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

Running title: sr39TK PET and suicide gene elimination of human HSCs

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Abstract

Engineering immunity against cancer by the adoptive transfer of hematopoietic stem cells (HSC) modified to express antigen-specific T-cell-receptors (TCR) or chimeric antigen receptors (CAR) generates a continual supply of effector T-cells, potentially providing superior anti-cancer efficacy compared with the infusion of terminally differentiated T-cells. Here we demonstrate the in vivo generation of functional effector T-cells from CD34-enriched human peripheral blood stem cells (PBSC) modified with a lentiviral vector designed for clinical use encoding a TCR recognizing the cancer/testes antigen NY-ESO-1, co-expressing the PET/suicide gene sr39TK. Ex vivo analysis of T-cells showed antigen- and HLA-restricted effector function against melanoma. Robust engraftment of gene-modified human cells was demonstrated with PET reporter imaging in hematopoietic niches such as femurs, humeri, vertebrae, and the thymus. Safety was demonstrated by the in vivo ablation of PET signal, NY-ESO-1-TCR bearing cells, and integrated lentiviral vector genomes upon treatment with ganciclovir (GCV), but not with vehicle control. Our study provides support for the efficacy and safety of gene-modified HSCs as a therapeutic modality for engineered cancer immunotherapy.
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 \textit{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 \textit{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 \textit{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 27-35 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-[18F]-fluoro-3-[hydroxymethyl]butyl)guanine ([18F]-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; 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 below. Immunological cytotoxicity assays were performed as previously described(41).

**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 ^{157-165}NY-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

[^18F]-FHBG was synthesized as described(42). Mice were injected IV with 250µCi[^18F]-FHBG in 50-100µl, 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 (43) 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).

Results

NY-ESO-1-TCR/sr39TK modified human HSCs engraft in NSG-A2.1 mice and generate functional NY-ESO-1 reactive T-cells 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-repeate 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 sub-fractioned 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 \textsuperscript{157-165}NY-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 \textit{in vivo} from transduced HSCs, experimental mice were harvested, splenocytes dissociated, and expanded by co-culture with artificial antigen presenting cells loaded with the \textsuperscript{157-165}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. \textit{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. \textsuperscript{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...
Minimal background cytotoxicity in non-transduced donor T-cells was observed (Figure 3C). 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 [¹⁸F]-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 (44). 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 [¹⁸F]-FHBG (Supplemental Figure 1B), and were selectively killed by GCV (Supplemental Figure 1C) confirming the functional activity of the ESO/TK vector.

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 [¹⁸F]-FHBG before and after treatment with the prodrug Ganciclovir (GCV) followed by investigation of cell composition by cell- and molecular-biological methods (Figure 3A). Non-transplanted NSG-A2.1 mice and transplant recipients of mock transduced or ESO/TK gene-modified human PBSC were injected with 250µCi [¹⁸F]-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 [¹⁸F]-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 (45). 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 3B). Evaluation of uptake in the spleen was occluded by GI signal. Non-transduced humanized mice showed similar background biodistribution of \(^{18}\text{F}\)-FHBG probe, and lack of hematopoietic niche signal (Figure 3C). 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 3D). 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).

**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 4A); 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 4B). 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 4C). GCV treated ESO/TK-transduced recipient mice showed significant ablation of $[^{18}F]$-FHBG PET signal in hematopoietic compartments in post-treatment scans (Figure 4D). 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 5A). 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 5B,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 5D).

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 5E). 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 5F). 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 5G) (P<0.001).

**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 (46). 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 $[^{18}\text{F}]$-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. The limit of detection using $[^{18}\text{F}]$-FHBG as a probe with the HSV-sr39TK PET reporter gene was previously determined to be $1 \times 10^6$ cells/mm$^3$ (47). The thymus of a well-engrafted humanized mouse is populated by approximately $2.5 \times 10^6$ human thymocytes, the majority of which are TCR positive in transduced cohorts, and is approximately 1 mm$^3$ in volume (EHG unpublished observation). In the clinical setting, the number of transduced cells along with the richer soil of a human host for transduced/transplanted human HSCs is likely to result in robust PET imaging in excess of seen in our humanized mouse study.

While the immunogenicity of sr39TK has been reported in human studies of gene modified T-cells (48, 49), in the setting of gene modified HSCs, de novo generated DCs may home to the thymus and induce tolerance to the introduced gene product (50). 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, 51).
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 (52). 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**

EHG, MNM, RPH, AR, ONW, and DBK 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.

**Disclosure of Conflicts of Interest**

The authors declare no competing financial interests.
Figure Legends

Figure 1. Experimental system to test ESO/TK PET reporter and suicide gene function in vivo. (A) Schematic of lentiviral vector used to engineer HSCs to express the ESO/TK transgene. (B) CD34 enriched G-CSF mobilized peripheral blood stem cells from healthy donors were stimulated overnight then transduced with a lentivirus encoding the ESO/TK vector. The next day, cells were 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 first gated on the characteristic lymphocyte SSC x FSC profile, followed by examination of murine and human CD45 to exclude non-nucleated cells. Human CD45+ cells were examined for hCD19 to identify B- and hCD3 to identify T-lineage cells. T-cells were gated into separate hCD4 helper and hCD8 effector subsets, and evaluated for their ability to bind the NY-ESO-1 tetramer as indicative of TCR expression.

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.

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. 51Cr 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.

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 [18F]-FHBG and PET/CT imaged. Scans of (B) non-transplanted NSG-A2.1, (C) non-transduced humanized, and (D) ESO/TKn-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.

Figure 5. GCV ablates gene modified cells hematopoietic niches. Mice were PET/CT scanned with [18F]-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 [18F]-FHBG PET signal in hematopoietic ROIs in ESO/TK mice treated with GCV (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.

References


44 Black ME, Kokoris MS, Sabo P. Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. Cancer research. 2001 Apr 1;61(7):3022-6.
51 McCracken MN, Gschweng EH, Nair-Gill E, et al. Long-term in vivo monitoring of mouse and human hematopoietic stem cell engraftment with a human positron emission tomography
Figure 2

A. CD45

B. CD19

C. CD3

D. CD4

E. CD8

F. NY-ESO-1-TCR

G. NY-ESO-1-TCR

％ of lymphocytes

P = ns

NT ESO/TK

NT ESO/TK

NT ESO/TK

NT ESO/TK

NT ESO/TK

NT ESO/TK
Figure 6

A  Human Chimerism

B  CD19

C  CD3

D  NY-ESO-1-TCR

E  Non-Transduced Humanized

F  ESO/TK - Vehicle

G  ESO/TK - GCV
HSV-sr39TK positron emission tomography and suicide gene elimination of human hematopoietic stem cells and their progeny in humanized mice


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