. A second PCR amplification was then performed to attach unique barcode sequences to the 3′ end. This allows multiple samples to be sequenced together and each sample to be precisely identified. Pooled samples underwent paired-end 50-nt Illumina sequencing to identify the unique integration site. Results were then aligned against genomic DNA to identify the exact integration location. Comparison of myeloid, B- and T-cell integrations were analyzed for each animal identifying individual and overlapping integrations (Fig. 5A).

Lineage-specific integrations identified committed progenitor cells. Integration sites found in all three populations are derived from a common transduced cell of origin (HSC) which then differentiated into all lineages (Fig. 5 B–D). Previous vector copy number per cell was determined by PCR to be ~0.5 (0.485, hdCK3mut; 0.494, YFP) after transduction. It is estimated that each HSC integration site represents a single engrafted clone.

A comparison of the total number of integrations from each sample in hdCK3mut and YFP animals confirms that the number of integrations detected is similar between vectors. This demonstrates that the expression of hdCK3mut does not prevent hHSCs from differentiating into all major hematopoietic lineages within the humanized mouse model. hdCK3mut also had no effect on long-term engraftment and was detected up to 5 mo post-HSC transplant with no lineage restriction due to gene toxicity or clonal expansion due to growth advantage.

Figure 5. Overlapping integration sites in hdCK3mut-labeled human hematopoietic cells defines a common cell of origin with multipotent lineage capacity *in vivo*.



(A) Schematic of integration site analysis. Cells from the spleens of engrafted animals are sorted into three populations. DNA is amplified from the LTR and a 3′ cap is attached. Unique index sequence is placed on all samples in a second amplification. Samples are sequenced, and genomic integration sites are determined. Overlapping integration sites from multiple lineages determines a common cell of origin differentiated into multiple hematopoietic lineages. (B) YFP-engrafted animal. (C and D) hdCK3mut-engrafted animals.

Discussion

We have demonstrated that an alternate dCK mutant (hdCK3mut) is well-tolerated, highly sensitive, and capable of monitoring long-term HSC engraftment.

Expression of HSV1-TK After Gene Transfer Provides a Safety Mechanism in Aberrant Reporter Cell Populations Through Reporter-Specific Cytoxicity (38, 39).

hdCK3mut provides an alternative PET reporter gene to sr39TK. One concern for alternate reporters is the loss of suicide gene function, which is gained when HSV1-TK and mutants are used (40). Gene transfer of HSV1-TK into select cell populations allowed for targeted cytotoxicity when treated with FDA-approved acycloguanosine-based antiviral drugs such as ganciclovir (41). This eliminates all cells expressing the reporter with limited off-target cytotoxicity (38). A limitation in HSV1-TK expression is that prophylaxis treatment with acycloguanosine antivirals to minimize cytomegalovirus infection in immunocompromised individuals cannot be administered without potentially eliminating the donor cells (42).

In previous studies, hdCK3mut also exhibited higher substrate specificity for several chemotherapeutic and antiviral nucleoside analogs in comparison with wild-type dCK (18–20). hdCK3mut had a higher activity with gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) with little activity toward acycloguanosine drugs. dFdC is an FDA-approved chemotherapeutic that works through self-potentiation in the diphosphate and triphosphate forms (43). The triphosphate form is then incorporated into DNA, causing chain termination. Treatment with drugs that have a higher affinity for hdCK3mut may allow a targeted suicide gene therapy. Proper *in vivo* models will need to be tested to validate hdCK3mut enhanced sensitivity and to evaluate the off-target effects from treatment.

Evaluating the Potential Immunogenicity of hdC3Kmut in Short-Term Therapeutic Cell Transplants.

Before transitioning hdCK3mut into a clinical PET reporter for stem cell or long-term therapeutic transplants experimental short-lived transplanted cells need to be investigated. The immunogenicity of HSV1-TK was found when reporter-labeled lymphocytes caused a CD8 immune response selectively killing reporter cells (8–10). A similar study could determine if expression of hdCK3mut will cause an immune response.

hdCK3mut contains only three point mutations and is expected to not cause an immunogenic epitope for MHC class 1 presentation. Using a predictive software for MHC class 1 presentation (44), hdCK3mut has only one additional peptide fragment, which is predicted to be different from WT hdCK. This peptide, which incorporates the mutated amino acid methionine at position 104, could use the methionine as an anchoring residue within the MHC (45). All other amino acids that are displayed in the MHC are natural, and therefore the peptide fragments displayed from hdCK3mut are predicted to be recognized as "self" avoiding immune cell detection.

Acknowledgments

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Authorship Contributions

Melissa N. McCracken, Donald B. Kohn, and Owen N. Witte designed research; Melissa N. McCracken, Eric H. Gschweng, Evan Nair-Gill, Jami McLaughlin, Aaron R. Cooper, Mireille Riedinger, Donghui Cheng, Chris Nosala, and Owen N. Witte performed research; Mireille Riedinger contributed new reagents/analytic tools; Melissa N. McCracken, Eric H. Gschweng, Evan Nair-Gill, Jami McLaughlin, Aaron R. Cooper, Donghui Cheng, Chris Nosala, and Owen N. Witte analyzed data; and Melissa N. McCracken and Owen N. Witte wrote the paper.

Methods and Materials

Animals

Immunocompetent C57BL/6 and C57BL/6 SJL female mice and immunedeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were bred and maintained according to the guidelines of the Department of Laboratory Animal Medicine (DLAM) at the University of California, Los Angeles. All animal studies were carried out by using protocols that had been approved by DLAM.

Constructs and Cloning

Codon optimized human deoxycytidine kinase (hdCK) sequence was purchased from DNA 2.0. PCR amplification added EcoR1 and Xho1 restriction sites. hdCK was then placed in murine stem cell virus (pMSCV) vector ahead of an internal ribosomal entry site (IRES) and yellow fluorescent protein (YFP). Amino acid mutations were made by multisite-directed mutagenesis (Stratagene) at the following locations: A100V, R104M, and D133A. A lentiviral vector (2F-X, pCCL based) was constructed for infection of human HSCs. hdCK3mut-IRES-YFP was cut from MSCV, and BamH1 sites were added by phosphate linkers. 2F-X was digested with BamH1 and hdCK3mut-IRESYFP was ligated into the vector. Helper plasmids for making ecotropic retrovirus was PCL-II. Third-generation nonreplicating lentiviral helper plasmids were used (VSVg, PREV, and PMDL). Cell Lines. L1210 and L1210-10K mouse leukemia cell lines were a gift from Charles Dumontet (Université Claude Bernard Lyon I, Lyon, France). All stable cell lines were made with retroviral transduction with ecotropic packaging of pMSCV constructs. Cell lines were sorted to equal expression of YFP by FACS and grown in cell-specific media conditions.

In Vitro Uptake Assay

Suspension cell lines: 1 x 10^5 cells in 100µL media were plated in triplicate in a 0.22µm filter bottom plates. [^{3}H]-probe was diluted to 5µCi/mL in media. A total of 100µL (0.5µCi) was added per well. Cells were incubated at 37°C for 1h, washed five times, and dried. Scintillation fluid was added and counted for counts per minute (cpm) for 1min on a BetaMax plate reader (PerkinElmer).

Recombinant Protein Production

For bacterial expression of Histagged human dCK, 5′ and 3′ primers (5′-CATGGATCCATGGCCACCCCGCCCAAGAG-3′ and 5′-GTAGGTACCTCACAAAGTACTCAAAAACTCTTTGACCTTTTC-3′) were designed introducing BamHI and KpnI sites for cloning into pQE-80L vector (Qiagen). TOP10 bacteria were transformed with pQE-80LhdCK3mut, dCKDM, or hdCK. A single colony was used for an overnight starter culture. A large-scale 1L culture was inoculated and induced at OD600: 0.6 with isopropylthio-β-galactoside (IPTG) to 1mM. Cells were harvested 4h later and cleared lysate was prepared according to Qiagen instructions. Lysate was combined with 1mL bed volume Ni-NTA agarose and transferred to a 20mL column. Beads were washed extensively and eluted in PBS 150mM NaCl, 250mM imidazole, 5% (wt/vol) glucose.

Enzyme Kinetic Assay

A coupled spectrophotometric kinase assay was adapted to determine kinetics for L-FMAU (44). Reads were performed in ~30s intervals at 37°C for 30min. An NADH standard curve was constructed. Nonlinear regression analysis and Michaelis–Menten plots determined the Km values.

Grafts

Cells were counted, washed, and resuspended in a 1:1 mixture of sterile RPMI and Matrigel. Each graft contained 2×10^5 cells in 100μL total volume. Grafts were implanted s.c. in 6- to 8-wk-old female NSG mice. [^{18}F]-FDG (2-deoxy-2-18fluoro- D-glucose) was performed on day 7 or 8, with sequential [^{18}F]-LFMAU (1-(2-deoxy-2-18fluoro-β-L-arabinofuranosyl)-5-methyluracil) scans on day 8 or 9 depending on tumor growth rate. Grafts were removed after [^{18}F]-L-FMAU imaging, weighed, and total cpm/g was determine by the amount of radioactivity in each graft measured using a Wallac Wizard 3″ 1480 Automatic Gamma Counter (PerkinElmer).

Mouse HSC Transplant

Six- to 10wk old C57BL/6 SJL mice were injected i.v. with 150mg/kg 5-fluorouracil (APP Pharmaceuticals). BM was harvested 5d after treatment and cultured with IL-3 (6ng/mL), IL-6 (10ng/mL), stem cell factor (SCF) (100ng/mL), and 5% (vol/vol) conditioned media from WeHi-3 cells (WeHI) as growth factors. After 24 h, the cells were infected with Murine Stem Cell Viruses (MSCV), MSCV-hdCK3mut-IRESYFP or MSCV-IRES-YFP and 1.6μg/mL of polybrene under spin conditions (2,500rpm, 90min, 30°C, Beckman CS-6R centrifuge) and then incubated overnight at 37°C. Cells were superinfected the next day, washed, and counted for total cell number. Six- to 8-wk-old C57BL/6 mice were lethally irradiated (900rad) before i.v. injection of 5×10^5 transduced BM cells on the same day. Each population was ∼40–60% transduced, with each animal transplanted with a mixed population of reporter and nonreporter cells.

Human HSC Transplant

Hematopoietic stem and progenitor cells were enriched from Ficoll fractionated cord blood or bone marrow with CD34+MACS beads (Miltenyi). Cells were thawed and prestimulated for

24h in RetroNectin (TaKaRa)-coated nontissue culture treated 24-well plates (Falcon) in X-VIVO-15 medium (Lonza) containing SCF (50ng/mL), fms-like tyrosine kinase-3 (Flt-3) ligand (50ng/mL), Thrombopoietin (TPO) (50ng/mL), and IL-3 (20ng/mL). The next day, cells were transduced at a lentiviral vector concentration of 2×10^8 transduction units/mL. Cells were injected 24h after transduction. Neonatal (1-3 days old) NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were conditioned with 150cGy total body irradiation. Twenty-four hours post-conditioning, mice were injected intrahepatically with CD34+-enriched cord blood or bone marrow cells at a dose of 3×10^5 cells in 50μL of medium.

Anti-hdCK Antibody Generation

Six His-tagged human dCK was produced in bacteria, purified by Ni-NTA (nickel-nitrilotriacetic acid) resin chromatography and used as immunogen. Four mice (BALB/c females 6–8wk) were immunized by i.p. injection of 200μg 6 His-hudCK in RIBI adjuvant (Sigma) followed by four monthly boosts of 100μg immunogen i.p. in RIBI. Antibody titer was determined in the serum by ELISA. Spleen of the highest titer mouse was excised and dissociated. Isolated splenocytes were fused to the myeloma cell line sp2/0 at a ratio of 5:1 splenocytes/myeloma using PEG1500 (Roche). Twenty percent of fusion was plated in hypoxanthine-aminopterin-thymidine medium (HAT medium) onto 10× flat bottom 96-well plates at 200μL/well, the remaining fusion was frozen down. Fusion was cultured until clones appeared in the wells and covered 25–50% of well. Supernatant was collected and ELISA performed: 96-well flat bottom assay plates (Nunc Maxisorp) were coated with 10μg/μL immunogen, blocked with PBS 1% (wt/vol) BSA. Supernatants were applied to wells, goat antimouse HRP was used to detect binding, and reaction was developed with 2,2′-Azinobis [^{3}-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS). Positive wells were replated in 24-well plates in hypoxanthine-thymidine (HT) medium.

ELISA was repeated on previously positive wells when cultures got 50% confluent. Positive supernatants were tested for ability to detect immunogen by Western blot at 1:10 dilution in PBST-5% (wt/vol) milk. Positive wells were subcloned by limiting dilution to obtain single clones per well in 96-well flat bottom plates. Subclones were tested by ELISA and highest positives were tested on Western blot. Clones were tested for IHC staining ability and clone 9D4 (plate 9 well D4) was determined to be the best in overall performance. Preparative amounts of antibody were produced in CELLine-1000 flasks (Integra Bioscience) and purified by affinity chromatography (protein G sepharose, Prosep-G; Millipore). Anti-dCK clone 9D4 is now commercially available with Millipore.

Antibodies and Western Blot

A total of 20μg of total cell lysate in RIPA buffer was run on SDS/PAGE using Precise Tris·Hepes protein gels, 4–20%. Gels were transferred onto 0.22μm nitrocellulose and blocked for 1 h at room temperature in 5% (wt/vol) milk in PBS with 0.05% Tween. Antibodies were diluted in 5% (wt/vol) milk in PBS with 0.05% Tween as follows: dCK (Witte Laboratory-9D4, see SI Materials and Methods; Millipore) 1:1,000, YFP (Witte Lab, polyclonal rAb) 1:5,000, ERK2 (Santa Cruz; SC-154) 1:5,000, goat anti-mouse IgG HRP (Bio-Rad; 172–1011) 1:10000, goat anti-rabbit IgG HRP (Bio-Rad; 170–6515)1:15000. ECL substrate (Millipore) was used for detection and development on GE/Amersham film.

Immunohistochemistry

Tissue was fixed in 10% phosphate-buffered formalin overnight. Sections were fixed in paraffin and cut at 0.4μm, with staining for hematoxylin and eosin for representative histology every five slides. Tissue sections were heated at 65°C for 1h to melt the paraffin followed by

rehydration. Antigen retrieval was performed using citric acid buffer and visualization was performed using a liquid DAB+ kit (Dako). Slides were blocked for endogenous peroxidase activity with 3% (vol/vol) H2O2 in PBS for 5min, then blocked for mouse IgG by using Vector Labs Mouse-on-Mouse (M.O.M.) kit (BMK-2202). Primary antibodies were diluted with M.O.M. diluent as follows: dCK (Witte Lab-94D, 1:2,000), YFP (Witte Lab-Mouse Monoclonal, 1:200), HLA (Santa Cruz; 1:100), mouse IgG (Santa Cruz; 0.4μg/μL), and incubated at 4°C overnight. Secondary antibody was added (ImmPRESS antimouse Ig (peroxidase) Polymer Detection kit).

Flow Cytometry and Fluorescent-Activated Cell Sorting

Single-cell suspensions from spleen, thymus, bone marrow, and peripheral blood were stained with the following fluorochrome-conjugated antibodies: anti-CD45.1, anti-CD4, anti-CD8, anti-Ter119, anti-CD71, anti-CD11b, anti-GR1, anti-B220, anti-CD19, anti-IgM, anti-CD43, and Hoechst for cell cycle analysis. Human engraftment was monitored with the following fluorochrome-conjugated antibodies: anti-hCD45, anti-hCD3, anti-hCD19, and anti-hCD33. In all reporter BM chimera animals, reporter-labeled cells were defined as YFP (FITC channel) positive. Cell sorting was performed on the FACSAria automated cell sorter (BD Biosciences), and flow cytometry was performed on the BD FACScanto II.

MicroPET and Image Analysis

Mice were warmed under gas anesthesia (2% (vol/vol) isoflurane) and injected i.v. with 200μCi of either [^{18}F]-FDG or [^{18}F]-L-FMAU (radiochemical synthesis described in ref. 45), followed by 1h unconscious or 3h conscious uptake. Mice were then positioned in an imaging chamber for sequential imaging with the Siemens Preclinical Solutions MicroPET Focus 220 and MicroCAT II CT systems (Siemens). MicroPET data were acquired for 10 min and reconstructed

with a filtered background projection probability algorithm. Micro-PET and CT images were coregistered. Quantification of PET signal was performed by drawing 3D region of interests (ROIs) around the area of interest using AMIDE software (http://amide.sourceforge.net/). The mean intensity of the ROI, based on the percent injected dose per gram, was normalized to control grafts of L1210-10K ROI drawn around untransduced grafts in the same animal. Data are presented as fold change over L1210-10K grafts. Images are presented here using a false-color scale that is proportional to tissue concentration (% injected dose/gram, %ID/g) of positron-labeled probe. Red represents the highest with yellow, green, and blue corresponding to lower concentrations.

***In Vivo* Uptake Assay**

BM chimera animals were placed under anesthesia with 2% (vol/vol) isoflurane in heated chambers. One microcurie of [^{18}F]-L-FMAU was injected i.v. with 1h unconscious probe uptake. Animals were euthanized and spleen, femur, tibia, and thymus were removed. Cells were then dissociated into single cell suspension and stained for CD45.1. In each tissue 2 x 10^5 cells of CD45.1, YFP+ (donor and reporter labeled), or CD45.1 (donor) were sorted. The amount of radioactivity in each cell type was measured using a Wallac Wizard 3- Inch 1480 Automatic Gamma Counter (PerkinElmer). Peripheral Blood Analysis. Peripheral blood was collected through serial retroorbital bleeds into Capiject EDTA collection tubes (T-MQK). Complete blood counts were performedby Department of Laboratory Animal Medicine, University of California, Los Angeles core laboratory with Hemavet. Engraftment was determined by flow cytometry.

Methylcellulose Assay

One hdCK3mut and one YFP BM chimera animal 6wk posttransplant were compared with a wild-type SJL (B6.SJL-Ptprc) mouse for each experiment in three independent experiments.

Bone marrow from both femurs and tibia was extracted. Total bone marrow was plated in duplicate. FACS was used to isolate donor cells through sorting CD45.1 positive and YFP (reporter) positive or YFP negative; cells were plated in duplicate. Commercial methylcellulose for CFC was used (R&D Systems). Colonies were analyzed on d11, and fluorescent analysis was analyzed by flow cytometry.

Integration Site Analysis

DNA was isolated from FACS-sorted cells using the Pure Link Genomic DNA Mini kit (Invitrogen). Dependent on availability, 1–100ng of DNA was used to perform nonrestrictive linear amplification-mediated PCR (46). Briefly, 100 cycles of linear amplification were performed with primer HIV3linear (biotin-AGTAGTGTGTGCCCGTCTGT). Linear reactions were purified using 1.5 volumes of AMPure XP beads (Beckman Genomics) and captured onto M-280 streptavidin Dynabeads (Invitrogen Dynal). Captured single strand DNA was ligated to read 2 linker (Phos-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3C spacer) using CircLigase II (Epicentre) in a 10μL reaction at 65°C for 2h. PCR was performed on these beads using primer HIV3-right (AATGATACGGCGACCACCGAGATCTACACTGATCCCTCAGACCCTTTTAGTC) and an appropriate indexed reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT). PCR products were mixed and quantified by probe-based qPCR and appropriate amounts were used to load Illumina v3 flow cells. Paired-end 50bp sequencing was performed on an Illumina HiSEq 2000 instrument using a custom read 1 primer (CCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA). Reads were aligned to the hg19 build of the human genome with Bowtie (47) and alignments were condensed and annotated using custom Perl and Python scripts to locate vector integrations. A custom Python script was

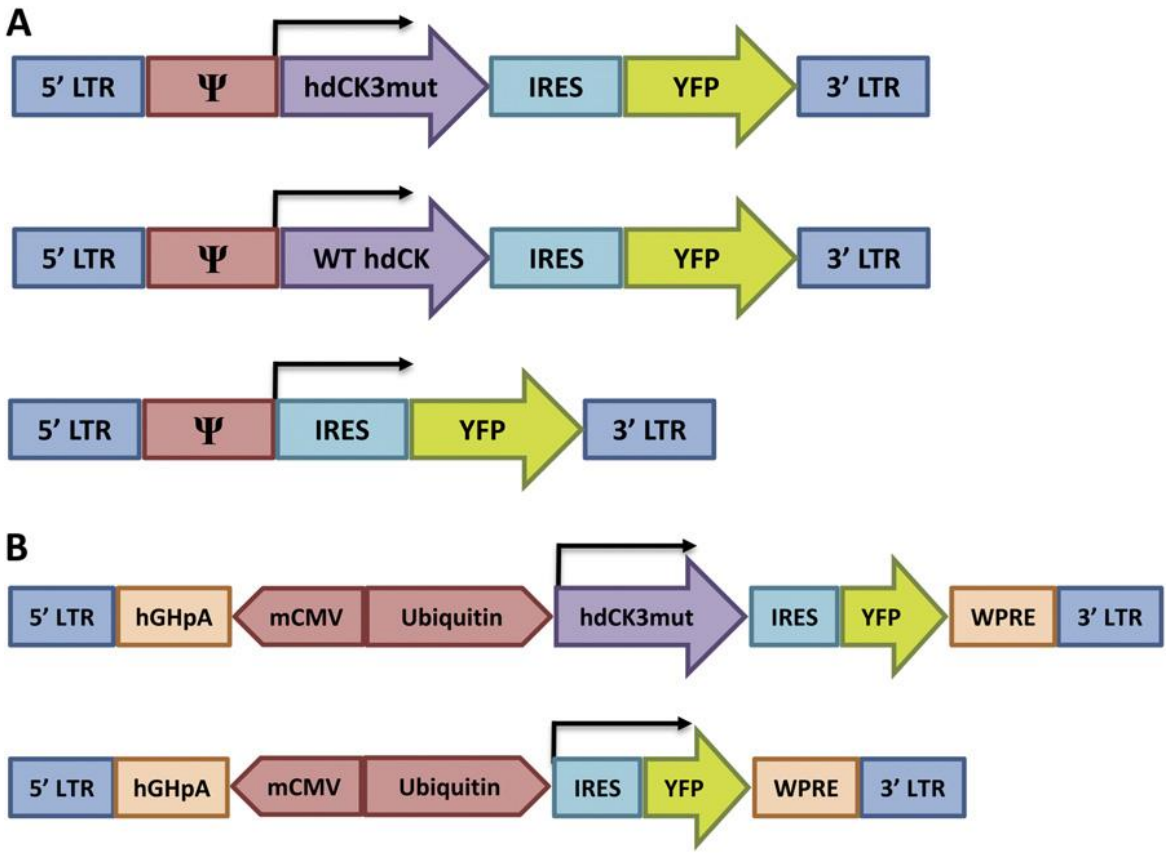
written to assess for overlapping integration sites between different samples, and a conservative estimate of 5% FACS sorting impurity was used to set a cutoff to eliminate overlaps with technical causes.

Graphs, Statistics, and Survival Analysis

Graphs are plotted as mean with SE of mean (SEM) for error bars. Statistics were analyzed using a Student's nonpaired t test. Survival analysis was plotted in Graphpad and determined based on overall length of animal survival. (Two animals were excluded from survival analysis due to premature death caused from malocclusion).

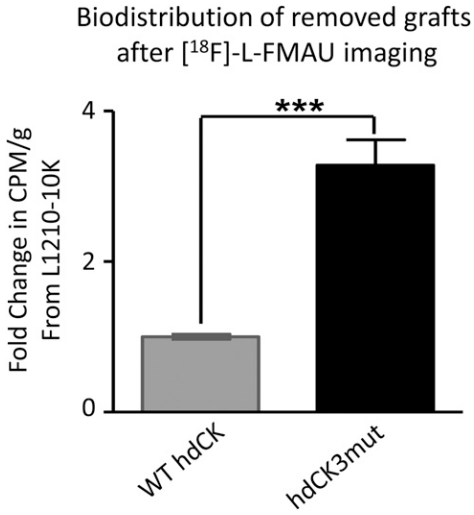
Supplemental Figures

Supplemental Figure 1. Vector maps.



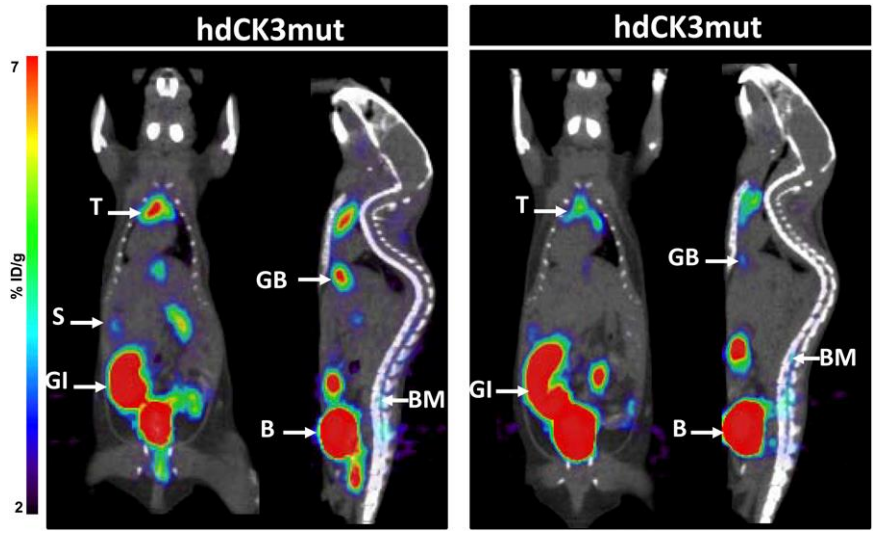
(A) Vector maps of pMSCV retroviruses used in generating stable ectopic expressing L1210-10K cell lines, and mouse HSC experiments. (B) 2F-X lentivirus vector maps used in human HSC experiments.

Supplemental Figure 2. Biodistribution.

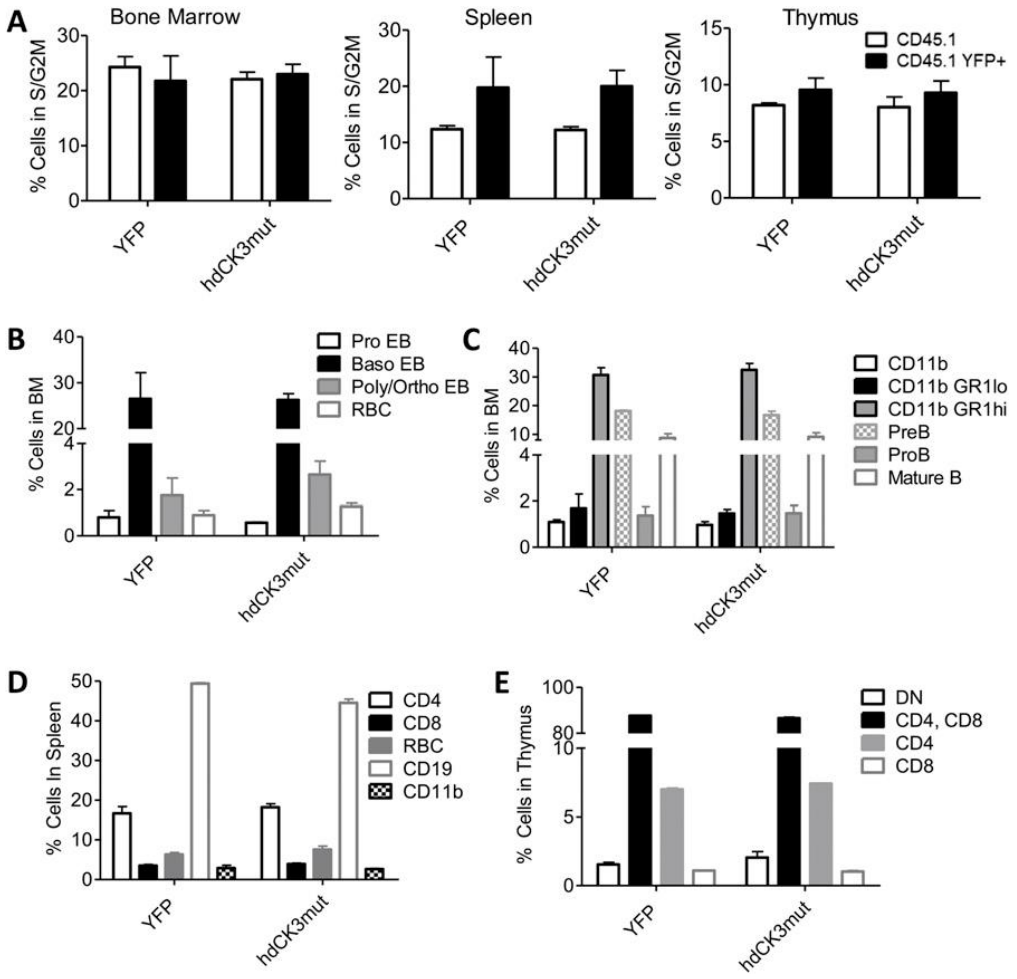


Grafts were excised after [^{18}F]-L-FMAU MicroPET and were weighed and placed in a gamma counter. Counts were normalized to counts per minute per gram (cpm/g) and compared with nontransduced tumors for background cpm ($P < 0.0001$).

Supplemental Figure 3. Additional [^{18}F]-L-FMAU MicroPET scans at 4wk post BMT in reporter chimeric mice.

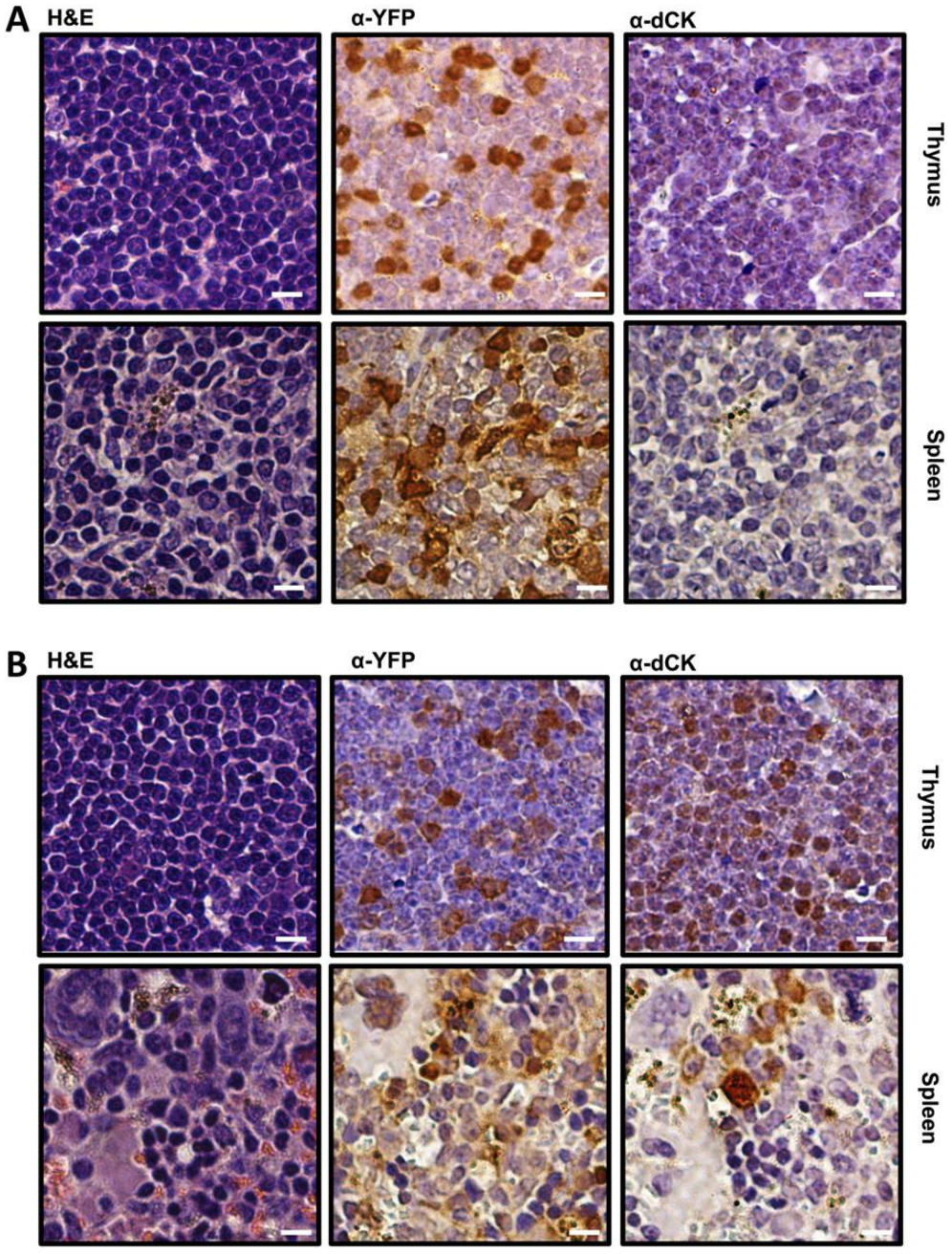


Supplemental Figure 4. Quantification of flow cytometry from hdCK3mut and YFP mHSC recipient mice.



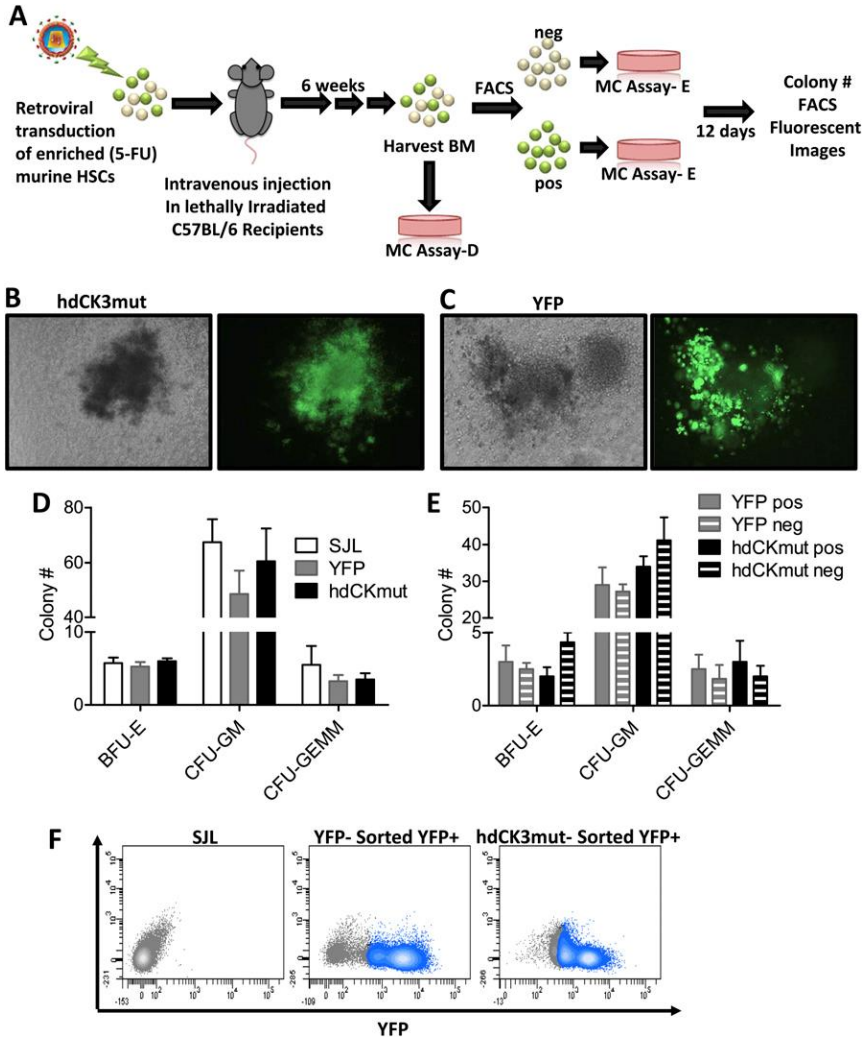
(A) Cell cycle analysis by Hoechst staining. (B and C) Analysis of bone marrow (BM) lineage distribution. (B) Red blood cell development with CD71 and Ter119 staining. Pro EB-CD71+, Ter119−; Baso EB-CD71+, Ter119+; Poly/Ortho EB-CD71 low, Ter119+; RBC-CD71−, Ter119+. (C) Myeloid and B-cell development Pre B-B220+, CD43−; Pro B-B220+, CD43+; mature B-B220+, IgM+. (D) Total engraftment within the spleen. (E) Thymus engraftment.

Supplemental Figure 5. IHC of mHSC recipient animals of (A) YFP and (B) hdCK3mut at 8wk post-BMT.



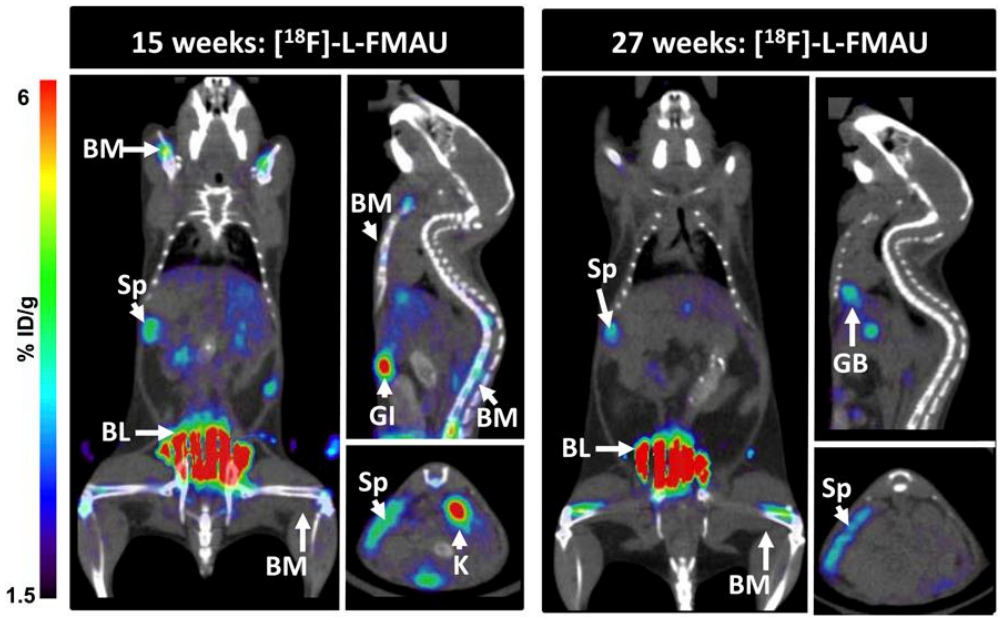
Spleen and thymus were analyzed for normal tissue architecture through H&E. IHC of α-YFP detected vector positive cells in YFP and hdCK3mut. α-dCK detected reporter cells only within hdCK3mut recipient mice. (Scale bar, 10μm).

Supplemental Figure 6.



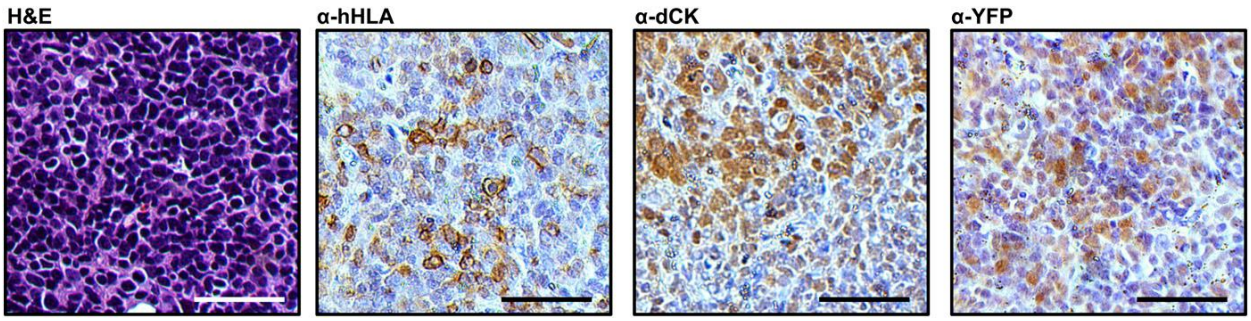
(A) Schematic of MC assay. hdCK3mut or YFP recipients at 6 wk post-BMT were harvested for total bone marrow. (B and C) Representative white and fluorescent images demonstrate the normal morphology of methylcellulose colonies for hdCK3mut and YFP. (D) Cells were placed in a MC assay, with an aged matched normal BL6 (CD45.1). (E) Remaining bone marrow was sorted based on CD45.1 YFP+ or CD45.1 YFP− and placed in MC assay. Total colonies were counted at 12d post-plating. (F) Cells from C were analyzed for retained YFP expression through flow cytometry.

Supplemental Figure 7. Sequential scans of hdCK3mut recipient mice at 15 and 27wk post-BMT.



Reporter signal is observed within the spleen (Sp) and bone marrow (BM). Probe metabolism is seen within the kidneys (K), gallbladder (GB), and bladder (Bl).

Supplemental Figure 8. IHC of hdCK3mut recipient hHSC recipient animal.



Thymus was analyzed for normal tissue architecture through H&E. IHC of α-hHLA detected total human engraftment. α-YFP detected vector positive cells with α-dCK detecting hdCK3mut reporter cells. (Scale bar, 50μm).

Supplemental Table 1. Enzyme kinetics for hdCK3mut and hdCKDM with L-FMAU

	L-FMAU		
Enzyme	K_m (μM)	V_{max}, nmol/min/μg	R^2
hdCK3mut	12.681 ± 3.893	0.724 ± 0.217	0.899
hdCKDM	55.970 ± 6.880	1.671 ± 0.312	0.975

Enzymatic studies were performed using His-tagged recombinant dCK in a coupled enzyme assay with LDH/PK. Values are an average of three independent experiments.

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CHAPTER 3:

HSV-sr39TK positron emission tomography and suicide gene elimination of human hematopoietic stem cells and their progeny in humanized mice.

Introduction

The genetic modification of hematopoietic stem cells (HSC) is an attractive approach for the treatment of disease, first demonstrated in primary immune deficiencies (1-3). Transplantation of gene-modified HSCs into patients resulted in long term correction of disease in the majority of subjects, and paved the way for future applications using viral vectors to modify hematopoietic cells (4). Gene therapy has also proven a promising modality for engineered immunity. Preclinical studies and clinical trials that engineered peripheral T-cells with cancer-antigen reactive T-cell-receptors (TCR) and chimeric antigen receptors (CAR) have achieved tumor regression in patients (5-8). Unfortunately, not all patients developed a lasting and complete response with most demonstrating transient anti-tumor reactivity. The observation that many patients initially responded with a reduction in tumor burden yet ultimately relapsed is hypothesized to be due to the nature of the *ex vivo* T-cell expansion protocol, which pushes T-cells to a differentiation state characterized by robust cytotoxic effector function at the cost of regenerative capacity (9-11). The ability to generate an antigen specific T-cell infusion product with long-lasting *in vivo* persistence, such as central memory T cells, is an area of active pre-clinical and clinical investigation (12-16).

HSCs represent the most primitive hematopoietic cells with the greatest regenerative potential, and recent preclinical studies have examined the modification of HSCs for cancer immunotherapy. The introduction of a pre-arranged TCR to HSCs was first demonstrated in mice (17), and later in humanized mouse models (18-20). These studies demonstrated that engineered HSCs give rise to progeny T-cells expressing the introduced transgenic TCR, and are reactive against cells expressing the target antigen. CARs have also been shown useful in the modification of HSCs for therapeutic immunotherapy, specifically against CD19 for B-cell malignancies (21).

The duration of *de novo* T-cell production in this chimeric setting is currently unknown, though clinical evidence supports the notion that HSCs support long-lasting thymopoiesis (22, 23).

The use of strong enhancer/promoter sequences within the vector necessary to achieve therapeutic levels of the introduced transgene can result in activation of proto-oncogenes in proximity of the integration site, and clonal expansion culminating in leukemic transformation of modified hematopoietic cells (24). These events, while rare, mandate the incorporation of safety elements in vector design including insulators (25) or internal promoters with self-inactivating long terminal repeats (LTR) lacking strong enhancers (26-28). An additional concern particular to T-cell immunotherapy is that the introduction of a self-antigen-specific TCR or CAR has the potential to induce an auto-immune reaction. There have been several reports of cytokine storm syndrome after the transplant of CAR-transduced T cells (29, 30) which may benefit from an approach to decrease the number of transgenic cells through the use of a suicide gene. Immunotherapy is designed to focus primarily on tumor-specific antigens, though low level of these antigens may be expressed by normal tissue leading to unintended off-target reactivity. In clinical trials targeting melanoma by transfer of T-cells engineered to express a human TCR against the $_{27\text{-}35}$MART-1 peptide, acute skin rash and auto-immune vitiligo are often observed due to reaction against normal melanocytes that also express the MART-1 antigen (31). More concerning is the recent report of the death of two patients in a clinical trial using autologous T-cells modified with an affinity-enhanced TCR against the MAGE3 antigen due to unpredicted reactivity to cardiac Titin (32). The possibility of occult cytotoxicity of the TCR or CAR further supports the inclusion of a method to eliminate gene-modified cells *in vivo*.

Suicide genes can be incorporated as a safety switch to selectively ablate gene-modified cells during an adverse event. These have been demonstrated in the setting of clonal outgrowth

from activation of a proto-oncogene (33) and graft versus host disease (GvHD) and on-target/off-organ cytotoxicity (34). Selective uptake of DNA replication chain terminator drugs by engineered nucleoside kinases such as native or modified herpes-simplex-virus-thymidine-kinase (sr39TK) (35), initiation of apoptosis mediated by inducible caspase systems by chemical dimerizers (36, 37), or surface proteins designed as antibody targets (38) have all been used to eliminate gene-modified cells. sr39TK (39) is advantageous over other modalities in that it additionally serves as a positron emission tomography (PET) reporter gene, allowing *in vivo* imaging to non-invasively track gene modified cells using radio-labeled substrates such as 9-(4-[^{18}F]-fluoro-3-[hydroxymethyl]butyl)guanine ([^{18}F]-FHBG) (40). Despite clear potential benefit, the characterization of the utility of sr39TK as both a PET reporter and suicide gene in human HSCs and their progeny has yet to be demonstrated.

Here we report the use of a lentiviral vector encoding sr39TK to gene-modify human HSCs, demonstrate a lack of developmental skewing due to the transgene; *ex vivo* functional activity of T cells developed from gene modified HSCs; visualization of gene-modified HSCs and their progeny at high resolution serial scans *in vivo*; and the ablation of gene-modified cells in hematopoietic tissues after a single course of the pro-drug GCV as evaluated by biochemical, cell-biological, and molecular biological techniques. These results lend support for the inclusion of sr39TK in clinical trials for the modification of HSCs with a cancer-antigen reactive TCR or CAR to both monitor successful transplant and provide a safety-feature allowing the ablation of cells during a serious adverse event.

Materials and Methods

HSC Transduction

Cells were thawed in a 37°C water bath and transferred to 50ml tubes. X-VIVO-15 was added drop-wise with agitation to dilute thawed cell product 1:10. Cells were spun at 500g for 5min and supernatant was aspirated. Cells were resuspended in 50ml X-VIVO-15 and counted using a ViCELL Cell Viability Analyzer (Beckman Coulter, Brea, CA). Cells were spun down at 500g for 5min, and supernatant was aspirated. Cells were resuspended in X-VIVO-15 + [50ng/ml] SCF, [50ng/ml] Flt3-L, [50ng/ml] TPO, and [20ng/ml] IL-3 (Peprotech, Rocky Hill, NJ) at a density of $4x10^6$ cells/ml. Twenty-four-well non-tissue-culture treated plates coated with RetroNectin (TaKaRa, Shiga, Japan) were seeded with 0.25ml ($1.0x10^6$ cells) of cell suspension and incubated overnight. The following day, concentrated NY-ESO-1-TCR/sr39TK lentiviral prep was added for a final vector concentration of $1.0x10^8$ TU/ml in a final volume of 500ul X-VIVO-15 + cytokines as described above. Cells were incubated overnight. The following day, cells were collected from wells and rinsed thrice in X-VIVO-15 without cytokines. Cells were counted, and resuspended at a density of $2.0x10^7$ cells/ml in X-VIVO-15 + cytokines as described above.

Generation of Humanized Mice

Humanized mice were generated by the intrahepatic transfer of $1.0x10^6$ NY-ESO-1-TCR/sr39TK- or mock-transduced CD34+ PBSCs to neonatal NSG-HLA-A2.1 mice on day 3-5 post-birth using a 28G tuberculin syringe (18). Neonates were preconditioned immediately before injection with 100cGy irradiation from a ^{137}Cs source (JL Shepherd, San Fernando, CA). For tissue harvest, animals were euthanized by 5% CO2 asphyxiation immediately before dissection. Single cell suspensions of thymus and spleen were prepared by dissociating organs with a 3ml syringe

plunger over 70um mesh in FACS buffer (DPBS, 2% FBS, 2mM EDTA) . Individual bones (femurs, humeri, and sternum) were kept separate to investigate potential differences in marrow spaces by flow cytometry and ddPCR. Marrow spaces were flushed with a 23G needle through 70uM mesh. Cells were enumerated and $1x10^6$ splenocytes, $1x10^6$ cells from the marrow, and $1x10^5$ thymocytes were stained with antibodies as described.

Flow Cytometry

Blood was drawn from the retro-orbital sinus using heparin coated capillary tubes (Thermo Fisher, Waltham, MA). The following antibodies (Becton Dickinson (BD), Laguna Hills, CA) were used to assess human engraftment: murine CD45-V500 clone 30-F11, human CD45-V450 clone HI30, human CD19-PE-Cy7 clone SJ25C1, human CD3-PerCP clone SK7, human CD4-APC clone RPA-T4, and human CD8-FITC clone HIT8a. Expression of the NY-ESO-1-TCR was determined by binding to a PE-labeled HLA-A2.1 MHC-tetramer loaded with the 157-165NY-ESO-1 SLLMWITQC (Beckman Coulter, Brea, CA). Antibodies were added to 80ul whole blood, incubated in the dark for 30min, RBC lysed with 1ml FACS Lyse (BD), washed with 3ml FACS buffer, spun at 500g for 5min, and resuspended in 250ul FACS buffer. Data were acquired on a FACS Fortessa (BD). Analysis was performed on an average of 2,000 to 10,000 hCD45+ cells per 80ul peripheral blood drawn per mouse.

PET Scan

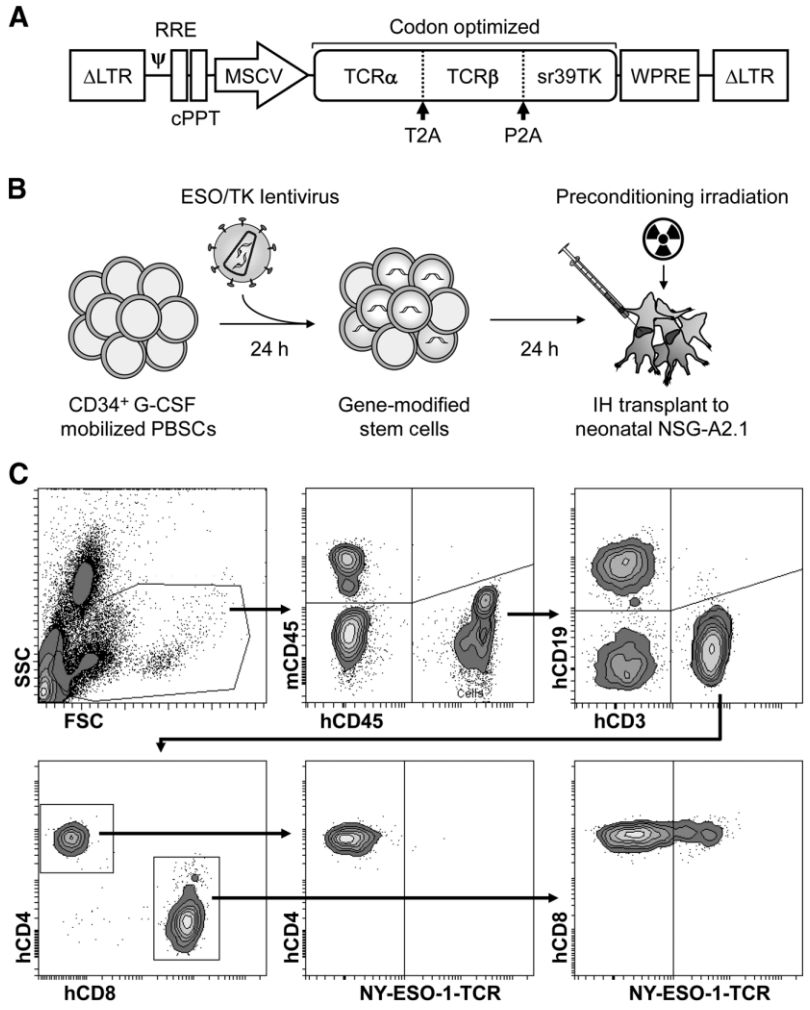
[^{18}F]-FHBG was synthesized as described (41). Mice were injected IV with 250µCi [^{18}F]-FHBG in 50-100ul, and allowed a 3h conscious uptake. Mice were anesthetized with 2% isoflurane for sequential imaging in the Siemens Preclinical Solutions MicroPET Focus 220 and MicroCAT IICT (Siemens Malvern, PA). PET data were acquired for 10 min and reconstructed with a filtered

background projection probability algorithm. PET/CT images were co-registered. Quantification of PET signal was performed by drawing 3D regions of interest (ROIs) using AMIDE software (http://amide.sourceforge.net/). MAP projections were generated for display in figures. The max intensity of the muscle ROI, based on the percent injected dose per gram, was subtracted from each hematopoietic niche ROI to normalize for background. Images are presented in false-color volumetric renderings generated in AMIDE.

Statistical Analysis

Descriptive statistics for quantitative variables such as the mean and standard error by experimental groups were summarized and presented. Differences between experimental groups were assessed by unpaired t-test (Figures 2a-2g) or pairwise comparison (Figures 6a-6d) within the framework of one-way analysis of variance (ANOVA). To account for variation from individual animals, linear mixed effect models with random intercept (42) were used to evaluate the between-experimental group difference (Figures 5a-5c, Figures 6e-6f) as well as pre- and post-treatment difference (Figures 5a-5c). For all statistical investigations, tests for significance were 2-tailed unless otherwise specified. A p-value less than the 0.05 significance level was considered to be statistically significant. All statistical analyses were performed using SAS version 9.3 (SAS, Cary, NC).

Figure 1. System to test ESO/TK PET reporter and suicide gene function *in vivo*.



(A) Lentiviral vector used to engineer HSCs to express ESO/TK transgene. (B) CD34+ G-CSF mobilized PBSCs were stimulated overnight, transduced with a lentivirus encoding the ESO/TK vector, then transplanted to irradiated NSG-A2.1 neonates by intrahepatic injection. Two months post-transplant, peripheral blood was screened for human chimerism and lymphoid development by flow cytometry. (C) Cells were gated on lymphocyte SSC x FSC profile, then murine and human CD45 to exclude non-nucleated cells. Human CD45+ cells were examined for hCD19 to identify B- and hCD3 to identify T cells. T cells were separated into CD4 and CD8 subsets, and evaluated for their ability to bind the NY-ESO-1 tetramer as indicative of TCR expression.

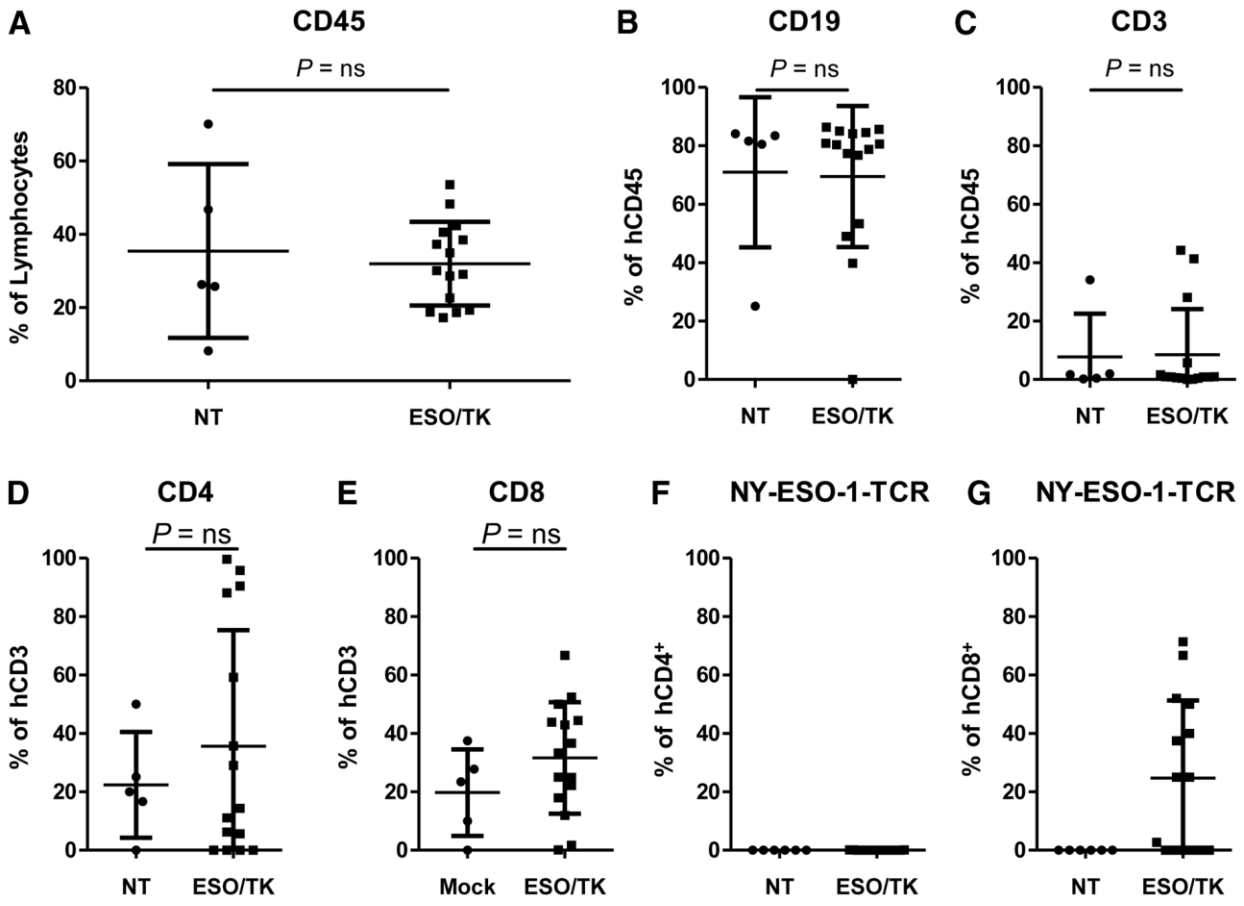
Results

NY-ESO-1-TCR/sr39TK modified human HSCs engraft *in vivo*

To test the function of sr39TK, we generated a lentiviral vector composed of a codon optimized NY-ESO-1-TCR linked by a 2A cleavage-peptide to sr39TK (ESO/TK) driven by the strong retroviral long-terminal-repeat promoter MSCV (Figure 1A). Humanized mice were generated by transplanting neonatal NSG-A2.1 mice with ESO/TK transduced CD34 enriched peripheral blood stem cells (PBSC) via intrahepatic injection (Figure 1B). At two months post-transplant, mice were screened by peripheral blood immunophenotyping. Human cell chimerism in the mice was determined by evaluating lymphocytes for human CD45% divided by total (human + murine) CD45%. Human cells were gated into hCD19+ B-cells and hCD3+ T-cells, and the CD3+ population was subfractioned to CD4 helper and CD8 cytotoxic subsets with the NY-ESO-1 tetramer binding activity of each assayed (Figure 1C). The transplant of PBSCs to neonatal NSG-A2.1 mice resulted in human chimerism in peripheral blood beginning at 2 months post-transplant. The transduction of PBSCs with an ESO/TK lentiviral vector did not result in a significant change in total human cell chimerism nor alter the composition of human lymphoid cells (Figure 2A-E and Supplemental Table 1). NY-ESO-1-TCR+ cells, identified by co-staining with the 157-165NY-ESO-1 HLA-A2.1 tetramer, were only observed in the animals transplanted with gene-modified cells. CD4+ T-cells bearing NY-ESO-1-TCR were not observed (Figure 2F). CD8+ NY-ESO-1-TCR bearing cells developed solely in the ESO/TK-transduced group, and 8 out of 15 mice had readily detectable TCR-positive CD8 T-cells in the periphery as early as 2 months post-transplant (Figure 2G).

To validate the effector function of NY-ESO-1-TCR bearing T cells developed *in vivo* from transduced HSCs, experimental mice were harvested, splenocytes dissociated, and expanded by

Figure 2. Human cells develop in NSG-A2.1 mice transplanted with PBSCs.



Non-transduced and ESO/TK transduced PBSC transplanted humanized mouse peripheral blood was assayed by flow cytometry at 2 months post-transplant. No significant difference was observed in proportions of (A) human chimerism, (B) B-cells, (C) T-cells, (D) the CD4 subset, (E) or the CD8 subset of T-cells. (F) NY-ESO-1-TCR bearing CD4 cells were not observed. (G) NY-ESO-1-TCR bearing CD8 T-cells developed only in the ESO/TK cohort.

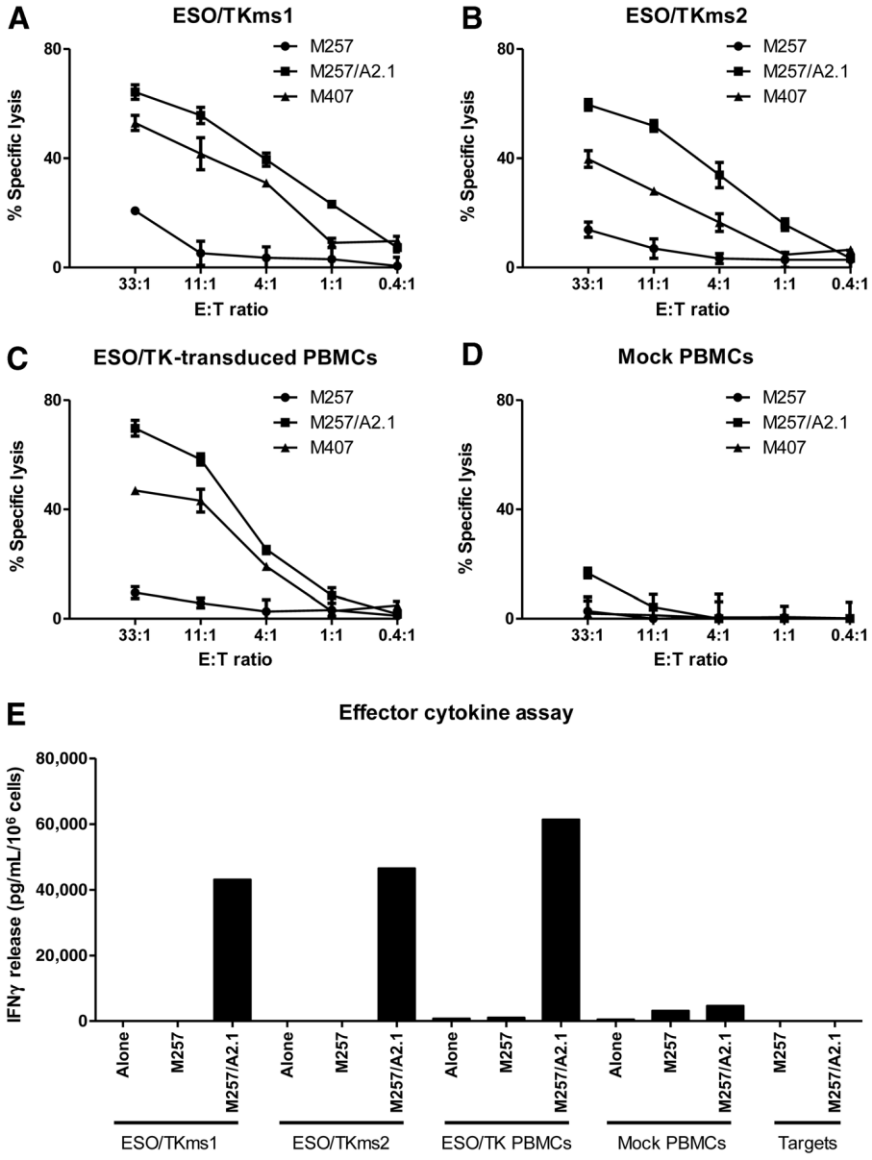
co-culture with artificial antigen presenting cells loaded with the $_{157\text{-}165}$NY-ESO-1 peptide. Controls were generated from healthy adult donor peripheral blood T-cells activated by CD3/CD28 beads and transduced with the ESO/TK vector or mock transduced. *Ex vivo* expanded splenocytes from humanized mice or control human T-cells were co-cultured with non-HLA-A2.1 (M257) or HLA-A2.1 (M257/A2.1 and M407) patient derived melanoma cell lines expressing the NY-ESO-1 antigen. ^{51}Chromium release assays to assess cytotoxicity revealed humanized mouse derived T-cells killed target cells in an HLA-restricted fashion (Figure 3A, 3B), comparable to control normal donor T-cells transduced with the NY-ESO-1-TCR (Figure 3C). Minimal background cytotoxicity in non-transduced donor T-cells was observed (Figure 3D). ELISA assays revealed similar results, with both humanized mouse derived- and healthy donor transduced NY-ESO-1 antigen-specific T-cells secreting the effector cytokine interferon-gamma when cultured in the presence of target cells (Figure 3E).

A subset of mice were selected for PET imaging studies (non-transduced humanized N=3, ESO/TK-transduced humanized N=10) based on equivalent human chimerism and lymphocyte composition, with an additional (N=3) non-transplanted age-matched NSG-A2.1 control animals to examine background biodistribution.

sr39TK shows selective uptake of [^{18}F]-FHBG *in vivo*

The PET reporter/suicide gene sr39TK is an engineered herpes-simplex-virus-thymidine-kinase with approximately 300x greater affinity for GCV than wild type HSV-TK (43). The ESO/TK vector was first tested in Jurkat cells *in vitro*. Cells transduced at an MOI of 10 and 100 expressed the NY-ESO-1-TCR (Supplemental Figure 1A), showed selective uptake of [^{18}F]-FHBG (Supplemental Figure 1B), and were selectively killed by GCV (Supplemental Figure 1C) confirming the functional activity of the ESO/TK vector.

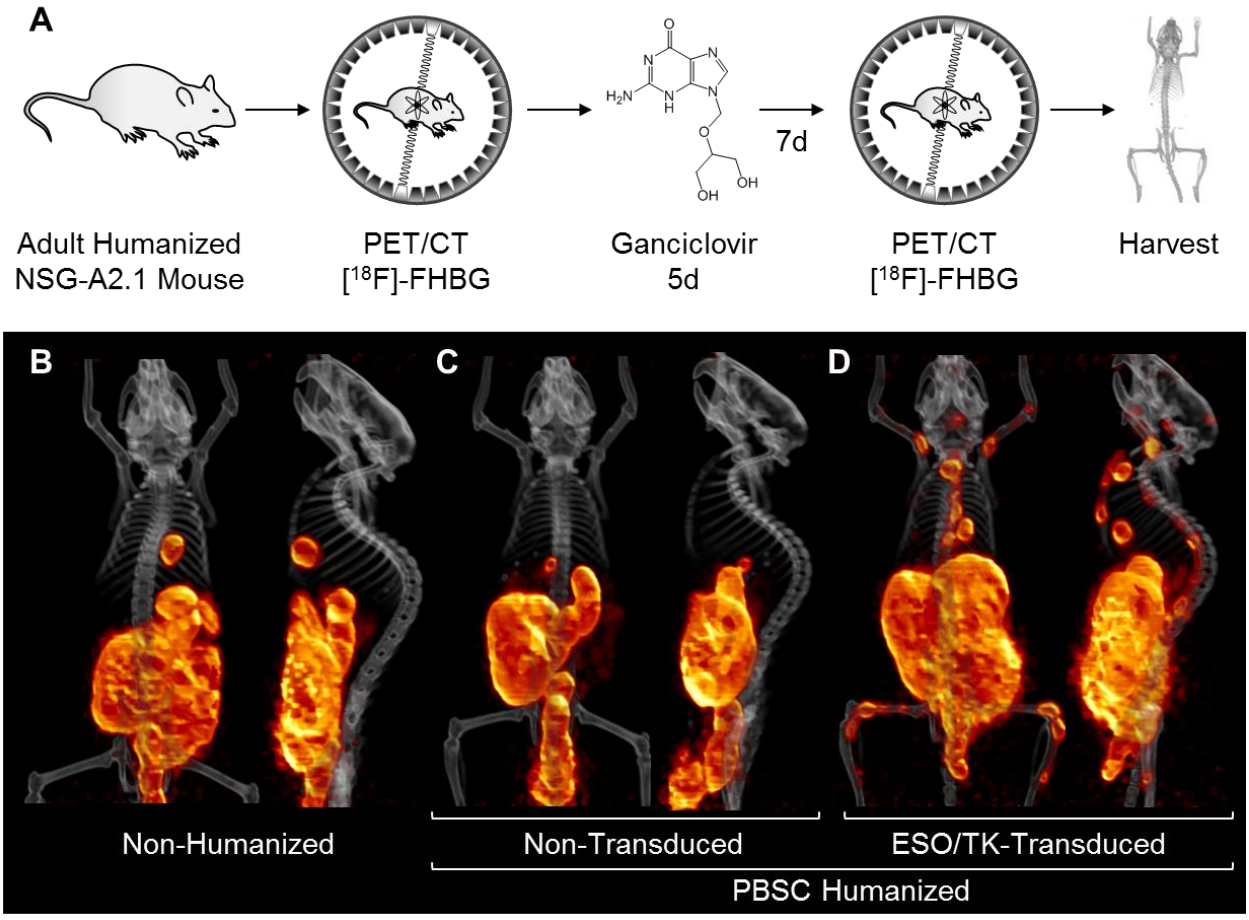
Figure 3. Effector function of *in vivo* derived NY-ESO-1-TCR bearing cells from HSCs.



Ex vivo expanded splenocytes from ESO/TK humanized mice were evaluated alongside ESO/TK transduced or mock transduced normal donor PBMCs. ^{51}Cr release assays were performed on (A,B) splenocytes from ESO/TK humanized mice (ms1 and ms2), (C) healthy donor ESO/TK transduced T-cells, and (D) mock transduced T-cells cocultured with HLA mismatched (M257) or HLA matched (M257/A2.1 and M407) melanoma cell lines. (E) IFNγ ELISA was performed to validate results from cytotoxicity assays.

To test the function of sr39TK as a PET reporter/suicide gene *in vivo*, we designed an experiment to serially scan humanized mice with the PET reporter [^{18}F]-FHBG before and after treatment with the prodrug Ganciclovir (GCV) followed by investigation of cell composition by cell- and molecular-biological methods (Figure 4A). Non-transplanted NSG-A2.1 mice and transplant recipients of mock transduced or ESO/TK gene-modified human PBSC were injected with 250µCi [^{18}F]-FHBG and imaged on a Siemens MicroPET scanner followed by CT scan for overlay. Non-transplanted NSG-A2.1 mice were imaged to determine background biodistribution of [^{18}F]-FHBG, which is known to have a high background in the abdominal area due to the probe elimination through the biliary tree and the GI tract in mice (44). As expected, non-humanized NSG-A2.1 mice exhibited predominantly gastrointestinal tract (GI), gall bladder, and bladder signal, with no signal in presumptive hematopoietic niches, or areas of high metabolic activity such as the brain or heart (Figure 4B). Evaluation of uptake in the spleen was occluded by GI signal. Non- transduced humanized mice showed similar background biodistribution of [^{18}F]-FHBG probe, and lack of hematopoietic niche signal (Figure 4C). In contrast, mice humanized with ESO/TK transduced PBSCs exhibited strong signal in hematopoietic compartments (i.e. long bones, skull, vertebrae, and thymus) in addition to background GI biodistribution (Figure 4D). Signal quantitation was performed in Amide software by drawing 3-dimensional regions of interest (ROI) on individual femurs, humeri, the thymus, and arm muscle (Supplemental Figure 2A). The maximum percent injected dose/g (%ID/g) was determined for each ROI, and muscle was subtracted from hematopoietic niche ROIs to normalize background tissue uptake. Significant accumulation of probe in ROIs was observed in hematopoietic compartments in the ESO/TK-transduced cohort vs. the non-transduced humanized group (Supplemental Figure 2B).

Figure 4. High-resolution sr39TK PET reporter imaging of gene-modified cells *in vivo*.



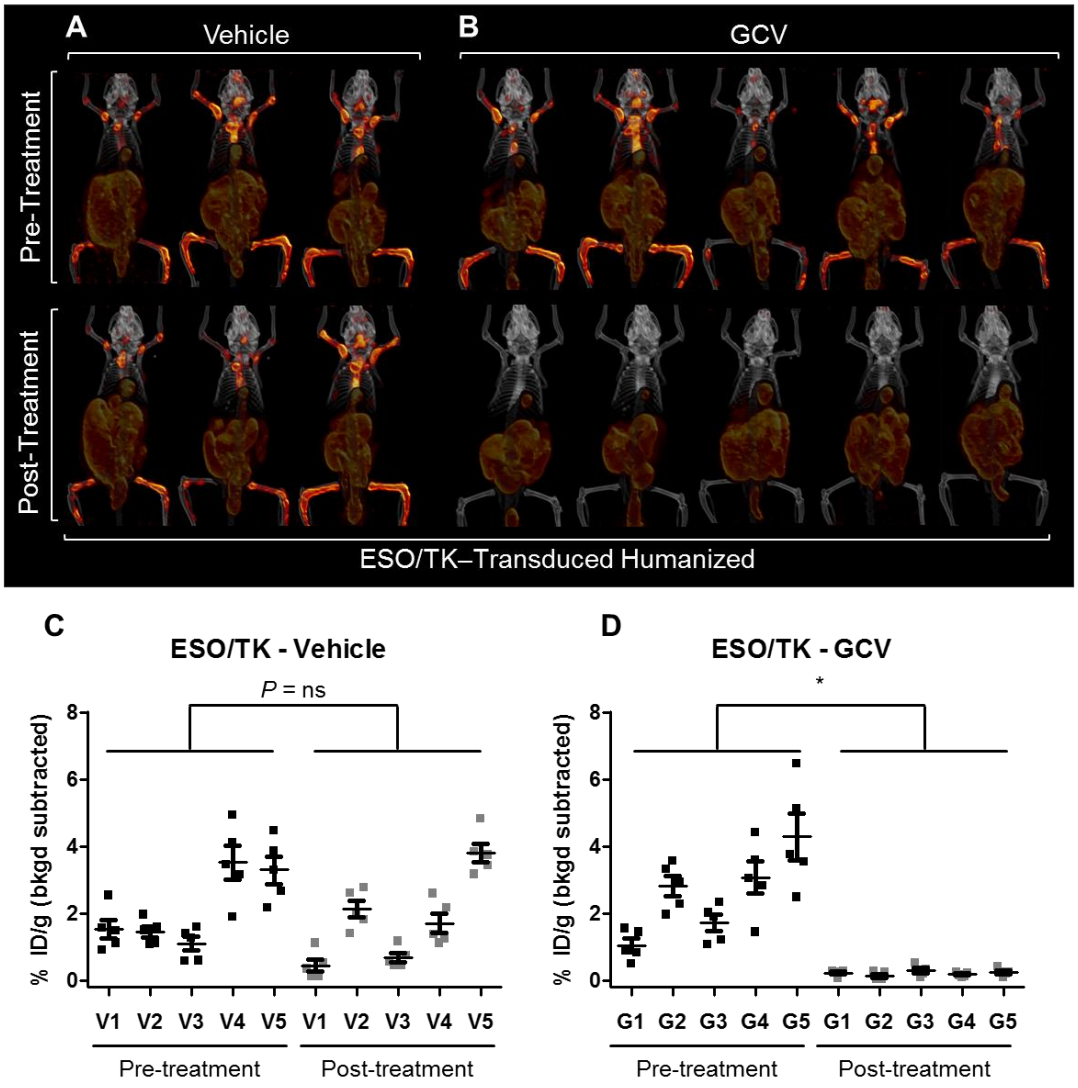
(A) Experimental procedure for PET imaging. Mice were injected with 250uCi [^{18}F]-FHBG and PET/CT imaged. Scans of (B) non-transplanted NSG-A2.1, (C) non-transduced humanized, and (D) ESO/TK-transduced humanized mice. Probe was detected in the gastrointestinal tract and gall bladder in all mice. In ESO/TK-transduced humanized mice, signal was detectable in the long bones of the arms and legs, the sternum, the thymus, and vertebrae.

Gene-modified cells are selectively ablated by GCV

To test the suicide gene function of sr39TK in transduced human cells *in vivo*, previously scanned non-transduced humanized mice and ESO/TK-transduced humanized mouse cohorts were treated intraperitoneally for 5 days with vehicle or [50mg/kg] of the nucleoside prodrug GCV which is converted to a cytotoxic nucleotide when phosphorylated by sr39TK. PET/CT imaging was performed one week after the final drug injection to allow ablation of gene-modified cells and clearance of residual GCV. Vehicle treated ESO/TK mice demonstrated specific uptake in hematopoietic niches in pre- and post-treatment scans (Figure 5A); however, GCV completely ablated PET signal in post-treatment scans in all hematopoietic niches previously observed to harbor probe accumulation in ESO/TK-transduced humanized mice (Figure 5B). No difference in signal accumulation was detected in pre- and post-treatment scans in the non-transduced humanized cohort (Supplemental Figure 2C). Vehicle treated ESO/TK-transduced recipient mice showed no significant difference in signal accumulation in hematopoietic compartments as determined by pre- and post-treatment scans (Figure 5C). GCV treated ESO/TK-transduced recipient mice showed significant ablation of [^{18}F]-FHBG PET signal in hematopoietic compartments in post-treatment scans (Figure 5D). The post-treatment signal of GCV treated ESO/TK mice were not significantly different than background uptake in non-transduced humanized mice.

Animals were euthanized one day after the final scan, and tissues were collected and dissociated. Cell suspensions were enumerated, and allocated for subsequent analyses. Flow cytometry of splenocytes to measure chimerism revealed human cells present in all cohorts; non-transduced humanized, vehicle treated- and GCV-treated ESO/TK-transduced humanized mice. There was not a significant reduction of human chimerism in GCV treated ESO/TK mice (Figure

Figure 5. GCV ablates gene modified cells hematopoietic niches.

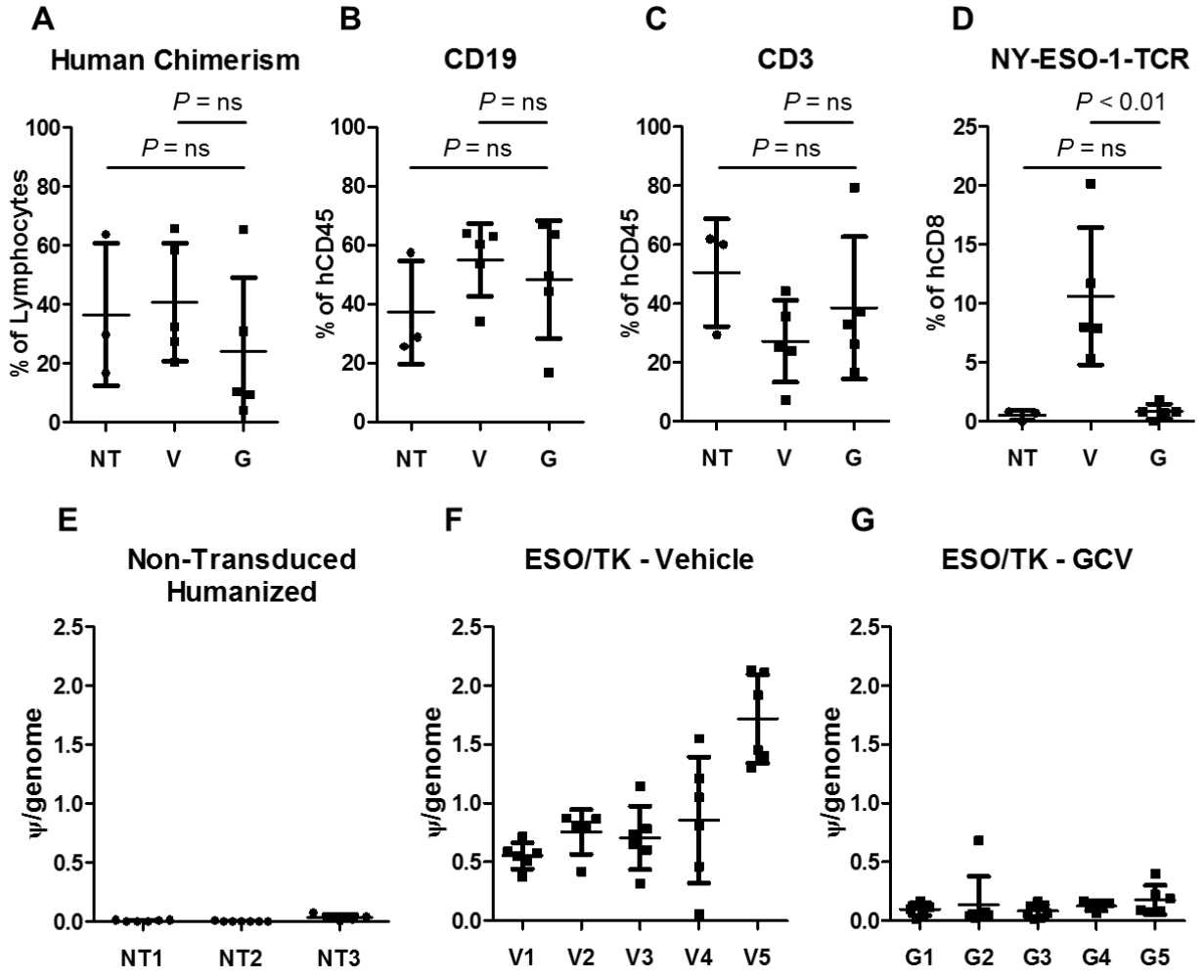


Mice were PET/CT scanned with [^{18}F]-FHBG before and 7d after treatment with (A) vehicle or (B) GCV. Three of five representative vehicle treated mice and five of five GCV treated mice are shown. Neutral density masks were drawn to visually mute background GB and GI signal. ROIs were drawn on femurs, humeri, and the thymus of each mouse in pre- and post-treatment scans. (C) ESO/TK mice treated with vehicle showed no significant difference between pre- and post-treatment scans (P=0.402). (D) There was a significant decrease in [^{18}F]-FHBG PET signal in hematopoietic ROIs in ESO/TK mice treated with GCV (P<0.001).

6A). CD19 B-cells and CD3 T-cells were detected in all cohorts at endpoint analysis with no significant difference between vehicle and GCV treated ESO/TK mice (Figures 6B,C). In contrast, NY-ESO-1-TCR bearing CD3+CD8+ T-cells were reduced to background levels in the GCV treated ESO/TK-transduced humanized mice (Figure 6D).

Quantitation of PET signal and flow cytometric analyses demonstrated ablation of gene-modified cells while sparing non-modified cells. However, cells with low metabolic activity may not be sensitive to drug selection nor show specific uptake of [^{18}F]-FHBG. In addition, as surface TCR expression requires co-expression of CD3, flow cytometry is unable to measure the presence of this transgene in non-T-cells. In order to investigate persistence of other gene-modified cells, quantitative PCR was performed to measure the amount of lentiviral vector psi element per human genome in each organ compartment. No vector genomes were detected in non-transduced humanized mice (Figure 6E). The amount of vector present in the ESO/TK-transduced mice treated with vehicle varied among different animals (mean=0.918±0.131, range=0.552–1.72), but was relatively consistent among the different tissues tested for each recipient (Figure 6F). In the cohort treated with a course of GCV, there was a significant reduction of integrated vector (mean=0.123±0.131) compared with the vehicle treated cohort (Figure 56) ($P<0.001$).

Figure 6. Immunophenotyping and VCN analysis after drug treatment.



Harvested splenocytes from non-transduced humanized, vehicle treated ESO/TK-transduced humanized, and GCV treated ESO/TK-transduced humanized mice were evaluated by flow cytometry. No significant difference was observed for (A) human chimerism, (B) human B-cell or (C) T-cell composition. (D) A significant decrease of CD8+NY-ESO-1-TCR+ cells was observed after GCV treatment in the ESO/TK group (P=0.006). (E-G) VCN analysis of gDNA harvested from the sternum, thymus, femurs, humeri, and spleen were measured for each treatment group.

Discussion

Gene therapy using HSCs has proven to be an efficacious treatment for monogenetic diseases, and is currently of interest for immunotherapy applications. Pre-clinical studies have provided evidence that HSCs transduced to express a transgenic TCR are capable of producing antigen specific effector T-cells *in vivo* paving the way for a first-in-man study nearing Phase I clinical trial (CIRM Disease Team Grant DR2A-05309). However, several questions remain. Enthusiasm for engineered immunity is tempered by the possibility of on-target/off-organ reactivity of the modified cells, and the cautionary tales of clonal outgrowth in HSC gene therapy patients merit the inclusion of safety measures in vector design. The inclusion of a suicide gene could provide a safety switch capable of ablating gene-modified cells in the event of undesirable off-target reactivity or clonal transformation. The ability to non-invasively track gene-modified cells *in vivo* would allow early detection of successful engraftment, active thymopoiesis, and homing to tumor tissue.

The humanized mouse allows the study of HSCs and development of their progeny *in vivo*. We used this model system to investigate the potential application of the PET reporter/suicide gene sr39TK in the setting of HSC based engineered immunotherapy to non-invasively locate and ablate gene modified cells. We observed no detrimental effect of lentiviral transduction with the ESO/TK vector on the engraftment of PBSCs as evidenced by equivalent human chimerism and lymphoid composition between transduced and mock transduced cohorts. Detection of gene-modified cells by PET was ubiquitous in ESO/TK transduced humanized mice (N=15), though only 8/15 (53.33%) had detectable NY-ESO-1-TCR+ cells in peripheral blood at 2-months post-transplant. Therefore, PET imaging allowed early assessment of engraftment of gene-modified cells before

NY-ESO-1-TCR+ cells have developed and migrated to the periphery in sufficient numbers for flow cytometric analysis.

A previous report used bioluminescent imaging and the luciferase reporter to visualize gene-modified human HSCs and their progeny residing in hematopoietic niches in a humanized mouse model (45). Our work expands on this pioneering study by using PET imaging, a higher-resolution, directly clinically translatable approach, to locate human HSCs *in vivo*. HSCs modified to express sr39TK were observed in hematopoietic niches, such as the long bones of the arms and legs and the thymus after dosing with [^{18}F]-FHBG. Strong sternal signal in mice led us to include this hematopoietic niche in our harvests, a practice not routinely performed in humanized mouse studies yet an abundant source of hematopoietic cells. Punctate murine vertebral marking with engraftment of vector-bearing cells (Supplemental Figure 3) directly demonstrates the high-resolution possible with this imaging technology.

While the immunogenicity of sr39TK has been reported in human studies of gene modified T-cells (46, 47), in the setting of gene modified HSCs, *de novo* generated DCs may home to the thymus and induce tolerance to the introduced gene product (48). Currently, only in silico predictive models of human immunogenicity exist, and the only true test is to evaluate the development of an immune reaction to a transgene in clinical trials. Still, there are alternative approaches that do not rely on viral-derived or otherwise xenogeneic reporter genes (37, 49).

Although PET signal was completely ablated after GCV treatment, we detected a small amount of vector-containing cells in harvested hematopoietic compartments by qPCR. This may indicate that some transduced HSCs were GCV resistant and generated new cells post-GCV treatment. Longitudinal studies to examine these possibilities in small animals are technically difficult owing to the paucity of human cells generated, though a recent study examining sr39TK

mediated ablation of rhesus macaque HSCs provides evidence that a single round of GCV is sufficient to ablate stem cells (50). The elimination of the majority of modified cells should be sufficient to control major toxicities.

sr39TK allows evaluation of successful engraftment of gene-modified HSCs *in vivo* with high resolution, and the detection of thymic engraftment indicative of developing anti-cancer TCR expressing T-cells. It may further be used to examine the homing of gene-modified T-cells to intended tumor targets and eradication of disease. In the event of off-target cytotoxicity by engineered T-cells, GvHD, or insertional oncogenesis, the suicide gene function of sr39TK could be harnessed to eliminate modified cells while importantly sparing the remaining unmodified graft. Our study supports the hypothesis that a clinical approach to engineered HSC immunotherapy would benefit from the inclusion of an imaging/suicide gene.

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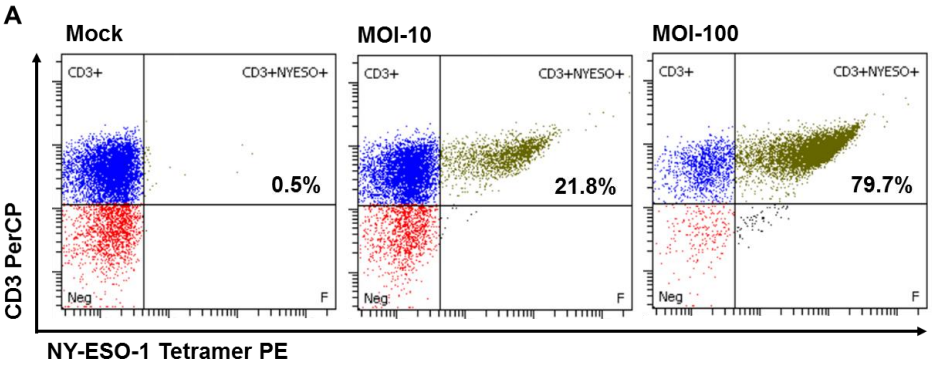
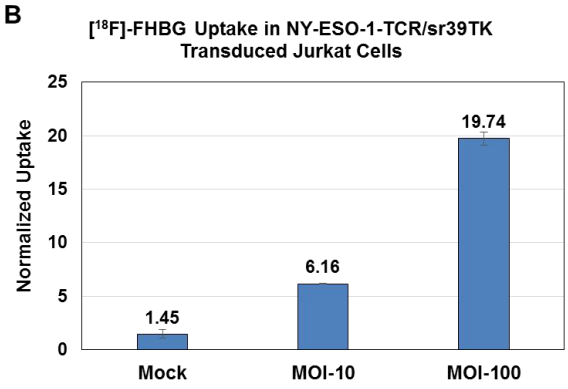
Authorship Contributions

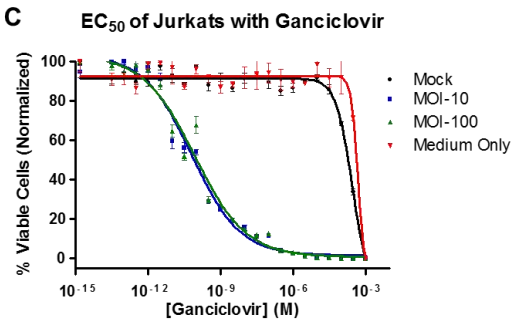
Eric H. Gschweng, Melissa N. McCracken, Roger P. Hollis, Antoni Ribas, Owen N. Witte, and Donald B. Kohn designed research. EHG, MNM, MLK, NS, RCK, and TC performed research. MLK, MH, RPH, RCK, TC, and AR contributed vital reagents. EHG, MNM, and XW analyzed data. EHG and DBK wrote the paper, and MNM, XW, AR, and ONW contributed significantly to manuscript preparation.

Supplemental Material

Supplemental Figure 1





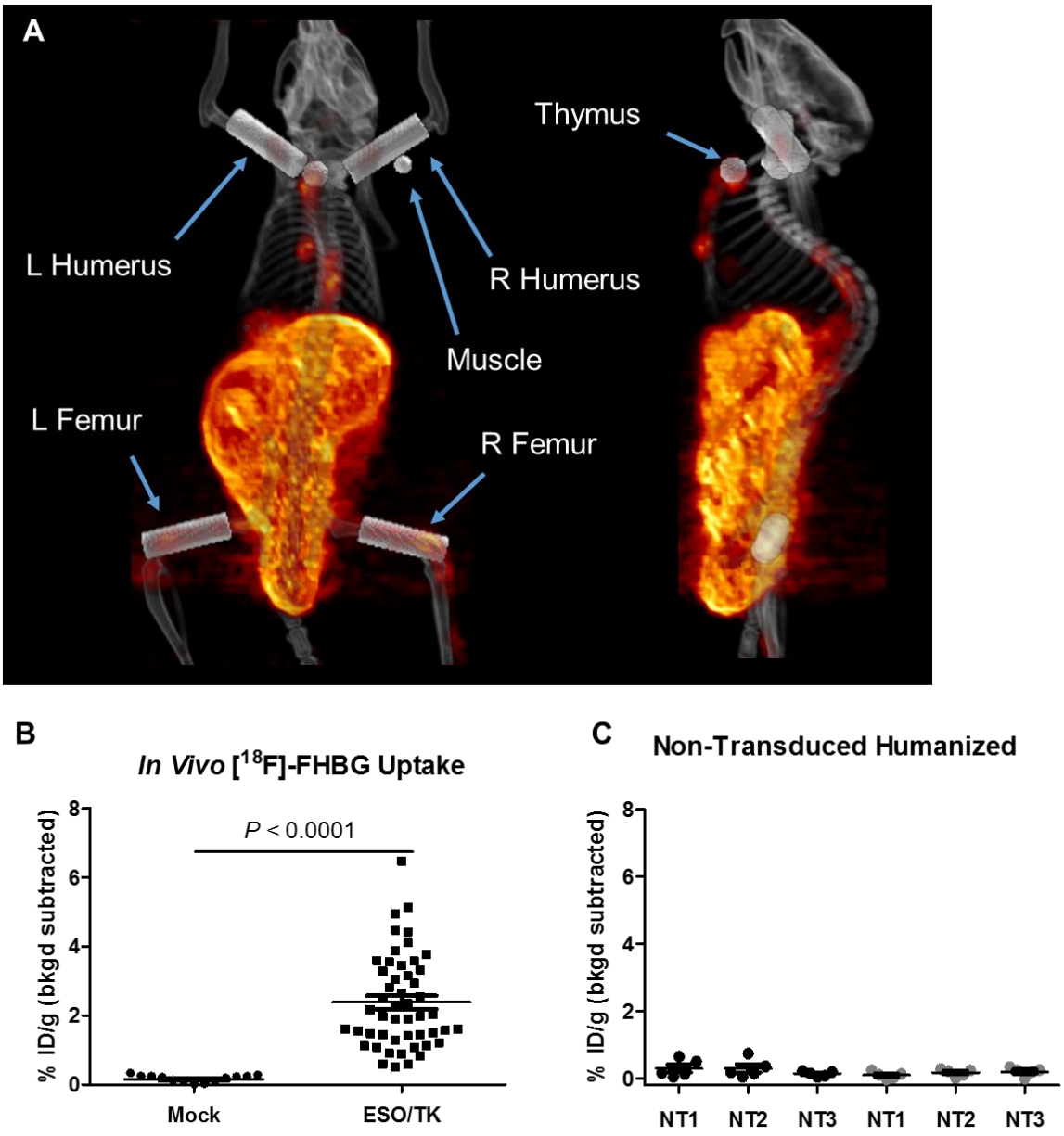


	Mock	MOI-10	MOI-100	Medium Only
EC50	0.0002888	6.370e-011	8.454e-011	0.0004830

	Mock	MOI-10	MOI-100	Medium Only
R square	0.9483	0.9773	0.9727	0.9144

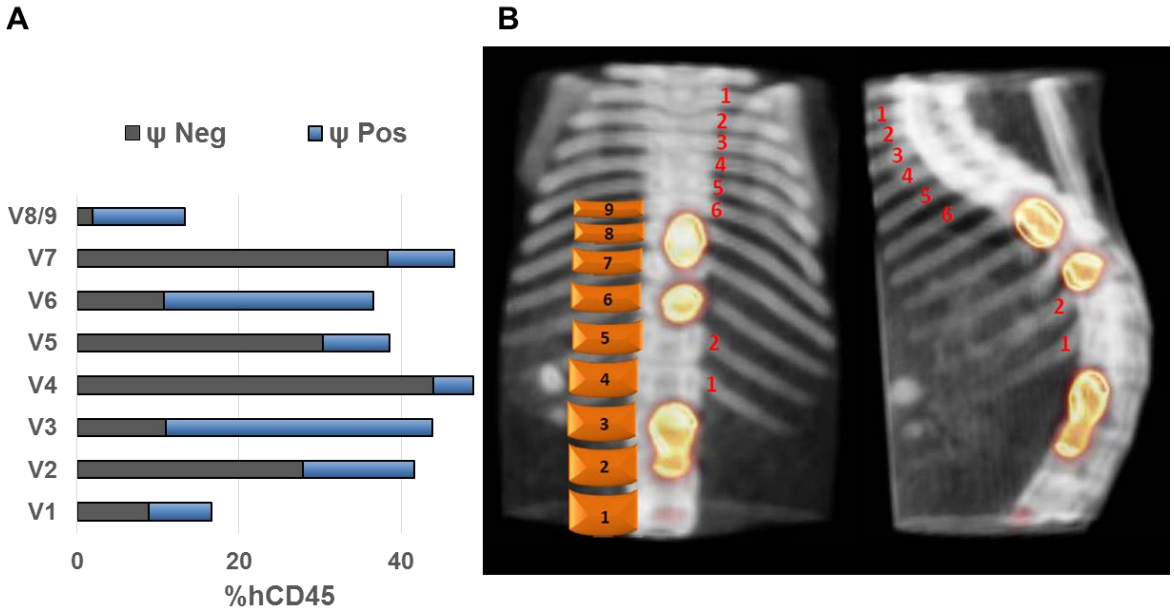
Validation of sr39TK function in Jurkat cells. (A) Mock or ESO/TK transduced Jurkats were evaluated by flow cytometry for NY-ESO-1 tetramer binding. (B) Cells were cultured with 0.5µCi [^{18}F]-FHBG for 1 hour, washed, and evaluated for uptake. (C) Cells were cultured in half-log increasing concentrations of GCV for 48h and evaluated for viability.

Supplemental Figure 2



ROI Analysis. (A) Schematic of ROIs drawn on PET imaged mice. 3D cylindrical or spherical ROIs were drawn using Amide software on hematopoietic niches to determine probe uptake and arm muscle for background subtraction. (B) There was significant [^{18}F]-FHBG uptake in the hematopoietic niches of ESO/TK mice compared with mock transduced controls. (C) [^{18}F]-FHBG uptake in non-transduced humanized mice before and after GCV treatment.

Supplemental Figure 3



Chimerism, vector marking, and PET probe signal in vertebrae. (A) Cells harvested from individual vertebrae were evaluated for human chimerism by flow cytometry and vector marking by qPCR. (B) Data were matched with [^{18}F]-FHBG PET scan.

Supplemental Table 1. Total human chimerism and lymphoid composition in NSG recipients of ESO/TK-transduced or non-transduced PBSC.

	Non-Transduced	ESO/TK-Transduced
Total PB Chimerism (% of lymphocytes)	35.44±10.60%	32.00±2.97%
CD19+ B Cells	70.94±15.09%	69.47±6.24%
CD3+ T Cells	7.70±5.55%	8.49±4.03%
% CD4+/CD3+ T Cells	22.36±8.09%	35.67±10.23%
% CD8+/CD3+ T Cells	19.74±6.63%	31.59±4.93%

Supplemental Methods

NY-ESO-1-TCR/sr39TK vector cloning and virus production

The TCR recognizing the NY-ESO-1 cancer/testes antigen has been previously described (1). Modifications to this self-inactivating (SIN) lentiviral vector include codon optimization of the 2A-linked TCR alpha and beta cDNA and 2A linkage to a codon-optimized sr39TK sequence, internal Murine Stem Cell Virus (MSCV) LTR promoter, and the WPRE (ESO/TK). Large-scale manufacture of concentrated lentivirus using a 2nd generation self-inactivating HIV-1 vector system was performed as described previously (2). Briefly, 239T cells were transfected with (150ug) ESO/TK transfer plasmid, (150ug) p8.9 HIV-1 gag-pol expression plasmid, and (30ug) pMD-G VSV-G expression plasmid. One day following transfection, sodium butyrate induction was performed for 8h. Viral supernatant was harvested on d4 and d5 of production, followed by 2000-fold concentration by tangential flow filtration (SpectrumLabs, CA) with diafiltration to 10% X-VIVO-15 medium (Lonza, Walkersville, MD). qPCR titers on HT29 cells of concentrated preps ranged from 7.6x10^8 TU/ml-4.0x10^9 TU/ml.

NSG-A2.1 Mice

NSG mice harboring a transgene encoding the human HLA-A2.1 protein covalently linked to human beta 2 microglobulin (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(HLA-A/H2-D/B2M)1Dvs/SzJ, Stock number 014570) were obtained from The Jackson Laboratory (Bar Harbor, ME) (3). Mice were bred, housed, and monitored according to UCLA Department of Laboratory Animal Medicine standards.

Transduction of Jurkat cells and uptake assay

Jurkat cells (ATCC TIB-152) were transduced with the ESO/TK lentiviral vector at an MOI of 10 and 100, cultured for 2 weeks, and assayed for surface TCR expression by tetramer staining to validate transduction. Mock transduced, MOI-10 and MOI-100 transduced Jurkats were cultured in 0.5µCi [^{18}F]-FHBG for 1hr, cells were washed 3X, and resuspended in 1ml culture medium. Uptake was measured on a Wallac WIZARD scintillation counter (Perkin Elmer, Waltham, MA) using RiaCalc WIZ software (Perkin Elmer).

HSC Isolation and Purification

G-CSF mobilized peripheral blood units were purchased from Cincinnati Children's Hospital Medical Center and processed with the CliniMACS CD34 Reagent System, CD34 Kit, and Tubing System (Miltenti, Auburn, CA) per manufacturer's instructions. Approximately 3.44x10^9 CD34+ cells were obtained from approximately 150ml apheresis product at a purity of >98% as assessed by flow cytometry. Aliquots of 5.0x10^6 cells were frozen in Pentastarch + 10% DMSO at -80C overnight then transferred to liquid nitrogen for long-term storage.

VCN Analysis

Vector copy number was determined by digital droplet quantitative PCR (ddPCR) for the lentiviral psi element (FWD: AAG TAG TGT GTG CCC GTC TG, REV: CCT CTG GTT TCC CTT TCG CT, PRO: 5'-FAM / CCC TCA GAC / ZEN / CCT TTT AGT) and normalized to the endogenous human SDC4 reference gene (FWD: CAG GGT CTG GGA GCC AAG T, REV: GCA CAG TGC TGG ACA TTG ACA, PRO: 5'-HEX / CCC ACC GAA CCC AAG AAA CTA). Genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey Nagel, Bethlehem, PA). PCR using 200ng genomic DNA template, [400nM] primers and [100nM] probe, and 1000U DraI

per reaction was digested for 1hr at 37C. Digested pre-PCR reactions were run through the QX100 Droplet Generator (BioRad, Hercules, CA) followed by the following reaction conditions: 95C for 10min, [94C for 30sec, 60C for 1min] for 55 cycles, 98C for 10min, and 12C hold on a T100 thermal cycler (BioRad). Droplets were read on a QX100 Droplet Reader (BioRad).

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CHAPTER 5:

Development of NY-ESO-1 TCR bearing T cells from human HSCs *in vivo* is dependent on HLA-A2.1.

Introduction

Engineering hematopoietic stem cells (HSC) for cancer immunotherapy has the potential to provide a life-long supply of engineered T cells, overcoming current limitations associated with the use of a differentiated T cell based infusion product. Affinity enhanced T cell receptors (TCR) are being designed to increase anti-cancer efficacy, for example to target cancer cells that have down-regulated major histocompatibility complex (MHC) expression to avoid immune cell detection. However, the development of T cells from an engineered HSC transplant may be negatively affected when using a TCR with supra-physiologic affinity by signaling during thymopoiesis and inducing negative selection.

We studied the development of T cells from HSCs transduced to express an affinity enhanced NY-ESO-1 TCR in a humanized mouse model to investigate the feasibility of this approach. While polyclonal T cells developed normally, we observed a failure of development of NY-ESO-1 TCR bearing cells from engineered HSCs in the absence of HLA-A2.1 in both the host and donor cells. A NOD.scid.Prkdc-/- (NSG) mouse engineered to express a single chain HLA-A2.1 covalently linked to B2M was sufficient to allow development of T cells bearing the NY-ESO-1 TCR. Accelerated development to CD8SP stage of engineered T cells was observed.

To investigate the *in vivo* effector ability of gene engineered T cells, we transplanted NY-ESO-1 HLA-A2.1 patient derived melanoma cell lines subcutaneously to engineered humanized mice. While previous studies showed these TCR-expressing T cells capable of *ex vivo* effector function, we were surprised to discover *in vivo* effector function was absent. Tumor growth as well as histological examination showed no retardation of tumor growth or presence of tumor infiltrating lymphocytes (TIL). To investigate if this phenomenon was intrinsic to the relatively lymphopenic src-SCID humanized mouse model, we conducted study in the thymic-liver organoid

(LT) implant mouse model. One of two gene therapy treated LT mice halted growth of an engineered tumor, but not the contralateral tumor xenograft. *Ex vivo* analysis showed the presence of activated TILs within tumor tissue in contact with target cells.

These studies demonstrate the ability of affinity enhanced TCRs to develop *in vivo,* as well as the necessity of HLA-A2.1 for optimum *in vivo* development of HLA restricted TCRs from HSCs. Further, we show *in vivo* efficacy from tumor challenge from an NY-ESO-1 peptide presenting tumor xenograft.

Materials and Methods

Mice

All animals were cared for under the supervision of the UCLA Department of Animal Laboratory Medicine (DLAM) using DLAM approved protocols. Mouse strains in this study were purchased from The Jackson Laboratory (Bar Harbor, ME): NOD.Cg-Prkdc^{scid} Il2rg$^{tm1Wjl/SzJ}$ (NSG) (Jax 005557) and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(HLA-A/H2-D/B2M)1Dvs/SzJ (NSG-A2.1) (Jax 014570). Animals were weaned at age 21d, and co-housed with same-sex littermates.

Src-SCID humanized mice were generated as previously described (1). Briefly, 1x10^6 CD34+ G-CSF mobilized peripheral blood stem cells in 50µl of X-VIVO-15+FST3 were transplanted to irradiated (100cGy) NSG neonatal pups via intrahepatic injection with a 30g tuberculin syringe. PBSCs were mock transduced, or transduced with a concentrated lentiviral vector encoding codon-optimized, 2A-linked αβ chains of the $_{157\text{-}165}$NY-ESO-1 TCR chains 2A linked to HSV-sr39K followed by the woodchuck hepatitis virus posttranscriptional response element (WPRE) (ESO/TK) at a concentration of 1x10^{8}TU/ml. Vectors were produced, concentrated, and titered as previously described (2).

LT humanized mice were generated as previously described (3). Briefly, transplant of human fetal thymus and liver to the subrenal capsule of NSG mice was performed under general anesthesia. Fetal liver was mock transduced or transduced with the ESO/TK vector as above.

Two months post-transplant, mice were sacrificed via CO_2 asphyxiation. Tissues were immediately harvested for dissociation and analysis.

Tissue dissociation

Thymuses were dissociated over a 70µm mesh strainer (Falcon) using a 3ml syringe plunger (BD) in RPMI+10%FBS. Spleens were dissociated using 23g needles by piercing the splenic capsule and applying pressure to force cells out. Extruded cells were pipetted with a p1000 to dissociate. Bone marrow was flushed from femurs using a 25g needle and pipetted with a p1000 to dissociate. All cell suspensions were filtered over a 70µm mesh strainer in RPMI+10%FBS and enumerated on a ViCell (Beckman Coulter) automated cell counter.

Flow cytometry

Analysis of humanized mice was performed as previously described (1). Briefly, 2-5x10^6 cells were resuspended in 100ul MACS buffer and stained using the following antibodies (Becton Dickinson (BD), Laguna Hills, CA) to assess human engraftment and development: murine CD45 clone 30-F11, human CD45 clone HI30, human CD19 clone SJ25C1, human CD3 clone SK7, human CD4 clone RPA-T4, human CD8 clone HIT8a, human CD69 clone FN50, and human CD5 clone L17F12. Dead cells were excluded by gating on the DAPI negative population. Expression of the NY-ESO-1-TCR was determined by binding to a PE-labeled HLA-A2.1 MHC-tetramer loaded with the $_{157\text{-}165}$NY-ESO-1 SLLMWITQC peptide (Beckman Coulter, Brea, CA). Antibodies were added to 2-5x10^6 cells from dissociated tissue, incubated in the dark for 30min, washed with 3ml MACS buffer with DAPI, spun at 500g for 5min, and resuspended in 250ul MACS buffer. Data were acquired on a FACS LSRII or Fortessa (BD). Analysis was performed using FlowJo (Tree Star Inc, Ashland, OR).

Tumor Challenge

The HLA-A2.1+ NY-ESO-1+ primary patient melanoma cell line M407 was subcutaneously injected to humanized mice at a dose of $2x10^6$ cells in a 100µl volume of 50/50 mixture of Matrigel and DMEM+10%FBS. K562 cells or K562 cells expressing a single chain trimer of HLA-A2.1, beta2microglobulin (β2M), and the $_{157\text{-}165}$NY-ESO-1 peptide (V0171) were subcutaneously transplanted to humanized mice on the left and right contralateral shoulder flank, respectively, at a dose of $3x10^6$ cells each in a 50/50 mixture of Matrigel (BD) and DMEM+10%FBS. Tumor growth was monitored using calipers. Mice were euthanized if tumor diameter along the longest axis exceeded 1.5cm per UCLA Division of Laboratory Animal Medicine guidelines.

Results

Development of thymocytes is linked to markers of activation

Newborn thymus was dissociated and investigated by flow cytometry for expression of CD69 and CD5, markers of thymocyte development. CD69 is expressed on a proportion of $CD4_{neg}$ $CD8_{neg}$ early thymocytes, and expression increases on TCRαβ thymocytes in the context of TCR-MHC signaling (4, 5). CD5 is recruited and clusters with CD3 at the immunological synapse and reduces the intensity of TCR signaling. The intensity of expression of CD5 on DP thymocytes is indicative of TCR engagement, and expression is maintained on mature thymocytes (6, 7). A developmental continuum was identified beginning with $CD69_{neg}$ $CD5_{neg}$, progression to $CD69_{int}$ $CD5_{neg}$, and ending with $CD5_{pos}$ (Fig 1A). Gating on the inverse population revealed DP cells were $CD69_{low/int}$ $CD5_{neg}$, while late stage CD4SP cells were predominantly $CD69_{pos}$ and CD8SP cells were $CD69_{low/neg}$ (Fig1B). These observations show a progressive phenotype that can be used to identify the developmental stage of thymocytes, and couple development to the engagement of TCR and MHC.

Normal human thymic development is recapitulated in LT and src-SCID mouse models

To investigate the fidelity of thymocyte development in humanized mice, both src-SCID and LT models were utilized. While developing thymocytes in the LT mouse model interact with normal human thymic stroma and form thymic architecture that resembles normal human thymus (Fig 2A, B), src-SCID model interact with murine thymic stroma. The src-SCID thymus is hypotrophic, with approximately 1000-fold less cellularity than the LT thymic organoid (not shown). Medullary thymic architecture is absent from src-SCID thymus (Fig 2C), while the LT mouse organoid develops proper thymic architecture and AIRE+ cells (Fig 2D). The presence of

Figure 1. The activation markers CD69 and CD5 delineate progressive developmental stages of human fetal thymocytes

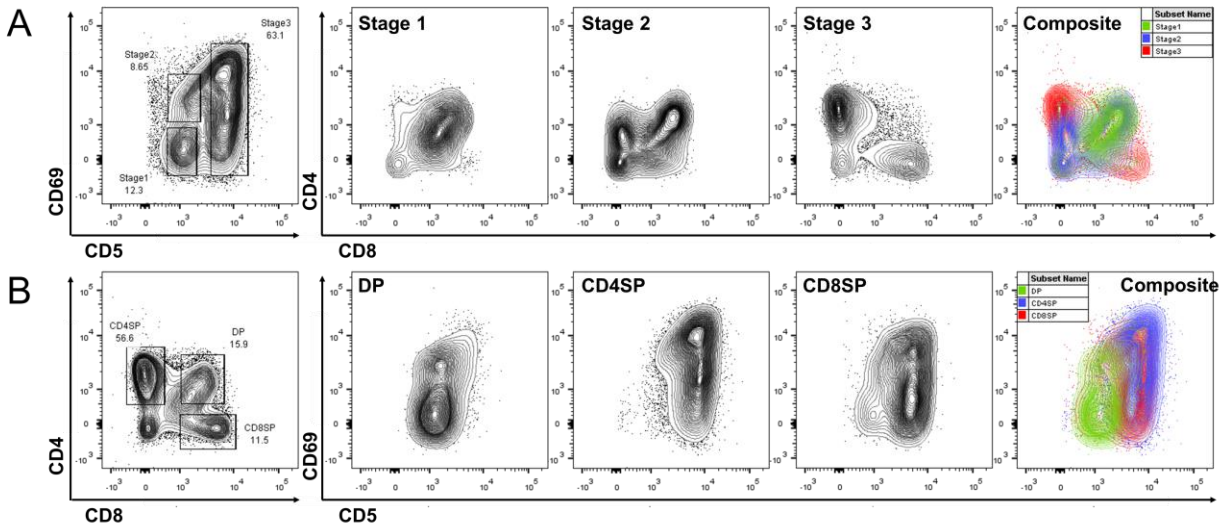

Fig 1. Fetal thymocytes display a continuous phenotype of CD69 and CD5 surface expression. A: Subgating on $CD69_{neg}$ $CD5_{neg}$ (Stage 1) shows this population is $CD4_{low}$ $CD8_{low}$ early DP. Gating on $CD69_{int}$ $CD5_{low}$ (Stage 2) shows a progression in development to CD4 CD8 DP. Gating on the $CD5_{pos}$ (Stage 3) population delineates mature CD4 or CD8 SP populations. B: Inverse gating strategy shows CD4 CD8 DP cells are $CD69_{neg/int}$ $CD5_{neg}$, while CD4SP and CD8SP mature thymocytes are $CD5_{pos}$ and $CD69_{neg/int/pos}$ expression.

AIRE+ cells is notable as they instruct normal T cell development, and shape the T cell repertoire by deleting self-reactive cells and inducing T_{reg} formation (Reviewed in (8)). Nonetheless, development of SP cells and the CD69 CD5 signaling continuum was observed in the src-SCID thymocytes (Fig 2E), indicating that communication between polyclonal human TCRs and murine MHC in this model is sufficient to educate the development of mature thymocytes.

NY-ESO-1 TCR bearing cells require HLA-A2.1 for development

Our studies used the NY-ESO-1 specific TCR, designated 1G4, with two mutations in the CDR3α region to increase affinity and enhance anti-cancer activity (9). This TCR is restricted to recognizing the $_{157\text{-}165}$NY-ESO-1 peptide in the context of HLA-A2.1. To study the role of HLA-A2.1 expression on the development of NY-ESO-1 TCR bearing thymocytes developing from engineered HSPCs, we investigated the expression of CD5 and CD69 in LT humanized mice generated from HLA-A2.1 positive tissue, and non HLA-A2.1 tissue. Analysis of the polyclonal, non-engineered thymocytes showed a similar developmental pattern as seen in normal human thymocytes (Fig1A) with the $CD69_{neg}$ $CD5_{neg}$ population in the DP stage, progressive development of CD4 and CD8SP cells in the $CD69_{int}$ $CD5_{neg}$ gate, and the bulk of mature CD4 and CD8SP cells in the $CD5_{pos}$ gate (Fig 3A).

When examining the NY-ESO-1 TCR bearing cells in non-HLA-A2.1 LT mice, a failure of progression past developmental stage 2 was observed with the majority of cells residing in the $CD69_{neg}$ $CD5_{neg}$ or $CD69_{int}$ $CD5_{neg}$ populations, and a lack of cells in the $CD5_{pos}$ population (Fig 3B). Accordingly, CD4 and CD8 expression analysis in these populations show a lack of NY-ESO-1 TCR bearing cells in the SP populations, with most cells halted in the DP stage.

Figure 2. Humanized mouse thymic architecture recapitulates normal human thymus development

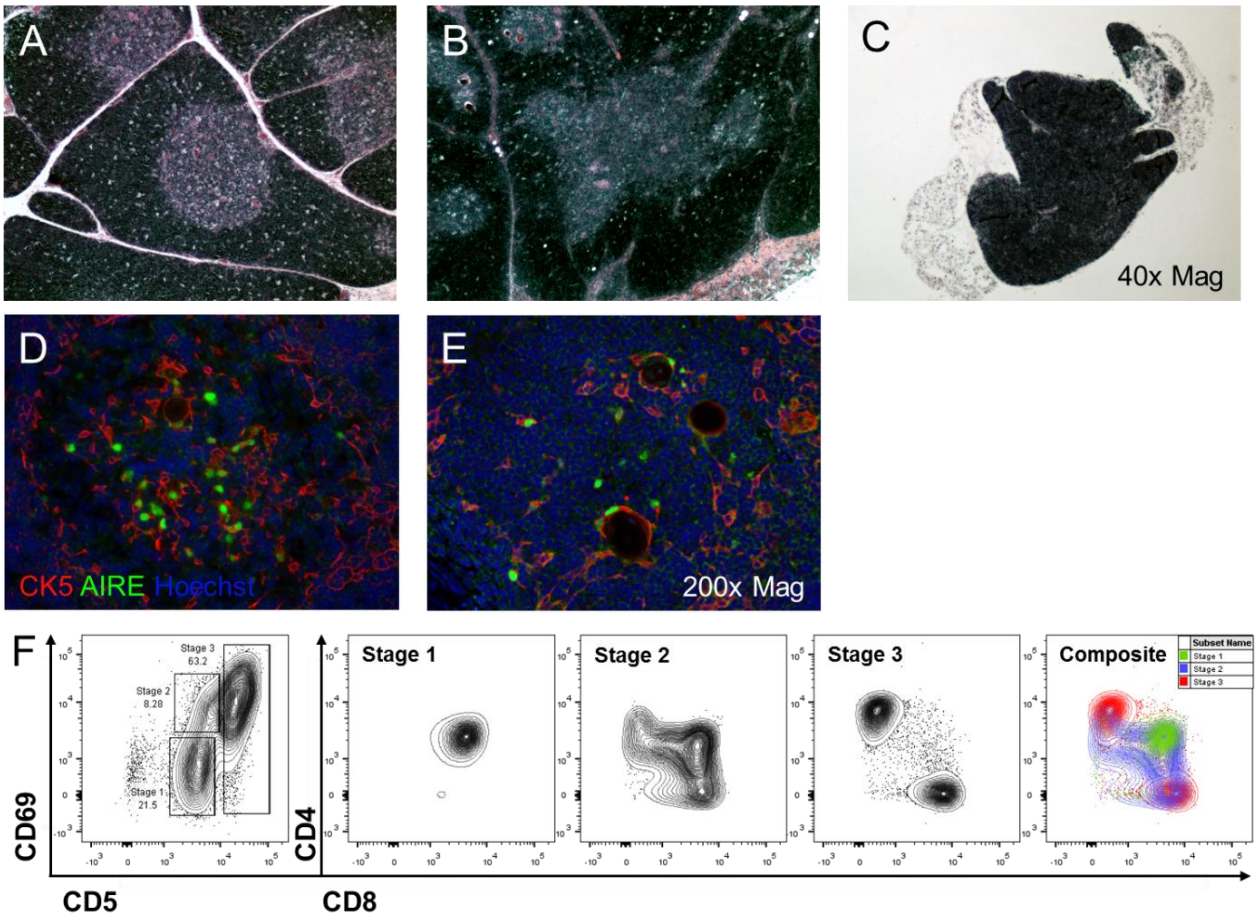

Fig2 A: Histology of a normal human thymus stained with hematoxylin and eosin (H&E) shows a thymocyte dense cortical region surrounding a relatively sparsely populated thymic medulla. B: H&E stained thymic organoid section from an LT mouse shows similar pattern to normal human thymus with dense cortex and lightly populated medulla. C. src-SCID thymic sections are markedly hypocellular with an absence of medullary region. D: IHC staining of normal human and E: LT thymic organoid shows presence of cytokeratin 5 positive mTECs and mature AIRE expressing cells. F: Flow cytometric examination of src-SCID thymocytes reveals a pattern of CD69, CD5, CD4, and CD8 expression that parallels normal human thymic development.

Conversely, examination of LT mice generated with HLA-A2.1 positive tissue showed thymocytes bearing the NY-ESO-1 TCR residing mostly in the $CD5_{pos}$ mature stage, and an

absence of $CD69_{neg}$ $CD5_{neg}$ cells (Fig 3C). Gating on these populations showed increased CD4 and CD8SP cells, indicative of accelerated development of engineered cells in agreement with previous studies of thymocyte development bearing a prearranged TCR(10-12).

NY-ESO-1 TCR bearing T cells developed from HSPCs are capable of robust anti-tumor activity *in vivo*

To assess the functional activity of T cells developed from engineered HSPCs, we designed an *in vivo* tumor challenge model. LT mice generated with mock transduced HSPCs or ESO/TK transduced HSPCs were generated as described above. At two months post-transplant, mice were injected with $3x10^6$ K562 cells subcutaneously in the left shoulder flank as a negative control. In the contralateral shoulder flank, mice were injected with $3x10^6$ K562 transduced with a vector expressing the $_{157\text{-}165}$NY-ESO-1 peptide covalently linked to HLA-A2.1 and β2M (V1071). Single chain trimer constructs ensure surface expressed MHC are stably loaded with the $_{157\text{-}165}$NY-ESO-1 peptide, and are targets of the 1G4 TCR (13).

K562 tumors grew rapidly in both the mock LT mice as well as ESO/TK generated LT mice (Fig 4A). At 16d post-transplant, 4 of 5 experimental animals had reached or approached UCLA DLAM limit of acceptable tumor size in either the K562 or V1071, and had to be euthanized, whereas one ESO/TK mouse was continued on study. Where mock LT mice were unable to control the growth of V1071 tumors, one of two ESO/TK mice was capable of completely preventing tumor growth (Fig4B). Residual tumor mass was present, showed evident neovascularization, and was white in coloration (data not shown). The hypocellular V1071 tumor

Figure 3. The development of NY-ESO-1 TCR bearing cells is dependent on the expression of HLA-A2.1

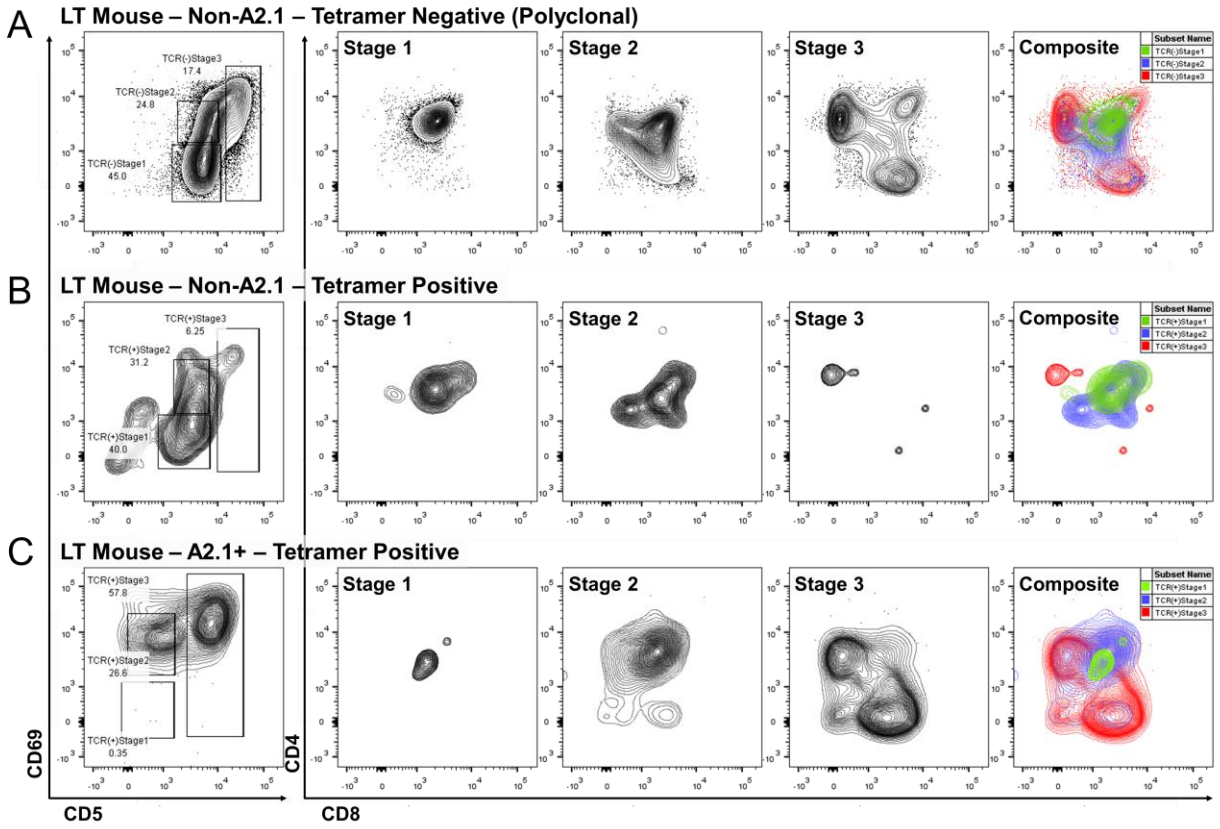

Fig3 A: Polyclonal (non-engineered) CD3+ cells in LT thymus showed a developmental continuum characteristic of normal human thymocyte development, beginning with $CD69_{neg}CD5_{neg}$, progressing to $CD69_{dim}CD5_{neg}$, and ending in $CD5_{pos}$ cells. B: NY-ESO-1 TCR bearing cells developing in non-A2.1+ LT mice reached a developmental block in Stage 2, with $CD69_{neg/low}$ phenotype, and did not progress to CD4 or CD8SP mature thymocytes. C: Thymocytes bearing the NY-ESO-1 TCR showed $CD69_{int/pos}CD5_{pos}$ phenotype and were predominantly Stage 3 CD8SP T cells.

was dissociated over a 70μm mesh, and cells were cultured in RPMI+10%FBS with 300IU/ml IL-2. At 4 days of culture, cells were stained to investigate immunological phenotype (Fig4C). Cells cultured from V1071 tumors were predominantly human CD45+ CD3+ lymphocytes. Of the CD3 positive population, 82.7% bound the $_{157\text{-}165}$NY-ESO-1 tetramer. Tetramer negative CD3 cells showed either $CD4_{pos}$ or $CD8_{pos}CD4_{dim}$ phenotype, characteristic of activated T cells (14, 15). The tetramer positive population was exclusively $CD8_{pos}CD4_{dim}$. Taken together, these data indicate that LT mice generated from ESO/TK transduced HSPCs generate an active immune system capable of eradicating an aggressive tumor burden *in vivo*.

Figure 4. NY-ESO-1 TCR engineered HSPCs developing in the context of HLA-A2.1 are capable of providing *in vivo* tumor protection

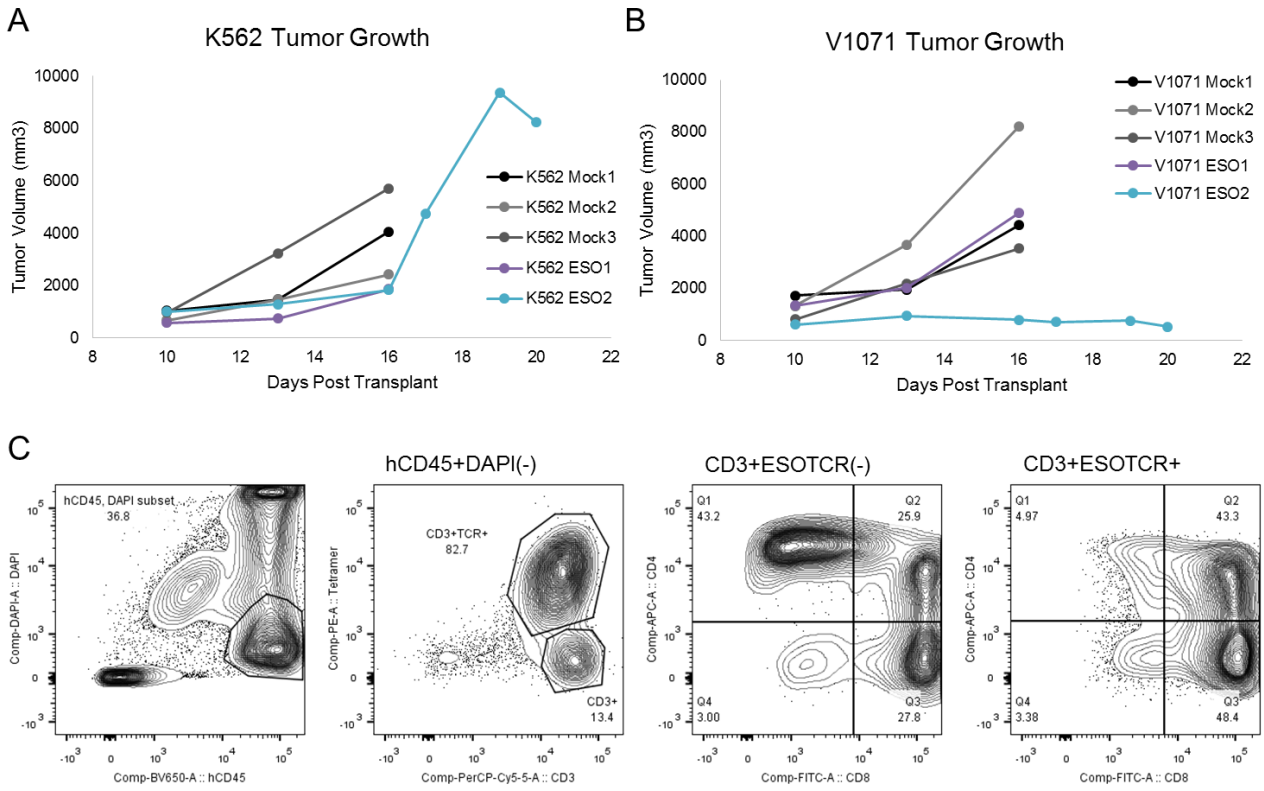

Fig 4A: K562 tumors grew rapidly in both Mock LT and ESO/TK LT mice. B: V1071 tumors grew aggressively in Mock LT mice, but one of two ESO/TK mice was capable of controlling V1071 tumor growth. C. The immunophenotype of cells grown from the explanted growth controlled V1071 tumor was determined by flow cytometry. The majority of cells were $hCD45_{pos}hCD3_{pos}$, with 82.7% of the CD3pos cells bearing the NY-ESO-1 TCR. NY-ESO-1 TCR(-) cells were both CD4 and CD8SP cells, while NY-ESO-1 TCR+ cells were entirely of an activated $CD4_{dim}CD8_{pos}$ phenotype.

Discussion

The development of mature T cells is dependent on the expression of TCRs consisting of αβ chain pairs generated by independent random genomic rearrangements. Further, these TCRs must interact with self-peptide loaded MHC class I or II expressed by the thymic stroma in a fashion that generates an intermediate strength signal. Too little signal, and the cells die of neglect; too much, and the cells are deleted to prevent autoimmunity (16, 17). The reactivity of a pre-arranged TCR with thymic stroma presenting MHC loaded with self-peptides is of critical concern when designing an HSC based approach to engineering immunity. As cancer target antigens are typically self-expressed normal antigens, and affinity enhanced TCRs generate stronger immune responses, the ability of these TCRs to undergo development without being negatively selected must be evaluated.

This study sought to study the development of T cells, either polyclonal non-transgenic or engineered to express an affinity enhanced NY-ESO-1 TCR, *in vivo* in humanized mouse models. We established that T cell development can be coupled to TCR – MHC interaction by investigating the expression of CD69, CD5, CD4, and CD8 in developing thymocytes in mice with or without the cognate human HLA A2.1. In both the src-SCID and LT humanized mouse models, polyclonal T cells displayed a signaling and development expression pattern reminiscent of thymocytes from normal human thymus. Histologically, LT mouse thymic organoids appear structurally identical to normal human thymus, with a clearly defined corext and medulla and mature, AIRE+ mTECs. Somewhat perplexing is the absence of the thymic medulla in histological examination of src-SCID thymus. Nevertheless, development of mature peripheral T cellswais observed in this model (1, 10, 18), suggesting a sufficient, albeit hypocellular, thymic architecture.

The presence of HLA-A2.1 was found critical for the development of mature NY-ESO-1 TCR bearing cells in LT mouse thymic organoids, with non-A2.1 tissue insufficient to generate TCR – MHC signal required for development. In A2.1+ LT mice, we observed markers of strong activation in the NY-ESO-1 TCR bearing thymocytes. This indicated activation was sufficient for development, yet apparently did not exceed the negative selection threshold. This observation is important from the consideration of using this specific TCR in humans in a trial for cancer immunotherapy via engineered HSCs, demonstrating the TCR will be positively selected only in the presence of HLA-A2.1 stroma, yet not deleted via central tolerance. Further, the output T cells were capable of eliminating an aggressive tumor lesion *in vivo*. Together, these data highlight the importance of selection of donor tissue when modeling a specific TCR using humanized mouse models, as thymic stroma mis-matched for the MHC restriction of the TCR can result in a failure of thymic development.

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CHAPTER 5:

Conclusions and Future Studies

In 2013, Science magazine named engineering immunity the breakthrough of the year (1). This paradigm shift diverted from previous therapies that sought to targeted cancer cells directly, carrying side-effects on normal tissue that were, in some cases, more devastating than the disease itself. Harnessing the immune system has coupled surgical precision with systemic surveillance. The clinical benefit of engineered immunity is evident in case reports of patients cured of disease when all other treatments have failed, and the explosive growth of the private sector in this field makes it clear that engineering the immune system to combat illness is the way of the future.

The studies that compose this thesis (2-4) detail the use of genetically engineered hematopoietic stem cells (HSC) as a means to deliver a long-term, potentially life-long, supply of immune cells that fight cancerous lesions, the ability to non-invasively monitor the graft with positron emission tomography (PET) imaging, and the ablation of gene modified cells using HSV-sr39TK as a suicide gene. In addition to a relatively young, albeit potent, body of work before this (5-7), there is strong support for the feasibility of HSCs in cancer immunotherapy. Indeed, a clinical trial led by Dr. Antoni Ribas at UCLA will enroll patients before the end of 2015 using NY-ESO-1 T cell receptor (TCR) transduced HSCs cure patients of melanoma: modeled by the work detailed in this thesis. This trial will no doubt be the harbinger of many future therapies to use stem cells to shape the immune system to cure disease.

Moving forward, there are several areas that merit discussion. New targets will be needed to expand on the successes seen with MART-1, NY-ESO-1, MAGE-A3, etc. The choice of target is no easy matter, as most cancer antigens are also expressed on normal tissue, and bring on-target / off-tumor cytotoxicity (Reviewed in (8, 9)). Approaches to minimize this undesired outcome by increasing specificity have been described, at least in the application of chimeric antigen receptors (CAR), by the use of TanCARs (10). In principle, a strong activation signal and cytotoxicity only

occurs when a TanCAR expressing effector recognizes two separate antigens on a single cell. The choice of these antigens is designed to increase specificity to cancer antigens, thereby reducing the likelihood of bystander cell death on normal tissue. It is easy to speculate on a similar design, using one strong activating CAR to recognize a cancer antigen, while including a second inhibitory CAR recognizing a normal tissue antigen to step on the brakes and reduce the killing of normal tissue. In the same vein, recent work describing "or-gate CARs," tandem recognition domains on a single CAR designed to allow targeting of multiple antigens by the same cell, will pave the road for "and-gate CARs" that require multiple antigens for activation (Y. Chen, personal communication).

Self-renewal and differentiation are the hallmarks of stem cells. While we demonstrated the use of human HSCs in cancer immunotherapy to provide a long-term supply of effector cells, the HSC may not be the only cell type capable of fulfilling this role. Central memory T cells (T_{CM}) have been demonstrated capable of self-renewal and the ability to generate all downstream cell types on the T cell continuum (11), even by stringent tests such as single cell adoptive transfer (12). Using T_{CM} reactive against common chronic human infections such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV) allows the isolation of T cells reactive against defined antigens, reducing the likelihood of off target cytotoxicity after engineering at least in the case of CAR (13). Further, endogenous anti-virus antigen TCR signaling against the chronic infection may support the long-term homeostasis of these cells, allowing persistence of the engineered cell infusion product. Unfortunately, in the case of TCR engineering, the introduction of an exogenous TCR to T cells can result in TCR mispairing and unpredictable antigen reactivity, in some cases with catastrophic results (14). Attempts to prevent the mispairing of endogenous and exogenous TCRs by engineering domains for preferential self-pairing and are steps towards the safe use of these cells ((15) and M. Bethune, personal communication). Another consideration is that using T_{CM}

will only yield the generation of T lineage cell types, thus losing the added benefit of generating myeloid cell types that may also have effector function in cancer immunotherapy (16, 17). HSCs may have an edge over T_{CM} in the arena of engineered immunotherapy in terms of safety and potency, though application in humans will provide a more definitive answer.

The broader application of any technology is made more palatable by refining its economy. Being able to engineer an infusion product at a limited number of central sites, and then distribute to multiple clinical sites for infusion will reduce cost by paring down the required number of specialized personnel and equipment. Memory cells with defined specificity, as mentioned above, may lack reactivity to allogeneic tissues, and the deletion of β2m to eliminate the surface expression of the major histocompatibility complex (MHC) (18), could prevent graft rejection. A long term goal of engineering the immune system should be to apply this technology from directed cytotoxicity to the creation of therapies for autoimmunity. Approaches to target regulatory T cells are under development (19). Taken together, these modifications could bring engineered immunity to a wider community.

The ability to non-invasively image the infused cell product is advantageous from the perspective of evaluating the success of engraftment and thymic reconstitution. Further, the ability to specifically image the immune system by metabolic specificities of activated immune cells (6, 20, 21) or specific labelling of immune cells themselves (22, 23) would allow early stratification of responders from non-responders of therapy. A step forward from the perspective of genetically engineering cells would be to include a human derived PET reporter gene (3) instead of a viral derived transgene to avoid the possibility of immunogenicity.

The use of HSCs for engineering immunity holds the promise to cure cancer by generating a theoretically limitless army of effector cells. Building the knowledge base of immunology and

cancer biology will broaden the approach, first to other cancers and eventually to other diseases. Advances in imaging technologies will allow early detection of disease, as well as the efficacy of genetic therapies and the ability to control them. In short time, these technological advances may move cancer from a devastating disease to a manageable malady treated on a routine, outpatient basis. The studies detailed herein sought to serve as a stepping stone to achieve this end, to add meaningful information to a growing body of work in this field, and to push engineered immunotherapy forward as a standard of care in the hope that the next generation might never know the devastation of having a family member with cancer.

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